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Evaluation of crosslinked chitosan hydrogels as carriers for prolonged delivery of some novel nitric oxide donor compounds based on theophylline and paracetamol

*Anca Cojocariu,[1] Lenuta Profire,[2] Catalina Cheaburu,[1] Ana-Maria Oprea,[1] Cornelia Vasile[1]**

[1*]Romanian Academy, "P. Poni" Institute of Macromolecular Chemistry, Department of Physical Chemistry of Polymers, 41A Grigore Ghica Voda Alley, Ro 700487 Iasi, Romania, fax: +40232211299; e-mail: cvasile@icmp.ro
[2] "Gr. T. Popa" Medicine and Pharmacy University, Faculty of Pharmacy, Department of Pharmaceutical Chemistry, 16 University Str., 700115, Iasi, Romania; fax:+40.232.211.820, e-mail: nprofire@yahoo.com.

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Abstract: The hydrogels of crosslinked chitosan with different glutaraldehyde amounts have been "in vitro" tested as carriers for prolonged release of some drugs for inflammatory and bronchopulomonary diseases. These hydrogels have been loaded with two novel nitric oxide donors, derivatives of *7-[2-nitroxyacethyl-oxy-3-(4-acethyl-amino-phenoxy)-propyl]-8-R-1,3-dimethyl-xanthine* compounds (R=H, NO_2 for 65 and respectively 77 compounds) designed as multitarget drugs and parent compounds as paracetamol, theophylline, two xanthine derivatives *7-[2-hydroxy-3-(4-acethyl-amino-phenoxy)-propyl]-8-R-1,3-dimethyl-xanthine derivatives* with (R=H, NO_2 for D_1 and respectively D_2) and their release was evaluated in an acidic solution (pH=2.2) simulating the gastric fluid. Results have been correlated with the swelling behaviour which was also followed in acidic media with pH=2.2. The relationships between the release profiles and kinetics and matrix characteristics and/or drug properties have been established.
Keywords: NO-donor drugs, paracetamol, theophylline, chitosan, hydrogels, drug delivery systems, kinetics.

Introduction

Most drugs commonly used in therapy have been developed on the basis of the reductionistic "one target–one disease" approach. They are able to address individual targets, and consequently they are successfully used in single-target therapy. They are also used in combination for the treatment of complex diseases, such as cardiovascular and inflammatory diseases, cancer, and AIDS, which require addressing more than one target. In this regard, today there is great interest in the use of multitarget drugs, also called polyvalent or multifunctional drugs, namely, single products capable of interacting simultaneously with multiple targets, directly or following metabolism. [1, 2]
The use of polyvalent drugs shows some advantages compared with a cocktail of drugs, including a lower risk of drug–drug interactions, improved compliance by the patient, and a more predictable pharmacokinetic profile.
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.[3] It dilates blood vessels, inhibits platelet adherence and aggregation,

attenuates leukocyte adherence and activation, and inhibits vascular smooth muscle cell proliferation. Delivery of exogenous NO is an attractive therapeutic option, particularly with a view to slowing progression of atherosclerosis and reducing the risk of thrombosis. [4] New compounds, in which NO donor groups are linked to the classical parent molecules, have been synthesized [5-7] with the purpose to identify new molecules with an improved pharmacological profile in terms of the increase of the therapeutically efficiency and of the reduction of the side effects.

In our previous works, new nitric oxide donors, where two parent molecules namely theophylline and paracetamol, are linked by a nitric oxide donor chain have been synthesized and it has demonstrated that they have a good pharmacological activity higher than parent molecules.[8-10] These compounds could be useful tools in the therapy of complex diseases such as bronchopulmonary and inflammatory diseases because they combine NO-donor nonsteroidal anti-inflammatory compounds and NO-donor bronchodilatators. The composed molecules could be useful agents in the treatment of anti-inflammatory diseases being devoid of gastro- and cardiotoxicity and also could be a valid approach to the treatment of many bronchopulomonary diseases.

Rapid release of NO can cause the adverse effects as cerebral or basilar artery vasospasm. Therefore we consider interesting to find solutions for their controlled/sustained delivery. Although these drugs have the same base compounds (paracetamol and theophylline) the other properties of them (as solubility and swelling) are different, therefore the carriers should be carefully selected.

Matrices based on covalently crosslinked *chitosan hydrogels* have been selected because they have many applications from food additives to pharmaceuticals [11-13] and biomedical purposes. Covalent crosslinking leads to the formation of a permanent network allowing the free diffusion of water and/or bioactive compounds without dissolution and permits drug release by diffusion and enhancing the mechanical properties of the hydrogel. Chitosan hydrogels (C) also are biocompatible, non-toxic and biodegradable.

Paracetamol (acetaminophen) (P) is a common analgesic and antipyretic drug having also weak anti-inflammatory activity. It is one of the most widely used drugs for the symptomatic treatment of affections with small and moderate intensity of pain like headache, dental neuralgia, surgical pain, cefalee, recently being considered for the therapy of neurodegenerative diseases such as Alzheimer's disease that are characterized by oxidant and inflammatory stress.[14] Unlike analgesics such as aspirin or NSAIDs, acetaminophen is not associated with gastrointestinal (GI) tract irritation and has no vaso-constrictive effects, Paracetamol reduces levels of prostaglandin metabolites in urine but does not reduce synthesis of prostaglandins by blood platelets or by the stomach mucosa and is also a weak inhibitor in vitro of both cyclooxygenase (COX)–1 and COX-2.[15, 16]

Theophylline (T) is a drug used for the treatment of asthma, due to its bronchodilatatory, anti-inflammatory and immunomodulatory effects. Theophylline relaxes directly smooth muscles of the bronchial and pulmonary blood vessels, so that acts largely as bronchodilatator and relaxing the smooth muscle. [17] Main mechanism by which theophylline exercises is the relaxation of smooth muscle by phosphodiesterases inhibiting and antagonistic adenosine, producing such bronchodilatation. [18]

We herein present the work on the controlled release of two novel NO-donor compounds *7-[2-nitroxyacethyl-oxy-3-(4-acethyl-amino-phenoxy)-propyl]-8-R-1,3-dimethyl-xanthine (R is H or NO_2 for 65 and respectively 77 compound)* from chitosan crosslinked with glutaraldehyde matrices. These new NO-donor compounds have been studied comparatively with their derivatives *7-[2-hydroxy-3-(4-acethyl-amino-phenoxy)-propyl]-8-R-1,3 -dimethyl-xanthine derivatives* (*R is H or NO_2 for* D1 and respectively D2 derivative) and parent drugs paracetamol and theophylline.

Many factors affecting *in vitro* drug release behavior of the new compounds from chitosan-based hydrogels were investigated, including different kinds of drugs and chemical composition of the hydrogel. By altering the composition of hydrogels swelling and *in vitro* drug release behavior of the prepared hydrogels could be controlled, which is of great importance for their further applications.

Results and discussion

"In vitro" drug release

Controlled release of the paracetamol and theophylline from polysaccharide containing matrices is extensively studied because they are very useful drugs and also are considered as model drugs. The paracetamol release was investigated by Ofori-Kwakye *et al.*[19] from pectin/chitosan/hydroxypropylmethylcellulose film-coated tablets, ***Säkkinen et al,*** [20] from microcrystalline chitosan or amylodextrin. [21] The *n* values for the coated tablet formulations in the various GIT conditions were close to unity indicating that the coated system provided zero order drug release or a non-Fickian diffusion was found. The zero-order release kinetics from amylodextrin matrix tablets are maintained under *in vivo* as well as *in vitro* conditions. In both cases of release profiles (*in vitro* and *in vivo*) showed an initial small burst effect, followed by an almost constant drug relase rate from the matrix tablets during 8 hours. Different controlled release dosage forms for simultaneous delivery of salbutamol and theophylline have been proposed.[22] The assessment of the release kinetics revealed that drug release from the ethylcellulose microspheres followed Higuchi model, it was diffusion-controlled

Acetaminophen, and theophylline kinetic release study from tamarind seed polysaccharide [23] and hydroxyethylcellulose [24] indicated in the case of theophylline a n value of 0.70, while from commercial xanthan (X) and the highly hydrophilic galactomannan (G) *n* values between 0.63 and 0.83 were obtained [25] These values are characteristic of anomalous kinetics (non Fickian), suggesting that more than one mechanism may be involved, but approaching Case-II transport.

Nunthanid et al., [26] evaluated the performance of spray-dried chitosan acetate. The mechanism of drug release up to 60–70 % was Fickian. Rokhade *et al*., [27] found that the theophylline release from crosslinked chitosan microspheres depends on the extent of matrix crosslinking, amount of drug loading and MC content of the matrix. The *n* values range from 0.363 to 0.563, indicating a slight deviation from the Fickian transport.

Crosslinking of the chitosan with glutaraldehyde (GA) [28] has been studied for controlled release of different drugs such as centchroman [29] 6-MP,[30] 5-Fluorouracil (5-FU) [31] sodium diclofenac, [32] propanolol HCl [33], oxprenolol HCl [34], etc. No studies on the release of the paracetamol and theophylline and

xanthine derivatives drugs under study from crosslinked chitosan matrices were found, neither for NO-donor compounds and xanthine derivatives**.**

In the Figs. 1 - 3 are given the release profiles of the paracetamol, theophylline (Fig.1), intermediary D1 and D2 xanthine derivatives (Fig. 2) and of the two novel NO-donor drugs (Fig. 3) studied, respectively.

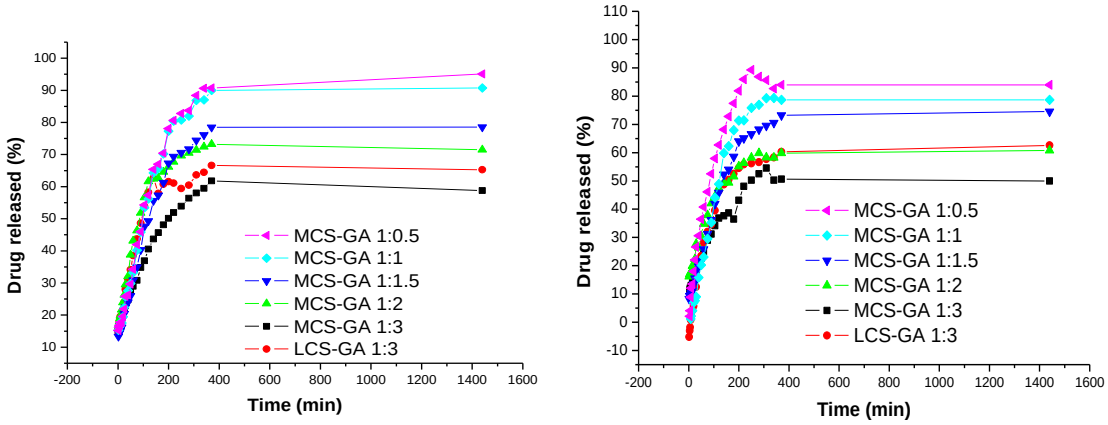


Fig. 1. *In vitro* cumulative release profiles of the paracetamol (a) and theophylline (b) from LCS:GA and MCS:GA matrices from LCS:GA and MCS:GA.

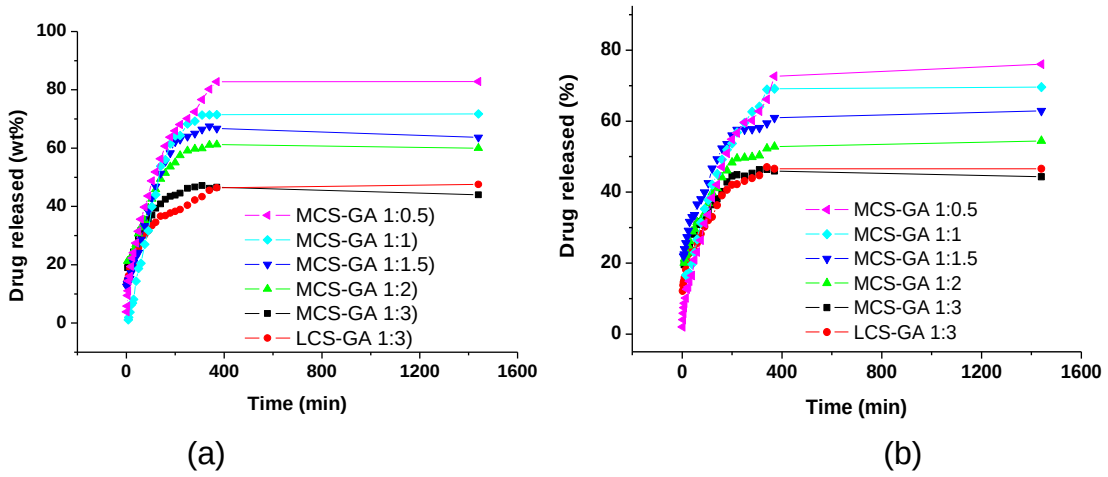
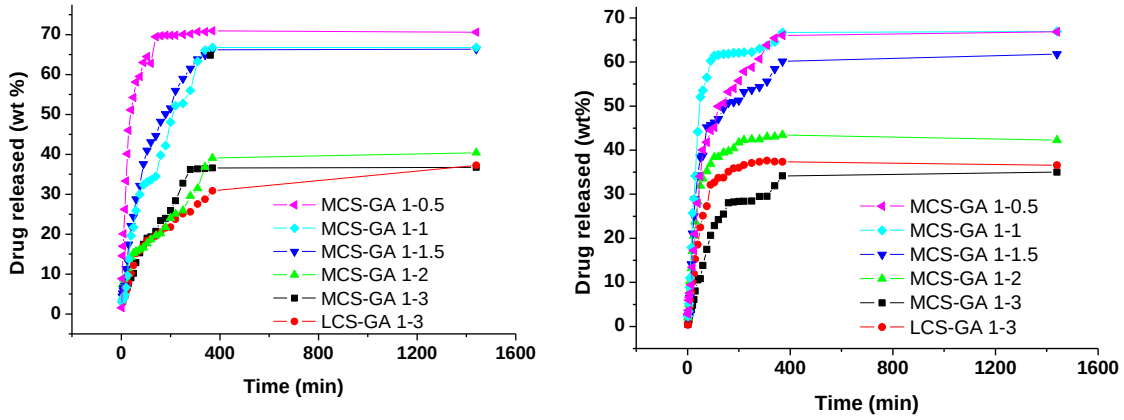


Fig. 2. *In vitro* cumulative release profiles of the D_1 (a) and D2 (b) xanthine derivative release from LCS:GA and MCS:GA matrices.

The dependence of the shape of the kinetic profiles both on the matrix characteristics, namely crosslinking density (crosslinking agent amount) and drugs properties can be easily observed. It has been shown for several drugs such as 5-fluoroucil, propanolol, rifamicin, oxprenolol that in hydrogels formed by chitosan crosslinked with itself, the release is mostly controlled by the crosslinking density; consequently, the higher the crosslinking density, the lower the release rate. [35-37]

Most of the hydrogels reached equilibrium after about 6.6 h for paracetamol, theophylline and xanthine derivatives D1 and D2 with a little influence of the crosslinking degree. For NO-donor compounds the crosslinking degree has a significant influence. A burst effect was observed initially for NO-donor compounds

release from slightly crosslinked chitosan, which may be due to rapid dissolution of the surface drug followed by the diffusion of the drug through the polymer network. The NO-donor compound 65 is released from slightly crosslinked chitosan in about 70 minutes, while at higher crosslinking degree the release duration increases at 6 – 7 hours and finally for the highest crosslinking density the equilibrium release not reached even after 20-24 hours. This means that when time of release may seem to be short for some applications, it can be controlled by the variation of the extent of crosslinking. It was noticed also from the release data that some drugs were not quantitatively released from the hydrogels. Some drug molecules may be deeply buried in the gel matrix and are more slowly released or indeed may never be released into the surrounding media as long as the hydrogel has not been practically dissolved after degradation in medium.



(a) (b)

Fig. 3. *In vitro* cumulative release profiles of the NO-donor drug 65 (a) and 77 (b) from LCS:GA and MCS:GA matrices.

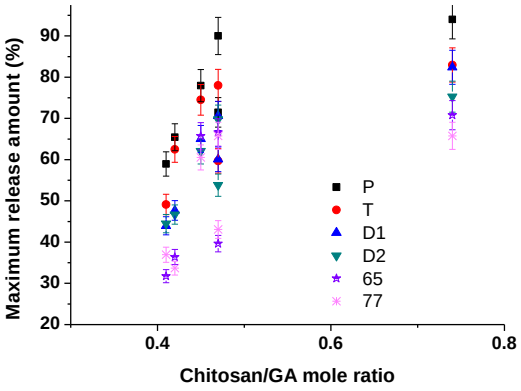


Fig. 4. Maximum release amount of various drugs vs chitosan/GA mole ratio.

For the compounds under study, the amount of the drug released also decreased with increasing crosslinking agent amount and maximum release amount depends on the properties of the drug. To evidence these dependences in Figs. 4 and 5 are given the plots of the maximum release amount versus chitosan mole ratios in hydrogels and molecular mass of the drugs, respectively. It appears very evident the increase

of the release amount in the case of each drug with increasing CS amount of the matrix (decreasing GA amount) and decreasing of the release amount with increasing molecular weight of the drug (Fig. 5).

There is also a dependence of the release amount on the solubility of the drugs in release medium for each kind of matrix (Fig. 6). The higher solubility the greater quantity of the drug is released.

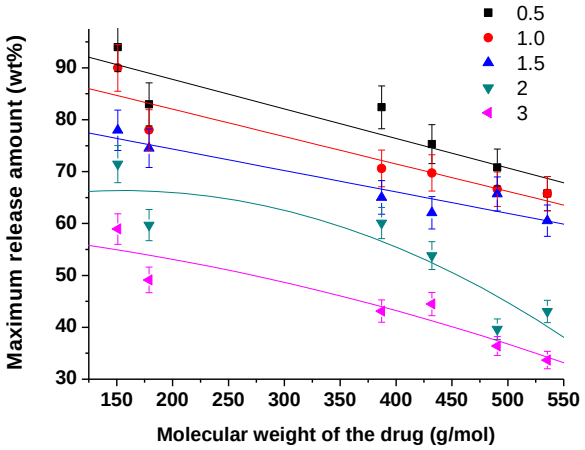


Fig. 5. Maximum release amount of various drugs vs their molecular weight from different types of matrices of crosslinked chitosan. C/GA volume ratios are indicated in legend.

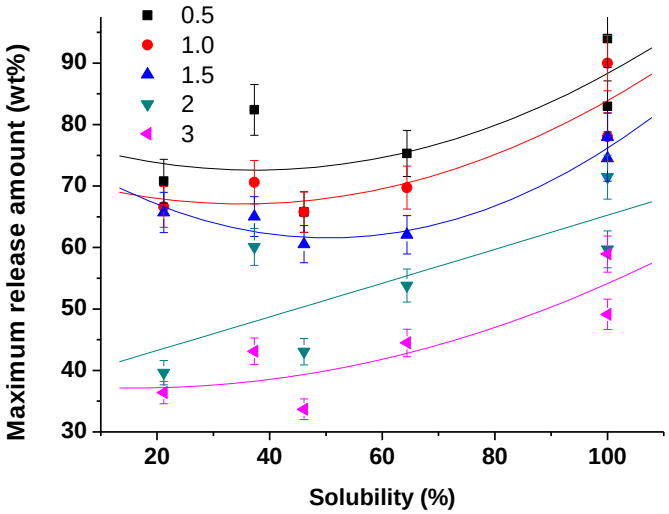


Fig. 6. Maximum release amount of various drugs vs their solubility in a medium of pH = 2.2 from different types of matrices of crosslinked chitosan.

Figures 1-4 shows that the drug released (%) from chitosan crosslinked with glutaraldehyde increases with the decreasing of crosslinking degree of the chitosan. A higher drug released (%) has been registered for MCS:GA 1:0.5 (85-90%) that has the lowest crosslinking degree with glutaraldehyde. Comparing the amounts of drug

released from hydrogels clearly confirmed that the extent of release at equilibrium was inversely related to the degree of crosslinking.

Drug release of the slightly cross-linked chitosan was significantly quicker than that of the highly cross-linked chitosan due to their higher chain-relaxation ability. The diffusivity of the penetrant diffusing through the swollen rubbery phase could be of the order 10^3–10^5. There are relative mobilities of the penetrating solvent and the drug in the presence of macromolecular relaxation in swollen hydrogel. Thus, a faster drug release rate was obtained from the highly swollen chitosan for all compounds studied. This result indicated that the higher the crosslinking density of chitosan, the lower the swelling ability due to the slower relaxation rate of the polymer chain, which results in the decreased drug-release rate.

It can be also noticed that by using chitosan crosslinked matrices we can modulate the release of each drug used for both commercial drugs such as paracetamol and theophylline and also new xanthine derivatives D1, D2 and new NO-donor compounds 65 and 77. The NO-donor compounds having the highest molecular weight and lowest solubility exhibit the lowest amount released (from 60 to 35% for paracetamol and NO-donor compounds, respectively) therefore their release can be more correctly controlled. The total release percent of soluble drugs decreases as the solubility of drug in water decreases. The rate of release of drugs decreases with decrease in solubility of the drugs. It is because the water dissolves the drug at the surface first, and then penetrates the matrix via pores, bringing about a gelling of the polymer. Dissolved drug is then released by diffusion through the gel and finally the release rate falls as the water reaches the center due to decreased drug concentration to less than its solubility [23].

In Table 1 the kinetic drug release parameters (the diffusion exponent n_r and the kinetic release constant k_r) for chitosan crosslinked with glutaraldehyde in different volume ratios as: LCS:GA 1:3, MCS:GA 1:0.5, MCS:GA 1:1, MCS:GA 1:1.5, MCS:GA 1:2, MCS:GA 1:3 are given.

Kinetic parameters of *paracetamol* release take approximately constant values of the exponent n ~ 0.35 and the kinetic constant is 70-90 min^{-n} and this indicates a non-Fickian release for all samples of chitosan crosslinked with glutaraldehyde. The similar values for the exponent n have been obtained for *theophylline* release from MCS:GA 1:3 and 1:2, for D_1 and D_2 release from LCS:GA 1:3, MCS:GA 1:3 and MCS:GA 1:2. These values are in accordance with those found for other systems containing chitosan.[38] Theophylline release from the matrices with low crosslinking amount the n values are close to those found in literature [25] of 0.6-0.8 which was mentioned above and are characteristic of anomalous kinetics (non Fickian), suggesting that more than one mechanism may be involved, but approaching Case-II transport. This case is found also for D_1 release from MCS:GA 1:0.5 and D_2 release from MCS:GA 1:1 and MCS:GA 1:0.5.

The release exponent for NO-donor drugs for low crosslinking degree are close to the unity therefore they are released by a kinetics of zero order which is the most efficient one. For high crosslinking degree the exponent decreases while the rate constant is almost unchanged. They also exhibit the slowest kinetic constant rates with 10 or 20 times smaller than those of paracetamol, theophylline and xanthine derivatives from high croslinked matrices. For paracetamol and theophylline the maximum release amount varies in close limits from 65 to 80 % by decreasing the crosslinking agent amount. The maximum release amount of NO-donor drugs may

be varied from 30 to 70-80 % by controlling the amount of the crosslinking agent amount. These results pointed out that the crosslinking degree along with solubility and molecular weight of the drugs are the most important parameters controlling release processes and swelling from chitosan hydrogels and that the swelling and dissolution kinetics were the predominant mechanisms of release of xanthine derivatives compounds and NO-donor drugs. Molecular mass of chitosan has only an insignificant influence.

Tab. 1. Kinetic parameters of the drug release from chitosan crosslinked with glutaraldehyde matrices.

Composition	Drug	n_r	R^2	$k_r \times 10^3$ in min^{-n}	R^2
LCS-GA 1:3	P	0.35	0.96	90	0.97
	T	1.0o	0.95	30	0.98
	D_1	0.49	0.95	90	0.98
	65	0.58	0.98	10	0.98
	D_2	0.25	0.99	130	0.99
	77	1.02	0.96	3	0.98
MCS-GA 1:0.5	P	0.34	0.97	93	0.99
	T	0.9	0.97	9	0.98
	D_1	0.8	0.99	20	0.99
	65	0.97	0.94	16	0.98
	D_2	0.65	0.99	10	0.99
	77	0.88	0.98	19	0.97
MCS-GA 1:1	P	0.33	0.92	90	0.95
	T	0.88	0.99	10	0.99
	D_1	0.96	0.99	25	0.99
	65	0.7	0.97	11	0.98
	D_2	0.52	0.98	30	0.99
	77	0.97	0.98	11	0.98
MCS-GA 1:1.5	P	0.36	0.94	70	0.96
	T	0.49	0.96	40	0.98
	D_1	0.4	0.96	50	0.98
	65	0.68	0.99	15	0.98
	D_2	0.27	0.99	110	0.99
	77	0.92	0.95	8	0.94
MCS-GA 1:2	P	0.34	0.96	100	0.97
	T	0.37	0.97	70	0.98
	D_1	0.31	0.97	90	0.98
	65	0.57	0.97	20	0.98
	D_2	0.27	0.99	120	0.99
	77	0.94	0.96	20	0.96
MCS-GA 1:3	P	0.31	0.97	90	0.98
	T	0.38	0.98	50	0.98
	D_1	0.26	0.98	100	0.99
	65	0.56	0.98	13	0.99
	D_2	0.25	0.99	120	0.99
	77	0.79	0.98	12	0.99

Matrix characterization

-Swelling behaviour

The equilibrium swelling of hydrogels is a result of the balance of osmotic forces determined by the affinity to the solvent and the network elasticity. All swelling kinetic curves are plotted on the average of three trials. In Figures 7 and 8 the variation of the swelling degree with crosslinking agent amount is plotted. Initially, the rate of water uptake sharply increases and then begins to level off. The sharp swelling capacity changes can be attributed to high repulsion of $-NH_3^+$ groups in acidic medium. The swelling kinetics profile shows that crosslinked chitosan samples attained equilibrium after different time periods depending on crosslinking amount. The sample MCS:GA 1:0.5 reached equilibrium in approximately 90 minutes, while the MCS:GA 1:3 sample in approximately 200 minutes. It can be noticed that the maximum swelling degree (%) decreases with the increasing of the crosslinking agent GA amount. A fast increase of the swelling degree is observed in the first 15-20 minutes (see insert) then slowly the equilibrium is reached. For the same crosslinking agent amount the maximum swelling degree does not depend on the molecular weight of chitosan (see MCS samples comparatively with LCS).

Q_{max} is higher for MCS:GA 1:0.5 of 3500 %, three times higher than that of MCS.GA 1:3 which has the lowest swelling degree of ~950 % - Figure 7 and 8.

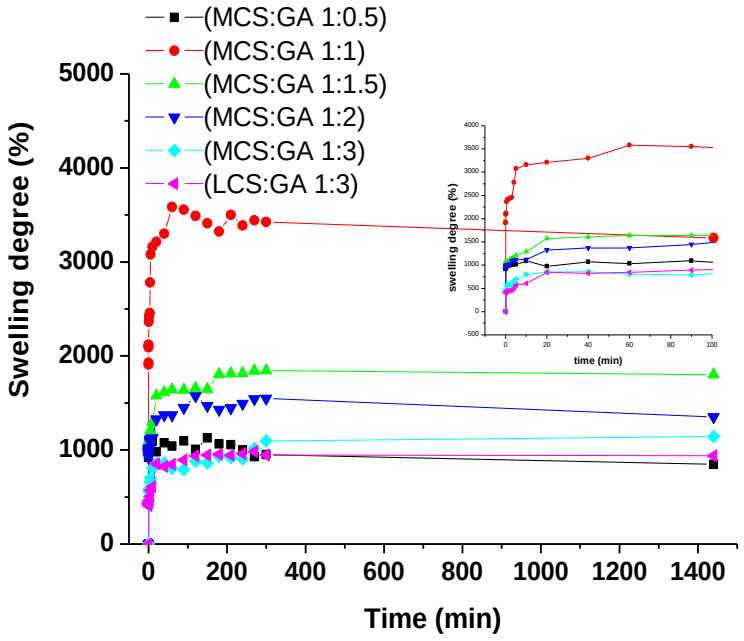


Fig. 7. The swelling kinetic curves LCS and MCS crosslinked with GA.

As the percentage of glutaraldehyde increases, the extent of crosslinking increases, and consequently the equilibrium swelling decreases. The higher the cross-linking density of chitosan hydrogel, the lower the swelling ability of chitosan hydrogel due to the slower relaxation rate of the polymer chains, which will result in the decreased drug-release rate. The swelling ratio change of chitosan hydrogels translates into a change in the mesh size of the gel pores, which modulates drug release. Lightly crosslinked systems form super adsorbing hydrogels in which crosslinking gives rise to a continuum of free water.

As the crosslinking density increases, water content, swelling capacity and the mesh size of the network decreases. The increasing amount of crosslinker decreases the ability of chitosan to form hydrogen bonds with water molecules.

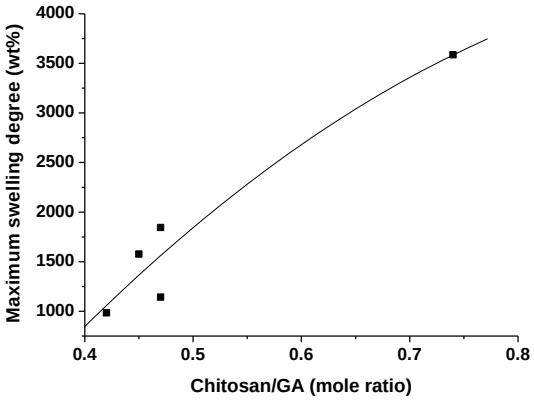


Fig. 8. Maximum swelling degree vs chitosan/GA mole ratio.

Moreover, the higher the crosslinking density, the lower the swelling ability of chitosan hydrogels due to the slower relaxation time of the polymeric chains, which results in a decreased drug-release rate.[39] The analysis of swelling ratios and equilibrium or dynamic swelling behaviour also allows one to gain insight into the drug release behaviour from the network.

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

$$F_t = \frac{W_t}{W_{eq}} = k_{sw} t^{n_{sw}} \quad (1)$$

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. 1 applies to initial states of swelling and linearity is observed when *ln Ft* as a function of *ln t* is represented with a high correlation factor R (see Table 2).

In Table 2 are summarized the swelling kinetic parameters n_{sw} and k_{sw} and the maximum swelling degree for LCS and MCS crosslinked with GA in different ratios.

The swelling kinetic parameters vary depending on the crosslinking degree of chitosan. From this equation, the value of *n* gives indication on the sorption mechanism. For $n = 0.5$, Fickian diffusion dominates; for $n > 0.5$, the solute transport is non-Fickian; and when $n = 1$, the swelling mechanism will be relaxation controlled. [38] The release exponent n_{sw} is close to zero value. It is seems that the swelling is not time dependent. It can conclude that it corresponds to an anomalous mechanism of swelling or that the water sorption in the hydrogels was more diffusion- than relaxation-controlled. The type of water transport through hydrogel is judged from n values.

Tab. 2. Swelling kinetic parameters for chitosan crosslinked with glutaraldehyde.

Composition	Q_{max}%	n_{sw}	R^2	k_{sw}	R^2
LCS-GA 1:3	1128.8	0.01	0.93	10.82	0.99
MCS-GA 1:0.5	3586.1	0.07	0.97	25.16	0.99
MCS-GA 1:1	1844.8	0.01	0.98	11.95	0.99
MCS-GA 1:1.5	1575.5	0.03	0.96	11.39	0.99
MCS-GA 1:2	1141.7	0.09	0.97	6.87	0.98
MCS-GA 1:3	982.9	0.01	0.82	5.37	0.99

The release depends on the swelling degree of the hydrogel matrix, in which the drug release may be due to the diffusion–dissolution mechanism through swollen gels – Figure 9. It is seems that the mechanism could be changed with advanced swelling degree as was also observed from the values of the kinetic parameters of release.

The swelling kinetic constant k_{sw} is the lowest for MCS:GA 1:3 (5.37) and the highest (25.16 five times higher) for MCS:GA 1:0.5 – Table 2. The kinetic constant k_{sw} increases with the decreasing of the glutaraldehyde amount in chitosan.

Cytotoxicity Testing

The main drawback of dialdehydes such as glutaraldehyde is that they are generally considered to be toxic. For example, glutaraldehyde is known to be neurotoxic, its fate in the human body is not fully understood. Therefore, even if hydrogels are purified before administration, the presence of free unreacted dialdehydes in hydrogels could not be completely excluded and may induce toxic effects. That is why the cytotoxicity testing is very important. Silva et al established that the GA-crosslinked chitosan samples having a GA content up to 20% are not cytotoxic.[40] Here we test the samples with higher amount of GA using similar procedure.

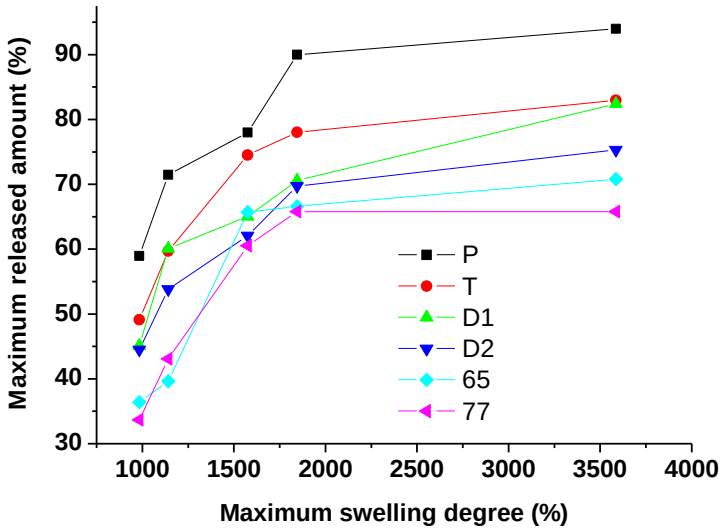
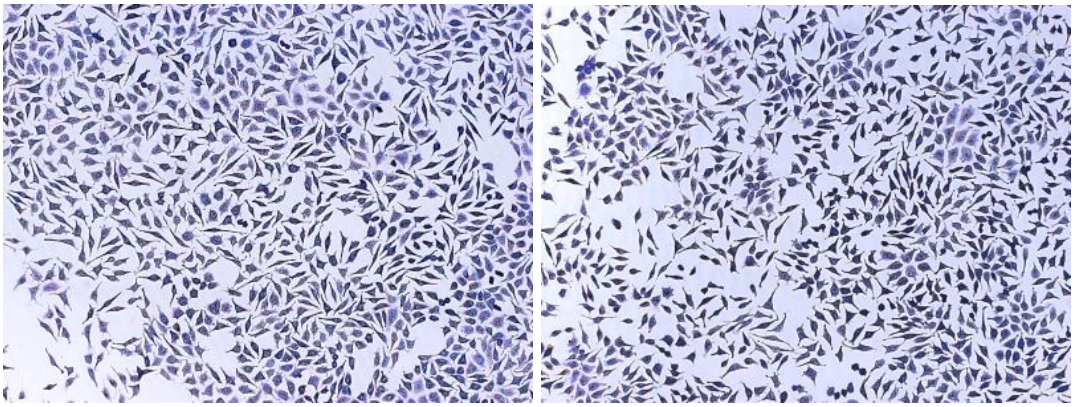


Fig. 9. Correlation between maximum release amount of different drugs studied and maximum swelling degree.

The hydrogels biocompatibility was evaluated by extract method in accordance with European Standard ISO 10993-5, by qualitative methods (cytochemical cells colouring with Giemsa) and quantitative methods (MTT assay).

The results for the studied samples are comparable with those for the control sample from the point of view of fibroblasts adhesion and of cellular viability (Fig. 10) and with literature results. [40]



(a) (b)

Fig. 10. Microscopic images (100 X) of the culture after 48 hours: control (cultivated cells in extracts absence) (a) and chitosan hydrogel containing matrix C/GA molar ratio 0.4 (cultivated cells in extract presence).

Light microscopy images showed that all tested polymeric samples caused no morphological or cytotoxical modifications of fibroblasts; after 24 h of incubation, the control and cells cultivated in presence of polymeric materials have retained their specific characteristics. The results obtained by MTT test evidenced that all the polymeric samples were not cytotoxics (the cellular viability is higher than > 83.13%) and the cellular viability has the biggest value of 98.3 %. The tests of toxicity and biocompatibility on mousses are in progress and their preliminary results indicate also non-toxicity and biocompatibility of the hydrogels with higher GA amount after intensive purification.

Conclusions

Several biocompatible matrices of crosslinked chitosan have been “in vitro” tested as carriers for prolonged release of some drugs for inflammatory and bronchopulomonary diseases such as novel NO-donor compounds comparatively with their parent molecules as paracetamol, theophylline, xanthine derivatives. The drug released amount from chitosan crosslinked with glutaraldehyde increases with the decreasing of amount of glutaraldehyde used for crosslinking. The two novel NO-donor compounds 65 and 77 are released by zero order kinetics from slightly crosslinked matrices. It has been shown that the maximum release amount of various drugs depends on matrix characteristics (crosslinking amount and swelling behaviour) and drugs’properties as molecular mass and solubility.

This preliminary investigation of chitosan-based hydrogels has shown that they may be used to expand the utilization of these systems in controlled release applications of NO-donor compounds. Prolonged delivery of NO-donor compounds via hydrogel

matrices is a safe and effective strategy for preventing secondary reactions as cerebral or basilar artery vasospasm.

Experimental part

Materials

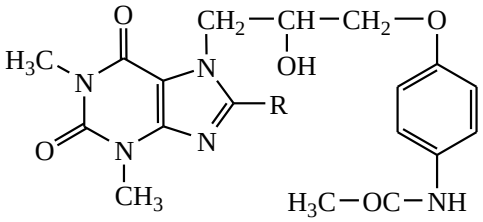
Chitosan samples (with low molecular weight LCS with degree of deacetylation of 68 %, η = 20-200 cP in 1 wt% of 1% acid acetic, and with medium molecular weight - MCS, M_n = 400 000, η ~ 200 mPas in 1 wt% of 1% acid acetic, Brookfield); glutaraldehyde (GA) aqueous solution of concentration 50%, were purchased from Fluka and Sigma Aldrich). All other chemicals and solvents were of analytical grade and were used without further purification.

Theophylline and paracetamol were purchased from Fluka.

The procedure for synthesis of *7-[2-hydroxy-3-(4-acethyl-amino-phenoxy)-propyl]-8-R-1,3-dimethyl-xanthine* derivatives where R is H or NO_2 for D1, D2 respectively and of *7-[2-nitroxyacethyl-oxy-3-(4-acethyl-amino-phenoxy)-propyl]-8-R-1,3-dimethyl-xanthine (*where R is H or NO_2 for 65 and 77, respectively*)* novel NO-donor compounds together with their chemical and pharmacological characterization has been previously reported. [8-10, 41]

The *new xanthine derivatives and NO-donor compounds* synthesized according with procedure previously described are white powders, soluble in acidic solutions are less toxic as paracetamol and theophyline and exhibit superior bronchodilatatory and anti-inflammatory effects (at least two times higher).[8-10]

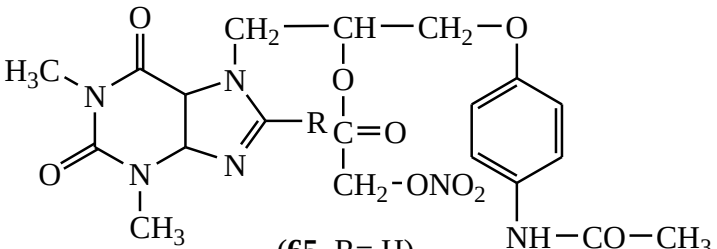
The structures of the two xanthine derivatives are:



(**D$_1$**, R = H)
(**D$_2$**, R = NO_2)

Scheme 1. The chemical structures of xanthine derivatives D1 and D2.

and those of the NO-donor compounds are:



(**65**, R= H)
(**77**, R= NO_2)

Scheme 2. Chemical structures of the novel NO-donor compounds 65 and 77.

The solubility of the studied drugs in solutions of pH = 2.2 varied from 100% for paracetamol and theophylline to 20-40% for NO-donor compound – see Fig. 6.

Procedure for obtaining of the chitosan hydrogels

Chitosan was dissolved in 1% aqueous acetic acid solution at room temperature and left overnight with continuous mechanical stirring to obtain a 1% (w/v) solution. The most commonly used crosslinking agent for chitosan is glutaraldehyde. [42] The reaction with chitosan amine groups produces covalent crosslinking through a Schiff base reaction. The 5 % (w/v) aqueous glutaraldehyde solution in different mole ratios was added to the clear chitosan solution under stirring at room temperature. Chitosan dissolved in acetic acid pH 3-4 reacts with glutaraldehyde quickly in less than 1 hour. Such hydrogels have been studied for lower crosslinking amount up to 20% [40] here we have increased the GA amount. Chitosan was crosslinked with an excess of glutaraldehyde in different ratios (v/v) as: MCS:GA 1:0.5; MCS:GA 1:1; MCS:GA 1:1.5, MCS:GA 1:2, MCS:GA 1:3, and. LCS:GA 1:3. The concentration of GA in the last hydrogels was about 40-50%. A high amount of crosslinking agent was used to create a compact structure required for retarded drug delivery. After 1 h the viscous solution was poured into Petri dishes and dried at room temperature overnight to form the hydrogel. Crosslinking took place at room temperature in a dark space to protect system from oxidative/photodegradation of GA for 4h. The hydrogels obtained were extensively washed with twice-distilled water to remove the excess of crosslinking agent (GA is easily water-soluble) and any residual monomer, then freeze-dried by means of a Labconco FreeZone device and stored until further use. The purity was checked by pH and spectroscopic measurements. The molar ratios between chitosan and GA was determined by weighing of the final product after extensive and careful purification and they ranges between 0.74 and 0.42 MCS (or LCS)/GA.

Methods of investigation

Dried hydrogels were loaded, at 37 ^{o}C, by immersion in drug solutions of concentrations 18 mg/mL, (the quantity being evaluated from maximum swelling degree) for 2 h inside capped containers while the drugs penetrate and/or attached into matrices. Finally, the loaded hydrogels were dried by freeze drying at low temperature and pressure using a Labconco FreeZone device, for two hours. Each experiment was repeated three times. The absorbance of each solution was measured at 240 nm (HP Agilent, USA) before and after immersion of the hydrogel. The absorbance at 290 nm of the solutions was used to calculate the absorbed amount.

The release experiments were carried out at 37 ◦C, in acidic solution (pH=2.2) where all compounds under study were soluble. The NO-donor compounds were not soluble in alkaline medium (pH= 7.4). *In vitro* release studies were conducted by a standard dissolution set-up. [43] The *kinetic release* of the drug-loaded hydrogels was evaluated also in acid solution (pH=2.2). Aliquots of the medium of 1 mL were withdrawn periodically at predetermined time intervals and analyzed using a Hewlett Packard 8540A spectrophotometer. In order to maintain the solution concentration, the sample was carefully reintroduced in the circuit after analysis. The concentration of the drugs were calculated based on previously measured calibration curves for each drug at their specific maximum absorption wavelengths using solutions of known concentrations in the range of loaded drug at different wave lengths

depending on the drug used, namely: λ=241-242 nm for paracetamol and λ= 270 - 271 nm for theophylline, D_1, D_2 , 65 and 77 NO-donor drugs.

A simple, semi-empirical equation was used to kinetically analyze the data regarding the drug release from crosslinked chitosan matrices at the initial stages (approximately 60 % fractional release): [44,45]

$$\frac{M_t}{M_\infty} = k_r t^{nr} \quad (2)$$

or

$$\mathrm{Ln}(M_t/M_0) = \mathrm{Ln}(k_r) + n_r\,\mathrm{Ln}(t) \quad (3)$$

where M_t and M_∞ are the cumulative amounts of drug released at time t and infinite time, respectively; k_r is a constant incorporating structural and geometric characteristics of the drug dosage form, and n_r is the release exponent, indicative of the mechanism of drug release. Drug release data were employed for determination of the release exponent and release rate constants. When the exponent n takes a limiting value of 0.5, it is in the case of diffusion-controlled drug release (Fickian release). Case II transport or relaxation controlled delivery (zero order), the exponent n is close to unity for release from cylinders. When n lies between 0.5 and 1 an anomalous transport is involved.[46,47] The non-Fickian kinetics is regarded as couple diffusion/polymer relaxation.[48]

Swelling tests

The hydrogels were swollen in acid solution (pH=2.2) and then weighed at predetermined periods of time. The equilibrium swelling degree was calculated according to the Eq. (1):

$$Q_{eq}\,(\%) = (W_{eq} - W_d) / W_d\ 100 \quad (4)$$

where W_{eq} is the weight of the swollen sample when thermodynamic equilibrium was reached and W_d is the dry weight of the sample.

Biological tests

The *in vitro cytotoxicity* of polymeric blends was evaluated on the basis of cell proliferation, viability and morphology. Fibroblasts primary culture obtained from human dermis explants were used. Human fibroblast cultures were established from explants of infant foreskins by standard techniques. Cells were grown in 100-mm diameter Petri dishes in Eagle's medium supplemented with 10% foetal calf serum. They are a well established system for *in vitro* analysis of fibroblast growth, migration and collagen metabolism in wound healing.

The dimensions of the studied films were 5x5 mm. All the samples were sterilized with UV radiation for 8 h.

To analyze cell proliferation human dermal fibroblasts (3.5 x 10^4 cells/mL) were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 UI/mL penicillin, 100µg /mL streptomycin and 50 µg/ml neomycin into 35 mm diameter culture dishes. After 24 h of incubation at 37 ^{0}C in a humidified atmosphere of 5 % CO_2 the culture medium was replaced with a fresh one and the samples were added into the dishes. Cells were allowed to grow for another 24h and 48h, respectively. After that, fibroblasts were trypsinised, stained with a 0.4

% Trypan blue solution and the viable cells were counted using a Burker Turk hemocytometer. Trypan blue negative cells indicated viable cells.

To analyse possible release of toxic products by the samples, dermal fibroblasts were grown in contact with polymeric samples. The culture medium was replaced every 3-4 days. The morphology and growth of cells were monitored with a phase contrast microscope. Reference sample was cellular suspension without polymer and is considered as having a cellular viability of 100.

MTT assay

Cell viability was spectrophotometrically measured by MTT test. The method is based on the conversion of tetrazolim salt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to purple formazan by the mitochondrial succinate dehydrogenase in viable cells. For experiment, cells were seeded onto 24-well plates at a density of 2.5 x 10^4 cells/mL, and after, there were incubated for 24 h at 37 ºC in humid atmosphere with 5% CO_2 the polymeric samples were added to allow the cells to adhere. After the incubation period the culture medium was discarded and replaced with extract medium. At 24 h and 48 h of cultivation in the presence of the extracts, medium was discarded and replaced with extract medium, 50 µL of MTT solution (0.25 mg/mL) dissolved in the culture medium were added in each well and then, cells were incubated at 37 ºC for 3 h at dark. Water-insoluble dark blue formazan crystals formed in viable cells were solubilized with isopropanol and the absorbance of every well was measured at 570 nm using an UV-VIS spectrophotometer (Jasco V-650, Japan).

Concentration of converted dye directly correlated to the number of metabolically active cells in culture. Cells viability was calculated by comparison with control samples (fibroblasts cultivated in absence of polymers), considered to be 100 % viable cells. The extracts were triplicate analysed.

Cellular morphology

Fibroblasts morphology was assessed by light microscopy. Human cells were incubated at 37 ^{0}C for 24 h. After this period of time, the matrices were added in cell culture. Fibroblasts cultured in the presence of polymeric samples for 48 h were washed with PBS, fixed in methanol, stained with Giemsa solution and pictures were taken using an inversed-phase microscope (Nikon Japan). In all experiments the control sample was represented by dermal fibroblasts cultivated in the absence of polymers.

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