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Synthesis and characterization of some cellulose/chondroitin sulphate hydrogels and their evaluation as carriers for drug delivery

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ABSTRACT

Mixed hydrogels based on natural, biodegradable and biocompatible polysaccharides, such as cellulose (C) and chondroitin sulphate (CS) in various mixing ratios were prepared by a crosslinking technique and characterized by swelling behaviour, FTIR spectroscopy, scanning electron microscopy, toxicity and biocompatibility tests.

The mixed cellulose/chondroitin sulphate hydrogels have been loaded with 7-[2-nitroxiacetyl-oxy-3-(4-acetyl-amino-phenoxy)-propyl]-8-morpholino-1,3-dimethyl-xanthine, a novel nitric oxide donor compound with a lower toxicity and a higher anti-inflammatory activity than its parent molecules, paracetamol and theophylline. Swelling and release kinetics have been also studied. It has been established that an increase of CS content in hydrogels composition leads to a higher swelling ratio for all formulations and to a decreased released amount of nitric oxide donor compound. It has been found that the swelling occurs by an anomalous swelling mechanism, while the release of nitric oxide donor compound follows a diffusion controlled mechanism.

1. Introduction

Hydrogels are three-dimensional, hydrophilic, polymeric networks, with chemical or physical cross-links, capable of imbibing large amounts of water or biological fluids. The characteristic of hydrogels, including sensitivity to the environment, tissue-like water content and elasticity afford the potential for biomedical applications. For instance, hydrogels are used as carriers for drugs delivery (Ichikawa & Fukumori, 2000; Matsumoto, Ikeda, Harada, & Kataoka, 2003).

Novel controlled drug delivery systems are designed to deliver drugs at predetermined rates for predefined periods at the target organ and to overcome the shortcomings of conventional drug formulations therefore could diminish the side effects and improve the life quality of the patients (Gong et al., 2009).

Among the numerous macromolecules that can be used for hydrogels formation, polysaccharides are extremely advantageous compared to synthetic polymers being widely present in living organisms and often being produced by recombinant DNA techniques. Coming from renewable sources, polysaccharides also have frequently economical advantages over synthetic polymers. Polysaccharides are usually non-toxic, exhibit high biocompatibility and biodegradation ability and show a number of peculiar physico-chemical properties that make them suitable for different applications in drug delivery systems mainly because of their high water content and soft rubbery consistency giving them a certain resemblance to living tissue (Coviello, Matricardi, Marianecci, & Alhaique, 2007).

Cellulose is degradable by enzymes (Märtson, Viljanto, Hurme, Laippala, & Saukko, 1999) and its solubility in water depends on its chain length (Klemm, Philipp, Heize, Heinze, & Wagenknecht, 1998) being available in a wide range of forms and shapes, e.g. as membrane sponges, microspheres and non-woven, woven or knitted textiles. Chang, Duan, Cai, and Zhang (2010) prepared superabsorbent hydrogels from sodium carboxymethylcellulose (CMC) and cellulose in the NaOH/urea aqueous system by using epichlorohydrin (ECH) as cross linking agent, for applications in biomaterials area. Macroporous hydrogels prepared by blending cellulose and sodium alginate solution cross linked with epichlorohydrin were also, tested for biomedical applications (Chang, Duan, & Zhang, 2009).

The most recent advances in cellulose-based hydrogels aim not only at the sustained release of a bioactive molecule over a long time period, ranging from hours to weeks, but also at a space-controlled delivery, directly at the site of interest. The need to encapsulate bioactive molecules into a hydrogel matrix or other delivery devices

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(e.g., microspheres) is also related to the short half-life displayed by many biomolecules *in vivo* (Sannino, Demitri, & Madaghiele, 2009).

Controlled release through oral drug delivery is usually based on the strong pH variations encountered when transitioning from the stomach to the intestine. Cellulose-based polyelectrolyte hydrogels (e.g., hydrogels containing NaCMC) are particularly suitable for this application. For instance, anionic hydrogels based on carboxymethyl cellulose have been investigated recently for colon-targeted drug delivery (El-Hag Ali, Abd El-Rehim, Kamal, & Hegazy, 2008;Chang & Zhang, 2011).

Chondroitin sulphate is an important structural component in connective tissues and cartilages. It provides compressive strength to connective tissues by regulating their water content, and possesses characteristic features, such as high water absorption, multifunctionality and biodegradability characteristics suitable for bioapplications (Wang et al., 2007; Comper & Zamparo, 1990). The readily water-soluble nature of chondroitin sulphate limits its application as a solid-state drug delivery vehicle. Therefore, it is usual to carry out a cross linking treatment to tailor the properties of chondroitin sulphate as reported in several works (Sintov, Di-Capua, & Rubinstein, 1995) or to combine it with other polymers, such as chitosan (Espirito Santo, Gomes, Mano, & Reis, 2009; Park et al., 2000), gelatin and hyaluronan (Chang, Liu, Lin, Chou, & Lin, 2003; Fan et al., 2006), collagen (Flanagan et al., 2006; Keskin, Tezcaner, Korkusuz, Korkusuz, & Hasirci, 2005), poly(vinyl alcohol) (Lee, Kung, & Lee, 2005) or poly-(lactic-co-glycolic acid) (Fan et al., 2006) in order to produce more stable materials.

Up to now, after our knowledge, the combination of the cellulose and chondroitin sulphate, for obtaining new hydrogels and their potential for biomedical applications, such as drug delivery, has not been yet exploited.

Nitric oxide synthesized in endothelial cells that line blood vessels has a wide range of functions vital for maintaining a healthy cardiovascular, nervous and immune systems (Bath, 1993). Delivery of exogenous NO is an attractive therapeutic option, particularly with a view to slowing progression of atherosclerosis and reducing the risk of thrombosis (Megson & Webb, 2002). Organic nitrates were first used to relieve the symptoms of angina over a century ago, long before the identification of NO as an endogenous messenger and, despite limitations, they remain the most commonly used NO donor drugs in cardiovascular medicine (Megson & Webb, 2002). New compounds, in which NO donor groups are linked to the various classical parent molecules like NO-aspirin (NCX-4016, NCX-4215), NO-flurbiprofen (HCT-1026, NCX-2216), NO-naproxen (AZT-3582), NO-diclofenac, NO-indometacin, NO-ketoprofen, NO-sulindac, NO-rofecoxib, NO-sildenafil, NO-enalapril, NO-salbutamol (NCX-950), have been synthesized (Napoli & Ignarro, 2003; Burgaud, Ongini, & Del Soldato, 2002; Ignarro, Napoli, & Loscalzo, 2002; Megson, 2000; Yamamoto & Bing, 2000). This strategy has as purpose to identify new molecules with an improved pharmacological profile in terms of increasing the therapeutically efficiency and the reduction of the side effects.

New nitric oxide donors, where two parent molecules namely theophylline and paracetamol, are linked by alkyl nitric oxide donor chain (as nitroxiacetyl-oxy-propyl) have been synthesized (Danila, Profire, Bumbu, & Vasile, 2000; Profire, Bumbu, Costuleanu, Danila, & Vasile, 2002; Profire, Lupascu, Sunel, Bibire, & Vasile, 2010; Profire, Sunel, et al., 2010).

In continuation of our previous research on the development of new drugs (Danila et al., 2000; Profire et al., 2002) with prolonged release utilizing carbohydrate polymers (Cheaburu, Vasile, Duraccio, & Cimmino, 2009; Dumitriu, Oprea, & Vasile, 2009; Vasile, Dumitriu, Cheaburu, & Oprea, 2009) we herein present the work on the release of a novel NO-donor compound namely 7-[2-nitroxiacetyl-oxy-3-(4-acetyl-amino-phenoxy)-propyl]-8-morpholino-1,3-dimethyl-xanthine from cellulose/chondroitin sulphate matrices.

Table 1
Characteristics of chondroitin sulphate.

Characteristic	Value
Uronic acids (g/100 g d.s.)	33,2
Nitrogen total % (g/100 g d.s.)	3,5
Hexozamine (g/100 g d.s.)	25,7
Sulphur as sulphate (g/100 g d.s.)	5,5
Average number molecular weight (Da)	35.000

2. Experimental

2.1. Materials

Microcrystalline cellulose–Avicel® PH-101 with the polymerisation degree of 183 was supplied by Sigma-Aldrich.

Chondroitin sulphate (CS) was purchased from Roth, Germany. It was obtained from bovine tracheal cartilage. The average characteristics of chondroitin sulphate were presented in Table 1.

Epichlorohydrin used as crosslinking agent of analytical purity was purchased from Sigma-Aldrich.

2.2. Preparation of cellulose/chondroitin sulphate hydrogels

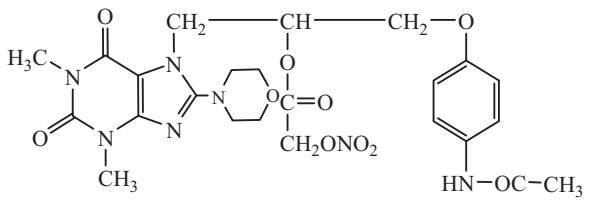
The hydrogel samples were prepared in various mixing ratios (wt%) as: 90/10, 80/20, 70/30, 60/40, 50/50 and produced by a cross linking technique.

The cellulose was initially dissolved in 6.725 ml of 9 wt%, NaOH solution and then it was frozen by holding at -30 °C for 24 h. By this way it is transformed in cellulose (C) (Klemm et al., 1998). In the next step the frozen mass was thawing at room temperature and then the chondroitin sulphate in different ratios was added. The resulted mixture was undergone to crosslinking reaction in presence of 2.1431 ml of freshly distilled epichlorohydrin, under vigorously continuous stirring (Isogai & Atalla, 1998).

The gel-like samples were lied on glass plates then the cross linking reaction was performed for 8 h at 80 °C. The cellulose/chondroitin sulphate hydrogels were extensively washed with warm water in order to remove unreacted compounds, especially epichlorohydrin traces. Obtained hydrogels were undergone to freeze drying for 10 h by means of a LABCONCO FreeZone device.

The synthesis, structure, characterization and pharmacological activity of NO-donor compound have been previously presented and studied (Profire et al., 2011). It was found that it presents lower toxicity and 5 times higher anti-inflammatory effect than its parent molecules, paracetamol and theophylline. The structure of the 7-[2-nitroxiacetyl-oxy-3-(4-acetyl-amino-phenoxy)-propyl]-8-morpholino-1,3-dimethyl-xanthine compound is given in Scheme 1.

The release of the 7-[2-nitroxiacetyl-oxy-3-(4-acetyl-amino-phenoxy)-propyl]-8-morpholino-1,3-dimethyl-xanthine



Scheme 1. Structure of the NO-donor compound 7-[2-nitroxiacetyl-oxy-3-(4-acetyl-amino-phenoxy)-propyl]-8-morpholino-1,3-dimethyl-xanthine.

compound from cellulose/chondroitin sulphate hydrogels of different compositions was followed.

Due to the fact that the NO-donor compound is partially soluble in fluids with physiological pH, its complete solubilisation was achieved in alcoholic medium. The necessary amount of NO-donor compound was dissolved in a small quantity of ethanol and after that the water was added to alcoholic solution to obtain a 90:10 water:ethanol mixture. The 7-[2-nitroxiacetyl-oxy-3-(4-acetyl-amino-phenoxy)-propyl]-8-morpholino-1,3-dimethyl-xanthine compound remains soluble in this mixture and solution was stable entire period of study. The swelling and *in vitro* drug release studies were also performed in the 90:10 water:ethanol mixture.

2.3. Investigation methods for hydrogels

2.3.1. Swelling tests

Swelling studies were performed for all formulations and carried out by direct immersion in 90:10 water:ethanol mixture at 37 °C, where the studied drug presents high solubility.

The hydrogels samples were maintained for 24 h at 37 °C, periodically removed from the solution, gently wiped with a soft tissue to remove excess surface solution, weighed and than placed back into the vessel as quickly as possible. The degree of swelling (S) was calculated according to the equation:

$$S(\%) = \frac{(W_t - W_d)}{W_d} \times 100 \quad (1)$$

where W_t is the weight of the swelled samples at time t and W_d is the weight of dry sample.

To determine the kinetics of solvent diffusion into the matrices (swelling) the following equation was used (Berens & Hopfenberg, 1978):

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

where W_t and W_{eq} represent the amount of water:ethanol solution, absorbed by the matrices at time t and at equilibrium, respectively, k_{sw} is the specific swelling rate or rate constant characteristic to system and n_{sw} is the power law diffusion exponent which takes into account the type of solvent transport. Eq. (2) is applied to initial states of swelling (swelling degree less than 60%) and linearity is observed when ln Ft as a function of ln t is represented.

2.3.2. Scanning electron microscopy

The hydrogel samples were frozen by direct immersion in liquid nitrogen and fractured. After metallization with gold the samples examination was performed on a Quanta 3D Scanning Electron Microscope (USA). Magnification is given on pictures.

2.3.3. FT-IR spectroscopy

The hydrogels loaded with NO-donor compound were analyzed by FT-IR spectroscopy, using the KBr pellet technique. The spectra were scanned on a Bruker VERTEX 70 (USA) device, over the 4000–500 cm^{-1} range, at a resolution of 4 cm^{-1}.

2.3.4. Toxicity tests

White Swiss strain male mice weighing between 20–25 g purchased from Cantacuzino Institute, Bucharest, Romania were used for the study. The animals were maintained in identical laboratory conditions for one week before starting the experiment having access at food and water *ad libitum*.

Each composition was tested on 5 mice.

The tested hydrogels have been intraperitoneal delivered as suspensions in sodium carboxymethylcellulose 0.5 wt%. The death rate has been recorded up to 2 weeks.

All the experiments were performed in accordance with the ethical rules stated in the paper "Ethical guidelines for investigations of experimental pain in conscious animals" (Zimmermann, 1983).

2.3.5. Biocompatibility test

The white Swiss strain male mice weighing between 23–34 g treated as before were randomly divided into three groups of seven animals each and noted as groups I to III: group I (control) received physiological serum, group II received 90/10 of C/CS and group III received 80/20 of C/CS.

The tested 90/10 of C/CS and 80/20 of C/CS hydrogels were intraperitoneal delivered as suspensions in sodium carboxymethylcellulose 0.5 wt%, in a single daily dose of 160 mg/kg body for 14 consecutive days, following the European Community guidelines. The control group was injected with in a daily dose of 0.2 ml physiological serum/10 g body. Each injection was performed through a syringe equipped with a gauge 21G needle.

The death rate has been monitorized up to 2 weeks.

Behavioral or physical changes such as abdominal swelling were not seen in treated mice following injection or on subsequent days. Throughout the study period, animals showed no signs of peritonitis, lethargy, muscle loss, dehydration or anorexia; symptoms which are associated with animal toxicity (Vassileva, Grant, De Souza, Allen, & Piquette-Miller, 2007; Stokes, 2002). The mice were euthanized 14 days after injection by an overdose of ethyl ether. All the protocols using the animals were carried out according with ethical rules presented by Zimmermann in "Ethical guidelines for investigations of experimental pain in conscious animals" (Zimmermann, 1983).

The biological samples, such as blood, intraperitoneal liquid and spleens were harvested in order to determine the hemogram, the phagocytic capacity of neutrophils (NBT test), the opsonic capacity of serum, the phagocytic and bactericid capacities of peritoneal macrophages and the hepatotoxic effects by determining the enzymatic levels of aspartate aminotransferase (AST), serum alanine aminotransferase (ALT) and lactate dehydrogenase (LD).

2.3.5.1. Hematological studies. Blood samples were collected from all the animals in each group by cardiac puncture after general anesthesia of animals with ethyl ether.

Blood was collected in EDTA tubes for estimation of hematological parameters such as total leukocyte count (TLC) and differential leukocyte count (DLC), hemoglobin concentration (HB), hematocrit (Ht), total erythrocyte count (TEC), using an HEMAVET 950 FS device from Drew Scientific Group (England).

2.3.5.2. Phagocytic capacity of peripheral neutrophils. The blood was collected into heparinized test tubes and evaluated by NBT test. The NBT test measures the percentage of actively phagocytosing (positively stained) neutrophils in blood smears (Peacock et al., 1980). Briefly, the cells were incubated for 30 min at 37 °C and then aliquots of NBT (Nitro Blue Tetrazolium—Sigma) solution was added into the cells and incubated for 1 h at 37 °C. Blood smears were observed through a light microscope in immerse magnification to determine the percentage of positively stained neutrophils (neutrophils which contained formazan deposits with size of 1 lobe, at least, of the nucleus were defined as positive) and showed active involvement in phagocytosis.

2.3.5.3. Serum opsonic capacity and peritoneal macrophages activity. Serum opsonic capacity and macrophages function (phagocytosis and bactericidal activity) were assessed after 14 days of treatment using the Shortmann and Palmer technique (Shortman & Palmer, 1971). Bacteria used were *Staphylococcus aureus*, grown in broth overnight, centrifuged and washed twice in phosphate-buffered saline. The final suspensions were adjusted with a nephelometer to

a standard concentration of approximately 1×10^6 colony forming units/mL.

2.3.5.4. Spleenic lymphocytes with rosetting capacity. Spleenic lymphocytes with rosetting capacity were assessed after 14 days of treatment (Jerne & Nordin, 1963).

2.3.5.5. Biochemical study. The collected serum was used to estimate biochemical parameters such as aspartate aminotransferase (AST), serum alanine aminotransferase (ALT) and lactate dehydrogenase (LD) using a CORMAY ACCENT 200 device (Poland).

2.3.5.6. Statistical analysis. Statistical analysis of the data was performed using the GraphPad Prism software (version 5.0). Statistical significance of differences between data was evaluated by one-way ANOVA using the Tukey test. A value of $p < 0.05$ was considered significant. All data are presented as mean ± standard deviation.

2.3.6. Drug loading and in vitro release studies

The drug loading of the hydrogel matrices was carried out by mixing 7-[2 nitroxiacethyl-oxy-3-(4-acetyl-amino-phenoxy)-propyl]-8-morpholino-1,3 dimethyl-xanthine derivative novel NO-donor compound with dried matrices in powdered form, and then a certain quantity of the appropriate solvent (maximum amount of liquid uptake during swelling) was added and left to swell at room temperature at least one hour, while the drug penetrates and/or attached into matrices. The drug concentrations solution was 18 mg/mL. At the end, the drug-loaded samples were freeze-dried using a Labconco FreeZone device.

In vitro release studies have been conducted by a standard dissolution set-up (Oh, Cho, & Choi, 2004). The dissolution medium was 90:10 water:ethanol. During dissolution testing, the media was maintained at 37 ± 0.5 °C. Aliquots of the medium of 1 mL were withdrawn periodically at predetermined time intervals and analyzed at λ_{max} value of 277 nm using a HP 8450A UV–visible spectrophotometer. In order to maintain the solution concentration the sample is carefully reintroduced in the circuit after analysis.

The concentrations of the drugs were calculated based on calibration curves determined for drug at specific maximum absorption wavelength.

A simple, semi-empirical equation using Korsmeyer and Peppas model was used to kinetically analyze the data regarding the drug release from studied matrices system which is applied at the initial stages (approximately 60% fractional release) (Peppas & Korsmeyer, 1986):

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

where M_t/M_∞ represents the fraction of the released drug, M_t and M_∞ are the absolute cumulative amount of drug released at time t and at infinite time (in this case maximum release amount in the experimental conditions used, at the plateau of the release curves), respectively, k_r is a constant incorporating characteristics of the macromolecular matrix and the drug n_r is the diffusion exponent, which suggests the release mechanism. In the equation above a value of $n_r = 0.5$ indicates a Fickian diffusion mechanism of the drug from matrix, while a value $0.5 < n_r < 1$ indicates an anomalous or non-Fickian behaviour. When $n_r = 1$ a case II transport mechanism is involved while $n_r > 1$ indicates a special case II transport mechanism (Korsmeyer & Peppas, 1984; Serra, Domenechc, & Peppas, 2006).

The corresponding drug-release profiles were represented through plots of the cumulative percentage of drug release versus time.

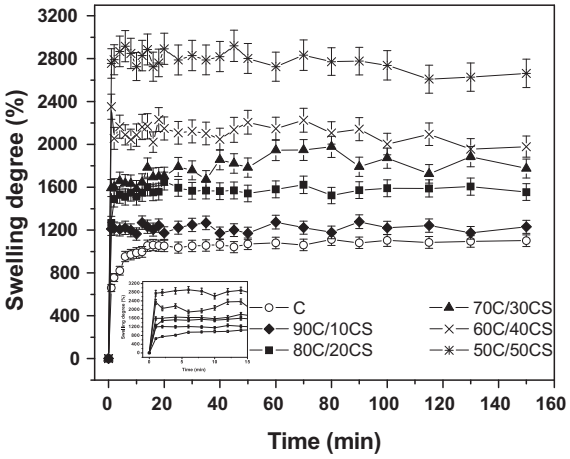


Fig. 1. Swelling profiles of C/CS hydrogels with different compositions.

3. Results and discussion

3.1. Matrix characterization

3.1.1. Swelling studies

The chemical composition of the hydrogels components affects the swelling ratio of the matrices which is directly related to loading and release of drugs from the formulations. In the hydrogels system, absorption of solvent from the environment changes the dimensions of the pores and physico-chemical properties of the system and thus influences the drug release kinetics. Swelling studies were performed in 90:10 water:ethanol solution, at 37 °C.

Swelling ratio of ionic gels such as C/CS hydrogels is determined by two main factors, one is the electrostatic repulsion between the polymer chains and other is ions present in swelling medium (Lee et al., 2005).

Examination the swelling profiles presented in Fig. 1 it appears that the swelling occurs rapidly in the first 2 min and is faster for hydrogels with increasing chondroitin sulphate content of hydrogels–see insert. The time to reach maximum swelling degree is about 16 min for cellulose and under 2 min for bicomponent hydrogels.

The swelling ratio of the C/CS hydrogels increases with the amount of CS. The 50/50 of C/CS hydrogel, containing 50% cellulose and 50% CS, has the highest degree of swelling of 2931.8 wt%. The maximum swelling degree for bicomponent C/CS hydrogels gradual decreases with increasing percentage of cellulose, reaching in case of cellulose-based hydrogels to a value of 1201.6 wt%. The high water quantity uptake and faster swelling can be attributed to the existence of groups with negative charges in CS structure, such as $-COO^-$ and $-SO_3^-$, which allow the gels to swell highly, conferring a high concentration of negative charge in the regions that contain them. The presence of ionization groups on CS chain causes the strong repulsion of negative charges and polar groups so the network chain segments apart and thus attract more water into the hydrogels, so a higher swelling ratio was observed. The swelling of the hydrogels will be much pronounced than that of non-polyelectrolyte hydrogels (Zhang, Luo, & Li, 2007; Comper & Zamparo, 1990).

3.1.2. Kinetic studies of swelling

In Table 2, the kinetic parameters of swelling are given for C/CS hydrogel samples with various compositions.

The values of kinetic parameter (n_{sw}) obtained for C/CS hydrogels indicates an anomalous swelling mechanism. The swelling kinetic constant increases with CS content in hydrogels

Table 2
Kinetic parameters of swelling for C/CS hydrogels.

Compositions (%)	n_{sw}	k_{sw} (min^{-n})
Cellulose C	0.16	0.58
90/10 C/CS	0.13	0.47
80/20 C/CS	0.04	0.63
70/30 C/CS	0.04	0.31
60/40 C/CS	0.06	0.62
50/50 C/CS	0.03	0.93

compositions, therefore the rate of swelling (k_{sw}) increases with CS content because increased repulsive forces and formation of a more relaxed network.

3.1.3. Scanning electron microscopy

Fig. 2 shows the SEM images of the cross-section of freeze-dried hydrogel samples.

The cross-sections of the samples exhibited a large numbers of pores. The cellulose hydrogel had a relatively smooth porous structure, whereas mixed hydrogels showed rough porous network structure. The micrographs revealed also that the CS contributed to the decreasing of pore size (Table 3), whereas cellulose acted as a backbone in the hydrogel to strengthen it. Therefore, the water molecules could easily diffuse into hydrogels, leading to higher swelling ratio.

3.1.4. Toxicity tests

All C/CS hydrogels were obtained by a cross linking technique using epichlorohydrin. Because there are many controversies concerning the carcinogenic effects of epichlorohydrin in unreacted state (Giri, 1997) being a direct acting mutagen (Sund & Kronberg, 2006) a very careful characterization of the matrices from the point of view of toxicity and biocompatibility have been made.

After synthesis, in order to be tested for their biocompatibility, the hydrogels were extensively washed with water at room temperature for 10 days, knowing that the hydrolytic half-life of epichlorohydrin being 8 days. Brönsted and Kilpatrick (1929) established that after 10 days higher than 96% of epichlorohydrin was removed, so it can suppose that its harmful effects will not more appear.

At the beginning, a test consisted on intraperitoneal administration of a single dose of 2000 mg/kg body hydrogels suspension at a mouse, for each composition, was performed. Because the mice survived up to 2 weeks after administration, for each composition, four other mice were injected and their survival was assessed. It was found that all mice survived 14 days after intraperitoneal administration of hydrogel suspensions.

The acute toxicity of a 5000 mg/kg body dose was tested according to OECD/OECD 425 guidelines (OECD guidelines for the testing of chemicals, Acute Oral Toxicity—Up-and-Down-Procedure (UDP), 2008). Due to technical problems for preparing the solutions with so high concentrations, a single dose of 3200 mg/kg body was tested. It was found that after intraperitoneal administration of a single dose of 3200 mg/kg body of hydrogels suspension to mice, for each composition, they survived 14 days after administration. It has been concluded that the LD_{50} for C/CS hydrogels, after intraperitoneal administration as suspensions, is bigger than 3200 mg/kg body. This is also a first proof that the unreacted ECH was totally removed from C/CS hydrogels compositions which are non-toxic.

Table 3
Pores dimensions for C/CS hydrogels with different compositions.

Hydrogel	Pores diameters (μm)
Cellulose	44.86
80/20 C/CS	40.3
60/40 C/CS	19.59
50/50 C/CS	5.54

3.1.5. Biocompatibility tests

The effects of tested compounds intraperitoneal injected at mice for 14 consecutive days on hematological and immune system parameters comparatively with a control group of mice which received physiological serum, are presented in Table 4.

3.1.5.1. Hematological parameters. The values of total leukocyte (TLC) and differential erythrocyte count (DLC), hematocrit values and hemoglobin concentration showed no significant variations between mice groups treated with 90/10 of C/CS and 80/20 of C/CS hydrogels compared to control mice group, being in range of normal limits reported for healthy mice (Schneck, Washington, Holder, Lodge, & Motzel, 2000; Weiss & Wardrop, 2010).

3.1.5.2. Phagocytic capacity of peripheral neutrophils. Neutrophils, being elements of the non-specific immune system, play a critical role in host defense mechanism against various bacterial infections. The reduction of the NBT dye is an indirect measure of bactericide function of neutrophils, O^{2-} radicals generation during phagocytosis process reducing the dye.

Statistical analysis revealed no significant influence of the studied compounds on the neutrophils phagocytic capacity, compared to control group, after 14 days of testing.

3.1.5.3. Serum opsonic capacity and peritoneal macrophages activity. Bacteria opsonization is an important incipient host defense mechanism required for optimal phagocytosis. The primary function of neutrophils and macrophages is the ingestion and killing of microorganisms.

Examination the data of Table 4, it results that the administration of 90/10 of C/CS and 80/20 of C/CS hydrogel suspensions did not influence the phagocytosis activity of immune cells. They had no significant influence on serum opsonic capacity, phagocytosis and bactericidal capacities of peritoneal macrophages compared to control group, after 14 days of testing.

3.1.5.4. Spleenic lymphocytes with rosetting capacity. The studied hydrogel suspensions had no significant statistically influence the spleenic lymphocytes with resetting capacity of tested mice compared to control group, after 14 days of testing.

3.1.5.5. Biochemical study. ALT and AST are two liver enzymes that are associated to the hepato-cellular damage. In liver diseases serum levels of AST and ALT rise and fall at the same time. Although both AST and ALT are common liver enzymes because of their higher concentrations in hepatocytes, only ALT is remarkably specific for liver function since AST is mostly present in the myocardium, skeletal muscle, brain and kidneys (Witthawaskul, Panthong, Kanjanapothi, Taesothikul, & Lertprasertsuke, 2003).

Data from Table 5 showed that it was not a significant statistically difference between the levels of AST, ALT and LD in the blood of tested mice injected with 90/10 of C/CS hydrogel suspension, comparing with the control mice group, after 14 days of testing.

There was a significant statistically increase of ALT and AST values in case of mice injected with 80/20 of C/CS hydrogel suspension after 14 days of testing but these values are in range of normal limits. There was also no significant effect on LD levels in case of mice injected with 80/20 of C/CS hydrogel suspension compared with that obtained for control mice group.

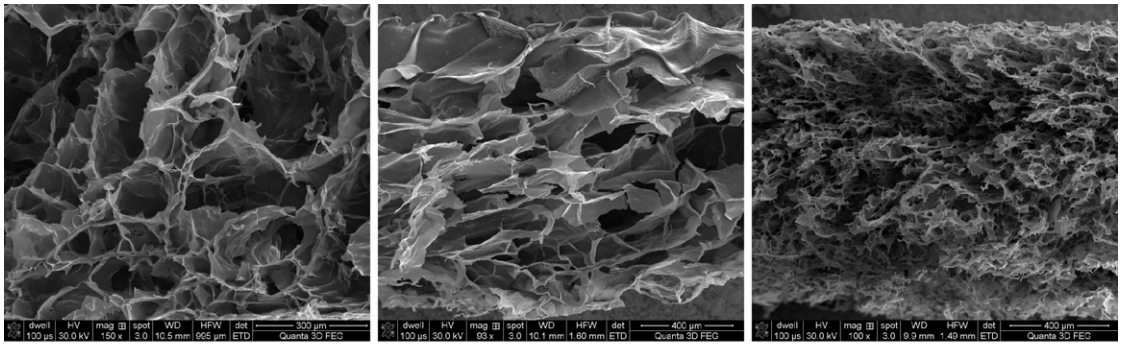


Fig. 2. SEM micrographs of the C/CS hydrogels with different compositions.

Table 4
Hematological and immune systems parameters (mean ± standard deviation STD) at mice intraperitoneal injected with 90/10 and 80/20 of C/CS hydrogel suspensions.

Hematological parameter	Tested mice groups		
	Control mice group	Mice group intraperitoneal injected with 90/10 C/CS hydrogel	Mice group intraperitoneal injected with 80/20 C/CS hydrogel
White blood cells ($\times 10^9$/L)	5.64 ± 0.13	5.61 ± 0.09	5.63 ± 0.1
Polymorphonuclear cells (PMN) ($\times 10^9$/L)	1.51 ± 0.06	1.49 ± 0.09	1.49 ± 0.11
Lymphocytes ($\times 10^9$/L))	3.68 ± 0.11	3.67 ± 0.11	3.67 ± 0.09
Monocytes ($\times 10^9$/L)	0.35 ± 0.05	0.35 ± 0.04	0.37 ± 0.02
Eosinophils ($\times 10^9$/L)	0.04 ± 0.02	0.05 ± 0.01	0.06 ± 0.02
Basophils ($\times 10^9$/L)	0.052 ± 0.03	0.053 ± 0.02	0.05 ± 0.02
Polymorphonuclear cells (PMN) (%)	26.82 ± 0.97	26.62 ± 1.71	27.26 ± 0.63
Lymphocytes (%)	65.26 ± 1.05	65.34 ± 1.3	65.64 ± 1.61
Monocytes (%)	6.23 ± 0.71	6.25 ± 0.7	6.57 ± 0.43
Eosinophils (%)	0.78 ± 0.36	0.84 ± 0.23	0.97 ± 0.28
Basophils (%)	0.93 ± 0.47	0.94 ± 0.36	0.89 ± 0.43
Red blood cells ($\times 10^9$/L)	9.39 ± 0.06	9.4 ± 0.07	9.39 ± 0.06
Hemoglobin level (g/dL)	11.47 ± 0.05	11.44 ± 0.05	11.38 ± 0.19
Hematocrit level (%)	41.03 ± 0.04	41.02 ± 0.07	41.01 ± 0.06
NBT test (%)	13.83 ± 0.75	13.71 ± 0.76	13.83 ± 0.75
Platelets ($\times 10^9$/L)	253 ± 38.82	252 ± 7.42	252 ± 9.57
Immune system parameter			
Serum opsonic capacity (*S. aureus* × 1000/mL)	771.67 ± 58.45	772.86 ± 52.19	766.67 ± 54.28
Phagocytic capacity of peritoneal macrophages (*S. aureus* × 1000/mL)	716.67 ± 51.64	727.14 ± 57.65	713.33 ± 53.54
Bactericidal capacity of peritoneal macrophages (*S. aureus* × 1000/mL)	696.67 ± 8.16	697.14 ± 9.51	695 ± 5.48
Splenic T lymphocytes (%)	12.5 ± 0.55	12.57 ± 0.53	12.5 ± 0.55

3.2. NO-donor compound loaded hydrogel characterization

3.2.1. FT-IR analysis

The FT-IR spectra of C/CS hydrogels loaded with 7-[2-nitroxiacetyl-oxy-3-(4-acetyl-amino-phenoxy)-propyl]-8-morpholino-1,3-dimethyl-xanthine NO-donor compound are shown in Fig. 3. In the FT-IR spectra the bands of both hydrogel components and NO-donor compound can be identified. They contain all characteristic bands of the components. The main band appearing at 2936 cm^{-1} is due to the aliphatic C–H stretching vibration. The IR bands about 1160 ± 30 cm^{-1} are dominated by the glycosidic linkage ν(C–O–C) contribution in polysaccharides. The band at 3335 cm^{-1}, is assigned to O_3–H. . .O_5 intramolecular hydrogen bonds. The band at about 2968 cm^{-1} is assigned to an asymmetric stretch vibration of CH_2 from the CH_2–OH group in cellulose, while the band at 2878 cm^{-1} derives from symmetric

Table 5
Serum biochemical parameters (mean ± standard deviation STD) at mice treated with 90/10 and 80/20 of C/CS hydrogel suspensions for 14 days.

Serum biochemical parameter	Tested mice groups		
	Control mice group	Mice group intraperitoneal injected with 90/10 C/CS hydrogel	Mice group intraperitoneal injected with 80/20 C/CS hydrogel
AST (U/L)			
Mean ± STD	23.17 ± 1.17	24.71 ± 1.98	32.17 ± 1.17
Range values	22.0–25.0	23.0–28.0	31.0–34.0
ALT (U/L)			
Mean ± STD	73.33 ± 1.75	74 ± 1.41	84.5 ± 1.64
Range values	72.0–76.0	72.0–76.0	82.0–87.0
LD (U/L)			
Mean ± STD	497.5 ± 3.33	499 ± 1	500.67 ± 1.51
Range values	492.0–500.0	498.0–500.0	499.0–503.0

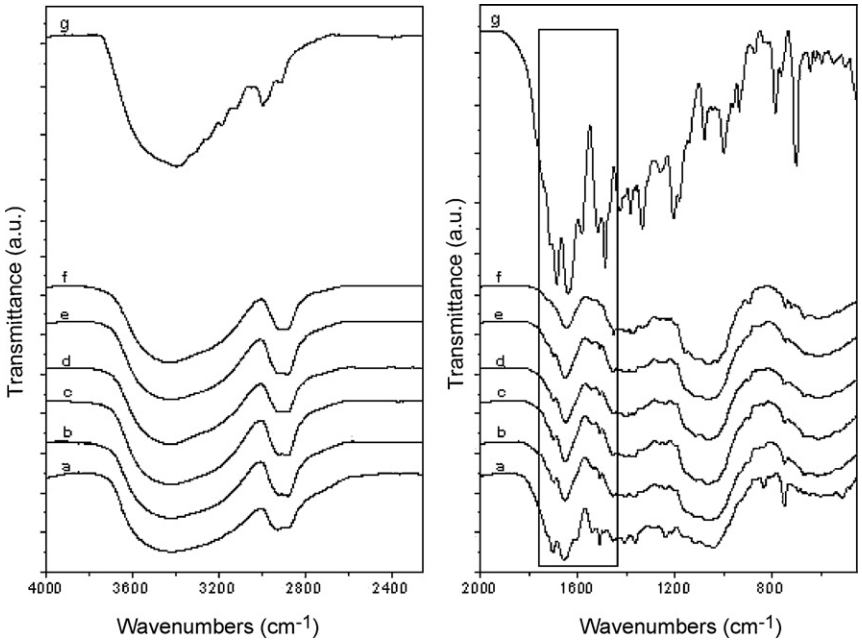


Fig. 3. FT-IR spectra of C/CS hydrogels loaded with NO-donor compound: (a) cellulose, (b) 90/10 of C/CS, (c) 80/20 of C/CS, (d) 70/30 of C/CS, (e) 60/40 of C/CS, (f) 50/50 of C/CS, and (g) NO-donor compound.

stretch vibration of the CH_2 group (Schwanninger, Rodrigues, Pereirac, & Hinterstoisse, 2004). The adsorbed or so-called "bound" water (Lojewska, Misĭkowiec, Lojewski, & Proniewicz, 2005), whose bending vibrations are observed around 1640 cm^{-1} and interfere with carbonyl bands also is present in all spectra.

The characteristic band of the presence of chondroitin sulphate are centred at the wavenumber 857 cm^{-1} and 826 cm^{-1} as chondroitin-4-sulphate and chondroitin-6-sulphate, respectively (Garnjanagoonchorn, Wongekalak, & Engkagul, 2007). The bands at 3410–3470 cm^{-1} originated by hydroxyl groups while those at 1644–1660 cm^{-1} by carbonyl group (Cavalcanti, da Silva, Gómez Pineda, & Winkler Hechenleitner, 2005). The band at 1240 cm^{-1} corresponds to the R–OSO_2–OR (Yuan et al., 2008). The occurrence of a peak at 1015 cm^{-1}, is related to the formation of C–O–C bonds between the cross linker and the polysaccharide.

The characteristic peaks for NO-donor compound appear at 3141 cm^{-1}, 2954 cm^{-1} (>N–CH_3); 2875 (–CH<); 2875 cm^{-1} (>C=O); 1606 cm^{-1} (>C=C< N–); 1540 cm^{-1} (>C=C< CO); 1704 cm^{-1}(>C=N); 1658 cm^{-1} (–NH–CO); 1511 cm^{-1}, 751 cm^{-1}, 675 cm^{-1} (–C_6H_4–); 1363 cm^{-1} (–CO–CH_3); 1289 cm^{-1} (–O–NO_2); 2908 cm^{-1} (CH_3, –CH_2–); 1453 cm^{-1} (–O–CH_2).

The shift of absorbance bands from 1540 cm^{-1} (>C=C< CO) and 1700–1703 cm^{-1} (>C=N) in the spectra of the loaded hydrogels could be due to the interactions between active principle and hydrogels.

3.2.2. *In vitro release studies of NO-donor compound 7-[2-nitroxiacetyl-oxy-3-(4-acetyl-amino-phenoxy)-propyl]-8-morpholino-1,3-dimethyl-xanthine from C/CS based-hydrogels*

The release profiles of NO-donor compound 7-[2-nitroxiacetyl-oxy-3-(4-acetyl-amino-phenoxy)-propyl]-8-morpholino-1,3-dimethyl-xanthine from C/CS hydrogels are shown in Fig. 4. The shape of the curves are almost similar varying only the percent release not only the total amount but the release amount at each moment of release process, the slope of the curves depends on hydrogel composition–Table 6.

Thus, an increase of CS content leads to the increase of the half release time from 67 min to 160 min and time to reach maximum release amount of NO-donor compound increased from 340 min to 550 min and to the decrease of NO-donor compound percent released. The released amount varied from 80% for 50/50 of C/CS hydrogel to 93% for the other compositions this behaviour being caused probably because of drug-matrix interactions for hydrogels containing a higher amount of CS through hydrogen bonding leading to slower release rate and smaller released amount.

3.2.3. *Release kinetics*

Testing many kinetic models, the most suitable one was found the Higuchi model (Korsmeyer & Peppas, 1984) according to the fitted results for some hydrogels with different composition shown in Fig. 5.

For a release factor $n_r = 0.5$ according to Higuchi model the constant k_r of NO-donor compound release in water:ethanol mixture are obtained from the linear regression–Table 6.

It can conclude that the release of the 7-[2-nitroxiacetyl-oxy-3-(4-acetyl-amino-phenoxy)-propyl]-8-morpholino-1,3-dimethyl-

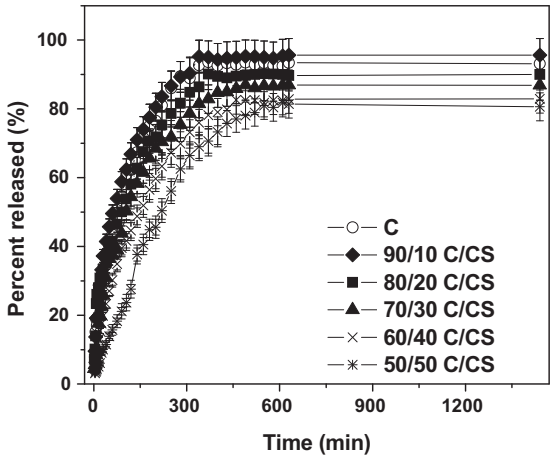


Fig. 4. The release profiles of 7-[2-nitroxiacetyl-oxy-3-(4-acetyl-amino-phenoxy)-propyl]-8-morpholino-1,3-dimethyl-xanthine from C/CS-based hydrogels in water:ethanol solution, at 37 °C.

Table 6
The kinetic parameters of 7-[2-nitroxiacetyl-oxy-3-(4-acetyl-amino-phenoxy)-propyl]-8-morpholino-1,3-dimethyl-xanthine NO-donor compound release from C/CS-based hydrogels according to Higuchi model.

Hydrogel	Half release time (min)	Time to reach maximum amount released (min)	Maximum release amount (%)	Release constant k_r 10^3 ($min^{-0.5}$)	R
Alkali cellulose	67	340	93	56.7	0.99
90/10	67	340	95	64.4	0.99
80/20	70	370	90	57.9	0.99
70/30	87	400	87	53.0	0.99
60/40	100	460	82	40.5	0.99
50/50	160	550	80	27.9	0.99

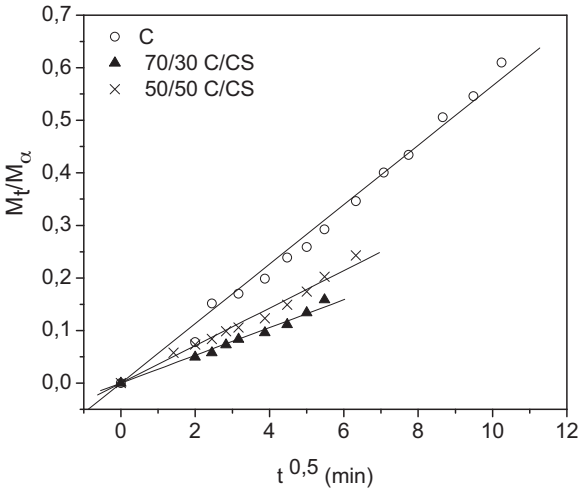


Fig. 5. Linear regression profiles of some C/CS hydrogels, with different mixing ratios, loaded with studied NO-donor compound in water:ethanol solution according to Higuchi model.

xanthine NO-donor compound in water:ethanol solution is described by a Fickian diffusion mechanism and can be controlled by CS content of the bicomponent hydrogels.

The values of the specific rate (k_r) decreased from 56.7 $min^{-0.5}$ to 27.9 $min^{-0.5}$ indicating a significant retarded delivery by increasing CS content of hydrogels.

It can conclude that polysaccharide bicomponent hydrogels offer a satisfactory solution for retarded delivery of 7-[2-nitroxiacetyl-oxy-3-(4-acetyl-amino-phenoxy)-propyl]-8-morpholino-1,3-dimethyl-xanthine NO-donor compound which can be controlled by hydrogel composition.

4. Conclusions

The bicomponent polysaccharide cellulose (C) and chondroitin sulphate (CS) hydrogels were prepared in various mixing ratios by a chemically cross linking technique.

The swelling behaviour depends on the CS content of the matrix showing that an increase of CS content in hydrogels composition leads to a higher swelling ratio for all formulations due to the strong repulsion of negative charges and polar groups existing in CS structure which make them more hydrophilic and absorbent.

The LD_{50} for C/CS-based hydrogels, after intraperitoneal administration as suspensions, is bigger than 3200 mg/kg. The LD_{50} obtained values demonstrated that the compositions have a low toxicity.

Blood analysis, performed after 14 days of administration, in order to detect acute manifestations of toxicity on immune system components and liver enzymes did not reveal major relevant abnormalities; moreover it indicated a good biocompatibility of tested hydrogels after intraperitoneal administration at mice.

Potential applications of the hydrogels matrices as drug delivery carriers were examined, the matrices being loaded with a novel nitric oxide donor compound, 7-[2-nitroxiacetyl-oxy-3-(4-acetyl-amino-phenoxy)-propyl]-8-morpholino-1,3-dimethyl-xanthine, which exhibits a lower toxicity and a higher anti-inflammatory activity than its parent molecules, paracetamol and theophylline. The compositions of hydrogels had an important effect on NO-donor compound release. The increase of CS content leads to a decrease of released amount of NO-donor compound and a decreased rate constant due to the possible interactions which appear between hydrogels and active principle and change in morphology of the hydrogels with different compositions.

It is found that the values of diffusion exponent (n_r) indicated a Fickian diffusion transport mechanism for all formulations.

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