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Carbon Dot-Enhanced Doxorubicin Liposomes: A Dual-Functional Nanoplatfom for Cancer Therapy

Corina-Lenuta Logigan ^{1,†}, Cristian Peptu ^{2,†}, Corneliu S. Stan ¹, Gabriel Luta ³, Crina Elena Tiron ^{3,‡}, Mariana Pinteala ², Aleksander Forys ⁴, Bogdan Simionescu ^{1,2,‡}, Constanta Ibanescu ¹, Adrian Tiron ^{3,*} and Catalina A. Peptu ^{1,*}

- ¹ Department of Natural and Synthetic Polymers, Faculty of Chemical Engineering and Environmental Protection, “Gheorghe Asachi” Technical University of Iasi, 700050 Iasi, Romania; savincorina@yahoo.com (C.-L.L.); corneliu-sergiu.stan@academic.tuiasi.ro (C.S.S.); bcsimion@icmpp.ro (B.S.); constantaib@yahoo.com (C.I.)
- ² “Petru Poni” Institute of Macromolecular Chemistry, 700487 Iasi, Romania; cristian.peptu@icmpp.ro (C.P.); pinteala@icmpp.ro (M.P.)
- ³ Regional Institute of Oncology, 700483 Iasi, Romania; gabriel.luta@iroiasi.ro (G.L.); transcendtiron@iroiasi.ro (C.E.T.)
- ⁴ Centre of Polymer and Carbon Materials of the Polish Academy of Sciences, 41-819 Zabrze, Poland; aforys@cmpw-pan.pl
- * Correspondence: adrian.tiron@iroiasi.ro (A.T.); catalina-anisoara.peptu@academic.tuiasi.ro (C.A.P.); Tel.: +40-765-677-151 (A.T.); +40-765-253-915 (C.A.P.)
- † These authors contributed equally to this work.
- ‡ Passed away.

Abstract

Liposomes (LPs) represent one of the most effective nanoscale platforms for drug delivery in cancer therapy due to their favorable pharmacokinetic and various body tissue compatibility profiles. Building on recent findings showing that carbon dots derived from N-hydroxyphthalimide (CDs-NHF) possess intrinsic antitumor activity, herein, we investigate the possibility of preparing complex nano-platforms composed of LPs encapsulating CDs-NHF and/or doxorubicin (DOX) for breast and lung cancer. Various LP formulations were prepared and characterized using Cryo-TEM and Cryo-SEM for morphological analysis, while zeta potential and fluorescence assessments confirmed their stability and optical properties. Cellular effects were evaluated through immunofluorescence microscopy and proliferation assays. LPs-CDs-NHF significantly reduced cancer cell viability at lower concentrations compared to free CDs-NHF, and this effect was further amplified when combined with doxorubicin. Mechanistically, the liposomal formulations downregulated key signaling molecules including pAKT, pmTOR, and pERK, indicating the disruption of cancer-related pathways. These findings suggest that LPs containing CDs-NHF, either alone or in combination with DOX, exhibit synergistic antitumor activity and hold strong promise as multifunctional nanocarriers for future oncological applications.

Keywords: liposomes; carbon dots (CDs-NHF); drug delivery; breast cancer; lung cancer; doxorubicin synergy; signal transduction inhibition



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1. Introduction

During the last decade, new, advanced, and promising applications have been developed based on carbon dots (CDs). The CDs are attractive nanomaterials due to their outstanding physicochemical and photoluminescent properties facilitating the synthesis

and encapsulation of novel materials, with potential applications in optoelectronics, catalysis, and biomedicine [1,2]. As well, CDs have unique structural, thermal, mechanical, electrical, and optical properties, respectively; facile functionalization, the possibility of entrapment in various polymeric matrices, low toxicity, and biocompatibility, which generate particular interest for different biomedical applications including drug/gene delivery, tissue engineering, bioimaging, respectively; and diagnoses and cancer therapy [2,3].

Within the last years, different types of drug delivery systems targeting breast and lung cancer were developed for imparting the selective delivery of drugs to the desired site [4–7]. Moreover, there is an increasing interest in the development of nano-theranostically engineered multimodal platforms for cancer treatment. In this context, few studies are focused on the usefulness of a natural/polymeric delivery system capable of integrating CDs' physicochemical and photoluminescent properties [8].

Liposomes are usually sphere-shaped vesicles with a size range of 10 nm–1 μ m or greater, consisting of multi-lipid layers surrounding an aqueous core enclosed by phospholipid bilayers of natural or synthetic origin. LPs present a good affinity for cell membranes and can easily bind to cell surfaces and facilitate drug transfer. Liposomal formulations have been extensively investigated as a carrier and a great choice for the delivery of therapeutic agents. Compared with other delivery systems, LPs possess good properties like cell-like membrane structure, biocompatibility, biodegradability, low toxicity, and a hydrophobic, hydrophilic character which facilitates the incorporation and targeted delivery of both hydrophobic and hydrophilic molecules [9,10].

Several approved LPs for the treatment of cancer include Onivyde™, a pegylated liposomal formulation for the treatment of pancreatic cancer; Marqibo®, a liposomal formulation based on vincristine liposome for the treatment of blood cancer; Doxil®, a liposomal doxorubicin formulation for the treatment of breast cancer; Visudyne®, a liposomal formulation containing verteporfin used along with laser light treatment to treat certain serious eye conditions (such as macular degeneration, pathologic myopia, and ocular histoplasmosis); and Depocyt®, used for the treatment of acute nonlymphocytic leukemia, meningeal leukemia, refractory leukemia, and lymphomatous meningitis [11].

All the recent advancements in the clinical management of many types of cancers, such as breast, lung, and colorectal cancers, are still challenging due to advanced evolution, escape from screening, and rapidly acquired resistance [12]. Cancer therapy, typically surgery, chemotherapy, and radiotherapy, have several limitations like nonspecific targeting, inducing multi-drug resistance, and excessive toxic effects leading to treatment failure and disease recurrence [13–15].

Various signaling pathways, like PI3K/AKT/mTOR and EGF/RAS/RAF/MEK/ERK, are pivotal in oncogenesis, associated with cancer progression and drug resistance in different types of human cancer [16–18]. Several drugs targeting those pathways are in clinical trials, mainly in combination with different therapies since monotherapy was not satisfactory [19,20].

One of the most popular anticancer agents, Doxorubicin (DOX), is used for the treatment of different types of cancers like ovarian, breast, prostate, lung, etc. [13]. One of our recent studies demonstrated that the pyrolytic processing of chemicals such as NHF (*N*-hydroxyphthalimide) and NHS (*N*-hydroxysuccinimide) results in CDs with interesting antitumor activities [21].

Nanomedicine progress concerning cancer therapy allows for a more personalized and precise delivery, and combined with other treatments, had a direct effect on local tumor development and outcome [22]. The nanomedicine approach may comprise nanostructured materials between 5 and 200 nm in diameter which carry therapeutic, diagnostic, and theranostic compounds. In oncology, clinically approved nanostructures are molecular drug

carriers designed to improve tumor drug delivery which includes polymeric/inorganic nanoparticles, liposomes (LPs), protein–drug conjugates dendrimers, etc. [23–25]. Due to the typically leaky and disorganized nature of tumor vasculature, cancer cells can readily escape systemic circulation and infiltrate the surrounding extracellular space. Because of that, nanomaterial drug carriers are preferentially entrapped in tumors and excluded by normal tissues. Also, nanocarrier surfaces can be decorated with diverse structures like polymers, proteins, and antibodies designed to prevent immune detection and improve targeting and uptake in specific cell populations [26–29].

Embedding CDs in different matrices such as LPs [9], natural/polymeric nanoparticles or capsules [30], and dendrimers [31] has great potential as a delivery system with enhanced anticancer activity of the CDs. Moreover, a theranostic system formed from a liposomal system including CDs or a drug or both can allow the observation of the CDs/drug localization at a particular site (through fluorescence detection), visualization of its bio-distribution, and evaluation of the therapeutic efficacy [32].

In this context, the literature includes only a few studies on liposomal formulations encapsulating CDs. Demir et al. demonstrated in a relatively recent study the use of liposomes for a theranostic purpose as a novel multimodal platform for a CDs, curcumin, and anti-CD44 antibody combination for cancer treatment. They found that the prepared theranostic LPs-loaded anti-CD44 antibody showed an enhanced effect on cancer cells compared to curcumin-loaded and CUR/CD-loaded LPs [33]. Guan et al. [34] succeeded in preparing glycosylated LPs-loaded CDs for the targeted recognition of HepG2 cells. The results showed that obtained Man-CDs-LPs have a dual use and can be used for specific recognition, but also for tracking the interaction between carbohydrates and glycoprotein by the fluorescence images of CDs. Another study concerning CDs-loaded LPs was performed by Ren et al. [35] The developed multifunctional LPs were loaded with a hydrophilic near-infrared CD as a nanocarrier and tracer of the lipophilic anticancer agent cinobufagin. The LPs displayed some advantages such as lysosomes-targeted cellular imaging, tumor-targeted delivery, enhanced photoluminescence emission/imaging, enhanced cellular uptake, and improved anticancer activity.

This study focuses on developing various LPs formulations as multifunctional delivery systems for CDs-NHF and/or DOX. We hypothesize that LPs formulations can enhance the efficacy of CDs-NHF by improving their water solubility and reducing aggregation, which otherwise limits their clinical antitumor potential. Liposomes (LPs) prepared using the reverse-phase evaporation method demonstrated an effective and reliable approach for liposome production. The resulting LPs showed an excellent capacity for encapsulating CDs-NHF/DOX and may be considered as a potential nano-formulation for antitumor applications. Moreover, our previous works [36,37] succeeded in proving that CDs-NHF entrapped in a polymeric matrix presented antitumoral activity. In this investigation, we describe a slightly modified method for preparing various LPs formulations based on Phospholipon G and cholesterol using the reverse-phase evaporation technique. LPs characterization was carried out in terms of size (dynamic light scattering—DLS), morphology visualized via Cryo-Transmission Electron Microscopy (Cryo-TEM), Cryo-Scanning Electron Microscopy (Cryo-SEM), fluorescence analysis, and drug loading properties. In vitro studies of prepared LPs formulations were performed. The results revealed that LPs-loaded CDs-NHF significantly reduced mammary and lung cancer cell viability at lower doses compared to control CDs-NHF. That effect was further potentiated by LPs-CDs-NHF association with DOX. CDs-NHF-loaded LPs affected the expression of major molecules involved in oncogenesis like AKT, mTOR, and ERK.

Among various clinically used chemotherapeutic agents, doxorubicin (DOX) was selected in this study due to its extensive application in breast and lung cancer therapies,

which represent the main focus of this investigation. Additionally, DOX is known for its compatibility with liposomal encapsulation—as evidenced by several approved liposomal formulations such as Doxil®—and possesses intrinsic fluorescence, which facilitates dual-tracking when combined with CDs-NHF. Furthermore, its amphiphilic nature allows for efficient entrapment within lipid bilayers, making it an ideal candidate for inclusion in a multifunctional liposomal delivery system with both therapeutic and diagnostic potential.

2. Results and Discussion

2.1. Preparation and Characterization of LPs Formulations

An important role in the preparation of LPs formulations is played by the selection of lipid composition which usually dictates the drug binding, distribution, and retention capacity of the LPs, respectively, and the drug release rate from the LPs. Also, another important parameter that can modulate membrane permeability and biological stability is cholesterol. This study aimed to examine the antitumoral activity of developed LPs loaded with CDs-NHF; DOX or CDs-NHF-DOX. LPs size, zeta potential, drug/lipid content, and the antitumoral effect using *in vitro* cancer cell line models were studied.

2.1.1. LPs Formulations Preparation

The reverse-phase evaporation (REV) method was selected for liposome preparation due to its versatility and high encapsulation efficiency, especially for amphiphilic and hydrophilic molecules such as CDs-NHF and DOX. Unlike other methods, like thin-film hydration or ethanol injection, the REV method facilitates the formation of multilamellar vesicles (MLVs) with large internal aqueous volumes, providing an ideal microenvironment for the co-loading of therapeutic agents with different solubility profiles. Additionally, the process allows for better control over vesicle size and lamellarity, which is critical for the reproducibility and scale-up of multifunctional liposomal systems. Given the goal of integrating two bioactive compounds with distinct mechanisms of action into a stable nanosystem, REV proved to be the most appropriate and efficient approach.

The proposed pathway to obtain LPs based on Phospholipon 90G and cholesterol was a reverse-phase evaporation method, slightly modified [38]. The selected method involves the dissolution of the PC-G and CHOL in 10 mL of solvent (diethyl ether).

Next, a fixed volume of the previously prepared PBS or CDs-NHF/CDs-NHF-DOX/DOX suspension was slowly added dropwise to the solvent phase using a 1 mL syringe, through a rubber septum. During this step, the lipids spontaneously rearranged at the water/oil interface, leading to the formation of a microemulsion. The microemulsion was then subjected to sonication for 2 min to ensure the formation of a homogeneous dispersion.

Subsequently, the resulting microemulsion was transferred to a rotary evaporator, where the solvent phase was slowly removed under reduced pressure. The gradual removal of the solvent facilitated the formation of liposomes (LPs). The resulting LPs suspension was further homogenized using a vortex mixer for 2 min and placed in an ultrasonic bath for 30 min to obtain a uniform dispersion and dissociate any pre-formed aggregates. To reduce the size of the LPs, the final aqueous suspension was sequentially extruded through 400 nm, 200 nm, and 100 nm polycarbonate membranes using a mini-extruder (Avanti Polar Lipids, Alabaster, AL, USA). The obtained LPs were then purified by dialysis against distilled water for five days. The parameters used in the LP preparation process are summarized in Table 1.

Table 1. LPs formulations preparation parameters.

Sample ID	Pre-Purification			Post-Purification	
	PC-G, mg/mL	CHOL, mg/mL	Diethyl Ether, mL	PC-G, mg/mL	CDs-NHF or DOX/Lipid, mg/mL
Control cells	-	-	-	-	-
Cells-loaded LPs	-	-	-	-	-
Cells-loaded LPs-CDs-NHF	-	-	-	-	-
CDs-NHF	-	-	-	-	-
Control LPs	60	40	10	23	-
LPs-CDs-NHF	60	40	10	17	0.13
LPs-DOX	60	40	10	13	0.35
LPs-CDs-NHF-DOX	60	40	10	12	0.219 (CDs)
					0.373 (DOX)

Next, the LPs PC-G concentration (mg/mL) was determined via a Stewart assay and the results can be seen in Table 1. The variation in the PC-G concentration after the purification step was between 12 and 23 mg/mL. Moreover, the LPs' potential for effective use as a controlled drug delivery system for different biomedical applications was analyzed. Considering the possibility of reducing the nonspecific organ toxicity, LPs were loaded with CDs-NHF, DOX, and CDs-NHF-DOX, respectively. The prepared liposomes were capable of including 98.2% of CDs-NHF and 97.7% of DOX, showing a high capacity of the LPs to entrap biologically active compounds.

DOX and CDs-NHF encapsulation efficiency (DEE) represents the drug quantity added (%) in the LPs preparation step and divided by the total drug quantity which is encapsulated in the LPs formulation. The DEE of DOX/CDs-NHF from LPs was calculated with Equation (1).

$$\text{DEE} = \frac{\text{Actual drug (DOX or CDs – NHF) content loaded in LPs}}{\text{Theoretical drug content (DOX)}} \times 100 \quad (1)$$

As shown in Table 1, the LP formulations exhibited a good encapsulation capacity. A higher drug concentration was observed for DOX (4.57 mg/mL) compared to CDs (2.27 mg/mL). However, when both CDs-NHF and DOX were co-loaded into the LPs, the overall concentration remained within a similar range.

2.1.2. LPs Formulations Morphology

LPs morphology was investigated by Cryo-TEM (Figure 1) and Cryo-SEM (Figure 2), respectively.

Cryo-TEM micrographs of the unloaded liposomes, as well as those loaded with CDs-NHF, DOX, or both, confirmed the successful preparation of liposomal formulations as multilamellar vesicles (MLVs) with a spherical morphology (Figure 1). Also, the obtained MLVs are presenting several concentric bilayers with a mean particle size ranging from 300 to 1000 nm, in concordance with the DLS analysis results (Table 2, Figure S1). Cryo-TEM images of the LPs revealed discrete and intact structures without aggregation. A further investigation via Cryo-SEM (Figure 2) for control LPs also demonstrated that the LPs were obtained and presented a spherical shape.

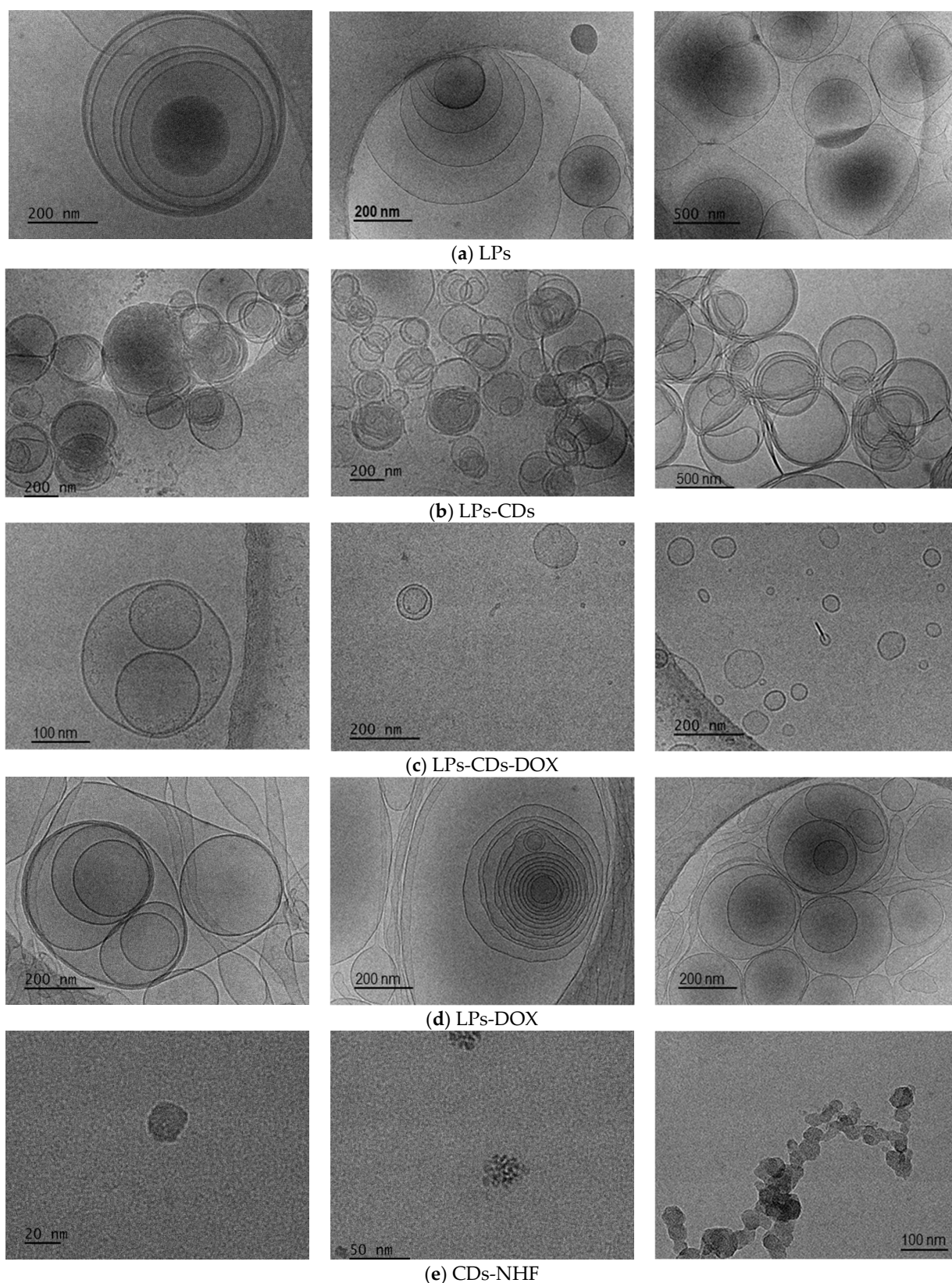


Figure 1. Cryo-TEM images for all LPs formulations and CDs-NHF. (a) Cryo-TEM images for LPs, (b) Cryo-TEM images for LPs-CDs, (c) Cryo-TEM images for LPs-CDs-DOX, (d) Cryo-TEM images for LPs-DOX, (e) Cryo-TEM images for the prepared CDs-NHF via the pyrolytic process revealed that obtained CDs-NHF are small entities in the 20–50 nm range.

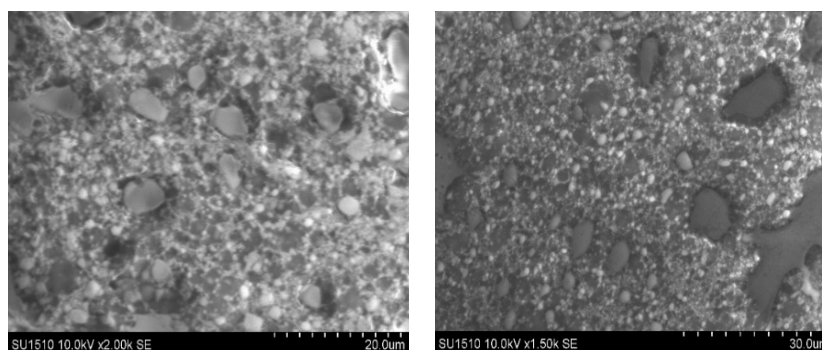


Figure 2. Cryo-SEM images of control LPs.

Table 2. Size distribution of LPs formulations.

Sample ID	Diameter (DLS $\pm$ SD), nm	Zeta Potential (mV)
Control cells	-	−8.11
Cells-loaded LPs	-	−6.10
Cells-loaded LPs-CDs-NHF	-	−11.98
CDs-NHF	44	−10.47
LPs	384	−33.12
LPs-CDs-NHF	1016	−24.86
LPs-DOX	917	−23.67
LPs-CDs-NHF-DOX	801	−26.57

2.1.3. LPs Formulations Size and Zeta Potential

The size and zeta potential (ZP) of the liposomal formulations were evaluated, and the results are summarized in Table 2 and Supplementary Figure S1. The measurements revealed an average vesicle size ranging from 384 to 1000 nm. These findings confirm that the employed preparation method successfully yielded multilamellar vesicles (MLVs) with a characteristic spherical morphology, composed of multiple lipid bilayers encapsulating an aqueous core. Dynamic light scattering (DLS) analysis further indicated a size increase for LPs loaded with CDs-NHF, DOX, and CDs-NHF-DOX compared to the unloaded (control) liposomes. This enlargement in vesicle size may be attributed to interactions between the encapsulated CDs-NHF and/or DOX molecules and the lipid bilayers, potentially affecting the structural organization of the vesicles. Similar effects have been reported in the literature, where the incorporation of hydrophilic or amphiphilic agents leads to modifications in liposomal size and membrane dynamics due to drug–lipid interactions [39,40].

ZP knowledge of the prepared liposomal formulation is critical for the LPs design and quality control. LPs' ZP was evaluated to establish surface charge stability via the Smoluchowski Equation (2):

$$\zeta = \frac{\eta\mu}{\varepsilon} \text{ and } k\alpha \gg 1 \quad (2)$$

where

η —viscosity;

ε —dielectric constant;

k and α —Debye–Hückel parameter, and LPs radius.

As a result, the LPs' ZP (Supplementary Figure S2 and Table 2) revealed that MLVs formulations are presenting a high dispersion stability, which leads to a strong electrostatic

repulsion interaction preventing the aggregation of MLVs and a higher entrapment capacity, respectively [40,41].

2.1.4. LPs Formulations Fluorescence Abilities

Figure 3 and Table 3 present the emissions profiles of CDs-NHF, unloaded liposomes (LPs), LPs loaded with CDs-NHF, and LPs loaded with CDs-NHF-DOX, recorded at an excitation wavelength of 370 nm. The results show that the control LPs exhibit no detectable emissions, in contrast to the pronounced photoluminescence of CDs-NHF and the corresponding loaded LP formulations. For free CDs-NHF, a maximum emission intensity was observed at 447 nm. In comparison, the emission peaks of CDs-NHF in the LPs-CDs-NHF and LPs-CDs-NHF-DOX systems were shifted to 408 nm and 405 nm, respectively, (as shown in Table 3). Furthermore, the emission intensity of CDs-NHF was reduced by approximately 50% upon encapsulation within the liposomal structure. This decrease in emission intensity could be attributed to the interaction with the liposomal bilayer, which may partially quench or shield the fluorescent signal, potentially through the interference with the optical pathway or limited accessibility of the excitation/emission light. These observations suggest that the phosphatidylcholine-based lipid (PC-G) matrix plays a significant role in modulating the photoluminescence behavior of CDs-NHF within the LP formulations.

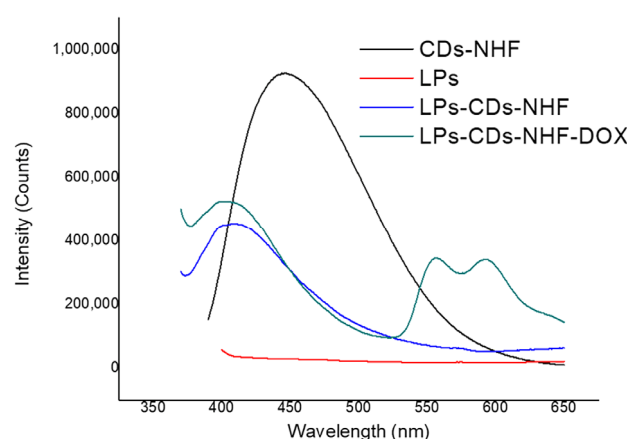


Figure 3. Emission spectra of CDs-NHF, LPs, LPs-CDs-NHF, and LPs-CDs-NHF-DOX formulations suspended in H₂O.

Table 3. Fluorescence results obtained for CDs-NHF, LPs, LPs-CDs-NHF, and LPs-CDs-NHF-DOX formulations.

Sample ID	Excitation (nm)	Emission Peaks (nm)	Intensity (Counts)
CDs-NHF	370	447	9.27641×10^5
Control LPs	370	0	0
LPs-CDs-NHF	370	408	4.55403×10^5
LPs-CDs-NHF-DOX	370	405	5.26137×10^5 (CDs-NHF)
LPs-CDs-NHF-DOX	370	557	3.46898×10^5 (DOX)
LPs-CDs-NHF-DOX	370	593	3.42876×10^5 (DOX)

In addition, the photoluminescent properties of the LPs formulations were visually assessed under both daylight and UV illumination using a Philips UVA TL4WBLB lamp (emission maximum at 370–390 nm) and a 50 mW laser diode at 440 nm (Supplementary Figure S3). The visual observations were consistent with the fluorescence spectroscopy results, further confirming the presence and emission behavior of the CDs.

2.2. In Vitro Studies

The liposomal formulations containing CDs-NHF significantly inhibited the viability of breast (MDA-MB-231) and lung (A549) cancer cells. Given the high mortality and incidence associated with these types of cancer, we evaluated the effects of several formulations: empty liposomes (LPs), unencapsulated CDs-NHF, and LPs-CDs-NHF. All were tested at concentrations of 0.1%, 1%, and 5%.

Figure 4 shows that CDs-NHF at a 5% concentration inhibited cancer cell proliferation, consistent with our previous findings [37]. Moreover, liposomal encapsulation of CDs-NHF amplified the antitumor effect, particularly in MDA-MB-231 and A549 cell lines (Figure 4A,B, columns 9–10). Notably, this treatment did not affect the viability of the normal breast epithelial cell line MCF-10A (Supplementary Figure S4).

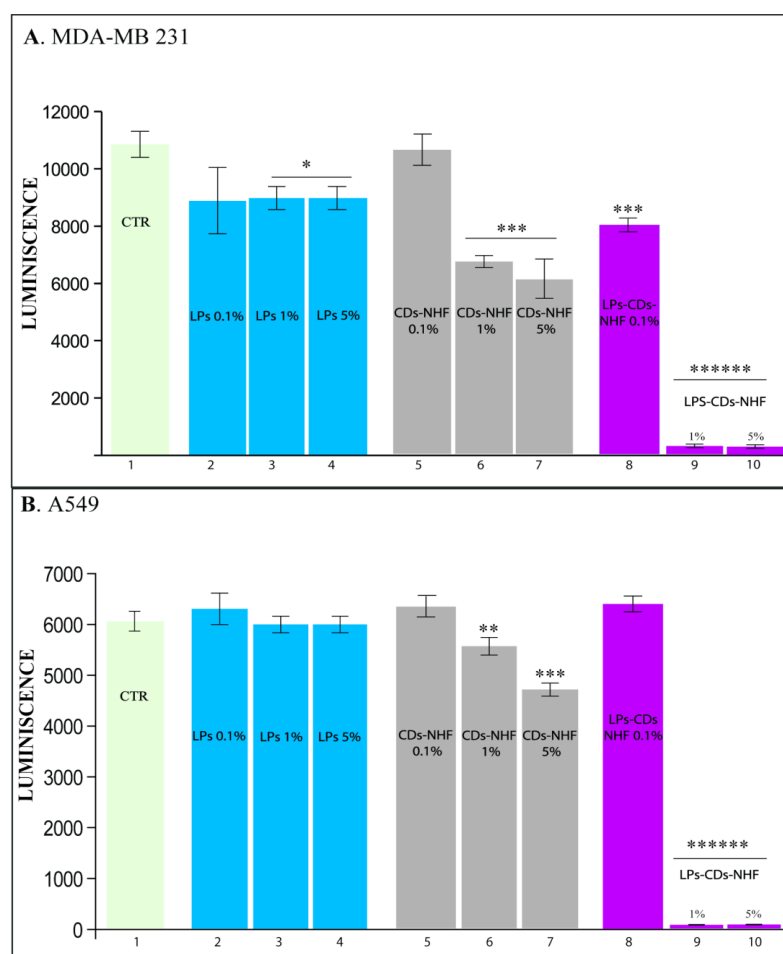


Figure 4. The effect of LPs-loaded CDs-NHF in cancer cell viability: (A) MDA-MDB231; (B) A549. 1 untreated; 2–4 empty LPs; 5–7 unencapsulated CDs-NHF; and 8–10 LPs encapsulated CDs-NHF. * $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$; and ***** $p < 0.00000005$.

DOX-loaded liposomes also reduced breast and lung cancer cell viability. While DOX is a widely used chemotherapeutic agent, its clinical application is limited by systemic side effects [22]. LPs have been shown to enhance the therapeutic index of drugs, and this effect was also evident in our study (Figure 5). Four different formulations were tested: free DOX, DOX-loaded LPs, LPs containing both CDs-NHF and DOX, and LPs with CDs-NHF suspended in a DOX solution.

DOX significantly reduced cancer cell viability (Figure 5A,B, columns 2–4), and this effect was further enhanced by DOX-loaded liposomes (columns 5–7). Interestingly, LPs

containing both CDs-NHF and DOX (columns 8–10) did not show further potentiation compared to DOX-LPs alone. However, when CDs-NHF-loaded LPs were administered alongside free DOX (columns 11–13), a marked reduction in cell viability was observed. This suggests that co-encapsulation may limit the efficacy of CDs-NHF, potentially due to interference within the same endocytic pathway, while separate administration allows each agent to exert its effect independently and synergistically.

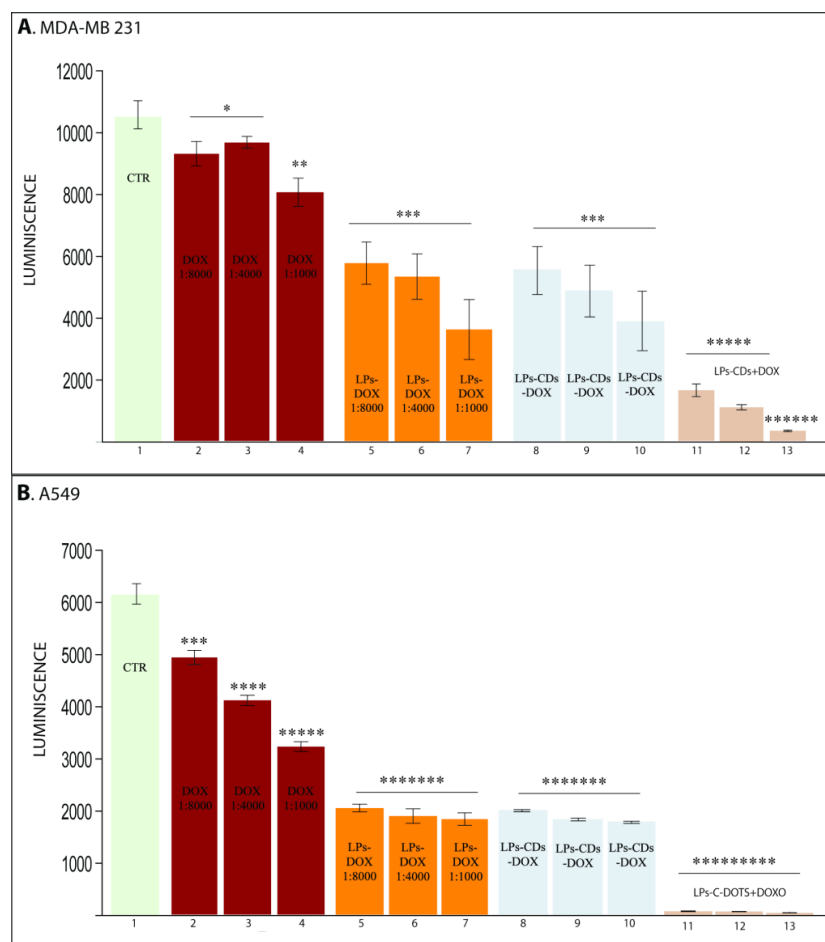


Figure 5. The effect of Doxorubicin in cancer cell viability: (A). MDA-MDB231; (B). A549. 1 untreated; 2–4 DOX; 5–7 LPs encapsulated DOX; 8–10 LPs encapsulated CDs-NHF—DOX (0.1%, 1%, and 5%)/1:8000; and 11–13 LPs encapsulated CDs-NHF (0.1%, 1%, and 5%) suspended in DOX (1:8000). * $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$; **** $p < 0.00005$; ***** $p < 0.000005$; ***** $p < 0.0000005$, and ***** $p < 0.000000005$.

CDs-NHF treatment also reduced the expression of phosphorylated Akt (pAkt 1/2/3), a key player in the PI3K/Akt/mTOR pathway implicated in tumor progression and treatment resistance [17,18]. The Akt family members—Akt 1,2,3—have different roles in invasion, migration, and metastasis dissemination [42].

Figure 6 shows that 5% CDs-NHF significantly downregulated pAkt in both MDA-MB-231 and A549 cells (Figure 6Ab,Bb) compared to untreated controls (Aa, Ba). DOX treatment, on the other hand, increased pAkt expression and altered cell morphology, particularly in A549 cells (Ad, Bd). The combination of LPs-loaded CDs-NHF with free DOX further reduced the pAkt expression, except in the MDA-MB-231 group treated with 1% CDs-NHF, likely due to insufficient concentration to overcome treatment resistance. All the obtained data highlights the important ability of CDs-NHF encapsulated in LPs to reduce metabolic activities in investigated malignant cells.

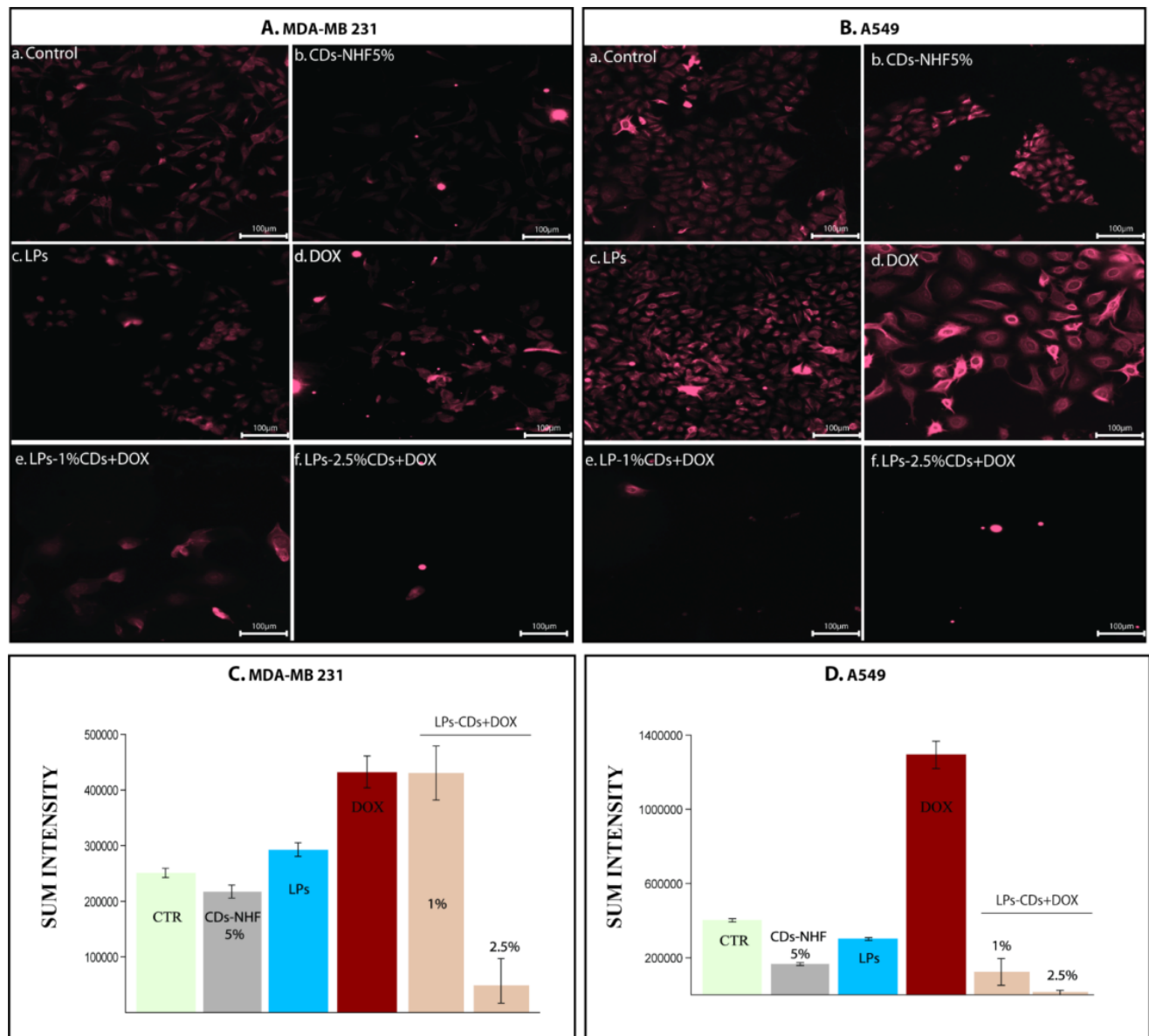


Figure 6. Level of pAkt (1,2,3): (A). MDA-MB231 cells; (B). A549 cells; (Aa,Ba) untreated; (Ab,Bb) un-encapsulated CDs-NHF 5%; (Ac,Bc) LPs; (Ad,Bd) free DOX; (Ae,Be) LPs encapsulated CDs-NHF 1% suspended in DOX; (Af,Bf) LPs encapsulated CDs-NHF 2.5% suspended in DOX; and (C,D) quantification of fluorescence straining.

To further investigate the underlying mechanisms, pmTOR expression was assessed and found to be reduced following CDs-NHF treatment. The mammalian target of rapamycin (mTOR), a downstream effector of Akt, plays a key role in cancer cell proliferation, apoptosis, and cell cycle regulation [43].

Downstream of pAkt, pmTOR expression was also reduced following CDs-NHF treatment Figure 7Ab,Bb, whereas it increased in DOX-treated groups (Ad,Bd). Co-administration of LPs-loaded CDs-NHF and soluble DOX reduced pmTOR expression across all treatment groups (Ae–f,Be–f), highlighting the potential of this combination to inhibit the PI3K/Akt/mTOR signaling axis.

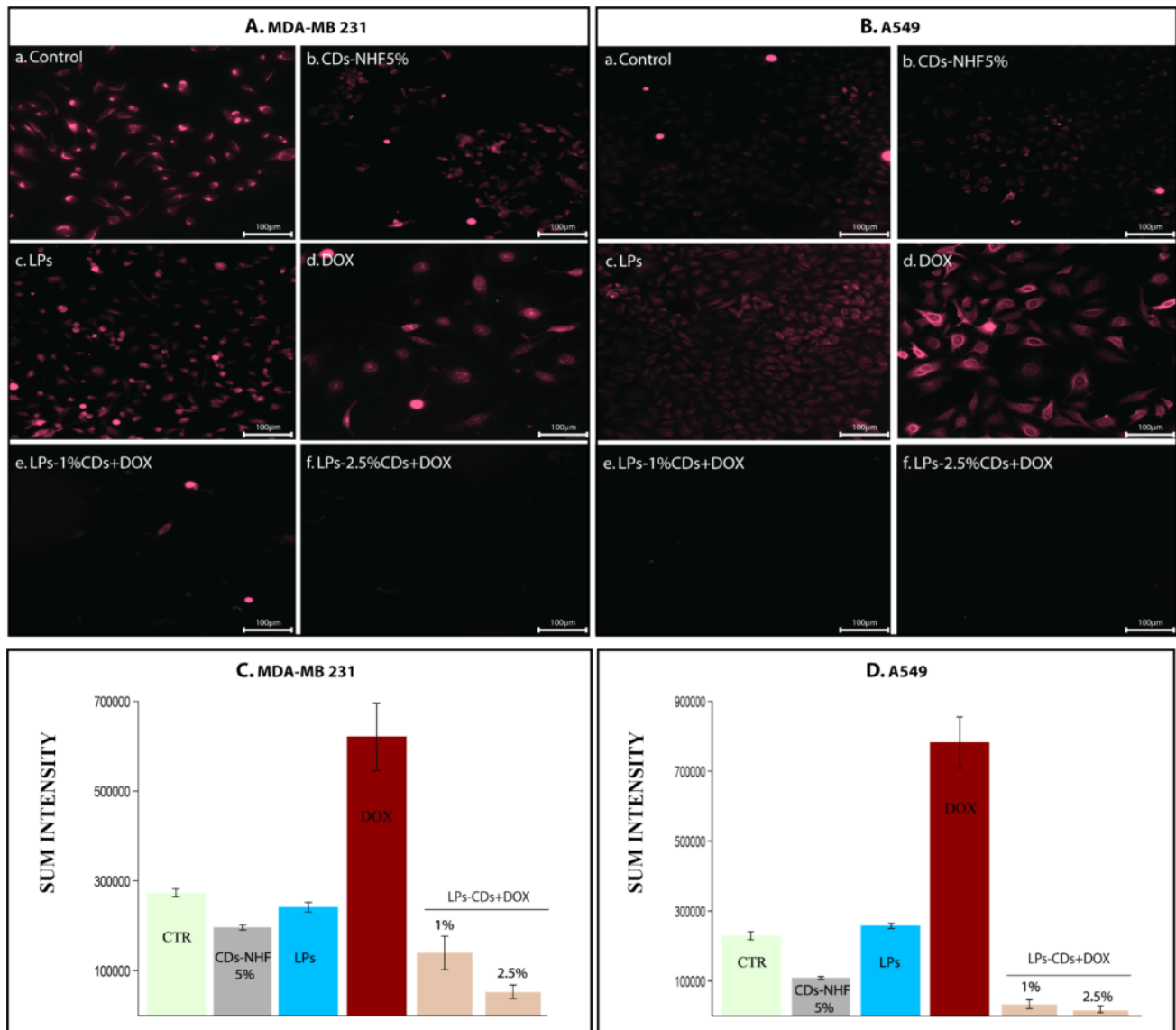


Figure 7. Expression levels of pmTOR: (A). MDA-MB231 cells; (B). A549cells; (Aa,Ba) untreated; (Ab,Bb) unencapsulated CDs-NHF 5%; (Ac,Bc) LPs; (Ad,Bd) free DOX; (Ae,Be) LPs encapsulated CDs-NHF 1% suspended in DOX; (Af,Bf) LPs encapsulated CDs-NHF 2.5% suspended in DOX; and (C,D) quantification of fluorescence straining.

Treatment-dependent changes were also observed in the pERK1/2 expression. This protein, part of the MAPK signaling cascade, is involved in cancer cell migration and invasion [44].

Moreover, triple-negative breast cancer (TNBC) patients with a high ERK expression have a poorer survival rate than those with ERK with a low expression [45–48]. Both cell lines exhibited high baseline pERK1/2 levels, which were significantly reduced by CDs-NHF (Figure 8Ab,Bb). DOX treatment increased the pERK expression in A549 cells only (Figure 8Ad,Bd), indicating cell-type-specific responses. Notably, LPs-loaded CDs-NHF combined with DOX reduced pERK1/2 levels in both cell types, although 1% CDs-NHF showed an unexpected increase, again suggesting a concentration-dependent effect.

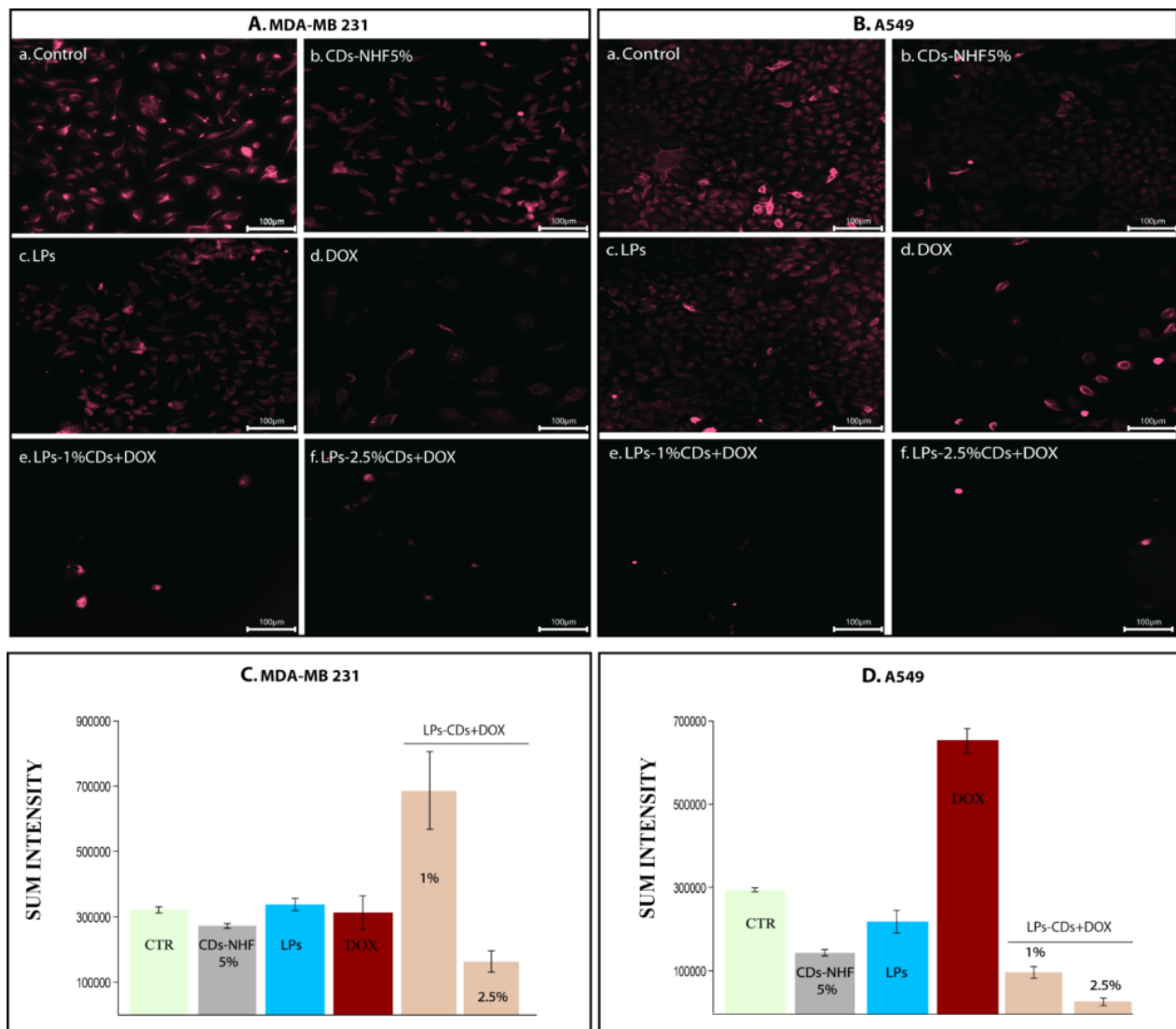


Figure 8. Level of pERK1/2: (A). MDA-MB231 cells; (B). A549 cells; (Aa,Ba) untreated; (Ab,Bb) un-encapsulated CDs-NHF; (Ac,Bc) LPs; (Ad,Bd) free DOX; (Ae,Be) LPs encapsulated CDs-NHF 1% suspended in DOX; (Af,Bf) LPs encapsulated CDs-NHF 2.5% suspended in DOX; and (C,D) quantification of fluorescence straining.

Our previous studies demonstrated that CDs-NHF reduce HSP90 expression in both in vivo and ex vivo cancer models, supporting their role in modulating oncogenic stress responses. Specifically, in a 4T1 murine breast cancer model, CDs-NHF treatment led to a decreased HSP90 and Ki67 expression as well as reduced metastatic dissemination, while in U87 glioma cells, CDs-NHF suppressed pAKT, p70S6Kinase, and other key signaling molecules involved in tumor survival and progression. These results suggest that the observed downregulation of HSP90 may be mediated indirectly through the inhibition of the PI3K/AKT axis and related pathways [45,46].

CDs-NHF treatment reduced HSP90 expression, a molecular chaperone involved in cancer progression (Figure 9). Combined treatment with encapsulated CDs-NHF and DOX further suppressed HSP90 levels, except in the group with 1% CDs-NHF, which was comparable to CDs-NHF alone. DOX treatment alone increased HSP90 expression.

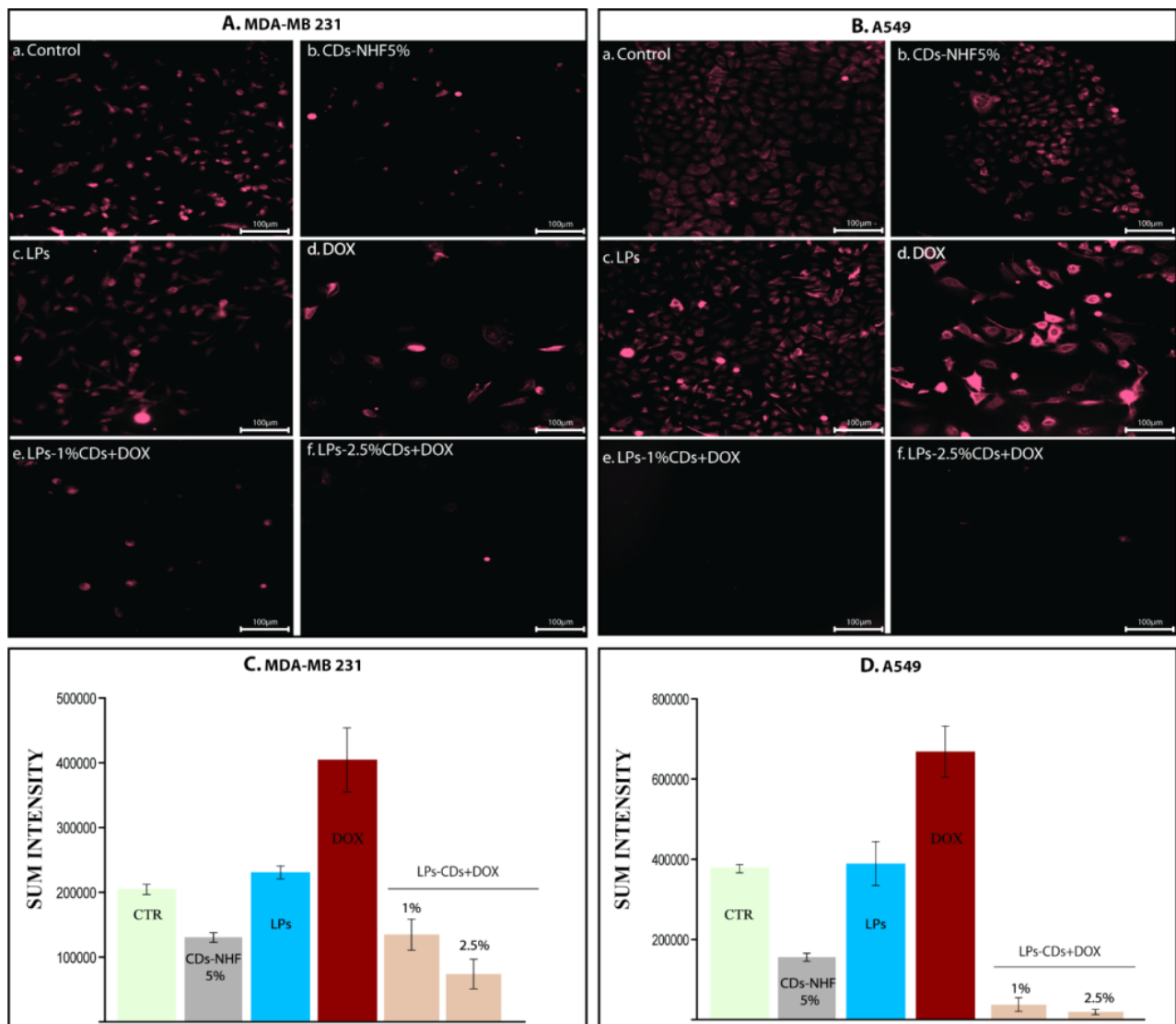


Figure 9. Expression levels of HSP90: (A). MDA-MB231 cells; (B). A549 cells; (Aa,Ba) untreated; (Ab,Bb) unencapsulated CDs-NHF; (Ac,Bc) LPs; (Ad,Bd) free DOX; (Ae,Be) LPs encapsulated CDs-NHF 1% suspended in DOX; (Af,Bf) LPs encapsulated CDs-NHF 2.5% suspended in DOX; and (C,D) quantification of fluorescence staining.

The intrinsic antitumor activity of CDs-NHF is likely mediated through a combination of mechanisms. Prior *in vivo* and *ex vivo* studies have demonstrated that CDs-NHF induce oxidative stress and mitochondrial dysfunction, while also modulating key oncogenic signaling cascades such as PI3K/Akt and MAPK. These effects result in the downregulation of HSP90, Ki67, pAKT, p70S6K, and pERK, impairing tumor cell viability, proliferation, and invasiveness [45,46]. Notably, these effects appear to be selective for malignant cells, as no significant cytotoxicity was observed in normal epithelial cells, further supporting the therapeutic potential of CDs-NHF as a multifunctional antitumor agent.

Together, these results indicate that CDs-NHF impacts key oncogenic pathways (PI3K/Akt/mTOR and MAPK) and that combining CDs-NHF with DOX enhances this effect. Nanotechnology-based formulations, such as LPs, offer promising strategies to improve drug efficacy and reduce toxicity [29,47–53].

3. Materials and Methods

3.1. Materials

The following materials were used for the experiments: Phospholipon 90G (phosphatidylcholine, from Lipoid), cholesterol, N-hydroxyphthalimide, ferric chloride hexahydrate, ammonium thiocyanate, and diethyl ether were purchased from Sigma-Aldrich (St. Louis, MO, USA), doxorubicin (from Teva Pharmaceuticals Srl, Bucharest, Romania) and Milli-Q ultrapure distilled water from Merck Chemicals (Darmstadt, Germany). MCF-10A (ATCC) (a gift from Prof. Dr. J. Lorens) was maintained in DMEM/F12 supplemented with 5% horse serum; 20 ng/mL EGF; 10 µg/mL insulin; 0.5 ng/mL hydrocortisone; 10 ng/mL cholera toxin; 1% Pen/Strep; and bovine serum were purchased from Sigma-Aldrich (St. Louis, MO, USA). MDA-MB-231 (ATCC) was cultured in F-12K Medium, supplemented with 100 U/mL of penicillin and 100 µg/mL of streptomycin and 5% bovine serum; A549 (ATCC) was cultured in F-12K Medium, supplemented with 100 U/mL of penicillin and 100 µg/mL of streptomycin and 5% bovine serum. Mouse anti-human pAkt sc-271966, Mouse anti-human pmTOR sc-293133, Mouse anti-human pERK1/2 sc-13652, and HSP90 (Cell Signaling Technology, Bucharest, Romania, E289) were used for immunofluorescence staining. All reagents were used as provided, without further purification or modification.

3.2. Instrumentation

The morphology of LPs, CDs, LPs-CDs-NHF, LPs-DOX, and LPs-CDs-NHF-DOX was characterized by Cryogenic Transmission Electron Microscopy (Cryo-TEM) images, which were obtained using a Tecnai F20 X TWIN microscope (FEI Company, Hillsboro, OR, USA) equipped with a field emission gun, operating at an acceleration voltage of 200 kV. Images were recorded on the Gatan Rio 16 CMOS 4k camera (Gatan Inc., Pleasanton, CA, USA) and processed with Gatan Microscopy Suite (GMS) software (Gatan Inc., Pleasanton, CA, USA). Specimen preparation was performed by vitrification of the aqueous solutions on grids with holey carbon film (Quantifoil R 2/2; Quantifoil Micro Tools GmbH, Großlobichau, Germany). Prior to use, the grids were activated for 15 s in oxygen plasma using a Femto plasma cleaner (Diener Electronic, Ebhausen, Germany). Cryo-samples were prepared by applying a droplet (3 µL) of the suspension to the grid, blotting with filter paper, and immediately freezing in liquid ethane using a fully automated blotting device VitroBot Mark IV (Thermo Fisher Scientific, Waltham, MA, USA). After preparation, the vitrified specimens were kept under liquid nitrogen until they were inserted into a Cryo-TEM-holder Gatan 626 (Gatan Inc., Pleasanton, CA, USA) and analyzed in the TEM at −178 °C.

Also, LPs' morphology was investigated by the Cryo-SEM technique. SEM images were recorded using a Hitachi SU 1510 (Hitachi Company, Japan) Scanning Electron Microscope, LPs were fixed on an aluminum stub and frozen at −20 °C with a Peltier stub before observation.

CDs-NHF, all LPs formulations, and cells mean diameter, size distribution, and zeta potential were analyzed via Delsa Nano C Submicron Particle Size Analyzer using the dynamic light diffusion (DLS), which measures the Brownian motion of the particles and correlates it with their size. The relationship between the size of a particle and its speed is given by the Stokes–Einstein Equation (3):

$$DH = \frac{kBT}{3\pi\eta D} \quad (3)$$

where

DH is the hydrodynamic diameter;

D is the diffusion coefficient;

k_B is Boltzmann's constant;
 T is the temperature;
 η is the viscosity of the medium.

For sample preparation, 1–2 drops of each concentrated LPs formulation were dispersed in 2 mL of distilled water, followed by ultrasonication for 1 min. The zeta potential (ZP) analysis was conducted using the same protocol as the particle size measurements. All measurements were performed in triplicate to ensure reproducibility.

The photostability of CDs-NHF and all LPs formulations was assessed using a Horiba Fluoromax-4P spectrofluorometer (Fluor Essence software, Version 35.1.20) equipped with a Quanta- ϕ integrating sphere. Additionally, visual testing of photoluminescence properties was performed under a Philips UVA TL4WBLB lamp (München, Germany; $\lambda_{\text{max}} = 370$ nm) and a 50 mW laser diode emitting at 440 nm.

Cell viability studies were carried out using a multi-mode plate reader (FilterMax F5), while immunofluorescence imaging was performed on a Zeiss Axio Observer Z1 microscope (TissueGnostics platform, Zeiss, Hilden, Germany).

3.3. Methods

3.3.1. Preparation of LPs Formulations

First, the carbon dots (CDs) were synthesized using a simple one-step pyrolytic method, as described by Stan et al. [54]. The resulting CDs exhibited a small average size of approximately 44 nm and were utilized in their suspension form for incorporation into the liposomal (LP) formulations.

To prepare the liposomal formulations—namely control liposomes (LPs), liposomes loaded with carbon dots (LPs-CDs-NHF), liposomes loaded with doxorubicin (LPs-DOX), and dual-loaded liposomes (LPs-CDs-NHF-DOX)—a slightly modified reverse-phase evaporation method, adapted from Xue et al. [38], was employed. Initially, Phospholipon 90G (phosphatidylcholine, PC-G; 60 mg) and cholesterol (CHOL; 40 mg) were dissolved in 10 mL of diethyl ether (in a 1:1.5 molar ratio) in a round-bottom flask.

For the preparation of control LPs, 1.5 mL of distilled water was added dropwise via a syringe through a rubber septum to form a water-in-oil emulsion. The mixture was then subjected to sonication for 2 min to facilitate emulsification. Subsequently, the solvent was removed by rotary evaporation (Heidolph Laborota 4002, 60 rpm, 35 °C, and under reduced pressure) until the disappearance of the gel phase was observed.

The resulting LPs suspension was homogenized by vortexing for 2 min and then placed in an ultrasound bath for 30 min to ensure uniform dispersion and to break potential aggregates. The liposomes were further size-reduced by sequential extrusion through polycarbonate membranes with pore sizes of 400 nm, 200 nm, and 100 nm using a mini-extruder (Avanti Polar Lipids, Alabaster, AL, USA).

For the preparation of LPs-CDs-NHF, LPs-DOX, and LPs-CDs-NHF-DOX, the same protocol was followed, with the exception that the 1.5 mL of distilled water was replaced with 1.5 mL of either an aqueous suspension of CDs-NHF (4 mg/mL), a DOX solution (2 mg/mL), or a combined CDs-NHF and DOX suspension.

Finally, all liposomal suspensions were purified by dialysis against Milli-Q water using cellulose ester membrane bags (molecular weight cutoff: 12,400 Da) for 5 days to remove unencapsulated components. The encapsulated amounts of carbon dots and/or doxorubicin were quantified by disrupting the liposomes with Triton X-100.

3.3.2. Determination of Phospholipids Concentration by Stewart Assay

Phosphatidylcholine (PC) content was determined using the Stewart assay [55], a colorimetric method based on the formation of a red-colored complex between the phos-

pholipid headgroups and ammonium ferrothiocyanate. This complex is extractable into an organic phase (chloroform) and can be quantitatively analyzed spectrophotometrically without interference from inorganic phosphate. All purified LPs formulations were analyzed using this technique.

The assay procedure included several key steps. Initially, a 0.1 M ammonium ferrothiocyanate solution was prepared, along with a standard calibration curve using known concentrations of PC-G. Standard solutions and six liposomal samples were transferred into 10 mL volumetric tubes, sealed with aluminum foil, and processed in triplicate. Each tube was vortexed vigorously for 15 s and left to stand at room temperature for 20 min to ensure complete reaction.

Following incubation, the aqueous phase (containing excess ammonium ferrothiocyanate) was carefully removed using a Pasteur pipette. The remaining organic (chloroform) phase, containing the PC-G complex, was analyzed at 485 nm using a UV-Vis spectrophotometer. The phosphatidylcholine concentration was calculated by comparing the absorbance values with the previously established calibration curve.

3.3.3. In Vitro Studies

Cell Viability

Cell viability was assessed using the CellTiter-Glo[®] Luminescent Cell Viability Assay. Cells were seeded in 96-well flat-bottom tissue culture plates at a density of 2000 cells/well and incubated overnight at 37 °C in a 5% CO₂ atmosphere to allow adherence. After attachment, the cells were treated with different concentrations (0.1%, 1%, 2.5%, and 5%) of the following: empty liposomes (LPs), unencapsulated CDs-NHF, liposomes loaded with CDs-NHF, and liposomes loaded with CDs and suspended in DOX solution. Control cells received the same volume of medium used as a vehicle, as per the manufacturer's instructions. After 72 h of treatment, 50 µL of the CellTiter-Glo[®] reagent was added to each well, followed by 4 h of incubation. Luminescence was then measured using a multi-well plate reader (FilterMax F5).

Immunofluorescence (IF)

Samples were fixed with 4% paraformaldehyde for 30 min, then blocked in PBS containing 5% normal goat serum. Permeabilization was performed using 0.1% Triton X-100. Samples were incubated with the following primary antibodies for 72 h at 4 °C: anti-human pAkt (1:50), pmTOR (1:25), pERK44/42 (1:50), and HSP90 (1:25). After washing, cells were incubated with Alexa Fluor 647-conjugated secondary antibodies (1:200; Invitrogen). Images were acquired with a Zeiss Axio Observer Z1 microscope using TissueFAXs 4.2 software, and fluorescence intensity was quantified with TissueQuest 6.0.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism, version Prism 8.0.0. The specific tests used are indicated in the figure legends. Grouped data were analyzed using one-way ANOVA, and statistical significance was considered at $p < 0.05$.

4. Conclusions

This study demonstrates the feasibility of liposome (LP) preparation using a slightly modified reverse-phase evaporation method. The resulting LPs formulations were characterized by Cryo-TEM and Cryo-SEM microscopy, which confirmed the formation of multilamellar vesicles (MLVs) with a spherical morphology. MLVs exhibited multiple concentric bilayers and had an average particle size ranging from 300 to 1000 nm. Furthermore, Cryo-TEM images revealed discrete, intact vesicles with no signs of aggregation, findings

that were further corroborated by Cryo-SEM analysis. The drug loading capacity of the LPs, evaluated using CDs-NHF and/or free DOX, demonstrated high encapsulation efficiencies of up to 98.2% for CDs-NHF and 97.7% for DOX. The LPs formulations also exhibited good polydispersity and a negative zeta potential, indicating a high colloidal stability, which contributes to their enhanced drug entrapment capacity.

Finally, the antitumoral potential of CDs-NHF, DOX, and the co-loaded formulation (CDs-NHF-DOX) encapsulated within LPs was investigated, highlighting the promising application of these nano-formulations as drug delivery systems in biomedical contexts.

The in vitro study demonstrates that liposomes encapsulating CDs-NHF significantly reduce cancer cell viability in breast and lung cancer models compared to unencapsulated CDs-NHF. DOX-loaded liposomes were also more effective than free DOX. The combination of LPs-CDs-NHF with soluble DOX showed the highest cytotoxicity, likely due to synergistic effects. Mechanistically, CDs-NHF reduced the expression of pAkt (1,2,3), pmTOR, and pERK1/2, particularly when co-administered with DOX. HSP90 expression followed a similar trend. These findings support the use of CDs-NHF-loaded LPs, especially in combination therapies, as a promising nano-delivery platform for anticancer applications. Compared to conventional DOX-liposomal systems, the CDs-NHF/DOX co-loaded formulation presented here offers the added value of integrating a bioactive nanomaterial with proven antitumor potential, enabling a dual-action strategy that may enhance therapeutic efficacy while reducing DOX-associated toxicity.

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Article

Chitosan Grafted Poly (Ethylene Glycol) Methyl Ether Acrylate Particulate Hydrogels for Drug Delivery Applications

Corina-Lenuța Logigan ¹, Christelle Delaite ², Crina-Elena Tiron ³, Cristian Peptu ⁴, Marcel Popa ^{1,5,6} and Cătălina Anișoara Peptu ^{1,*}

- ¹ Department of Natural and Synthetic Polymers, Faculty of Chemical Engineering and Environmental Protection “Cristofor Simionescu”, “Gheorghe Asachi” Technical University of Iași, 700483 Iași, Romania
 - ² Laboratory of Photochemistry and Macromolecular Engineering, Institute J.B. Donnet, University of Haute Alsace, Mulhouse, Street des Frères Lumière, F-68093 Mulhouse, France
 - ³ Regional Institute of Oncology, General Henri Mathias Berthelot Street, 2–4, 700483 Iași, Romania
 - ⁴ “Petru Poni” Institute of Macromolecular Chemistry, Aleea Grigore Ghica Voda, 41A, 700487 Iași, Romania
 - ⁵ Faculty of Medical Dentistry, “Apollonia” University of Iasi, Pacurari Street, 11, Iasi 6600, Romania Muzicii Street, No. 2, 700511 Iași, Romania
 - ⁶ Academy of Romanian Scientists, Splaiul Independentei Street, No 54, 050094 Bucharest, Romania
- * Correspondence: catipeptu@ch.tuiasi.ro

Abstract: Chitosan (CS) crosslinking has been thoroughly investigated, but the chemical reactions leading to submicronic hydrogel formulations pose problems due to various physical/chemical interactions that limit chitosan processability. The current study employs the chemical modification of chitosan by Michael addition of poly (ethylene glycol) methyl ether acrylate (PEGA) to the amine groups to further prepare chitosan particulate hydrogels (CPH). Thus, modified CS is subjected to a double crosslinking, ionic and covalent, in water/oil emulsion. The studied process parameters are polymer concentration, stirring speed, and quantity of ionic crosslinker. The CPH were structurally and morphologically characterized through infrared spectroscopy, scanning electron microscopy, light scattering granulometry, and zeta potential, showing that modified CS allows better control of dimensional properties and morphology as compared with neat CS. Swelling properties were studied in acidic and neutral pH conditions, showing that pH-dependent behavior was maintained after grafting and double crosslinking. The applicability of the prepared materials was further tested for drug loading and in vitro delivery of levofloxacin (LEV), showing excellent capacity. CPH were found to be cyto- and hemocompatible demonstrating their potential for effective use as a controlled release system for different biomedical applications.

Keywords: chitosan particulate hydrogels; poly (ethylene glycol) methyl ether acrylate; double crosslinking; levofloxacin; biomedical applications



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1. Introduction

The nanotechnology field is dedicated to designing and producing materials with nanometric dimensions by using different devices, structures, and systems that are inspired constantly by nature. The rapid progress of nanotechnology involved the development of new materials with different properties, such as biodegradability, biocompatibility, versatility, stimuli-sensitive properties, and the ability to deliver therapeutic biomolecules (drugs, proteins, genes, and photosensitizer) to a target in a controlled manner. Nanotechnology is already used in biomedicine applications through the use of liposomes, nanoparticles, and nanotubes, which are currently interesting and promising alternatives. Moreover, the chemical functionalization of natural/synthetic polymer nanomaterials offers a variety of opportunities such as tolerability of the polymer properties such as solubility, bioadhesivity, antibacterial properties, and antifungal activity [1].

Advanced nanomedicine systems require the combination of targeted delivery, imaging, and therapeutics in a single nanocarrier system. Hydrogel nanoparticles are promising nanomaterials due to advantages such as targeting, decreasing drug dose, the possibility of maintaining a desired therapeutic concentration, and low toxicity effects [2]. Moreover, a hydrogel is a polymer three-dimensional network, capable of capturing large amounts of water or active principles due to its hydrophilic structure, presenting excellent properties such as a porous structure, sensitivity to different environmental factors (pH, temperature, light), biocompatibility, and the similarity of their physical properties to natural tissue, which led to its use as support material in tissue engineering [3–8].

Chitosan (CS) is a cationic polysaccharide obtained via deacetylation and partial depolymerization of chitin under alkaline conditions or enzymatic hydrolysis, and is the second most common natural polysaccharide after cellulose”.

Although chitin is insoluble in most organic solvents, CS is soluble in dilute acidic solutions with $\text{pH} < 6$ due to the presence of primary amino groups (with $\text{pK}_a = 6.3$) which become protonated. An increase in pH above 6, leads to the deprotonation of amino groups and CS loses its soluble character [9]. Chitosan represents a natural polymer with outstanding properties such as bioadhesivity, biocompatibility, biodegradability, non-toxicity, bioavailability, etc., which justify its attractiveness, especially for bio-related applications [2,9]. Moreover, CS shows important biological activity, including antiviral, antifungal, and antibacterial properties; antihyperglycemic, hypocholesterolemic, and antitumoral effects; genetic material transfection; and wound healing promotion [2].

The highly reactive amine moieties may be employed in a wide range of physical/chemical reactions for CS modification to gain a specific effect. One example is ionotropic gelation through ionic interactions between chitosan's amine groups and negatively charged crosslinking agents, which results in a simple and flexible materials preparation procedure [10].

The diversity and efficiency of chitosan-based micro/nano-particulate hydrogel systems for drug targeting and controlled drug release applications have been extensively recorded in the literature [9,11–13]. Thus, physical crosslinking using small molecular weight anionic crosslinkers, such as sodium tripolyphosphate (TPP), has been employed in many studies [14]. However, the crosslinking process is reversible, and a pH increase leads to fast destructuring of the hydrogels through dissociation of the TPP/CS complexes into free chitosan chains [15]. Such phenomena are creating problems for applications that involve high pH variations, e.g., the passage through the gastrointestinal tract. A solution to alleviate the problems associated with the reversibility of ionic crosslinking in chitosan-based systems employs a supplementary covalent crosslinking step [13,15–17]. Chitosan's particular chemistry favors crosslinking processes using aldehydes which form imine bonds with the amine functions [18]. Chitosan double ionic and covalent crosslinking has rarely been performed [18,19].

CS nanoparticles prepared via emulsification and crosslinking using the amino group of CS and the aldehyde group of glutaraldehyde (GA) were first obtained and characterized by Ohya et al. The nanoparticles were used as a system for circulatory delivery of 5-fluorouracil [20]. Furthermore, as an anionic crosslinker for CS, TPP is widely used and has been extensively investigated for the preparation of micro/nanoparticles, hydrogels, and scaffold systems in biomedical applications. For increasing micro/nanoparticle systems stability, both types of crosslinkers (e.g., TPP and GA) are needed [21]. Few studies are mentioned in the literature concerning the preparation of nanoparticle system-based CS or modified CS via the double ionic/covalent crosslinking method [16,17,22–25]. Adrenalin-loaded gelatin/CS particles for application in ocular drug administration were prepared using a two-step cross-linking process performed in an emulsion-phase separation system by Peptu et. al., (2010) [17]. Another interesting study concerning the obtaining of nanoparticles-based CS and gelatin via double crosslinking for drug targeting was realized by Jatariu (Cadinoiu) et. al, (2012) [22]. For the first time, polymer magnetic nanoparticles based on the chitosan-maltose derivative and magnetite double crosslinking technique

(ionic and covalent) in reverse emulsion for antitumor drug delivery were prepared by Alupe et al., (2016) [16].

Damour I. and Muti H., (2021) succeeded in obtaining stable CPH based on lecithin/CS conjugates via a dual ionic/covalent crosslinking method. They found that the dual ionic/covalent crosslinking improved nanoparticles' drug loading/releasing properties [26].

On the other hand, the chemical modification of CS is a promising method for achieving desired polymer properties. CS solubility in acidic solutions, its polycationic character, and the presence of NH_2 groups in its structure, allow a variety of chemical modification strategies leading to new CS derivatives with new physical or biological properties [27].

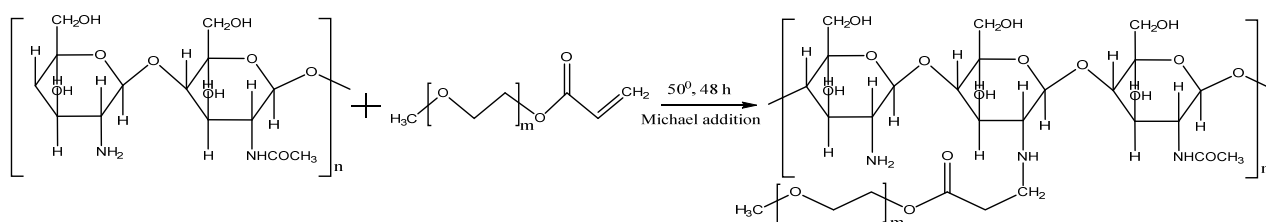
The aim of this work is the chemical modification of chitosan by Michael addition of poly (ethylene glycol) methyl ether acrylate (PEGA) to the amine groups to further prepare submicronic particulate hydrogels. In this investigation, the synthesis of new submicronic particulate hydrogels based on CS grafted with poly (ethylene glycol) methyl ether acrylate (CS-PEGA) through a two-step cross-linking technique (ionic followed by covalent), in a reverse emulsion, is described for the first time. CPH characterization was carried out in terms of structural, size, morphological, swelling, and drug loading/release properties which were controlled by varying the synthesis parameters (polymer concentration, dispersion speed, and amount of ionic cross-linking agent). In vitro tests were performed to demonstrate CPH capacity for drug loading and release, hemocompatibility, and cytotoxicity. The two-step cross-linking technique was revealed to be a valid method for the synthesis of nontoxic CPH-based materials.

2. Results and Discussion

The work presented herein is focused on the effect of CS modification with PEG-acrylate on polymer processability during the preparation of submicronic nanoparticulate hydrogel drug formulations. The use of PEGA to chemically modify CS may lead to the improvement of its solubility in water and diminish its pH-dependent water swelling character by adding hydrophilic segments to the CS linear chain. In principle, native chitosan may be used for emulsion double crosslinking using ionic gelation in the first step followed by network fixation via covalent crosslinking at the emulsion interface level in the second step. Amine groups of CS are involved in both crosslinking steps and partial grafting with PEG-acrylate may be the source of uncertainties concerning the double crosslinking process. Moreover, the pegylated chitosan will further affect pH-dependent water swelling and drug release properties.

2.1. Synthesis and Characterization of CS-PEGA

The proposed pathway to synthesize CS-PEGA is a Michael addition reaction, which involves the addition of the acrylate PEGA end chains to the amino groups of CS, at 50°C , as presented in Scheme 1.



Scheme 1. The proposed route for chitosan grafted poly (ethylene glycol) methyl ether acrylate synthesis.

CS-PEGA structural characterization was performed via FTIR and ^1H NMR aiming to have qualitative and quantitative proofs of CS grafting. Figure 1 illustrates the overlapped FTIR spectra of the starting precursors (CS and PEGA) and the synthesized polymer (CS-PEGA obtained using a molar ratio of NH_2 :PEGA = 1:0.5). The assigned characteristic bands from the obtained spectra are presented in Table S1.

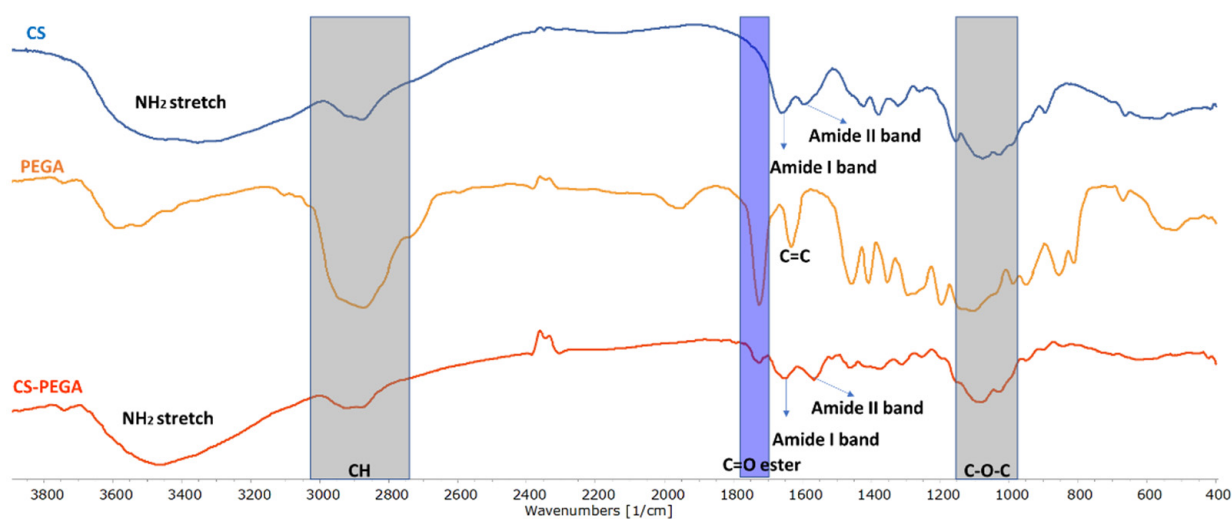


Figure 1. CS, PEGA, and CS-PEGA-FTIR spectra.

FTIR CS spectra (Figure 1) showed absorption bands at 1076 cm^{-1} (C-O-C stretching vibration) [28], 1659 cm^{-1} , 1423 cm^{-1} , and 1378 cm^{-1} (NHAc units, amide I, NH_2 bending, and amide III, respectively) [29]; and a strong peak at 3355 cm^{-1} attributed to the axial stretching vibration of (OH) overlapped with the (NH_2) stretching band of CS molecules. The PEGA spectra show characteristic signals at 1631 cm^{-1} (C=C stretching vibration), 1724 cm^{-1} (C=O asymmetrical and symmetrical stretching), and intense peaks at 2874 cm^{-1} (CH) and 3522 cm^{-1} (OH stretching vibration). The FTIR spectrum of CS-PEGA proves PEGA grafting onto CS through the appearance of the specific signal of (C=O) at 1733 cm^{-1} , as highlighted.

^1H NMR spectroscopy was employed to obtain more information regarding the chemical composition of the synthesized polymer. Figure 2 displays the ^1H -NMR spectra of CS, PEGA, and CS-PEGA (molar ratio $\text{NH}_2\text{:PEGA} = 1\text{:}0.5$ in PEGA synthesis feed).

The ^1H NMR spectrum recorded for CS (Figure 2a) reveals the characteristic signal of methyl protons from the chitin residue, the resonance peaks of the acetylated group found at 1.90 ppm, and the peak corresponding to the H_2 methine protons of the deacetylated groups at 3.01 ppm. H_3 , H_4 , H_5 , and H_6 protons of the deacetylated and acetylated groups were observed in the 3.5–3.8 ppm range. Based on the spectrum, the hydrolysis degree of the CS was determined to be $D = 88\%$.

The PEGA ^1H NMR spectrum (Figure 2b) was recorded in D_2O and the characteristic observed peaks were as follows: 6.44 ppm ($\text{CH}_2 = \text{CHCOO}$), 6.24 ppm ($\text{CH}_2 = \text{CHCOO}$), 6.005 ppm ($\text{CH}_2 = \text{CHCOO}$), 4.35 ppm ($-\text{COOCH}_2-$), 3.6–3.83 ppm ($-\text{COOCH}_2\text{CH}_2-$, $-\text{OCH}_2\text{CH}_2-$, $-\text{CH}_2\text{CH}_2\text{OCH}_3$), and 3.38 ppm ($-\text{OCH}_3$).

In the case of CS-PEGA, compared to CS, the peaks corresponding to the ($-\text{COOCH}_2\text{CH}_2-$) and ($-\text{NH-CH}_2\text{CH}_2\text{COO-}$) appear at 4.33 ppm and 2.60 ppm, respectively. A clear signal at 3.37 ppm corresponds to the ($-\text{OCH}_3$) group of the PEGA structural unit. The signals of PEGA and in-chain methylene groups overlap with the signals of H_3 , H_4 , H_5 , and H_6 protons of CS. The CS-PEGA spectrum (Figure 2c) was similar to that obtained by Han et al., also confirming the successful synthesis of CS-PEGA [30].

The obtained spectrum was further used to calculate the CS substitution yield. Previously, the calculation of DS used the peak intensity of the PEG graft ($-\text{COOCH}_2-$, 4.33 ppm) and ($-\text{CH}_3$, 2.00 ppm) specific to chitin residues. Thus, DS calculated using Equation (1) was around 16%.

$$\text{DS} = \frac{3 \cdot I_{4.3\text{ ppm}} \cdot (1 - \text{DD})}{2 \cdot I_{2.0\text{ ppm}}} \quad (1)$$

However, because of the influence of neighboring peaks, such calculations may be highly biased, and a different protocol was used for DS calculation taking into consideration only the CS backbone peaks:

$$DS = \frac{I_{2'}}{I_2 + I_{2'}} 100 \quad (2)$$

where I_2 is the integration of the peak found at 3.17 ppm, corresponding to the unmodified chitosan rings, and $I_{2'}$ represents the integration of the modified chitosan, probably situated at 2.6 ppm. However, the NMR spectrum previously reported was slightly different [30] and in our case, the 2' peak is overlapping with the peaks corresponding to the PEG methylene protons, a and b. Therefore, the 2' integration value was indirectly obtained using the calculated CS hydrolysis degree, through Equation (3):

$$I_{2'} = \frac{I_a \times D}{3 \times (100 - D)} - I_2 \quad (3)$$

where D represents the calculated hydrolysis degree.

Finally, the obtained functionalization degree using Equation (2) was $DS = 20\%$, much lower than that previously obtained by Han et al. In principle, higher substitution degrees may be reached (e.g., a 1:1 NH_2 :PEG molar ratio led to a DS of 51%) but our goal was to leave a certain amount of amine groups available while achieving complete water solubilization of CS-PEGA.

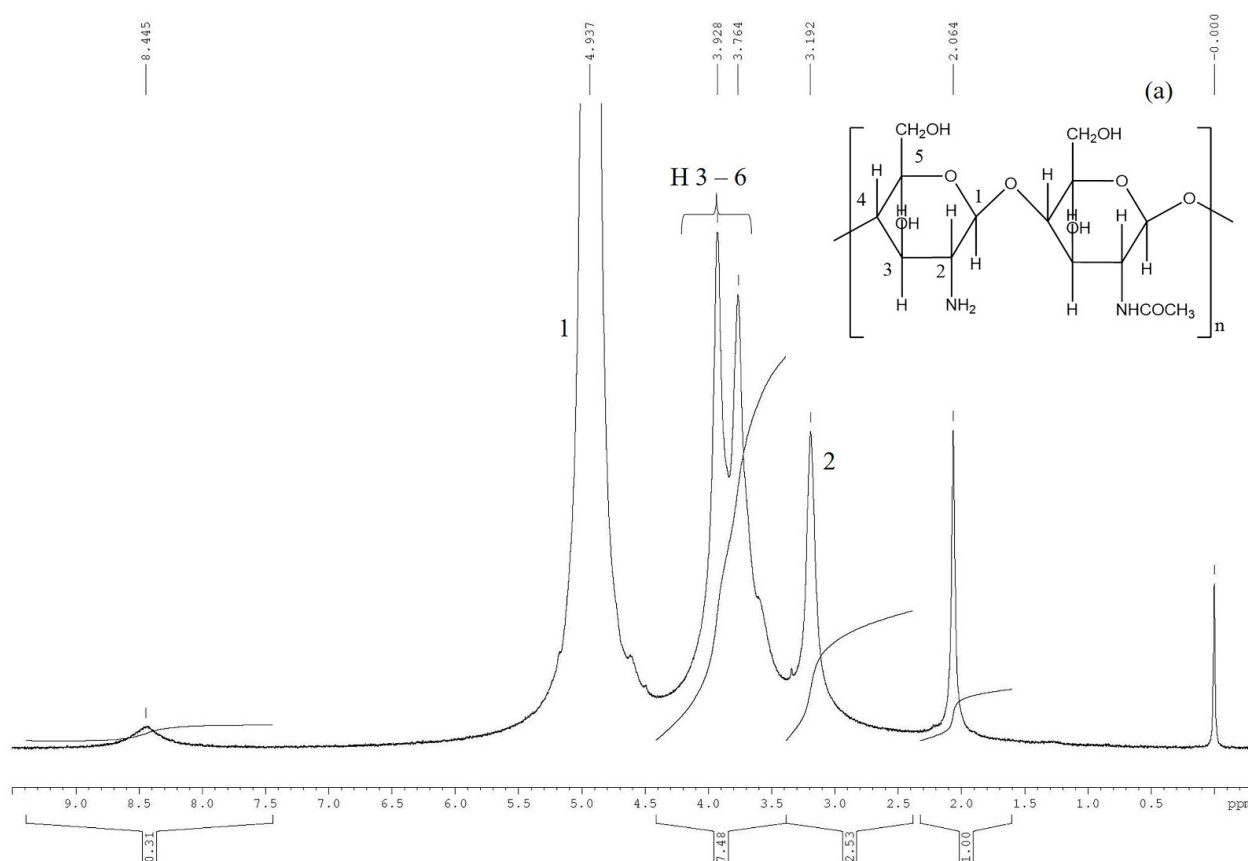


Figure 2. Cont.



Figure 2. ^1H RMN spectra of (a) CS; (b) PEGA; (c) CS-PEGA.

2.2. Preparation and Characterization of CPH

The proposed pathway to obtain CPH is a double crosslinking technique in reverse emulsion (*w/o*) [10,11,16–19]. The main problem related to the crosslinking procedure consists of the CS chemical modification impact on amine groups' availability for further reactions. Furthermore, the improved water solubility of CS-PEGA should provide better preparation conditions for the particulate hydrogel's formulation. The prepared systems were evaluated according to the following parameters: size and polydispersity, morphology, and zeta potential FTIR.

The selected method for particle preparation was the double crosslinking (ionic and covalent) technique in a water-in-oil emulsion, the advantage being the use of a lower amount of covalent crosslinker, which leads to a decrease in final product toxicity. The fundamental principle of the process is the formation of an interconnected/interpenetrated network in the first stage through the majority ionic crosslinking process, followed by the covalent crosslinking step. The experimental program concerning the preparation of CPH is presented in Table 1.

Table 1. CPH—preparation parameters and size measurements.

Sample Code	Polymer Solution Concentration, %	Molar Ratio $\text{NH}_2/\text{Na}_5\text{P}_3\text{O}_{10}$	Speed, Rpm	Water Phase, mL	The Organic Phase, mL	Surfactants Concentration, %	Ionic Crosslinking Time, min	Yields %	Average Diameter (LD), μm	Potential Zeta (mV)
A1	0.5	1:05	5000	50	200	2	60	32	Aggregates	-
A2	0.5	1:1	5000	50	200	2	60	41	Aggregates	-
A3	0.5	1:2	5000	50	200	2	20	36	Aggregates	-
A4	0.5	1:2	5000	50	200	2	40	44	Aggregates	-
A5	0.5	1:2	5000	50	200	2	60	79	3.548	15.4 ± 0.2
A6	0.5	1:2	9000	50	200	2	60	80	3.263	15.2 ± 0.1
A7	0.5	1:2	12,000	50	200	2	60	76	0.708	14.9 ± 0.2
A8	0.5	1:2	15,000	50	200	2	60	78	0.562	14.8 ± 0.2
A9	0.35	1:2	15,000	50	200	2	60	56	0.089	11.9 ± 0.2
A10	0.5	1:3	15,000	50	200	2	60	59	0.141	15.5 ± 0.1
A11	0.75	1:2	15,000	50	200	2	60	51	0.730	15.8 ± 0.1

The parameters taken into consideration for the preparation process are CS-PEGA initial concentration, NH_2 /TPP molar ratio, and stirring speed in the emulsion formation phase. The initial water/oil phase ratio, surfactants concentration, and GA content in the organic phase were kept constant throughout the study.

Samples from A1 to A4 were obtained in the first experiments of the study and the molar ratio $\text{NH}_2/\text{Na}_5\text{P}_3\text{O}_{10}$ was varied at 1:0.5 (A1) and 1:1 (A2), the time of the ionic crosslinking process was varied at 20 min (A3) and 40 min (A4), while the water/oil phase ratio, surfactants concentration, and GA content in the organic phase were kept constant. SEM microphotographs showed a nonuniform spherical shape, which suggests that the amount of the ionic crosslinker (A1 and A2) or ionic reaction time (A3 and A4) were insufficient for their favorable formation. In addition, the formation of aggregates with average sizes of 2–3 μm and a fairly wide dimensional polydispersity, proves that they couldn't be used in further investigations. After the first stages of optimization, which involved increasing the time of the ionic cross-linking/polymer ratio and molar ratio $\text{NH}_2/\text{Na}_5\text{P}_3\text{O}_{10}$, the prepared CPH presented a spherical shape, were individualized, and the diameter and polydispersity were much reduced compared to the first attempts.

The preparation yields (Table 1) revealed that the initial CS-PEGA amount was diminished during thorough purification procedures. Final yields were around 80% (samples A5, A6) at lower speeds and decreased at higher speeds to 76% (sample A7). Although yield variation is almost negligible, it could be considered that the centrifugation-washing cycles led to fractionation through the loss of the particle populations having the smallest

size. Thus, after correlating the preparation yields with the preparation parameters, it may be observed that the highest yields are obtained at the lowest emulsification speeds because the emulsification conditions (stirring speed) favor the formation of larger size particles. The formation of larger size particles may lead to the encapsulation of CS-PEGA material with low network connectivity as compared with lower size particles. In principle, the polymers which do not benefit from crosslinking during the preparation process are eliminated during the purification step, contributing to lower yields. Thus, larger-sized particles may encapsulate free chains that are not eliminated by purification, increasing the final yields.

Particle size measurements confirm that preparations performed at lower emulsification speeds result in larger particle size. Furthermore, the initial concentration of the CS-PEGA affected the preparation yields. Thus, switching the initial concentration from 0.5% to 0.35% (wt) led to a decrease in the preparation yields from 78% to 56% together with a drastic reduction in particle average size from A8 to A9. On the other hand, increasing the polymer concentration to 0.75% (wt) also led to diminished yields associated with the loss of free polymer. Higher polymer density may affect the crosslinking processes, both ionic and covalent. Finally, the increase in TPP amount is correlated with a particle size reduction, from 562 nm (A8) to 141 nm (A10) and, consequently, with diminished yields from 78 to 59%.

CPH samples were also analyzed by zeta potential measurements and Table 1 summarizes the arithmetic average of zeta potentials. The analyzed samples were dispersed in distilled water at a mass concentration of 1.25 g/L and sonicated for 15 min to avoid agglomeration. In principle, a large negative or positive value of zeta potential for suspended particles in an aqueous medium indicates that the system is stable against flocculation or coagulation. As shown in Table 1, the obtained results for samples A8, A9, and A10 indicate that CPH have good stability in aqueous suspension. The measured zeta potential values are explained by the lower availability of the amine groups due to PEG grafting and covalent crosslinking.

2.2.1. CPH Characterization

To better understand the polymer network formation after the double crosslinking process, CPH were first investigated by FTIR spectroscopy. The recorded spectra revealed a similar profile for all CPH and confirmed the success of both crosslinking processes, thus only the FTIR spectra for sample A8 are displayed (Figure 3). Table S2 presents in detail the characteristic absorption bands. The first crosslinking process confirmed was the ionic one, the spectra revealing at 846 cm^{-1} the presence of a new bond formed between $\text{P}_3\text{O}_{10}^{5-}$ anions of TPP and NH_3^+ cations from CS-PEGA. The characteristic absorption bands corresponding to the covalent crosslinking process, namely C=N, were observed at 1573 cm^{-1} [13,16,31–34].

Furthermore, the CPH were also subjected to TGA analysis (Figure 4). The data results from the TGA analysis indicated that CPH decomposition proceeds in three steps as shown in Figure 4. TGA thermograms (Figure 4) showed a steady weight loss in the temperature range of 100–500 °C. The slight weight losses (9.514% and 10.13%) up to 116 °C, and 119 °C respectively, correspond to adsorbed water and moisture loss by the CS and CPH. The second decomposition step (A8—54.65%, CS—52.73%) in the temperature range of 116/119–500 °C is mainly due to the degradation of oligosaccharides and PEG. The main difference between CS and A8 samples is related to the early onset of A8 degradation due to the chemical modification in correlation with the native CS. Thus, the presence of covalent GA bridges is probably the reason for the decrease in thermal stability. Moreover, the A8 sample presents a distinct extra shoulder in DTG corresponding to the additional PEG, between 350 and 400 °C. Overall, thermal analysis confirms the structural composition of A8 concerning CS-PEGA content and covalent crosslinking. Supplementary TGA analysis performed on A9 and A10 samples (Figure S1, Supplementary Material) showed that differ-

ent preparation conditions, such as concentration and TPP amount, do not significantly influence the TGA profile.

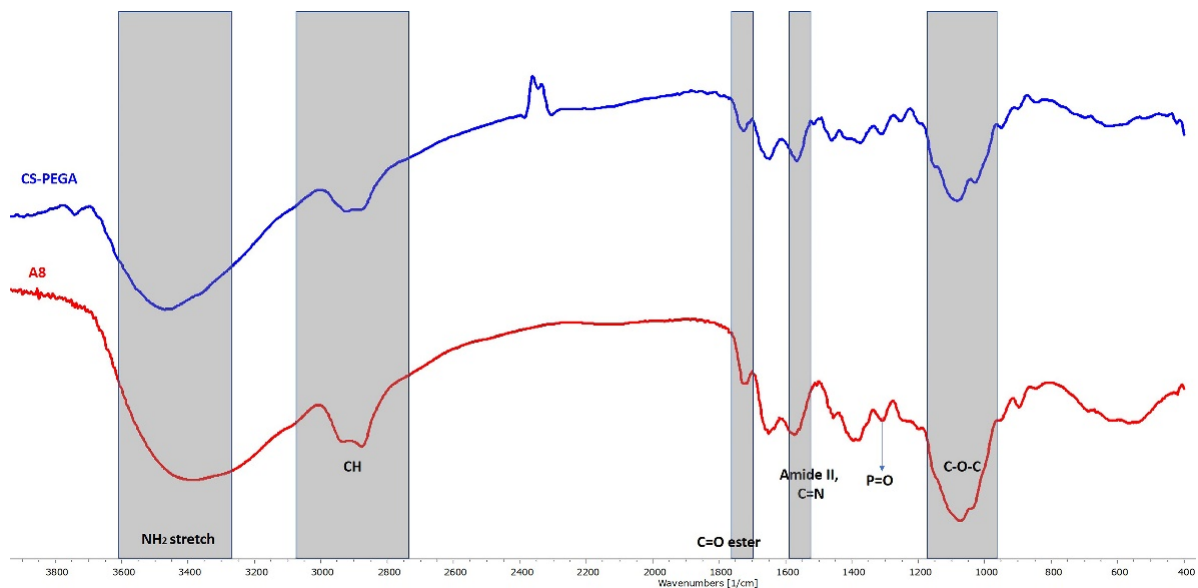


Figure 3. FTIR spectra of the modified chitosan (CS-PGA) and chitosan particulate hydrogels (A8 sample).

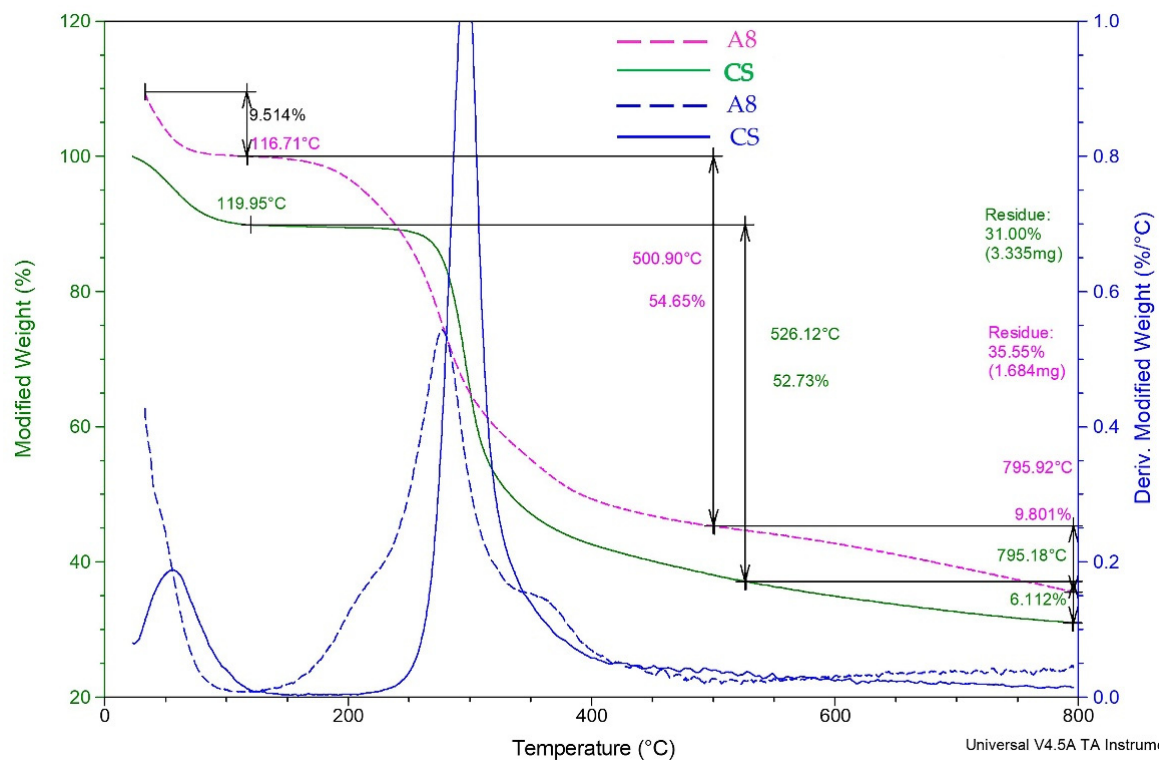


Figure 4. TGA analysis of chitosan (CS) and chitosan particulate hydrogels (A8 sample).

2.2.2. CPH Morphology

SEM technique was used to inspect the morphology of the prepared submicronic gel systems, Figure 5. Results indicate that they have a spherical shape, are well individualized, generally have a submicronic diameter, and reduced dimensional polydispersity. The adapted emulsion double crosslinking procedure allows the obtaining of clear particulate

systems with relatively low dimensional dispersity, as observed from SEM micrographs. Furthermore, it may be remarked that particles are homogeneous, separated, and lack impurities resulting from surfactants, crosslinkers, or CS-PEGA residues from faulty emulsion crosslinking.

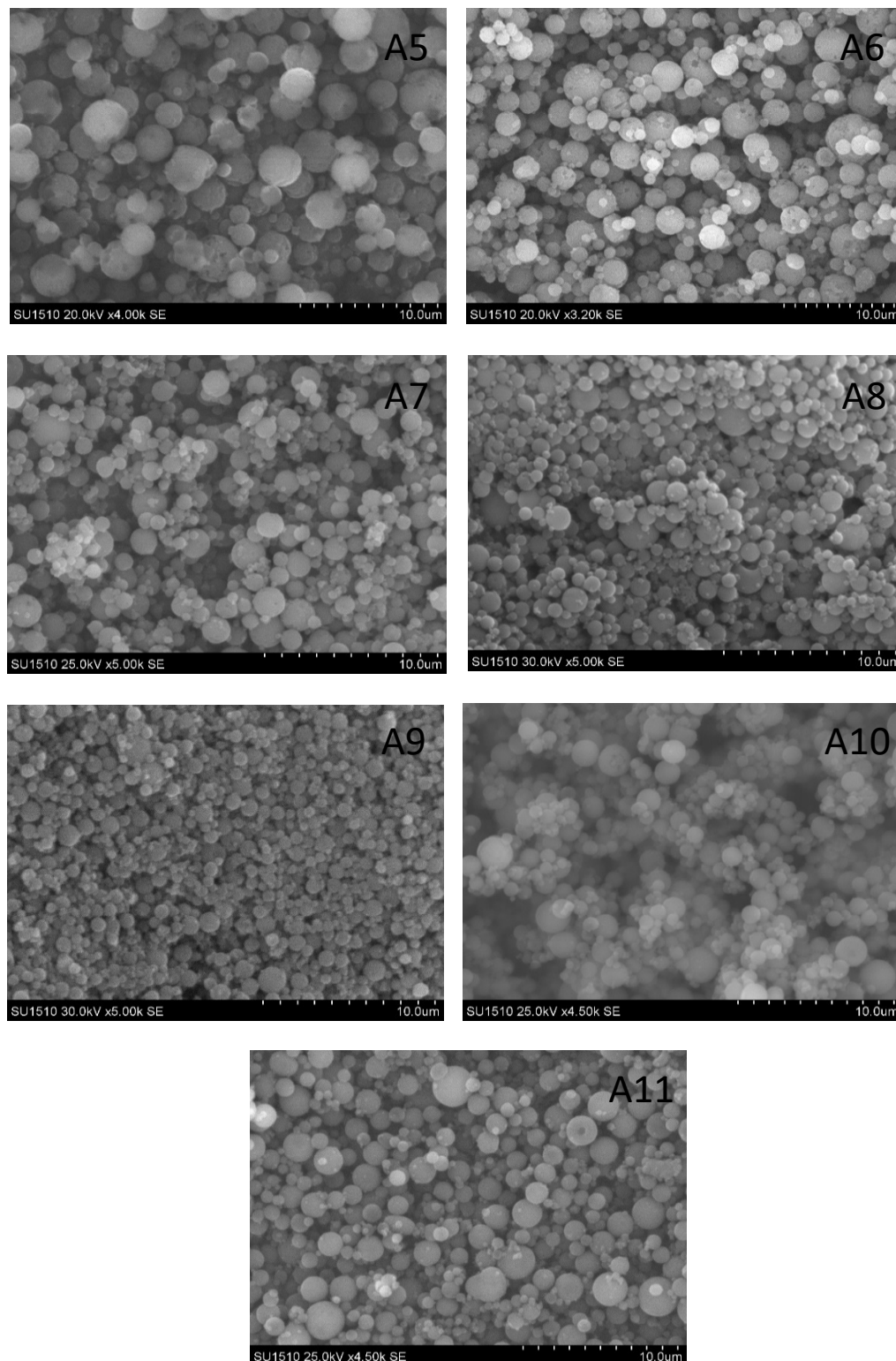


Figure 5. Micrographs SEM of CPH (magnification graphical bar length A5–A11:10 μm).

The influence of the preparation parameters is well reflected in the size of CPH, especially the lowering of the concentration of CS-PEGA, which produced particulate gels with the lowest dimensions. A visual comparison between the A8 and the A9 samples (Figure 5) clearly shows the dimensional decrease.

Furthermore, a more quantitative idea of the dimensional distribution of particle populations is obtained by dimensional analysis via laser diffraction (Figure 6), which provides the diameter and dimensional polydispersity of CPH. The analysis was carried out in acetone, known as a poor solvent for CS, and the measured average diameters were in good agreement with SEM observations. The experimental data confirmed that the particle size is dependent on the polymer solution concentration and stirring speed used during preparation. Thus, a higher stirring speed implies higher energy dissipated in the stirred emulsion, i.e., a higher energy transfer, which causes the emulsion to break into smaller droplets, resulting in nanometric particles. The granulometric distribution curves of the CPH obtained (determined by laser beam diffraction (LD)) have a unimodal allure and a diameter falling in the submicronic range, depending on the preparation conditions (Figure 6 and Table 1). Thus, for samples A5–A8, after increasing the stirring rate from 5000 to 15,000 rpm/min, it was noticed that particle size decreases due to better dispersion and formation of small droplets in the emulsion phase.

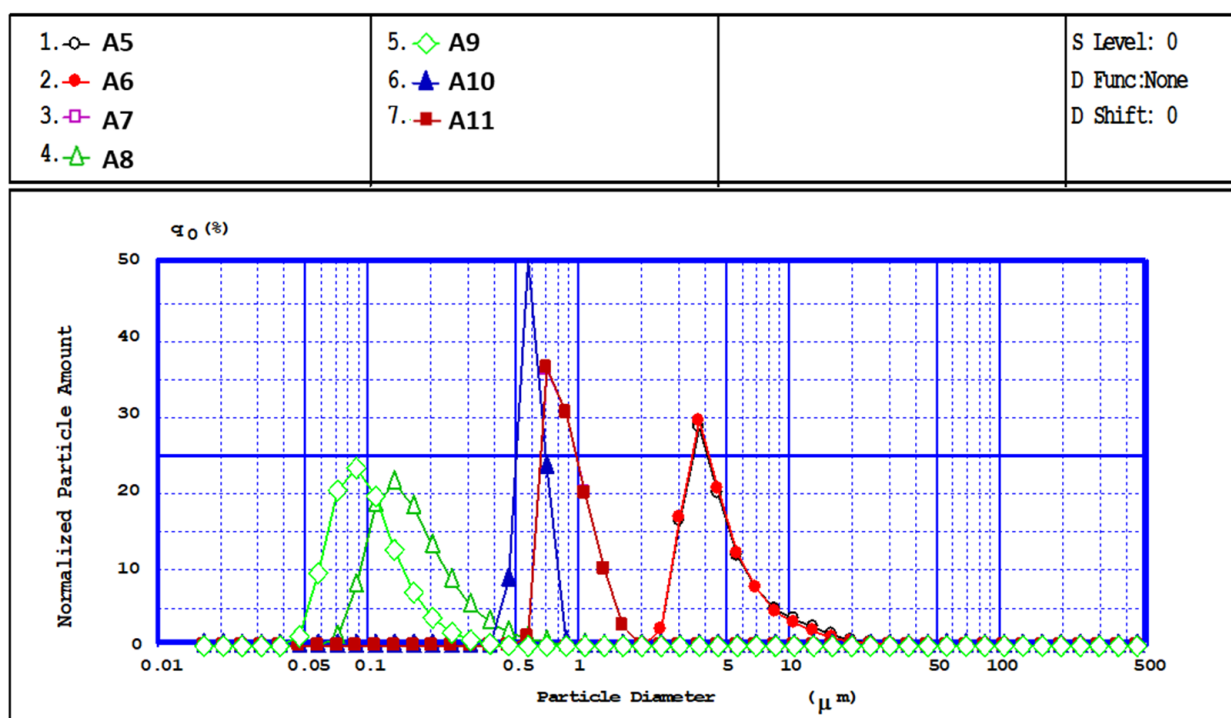


Figure 6. Granulometric distribution curves of CPH.

The influence of the concentration of CS-PEGA solution on particle size was also analyzed for three different polymer concentrations, namely 0.35%, 0.5%, and 0.75%, while the other parameters were kept constant (samples A 8, A9, A11, Table 1). The lowest polymer concentration led to particles with sizes in the nanometric range and an average diameter of 89 nm, and higher concentrations resulted in larger particles. Additionally, increasing the polymer solution concentration led to a considerable increase in the mean diameter and particle polydispersity, as well as a decrease in agglomeration tendency. The observed tendency is explained by the fact that higher concentrations lead to an increase in solution viscosity, and therefore to the formation of larger emulsion droplets, as presented by other studies [35,36].

Another studied parameter, the increased polymer/ionic crosslinker ratio (A8 vs. A10), caused a noticeable decrease in particle diameter, from 562 to 141 nm. Better availability of TPP may favor ionic gelation in smaller colloids in the early emulsification process. Furthermore, in the ionic crosslinking phase, due to the increase in the cohesion of the polymer matrix, the emulsion droplets should better maintain their colloidal stability. Covalent crosslinking improves and fixates the resulted particles which otherwise may suffer modifications in size.

Overall, considering the small size and dimensional uniformity of the prepared particulate hydrogels, the use of CS-PEGA in double crosslinking processes is an improvement on previous efforts. TPP ionotropic gelation alone leads to particle sizes of around 300 nm [14] while previous double crosslinking attempts on native or modified CS led to a minimum particle size of 500 nm. The current work shows that PEG grafting up to 20% does not affect the capacity of CS to undergo crosslinking reactions because of sterical effects and amine moieties coverage.

2.2.3. Water Uptake

The swelling capacity was studied in two environments, which were closely related in terms of CPH drug loading and release potential. The acidic/basic standard buffer medium was chosen because, in general, the swelling behavior of CS hydrogels can be influenced/dependent on external triggers such as pH, ionic strength, the temperature of the environment, and the degree of cross-linking. Furthermore, in the case of CS hydrogels which are pH-dependent, the loading/release behavior of active principles is related to a higher swelling capacity in the acidic environment due to a significant increase in their volume which leads to an accelerated drug caption inside the network [36].

To better understand the interaction between CPH and the aqueous environment, swelling studies were performed in acidic (pH = 3.3) and respectively alkaline (pH = 7.4) mediums, using the gravimetric method. The water swelling behavior gives insights into doubly crosslinked CS-PEGA interactions with selected environments and its adequacy for the drug loading and release process.

The kinetic data are presented as an example for sample A9 (Figure 7). The results revealed two phases in the acidic environment: a fast increase up to 800% (sample A9) in the first 90 min, followed by a slower water uptake increase rate (90–480 min); the maximum value is reached after 24 h at 1144%. This behavior may reflect the double crosslinked nature of CPH. In the case of PBS, the maximum value of the swelling degree was smaller at 669%. Swelling kinetics presented in Figure 7 reveal that an equilibrium phase is established in both acidic and alkaline environments, showing good stability of the particulate hydrogel's formulations. Although in the alkaline environment such behavior would be expected because of diminished electrostatic repulsions among polysaccharide chains, the stability in the acidic environment confirms successful covalent crosslinking.

The water uptake in both environments proceeded quickly, demonstrating good water-CS interactions, most likely favored by hydrophilic PEG grafts. However, the remaining free amine groups dictate the swelling behavior as acidic ABS determined significant water swelling. Therefore, we can infer that CS-PEGA provides a good prospect for nanoparticulate hydrogels by diminishing the large difference in CS behaviors in acidic and alkaline environments.

Furthermore, the water interactions of CS-PEGA nanoparticulate hydrogels are discussed according to the maximal water uptake reached in the equilibrium swelling phase (Table 2). Generally, water swelling of homogeneously crosslinked polymer networks is influenced by polymer–water interactions and the network's capacity to expand its volume, influenced in turn by chain conformations, flexibility, and network crosslinking density. However, in the case of double crosslinked particulate hydrogels, the crosslinking is heterogeneous. GA covalent bridges are formed starting from the organic phase–water interface. In principle, because of its high reactivity with amine groups, GA diffusion into the water phase is limited and it can be considered that a layer on the surface of particles benefits

from the covalent crosslinking while the inner polymer matrix remains mostly ionically crosslinked. The lack of crosslinking anisotropy further generates specific particle–water interactions. The outer layer of covalently crosslinked polymer is the key factor that determines the swelling behavior. By comparing the water uptake of samples A8 and A10 (Table 2), with an increasing amount of ionic crosslinker, it was noticed that there were insignificant differences between the acidic and alkaline environments. Such behavior is in opposition to the increasing network crosslinking density and also to the possible network dissociation due to the neutralization of the ionic bridges in an alkaline environment.

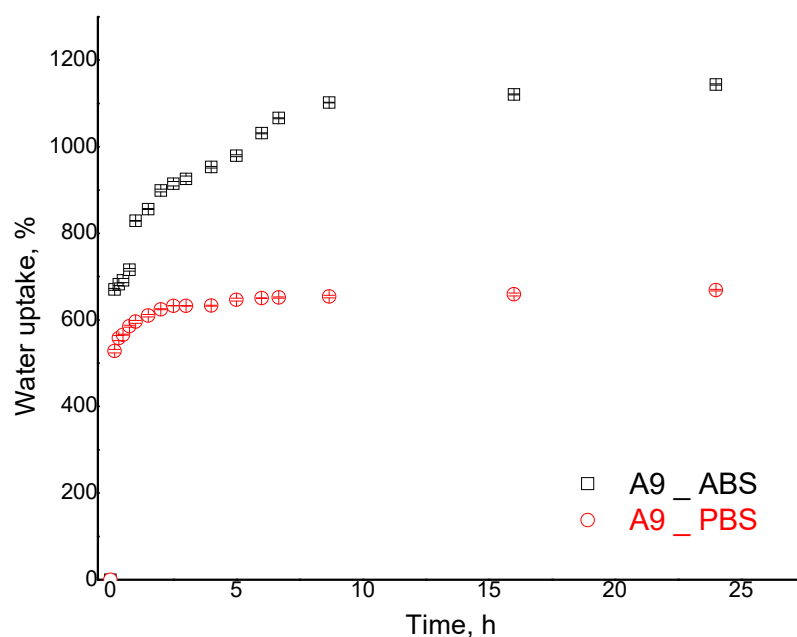


Figure 7. Chitosan particulate hydrogels (A9 sample) pH-dependent water uptake in pH = 3.3 (ABS) and pH = 7.4 (PBS).

Table 2. Maximum water uptake of A5–A11 samples.

Sample Code	A5	A6	A7	A8	A9	A10	A11
$Q_{\text{ABS}}, \%$	835	838	886	985	1144	995	904
$Q_{\text{PBS}}, \%$	511	541	580	584	669	559	598

In principle, an increase in the surface of the particles is associated with a decrease in the particles' average diameter. Taking this into consideration it can be inferred that particle size is influencing the swelling behavior. The evolution of water uptake according to particle size is presented in Figure 8.

The decrease in particle size of the considered samples is only due to a more energetic initial emulsion stirring and the other parameters influencing crosslinking, such as ionic/covalent crosslinkers and polymer concentration, were kept constant. The acidic swelling for particles with an average diameter of 3540 nm is around 835% (sample A5), and a decrease in particle size to 562 nm leads to higher swelling, up to 985% (sample A8). Furthermore, in the alkaline environment, the swelling increases from 511 to 585%. Thus, water uptake shows a clear dependency on the average diameter of particles in both acidic and alkaline environments, but the effect is more pronounced in ABS swelling.

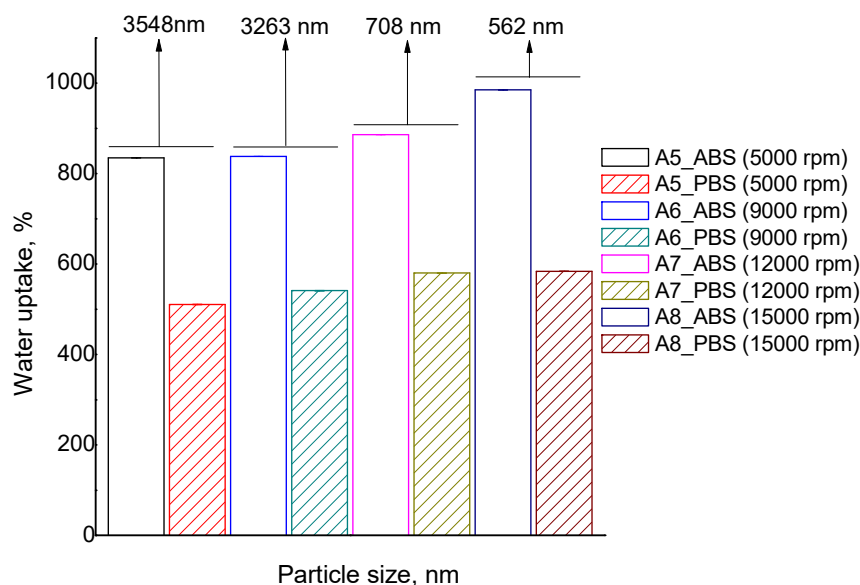


Figure 8. The influence of CPH size on the swelling process for CPH: A5 (5000 rpm); A6 (9000 rpm); A7 (12,000 rpm); A8 (15,000 rpm).

The water uptake experiments were performed to investigate the dependence between the swelling degree and polymer concentration (Table 2 and Figure 9). The data presented in Figure 9 show the evolution of the swelling degree for three different concentrations and it is observed that increasing the CS-PEGA concentration in the emulsion feed diminishes the particle water uptake in both ABS and PBS. However, the swelling decrease is more obvious in an acidic environment, from 1175% (sample A9) to 900% (sample A11). This behavior is to be expected due to more dense polymer networks with a concentration increase.

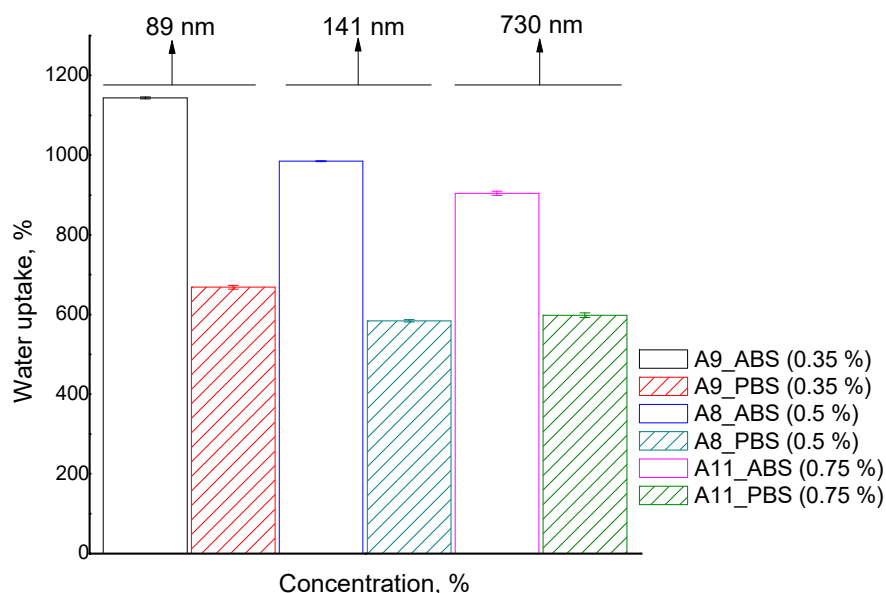


Figure 9. Polymer concentration influence on water uptake for CPH: A9 (0.35%); A8 (0.5%); A11 (0.75%).

Taking into consideration the significant differences observed in water uptake behavior in ABS and PBS, it can be affirmed that CS-PEGA particulate hydrogels have a pH-sensitive character. The effect is determined by the amine groups of the CS derivative which didn't participate in the crosslinking process (both ionic and covalent). In an acidic environment, the amine groups are quaternized by passing into ammonium cations, and the electrostatic

rejections between them increase the distance between network chains, and their meshes, leading to higher water uptake. The maximum swelling values are lower in a basic pH compared to an acidic pH, because the amine groups of the CS derivative are deprotonated, therefore, hydrogen bonds are formed in the newly formed polymer network.

The best swelling ratio was achieved by sample A9, obtained at a low polymer solution concentration (0.35%) and with the smallest particle size (89 nm). The higher water uptake for CPH obtained is probably due to the increased agglomeration tendency, which is mainly evident in sample A9, and a low cross-linking density of the polymer matrix.

2.2.4. Drug Loading and Release

The ability of CPH to encapsulate active principles was analyzed using levofloxacin (LEV) as a model drug. LEV is a synthetic antibacterial drug of a broad-spectrum antibiotic belonging to the class of fluoroquinolones, generally used to treat antibacterial infections. LEV is a hydrophilic drug and therefore a suitable candidate for hydrogel particulate systems designed for the treatment of bacterial infections [35,37]. Taking into account the swelling results, which demonstrate that in acid pH the swelling ability of CPH is much higher than in weak alkaline conditions, it was decided to load the LEV in acid conditions ensuring a higher amount of loaded LEV. The LEV content in the supernatant was determined by UV-Vis spectrophotometry (at 287 nm wavelength) based on an established calibration curve. CPH were able to encapsulate between 0.48–0.75 mg drug/mg particles (Equation (6)). Therefore, the LEV loading efficiency values calculated with Equation (7) were between 47.0–76.0%, showing a high capacity of the CPH to support the biologically active compound. The amounts of encapsulated LEV in CPH and loading efficiencies are outlined in Table 3.

Table 3. Encapsulated LEV and efficiency.

Sample Code	A5	A6	A7	A8	A9	A10	A11
mg LEV/mg CPH	0.63	0.69	0.62	0.75	0.67	0.48	0.66
Encapsulation efficiency, %	62.34	66.36	58.76	76.41	66.36	47.32	66.53

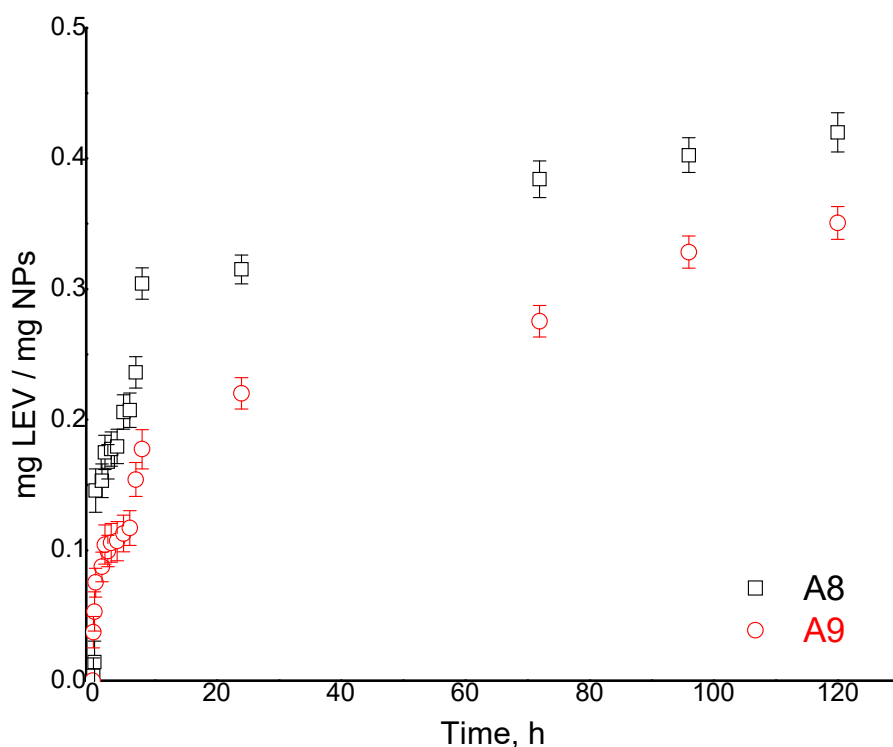
The values obtained at different time intervals showed that after 72 h the amount of encapsulated LEV ranged from 0.48–0.75 mg per mg of CPH. The kinetic data presented as an example for samples A8 and A9 (Figure S2), showed that LEV is loaded in two phases, a fast process during the first 10 h, followed by a slower drug loading until 72 h. When comparing the drug loading profile with the swelling results, a similarity could be observed.

For the LEV release study, pH = 7.4 was considered appropriate for a future systemic administration, acidic release conditions being used only for oral formulations. The LEV release capacity of CPH was analyzed by the diffusion method in phosphate buffer pH = 7.4 at 37 °C. The released LEV for all samples is presented in Table 4 and the kinetic data for two representative samples, A8 and A9, are displayed in Figure 10. One can observe a fast release phase, which is reached within the initial 10 h, followed by a slower phase (characterized by a slow release) until 120 h. The sustained release of LEV can be explained by the fact that the released drug was adsorbed in the CPH network due to the excellent hydrophilicity of the polymer network, which it seems is retained after the crosslinking procedures.

By analyzing the influence of preparation parameters on the release ability of the CPH, similar behavior as for drug loading was observed. The maximum released amount of LEV varied between 0.27 and 0.42 mg/mg CPH (Table 4). Furthermore, when calculating the efficiency of LEV release for all samples, values between 41 and 60% were obtained (Figure S3), the highest efficiency being in sample A8, which has also shown a high water uptake. The analysis of the release kinetics was achieved based on the Korsmeyer–Peppas mathematical model (Figure S4).

Table 4. Released drug and efficiency.

Sample Code	A5	A6	A7	A8	A9	A10	A11
mg LEV/mg CPH	0.31	0.30	0.31	0.42	0.35	0.27	0.27
Release efficiency, %	49	44	49	50	60	57	41

**Figure 10.** Chitosan particulate hydrogel in vitro LEV release.

According to the literature [38–41], the drug release process is dependent on the method of obtaining the particulate systems, their dimensions, and the physical–chemical properties of the polymer support used. After analyzing the experimental kinetic data for the interval 0–480 min, it was possible to determine the diffusion exponent based on the equations listed in Table S3. As an example, Figure S4 shows the results obtained for samples A8 and A9. One can observe that for sample A8, the value of the exponent n , which characterized the release mechanism, is quite close to 0.5 ($0.5 < n < 1.0$), indicating a transport/release mechanism dominated by diffusion. Furthermore, it can be stated that sample A11 exhibits an abnormal, non-Fickian diffusion, the drug transport process through the polymer matrix being governed by both diffusion and swelling.

The CPH hemolytic potential test is a mandatory requirement for blood-borne materials, since their interaction with blood components may lead to erythrocytes lysis. For this reason, the effects of the prepared CPH on blood were evaluated using a hemolysis assay. The results obtained from this study revealed that the degree of hemolysis increases with an increase in the CPH concentration (Figure 11). The degree of hemolysis for three different samples (A8, A9, A10) was analyzed and the results showed that there were no significant differences between them at the same concentration. It has been found that the prepared CPH shows very good compatibility with normal blood (<10% compared to the positive control) [41].

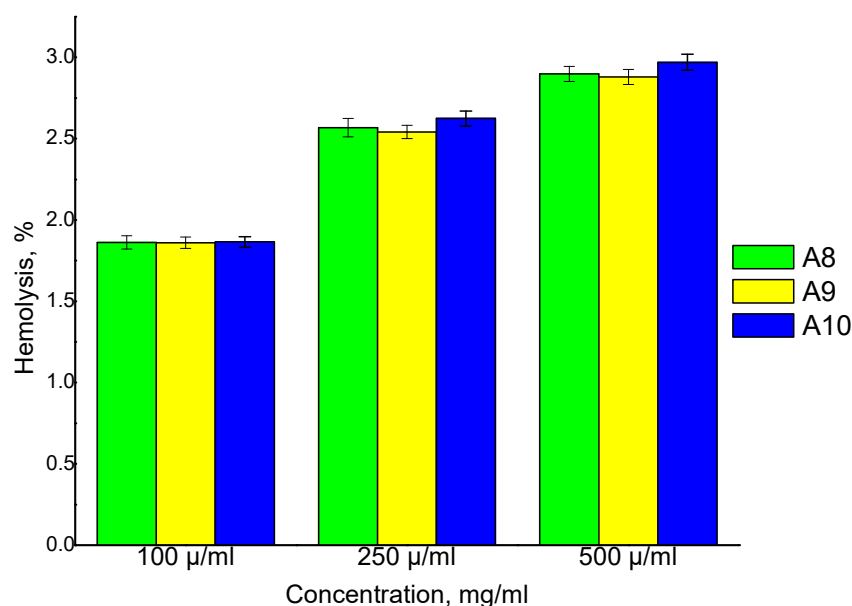


Figure 11. The influence of CPH concentration on the degree of hemolysis (samples A8, A9, A10).

The viability of HDMVEC cells (Primary Dermal Microvascular Endothelial Cells) in 2D culture treated with nanoparticulated formulations (A9) was not affected by the presence of glutaraldehyde in the CPH structure (Figure S5).

2.2.5. Cell Proliferation

For cell viability, a CellTiter-Blue® Cell Viability Assay (Promega, Madison, WI, USA) was used. Cells were seeded into a 96-well flat-bottomed tissue culture plate at a density of 3000 cells/well and allowed to adhere to the plate by incubating at 37 °C under 5% CO₂ overnight. Following cell attachment, the cells were incubated with 0.5 and 1% CPH for 72 h. After 72 h treatment periods, 50 µL of cell viability solution was added to each well and the plate was reincubated for 4 h before fluorescence recording using a multiplate microplate reader (FilterMax F5, Sunnyvale, CA, USA). As one can see from Figure S5, analyzed CPH does not affect cell viability according to statistical analysis. The statistical analysis of significance in differences was performed using a GraphPad Prism 7 Anova *t*-test.

CPH formulation-based CS-PEGA does not affect cell viability, thus confirming that they are cytocompatible and can be used as a drug delivery system.

3. Conclusions

This work demonstrates the suitability of CS-PEGA for the preparation of particulate hydrogel systems through double emulsion crosslinking. The relatively low substitution degree of chitosan with PEG-acrylate, about 20% as determined by ¹H NMR, ensures sufficient availability of the remaining amino functions for further ionotropic gelation with TPP and covalent crosslinking with glutaraldehyde in water in oil emulsions. The outcome of the particle preparation procedure may be finely tuned by playing with process parameters such as water phase concentration, emulsification stirring speed, and amount of ionic crosslinker. According to SEM analysis, the CPH have a spherical shape, are homogeneous, separated, and lack impurities. The particle average diameter, measured by laser diffractometry, depends on specific preparation conditions and ranges from 3500 to 90 nm, in agreement with SEM estimations. Particularly, emulsification speed and water phase concentration significantly influence the CPH size and allow fine-tuning, according to the envisaged applications.

CPH behavior in two different aqueous media, acid (pH = 3.3) and basic (pH = 7.4,) was studied, demonstrating a higher water retention capacity for the acidic media, compared

to basic media. Furthermore, the water uptake properties are significantly influenced by polymer concentration and ionic crosslinker amount employed in the preparation stage, and they are correlated with the particle size. The drug loading and release capacity of CPH, tested using levofloxacin, showed a good diffusional loading capacity, up to 0.75 mg of LEV per mg of CPH in an acidic environment. Drug rapid release in physiological pH was observed within the first 10 h, followed by a slower phase, characterized by a constant release up to 120 h. Drug release kinetics analysis demonstrated that the drug transport/release mechanism is predominantly diffusional.

CPH have also been tested by hemocompatibility analysis, the results showing a hemolysis percentage of less than 3%, indicating that they are hemocompatible. Cytocompatibility studies demonstrated that CPH formulations based on CS-PEGA do not affect cell viability, thus confirming their low cytotoxicity and recommending them as pH-dependent drug delivery systems. Further experiments will consider the antibacterial potential of the levofloxacin-loaded chitosan particulate hydrogels.

4. Materials and Methods

4.1. Materials

Chitosan (CS, low molecular weight, 88% hydrolysis degree); Poly (ethylene glycol) methyl ether acrylate (PEGA, $M_n = 480$); Acetic acid (99%); Glutaraldehyde (GA) (25% aqueous solution); Sodium tripolyphosphate (TPP); levofloxacin (LEV); Tween 80; Span 80; acetate and phosphate-buffered saline (PBS) were purchased from Sigma-Aldrich, Saint Louis, MO, USA; MCF-10A cells were purchased from American Type Culture Collection, Manassas, VA, USA; DMEM/F12 supplemented with 5% horse serum (Sigma Aldrich, Saint Louis, MO, USA); 20 ng/mL EGF (Sigma Aldrich, Saint Louis, MO, USA); HDMVEC (Primary Dermal Microvascular Endothelial Cells) are from ATCC, Manassas, VA, USA; Milli-Q ultrapure distilled water (Merck, Rahway, NJ, USA). All other reagents used in this study were of analytical grade purity and were used without further purification. The human blood samples used were freshly obtained from one healthy nonsmoker volunteer.

4.2. Methods

4.2.1. Synthesis of Chitosan Grafted Poly (Ethylene Glycol) Methyl Ether Acrylate

Chemically modified CS with PEGA via Michael addition reaction was performed according to a protocol reported by Han et al. [30]. Briefly, CS (2.0 g) was dissolved in 100 mL CH_3COOH solution 1% (w/w) and added to a 250 mL reaction flask equipped with a reflux condenser. Next, the reaction solution was immersed in a water bath and heated at 50 °C, purged with nitrogen, and stirred for 30 min. Subsequently, PEGA was added dropwise (molar ratio $\text{NH}_2:\text{PEG} = 1:0.5$). After 48 h the reaction was complete. The reaction mixture pH was raised from 3.4 to 8 with NaHCO_3 saturated solution. Subsequently, the polymer solution was filtered via centrifugation and precipitated into acetone. The polymer was purified three times with acetone, followed by dialysis against distilled water for 3 days. Finally, the obtained polymer was lyophilized. The reaction yield reached 61%.

4.2.2. Chitosan Particulate Hydrogels Preparation

The selected method for particle preparation was the double crosslinking (ionic and covalent) technique in a water-in-oil emulsion, the advantage being the use of a lower amount of covalent crosslinker, which leads to a decrease in CPH toxicity. The fundamental principle of the process is the formation of an interconnected/interpenetrated network in the first stage through the majority ionic crosslinking process, followed by the covalent crosslinking step. CPH-based CS-PEGA was prepared in reverse emulsion (w/o) according to our previous study [17]. At first, CS-PEGA was dissolved in 50 mL CH_3COOH (1% w/w) to produce solutions with concentrations of 0.35%; 0.5%; and 0.75% wt. Then, Tween 80 was added to the solution (2% wt) and stirred vigorously for 5 min. Next, the organic phase was prepared: 200 mL toluene and Span 80 (2% wt). Aliquots (250 μL) of polymer solution were added into the organic phase, under ultra Turrax stirring (5000–15,000 rpm), leading to

emulsion formation. Subsequently, freshly prepared crosslinker aqueous solutions, namely TPP (5% wt), were added according to the calculated amounts. After 10 min, the obtained emulsion was transferred to a mechanical stirring-equipped reactor, where the ionic cross-linking process continued at 500 rpm for 1 h at room temperature. Afterward, the covalent cross-linking process was carried out by adding 5 mL glutaraldehyde (GA) extracted in toluene ($c = 1.12 \text{ mg/mL}$) for 1 h. Obtained CPH were centrifuged at 5000 rpm for 1 h and resuspended in acetone after removing the organic phase. The unreacted reagents (TPP, GA, respectively surfactants) were removed by successive washing with water, acetone, and hexane. The purified CPH were weighted and characterized to determine their composition (FTIR, TGA), size distribution (laser diffractometry), zeta potential, morphology (scanning electron microscopy (SEM)), swelling behavior in an aqueous environment, hemocompatibility, cytotoxicity, drug loading, and in vitro drug release.

4.2.3. Characterization of Chitosan Derivatives and Micro/Nanoparticles Techniques of Characterization Used

Fourier Transform Infrared Spectroscopy (FT-IR) spectra of CS, PEGA, CS-PEGA derivative, and CPH were recorded with a DIGILAB SCIMITAR FTS 2000 spectrometer. The samples were prepared as KBr pellets and scanned over the wave number range of $4000\text{--}450 \text{ cm}^{-1}$ at a resolution of 4.0 cm^{-1} .

^1H NMR spectra were obtained using a Bruker Avance DRX 400 (Bruker, Rheinstetten, Germany). CS, PEGA ($M_n = 480$), and CS-PEGA derivatives were dissolved in $\text{D}_2\text{O} + \text{HCl}$ or D_2O according to their solubility. The substitution degree (DS) was calculated from the peak area at the chemical shift of $-\text{CH}_2$ -proton against that of NHAc proton. NMR spectra were recorded in $\text{HCl}/\text{D}_2\text{O}$ using a 400 MHz spectrometer (experimental section).

Thermo Gravimetric Analysis (TGA) was carried out with a Mettler Toledo model TGA/SDTA 851 (TGA Q 500–1285 system). Dried CPH (5 mg) were loaded in an open aluminum pan, heated under a dynamic N_2 atmosphere, with a heating rate of 10°C/min , in a temperature range from room temperature to 800°C .

CPH morphology was investigated by SEM technique. SEM images were recorded with a HITACHI SU 1510 (Hitachi SU-1510, Hitachi Company, Tokyo, Japan) Scanning Electron Microscope, CPH were fixed on an aluminum stub and coated with a 7 nm thick gold layer using a Cressington 108 device before observation.

CPH sizes were analyzed by laser diffractometry technique (SHIMADZU SALD 7001, Shimadzu Company, Kyoto, Japan). CPH (3 mg) were immersed in acetone and sonicated for 15 min at room temperature using a sonication bath (Bandelin Sonorex, Bandelin Company, Berlin, Germany). CPH suspension was added to a quartz cuvette equipped with a stirring mechanism and analyzed. All measurements were performed in triplicate.

CPH Zeta potential was analyzed with Zetasizer Nano ZS Series from Malvern (Malvern, UK). CPH were dispersed in distilled water at a mass concentration of 1.25 g/L and sonicated. The determinations were made in triplicate at 25°C with a Zetasizer Nano Series ZS from Malvern.

4.2.4. CPH Swelling Behavior in Aqueous Media

CPH swelling characteristics in an aqueous environment were evaluated by the gravimetric method using buffer solutions with different pH (slightly alkaline aqueous medium with pH 7.4 (Phosphate Buffer Solution, PBS) and acidic aqueous medium with pH 3.3 (Acetate Buffer Solution, ABS)).

0.030 g of CPH were immersed in a 2 mL Eppendorf tube containing 1 mL of ABS or PBS. The suspensions were monitored and maintained at room temperature under mechanical stirring (100 rpm) for 24 h when equilibrium swelling was attained. CPH suspensions were ultra-centrifuged (15,000 rpm), withdrawn from the buffer solutions, and

weighed at specified time intervals, and immediately replaced with an equivalent volume of fresh buffer. The maximum swelling degree was determined using (Equation (4)):

$$Q\% = \frac{w_s - w_0}{w_0} \times 100 \quad (4)$$

where w_s is the weight of swollen CPH; w_0 is the weight of dry CPH.

4.2.5. CPH Drug Loading Capacity

Levofloxacin (LEV) a fluoroquinolone antibacterial used as a model drug for loading into the CPH drug delivery system. The drug loading process was carried out through a diffusional mechanism. An accurately weighed amount of CPH (0.030g) was suspended in a 1 mL aqueous solution of LEV ($c = 25 \text{ mg/mL}$) and dispersed by sonication for 15 min. The suspensions were maintained at room temperature, under gentle mechanical stirring for 72 h. After preset time intervals CPH samples were centrifuged (15,000 rpm, 5 min). The quantity of LEV encapsulated by CPH was determined by calculating the drug content from the supernatant with Equation (5) based on a calibration curve, previously prepared.

$$y = 6.0424 \cdot x; R^2 = 0.9973 \quad (5)$$

Drug encapsulation efficiency (DEE) represents the quantity of added drug (%) that is encapsulated in the formulation. The DEE of LEV from CPH was calculated with Equation (6). The LEV entrapment efficiency of CPH was determined with Equation (7).

$$DEE = \frac{\text{Actual drug (LEV) content loaded in CPH}}{\text{Theoretical drug content (LEV)}} \times 100 \quad (6)$$

$$\text{Entrapment efficiency} = \frac{\text{Total amount of LEV} - \text{Amount of LEV from supernatant}}{\text{Total amount of LEV}} \quad (7)$$

All tests were accomplished with a spectrophotometer UV-VIS NanoDrop ND-1000. LEV-loaded CPH were freeze-dried. The tests were made in triplicate and the results were averaged.

4.2.6. In Vitro LEV Release from CPH

To evaluate the in vitro drug release property, the release of LEV-loaded CPH was performed in PBS ($\text{pH} = 7.4$); this environment ensures a slower release rate of the drug. The LEV release process was carried out by immersing 0.030 g of CPH in a 2 mL Eppendorf tube containing 1 mL of PBS. The suspensions were maintained at 37°C , under continuous gentle stirring (50 rpm). After pre-established time intervals, the suspensions were ultra-centrifuged (15,000 rpm) and the drug quantity was determined by UV-Vis spectrometry at 287 nm. The determinations were made in triplicate and the results were averaged.

4.2.7. CPH Hemocompatibility

Particle hemolysis tests were performed according to a method suggested by Vuddanda et al. [42] presented in our previous study [16,17]. Briefly, 2 mL of CPH were suspended in saline solution with different concentrations and mixed with purified red blood cells (RBC, 2 mL, at final concentrations of 100, 250, and 500 mg CPH/mL). Samples were incubated at 37°C for 2, 4, and 6 h and gently shaken every 30 min for re-suspension. After a preset time, samples were centrifuged (2000 rpm, 5 min) and 1.5 mL of supernatant was incubated for another 30 min at room temperature, allowing the hemoglobin oxidation. Finally, the oxyhemoglobin from the supernatant was spectrophotometrically analyzed with a spectrophotometer (PG Instruments T60 UV-Vis Spectrophotometer) at

540 nm. Hemolysis was determined using Equation (8). The determinations were made in triplicate and the results were averaged.

$$\% \text{ Hemolysis} = \frac{\text{Abs sample} - \text{Abs negativ control}}{\text{Abs pozitiv control} - \text{Abs negativ control}} \quad (8)$$

4.2.8. Cytotoxicity

Cell Culture

All cells were cultured in a humidified atmosphere at 37 °C with 5% CO₂. MCF-10A cells were cultured in DMEM/F12 supplemented with 5% horse serum, 20 ng/mL EGF, 10 µg/mL insulin, 0.5 ng/mL hydrocortisone, 10 ng/mL cholera toxin, 1% Pen/Strep; HDMVEC (primary dermal microvascular endothelial cells) were cultured in Vascular Cell Basal Medium supplemented with Microvascular Endothelial Cell Growth Kit-BBE ATCC[®] PCS-110-040.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/gels8080494/s1>, Figure S1: TGA analysis of chitosan particulate hydrogels (A8, A9, A10 samples); Figure S2: Kinetics of the LEV loading into the ABS from chitosan particulate hydrogels (A8, A9 samples); Figure S3: Kinetics of the LEV release efficiency into the PBS from chitosan particulate hydrogels (A8, A9 samples); Figure S4: Korsmeyer-Peppas model for A8, 9 samples; Figure S5: Viability of HDMVEC and MCF-10A cells for submi chitosan particulate hydrogels (sample A9); Table S1: Characteristic absorption bands of CS, PEGA, CS-PEGA; Table S2: CPH characteristic absorption bands; Table S3: n and k values obtained based on the Korsmeyer-Peppas model (0–480 min).

Author Contributions: Nanogels experimental part, data interpretation, writing and editing original draft preparation C.-L.L., C.A.P. and C.P.; Conceptualization and supervision C.A.P., M.P. and C.D.; cytotoxicity investigation—C.-E.T. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

ABS—Acetate Buffer Solution
 CH₃COOH—Acetic acid
 CS—Chitosan
 DEE—Drug Encapsulation Efficiency
 DS—Degree of substitution
 Eq.—Equation
 FTIR—Fourier Transform Infrared Spectroscopy
 GA—Glutaraldehyde
 GlcN—2-amino-2-deoxy-β-d-glucopyranosyl
 GlcNAc—2-acetamido-2-deoxy-β-d-glucopyranosyl

LEV—Levofloxacin
 CPH—Chitosan particulate hydrogels
 NMR—Nuclear Magnetic Resonance
 PBS—Phosphate buffer solution
 PEG—Poly (ethylene glycol)
 PEGMA—Poly (ethylene glycol) methacrylate
 PEGA—Poly (ethylene glycol) methyl ether acrylate
 CS-PEGA—Chitosan grafted poly (ethylene glycol) methyl ether acrylate
 RBC—Red blood cells
 RT—Room temperature
 SEM—Scanning Electron Microscope
 Span 80—Nonionic surfactant
 TGA—Thermo Gravimetric Analysis
 Tween 80—Nonionic Surfactant
 TPP—Sodium tripolyphosphate

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Article

Hydrogels Based on Alginates and Carboxymethyl Cellulose with Modulated Drug Release—An Experimental and Theoretical Study

Cătălina Anișoara Peptu ¹, Elena Simona Băcăiță ^{2,*} , Corina-Lenuta Savin (Logigan) ¹ , Marian Luțcanu ³ and Maricel Agop ²

- ¹ Department of Natural and Synthetic Polymers, Faculty of Chemical Engineering and Environmental Protection, “Gheorghe Asachi” Technical University of Iasi, 71, Prof. Dr. Docent Dimitrie Mangeron Street, 700050 Iasi, Romania; catipeptu@ch.tuiasi.ro (C.A.P.); savincorina@yahoo.com (C.-L.S.)
- ² Department of Physics, Faculty of Machine Manufacturing and Industrial Management, “Gheorghe Asachi” Technical University of Iasi, Bd. Prof. Dr. Docent Dimitrie Mangeron 73, 700050 Iasi, Romania; maricel.agop@academic.tuiasi.ro
- ³ Materials Science Department, Faculty of Materials Science and Engineering, “Gheorghe Asachi” Technical University of Iasi, 71, Prof. Dr. Docent Dimitrie Mangeron Street, 700050 Iasi, Romania; marian.lutcanu@staff.tuiasi.ro
- * Correspondence: elena-simona.bacaita@academic.tuiasi.ro or bsimona77@yahoo.com



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Abstract: New hydrogels films crosslinked with epichlorohydrin were prepared based on alginates and carboxymethyl cellulose with properties that recommend them as potential drug delivery systems (e.g., biocompatibility, low toxicity, non-immunogenicity, hemostatic activity and the ability to absorb large amounts of water). The characterization of their structural, morphological, swelling capacity, loading/release and drug efficiency traits proved that these new hydrogels are promising materials for controlled drug delivery systems. Further, a new theoretical model, in the framework of Scale Relativity Theory, was built with to offer insights on the release process at the microscopic level and to simplify the analysis of the release process.

Keywords: alginic acid; carboxymethyl cellulose; films; epichlorohydrin; drug delivery; fractalization

1. Introduction

In recent years, significant progress has been made in the field of biology, medicine and materials science, which has led to the development of innovative systems, including films, hydrogels and micro- and nanoparticulate systems based on natural/synthetic polymers as drug carriers. The advantages of such drug delivery systems are the improved therapeutic efficacy of drugs and reduced side effects and associated costs [1–3]. Generally, polymeric films are biocompatible and can be used as supports for the controlled release of bioactive compounds. They can be obtained through the chemical and/or physical crosslinking of hydrophilic polymers, and their physical-chemical properties depend on the type and density of crosslinking in addition to the molecular weight and chemical composition [4].

In recent years, a wide variety of sources for drug delivery systems have been developed and applied, such as proteins, polysaccharides, synthetic polymers and organo-inorganic hybrids. Of these, polysaccharides present important advantages as they can be obtained from various natural renewable resources (ocean and plant) and they present an abundance of functional groups, such as hydroxyl (-OH), amino (-NH₂) and carboxyl (-COOH), that make them suitable for chemical modification in order to adjust certain properties, biocompatibility, bioactivity and nontoxicity [5].

Alginates (AG) are natural hydrophilic colloidal polysaccharides obtained from various species of brown algae. They are extensively investigated and used for many biomedical applications, due to several unique properties, including biocompatibility, low toxicity,

non-immunogenicity, hemostatic activity and accessibility at low prices. In addition to these, they form gels easily by adding bivalent cations as Ca^{2+} . Alginates are known as natural block copolymers containing 1,4-linked residues of β -D-manuronate (M) and α -L-guluronate (G). The content in the residues of D-manuronate and α -L-guluronate alginates and the length of each block may differ depending on the species of algae used for their extraction. Alginates are excellent candidates for the development of different systems, such as films, membranes, hydrogels, particles and nanofibers [1,4,6].

Carboxymethyl cellulose (CMC) is another polysaccharide widely used in the biomedical field. It is cellulosic, which, when in contact with aqueous media, leads to the formation of colloidal solutions that contain, at low concentrations, filiform macromolecules and gels at high concentrations. CMC solutions are viscous at acidic pH, and the viscosity increases with the decreasing pH value. The solution viscosity decreases with temperature but returns to the initial value after cooling. CMC has desirable characteristics for a drug delivery system, such as biocompatibility, non-toxicity and the ability to absorb large amounts of water, and CMC has been applied as a stabilizer and as protective colloids in pharmaceuticals [7–9].

Despite the excellent properties of both alginate and carboxymethyl cellulose, their combination as hydrophilic/hydrophilic polymers in the formation of new materials represents a challenging task due to their high-water solubility [10]. To overcome this limitation, an alternative is to add in the polymeric matrix a crosslinking agent, such as epichlorohydrin (ECH). As result, a film with increased resistance in contact with aqueous media, and thus a higher tensile strength, is obtained. Moreover, the crosslinking step allows control of the release rate of bioactive compounds through the film matrix [11] leading to new materials with synergetic properties.

Aiming to exploit the AG and CMC properties that recommend them as potential drug delivery systems (i.e., biocompatibility, low toxicity (AG and CMC), non-immunogenicity, hemostatic activity, accessibility at low prices (AG) and the ability to absorb large amounts of water (CMC)), new hydrogel films, based on AG and CMC with epichlorohydrin (ECH) as a crosslinker, were prepared and characterized in order to evaluate their properties as drug delivery systems [12].

Fourier-transform infrared spectroscopy (FT-IR) and scanning electron microscopy (SEM) were used to characterize the structures and morphology. Further, the swelling capacity and loading/release drug efficiency, using metronidazole (MT) as a model drug, were evaluated. The further goal of the present study is to use these formulations as wound dressing materials and, for this purpose in this preliminary study, MT was chosen since it is widely used for its ability to treat a large variety of infections.

2. Experimental Procedure

2.1. Materials

The following materials: AG (alginic acid sodium salt from brown algae with medium viscosity, Brookfield viscosity 2% in water at 25 °C, >2000 cps), CMC (medium viscosity, sodium (Na) 6.5–9.5%. Brookfield viscosity 2% in water at 25 °C, 400–800 cps. Degree of substitution 0.65–0.90), NaOH and ECH were purchased from Sigma-Aldrich; Metronidazole 99% was purchased from Alfa Aesar; and Milli-Q ultrapure distilled water (DW) was purchased from Merck Chemicals (Darmstadt, Germany). Epichlorohydrin (ECH) was used as a crosslinking agent without any pretreatment. All chemical reagents were of analytical-grade purity and were used without further purification.

2.2. Instrumentation

The film morphology was investigated via the SEM technique: films were fixed on an aluminum stub and coated with a 7 nm thick gold layer using a Cressington 108 device before observation, and SEM images were recorded with a HITACHI SU 1510 (Hitachi SU-1510, Hitachi Company, Tokyo, Japan) Scanning Electron Microscope at an accelerating voltage of 25 kV.

Fourier Transform Infrared Spectroscopy spectra of films were recorded with a DIGILAB SCIMITAR FTS 2000 spectrometer. The samples were prepared as KBr pellets and scanned over the wave number range of 4000–450 cm^{-1} at a resolution of 4.0 cm^{-1} .

2.3. Methods

2.3.1. Preparation of AG/CMC Films

The AG/CMC films were synthesized via the Jeong et al. method with certain modifications. Films based on AG/CMC were prepared as follows: 500 mg of CMC was dissolved in 10, 15 and 20 mL of 9 wt.% NaOH solution. Subsequently, 500 mg of AG was added to the CMC solution, and, after the complete dissolution of both polymers, 1.5 mL of ECH was slowly added to the solution while stirring at 600 rpm at 25 °C for 25 min. The resulting films were washed with ethanol solution. Finally, all the samples were freeze-dried. The initial reactants composition is shown in Table 1. The percent hydrogel yield was obtained using the following equation:

$$\text{Films yield, \%} = \frac{\text{Dry weight of film}}{\text{Total weight of reactant in feed}} \times 100. \quad (1)$$

Table 1. The initial reactants composition for hydrogel preparation and yields.

Sample Code	Concentration (%)	Polymer Ratio (g/g)	AG(g)	CMC(g)	NaOH (mL)	ECH (mL)	Yields (%)
F1	6.6	1:1	0.5	0.5	15	1.5	79.7
F2	15	1:2	0.5	1	15	1.5	54.1
F3	15	2:1	1	0.5	15	1.5	89.7
F4	10	1:1	0.5	0.5	10	1.5	69.5
F5	5	1:1	0.5	0.5	20	1.5	67.2
F6	6.6	1:1	0.5	0.5	15	0.75	71.7
F7	6.6	1:1	0.5	0.5	15	3	43.5

2.3.2. Evaluation of Swelling Characteristic

The film swelling properties were evaluated in order to predict their loading and releasing capacity. All synthesized films were immersed in vials (glass bottles of 10 mL) containing 3 mL of DW. They were maintained at room temperature for 4 days until the swelling equilibrium was reached. The swelling properties were monitored at different times, the films were weighed, and the DW was gently removed from the vial and the film surface with laboratory tissues.

The water swelling ratio was determined with the equation:

$$Q(\%) = \frac{w_s - w_0}{w_0} \times 100 \quad (2)$$

where w_s is the weight of the swollen probe and w_0 is the weight of the dry probe.

2.3.3. Evaluation of Metronidazole Loading and Release

Drug loading and release studies were performed via a diffusional mechanism [13]. MT as a drug model was used. First, samples of dried films were placed in a glass vial (10 mL volume), and 3 mL of MT aqueous solution (10 mg/mL) was added and maintained at room temperature. The glass vials containing the films and MT solution were left in the solution during 48 h and gently stirred by a small Teflon bar. The quantity of MT retained was determined spectrophotometrically based on the calibration curve previously obtained [14]. After 2 days, the MT-loaded films were washed with DW, and the remaining water on the films surface was gently wiped away with a laboratory tissue.

Next, the release profiles of MT were evaluated in DW at room temperature. Periodically, 20 μ L of volume-releasing solution was withdrawn from the DW. The volume of DW was held constant by adding fresh DW. The amount of released MT was calculated spectrophotometrically with a spectrophotometer, UV-VIS NanoDrop ND –1000, at a wavelength of 320 nm. All measurements were performed in triplicate and averaged. The equations used are as follows, where W_{MT} is the amount of MT:

$$\text{Drug loading efficiency (\%)} = \frac{W_{MT\text{crude}} - W_{MT\text{supernatant}}}{W_{MT\text{plain}}} \times 100 \quad (3)$$

$$\text{Release efficiency (\%)} = \frac{W_{MT\text{released}}}{W_{MT\text{total}}} \times 100 \quad (4)$$

3. Results and Discussions

A first important step in the design of a hydrogel type system is represented by the selection of precursors and the method of preparation, which strongly influences the speed and mechanism of the release of bioactive compounds encapsulated in the hydrogel. In order to analyze the influence of reaction parameters on the final hydrogel properties and to select the best formulation for drug delivery systems, varied ratios between AG and CMC were tested regarding the ECH concentration and NaOH reaction volume. The sample F1, which was prepared using the following parameters, AG/CMC ratio 1:1, 0.02 mol of ECH, 15 mL of NaOH 9 wt.% and a 25 min reaction time, was chosen for further experiments due to the medium values of most of the considered variable parameters (Table 1).

After the purification step with ethanol and freeze drying, the recovered fractions were found to be relatively high at around 90% in the case of sample F2, with a ratio of 1:2 (AG: CMC), and lower than 43% for the F7 sample, which had a high quantity of ECH crosslinking agent. The high recovered fraction in the case of sample F2 may be explained by the high CMC quantity compared to AG, which could provide a high mechanical strength of the film, due to a longer polymer chain. In the case of sample F2, the amount of crosslinker, referring to the total reaction component, was reduced, which favors the obtaining of a higher yield compared to the F7 sample that had a high ECH concentration. Therefore, we can state that the yields of films are dependent on the polymer and crosslinking agent concentration (see Table 1).

3.1. Structural Characterization

3.1.1. FTIR Characterization

The Fourier transform infrared (FTIR) spectra of the AG, CMC and synthesized film were recorded and compared (Figure 1). First, the recorded AG spectra revealed important absorption bands concerning the hydroxyl and carboxylic functional groups. In the range of 3107–3579 cm^{-1} , stretching vibrations of O–H bonds of AG appeared. Stretching vibrations of C–H were observed at 2854–2960 cm^{-1} . The bands observed at 1598 and 1409 cm^{-1} were attributed to asymmetric and symmetric stretching vibrations of COO– groups. The bands at 1028 and 947 cm^{-1} were attributed to the C–O stretching with contributions from C–C–H and C–O–H deformation.

For CMC, a characteristic peak at 2922 cm^{-1} was observed corresponding to C–H anti-symmetrical stretching. The bands observed at 1413 and 1591 cm^{-1} were attributed to the carboxylate group stretching vibrations (symmetric and asymmetric). Moreover, a visible band was observed at 3350 cm^{-1} characteristic to –OH groups.

In the case of the synthesized film spectrum, significant differences in bands were observed, compared with those of the plain AG and CMC, as characteristic peaks were observed at 1620, 1336 and 1058 cm^{-1} , which can be attributed to the stretching modes of the (C=O), (C–C–O) and (O–C–C) bonds of the ester group, respectively. At 877 cm^{-1} , a band specific to the C–H bending was observed. Further, at 1415 cm^{-1} , a higher intensity of the O–H bending peak compared to AG and CMC spectrum, which may be attributed to a cross-linked network, was observed.

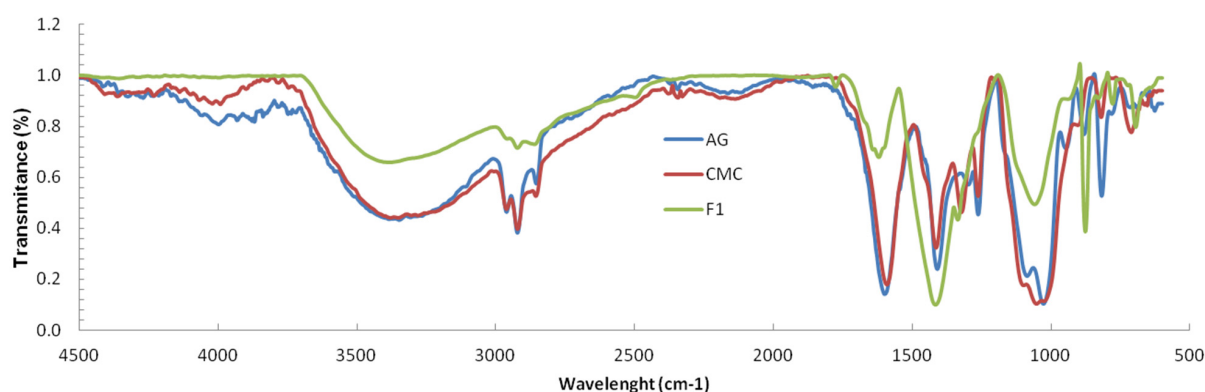


Figure 1. Fourier transform infrared (FTIR) spectra of the AG, CMC and F1 synthesized film.

In the case of the synthesized film spectrum, significant differences in bands were observed, compared with those of the plain AG and CMC, as characteristic peaks were observed at 1620 , 1336 and 1058 cm^{-1} , which can be attributed to the stretching modes of the (C=O), (C-C-O) and (O-C-C) bonds of the ester group, respectively. At 877 cm^{-1} , a band specific to the C-H bending was observed. Further, at 1415 cm^{-1} , a higher intensity of the O-H bending peak compared to AG and CMC spectrum, which may be attributed to a cross-linked network, was observed.

3.1.2. Morphological Characterization by SEM

The SEM technique was used to visualize the morphological characteristics of lyophilized hydrogel films. Images of the films surface and section morphology are highlighted in Figure 2, for F2 and F7 samples, and in Figure S1, for all other samples. We observed that all films were compact and presented surfaces with aggregates and roughness. However, as can be observed from the cross-section images, all films presented areas with large pores of irregular sizes and shapes.

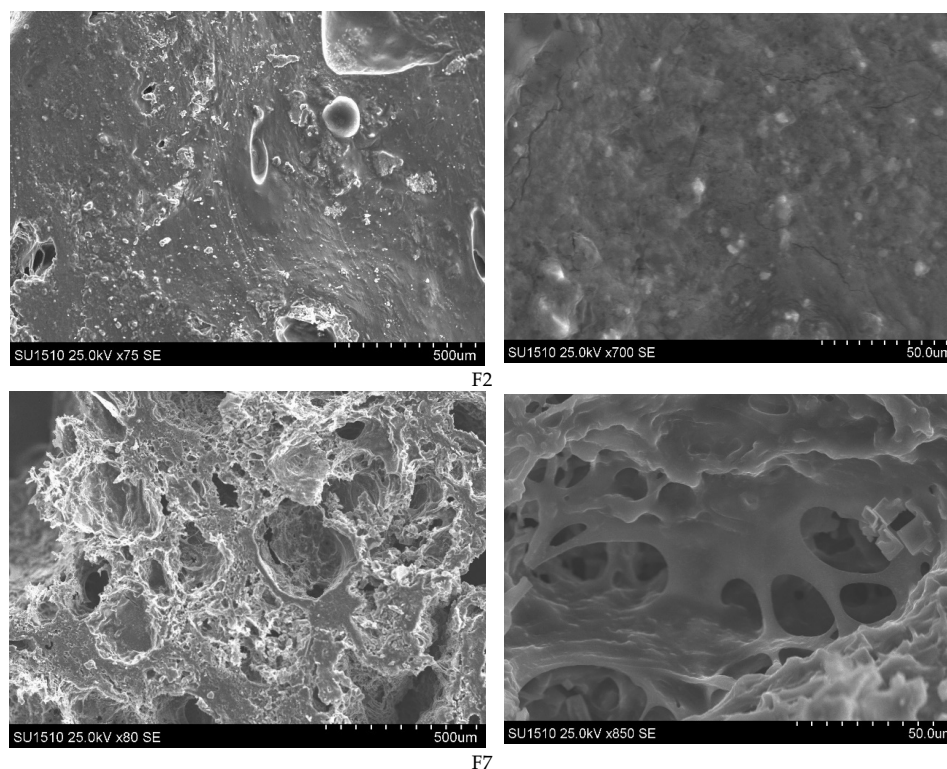


Figure 2. SEM images of the AG/CMC films (samples F2 and F7).

Comparing SEM pictures for the F2 and F3 samples, one can see that the porosity of the film decreased with increasing polymer concentration; therefore, we can state that the formation of the porous structures depends on the polymer's initial concentration. Moreover, SEM images revealed that the F1 sample had a surface without pores in contrast with samples F6 and F7, which had porous structures at the same polymer concentration. In conclusion, the morphology of polymer hydrogels, which influences further properties, can be adjusted by varying the crosslinking reaction parameters.

3.2. Evaluation of Hydrogels Behavior in Aqueous Media

Considering the hydrophilicity of the initial polymers, swelling properties of the AG/CMC films are a key factor regarding their ability to load/release bioactive components. Swelling studies were performed for all prepared samples in DW. The swelling ratios $Q(\%)$ were calculated, and the results can be seen in Table 2.

Table 2. The maximum swelling degree of the synthesized films.

	F1	F2	F3	F4	F5	F6	F7
$Q, \%$	587	316	398	299	953	1273	362

The obtained results reveal a connection between the swelling degree and sample preparation parameters, such as the polymer and crosslinker concentration and reaction volume. The best swelling ratio of 1273%, indicating high superabsorbent properties, was observed for the F6 sample (1:1 (AG: CMC) molar ratio and 6.6% polymer concentration). This result was expected, since the SEM images revealed, for the F6 sample, a very porous structure. Still, despite its porous structure, as shown by SEM images, the sample F7 had a swelling ratio of only 362%.

This can be explained due to the higher ECH crosslinker concentration of sample F7, which determined a more extensively crosslinked polymeric network. The polymer concentration influenced the swelling ratio: at low polymer concentrations, higher swelling ratios were obtained compared to the samples with high concentrations that showed low swelling ratios. These results demonstrated that the swelling ratio $Q(\%)$ was dependent both on the polymer and crosslinking agent concentration (Figure 3).

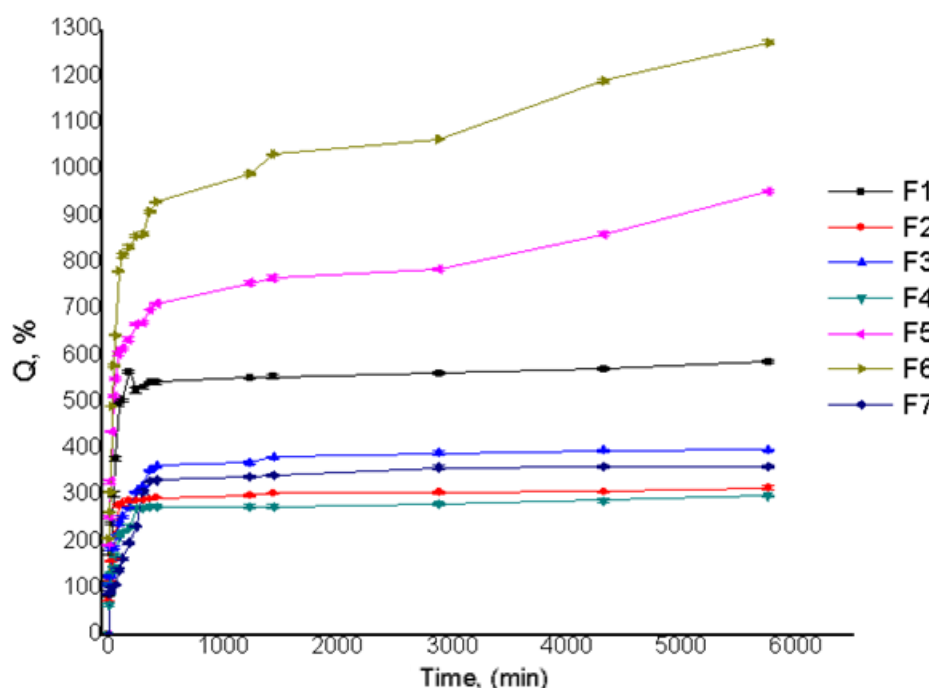


Figure 3. Film swelling ratios for films with different reaction parameters.

3.3. Evaluation of Metronidazole Loading and Release Kinetics

The potential of AG/CMC hydrogels as drug delivery systems was investigated by evaluating their drug loading and release efficiency. MT, a limited spectrum antibiotic that actively deters the growth of protozoa, anaerobic gram-positive and anaerobic gram-negative bacteria, was used as the model drug. All prepared films were loaded with a MT solution (10 mg/mL), and subsequently the released MT from the loaded films in DW was determined.

The amount of MT loaded in hydrogel films after 48 h was between 10.8 mg and 18 mg, corresponding to a loading efficiency between 40% and 64% (Table 3). As expected, sample F6, which had the higher swelling ratio, also presented the higher MT-loading capacity (18 mg, 64%) in comparison to sample F7 (10.8 mg, 40%) most likely due to a stronger complexation capability of sample F6 compared to F7. We can conclude that the amount of MT loaded in hydrogel films is dependent on the polymers and ECH concentration used in the preparation procedure (Figure 4).

Table 3. MT loaded and released film profiles.

	F1	F2	F3	F4	F5	F6	F7
MT-loaded films, mg	16.6	14.9	11.9	13.4	15.8	18.0	10.8
MT-loaded films Efficiency, %	44.63	50.22	60.39	55.40	47.48	39.86	63.99
MT-released films, mg/g	15.03	12.36	7.11	9.07	12.13	13.54	9.88
MT-released films Efficiency MT, %	90.49	82.78	59.83	67.82	76.97	75.04	91.41

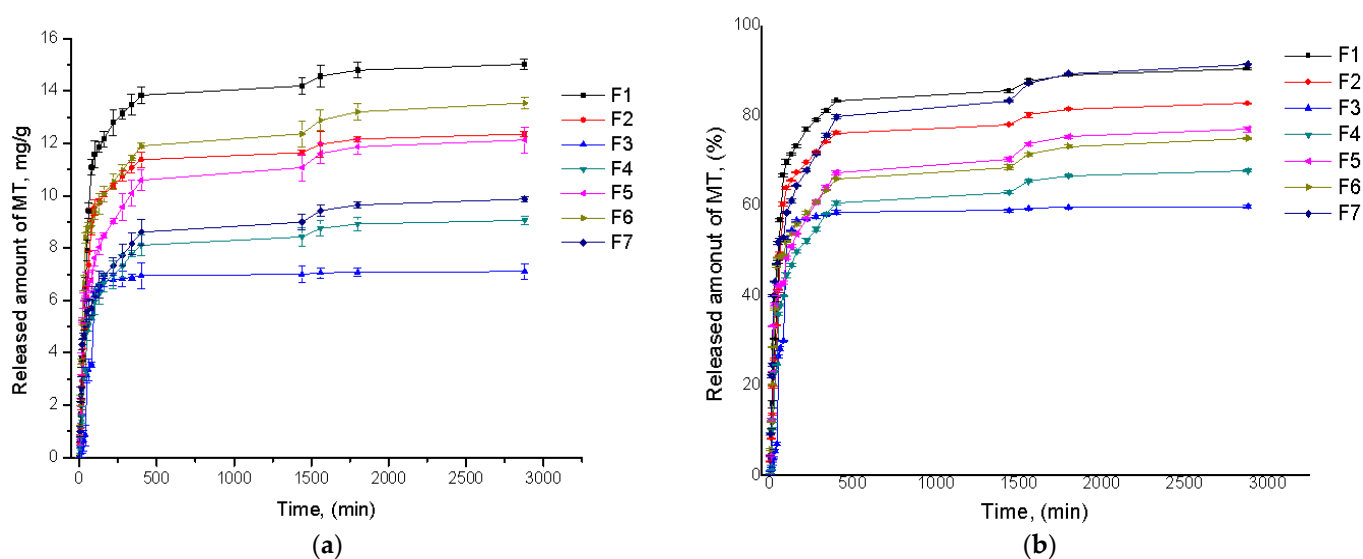


Figure 4. The MT release kinetics from synthesized films expressed in terms of mg/g (a) and released efficiency (b).

Regarding the drug release capacity, all hydrogel films released between 9.88 mg/g and 15.03 mg/g MT or, in terms of efficiency, between 59% and 91% MT (Table 3), demonstrating a good ability to release the active compound. The release kinetics showed an initial burst release phase in the initial approximately 100 min, followed by a slower release phase within the next 9 h, characterized by a final constant release until 48 h.

By analyzing the influence of synthesis parameters on the film's release capacity, we noticed that the sample that had the highest amount of released MT was F1 (15.03 mg/g), in contrast to sample F6 (13.54 mg/g). This result, somehow unexpected, may be due to the host–guest complex formation between hydrogel network and MT: a high amount of MT inside the film may have caused a recomplexation between the crosslinked hydrogel film and MT. These results suggest that samples F1 and F6 are more effective as drug delivery systems that are capable of encapsulating a large amount of MT and releasing it effectively.

4. Theoretical Evaluation of Drug Release

4.1. Justification for the Need for a New Mathematical Model

The experimental results, previously discussed, led to some unexpected results in respect to the dependence of drug release on the crosslinker amount. For example, the expected behavior was that, as the amount of crosslinker increased, the swelling ratio and the amount of drug loaded/released into/from the polymer network would decrease due to the higher network strength. The experimental results confirmed this hypothesis for the swelling ratio and the loaded drug but failed for the released drug amount/efficiency; moreover, the expected similarity in terms of swelling and loading/releasing efficiency was not encountered (Table 4).

Table 4. The dependence of the swelling, loading and releasing capacity on the crosslinker amount (the other reaction parameters are considered constant).

Sample Code	ECH Crosslinker (mL)	Swelling Ratio (%)	Loaded MT		Released MT	
			mg	%	mg	%
F6	0.75	1273	18.0	39.86	13.54	75.04
F1	1.5	587	16.6	44.63	15.03	90.49
F7	3	362	10.8	63.99	9.88	91.41

The given explanation implies a certain degree of uncertainty, i.e., a possible host-guest complex formation between the hydrogel network and drug. The above issue raises, once again, the problem of the complexity of the release processes and the interdependence of the variables that appear: the strength of the hydrogel network and the concentration of the loaded drug; in turn, the strength of the network is determined by the amount of crosslinker used, the polymer concentration, their molar ratio etc.

4.2. The Theoretical Model of Drug Release in the Framework of Scale Relativity Theory

As a consequence of the information presented above, the need to understand the release process adds the idea of building a theoretical model of drug release able to offer insights on the release process at microscopic level and to simplify the analysis of the release process. The main purpose of the presented model is to be an alternative to the classical models of drug release, which analyze the phenomenon only empirically at the macroscopic level [15] as if ignoring that matter obtains its properties from the molecules it is made of and their interactions.

The starting point of the theoretical model is the Scale Relativity Theory [16,17] that, in recent years, has been used successfully in describing the dynamics of complex systems, such as nanofluids [17] and plasma [18–23]. The hypothesis underlying this theory is that the entities of any complex system move on continuous and non-differential curves named fractal curves, i.e., three dimensional fractured lines, whose non-linearity is dependent and proportional with the number of interactions within the system. In this context, the fractalization degree will be defined as a measure of the system complexity, and the physical quantities, characterizing the system evolution, will be fractal functions dependent both on the spatio-temporal coordinates and resolution scales [24].

Further, we will use this hypothesis in the case of our system, which will be considered as a complex system consisting of a hydrogel, drug and release medium. Previous studies showed that the theoretical models built on this hypothesis allowed insights concerning drug release at the microscopic level, which has been unaddressed thus far due to the complexity of the phenomena involved. For example, this revealed that, in the case of polymeric particles based on chitosan and gelatin, the trajectories of drug molecules follow cnoidal oscillation modes [25], while, for polysaccharide-based hydrogels, their trajectories evolved from a normal period doubling state towards damped oscillating via strong modulated dynamics [26].

For chitosan-gelatin and chitosan-poly(vinyl alcohol) hydrogels, analyzing the release kinetics at long time scales (i.e., up to 25 days), when the hydrogel films degrade, it was found that the release efficiency was dependent on the time and system nonlinearity, i.e., the fractalization degree [27]. Moreover, it was found that, along with the evolution through all the phases of the release process (i.e., burst, swelling, equilibrium and degradation phases), the fractalization degree increased, thus, reflecting the increasing system complexity [28].

In this study, we will consolidate this hypothesis by analyzing the release dynamics in two directions: (i) the release dynamics in the Schrodinger fractal representation and (ii) the release dynamics in the Mandelung fractal hydrodynamic representation [24].

In our opinion, the two representations describing the dynamics of release are not excluded; on the contrary, they are complementary. As the problem of compatibility of the two representations in the description of the release dynamics has not been analyzed thus far, next we will analyze such a problem and its implications.

4.2.1. Two Models of Describing the Release Dynamics through the Scale Relativity Theory

In all the above assumptions, the motion equation of any of the system component (either drug and either release medium molecules) can be written in the form:

$$\frac{d\hat{V}^i}{dt} = \partial_t \hat{V}^i + \hat{V}^l \partial_l \hat{V}^i + \frac{1}{4} (dt)^{[\frac{2}{f(\alpha)}]-1} D^{lk} \partial_l \partial_k \hat{V}^i = 0 \quad (5)$$

where

$$\begin{aligned} \hat{V}^l &= V_D^l - V_F^l \\ D^{lk} &= d^{lk} - i d^{lk} \\ d^{lk} &= \lambda_+^l \lambda_+^k - \lambda_-^l \lambda_-^k \\ \hat{d}^{lk} &= \lambda_+^l \lambda_+^k + \lambda_-^l \lambda_-^k \\ \partial_t &= \frac{\partial}{\partial t}, \partial_l = \frac{\partial}{\partial x^l}, \partial_l \partial_k = \frac{\partial}{\partial x^l} \frac{\partial}{\partial x^k}, i = \sqrt{-1}, l, k = 1, 2, 3 \end{aligned} \quad (6)$$

where x^l is the multifractal spatial coordinate, t is the non-fractal time with the role of an affine parameter of the motion curves, $\hat{V}^l$ is the complex velocity, V_D^l is the differential velocity independent of scale resolution, V_F^l is the non-differential/fractal velocity dependent of scale resolution, dt is the scale resolution, $f(\alpha)$ is the singularity spectrum of order α , α is the singularity index and a function of fractal dimension D_f , D^{lk} is the constant tensor associated with the differentiable–non-differentiable transition, $\lambda_+^l (\lambda_+^k)$ is the constant vector associated with the backward differentiable–non-differentiable dynamic processes, $\lambda_-^l (\lambda_-^k)$ is the constant vector associated with the backward differentiable–non-differentiable dynamic processes, and F is a multifractal function.

In the case of our drug release system, multifractalization will be considered by means of Markovian stochastic processes, i.e., processes for which the probability of possible “events” in system evolution depends only on the state attained in the previous “event”. From a mathematical point of view, the following restrictions must be fulfilled:

$$\lambda_+^i \lambda_+^l = \lambda_-^i \lambda_-^l = 2\lambda \delta^{il}, \quad i, l = 1, 2, 3 \quad (7)$$

where λ is a constant associated with the differentiable–non-differentiable transition, which, in the case of release dynamics, is actually the transition from Fickian to non-Fickian diffusion, and δ^{ir} is Kronecker’s pseudo-tensor [16,17,24].

Imposing the restrictions from (7), Equation (5) becomes:

$$\frac{d\hat{V}^i}{dt} = \partial_t \hat{V}^i + \hat{V}^l \partial_l \hat{V}^i - i\lambda (dt)^{[\frac{2}{f(\alpha)}]-1} \partial_l \partial_l \hat{V}^i = 0 \quad (8)$$

where $\partial_t \hat{V}^i$ represents the local multifractal acceleration, $\hat{V}^l \partial_l \hat{V}^i$ the multifractal convection and $i\lambda(dt)^{[\frac{2}{f(\alpha)}]-1} \partial_l \partial^l \hat{V}^i$ the multifractal dissipation. Equality with zero from Equation (8) reflects the fact that the local multifractal acceleration, the local multifractal convection and the multifractal dissipation are in equilibrium at all points of the motion curve. Moreover, the existence of the complex dissipation term confirms that the multifractal fluid with which the drug release complex system can be assimilated exhibits memory according to the assumption of Markovian stochastic processes.

Considering that, in the release flow, each element of the moving fluid undergoes no net rotation (i.e., rotational movements of system structural units), the complex velocity field from (6) becomes

$$\hat{V}^i = -2i\lambda(dt)^{[\frac{2}{f(\alpha)}]-1} \partial^i \ln \Psi \quad (9)$$

where

$$\chi = -2i\lambda(dt)^{[\frac{2}{f(\alpha)}]-1} \ln \Psi \quad (10)$$

is the complex scalar potential of the complex velocity fields from (6), and Ψ is the state function that describes the equilibrium state of the system. Substituting (9) in (8) and using the mathematical procedure from [24], Equation (8) takes the form of the multifractal Schrödinger type equation:

$$\lambda^2(dt)^{[\frac{4}{f(\alpha)}]-2} \partial^l \partial_l \Psi + i\lambda(dt)^{[\frac{2}{f(\alpha)}]-1} \partial_t \Psi = 0 \quad (11)$$

Therefore, for the complex velocity field (9), the dynamics of any complex system entities are described through multifractal Schrödinger “regimes”, i.e., Schrödinger equations at various scale resolutions.

From such a perspective, the meaning of Ψ can be given based on a conservation law. Indeed, multiplying Equation (11) with $\bar{\Psi}$ (complex conjugate of Ψ) and the complex conjugate of Equation (11) with Ψ , followed by their difference, the multifractal law of conservation for the state density is obtained in the form [29]:

$$\partial_t \rho + \partial_l J^l = 0 \quad (12)$$

where

$$\rho = \Psi \bar{\Psi}, J^l = \frac{\lambda(dt)^{[\frac{2}{f(\alpha)}]-1}}{i} (\bar{\Psi} \partial^l \Psi - \Psi \partial^l \bar{\Psi}) \quad (13)$$

In the relations (12) and (13), ρ is the multifractal state density, while J^l is the multifractal current density. In the case of the existence of a constraint, such as the hydrogel network-drug interactions, in the form $\mathbf{B} = \text{rot} \mathbf{A}$, the usual derivative from the Scale Relativity Theory is substituted with the covariant derivative, ∇^l :

$$\nabla^l = \partial^l - i \frac{g}{2} A^l \quad (14)$$

where A^l is a vector potential and g is a coupling constant between Ψ and A^l . In such a context, (12) maintains its validity, with the difference that J^l from (13) can be expressed as

$$J^l = \frac{\lambda(dt)^{[\frac{2}{f(\alpha)}]-1}}{i} (\bar{\Psi} \partial^l \Psi - \Psi \partial^l \bar{\Psi}) - g A^l \Psi \bar{\Psi} \quad (15)$$

Moreover, if Ψ is chosen of the form (Mandelung’s choice):

$$\Psi = \sqrt{\rho} e^{is} \quad (16)$$

where $\sqrt{\rho}$ is the amplitude of the state quantities and s the phase, then the complex velocity field (9) takes the explicit form:

$$\hat{V}^i = 2\lambda(dt)^{[\frac{2}{g(\alpha)}]-1} \partial^i s - i\lambda(dt)^{[\frac{2}{g(\alpha)}]-1} \partial^i \ln \rho, \quad (17)$$

which leads to the determination of velocity fields:

$$\begin{aligned} V_D^i &= 2\lambda(dt)^{[\frac{2}{f(\alpha)}]-1} \partial^i s \\ V_F^i &= \lambda(dt)^{[\frac{2}{f(\alpha)}]-1} \partial^i \ln \rho \end{aligned} \quad (18)$$

By (17) and (18) and using the mathematical approaches from [24,29], the Equation (8) reduces to the multifractal Mandelung's equations:

$$\partial_t V_D^i + V_D^l \partial_l V_D^i = -\partial^i Q \quad (19)$$

$$\partial_t \rho + \partial_l (\rho V_D^l) = 0 \quad (20)$$

where Q denotes the multifractal specific potential:

$$Q = -2\lambda^2(dt)^{[\frac{4}{f(\alpha)}]-2} \frac{\partial^l \partial_l \sqrt{\rho}}{\sqrt{\rho}} = -V_F^i V_F^i - \frac{1}{2}(dt)^{[\frac{2}{f(\alpha)}]-1} \partial_l V_F^l \quad (21)$$

Equation (19) corresponds to the multifractal specific momentum conservation law, while Equation (20) corresponds to the multifractal density conservation law [24]. The specific multifractal potential Q expressed by (21) implies the existence of a specific multifractal force:

$$F^i = -\partial^i Q = -2\lambda^2(dt)^{[\frac{4}{f(\alpha)}]-2} \partial^i \frac{\partial^l \partial_l \sqrt{\rho}}{\sqrt{\rho}} \quad (22)$$

that quantifies the multifractality degrees of the motion trajectories.

Therefore, for the complex velocity fields (18), the dynamics of any complex system are described through multifractal Madelung "regimes" (i.e., Madelung equations at various scale resolutions). In this last context, the following consequences result:

- (i) Any complex system entities are in permanent interaction with a multifractal medium through the multifractal specific force (18) [29].
- (ii) Any complex system can be identified with a multifractal fluid, the dynamics of which is described by the multifractal Madelung model (Equations (19) and (20)).
- (iii) The velocity field V_F^i does not represent the contemporary dynamics; since V_F^i is missing from (20), this velocity field contributes to the transfer of the multifractal specific momentum and to the multifractal energy.
- (iv) Any analysis of Q should consider the "self" nature of the multifractal specific momentum transfer; then, the conservation of the multifractal energy and the multifractal momentum ensure the reversibility and the existence of the multifractal eigenstates.
- (v) If a multifractal tensor is considered:

$$\hat{\tau}^{il} = 2\lambda^2(dt)^{[\frac{4}{f(\alpha)}]-2} \rho \partial^i \partial^l \ln \rho \quad (23)$$

the equation defining the multifractal "forces" that derive from Q can be written in the form of a multifractal equilibrium equation:

$$\rho \partial^i Q = \partial_l \hat{\tau}^{il}. \quad (24)$$

Since $\hat{\tau}^{il}$ can be also written in the form:

$$\hat{\tau}^{il} = \eta(\partial_l V_F^i + \partial_i V_F^l) \quad (25)$$

with

$$\eta = \lambda(dt)^{[\frac{2}{f(\alpha)}]-1} \rho \quad (26)$$

a multifractal linear constitutive equation for a multifractal “viscous fluid” can be highlighted. In such a context, the coefficient η can be interpreted as multifractal dynamic viscosity coefficient of multifractal fluid.

4.2.2. The Compatibility of the Two Models of Describing the Release Dynamics through the Scale Relativity Theory

As anticipated, the two multifractal descriptions of the dynamics of complex systems, the multifractal Schrödinger description and the multifractal Madelung one, are not mutually exclusive but, on the contrary, are complementary. Let us explain this on the basis of the following hypotheses:

- (a) The dynamics of any complex system, independent of the two scale resolutions (differentiable and non-differentiable scale resolutions), are one-dimensional dynamics. It thus results that the conservation law for the multifractal states density (12) becomes

$$\partial_t \rho + \partial_x (J_x) = 0 \quad (27)$$

where

$$J_x = \frac{\lambda(dt)^{[\frac{2}{f(\alpha)}]-1}}{i} \left(\bar{\Psi} \frac{d\Psi}{dx} - \Psi \frac{d\bar{\Psi}}{dx} \right) - g A_x \Psi \bar{\Psi} \quad (28)$$

In particular, for the state function in the form (16), Equation (28) becomes

$$J_x = 2\lambda(dt)^{[\frac{2}{f(\alpha)}]-1} \left(\frac{ds}{dx} \right) \rho - g A_x \rho \quad (29)$$

- (b) The synchronization of the dynamics of any complex system at the two scale resolutions is achieved by “compensating” the speed fields V_D^l and V_F^l given by the restriction:

$$V_D^l = -V_F^l \quad (30)$$

Through (18) and (19), a dependence results between the amplitude $\sqrt{\rho}$ and phase s of the state function Ψ in the form:

$$\rho = \rho_0 e^{-2s} \quad (31)$$

with ρ_0 an integration constant.

- (c) The vectorial field $\mathbf{B}$ is uniform, a situation in which the potential vector $\mathbf{A}$ has the expression:

$$\mathbf{A} = \frac{1}{2}(\mathbf{r} \times \mathbf{B}) \quad (32)$$

In particular, for one-dimensional dynamics of any complex system, A_x can be chosen in the form:

$$A_x = \frac{1}{2} Bx, B = \text{const.} \quad (33)$$

Since, on the basis of the above hypotheses (a)–(c), Equation (29) takes the form:

$$J_x = -\lambda(dt)^{[\frac{2}{f(\alpha)}]-1} \frac{d\rho}{dx} - \frac{1}{2} g Bx \rho \quad (34)$$

Equation (27) becomes the multifractal equation Fokker–Planck:

$$\partial_t \rho + \partial_x \left(-\frac{1}{2} g B x \rho \right) + \partial_{xx} \left[-\lambda (dt)^{\left[\frac{2}{f(a)} \right] - 1} \rho \right] = 0 \quad (35)$$

Therefore, the correlations between the two descriptions, i.e., the multifractal Schrödinger and the multifractal Mandelung one, imply multifractal Fokker–Planck description of complex system dynamics.

With the notations:

$$\eta = \frac{1}{2} g B, D = 2\lambda (dt)^{\left[\frac{2}{f(a)} \right] - 1} \quad (36)$$

where η can be correlated with the strength of the hydrogel network and D is the fractalization degree, as previously defined, the solution of Equation (35) has the expression:

$$\rho(x, t) = \left(\frac{1}{2\pi(D/\eta)[1 - \exp(-2\eta t)]} \right)^{1/2} \exp \left[-\frac{[x - x_0 \exp(-\eta t)]^2}{2(D/\eta)[1 - \exp(-2\eta t)]} \right] \quad (37)$$

signifying that the density of states is a multifractal Gaussian whose average multifractal value decreases exponentially to zero and whose multifractal variance tends asymptotically towards (D/η) .

4.3. Implications of the Compatibility between the Two Models for Describing the Release Dynamics through Scale Relativity Theory. Model Validation

In the framework of the presented theoretical model, considering our aim to simplify the analysis of the release process, we will incorporate all the process variables, i.e., the synthesis parameters, that influence the hydrogel structure into one named fractalization degree.

The theoretical model established, through Equation (37), a direct correlation between the fractalization degree and the maximum efficiency of the polymer–drug system. Therefore, when calibrating the theoretical model on the experimental data of the release efficiency, the fractalization degree for each sample can be extracted (Table 5).

Table 5. Fractalization degree vs. reaction parameters.

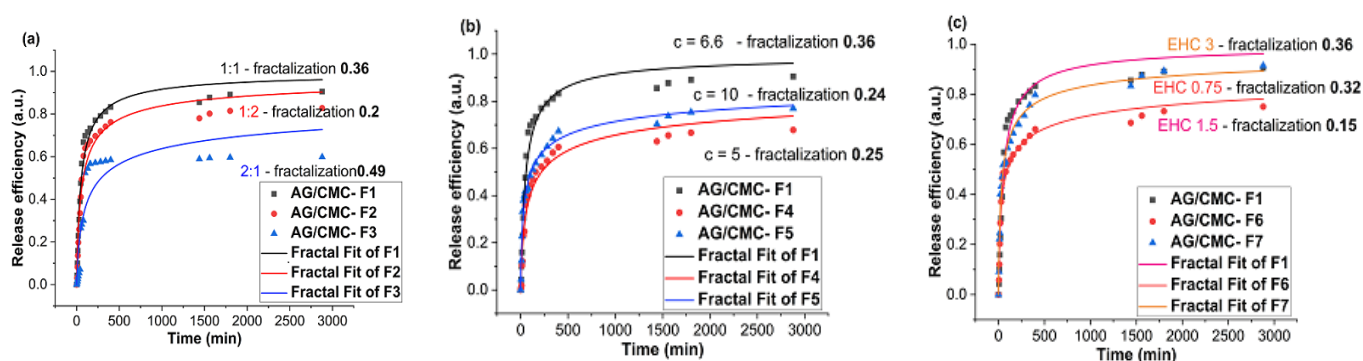
Sample Code	Process Variables						Fractalization Degree
	Concentration (%)	Molar Ratio	AG (g)	CMC (g)	NaOH (mL)	ECH (mL)	
F1	6.6	1:1	0.5	0.5	15	1.5	0.36
F2	15	1:2	0.5	1	15	1.5	0.20
F3	15	2:1	1	0.5	15	1.5	0.49
F4	10	1:1	0.5	0.5	10	1.5	0.24
F5	5	1:1	0.5	0.5	20	1.5	0.25
F6	6.6	1:1	0.5	0.5	15	0.75	0.15
F7	6.6	1:1	0.5	0.5	15	3	0.32

Regarding the issues mentioned previously related to unexpected behavior in respect to the dependence of released drug efficiency on the crosslinker amount, the values summarized in Table 6 show that the fractalization degree, which cumulates all the reaction parameters, can explain these, i.e., a proportionality between the fractalization degree and release efficiency.

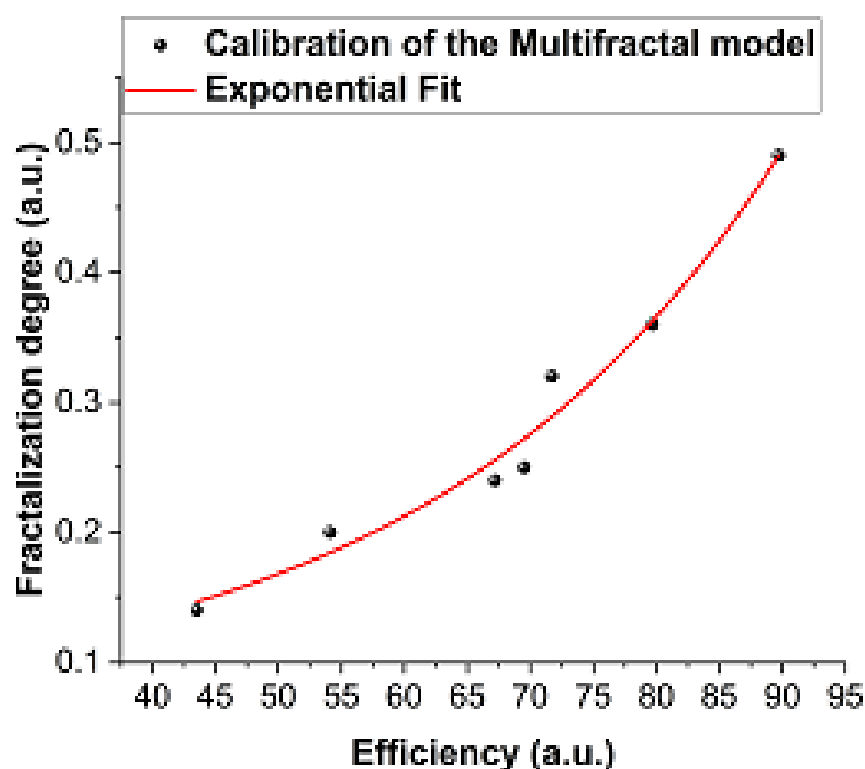
Table 6. Fractalization degree vs. crosslinker amount and released drug amount.

Sample Code	ECH Crosslinker (mL)	Swelling Ratio (%)	Release MT (%)	Fractalization Degree
F6	0.75	1273	75.04	0.15
F1	1.5	587	90.49	0.36
F7	3	362	91.41	0.32

This multivariable empirical approach emphasizes that, in the multifractal analysis plane, the fractalization degree of the system is one parameter that can quantify the overall outcome of all system interactions, generating a single parameter that can predict the behavior of certain polymer–drug combinations (Figure 5).

**Figure 5.** Comparative plots of fractal fits for different release kinetics: (a) F1, F2, F3 films, (b) F1, F4, F5 films, (c) F1, F6, F7 films.

The calibration of the model is described in Figure 6 where the efficiency of the system is plotted as a function of the fractalization degree as given by the model. An exponential function proved to be the best calibration fit.

**Figure 6.** The calibration to the multifractal model.

We can conclude that the presented multifractal model is able to describe the system behavior in regard to the release efficiency through a global parameter, the fractalization degree, that encompasses all reaction parameters.

5. Conclusions

This study proposed the preparation of a new hydrogel film based on crosslinking with epichlorohydrin of alginates and carboxymethyl cellulose. The obtained films were characterized in terms of their swelling capacity and loading/release drug ability as well as in the way in which they were influenced by the synthesis reaction parameters, such as the polymer concentration and crosslinker amount. The films' recovered fractions were high and ranged between 43% and 89%. The results showed that all films presented good swelling properties in water and a good ability to encapsulate and release the drug.

The drug release profiles revealed a fast release over 9 h followed by a slower phase (characterized by a constant release) until 48 h, with all values being in good concordance with the variable parameters considered. Therefore, we consider that these new hydrogel films have the potential to become systems for the controlled release of bioactive compounds with modulable properties in terms of released amount.

The proposed theoretical model analyzed the release process at the microscopic level and we described this process in regard to the release efficiency through a global parameter—the fractalization degree—associated with the system complexity. This encompasses all reaction parameters that influence the release process, thus, simplifying the release analysis. The calibration of the model on the experimental data revealed an exponential dependence of the release efficiency on the fractalization degree.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/polym13244461/s1>, Figure S1: SEM images of the AG/CMC films of all samples F1–F7.

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Article

Entrapment of *N*-Hydroxyphthalimide Carbon Dots in Different Topical Gel Formulations: New Composites with Anticancer Activity

Corina-Lenuta Savin ¹, Crina Tiron ^{2,*}, Eugen Carasevici ², Corneliu S. Stan ¹, Sorin Alexandru Ibanescu ³, Bogdan C. Simionescu ^{1,3} and Catalina A. Peptu ^{1,*}

¹ Department of Natural and Synthetic Polymers, Faculty of Chemical Engineering and Environmental Protection, "Gheorghe Asachi" Technical University of Iasi, 71, Prof., Dr. Docent Dimitrie Mangeron Street, 700050 Iasi, Romania

² Regional Institute of Oncology, 2-4, General Henri Mathias Berthelot Street, 700483 Iasi, Romania

³ "Petru Poni" Institute of Macromolecular Chemistry, 41A Aleea Grigore Ghica Vodă street, 700487 Iasi, Romania

* Correspondence: transcendenttiron@iroiasi.ro (C.T.); catipeptu@ch.tuiasi.ro (C.A.P.); Tel.: +40-765-677-356 (C.T.); +40-765-253-915 (C.A.P.)

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Abstract: In the present study, the antitumoral potential of three gel formulations loaded with carbon dots prepared from *N*-hydroxyphthalimide (CD-NHF) was examined and the influence of the gels on two types of skin melanoma cell lines and two types of breast cancer cell lines in 2D (cultured cells in normal plastic plates) and 3D (Matrigel) models was investigated. Antitumoral gels based on sodium alginate (AS), carboxymethyl cellulose (CMC), and the carbomer Ultrez 10 (CARB) loaded with CD-NHF were developed according to an adapted method reported by Hellerbach. Viscoelastic properties of CD-NHF-loaded gels were analyzed by rheological analysis. Also, for both CD-NHF and CD-NHF-loaded gels, the fluorescence properties were analyzed. Cell proliferation, apoptosis, and mitochondrial activity were analyzed according to basic methods used to evaluate modulatory activities of putative anticancer agents, which include reference cancer cell line culture assays in both classic 2D and 3D cultures. Using the rheological measurements, the mechanical properties of gel formulations were analyzed; all samples presented gel-like rheological characteristics. The presence of CD-NHF within the gels induces a slight decrease of the dynamic moduli, indicating a flexible gel structure. The fluorescence investigations showed that for the gel-loaded CD-NHF, the most intense emission peak was located at 370 nm (upon excitation at 330 nm). 3D cell cultures displayed visibly larger structure of tumor cells with less active phenotype appearance. The *in vitro* results for tested CD-NHF-loaded gel formulations revealed that the new composites are able to affect the number, size, and cellular organization of spheroids and impact individual tumor cell ability to proliferate and aggregate in spheroids.

Keywords: composite; *N*-hydroxyphthalimide; carbon dots; polymer gels; antitumoral activity

1. Introduction

Statistically, it has been reported that approximately 8 million people die from different types of cancer each year in the world; including breast, lung, liver, skin, and brain cancers, etc. So far, the main therapeutic methods for cancer treatment remain surgery and chemotherapy; however, researchers have made enormous efforts in the last years to develop new compounds that are better tolerated by patients during cancer therapy. In order to attack cancer cells and to reduce the same effect in the healthy cells, new treatments methods have been investigated. Among them, transdermal drug

delivery systems appear as a promising alternative strategy to carry antineoplastic agents due to certain several advantages such as increased drug solubility, better bioavailability, high stability, controlled drug release, prolonged half-life, selective organ or tissue distribution, and reduction of the total required dose [1]. Enhanced topical delivery of the active principle to the target site, including those for cancer therapy, can be achieved by noninvasive drug delivery systems which can ensure sustained therapy with a single application, thus avoiding first-pass hepatic metabolism, gastric degradation, or frequent dosing and also the inconvenience of parenterals [2].

Carbon dots (CD) are a new star of the carbon nanomaterials family; their unique properties mean they are attractive materials for a wide range of applications such as bioimaging, biosensing, drug delivery, optoelectronics, photovoltaics, and photocatalysis [3,4]. Therefore, more and more researchers are paying significant interest to the synthesis, properties, and applications of these carbon nanostructured materials. However, the investigations toward drug delivery for biomedical applications are still at their beginning [5], along with their putative antitumoral properties. Precursors used to synthesize carbon dots determine their properties, creating a new opportunity for finding new anticancer molecules for certain types of cancer (personalized cancer therapy) [6]. Gels based on natural and/or synthetic polymers represent a good pathway for biomedical applications, due to their hydrophilic properties and their biocompatibility. Physical gels are usually prepared by mixing a polymer and a solvent, with or without thermal treatment, resulting a homogeneous material with remarkable mechanical properties related to the aggregation process of the polymer chains [7]. So far, different types of natural or synthetic polymers have been used to prepare polymer–drug conjugates, produced mainly by physical entrapment of the biologically active principle into macromolecular hydrogels, micro/nanoparticles, or liposomes in therapeutic approaches for the treatment of various cancers.

Polysaccharides are renewable resources found in all living organisms, which have been widely used in different important fields such as the biomedical, pharmaceutical, and food industries and tenvironmental remediation. Their unique properties, such as biocompatibility, biodegradability, nontoxicity, hydrophilicity, stability, and structural variability, allow a high capacity for carrying biological information, making them suitable as a promising natural biomaterial [8]. In recent years, the scientific community has used polysaccharides in a broad range of biomedical applications, such as drug delivery systems for the treatment of inflammation, cell–cell recognition, immune responses, metastasis (tumor cells are spreading from primary tumor to secondary organs e.g., from breast to lungs), and in tissue engineering in scaffolds and wound dressings [9]. Sodium alginate (AS) is a natural polysaccharide, extracted from brown seaweed, used in various biomedical applications such as drug delivery, tissue engineering, and wound healing, due to the attractive properties such as biocompatibility, low toxicity, ease of manipulation, and mild gelation [10]. Another attractive polysaccharide is carboxymethylcellulose (CMC) in its sodium salt form. CMC is the major cellulose ether, also known as cellulose gum, formed from carboxymethyl ether groups. CMC's valuable properties, such as its hydrophilic nature, capacity to form gels at high concentration, and thixotropy, make it suitable for biomedical applications such as drug delivery and tissue engineering [11]. From the synthetic polymers category, carbomers (CARB) are a high-molecular-weight crosslinked polymer of acrylic acid, which are playing an important role in many commercial products such as gels, creams, and lotions, providing viscosity, stabilization, and suspension properties [12].

In this article, we focus on the embedding of *N*-hydroxyphthalimide carbon dots (CD-NHF) in different continuous matrices formed of natural (alginate or carboxymethyl cellulose) or synthetic (CARB) polymers. Due to the recently proven antitumoral activity of NHF [13], CD-NHF were developed and the composite was proven also to induce breast cancer cell apoptosis at doses that only marginally affect normal cell counterparts. The reason for embedding CD-NHF is on one hand, to reduce the aggregation tendency of CD which can affect the treatment efficacy, and on the other hand, to be protected by a polymer environment against chemical modification, which can also affect their properties. The fluorescence analysis demonstrates the CD-NHF presence within the gel.

Appropriate gel properties were investigated from a rheological point of view considering different polymer concentrations; the optimum formulation has been used as the matrix for CD-NHF and further biological investigations.

Cancer is a disease that causes cells to change and grow in an uncontrolled manner. Many—if not all—of the tumor aggressivity behaviors are caused by key molecular defects in multiple biochemical pathways controlling cell survival, proliferation, differentiation, and integration in histological tisular structures and immune interactions. Apoptosis is a cell suicide program that plays an important role in tissue homeostasis by eliminating unnecessary cells [14,15]. The deregulation of the apoptotic pathway results in variety of diseases and has been shown to be involved in cancer cell resistance to conventional anticancer therapy [16]. There are two critical pathways of apoptosis—the extrinsic pathway (death receptor-mediated pathway) and the intrinsic pathway (mitochondria-mediated pathway)—and caspases are fundamental players in both pathways [17]. Mitochondria are vital organelles for energy production and intracellular Ca^{2+} homeostasis and they are involved in a variety of cellular processes, including differentiation, proliferation, and apoptosis. It has been shown that abnormal mitochondrial dynamics have an important role in tumorigenesis. Also, the increased anaerobic glycolysis activity in neoplastic cells was the result of a dysfunction of the mitochondrial activity [18].

We consider that CD-NHF incorporation in gels could be a convenient way to manipulate its local availability (and effects) and its persistence. While the actual mechanism of action is still incompletely understood for many emerging nanoformulations, interactions with extracellular matrix components are plausible objectives in many drug designs. The incorporation of CD-NHF in both gel varieties utilized in our assay was thought to be promising for possible targeted applications in certain malignancies (perhaps including basocellular and spinocellular skin carcinomas). The effect of gels with CD-NHF in 4T1 (mouse mammary breast cancer), MDA-MB-231 (human mammary breast cancer), HDMVECN (primary dermal microvascular endothelial cells), Balb/c-5064 (mouse dermal microvascular endothelial cells), A375 (human malignant melanoma), and B16F10 (mouse malignant melanoma) cells in 2D (cultured cells in normal plastic plates) and 3D (Matrigel) models was evaluated by assessing cell viability, mitochondrial activity, and apoptosis.

2. Materials and Methods

2.1. Materials

The following materials were used for the experiments: the carbomer Ultrez 10 was provided by Antibiotice SA Iasi (Iasi, Romania); *N*-hydroxyphthalimide, medium-viscosity sodium alginate (extracted from *Macrocystis pyrifera*), and glycerin (99.9%) were purchased from Sigma-Aldrich (St. Louis, MO, USA); medium-viscosity carboxymethyl cellulose from Fluka (Buchs, Switzerland); and reagent-grade ethanol (EtOH) and Milli-Q ultrapure distilled water from Merck Chemicals (Darmstadt, Germany). The chemical reagents used in this study were of analytical-grade purity and were used without further purification. MDA-MB-231 cells were purchased from the American Type Culture Collection, Rockville (ATCC), VA, USA; 4T1 cells were from the American Type Culture Collection, Rockville, MD, USA; bovine serum was purchased from Sigma-Aldrich; HDMVECN (primary dermal microvascular endothelial cells) were from ATCC; Balb/c-5064 (mouse dermal microvascular endothelial cells) were from Cell Biologics (Chicago, IL, USA); and Matrigel Matrix from BD Biosciences (356234), Bedford, MA, USA. For biological tests, the Live and Dead Cell Assay (Abcam ab115347), CellTiter-Blue® Cell Viability Assay (Promega, (Madison, WI, USA), MitoTracker™ Red (Molecular Probe, Eugene, OR, USA), Matrigel (Corning, Bedford, MA, USA), and Caspase-3/7 Assay (Promega, Madison, WI, USA) were used according to the manufacturer's recommendations.

2.2. Methods

2.2.1. Preparation of Gel Formulations Based on CARB, AS, and CMC

Antitumoral gels based on CARB, AS, and CMC, respectively, and loaded with CD-NHF were prepared according to the method reported by Hellerbach et al. [19] with slight modification. CD-NHF were prepared according to a protocol reported by Stan et al. [4]. Prior to gel embedment, the carbon dots were prepared through pyrolytic processing of an NHF precursor using an experimental setup presented in Figure S1. Briefly, in a typical synthesis procedure, 0.1 g of NHF is thermally processed in carefully controlled conditions (temperature of the main sequence of the pyrolytic process is 253 °C within 30 min), leading to a typical configuration of the carbon dots consisting of a carbonaceous core with a surface highly decorated by various functional groups. The slightly different processing parameters compared with the previously reported preparation path are due to the different experimental setup, which in this work, was reconfigured and extended in order to increase both the reproducibility of the carbon dots' preparation path and the resultant per batch quantities. The resultant reaction mass is primarily dispersed in high-purity water kept cooled at 4–5 °C. In the next stages, the suspension is centrifuged at 10,000 rpm for 15 min and the supernatant is collected and freeze-dried, with a fine powder of carbon dots being obtained. A series of gel formulations were prepared by mixing different quantities of CARB, AS, or CMC (Table 1) in a solution mixture consisting of 10 mL distilled water and 3 mL ethanol in glass vessels at room temperature. In order to achieve a uniform gel formulation, a T 18 digital ULTRA-TURRAX disperser by IKA was used (10 min, 10,000 rpm). The gelling agent's concentration ranged from 3.8 to 5.8% (Table 1). In order to prepare the gel formulations containing CD-NHF (CARB-F4, AS-F6, or CMC-F6, respectively), the CD-NHF (3.84 mg/mL) was added into the water–ethanol solution and dispersed by sonication for 1 min at room temperature using a sonication bath (Bandelin Sonorex, Berlin, Germany), followed by adding CARB, AS, or CMC. The obtained gel formulations and CD-NHF gel (Figure S3) were stored at 4 °C in a refrigerator. The basic molecular formulas for the polymers and CD-NHF used in order to obtain the gel formulations with and without CD-NHF are: sodium alginate (AS)— $(C_6H_8O_6)_n$, carboxymethyl cellulose (CMC)— $[C_6H_7O_2(OH)_2OCH_2COONa]_n$, Ultrez 10 carbomer (CARB)— $(C_3H_4O_2)_n$, and *N*-hydroxyphthalimide (NHF)— $C_8H_5NO_3$.

Table 1. Polymeric composition of various CARB, AS, and CMC gel formulations. CARB: carbomer Ultrez 10; AS: sodium alginate; CMC: carboxymethyl cellulose; GLY: glycerin.

Sample Code	CARB, %	AS, %	CMC, %	GLY, %	Distilled Water (mL)	Ethanol (mL)	CD-NHF (g)
CARB-F1	3.8	-	-	-	10	3	-
CARB-F2	4.6	-	-	-			-
CARB-F3	5.8	-	-	-			-
CARB-F4	4.6	-	-	-			0.050
AS-F1	4.6	4.6	-	-			-
AS-F2	4.6	4.6	-	9.2			-
AS-F3	3.5	4.6	-	8.1			-
AS-F4	2.3	4.6	-	6.9			-
AS-F5	1.2	4.6	-	5.8			-
AS-F6	4.6	4.6	-	5.8			0.050
CMC-F1	2.9	-	2.9	-			-
CMC-F2	2.9	-	2.9	5.8			-
CMC-F3	3.6	-	2.9	6.5			-
CMC-F4	4.3	-	2.9	7.2			-
CMC-F5	5.8	-	2.9	8.7			-
CMC-F6	3.6	-	2.9	6.5			0.050

2.2.2. Characterization of the Prepared NHF Carbon Dots

The morpho-structural characteristics of the prepared NHF carbon dots were essentially similar to those previously reported [4]. In brief, the XPS survey and high-resolution O1s, N1s, and C1s spectra

revealed the presence of the defect-rich graphitic core highly decorated with various terminal functional groups, while the recorded FT-IR spectra revealed the modifications/reconfigurations occurring through thermal processing of the NHF precursor and confirmed by XPS results which were revealing the presence of various functional groups attached to the graphitic core, which are essentially responsible for both optical and antitumoral properties of this type of carbon dots. In Figure S2 are presented the recorded spectra of the NHF precursor and resultant carbon dots, and several specific vibrations of various groups are detailed. Interesting details regarding the morphology of the prepared carbon dots were highlighted by the HR-TEM investigation. Figure 1 presents the recorded micrographs of two samples obtained by depositing a highly diluted chloroform-dispersed carbon dot solution on 300-mesh carbon-plated copper grids. Both images suggest a cluster organization of smaller entities, which presumably are individual carbon dots. While their aspect could suggest individual carbon dots, it is also possible to be attributed to even smaller clusters, as can be seen in Figure 1b.

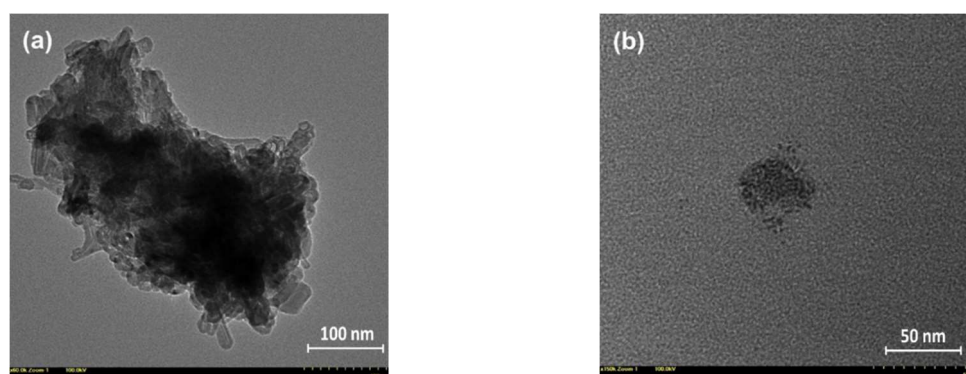


Figure 1. Recorded HR-TEM images of the carbon dots prepared from *N*-hydroxyphthalimide (CD-NHF): (a) CD-NHF with an average size of the clusters in 100–300 nm range and (b) CD-NHF with an average size of the smaller entities in 2–4 nm range

2.2.3. Rheological Studies

Viscoelastic properties of all gel formulations were evaluated in triplicate by using an Anton Paar Physica MCR 501 (Graz, Austria) rheometer. The modular rheometer is equipped with an electronically commutated synchronous motor (ECMotor, Graz, Austria), allowing rheological testing in controlled stress (CS) and controlled strain (CR) modes. Also, the instrument allows the individual creation of complex real-time tests containing a large number of different intervals under CS or CR control, both in rotational and oscillatory modes. The rheometer is provided with an H-PTD200 system for the temperature control. A parallel-plate geometry specially designed for gels (with serrated plates to avoid slippage) with a diameter of 50 mm was chosen as the measuring system. The freshly prepared, well-homogenized gel formulations were introduced into the measuring cell and analyzed. The rheometer has an intelligent configuration system for automatic identification and configuration—“Tool Master”—which performs automatic transfer of the parameters of the measuring system (constructive characteristics, operating constants, geometry) and temperature control to the Rheoplus program. The chip integrated into the geometry contains all data connected to that and transfers them automatically to the program.

2.2.4. Fluorescence Analysis

The fluorescence analysis of CD-NHF and CD-NHF-loaded gel formulations were recorded on a Horiba Fluoromax 4P (Fluor Essence Version 35.1.20) provided with the Quanta-φ integration sphere. Also, a visual testing of photoluminescence properties was performed using a Philips UVA TL4WBLB lamp (München, Germany) with the emission maximum located in the 370–390 nm range and a 50 mW, 440 nm laser diode.

2.2.5. Cell Culture

MDA-MB-231 cells were cultured in F-12K medium supplemented with 100 U/mL of penicillin and 100 µg/mL of streptomycin and 5% bovine serum; 4T1 cells were cultured in RPMI-1640 supplemented with 100 U/mL of penicillin and 100 µg/mL of streptomycin and 10% bovine serum; HDMVECn (primary dermal microvascular endothelial cells) and Balb/c-5064 (mouse dermal microvascular endothelial cells) were cultured in Vascular Cell Basal Medium supplemented with Microvascular Endothelial Cell Growth Kit-BBE ATCC® PCS-110-040; A375 (human malignant melanoma) and B16F10 (mouse malignant melanoma) were cultured in Dulbecco's Modified Eagle's Medium supplemented with 100 U/mL of penicillin and 100 µg/mL of streptomycin and 10% bovine serum.

2.2.6. Cell Proliferation and Apoptosis Activity

For cell viability estimation, we used the CellTiter-Blue® Cell Viability Assay (Promega). Cells were seeded into a 96-well flat-bottomed tissue culture plate at a density of 2000 cells/well and allowed to adhere to the plate by incubating at 37 °C under 5% CO₂ overnight. Following cell attachment, the cells were incubated with the tested CD-NHF at 5% concentrations (50 µg/mL) for 72 h. For all in vitro experiments, we used the 5% CD-NHF concentration, based on our preliminary results. Control cells were treated with phosphate-buffered saline (PBS), which was equivalent to the amount of PBS used as vehicle. After each of the 72 h treatment time periods, 50 µL of cell viability solution was added to each well and the plate was reincubated for 4 h before fluorescence recording using a multiplate microplate reader (FilterMax F5, Sunnyvale, CA, USA). Apoptosis was assessed using the Caspase-3/7 Assay (Promega) according to the manufacturer's indications.

2.2.7. Mitochondrial Activity

For mitochondrial assay, MitoTracker™ Red (Molecular Probe, Eugene, OR, USA) cells were incubated for 72 h with CD-NHF and then subjected to 1 µg/mL MitoTracker and incubated for 30 min before the fluorescence of the resultant solutions was determined at 590 nm using a multiplate microplate reader.

2.2.8. 3D Matrigel Assays

The 3D Matrigel assays were conducted with 1000 cells seeded in Ibidi plates between 2 layers of Matrigel (BD Matrigel Matrix, Growth Factor Reduced (BD Biosciences)) and cultured for 14 days before microscopy analysis (TissueGnostic rig, Vienna, Austria, Europe). Twelve hours post-seeding, 3D embedded cells began to be treated with gels (CARB-F2, CMC-F3, AS-F5) alone and with gels containing 5% (50 µg/mL) CD-NHF (CARB-F4, CMC-F6, AS-F6) for 72 h. After 72 h, the treatments were removed and replaced with normal 3D Matrigel medium (medium corresponding to every cell type supplemented with 2% fetal bovine serum (FBS) and 1% Matrigel). The Live and Dead Cell Assay (Abcam) was used according to the manufacturer's instructions. Nuclei were counterstained with NucBlue Live Ready Probes Reagent (Thermo Fisher Scientific, Eugene, OR, USA). Fluorescence pictures were acquired at 20× magnification using a Zeiss Axio Observer Z1 Fluorescence Microscope from TissueGnostics rig. Single focal plane images were acquired using Tissue FAXS 4.2 software. The TissueQuest 6.0 software was used for total area segmentation analysis and to quantify the area and sum intensity of fluorescence signal for each spheroid (Figures S4 and S5).

2.2.9. Statistical Analysis

GraphPad Prism was used for statistical analysis using tests stated in the figure legends. Grouped analyses were performed by *t*-test. Significance was established when $p < 0.05$.

3. Results and Discussion

3.1. Preparation of Gel Formulations

Previous studies concerning the preparation of physical gels based on natural or synthetic polymers, namely carbomers, sodium alginate, and carboxymethyl cellulose, have pointed out that these materials are nontoxic and nonhazardous and easily synthesized. We aimed to prepare different topical CD-NHF-loaded gel formulations based on polymers with antitumoral activity. Herein, physical gel formulations based on CARB, AS, and CMC were synthesized according to a slightly modified version of the method reported by Hellerbach et al. [19]. This method offers some advantages compared with other methods reported, such as being simple, rapid, and consistent; performed in the absence of crosslinkers (hence, we eliminated the possibility of toxic chemical contamination commonly associated with methods which use covalent crosslinkers); and the product is a homogeneous soft material formed at room temperature.

Different concentrations of CARB, AS, or CMC were tailored and their effects were observed. First, simple gels were prepared and analyzed before loading with CD-NHF. The gel formulations with or without CD-NHF obtained were stable, odorless, transparent, and highly viscous. Of them, the best formulations presenting a suitable consistency and the best viscoelastic properties, respectively, were considered for further study and loading with CD-NHF, namely samples CARB-F2, AS-F5, and CMC-F3. The carbon-dot-loaded gel samples were denoted as CARB-F4, AS-F6, and CMC-F6.

3.2. Rheology Studies

The viscoelastic nature of polymer gels plays an important role in their adhesion properties; a very important characteristic considering different biomedical applications. Gels based on natural or synthetic polymers exhibit an excellent adhesion property due to both the elastic and viscoelastic properties.

Using the rheological measurements, the mechanical properties of the best formulations were analyzed. All analyzed samples presented rheological characteristics of a gel. The obtained results showed that viscosity was directly dependent on the polymeric content of the formulations. During the rheological measurements, both dynamic moduli were constant. As can be observed from Figure 2, the storage modulus (G') exhibited higher values than the loss modulus (G'') over the entire strain range of 0.01 to 100%, indicating the gel-like behavior (Figure 2). The results obtained by rheological analysis confirmed that CARB-F2, AS-F5, and CMC-F3 samples were optimized and further considered for loading with CD-NHF, due to the gel equilibrium modulus. The presence of CD-NHF (for samples CARB-F4, AS-F6, and CMC-F6) in the gel structure has a significant influence over both dynamic moduli. In the case of the AS-F6 sample, the CD-NHF addition induced a slight increase of the dynamic moduli, indicating stiffness of the gel structure, whereas in CARB-F4 and CMC-F6 gels, the effect of CD-NHF addition was exactly the opposite, leading to a slight decrease of the dynamic moduli, indicating a flexible gel structure. Also, the amplitude sweep allows determination of the limits of the linear viscoelastic range for the prepared gels. These values are not affected by the presence of CD-NHF (Table 2).

Table 2. The limits of the linear viscoelastic range (γ_{LVE}) for the tested gel formulations.

Sample Code	γ_{LVE} (%)
CARB-F2	0.25
CARB-F4	0.25
AS-F5	5
AS-F6	5
CMC-F3	10
CMC-F6	10

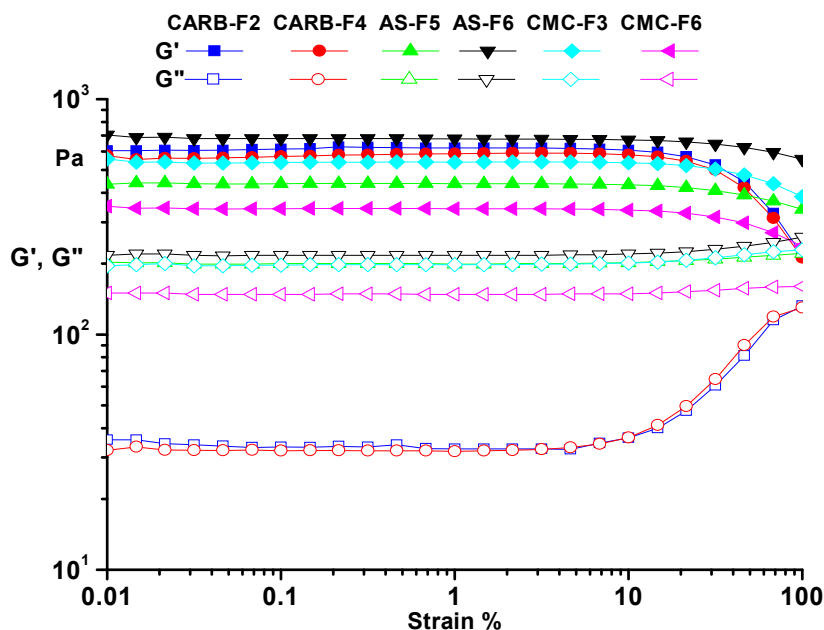


Figure 2. Amplitude sweep for simple gels (CARB-F2, AS-F5, and CMC-F3) and gels with CD-NHF (CARB-F4, AS-F6, and CMC-F6). G' : storage modulus; G'' : loss modulus.

All tested gels showed a solid-like behavior, as the storage modulus (G') was always larger than the loss modulus (G''). As one can observe from Figure 2, the CARB and CMC gel formulations have a more flexible structure than the AS gel, confirmed by storage modulus (G') values (G'_{CARB} (620 Pa) > G'_{CMC} (540 Pa) > G'_{AS} (437 Pa)). Frequency sweep tests allow observation of the elastic response of gels. The presence of the hydrogen bonding is evidenced by the frequency dependence of the moduli over the entire range of 0.05–500 1/s ($\gamma_{\text{LVE}}(\text{CARB}) > \gamma_{\text{LVE}}(\text{CMC}) > \gamma_{\text{LVE}}(\text{AS})$) (Figure 3).

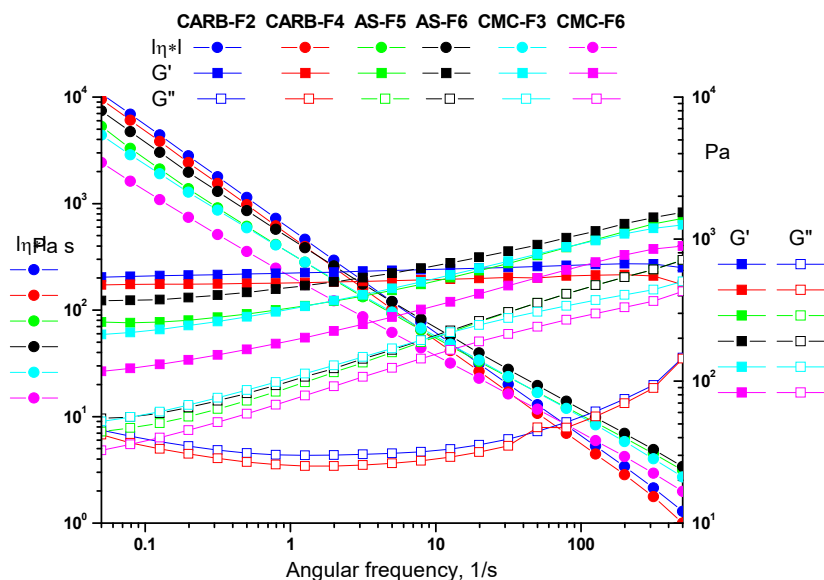


Figure 3. Frequency sweep for simple gels (CARB-F2, AS-F5, and CMC-F3) and gels with CD-NHF (CARB-F4, AS-F6, and CMC-F6).

3.3. Fluorescence Analysis

In order to confirm the presence of CD-NHF in the gel matrices, the formulations were further evaluated through fluorescence analysis. Fluorescence spectra exhibit the maximum emission (λ_{em}) at 424 nm when using an excitation wavelength (λ_{ex}) of 370 nm for CD-NHF (Figure 4 and Table S1).

Also, Figure 4 illustrates the emission profile of CD-NHF-loaded gels (CARB-F4, AS-F6, and CMC-F6) at three different excitation wavelengths ranging from 370 to 410 nm. Moreover, the recorded results (Table S1) revealed that at 370 nm, in the case of CARB, the blue-light emission was not significantly affected by the CARB matrix. The observed difference between CD-NHF blue-light emission and CARB matrix was only 1.5% (Table S1). When AS and CMC were used as a matrix for the entrapment of CD-NHF, an 11% decrease of the blue-light emission was observed, compared to the case of the CARB matrix and CD-NHF. Herein, we can conclude that in this case, the polymer matrix clearly plays an important role for the modification of CD-NHF optical properties.

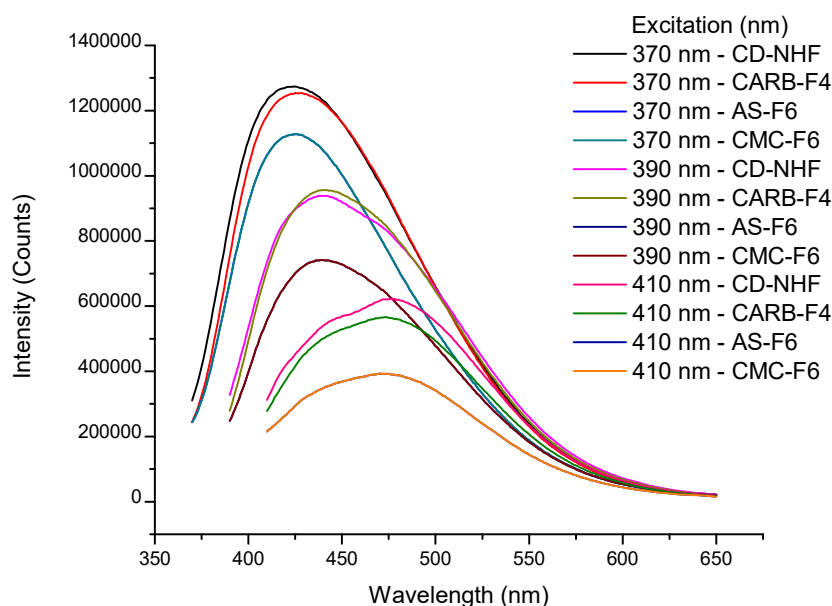


Figure 4. Emission spectra of CD-NHF suspended in H₂O: CARB-F4, AS-F6, and CMC-F6 samples.

3.4. In Vitro Studies

Analysis of the cytostatic or cytolytic or growing pattern modulatory activities may be done with end point assays or live cell imaging techniques. 3D cultures create a better similarity between the cultured cells and the living organism. The basic methods to evaluate modulatory activities of putative anticancer agents include reference cancer cell line cultures assays in both classic 2D and 3D cultures.

Viability of HDMVECN cells (primary dermal microvascular endothelial cells) in 2D culture treated with simple gel formulations (CARB-F2, AS-F5, and CMC-F3) and gel formulations containing CD-NHF (conc. 5%; CARB-F4, AS-F6, and CMC-F6) was not affected by CARB-F4 and CMC-F6, whereas, interestingly, AS-F6 had a significant effect (Figure 5).

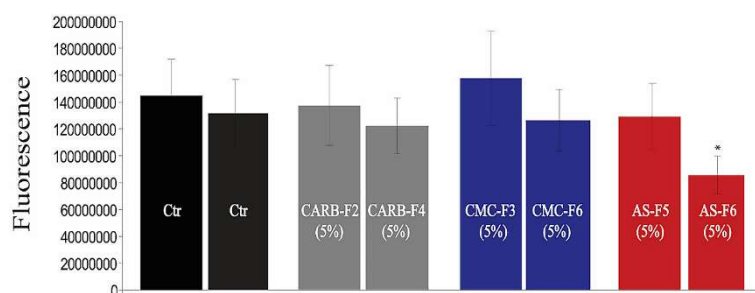


Figure 5. Viability of normal dermal cells (Primary Dermal Endothelial Cells (PDMC)) for gel formulations without and with CD-NHF ($N = 15$ wells/column from two independent experiments). Ctr (Control).

Proliferation activity of melanoma cell lines (2D system) was not affected by the presence of CARB-F2, CMC-F3, or AS-F5 formulations (Figure 6a), respectively, while cell viability was affected in a gradual manner in cells treated with CARB-F4, CMC-F6, and AS-F6 formulations (Figure 6b,c).

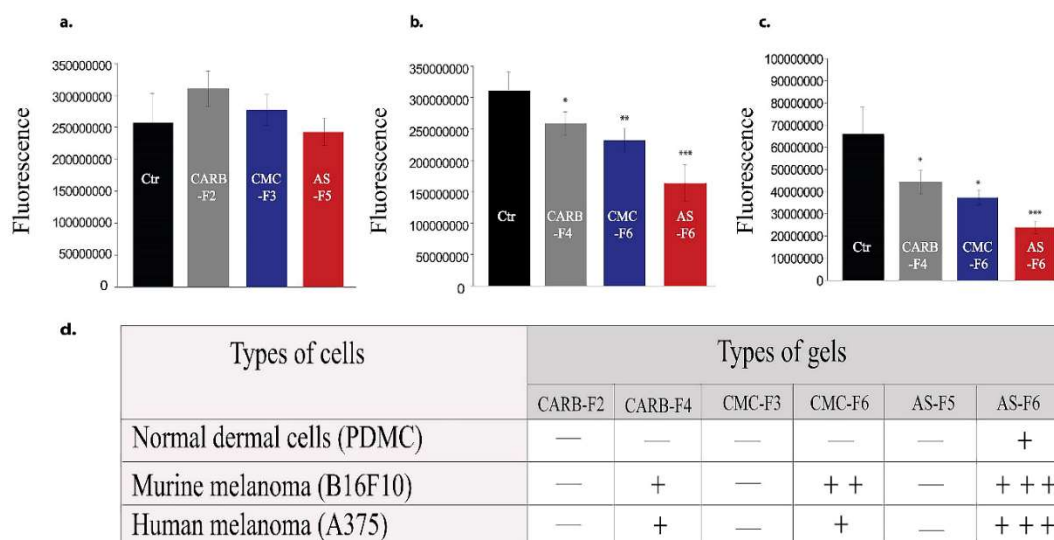


Figure 6. Murine and human melanoma cell viability for gel formulations without (a) and with (b,c) CD-NHF (conc. 5%). $N = 15$ wells/column from two independent experiments; viability of treated groups is expressed relative to the control group. (a) Skin cancer; (b) murine melanoma (B16F10); (c) human melanoma (A375); (d) scoring of significance effect, where +++ (***) $p = 0.0003$, ++ (**) $p = 0.01$, + (*) $p = 0.04$, and — = no effect.

In the 3D Matrigel assay, the human melanoma cells form visible and consistently larger colonies compared with the same cells treated with CD-NHF-loaded gel formulations (conc. 5%) (Figure 7 and Figure S4).

By comparing the left column (Matrigel) with the median (simple gel formulations), it can be observed that spheroids are less compact, with decreased cellular uniformity and a tendency to dissociate. In the median column, we find that CMC and AS gel formulations are more unfavorable for maintaining a homogeneity of metabolic activity (the green shade in the L/D kit reflecting live cell population) and induce (or amplify) the dissociation tendency of cells from differentiated proliferation aggregates. The differences found in the analysis of 3D spheroids can be caused by the fact that the different types of gel formulations used either provide different stability conditions for extracellular molecules that favor maintaining a differentiated cellular phenotype, or employ different receptors for adhesion to cell membranes favorable to maintaining both differentiation and inhibition of the cellular dissemination tendency [20,21]. Comparing the median column (single gel formulations) with that on the right (gel formulations supplemented with 5% CD-NHF), we find that exposure to CD was unfavorable to cell proliferation, with global cell counts being significantly impaired and the quantitative weight of cells in invasion reduced. We specify that the shade of the green marker is proportional (L/D kit) to the level of cell metabolic activity. Smaller color intensities can be determined by both a decreased number of cells and more restricted metabolic activity in CD-NHF exposure. Also, the morphological aspect of spheroids under the influence of gel formulations containing CD-NHF is presented in Figure 8.

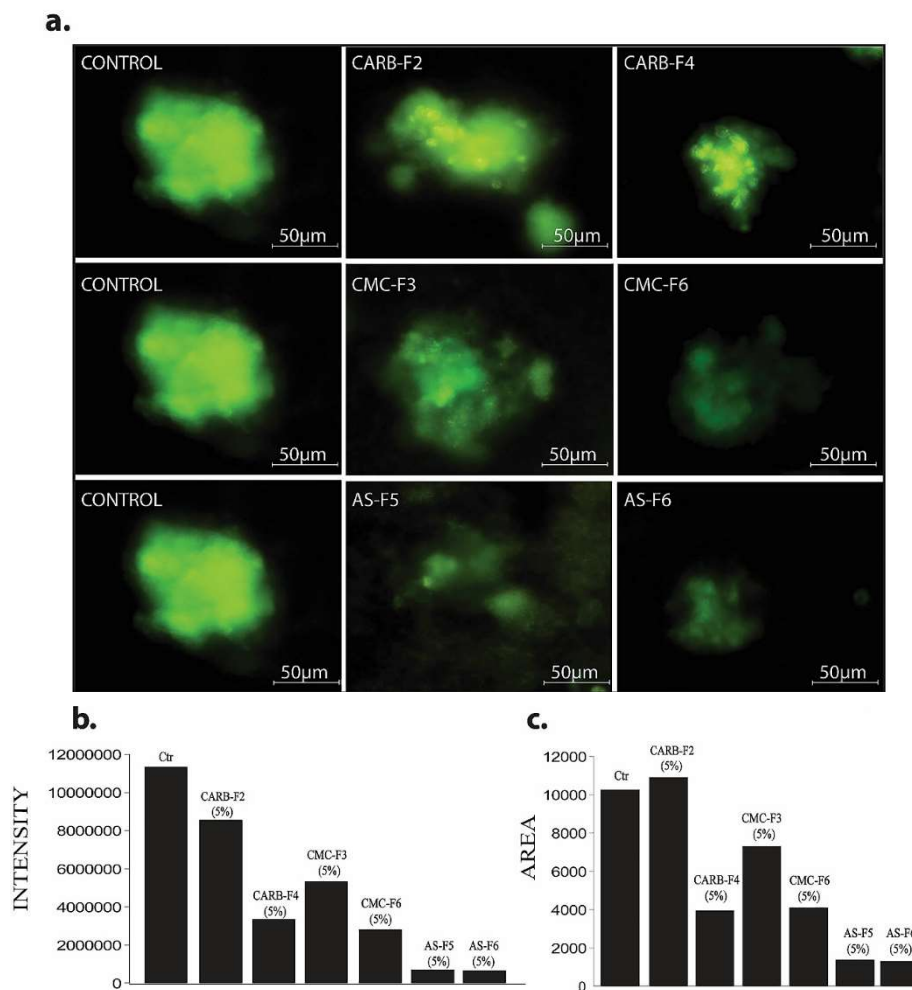


Figure 7. (a) 3D Matrigel assay for human melanoma cell cultures in standard conditions (left) and in the presence of added CD-NHF-free gel (middle) versus 5% CD-NHF-loaded gel (right). $N = 3$ 3D Matrigel cultures per type of gel with or without CD-NHF; 20 $\times$ microscope objective. (b) Sum intensity of fluorescence signal; (c) area of spheroids expressed in μm^2 .

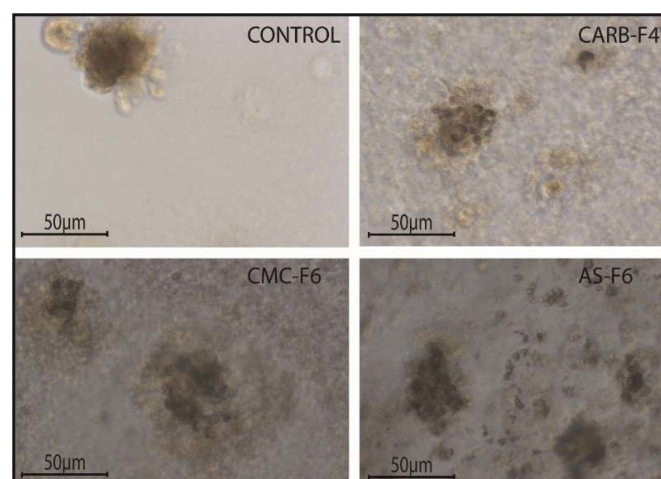


Figure 8. The morphological aspect of spheroids under the influence of gel formulations. $N = 3$ 3D Matrigel cultures per type of gel with or without CD-NHF; 20 $\times$ magnification.

Also tested was the viability potential of two different breast cancer cell lines, namely 4T1 (mouse breast cancer) and MDA-MB-231 (human breast adenocarcinoma), in 2D and 3D culture models.

The viability of the breast cancer cell lines 4T1 (mouse breast cancer) and MDA-MB-231 (human breast adenocarcinoma) in the 2D culture system treated with CARB-F4 was affected (Figure 9a). Mitochondrial activity was affected upon CARB-F4 treatment (Figure 9b). In the 3D Matrigel assay, the malignant cells form visible and consistently larger colonies compared with same cells treated with CARB-F4 (Figure 9c). Moreover, the apoptotic foci in 3D cultures treated with gels containing CD-NHF were significantly higher (Figure 9c; white squares in boxes 2 and 4). The apoptotic pattern in the whole spheroid body is also distorted: while in native culturing, inner cells (typically associated with hypoxic conditions inside large spheroids) in spheroids began the apoptotic program, at 5% CD-NHF in exposed spheroids, this form of cell death is initiated in all spheroid compartments. 3D cell cultures displayed visibly larger structure of cancer cells with reduced active phenotype appearance. This suggests that treatment with the CARB-F4 formulation could also either induce cell senescence or cell dormancy—a topic that deserves further clarification. Together, these data suggest that CD-NHF-loaded gels affect the number, size, and cellular organization of spheroids and impacts individual cancer cell ability to proliferate and aggregate in spheroids.

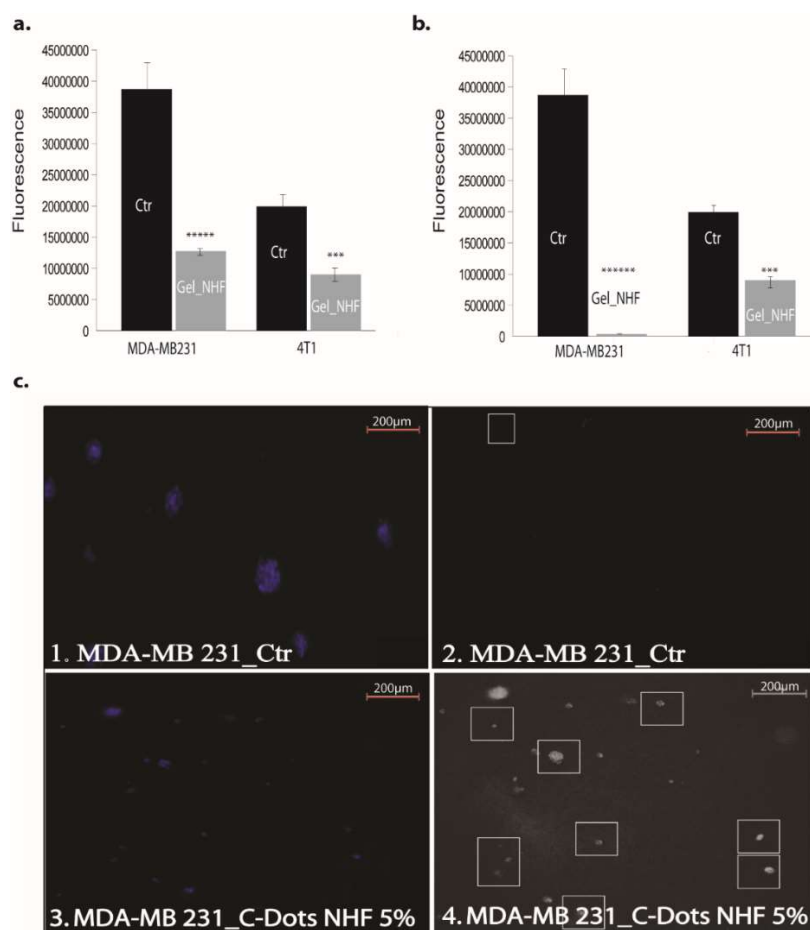


Figure 9. The effects of gels with 5% CD-NHF in 2D and 3D culture. (a) Viability of MDA-MB-231 (human breast adenocarcinoma 33.3%) and 4T1 (mouse mammary breast cancer 44.87%) cell lines treated with gels containing 5% CD-NHF ($N = 8$ wells/column from two independent experiments); (b) mitochondrial activity in MDA-MB-231 (human breast adenocarcinoma 2%) and 4T1 (mouse mammary breast cancer 49.2%) treated with gels containing 5% CD-NHF ($N = 8$ wells/column from two independent experiments); (c) apoptosis evidence in MDA-MB-231 by immunofluorescence microscopy and 3D Matrigel culture treated with gels containing 5% CD-NHF ($N = 6$; 3 Matrigel culture controls and 3 Matrigel cultures treated with 5% NHF). (c1,c3) represent NucBlue nuclei staining; (c2,c4) reflect apoptosis. Picture magnification 5 $\times$; where *** $p < 0.0005$, ***** $p < 0.000005$, ***** $p < 0.0000005$.

4. Conclusions

This paper reported the development via a physical method of three topical gel formulations loaded with CD-NHF, which were evaluated from the point of view of the modulatory activities of putative anticancer agents. The obtained results indicate that CD-NHF-loaded gels matrices present complex and interesting cell modulatory activities which are relevant for cancer control in potential clinical applications. The mechanical properties of gels were investigated, concluding that the presence of CD-NHF in the gels induced a slight decrease of the dynamic moduli, indicating a more flexible gel structure. The fluorescence analysis confirmed the presence of the embedded CD-NHF in all gel formulations. Also, worth noting is the potential antitumoral activity of the gel formulations loaded with CD-NHF. The most important finding is that the CD-NHF-loaded gel formulations are able to affect the number, size, and cellular organization of spheroids, while also having a significant impact on the individual tumor cell's ability to proliferate and aggregate in spheroids.

Supplementary Materials: The following are available online at <http://www.mdpi.com/1999-4923/11/7/303/s1>, Figure S1. Experimental setup used to prepare carbon dots. Figure S2. FT-IR spectra for CD-NHF. Figure S3. Photos of gels without and with CD-NHF under (a) white and (b) UV light. Figure S4. Spheroid fluorescent staining using green/FITC (left column) for live cells, blue/NucBlue for nuclei (middle column), and merged signals (right column) from 3D human melanoma cell cultures. Figure S5. The analytical segmentation procedure for a typical spheroid image. Table S1. Fluorescence results obtained for CD-NHF-loaded gels at different excitation wavelengths: 370–410 nm.

Author Contributions: Gel formulation experimental part, results interpretation, and writing and editing of the manuscript: C.-L.S. and C.A.P.; carbon dot preparation and fluorescence analysis: C.S.S.; rheological analysis and rheology results interpretation: C.A.I.; conceptualization and supervision: B.C.S.; in vitro experiments, biochemical tests, writing, and results interpretation: C.T. and E.C. All authors have given approval to the final version of the manuscript.

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Abbreviations

A375	human malignant melanoma
AS	sodium alginate
B16F10	mouse malignant melanoma cells
Balb/c-5064	mouse dermal microvascular endothelial cells
CARB	Ultrez 10 carbomer
CD	carbon dots
CD-NHF	N-hydroxyphthalimide carbon dots
Conc.	concentration
CMC	carboxymethyl cellulose
CR	controlled strain mode
CS	controlled stress mode
FBS	fetal bovine serum
GLY	glycerin
HDMVECn	primary dermal microvascular endothelial cells
MDA-MB-231	human mammary breast cancer
PBS	phosphate-buffered saline
λ_{em}	emission maximum
λ_{ex}	excitation wavelength

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Pharmaceutical nanotechnology

Formulation and evaluation of cefuroxim loaded submicron particles for ophthalmic delivery



Gabriela Andrei^a, Cătălina A. Peptu^{a,*}, Marcel Popa^a, Jacques Desbrieres^b,
Cristian Peptu^c, Fotios Gardikiotis^d, Marcel Costuleanu^d, Dănut Costin^d,
Jean Charles Dupin^b, Arnaud Uhart^b, Bogdan I. Tamba^d

^a Gheorghe Asachi Technical University of Iasi, Department of Natural and Synthetic Polymers, Prof. dr. docent Dimitrie Mangeron street, Iasi 73, Romania

^b Université de Pau et des Pays de l'Adour, Institut des Sciences Analytiques et de Physico-Chimie pour l'Environnement et les Matériaux, IPREM, Hélioparc Pau Pyrénées, 2 Avenue P. Angot, Pau Cedex 09, 64053 France

^c "Petru Poni" Institute of Macromolecular Chemistry, Aleea Grigore Ghica Voda 41A, Iasi 700487, Romania

^d "Grigore T. Popa" University of Medicine and Pharmacy, University Str., Iasi 16, Romania

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ABSTRACT

Chitosan gelatin particles could be the ideal candidate for intraocular drug delivery due to their desirable properties. Double crosslinking in double emulsion has been used as an original and reliable method for particles preparation and their morphology has been optimized considering the main synthesis parameters such as polymers ratio, crosslinker amount, stirring speed, tensioactive amount and ionic crosslinking time, respectively. The particles have been analyzed for their physical–chemical properties (swelling degree, drug loading and release capacity, surface characteristics, etc.), the enzymatic degradation properties along with *in vivo* ocular investigations (ocular biodistribution, *in vivo* drug release). In the present study cefuroxim was used as a model drug, which is generally used in the prophylaxis of postoperative endophthalmitis following cataract surgery after intraocular administration. The present study proved that the dimensions and the physical–chemical properties of the particles can be modulated (by varying the preparation parameters) to facilitate the administration, the biodistribution and the drug release in the specific segment of the eye. This experimental study demonstrated also the ability of fluorescent nanoparticles to penetrate ocular tissues close to the administration site (intravitreal injection) and especially their tendency to migrate deep in the retina at time intervals of 72 h.

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1. Introduction

The diseases of the posterior segment of the eye (glaucoma, diabetic retinopathy and age-related macular degeneration, etc.) are responsible for compromising eyesight of a large number of subjects. The therapeutic efficiency of pharmacological treatments in this area is limited mainly due to the difficulty of the active substance to reach the target tissues. That is the reason the delivery of therapeutic doses of drugs to the tissues in the posterior segment of the eye, however, represents a significant challenge. When a topical path is chosen, there are a lot of barriers (cornea, lens, haematoaqueous and haemoretinal barriers) which stop the medication access to the vitreous, the retina and the choroid.

On the other hand, if the oral pathway is selected, the amount of drug reaching the posterior segment of the eye coming from general blood flow is very low (Alarcón and Martínez, 2006). Also, therapeutic drug levels cannot be maintained for longer periods in target tissues.

In the last years, many pharmaceutical formulations have been used for the treatment of ocular diseases. Micro and nanotechnology ophthalmic formulations represent one of the approaches which are currently being designed for both anterior and posterior segment drug delivery. Several carriers, such as micro/nanoparticles, nanosuspensions, liposomes, nanomicelles and dendrimers have been developed for ocular drug delivery, some of them showing promising results for improving ocular bioavailability (Patel et al., 2013). Nanomicelles are carrier systems able to formulate therapeutic agents into clear aqueous solutions with high drug encapsulation efficiency. They are easy to prepare and present a small size and they have been proved to enhance the

* Corresponding author.

E-mail address: catiipeptu@tuiasi.ro (C.A. Peptu).

bioavailability of the therapeutic drugs in ocular tissues (Trivedi and Kompella 2010). Nanosuspensions, a promising strategy for delivery of hydrophobic drugs, are colloidal systems of submicron drug particles stabilized by polymer or surfactant and they present some important advantages for ocular delivery such as sterilization, ease of eye drop formulation, less irritation, increase precorneal residence time and enhancement in ocular bioavailability of drugs which are insoluble in tear fluid (Patravale and Kulkarni, 2004). Liposomes, vesicular systems composed of aqueous core enclosed by phospholipid bilayers of natural or synthetic origin, were intensively explored over the years for the ophthalmic drug delivery applications. Liposomes are biodegradable and biocompatible and they can enhance the permeation of poorly absorbed drug molecules by binding to the corneal surface and improving residence time. Liposomes are able to encapsulate both the hydrophilic and hydrophobic drug molecules. In addition, liposomes can improve pharmacokinetic profile, enhance therapeutic effect, and reduce toxicity associated with higher dose. Liposomes have been widely investigated for the treatment of both anterior and posterior segment eye disorders (Mishra et al., 2011). Micro/nanoparticles based systems with an appropriate morphology can be designed to ensure low irritation, adequate bioavailability, and ocular tissue compatibility. For ophthalmic delivery, particles are generally composed of lipids, proteins, natural or synthetic polymers such poly(alkyl cyanoacrylates)(PACA) (Kreuter, 1993), poly(caprolactone) (PCL) (Gagandeep et al., 2014), poly(lactic acid) (PLA) (Nagarwal et al., 2011), poly(lactic-co-glycolic acid) (PLGA) (Wagh and Apar, 2014), chitosan (CS) (Jayaraman et al., 2012), poly(acrylic acid) (PAA) (De et al., 2003; Hornof et al., 2003) and hyaluronic acid (Apaolaza et al., 2014). Among these polymers, chitosan is most widely studied due to its unique properties besides the well known biocompatibility and biodegradability—the chitosan is positively charged and hence it binds to negatively charged corneal surface and thereby improves precorneal residence and decreases clearance. These are the main reasons to explain why chitosan was chosen as the main polymer for our research. In order to better modulate the particles morphology we introduced a second polymer, gelatin, which presents free amine groups and gives similar chemical reactions as chitosan. Moreover, gelatin is well known for its properties being

intensively used in advanced drug delivery field (Elzoghby, 2013). Previous studies on chitosan–gelatin hydrogel films and particles were already published by our research group (Cadinioiu et al., 2011; Peptu et al., 2010).

The purpose of this study was to develop and characterize a new ophthalmic formulation of cefuroxime, a drug generally used in the prophylaxis of postoperative endophthalmitis following cataract surgery after intraocular administration (Delyfer et al., 2011), using polymer based nanoparticles. Particles were synthesized using a double crosslinking in double emulsion technique and the effects of crosslinkers amount and concentration, ionic crosslinking time, surfactant amount, stirring speed, polymer ratio on their morphology and physical–chemical properties (swelling degree, drug loading and release capacity, surface characteristics, etc.). The optimum considered particles has been analysed from the point of view of the enzymatic degradation properties along with *in vivo* ocular investigations (ocular biodistribution, *in vivo* drug release).

We consider that our work bring original elements from many points of view: (i) it was developed an improved method comparing to the one previously reported (Peptu et al., 2010); (ii) the *in vivo* evaluation of cefuroxime release was performed by HPLC-MS, the method being developed and validated by the authors; (iii) to our knowledge, this is one of the few papers which include preparation of the polymer particles, optimization of the preparation method, physical–chemical characterization of the particles and also from the point of view of biomaterial characteristics and *in vitro* and *in vivo* tests of the new polymer-drug nanosystems specially designed for targeting the posterior segment of the eye.

2. Materials and methods

2.1. Materials

Chitosan (CS)—low molecular weight (Sigma–Aldrich), molecular weight—70,000 (determined by viscosimetry), deacetylation degree 91%, Gelatin type A (Sigma–Aldrich), glutaraldehyde (25%—Sigma–Aldrich), sodium fluorescein (Sigma–Aldrich), *N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDAC) (Fluka), Sodium triphosphate pentabasic (TPP) (Sigma–Aldrich), sorbitan monooleate—Span 80 and Polyoxyethylenesorbitan

Table 1
Varying parameters of microparticles preparation.

Sample code	CS/G (w/w)	TPP concentration (%)	NH ₃ /TPP (mol/mol)	NH ₃ /GA (mol/mol)	TPP crosslinking time (h)	Ultraturrax speed, (rpm)	Surfactant, (% in respect with phase volume)	Mean diameter in acetone (microns)
C1	1/1	5	2/1	14/1	1	9000	2	0.967
C2		15						4.154
C3		1						5.116
C4		10						6.3
C5		5	4.7/1					2.547
C6			11.74/1					0.667
C7			1.175/1					1.466
C7.1					2			2.224
C7.2					3			0.648
C 7.3					4			0.755
C7.4				28/1				0.225
C 7.5				7/1				0.742
C7.6				14/1		15,000		0.889
C7.7						9000	3	0.12
C7.8							4	0.166
C7.9				7/1	4	15,000	3	0.225
C 7.9.1	1/0							0.345
C7.9.2	3/1							0.42
C7.9.3	1/3							0.637
C8	1/0		5/1	14/1	1	9000	2	0.82
C9	3/1							0.341
C10	1/1							0.637
C11	1/3							1.190

monooleate—Tween 80 (Sigma—Aldrich); cefuroxim (CF) collagenase from *Clostridium histolyticum* (EC 3.4.24.3, US Biological, USA) with collagenase activity of 125 units/mg dry weight. All other chemicals used (solvents and other substances) were at least analytical grade.

2.2. Methods

2.2.1. Micro/nanocapsules preparation

Nano and microcapsules were obtained by two steps cross-linking of the two polymers (chitosan and gelatin) in a double emulsion system. The first step, represented by gelation through ionic crosslinking of the polymers solution was followed by a second step, covalent crosslinking, in order to harden the unstable gel particles.

Briefly, the procedure is the following: 0.25 g polymer mixture (depending on the sample) is dissolved in a specific amount of 1% acetic acid; afterwards, the amount of hydrophilic surfactant (Tween 80) is also dissolved. In order to prepare the primary emulsion, a certain amount of toluene (aqueous phase to organic phase ratio was 4/1) containing the specific surfactant, Span 80 has been dropped under vigorous stirring to the aqueous phase containing the two polymers. The primary emulsion has been added drop by drop into the organic phase (consisting in toluene and the certain amount of the hydrophobic surfactant—Span 80) under stirring at 9000–15,000 rpm. After 10 min the TPP solution was added also under stirring but only after the stirring speed was reduced at 5000 rpm (in order to ensure uniform ionic crosslinking and to diminish the aggregates formation due to the coalescence which can appear at a higher speed). The ionic crosslinking was allowed to take place for different time intervals (between 1 and 4 h). The following step was the addition of the covalent crosslinker (glutaraldehyde toluene solution) and left to react for 1 h. The micro and nanocapsules were separated by successive cycles of centrifugation—washing (with different solvents—acetone, water and finally hexane) and dried at room temperature.

As it is already known, the use of glutaraldehyde may increase the toxicity, thus limiting the possible medical applications of the prepared system. Therefore the neutralization of unreacted glutaraldehyde sites was performed as a supplementary step by treating the prepared nanoparticles repeatedly with L-glutamic acid solution (low pH) as reported by Goswami et al. (Goswami et al., 2010).

In order to establish the best formulation different parameters of both crosslinking reactions have been considered: the mass polymer ratio, TPP concentration, the molar ratio between the two polymers (in terms of free amine groups) and each of the two crosslinkers (TPP and GA), ionic crosslinking time, surfactant amount (in percent in respect with the aqueous or organic phase weight) and the stirring speed used at the beginning when the emulsion was formed. All the values of these parameters as well as the yields of each reaction are presented in Table 1.

2.2.2. Particles morphology

The prepared nanoparticles were observed by scanning electron microscopy (HITACHI SU 1510). A small amount of particles in powder form was layered on the SEM stub and then coated with gold metal using a sputter coater (Cressington 108 auto).

The mean diameter of the particles and the size distribution were analyzed by laser light diffractometry technique (SHIMADZU—SALD 7001). All measurements were carried out on suspensions of particles in acetone after sonication for 15 min at room temperature using a sonication bath (Bandelin Sonorex).

2.2.3. X-ray photoelectron spectroscopy

In the aim to characterize the chemical environments and compositions, X-ray photoelectron spectrometry (XPS) measurements were performed on a Thermo K-alpha spectrometer

with a hemispherical analyzer and a microfocussed (analysis area was c.a. $40 \mu\text{m}^2$) monochromatized radiation (AlK α , 1486.6 eV) operating at 72 W under a residual pressure of 1×10^{-9} mbar. The pass energy was set to 20 eV for high resolution core levels spectra and 150 eV for large scale spectra. The instrument work function was calibrated to give a Binding Energy (BE) of 83.9(6) eV for the Au 4f $_{7/2}$ line for metallic gold and 932.6(7) eV BE for the Cu 2p $_{3/2}$ line of metal copper. Charge effects were compensated by the use of a charge neutralisation system (low energy electrons) which had the unique ability to provide consistent charge compensation. All the neutraliser parameters remained constant during analysis and allow ones to find a 285.0 eV C1s binding energy for hydrocarbon. Peaks convolution was processed with a nonlinear Shirley-type background (Shirley, 1972) and mathematical components were optimized with a weighted least-square fitting method using 70% Gaussian, 30% Lorentzian line shapes. Quantification was performed with CasaXPS processing software (CasaXPS Ltd., Teignmouth, UK) using Kratos Relative Sensitivity Factors's library (Scofield, 1976). Binding energies are given with a ± 0.2 eV precision. The mounting of the nanoparticles has been limited to a thin powder deposition onto a conductive copper paste for the XPS analysis. To prevent the samples from moisture/air exposure, the XPS spectrometer is directly connected to an argon dry-box working at low H $_2$ O /O $_2$ level (<1 ppm through a fast load transfer chamber).

2.2.4. Maximum swelling degree

The gravimetric method was used in order to determine the swelling degree of the nanocapsules in slightly alkaline aqueous medium (phosphate buffer solution, pH 7.4) and in the acidic medium (acetate buffer solution, pH 3.3). For this, a certain amount of dried sample (w_0) was immersed into the buffer solution and maintained under magnetic stirring at 25 °C. After 24, 48 and 72 h the nanocapsules suspension was ultracentrifuged (15,000 rpm) and the swollen sample (w_1) was weighed. The traces of water were removed by using a piece of filter paper. The swelling degree was calculated with the following equation:

$$Q(\%) = \frac{w_1 - w_0}{w_0} \times 100 \quad (1)$$

In order to diminish the errors which can interfere with the real measurements, the swelling degree was measured after 24, 48, 72 and 96 h. The results represent the arithmetic average of three different determinations, the standard deviation being lower than 10%.

2.2.5. CF loading and in vitro release

CF was used as drug for the *in vivo* and *in vitro* release tests. For loading experiments 20 mg of capsules (sample C7.9) were suspended in 1 mL CF solution (25 mg/mL) and dispersed in a sonication bath for 15 min. The suspension was maintained under stirring for 24 h and then centrifuged for 15 min at 15,000 rpm and finally freeze-dried (Laboratory freeze-dryer Christ Alpha 1–4). The CF encapsulation efficiency has been calculated by determining the CF content in the supernatant in correlation with the standard calibration curve by using UV–vis spectroscopy (Nanodrop ND 1000) at 275 nm wavelength.

Concerning the release experiments a certain amount of CF loaded particles were introduced in cellulose dialysis tubes immersed in 20 mL buffer solution at 37 °C, in a water bath, under continuous shaking. Small aliquots of buffer solution were withdrawn at appropriate time intervals and the CF concentration was determined by UV–vis spectroscopy (Nanodrop ND 1000) at 275 nm.

2.2.6. In vitro enzymatic degradation

Starting from the idea that gelatin is in fact a collagen hydrolysate, the ability of capsules to degrade on exposure to enzymes was studied by using *collagenase* which represents the main enzyme responsible for *in vivo* degradation of native collagen fibrils in different eye diseases such as Keratomalacia (rapid corneal ulceration that occurs in children who are severely deficient in vitamin A) (Seng et al., 1980), corneal destruction after injury and alkali-induced burns. (Elner et al., 2003a) or associated with other diseases (Riley et al., 1995).

The process was evaluated by measuring the amount of α -NH₂ groups (mmol/mL $\times 10^{-10}$) after enzyme action, by means of ninyhydrin test.

Briefly, the ninyhydrin reagent was prepared by dissolution of SnCl₂·2H₂O (0.032 M) in citrate buffer solution pH 5.0, (500 mL), and the addition of 500 mL solution of ninyhydrin (20 g) in 2-methoxyethanol.

The initial samples were incubated in 30 mL PBS (0.01 M) pH 7.2, containing 0.01% collagenase in a shaking water bath at 37 °C. At preestablished time intervals, 1 mL of sample solution was withdrawn, mixed with ninyhydrin reagent (5 mL) and quenched in boiling water for 20 min, followed by a rapid cooling (within 5 min; ice bath) to room temperature. Then, water/2-propanol solution (1:1, v:v), (25 mL) was added and optical absorbance was recorded (570 nm using a UV-vis spectrophotometer—BOECO S 30).

The degradation degree was calculated from the calibration curve of gelatin degradation and the equations:

$$DD = \frac{C_n}{C_0} \times 100, \quad (2)$$

where DD is the degradation degree (%), C_n the nanocapsule concentration in primary amino groups (mmol NH₂/mL), C_0 the maximal concentration in primary amino groups from gelatin (mmol NH₂/mL) and

$$DE = \frac{C_n}{C_{nmax}} \times 100 \quad (3)$$

where DE is the degradation efficiency (%) and C_{nmax} the maximal concentration in primary amino groups of nanocapsules (mmol NH₂/mL).

2.2.7. In vivo ocular biodistribution

For preparing fluorescent labelled capsules the chitosan has been labelled with fluorescein by following the method of De Campos et al. (2004). Fluorescein was covalently attached to chitosan: the reaction has been taken place between the amine groups of chitosan and carboxyl groups of fluorescein (Fig. 1), the amidation reaction being catalysed by EDAC.

Briefly, a certain amount of CS was dissolved in 1% acetic acid solution to obtain a final concentration of 1%. The amounts of fluorescein and EDAC have been calculated in order to have half of the chitosan free amine groups to react with fluorescein, the rest being necessary later for reaction with TPP and GA, respectively, the pH of the solution has been adjusted to 6 with NaOH (1 M). The demineralised water has been added in order to have a final solution volume of 250 mL. Separately, the specific amount of fluorescein has been dissolved in ethanol in order to have 10 mL with a concentration of 10 mg/mL. These two solutions were mixed together and the specific amount of EDAC has been added in a final concentration of 0.05 M. The mixture was allowed to react in the dark for 24 h at room temperature. The conjugate was purified by dialysis (cellulose dialysis tubing, pore size 12,400 Da) against demineralized water, 0.05 N NaOH solution, and finally against de-mineralized water and then lyophilized. With this derivatized chitosan, particles have been obtained following the procedure presented above.

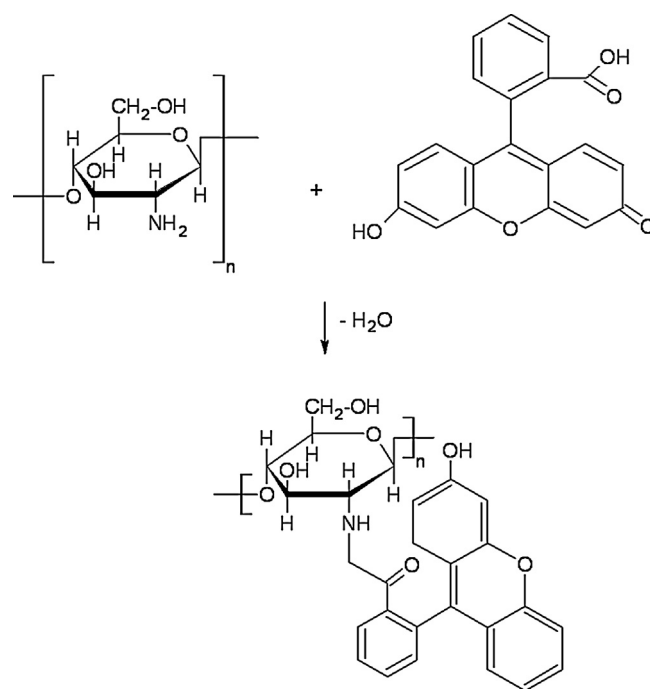


Fig. 1. Chitosan–fluorescein reaction.

For *in vivo* ocular biodistribution test, one series of 9 Wistar rats have been used, weighing 150–180 g. The left eye was used as control: it has not received any treatment and the fluorescent nanoparticles suspension (the concentration of nanoparticles in physiological serum, being equal to 1%) has been intravitreal administered in the right eye after a general anaesthesia with Ketamin (0.1 mL/100 g b.w.). The injected eyes have been enucleated and the animals euthanized at the end of the experiment. The eyes have been transferred on ice and sectioned immediately after the enucleation. All ocular tissue sections have been treated with monoclonal anti-fluorescein antibodies (produced in mouse—FL-D6 clone—Sigma—Aldrich) using a dilution of 1/400 at room temperature. After 48 h, the primary antibodies have been removed by successive washings with PBS and the samples were treated with anti-mouse secondary antibodies (the whole molecule of IgG; produced in goat and labelled with R-phycoerythrin—Sigma—Aldrich) for 1 h at a dilution of 1/100 and room temperature.

All sections have been transferred on microscope slides and covered with specific medium for fluorescence (0.1% DABCO in PBS; glycerol for microscopy 1:1). For fluorescence analysis a Nikon Eclipse TE300 Inverted Microscope has been used (40 $\times$). The excitation wavelength was setup at 514 nm and for emission a filter having 570 nm wavelength has been used for R-phycoerythrin. The images have been captured using the software associated to the microscope in two modes: red fluorescence for R-phycoerythrin and transmitted light for morphological structures of the samples. The final processing has been performed using the software Image J (NIH) for the superposition of the two types of images and measurement of red intensities (arbitrary units). The red fluorescence intensities were calculated starting from RGB diagrams.

2.2.8. In vivo cefuroxim release study

The *in vivo* tests were performed on Wistar rats having each a weight of 300 g. Each subject was sedated by intraperitoneal injection of a Thiopental solution, 5 mg/kg body weight. When the anesthesia was effective the drug loaded particles were injected in the left eye, 50 μ L of 1% particles suspension containing an

estimated drug quantity of 4×10^{-4} g. The same amount of drug was injected also in the right eye but as free cefuroxim in a 20 μ L solution. Both eyes of each animal were eviscerated under anesthesia at fixed time intervals, 15 min, 30 min, 1 h, 2, 3, 4, 5, 6, 24, 48 and 92 h, respectively. At specific time points (during 5 h) the same amount of vitreous was extracted and analyzed by HPLC-MS technique (the rats were euthanatized at the end of the extraction). The samples were suspended in 2 mL water/acetonitrile mixture 1/2 using an Ultraturrax at 5000 rpm. The obtained eye homogenate was centrifuged at 11,000 rpm and 0.5 mL of the supernatant was filtered through 200 μ m PTFE filters and further used for quantitative measurements by LC MS (10 μ L per injection).

For the LC-MS analyses of cefuroxim a standard quantitative curve was established using eye homogenate without any addition of cefuroxim. The analyzed samples were obtained by spiking clean homogenate extract, obtained as previously described, with fixed amounts of cefuroxim (CF) stock solution, thus resulting solutions of 0.2, 0.8, 1, 1.6, 5, 10, 20 μ g/mL, respectively. This resulted in a standard curve used for quantitative measurements. The solutions were stored at 4°C avoiding light for using.

2.3. Sample extraction procedures

The samples were suspended in 2 mL water/acetonitrile mixture 1/2 using an Ultraturrax at 5000 rpm for 1 min. The obtained eye homogenate was centrifuged at 11,000 rpm and 0.5 mL of the supernatant was filtered through 200 μ m PTFE filters and further used for quantitative measurements by LC MS (10 μ L per injection).

2.4. Method validation

To evaluate selectivity, drug-free eye homogenate samples from 6 individuals were analyzed to check for the presence of any interfering peaks at the elution times of cefuroxim. The calibration curves were constructed using 7 standards ranging in concentration from 0.2 to 20 μ g/mL. The validity of the linear regression equation was indicated by the correlation coefficient ($R^2=0.995$).

The intraday and interday precision and accuracy were evaluated by assessing QC samples at the following concentrations: LLOQ (0.1 μ g/mL), low (0.2 μ g/mL), medium (1.6 μ g/mL), and high (20 μ g/mL).

The extraction recovery and matrix effect of cefuroxim for three concentrations of QC samples was determined by comparing the

response of CF spiked eye homogenate after extraction to that of analyte spiked into the solution extracted from blank eye homogenate and the response of CF spiked after extraction to that of CF dissolved in mobile phase, respectively.

Stability experiments were performed by leaving the eye homogenate sample at room temperature for 6 h (no light), placing the treated eye homogenate in an autosampler for 24 h and storing for 100 days at -20°C , using three similar samples of each QC sample with three different concentrations.

2.5. Mass spectrometry measurements

The mass spectrometry setup for the quantitative measurements consists in an Agilent 6510 Triple Quad LC MS system (Agilent, USA) controlled using the MassHunter software. The CF samples were analyzed by MRM. CF fragmentation (Fig. 2) in negative mode was found to be the most sensitive in the given MS conditions and was used for further quantitation.

The chromatographic conditions for the CF separation were as follows: isocratic solvent flow 1 mL/min, constant temperature of 30°C , mobile phase—acetonitrile/water 7/3 spiked with 1 mM HCOOH and Agilent ZORBAX 300SB-C18 4.6×150 mm, 5 μ m C18 column. ESI source parameters were established for the best sensitivity: Vcap = 4000 V, fragmentor voltage = 200 V, drying gas temperature = 325°C , drying gas flow = 12 L/min, spraying pressure = 35 psi g. The gas used by ESI source is nitrogen provided by an external source, Peak Scientific. The CF fragmentation performed in the collision cell were obtained at $E_{\text{lab}}=5$ eV using high purity nitrogen as collision gas. The monitored fragmentation reaction characteristic for CF (Fig. 3) was m/z 423 $\rightarrow$ (m/z 207 + 318 + 362). The LC-MS/MS conditions were established for best sensitivity by elimination as much as possible the matrix effects. LC-MS/MS peak present at retention time 1.85 min (Fig. 3A) was integrated and further used for quantitation (quantitation curve $y=6E+09x+1328.9$). Thus, the LC-MS experiment was divided in three segments (see also Fig. 3B):

- first segment—the flow is directed to ESI source (0–0.9 min).
- second segment—the flow is diverted to waste in order to avoid the matrix effects over the ESI measurements (0.9–1.5 min).
- third segment—the flow is directed to ESI source and the MRM of CF is performed (only MS/MS spectra of 423 peak are quantified at 1.85 min) (1.5–2.4).

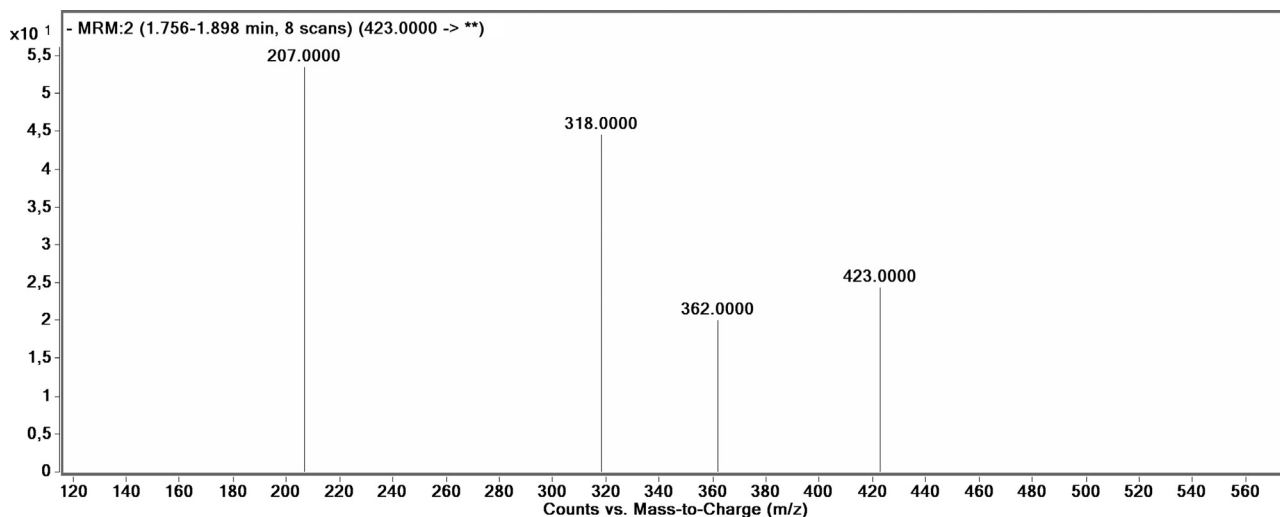


Fig. 2. ESI MS/MS of cefuroxim.

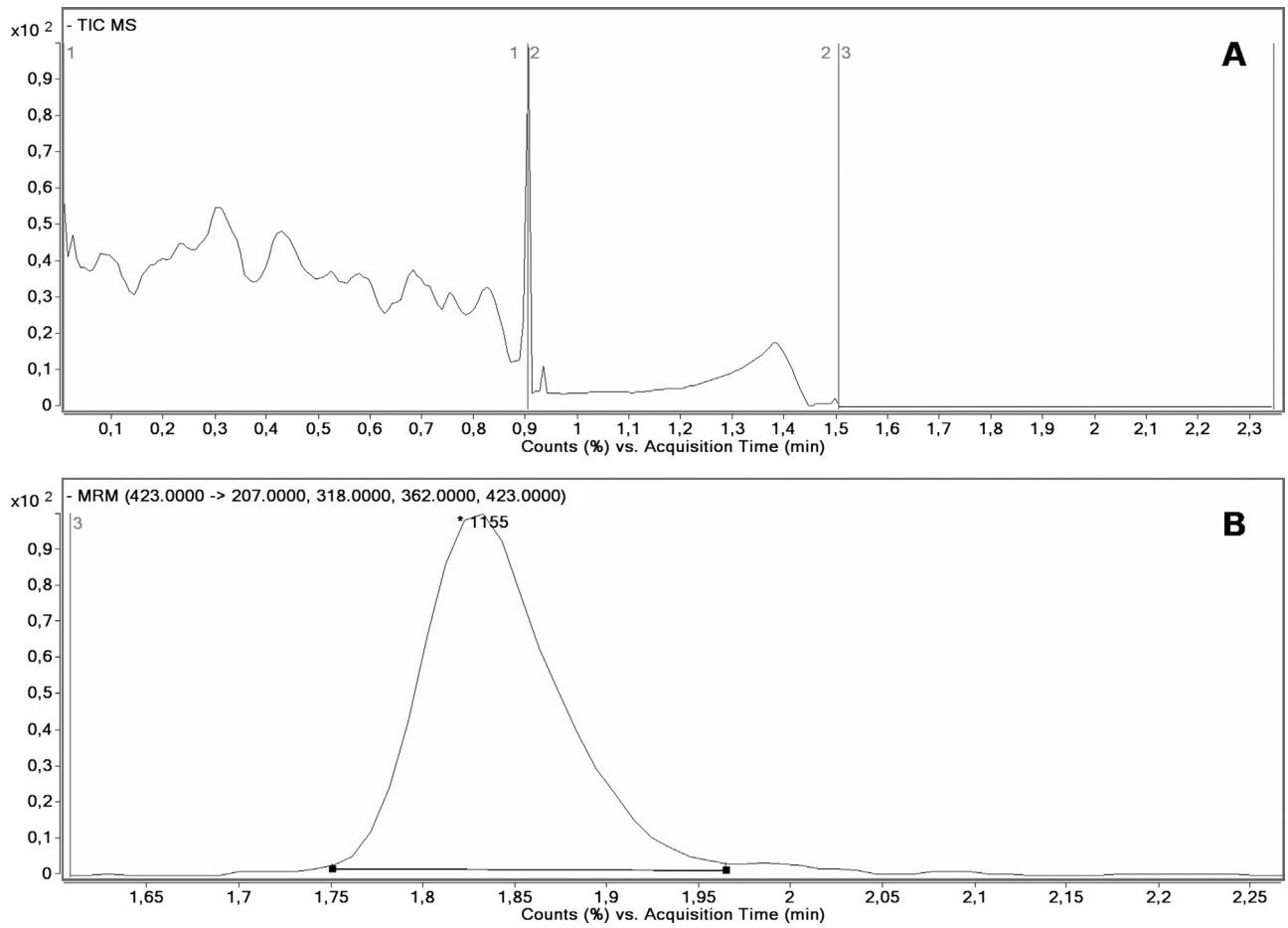


Fig. 3. LC MS chromatograms (A) total ion chromatogram; (B) MRM chromatogram for CF detection.

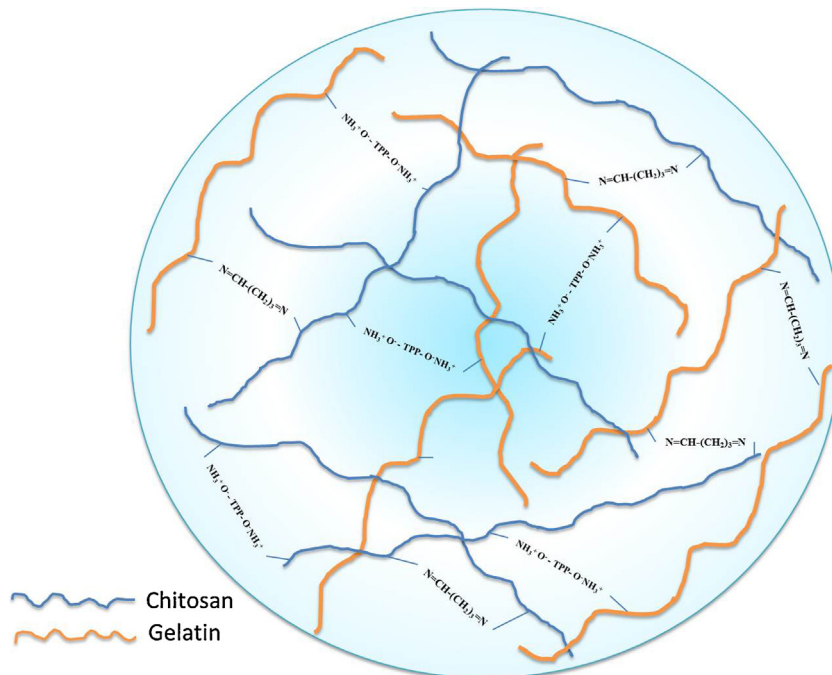


Fig. 4. Double crosslinking mechanism.

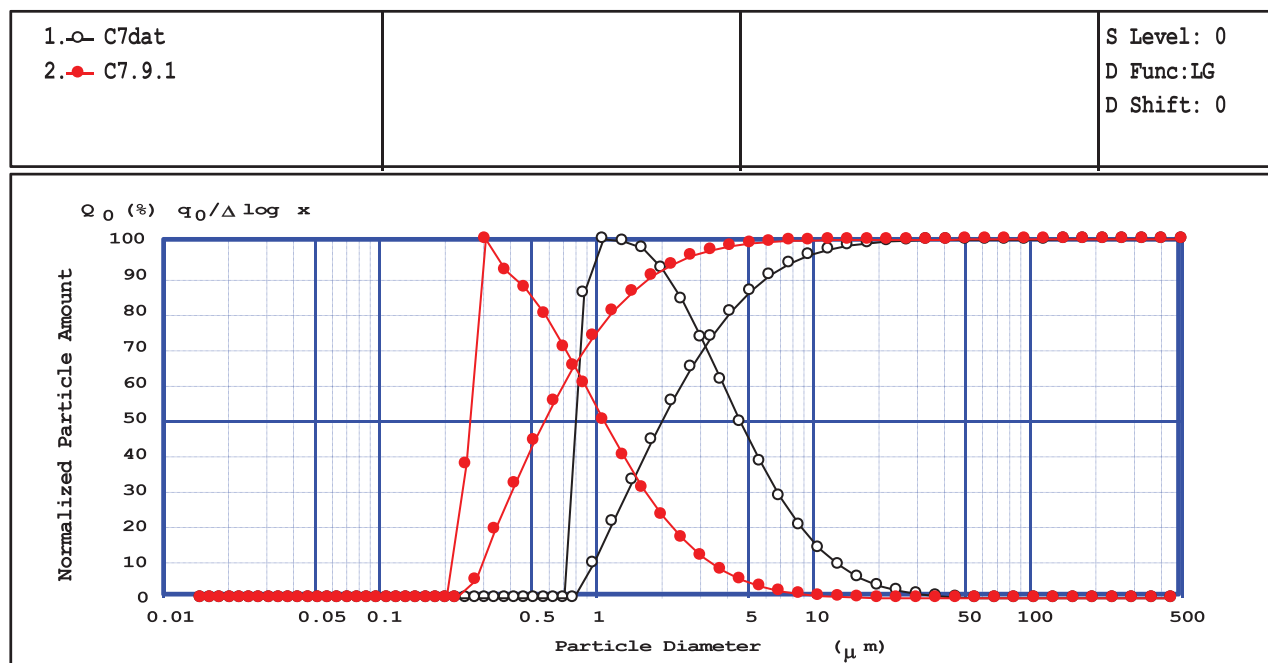


Fig. 5. Dimensional distribution of the samples C7 and C7.9.

3. Results and discussions

The capsules preparation process is an original one derived from another method previously developed in our group (Peptu et al., 2010) starting from the point that we wanted to prepare superior particles in terms of morphology and encapsulation efficiency. The double crosslinking was performed in order to maintain the stability of the polymer particles (which is generally given by the crosslinker bridges between the two types of macromolecules) without the increase of the toxicity of the particulate system. In this case that means the use of a minimal amount of the covalent crosslinker (GA) enough to stabilize the already formed gel particles (obtained after the ionic bonding of the polymers with TPP). The crosslinking mechanism is presented graphically in the Fig. 4.

One of the major advantages of the present method is represented by two natural polymers used both presenting excellent properties which make them suitable for many kinds of biomedical applications. In addition, in this case, we have tried to ensure a very good stability of the particles by using chitosan which will form the majority of the chemical bonds (both ionic and covalent) because it presents a higher number of free amine groups

(susceptible for ionic/covalent crosslinking) in comparison with gelatin which present a very small amount of free amount groups but which will ensure the formation of the interpenetrated network. Moreover, the addition of gelatin will ensure the high swelling capacity of the particles which will determine the high drug encapsulation efficiency (see the following sections).

3.1. Morphological analysis (size and shape)

The morphology represents the main characteristic which has been considered for the capsules optimization process. For this, the first studied parameters of the double crosslinking process were the TPP concentration (samples C1–C4) and the molar ratio between polymers (free amine groups) and the ionic crosslinking agent (TPP) (samples C5–C7). After these two steps an optimal sample from the morphological point of view has been selected (C7). The following steps were represented by the samples obtained after the variation of the ionic crosslinking time (C7.1–C7.3), the molar ratio between polymers (free amine groups) and the covalent crosslinking agent, GA (C7.4, C7.5), the stirring speed (C7.6) and the surfactant amount (C7.7, C7.8). The optimal sample C7.9 has been obtained by using for the preparation of the particles

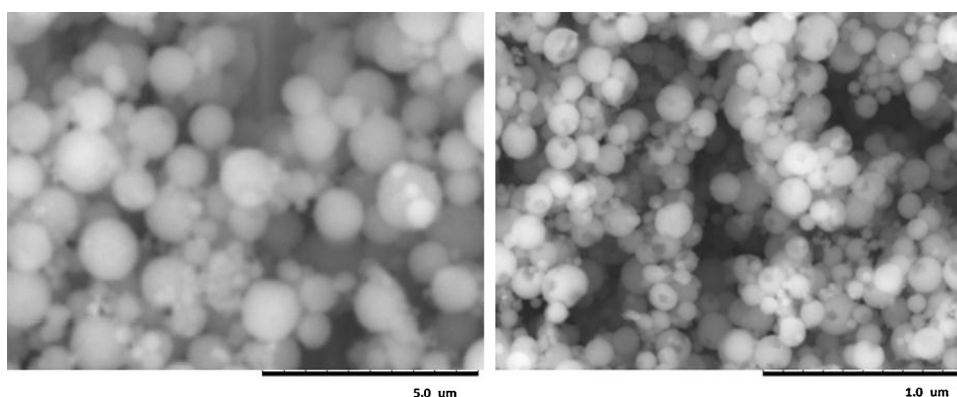


Fig. 6. Scanning electron microscopy images for the optimum samples C7 and C7.9.

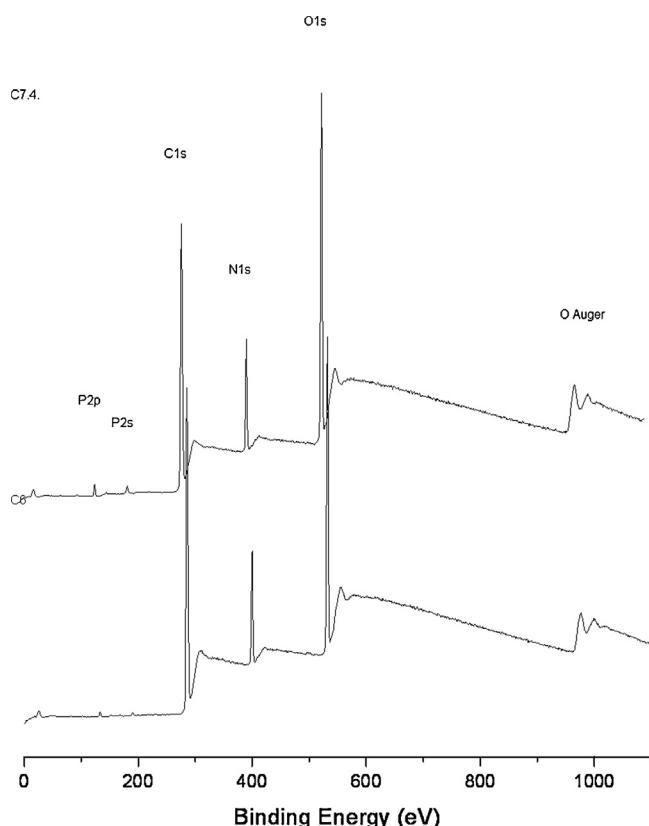


Fig. 7. Large scale spectra of samples with different ratios between ionic (TPP) and covalent (GA) crosslinking agents (C7.4 and C6).

the optimal values of the previously described parameters. In the end we have tried to see if changing the initial polymer ratio (C7.9.1–C7.9.3) allows obtaining a better morphology.

In the Table 1 the mean diameter for each sample has been presented. The sample C7 followed by the optimum sample C7.9 have been chosen taking into account both size and spherical shape characteristics as well as the aggregation tendency which can further cause problems during the *in vivo* tests. The dimensional distribution of the optimum samples C7, respectively C7.9 is represented in the Fig. 5.

SEM images of the first samples (C1–C4) indicated the formation of aggregates and a relative high polydispersity, but they present a good dispersability in water and organic solvents after stirring or ultrasonication. Dimensional polydispersity is high and aggregates are observed for the samples with different

polymer ratios (C7.1–C7.3). After the first optimization steps, the particles are better individualized (probably due to the increasing of the ionic crosslinking time or the amount of the covalent crosslinker (C7.4, C7.5) allowing the maturation of the particles). By changing the initial amount of the surfactant and the stirring speed, particles with nanometric size with a clear round shape and without aggregates have been obtained. In the Fig. 6 SEM photographs for the most representative samples are presented.

3.2. X-ray photoelectron spectroscopy

In Fig. 7, spectra of samples with different ratios between ionic (TPP) and covalent (GA) crosslinking agents (samples C7.4. and C6) were recorded in a large energy scale (0 eV–1100 eV). P_{2p} peaks are observed confirming the ionic crosslinking and the signal is lower for GA/TPP=0.83 as expected. For high energy resolution, the spectra are better resolved and mathematical convolutions give access to the different chemical functions in the composites. Samples were compared considering the difference in the content of specific crosslinking agents. Specific C_{1s} , P_{2p} , N_{1s} and O_{1s} regions were then examined and results are reported in Table 2.

Concerning the nitrogen species detected, the N_{1s} spectra (Fig. 8) exhibit three components which were assigned to: N–C bonds (Wagner et al., 2003) at 400.0 eV, amino groups in the ammonium form (Amaral et al., 2005) at 401.9 eV. The supplementary peak at 398.6 eV is the signature of the covalent crosslinking (N=C, (Beamson and Briggs, 1992) with glutaric aldehyde as given in the scheme.

For phosphorus entities present in the composites, all spectra revealed a doublet ($2p_{3/2-1/2}$ components at respectively c.a. 133.5 eV and 134.4 eV) clearly associated with HPO_4^- chemical groups (Wagner et al., 1979) operating in the ionic crosslinking. Moreover the O_{1s} peak at 533.9 eV corresponds to same HPO_4^- units (Quan et al., 1989) confirming the ionic crosslinking.

Comparing the relative abundances (atomic percentages) of elements, a correlation exists between the ratio of specific peaks and the ratio between covalent and ionic crosslinking agents. As examples, the relative abundance of the peak assigned to N=C groups (N_{1s} spectrum) is higher for the sample with an important covalent crosslinking agent content (sample a), whereas highest intensities of O_{1s} peak at 533.9 eV and N_{1s} peak at 401.9 eV (interactions between HPO_4^- and NH_3^+) were observed for sample b with a high content of ionic crosslinking agent. In the same time the relative abundances of each of the elements are in good agreement with the ratio between the different crosslinking agents (Table 3)

Calculating the ratio amine groups ionically bonded/amine groups covalently bonded, close to 1 (Table 4, line 8), results that,

Table 2

Peak positions and relative abundance (atomic percentages) for Samples a (GA/TPP=0.042—sample C7.4) and b (GA/TPP=0.83—sample C6).

Peak	GA/TPP=0.042 (Sample a)			GA/TPP=0.83 (Sample b)		
	Position (eV)	FWHM (eV)	at. %	Position (eV)	FWHM (eV)	at. %
C 1s	285.0	1.5	28.1	285.0	1.4	43.4
	286.3	1.4	21.2	286.4	1.4	19.1
	287.9	1.5	12.3	287.9	1.3	8.8
	288.9	1.5	3.2	288.9	1.3	2.4
N 1s	398.6	1.4	0.4	398.6	1.5	0.6
	400.0	1.6	8.5	400.0	1.6	8.1
	401.8	1.6	1.4	402.0	1.7	0.7
	531.5	1.8	10.6	531.6	1.9	8.8
O 1s	532.7	1.5	9.5	532.8	1.5	6.3
	533.9	1.6	2.9	533.9	1.4	1.2
	133.5	1.6	1.3	133.4	1.5	0.4
P 2p $_{3/2-1/2}$	134.4	1.6	0.6	134.3	1.6	0.2

FWHM: full width of peaks at half maximum.

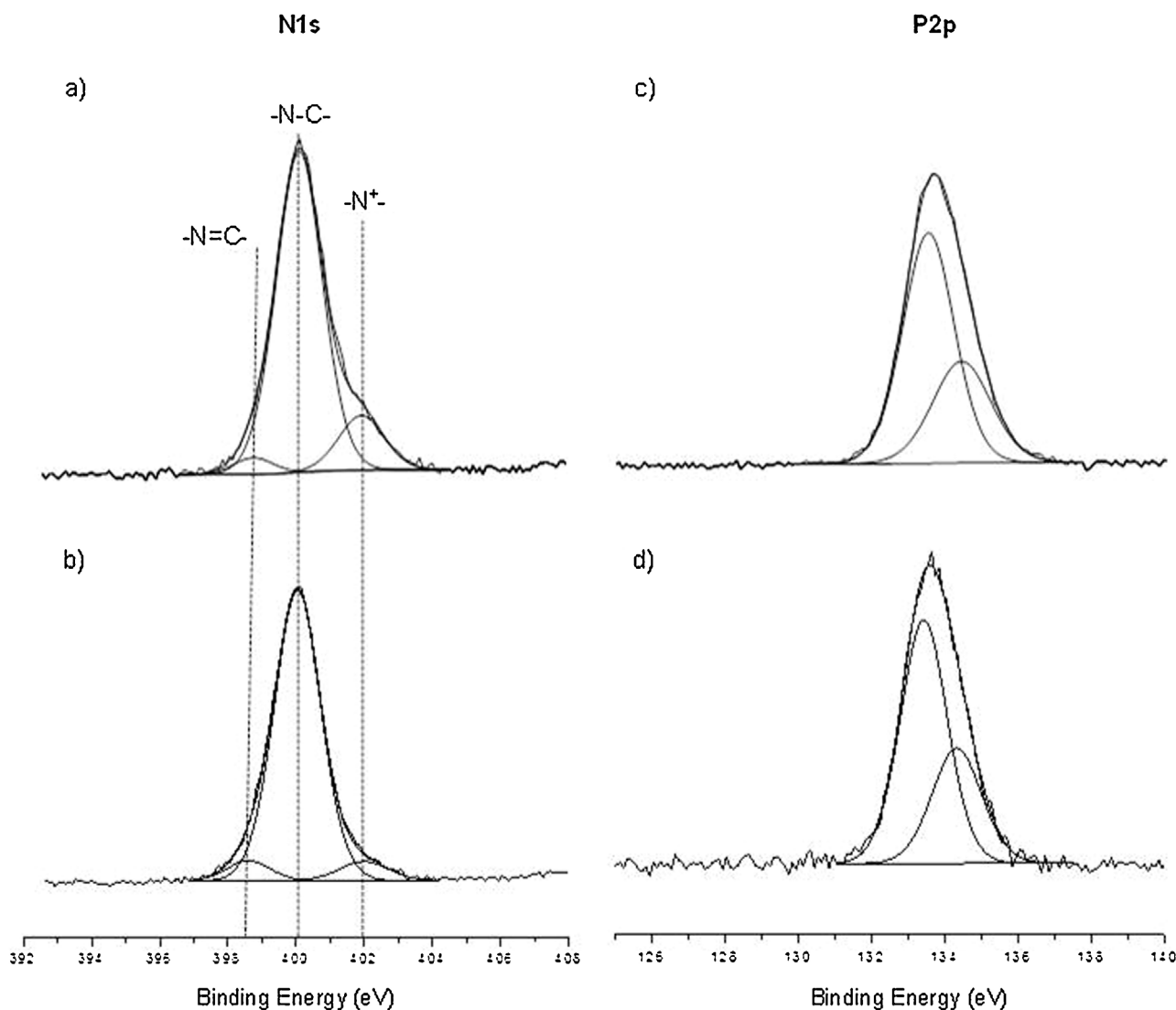


Fig. 8. XPS spectra of GA-TPP composite with: GA/TPP = 0.042 ((a) N1s peak and (c) P2p_{3/2-1/2} peak); GA/TPP = 0.042 ((b) N1s peak and (d) P2p_{3/2-1/2} peak).

on particles surface, the same number of amine groups participates to each type of crosslinking bond. The GA is added in a smaller amount compared with TPP and, as expected, the covalent crosslinking has taken place at the particle surface not in the entire volume, whereas the ionic crosslinking is taking place in the whole volume of the aqueous polymer drops.

3.3. Swelling degree

Swelling experiments lead to interesting results showing that the maximal swelling degree is in very good correlation with particles preparation parameters. In Fig. 9 a general view over these parameters is presented and the first conclusion is that for all samples, the maximal swelling degree in acetate buffer (pH 3.3) is higher (between 804 and 1587%) than in phosphate buffer (pH 7.4) (between 516 and 1121%). This effect is determined by the amine groups of the two polymers which have not participated to the crosslinking process (both ionic and covalent). In acid environment the amine groups are quaternized into ammonium groups; the electrostatic repulsions between these groups increase the distances between the network chains determining the accumulation of high amount of water. The maximal swelling degree is

high in alkaline pH too: in these conditions an important role is given to the carboxylic groups of gelatin coming from the dicarboxylic acids present in its structure. In basic media, these groups are as carboxylate anions which determine electrostatic repulsions but not as strong as those developed in acidic pH giving a high maximal swelling degree but lower than in alkaline pH. These results are in good agreement with other previous studies (Mi et al., 2003; Shu and Zhu, 2002a; Boral et al., 2006)

Analysing the C1–C4 series one can notice that increasing TPP concentration reduces the maximal swelling degree, as a logical consequence of increasing the network crosslinking density. A similar effect was noticed in the series C1, C5, C7 when increasing the amount of TPP, the maximum swelling degree is reduced.

Table 3

Relative abundance (atomic percentages) of elements within the particles with different crosslinking agents ratios (Samples C7.4 (GA/TPP = 0.042) and Sample C6 (GA/TPP = 0.83)).

	at.% C	at.% N	at.% P	at.% O
Sample C7.4	64.8	10.3	1.9	23.0
Sample C6	73.7	9.4	0.6%	16.3

Table 4

XPS quantitative aspects regarding surface structure of two types of particles.

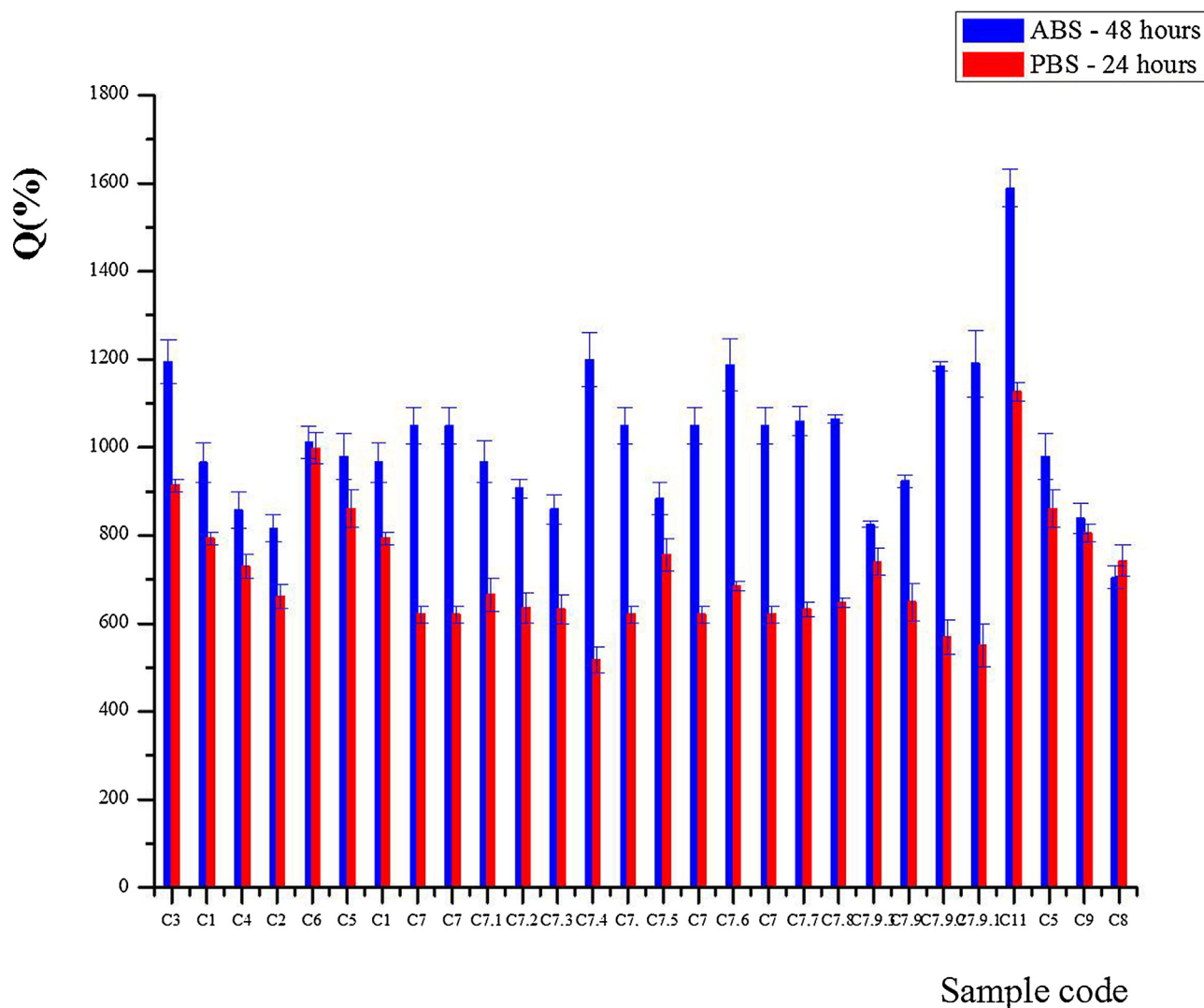
	Property	Value	
		C7.4	C6
1.	TPP in initial mixture, (mol)	6.2×10^{-4}	6.21×10^{-5}
2.	TPP chemically bonded, on the surface (mol)	2.45×10^{-5}	2.56×10^{-5}
3.	TPP bonded on the surface, (reacted mols/initial mol) $\times 100$, (%)	3.95	41.22
4.	GA added initially, (mol)	11.18×10^{-5}	22.37×10^{-5}
5.	GA covalently bonded (mol)	7.57×10^{-5}	7.93×10^{-5}
6.	% GA chemically bonded reacted mol/total mol $\times 100$, (%)	67.71	35.44
7.	Nitrogene amount participating to ionic crosslinking, (mol)	8.14×10^{-5}	8.52×10^{-5}
8.	Amine groups ionically bonded Amine groups covalently bonded	$\frac{8.14 \times 10^{-5}}{7.57 \times 10^{-5}} \times 1.075$	$\frac{8.52 \times 10^{-5}}{7.93 \times 10^{-5}} \times 1.074$

Other researchers have obtained similar results (Shu and Zhu, 2002b).

The C7–C7.4 samples are different by the ionic crosslinking time from 1 h to 4 h. As it was predictable, increasing the ionic crosslinking time will determine obtaining denser networks with lower maximal swelling degree values in total agreement with other researches (Ko et al., 2002). Following the same reasoning in the series C7.4, C7.8, C7.5 when an increasingly higher amount of GA was used the maximal swelling degree was lower as observed in other similar studies (Silva et al., 2004). Stirring speed (used for

emulsion preparation) and the amount of surfactant does not present an influence on the swelling degree, since these parameters do not interfere in the chemistry of the crosslinking process.

The composition of initial polymer mixture represents an important parameter for swelling degree evaluation. Reducing CS amount from 100% (C8) to 25% (C11) leads to higher swelling degree, the phenomenon having a simple explanation: reducing the amount of CS means a reduced number of amine groups participating to both types of crosslinking sites, then the

**Fig. 9.** Swelling degree (SD) in different pH conditions.

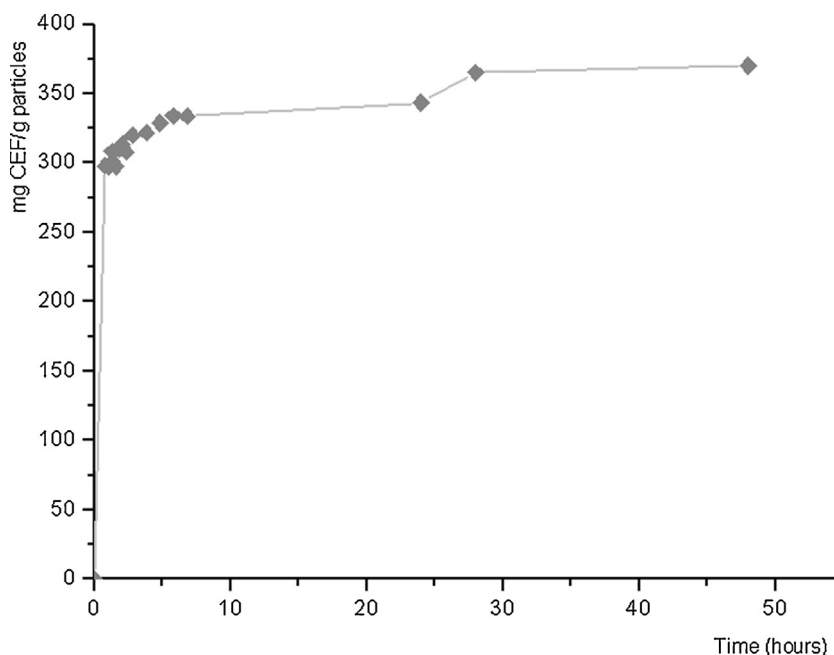


Fig. 10. CF release kinetic in pH 7.4 environment.

crosslinking density is also reduced which makes the swelling degree to be higher. In addition, G presents a superior hydrophilicity determining the accumulation of higher amount of water.

3.4. In vitro drug release

For loading and release studies sample C7.9 was considered optimal. For this sample a toxicity test has been performed using the determination of average lethal dose (DL50) according to a method which was previously described in one of our published paper (Peptu et al., 2010). In conformity with Lodge and Sterner scale (Danila, 1984) the tested nanoparticles are in the category of “practically nontoxic”.

The cefuroxim encapsulation efficiency for tested sample was around 47%. The dried sample was then tested for *in vitro* release characteristics. The release kinetics in alkaline environment (pH

7.4) is presented in Fig. 10. The release behavior is typical of diffusional systems like this one. A relative strong burst effect has been noticed in the first hour, afterwards the system is equilibrating and slowly release CF for 48 h tested.

3.5. In vitro enzymatic degradation

The particles composition includes two natural polymers known as being degradable under the effect of specific enzymes. CS is degraded by chitosanase by endohydrolysis of beta-(1 → 4)-linkages between D-glucosamine residues to monosaccharide level G, which is resulted from collagen by a controlled hydrolysis, is sensitive to collagenase which is catalyzing the hydrolysis of peptide bond in the main chain determining the appearance of ending alpha amine groups. The latter can be identified by a color reaction with ninhydrin; depending on the color intensity

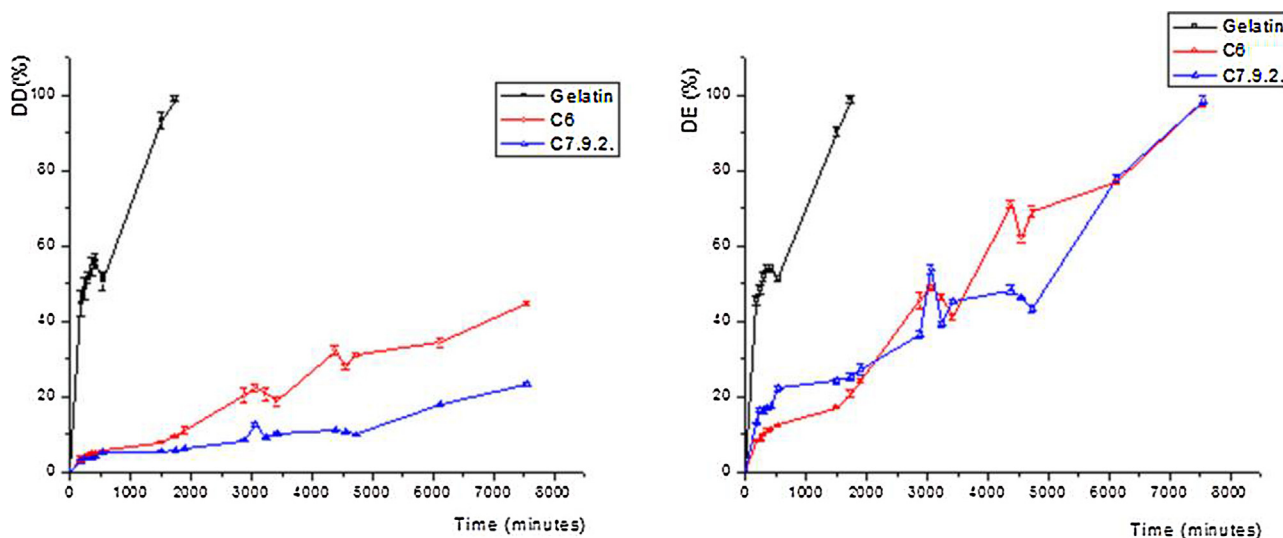


Fig. 11. (A) The variation in time of degradation degree (DD) for samples with different gelatin amount in composition (C6–50% Gelatin and C7.9.2–25% gelatin) in comparison with pure gelatin; (B) the efficiency of enzymatic degradation process (DE) as a function of time for particles with different gelatin composition (C6–50% Gelatin and C7.9.2–25% gelatin) in comparison with pure gelatin.

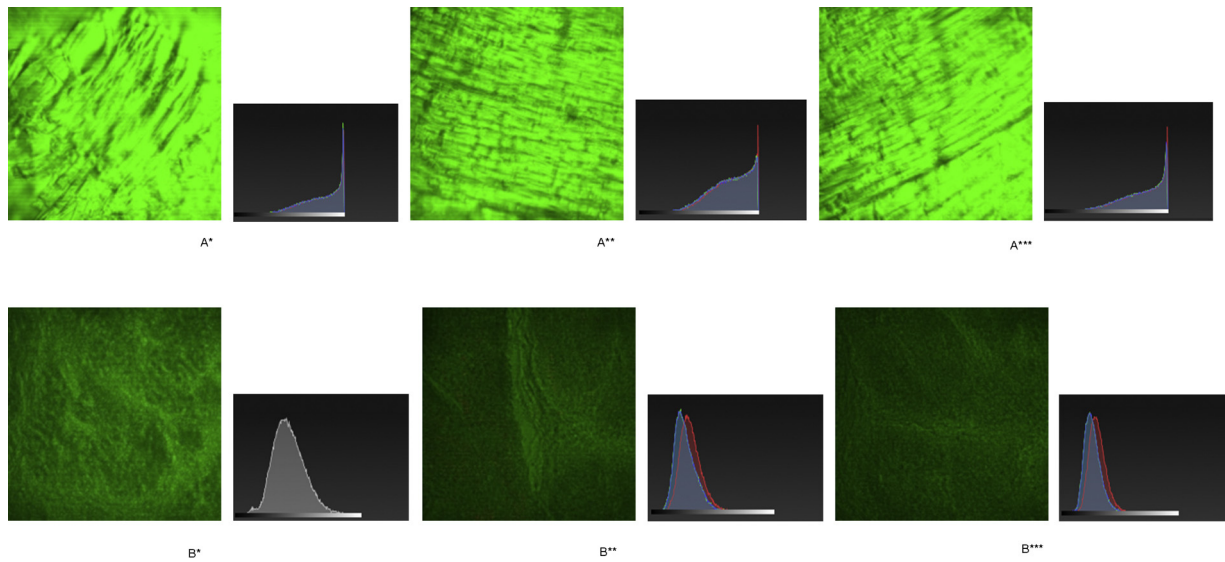


Fig. 12. Analysis of red fluorescence intensity (RGB diagram on morphology) from lens (A) and retina (B) sections, 24 h (**) and 72 h (***) as compared to the control group (*).

(spectrophotometrically determined) it is possible to analyze the degradation degree of the particles and the gelatin degradation efficiency. Collagenase has been associated with the phenomenon of cornea destruction following accidents or alkaline burns (Elner et al., 2003b). In our experiment, the gelatin degradation efficiency (blank sample) is maximal (100%) after 29 h (Fig. 11), when the maximum alpha amine end group concentration is achieved. Gelatin from capsules is achieved after longer periods of time (126 h), this effect being due to the fact that gelatin being trapped within the interconnected network, the access of the enzyme to the amide bond is decreased. By analyzing the results presented in the Fig. 10, one can observe that after this time interval, the particles degradation degree is different depending on their composition. The degradation process presents a linear evolution,

the particles are 47% degraded for sample C6 and 25% for sample C7.9.2. These values of degradation degree are identical with the amount of gelatin in initial composition, indicating that under the action of collagenase only the protein is degrading.

3.6. In vivo ocular biodistribution

Analyzing the results of particles eye biodistribution after intravitreal administration, one can observe that the particles can reach in large quantities the lens and retina 24 h after administration (Fig. 12). This is based on the fluorescence intensity peak and surface of the fluorescence diagram analysis which allows only the qualitative estimation of the particles concentration which penetrated a certain segment of the eye. Smaller amounts (fluorescence

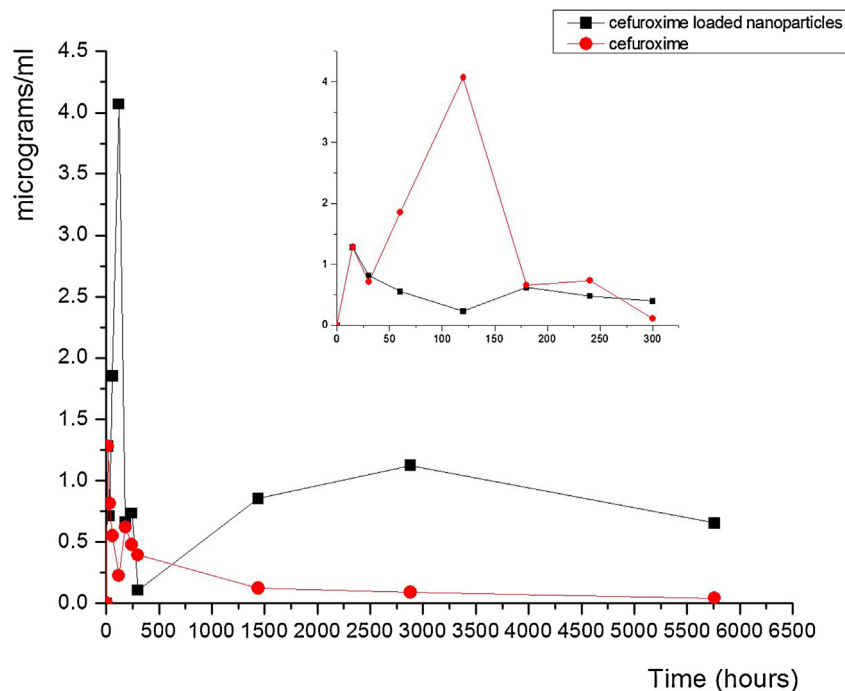


Fig. 13. In vivo cefuroxime release profile kinetic for free (red line) and encapsulated (black line) cefuroxime. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

reduced intensity) were found at cornea and lens level also 24 h after administration (personal observation, data not shown). Although the intensity of fluorescence is lower, at 72 h the particles are still present at retinal, lens, scleral and corneal level. Thus, analyzing the biodistribution of particles it has been demonstrated their potential to penetrate the adjacent tissues of the administration site and also the deep migration to cornea and retina after longer time.

3.7. In vivo cefuroxime release behavior

The evolution in time of the CF accumulated at the level of the eye is presented in Fig. 13.

The CF was administrated as free drug solution or as CF loaded nanoparticles. The free drug administration leads to a burst effect in the first 15 min followed by relatively constant CF concentration for about 5 h. When CF is administered as nanoparticles a different kinetics is displayed. In the first 15 min the drug may be found at similar levels as in the case of free CF administration but in the interval between 50 and 200 min a sustained release may be observed. As it may be observed the difference in terms of maximal concentration of the released CF between the two administration pathways is 8 times bigger in favor of nanoparticles. This fact can be explained by a fast clearance of the drug when administered as free CF, possibly occurring during the first 15 min after administration. The nanoparticles continue to release CF even after the initial burst release up to 96 h.

4. Conclusions

Various efforts have been made by the scientific community to improve the bioavailability and the drug release rate from formulations. We consider the present results as an important step in achieving an appropriate polymer particulated system for cefuroxime release at intraocular level. The dimensions and the physical–chemical properties of the particles can be modulated (by varying the preparation parameters) to facilitate the administration, the biodistribution and the drug release in the specific segment of the eye. Therefore, the optimum reaction conditions considered for preparing nanoparticles appropriate for intravitreal administration are the following: CS/G ratio equal to 1.5% TPP concentration, $\text{NH}_3^+/\text{TPP} = 1.175$ (molar ratio), $\text{NH}_3^+/\text{GA} = 7/1$ (molar ratio), the crosslinking time—4 h, stirring speed—9000 rpm and 4% surfactant (in respect with each phase volume). This experimental study demonstrated also the ability of fluorescent nanoparticles to penetrate ocular tissues close to the administration site (intravitreal injection) and especially their tendency to migrate deep in the retina at time intervals of 72 h.

To conclude, although further experiments are warranted, all these results point out CS–G particles as potentially useful candidates for drug delivery at intraocular level.

Acknowledgement

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Modern Drug Delivery Systems for Targeting the Posterior Segment of the Eye

C.A. Peptu¹, M.Popa^{1,4*}, C. Savin¹, R.F. Popa² and L.Ochiuz³

¹Faculty of Chemical Engineering and Protection of the Environment, Department of Natural and Synthetic Polymers, "Gheorghe Asachi" Technical University of Iasi, Professor dr. docent Dimitrie Mangeron Str, nr. 73, 700050 Iasi, Romania; ² Faculty of Medicine, Department of Surgery, "Grigore T. Popa" University of Medicine and Pharmacy, Iasi, 700115, Romania; ³Faculty of Pharmacy, Department of Pharmaceutical Technology, "Grigore T. Popa" University of Medicine and Pharmacy, Iasi, 700115, Romania; ⁴Academy of Romanian Scientists, Splaiul Independentei Street 050094 Bucuresti, Romania



Abstract: Some of the most dangerous diseases of the eye are related to the posterior segment. Diseases such as age-related macular degeneration, cytomegalovirus retinitis, diabetic retinopathy, posterior uveitis and retinitis pigmentosa are difficult to treat using classical methods because of the many internal barriers of the eye which affect the drug efficiency. In this review, we will summarize the main research directions in the field of medicamentous treatment of posterior eye disorders belonging to the controlled drug delivery concept.

The review is starting with the most important knowledge regarding anatomy and pathology of the posterior segment of the eye and is continuing with the current treatment methods of the eye posterior segment illnesses and drawbacks of these methods, the drugs administration pathways to the posterior segment of the eye. The last three sections present the state of the art regarding the latest discoveries including the commercial products in the modern drug delivery systems; the main classes of materials treated in the present review are implants, hydrogels and nano- microparticulate systems.

Keywords: Drug delivery, posterior segment of the eye, micro- and nanoparticles, sustained release, intravitreal, implants, hydrogels, liposomes.

1. INTRODUCTION

Posterior segment ocular disorders represent the most frequent causes of visual impairment which untreated can lead to blindness. The most important diseases are age-related macular degeneration, cytomegalovirus retinitis, diabetic retinopathy, posterior uveitis and retinitis pigmentosa. Currently, the treatment of posterior segment diseases is limited by the difficulty to achieve sufficient effective drug level within the target tissues, most of the methods requiring repeated long-term administration of therapeutic agents. Most of the commercial drugs or formulations for ophthalmological disorders are designed for the treatment of the anterior segment illnesses so there is a permanent need for the medications for the posterior ocular segment. In the case of the existing ones the drug is released after repeated intravitreal injections which are generally associated with ocular complications and with a reduced compliance of the patient [1,2]. The challenge in developing a new formulation for the treatment of posterior eye disorders is to reach the effective drug dose into the affected tissue and in the same time to minimize the systemic and local side effects. The difficulty is given by the unique anatomy and physiology of the eye with its protective barriers which complicate all pharmacokinetic processes [3]. The literature reports many studies for different types of formulations, all of them are trying to contribute to the development of a new and reliable system. The present review presents the latest progresses and trends in ocular drug delivery systems for treating posterior eye segment disorders, with an emphasis on implants, hydrogels, micro- and nano- particulate systems.

Currently, a limited number of reviews have been reported in the literature focused on the modern drug delivery systems

dedicated strictly to the posterior segment of the eye. In the Table 1 are presented the most relevant reviews related to the topic in discussion. In addition, in the table are mentioned the most important aspects which are discussed in the reviews already published until now, as well as the year of publication revealing this way the novelty and the necessity of this new review outlining the current state of the art in the above mentioned field. As it can be seen, except one paper published in 2014 (which covers only the nano carriers for the treatment of the posterior eye disorders) all the rest are from 2010 and before.

2. ANATOMY AND PATHOLOGY OF THE POSTERIOR SEGMENT OF THE EYE

The eye is a unique organ, physically protected from the resistant structures of the sclera and cornea and anatomically isolated by the blood-retinal barrier. The surface of the eye does not favor drug penetration because the drainage and secretion of tears, the aqueous humor and the corneal structure generally prevent the passage of the drug substances to the internal structures of the eye [11]. As a result of several studies conducted in recent years, various drug delivery systems which provide effective concentrations of drug substance both in the anterior and the posterior region of the eye were introduced in therapy. There are three major factors to be taken into account for the optimization of the bioavailability in the posterior segment of the eye: 1. going across the blood-retinal barrier or the cornea and getting inside the eye; 2. obtaining a local effect; 3. extending the duration of the drug substance action, for a reduced frequency of administrations [9, 12].

The systemic penetration of ophthalmic drug is achieved when the passage occurs by drug substance diffusion starting in the blood and ocular fluids (aqueous humor), through the eye capillaries of the conjunctiva. For this reason, this route is also called conjunctival route. A second route is the topical application by instillation of eye drops, also called transcorneal route. Another mechanism that achieves drug substance penetration in the eye consists in its absorption through the palpebral and bulbar conjunctiva, followed by

*Address correspondence to this author at the Faculty of Chemical Engineering and Protection of the Environment, Department of Natural and Synthetic Polymers, "Gheorghe Asachi" Technical University of Iasi, Professor dr. docent Dimitrie Mangeron Str, nr. 73, 700050 Iasi, Romania; E-mail: marpopa2001@yahoo.fr

Table 1. The most relevant reviews.

Title of the review	Aspects treated within the review	Year of publication	References
Drug delivery to the posterior segment of the eye for pharmacologic therapy	- advances in ocular drug delivery techniques - implantable devices as well as micro- and nanoparticles.	2010	[4]
Current and future ophthalmic drug delivery systems A shift to the posterior segment	- ocular pharmacokinetics: barriers in drug delivery - progress and trends in ocular drug delivery systems - advantages and disadvantages of drug delivery systems to treat ocular diseases	2008	[15]
New Techniques for Drug Delivery to the Posterior Eye Segment	- Progress and trends in ocular drug delivery systems for treating posterior eye segment diseases, with an emphasis on transscleral iontophoresis - conventional dosage forms and advanced delivery systems for posterior segment disorders	2010	[5]
Topical and systemic drug delivery to the posterior segments	- pathology of the posterior segment of the eye - pharmacokinetic aspects of topical and systemic delivery to posterior segment of the eye	2005	[6]
Topical drug delivery to the posterior segment of the eye: anatomical and physiological considerations	- the four approaches for drug delivery to reach the posterior segment of the eye (topical, systemic, intraocular, and periocular release) and anatomical arguments	2000	[7]
Nanotherapy for posterior eye diseases	- The available routes to target posterior segment of the eye with barrier descriptions, advantages and disadvantages - Nanocarriers for targeting posterior segment of the eye	2014	[8]
A Review of Implantable Intravitreal Drug Delivery Technologies for the Treatment of Posterior Segment Eye Diseases	- The main sites and barriers for intraocular drug delivery device implantation and mechanisms of drug release - Implantable technology and examples	2010	[38]
Drug delivery to the posterior segment of the eye	- barriers of the posterior eye drug delivery and paths of the administration - ocular drug-delivery systems	2011	[44]
Drug Delivery to the Posterior Segment of the Eye: Challenges and Opportunities	- barriers to posterior eye drug delivery - current strategies used to overcome these barriers	2011	[9]
Topical drug delivery to the posterior segment of the eye: anatomical and physiological considerations	- Structures of the eye - barriers to topical drug delivery to the eye - topical drug delivery to the posterior segment of the eye - cyclodextrin-based delivery system	2008	[10]

rapid transport into the peripheral blood circulation at the level of the eye tissue (non-corneal absorption) [13, 14].

Unlike non-corneal absorption, corneal absorption is well studied and takes into account the role played by the various corneal layers in drug penetration. In Fig. 1 a schematic presentation of ocular structure is shown. Anterior segment comprises of the cornea, conjunctiva, aqueous humor, pupil and lens. The posterior segment includes vitreous humor, retina, choroid and optic nerve. Sclera is partly in the anterior and partly in the posterior segment.

The cornea is a thin transparent tissue, non-vascularized but rich in nerve endings, which makes it the most sensitive tissue in the body. It consists of five layers of tissue: the outermost region is the *pre-corneal film*/the anterior epithelium which is lipophilic,

very fine and very fragile and allows the penetration of the lipophilic substances; an elastic sheet of tissue called *Bowman's layer* links the epithelium to the corneal tissue proper; the *corneal stroma* comprises almost 90% of the total thickness of the cornea and consists of collagen fibers and no blood capillaries (which makes it perfectly transparent) and at the same time permeable only to hydrophilic substances and electrolytes and not to the lipophilic products; *Descemet's basal membrane*; *endothelium*, the last layer, is very rich in phospholipids and only allows the passage of lipophilic substances. Therefore, in order to cross the cornea, a drug must present amphiphilic properties. Sclera, a fibrous, opaque and vascularized membrane, also has nerve endings. The ciliary body joins the iris, which acts as a diaphragm causing the circular opening of the pupil [9, 15].

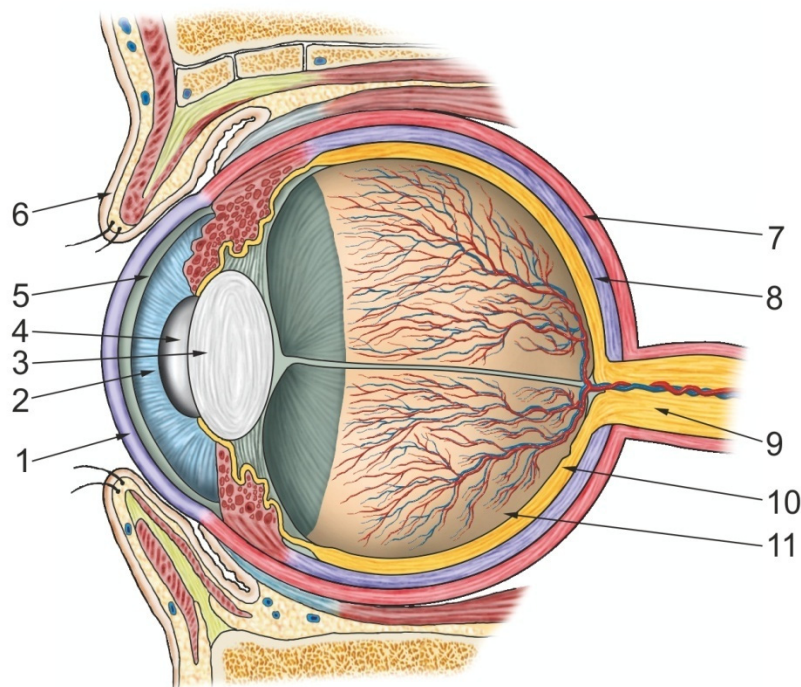


Fig. (1). The general anatomical features of the eye

1. Conjunctiva; 2. Aqueous humor; 3. Lens; 4. Pupil; 5. Cornea; 6. Eyelids; 7. Sclera; 8. Choroid; 9. Optic nerve; 10. Retina; 11. Vitreous humor.

When the drugs are administered topically in the eye, they first get into contact with the cornea and conjunctiva, which are the first barriers against the penetration of the active substances. The epithelium and the corneal endothelium are rich in lipids, which become a barrier against the permeation of the water-soluble polar compounds, whereas the corneal stroma is hydrophilic, this layer containing 70-80% water, and is a barrier against the permeation of fat-soluble nonpolar compounds. On the opposite side, the cornea adjoins the sclera, a white opaque layer that contains several blood vessels supplying the anterior tissues of the eye. However, the sclera does not act as a barrier for substances with different solubilities. The external surface of the sclera is covered by the conjunctiva which continues on the internal side of the eyelids and thus acts as a significant permeability barrier to several drugs. For the substances which enter the vascular system of the sclera and the conjunctiva, the transport tends to get away from eyes, into the general circulation [16]. The blood vessels of the iris have thick walls, thus being another part of the hemato-ocular barrier (Fig. 2).

The ciliary epithelium of the ciliary processes is a unique barrier that prevents the movement of most molecules such as antibiotics and proteins [6].

Other molecules cross this barrier due to the secretory process forming the aqueous humor. Another major physiological barrier mechanism is given by the tear production and the blink reflex. Blinking continuously displays the tear film on the surface of the eye and drains the tears via the nasolacrimal canal into the nose and further into the gastrointestinal tract [17].

The blood-retinal barrier may be damaged by injury or inflammation. The drug administered on the anterior segment of the eye is effectively swept by its diffusion into the circulation and/or the flow through Schlemm's canal. Thus, the topically instilled drug will not reach effective concentrations in the posterior segment of the eye [19]. In other cases, the only method used in targeting the posterior segment of the eye is the ocular injections that can be subconjunctival, retrobulbar, intraocular, intravitreal, etc. [18].

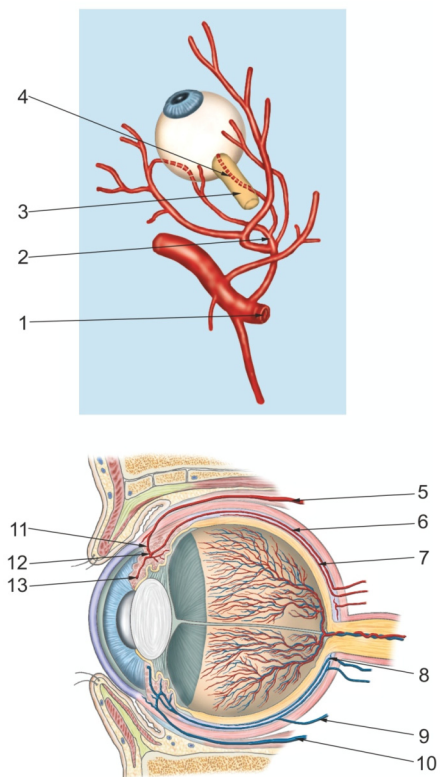


Fig. (2). The vascular system of the eye

1. Internal carotid artery; 2. Ophthalmic artery; 3. Optic nerve; 4. Central retinal artery; 5. Anterior ciliary artery; 6. Long posterior ciliary artery; 7. Short posterior ciliary artery; 8. Short posterior vein; 9. Vortex vein; 10. Anterior ciliary vein; 11. Anterior episcleral arterial circle; 12. Major arterial circle of iris; 13. Minor arterial circle of iris

3. CURRENT TREATMENT OF THE EYE POSTERIOR SEGMENT ILLNESSES AND DRAWBACKS

Targeting the posterior region of the ocular globe is necessary for the treatment of certain degenerative diseases which may even lead to total loss of vision. The diseases of the posterior segment of the eye generally have a rapidly evolution, as seen in the description of macular degeneration and diabetic retinopathy.

Diabetic retinopathy is a common complication of diabetes that has a prevalence of over 33% among diabetic patients. In developed countries, diabetic retinopathy is the leading cause of blindness in people between 20 and 65 years. Recent statistics show that most patients who have had diabetes for at least 20 years develop diabetic retinopathy.

Diabetic retinopathy has two distinct forms: the nonproliferative form and the proliferative form. Macular edema is a complication that can occur in each of the two forms. In the early stages of the disease, direct laser photocoagulation of the micro-aneurysms leads to good therapeutic results. It is estimated that photocoagulation during the first 3 years achieves a 50% reduction in the risk of moderate vision loss [20]. The complications of the proliferative retinopathy are extremely serious and may include vitreous hemorrhage, retinal detachment and neovascular glaucoma. The most recent therapies target the intravitreal administration of bevacizumab, a monoclonal antibody which is an endothelial growth factor inhibitor [21-23]. This active substance administered intravitreally exerts an antiproliferative effect by inhibiting the formation of new vessels. Encouraging results were obtained with the dexamethasone intravitreal implant which reduces macular edema [24].

Macular degeneration is another common condition characterized by the destruction of central vision as a result of lesions in the macula. The risk of macular degeneration increases with age, starting from 50 years. Nonexudative (dry) age-related macular degeneration (AMD) is the most common form, occurring in about 90% of cases. In this form, the cells and blood vessels within the macula are destroyed and hypertrophic deposits accumulate in the posterior segment of the eye. This causes lesions to the macula and affects its ability to send messages to the brain. Exudative (wet) age-related macular degeneration is less common and usually occurs with patients whose dry AMD progresses to wet AMD. In this form, new abnormal (neofornation) blood vessels develop; they are frail, bleed and cause the destruction of the macula [25, 26]. At present there are few drugs with proven efficacy for the treatment of macular degeneration. The latest research data on macular degeneration therapy report the results obtained after the intravitreal injection of inhibitors of vascular endothelial growth factor such as bevacizumab and ranibizumab [27].

Serous chorioretinitis (CRS) is a relatively common condition that consists in the serous detachment of the sensory retina at the level of the macular region, through the building-up of serous, non-inflammatory fluid between the sensory retina and retinal pigment epithelium affected by a focal defect of the coriocalpillary. The CRS therapy currently provides very good alternatives for rapid recovery by direct argon laser photocoagulation of the point of separation. Likewise, intravitreal injection of triamcinolone acetonide and bevacizumab are modern treatment options, indicated especially in cases of prolonged or recurrent evolution [22, 28].

4. DRUG DELIVERY TO THE POSTERIOR SEGMENT OF THE EYE

Achieving the therapeutic drug concentrations in the posterior segment of the eye continues to be a challenge in medical research, although in recent years important progress in this area have been made. Four drug administration routes are currently used in targeting the posterior segment of the eye: topical, systemic, intravitreal and periocular administration (Fig. 3).

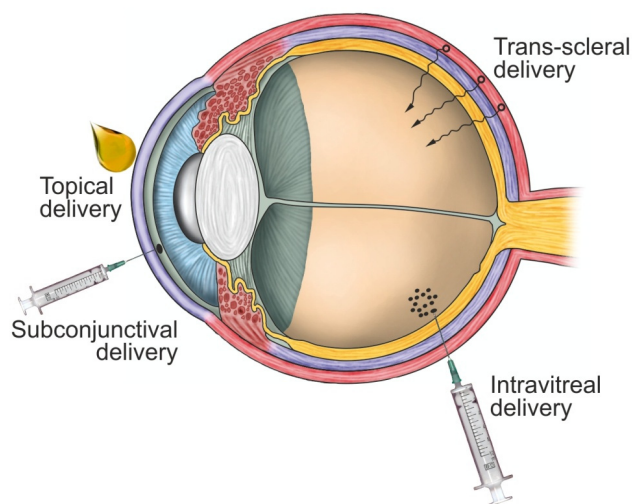


Fig. (3). Drug delivery routes to the posterior eye (systemic route is not shown).

Topical administration has the highest compliance with patients, but the therapeutic results of targeting the posterior segment are minimal on this route. Basically, due to the anatomical characteristics described above (the low permeability of the cornea, lacrimal secretion and nasolacrimal drainage), transcorneal drug absorption is very low. It is estimated that less than 5% of the topically administered drug substance concentration comes into contact with the intraocular tissues [19].

Targeting the posterior segment of the eye by the *systemic route* following drug administration in different dosage forms (tablets, capsules, intravenous solutions, etc.) is limited by the existence of the blood-retinal barrier which is impermeable to hydrophilic substances [29]. The reduced bioavailability of 1-2% drugs across the blood-retinal barrier requires the administration of high doses of drugs, and that can lead to serious side effects. However, some drugs with lipophilic properties (egg chloramphenicol, minocycline, cephalosporins) that can penetrate the barrier are administered systemically in the treatment of serious ocular infections. Likewise, mannitol and carbonic anhydrase inhibitors, such as acetazolamide are administered intravenously to reduce intraocular pressure [30].

Intravitreal delivery involves injecting the drug directly into the vitreous humor using a 30-G needle [31]. Today, various pharmaceutical forms are administered by intravitreal injection, such as aqueous solutions, viscous solutions prepared with hydrophilic polymers, suspensions, micro- and nano- particulate systems, biodegradable and non-biodegradable implants [32]. However, the release and distribution of the medicinal products administered intravitreally depend both on the properties of substances and on certain physiological parameters. It is known that low molecular weight drug substances diffuse quickly and require repeated administrations, while large molecules (greater than 500 Da) can persist in the vitreous humor for several days or even weeks. Accordingly, in formulating a drug delivery system for intravitreal administration, the pharmacokinetics of the drug substance must be considered so as to avoid repeated administrations. However, significant side effects have been reported, including retinal detachment caused by repeated injections, retinal hemorrhage, endophthalmitis, uveitis etc., due to high concentrations upon bolus dose administration [33].

Periocular injections are a less traumatic route for patients and have minimal risk of adverse reactions compared with the intravitreal route. The accumulation of a drug "deposit" on the surface of the sclera can occur following the subconjunctival administration

of a drug delivery system. The sclera has a much higher permeability to drug substances than the conjunctiva, which allows a facile trans-scleral penetration and diffusion of the drug substances [34], [35]. Nonetheless, frequent administration of periocular injections may lead to nonspecific absorption and undesirable systemic toxicity, especially when low therapeutic index drugs are used [5].

The development of new modified release ophthalmic drug delivery systems is a priority in medical research required by the current therapeutic needs and by the pathology of the posterior segment of the eye.

5. IMPLANTS

Conventional ophthalmic drug delivery systems such as topical eye-drops, systemic drug administration or direct intravitreal injections do not provide concentrations in the therapeutic range, which are essential for an efficient treatment in posterior segment eye disease. The main cause is the limitations of the restrictive blood-ocular barriers located at the level of the retinal vascular endothelial cells and retinal pigment epithelium (the most important are blood-aqueous barrier and blood-retinal barrier).

Subconjunctival, scleral and intravitreal routes are considered the most suitable for the purpose of drug delivery to the posterior segment of the eye *via* implants as they offer direct drug delivery to the target site, with minimal systemic loss [36]. Intraocular administration of the implants always requires minor surgery. In general, they are placed intravitreally, at the pars plana of the eye (posterior to the lens and anterior to the retina) [15]. Drug release occurs either by diffusion across a permeable membrane or by degradation of the polymer block [37, 38]. Although this is an invasive technique, the implants have some benefits: by-passing the blood-ocular barriers to deliver constant therapeutic levels of drug directly to the site of action, avoidance of the side effects associated with frequent systemic and intravitreal injections, smaller quantity of drug needed during the treatment, prolonged activity, etc. The main commercial implants currently used for the treatment of the posterior eye diseases are represented in the Fig. 4.

An intraocular device can be designed as either a **reservoir** or **matrix** system. Reservoir systems are non-eroding devices that

provide superior control of drug release by virtue of a specific physical rate control feature linked with the internal drug reservoir. This feature may be an opening in the device, a porous membrane or a coating through which drug diffusion is retarded. With such systems a "zero" order release kinetics can easily be reached, and the release rate is controlled only by a single variable [39]. A matrix device can be designed in such a manner that the drug to be released after a certain kinetic. For example, if the polymer is non-eroding and the release is then governed by polymeric chain relaxation and the dissolution rate of the drug, the release kinetic has a "zero" order. However, even in the case of this type of matrix, the strength or elasticity of the device can be compromised as the released drug produces a series of interconnected gaps through the device. If the polymer matrix is bio-erodible (either by bulk and/or surface erosion), then the device may exhibit more complex drug release kinetics. The erosion factor of the polymer may contribute independently to the drug dissolution or diffusion behavior, unless the geometry of the device is considered to produce a constantly eroding drug releasing surface. Table 2 shows some aspects of certain types of implants currently used in drug delivery for targeting the posterior segment of the eye.

5.1. Non-biodegradable Implants

Non-biodegradable implants (i.e., reservoir type) typically contain a drug core surrounded by a semipermeable membrane allowing steady release of drug with zero-order kinetics for up to months to years [48]. Minor surgery is required to place the implant at the pars plana and typically anchor it to the sclera via a suture. Due to this surgical procedure, non-biodegradable implants are more susceptible to complications, such as retinal detachment [49]. One problem is the fact that these implants must be removed or refilled, step which can involve a second surgical intervention, after the drug was consumed. Currently, there are numerous clinically approved non-biodegradable implants available on the market, which release small-molecule drugs into the vitreous for a prolonged period of time. Non-biodegradable implants for macromolecular weight drugs release have not yet reached the market [50].

An osmotic pump implant has been used to deliver IgG across sclera for 28 days. However, due to large size of the implant, the



Fig. (4). The main commercial implants currently used for the treatment of the posterior eye diseases.

Table 2. Examples of biodegradable and non-biodegradable implants - advantages, disadvantages, site of implantation, the drug release, targeted disease and time of release

Implant type	Advantages	Disadvantages	Place of implantation	Drug	Disease targeted	Time of action	Refs.
Non-biodegradable	- More controllable delivery profile and longer periods of drug release than biodegradable ones - Increased half-life of drugs - - Drug stabilization - Improved patient compliance	- Requires surgical implantation - Requires surgery to harvest the device once is depleted of the drug - Side effects: insertion of devices is invasive and with associated ocular complications (retinal detachment and intravitreal hemorrhage for intravitreal implant)	Intravitreal	Fluocinolone acetonide	- CMC retinitis	Months to years	[40],
			Intravitreal	Triamcinolone acetonide	-Chronic non-infectious uveitis		[41]
				Ciliary neurotropic factor	- Diabetic macular oedema - Wet AMD -Retinitis pigmentosa and AMD		[42] [43]
Biodegradable	- Controlled release of drug over a moderate period of time - Alternative to repeated injections because they increase half-life of the drug and may help to minimize peak plasma level - Drug stabilization - Improved patient compliance - Do not need to be removed - Various shapes	- Surgical implantation or injection associated with ocular complications - Final uncontrolled 'burst' effect in the drug-release profile	Intravitreal	Dexamethazone	- Diabetic macular oedema	Weeks to months	[44],
			Intraclear	Betamethasone	- Uveitis, - Post-cataract surgery -Uveitis		[45], [37] [46], [47]

main compartment was implanted in the subcutaneous space and connected using a brain infusion kit to the sclera [51].

Reservoir-type implant based on non-biodegradable polymers, - Vitrasert[®]-, was first used clinically for intraocular controlled release of ganciclovir in the treatment of CMV retinitis (approved by the FDA in 1996) [52]. The implant, composed of drug and coating polymers of poly(vinyl alcohol) (PVA) and poly(ethylene-co-vinyl acetate) (EVA), was implanted in eyes with CMV. PVA, which is permeable, acts as the framework of the device but it also, regulates the rate of ganciclovir permeation. EVA or silicone laminate, which are permeable, limit the practical surface area of the device through which the drug can be released. Drug release occurs when water diffuses through the outer EVA coating and partially dissolves the enclosed drug, forming a saturated drug solution that is then released into the surrounding tissue via diffusion [53]. The drug release rate can be slowed by reducing the surface area or thickness of the drug restricting polymer, and drug delivery can be increased by maximizing the surface area available for drug diffusion or by using a more permeable membrane. This implant enables well-controlled (zero-order) sustained drug release, is superior to other types of devices and has no initial burst effect of drug. As long as solid drug remains within the core, the drug release will be relatively constant. After the drug is completely released the implant must be removed during a second surgery and implant another device. Okabe developed an intrascleral implant made of same polymers (PVA and EVA) containing betamethasone in the form of a disc (4 mg in weight, 1 mm thick, 4 mm in diameter) [54]. The

betamethasone is released at a therapeutic level more than 4 weeks without burst effect. An advantage is represented by the fact that this implant may reduce complications after implantation because intrascleral implantation does not require perforation of the eye wall.

Some candidate drugs for the treatment of AMD were using the sustained release Vitrasert[®] device: fluocinolone acetonide or cyclosporine (to treat severe uveitis [55, 56]). The sustained release of both triamcinolone and 5-fluorouracil (5-FU) was studied in the treatment of experimental proliferative vitreoretinopathy (PVR) [57]. After implantation Vitrasert[®] device, the sustained release of these steroids may cause cataracts and glaucoma; in some cases patients developed endophthalmitis and retinal detachments with an increased rate.

Retisert[®] represents another intravitreal implant approved by Food and Drug Administration (FDA) that contains fluocinolone acetonide and releases the drug at a constant rate over a three year period of time [58]. It is currently used for the treatment of chronic uveitis or diabetic macular edema off-label. Implantation of this device requires surgery and the implant is placed through a pars plana incision and anchored to the sclera with sutures [59]. Clinical studies have shown a significant decrease in disease recurrence in fluocinolone implanted eyes (from 51.4% to 6.1% post implantation) and also a reduction in the need for adjunctive therapy. Nonetheless, the implant has been shown to increase intraocular pressure and cataract progression, with 51.1% of implant-treated eyes requir-

ing ocular antihypertensive medication and 9.9% of patients requiring cataract surgery [44].

The ILUVIEN intravitreal implant is a small, cylindrical tube (3.5 mm in length; 0.37 mm in diameter) designed to provide a low daily dose of 0.2–0.45 µg fluocinolone acetonide for 24 to 36 months after injection, and is composed of an inert, non-erodible, polyimide material that is often used in the manufacturing of intraocular lenses [5]. The implant is used for the treatment of chronic diabetic macular edema and can be inserted intravitreally as an injection, instead of via surgery [60]. It does not require removal, potentially resulting in multiple devices in the eye if subsequent implants are required, and it could represent in the future a more convenient treatment option for treating non-infectious uveitis.

I-VATION is a helical sustained-release implant containing 0.925 mcg triamcinolone acetonide coated in titanium, PVA, and EVA. The implant elutes drug for up to 2 years. The helical shape is designed to increase surface area available for drug diffusion and anchor the device to the sclera, while the flat cap is meant to sit just beneath the conjunctiva. Clinical tests have shown that this implant is effective in treating diabetic macular edema, but can cause some complications, such as increased IOP (intraocular pressure) and cataract [61].

A drug delivery system able to release topotecan (TPT) for several days has been developed and studied as an alternative for the treatment of retinoblastoma [62]. The aim was to design a method for a more selective periocular administration of topotecan that would allow the safe use of this drug alone or in association with carboplatin for the treatment of retinoblastoma with vitreous seeding. The authors hypothesized that a drug delivery system implanted locally could saturate the adjacent eye barriers and achieve selective drug delivery to the vitreous of the treated eye while minimizing systemic exposure. The implant consists in a topotecan-loaded “double-faced” polymeric drug delivery system composed of a free drug face designed to isolate the drug reservoir from the orbital conjunctival tissues and a drug-loaded face for TPT concentration in the adjacent scleral surface when the system is inserted episclerally. Poly(ϵ -caprolactone) was used as polymer matrix, the implant being manufactured by a simple, fast, and reproducible melt-molding-compression method. *In vivo* tests have revealed that the percentage of drug released from the total amount of encapsulated drug was 12% after 5 hours after implantation in vitreous, increasing to 20 and 40% after 24, 48 hours. This result highlights the capacity of the local barriers to impede local drug penetration even at high concentration gradients and supports the saturation of the barriers over time with continuously released drug.

A polymeric device able to administer multiple compounds with distinct kinetics to the posterior segment of the eye was recently reported. The device comprises a micro-fabricated reservoir, controlled-release cover and three different fluorescent formulations, which were made of photo polymerized tri-ethyleneglycol dimethacrylate (TEGDM) and poly(ethylene glycol) dimethacrylate (PEGDM) [63]. The release rate of each fluorescent compound is controlled by varying the PEGDM/TEGDM ratio in its formulation and the cover. The release kinetics appeared to be related to the swelling ratio of the PEGDM/TEGDM polymers. When the devices were implanted onto rat sclera, fluorescence was observable in the ocular tissues during 4 weeks, implantation and distributed locally around the implantation site. This system can administer multiple compounds with distinct kinetics, provides prolonged action and less invasive transscleral administration, and is expected to provide new tools for the treatment of chronic retinal diseases with new therapeutic modalities.

5.2. Biodegradable Implants

Biodegradable ocular drug delivery devices are composed of biocompatible polymers, which degrade into nontoxic by products, or polymers that solubilize *in vivo* and can be eliminated safely by

the human body. The main advantage of using biodegradable implants is the higher drug loading, due to the larger dosage form size, and the smaller surface to volume ratio allows prolonged drug release. Implants can be introduced into the body by minor surgical techniques and many implants are placed directly in the vitreous or on the sclera for the drug formulation to release into the eye for many months. Devices made from these polymers have a distinct advantage over non-biodegradable drug delivery systems: they do not elicit permanent chronic foreign body reactions and do not require removal after the drug supply has been exhausted [64]. Typically, the biodegradable devices are composed from synthetic aliphatic polyesters of the poly(α -hydroxy acid) family, including poly(glycolic acid) (PGA), poly(lactic acid) (PLA), and poly(lactico-glycolic acid) (PLGA) [31]. These polymers, which are hydrophilic, are degraded by non-enzymatic hydrolysis of their ester bonds, by reaction with water from the surrounding tissue (bulk erosion). From hydrolytic degradation result monomers such as lactic acid and / or glycolic acid which are nontoxic and eliminated safely via the Krebs cycle by conversion to CO₂ and H₂O. Other polymers used for the preparation of biodegradable implants but having hydrophobic character, are poly(ϵ -caprolactone) (PCL), polyanhydrides (PA) and poly(ortho esters) (POE). The PCL is often combined with PLGA to fabricate PLGC implants which are less immunogenic than pure PLGA devices [65].

For instance, a biodegradable scleral implant was prepared from PLA or PLGA and contains various quantities of ganciclovir [66]. It can be implanted at the pars plana and gradually release effective doses of drugs with polymer biodegradation over several months. The release profiles of drug were dependent on the type of polymers used, the polymer molecular weight, and the amount of loaded drug. Electroretinography and histological studies revealed minimal retinal toxicity. The implant may be advantageous for diseases such as cytomegalovirus and for vitreoretinal disorders such as proliferative vitreoretinopathy that require vitrectomy.

A hyaluronic acid (HA) intravitreal plug device implanted through a sclerotomy at 3.5mm from the limbus of rabbit eyes was developed, comprising three different HA esters namely, 100% ethyl ester, 100% benzyl ester and 75% benzyl ester [67]. The biodegradation rate of each device was related to the chemical structure of the three types of HA. For example, the partial benzyl ester was completely reabsorbed after 15 days. The study suggested that the intravitreal plug devices based on HA esters may provide useful biocompatible and biodegradable devices for potential drug delivery in the treatment of posterior segment eye diseases.

A doughnut-shaped minitablet was developed and evaluated as a biodegradable intraocular drug delivery system of antiviral drugs, using PLGA. The device gradually eroded when immersed in simulated vitreous humor and released drugs in a sustained manner [68]. It was implanted through the pars plana/peripheral retina of the right eye of rabbits, and no retinal detachments or significant vitreous hemorrhages were observed in the vitreous, retina and choroid. The implant has been demonstrated to be a flexible and versatile biodegradable intraocular device able to provide a modulated release of drugs to the posterior segment of the eye.

Ozurdex, the first biodegradable intravitreal implant FDA approved, is a cylindrical implant comprising dexamethasone dispersed in a PLGA matrix which is clinically effective up to six months in the treatment of diabetic macular edema and non-infectious uveitis. It is injected into the vitreous and exhibits fast drug release over the first two months due to diffusion of the drug and degradation of the polymer after which solely polymer degradation is responsible for the slower drug release into its target tissues, the vitreous and retina, over the following four months [69, 70]. The implant is contraindicated in patients with periocular infections and advanced glaucoma and in patients whose posterior lens capsule is not intact because of the risk of implant migration into the anterior chamber [71].

The formulation approach used in Ozurdex was also evaluated for delivery of brimonidine tartrate in clinical trials, for the treatment of retinitis pigmentosa [49]. Similarly to Ozurdex, this implant is injected into the vitreous and releases the drug for up to six months preventing cell death in the retinal pigment epithelium [72]. It has been shown that the low micro-pH of PLGA degradation products can cause inflammation of the ocular tissues which may be hidden in the case of delivering a steroid (Ozurdex), but may become more apparent with the brimonidine implant.

Natural lipids, e.g. triglycerides and cholesterol, may have potential to be used as biocompatible and biodegradable carriers for many of the therapeutics [73]. Recently, a sustained release device made of glyceryl tripalmitate was reported, which was evaluated in terms of its drug release profile and *in vivo* tolerance on rabbit eyes [74]. Glyceryl tripalmitate was selected as a matrix for clyndamicin phosphate because of its advantages in comparison with polymers: easy processability, low swelling ratio in aqueous media and lower melting point. The systems showed good biocompatibility with retinal tissue in mice and had promising drug release profiles; it represents a promising alternative for the ocular sustained release drug delivery systems.

Another biodegradable drug delivery platform which contains drug within a lipid/oil layer is Verisome, which is injected into the vitreous. The triamcinolone containing lipid gels *in situ* is forming a sphere in the vitreous humor which releases therapeutic levels of drug for up to 12 months. The formed sphere degrades slowly as the drug releases out of the *in situ* forming implant [75, 76]. Clinical trials proved that the implant is safe and effective in the treatment of macular edema. According to the manufacturer, its versatility allows it to deliver small molecules, peptides, proteins and monoclonal antibodies. It can be formulated also as a liquid, gel and solid.

To conclude, biodegradable polymer-based drug delivery systems show a considerable potential for the treatment of ocular diseases. Their versatility allows for construction of delivery systems to tailor the drug release for treating a variety of back-of-the-eye diseases, especially for sight-threatening retinal diseases. In addition, biodegradable drug delivery systems can also be used to adapt well-established drugs that may be ineffective by conventional administration routes. Clinical studies are still in progress examining the efficacy and safety of such delivery systems.

5.3. Other Implants

Other biocompatible implantable devices for sustained or controlled intravitreal drug delivery to the posterior segment of the eye have been developed employing diverse approaches: osmotic minipumps, configured capillary fibers, scleral discs, stimuli responsive implants, providing unique mechanisms of drug release for the delivery of drugs to the posterior segment of the eye [39, 65, 68].

Micro- Electro Mechanical System based implants actuate to perform their function, with actuation achieved by various stimuli including temperature, electrical stimulus, magnetic field [77] or osmotic pressure [78]. They consist of one or more drug reservoirs containing either a single drug or multiple drugs in different reservoirs and actuators which are responsible to push the drug out of the reservoir by mechanical means in response to the stimulus. For the purpose of drug delivery to the posterior segment of the eye, such implants have been explored with manual mechanical, electrolysis based mechanical and magnetic actuation. For example, Pirmoradi [77] obtained an implant containing docetaxel, for the treatment of proliferative vitreoretinopathy based on poly(dimethyl siloxane) with one side of the device having a magnetic field responsive membrane incorporating iron oxide nanoparticles in the polymeric matrix. A small incision of approximately $100 \times 100 \mu\text{m}^2$ was performed in the membrane to allow the drug release. The device provided precise control over drug release dependent upon the strength and duration of the magnetic field applied; however,

additional research is required to determine the suitability of these systems for the purpose of long term stimuli-responsive drug delivery to the posterior segment of the eye.

6. HYDROGELS

Since the development of hydrogels in 1960, they have been of great interest to biomaterial scientists and tissue engineers [79]. Hydrogels are polymeric networks of natural or engineered hydrophilic biopolymers that have the ability to swell in water or aqueous solvent system, and they hold the solvents in a swollen crosslinked gel system for delivery. By varying the preparation parameters (the ratio of biopolymer and crosslinking, duration and temperature of the crosslinking reaction etc), it is possible to obtain networks with variable crosslinking density, allowing the manipulation of permeation and diffusion characteristics. Therefore, they can retain hydrophobic and hydrophilic agents, small molecules, macromolecules, growth factors, antibodies and cells. Numerous advantages make hydrogels an attractive biomaterial. The aqueous environment of hydrogels can protect cells and fragile drugs (such as peptides, proteins, oligonucleotides and DNA). They serve as carrier of nutrients to cells and products from cells. They can be modified with cell adhesion ligands and can change physical state (liquid to solid) in response to pH or temperature changes. Depending on the specific structure, they can be nondegradable or degradable and their numerous advantages make hydrogels an attractive system. Most important, they are highly biocompatible [80].

Several hydrogels have been used as excipients for FDA-approved ocular small molecule therapeutics to increase their residence time on the eye surface through increased viscosity and/or mucoadhesion [81]. Examples include carboxymethylcellulose, gellan gum, crosslinked poly(acrylic acid), physical blend of methylcellulose, high molecular weight hyaluronic acid, poly(isopropyl acrylamide-*co*-acrylic acid N-hydroxysuccinimide-*co*- hyaluronic acid) [82] etc.

In situ hydrogels refer to the polymeric solutions which undergo sol-gel phase transition to form viscoelastic gel in response to environmental stimuli. The phase transition can be reached by changes in temperature, pH, ionic composition or by UV irradiation. For ocular delivery, research studies have been more focused toward development of thermosensitive gels which respond to changes in temperature [83]. Several thermogelling polymers have been reported for ocular delivery which includes poloxamers [84], multiblock copolymers made of poly(ϵ -caprolactone) [85]; poly(ethylene glycol) (PEG), PLA, PLG, poly(*N*-isopropylacrylamide), chitosan [86]. These thermosensitive polymers form temperature dependent micellar aggregates which form a gel after a further temperature increment due to aggregation or packing [87].

A thermosensitive gel made of triblock polymer PLGA-PEG-PLGA as an ocular delivery carrier for dexamethasone acetate (DXA) was developed by Gao *et al.* [88]. It was formulated as a DXA solution in a triblock copolymer *in situ* gel forming solution and administered topically in rabbit eye. In comparison with topical administration of conventional DXA drops in the anterior chamber of the eye, in this case the drug concentration achieved by administering eye gel was by far superior, suggesting the potential of PLGA-PEG-PLGA thermosensitive gel forming solution for enhancing ocular bioavailability.

In situ gelling systems are generally used as a method to increase the precorneal residence time of topically applied drugs, which can increase bioavailability of small-molecule drugs [89], [90], but may not be applicable to macromolecules because of their extremely poor permeability across the corneal epithelium. A thermosensitive *in situ* gelling injectable hydrogel was developed for ocular delivery of bevacizumab and demonstrated *in vitro* release of bevacizumab for 18 days [91]. However, it is difficult to get long-term release at a therapeutic level of macromolecules for more than few weeks or a month.

Based on the unique ability of silk fibroin to deliver therapeutic proteins with controlled release kinetics and biodegradation rates while preserving bioactivity, Lovett *et al.* proposed incorporating anti-VEGF drugs such as bevacizumab into silk hydrogels [92]. Analyzing the silk fibroin hydrogels loaded with the standard dose of bevacizumab it was found that bevacizumab concentrations in the vitreous humor after 90 day were equivalent to those levels for the positive solution control at 1 month. This system has a promising potential for antibody, peptide, small molecule, and/or RNAi therapeutics and may be used for the treatment of a wide range of diseases beyond age-related macular degeneration, offering enhanced patient comfort, reduced costs, and improved clinical outcomes.

A biodegradable, thermally responsive hydrogel, based on poly(ethylene glycol)-poly-(serinol hexamethylene urethane) was developed to deliver bevacizumab in rabbit eyes. The gel was well tolerated *in vivo* with just a little to no evidence of inflammation, and it was capable of sustaining release of bevacizumab over 9 weeks. This is the first study to date that compares the long-term *in vivo* release of bevacizumab from thermoresponsive biodegradable hydrogels to bolus intravitreal administration [93].

A newly synthesized biodegradable and thermo-sensitive triblock copolymer consisting of poly(2-ethyl-2-oxazoline) (PEOz) and poly(ϵ -caprolactone) (PCL) segments (PEOz-PCL-PEOz) that can sustain the release of bevacizumab *in vitro* and *in vivo* was recently reported by Wang *et al.* [94]. The electrophysiology examinations showed a good biocompatibility of hydrogel, and suggests it is an appropriate biomaterial for potentially use in intraocular application [95].

A biodegradable thermosensitive gel formed by a triblock copolymer based on PLGA and PEG has demonstrated its potential in sustained release of a large ovalbumin molecule to the choroid and retina following subconjunctival administration in the rat eye [96]. The ovalbumin concentrations were maintained at measurable levels in the sclera, choroid, and retina of rats over a period of 14 days, and these results suggest feasibility of thermosetting gel system in providing sustained delivery of macromolecules to the posterior segment ocular tissues such as choroid and retina.

Poly(*N*-isopropylacrylamide) (PNIPAAm) hydrogel is a well-known thermosensitive materials that has a lower critical solution temperature (LCST) or transition temperature at $\sim 32^{\circ}\text{C}$. Below the LCST the hydrogel is swollen, and above the LCST the hydrogel will collapse (shrink). The change in physical state is rapid and reversible, which makes the thermoresponsive hydrogel an attractive means of drug delivery. With a fast response to temperature stimuli, the drug can be released from the hydrogel quickly and act as an on-off switching release system. In order to induce the ability of sustained release of encapsulated drug to the new material, recent studies are proposing the use of poly(ethylene glycol) (PEG) as a pore-forming agent to obtain macroporous hydrogel. Zhang and Chu demonstrated that by introducing poly(ethylene glycol) diacrylate (PEGDA) to PNIPAAm, the hydrogel preserves better thermoresponsive characteristics and had homogeneous pores [97]. The pore size of the hydrogels can be modified by the amount of PEGDA introduced in the system. PNIPAAm-PEGDA hydrogels showed a significant improvement of mechanical properties. Kang and Mieler demonstrated that PNIPAAm-PEGDA hydrogels can be used to encapsulate and release some proteins (bovin serum albumin, immunoglobulin, bevacizumab, ranibumab) for ocular delivery to the posterior segment [98].

Crosslinked poly(*N*-isopropylacrylamide) (PNIPAAm)-poly(ethylene glycol) diacrylate hydrogels were also synthesized for sustained release of macromolecules such as bovine serum albumin (BSA) and immunoglobulin G (IgG) [98]. The gel system has provided nearly 3 weeks of sustained BSA release under *in vitro* condition. The results of research studies clearly signify the

advantages of thermosensitive gels in providing sustained drug release, prolonging the contact time of drugs with the cornea, reducing frequency of applications and side effects and increasing the ocular bioavailability over aqueous drops. In conclusion, the thermosensitive gels may be a viable option for the delivery of drugs for treating chronic ocular diseases.

Another hydrogel based on poly(ethylene glycol) diacrylate (PEGDA) crosslinked PNIPAAm was obtained by Turturro *et al.* [99]. Hydrogel was injected into the vitreous of the eye of rat, in order to determine the effect on retinal function. It was found that in region in the vicinity of the hydrogel, there was a significant decrease in arterial and venous diameters (4%) and an increase in venous blood velocity (8%) 1 week post-injection. Retinal thickness decreased (6%) at 1 week and the maximum a- and b-wave amplitudes of ERG decreased (15%). All data returned to baseline values after first week. These data suggest that the injection of PEGDA crosslinked PNIPAAm hydrogel result in a small temporary effect on retinal function without any long-term effects, and prove the potential of PNIPAAm-based materials as an ocular drug delivery platform.

Subconjunctivally implantable, biodegradable hydrogels for sustained release of intact insulin to the retina to prevent and treat retinal neurovascular degeneration, such as diabetic retinopathy, were obtained by Misra *et al.* by UV photopolymerization of NIPAAm monomer and a dextran macromer containing multiple hydrolytically degradable oligolactate-(2-hydroxyethyl methacrylate) units [100]. Insulin was loaded into the hydrogels during the synthesis process with loading efficiency up to 98%. The hydrogels can release biologically active insulin *in vitro* for at least one week and the release kinetics can be modulated by varying the ratio between NIPAAm and Dex-lactateHEMA and altering the physical size of the hydrogels. The hydrogels are non-toxic, and can be implanted under the conjunctiva without causing adverse effects to the retina. These hydrogels have potential for long-term periocular delivery of insulin or other drugs to treat diabetic retinopathy and other retinal diseases.

The hydrogel formulations has some key advantages for ophthalmic use, but none of them have all of the characteristics required for both successful delivery of both complex, fragile macromolecules and cells and for ease of use by the physician. Recently, a hydrogel based on thiol-modified derivatives of hyaluronic acid (HA) and porcine gelatin crosslinked with polyethylene glycol diacrylate (PEGDA) has been developed to meet these criteria [101, 102]. This system has been successfully used in animal models for retinal repair [103]. The gelation time in this case is too high and in order to reduce it one possibility is to crosslink the two thiolated biopolymers through disulfide bonds using the thiol-disulfide exchange reaction with a physiologically relevant disulfide-containing molecule. A thiolated-based hydrogel crosslinked using glutathione, which is gelling in less than 5 minutes, was recently reported by Zarebinski *et al.* [104]. This hydrogel supports the 3-D culture of adipose-derived stem cells *in vitro* and shows biocompatibility in preliminary intracutaneous and subconjunctival experiments *in vivo*. The obtained results suggest that this hydrogel could be well suited for local ocular delivery, focusing on front of the eye therapies, as well as on back of the eye treatments.

Light-sensitive hydrogels also have potential application in ocular drug delivery. These systems can be stimulated by either UV light or visible light, the latter of which is inexpensive, safe and readily available. Light-sensitive drug-delivery systems have opened up new perspectives in the targeted control delivery of therapeutics to the posterior ocular tissue, with minimal systemic adverse effects. Nonetheless, the clinical application of these hydrogels for treating posterior eye diseases is reduced by their slow response time [105].

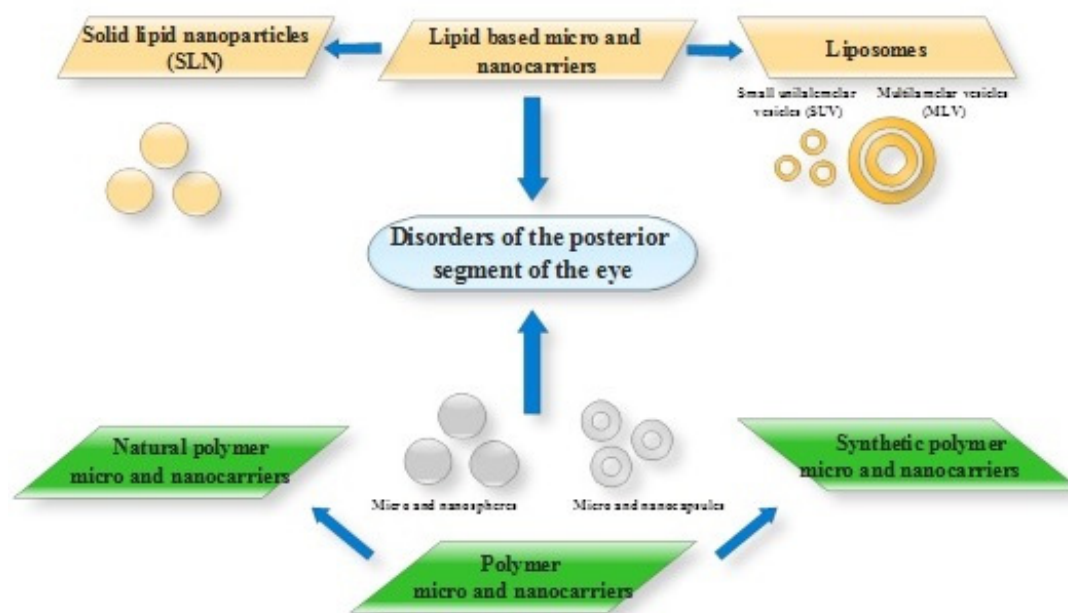


Fig. (5). Schematic representation of the micro- and nanocarriers with potential in the treatment of posterior eye disorders.

In conclusion, the potential for the development of sustained drug delivery systems involving polymeric systems is limitless, and newer polymers would serve the purpose of controlled and sustained delivery for treating posterior segment of the eye diseases.

7. MICRO- AND NANO CARRIERS

As literature reports, micro- and nanocarriers represent one of the most reliable solution for the delivering drugs specifically to the posterior segment of the eye. In the following subchapters the most representative classes of micro- and nanocarriers will be treated for underlining their potential in the treatment of posterior eye disorders. In the Fig. 5 the micro- and nanocarriers types discussed further are schematically represented.

7.1. Lipid Nanocarriers (LN) and Liposomes

Lipids represent amphiphilic molecules with one hydrophilic part and the other hydrophobic. When the lipids are placed in water, the interactions of the hydrophobic segments of the molecule with water, the lipids are self-assembling and liposomes are born. Liposomes, first discovered by Bangham and his co-workers in 1961 [106], present an aqueous core where water soluble drugs can be dissolved, which is surrounded by a lipid bilayer in which liposoluble drugs are usually entrapped. The liposomes, depending on the method of preparation, can be small, large or giant, and can be unilamellar (SUV – small unilamellar vesicles) or multilamellar (MLV – multilamellar vesicles). Liposomes have been studied as a viable alternative for ocular drug delivery since the beginning of 1980 and ever after the researchers are looking for the perfect solution. Regarding the applications for the posterior segment of the eye, it seems that liposomes can improve the clearance time and the specificity of the drug release in the same time with the reducing of the toxicity [107]. To our knowledge there was only one available liposomal formulation on the market for posterior eye disorders, Visudyne from Novartis Ophthalmics used for releasing Verteporfin (a hydrophobic chlorin-like photosensitizer, highly effective for photodynamic therapy *in vivo*) and it was the only drug approved by the FDA for the photodynamic treatment of age-related macular degeneration. Its application was limited since it was observed that the liposomes are losing their stability in sanguine plasma after intravenous administration [108].

In addition, intravitreal administration of liposomes leads to vitreal condensation, vitreal bodies in the lower part of eye, and retinal abnormalities. Therefore, this factor together with other factors such as formulation, difficult storage of liposomes, long-term side effects, must be taken into consideration while developing new liposomal formulation for ophthalmic application [109].

Other lipid particles, solid lipid nanoparticles were developed as an alternative to other traditional colloidal carriers like liposomes, polymeric nanoparticles and emulsions and they present advantages like controlled drug release and targeted drug delivery with increased stability. LNs are colloidal carrier system based on high melting point lipid as a solid core coated by aqueous surfactant. Generally, LNs present a mean diameter between 50 and 400 nm and they are made from lipids like triglycerides, partial glycerides, fatty acids, hard fats & waxes which are suitable for the incorporation of lipophilic and hydrophilic drugs [110]. LNs proved their efficacy in ocular drug delivery as they can enhance the ocular retention and corneal absorption and can also improve the ocular bioavailability [111]. The first generation of lipid carrier is represented by solid lipid nanoparticles which consist in a nanosized lipidic core stabilized by a layer of surfactants and the second generation, nanostructured lipid carriers, designed to improve the low drug loading and release ability of the first type, consisting of a mixture of solid and liquid lipids. Only few examples of the latest discoveries regarding the targeting of eye posterior segment using lipid based particulates are presented in the Table 3.

7.2. Polymeric Micro and Nanoparticles

As it was already mentioned in the previous sections of the present review, in order to deliver drugs to the posterior segment, periocular or intraocular routes are currently investigated as alternative routes for drug administration. The drawbacks of this include the invasive administration and frequent administrations which further affect the patient safety and compliance [117]. It seems that polymer based micro- and nanoparticles due to their diversity and reliability represent an alternative solution for drug prolongation of the posterior segment drug delivery.

Many techniques have been developed for micro- and nanoparticles preparation in respect with the envisaged ocular application.

Table 3. Examples of lipid based particulates with applications in the treatment of posterior eye disorders.

No.	Anti inflammatory drug	Lipid carrier composition	Observations	References
	Lutein (uveitis, retinitis)	Capric acid, with or without cyclodextrin	- The partition coefficient of lutein in vehicle of lipid carrier and HPβCD was about 15 - fold higher than that of HPβCD suspension, enhancing the effect of corneal accumulation. - Higher drug loading in the LNs increasing the concentration gradient towards the cornea. - LNs had more corneal lutein accumulation and penetration	[111]
	Diclofenac sodium	Goat fat and phospholipid	- high encapsulation efficiency and high corneal permeation	[112]
	Resina Draconis (blood stasis syndrome, trauma, tumors, inflammation, etc.)	composite hybrid thermosensitive gel containing solid lipid nanoparticles	- higher drug bioavailability - prolong the precorneal residence time	[113]
	Triamcinolone acetonide	nanostructured lipid carrier based on Precirol®ATO5 and Lutrol F68	- reduced toxicity	[114]
	Coumarin-6	poly-L-lysine surface modified liposomes	- poly-L-lysine molecular weight influence the efficiency of liposomal delivery to the retina - PLL solutions have been shown to be toxic for cells but the toxicity of poly-L-lysine liposomes was only slightly reduced	[115]
	plasmid DNA.	Phospholipid and cholesterol based liposomes transferrin grafted	- very small liposomes were obtained and they were stable for several months - only very small liposomes, under 85 nm, were retained in retinal epithelial tissue was observed	[116]

The methods are either physical, chemical or mechanical taking into consideration the nature of the polymer; polymer nanoparticles can be made from natural or synthetic polymers. As natural polymers the most frequently used are polysaccharides such as chitosan, alginate, hyaluronic acid and dextran and polypeptides like gelatin and other collagen materials and as synthetic polymers poly (lactic co glycolic) copolymer, polycaprolactone, poly(2-hydroxyethyl methacrylate) (PHEMA), poly orthoesters, etc. are very often taken into account by researchers for micro and nanocarriers preparation [118].

Also, the choice of the preparation method depends on the drug molecule which will be included, meaning it hydrosoluble or liposoluble character, and as a consequence on the particles type (microspheres and microcapsules, respectively, nanospheres and nanocapsules) which can ensure the desired amount of a certain drug which will be released upon a certain kinetic. All the components involved in particles preparation (polymers, solvents, reagents, surfactants, etc.) must be non-toxic or at least their use must be limited as much as possible. In general, the particles are obtained by one of the following methods taking into accounts the factors shortly presented above: single or double emulsion solvent evaporation, precipitation, coacervation, spray drying, ionic gelation, polymer cross-linking in emulsion system.

The use of micro- and nanoparticles for treating ocular disorders bring some important advantages such as [15]:

- Ensure the localized drug delivery
- Diminish the peak concentration resulting in minimizing the burst effect in drug release kinetic having as a main consequence the decrease of the system toxicity

- Decrease the frequency of administration by increasing the drugs half-life
- Improve the patient compliance

7.3. Natural Polymers Based Micro- and Nanoparticles For Treatment of Posterior Eye Disorders

Polysaccharides from human (hyaluronic acid) and non human (chitosan, alginic acid) sources are probably the most frequently used natural material for ocular drug delivery [119], and from these we think it is not overestimated to consider chitosan on the first position. The reason is that chitosan (CS) is a cationic polysaccharide which presents many exceptional biological properties such as biodegradability, nontoxicity, biocompatibility and mucoadhesiveness [120].

Ketorolac loaded chitosan nanoparticles have been developed by Aşık *et al.* as an potential alternative to repetitive intravitreal injections for treating ocular diseases such as pseudophakic cystoid macular edema [121].

Chitosan nanoparticles obtained by ionotropic gelation realised by Rajendran *et al.* had shown an excellent capacity for the association of acyclovir used for treating a wide ocular infections [122]. The results showed an improved interaction of chitosan nanoparticles with the cornea and other tissues due to the mucoadhesive properties of chitosan because of the electrostatic interaction between the positively charged chitosan nanoparticles and the negatively charged corneal cells. The authors proved in their work that chitosan nanoparticles can improve the corneal permeation, contact time and bioavailability of acyclovir.

The same method, ionotropic gelation, has been used for preparing daptomycin encapsulated chitosan nanoparticles as a new formulation for the treatment of bacterial ocular endophthalmitis [123]. Loaded nanoparticles were found to interact with mucin (a component of ocular fluids) and it seems that they are able to release the antibiotic directly to the site of infection and increasing in the same time the residence time in the eye. *In situ* gelling chitosan nanoparticles have found to be efficient for enhancing the bioavailability and efficacy of dorzolamide in the glaucoma treatment [124]. The formulation is mucoadhesive, safe and non-irritant and demonstrated a good pre corneal retention.

Chitosan was also used for improving the properties of other particles carriers. For example, Tsukamoto *et al.* developed bromfenac loaded liposomes modified with chitosan for the treatment of inflammations and other diseases of the ocular posterior segment, such as retinal and choroidal neovascularization and cystoid macular edema [125]. The authors of this study have proven that covering nanometric sized liposomes with cationic chitosan can be improved the delivery efficiency to the retina.

An association of oppositely charged polysaccharides, chitosan and alginate, has been used by Nagarwal *et al.* for obtaining nanoparticles loaded with 5-fluorouracil designed for topical chemotherapy of conjunctival/corneal squamous cell carcinoma [126]. They have found 5-FU in both aqueous and vitreous humour which means that this new formulation could be utilized for ocular application in both anterior as well as posterior segments. Nanoparticles based on chitosan–alginate polyionic complexes were developed again by ionotropic gelation by the group of Motwani [127]. The formed complex played the role of limiting the release of encapsulated drug in respect with each polysaccharide alone. The nanoparticles were loaded with Gatifloxacin (fourth generation fluoroquinolone – antibacterial agent used in the treatment of ocular infections), the release results are encouraging since the drug is sustained released over a by non-Fickian diffusion.

Another polysaccharides combination has been performed by Wadhwa *et al.* between chitosan and hyaluronic acid obtaining nanoparticles loaded with antiglaucoma drugs (dorzolamide hydrochloride and timolol maléate) [128]. It seems that hyaluronic acid enhance the mucoadhesiveness and efficiency of nanoparticles and measuring the intraocular pressure for this formulation in comparison with a commercial product, the decrease of this parameter was by far more effective in the case of chitosan – hyaluronic acid nanoparticles.

7.4. Synthetic Polymers Based Micro and Nanoparticles for Treatment of Posterior Eye Disorders

Natural polymers present variable purity and it is difficult to obtain reproducible particles and sometimes controlled release profiles for the encapsulated drug. On the contrary, synthetic polymers are generally provided with good purity and the drug release kinetic from synthetic polymeric nanoparticles can be also be modulated. Commonly used synthetic polymers for drug delivery applications include biodegradable aliphatic polyesters such as polylactides (PLA), poly(lactide-co-glycolide) copolymers (PLGA), and poly(ϵ -caprolactone) as well as non-degradable polymers such as poly(methyl methacrylate) and polyacrylates. Also in the case of synthetic polymers there is a winner regarding the number of studies which are describing the possibility to obtain micro- and nanoparticles for the treatment of posterior eye disorders and this is poly(lactide-co-glycolide) copolymers. For all the other polymers the number of literature references regarding micro- or nanodevices for this application are very limited, most of the studies are using these polymers for implants or inserts. Only few examples concerning PLGA micro- and nanoparticles with applications in the field of treatment of posterior eye disorders will be shortly presented.

Core-shell chitosan surface-modified poly(lactide-co-glycolides) nanoparticles for targeting glioma have been developed;

PLGA was used as the core of nanoparticles, while CS was used as a coating shell [129]. Carmustine and O6-benzylguanine were simultaneously loaded in the nanoparticles and tested *in vitro*, the results showed that nanoparticles improve the plasma stability of carmustine and enhance the cellular uptake of nanoparticles. Dexamethasone (anti-inflammatory drugs for the treatment of acute and chronic posterior segment eye diseases such as uveitis) entrapped in biodegradable poly(lactide-co glycolide) nanoparticles were prepared by the solvent evaporation process [130].

The results proved their applicative potential because the amount of dexamethasone encapsulated within nanoparticles seems to be sufficient for obtaining a therapeutic effect; the object of this study was to improve the encapsulation ability of PLGA nanoparticles to encapsulate dexamethasone.

Janoria *et al.* studied the effect of the lactide/glycolide ratio on the *in vitro* release of ganciclovir (in two forms: one hydrophilic and one lipophilic prodrug) from PLGA microspheres for the treatment of cytomegalovirus (CMV) retinitis [131]. Microspheres were prepared by the + (O/O) solvent evaporation method. One of the most interesting conclusions of the study was that lipophilic prodrug encapsulation into microspheres leads to longer residence time at the administration site due to multiple processes involved in drug release at target tissue.

CONCLUSION

Posterior eye disorders affect patient's quality of life and in many cases lead to different degree of vision loss. Even if some of the biochemical and biological mechanisms are still uncertain, the strategies for treating these diseases are lately oriented to the development of sustained and prolong action non-toxic drug delivery systems which are proved that are able to overcome the complicated eye barriers and to provide more efficient therapy with an improved compliance for the patient.

New technologies have been developed in order to provide prolonged action, increased bioavailability of the drugs, improved patient safety and minimal side effects. The present review is proposing to describe the latest discoveries in the field of drug delivery systems together with the basic knowledge of the posterior segment eye anatomy and pathology and with the current therapeutical strategies and their drawbacks. Modern drug delivery systems such as implants, hydrogels, micro- and nanocarriers, respectively, liposomes, solid lipid nanoparticles, natural and synthetic polymer micro- and nanoparticles have been presented together with the level of progress in founding reliable solutions for treating posterior eye diseases have been detailed. The overall conclusion of this study is that the potential in developing new systems is endless due to the diversity of the materials but also due to the high level of the knowledge in the biomedical sciences which finally will lead to find the perfect solution.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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Modulated release from liposomes entrapped in chitosan/gelatin hydrogels



Bogdan C. Ciobanu^{a,b}, Anca N. Cadinoiu^a, Marcel Popa^a, Jacques Desbrières^b, Cătălina A. Peptu^{a,*}

^a Department of Natural and Synthetic Polymers, Faculty of Chemical Engineering and Environmental Protection, "Gheorghe Asachi" Technical University of Iași, 73, Prof. dr. docent Dimitrie Mangeron Street, 700050 Iași, Romania

^b Université de Pau et des Pays de l'Adour, Institut Pluridisciplinaire de Recherche sur l'Environnement et les Matériaux, Equipe de Physique et Chimie des Polymères, IPREM, Hélicoparc Pau Pyrénées, 2 Avenue P. Angot, 64053 Pau Cedex 09, France

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ABSTRACT

The paper describes the preparation of chitosan/gelatin hydrogels, obtained by double crosslinking with glutaraldehyde and sodium sulphate/sodium tripolyphosphate that may be used as matrices for the inclusion of drug loaded liposomes composed of phosphatidylcholine. The main objective was to create a protective layer to stabilize the liposomal surface and to prolong/control the release of drugs from such systems. Therefore, complex systems capable of prolonged drug release and controlled release kinetics were obtained. Samples consisting of different chitosan/gelatin ratios and type/amount of ionic crosslinker have been prepared and characterized. The present study shows that calcein (used as a model hydrophilic drug) release from polymeric hydrogels has been retarded from several days to weeks after calcein inclusion in small unilamellar vesicles (SUVs) and multilamellar vesicles (MLVs) entrapped subsequently in hydrogels with variable composition. The calcein release kinetics of complex systems were compared to simple systems (control hydrogels) and important changes were observed thus proving that the mechanism of the process increases in complexity. Also, it is demonstrated that liposomes' stability can be greatly improved by inclusion in polymeric matrices. Multilamellar liposomes showed a better release behaviour, which indicates that these calcein loaded vesicles remained intact to some extent after release from the matrix, due to their improved stability provided by the multiple layers. When small unilamellar liposomes were tested, calcein have been released from hydrogels predominantly in a free form (due to their unilamellarity related instability even inside the hydrogel) but in a sustained and controllable manner. The main applications of the systems obtained are in the area of drug release for tissue engineering/tissue repair (topical administration of drugs for wound therapy – burns, for example). Hydrogels capable of delivering drugs over prolonged periods of time represent a step forward in wound management and many diseases that request long term and sustained delivery of drugs. These hydrogels could be used as tissue replacement or injectable depot systems in many high risk diseases including cancer.

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1. Introduction

Liposomes are spherical self-closed structures, composed of curved lipid bilayers, which enclose part of the surrounding solvent into their interior. The simplicity of production, their biocompatibility, the absence of toxicity [1,2], the size and similar composition to cells and

cellular organites, the possibility of size adjustment for specific applications [3], the ability of hydrophilic or/and hydrophobic drug loading (if we consider liposomes a biphasic system), and the stimuli sensitivity (temperature, pH and magnetic field) [4] make them a revolutionary tool in biomedical domain. Liposomes have been developed for a variety of applications. One promising area is drug delivery (small molecules, genes and macromolecules) [5,6]. Liposomes have been used as artificial cells for haemoglobin encapsulation and oxygen transport [7], as models in cell physiology research, as imaging agents for malignant tissue labelling [8], in textile industry [9] and cosmetics [10]. Liposomes are among the few classes of nanoscale materials used as drug delivery systems that have already reached the market. Other nanoscale systems as nanoemulsions, polymeric or ceramic nanoparticulate systems, micelles or dendrimers are in preclinical or clinical evaluation stage [11].

* Corresponding author at: "Gheorghe Asachi" Technical University of Iași, Faculty of Chemical Engineering and Environmental Protection, Department of Natural and Synthetic Polymers, Prof.dr. docent Dimitrie Mangeron Avenue, 73, 700050 Iași, Romania. Tel.: +40 232 278683 2332.

E-mail addresses: ciobi_b@yahoo.com (B.C. Ciobanu), jancaniculina@yahoo.com (A.N. Cadinoiu), marpopa2001@yahoo.fr (M. Popa), jacques.desbrieres@univ-pau.fr (J. Desbrières), catipeptu@ch.tuiasi.ro, catipeptu@yahoo.co.uk (C.A. Peptu).

For modulating the drug release kinetics from liposomes the dispersion of drug loaded liposomes in hydrogel matrices has been proposed by several authors in the last decade with the aim of producing controlled release systems [1,11–26]. Natural and synthetic polymers were used as matrix systems for the inclusion of drug loaded liposomes. Collagen, chitosan, gelatin, fibrin, alginate and dextran are the most used natural biopolymers [1,11]. The release time of active compounds could be easily modified according to the hydrogel preparation parameters (e.g. by modulating the film composition, liposome composition or the crosslinker type and concentration) [1,12–15]. Composite materials (liposomes in the hydrogel approach) provide the opportunity to control the release of incorporated low molecular weight drugs with fast diffusion from polymeric matrices [14].

Prolonged release of liposome incorporated drugs from polymeric matrices has been achieved by many research groups during the last years [12–26]. These systems are recommended for the treatment of many injuries located in various body areas. These composites could take different forms such as wound dressings or injectable depot systems. Depot systems based on chitosan incorporating liposomes were obtained recently by Hurler et al. providing prolonged release of mupirocin, an antimicrobial agent used in the treatment of burns [15]. The inclusion of drugs into liposomes and the dispersion of such vesicles into the polymeric matrix have multiple purposes like increasing drug concentration at the site of action, decreasing systemic drug concentration, maintaining a moist environment at the wound site, creating a barrier against pathogens and many others.

Injectable depot systems based on chitosan and drug loaded liposomes have been tested by many research teams [14–19]. Alinaghi et al. prepared composite systems that can prolong the release of drug loaded liposomes from such polymeric system [16]. Drug release (doxorubicin) modulation from an injectable composite system based on chitosan has been described recently by López-Noriega et al. [17]. The drug release from another thermosensitive chitosan hydrogel was studied by Wang et al. observing a reduced burst effect with improved antitumor activity and reduced systemic toxicity [18]. Another *in situ* gelling chitosan based hydrogel containing liposomes has been developed by Mulik et al. [19]. Recently, depot systems containing antimicrobial drugs metronidazole or clotrimazole included in highly deformable liposomes were prepared and evaluated for the treatment of vaginal microbial infections [20]. In order to reduce the toxicity of Amphotericin B used in the treatment of fungal infections Kang et al. incorporated the drug into a composite system based on a matrix composed of poloxamer 407 and poloxamer 188 [21].

The great versatility of polymers, crosslinking agents and liposomes creates a vast domain of choice when designing polymer matrix–liposome composites. Therefore, this area of research has gained more interest in recent era.

Our group proposed novel matrices for the modulation of drug release from chitosan/gelatin hydrogels containing drug loaded liposomes. The reason for choosing these natural polymers is based on their intrinsic properties like biocompatibility [22], biodegradability [23], low toxicity [24], versatility in functionalization [25–28] and so on. Previous toxicity tests have been performed by our group on chitosan/gelatin double crosslinked micro- and nanoparticles designed for ocular applications and the results have shown that the material is “practically nontoxic” [29]. Also, hydrogels consisting of chitosan and gelatin, two polymeric hydrogel systems previously proposed, were prepared via double crosslinking method that allows to diminish the potential toxicity of glutaraldehyde by partially replacing it with non-toxic ionic crosslinkers (sodium tripolyphosphate [27] or sodium sulphate) [28]. All the prepared hydrogels showed good mechanical stability and high loading/release rate of low molecular weight compounds, which are suitable properties for drug delivery applications.

In this study, the *in vitro* release of drug-in-liposomes from different biocompatible and biodegradable hydrogels, with potential applications as controlled drug delivery systems for tissue engineering/tissue repair,

is compared. The possibility of modulating the *in vitro* release profile of calcein (model drug) incorporated into two types of first generation liposomes, dispersed in different hydrogels, was analyzed. The influence of the liposome size and the hydrogel characteristics (composition and crosslinking density) over the calcein release kinetics was determined. We consider that this study contributes to the general direction of all controlled drug delivery systems to modulate the drug release kinetics by varying the properties of the carrier itself.

2. Materials and methods

2.1. Materials

Chitosan High Molecular Weight (C), Brookfield viscosity 200,000 cps, degree of deacetylation (DD ~75% determined by ¹HNMR technique), gelatin B (G) of bovine origin, glutaraldehyde (GA) 25% aqueous solution, sodium tripolyphosphate (TPP), Sephadex G-25 presenting 50–150 µm bead diameter, rhodamine B (fluorescent hydrophobic tracer), calcein (fluorescent hydrophilic model drug) and Triton X-100 were purchased from Sigma-Aldrich (St. Luis, MO, USA). Calcein and rhodamine B were used without further purification. Phospholipon-90G was received as a gift sample from Phospholipid GmbH, Nattermannallee 1, D-50829, Köln. Sodium sulphate was obtained from PrimexChim (Bucharest, Romania). All other chemicals used were of analytical grade.

2.2. Preparation methods

2.2.1. Preparation of liposomes

Calcein loaded MLVs and SUVs composed of phosphatidylcholine (PC) were used for complex hydrogel formation, in order to check the effect of the liposomes as an extra barrier for the calcein release. Plain liposomes (not containing calcein) were used for size determinations. For the preparation of complex systems, in each hydrogel a certain volume of liposome suspension was added before the GA addition and the total amount of included calcein was calculated by breaking a liposomal suspension with Triton X-100 surfactant. Also, rhodamine B labelled liposomes were prepared for the studies concerning the liposomal distribution throughout the polymeric matrix.

MLVs were prepared by the thin film hydration method [30] with minor modifications. The lipids were dissolved in a chloroform/methanol mixture (2:1 v/v). The organic solvents were evaporated (using a rotary evaporator – Heidolph Laborota 4002 – at 60 rpm, 30 °C and reduced pressure) from the round bottom flask (250 mL) resulting in the formation of a thin film on the walls of the flask. Then, the lipid film was hydrated with 2 mL of 30 mg/mL calcein solution (prepared in phosphate buffer saline [PBS], pH 7.4), at 25 °C. After the vortex shaking, a spontaneous formation of MLVs occurred. The dispersion was placed in a bath sonicator for 30 min at 25 °C, in order to break the existing aggregates. Finally, the liposomal dispersions were incubated for 1–2 h at a temperature above the lipid transition temperature (at 25 °C) for annealing membrane structural defects. The separation of liposomes from non-encapsulated calcein was achieved by centrifugation (3 spins at 15,000 rpm for 40 min) (Hettich Zentrifugen Universal 320R). A small amount of free calcein, still present in the supernatant of the centrifugation, was measured spectrophotometrically. The amount of calcein entrapped in the liposomes was determined by breaking them with Triton X-100. The latter is the total amount of calcein included in complex systems.

SUVs were prepared by sonication method [31]. A MLV suspension prepared as explained above was sonicated using a Bandelin Sonopuls GM 2200 probe tip sonicator (uniform sonic waves), 20 kHz processing frequency (10 pulses of 60 s duration and 30 s break to allow the sample to cool down, the probe was immersed 0.5 cm below the surface of the lipid suspension) to obtain the SUVs. The sonication temperature was above the transition temperature of the lipids to ensure a uniform bilayer of the vesicle and the

sample was kept cool by immersing the tube in a beaker with ice water. The SUV separation from MLVs and free calcein was achieved using Sephadex G-25 column chromatography (25 cm length, 1 cm diameter) eluted with PBS buffer (2.2 mL/min). Liposomes were used in the same day of preparation or the day after, keeping them sealed and protected from light at 4 °C. The model hydrophilic drug calcein (an analogue for low molecular weight drugs such as antibiotics) was selected due to its self-quenching property that allows it to distinguish the leaked out dye from the free dye (using Triton X-100 which disrupts the liposomal walls) [32] and by subsequent calcein dilution in PBS. The total calcein included in complex systems represents, as in the case of MLVs, the amount of calcein dosed after Triton X-100 treatment of SUV suspension.

2.2.2. Preparation of hydrogels loaded with calcein

Briefly, the two polymers were dissolved overnight in 5 mL of 2% acetic acid solution (v/v) at room temperature (2.5% final concentration). The solutions were filtered by using a reusable syringe filter holder coated with a filter paper. The calcein solution (32 µL of stock solution containing 640 µg calcein) was added dropwise under stirring in the polymer mixture. To ensure a minimal mechanical stability, glutaraldehyde amount was adjusted to crosslink approximately 20% of amino groups of both polymers (considering the deacetylation degree of chitosan and calculating the composition of diamino monocarboxylic acids from the gelatin) [33]. The optimal glutaraldehyde quantity was fixed by preliminary tests. The hydrogels were then placed in the oven at 50 °C for 1 h to partially eliminate water, thus allowing subsequent swelling of the hydrogel in the ionic crosslinking solution. The obtained hydrogels were immersed in 12 mL of different concentrations of ionic crosslinking agent (sodium sulphate and sodium tripolyphosphate) (Table 1), maintained for 30 min in these solutions and then placed in containers with buffer solution. The calcein was dosed in the ionic crosslinking solution remained after the preparation of hydrogels. Based on these calculations, the amount of calcein left in hydrogels was quantified (Table 1).

2.2.3. Preparation of polymer–liposomes–calcein systems

Complex polymer–liposomes–calcein systems were prepared in order to achieve the controlled release of calcein. In a first stage, calcein was encapsulated in liposomes (as it was indicated above). The suspension of calcein loaded liposomes in PBS was then added to the polymer solution prior to the introduction of GA [34]. As in the case of control samples, the hydrogels were then placed in the oven at 50 °C for 1 h. Subsequently, the aqueous solutions with specific amounts of GA were added under vigorous stirring and the crosslinking process continued with the introduction of ionic crosslinker according to the procedure described above. Eight complex systems were obtained in duplicate using the preparation parameters presented in Table 1.

Table 1
The hydrogel preparation protocol.

Sample code	C/G (w/w)	GA (µL)	TPP (mg)	Na ₂ SO ₄ (mg)	Calcein loaded (µg)
CG-S1	1.9	31.5		60.5	579.0
CG-S2	9	40		64	545.6
CG-S3	4.5	37.5		79	562.8
CG-S4	4.5	37.5		59.5	585.9
CG-T1	1.9	31.5	78		503.7
CG-T2	9	40	83		496.5
CG-T3	4.5	37.5	102		503.0
CG-T4	4.5	37.5	77		508.9

2.3. Characterization methods

2.3.1. Liposome size measurements

The mean diameter of the liposomes was determined by a laser light diffractometry technique (Shimadzu – SALD 7001). Three successive measurements were performed and standard deviation was calculated.

2.3.2. Liposome distribution within C/G hydrogels

In order to assess the distribution of entrapped liposomes into hydrogels, rhodamine B labelled MLVs consisting of PC were prepared. Complex systems based on hydrogel CG-S1 loaded with liposomes (MLVs and SUVs) containing rhodamine B were prepared in order to visualize the distribution of liposomes throughout the polymeric matrix. These rhodamine B labelled liposomes trapped in hydrogels were visualized by confocal microscopy (Eclipse TE300, using IBM Warp-40 operating system and BioRad Lasersharp soft). The samples were analyzed at a 10× magnification after ice cutoff. Control hydrogels without rhodamine B labelled liposomes were also prepared and analyzed.

2.3.3. Calcein release from hydrogels and from polymer–liposome complex

After the liposome preparation and their entrapment in C/G hydrogels, the release of fluorescent dye (calcein was a small molecular weight model drug) was studied to determine the release kinetics of the prepared systems. Each hydrogel (0.125 mg) was immersed in covered containers with 20 mL PBS solution and placed in a GFL 1092 water bath (60 rpm, 37 ± 0.1 °C). This device ensured the lack of light, protecting the fluorophore against photobleaching. The calcein release has been spectrophotometrically monitored at predefined times (24 h) using a Perkin Elmer LS 50B fluorescence spectrophotometer (excitation/emission = 490/515 nm, bandwidth 5 nm). In the case of hydrogels containing calcein loaded liposomes spectrophotometric determinations have been performed before and after the addition of Triton X-100 at a final concentration of 1% w/v. For each measurement, 1 mL of supernatant was removed from the container and replaced with fresh PBS buffer.

The calcein released (m_{ci}) was calculated using the calibrating plot and Eq. (1):

$$m_{ci} = C_{ci} \times V + \sum_{i=1}^{i-1} m_{ci-1} \times 100 \quad (1)$$

where: m_{ci} – total calcein released at the moment i , C_{ci} – calcein concentration at the moment i (ng/mL); V – the buffer volume where the calcein was released (20 mL); m_{ci-1} – calcein content in 1 mL of suspension or solution equal with concentration (C_{ci}) removed from supernatant for determinations and replaced with fresh PBS buffer at previous moments of study (t_{i-1}) (g).

The release efficiency was calculated using the following equation:

$$E_i(\%) = \frac{m_{ci}}{m_{ct}} \times 100 \quad (2)$$

where: m_{ci} is the total calcein released at moment i and m_{ct} is the total calcein included into hydrogels.

2.3.4. Measurement of liposome integrity

Calcein presents a high emission when its concentration is lower than the self-quenching concentration, so the calcein from the vesicles' interior which was found at 30 mg/mL (higher than self-quenched concentration) could not be detected spectrophotometrically [35]. After the addition of Triton X-100 the vesicles disintegrate and release the calcein which is subsequently diluted in the surrounding media. The total calcein was measured and the *latency* calculated. Information about the calcein release mechanism from complex systems (in the form of intact liposomes or free calcein) was obtained by measuring the *latency* in the hydrogel incubation medium. Calcein *latency* was measured after the separation of SUVs or MLVs from non-encapsulated calcein to

ensure the purity of calcein loaded liposomes, just before their use for the preparation of complex systems; thus, it was ensured that calcein could not have leaked out of the vesicles during storage. In the case of MLVs, if the calcein *latency* (%) was lower than 95%, the liposomes were re-centrifuged and re-dispersed before being used. For SUVs, when multiple fractions were collected from the separation column, the determination of *latency* was useful to evaluate which fractions present the highest *latency* and which will be further used for entrapment into hydrogels. Finally, calcein *latency* determination was used as a method for quantitative evaluation of the intact liposomes released from the films (the calcein *latency* was calculated during the release experiments).

For calcein *latency* calculations, 20 μL of the liposome dispersions was diluted with 4 mL buffer, pH 7.40 and the fluorescence was measured. These diluted dispersions of liposomes were treated with surfactant and the calcein fluorescence was measured again. The percent *latency* (%) was calculated from Eq. (3) [36]:

$$\text{Latency (\%)} = \frac{1, 1 \cdot I_{AT} - I_{BT}}{1, 1 \cdot I_{AT}} \times 100 \quad (3)$$

where: I_{BT} and I_{AT} are the calcein intensity emitted before and after the addition of Triton X-100.

3. Results and discussions

Reported hydrogels have been analyzed from the point of view of drug loaded liposome entrapment. The formation of polymeric networks has been previously proved by FT-IR spectra and the hydrogel character has been demonstrated by swelling degree measurements [27,28]. These hydrogels presented high swelling degree values in weak basic environment, ability to load/release water soluble drugs depending on the composition of the initial mixture and the type/amount of ionic crosslinker. In order to achieve a sustained release, hydrophilic compound calcein was initially entrapped in liposomes which were subsequently dispersed in the polymeric matrix (hydrogels) during the preparation process. The main challenge for preparation of such complex systems was to achieve high drug loading into liposomes and to incorporate the liposomes in the polymeric matrix without affecting the hydrogel formation and the liposome integrity. Moreover, it was expected to achieve a greater control of the drug release process from prepared systems by modulating the liposome size and matrix preparation parameters (steady-state release rate over time, reducing or even eliminating the “burst effect”).

However, it remained to evaluate whether such vesicles will retain their integrity if mixed with C/G blends during the crosslinking procedure leading to a polymeric network formation. The presence of the liposomes in supernatant (phosphate buffer saline, where the release take place) has been checked to establish if the drug was released also in an encapsulated form. If the liposomes would be found in the supernatant may prove that the polymer matrix plays a role in their protection, as was initially assumed.

3.1. Liposome size distribution

The mean diameters measured for the vesicles used in this study were $1.266 \pm 0.237 \mu\text{m}$ for MLVs and $0.112 \pm 0.014 \mu\text{m}$ for SUVs. As seen, MLV liposomes were large vesicles of micrometre dimension and SUV liposomes were small vesicles of nanometre size. Fig. 1 shows the distribution curves of the two types of liposomes used for complex system preparation. It may be observed that dimensional dispersity is a single-mode and presents relatively narrow distributions.

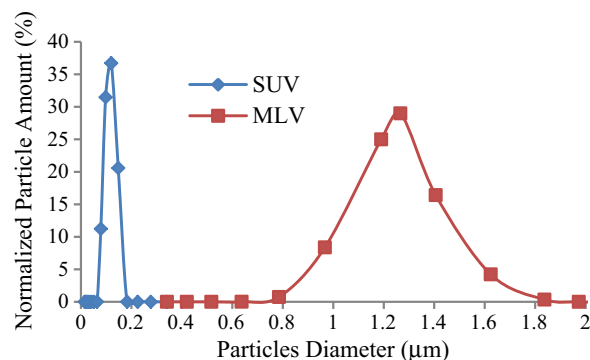


Fig. 1. Dimensional distribution curves for the two types of liposomes.

3.2. Liposome distribution within C/G hydrogels

The hydrogels used in the present work have been characterized recently by our group. The structural and morphological characteristics, the maximum swelling degree at 24 h and the drug loading/releasing capacity were presented in a previously published paper [27]. These results have led to the current work.

Fig. 2 presents micrographs from confocal microscopy observation of the double crosslinked hydrogels that entrap rhodamine B labelled MLV liposomes. The liposomes are well distributed throughout the whole surface area of the hydrogels (Fig. 2A). Similar micrographs were obtained for hydrogels containing SUVs. No fluorescence was detected in control hydrogels (Fig. 2B).

3.3. Release of calcein from hydrogels loaded with calcein

The release of calcein from control hydrogels (Fig. 3) was studied. In the case of hydrogels in which calcein was directly included first, it appeared as an intense burst release (in the first 3–4 h of the process) due to calcein release into the supernatant from the film surface or from superficial layers (which can reach up to 30% of the total calcein included into the hydrogel). In the next period, first 2 to 3 days, the in vitro release profile presents an increasing concentration for both

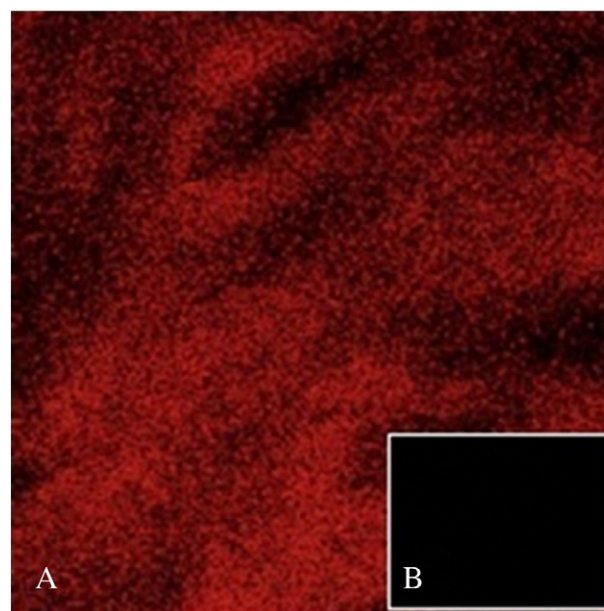


Fig. 2. Confocal microscopy images of CG-S1-MLV that entrap rhodamine B (A) and CG-S1 control hydrogels (B) observed at 100 $\times$ magnification.

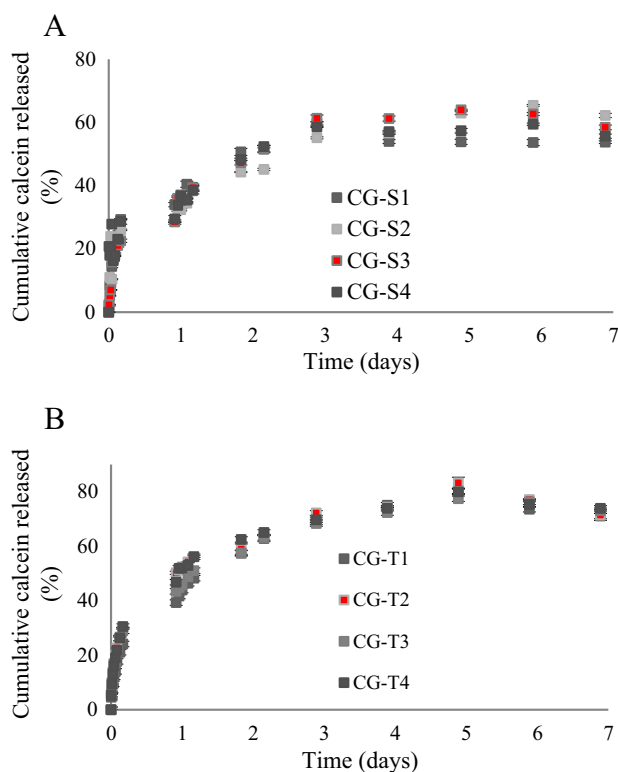


Fig. 3. Kinetics of calcein release efficiency at pH = 7.4 from control hydrogels crosslinked with GA/Na₂SO₄ (A) and GA/TPP (B).

studied systems, reaching 50–80% efficiency (depending on hydrogel composition and crosslinker type). Calcein was included into the hydrogels during the preparation process which took place in an acid environment that ensure the solubility of the polysaccharide. Calcein pK_a varies between 2.1 and 5.5 according to four dissociation steps determined by the existence of four carboxyl groups within the structure. Therefore, during the first stage of ionization calcein acted as a strong acid and its strength gradually decreased in the other stages of dissociation. In the conditions of hydrogel preparation certain ionic bonds occur that caused calcein retention inside the hydrogel. The ionic crosslinking may undergo between some of the ammonium ions of chitosan and gelatin (however, mostly participating in the crosslinking reaction with sulphate and triphosphosphate anions) and carboxylate anions of calcein. When the hydrogel loaded with calcein was suspended in phosphate buffer (pH 7.4), ammonium cations from the surface and from the superficial layers of the hydrogel were quickly neutralized. Therefore, ionic interactions between calcein and ammonium ions disappear, so calcein is quickly released, generating the “burst effect”. As the buffer diffuses inside the hydrogel, ammonium ions within are also gradually neutralized, causing the calcein release. The process underwent slower this time conditioned on the one hand by the buffer diffusion in hydrogel matrix and on the other hand by the diffusion of calcein itself outside the matrix, processes that depend, in principle, on hydrogel composition and crosslinking density. To these processes we added possible hydrogen bonds formed between the amino groups of the macromolecular substrate (H donor) and carboxylate anions of calcein (H acceptor). These links were more numerous as the content in the chitosan hydrogel was higher; therefore, in the first three days of experiments, a slight difference between the kinetic curves can be observed, specifically, slightly lower release efficiency at a higher C/G ratio. Even if the calcein release rate was reduced after manifesting the “burst effect”, the release efficiency reached 70% (for sulphate anion crosslinking) and even 80% in the case of triphosphosphate crosslinking after 5 days. Green et al. observed a similar phenomenon when analyzing the release of calcein from polymeric matrices containing chitosan [37].

The hydrogel matrix represents a diffusional barrier for calcein release causing, as it was expected, a gradual release except for the period when the “burst effect” was noticed. However, it was found that the release efficiency was not greatly influenced by the diffusion, but mostly by crosslinking density of the network, considering that between the curves shown in Fig. 3 the difference of release efficiency after 7 days was ≈10%. It can be noticed that crosslinking with higher amounts of sulphate ions (CG-S3) leads to a calcein release with a slightly higher efficacy compared to the hydrogels crosslinked with less sulphate (CG-S4). The results were consistent with the determined amounts of calcein included in hydrogels (Table 1), which decreases with the higher amount of sodium sulphate.

Comparing the calcein release kinetics from two types of matrices it was found that the release efficiency is higher for TPP crosslinked matrices, which may seem surprising, given the fact that this crosslinking agent is able to accomplish a superior crosslinking density than the sulphate ion due to its higher functionality (5 per molecule, compared with 2 for sulphate anion). In order to explain the apparent differences that occur between the two matrices in terms of release kinetics estimated by the process efficiency, it should be kept in mind that hydrogels crosslinked with TPP retained a reduced amount of calcein compared with those crosslinked with sulphate anions (Table 1). Taking into account the average values of calcein included in the two types of hydrogels and also the maximum release efficiency, we could obtain the amount of calcein released, which is practically the same for both types of hydrogels (approx. 400 μg). Lower amounts of calcein included into hydrogels crosslinked with TPP can be explained. TPP, through its superior functionality binds ionically to a larger number of ammonium cations from C/G blend. Accepting the hypothesis that calcein also binds ionically to both substrates (see explanation above), clearly it will bind in smaller quantities given the lower number of available ammonium sites. The immersion of hydrogels in phosphate buffer in order to study the release, in absolute value, will release the same amount of calcein as sulphate crosslinked hydrogels (≈400 μg), but in terms of process efficiency, it will be higher (Fig. 3B). Close values of the release efficiency corresponding to hydrogels crosslinked with TPP are further evidence that the diffusion is not the determining factor in the process. The fact that, in terms of released calcein amount, there are no differences between the two types of hydrogels (although the TPP crosslinked hydrogels are more dense as shown by the swelling kinetics studies reported previously) leads to the same conclusion [27,28].

In conclusion, it seems that the studied hydrogel preparation parameters (polymer ratio, type and amount of ionic crosslinker) do not have an important impact on the release behaviour of calcein from control hydrogels. Therefore, the use of liposomes as a supplementary barrier against the rapid and uncontrolled diffusion of small molecular drugs is justified.

3.4. Release of calcein from calcein loaded MLVs

The release of calcein from liposomes was studied to determine how calcein is released from liposomes. We found that in less than 24 h all the loaded calcein was released in PBS buffer under the same conditions of stirring and temperature used to study the complex systems. When calcein was loaded in MLVs and then dispersed within the hydrogels during the preparation process, the release kinetics was modified.

Calcein loaded in dispersed liposomes and then in hydrogels encounters two barriers in the release process: liposome membrane itself and the polymer matrix. As a result, the burst release attenuates by achieving a better control of the release process compared to the situation of the compound encapsulated in liposomes or just in hydrogel matrix. In this case it is found, however, that the calcein release efficiency was reduced for TPP crosslinked hydrogels compared with sulphate crosslinked hydrogels, oppositely from the case of calcein included directly in hydrogel. The phenomenon is related to the calcein release mechanisms that occur in these systems: (i) the most part of calcein is

transported by liposomes in which is encapsulated, driven by PBS solution entered in hydrogels causing its swelling; these liposomes gradually diffuse outside the matrix where they encounter the network resistance which causes their breakage; calcein continues to diffuse in free form to the supernatant; (ii) another amount of calcein is transported by liposomes that remain intact during diffusion through polymer hydrogel and they are released in supernatant – this amount is emphasized by breaking the liposomes with a surfactant, Triton X-100 (see [Measurements of liposome integrity](#) section); (iii) a lower quantity of calcein exists in free form from the beginning within the matrix, either released from liposomes that are broken during the crosslinking process or existing as such in the liposome suspension introduced into the polymeric solution before crosslinking – in our opinion, it is responsible for the “burst effect” that appears also in complex systems, but is highly attenuated compared to hydrogel–calcein simple systems. We must mention that this amount of calcein was determined before preparing complex systems according the same method for measuring the integrity of liposomes in order to determine the *latency*. It represents a maximum of 5% of the total calcein used in the loading of liposomes and it cannot be completely separated from the liposome suspension included in hydrogels. As it can be observed, this amount (about 5%, the remaining 95% being encapsulated in liposomes) is comparable to that released by the “burst effect” (Figs. 4 and 5). Thus, in the case of TPP crosslinked hydrogels, when a superior crosslinking density is involved, the diffusion of MLV liposomes is slowed down because of the matrix rigidity and especially the higher crosslinking density, mainly a smaller mesh size of the network. Therefore, liposomes reach outside the hydrogel matrix with difficulty due to their reduced mobility; consequently the percentage of calcein released is lower as compared with sulphate ion crosslinked hydrogels.

Furthermore, the hydrogel CG-S2-MLV releases calcein less efficiently, obviously due to more chitosan in its composition. On the one hand, due

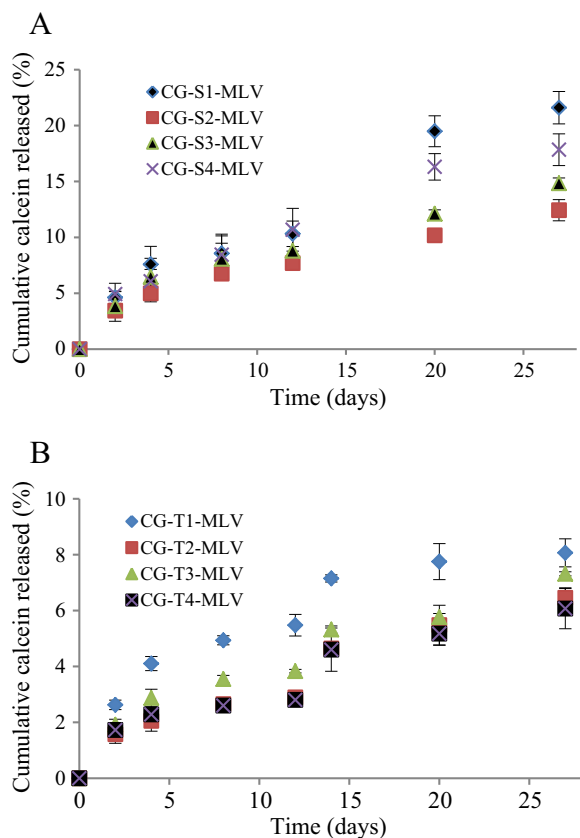


Fig. 4. Kinetics of calcein release efficiency at pH = 7.4 from CG-hydrogels entrapping MLVs crosslinked with GA/Na₂SO₄ (A) and GA/TPP (B) after Triton X-100 treatment.

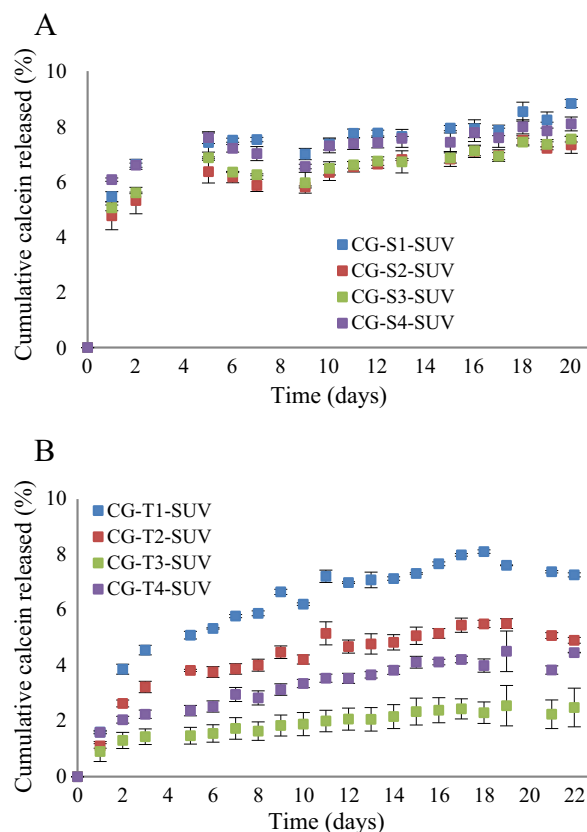


Fig. 5. Kinetics of calcein release efficiency at pH = 7.4 from hydrogels crosslinked with GA/Na₂SO₄ (A) and GA/TPP (B) entrapping SUVs after Triton X-100 treatment.

to the increased number of amino groups, the network is denser slowing the diffusion of liposomes; on the other hand, the large number of free amino groups that forms in phosphate buffer interacts with free calcein from the network (from the two sources, as mentioned above) by possible hydrogen bonds. Accordingly, hydrogel CG-S1-MLV with less chitosan in composition releases more calcein; the effect is the same for both hydrogels crosslinked with sulphate anions and TPP.

An interesting observation stands in the sharp increase of the calcein concentration in supernatant after 12–13 days for both types of hydrogels, especially for TPP crosslinked hydrogels. A possible explanation is the fact that a larger amount of liposomes that diffuse through the hydrogel matrix is destabilized after this time interval, releasing some of the calcein inside the polymeric matrix; then, a small molecular compound quickly diffuses in the supernatant. It should be mentioned also that calcein release is more difficult with increasing crosslinking density of the network (namely the ionic crosslinking agent) for the systems crosslinked with sodium sulphate (CG-S3-MLV compared with CG-S4-MLV).

3.5. Release of calcein from SUVs in hydrogels

Unlike the previous case, the release kinetic curves reveals the presence of a “burst effect”, more pronounced in the case of crosslinked networks with sulphate anions. Two explanations could be taken into consideration for this effect: a) the lower size SUV liposomes enable their easier diffusion through the hydrogel matrix (this effect is observed only for sodium sulphate crosslinked systems when the reduced crosslinking density is responsible); those from the superficial layers of the matrix quickly reach the surface where they destabilize, immediately releasing high levels of calcein; b) the mechanical stability of unilamellar liposomes, even in hydrogel matrix, is lower compared to the MLVs. It is possible that during the diffusion through the polymeric

network or even during the crosslinking procedure, the rupture of SUVs determines the calcein release, which then diffuses easier to the surface of the hydrogel. In this case, however, the calcein release efficiency is lower and the kinetic is slower in the case of TPP crosslinked hydrogels, the explanation being similar as the one in the case of MLVs. The diminished burst release in the case of systems crosslinked with TPP may be explained in the same way.

We noticed that an increasing amount of chitosan in hydrogel composition determines a reduction in calcein release efficiency. As discussed above, the larger amounts of free amino groups, in the presence of buffer solution, make possible strong interactions (hydrogen bonding) with free calcein resulted through the breakage of liposomes. In these samples, the influence of the crosslinking agent concentration on calcein release can be also observed. Hydrogels prepared by TPP crosslinking show an almost direct dependence between TPP concentration and calcein release. Keeping constant the polymer ratio and GA concentration, CG-T3-SUV releases a reduced amount of calcein compared to the CG-T4-SUV (Fig. 5B) due to greater ionic crosslinking (similar behaviour was observed in the case of MLVs).

Systems containing MLVs release higher amounts of calcein compared to systems containing SUVs, although these liposomes are more stable in the matrix (being mainly multilamellar) and diffuses with difficulty. The likely explanation is that, although MLV liposomes are more stable than SUVs, they transport a much higher quantity of calcein. The MLV diameter is about 10 times bigger than that of the SUVs (if we neglect the lipid membrane thickness) which means that a single MLV can encapsulate amounts of calcein 1000 times higher than a SUV. A smaller number of MLVs diffuse through the hydrogel matrix and their higher stability is compensated by the high amount of calcein transported and released through destabilization.

The slow release of calcein from the liposomes entrapped in hydrogels (SUVs and MLVs) compared to the controls as well as reduced “burst effect”, prove that liposomes largely remain intact during the hydrogel preparation process. All these results demonstrate that the hydrogel–liposomes–drug complex systems provide a better control of the drug diffusion process and allow a better control of release kinetics.

3.6. Measurement of liposome integrity

As discussed above, Fig. 5(A, B) shows the kinetic curves corresponding to the total calcein release efficiency (free calcein in the supernatant at different times, to which is added that of the liposomes remained intact after diffusion through hydrogel). As a result of the diffusion, part of calcein loaded liposomes reach outside the hydrogel and remain suspended in PBS buffer. For a calcein content determination it is necessary to break up the existing liposomes by using a tensioactive, Triton X-100. The calcein concentration in supernatant is measured, first, before breaking the liposomes and, second, after their breaking using Triton X-100.

The results obtained for CG-S2-MLV (arbitrarily selected) compared to the values of calcein concentration before and after the Triton X-100 treatment are presented in Fig. 6. The kinetic curve corresponding to CG-S2-MLV-AT is composed of free calcein released from the hydrogel and from calcein resulted from liposome destruction, observing the superior values of the calcein concentration. Based on measurements of calcein released before (BT) and after the Triton X-100 treatment (AT), the so called *latency* could be determined; this provides information about the stability of liposomes in PBS. The variation of this parameter at different moments of the release process is represented for hydrogels containing MLVs for all the polymer–liposomes–calcein systems studied and for both ionic crosslinking agents used (Fig. 7). These results confirm the stability of MLVs composed of multiple lipid layers. The disruption of MLV liposome membrane is a multiple-stage phenomenon (layers can break up one by one) that contributes to the drug protection inside the hydrogels.

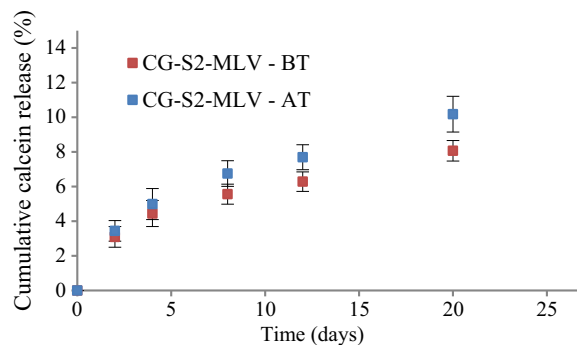


Fig. 6. The kinetics of calcein release efficiency before (BT) and after the addition of Triton X-100 (AT) for a randomly selected sample.

The values of the *latency* parameter were lower for hydrogels crosslinked with TPP compared with those of hydrogels crosslinked with Na_2SO_4 . This can be observed as a general trend for prepared hydrogels that contain MLVs. The hydrogels based on C/G ionically crosslinked with TPP form compact matrices that retain liposomes for long periods. Liposomes surrounded by polymer matrix are protected against destabilization as it can be seen by comparing the total calcein released (diagrams from Fig. 4) and calcein *latency* (Fig. 7). Contrary to the hydrogels entrapping MLVs, hydrogels containing SUVs present lower *latency* values (near zero, no-show results). In the case of SUVs, where one lipid layer covers the aqueous phase, a single rupture of liposomal membrane results in a total release of calcein inside the hydrogel, partially during the crosslinking process, but mostly during the diffusion process through the polymeric matrix.

3.7. The mechanism of calcein release from complex systems

The formulation of the calcein release mechanism from hydrogel–liposomes–calcein complex systems must take into account the

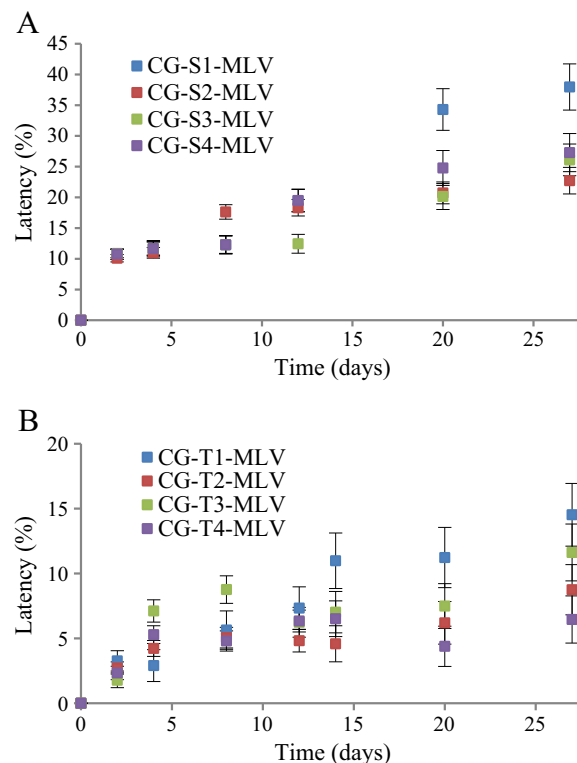


Fig. 7. The latency parameter for hydrogels crosslinked with GA/ Na_2SO_4 (A) and GA/TPP (B) entrapping MLVs.

local physical and chemical environments of the small molecular compound included in the matrix. After suspending the loaded hydrogel in phosphate buffer in which the release is carried out, the calcein that may be detected spectrophotometrically is the non-encapsulated form (free form) (considering two categories of calcein: encapsulated in liposomes which is not spectrophotometrically detectable and free calcein, a solution with increasing concentration due to liposome content of calcein). This free calcein detected in the supernatant originates, on the one hand, from the suspension of liposomes encapsulated in the hydrogel during the preparation process (this calcein cannot be removed by a single pass through column because of the separation efficiency – few percents under 100%) and, on the other hand, from the liposomes which were broken inside the matrix during crosslinking. The free calcein diffuses quickly outwards the matrix (even if it interacts with amino groups from macromolecular support) and generates the “burst effect” more or less pronounced, depending on the hydrogel composition, nature and amount of the crosslinker, type of liposome. When the supernatant penetrates the hydrogel mass causing its swelling to the maximum degree, the diffusion of liposomes inside the matrix begins, but slower in the case of MLVs compared to SUVs. In this process, liposomes encounter resistance from the rigid chains of the polymer network (possibly lower resistance compared with the dry state) and its mesh size. As a result, they may destabilize and release calcein which then begins to diffuse freely towards the outside of the matrix. A smaller number of intact liposomes can reach outside the matrix; the calcein that they carry is emphasized by breaking the liposomes with a surfactant (Triton X-100). It is obvious that the release rate determining the first step of the whole process is given by the diffusion of liposomes; this represents a potential way to adjust the kinetics of drug delivery and to eliminate the “burst effect” specific to drug release systems controlled by diffusion.

4. Conclusions

Small unilamellar and multilamellar liposomes loaded with model hydrophilic drug, calcein, were entrapped in polymeric hydrogels consisting of chitosan and gelatin mixtures for modulating the release profile of hydrophilic drugs with a fast diffusion from hydrogel matrices. It has been shown that the liposome integrity is highly protected during the double crosslinking procedure, especially in the case of MLV liposomes where multiple layers cover the drug content. The poor control of drug diffusion from hydrogel films can be avoided in therapeutic approaches by liposome inclusion in composite systems like these. By modulating the different parameters of the system (hydrogel composition, type and amount of ionic crosslinking agent and also vesicle size) one can prepare hydrogel systems capable of releasing the liposome-encapsulated active substance in a controlled manner over prolonged periods. In this study it was demonstrated that liposomes containing calcein, especially MLVs, were still present in hydrogel surrounding media even after three weeks. As it was expected, TPP performs a higher crosslinking density that reduces the mobility of liposomes, more pronounced in the case of larger vesicles which remain entrapped in hydrogel meshes for longer periods of time. Therefore, the percentage of calcein released is finally inferior to the one released from hydrogels crosslinked with sulphate anions. The kinetic data suggests that calcein release mechanism is complex and the calcein reaches the hydrogel surface in two main ways. First, the intact liposome diffusion through hydrogel (free calcein from liposome suspension and that released by breaking the liposomes inside the hydrogel matrix) should be considered. The second component consists in destabilization of vesicles before leaving hydrogel due to network resistance.

The great variability in preparation of liposomes (different sizes or lamellarity) and hydrogel biomaterials (the ratio of the components, the type and amount of crosslinking agent) gives the possibility to fine tune the ability of these systems to regulate drug release kinetics of water soluble drugs. Thereby, depending on the therapeutic need,

such composite materials may be useful in different medical applications for the long term delivery of drugs (in tissue engineering/tissue repair or replacement and injectable depot systems). The inclusion of liposomes loaded with drugs in polymer networks represents a solution for a slower and better controlled release which can be extrapolated to other water-soluble drugs.

Acknowledgements

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Biomaterial properties evaluation of poly(vinyl acetate-*alt*-maleic anhydride)/chitosan nanocapsules

Delia Mihaela Rață · Marcel Popa ·
Jean-François Chailan · Carmen
Lăcrămioara Zamfir · Cătălina Anișoara Peptu

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Abstract Nanocapsules with diameter around 100 nm based on a natural polymer (chitosan) and a synthetic polymer poly(vinyl acetate-*alt*-maleic anhydride) [poly(MAVA)] by interfacial condensation method were prepared. The present study proposes a new type of biocompatible nanocapsules based on poly(vinyl acetate-*alt*-maleic anhydride-chitosan) (MCS) able to become a reliable support for inclusion and release of drugs. The spherical shape of the nanocapsules was evidenced by scanning electron microscopy. Nanocapsules presented a good Norfloxacin loading and release capacity. Haemocompatibility tests have demonstrated that the nanocapsules present a low toxicity and a good compatibility with sanguine medium. The biocompatibility properties of the nanocapsules after their intraperitoneal

administration in rats were evidenced by histopathological examination of different organs (brain, liver, kidney, and lung). The results are encouraging and the nanocapsules can be used as controlled drug delivery systems.

Keywords Nanocapsules · Interfacial condensation · Controlled release · Haemocompatibility · Biocompatibility

Introduction

Carrier-based drug delivery has already an enormous impact on medical technology and represents an alternative approach to improve the bioavailability

D. M. Rață · M. Popa · C. A. Peptu (✉)
Faculty of Chemical Engineering and Environmental
Protection, Department of Natural and Synthetic
Polymers, “Gheorghe Asachi” Technical University of
Iasi, Prof.dr. docent Dimitrie Mangeron Avenue, 73,
700050 Iasi, Romania
e-mail: catipeptu@yahoo.co.uk

D. M. Rață
e-mail: iureadeliamihaela@yahoo.com

M. Popa
e-mail: marpopa2001@yahoo.fr

D. M. Rață
Faculty of Medical Dentistry, “Apollonia” University of
Iasi, Muzicii Street, No. 2, 700511 Iasi, Romania

J.-F. Chailan
Matériaux-Polymères-Interfaces-Environnement Marin
(MAPIEM) Laboratory, University of Sud Toulon-Var,
Avenue de l'Université, BP 20132,
83957 La Garde Cedex, France
e-mail: chailan@univ-tln.fr

C. L. Zamfir
Department of Morpho-Functional Sciences, Faculty of
Medicine, “Grigore T. Popa” University of Medicine and
Pharmacy, 16 University Street, 700115 Iasi, Romania
e-mail: zamfircia@yahoo.com

of drugs (Rieger et al. 2005); the use of carriers such as nanocapsules has been reported to be a promising solution in this sense (Prego et al. 2006). Nanocapsules can be used to encapsulate various hydrophilic drugs, proteins, and nucleic acids and represent very promising candidates for the treatment of genetic diseases, viral infections, or cancers (Fua et al. 2011). Ideally, a release system should encapsulate and protect the active substance, to prevent undesired release and to deliver the active substance in a controlled manner at the desired site (Irfan and Seiler 2010). Biocompatibility is the basic requirement which makes possible the application of a biomaterial for a therapeutic treatment. A large variety of synthetic and natural polymers can be used for the preparation of these release systems (Watanabe et al. 2005), even the polymers which form the membrane of the nanocapsules used in biomedical field should be biodegradable, nontoxic, and nonreactive with the inner core (Singh et al. 2010). For this reason, the natural polymers such as polysaccharides or proteins are widely used for the preparation of drug delivery systems (Watanabe et al. 2005; Jatariu (Cadinoiu) et al. (2012); Anitha et al. 2011; Ciofani et al. 2008; Loh et al. 2010). On the other hand, the use of the synthetic polymers, which are fulfilling the above mentioned conditions, has the advantage of high purity and good reproducibility over the natural polymers. Also, they have an important advantage to achieve a sustained release of the therapeutic agent over a longer period of time in comparison with the natural ones which has a relatively short duration of drug release (Irache et al. 2011).

Considering this previous information, the main purpose of this work was to explore the possibilities to obtain nanocapsules based on a new concept, in order to improve the biological properties and the drug-loading capacity and to avoid direct contact of the drug with the tissues, facts that should lead to the reduction of side effects at the administration site. For this purpose, we chose as preparation method interfacial condensation, because this method offers the possibility of rapid formations of the nanocapsules, at ambient temperature (20 °C) and at atmospheric pressure, without using additional cross-linking agents (Sunil Dhumal et al. 2008) which are frequently toxic and difficult to remove from the synthesis products.

For the preparation of these nanocapsules, we used a combination between a natural hydrosoluble

polymer (chitosan) and a synthetic organosoluble polymer, {poly(maleic anhydride-*alt*-vinyl acetate) [poly(MAVA)]}. The reaction took place at interface between the two immiscible solutions by the opening of anhydride cycles from the copolymer, under the action of the $-NH_2$ groups from chitosan, finally forming a thin polymer membrane. In the membrane composition, chitosan is found outside of the nanocapsule and the copolymer inside. The presence of numerous and powerful chemical amide bonds between the two polymers makes it impossible detachment of chitosan from the membrane surface during nanocapsules processing or their stationing in physiological fluids from the body. We chose chitosan as natural polymer because it presents good biocompatibility, biodegradability, and capacity to form membranes, gels, or nanoparticles, and inhibits the growth of a wide variety of bacteria (Tripathi et al. 2009). On the other hand, poly(MAVA) is an alternant copolymer insoluble in water but very soluble in acetone, which exhibits a functional role in neoplastic processes in immunology and presents a great resistance to viruses (Popa et al. 2007; Spiridon et al. 1997). This copolymer has showed interesting properties as multi-functional material for advanced applications (Chitanu et al. 2006) in industrial processes or in medicine and pharmacy. The combination of these two polymers leads to a new material type interconnected network with advanced properties in terms of biocompatibility, biodegradability, respectively, capacity to include and release active principles.

Prepared nanocapsules were loaded with Norfloxacin which was used as model drug. Norfloxacin is a synthetic antibacterial compound which belongs to the second generation fluoroquinolones group. In vitro, this compound is active against a high spectrum of aerobic gram-positive and gram-negative bacteria, and is used to treat gonorrhea, prostate, and urinary tract infections (Barbas et al. 2007). However, because of low lipophilicity, it very slowly diffuses through cell membrane and cannot reach at the desired therapeutic level (Dhaneshwara et al. 2011). Our goal was to improve the bioavailability of Norfloxacin through the inclusion of the drug in the obtained nanocapsules.

This work contains also a very important study concerning the in vitro haemocompatibility, toxicity, and biocompatibility of the nanocapsules at different organs level after intraperitoneal administration.

Experimental part

Materials

Chitosan (degree of deacetylation 91 %), acetone, Norfloxacin (NFX), hexane, and surfactants (Tween 80, Span 80) were purchased from Sigma Aldrich. The alternant copolymer—poly(MAVA) with a molecular weight of 140.000 g/mol was synthesized by radical copolymerization and characterized in laboratory (Sunel et al. 2005).

Nanocapsules preparation

The nanocapsules were prepared by an interfacial condensation method. Three types of nanocapsules were obtained and detailed in the Table 1. The experiment begins with the preparation of polymer solutions. In a beaker, 50 ml acetone was introduced and 0.375 g of copolymer has been added under magnetic stirring. After complete dissolution of the copolymer, the surfactant (Span 80) with a final concentration of 2 % has been added under continuous stirring. Separately, a certain amount of chitosan was dissolved in 200 ml of 2 % acetic acid solution at a temperature of 40 °C, and the specific amount of surfactant for aqueous phase (Tween 80) was added in a final concentration of 2 %.

Copolymer solution was introduced dropwise in aqueous solution of chitosan under continuous magnetic stirring (750 rpm). After 3 h, the nanocapsules suspension was centrifuged for 25 min at 14.000 rpm.

In order to remove any possible unreacted products and the surfactants, the nanocapsules were purified by repeated washings with water, acetone, and hexane. Finally, obtained nanocapsules were dried from hexane at room temperature for 24 h.

Table 1 Experimental program and codification of synthesized samples

Sample code	Molar ratio (moles NH ₂ from chitosan/ moles (MAVA)	Poly(MAVA) amount in acetone (g)	Chitosan amount in water (g)
MCS-1	1/1.44	0.375	0.250
MCS-2	1/1		0.375
MCS-3	1/0.72		0.5

Nanocapsules characterization

Determination of total nitrogen content from nanocapsules

Kjeldahl method was used to determine the nitrogen content from nanocapsules and gives information about nanoparticles final content in chitosan. This test involves the thermal decomposition of the sample with nitrogen in the environment of concentrated sulfuric acid, when is formed ammonium sulfate based on existing nitrogen in the sample. This introduced in the Parnas–Wagner's distillation apparatus, is decomposed with concentrated NaOH, repostulating the ammonia in liberty, which is entrained by steam distillation into a recipient that contains a known volume of sulfuric acid (a from equation) with known titer, T_a , which will retain ammonia. Finally, the excess sulfuric acid remained unused is titrated and the percentage of nitrogen in the sample is calculated (based on the Eq. 1).

The amount of nitrogen in the analyzed sample was calculated with the following equation:

$$\%N = \frac{a \cdot T_a \cdot E_N}{E_{\text{acid}} \cdot m_p}, \quad (1)$$

where a = volume of H₂SO₄, T_a = acid titer, E_N = gram equivalent of nitrogen, E_{acid} = gram equivalent of acid, m_p = amount of analyzed sample (approximately 50 mg)

Morphological characterization

Surface morphology of the nanocapsules was investigated using Scanning Electron Microscopy (SEM) type TESCAN Vega. Nanocapsules were analyzed in a powder state. This analysis provides information on the shape and size of the nanocapsules and allows evaluation (qualitative only) of the agglomeration capacity of them. Nanocapsules mean diameter has been analyzed in duplicate using laser light diffraction method (SHIMADZU—SALD 7001). Nanocapsules were suspended in acetone and then dispersed by ultrasonication. A droplet of Span 80 was added in order to avoid nanocapsules aggregation.

Capsules NFX loading capacity

Each sample precisely weighed (30 mg) was dispersed in 1 ml drug solution with a concentration of 5 mg/ml

and kept for 24 h under magnetic stirring at 120 rpm. The nanocapsules were separated by ultracentrifugation at 14,000 rpm at 25 °C for 20 min using a SIGMA 3–30 K Laborzentrifugen ultracentrifuge. The amount of drug from nanocapsules was calculated by the difference between the initial amount of drug and the amount of drug from supernatant. Measurements were performed at a wavelength of 273 nm using a UV–Vis spectrophotometer NanoDrop ND 1000. The encapsulation efficiency of drug in nanocapsules (E_{ef} %) was calculated as follows:

$$E_{\text{ef}} \% = \frac{\text{initial amount of NFX (mg)} - \text{amount of NFX in supernatant (mg)}}{\text{initial amount of NFX (mg)}} \times 100 \quad (2)$$

In vitro release of NFX

The release of active principles from nanocapsules in a phosphate buffer solution (pH 7.4) was monitored by UV–Vis spectroscopic measurements using a NanoDrop ND UV–Vis 1000 spectrometer. Each sample loaded with NFX was introduced in a dialysis membrane which was then placed in buffer solution. This system was maintained at a constant temperature (37 °C) under constant magnetic stirring. 100 µl of solution was taken at certain time intervals, and the absorbance has been measured using a UV–Vis spectrophotometer. Release efficiency of NFX (E_{ef} %) was calculated with the formula:

$$E_{\text{ef}} \% = \frac{\text{the amount of released norfloxacin (mg)}}{\text{the amount of norfloxacin loaded into capsules (mg)}} \times 100 \quad (3)$$

serum were added to 3 ml blood. Control samples were prepared by adding 0.5 ml of physiological serum to the same volume of integral blood. Samples were incubated at 37 °C for 30 min under gentle stirring, then the nanocapsules were separated from blood cells by centrifugation (1,000 rpm, 10 min). PT in blood was determined using an ACL 100 blood tester and a PT-Fibrinogen kit (International Sensitivity Index (ISI) 1.06) (Biodevice, Italy). INR was calculated as follows:

$$\text{INR} = \left(\frac{\text{PT}_{\text{NC}}}{\text{PT}_{\text{m}}} \right)^{\text{ISI}}, \quad (4)$$

where, PT_{NC} : prothrombin time of nanocapsules, PT_{m} : prothrombin time of control samples, ISI: International Sensitivity Index = 1.06.

Calculation formula for Quick index (IQ) is

$$\text{IQ} = \frac{\text{PT}_{\text{m}}}{\text{PT}_{\text{NC}}} * 100 \quad (5)$$

Normal values of followed parameters are $\text{IQ} = 70\text{--}100\%$, $\text{INR} = 1\text{--}4$

Haemocompatibility tests

Haemocompatibility test consisted in the determination of prothrombin time (PT) and international normalized index (INR) of the blood in contact with nanocapsules. Integral blood from volunteers (healthy, nonsmoker) was incubated with anticoagulant (aqueous sodium citrate 3.8 % w/v; ratio 1/9 v/v). 20 mg nanocapsules suspended in 0.5 ml physiological

Toxicity tests

“Up and down method” allows the estimation of median lethal dose (DL50) with a satisfactory precision, in the same time respecting one of the current trends and requirements of experimental studies with laboratory animals—to involve a small number of experimental animals. The obtained results allow classification of the substance in terms of acute

toxicity, according to the International Classification of *Globally Harmonized System*. This test aimed the determination of nanocapsules LD50 after intraperitoneal administration using Swiss mice with a weight of 20–25 g. The animals were grouped on batches, in special cages and were maintained on a cycle of light/dark for 12 h at a temperature of 22 ± 3 °C and a relative humidity of 30–70 %. All animals received standardized food and had unlimited access to water during of the experiment.

Principle of the method

Initially, it was performed a *limit test* which consists in the intraperitoneal administration of a single dose of 2,000 mg substance/kg body weight. If the animal dies within 24 h after administration, the main test to determine LD50 is performed. If the animal does not die within 24 h, the same dose is injected to 4 different animals, so that finally 5 animals will be tested. It was noted with X the animals that died and with O the surviving animals. However, if three animals die within 24 h, limit test is considered finished because LD50 is less than 2,000 mg/kg and then is performed the main test to determine LD50. If an animal dies unexpectedly after a few days, all animals should be observed for a longer period of time. If all animals survive after administration of 2,000 mg/kg body weight than the dose is increased to 5,000 mg/kg.

After the limit test, the *main test* is performed. This one is performed by administration of a dose from a range of doses equally spaced on a geometric scale. Each animal is carefully pursued for a period of 48 h. The signs of acute toxicity which appear are noted. For an animal, a dose considered to be close to LD50 is used. If this animal dies, the second is injected with the next lower dose. If the first animal survives, it is injected with next greater dose.

The test is stopped when performed one of the following criteria:

- the maximum number of chosen animals was injected;
- 3 consecutive animals survive or die at the maximum chosen dose;
- for five from six consecutive tested animals, reversed results are obtained.

The obtained results at main test serve as starting point for calculating LD50 by probability method.

Calculation of LD50

Calculation of LD50 has been performed using probability method. LD50 is equal with m antilogarithm; m was calculated as

$$m = y + d(A/N + 1/2) \quad (6)$$

the standard deviation was calculated as

$$S = 1.62 \times d \times [(NB - A^2)/N^2 + 0.029], \quad (7)$$

where, y = logarithm of the lowest dose at which was produced the less frequently phenomenon (e.g., survival); $A = \sum i * n_j$, $B = \sum i^2 * n_j$, where i is the number that corresponds to the frequency of phenomenon (for the less frequently phenomenon, then successively up with 1, 2, 3, etc.), n_i : number of dead or survivors animals (the column which has a lower total); Reliability limit is calculated as m + standard error, respectively, m – standard error.

Nanocapsules in vivo biocompatibility: histopathology exam

For in vivo test of nanocapsules biocompatibility, 20 male Wistar rats were used, weighing 200–220 g, divided into 2 groups:

- control batch—the rats have received anything
- test batch—the rats were injected intraperitoneally with a nanoparticles suspension (0.5 mg/kg for 21 days), a single administration/day, same dose at the same time.

At the end of the experiment, after the intraperitoneal administration of the nanocapsules, animals were euthanized, and the tissue fragments from major organs were prelevated, processed, and included in paraffin, then sectioned and colored (hematoxylin and eosin) to be examined microscopically.

Results and discussion

The membrane of capsules was produced by condensation reaction between amino groups of chitosan and anhydrides cycles of poly(MAVA). Therefore, the membrane is made from an interpenetrated/interconnected network of natural and synthetic polymers, the reticulation being assured by amide bonds, as demonstrated in our previous work (Iurea (Rata) et al. (2013)).

Preparation conditions for the samples used in the present study were selected to lead to nanometric particles with diameter at around 100 nm which can be administered into the body by injection from suspensions in physiological fluids.

Determination of nanocapsules composition

Evaluation of nanocapsules membrane composition was possible by determination of nitrogen content (organogenic element brought only by chitosan in the network). As it can be observed in the Fig. 1, chitosan was found in nanocapsules composition in a proportion between 30.6 and 48.7 %, whereas chitosan percentage values in initial polymers mixture vary between 40 and 57 %.

These results have demonstrated that approximately 85 % of chitosan which entered in reaction was found in nanocapsules composition. Polysaccharide was found at lower levels in membrane composition compared to the original mixture. As expected, the content of polysaccharide from capsules increases with the increase of chitosan concentrations from polymer mixture.

Nanocapsules morphology

Scanning electron microscopy (SEM) brought additional information about the shape and diameter of nanocapsules (Fig. 2). Microscopy images have revealed that (1) synthesized nanocapsules are spherical, (2) in dry state, nanocapsules present an agglomeration tendency, but when were placed in an aqueous medium (under stirring) nanocapsules dispersed very

well, (3) medium nanocapsules diameter is around 100 nm. After the samples drying and re-dispersing in acetone, it was found that the observed aggregates by SEM are redispersed in particles with mean diameter slightly increased, the increase not exceeding 10 %. The dispersion of aggregates in water and the measuring of the mean diameter showed an increase of approximately 50 %, the diameter value being much lower than the volume of an aggregate.

In Fig. 3, the granulometric distribution curves are exhibited for MCS-3 sample (in duplicate) which present a mononodal character and a mean diameter around 120 nm.

Norfloxacin encapsulation in nanocapsules

In the Table 2, the encapsulation efficiency of NFX in nanocapsules and the amount of NFX (mg) per 1 g of nanocapsules are listed. Amount of NFX loaded into nanocapsules decreased with the increasing of chitosan amount in the initial polymers mixture, membrane composition, respectively. This phenomenon has been due to the reduction of diffusion process of drug solution through polymeric membrane and it was caused by increasing of polymer network density. A part of the drug remains included in the membrane with hydrogel character. In the release process, the drug from the surface of the membrane is fast released, generating the “burst” effect. As physiological liquid penetrates the capsule, the encapsulated drug starts to diffuse to the outside, but with a slower and approximately constant speed.

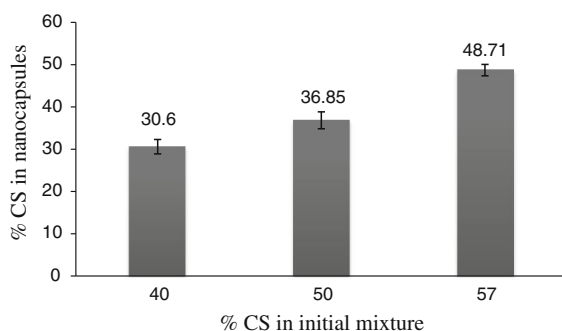


Fig. 1 Influence of chitosan concentration from initial mixture of polymers on the nanocapsules composition in chitosan

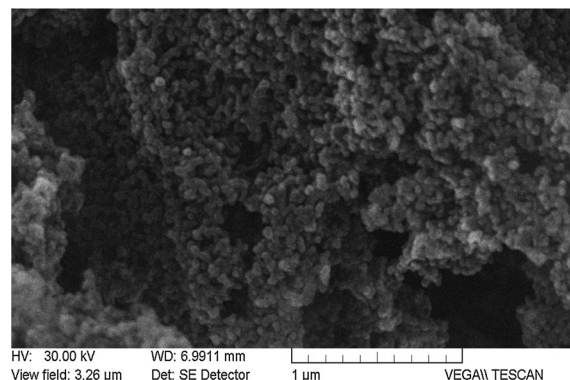
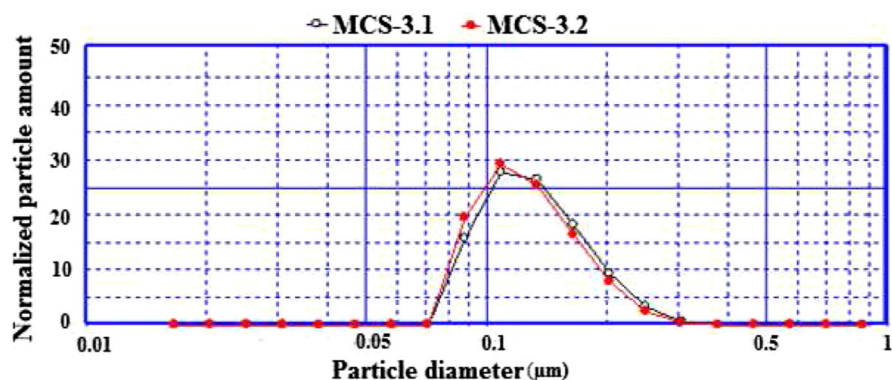
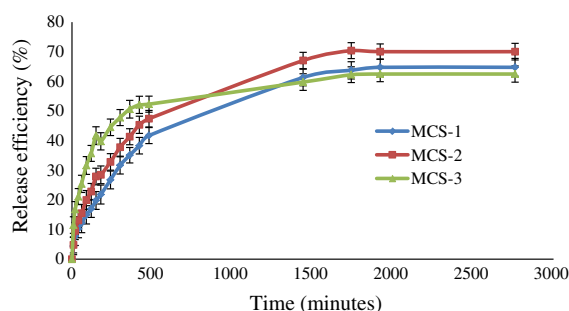


Fig. 2 Scanning electron microscopy for nanocapsules type MCS-3

Fig. 3 Nanocapsules granulometric distribution**Table 2** Encapsulation of NFX into nanocapsules

Sample code	NFX loaded mg/g nanocapsules	NFX encapsulation efficiency in nanocapsules (%)
MCS-1	92	55
MCS-2	80	48.4
MCS-3	81.8	49

**Fig. 4** In vitro release kinetics of NFX for obtained nanocapsules

Encapsulation efficiency was found to be between 48.4 and 55 %, and it was slightly influenced by the polymers ratio.

NFX release profile

Release profiles of NFX in phosphate buffer solution (pH 7.4) are represented in Fig. 4. Analyzing the results, one can observe that NFX release efficiency in alkaline conditions ranged from 61 to 70 %. The release of NFX from nanocapsules occurred by a diffusional mechanism of drug through polymeric membranes. Another interesting observation was that in the first 8 h, a rapid release of NFX from

Table 3 The results of hemocompatibility tests

Sample code	Fb (g/l)	PT (s)	PT (s) medium	IQ	INR
MCS-3	1.93	16.1	17.3	93.64	1.07
MCS-3	1.47	18.5			
Control sample 1	1.81	16.6	16.2	100	1
Control sample 2	2.26	15.8			

nanocapsules has taken place (“burst” effect), this behavior being due to the release of the drug absorbed in the membrane nanocapsules. After this time interval, a slow release of the encapsulated drug was observed and after approximately 30 h, the system reached an equilibrium state, the release efficiency value of NFX is depending on nanocapsules membrane composition. NFX release rate of the process increased in the first 8 h with the amount of chitosan from membrane composition which led to the increasing of its thickness (Iurea (Rata) et al. (2013)). The results did not revealed significant differences between the samples that differ by chitosan amount in composition, the encapsulation efficiency, and release kinetics of norfloxacin.

In these conditions, the amount of NFX retained in the capsule membrane increased, the drug is released faster, explaining this way the order in which are disposed the curves from Fig. 4.

Haemocompatibility tests

The interaction of materials with blood components can lead to the modification of the properties of plasma coagulation (Balan et al. 2012). For this reason, the

Table 4 Data for calculation of LD50 at 24 h

Dose (mg/kg)	Log	1	2	3	4	5	6	7	8	9	10	11	12	13
3,000	3.48	0												
4,000	3.60		0		0									
5,000	3.70			X		0		0		0		0		0
6,000	3.78						0		0		0		0	
LD ₅₀ = 4,672 mg/kg														
Total														
<i>y</i>											3.48			
<i>d</i>											0.10			
<i>A</i>											5			
<i>B</i>											13			
<i>N</i>											2			
<i>m</i>											3.67781			
LD50 = 4,672 mg/kg body weight														

0 survive, X death

effects of the nanocapsules on the blood using a standard coagulation test were evaluated (Zhu et al. 2009; Amarnath et al. 2006). In this sense, the prothrombin time (PT), Quick index (IQ), and international normalized index (INI) were determined. The obtained results are presented in Table 3.

Analyzing the results, the following conclusions can be evidenced:

- There is no significant difference between the values of coagulation control sample and the sample of nanocapsules;
- All coagulation indices evaluated are located in the normal limits.

This information suggests that the obtained nanocapsules exhibit a good compatibility with the sanguine environment.

Toxicity tests

Acute toxicity evaluation of nanocapsules type MCS-3 by “up and down” method

Initially, limit test to 2,000 mg/kg body weight has been performed, followed by the main test.

Limit test to 2,000 mg/kg body weight consisted in a single intraperitoneal administration (dose of 2,000 mg/kg) for a mouse. Because the mouse died within 24 h, the main test was performed.

Main test for determination of LD50

Because the mouse died within 24 h after the administration of a dose of 2000 mg/kg, it was decided to administer smaller doses in geometrical progression according to the guidelines OECD/OECD 425.

Data for calculation of LD50 at 24 h are listed in the Table 4 together with the results of LD50. In conclusion, after the limit and the main tests, it was found that LD50 for the tested nanocapsules at 24, 48, 72 h, 7, and 14 days after their intraperitoneal administration was 4,672 mg/kg body weight. This value of LD50 includes the substance in the group with *low toxicity*.

In vivo biocompatibility of MCS-3 nanocapsules: Histopathology exam

The histopathological exam was realized on both control (a) and nanoparticles-exposed (b) group of rats, in order to detect their impact on different tissues after their administration. The study investigated all the major organs and tissues which are susceptible to reveal the existence or not of a proper nanocapsules biocompatibility. The microscopic exam highlighted a perfect concordance of cerebral (1), pulmonary (2), and hepatic tissues (3) from the examined groups, the normal morphology being a common feature for all these tissues. It was observed that the tested nanoparticles didnot change the cellular architecture of the

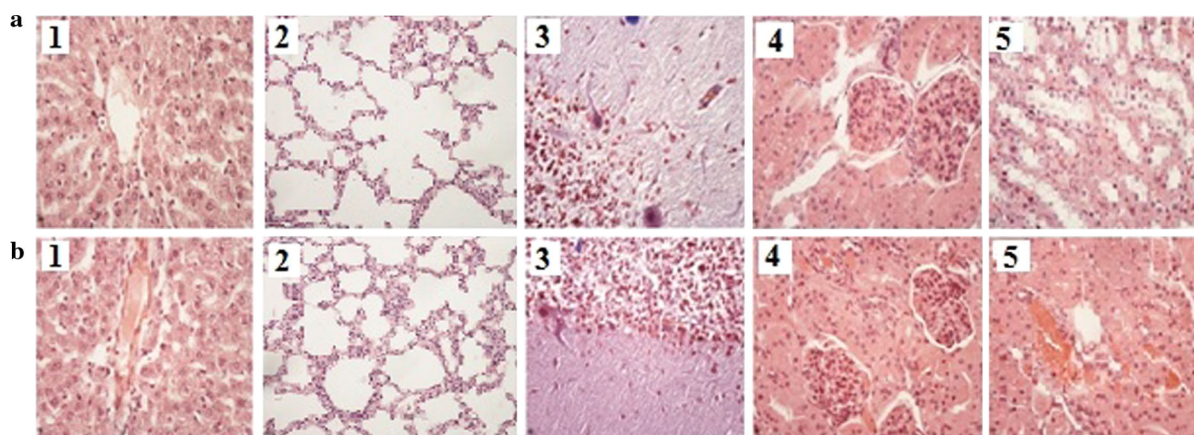


Fig. 5 Tissue morphology of 1: liver, 2 lung, 3 cerebellum, 4 renal cortex, 5 uriniferous tubes; for **a** control group and **b** nanoparticles-exposed group; (H&E stain, ob. $\times 20$)

normal tissue. Biocompatibility test was carried out by intraperitoneal injection with particles suspension to the test animals. As has been stated above, in the re-suspension of drug-loaded particles in aqueous media, destruction of the aggregates and the individual dispersion of the particle take place. Histological analysis reveals the presence of these particles (not aggregates) to the brain, proving that the particles are crossing the blood–brain barrier. The hepatic samples prelevated from the control group, presenting a normal morphology (Fig. 5a1) were compared with those from the nanoparticles-exposed group, which did not reveal structural alterations: the central veins and the sinusoids were normal configured, the hepatic cords were radiating out from the central veins, and the hepatocytes had a normal morphology, according to their function (Fig. 5b1). The normal aspect of the lung samples prelevated from the control group (Fig. 5a2) was also compared with that of the nanoparticles-exposed group, which presented normal thin-walled alveoli, blood vessels, and pulmonary air spaces without alterations (Fig. 5b2). The nervous structures examined in the control group (Fig. 5a3) were compared with those of investigated group, which did not reveal distinct damages. For example, the cerebellar structures had a normal appearance, the aspect of its layers and their different types of cells was normal (Fig. 5b3). The normal renal structure of the control group (Fig. 5a4) presented some distinct features in the second group. While in renal cortex, the renal corpuscles were normal and the renal tubules kept their specific features (Fig. 5b4), despite the

tubuloglomerular integrity (Fig. 5a5), the renal vasculature, especially that one along the boundary between the renal cortex and the renal medulla, where large arteries are visible, presented a high degree of vasodilatation, with red blood cells dispersed in the renal parenchyma (Fig. 5b5). These results, together with the rest of investigations sustain the existence of a distinct biocompatibility of MCS-3 nanocapsules. All these data suggest that despite an evident biocompatibility, we found a renal degree of susceptibility, which will represent a significant factor to take in consideration during the definition of structural parameters of nanoparticles.

Conclusions

The present study aimed the evaluation of biomaterial characteristics of a nanometric capsules based on chitosan and poly(MAVA) and the validation of the obtaining method in order to encapsulate, transport, and release of hydrosoluble drug in the body to some organs. Combination by interfacial condensation of the two polymers led to the formation of a membrane type interpenetrated/interconnected network with hydrogel characteristics, biocompatible, and nontoxic.

Scanning electron microscopy (SEM) revealed that nanocapsules are spherical and have a diameter of about 100 nm. Nanocapsules allowed the encapsulation of an increased amount of Norfloxacin and presented a good drug release capacity. Haemocompatibility and toxicity tests of the nanocapsules exhibit

a good compatibility with the sanguine environment, and a low toxicity which makes them suitable for medical applications. Histopathological examination revealed that the nanocapsules are biocompatible because does not appear modifications in the liver, brain, and lung. Tests also revealed that there is sensitivity at the kidney level.

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Covalent and ionic co-cross-linking—An original way to prepare chitosan–gelatin hydrogels for biomedical applications

Anca N. Jătaru (Cadinoiu),¹ Marcel Popa,¹ Silvia Curteanu,² Cătălina A. Peptu¹

¹Technical University “Gheorghe Asachi,” Faculty of Chemical Engineering and Environmental Protection, Department of Natural and Synthetic Polymers, Prof. Dr. Docent Dimitrie Mangeron Street, 73 Iasi 700050, Romania

²Technical University “Gheorghe Asachi,” Faculty of Chemical Engineering and Environmental Protection, Department of Chemical Engineering, Iasi 700050, Romania

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Abstract: The first goal of this work was to develop a method for obtaining interpenetrating gelatin (G)–chitosan (CS) networks prepared by double cross-linking (covalent followed by ionic) that exhibit hydrogel character. The second goal was to modulate their properties as a function of the preparation parameters by using neural network models. This study was therefore carried out by experiment and simulation. The covalent cross-linking resulted from the reaction between the carbonyl groups of glutaraldehyde with amino groups belonging to both polymers; the ionic cross-linking is based on the interaction between tripolyphosphate anions and protonated amine groups (ammonium ions) of the polymers. The total cross-linking density (indirectly assessed by estimating the water swelling capacity) and the ability to include hydro-soluble bioactive principles are influenced by the following process parameters: the CS/G ratio, the amount of ionic cross-linker, and the ionic cross-linking time. The prepared

hydrogels were characterized with respect to their structural, morphological, and some physical properties. The hydrogels ability to load high amounts of water-soluble drugs indicates their potential use as carriers for biologically active principles in the human body. A neural network methodology was applied to model the swelling degree and caffeine loading/release capacity depending on reaction conditions; in addition, applying this method, the optimal preparation conditions have been determined, targeting pre-established values for swelling degree or maximum caffeine value. The accuracy of the results obtained through this technique proves that the neural networks are suitable tools for modeling cross-linking processes taking place complex nonlinear polymers. © 2011 Wiley Periodicals, Inc. *J Biomed Mater Res Part A*: 98A: 342–350, 2011.

Key Words: hydrogels, double cross-linking, gelatin, chitosan, drug release, neural networks

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INTRODUCTION

Polysaccharide–protein systems show promising applications in the biomedical field due to their proven biocompatibility, biodegradability and nontoxicity.^{1–6} Nonetheless, developing a method to prepare hydrogels based on chitosan (CS) and gelatin (G) with good mechanical stability, adequate aqueous solution interaction and morphological characteristics has been a challenge.

CS is a cationic polysaccharide, produced by deacetylation of chitin, which is the structural element in the exoskeleton of crustaceans (crabs, shrimp etc.). CS offers special characteristics and advantages, such as biocompatibility, biodegradability, low toxicity, antibacterial properties, low cost, good mechanical properties, swelling capacity, and ease of preparation in various forms.^{7–10} Due to these properties, it

is frequently used in medical applications as a biomaterial or constituent of drug delivery systems.

G, also a natural polymer, is obtained by a controlled acid or alkaline hydrolysis of native collagen,¹¹ a protein that is the major constituent of skin, bones, and connective tissue. Due to its excellent biocompatibility, biodegradability, nontoxicity, G has been used in medical application, especially in pharmaceuticals¹² as a material for drug controlled release. G, when compared with CS, shows poor mechanical properties and very high swelling capacity in aqueous solutions.¹³

The first purpose of this study was to obtain double cross-linked hydrogels based on G and CS. The choice of these polymers is justified by the benefits of the two natural components regarding their application in the biomedical

Correspondence to: C. A. Peptu; e-mail: catipeptu@yahoo.co.uk (or) catipeptu@ch.tuiasi.ro

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field. The use of cross-linking agents is imposed by the properties of the G/CS non cross-linked gels, such as the lack of shape and mechanical stability.¹ It is also well known that the covalent cross-linkers like formaldehyde¹⁴ and glutaraldehyde present a higher toxicity¹⁵ than ionic cross-linkers like sodium sulphate, magnesium sulphate, and sodium tripolyphosphate (TPP). Gomez et al. have already shown long time ago that the glutaraldehyde used for the preparation of cross-linked CS microspheres made them cytotoxic toward B16 F10 cells (melanoma cells).¹⁶ On the other hand, covalently cross-linked hydrogels show superior mechanical properties compared with the ionic ones, but their swelling properties and the loading/release capacity are inferior.¹⁷ For example, Mao et al. have studied the mechanical behavior of CS/G hydrogels prepared by ionic cross-linking with TPP showing that it is very difficult to obtain uniform and stable hydrogels by using with this ionic cross-linker alone.¹⁸

To reduce the amount of toxic covalent cross-linker without substantially changing the mechanical stability and at the same time maintaining a high swelling capacity in aqueous media that will further induce the possibility of high hydrosoluble drug loading, this study has proposed an original method for the preparation of CS/G double cross-linked hydrogels.

The double cross-linking method has previously been used in our research group to prepare low toxicity micro and nanoparticles based on G and CS with potential application in ophthalmology.¹⁹ The method of double cross-linking of G and CS proposed in this work is completely different compared with the one published before by the group, namely: (1) the type of formulation which completely change the main principle of the preparation method (micro/nanoparticles vs. hydrogels), (2) the ionic cross-linker type (sodium sulphate was used for micro/nanoparticles preparation, while sodium TPP was chosen for hydrogels), and (3) the cross-linking reaction sequence was reversed: the first step was the covalent cross-linking and the ionic one was the second.

The second purpose of this study was to model the main properties of the hydrogels (the swelling degree and the maximum amount of caffeine released in phosphate buffer solution, phosphate buffered saline (PBS), or in citrate buffer solution (CBS) as a function of the reaction parameters [CS/G (g/g), TPP/free NH₂ moles/moles, cross-linking time (min)] using neural network modeling methods. Direct and inverse neural network modeling allow evaluation of the process in both directions: predicting the values of the product characteristics and identifying the optimal initial conditions that lead to imposed final values. A general modeling methodology was developed, with the possibility to apply it to other complex cross-linking processes.

Artificial intelligence based on neural networks and hybrid models has become an encouraging alternative for classical processes modeling techniques applied in industry. The difficulties encountered during chemical process development when a phenomenological model is used, are related to limited knowledge of the complex interdepend-

ence between variables. In these conditions, neural networks may overcome the modeling difficulties, presenting a few advantages: they can be applied to complex nonlinear processes, are easy to develop and use, and they can substitute experiments with predictions.²⁰ Neural networks are frequently used in polymer reaction engineering, in different types of applications. The open literature presents many attempts concerning neural network applications for polymerization processes: direct modeling with different types of neural networks,^{21,22} neural network based soft sensors,²³ inferential modeling,²⁴ inverse neural network modelling,^{25,26} optimization,^{27,28} process control.^{29,30} These types of applications were reviewed in our precedent work.³¹

EXPERIMENTAL PROCEDURES

Materials

CS—Sigma Aldrich, MW—310,000 to 375,000 g mol⁻¹, deacetylation degree 75%; Gelatin—Type B (G)—Sigma Aldrich; Glutaraldehyde (GA)—Merck; Sodium TPP—Sigma Aldrich; Caffeine (Cf)—Sigma Aldrich. All other reagents and solvents used were of analytical grade and were purchased from Chimreactiv and Merck.

Hydrogels preparation

0.5 g polymer (CS/G in various weight ratios) was dissolved in 25 mL of 2% (v/v) acetic acid solution to obtain 20 samples. Six samples were prepared under identical conditions to evaluate the reproducibility of this experimental plan. The calculated GA quantity required for cross-linking 20% of the total free NH₂ groups was added dropwise in each polymer solution under vigorous stirring.

The number of total free NH₂ groups belonging to G was calculated considering the G composition mentioned by Schrieber and Gareis in "Gelatine Handbook: Theory and Industrial Practice."¹¹ Preliminary tests were performed to determine the optimum quantity of GA yielding films that may be settled. The mixtures were transferred in Petri dishes with 90-mm diameter, ultrasonicated to remove the air bubbles, and then placed in the oven for 1 hour at 50°C. The covalently cross-linked hydrogels were immersed for certain time intervals in TPP aqueous solutions of various concentrations (Table I) for ionic cross-linking to take place. The non reacted reagents (GA and TPP) were extracted by successive washing steps using acetone and double distilled water.

Hydrogels characterization

Structural characteristics. The structural characteristics of the hydrogels, especially the ionic and covalent new bonds, were analyzed with an Digilab Scimitar FTS 2000 FTIR Spectrometer in the transmittance mode, ranging from 400 to 4000 cm⁻¹, using the KBr pellet procedure.

Morphological characteristics. The hydrogels were analyzed by scanning electron microscopy (FEI Quanta 200 Scanning Electron Microscope) for morphology determination. For ESEM evaluations of the hydrogels, the slices of

TABLE I. The Central Composite Rotatable Second Order Design with Three Variables in Terms of Coded and Experimental Data Used as Experimental Design

Variables	Code					
Real	Coded	−1.682	−1	0	1	1.682
The ratio between polymers CS/G (g/g)	x_1	0.11	1.92	4.56	7.2	9
The amount of ionic cross-linker (moles TPP/moles free NH_2)	x_2	0.3	0.361	0.45	0.539	0.6
The ionic cross-linking time (minutes)	x_3	60	97	150	204	240

each sample were placed on carbon discs, on an aluminium plate.

Maximum swelling degree— $Q_{\max}$ (%). The maximum swelling degrees of the hydrogels in buffer solutions with pH = 7.4 and pH = 3, respectively, were determined using the thermogravimetric method, following the steps described below:

- Swelling the sample in an alkaline solution (phosphate buffer) until equilibrium swelling was reached.
- Measuring the weight of the swollen sample after equilibrium swelling (24 h)— W_1 (the aqueous solution was removed from the sample surface using filter paper).
- Drying the sample in the oven at 40°C until equilibrium drying was reached, for more than 24 h.
- Measuring the weight of the dried sample after equilibrium drying— W_0 ;

The maximum swelling degree was calculated using the following equation:

$$Q_{\max} (\%) = \frac{W_1 - W_0}{W_0} \times 100. \quad (1)$$

Results are reported as the arithmetic mean of three determinations.

Drug loading using caffeine as model drug. Certain amounts of dried samples were immersed into a caffeine solution (10 mL, 10% w/w; aqueous solution) for 24 h, to complete the diffusional process. The amount of caffeine loaded in each CSG samples was calculated by difference, using spectrophotometric analysis of the remaining drug solutions after the sample removal correlated with the calibration curve of caffeine/water equation.

Drug release using caffeine as model drug. The caffeine release kinetics from CSG hydrogels in the PBS (pH = 7.4) or CBS (pH = 3) buffer solution was analyzed using UV-VIS spectroscopy. Caffeine-loaded samples were immersed in covered beakers containing 50 mL buffer solution and then placed in a GFL 1092 Water-Bath shaking incubator at 37°C with a rotation speed of 60 rpm. The released caffeine amount was monitored at certain time intervals using a NanoDrop 1000 UV-VIS Spectrophotometer from Thermo Scientific (the wavelength used for detection of caffeine was 272 nm). The results were reported as the arithmetic mean of three determinations.

The maximum amount of released caffeine was calculated by taking into account the equation of the calibration curve and the weight of each sample as follows:

$$m_{\text{Cf}} = \frac{C_{\text{Cf}} \times 50 \text{ mL}}{m_s} \quad (2)$$

where m_{Cf} is the maximum amount of released caffeine, C_{Cf} is the caffeine concentration calculated with the calibration curve ($A_{\text{Cf}} = C_{\text{Cf}} \times 0.0042$) (mg/mL), and m_s is the sample weight (mg).

The drug release efficiency (%) was calculated as shown below:

$$R_{\text{ef}} = \frac{m_r}{m_l} \times 100 \quad (3)$$

where m_r is the maximum amount of released caffeine and m_l is the maximum loaded caffeine.

MODELLING

The experimental programme based on a first order central composite rotatable design

Hydrogels containing CS and G (CSG) were prepared by partial covalent cross-linking using GA, followed by ionic

TABLE II. The Experimental Plan Using Coded Variables

Sample	x_1 —The Ratio Between Polymers CS/G (g/g)	x_2 —TPP/Free NH_2 (moles/moles)	x_3 —The Ionic Cross-linking Time (min)
CSG-1	−1	−1	−1
CSG-2	1	−1	−1
CSG-3	−1	1	−1
CSG-4	1	1	−1
CSG-5	−1	−1	1
CSG-6	1	−1	1
CSG-7	−1	1	1
CSG-8	1	1	1
CSG-9	−1.682	0	0
CSG-10	1.682	0	0
CSG-11	0	−1.682	0
CSG-12	0	1.682	0
CSG-13	0	0	−1.682
CSG-14	0	0	1.682
CSG-15	0	0	0
CSG-16	0	0	0
CSG-17	0	0	0
CSG-18	0	0	0
CSG-19	0	0	0
CSG-20	0	0	0

cross-linking with TPP based on first order central composite rotatable design for modeling and optimization of the influence of three operating variables (Table I). The preparation of the hydrogels samples followed the experimental plan presented in Table II.

Neural network modeling

The goal of the neural network modeling was to establish the influence of the reaction conditions upon the characteristics of the final product. Consequently, the designed networks have the following input variables: CS/G (g/g) – x_1 , moles TPP/2 moles NH_2 free – x_2 , cross-linking time (min) – x_3 and the subsequent output parameters: the swelling degree in PBS (pH = 7.4) (SDPBS, %) – y_1 , the swelling degree in CBS (pH = 3) (SDCBS, %) – y_2 , the maximum amount of released caffeine in PBS (mg caffeine/g hydrogel) – y_3 , and the maximum amount of released caffeine in CBS (mg caffeine/g hydrogel) – y_4 . Each of the output parameters is correlated through neural network modeling with the three input variables. Therefore, four models have to be developed for this case study.

The neural network models are obtained by training with input/output pairs, which have to be related to the transformation that is being modeled. The adjustment of the neural network function to the experimental data (learning process or training) is based on a nonlinear regression procedure. Training is done by assigning random weights to each neuron, evaluating the output of the network and calculating the error between the output of the network and the known results by means of an error or objective func-

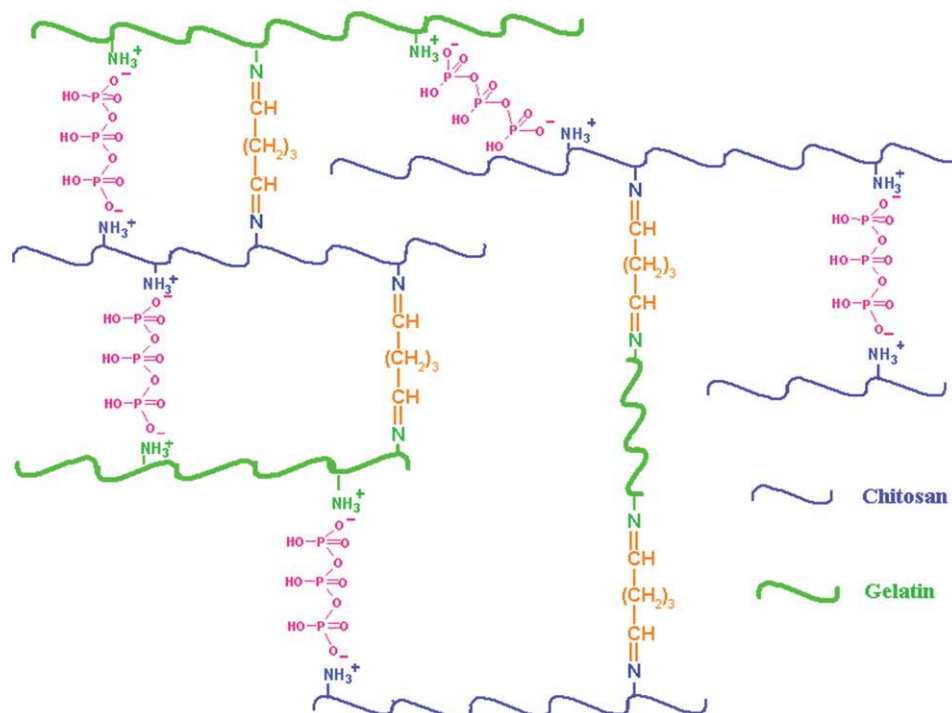
tion. When the error becomes too high, the weights have to be adjusted and the process returns to network output evaluation. This cycle is repeated until the error becomes low or the stop criterion is satisfied.³²

One major problem during the development of neural network models is determining the network architecture, that is, the number of hidden layers and the number of neurons in each hidden layer. The trial-and-error method is applied by testing several topologies and comparing the prediction errors. Lower errors indicate potentially good architectures, that is, neural network topologies with chances to train well and to output good results.

Most studies concerning the use of neural networks have applied a multilayered, feed-forward, fully connected network of perceptions.³³ The use of simple neural networks is justified by the simplicity of the theory, ease of programming, and good results. Also, this type of network represents an universal function in the sense that it can take the shape of any broken curve if the topology of the network is allowed to vary freely.³⁴

Many neural networks were trained and evaluated based on mean square error (MSE) correlation (r)—the concordance between experimental data and neural network prediction and percent error (E_p). The best performances were registered by MLP (3:5:1), a multilayer perceptron network with three neurons in input layer corresponding to the three input variables (x_1 , x_2 , x_3), a hidden layer with five neurons and one output layer with one neuron for y_1 , y_2 , y_3 , and y_4 , respectively (four neural models).

It is important to notice that even though the number of experimental data is not very large, it allows attaining a



SCHEME 1. Proposed structure of CSG hydrogels. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

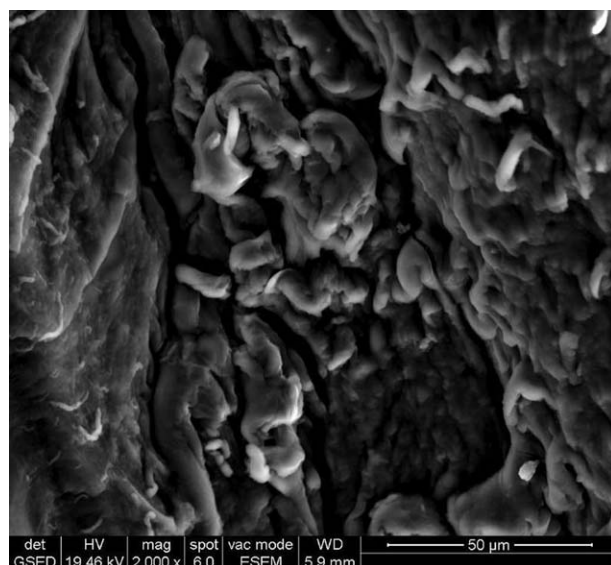


FIGURE 1. Electronic microscopy image for the sample CSG-2.

good neural network model because the data are uniformly distributed in the investigated domain. The experiments were planned in such a way that a minimum number of data were required.

RESULTS AND DISCUSSION

The theoretical double cross-linked structure of hydrogels is represented in the following diagram (Scheme 1):

Structural characteristics

The Fourier transform infrared (FTIR) spectra performed for the two polymers and for the prepared hydrogels evi-

denced the presence of the covalent cross-linking bond located at $\sim 1730\text{ cm}^{-1}$ indicating the presence of imine groups (—C=N—). The ionic cross-linking was identified at $\sim 893\text{ cm}^{-1}$, associated with the presence of the —P—O—P— groups.

The hydrogels morphology

Using scanning electron microscopy analysis, the compact morphology of hydrogels was observed, without obvious pores (Fig. 1). This may be due to the high density of the hydrogel networks taking into account the amount of the amine groups (in CS there is one amine group for each structural unit) and the reactivity of the cross-linkers toward these functional groups.

Swelling degree

The maximum swelling degree was less than 1600% due to the compact structure obtained as a result of a high cross-linking degree.

The maximum swelling degree (Q_{max}), measured in acidic conditions ($\text{pH} = 3$) (Fig. 2) was generally lower than that measured in weak basic medium ($\text{pH} = 7.4$) (Fig. 3), for each hydrogel sample.

In alkaline conditions, carboxylate anions that are formed from protein chains determine electrostatic repulsion allowing the aqueous solution to get inside the network.

An interesting observation is that the ionic cross-linking time has no significant influence upon the maximum swelling degree in alkaline or acidic medium for CSG hydrogels. The high reactivity of the ionic cross-linking agent causes it

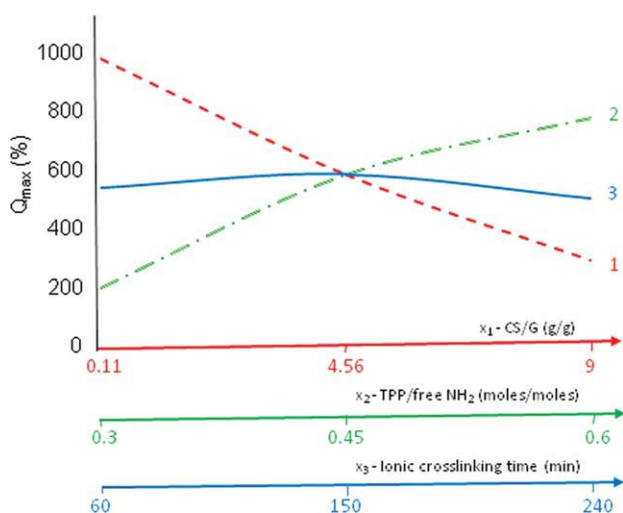


FIGURE 2. The variation of the hydrogels maximum swelling degree at $\text{pH} = 3$ depending on the parameters of cross-linking reaction: 1—the ratio CS/G (g/g) ($\text{TPP/free NH}_2 = 0.45$ moles/moles and the ionic cross-linking time = 150 min); 2— TPP/free NH_2 (moles/moles) ($\text{CS/G} = 4.56$ g/g, the ionic cross-linking time = 150 min); 3—the ionic cross-linking time (min) ($\text{CS/G} = 4.56$ g/g, $\text{TPP/free NH}_2 = 0.45$ moles/moles). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

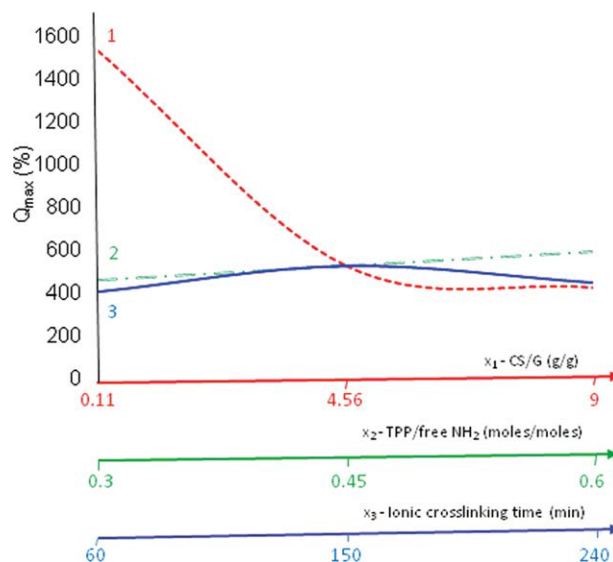


FIGURE 3. The variation of the hydrogels maximum swelling degree at $\text{pH} = 7.4$ depending on the parameters of cross-linking reaction: 1—the ratio CS/G (g/g) ($\text{TPP/free NH}_2 = 0.45$ moles/moles and the ionic cross-linking time = 150 min); 2— TPP/free NH_2 (moles/moles) ($\text{CS/G} = 4.56$ g/g, the ionic cross-linking time = 150 min); 3—the ionic cross-linking time (min) ($\text{CS/G} = 4.56$ g/g, $\text{TPP/free NH}_2 = 0.45$ moles/moles). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

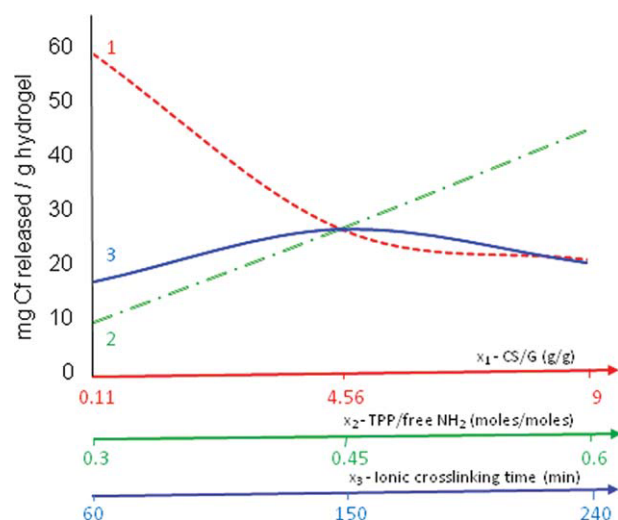


FIGURE 4. The variation of the maximum amount of caffeine released at pH = 3 depending on the parameters of cross-linking reaction: 1—the ratio CS/G (g/g) (TPP/free NH_2 = 0.45 moles/moles and the ionic cross-linking time = 150 min); 2—TPP/free NH_2 (moles/moles) (CS/G = 4.56 g/g, the ionic cross-linking time = 150 min); 3—the ionic cross-linking time (min) (CS/G = 4.56 g/g, TPP/free NH_2 = 0.45 moles/moles). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

to react within the first hour, despite the large size of the TPP molecule.

The experimental data show that higher amounts of ionic cross-linking agent result in hydrogels with higher swelling degrees in acidic conditions. This is a consequence of the fact that under these circumstances the triphosphate anions partially hydrolyze to their weakly acidic form.³⁵ The net result is that an increase in the cross-linking agent/polymer ratio, expressed as the TPP mol/2 moles free NH_2 (x_2) parameter causes an enhancement of the maximum swelling degree measured at low pH.

Apparently, the swelling degree in acidic medium should be higher due to the electrostatic repulsions between the numerous ammonium cations located on both CS and G chains. This effect was not actually observed; due to the fact that the ions are trapped in the network formed by ionic and covalent cross-linking, they cannot exert rejection effects that would be evidenced in higher Q_{max} values.

Increasing the CS/G ratio (x_1) results in lower values of the maximum swelling degree in both acidic and basic media.

In vitro caffeine release

The maximum amount of released caffeine (after 24 h) from CSG hydrogels in acidic conditions was in the range of 9–59 mg/g hydrogel (Fig. 4), while in alkaline conditions it was between 17 and 115 mg/g hydrogel (Fig. 5). The caffeine release efficiency is very high in alkaline medium, ranging between 83% and 94%, while in acidic conditions it is only in the range of 46–62%.

The caffeine release behavior followed a similar pattern to the swelling degree in both release environments (acidic

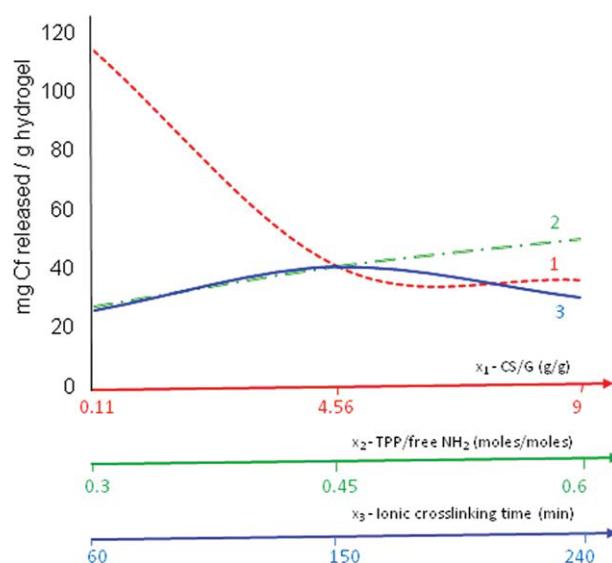


FIGURE 5. The variation of the maximum amount of caffeine released at pH = 7.4 depending on the parameters of cross-linking reaction: 1—the ratio CS/G (g/g) (TPP/free NH_2 = 0.45 moles/moles and the ionic cross-linking time = 150 min); 2—TPP/free NH_2 (moles/moles) (CS/G = 4.56 g/g, the ionic cross-linking time = 150 min); 3—the ionic cross-linking time (min) (CS/G = 4.56 g/g, TPP/free NH_2 = 0.45 moles/moles). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

and alkaline). As it was expected, the total amount of released caffeine was lower in acidic environment than in alkaline environment.

Simulation results

For the parameter of interest in this case study, the feed forward neural networks with the topology (3:5:1) were developed. They were obtained with a trial-and-error method and were trained with the well known back propagation algorithm. The main indicators of performances in the training phase were MSE, r and E_p . Table III shows the values of these criteria for the neural models based on which the neural models were chosen.

The predicted neural network values were in good agreement with experimental data for all four parameters, proving that the neural models learned the behavior of the process well.

The main advantage of a neural network consists in the capacity of generalization to other inputs which were not seen yet. Six experimental data were kept for the validation phase to check the generalization capability of the neural

TABLE III. The Neural Network Performances in the Training Phase

Nr.crt.	Output Parameters	MSE	r	E_p %
1	SDPBS (y_1)	0.00021	0.99942	1.4932
2	SDCBS (y_2)	0.000055	0.99979	0.56361
3	Max Caffeine released in PBS (y_3)	0.00067	0.98087	2.16925
4	Max Caffeine released in CBS (y_4)	0.000048	0.99997	0.66138

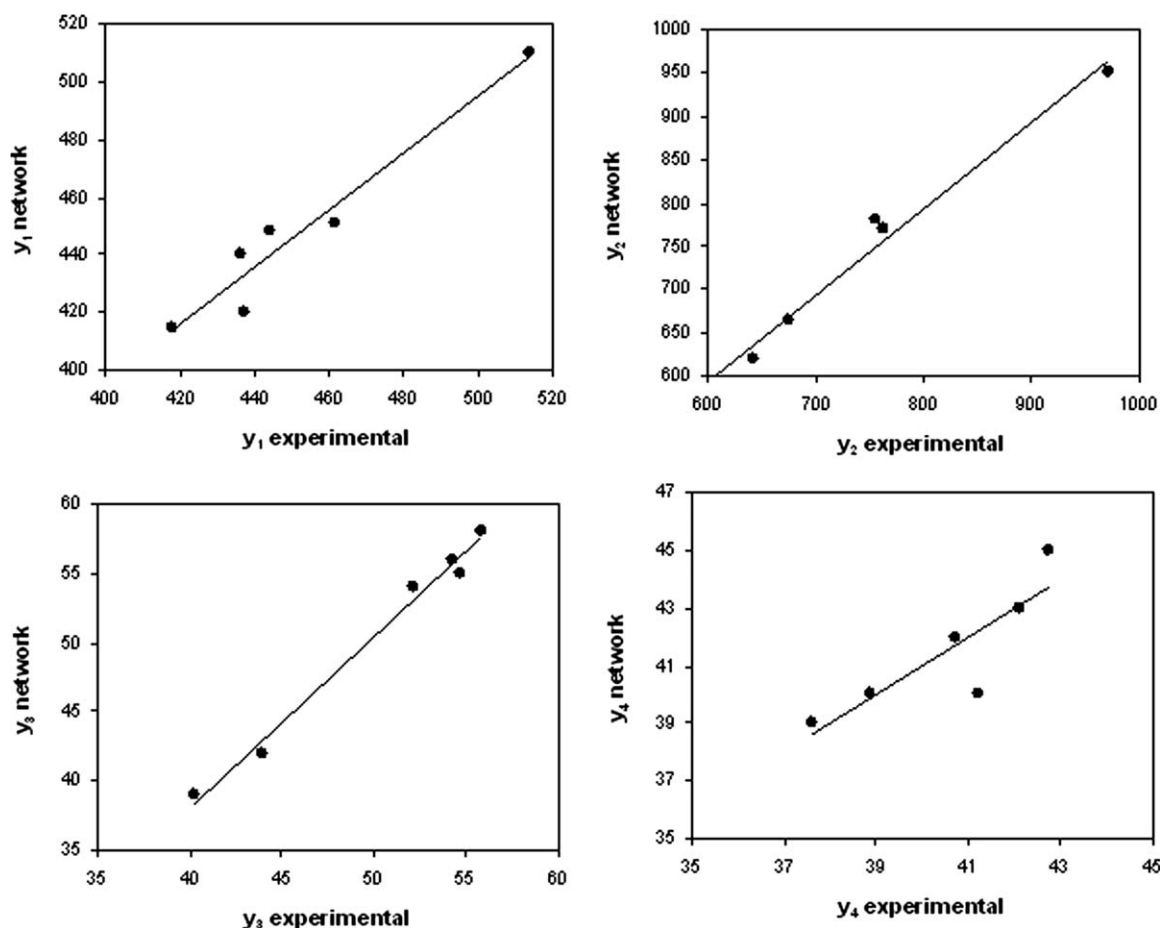


FIGURE 6. The experimental data compared with neural network results for the validation stage: y_1 —swelling degree in PBS, y_2 —swelling degree in CBS, y_3 —maximum caffeine released in PBS, and y_4 —maximum caffeine released in CBS.

models. Figure 6 shows a comparison between experimental data (not included in the training set) and MLP (3:5:1) predictions for all the outputs of the neural networks. A maximum relative error of 6% means accurate prediction for neural models.

Consequently, the neural models can be used to make predictions for other new experimental conditions, substituting the experiments or providing useful information for the experimental practice.

The direct neural modeling performed before allows the estimation of the reaction yields and swelling degrees in

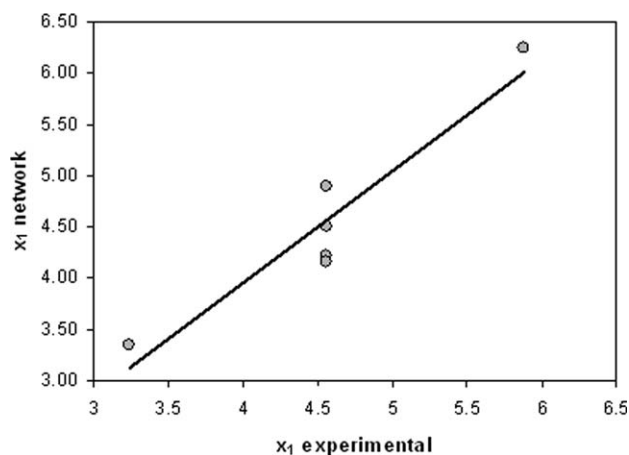


FIGURE 7. Validation of the inverse neural network model, MLP (3:12:4:1): experimental data compared with neural network predictions for some “unseen” data of CS/G.

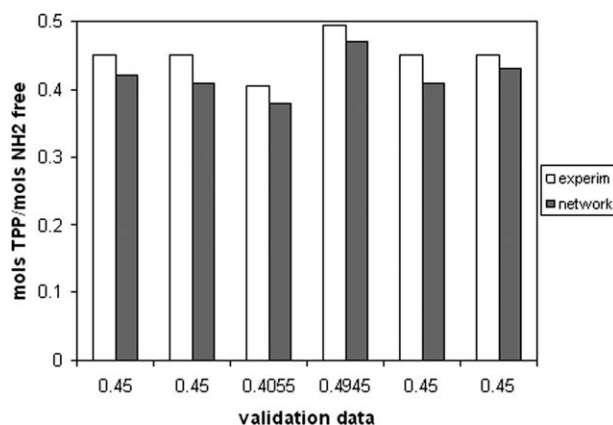


FIGURE 8. Experimental and neural network data for the validation phase of inverse modeling with MLP (3:33:11:1).

various reaction conditions, even under circumstances in which experimental data are not available.

Supplementary information is obtained by inverse neural network modeling, which is an optimization problem for identifying reaction conditions that lead to imposed final values for y_1 , y_2 , y_3 , or y_4 . Different optimization problems can be formulated and solved by inverse neural modeling.

For instance, what is the value of the CS/Gel (x_1) ratio that leads to imposed values for the swelling degree SDPBS (y_1) if the x_2 (mols TPP/2 mols NH_2 free) and x_3 (cross-linking time) parameters are pre-established?

A neural network which answers to such a problem has x_2 , x_3 , and y_1 as inputs and x_1 as the output. Several tests performed in inverse neural network modeling led to an optimal topology MLP (3:12:4:1) with $\text{MSE} = 0.000006$, $r = 0.99991$, and $E_p = 0.181415\%$ for the training phase. Figure 7 presents the predictions of the MLP (3:12:4:1) for a series of data with unknown experimental answers for the model.

Another example is represented by the following problem: what is the value of x_2 (moles TPP/2 moles NH_2 free) leading to an imposed value for y_3 (maximum amount of caffeine released in PBS) when x_1 (CS/Gel) and x_3 (cross-linking time) parameters are pre-established?

The answer to the question shown above is given by a neural network with two hidden layers, MLP (3:33:11:1), with x_1 , x_3 , and y_3 as inputs and x_2 as the output. The simulation results are in satisfactory agreement with the experimental data (Fig. 8), with a maximum relative error of about 9%.

CONCLUSIONS

The double cross-linking method may be an alternative way for obtaining stable hydrogels, thus reducing the amount of covalent cross-linking agents that are generally toxic. The hydrogel characteristics, correlated to their ability to load/release water-soluble drugs are sometimes influenced in an unexpected way by the cross-linking reaction parameters. The swelling degree presents higher values in alkaline conditions than in acidic ones. The ionic cross-linking time does not influence the maximum swelling degree, especially in alkaline environment. The higher amount of CS in the initial mixture increases the maximum swelling degree and the maximum amount of loaded/released caffeine.

The modeling procedure for the process under study is conducted based on the experimental data aiming to evaluate the influence of the reaction conditions upon the swelling degree and the reaction yield. Simple architecture of neural networks and simple methods of establishing the network structure are proposed. Good predictions are obtained with all the neural models that were considered, with an average error of less than 9%; thus, these neural models give a very good representation for the synthesis process of double cross-linked hydrogels based on CS and G.

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Double Cross-linked Chitosan–Gelatin Particulate Systems for Ophthalmic Applications

C. A. PEPTU, G. BUHUS AND MARCEL POPA*

*“Gh. Asachi” Technical University of Iasi, Department of Natural
and Synthetic Polymers, D.Mangeron, 71A, 700050, Iasi, Romania*

A. PERICHAUD

*University of Provence, Department of Macromolecular Chemistry
Pl. Victor Hugo, 13331, Marseille, France*

D. COSTIN

*The University of Medicine and Pharmacy “Gr.T.Popa,”
University Street, no. 16, 700115, Iasi, Romania*

ABSTRACT: Gelatin/chitosan particles suitable for application in ocular drug administration were prepared by a two-step cross-linking process performed in an emulsion-phase separation system. The particles were characterized by scanning electron microscopy and laser diffractometry, and the diameters were 0.202–4.596 μm . The microparticles pH-dependent behavior was monitored by their mean diameter changes in aqueous environment. Adrenalin was drug used to study loading and release characteristics. The prepared particles were nontoxic, with the DL_{50} values of 6.9–8.19 g/kg body mass. The *in vivo* biocompatibility tests consisted of subcutaneous administration of a microparticle suspension in physiological serum followed by morpho histological analysis of the implantation site. The *in vivo* adrenalin ocular delivery was tested on both animals and a voluntary human patient to determine the adrenalin action and by tears. The particles showed good adherent properties without irritation to the patient; adrenalin was released cleared the ocular congestion.

*Author to whom correspondence should be addressed.

E-mail: marpopa2001@yahoo.fr, marpopa@ch.tuiasi.ro

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KEY WORDS: gelatin, chitosan, double cross-linking, microparticles, *in vivo* ophthalmic tests, bulbar conjunctiva, fornix, palpebral conjunctiva.

INTRODUCTION

Particulate systems have been found to be potential carriers for ocular delivery following the observation that various types of nanoparticles tend to adhere to the ocular epithelial surface. The prolonged residence time of nanoparticles leads to a much lower elimination rate compared to conventional ophthalmic formulations, thereby improving drug bioavailability. As a consequence, nanoparticles have been developed for targeted ophthalmic delivery of anti-inflammatory, antiallergic, and beta-blocker drugs [1]. Among the mucoadhesive polymers, chitosan has attracted attention as an ophthalmic drug delivery carrier due to its absorption promoting effect. It has been proven that chitosan not only prolongs cornea contact time through mucoadhesion effects mediated by electrostatic interaction between its positive charges and mucin negatively charged residues but also has the ability to improve drug bioavailability through transient tight opening junction. It was found that chitosan solutions prolonged antibiotic residence time in rabbit cornea [2] and that the chitosan particles remained attached to the rabbit cornea and conjunctiva for at least 24 h [1]. Chitosan in ophthalmic formulations exhibited an excellent tolerance by rabbit corneal tissue [2]. Chitosan-coated particles also enhance corneal penetration [3]. After ocular administration of chitosan particles in rabbits, most of the drug was found in the extraocular tissue, cornea, and conjunctiva, while negligible drug amounts were found in intraocular tissues, iris/ciliary body, and aqueous humor. Together, these results indicate that chitosan is an attractive material as an ocular drug delivery vehicle with potential application at extraocular level.

Chitosan is a natural hydrophilic polysaccharide [4] that has nontoxic, mucoadhesive, and permeation enhancing effects across biological surfaces. Chitosan microspheres are used in oral or other mucosal administration to improve the transport of biomacromolecules across biological surfaces as chitosan microspheres enhance drug adsorption on paracellular route [5]. Only a few methods have been developed for the preparation of chitosan microspheres, including emulsification/solvent evaporation [6,7], spray drying [8,9], ionotropic gelation [10,11], and coacervation technique [12,13].

Controlled drug delivery systems based on natural polymers are attractive due to their biocompatibility and use in various formulations.

Although several drugs were successfully included in various chitosan-based delivery systems, a major drawback is reduced capacity to interact in hydrophilic environments and by the relatively low swelling. However, gelatin is surface active, and it is used as an emulsifier for various oils. Therefore, it is an ideal candidate as a biocompatible and biodegradable matrix material in microparticle formulations [14]. Water stability of chitosan/gelatin blends was found to decrease with greater gelatin content and so chemical cross-linking is necessary for long-term biomedical applications [15]. The emulsion-phase separation technique consists in the formation of water-in-oil (w/o) emulsion aided by the use of a surfactant. The chitosan solution droplets suspended in the oil phase can be hardened by cross-linking as well as several well-known techniques [16–18].

The main objective of this work is the preparation of micro- and nanoparticles based on gelatin (GEL) and chitosan (CHI) mixtures of interpenetrated/interconnected network type, prepared by a two-step cross-linking technique applied in reverse emulsion system. In the first step, the ionic cross-linking is achieved by using nontoxic cross-linking agents like sodium sulfate. Subsequently, a low amount of covalent cross-linking (glutaraldehyde (GA)) was used to stabilize the shape and dimensions of the obtained particles. For drug loading and release, adrenalin (ADR) was used. It is a hydrosoluble drug that is usually applied for maintaining the mydriasis during eye surgery.

MATERIALS AND METHODS

Materials

The following materials were used: low molecular weight CHI, deacetylation degree 75–80% (Aldrich), GEL (Aldrich), GA (Aldrich) – 25% aqueous solution, sodium sulfate (Primexchim, Bucharest), ADR pharmaceutical grade (SC Terapia SA, Cluj Napoca), Tween 80 (Aldrich), and Span 80 (Aldrich).

Preparation of Microparticles by Ionic Gelation and Covalent Cross-Linking in w/o Emulsion

The preparation of CHI solution was performed by dissolving the polymer in aqueous acetic acid solution 2% (w/w). GEL was dissolved in water at 40°C. Based on specific polymer ratios, the two solutions were mixed and then Tween 80 was added to make a 1% (w/w) surfactant in the solution. The mixture was magnetically stirred until the surfactant was completely dissolved. Separately, a 1% (w/w) solution of Span 80 in

toluene was prepared by dropwise addition of the aqueous phase to the organic phase under homogenization with an UltraTurax device at 20,000 rpm. The final water/oil ratio was 1/4 and the emulsion total volume was 125 mL, which was stirred for 10 more min. The emulsion was then gelled by slowly adding a 10% sodium sulfate solution at a rate of 2 mL/min with continuous stirring for an extra 10 min. The suspension was transferred to a round-bottom flask and mechanically stirred at 1000 rpm. Consecutively, 10 mL of a saturated solution of GA in toluene was added with stirring for 60 min. The particles were separated by centrifugation at 6000 rpm, repeatedly washed with acetone and water to eliminate the unreacted compounds. After washing with hexane, the particles were dried at room temperature.

Particle Characterization

Morphological Studies – Microscopy and Granulometry

The morphology of GEL/CHI microspheres was evaluated by scanning electron microscopy (SEM; ESEM Philips XL 30). The particle size distribution of the products, after drying, were measured by laser diffractometry; to avoid the swelling during the measurements, the particles were redispersed in toluene and analyzed by using a Shimadzu laser diffractometer (SALD 7010).

Particle Behavior in Aqueous Environment

The changes in the particle size, in an aqueous environment, were monitored by laser diffractometry (Shimadzu – SALD 7001). The influence of the pH was carried out in double distilled water (with pH adjusted to 5) and phosphate buffer solution (pH = 7.4) for alkaline conditions. A specific amount of microparticle powder was suspended in the quartz cuvette of the diffractometer filled with aqueous medium with stirring. All experiments were performed in duplicates.

Loading and Release of ADR

Microparticle drug loading and release profiles were studied by UV-VIS spectroscopy. The instrument used, Nanodrop ND-1000, allows analysis of very low sample volumes, in microliter range. Drug loading was studied in interval of 24 h. An aliquot of 0.1 mg of microparticle dry powder was suspended in a 0.5 mg/mL solution of ADR in double-distilled water. Samples were collected at specific time intervals and measured by UV-VIS. A calibration curve was prepared to determine the loaded drug concentration. After 24 h, the samples were centrifuged and added to

20 mL a phosphate buffer solution to determine the release profiles in the same manner as the loading. ADR is a catecholamine which, in physiological conditions, could become toxic by its auto-oxidation into ortho-quinones. It has been proved that this process is very slow at physiological pH [19]; this fact has already been taken into consideration when planning the release experiments. All loading and release tests were performed in duplicate, and the results shown are average values.

Particles Toxicity

The acute toxicity of the microparticles was evaluated by determining the average lethal dose (DL_{50}). The DL_{50} was measured in milligrams of active substance on kilograms of body weight. According to Hodge–Sterner toxicity scale [20], a DL_{50} value under 1 mg/kg body mass is considered extremely toxic, between 1 and 50 mg/kg body mass *very toxic*, between 50 and 500 mg/kg body mass *moderately toxic*, between 500 and 5000 mg/kg body mass *low toxic*, between 5000 and 15,000 mg/kg body mass is *practically nontoxic*, and above 15,000 mg/kg of body mass is considered *nontoxic*.

In Vivo Biocompatibility

The biocompatibility tests were performed on 27 one-year-old Swiss male mice. The evaluations on animals were performed in conformity with the European law. The typical testing protocol consisted in subcutaneous administration of a microparticle suspension in physiological serum, in the left thoracic area through a small incision. Samples were then collected from the affected region. The same procedure was applied to witness a lot of animals. The samples were collected by excision of the implanted area or edema containing microparticles, at days 7 and 14. The tissue fragments were inserted in paraffin and sliced at 0.005 mm thickness. The staining solutions were as follows: Novelli, PAS, Alscian blue, Steedman-Mowry, Papanicolaou, and Giemsa. The samples were then observed by optical microscopy and the most representative ones are presented.

In Vivo Ophthalmic Tests

The *in vivo* study has followed two steps: the experimental study on lab animals (New Zealand rabbits weighing 3.0–3.2 kg and Wistar rats weighing 250–300 g) followed by a human patient. Animals were randomly divided in two batches as follows: one was used for testing the ADR itself (animals received 30 μ L ADR solution, 0.25 g/mL in the right eye) and the other one for testing the ADR-loaded GEL/CHI microparticles (loaded for 24 h in 0.5 mg/mL ADR, the microparticles were loaded with ADR 0.025 mg/g microparticles and instilled in the

right eye). The left eye was used as control and received no treatment. Animal discomfort symptoms and various signs in conjunctiva, cornea, and lids were evaluated by an experienced ophthalmologist immediately after the administration, then after 24, 48, and 72 h, respectively. A scoring system established by Organization for Economic Cooperation and Development guidelines for ocular irritation testing [21] was used. The ADR-loaded microparticles were tested for biocompatibility and controlled drug release. No negative incidents were observed.

The human patient received detailed information about the experimental procedure and the substances used. A written consent form was signed by both patient and doctor.

The ADR-loaded microparticles were tested on a human eye to determine the adhesiveness (there was no elimination by tears flow). For human test protocol, the clinical effect of ADR release from gelatin–chitosan microparticles on congestive inflammation of eye conjunctiva was tested. Particles were loaded in ADR solution (0.5 mg/mL) 24 h before the usage. The right eye was considered as blank and the left eye was tested. The eyebrow was gently pulled from the medium inferior side of the left eye at 10 mm of cilia side to uncover the tarsal and bulbar conjunctivae and eye fornix. ADR-loaded particle suspension (0.025 g ADR/g microparticles) was uniformly applied with a syringe without needle. After closing the eyebrow, the area entrapping the particles was gently pressured in order to spread the drug containing suspension.

RESULTS AND DISCUSSION

Particle Preparation

The method employed for particle preparation involved a two-step solidification method. The first step, which has a critical influence over the future shape and size of particles, involved an ionic cross-linking effect achieved by sulfate bridges between amino functionalities from both types of polymeric chains. At this stage, an important role was played by the macromolecular surfactants present in oil and water phase to ensure emulsion stabilization prior to cross-linking.

One of the most important parameters influencing the cross-linking efficiency is the pH of the polymer solution [22]. At acidic pHs, the ionic cross-linking effected by both components of the polymer system. In the case of gelatin, the cationic density was higher when the pH value was ~ 4 ; in this pH range, chitosan is soluble in water and has a looser chain configuration due to electrostatic repulsions. Therefore, the choice of pH for water polymer solution was in the range of 3–6. The ratio

Table 1. Sample codes, preparation parameters^a and the mean diameter of the particles.

Sample code	% Gel	% CHI	Concentration C (%)	Mean diameter ^b (μm)
GC0	0	100	0.5	1.232
GC 10/90	10	90	0.5	0.759
GC 30/70	30	70	0.5	1.173
GC 50/50	50	50	0.5	1.108
GC 70/30	70	30	0.5	1.094
GC 100	100	0	0.5	0.202
GC-1-10/90	10	90	1	0.524
GC-1-30/70	30	70	1	0.467
GC-1-50/50	50	50	1	3.636
GC-1-70/30	70	30	1	4.596
GC-1.5-10/90	10	90	1.5	0.643
GC-1.5-30/70	30	70	1.5	0.595
GC-1.5-50/50	50	50	1.5	0.550
GC-1.5-70/30	70	30	1.5	1.36

^aFor all samples, preparation were used 20 mL Na₂SO₄ (10%) and 10 mL GA extracted toluene.

^bThe mean diameter value represents the arithmetic average of the measurements (the standard deviation values in this case has no physical meaning).

between the polymers also affected the cross-linking, decreasing with increases in the GEL content. For high amounts of GEL (GC 100) and a pH close to 6, the extent of ionic cross-linking should be lower (Table 1).

This fact has a crucial influence over the second cross-linking step achieved by covalent bridges with GA. Since the cross-linking agent is in the oil phase, the extent of cross-linking is dependent on its diffusion through the previously formed particles. A dense network would induce cross-linking mostly at the surface of the particles creating a shell stabilizing the particle size and shape (GC-1-70/30, GC 50/50, GC 30/70, and GC-1-50/50).

The SEM photographs (Figure 1) showed that the particle systems obtained were dimensionally polydisperse for all formulations. This was expected since the critical cross-linking step, with sodium sulfate, was carried out in heterogeneous system. The addition of the cross-linking agent in aqueous solution effected rapid gelation of polymeric vesicles in the emulsion. Although this was not thoroughly followed in this study, it could be presumed that ionic cross-linking was not equally performed for all the polymer vesicles and eventually that the particle forming vesicles were undergoing coalescence. This may result in relative dimensional polydispersity as it can be observed in the microscopy images. The granulometry light scattering studies show a bimodal distribution of the particles prepared using both polymers (Figure 2).

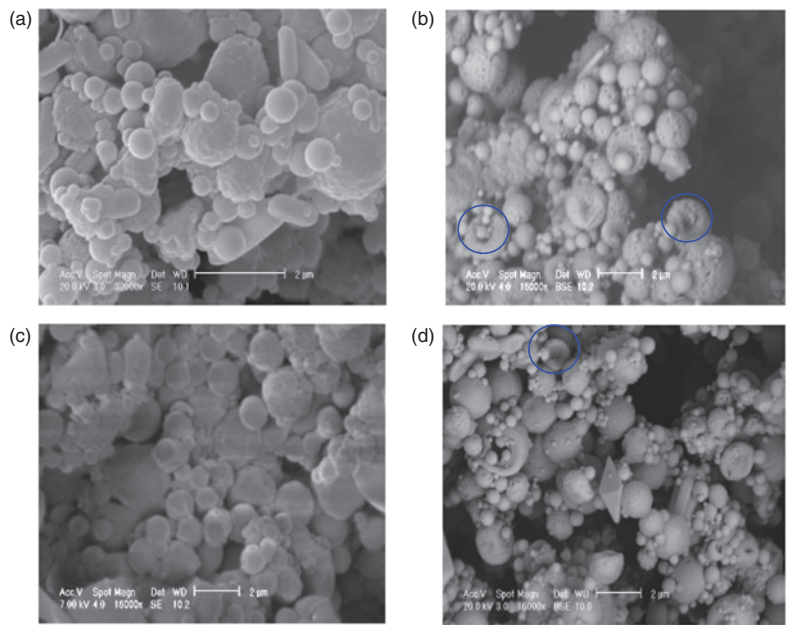


Figure 1. SEM photomicrographs for the samples (a) GC-1-70/30, (b) GC 50/50, (c) GC 30/70, and (d) GC-1-50/50. The small circles indicate the ‘doughnut-like’ microparticles.

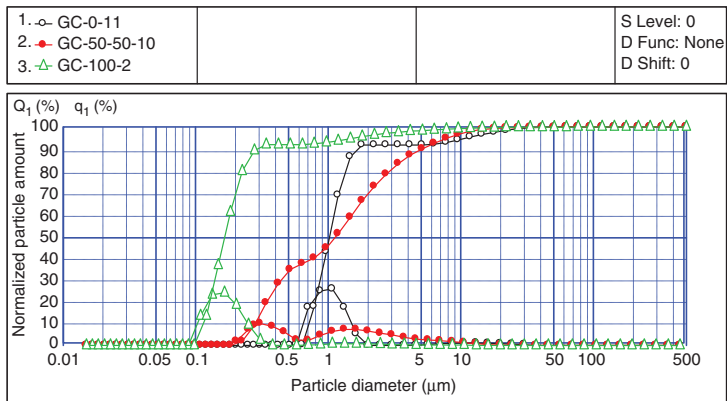


Figure 2. Typical dimensional distribution curves for samples prepared at different GEL–CHI ratios and at $C = 0.5\%$ concentration of polymer solution.

The particles were dispersed in toluene in order to obtain close dimensions to those in dry state (by swelling limitation) and then analyzed by laser diffraction granulometry. The obtained values for all preparations are presented in Table 1.

The size distribution curves, as given by the analysis software, are presented in Figure 2. It can be observed that the particles obtained only from chitosan or gelatin are monodisperse, while the combination of both polymers (sample GC 50/50) have a bimodal size distribution. All the other samples, prepared using both GEL and CHI and analyzed by laser diffractometry, have shown a similar bimodal distribution (data not shown).

The highest values of the mean diameter were obtained for chitosan particles, while the lowest values were the ones obtained for gelatin particles. This fact may be explained by faster occurring ionic cross-linking of chitosan compared with gelatin due differences in amino group density on both types of polymer chains. The influence of the polymer ratio could not be established from the analysis of the particle size, while the dimensional polydispersity is probably related to stirring conditions.

In the SEM microphotographs, two main particle populations were observable, the first was in the micron range, with rough surfaces and approximately spherical in shape, while the second population was submicron dimensions with clear spherical shape and smooth surfaces. The spherical-shaped particles there were 'doughnut-like' particles probably due to surface cross-linking. This fact supported the hypothesis that the particle outer layer is heavily cross-linked, while the core may actually be partially lost during the washing process. Some rod-like particles were also observed.

Covalent cross-linking was followed by Fourier transform infrared spectroscopy. The chitosan absorptions were 3417 cm^{-1} assigned to OH groups, 1635 cm^{-1} specific to -C=O (proving that chitosan is partially deacetylated), the 1114 cm^{-1} polysaccharide specific band, and the 1539 cm^{-1} absorption related to -NH_2 free functions. The gelatin absorptions were as follows: 1641 cm^{-1} amide I band, 1521 cm^{-1} amide II band, and free amino groups. The peak at 1100 cm^{-1} corresponds to -N=CH- vibrations of chitosan and collagenous chains indicating covalent cross-linking.

Particles Interactions in Aqueous Environment

Evaluation in Acid Environment

To quantify the interaction of particles with the aqueous environment, the modification of the mean diameter was measured by light scattering. This study did not take into account the eventual loss of polymers that are not cross-linked, cross-linked, gelatin or chitosan, during the swelling. The swelling behavior was evaluated at two different pH values, acidic and alkaline, respectively, based on the pH sensitive behavior for

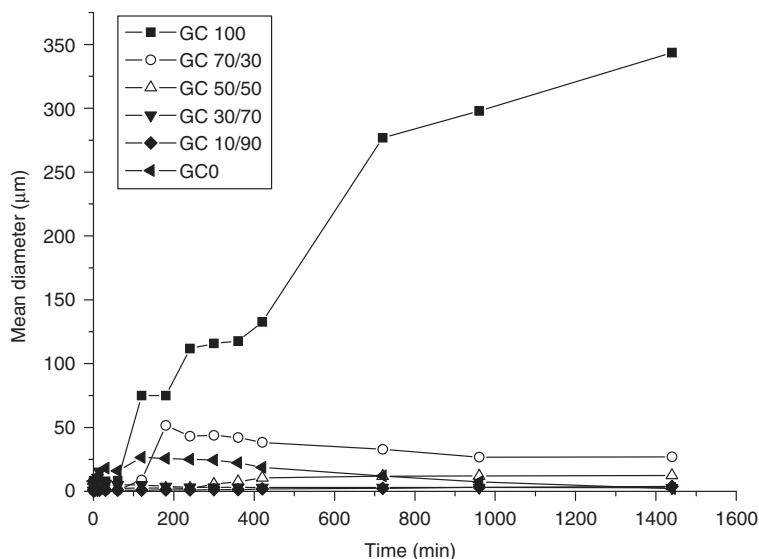


Figure 3. Mean diameter variation in time in acid aqueous environment for samples obtained at different GEL–CHI ratios and the total polymer concentration $C = 0.5\%$.

chitosan/gelatin particles [23,24]. At pH 5.5, most of the formulations showed a certain swelling with mean diameter modifications.

For a major of the samples, a decrease in mean particle diameter was observed after a certain period (Figure 3). This may be occurring due to the fact that in acidic pH, chitosan becomes soluble and a loss of material may occur, leading to a decreased mean diameter, as confirmed also by previous studies [25]. This could increase the GEL amount in the network composition, the swelling degree would be higher due to its higher hydrophilicity compared with CHI.

Another explanation is related to the morphology of the particles. As shown in SEM micrographs, some of the particles are doughnut shaped and some are broken microcapsules. This appearance may be due to the covalent cross-linking occurring mostly at the surface of the particles and encouraging a core shell structure formation. The doughnut shape may occur by the collapsing of the shell during the loss of the soluble GEL from the core during the rinsing procedures involving water washing. Also, during the swelling in aqueous environment with acidic pH, the particle shell may crack and release the inner core, ultimately leading to a drop in mean particle diameter.

The observed drug-loading capacity values show that the water interaction of the microparticles is not only due to swelling but there are also losses caused by particles breakup. This fact was confirmed

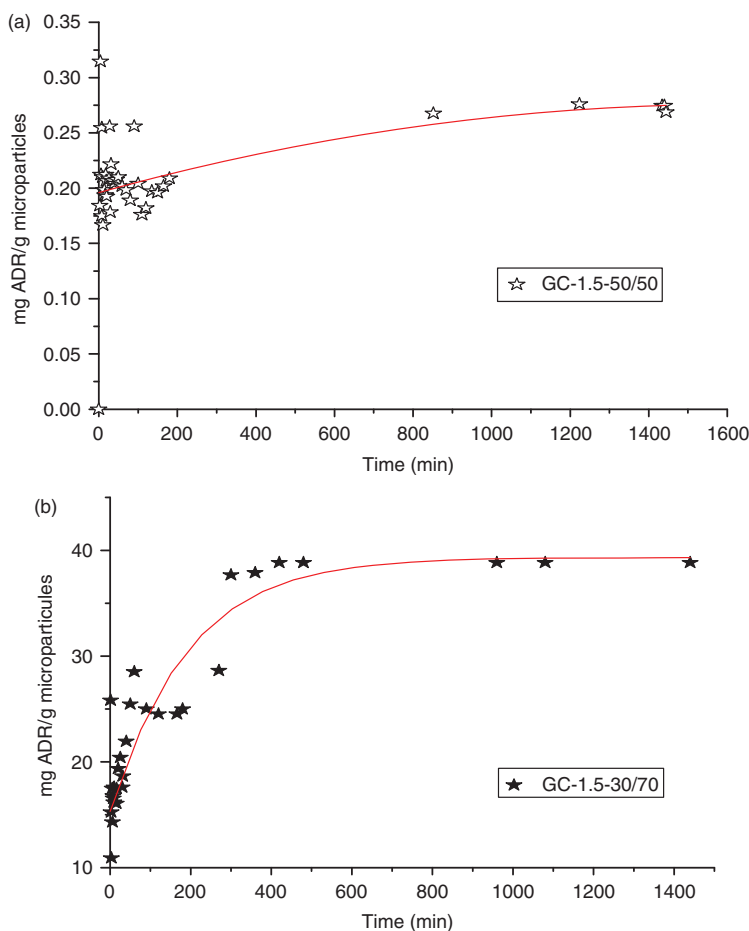


Figure 4. ADR-loading kinetics of microparticles presenting the minimum, respectively, maximum loading capacity: (a) ADR-loading profile for GC-1.5-50/50 and (b) ADR-loading profile for GC-1.5-30/70.

by the mean diameter variation measurements by laser diffractometry. The quantity of ADR incorporated in the particles by simple diffusion varied from 0.27 mg ADR/g of microparticles, for GC-1.5-50/50 (Figure 4(a)), to 38.84 mg ADR/g for GC-1.5-30/70 (Figure 4(b)). The particles prepared using a high concentration of polymer solution formed a more relaxed network (considering the acidic pH) due to the covalent cross-linking that is taking place at the superficial layers of the particles. Both the concentration of initial aqueous phase and the pH of the loading environment influence the drug-loading capacity.

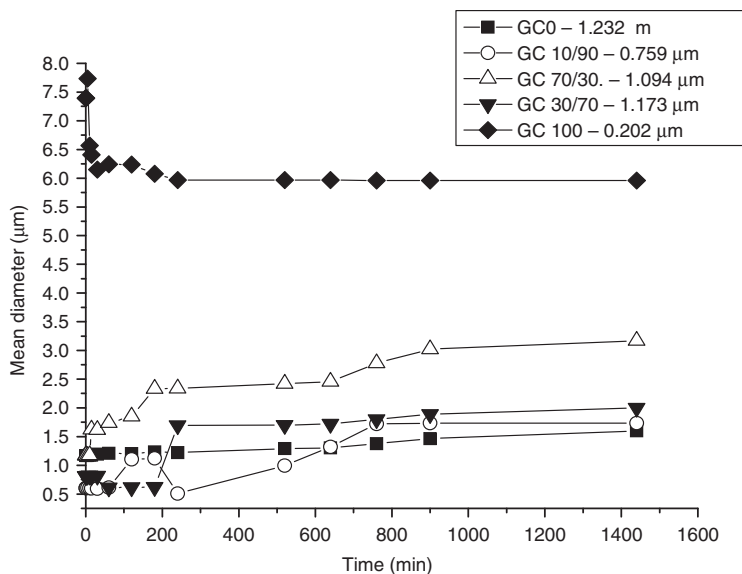


Figure 5. Mean diameter variation in time in aqueous alkaline environment for samples prepared at the same total initial polymer concentration ($C = 0.5\%$) and different polymer ratios (the legend contain the initial medium diameter measured in dry state for all analyzed samples).

Evaluation in Alkaline Environment

The swelling capacity in alkaline environment was investigated for all particle formulations. The particle interaction in water was quantified by determining the average particle diameter by laser diffractometry at specific time points (Figure 5). For the low concentrations of the initial polymer solution, the particle diameters were not modified very much in the studied swelling time range. However, a certain instability was observed, probably due to the amount of gelatin incorporated in the particles. Considering the diameter modifications observed in acidic pH, in a basic environment, the particles maintained their average size for a longer period of time.

In the basic pH, the mean particle diameter varied according to the interactions of both polymers in the composition of the particles. The main processes that govern the interactions of polymers from the particles system with the aqueous environment are the electrostatic repulsions between the gelatin carboxyl anions and ionic destabilization of the ionic cross-linked network. Since chitosan was less prone to interact in an aqueous environment at basic pH, it is obvious that gelatin was mainly responsible for particle swelling behavior. In Figure 5, the highest values for the mean diameter were obtained from samples with high amounts

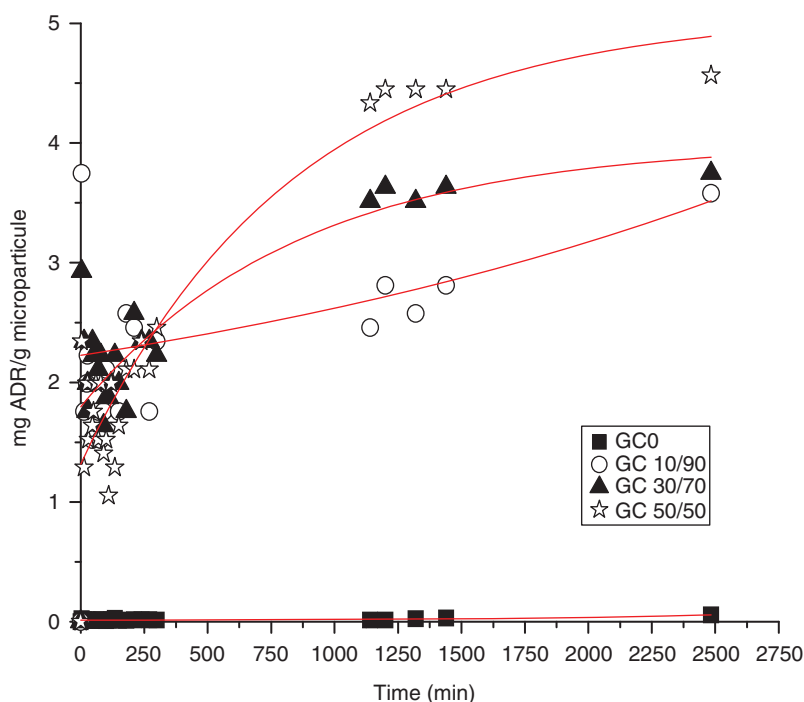


Figure 6. ADR release for particles prepared at different GEL–CHI ratios at a concentration of polymer aqueous phase $C = 0.5\%$.

of GEL. The destabilization was also favored by the relatively low molecular weight of the chitosan; consequently, the destabilization was followed by decomposition of particles. In the case of studied particle systems, it was noticed that a certain stability was maintained over 24 h.

The *in vitro* release of ADR was studied in phosphate buffer solution at $\text{pH} = 7.4$. The drug release kinetics are presented in Figure 6 together with the influence of the initial GEL:CHI ratio upon the amount of the released ADR. All analyzed systems showed concentration variations that were probably due to the modifications of microparticle dimensions in alkaline environment. The individual polymer ratio affected the amount of drug released; chitosan alone released the amount of lowest drug. It is well known that the diffusion of the drug from microparticles into external medium is influenced by their swelling capacity. This behavior is related to the modifications of the microparticle dimensions (due to swelling) in alkaline aqueous solutions; the GEL chains were mostly responsible for the swelling due to its higher hydrophilicity and the repulsive interactions between carboxylate groups.

Table 2. Average lethal dose (DL_{50}) for the samples prepared at different ratios between GEL and CHI and at 0.5% concentration of the polymer aqueous solution.

Sample	Gel	CHI	GC-10/90	GC-30/70	GC-50/50	GC-70/30
DL_{50} (mg/kg body mass)	8190	7700	6900	6990	7140	7980

Evaluation of Microparticles Toxicity

The toxicity microparticle systems were determined based on the average lethal dose (DL_{50}). The results are presented in Table 2. The particle systems were compared with the pure polymers used for synthesis. The values obtained did not vary much compared with pure polymers; therefore, the particle systems are in the category of practically nontoxic.

Evaluation of Particles Biocompatibility

In vivo biocompatibility tests were performed for three samples as follows: (1) gelatin particles (GC 100), (2) chitosan particles (GC0), and (3) gelatin–chitosan particles (GC 50/50), respectively. The tissue around the edema area created at the particle implantation site was analyzed on days 7 and 14, respectively.

The morphohistological photographs show the tissue reaction for GC0 at 7 days (Figure 7(b)); the edema was surrounded by lymphocytes giving an intense reaction. At the edema site, numerous fibroblasts were present performing an intense fibrillogenesis, similar results were obtained in previous studies [15]. The implanted particles were encapsulated with an increase in the macrophage activity at the edema site. At day 14, the edema was reduced and the particles were entrapped in the macrophages. The tissue surrounding the reduced edema contained macrophages with captured particles entrapped in thin conjunctive tissue (Figure 7(d)). This shows that the nonself elements were trapped and isolated by the macrophages but not metabolized.

In the case of the GC 100 sample, the histological examination at day 7, an intense peripheral reaction is revealed, numerous macrophages are carrying entrapped particles (Figure 7(a)). In the area that is limiting the edema, the fibroblast reaction is intense. At day 14, the macrophages were grouped in small nests separated by delicate conjunctive tissue (Figure 7(e)). The GC 50/50 microparticles had similar reactions at day 7 as the previous samples (Figure 7(c)). However, at day 14, the newly formed conjunctive tissue, surrounding the particle loaded macrophage region was more intense as well as intense fibrillogenesis (Figure 7(f) and (g)).

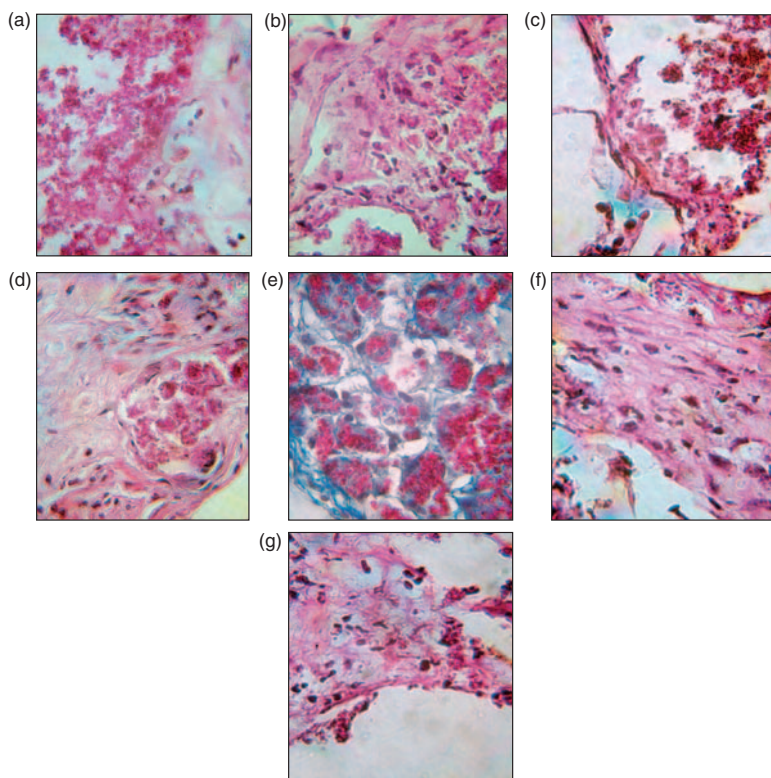


Figure 7. Histomorphological microphotographs after the subcutaneous administration of different types of particles on Swiss mice after certain period of time: (a) perifocal reaction with numerous macrophages, which engulfed the particle powder (PAS coloration; $\times 900$; GC 100, 7 days), (b) macrophages from the monocyte-macrophage system (MMS) containing numerous phagocytized powders (PAS coloration; $\times 900$; GC0, 7 days), (c) dilacerations of subcutaneous conjunctive tissue around the edema area and powder phagocytizing macrophages (PAS coloration; $\times 900$; GC 50/50, 7 days), (d) focal areas of macrophages surrounded by conjunctive tissue (PAS coloration; $\times 900$; GC0, 14 days), (e) intense perifocal reaction with numerous macrophages surrounded by conjunctive tissue (Papanicolaou coloration; $\times 900$; GC 100, 14 days), (f) numerous macrophages in the conjunctive spurs of the edema (PAS coloration; $\times 900$; GC 50/50, 14 days), and (g) fibroblasts fibrillogenesis and MMS reaction (PAS coloration; $\times 900$; GC 50/50, 14 days).

All of the presented elements showed that the prepared particles were inducing a lymphatic counter reaction occurring both at 7- and 14-day periods. The reaction was mainly located at the boundaries of the edema containing the implanted particles. In all cases, the fibrillogenesis was intense. The newly formed collagen fibers are grouped peripherally in fascicules limiting the macrophage nests. This is proof of an intense phagocytosis favored by peripheral capillary dilatation. The GC 50/50

Table 3. Macroscopically evaluated signs during the *in vivo* tolerance study.

Grade	Discomfort	Cornea	Conjunctiva	Discharge	Lids
0	No reaction	No alteration	No alteration	No discharge	No swelling
1	Blinking	Mild opacity	Mild hyperemia, mild edema	Mild discharge without moistened hair	Mild swelling
2	Enhanced blinking, intense tearing, vocalizations	Intense opacity	Intense hyperemia, mild edema	Intense discharge with moistened hair	Obvious swelling

was best tolerated as the nonself was surrounded by collagen fibers due to intense fibrilogenesis proving the particles biocompatibility.

In Vivo Ophthalmic Evaluation

The *in vivo* tolerance assay on animals for pure ADR and for ADR-loaded microparticles, according to the grading system of the macroscopic signs, the grades were 0 or 1 for all animals (Table 3); the conjunctiva had a mild hyperemia and edema, the lids suffered a very mild swelling. All of the animal tests were encouraging.

Subsequently, the ADR-loaded microparticles were tested on a human with discrete congestion observed at the bulbar conjunctiva level and accentuated at the fornix and palpebral conjunctiva levels. For the clinical study, ADR-loaded microparticles using 0.5% polymer concentration with a 1/1 gelatin/chitosan ratio. The microparticle suspension was injected using a syringe without needle directly in the fornix (Figure 8(a)) of the left eye only, the right eye was considered the control. After 1 minute, the bulbar conjunctiva was inspected and no adverse reaction was observed. The particles were still present on the fornix indicating adhesion to the conjunctive membrane. Clinically, a vasoconstriction was observed immediately at the place of injection, probably due to the release of the ADR from the microparticle surface. After 2 minutes, there were no more reactions (Figure 8(b)). The inspection after 4 minutes revealed a decrease in the congestion in all regions except the palpebral conjunctiva indicating a slow release rate. After 8 minutes, the congestion had disappeared completely (Figure 8(c) and (d)).

The conclusions after this test were very encouraging: the particles were easy to administer and well tolerated by the patient; the drug effect was rapid at the place of administration, as evidenced by the disappearance of congestion; there were no adverse reactions like intense congestion with tearfulness, conjunctive ulceration, and pupilar miosis/midriasis. The loaded ADR activity was as expected, but in a slow manner, thereby avoiding the disadvantages experienced with classical administrations.

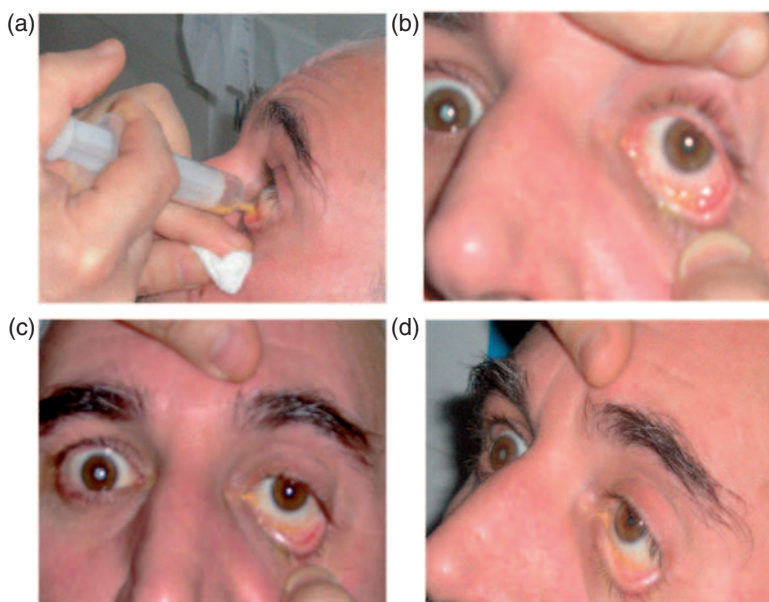


Figure 8. Human *in vivo* test of ADR release from GEL-CHI particles: (a) microparticles suspension administration in the eye fornix, (b) the eye inoculated with ADR-loaded microparticles at 2 min after the administration, (c and d) the congestion decrease in the external, medium third conjunctiva, and fornix areas.

CONCLUSIONS

Gelatin–chitosan microparticles were prepared by a w/o emulsion method with two-step cross-linking. Ionic gelation was induced by emulsifying the aqueous solution together with polymer emulsion, while covalent cross-linking was performed interfacially. The SEM micrographs of the microparticles indicated polydisperse sizes, which was supported by laser diffractometry. The sizes ranged from 0.202 to 4.596 μm . Microscopy showed collapsed microcapsules in dry state. The microparticles manifested pH sensitive interactions in aqueous environments. The swelling capacity, the ADR loading and release, respectively, can be controlled by modulating the initial ratio of the two polymers in aqueous mixture. This aspect is one of the advantages of this particulate system, compared with CHI or GEL systems alone. ADR loading and *in vitro* release were shown to be related to the swelling behavior in aqueous environment. Microparticle acute toxicity was similar to the individual polymers, *practically nontoxic* degree of toxicity. The *in vivo* biocompatibility tests showed that microparticles with both gelatin and chitosan were tolerated best and promoted fibrillogenesis. Potential usage of these microparticles

as an ophthalmic drug delivery system for ADR was demonstrated. Clinical tests showed that the microparticles were effective as anti-inflammatory agents. The gelatin–chitosan microparticles were easy to administer and well tolerated with no apparent adverse effects.

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