CONTROLLED RELEASE OF WATER SOLUBLE ANTIBIOTICS BY CARBOXYMETHYLCELLULOSE- AND GELATIN-BASED HYDROGELS CROSSLINKED WITH EPICHLOROHYDRIN

GABRIELA BUHUS*, CATALINA PEPTU, MARCEL POPA and JACQUES DESBRIÈRES*

Department of Natural and Synthetic Polymers,
"Gheorghe Asachi" Technical University of Iaşi, 71, Blvd. D. Mangeron,
700050 Iaşi, Romania
**Université de Pau et des Pays de l'Adour, IPREM/EPCP (UMR CNRS 5254), Hélioparc Pau*
Pyrénées, 2, Avenue Président Angot, 64053 Pau Cedex 09, France

Received March 9, 2009

Hydrogels based on carboxymethylcellulose (CMC) and gelatin (GEL) crosslinked with epichlorohydrin in alkaline environment are polymeric interpenetrated-interconnected network materials, designed for obtaining controlled release polymer-drug systems. CMC and GEL are chosen due to their biocompatibility and non-toxicity – compulsory conditions for the polymers used in biomedical applications. By modifying the parameters of the crosslinking reaction, the obtained networks present different crosslinking degrees and hence different swelling capacities, properties determining the quantity of the drug to be included. Hydrogels with the highest swelling degree were loaded with water soluble drugs (chloramphenicol - sodium hemisuccinate, ClPh). We have thus obtained systems with diffusion-controlled release, with zero-order kinetics during most of the release period. These systems prove a high bactericide activity, comparable to that of free drugs.

Keywords: Carboxymethylcellulose/gelatin hydrogel, interpenetrated network, drug delivery, microbiological test, epichlorohydrin

INTRODUCTION

By definition, hydrogels represent polymeric networks capable of absorbing large quantities of water, yet remaining insoluble due to chemical or physical crosslinks between individual polymeric chains.[1,2]

Compared to hydrophobic polymeric networks – such as those based on poly(lactic acid) or poly(lactic acid-co-glycolic acid) – presenting a low water absorption capacity, hydrogels possess a series of unique properties, which constitute great advantages for their use in biomedical applications: the ability to encapsulate biomacromolecules (including proteins and DNA) due to the absence of hydrophobic interaction, which may lead to the denaturation of these fragile species;[3] relatively accessible achievement conditions: most of the reactions are carried out at room temperature and the use of organic solvents is rarely necessary; the ability to gelate *in situ*, as well as to encapsulate active matter; possible sensitivity to different environment stimuli (pH, temperature[4,5]); possible bioadhesiveness for the release of the active matter, mostly by mucus membranes;[6] possible *in vivo* masking of the active matter, due to their hydrophobicity; an increasing circulating time of the release system avoiding immune response and the decrease of phagocytar activity;[7] possible inclusion of cells and growth factors.[8]

Hydrogels may be prepared from either natural or synthetic polymers. Generally, natural polymer-based ones present weak mechanical properties, a shortcoming that may be corrected, on the one hand, by their biocompatibility and biodegradability, and on the other, by the fact that they allow the sequence of cellular activity

without any repelling inflammatory response from the "host" organism. Among natural polymers, polysaccharides are interesting, compared to synthetic polymers, by the fact that they come from living organisms, are biocompatible, non-toxic and present major physico-chemical properties necessary for controlled release applications.[9] From this point of view, the most extensively studied polysaccharides are alginate,[10-13] dextran,[14-16] gellan,[17] xanthan[18] and hyaluronic acid.[19,20] The choice of the material and the synthesis of the polymeric network govern the rate and the release of the active matter from the hydrogel.[2,9]

The present investigation is devoted to the elaboration of hydrogels based on natural polymers, such as gelatin (as a protein, obtained from the hydrolysis of collagen) and carboxymethylcellulose (polysaccharide derived from cellulose). Due to their biocompatibility and biodegradability properties, proteins and polysaccharides have attracted considerable attention in biomedical and pharmaceutical domains in the last fifteen years.[1,20-23]

Gelatin is a very attractive candidate as a raw material for obtaining hydrogels, due to its gelation ability. Moreover, due to the large number of functional groups, gelatin may crosslink easily. As a consequence, controlled release systems based on gelatin are applicable in very wide domains, from tissular engineering[24,25] to controlled release and gene therapy.[26,27] Gelatin, a natural proteic polymer obtained from the hydrolysis of collagen, has an amphoteric character, due to the presence of amino and carboxylic groups within the macromolecular chain. The ratio between the number of acidic and basic groups determines the isoelectric pH (pH_{is}). This parameter is very important for proteins because around this value the protein solutions may be anionic ($pH > pH_{is}$) or cationic ($pH < pH_{is}$).[28,29] The isoelectric point of gelatin is around 4.6.

The sodium salt of carboxymethylcellulose (CMC) is a cellulose ether. According to its preparation, the degree of substitution may vary, but generally it ranges[30] between 0.6-0.95. This degree of substitution, as well as the degree of polymerization, will determine the solubility, viscosity and hardness of the gel.[31] CMC with low degrees of substitution manifests a thixotropic behavior, while the samples with higher degrees of substitution lead to pseudo-plastic fluids. At low pH values, CMC may be crosslinked using a lactone forming reaction of free carboxylic and hydroxyl groups.[32]

The objective of the present study was to obtain biocompatible chemical hydrogels with a high swelling ratio in water, which can be modulated with the parameters of the crosslinking reaction and with the ratio between the two polymers (gelatin and carboxymethylcellulose) and which will be able to include and release biologically active chemicals. The method selected for the elaboration of such hydrogels is the covalent crosslinking of polymers in aqueous solution using epichlorohydrin as a crosslinking agent. So far, the literature devoted to such hydrogels described them only as microparticles by inverse emulsion crosslinking.[33]

The work also discusses the influence of several crosslinking reaction parameters on the gel composition – decisive characteristics of its ability to swell in water – and hence its capacity to include and release biologically active matter. Some systems based on these hydrogels and chloramphenicol will be finally elaborated and characterized by their bactericide activity and drug release kinetics. Chloramphenicol was chosen as biologically active matter for several reasons: it is water soluble and hence easily loaded within hydrogels (acting as a model molecule for water soluble drugs), it presents a wide antibacterial spectrum (with a bactericide activity on gram-negative and gram-positive germs). Moreover, as these hydrogels are elaborated for ophthalmic applications, they are often used for treating some eye infections, in association with other ophthalmic drugs.

MATERIALS AND METHODS

Materials

Sodium carboxymethylcellulose salt (CMC:DS = 0.75) was supplied by Fluka and A-type gelatin ($GEL:NH_2$ = 0.954 mmol/g, pH_{izo} = 6.73, average molar mass 100,000 g/mol) by Merck. Epichlorohydrin (EpCl), from Sigma Aldrich, and chloramphenicol (or sodium hemisuccinate, ClPh), from S.C. Antibiotice (Iaşi, Romania), were used without further purification.

Obtention of hydrogels

Adequate quantities of CMC, GEL and distilled water were introduced into a beaker. The mixture was stirred until a homogeneous viscous solution was obtained. 0.8 mL NaOH (40%) was added dropwise to reach an intensely alkaline environment (pH = 10 ÷ 12). The crosslinking agent (epichlorohydrin, EpCl) was added dropwise under continuous stirring. The reaction medium was spread out as a paste between

two glass plates and then introduced in an oven at 45 °C.

When the reaction was finished, the hydrogel films were separated from the glass plates and washed with distilled water to remove unreacted chemicals. Three successive washing–extraction steps with 400 ml of hot water (45 °C) were carried out for 24 hours. Finally, water traces were extracted from the films with acetone, in a Soxhlet apparatus, and the films were dried in an oven at 45 °C. The influence that different crosslinking reaction parameters (such as the GEL concentration in the initial mixture, the ratio between the crosslinking agent and the polymer mixture (r_{AP}), the polymer solution concentration (C_p) and the reaction duration (t_r)) exert on the properties of hydrogels was studied (Table 1).

Methods

The hydrogels were characterized by the composition of the obtained network, the swelling properties in water, the inclusion and release kinetics of chloramphenicol (ClPh) and the bactericide activity of hydrogels charged with ClPh.

Characterization methods

Scanning Electron Microscopy: images were obtained from a TESLA BS 3001 microscope (from the Czech Republic). The sample, partially swollen in ethyleneglycol, was fixed with an electroconducting glue and covered with gold powder.

FTIR spectroscopy was carried out on KBr pellets using a FT-IR BONEM 104B spectrometer (Canada).

The composition of the hydrogels was determined from the nitrogen content (contained within gelatin) using the Kjeldahl method.[34]

Swelling kinetics and the maximum swelling degree, determined by the Dogadkin method,[35] were calculated (in %) by the relation:

$Q_t = \frac{m_s - m_d}{m_d} \cdot 100$ where Q_t is the swelling degree at time t, m_s is the weight of swollen hydrogel at time t and m_d is the weight of dry hydrogel.

Table 1
Reaction parameters for hydrogel synthesis based on CMC and GEL

Parameter code	Sample code	CMC, %	GEL, %	Polymer/Crosslinking agent ratio, r_{PE}, wt/wt	Crosslinking time, t_R, h	Total polymer concentration of solution C_P, %; (wt/v)
1G	G1	20	80	2.5	4	11.135
	G2	40	60			
	G3	60	40			
	G4	80	20			
	G5	50	50			
	G6	0	100			
	G7	100	0			
2G	G-lllA	60	40	3.125	4	11.135
	G3			2.5		
	G-lllB			2.083		
	G-lllC			1.7857		
3G	G-lll1	60	40	2.5	3	11.135
	G3				4	
	G-lll2				5	
	G-lll3				2	
4G	G-llla	60	40	2.5	4	12.755
	G3					11.135
	G-lllb					8.9606
	G-lllc					7.3206

The measurement of the swelling degree was performed at 25 °C using twice distilled water as a swelling agent

Inclusion and release of chloramphenicol in/from the hydrogel

The inclusion of chloramphenicol was carried out by diffusion. The dry hydrogel was suspended in 25 ml of 2% (wt/v) chloramphenicol solution. The concentration of the drug in the initial solution was determined by UV spectroscopy, using a UV CADAS 100 spectrophotometer (Germany), at a wavelength of 279 nm (after a calibration curve was established).

The kinetics of drug diffusion in the hydrogel was evaluated from the measurement of the drug concentration in the supernatant at different time periods. The difference between the initial drug concentration and its concentration at time t in the

solution allows the calculation of the quantity of the drug included in the hydrogel.

The drug release was studied under static conditions. The dry sample (with the included drug) was suspended in twice distilled water. From time to time, a 0.1 ml aliquot of solution was taken, diluted to 10 ml with water, while the drug concentration was measured from the UV calibration curve.

Antimicrobial activity

The antimicrobial activity of hydrogels was evaluated from their capacity to inhibit gram-positive germs (*Staphylococcus aureus* ATCC25923). Bacterial cultures were sowed on a glass support, according to the Mueller-Hinton method,[36] and inhibited for 24 hours at 37 °C. After 24 hours, the diameter of the inhibitory zone of the bacterial culture was measured.

RESULTS AND DISCUSSION

As the polymers under analysis have functional groups able to react with epichlorohydrin, the material obtained will have a complex structure, as an interpenetrated–interconnected network. The reactions that may occur are schematized in Figure 1.

FTIR spectral data were used to confirm the crosslinking of gelatin and CMC chains by EpCl. FTIR spectra of the NaCMC (curve a), gelatin (curve b) and G3 hydrogel (curve c) are compared in Figure 2. In the case of gelatin, a characteristic band due to N–H stretching is observed at 3416 cm^{-1}. The N–H bending vibration is assigned to the band observed at 1402 cm^{-1}. Aliphatic C–H stretching is observed at 2926 cm^{-1}, while aliphatic C–H bending vibrations are observed at 1450 and 1402 cm^{-1}. The band appearing at 1638 cm^{-1} indicates amide I band, while the band at 1327 cm^{-1} is assigned to the C–N bond stretching vibrations. NaCMC shows bands at 3416 and 3238 cm^{-1} due to O–H stretching vibrations. The distant band at 2986 cm^{-1} shows aliphatic C–H stretching vibrations, but those appearing at 1618 and 1420 cm^{-1} are due to the asymmetric and symmetric stretching of the carboxylate group, respectively. The bands found at 1108 and 1060 cm^{-1} represent C–O–C stretching vibrations.[37,38]

Hydrogel morphology was macroporous, as evidenced by scanning electron microscopy photographs (Fig. 3).

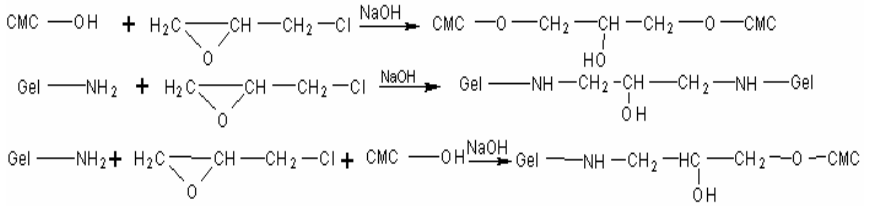

Figure 1: Crosslinking reactions with epichlorohydrin, occurring in carboxymethylcellulose-gelatin mixture

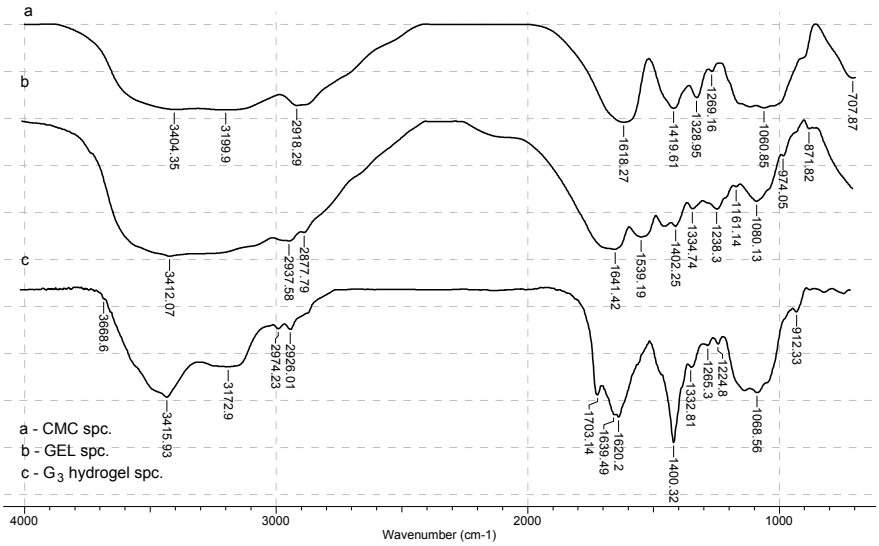

Figure 2: FTIR spectra for gelatine (GEL), carboxymethylcellulose (CMC) and gelatin-carboxymethylcellulose based hydrogel (CMC:GEL = 60:40, r_{PE} = 2.5, t_R = 4 h, C_p = 11.135%, T = 45 °C)

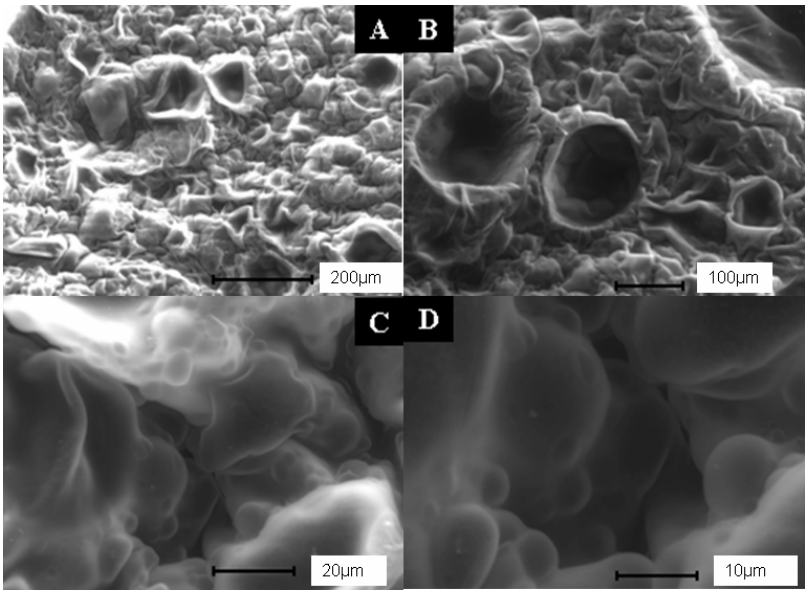

Figure 3: Scanning Electronic Microscopy images of G3 hydrogel
(A – 305 x, B – 410 x, C – 2440 x, D – 4880 x)

Hydrogel composition

Hydrogel composition is strongly influenced by the crosslinking reaction parameters, including the initial composition of the polymer mixture. For 1G hydrogels, it was noticed that the increase of the initial GEL content leads to an increase of the protein amount in the final hydrogel, which never exceeded 10% (Fig. 4).

Apparently, the free amine groups of GEL present a higher reactivity for EpCl, with respect to hydroxyl groups of CMC; thus the results obtained could be surprising. A valid explanation is that the strong alkaline environment and the temperature (45 °C) will affect the protein, causing its degradation and the formation of shorter chains with increased solubility, which are removed during the purification procedures.[39-43]

The networks, which present a high content of GEL, are obtained by increasing the amount of the crosslinking agent with respect to the total amount of the polymers (Fig. 5). The decreasing polymer/crosslinking agent value (r_{PE}), which means the increase of EpCl quantity in the system, will contribute to the GEL enrichment of the network.

An increased amount of the crosslinking agent implies the increase of the crosslinking density. Consequently, many gelatin chains are immobilized within the network and cannot be removed by successive washings with water. Finally, while the ratio between polymers (40% GEL), that between polymers and epichlorohydrin (r_{PE} = 2.5), and the reaction time (4 hours) were kept constant, the total polymer concentration – a parameter with a low influence on the gelatin content in the hydrogel – was varied (Table 2).

Table 2
GEL content in hydrogels as a function of polymer concentration in solution

Polymer concentration in solution (%)	GEL content in hydrogel (%)
7.32	8.8
8.96	9.5
11.16	6.6
12.76	5.7

Crosslinking conditions: T = 45 ˚C; t_R = 4 h; r_{PE} = 2.5; CMC:GEL = 60:40

At the highest polymer concentration, the decrease in the GEL content may be explained by the greater participation of the polysaccharide (CMC) in the reaction, due to the higher proximity of the polysaccharide chains within the solution, leading to an increase in the crosslinking ability of CMC chains (as previously demonstrated in the typical crosslinking reactions of polysaccharides). It was observed that the yield in the hydrogel formation (defined as the ratio

between the weight of dry hydrogel and the total weight of reagents) decreased with total polymer concentration (8.8% for a total polymer content of 7.3%, compared to 5.7% for a total polymer content of 12.7%).

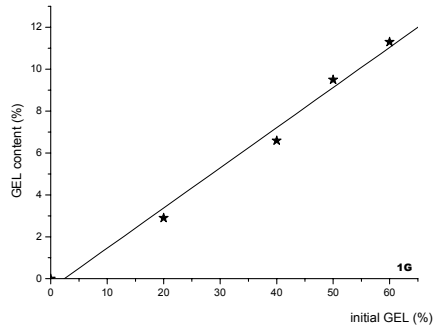

Figure 4: Gelatin (GEL) composition of hydrogels as a function of the initial gelatine (GEL) concentration in the polymer mixture (crosslinking conditions: T = 45 ˚C; t_R = 4 h; r_{PE} = 2.5; Cp = 11.135%)

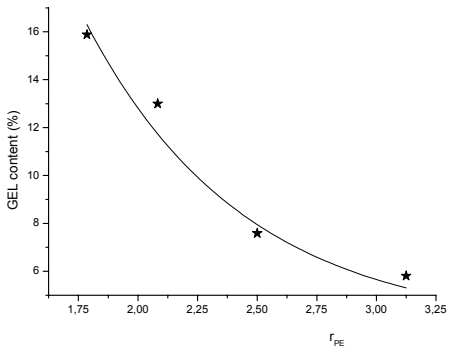

Figure 5: Influence of epichlorhydrin concentration on the gelatin (GEL) content of hydrogels (crosslinking conditions: T = 45 ˚C; t_R = 4 h; CMC:GEL = 60:40; Cp = 11.135%)

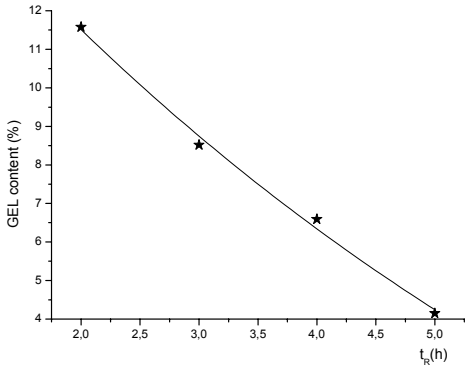

Figure 6: Variation of gelatin (GEL) content in hydrogels with crosslinking time (crosslinking conditions: T = 45 ˚C; CMC:GEL = 60:40; r_{PE} = 2.5; Cp = 11.135%)

Hydrogel swelling

The experimental data shown in Figure 7 indicate a kinetics of the swelling process typical of hydrogels. The swelling degree tends towards a limit value, which is specific to each of the hydrogels. In the same manner, the time necessary to reach this threshold depends on the elaboration conditions of the hydrogel. The large value of the maximum swelling degree (up to 5200%) classifies these hydrogels within the "superabsorbant" grade. In Figure 8, the maximum swelling degree (measured from Fig. 7) was plotted as a function of the crosslinking reaction parameters. It was observed that Figures 8a and 8c are very similar to Figures 4 and 6. This demonstrates that the major parameter is the GEL content. Davidson and Sittig [44] have studied the hygroscopy of polysaccharides and they found that proteins (among them, gelatin) absorb a higher amount of water than carboxymethylcellulose, due to the higher quantity of polar groups per monomeric unit. Due to this difference in water affinity, the higher the gelatin content is in the hydrogel, the higher its swelling degree.

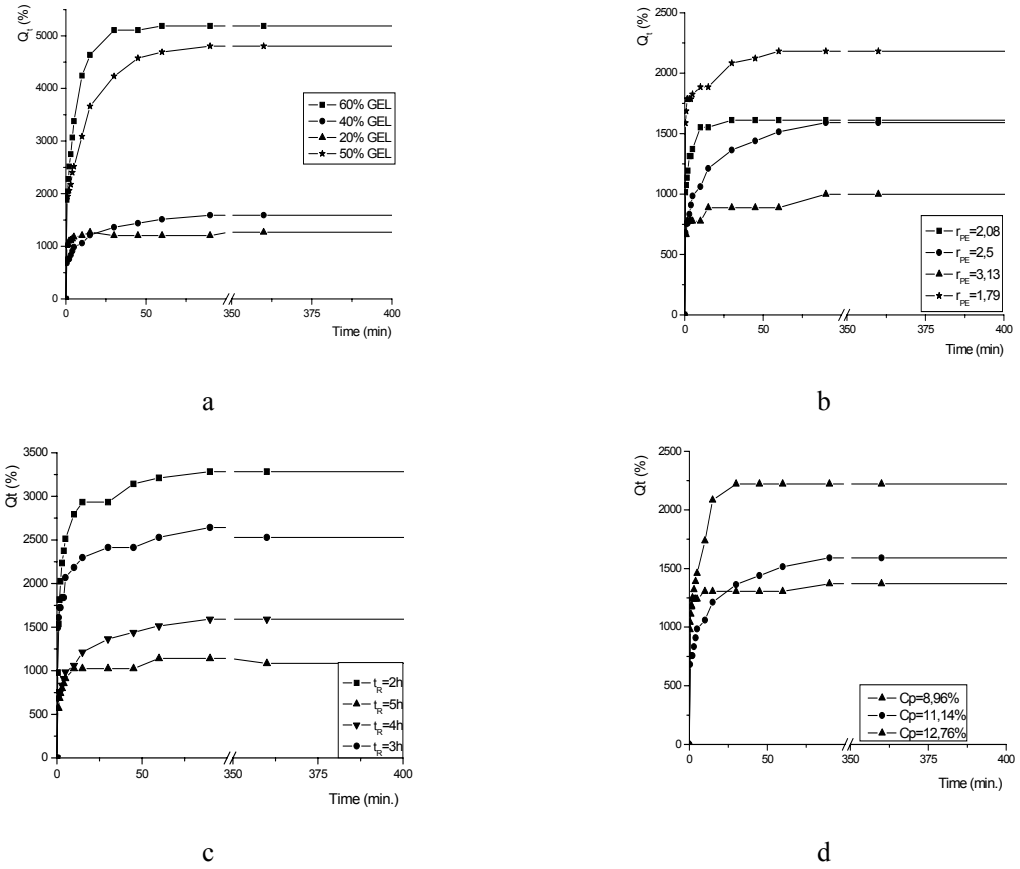

Figure 8: Variation of maximum swelling ratio as a function of the gelatine (GEL) content for different crosslinking reaction parameters: a – gelatin content in the polymer mixture; b – polymer mixture/crosslinking agent ratio; c – reaction duration; d – total polymer concentration in the solution

Hydrogels and chloramphenicol

Inclusion of chloramphenicol

The inclusion of chloramphenicol was performed through a diffusion process in the elaborated networks. The kinetic profile of the inclusion curves is typical of the absorption process of a chemical dissolved by a solid support. After the equilibrium is installed, from the initial and final values of the drug amount in the solution, the amount of immobilized drug per gram of dry hydrogel was calculated (Fig. 9).

Hydrogels can include high amounts of drug, varying from 25 to 198.7mg ClPh/g hydrogel. The time needed to reach the diffusion equilibrium varies from 200 to 250 minutes depending on the hydrogel composition. It can be also observed that there is a similarity between swelling and ClPh loading kinetics, the only difference being the time necessary to reach the equilibrium. This last aspect could be explained by the difference in the dimensions of the two molecules: the water having much smaller molecules than ClPh has, its diffusion capacity is higher.

The higher the GEL contents of the hydrogel, the higher the swelling degree and the quantity of included ClPh. Moreover, CMC is an anionic polymer like ClPh. As a consequence, repulsive interactions occur between these chemicals and the quantity of included drug decreases. When the variation of the maximum content of included drug was plotted as a function of the parameters of the crosslinking reaction and as a function of the gelatin content, a similar evolution with that of the maximum swelling degree was observed (Fig. 10).

Drug release from hydrogels

The variation in the released drug quantity with time was plotted in Figure 11. The release process is slower than the inclusion one (compare with Fig. 9) and the quantity of the released drug reaches a constant value after about 90-120 minutes. Large discrepancies exist as to the

quantity of GEL in the hydrogel. This is due to the repulsive electrostatic interactions between CMC and chloramphenicol and hence the weaker binding of the drug within the CMC based hydrogel.

The major part of chloramphenicol is released during the first 150 minutes, as demonstrated by the release rate (Fig. 12). A "burst effect" appears clearly in the first release minutes, due to a very quick release of the drug fixed to the surface or within the upper layers of the hydrogel, which is a typical effect of diffusion-controlled polymer-drug systems. After this effect, the release rate decreases and stays constant during a wide time range (up to a minimum of 1200 minutes).

It is well known that the release processes of small size molecules within or from hydrogels are controlled by diffusion. Many transport models were proposed. Among them, the most often cited and used is the Korsemeyer-Peppas one. According to this model, the drug transport process by the hydrogel may be described by the relation[45] $M_t/M_\infty = k \cdot t^n$,where M_t and M_∞ represent the total quantity of low molecular weight chemical released at time t, respectively at the end of the release process. The n value may be determined from the experimental data, while the deviation from the 0.5 value informs on the validity of the Fickian transport mechanism. The results are plotted for different hydrogels with the same composition, but at different crosslinking time values, in Figure 13 and n values are given in Table 3. The transport mechanism differs from the Fickian model, with the gels presenting a crosslinking time t_R of 5 hours. The deviation may be due to the porosity of hydrogels, which perturbed the diffusion laws related to the relaxation of the chain segments between crosslinking sites (we have demonstrated that our hydrogels are macroporous, see Fig. 3), or the consequence of ionic interactions between the drug and the network.

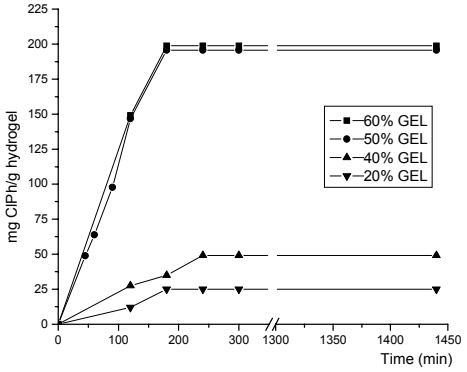

Figure 9: Inclusion kinetics of chloramphenicol (ClPh) in hydrogels with different gelatin (GEL) contents in the polymer mixture

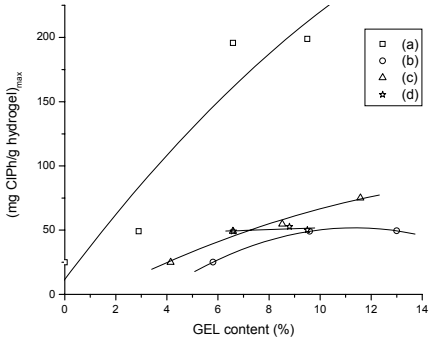

Figure 10: Variation of the maximum drug amount included as a function of the gelatin (GEL) content for different crosslinking reaction parameters: a – gelatin content in the polymer mixture; b – polymer mixture/crosslinking agent ratio; c – reaction duration; d – total polymer concentration in solution

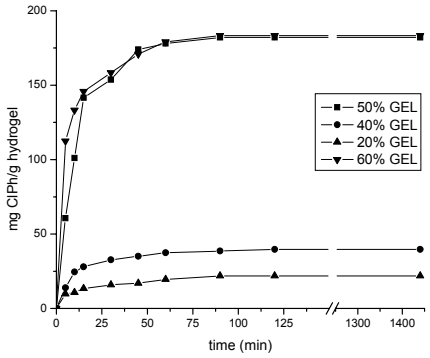

Figure 11: Chloramphenicol (ClPh) release kinetics of hydrogels differing as to the gelatin (GEL) content in the polymer mixture

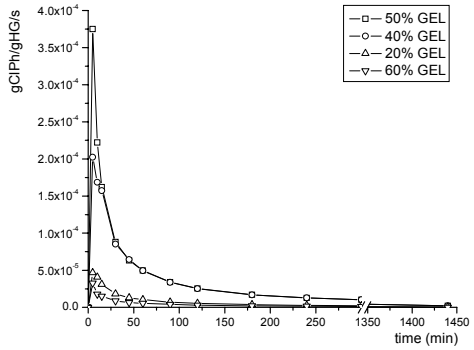

Figure 12: Chloramphenicol (ClPh) release kinetics of hydrogels differing as to the gelatin (GEL) composition of the initial polymer mixtures

Table 3
Values of n coefficient in the Korsemeyer-Peppas model equation

Code	t_R	Release n
G-III3	2	0.57
G-III1	3	0.63
G3	4	0.65
G-III2	5	0.51

Considering the polysaccharide-based hydrogels we have previously studied,[46-52] these hydrogels do not present a different behavior. But they are interesting for the ophthalmic applications considered. Indeed, such materials do have a certain grip – called mucoadhesivity – to the eye conjunctival mucous membrane, avoiding to be quickly removed by the reflex winking when a foreign body is introduced in the conjunctival bag. Even if polysaccharides have a sufficiently mucoadhesive character, the association with gelatin increases it. From this point of view, the hydrogels we have studied in this paper are superior to those based on polysaccharides and poly(vinyl alcohol).

Bactericide activity of the polymer-drug systems obtained

On the plates sown with the microbial culture, an inhibition zone appears either near the free drug or near the drug-charged hydrogel (Fig. 13). The inhibition zone is smaller for the polymer-drug system, as compared to the free drug, due to the fact that the drug was not completely released after 24 hours. No inhibition zone was observed for the gel without any included drug, which demonstrates that our polymer-drug systems manifest an antibacterial activity.[53]

CONCLUSIONS

GEL- and CMC-based hydrogels, crosslinked with EpCl, were developed. Crosslinking was proved by FTIR spectroscopy and between 3 and 16% GEL is present in the elaborated hydrogels. The differences in the composition of hydrogels were correlated with the crosslinking agent (and the highly alkaline environment) and with the parameters of the crosslinking reaction.

The maximum swelling degree varies between 1140% and 5190%, the characteristics of hydrogel swelling depending on the composition and the parameters of the crosslinking reaction.

The hydrogels load and release the ClPh by diffusion. The loading and release degrees were correlated with different swelling degrees, which depend on the crosslinking reaction conditions. The release of the ClPh follows *zero*-order release kinetics.

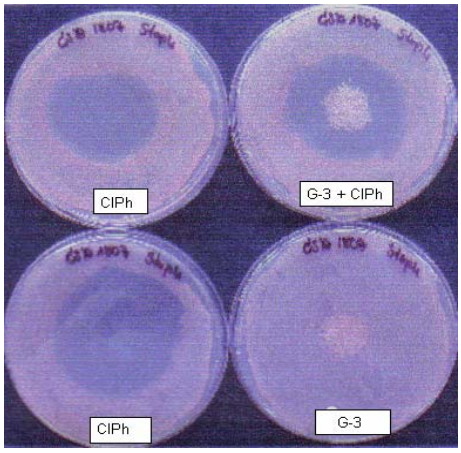

Figure 13: Microbiological tests on G3 hydrogel with and without chloramphenicol towards *Staphylococcus aureus* ATCC25923 cultures (0.509 g G3 hydrogel, 0.0245 g ClPh)

All these properties (swelling, inclusion and release of chloramphenicol) depend directly on the gelatin content of hydrogels.

The hydrogels loaded with chloramphenicol present a high bactericide activity.

REFERENCES

[1] N. Kashyap, N. Kumar and M. Kumar, *Crit. Rev. Ther. Drug Carr. Syst.*, **22**, 107 (2005).
[2] N. A. Peppas, P. Burns, W. Leobandung and H. Ichikawa, *Eur. J. Pharm. Biopharm.*, **50**, 27 (2000).
[3] S. Young, M. Wong, Y. Tabata and A. G. Mikos, *J. Control. Release*, **109**, 256 (2005).
[4] Y. Qiu and K. Park, *Adv. Drug Deliv. Rev.*, **53**, 321 (2001).
[5] T. J. Koob and D. J. Hernandez, *Biomaterials*, **24**, 1285 (2003).
[6] U. Bertram and R. Bodmeier, *Eur. J. Pharm. Sci.*, **27**, 62 (2006).
[7] S. Frokjaer and D. E. Otzen, *Nat. Rev. Drug Discov.*, **4**, 298 (2005).
[8] S. Cai, Y. Liu, X. Z. Shu and G. D. Prestwich, *Biomaterials*, **26**, 6054 (2005).
[9] T. Coviello, P. Matricardi, C. Marianecci and F. Alhaique, *J. Control. Release*, **119**, 5 (2007).
[10] H. H. Tonnesen and J. Karlsen, *Drug Dev. Ind. Pharm.*, **28**, 621 (2002).
[11] C. V. Liew, L. W. Chan, A. L. Ching and P. W. S. Heng, *Int. J. Pharm.*, **309**, 25 (2006).
[12] D. Bhopatkar, A. K. Anal and W. F. Stevens, *J. Microencapsul.*, **22**, 91 (2005).
[13] H. F. Liang, M. H. Hong, R. M. Ho, C. K. Chung, Y. H. Lin, C. H. Chen and H. W. Sung, *Biomacromolecules*, **5**, 1917 (2004).
[14] G. Fundueanu, M. Constantin, E. Esposito, R. Cortesi, C. Nastruzzi and E. Menegatti, *Biomaterials*, **26**, 4337 (2005).
[15] B. Stubbe, B. Maris, G. Van Den Mooter, S. C. De Smedt and J. Demeester, *J. Control. Release*, **75**, 103 (2001).
[16] S. A. Agnihotri and T. M. Aminabhavi, *Drug Dev. Ind. Pharm.*, **31**, 491 (2005).
[17] S. Dumitriu and E. Chornet, *Adv. Drug Deliv. Rev.*, **31**, 223 (1998).
[18] R. Barbucci, M. Consumi, S. Lamponi and G. Leone, *Macromol. Symp.*, **204**, 37 (2003).
[19] D. I. Ha, S. B. Lee, M. S. Chong, Y. M. Lee, S. Y. Kim and Y. H. Park, *Macromol. Res.*, **14**, 87 (2006).
[20] P. L. Soo, J. Cho, J. Grant, E. Ho, M. Piquette-Miller and C. Allen, *Eur. J. Pharm. Biopharm.*, **69**, 149 (2007).
[21] Y. S. Choi, S. R. Hong, Y. M. Lee, K. W. Song, M. H. Park and Y. S. Nam, *J. Biomed. Mater. Res.*, **48**, 631 (1999).
[22] K. Fujioka, M. Maeda, T. Hojo and A. Sano, *Adv. Drug Deliv. Rev.*, **31**, 247 (1998).
[23] B. Balakrishnan and A. Jayakrishnan, *Biomaterials*, **26**, 3941 (2005).
[24] H. Okino, Y. Nakayama, M. Tanaka and T. Matsuda, *J. Biomed. Mater. Res.*, **59**, 233 (2002).
[25] M. Changez, V. Koul and A. K. Dinda, *Biomaterials*, **26**, 2095 (2005).
[26] H. Yu and C. Xiao, *Carbohydr. Polym.*, **72**, 479 (2008).
[27] N. J. Einerson, K. R. Stevens and W. J. Kao, *Biomaterials*, **24**, 509 (2002).
[28] H. C. Liang, W. H. Chang, H. F. Liang, M. H. Lee and H. W. Sung, *J. Appl. Polym. Sci.*, **91**, 4017 (2004).
[29] R. Schrieber and H. Gareis, in "Gelatine Handbook –

Theory and Industrial Practice", Wiley-VCH, 2007, p. 138.
[30] http://www.lsbu.ac.uk/water/hycmc.html
[31] D. Klemm, B. Philipp, T. Heinze, U. Heinze and W. Wagenknecht, in "Comprehensive Cellulose Chemistry", Wiley-VCH, 1998, Vol. 2, p. 221.
[32] M. O. Emeje, O. O. Kunle and S. I. Ofoefule, *Acta Pharm*., **56**, 325 (2006).
[33] A. P. Rokhade, S. A. Agnihotri, S. A. Patil, N. N. Mallikarjuna, P. V. Kulkarni and T. M. Aminabhavi, *Carbohydr*. *Polym*., **65**, 243 (2006).
[34]http://www.rosesci.com/Products/Chemical Analysis/ KjeldahlChemistryOverview.htm
[35] C. Alupei, M. Popa, M. Hamcerencu and J. M. Abadie, *Eur*. *Polym*. *J*., **38**, 2313 (2002).
[36] http://fr.wikipedia.org/wiki/Mueller-Hinton _Gelose)
[37] J. Coates, in Encyclopedia of Chemistry, edited by R. A. Mayers, John Wiley & Sons Ltd, Chichester, 2000, p. 10815.
[38] H. S. Mansur, R. L. Orefice, M. M. Pereira, Z. I. P. Lobato, W. L. Vasconcelos and L. J. C. Machado, *Spectrosc*. *Int*. *J*., **16**, 351 (2002).
[39] M. Laguerre, C. Boyer, J.-M. Leger and A. Carpy, *Can*. *J*. *Chem*., **67**, 1514 (1989).
[40] R. M. Smith and D. E. Hansen, *J*. *Am*. *Chem*. *Soc*., **120**, 8910 (1998).
[41] D. Zahn, *Eur*. *J*. *Org*. *Chem*., **19**, 4020 (2004).
[42] R. Schrieber and H. Gareis, in "Gelatine Handbook – Theory and Industrial Practice", Wiley-VCH, 2007, p. 138.
[43] G. O. Phillips and P. A. Williams, in "Handbook of Hydrocolloids", CRC Press North East Wales Institute, UK, 2000, p. 86.
[44] R. Davidson and M. Sitting, in "Water Soluble Resins", Reinhold Book Corp., New York, U.S.A., 1962, p. 191.
[45] M. Delgado, C. Spanka, L. D. Kerwin, Jr., P. Wentworth, K. D. Janka and A. Tunable, *Biomacromolecules*, **3**, 262 (2002).
[46] C. L. Dumitriu, M. Popa, S. Vasiliu and V. Sunel, *J*. *Macromol*. *Sci*., *A*, **41**, 727 (2004).
[47] M. Popa, C. L. Dumitriu and V. Sunel, *Polymer Plast*. *Tech*. *Eng*., **43**, 1503 (2004).
[48] M. Popa, N. Bajan, M. I. Popa and A. A. Popa, *Polymer Plast*. *Tech*. *Eng*., **45**, 23 (2006).
[49] M. Popa, N. Bajan, A. A. Popa and L. Verestiuc, *J*. *Macromol*. *Sci*., *A*, **44**, 483 (2007).
[50] G. Buhus, M. Popa, C. Peptu and J. Desbrieres, *JOAM*, **9**, 3445 (2007).
[51] C. Peptu, M. Popa and S. G. Antimisiaris, *JNN*, **8**, 1 (2008).
[52] A. Bejenariu, M. Popa, D. Le Cerf and L. Picton, *Polym. Bull*., **61**, 631 (2008).
[53] D. Buiuc, "Microbiologie medicală" (in Romanian), Ed. Didactică şi Pedagogică, Bucureşti, 1992, 249 pp.