CONTROLLING THE RELEASE KINETICS OF CALCEIN LOADED LIPOSOMES FROM CHITOSAN/TANNIC ACID AND CHITOSAN/POLY(VINYL ALCOHOL)/TANNIC ACID HYDROGELS

MARCEL POPA,[*,***] BOGDAN C. CIOBANU,[*] LACRAMIOARA OCHIUZ,[****] JACQUES DESBRIERES,[**] CORNEL S. STAN[*] and CĂTĂLINA A. PEPTU[*]

[*]*"Gheorghe Asachi" Technical University of Iaşi, 73, Prof. D. Mangeron Str., 700050 Iaşi, Romania*
[**]*Université de Pau et des Pays de l'Adour, Institut Plurisdisciplinaire de Recherche sur l'Environnement et les Matériaux, IPREM, Hélioparc Pau Pyrénées, 2, P. Angot Av., 64053 PAU Cedex 09, France*
[***]*Academy of Romanian Scientists, Splaiul Independentei Str., 050094 Bucuresti, Romania*
[****]*"Grigore T. Popa" University of Medicine and Pharmacy, 700115 Iasi, Romania*
✉ *Corresponding author: C.A. Peptu, catipeptu@tuiasi.ro*
All authors have contributed equally to the present manuscript

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The paper describes the preparation of novel hydrogels based on chitosan or chitosan/poly(vinyl alcohol) crosslinked with tannic acid for the inclusion of phosphatidylcholine liposomes loaded with calcein as model fluorescent hydrophilic drug. This procedure ensured the reduction of the "burst effect" or even its elimination. The originality of the paper consists in the utilization of the natural compound tannic acid as crosslinker for the formation of complex hydrogels, which ensures biocompatibility and excellent biological properties. In order to modulate the properties of the hydrogel, the molecular weight of chitosan, the chitosan/tannic acid molar ratio, the molar ratio between the polymers and the crosslinking time have been taken into account as variable parameters.
Hydrogels based on medium molecular weight showed a reduced degree of swelling, as compared to those containing high molecular weight chitosan. More tannic acid in the hydrogel composition resulted in a higher crosslinking density in the hydrogels and reduced the swelling degree. As it was expected, the use of the synthetic polymer reduced the hydrophilicity of the materials and, as a consequence, the drug release capacity. Surprisingly, the crosslinking time did not reduce significantly the maximum degree of swelling. In good correlation with the characteristics of the hydrogels, the calcein release from the complex hydrogels could be delayed and better controlled. The release time was prolonged from several days (control hydrogels) to 21 days. The latency parameter showed that more that 40% of the calcein was released in the form of liposomes, which constitutes a second release barrier for the included drug.

Keywords: chitosan, poly(vinyl alcohol), liposomes, tannic acid, hydrogels, controlled drug release

INTRODUCTION

In recent years, drug delivery technologies have evolved continuously even up to the point where a biologically active compound can be released for a long time (even a year) with a relatively constant rate. Such a release process has numerous advantages, including reduction of side effects, maintaining a constant drug concentration in the body (at the disease site), administering the drug with lower frequency, increasing patient compliance and many others. Such effects could be obtained by associating drugs especially with polymers, the interaction of the two compounds being ensured by covalent bonds (polymer conjugates), ionic bonds, hydrogen bonding, van der Waals forces and so on. A polymer-drug system often reported in the literature is represented by hydrogels, polymeric networks capable of simultaneously absorbing large quantities of water and compounds dissolved in this aqueous medium. Both the characteristic drug release and loading processes of such materials are mainly controlled through diffusion, sometimes accompanied by erosion processes (chemical degradation). However, the use of

hydrogels for this purpose presents some drawbacks and the current interest of many research groups is to minimize or eliminate them. One of these is represented by the toxicity of residual traces of certain compounds used to crosslink the polymers and to obtain the polymeric network. A possibility to solve such a problem is to reduce the amount of covalent crosslinker (dialdehyde, epichlorohydrin *etc*.) and to partially substitute this amount with ionic crosslinkers, such as sodium tripolyphosphate[1,2] or sulphate anions.[3] The double crosslinking achieved in this case is limited only to polymers with ionisable functional groups capable of reacting with ionic crosslinker of the opposite type. Researchers have shown interest in completely removing the covalent crosslinker, which is dangerous for the human body. Some methods use natural products, such as citric acid,[4,5] caffeic acid, tannic acid,[6] or compounds able to produce crosslinking through other types of interaction with the macromolecules. The second problem is connected to the kinetics of the release process from the hydrogels through diffusion, which is manifested in most cases by an initial "burst effect", after which the release kinetics is described by specific diffusion equations. To reduce or to eliminate the "burst effect", various approaches were proposed, such as associating polymers with different hydrophilicity,[7,8] increasing the molecular weight of the polymers,[9,10] adjusting the porosity of the hydrogel,[11,12,13] and the crosslinking density of the network.[14,15] Other researchers[16] reported the possibility to reduce the "burst effect" and to obtain a better control of the release kinetics by loading the active compound in liposomes, followed by their inclusion in a crosslinked polymeric matrix.

Starting from these premises, this paper proposes the development of complex drug delivery systems by including small unilamellar liposomes (SUVs) loaded with a hydrophilic drug into hydrogels. Thus, chitosan and a chitosan-poly(vinyl alchohol) mixture were crosslinked through hydrogen bonds using tannic acid (TA), a natural polyphenolic compound, as crosslinker. Calcein was used as a biologically active model drug. The main purpose was to solve simultaneously two problems: the elimination of the chemical crosslinkers (often toxic) and the modulation of the drug release kinetics.

Tannic acid is a diet component, but also possesses remarkable biological activity.[17,18] This reagent is composed of a glucose esterified at all five hydroxyl functional groups by gallic acid molecules, which themselves bind other five gallic molecules through ester bonds (Fig. 1). Therefore, TA contains one glucose core and ten gallic ester moieties.

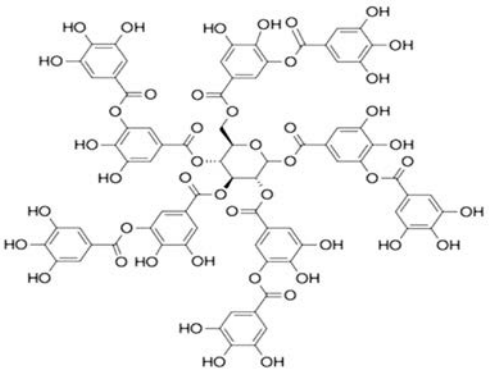


Figure 1: Tannic acid chemical structure

Although it is not a carboxylic acid, it is called "acid" due to numerous phenol groups, which confer strong acidic character. TA is a polyphenol and together with catechins and other classes of tannins presents a direct antioxidant effect by binding free radicals, such as phenol bioflavonoids.[19] Oral consumption of green tea containing TA was found to decrease the tumor size in mice and to inhibit induced tumor growth.[20] Used for decades as tanning agent for leather, due to its ability to bind and precipitate proteins and complex polysaccharides, TA was also proposed later to treat burned skin.[21,22] In addition, the use of TA can help to stabilize and preserve the dermal matrix.[21] TA has been also used in other medical areas besides burns. Isenburg *et al.*[23] proposed the crosslinking properties of the TA in the treatment of aortic

aneurysm. This polyphenol presents anti-bacterial, anti-enzymatic and astringent properties, and may be used as medicine against diarrhea, in hemostatic and anti-haemorrhoidal formulations.[24] TA compound is able to inhibit the development of skin tumors induced by means of UV-B radiation in mice by about 70%.[25] The study findings of Terao *et al.*[26] support the hypothesis that the administration of a daily dose of TA dissolved in drinking water leads to a decrease in the incidence of liver neoplasms. The cytotoxic triggering apoptosis in human cancer cell lines induced by gallic acid, the major constituent of TA, was also reported.[27] Naus *et al.*[28] studied the interaction of some chemo-therapeutic agents with TA given the polyphenol potential use as an adjuvant in anticancer therapy. The authors concluded that TA allows dose reduction when combined with 5-fluorouracil and mitomycin C. Some authors suggested the involvement of polyphenols in the prevention of neurodegenerative diseases[29] and cancer.[30] Tikoo *et al.*[31] have shown by *in vitro* and *in vivo* studies that TA presents antioxidant and anticancer effect on embryonic mioblasts of rats. It was also found to reduce cell viability of breast cancer cells and to enhance the anticancer activity of doxorubicin.

TA was used already to crosslink gelatin,[32] chitosan,[33] collagen,[34] copolymers like poly(allyl glycidyleter - acrylamide),[35] in order to obtain non-toxic hydrogels.

One may expect that a hydrogel crosslinked with TA will benefit of the above mentioned properties of the crosslinker. Thus, TA will have a double role, of crosslinking and therapeutic agent. Moreover, it seems that TA is able to maximize the stability of liposomes.[26]

In view of previous studies carried out on this polyphenol and the promising results with regard to its applications in contact with human tissues (biocompatibility, biodegradability, anti-oxidant, anti-bacterial and anti-microbial properties), in this work, a systematic study was carried out on the use of TA as a crosslinking agent, a process based both on the formation of multiple hydrophobic links and hydrogen bonds between chitosan – poly(vinyl alcohol) macromolecular chains (phenol groups are an excellent hydrogen donor) and TA.

The choice of chitosan for this study is not random, but it is due to its versatility in modeling biological and physico-chemical properties under moderate conditions.[36,37,38] Chitosan is a natural cationic polysaccharide obtained industrially by hydrolysis of the aminoacetyl groups of chitin. This biopolymer is currently extracted from the exoskeleton of crustaceans, mollusks, insects and certain fungi families.[16] Chitin and chitosan are the raw materials used to fabricate biomaterials. Both polysaccharides present interest for the drug delivery area due to their excellent biocompatibility, biodegradability and non-toxicity.[39]

Poly(vinyl alcohol) is a water-soluble synthetic polymer used in a wide variety of food, industrial, commercial and medical applications.[40,41]The formation of hydrogen bonds between PVA and chitosan leads to polymeric networks with superior mechanical and chemical resistance. Polymeric systems containing PVA have been evaluated in view of the therapeutic applications due to non-toxic, non-carcinogenic and bioadhesive properties.[42,43]

EXPERIMENTAL

Materials

High molecular weight chitosan (HC) (Brookfield viscosity 800.000 cps) and medium molecular weight chitosan (MC) (Brookfield viscosity 200.000 cps), poly(vinyl alcohol) (PVA), 80% degree of hydrolysis, molar mass 9000-10000 g/mol^{-1}, calcein (fluorescent hydrophilic tracer), tannic acid (TA) and extrapure Triton X-100 were purchased from Sigma-Aldrich (St. Luis, MO, USA). Calcein was used without further purification. Phospholipon-90G (phosphatidylcholine – PC) was received as a gift sample from Phospholipid GmbH, Nattermannallee 1, D-50829, Köln. All other chemicals used were of analytical grade. The degree of deacetylation for both chitosan types was ~76% as shown by ^{1}HNMR.

Preparation methods

Preparation of small unilamellar vesicles charged with calcein (SUVs)

First, a suspension of large liposomes was prepared by the thin film hydration method. The PC was dissolved in chloroform/methanol (2/1 v/v) and the solvent was evaporated at 30 °C by rotary evaporation until a thin film was formed on the walls of a 250 mL round-bottomed flask. The film was hydrated by vortex agitation with 2 mL of calcein solution (32 mg/mL). The resulting suspension of large liposomes was sonicated using a Bandelin Sonopuls GM 2200 probe tip sonicator (10 pulses of 60 s duration and 30 s break to allow the sample to cool down) to obtain SUVs. The sample was kept in an ice bath to avoid lipid breakage. To eliminate unentrapped calcein, size exclusion chromatography (SEC) (column of 25 cm length and 1 cm diameter) was used, with Sephadex G-25 eluted with PBS buffer (2.2 mL/min). By using this method, SUVs of an average diameter of 120±14 nm and a

quite narrow dimensional polydispersity were obtained.

For quantification of liposomes loading, the suspension of liposomes was treated with Triton X-100, which ensures the calcein release and enables the calculation of the total calcein content. Calcein fluorescence latency was calculated using Equation (1):

$$\% \, Latency = \frac{1.1 \, F_{AT} - F_{BT}}{1.1 \, F_{AT}} \, x \, 100 \quad (1)$$

where F_{BT} and F_{AT} are the calcein fluorescence intensity (in direct correlation with the concentration of calcein) before and after the addition of Triton X-100, respectively.

The charged liposomes were used in the same day of preparation or the day after, keeping them sealed at 4 °C and protected from light. The calcein latency should be higher than 95% to ensure the minimum free calcein before hydrogel preparation.

The lipid concentration of the liposome dispersions was measured by the Stewart colorimetric assay.[44] Phospholipids with ammonium ferrothiocyanate form a colored complex (OD 485 nm), which is extracted in chloroform. After measurement, the lipid concentrations of the liposome dispersions were adjusted to the desired value in order to prepare the liposome containing films, as further described.

Preparation of hydrogels

1.2% HC or MC, HC-PVA, MC-PVA stock solutions were prepared in 2% acetic acid aqueous solutions by stirring overnight the mixture and filtering to remove the impurities by using a reusable syringe filter holder coated with filter paper. The crosslinking agent, TA, was also dissolved in 2% acetic acid solution in different concentrations. HC and MC, HC-PVA and MC-PVA hydrogels were prepared based on two different molecular weights and C-PVA at various NH_2/OH molar ratios were prepared by dissolving 200 mg of polymers in a 2% acetic acid solution (16 mL) at room temperature overnight. The resulted solutions were filtered. 4 mL of TA solution (in different concentrations) was added dropwise over the polymer solution under stirring to give a final polymer concentration of 1%. The crosslinking agent and the polymers were mixed in different molar ratios, considering that the TA participates in the crosslinking process only with the outer hydroxyl groups (10 out of the 25 present) due to the sterical hindrance that occurs. Therefore, it was considered that for every esterified gallic acid residue only two phenol groups (of the three present on the aromatic ring) can interact with the polymer, the remainder being shielded and thus sterically hindered. Crosslinking by hydrogen bonding takes place at the amino groups of the polysaccharide, as well as at the hydroxyl groups of both polymers. Hydrophobic interactions could also be formed in such networks.

The experimental parameters that were varied in the preparation of the hydrogels based on C and C-PVA mixture can be seen in Tables 1 and 2. The obtained solutions were kept under vigorous stirring for 30 minutes, then allowed to rest to remove air bubbles. Then, the solutions were cast on round polyethylene plates, with 60 mm diameter and 25 mm height. Subsequently, the gels were placed in an oven at 60 °C for predetermined time intervals to remove water and obtain the films. The value of t_0 was determined when the films were mechanically stable and could be removed from the plates and washed. In all the cases, 12 hours were sufficient to dry and obtain a stable film. The other analyzed times were t_1 = 18 h and t_2 = 20 h. Then, the films were washed three times with water (two hours each) and with methanol (30 minutes), and then kept for drying at room temperature for 24 hours.

The hydrogels based on HC/PVA were prepared at t_2 = 20 hours after observing that the films obtained at different crosslinking time intervals present almost the same swelling behaviour.

Table 1
Parameters for C-TA hydrogel preparation

Film code	Chitosan molecular weight	C/TA molar ratio	Crosslinking time (hours)
HCT2	High molecular weight	20/1	t_0=12; t_1=18; t_2=20
HCT3		30/1	t_0, t_1, t_2
HCT4		40/1	t_0, t_1, t_2
HCT5		50/1	t_0, t_1, t_2
MCT2	Medium molecular weight	20/1	t_0, t_1, t_2
MCT3		30/1	t_0, t_1, t_2
MCT4		40/1	t_0, t_1, t_2
MCT5		50/1	t_0, t_1, t_2

Preparation of calcein and liposomal calcein charged hydrogels

For the preparation of control/liposomal hydrogels, 500 µg calcein from a stock solution (32 mg/mL) and an amount of SUV suspension containing the same amount of calcein were diluted in 1 mL acetic acid solution (2%) and added to the polymer solutions before the addition of crosslinker.

Characterization methods

Liposome size measurements

The mean diameter of the liposomes was analyzed by the laser light diffractometry technique (SHIMADZU – SALD 7001). Samples were analyzed in triplicate and standard deviation was calculated.

Table 2
Parameters for HC/PVA-TA hydrogel preparation

Sample code	C/PVA molar ratio	Polymers/TA molar ratio	Polymers/TA functional groups ratio*
A20	1:1	20/1	3.75:1
A30	1:1	30/1	4.2:1
A40	1:1	40/1	5.5:1
A50	1:1	50/1	6.9:1
B20	3:1	20/1	4.7:1
B30	3:1	30/1	7:1
B40	3:1	40/1	9.3:1
B50	3:1	50/1	11.5:1
C20	1:3	20/1	2.9:1
C30	1:3	30/1	4.3:1
C40	1:3	40/1	5.8:1
C50	1:3	50/1	7.2:1

*It was considered that only 10 -OH groups from TA participate in crosslinking

FT-IR spectroscopy

FTIR spectroscopy analysis was carried out using the KBr technique on Digilab Scimitar FTS2000, 64 scans were recorded at a resolution of 4 cm^{-1}.

Scanning electron microscopy (SEM)

For hydrogel morphology analysis, a Hitachi SU1510 scanning electron microscope was used. The samples were deposited on a carbon strip on aluminum stubs and covered with gold.

Swelling degree

Swelling kinetic in aqueous medium was determined by gravimetric method. The principle of the method was as follows: the samples were dried at constant weight for three hours in an oven at 100°C to completely remove water traces. The dried samples were weighted and then allowed to swell in double distilled water for predetermined time intervals (5, 15, 30, 60, 120 and 200 min), when the samples were removed from the swelling medium, gently pressed with filter paper to remove excess water and weighted again. The degree of swelling was calculated using the equation:

$$Q\ (\%) = \frac{W_1 - W_0}{W_0}\ x\ 100 \quad (2)$$

where W_1 – swollen sample weight at time t; W_0 – dry sample weight.

For each sample, five determinations were carried out. Standard deviation was subsequently calculated and plotted on the graph.

Tannic acid release

The kinetics of TA release from the hydrogels was determined by UV-VIS spectroscopy on a Nanodrop ND 1000 (Thermo Fisher Scientific) instrument based on the absorption intensity of TA at 280 nm. First, a calibration curve was built by measuring the absorbance of TA aqueous solutions of known concentration (between 0.01 and 0.3 mg/mL). Subsequently, the hydrogel films were immersed in 200 mL of distilled water and the TA release at different time intervals (up to 200 min) was determined by using the calibration curve. After 200 min, daily measurements were made until reaching the steady state. Each point on the graph represents the average of three measurements.

Calcein release from hydrogels

The calcein release from the hydrogels was assessed in phosphate buffer saline (PBS) at pH = 7.4. Each hydrogel was immersed in covered plastic containers with 50 mL PBS solution and placed in a GFL 1092 water bath (60 rpm, 37 ± 0.1 ºC). The calcein release was spectrophotometrically monitored at predefined time intervals using a Fluoromax-4 Spectrofluorometer Horiba Scientific (excitation/emission = 490/515 nm). In the case of

control hydrogels, 3 mL of supernatant was removed for each measurement and replaced with fresh PBS buffer. For complex hydrogels study, 20 µL of SUV suspension from the 3 mL was removed and diluted with 4 mL PBS buffer for measuring the unentrapped calcein. After each measurement, Triton X-100 surfactant was added to a final concentration of 1% in order to disrupt the liposomal membrane and release the entrapped calcein. Then, the total calcein concentration was analyzed.

The release efficiency (E (%)) was calculated using the following formula:

$$E\ (\%) = \frac{m_{ci}}{m_{ct}}\ x\ 100 \qquad (3)$$

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

RESULTS AND DISCUSSION

The main objective of the present study was the preparation of new hydrogels of low toxicity for drug loading/release systems, with potential application in pathologies when a long treatment period is desired. To achieve this objective (an alternative to chemical and ionic compounds) the release kinetics of hydrophilic drugs (calcein was tested as model drug), to eliminate the "burst" effect and to prolong the release period, TA was used as crosslinker in different concentrations and a second release barrier in drug transport across the polymeric matrix was added to the hydrogel system. Thus, calcein-encapsulated small unilamellar vesicles (SUVs) composed of PC were used for the preparation of complex systems, in order to check the effect of liposomes as an extra barrier in calcein release. The ability of TA to crosslink C was previously reported,[45] but no systematic studies on the preparation of hydrogels are available. In this paper, we analyzed some factors, such as C/TA molar ratio, crosslinking duration, C molecular weight and blending with another hydrophilic polymer (PVA). These parameters were expected to influence both the physical-chemical characteristics and calcein loading/release capacity of the hydrogels in the form of films.

Between TA and C (and PVA when blends were prepared) no chemical reactions arise allowing ionic crosslinking. The fact that TA does not ionize in an acid environment (unlike chitosan) makes the achievement of ionic bonds impossible. However, the presence in all system components of hydrogen atoms bonded to electronegative elements (O and N) sustains the idea of hydrogen bonding. Although lower in terms of energy than covalent and ionic bonds, hydrogen bonds are very numerous in these systems, ensuring the stability of an interpenetrated/interconnected network. Chitosan contains both amine and hydroxyl groups. Tannic acid contains only phenol hydroxyl groups, which, given their high number, confer a weak acid character to TA, hence its name. Under the acid conditions imposed by chitosan dissolution, its amino groups are protonated. The polysaccharide pKa is 6.2.[44] Therefore, at values of pH less than 6.2, the polymer contains dominating ammonium ions as substitute, their number increasing by reducing the pH. Unprotonated amino groups coexist and are able to engage hydrogen bonds with the polyphenol. They are added to the -OH groups of the polysaccharide, also capable of this effect. Under these conditions, one may expect that the gel structure is as depicted in Figure 2 (right). When PVA is present in the system, hydrogen bonds are also formed between its alcohol groups and the phenol groups of TA (Fig. 2, center and left).

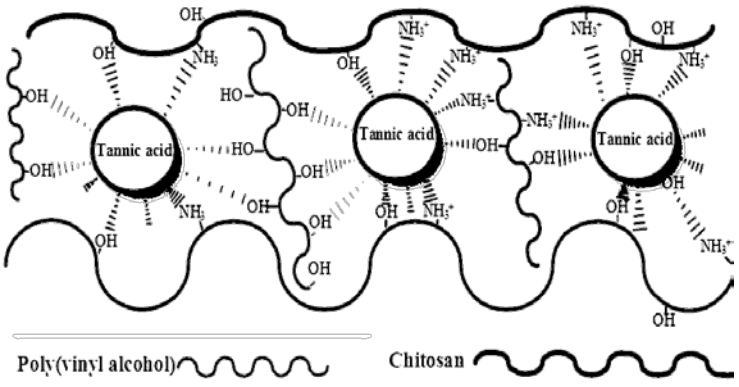


Figure 2: Schematic representation of hydrogels based on C-TA (left) and C/PVA-TA (centre and right)

FT-IR spectroscopy

Chitosan macromolecules can be folded on the TA, employing crosslinks through both functional groups. Sterical hindrance from the macromolecule, as well as from neighbouring phenol groups on each aromatic ring belonging to gallic acid dimer from the TA structure, makes impossible the participation of all the functional groups of the two compounds in the network formation. When poly(vinyl alcohol) participates in network formation through its hydroxyl groups, the network can become more relaxed, chitosan being hindered in some way to enter hydrogen bonding. Structural analysis by FT-IR spectroscopy (spectra not shown) revealed the presence of specific bands of the hydrogel components in the analyzed films. The FT-IR spectrum of chitosan shows bands characteristic of -OH groups in the 3000-3750 cm^{-1} interval, of the –NH and -CH groups at 2920 cm^{-1} and 2875 cm^{-1}, respectively, of -CH_3 groups (from acetate residues from chitin) at 1375 cm^{-1} and 1476 cm^{-1}. The interval 1480-1680 cm^{-1} reveals the presence of several carbonyl group vibration bands characteristic of secondary amide (-CONHR), which was deacetylated after hydrolysis of chitin to chitosan. Very prominent is the vibration band of the 1025-1080 cm^{-1}, attributed to groups -COH,-COC- and -CH from the glucosidic cycle. FT-IR absorption spectra of PVA also highlights the polymer characteristic bands: a broad absorption band in the 3200-3600 cm^{-1} region (attributed to free -OH groups or involved in hydrogen bonds), two absorption bands in the domain 2840-3200 cm^{-1} corresponding to -CH bonds from the polymer backbone, the absorption bands at 1760 and 1650 cm^{-1} characteristic of the C=O group from acetate residues (esteric) remaining after hydrolysis of poly(vinyl acetate) from which the polymer is obtained. The TA characteristic absorption bands associated to -OH groups (at 3388 cm^{-1}), to C=O groups (at 1715 cm^{-1}), and to ether groups (at 1198-1025 cm^{-1}) are also present. The existence of numerous -OH and -NH_2 groups that absorb in the wide region of 3000-3750 cm^{-1} forms a wide spectrum that covers the characteristic absorption bands of the hydrogen bonds.[33]

Liposome size measurements

It has been found that the SUVs of an average diameter of 120±14 nm and a quite narrow dimensional polydispersity were obtained, as can be visualized in Figure 3.

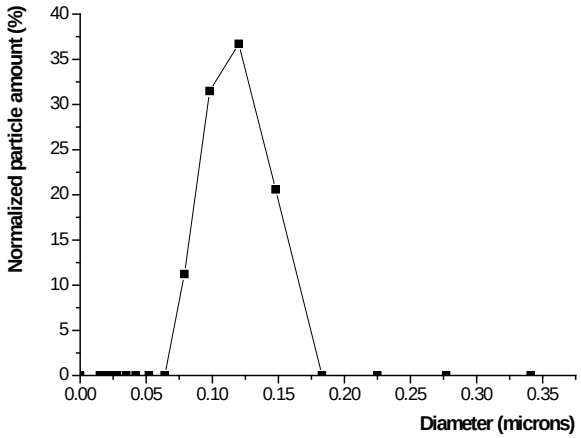


Figure 3: Liposome distribution curve determined by laser light diffractometry

Scanning electron microscopy (SEM)

The films obtained by TA crosslinking were morphologically characterized by scanning electron microscopy. Figure 4 highlights the surface appearance of the hydrogels based on HC crosslinked with TA at various molar ratios between the components. The presence of large crosslinker amounts in the films composition results in obtaining a rough surface (Fig. 4a). The rough surface appearance attenuates by reducing the amount of polyphenol, thereby the films with minimal amounts of crosslinker present a smooth surface (Fig. 4d), which can also be observed macroscopically. The films become more and more glossy and non-slippery with the reduction of TA amount. The surface appearance of the sample crosslinked with a minimum amount of TA (Fig. 4d) is basically identical to that reported in the literature for uncrosslinked chitosan films.[46] The explanation that we propose in order to

achieve such morphologies is further presented. Large amounts of TA lead to more compact networks, forcing chitosan macromolecules to adopt rigid conformations, which fold around the crosslinker molecule, generating a rough look at a macroscopic level. As the amount of crosslinker is reduced, the polysaccharide macromolecules are attached within a decreasing number of hydrogen bonds, they become more flexible and able to relax, which causes the network to become looser and, at a macroscopic level, the film surface becomes increasingly smoother. On the microphotographs, the presence of some fine particles can be observed, which we attribute to chitin residues passed from the filtration of the C solutions through the filter.

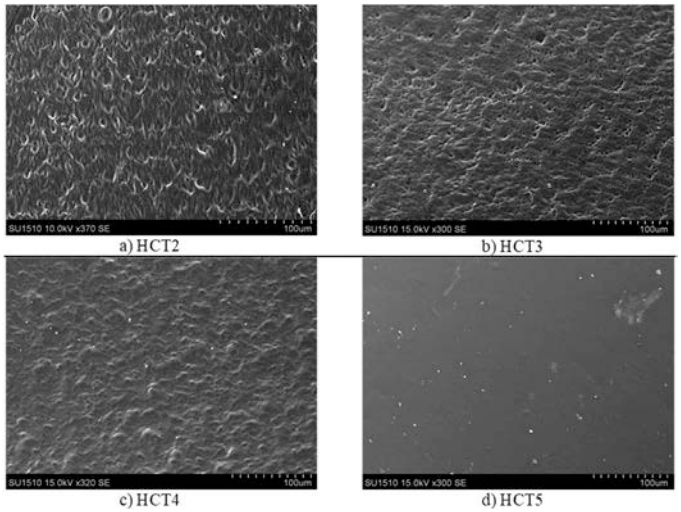


Figure 4: Scanning electron microscopy microphotographs presenting films based on HC crosslinked with TA (in different ratios between partners)

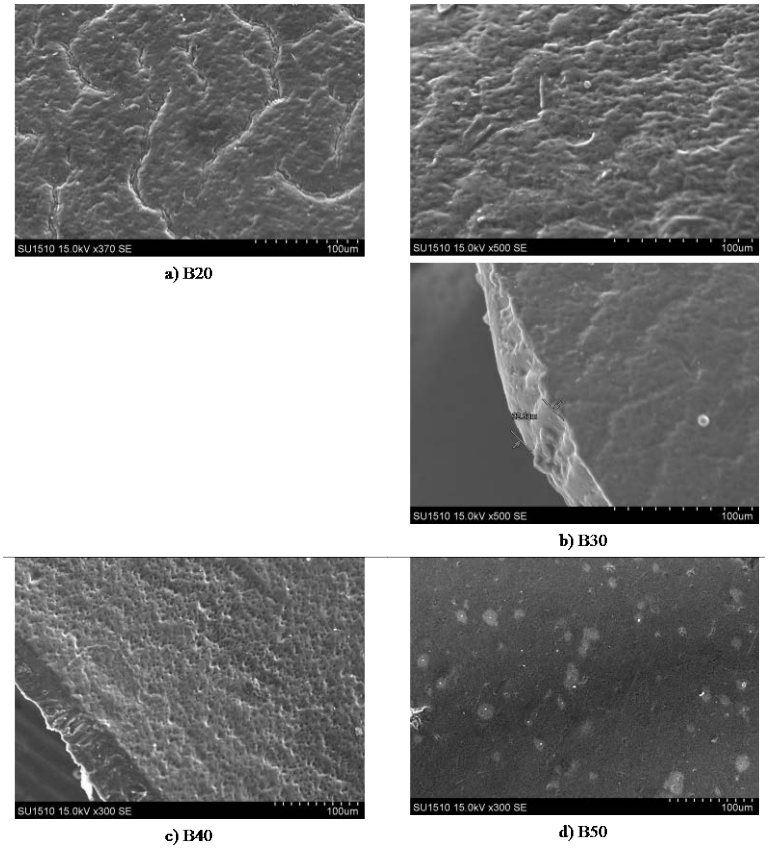


Figure 5: Scanning electron microscopy microphotographs presenting films based on HC/PVA (3/1) crosslinked with TA (in different ratios between partners)

The morphology of a film based on MC obtained at a MC/TA ratio = 30 is similar to that of the film based on HC and this is the reason why only the images corresponding to the HC films are presented.

The films based on chitosan blended with PVA (3/1 molar ratio) crosslinked with TA presented a different appearance than those discussed above (Fig. 5). The topography is less rough, as compared to the chitosan based films crosslinked with the same amount of TA. The porous appearance disappears, but at large amounts of crosslinker, visible artefacts occur, mostly at the ratio polymers/TA = 20.

The explanation may be related to the fact that PVA participates too, but to a lesser extent, in the formation of hydrogen bonds with TA (the nucleophilicity of the oxygen atoms is lower than that of the nitrogen atoms). However, intervening in the formation of an interpenetrated network, PVA can insert between chitosan macromolecules and crosslinker, preventing the participation of the two components with the maximum number of functional groups that can interact. Therefore, chitosan macromolecules (which represent the majority in the mixture) no longer adopt a rigid conformation, surrounding the crosslinker molecule, as in the case of the films not containing PVA, and the network becomes looser as described above (Fig. 5a compared to Fig. 4a). Reducing the TA amount leads to films with less rough appearance that become smooth for the minimum quantity of polyphenol (Fig. 5d). For the sample in which the ratio of the polymer mixture and crosslinker was 30, a microphotograph was performed that shows the appearance of its section. It is interesting to note that the film exhibits a compact appearance, devoid of pores, which is useful in developing the drug transport mechanism through the hydrogel.

Swelling capacity

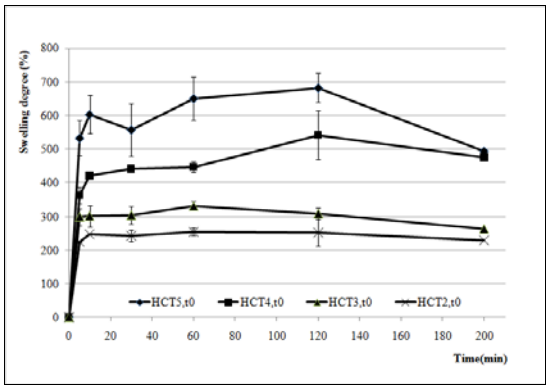
Swelling capacity of C-TA films

An important feature in the characterization of hydrogels is their behaviour in aqueous solutions. Three types of films were analyzed in terms of the water retention process, two for both types of chitosan crosslinked with TA, and the third – films resulted from co-crosslinking of a mixture of HC/PVA. The results obtained are shown in Figures 6, 7 and 8, respectively. A first observation is the fact that all the hydrogels presented high water retention capacity. The maximum degree of swelling varies depending on the molecular weight of chitosan, the amount of crosslinking agent and the mixture ratio of the polymers in the case of HC/PVA based hydrogels. The swelling process is almost instantaneous, determined, of course, by the hydrophilic character of chitosan. In most cases, after large amounts of water retention in the first 5-10 minutes of the process, the degree of swelling does not vary greatly in time, the maximum degree of swelling, in most cases, being reached in about two hours. Moreover, together with a logical increase of the degree of swelling with decreasing amounts of crosslinking TA agent, a more or less pronounced maximum in the variation of the degree of swelling *vs.* time is evidenced.

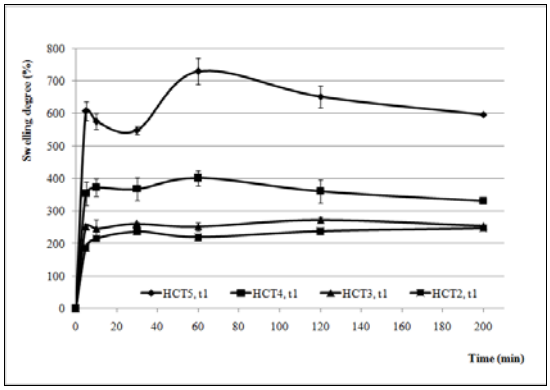
A first group of the analyzed hydrogels is represented by the HC in different molar ratios with the crosslinking agent, as well as for different times (12, 18 and 20 h). Analyzing the results shown in Figure 6, a logical progression of the maximum degree of swelling may be established on the basis of the amount of TA, in the sense that the degree of swelling increases by reducing the amount of crosslinking agent (as a result of the lower crosslinking density). As already mentioned above, on the majority of the kinetic curves, a maximum degree of swelling can be identified, more or less pronounced, depending on the parameters of film preparation. After achieving this level, the degree of swelling slightly decreases sometimes, especially in the case of the hydrogels with lower crosslinking density. Such a situation is also encountered in chemically crosslinked hydrogels, being characteristic of the cases when, after a certain duration, low molecular weight fractions not caught in crosslinks are solubilized.[47] In the case of our hydrogels, the species that can be gradually solubilized and removed from the hydrogels are TA and lower molecular weight chitosan fractions containing ammonium ions as a substituent (formed in weakly acidic solution subjected to crosslinking) by virtue of the polydispersity of the polysaccharide. To elucidate the nature of the soluble fractions, first, the amount of TA accumulated in time in the swelling medium (double distilled water) was monitored. The results presented below (in “Tannic acid release from hydrogels”) for a set of hydrogels prove the release of a quantity of TA from the hydrogels right from their first moments of immersion in water. This process may explain the fluctuations in the swelling degree of some hydrogels

(particularly those with minimal amounts of crosslinker). Secondly, some of the swollen films, randomly selected were re-dried and weighed. There was a reduction in the weight of the dry film, as compared to the initial dry mass, in some cases over 20%. Admitting that the weight loss corresponding to TA is up to 14% (see Fig. 9) from the total of the crosslinker, the remainder should represent the polymer loss that emerges partly by removing the crosslinker. Although neutral chitosan is not soluble in water, as it is ionized in the hydrogel, its solubility in water is increased. It must be also noted that between the hydrogels with the same composition, but crosslinked at different durations, there are no major differences in terms of the swelling degree values recorded over time. However, a very slight reduction in the value of this property can be seen with increasing the preparation time. The conclusion is that in the first 12 hours basically the maximum density of the network crosslinking is reached, suggesting that longer durations of time are not necessary.

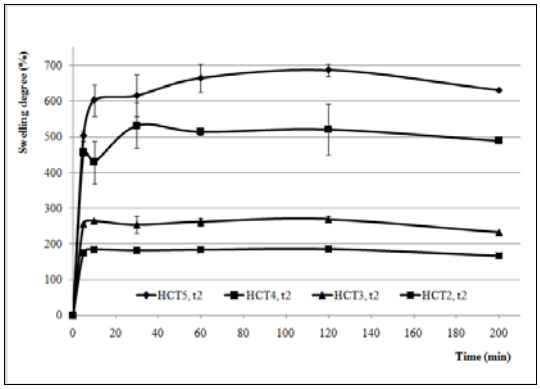
The films based on MC crosslinked with TA presented a higher degree of heterogeneity, as reflected by Figure 7. In this case, the sets of hydrogels crosslinked for 18 h and 20 h were retained for exemplification. As a general observation, the values of the swelling degree range between 200-600%, depending on the composition of the hydrogel analyzed. Regarding the influence of the starting composition on the hydrogels swelling characteristics, the previous conclusion is confirmed, *i.e.* the degree of swelling throughout its evolution decreases with the amount of crosslinking. The lower values of the maximum degree of swelling for the hydrogels of the same composition, as those obtained with HC, are probably explained by the lower number of conformations that can be achieved in the rubbery state (characteristic to swollen state) by shorter macromolecules of HC. Their flexibility is lower, the mesh network is less elastic, and thus the amount of water retained is reduced.



A



B



C

Figure 6: Variation in time of the swelling degree in double distilled water of HC/TA hydrogels of different HC-TA ratio and different crosslinking time intervals, (A) t_0, (B) t_1 and (C) t_2

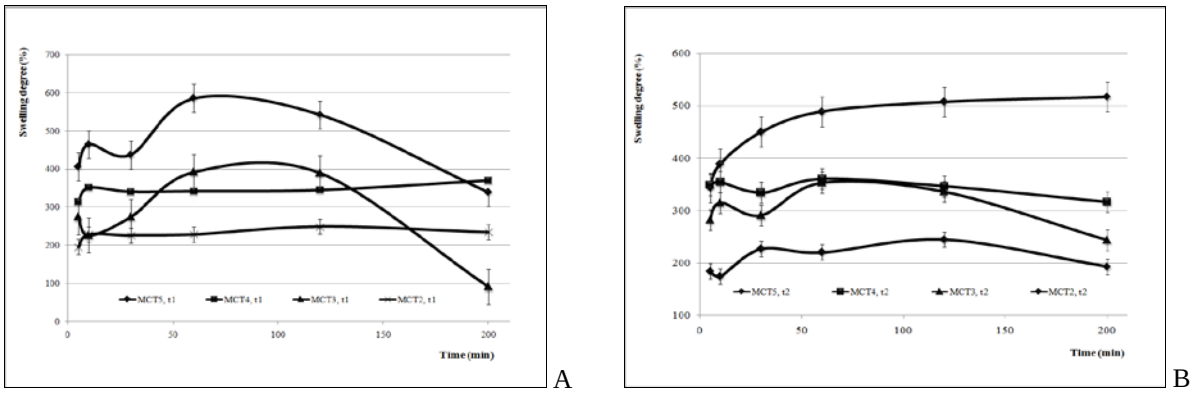


Figure 7: Variation in time of the swelling degree in double distilled water of MC/TA hydrogels of different C-TA ratio, (A) t_1 and (B) t_2

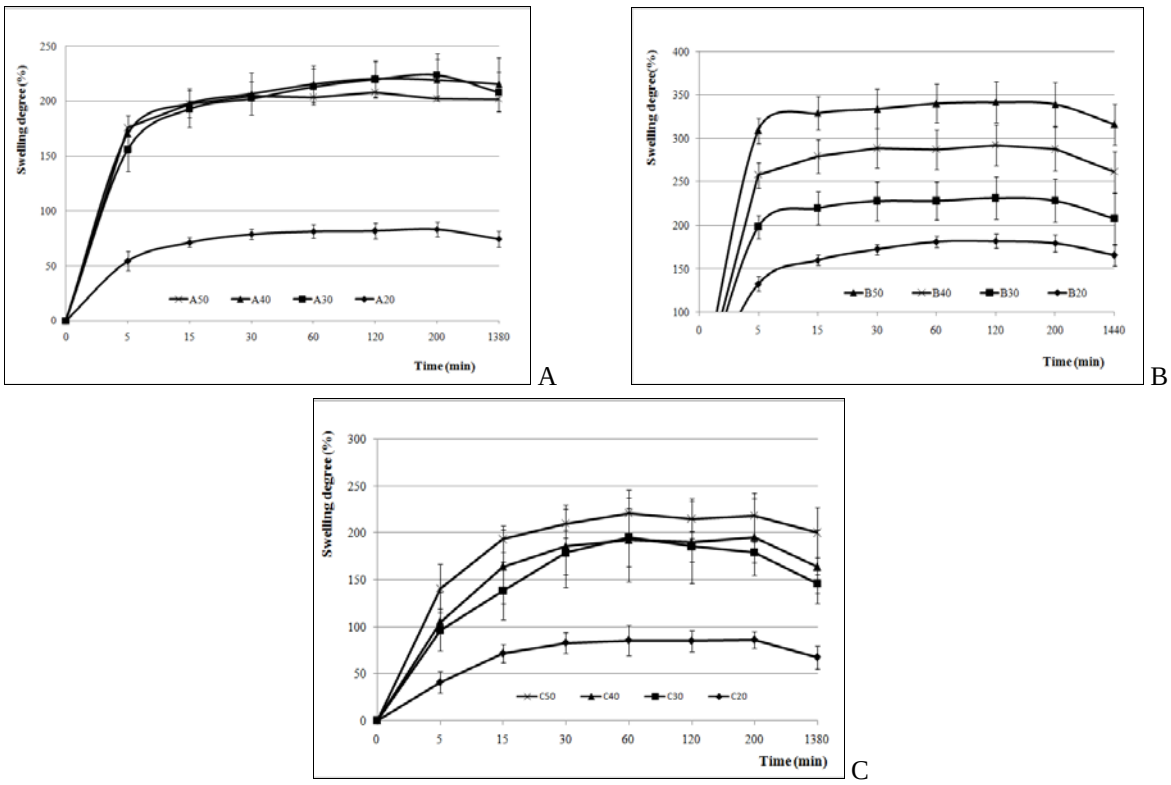


Figure 8: Variation in time of the swelling degree in double distilled water of HC/PVA-TA hydrogels of different polymers-TA ratio prepared at t_2, (A) HC/PVA (1/3), (B) HC/PVA (3/1) and (C) HC/PVA (1/1)

Swelling capacity of HC/PVA-TA films

The third group of films analyzed was that based on HC/PVA crosslinked with TA. The rationale of their study was the biocompatible character of PVA, on the one hand, and the ability to adjust downwards the films hydrophilicity by PVA intake, on the other hand. In addition, both polymers present processing versatility and capability to form films under moderate conditions. Since, in the previous studies, the films based on HC showed better characteristics and favorable results were obtained in terms of correlation of the swelling degree with the studied parameters, HC was chosen for the studies that focused on its mixing with PVA. As already mentioned, the presence of PVA in the film composition greatly reduces hydrophilicity, resulting in much lower values of the swelling degree (Fig. 8). Keeping constant the polymers molar ratio, the influence of the crosslinking agent amount has been monitored and it was concluded that the hydrogels with high TA content swell least in distilled water (Fig. 8). This behaviour is similar to that seen in the case of HC based films. With regard to series B, where HC is the major polymer in the hydrogel matrix, the maximum

degree of swelling is superior to the other two series (A and C), outlining the idea that the polysaccharide has higher affinity for water, compared with PVA. This series of hydrogels presented a maximum swelling degree of 341% for sample B50, which decreases to 208% for sample A50 and to 221% for sample C50. Furthermore, this clearly confirms the conclusion determined for the previous series of films, the evident reduction of the swelling degree by increasing the quantity of polyphenol.

This conclusion also applies to the series C, indicating that the influence of the amount of PVA in this case is not so obvious. A final observation is the fact that all the three series of films presented a decrease in the swelling degree after more than 200 minutes from the start of the process, but at a lower rate than in the case of the films based only on chitosan. The reduced amount of soluble fractions could be due to the interconnected/interpenetrated character of the hydrogels prepared. In these networks, the macromolecules not participating at the reaction are difficult to extract. The solubilization in time of low molecular weight fractions of PVA is not excluded.

Tannic acid release from hydrogels

During the swelling studies performed on the hydrogels based on chitosan crosslinked with TA, a decrease of their mass after reaching maximum values of the swelling degree was noticed. The phenomenon is obviously due to solubilization of small amounts of the components involved in network formation and this may be explained by the fact that the links established between the crosslinker and polymers in this case are not covalent, but weaker hydrogen bonds. In principle, the soluble fractions that are released from the polymeric matrix can be represented by TA and chitosan. As a result, a series of films composed of HC have been studied in terms of the TA release (Fig. 9). It should be mentioned that the release of TA may be partially considered even as beneficial, being known the biological activity of this compound, as mentioned in the introduction section.

The analysis of the release kinetic curves allows the following conclusions. At least until the 8th day, TA release takes place according to a typical diffusional kinetic system with a pronounced "burst" effect within the first 3-4 hours of the process. The effect is caused probably by the TA release from the surface of the film or from its superficial layers where the crosslinker molecules participate in the formation of the network only through a small number of phenol groups among the 25 available. The TA release from matrices based on chitosan in the form of particles has been reported by Aelenei *et al*.[24] The TA amount released after 8 days is closely related to that from the composition of the mixture subjected to crosslinking. As the HC/TA ratio decreases, the amount of TA released decreases as well.

Another finding that should be mentioned is that, in about 2 days after the start of the releasing process, the TA amount remains practically constant, but after 5 days it begins to gradually decrease. In principle, the diffusion process is determined by the existence of a concentration gradient of the compound released within the gel from the supernatant in which it is suspended, which is the driving force that causes the release. The process is dynamic, and takes place in both directions until it reaches a steady concentration of the compound within the hydrogel and in the supernatant. This equilibrium can be reached at a TA amount released from 4 to 15%, depending on the composition of the network. The fact that after about 5 days the amount of TA diffused in the supernatant begins to decrease can only be explained by the TA hydrolysis at simpler polyphenol molecules, such as gallic acid, which absorb in the UV spectrum at different wavelength compared to TA. Thus, gallic acid presents two absorbance peaks located at 220 and 260 nm, while the TA absorption wavelength is located at 280 nm.[24] UV-VIS qualitatively recorded spectra for the supernatant corresponding to hydrogel HCT5 showed a weak absorption at this wavelength value, proving this hypothesis. The elimination in time of certain amounts of crosslinker, which is found within the network either in free state or anchored by a few hydrogen bonds, can lead to their slight deterioration, eliminating chitosan macromolecules of low molecular weight, which may explain the achievement of the maximum on the swelling kinetic curves of some samples (as outlined above).

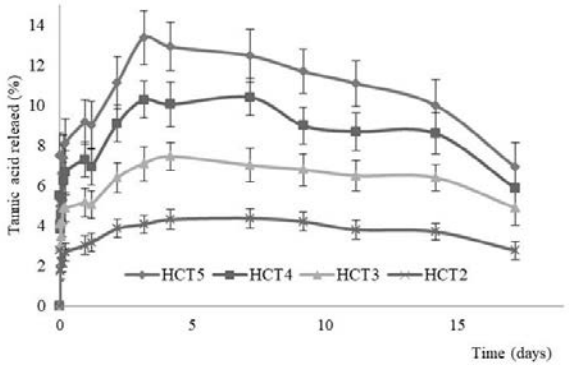


Figure 9: Tannic acid release from HC-TA films

Calcein release from hydrogels

Calcein release from control hydrogels

The main problem with hydrogel-type macromolecular carriers, in view of biomedical applications, is the rapid diffusion of low molecular weight drugs through these matrices, which is due to the mesh size of the polymer network, larger than the size of the drug molecules. It is expected that such behaviour should be also recorded when the hydrogels were obtained by TA crosslinking. We studied from the point of view of calcein release capacity the hydrogels based on HC, loaded with calcein during the hydrogel preparation process, and then immersed in the release medium (phosphate buffer, pH = 7.4). The results are shown in Figure 10. From the analysis of the variation of calcein release efficiency from the hydrogels over time, a sudden release of the model active compound can be observed within 24 hours, which is valid for both the HC-based hydrogels and for those obtained from the HC/PVA mixture. The "burst" effect is more pronounced in the case of the hydrogels containing small quantities of crosslinking agent. It should be noted that calcein was included in the hydrogel during preparation, thus in acidic solution, the existence of ammonium cations and carboxylate anions (pKa of calcein, for the 4 carboxyl groups varies between 2.1 and 5.5) makes the two components engage in ionic bonds. When placing the hydrogel in PBS (pH = 7.4), ammonium ions converge to amine groups and therefore calcein from the superficial layers and gel surface is suddenly released. As the buffer penetrates the hydrogel, a new quantity of calcein is released from the interactions with the support and begins to diffuse through the gel to the outside. The release rate decreases, consequently, from the baseline. The release process continues intensively for approx. 24 hours, especially for the hydrogels with less TA in the composition. For example, the hydrogel obtained corresponding to a polymers/crosslinker molar ratio of 50/1 releases approximately 90% of the calcein included in less than 24 hours.

The burst effect and, generally, the release efficiency reduce with the degree of hydrogel crosslinking, logically given the slow diffusion of drugs in denser networks. It is worth mentioning the good correlation of these results with those of the hydrogels swelling in water. The hydrogels based on HC/PVA behave in a similar manner. The burst effect is even more pronounced in this case, as well as the release intensity of all the hydrogels, as compared with those based solely on the HC at the same TA content. Of course, the explanation may lie in the lower crosslinking density of these hydrogels and the fact that by reducing the amount of HC (compensated by PVA) the number of the hydrogen bonding interactions of $–NH_2$ groups with calcein reduces, facilitating the easy release thereof. In this case, again, the release intensity decreased by increasing the amount of crosslinker, also correlated with the variation in the degree of swelling. It should be noted, however, that although the degree of swelling of this hydrogel is lower than that of the corresponding hydrogel based on chitosan only, it releases more calcein and faster. This phenomenon is linked to the lower hydrophilicity of the synthetic polymer, which reduces the overall affinity to water of the material, and therefore its swelling degree.

Calcein release from complex hydrogels

The objective from which we started at the beginning of this work was to test the possibility to replace the covalent crosslinker routinely used to obtain hydrogels for controlled drug delivery through body-friendly methods, trying to obtain a

system able to release the drug over a long period of time. We have chosen to achieve this goal in two ways. The first consists in replacing the covalent crosslinker with tannic acid, which is a biocompatible product. The second method to develop acceptable biological matrices able to constantly release a drug was to include low molecular weight active principle in liposomes. The rationale for choosing this composite system was slow diffusion through polymeric network of liposomes loaded with drugs.

Among all the hydrogels obtained using TA those based on HC and those based on CP (3:1 molar ratio) has been selected. Figure 11 shows the curves of variation over time of the efficiency of the calcein release from chitosan-based complex hydrogels. One can see a pronounced reduction of the "burst" effect up to its disappearance; also, calcein release is significantly delayed, the process efficiency ranging within 5-7% (depending on the HC/TA ratio) after 21 days. The four calcein release curves are placed in a logical order, the release efficiency thereby reducing with the amount of crosslinker (crosslinking density, respectively), in full compliance with the swelling kinetic curves corresponding to these hydrogels.

The liposomal integrity parameter indicates that, during the first 7 days, the liposomes reach a high proportion in the supernatant, destabilizing here and releasing the calcein (Fig. 12). Surprising is the fact that the hydrogels with higher crosslinking density presented higher values of this parameter. It would be expected that the hydrogels with looser crosslinking density would release more easily the SUV liposomes loaded, not those with high crosslinking density. To explain this effect, we advance the following hypothesis, which is based on the meaning of liposomal stability parameter – latency. Its value expresses, in fact, the calcein percentage that came from the liposomes from the total amount of calcein released.

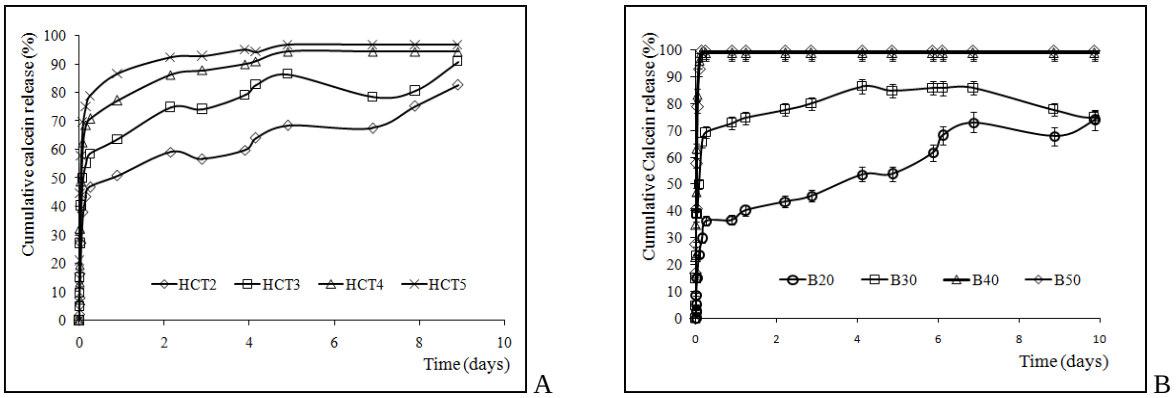


Figure 10: Calcein release efficiency from control hydrogels composed of A) HC and B) HC/PVA

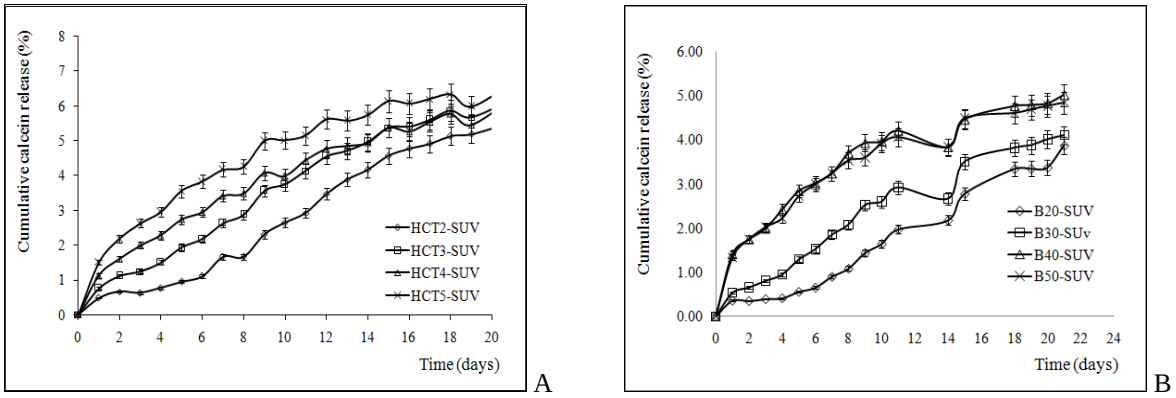
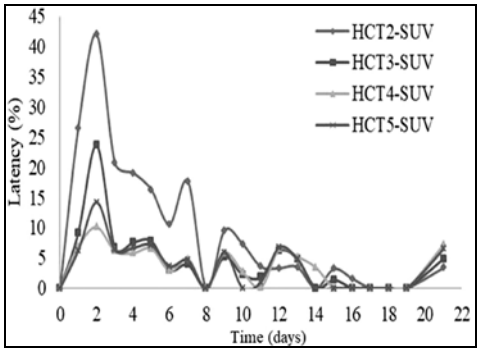
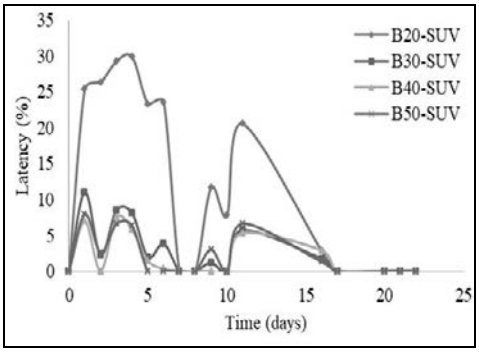


Figure 11: Calcein release efficiency from complex hydrogels composed of A) HC crosslinked with TA and B) HC/PVA crosslinked with TA



A



B

Figure 12: Latency parameter for the complex hydrogels composed of A) HC crosslinked with TA and B) HC/PVA crosslinked with TA

As noted in the release kinetics study of calcein included directly in hydrogels, the smallest amount of calcein (and with the lowest speed) is released from the highest crosslinked networks (using the highest amount of TA). This conclusion is confirmed by the results obtained for complex systems (Fig. 12). In the total amount of calcein released from the network, we find both calcein in free form and encapsulated in liposomes, which reach intact outside the matrix. Since the efficiency of calcein release included directly in the hydrogels is reduced in the case of the hydrogels with high crosslinking density, it is logical to assume that in complex systems the lowest amount of free calcein is released. Under these circumstances, even if the number of liposomes that diffuse through the matrix, reaching intact outside is reduced, the amount of calcein that is carried (which is released from them by treatment with Triton X-100 surfactant) can be high (up to approx. 40% of the total, Fig. 12A). As the network density decreases, more and more calcein is released in free form, so the share of calcein released from the liposomes (even if the number of liposomes that arrive intact in the supernatant increases) is reduced (latency decreases).

Another important observation is that, regardless of the hydrogels composition, the "burst" effect disappears and the release is much more extended, compared to the control hydrogels, where calcein was included in the free state only. The gradual decrease in latency time values could be explained as follows. In the early days of the process, particularly, the liposomes immobilized in the superficial layers diffuse outward the matrix. At longer durations of the process, in particular, more than 7 days, the liposomes of the inner layers reach the outside of the matrix, which on the one hand are increasingly reduced in number, and on the other hand are facing the increasing resistance of the thick layers of the network, destabilizing into the hydrogel network releasing calcein. This explains why, although latency is decreased, the amount of calcein released (expressed in terms of the release efficiency) is continuously growing. The release from the film and maintaining the integrity of the liposomes become important if you intend to use these hydrogels with drug loaded liposomes as drug carriers in targeted passive therapy. Otherwise, the liposomes fulfill only the role of an additional drug diffusion barrier, delaying the drug release from the hydrogels (*e.g.* drug loaded dressings used in burns treatment).

A similar behaviour is exhibited by the hydrogels with the CP mixture. In this case, the release efficiency is lower, maximum values being achieved in the hydrogels with low crosslinking density (maximum 5%) (Fig. 12B). However, the arrangement order of the kinetic curves of this set of systems, in the sense that the release efficiency decreases by increasing the amount of release of crosslinker, is maintained.

The ability of these hydrogels to swell in water is lower than that of chitosan-only hydrogels and therefore there has been a corresponding lower amount of calcein than for the HC-TA hydrogels. The calcein release from the control films was achieved according to the film composition. Therefore, the results presented clearly indicate that the crosslinking density plays an important role in the diffusion throughout the matrix both of the calcein and of the calcein loaded liposomes. Therefore, in order to extend the release of drugs from polymeric matrices, the use of liposomes as an additional barrier is an effective method. In the case of our systems, this process led to linear drug release and therefore in accordance with zero-order kinetics.[48] Just as in the case of plain

chitosan-based films, higher values of the liposomal stability parameter were recorded in the case of the films with high crosslinking density, the explanation being discussed previously. Also, with increasing duration of the process, the latency values shall be reduced, at times being even null. It is possible, particularly over 14 days, that intact liposomes would not reach outside the polymeric matrix. They destabilize inside it, further determining the diffusion of calcein only in free form.

CONCLUSION

The research reported in this paper had as its main objective the investigation of alternative ways for modeling the release kinetics of hydrosoluble drugs from matrices with hydrogel character. Although our investigation was focused on films, the method applied can be also extended to particles, sponges, implants, inserts *etc*. The goal was to eliminate or reduce the "burst" effect recorded in the release process of simple systems drug-polymeric network, respectively, a better control of the process over a longer time period. The strategy used to achieve this goal was the development of complex systems consisting of a hydrogel matrix in which liposomes containing calcein have been included, essentially, to create an additional barrier to the release process of the hydrosoluble compound. As a secondary objective, the paper has proposed the development of new interpenetrated/interconnected hydrogel networks totally non-toxic, bio- and cytocompatible, using components of natural origin as raw materials. New hydrogels based on chitosan and chitosan/poly(vinyl alcohol) crosslinked with tannic acid (polyphenol with remarkable biological properties) were synthesized and characterized physico-chemically and from the point of view of their behaviour in aqueous media. The crosslinking is carried out by numerous hydrogen bonds engaged between the phenolic compound and amino, respectively hydroxyl groups of the polymers.

The hydrogels prepared in the form of films were characterized by FT-IR spectroscopy, showing the presence of all the components of the starting composition; the presence of hydrogen bonds could not be highlighted being masked by many hydroxyl groups from the partners that interact. The hydrogels have compact morphology, as evidenced by scanning electron microscopy; the surface presents a rough appearance (less obvious in those with poly(vinyl alcohol) in the composition), which is attenuated by reducing the crosslinker amount. Differential thermal analysis performed shows superior stability of the chitosan-based films to polysaccharide alone, but lower than that of the synthetic polymer, poly(vinyl alcohol). The hydrogels presented different water retention capacity depending on the polymeric matrix composition – the amount of tannic acid used and the ratio of the two polymers participating in crosslinking. The maximum degree of swelling is reduced by increasing the amount of crosslinker, respectively, the amount of poly(vinyl alcohol) from the initial mixture. The crosslinking time does not significantly affect the swelling process. The hydrogels tend to release tannic acid in the aqueous media, the quantity of the crosslinker released being in close accordance with the ratio in the initial mixture subjected to crosslinking. Weakly crosslinked networks are likely to eliminate, along with tannic acid, some low molecular weight polymer fractions. Partial release of tannic acid from the hydrogels may constitute a positive factor, given the biological properties of polyphenols. Possible hydrolysis of tannic acid to gallic acid does not necessarily constitute a shortcoming of these products, knowing that the latter also has remarkable biological properties.[27] Depending on intended use of the hydrogels (in our opinion, especially for treatment of dermal diseases or transdermal administration of incorporated drugs), if tannic acid may enhance or supplement their action, then the amount that is released from the hydrogel can be adjusted from the stage of preparation. One last important observation for the application of these systems is the fact that although tannic acid can be released at a rate of 4-14%, the integrity of the films is not affected. This effect is of importance because it leads to the idea that such films can be used as dressings loaded with active compounds for long periods of time.

Calcein release from control hydrogels takes place in a very short time – of 24 hours in those with low crosslinking density – but in direct correlation with the amount of crosslinker. This finding is valid both for the systems based on the polysaccharide and for those based on the chitosan/poly(vinyl alcohol) mixture. The release of the model compound from the hydrogels crosslinked with tannic acid-liposome-calcein shows, in the case of denser networks, "zero order" kinetics during the testing period (21 days).

Reducing the amount of crosslinker leads to looser networks and to the manifestation of a more pronounced "burst" effect.

In the first 7 days of the calcein release process, the liposomes diffuse outward the polymeric matrix. These liposomes loaded with drugs represent in some cases 30-40% of the total released. This observation is of therapeutic value, being an advantage of these systems in terms of the possibility of passive targeting of liposomes loaded with drugs. The use of tannic acid as a crosslinker is a promising and prospective method for obtaining hydrogels totally non-toxic that can be potentially used as drug carriers. Even if the immersion in a biological fluid leads to the release of a small amount of tannic acid and chitosan, the integrity of the films is maintained, and the compounds released can contribute to an increase of the biological activity of the whole complex. Calcein encapsulation in liposomes and their subsequent inclusion into a polymeric matrix deeply modifies the release kinetics, the "burst" effect is drastically reduced or even disappears and the process continues for a long time with a constant speed.

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