**Journal of Macromolecular Science, Part A**
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597274

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Online publication date: 26 April 2010

**To cite this Article** Flocea, Petruta , Verestiuc, Liliana , Popa, Marcel , Sunel, Valeriu and Lungu, Adriana(2010) 'Crosslinked Networks Based on Polysaccharides and Collagen for Pilocarpine Sustained Release', Journal of Macromolecular Science, Part A, 47: 6, 616 — 625
**To link to this Article: DOI:** 10.1080/10601321003742196
**URL:** http://dx.doi.org/10.1080/10601321003742196

*Journal of Macromolecular Science, Part A: Pure and Applied Chemistry* (2010) **47**, 616–625

ISSN: 1060-1325 print / 1520-5738 online
DOI: 10.1080/10601321003742196

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# Crosslinked Networks Based on Polysaccharides and Collagen for Pilocarpine Sustained Release

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Received June 2009, Accepted December 2009



The paper presents the experimental studies regarding synthesis and characterization of hydrogels based on gellan (Gel)/chitosan (CS) and collagen (Col), obtained by crosslinking with glutaraldehyde (GLA). The influence of the polysaccharide content and GLA ratio on the final composition and swelling characteristics was evaluated. Hydrogels swelling analysis, in distilled water and phosphate buffer (PBS, pH 7.2) has shown higher swelling degrees at increased concentration of polysaccharide into hydrogels. *In vitro* release of pilocarpine has demonstrated the possibility to use gellan-collagen and chitosan-collagen hydrogels as ophthalmic drug delivery matrix.

**Keywords:** Gellan, chitosan, collagen, hydrogels, ophthalmic drug delivery

## 1 Introduction

Pilocarpine is a common drug used in topical treatment for intraocular pressure decreasing in glaucoma disease (1). However, this drug has a very low availability, poor corneal penetration and extensive precorneal loss is produced. Therefore, pilocarpine has to be frequently administered in order to achieve effective therapeutic results; side effects are often yielded by drug excess and poorly patient compliance is associated with drug administration (2). Sustained - release polymeric systems have been tested as pilocarpine carrier, from swellable polymeric matrices, with diffusional release of the actives to erodible carriers, or matrices with combinatorial mechanisms of drug release (3, 4).

A variety of polymeric carriers have been investigated to modify the drug response including poly(vinyl alcohol) (PVA) (5), poly(N-vinyl pyrrolidone) and poly(2-hydroxyethyl methacrylate) films (6), poly(acrylic acid) (7) and hyaluronic acid and gellan viscous solutions (8), chitosan particles (9), bioerodible systems such as gelatin and collagen (10, 11). However, only few carriers had really been proved for wide clinical applications. In the aim to obtain devices with synergetic properties, some polymeric materials have been proposed such as hydrogels, interpolymeric complexes or polymeric blends (12, 13).

Hydrogels are hydrophilic polymeric networks able to swell in water and aqueous solutions and form elastic gels (14). The hydrophilicity is due to functional groups (-OH, -COOH, $-CONH_2$ or $-SO_3H$) situated on side positions of the macromolecules. The water content in hydrogels is responsible for mechanical, diffusional and absorptive properties and makes these networks able to mimic live tissues (15–17). Some physical or chemical crosslinking methods have been studied for obtaining polymeric three-dimensional structures as a function of a chemical structure of the polymer (18).

Due to their biocompatibility and biodegradability, collagenic hydrogels have been successfully tested in soft tissue repair or in ophthalmic applications (19). Commercial products with promising results, for example Ocusert inserts, have been assessed for ophthalmic drug delivery (20). Also, polysaccharides, like chitosan and gellan have been intensely investigated as ophthalmic drug carriers (21–24).

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In the aim to obtain materials with improved properties the paper presents the experimental results about synthesis of chemical crosslinked hydrogels based on gellan and collagen, respectively chitosan and collagen, for pilocarpine controlled release, as well as hydrogels characterization (composition and swelling properties) and *in vitro* drug release study.

## 2 Experimental

### 2.1 General Remarks

Soluble collagen (Col) has been obtained by extraction from bovine hides with a solution of acetic acid 0.5 M ($[\eta] = 4.256$ dl/g and isoelectric pH = 4.82). Gellan (Gel, $M_w = 1.8 \times 10^5$g/mol; $M_n = 1.0*10^5$ g/mol), was supplied by Kelko Biopolymers. Chitosan (CS, $M_W$ =100.000 g/mol; 85.2% deacetylation degree) was obtained from Sigma. Glutaraldehyde - GLA (Merck) as crosslinking agent and Pilocarpine chlorhidrate (Sigma), as a miotic drug, have been used. Final materials composition was calculated from elemental analysis data. The kinetic of *in vitro* drug release was performed with an UV Cary 50 spectrophotometer – Varian.

### 2.2 Hydrogels Synthesis

A 1% (w/vol) gellan solution was prepared by polysaccharide dissolution in distilled water; then, a 1M NaOH solution was added until pH 8. Chitosan solution was prepared by 1% polysaccharide dissolution in 1% (vol/vol) acetic acid in distilled water. Two collagenic solutions were prepared as follow: a 1% (w/vol) collagen at pH 8 and 1% collagen in 1% (vol/vol) acetic acid in distilled water. Synthesis of hydrogels followed a factorial experimental matrix (Table 1) where the independent variables were: the gellan or chitosan concentration in polysaccharide-collagen solution and the ratio of glutaraldehyde. Collagen and polysaccharide solutions were mixed under strong stirring to form a homogenous system. GLA was added to the mixture and then stirred for another 20 min. After that the formed mixture was poured into glass molds and kept at 20°C for 72 h. The synthesized hydrogels were repeatedly washed by soaking in distilled water and dried at 40°C for 12 h.

**Table 1.** Experimental matrix for polysaccharide-collagen matrix synthesis

| | $x_1$ | | $x_2$ | |
|---|---|---|---|---|
| *Hydrogel* | *encoded* | *Gel or CS (%)* | *encoded* | *GLA (%)* |
| PZ-Col 1 | –1 | 25 | –1 | 2 |
| PZ-Col 2 | 1 | 75 | –1 | 2 |
| PZ-Col 3 | –1 | 25 | 1 | 4 |
| PZ-Col 4 | 1 | 75 | 1 | 4 |
| PZ-Col 5 | –1.414 | 14.65 | 0 | 3 |
| PZ-Col 6 | 1.414 | 85.35 | 0 | 3 |
| PZ-Col 7 | 0 | 50 | –1.414 | 1.414 |
| PZ-Col 8 | 0 | 50 | 1.414 | 4.414 |
| PZ-Col 9 | 0 | 50 | 0 | 3 |
| PZ-Col 10 | 0 | 50 | 0 | 3 |
| PZ-Col 11 | 0 | 50 | 0 | 3 |
| PZ-Col 12 | 0 | 50 | 0 | 3 |
| PZ-Col 13 | 0 | 50 | 0 | 3 |

### 2.3 Swelling Properties Measurements

For swelling studies, phosphate buffered solution (PBS, pH 7) was prepared by adding appropriate quantities of disodium orthophosphate (19.1 g/L), sodium hydroxyl orthophosphate (2.1) and sodium chloride (4.2 g/L). A 0.1N HCl solution was used for pH correction. The swelling parameters were measured gravimetrically. The equilibrium swelling degree, $SD_{eq}$, %, was calculated by using relation:

$$SD_{eq}\frac{m_{eq} - m_0}{m_0} \times 100$$

Where: $m_0$ = initial sample weight (g); $m_{eq}$ = sample weight at swelling equilibrium (g); Swelling characteristics in distilled water were also analyzed. Presented data are the media of three experiments.

### 2.4 Pilocarpine Chlorohydrate Loading into Hydrogels

A quantity of 0.5 g of dry hydrogel was swelled to equilibrium in distilled water and then kept in 2% (w/vol) Pilocarpine chlorohydrate aqueous solution. Hydrogels were maintained in drug solution for 72 h, until equilibrium drug diffusion was obtained. Afterwards, loaded hydrogels were dried at 30°C, for 48 h, under vacuum. The quantity of loaded drug has been calculated from gravimetrical data.

### 2.5 Drug Release Experiments

The *in vitro* release profile of pilocarpine chlorohydrate from hydrogels was obtained spectrophotometrically: a drug-loaded hydrogel was placed into a 50 mL phosphate buffer solution and pilocarpine release profile vs. time was registered by absorption solution monitoring at 222 nm. Calibration was performed using a series of phosphate buffer solutions containing well-known amounts of pilocarpine chlorohydrate (concentrations range 0.001–0.02%, w/w). Blank experiments, using polymer-only films, confirmed that the hydrogels did not contribute to the 222 nm absorption.

## 3 Results and Discussions

Polymeric three-dimensional networks have been obtained by crosslinking of gellan/chitosan and collagen with glutaraldehide. In these swellable matrices water-soluble drugs

such as pilocarpine cholohydrate are able to diffuse and interact by ionic and hydrogen bonds with hydrophilic and ionizable groups from polymers. By adding glutaraldehyde to the collagen-gellan mixture, a crosslinking reaction is produced, which can yield three types of networks (Figure 1):

**a)** **Compound I** is yielded due to involving one aldehyde group from the bifunctional crosslinking agent with the amine function of the collagen, in - CH=N- bond, and of the other in acetal bond with two –OH groups on the gellan;
**b)** **Compound II** is yielded due to the reaction of the two aldehyde groups from the crosslinking agent with two protein $–NH_2$ groups, with the formation of two –CH=N- bonds;
**c)** **Compound III** is yielded due to the reaction of the two aldehyde groups in the crosslinking agent with –OH protein groups and the acetal bonding forming.

Because of increased nucleophilicity of the $–NH_2^{25}$ in relation to the –OH group, in the established synthesis conditions (pH=8), the probability of the hydroxyl function in the lateral groups of the collagen macromolecular chain to participate in the crosslinking reaction is much lower. Also, compound III is yielded only in very low amounts, as the steric hindrances and the need for multiple hydroxyl functions participation in the crosslinking relation, which significantly reduces its possible formation.

The collagen and chitosan crosslinking reaction, in the presence of glutaraldehyde, can yield the products from Figure 2.

In the reaction of conditions for synthesis of hydrogels based on collagen and chitosan, pH=3.5 and 20°C, the compounds which can be obtained are as follow:

**a)** **Compound I** is yielded due to the participation of one of the aldehyde groups from the bifunctional crosslinking agent in –CH=N- bonds with the collagen amine function and another one in –CH=N- bonding with the chitosan amine function. The literature data also indicate this type of bonding in the acid range, where the polymer amine function is in ionized state ($-NH_3^+$), the crosslinking reaction mechanism being considered ionic (26, 27).
**b)** **Compound II** is yielded due to the reaction of the two aldehyde groups in the crosslinking agent with two $–NH_2$ groups on the proteic material, with the formation of two –CH=N- bonds.
**c)** **Compound III** is yielded due to the reaction of the two aldehyde groups in the crosslinking agent with chitosan $–NH_2$ groups.

The acid medium and the rigidifying of the chitosan macromolecular chain do not allow the simultaneous participation of the polysaccharide –OH groups in the formation of crosslinking bridges (28).

The FTIR spectra of pure biopolymers, collagen – gellan hydrogels and collagen – chitosan hydrogels are illustrated in Figure 3.

The absorption bands characteristic of collagen occur at wave lengths as follows:

–OH valence vibrations – 3284.7 $cm^{-1}$; –C-H of $–CH_2$ valence vibrations – 2995.4 $cm^{-1}$; Amide I – 1633.7 $cm^{-1}$; 1450.4 $cm^{-1}$; Amide III – 1238.4 $cm^{-1}$; Amide IV – 622.1 $cm^{-1}$.

Gellan exhibits absorption bands at: C-O-C valence vibrations at 802 $cm^{-1}$, -OH secondary aliphatic valence vibrations at 1122 $cm^{-1}$, from $-CH_2-$ at 1463 $cm^{-1}$, from R'-$CH_2$-R" at 2850 – 2920 $cm^{-1}$ and $(R)_2CH$-OH- valence vibrations at 3429 $cm^{-1}$.

For chitosan, the characteristic absorptions are observed at: 3354.2 $cm^{-1}$ (–OH valence vibrations), 2877.7 $cm^{-1}$(–C-H of $–CH_2$ valence vibrations), 1646.2 $cm^{-1}$(the amide I band), 1377.2 $cm^{-1}$(–$CH_2$vibrationabsorption), 1022.27 $cm^{-1}$(–CO absorption).

Hydrogels based on collagen and gellan exhibit absorption characteristics of the two polymers, the pronounced absorption bands indicating polymer crosslinking. The flattened –OH bands show hydrogen bonding interactions among the natural polymer macromolecular chains.

In the case of collagen-chitosan hydrogels, the FTIR spectroscopy data show the occurrence of certain interactions between the two polymers. Thus, the -OH absorption peaks are slightly flattened and those corresponding to the collagen amide III bands exhibit the same tendency. Increasing the chitosan composition content causes an absorption identification at 1079.9 $cm^{-1}$which corresponds to a continuous increase of the –CO function.

The final composition of the collagen–gellan hydrogel, calculated from elemental analysis data, is strongly influenced by the composition ratio of the biopolymers in the initial mixture and the glutaraldehyde concentration, as can be observed in Figure 4.

As expected, the increased gellan concentration in the initial mixture causes increased polysaccharide content in the synthesized hydrogels. A maximum value of 68.75% gellan in composition is obtained for 85% gellan in the initial protein – polysaccharide mixture and high concentration of crosslinking agent. Smaller quantities of gellan determined in the formed network structure is a consequence of its lower reactivity, by comparison with protein reactivity, amine groups from collagen having an increased nucleophilic character and react easier then hydroxyl groups form polysaccharide. The quantity of gellan is slowly increasing with the amount of crosslinker but is still inferior to those in the initial polymeric mixture.

The crosslinking degree increasing leads to more stable gels with compact structure which does not allow the diffusion of possible non-reacted polysaccharide molecules outside of the three-dimensional macromolecular structure, during the purification process. Low crosslinking density leads to "weak" hydrogels, with certain gellan amounts not

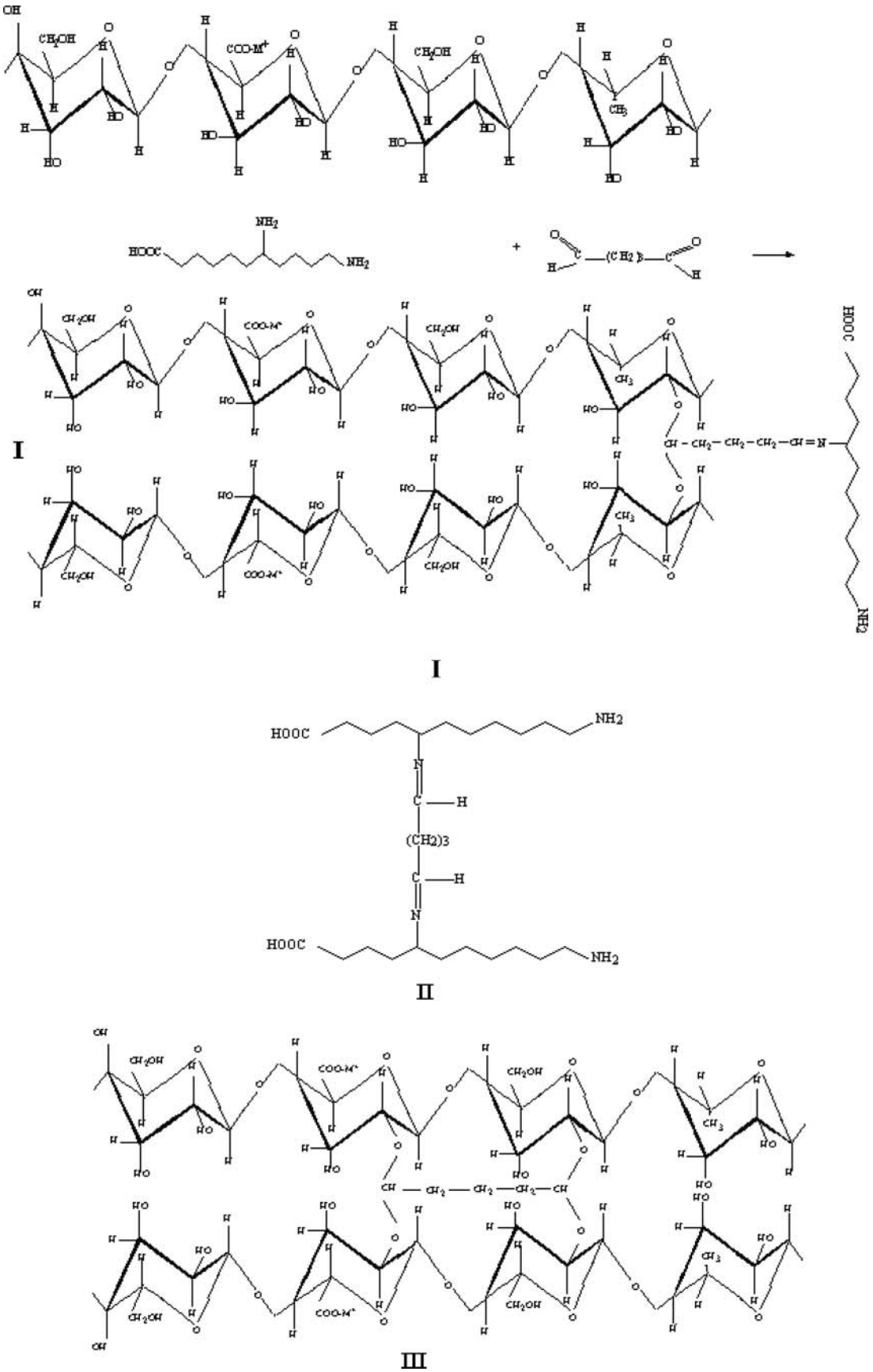

**Fig. 1.** Collagen and gellan crosslinking reactions in the presence of GLA.

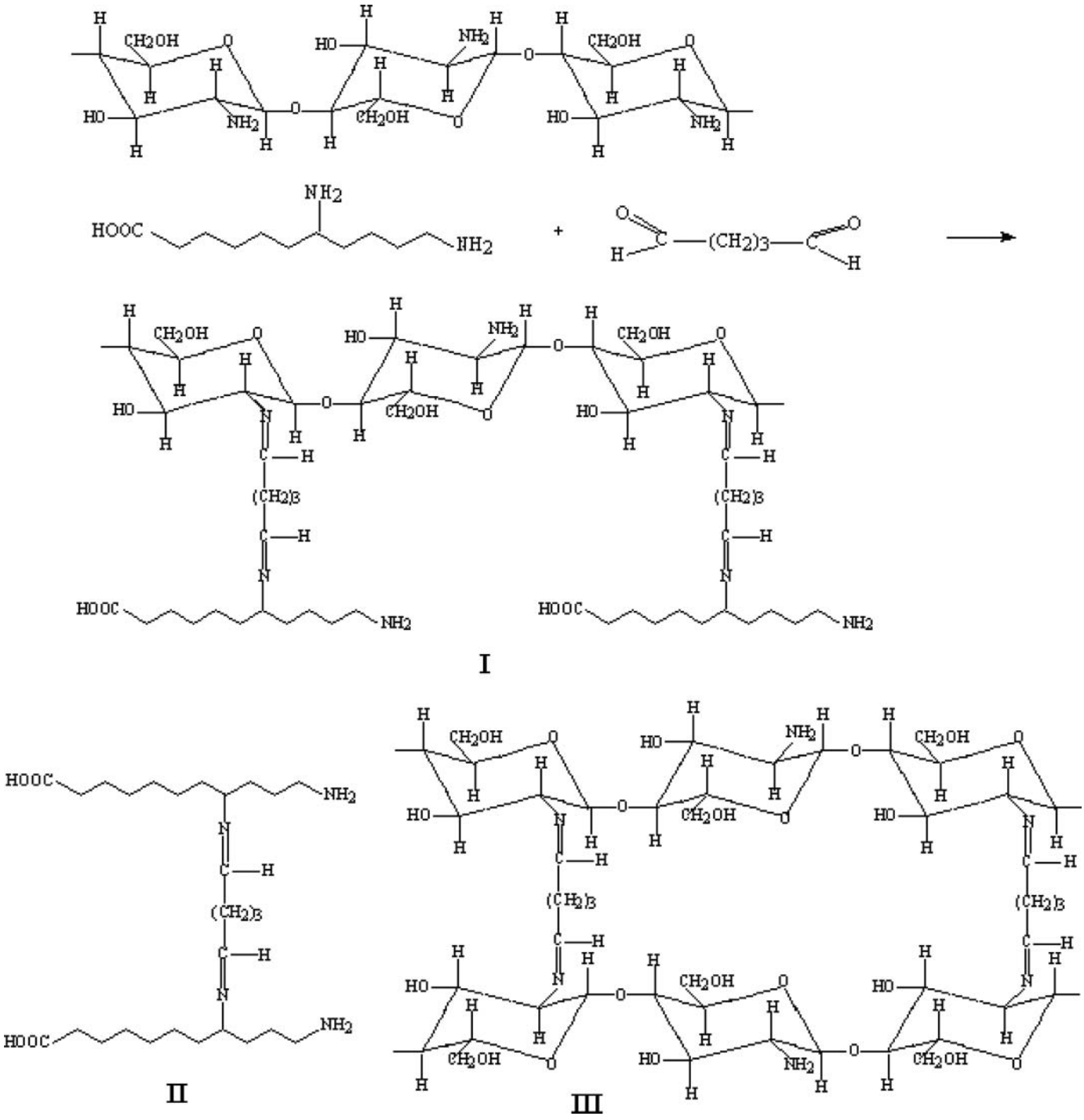

**Fig. 2.** Collagen and chitosan crosslinking reactions in the presence of GLA.

participating in the crossslinking reaction; only half of the gellan amount introduced in the reaction is identified in the final composition of the hydrogel.

Scanning electron microscopy (SEM) analysis on hydrogels based on collagen and gellan, and based on collagen and chitosan, respectively, are presented in Figure 5. The scanning electron microscopy images were sampled from the surface of the hydrogels. It is observed that gellan hydrogels have a more uniform surface and lower roughness, indicative of increased polymer compatibility; hydrogen bonding interactions contribute to hydrogel uniformizing and to the formation of a more compact three-dimensional structure.

Swelling tests were carried out in order to reveal the capacity of interaction with mediums imitating biological fluids. Thus, a buffer solution with pH values which are

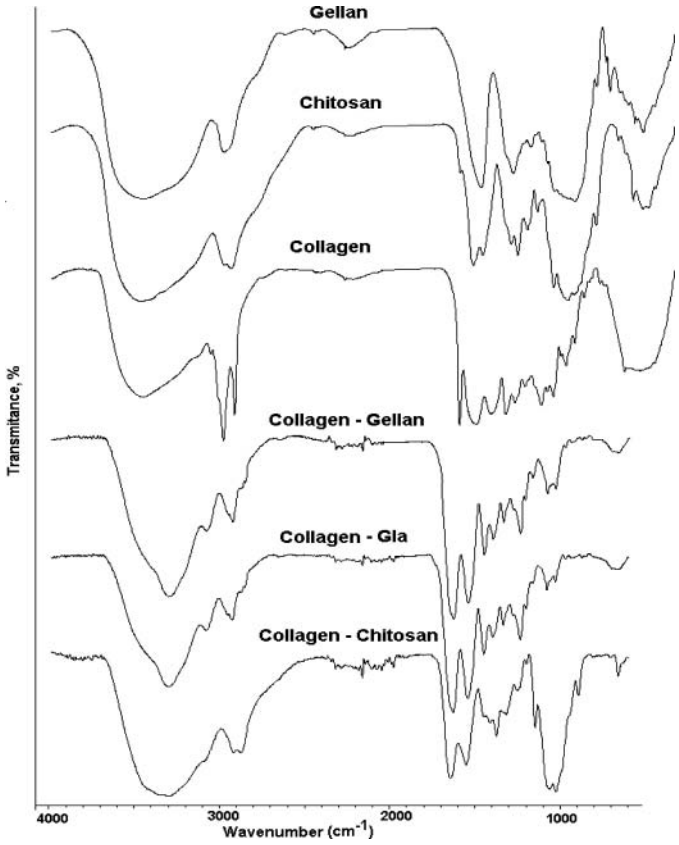


**Fig. 3.** FT-IR spectra of collagen-gellan and collagen-chitosan crosslinked hydrogels.

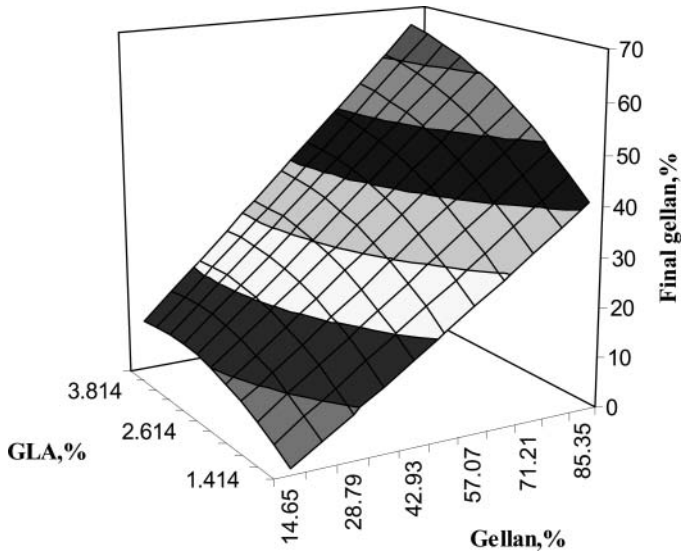


**Fig. 4.** Final composition of hydrogels based on collagen and gellan.



of interest to the ophthalmologic applications of the materials was selected (pH=7). Equilibrium swelling characteristics for the collagen-gellan-based hydrogels, in water and phosphate buffer solution are illustrated by the data in Figures 6 and 7.

The data concerning water hydrogel swelling indicates a decreasing of the equilibrium swelling degree with the increase of the crosslinking agent concentration. The phenomenon is due to the increased number of crosslinking bridges among the macromolecular chains, which diminish hydrogel water absorption; under these conditions, water diffusion into the three-dimensional macromolecular matrix is difficult. The effect is intensified as the gellan concentration in the polymer hydrogel increases. Although both polymers are hydrophilic, the polysaccharide creates higher wetability for the hydrogel, as the increased gellan proportion favors supplementary water absorption; the swelling degree reaches values of 2400% at low crosslinking agent concentration and 85% gellan in the initial mixture.

At pH=7 (Fig. 7), a decreased hydrogel swelling degree is observed, the maximum values reaching half of the value obtained for the same conditions of synthesis for three-dimensional intramolecular matrix.

Gellan is a polysaccharide which exhibits ionizable carboxylic functions (pKa ≈3.05) (29); at pH values higher than 3.05, the carboxylic groups are ionized (negatively charged). In the pH range chosen for the evaluation of swelling characteristics, collagen is also situated above the isoelectric pH value; accordingly, weak electrostatic repulsions appear between the two polymers. The presence of the electrolytes in the pH = 7 buffer solution causes a "salting out" effect on the macromolecular matrix which allows limited water diffusion in the hydrogel structure and the swelling degree decreases.

The equilibrium swelling characteristics for hydrogels based on collagen and chitosan, in pure water and buffered solution (pH = 7) are illustrated by the data from Figures 8 and 9.

Chitosan, due to its ionic nature, is very sensitive to medium pH variations. At acid pH values, lower than pKa (pKa≈6,5), the chitosan electric charge changes and the amine groups change to an ionized state (amonium ions), which favors ionic bonding between the chitosan and the collagen chains (30). At pH > 4,85 (the isoelectric pH value) the collagen amine functional groups quit the Zwiterionic state and change to an un-ionized state, and can participate in hydrogen bonds forming with hydroxyl groups found in the chitosan macromolecule. Consequently, hydrogel stabilization is achieved by ionic as well as by hydrogen bonds.

At pH = 7, hydrogels yielded at low reticulation agent concentrations exhibit equilibrium expansion degrees higher than those exhibited at pH = 5.5 (Fig. 9). The phenomenon is considered a consequence of the chitosan amine functions changing to an un-ionized state, as well as of the collagen –COOH groups changing to -$COO^-$. The interactions in this case are only achieved by hydrogen bonding; no macromolecular associates are formed that can diminish the water-enclosing capacity.

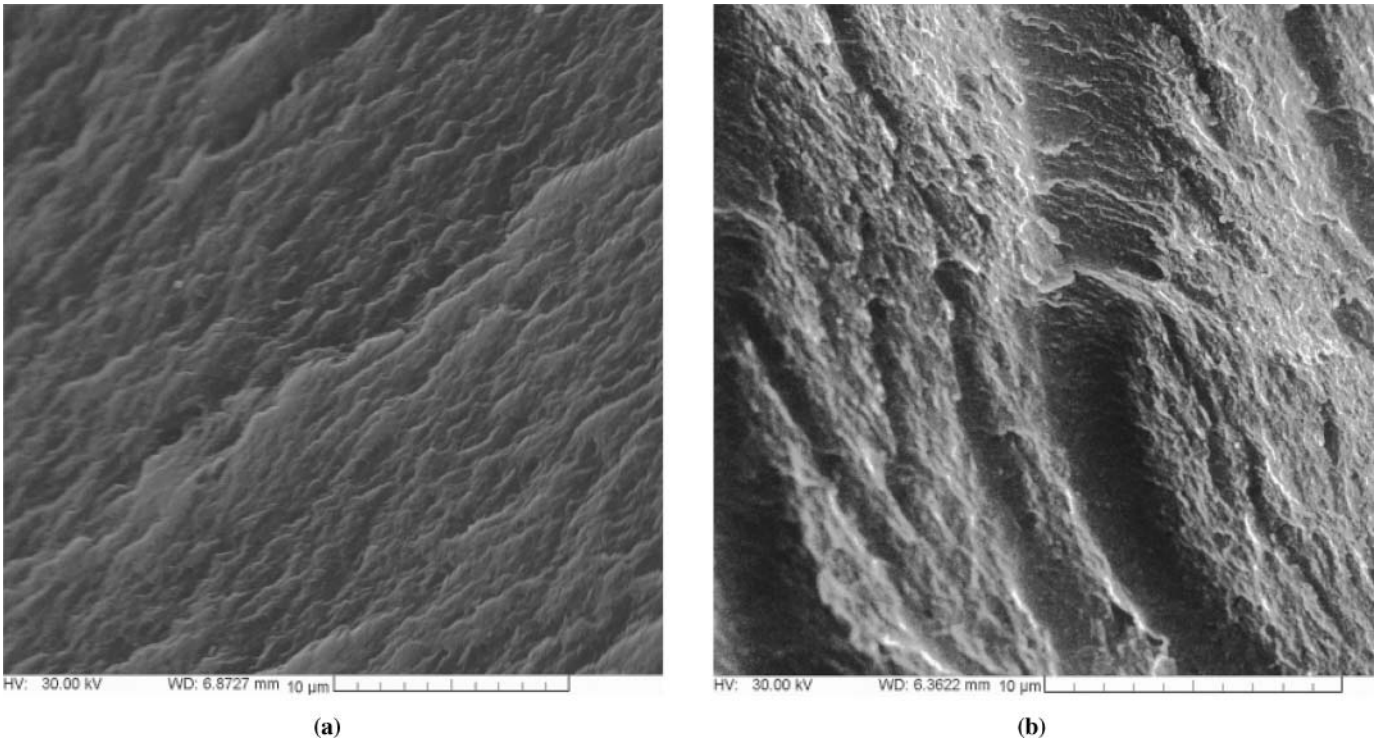


**Fig. 5.** SEM image for Collagen–Gellan hydrogel (a) (50% Gellan, 3% GLA) and Collagen–Chitosan hydrogel (b) (50% Chitosan, 3% GLA).



Increasing hydrogel chitosan proportion causes the hydrogel swelling capacity to decrease, followed by the occurrence of intramolecular hydrogen bonding among the polysaccharide molecules; at this pH value, the chitosan exhibits insolubilization (31). Also, by diminishing the collagen concentration into network the number of ionizable carboxylic groups decreases; the rejection forces between macromolecules are weaker, the network is more compact and the swelling degree is diminished too.

The crosslinker concentration used during the synthesis has significant importance in the case of buffer solution swelling, as an increased crosslinking degree causes a pronounced decrease of the water absorption capacity. The

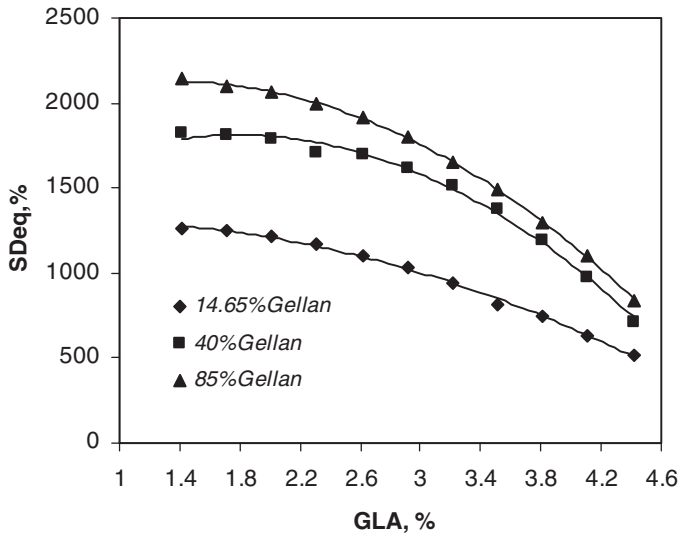


**Fig. 6.** Swelling degree for collagen-gellan hydrogels, in distilled water, as a function of GLA concentration.

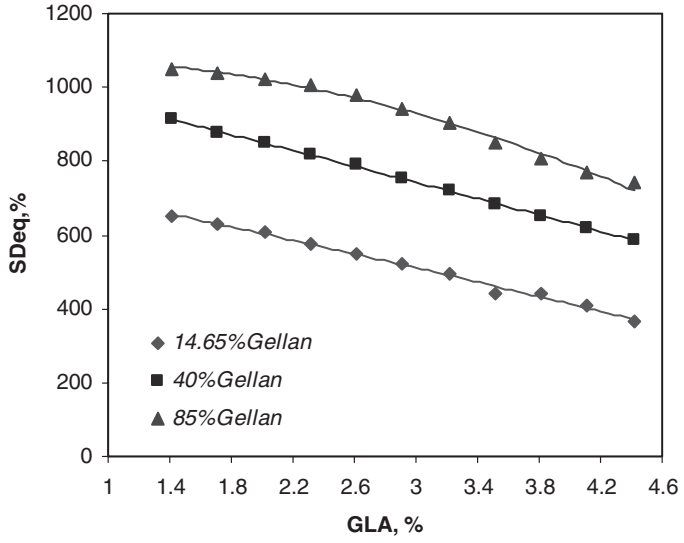


**Fig. 7.** Swelling degree in buffered solution (pH=7) for collagen-gellan hydrogels as a function of GLA concentration.

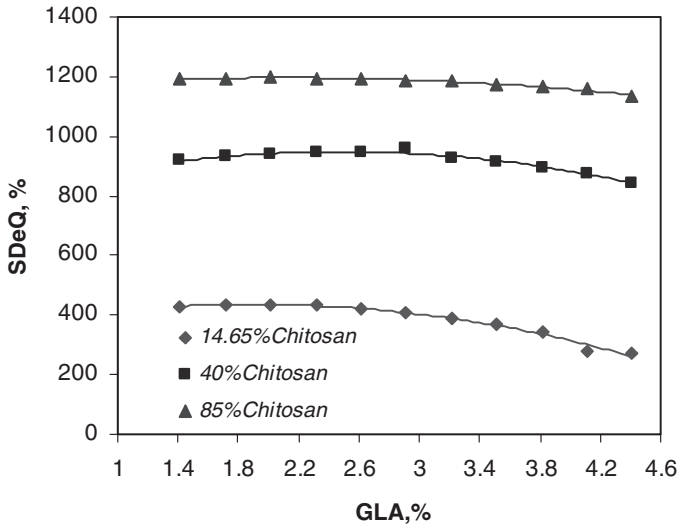


**Fig. 8.** Swelling degree for collagen-chitosan hydrogels in pure water, as a function of GLA concentration.

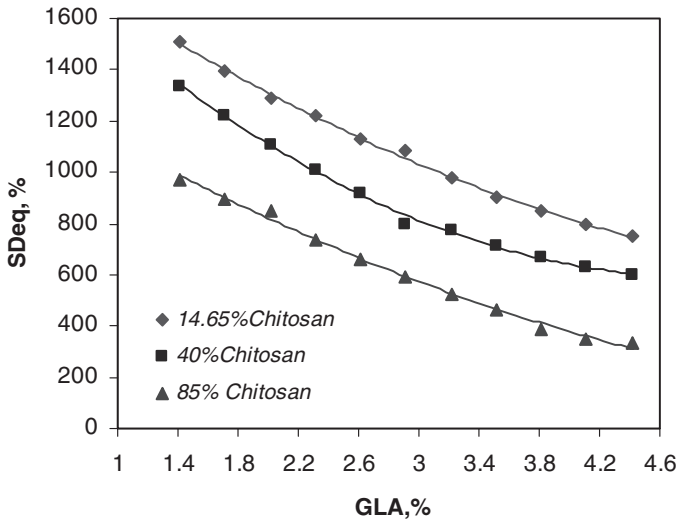


**Fig. 9.** Swelling degree for collagen-chitosan hydrogels in buffered solution (pH=7), as a function of GLA concentration.

polymer interaction mode inside the hydrogel as function of pH is illustrated in Table 2.

The Pilocarpine hydrochloride load kinetics in the synthesized hydrogels is illustrated by the data from Figures 10 and 11.

Figure 10 shows that, in collagen- gellan-based hydrogels obtained by crosslinking with glutharaldehyde, the Pilocarpine chlorohydrate loading process reaches equilibrium after approximately 24 h, for all biopolymer ratios.

**Table 2.** Polymers interactions as function of pH

| Polymer | Electrical charges and interactions | | | |
|---|---|---|---|---|
| | pKa/pH iz: ..... 3.05 ..... 4.85 ..... 6.5 ..... basic | | | |
| Gellan | n n n n n n n | - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - | | |
| Collagen | + + + + + + + + + + + | - - - - - - - - - - - - - - - - - - - - - - - - - | | |
| Chitosan | + + + + + + + + + + + + + + + + + + + + + | n n n n n n n n n n n | | |
| Collagen - Chitosan | H - - - O; H - - - N (+) : (+) | H - - - O; H - - - N (+ : -) | H - - - O: H - - - N (-) : (-) | |
| Collagen - Gellan | H - - - O; H - - - N (+) : (+) | H - - - O H - - - N (+ : -) | H - - - O: H - - - N (-) : (-) | |

H - - O; H - - N - hydrogen bonds; ( + : - ) - ionic interactions; ( + ) : ( + ) , ( - ) : ( - )- electrostatic repulsions

| Polimeric material | pH, electrical charges and interactions<br>acid medium.... 3.05.....4.85.....6.5.....basic medium | | | |
|---|---|---|---|---|
| Collagen | + + + + + + + + + + + + + + + | - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - | | |
| Gellan | n n n n n n | - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - | | |
| Chitosan | + + + + + + + + + + + + + + + + + + + + + + + + | n n n n n n n n n | | |
| Collagen - Gellan | hydrogen bonds weak electrostatic repulsions (+) | hydrogen and ionic bonds | hydrogen bonds weak electrostatic repulsions (-) | |
| Collagen - Chitosan | hydrogen bonds electrostatic repulsions | | hydrogen bonds ionic interactions | hydrogen bonds weak electrostatic repulsions |

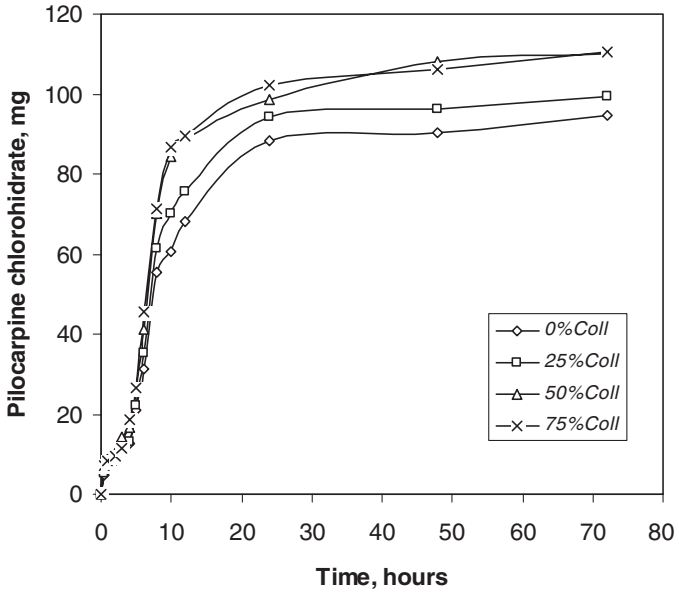


**Fig. 10.** Pilocarpine chlorohydrate load kinetics into collagen-gellan-based hydrogels crosslinked with glutaraldehyde.

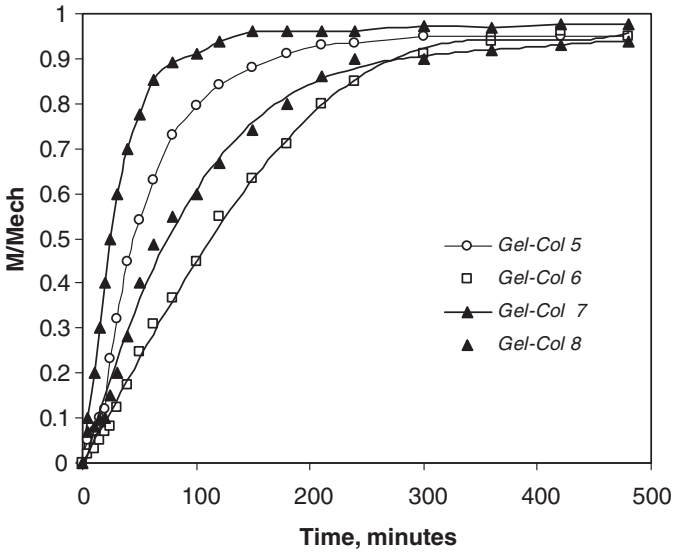


**Fig. 12.** Kinetics of Pilocarpine chlorohydrate delivery from collagen-gellan hydrogels crosslinked with glutharaldehyde.



The loading process has two stages, which can be observed due to the occurrence of two inflection points on the kinetic curve. During the first stage the Pilocarpine chlorohydrate (a highly hydrosoluble drug) diffuses into the collagen-gellan matrix, which process is achieved due to the rapid drug diffusion into the three-dimensional matrix; this stage lasts for two or three–four hours.

Subsequently, during the second stage (the second section on the kinetic curve), simultaneously with new drug quantities diffusion into polymeric matrix the ionic polymer–drug interactions occur, which fixate the Pilocarpine chlorohydrate on the free polymer chains. The drug–polymer matrix interactions are more intense for systems containing high collagen amounts, due to the protein aminoacid composition, which favors further collagen-drug bonding.

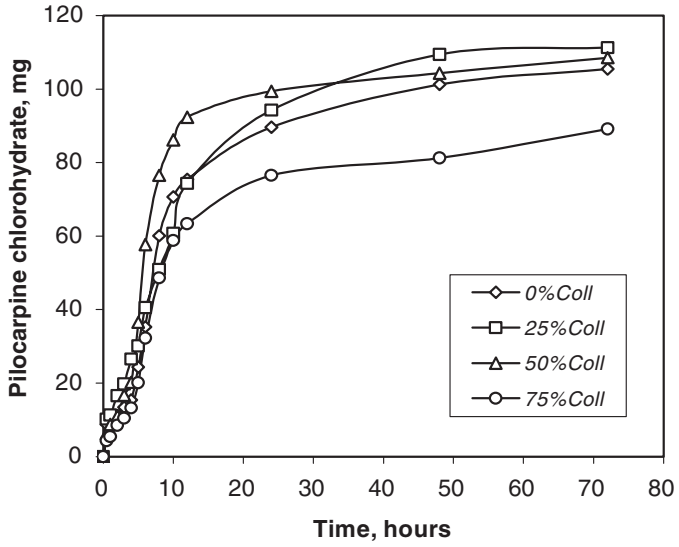


**Fig. 11.** Pilocarpine chlorohydrate load kinetics into collagen-chitosan-based hydrogels crosslinked with glutharaldehyde.

Collagen and chitosan – containing hydrogels, crosslinked with glutaraldehyde, exhibit similarly profiled load kinetics, the difference being that the two stages that occur when Pilocarpine chlorohydrate loads into the equilibrium swelled materials are not as clearly delineated on the kinetic curves. The drug amount enclosed by hydrogels reaches 100 mg, which implies a 5:1 polymer-drug ratio, enough to ensure the therapeutic dosage in the ophthalmologic application of Pilocarpine chlorohydrate.

The investigations on hydrogel drug delivery from drug-loaded hydrogels were carried out into buffered phosphate solution, thermostated at 37°C . The *in vitro* Pilocarpine chlorohydrate delivery data are presented in Figures 12 and Figure 13. Pilocarpine is a cholinergic antagonist with a pK = 6.6 and good water and aqueous solution solubility. At pH 7 the drug is able to interact only through hydrogen bonds with functional groups from polysaccharide and collagen. Therefore, the drug release from hydrogels has two components:

- drug interaction with the hydroxyl functional groups from gellan and hydroxyl or amino or carboxyl group from collagen – which delays drug release;
- molecular diffusion of the pilocarpine from crosslinked network –slower as the induced crosslinking degree is diminished.

Hydrogels based on chitosan and collagen have a drug release profile more appropriate of zero order and, for these hydrogels, polysaccharide improves controlled release capacity. Hydrogels with 85% chitosan release the drug for

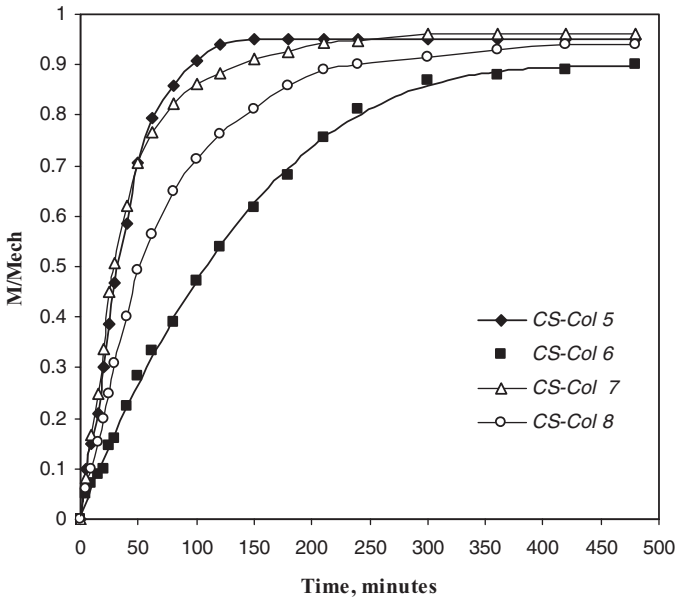


**Fig. 13.** Kinetics of Pilocarpine chlorohydrate delivery from collagen-chitosan hydrogels crosslinked with glutharaldehyde.

more than 5 h and made them very good candidates for sustained release of Pilocarpine chlorohydrate.

## 4 Conclusions

The hydrogels based on gellan/collagen and chitosan/collagen have been synthesized by crosslinking with glutaraldehyde. The network structure and composition is influenced by the nature and amount of polysaccharide used in synthesis and the quantity of crosslinker; densely packed and open networks are obtained as a function of these parameters. Hydrogels swelling characteristics, in distilled water and phosphate buffer (pH 7) vary with their composition; generally, hydrogels are more swellable in water and collagen–gellan matrices absorb increased water quantities. Synthesized hydrogels are able to include ophthalmic drugs (pilocarpine chlorohydrate) and drug release profiles recommend these hydrogels, especially those based on collagen and chitosan, as sustained-release matrices of water-soluble drugs.

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