CELLULOSE/CHONDROITIN SULPHATE HYDROGELS AS CARRIERS FOR DRUG DELIVERY APPLICATIONS

ANA-MARIA OPREA[1*], ANDREI NEAMTU[2], BOGDAN STOICA[2], CORNELIA VASILE[1]

Keywords: drug delivery, hydrogels, biocompatibility, codeine

Abstract: The potential of the hydrogels based on natural, biodegradable and biocompatible polysaccharides, cellulose (C) and chondrotin sulphate (GAG), as sustained release vehicles, has been followed by *in vitro* swelling and drug release studies. The swelling studies were performed by mass measurements at 37 ^{0}C in twice distilled water. The release profiles and release kinetics of codeine, an opiate used for its analgesic, antitussive and antidiarrheal properties, were determined. Water-uptake data and drug release measurements are given for characterization of new solid dosage forms, the importance of the chondroitin sulphate presence being also discussed. The biocompatibility testing was made by hemolysis (plasma hemoglobin) technique. It seems that cellulose/chondroitin sulphate hydrogels are promising formulations for drug delivery.

INTRODUCTION

Hydrogels, highly hydrated polymer networks, are formed by the chemical or physical crosslinking of the hydrophilic polymer. The characteristics of hydrogels, including sensitivity to the environment, tissue-like water content and elasticity afford the potential for biomedical application. For instance, hydrogels are used for delivering drugs (Ichikawa, 2000; Matsumoto, 2003), artificially dressing burns (Choi, 1999; Yannas, 1989), cell encapsulation (Lim, 1980; Uludag, 2000), and constructing a scaffold for use in tissue engineering (Lee, 2001; Tateishi, 2002).

Among the numerous polymers that have been proposed for the preparation of hydrogels, polysaccharides have a number of advantages over the synthetic polymers which were initially employed in the field of pharmaceutics (Wichterle, 1960).

Chondroitin sulphate (CS) consists of repeating disaccharide units of D-glucuronic acid and N-acetyl galatosamine, sulfated at either 4- or 6-positions (figure 1)

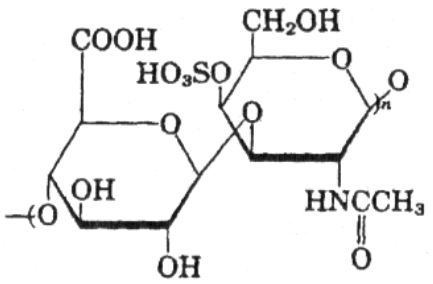

Figure 1. Chondroitin sulphate structure

The CS chains are roughly classified into types A, C, D, E, K and H (Yamada, 2000). They are named chondroitin sulfates A (C-4-S), C (C-6-S), D (C-2,6-S), E (C-4,6-S), K (C-3,4-S) and H (IdoAa1–3Gal-NAc(4S,6S)), of which C-4-S and C-6-S are the most common (Zou, 2009).

CS can bind with core protein to produce highly absorbent aggregan, which is a major structure inside cartilage and acts as a shock absorber, or it can produce sydecan, which is a cell receptor which can interact with adhesion proteins, cells and the extracellular matrix (ECM) (Bukalo, 2001). In biomedical applications, CS has shown *in vivo* anti-inflammatory effect in animal models. It also regulates metabolism *in vitro* (Bali, 2001). CS can be used for treating autoimmune and wasting joint diseases. VISCOAT^w (4% chondroitin sulphate $_{(aq)}$ and 3% sodium hyaluronate$_{(aq)}$) is used as a surgical aid in cataract extraction and lens implantation (Tomita, 2004). CS is also a component of the dermal layer of the FDA-approved skin substitute for treating burns (Phillips, 1998; Lee, 2005).

It has been demonstrated that the half-life of CS in the circulatory system is 3–15 min, based on the pharmacokinetic study of intravenously administered CS (Sakai, 2002). This indicates that orally administered CS is not systemically distributed to connective tissues such as cartilage and skin, and that exogenously administered CS may indirectly stimulate chondrocytes to synthesize ECM components. The mechanism of action of orally administered CS might be mediated by other systems (Yamada, 2008).

CS-coated poly(2-hydroxyethyl methacrylate) membranes were found to prevent adhesion in fullthickness tendon tears of rabbits (Gudemez, 2002). GAG hydrogels composed of poly(ethylene glycol) dialdehyde cross-linked with adipic dihydrazide derivatives of CS and hyaluronic acid have been evaluated as bio-interactive dressings for wound healing by Kirker et al (Kirker, 2002).

Lee et al. (Lee, 2005) prepared hydrogels based on poly(vinyl alcohol) (PVA)-chondroitin sulphate (CS) using glutaraldehyde as the crosslinking agent. These hydrogels, which have the advantages of both PVA and CS, can be used as a material for scaffolds in tissue engineering, promoting not only cell adsorption, but also cell growth. The mechanical properties of composite hydrogels facilitate the culturing of cells and make them bioactive toward the cells.

The hydrogels based on CS formed directly by crosslinking CS with poly(ethylene glycol) diglycidyl ether (EX-810) abbreviated as CS-EX or an interpenetrating polymer network named CS-EX-IPN were studied by Wang et al. (Wang, 2007) characterizing, also, the release of a model drug, diclofenac sodium (DS) and a model protein, bovine serum albumin (BSA). Diclofenac sodium released from the CS-EX and CS-EX-IPN hydrogels was rapid but BSA could be moderately controlled. The release profiles of both drugs fit in the diffusion-controlled mechanism, these preliminary results indicating that the CS-based hydrogels were more effective to sustain the release of large molecules like BSA.

A fast thermoresponsive hydrogel composed of poly(N-isopropylacrylamide) (PNIPAm) and chondroitin sulphate (CS) was synthesized using precipitation polymerization. CS was introduced to increase the water absorption of the PNIPAm hydrogel, and the precipitation polymerization method was used to induce a porous network morphology to enhance the thermal response of the hydrogel.

This hydrogel could be suitable for sensors, actuators or artificial muscle applications. In addition, the high porosity and negatively charged internal structure of PNIPAm/ChS hydrogels have the potential to load cationic drugs for controlled delivery applications (Varghesea, 2008).

Bacterial cellulose has long been used in a variety of applications in the paper, food, and electronic industries (Jonas, 1998; Miranda, 1965; Nishi, 1990; Shah, 2005; Yano, 2005).

Owing to its high porosity, water absorbance, mechanical properties, formability, and biocompatibility, bacterial cellulose has also recently attracted a great deal of attention for biomedical applications (Czaja, 2007). The structure of cellulose is showed in figure 2.

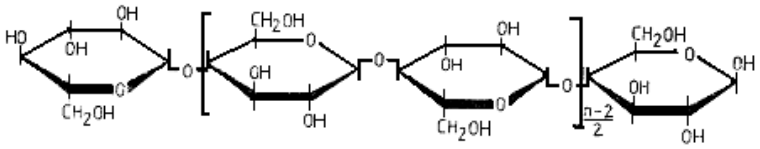

Figure 2. Cellulose structure

For instance, bacterial cellulose has been successfully used for wound dressings (Ciechanska, 1998; Czaja, 2006; Legeza, 2004) and for vascular implants (Klemm, 1999, 2001). The potential of bacterial cellulose for *in vitro* and *in vivo* tissue regeneration also continues to be explored and shows great promise (Backdahl, 2006; Helenius, 2006; Svensson, 2005; Watanabe, 1993).

Bacterial cellulose has been soaked into hydroxyapatite to develop a composite scaffold for bone regeneration (Hong, 200; Wan, 2006). Bacterial cellulose has also been augmented by immersion in solutions of polyacrylamide and gelatin, yielding hydrogels with improved toughness (Yasuda, 2005). Similarly, the immersion of bacterial cellulose into poly(vinyl alcohol) has yielded hydrogels having a wide range of mechanical properties of interest for cardiovascular implants (Millon, 2006).

In a study by Yasuda et al. microbial cellulose was immersed in two types of polymer solutions (2-acrylamide-2-methyl-propane sulfonic acid and gelatin) in order to create a hydrogel with enhanced mechanical toughness (Yasuda, 2005). The resulting double-network hydrogels (DN), consisting of two independently cross-linked networks of different polymers, can withstand high frictional forces, showing that they are resistant to wear. Thus, these microbial cellulose composites could function as replacement cartilage tissue in damaged joints (Czaja, 2007).

Sannino et al. (Sannino, 2000) tested cellulose-based hydrogels for their potential use in biomedical applications. This hydrogels main clinical application may be the treatment of oedemas of cardiac, hepatic and renal origin, which are resistant to diuretic therapy, evaluating, also, the long-term gel compatibility, in terms of morphological variations, toxicity and carcinogenic potential.

Controlled drug release systems offer numerous advantages compared to conventional dosage forms, including improved efficiency, reduced toxicity and improved patient compliance and convenience (Uhrich, 1999).

Controlled drug delivery occurs when a polymer, whether natural or synthetic, is judiciously combined with a drug or other active agent in such a way that the active agent is released from the material in a pre-designed manner. The release of the active agent may be constant over a long period, it may be cyclic over a long period, or it may be triggered

by the environment or other external events. Providing control over the drug delivery can be the most important factor at times when traditional oral or injectable drug formulations cannot be used. These include situations requiring the slow release of water-soluble drugs, the fast release of low-solubility drugs, drug delivery to specific sites, drug delivery using nanoparticulate systems, delivery of two or more agents with the same formulation, and systems based on carriers that can dissolve or degrade and be readily eliminated. The ideal drug delivery system should be inert, biocompatible, mechanically strong, comfortable for the patient, capable of achieving high drug loading, safe from accidental release, simple to administer and remove, and easy to fabricate and sterilize.

In this study it was aimed to characterize the C/GAG hydrogels containing codeine to achieve a controlled release profile suitable for subcutaneous administration so the investigation were made in pure water.

It may be mentioned that these types of formulations based on cellulose and condroitin sulphate have not been studied or proposed as hydrogels for biomedical applications.

MATERIALS AND METHODS

Microcrystalline cellulose (C) - Avicel HP-101 (Fluka) and chondroitin sulphat (GAG) powder were both separated in National Institute of Searching and Development for Biological Science laboratories, Bucharest.

The hydrogel samples were prepared in various mixing ratios: 90/10, 80/20, 70/30, 60/40, 50/50 C/GAG purified by washing with warm water and dried in air, at room temperature.

Cellulose/chondroitin sulphate hydrogels were produced by a crosslinking technique. In other study of ours the cellulose/chondroitin sulphate hydrogels have been tested for the controlled release of paracetamol and theophylline in a phosphate buffer solution (Oprea, 2008) with promising results.

In this study were used 80/20, 60/40 and 50/50 C/GAG formulations.

Codeine or methylmorphine is an opiate used for its analgesic, antitussive and antidiarrheal properties. It is one of the most effective orally-administered opioid analgesics and has a wide safety margin. It is from 8 to 12 percent of the strength of morphine in most people; differences in metabolism can change this figure as can other medications.

The *kinetics of the swelling* was carried out by weight measurements performed at 37 ^{0}C, in bidistilated water, the hydrogels samples being periodically removed and changes in weight were measured before and during swelling. The swelling degree was calculated according to the equation (1).

$$Q_{\max} = (W_t - W_d) / W_d \times 100(\%) \quad (1)$$

where W_t is the weight of the samples after swelling in water at time t and W_d is the dry weight of the sample.

The *drug loading* of the hydrogel matrices was carried out by mixing the drug used (codeine) with dried hydrogel in powder form using as a release medium physiological serum and then a certain quantity of was added and left to swell at room temperature, while the drug penetrate and/or attached into matrices. The drug-loaded samples were freeze-dried using a Labconco FreeZone device. During the *drug release* study, at predetermined time intervals, 1 ml sample was withdrawn from the release medium and concentration of codeine at 284 nm in the release medium were determined using a UV-VIS spectrophotometer HP 8450A.

To determine the kinetics of solvent diffusion into the hydrogels the following equation was used:

$$F_t = \frac{W_t}{W_{eq}} = k_{sw} t^{n_{sw}} \quad (2)$$

where W_t and W_{eq} represent the amount of water absorbed by the hydrogel at time t and at equilibrium respectively, k_{sw} is the swelling constant characteristic of the system and n_{sw} is the power law diffusion exponent which takes into account the type of solvent transport. Eq. 2 applies to initial states of swelling and linearity is observed when log F_t as a function of log t is represented.

In order to elucidate the kinetics of drug release, the data were further analyzed using the equation proposed by Korsmeyer and Peppas (Eq.3):

$$M_t / M_\infty = k_r t^{n_r} \quad (3)$$

where Mt/Mα represents the fraction of the drug released at time t, k_r is a constant incorporating characteristics of the macromolecular network system and n_r is the diffusion exponent, which is indicative of the release mechanism.

In the equations above a value of $n_{sw}/n_r = 0.5$ indicates a Fickian diffusion mechanism of solvent/drug in hydrogels, while a value $0.5 < n_{sw}/n_r < 1$ indicates an anomalous or non-Fickian behavior. When $n_r = 1$ a case II transport mechanism is involved while $n_r > 1$ indicates a special case II transport mechanism (Katime, 2001; Korsmeyer, 1984).

Percent Hemolysis Test

Blood was obtained from healthy patients drawn by routine venipuncture from the antecubital vein in tubes containing EDTA. The blood was stored refrigerated for no more than 2 days until its use. Prior to hemolysis testing all the hydrogel samples were sterilized by ultraviolet light trans-illumination for 2 min. Each hydrogel preparation was tested with blood from a single patient. Distilled water was used as positive control and plasma separated from the same blood as negative control. From each tube, 1.5 mL of blood were drawn and put into contact with hydrogels in Eppendorf centrifuge tubes (2 mL). The blood samples in contact with the biomaterials were incubated at 37 °C for 2 h. After the incubation time the samples were centrifuged at 5000 rpm for 6 min. The separated plasmas were diluted 11 fold with Tris (62.5 mmol/L, pH 8.0 adjusted with HCl) prior to spectrophotometrical measurements. The remaining 0.5 ml of blood in each tube were centrifuged at 5000 rpm and separated plasmas were diluted 11 fold with Tris, the resulting solutions being used as negative controls. The positive control was prepared by hemolysing blood with distilled water (1:11 dilution). The hemolysed solution was also incubated at 37 °C for 2 h. Finally, the positive control solution was diluted 100 fold for spectrophotometric analysis. The method used for measuring plasma hemoglobin concentration in all the specimens was the polychromatic method of Noe et al. (Noe, 1984). Absorbance was measured at 380nm, 415nm and 470nm and the formula used for evaluation was:

$$C(mg/L) = 1.65\ mA_{415} - 0.93\ mA_{380} - 0.73\ mA_{470}$$

where C is the hemoglobin concentration in mg/L, mA_{380}, mA_{415} and mA_{470} are the absorbances at 380nm, 415nm and 470nm expressed in miliabsorbance units. The results were expressed as:

$$\text{hemolysis percent (\%)} = (C - C_n)/(C_p - C_n) \times 100$$

where C is the concentration of hemoglobin in the sample, C_n the concentration of hemoglobin in the negative control and C_p the concentration of hemoglobin in the positive control.

RESULTS AND DISCUSSION

Swelling kinetic studies

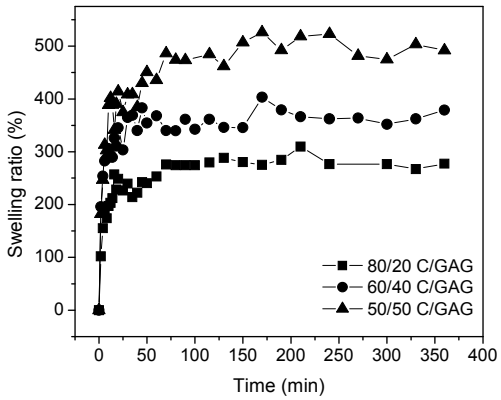

Figure 3. Swelling profiles of C/GAG hydrogels in physiological serum, at 37 ^{0}C

Table 1 presents the kinetic parameters of swelling, performed in bidistillated water, for C/GAG hydrogel samples with various compositions.

Table 1. Kinetic parameters of swelling for C/GAG hydrogels

Hydrogels	k_{sw} (min^{-1})	n_{sw}
80/20 C/GAG	0.41	0.21
60/40 C/GAG	0.58	0.14
50/50 C/GAG	0.41	0.22

The hydrogels swelling ratio (figure 3) increases with increasing GAG content and the values obtained for swelling parameter (n_{sw}) (table 1) in bidistillated water varies in range between 0.14-0.22 indicating an anomalous mechanism of swelling.

Kinetic of drug released

Codeine release

The release kinetic profiles and release rate profiles of codeine from C/GAG-based hydrogels, with different compositions, are showed in figure 4 and 5.

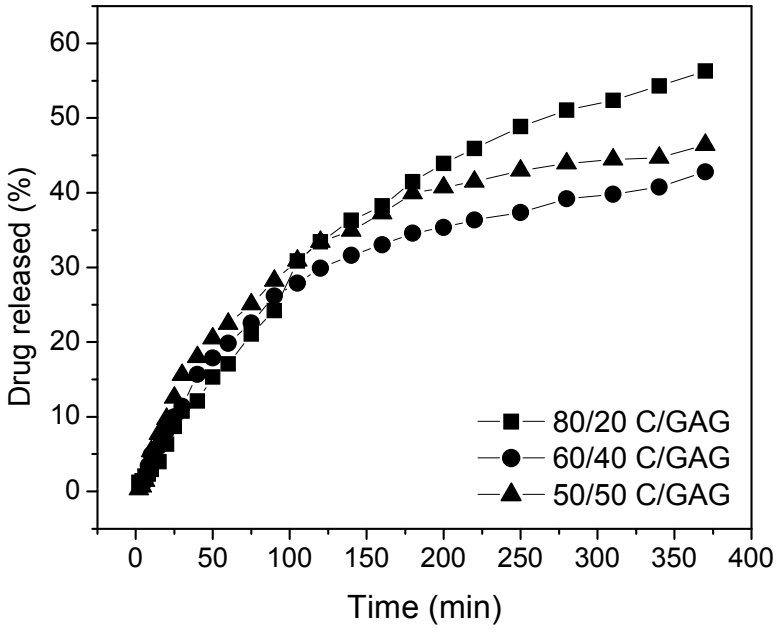

Figure 4. Release profiles of codeine from C/GAG-based hydrogels with different compositions, in physiological serum at 37 ^{0}C

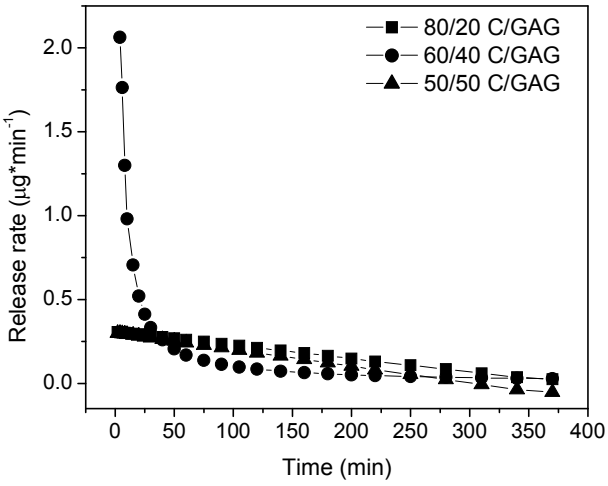

Figure 5. Release rate profiles of codeine from C/GAG-based hydrogels in, physiological serum, at 37 ^{0}C

The results showed that the release of codeine from C/GAG-based hydrogels depends on GAG content, so an increase of GAG content leads to a decrease of codeine percent released (case of 60/40 and 50/50 C/GAG compositions) with more than 15 % and a slower release rate.

The kinetic parameters for codeine released in physiological serum from C/GAG-based hydrogels with various compositions are presented in table 2.

Table 2. The kinetic parameters of codeine released from C/GAG hydrogels

Hydrogels	n_r	$k_r(min^{-n})$
80/20C/GAG	0.91	0.004
60/40C/GAG	0.78	0.008
50/50C/GAG	0.71	0.01

The values of n_r obtained for C/GAG hydrogels loaded with codeine indicate an anomalous transport mechanism for all formulations and rate coefficient (k_r) increases 100 times with increasing GAG content.

Hemolysis test

The hemolysis test showed that the hemolysis percentages of all the blood samples in contact with the hydrogels were negative. All percentages were less than 1% (table 3) compared to the positive control which is not significantly different than the negative control. The errors below 5% are admitted for this test.

Table 3. Hemolysis percentage of the three hydrogel formulations tested

Sample	Hemolysis percentage (%)
80/20 C/GAG	0.0512
60/40 C/GAG	-0.1136
50/50 C/GAG	0.1561

CONCLUSIONS

Cellulose/chondroitin sulphate hydrogels were produced by a crosslinking technique.

The swelling and drug release studies in pure water showed that an increase of GAG content in hydrogels composition leads to a higher swelling ratio and a decrease of codeine percent released and very small release rate.

The biocompatibility testing was made by hemolysis (plasma hemoglobin) technique, the results obtained showed a good biocompatibility between hydrogels and blood.

REFERENCES

Ichikawa, H., Fukumori, Y., 2000. *Journal of Controlled Release*, 63, 107–119.
Matsumoto, A., Ikeda, S., Harada, A., & Kataoka, K., 2003. *Biomacromolecules*, 4, 1410–141.
Choi, Y.S., Hong, S.R., Lee, Y.M., Song, K.W., Park, M.H., Nam, Y.S., 1999. *Biomaterials*, 20, 409–417.
Yannas, I.V., Lee, E., Orgill, D.P., Skrabut, E.M., Murphy, G.F., 1989. *Proceedings of the National Academy of Sciences of the United States of America*, 86, 933–937.
Lim, F., Sun, A.M., 1980. *Science*, 210, 908–910.
Uludag, H., Vos, P.D., Tresco, P.A., 2000. *Advanced Drug Delivery Reviews*, 42, 29–64.
Lee, K.Y.,Mooney, D.J., 2001. *Chemical Reviews*, 101, 1869–1880.
Tateishi, T., Chen, G., Ushida, T., 2002. *Journal of Artificial Organs*, 5, 77–83.
Wichterle, O., Lim, D., 1960. *Nature*, 18, 117–118.
Yamada, K., 2000. *Trends Glycosci Glycotechnol*, 12(67), 321–49.
Zou, X.H., Jiang, Y.Z., Zhang, G.R., Jin, H.M., Hieu, N.T.M., Ouyang, H.W., 2009. *Acta Biomaterialia,* xxx, xxx–xxx.
Bukalo, O., Schachner, M., Dityatev, A., 2001. *Neuroscience*, 104, 359–369.
Bali, J.P., Cousse, H., Neuzil, E.S., 2001. *Seminars in Arthritis and Rheumatism*, 31, 58–68.
Tomita, N., Sando, S., Sera, T., Aoyama, Y., 2004. *Bioorganic and Medicinal Chemistry letters*, 14, 2087.
Phillips, T., 1998. *Archives of Dermatology*, 134, 344–348.
Lee, C.T., Kung, P.H., Lee, Y.D., 2005. *Carbohydrate Polymers*, 61, 348–354.
Sakai, S., Onose, J., Nakamura, N., Toyoda, H., Toida, T., Imanari, I., Linhardt, R.J., 2002. *Anal. Biochem*., 302(2), 169-174.
Yamada, S., Sugahara, K., 2008. *Current Drug Discovery Technologies*, 5, 289-301.
Gudemez, E., Eksioglu, F., Korkusuz, P., Asan, E., Gursel, I., Hasirci, V., 2002. *J. Hand. Surg*., 27, 293-306.
Kirker, K.R., Nielson, J.H., Shelby, J., Prestwich, G.D., 2002. *Biomaterials,* 23, 3661-71.
Wang, S.C., Chena, B.H., Wang L.F., Chenb, J.S., 2007. *International Journal of Pharmaceutics*, 329, 103–109.
Varghesea, J.M., Ismail, Y.A., Lee, C.K., Shin, K.M., Shin, M.K., Kim, S.I., So, I., Kim, S.J., 2008. *Sensors and Actuators B,* 135, 336–341.
Jonas, R., Farah, L.F., 1998. *Polym. Degrad. Stab*., 59, 101-106.
Miranda, B.T., Miranda, S.R., Chan, L.P. Saqueton, E.R., 1965. *Nat. Appl. Sci. Bull*., 19(1), 67-79.
Nishi, Y., Uryu, M., Yamanaka, S., Watanabe, K., Kitamura, N., Iguchi, M., Mitsuhashi, S., 1990. *J. Mater. Sci*., 25(6), 2997-3001.
Shah, J., Brown, R.M., 2005. *Appl. Microbiol. Biotechnol*., 66(4), 352-355.
Yano, H., Sugiyama, J., Nakagaito, A.N., Nogi, M., Matsuura, T., Hikita, M., Handa, K., 2005. *Adv. Mater.*, 17(2), 153-5.
Czaja, W.K., Young, D.J., Kawecki, M., Brown, R.M., 2007. *Biomacromolecules*, 8(1), 1-12.
Ciechanska, D., Struszczyk, H., Guzinska, K., 1998. *Fibres Text. East. Eur*. 6(4), 61-65.
Czaja, W., Krystynowicz, A., Bielecki, S., Brown, R.M., 2006. *Biomaterials*, 27(2), 145-151.
Legeza, V.I., Galenko-Yaroshevskii, V.P., Zinovev, E.V., Paramonov, B.A., Kreichman, G.S., Turkovskii, I.I., Gumenyuk, E.S., Karnovich, A.G., Khripunov, A.K., 2004. *Bull. Exp. Biol. Med.*, 138(3), 311-315.
Klemm, D., Udhardt, U., Marsch, S., Schumann, D.I., 1999. *Polym. News*, 24(11), 377-378.
Klemm, D., Schumann, D., Udhardt, U., Marsch, S., 2001. *Prog. Polym. Sci.* 26(9), 1561-1603.
Backdahl, H., Helenius, G., Bodin, A., Nannmark, U., Johansson, B.R., Risberg, B., Gatenholm, P., 2006. *Biomaterials*, 27(9), 2141-2149.
Helenius, G., Backdahl, H., Bodin, A., Nannmark, U., Gatenholm, P., Risberg, B., 2006. *J. Biomed. Mater. Res. Part A*, 76(2), 431-438.
Svensson, A., Nicklasson, E., Harrah, T., Panilaitis, B., Kaplan, D.L., Brittberg, M., Gatenholm, P., 2005. *Biomaterials*, 26, 419-431.

Watanabe, K., Eto, Y., Takano, S., Nakamori, S., Shibai, H., Yamanaka, S., 1993. *Cytotechnology*, 13(2), 107-114.
Hong, L., Wang, Y.L., Jia, S.R., Huang, Y., Gao, C., Wan, Y.Z., 2006. *Mater. Lett.* 60(13-14), 1710-1713.
Wan, Y.Z., Hong, L., Jia, S.R., Huang, Y., Zhu, Y., Wang, Y.L., Jiang, H.J., 2006. *Compos. Sci. Technol.*, 66(11-12), 1825-1832.
Yasuda, K., Gong, J.P., Katsuyama, Y., Nakayama, A., Tanabe, Y., Kondo, E., Ueno, M., Osada, Y., 2005. *Biomaterials*, 26(21), 4468-4475.
Millon, L.E., Mohammadi, H., Wan, W.K., 2006. *J. Biomed. Mater. Res. Part B*, 79(2), 305-311.
Sannino, A., Esposito, A., Nicolais, L., Del Nobile, M. A., Giovane, A., Balestrieri, C., Esposito, R., Agresti, M., 2000. *Journal of materials science: materials in medicine*, 11, 247-253.
Uhrich, K.E., Cannizzaro, S.M., Langer, R.S., Shakesheff, K.M., 1999. *Chem. Rev.*, 99, 3181-3198.
Oprea, A.M., Ciolacu, D., Profire, L., Vasile, C., 2008. Days of the Faculty of Chemical Engineering and Environmental Protection, November 19-21, Iaşi.
Katime, I., Novoa, R., Zuluaga, F., 2001. *European Polymer Journal*, 37, 1465-1471.
Korsmeyer, R.W., Peppas, N.A., 1984. *J. Control. Rel.*, 1, 89.
Noe, D.A., Weedn, V., Beli W.R., 1984. *Clin. Chem.,* 30, 627-630.

1 „P. Poni" Institute of Macromolecular Chemistry, Physical Chemistry of Polymers Department, 41 A Gr. Ghica Voda Alley, 700487, Iasi, Romania, Tel: +40 232 217454, Fax: +40 232 211299
2 "Gr.T.Popa" Medicine and Pharmacy University, 16 University Street, 700115, Iasi, Romania, Tel: +40 - 0232/ 267.801, Fax: +40 - 0232/ 211.820
*aoprea@icmpp.ro

Acknowledgements: The authors thank for financial support of Romanian ANCS by the national grants IDEI 2561/2008 and IDEI 17/2007.