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# DAUNORUBICIN-LOADED CHITOSAN MICROPARTICLES - PREPARATION AND PHYSICOCHEMICAL CHARACTERIZATION

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## ABSTRACT

*The present study deals with the preparation and characterization of chitosan microparticles as a drug delivery system for daunorubicin. The microparticles are prepared by the suspension method and their properties are improved by covalent crosslinking using glutaraldehyde. The obtained spherical microparticles with size of 300 μm are loaded with daunorubicin. The morphology and chemical composition of the microparticles obtained are investigated by scanning electron microscopy and FTIR. The effect of the crosslinking and the amount of the encapsulated drug on the in vitro release kinetics is investigated by VIS spectroscopy. The release rate of daunorubicin (physically entrapped) is controlled by the degree of swelling of the microparticles hydrogel matrix. The release rate of the covalently bounded daunorubicin from chitosan microparticles in simulated intestinal fluids containing lysozyme depends on the density of crosslinking. It is found that slower drug release rates are obtained from highly crosslinked microparticles. These results indicate that chitosan microparticles have a potential as a drug delivery system for oral administration because they provide controlled release with the participation of the lysozyme enzymes in the digestive tract.*

*Keywords: chitosan microparticles, drug delivery system, daunorubicin.*

## INTRODUCTION

Daunorubicin or daunomycin (Dau), isolated from *Streptomyces peucetius* is the anthracycline antibiotic whose structure consists of a tetracycline aglycone attached to an amino-sugar daunosamine by a glycosidic linkage (Fig. 1-a). A prerequisite for its activity is the coupling of a coplanar hydrophobic region (anthraquinone) with an angled hydrophilic structure (OH groups and protonated amino-sugars), while the quinone and hydroquinone moieties allow them to function as electron accepting and donating agents. Dau is a chemotherapeutic that is given as a treatment for specific types of leukemia (acute myeloid leukemia and acute lymphocytic leukemia) to slow or stop the growth of cancer cells in the body [1 - 3]. Dau exhibits cytotoxic activity through topoisomerase-mediated interaction with DNA, thereby inhibiting DNA replication and repair, as well as RNA and protein synthesis [4, 5]. Unfortunately, Dau itself is haematological and cardiac toxic agent, and as most anticancer drugs needs an improvement of its therapeutic index and efficacy of treatment.

The drug delivery systems allow controlled release of the drug at the required site of action which leads to improved drug pharmacokinetics and pharmacodynamics. A wide range of materials have been employed as drug carriers. Among these, polysaccharides receive increasing attention because of their availability, mucoadhesivity, biocompatibility, biodegradability, nontoxicity and low imunogenicity.

Chitosan is a naturally occurring polymer that can be obtained from chitin by partial N-deacetylation in a strong alkali medium. It is a linear aminopolysaccharide composed of randomly distributed (1→4) linked D-glucosamine and N-acetyl-D-glucosamine units (Fig. 1-b). Chitosan is insoluble in water but it is soluble in

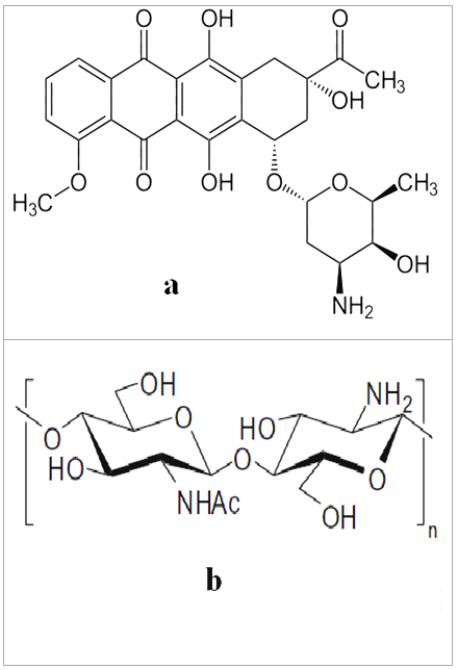


Fig. 1. Structure of: a) daunorubicin and b) chitosan.

acidic solutions with pH lower than 6.5. The primary hydroxyl and amine groups allow chemical modifications to control the chitosan's physical properties. Due to the availability of free amino groups, it carries a positive charge and reacts with negatively charged surfaces or polymers. Chitosan has been extensively studied as a carrier for drugs in the pharmaceutical industry [6]. Chitosan microparticles activated and covalently crosslinked with glutaraldehyde are used for trypsin immobilization [7]. Agnihotri et al. [8] describe various chitosan beads loaded by different drugs, which may release them in a controlled manner to a specific body area. Due to the gradual swelling in an acidic medium the chitosan beads are formulated for controlled drugs release [9].

Chemical attachment of the drug to the chitosan throughout the functional linker may produce useful prodrugs exhibiting biological activity at the target site [10]. Covalently attached drugs might be released by degradation of chitosan. The release of an entrapped drug from the microparticles involves its diffusion in a controlled manner. Chitosan microparticles usually need to be cross-linked by chemical crosslinking agents such as glutaraldehyde or glyoxal to achieve the controlled drugs release.

The oral administration of pharmaceuticals is the more usual route for active drug delivery to the body. An oral controlled release formulation is subjected to frequent changes of environment in the gastrointestinal tract. Chitosan and chitosan derivatives have been extensively studied for delivery and controlled release of therapeutic agents particularly via mucosal routes. Chitosan possesses mucoadhesive properties due to *i*. molecular forces formed by electrostatic interaction between positively charged chitosan and negatively charged mucosal surfaces and *ii*. hydrogen bonding groups like – OH, and COOH [11].

Chitosan-based derivatives have been studied for colonic drug targeting. They can protect therapeutic agents from an inactivation in the upper gastrointestinal tract and release the entrapped agents specifically at the colon through degradation of the glycosidic linkages of chitosan by colonic microflora. Lam et al. [12] report a possible mode of Dau encapsulation into chitosan microcapsules designed for oral administration. They find that the main quantity of the immobilized Dau is released (about 40 %) in the stomach where pH is acidic, but in the phosphate buffered saline (pH 7.4, containing trypsin and lipase) the drug releases slowly.

The aim of the present investigation was the preparation, cross-linking and activation of chitosan microparticles using glutaraldehyde and evaluation of their potential as a delivery system for controlled-release application of daunorubicin.

## EXPERIMENTAL

### Materials

Daunorubicin hydrochloride and chitosan from shrimp shells (low-viscosity) were obtained from Fluka. Glutaraldehyde, 25 % (Glu) was from Merck. Lysozyme (EC 3.2.1.17) was purchased from Sigma. All other chemicals used were of analytical grade.

### Preparation of microparticles

Chitosan microparticles were prepared by dissolving 0,6 g chitosan in 10 mL of 2 % (w/v) aqueous solution of acetic acid under vigorous agitation. The chitosan solution was dropped through a needle into an aqueous sodium hydroxide solution (10 %) under magnetic stirring. Since chitosan is not soluble at high pH, the drops solidified upon polymer precipitation. The obtained microparticles were filtered and washed with distilled water to remove residual sodium hydroxide and were stored in water. Subsequently the microparticles were

processed with glutaraldehyde solutions in order to form a covalent network and to activate them. Practically, 2 g of the swollen microparticles were suspended in 20 ml of distilled water under mild magnetic agitation and were exposed to the glutaraldehyde solutions for 4 h at 20°C. Molar ratios of glutaraldehyde molecules: chitosan amine groups of 0:1; 0.5:1; 1:1; 5:1 and 10:1 were used. The crosslinked microparticles obtained were finally washed with distilled water to remove the residual glutaraldehyde.

**Degree of swelling of chitosan microparticles**

The degree of swelling (DS) of crosslinked chitosan microparticles was determined by keeping 100 mg of them in 10 mL of a solution simulating a gastric fluid (pH 1.2) for 10 h. The reservoir kept at 37ºC was shaken at 100 rpm. The weight of each swollen sample ($W_t$) was determined. The current microparticles weight measured at 1 h intervals was compared to their initial weight ($W_0$). This provided the calculation of the degree of swelling in accordance with:

$$DS(\%) = \frac{(W_t - W_0)}{W_0} \times 100 \quad (1)$$

where $W_0$ and $W_t$ represent the weights of dry and swollen chitosan microparticles, respectively.

**Preparation of Daunorubicin-loaded microparticles**

The Dau loading was done after the crosslinking and activation of the microparticles with glutaraldehyde (at different molar excess). The loading of Dau on wet chitosan microparticles was carried out by immersing of 2 g of microparticles in 20 mL of phosphate buffered solutions (pH 7,0) of Dau of different concentrations (0.75; 1.5; 2.5 mg $ml^{-1}$) for 24 h under gentle stirring. The amount loaded on the microparticles was determined spectrophotometrically using a Perkin Elmer Lambda 2 UV/VIS spectrometer ($\varepsilon_{482nm} = 9420\ M^{-1}cm^{-1}$). The Dau-loaded microparticles were freeze-dried.

**Determination of Dau entrapment efficiency and percent loading**

The entrapment efficiency (EE) of the microparticles was expressed as percentage of the actual Dau loading of the microparticles referred to the initial amount of Dau used. EE (%) was determined using the following equation (2):

$$EE(\%) = \frac{\text{Amount of Dau in microparticles}}{\text{Amount of initial Dau used}} \times 100 \quad (2)$$

The loading of Dau in the microparticles was calculated as percentage loading (PL) using the following equation (3):

$$PL = \frac{\text{Amount of Dau loaded}}{\text{Amount of microparticles}} \times 100 \quad (3)$$

**Scanning electron microscopy studies**

The morphology of the freeze-dried chitosan microparticles was studied by scanning electron microscopy. The samples were coated by a thin gold layer using a fine coater JEOL JFC-1200 and the surface morphology of the microbeads was examined by means of a JEOL JSM-5510 scanning electron microscope (SEM).

**Fourier transform-infrared spectroscopy (FT-IR)**

FT-IR spectra were recorded on a Varian 660-IR spectrophotometer. Chitosan, Dau and Dau-loaded microparticles were prepared by processing KBr disks.

***In vitro* drug release studies**

To determine the quantity of Dau released, 100 mg of freeze-dried microparticles were introduced at 37°C to 50 mL of buffer solution simulating a gastric fluid (pH 1.2). This took place in a shaker at 100 rpm. After 10 h the solution was changed for 50 mL of a solution simulating intestinal fluids. It was in fact a buffer solution (phosphate buffer saline, pH 7.5) containing 10 mg $ml^{-1}$ lysozyme. The microparticles used were kept for additional 10 h. Then samples (1 mL) were withdrawn at fixed time intervals and were analysed spectrophotometrically by measuring the absorbance at 482 nm. The release of Dau from the loaded microparticles is expressed as efficiency of release (ER) using the following equation (4):

$$ER(\%) = \frac{\text{mg of Dau released}}{\text{mg of Dau loaded per 100 mg microparticles}} \times 100 \quad (4)$$

## RESULTS AND DISCUSSION

Chitosan microspheres are prepared by glutaraldehyde activation and crosslinking and subsequently loaded by Dau (Table 1). The influence of the different

Table 1. Activated and crosslinked with Glu chitosan microparticles loaded with daunorubicin.

| Sample | Molar ratio Glu:$NH_2$-chitosan | Amount of Dau used [mg] | Amount of Dau in microparticles [mg] | EE [%] | PL [%] |
|---|---|---|---|---|---|
| 1 | 0 | 15 | 7,0 | 46,7 | 3,5 |
| 2 | 0,5 | 15 | 10,2 | 68,4 | 5,0 |
| 3 | 1 | 15 | 12,5 | 83,3 | 6,2 |
| 4 | 5 | 15 | 14,9 | 99,3 | 7,4 |
| 5 | 10 | 15 | 14,9 | 99,1 | 7,3 |
| 6 | 10 | 30 | 27,0 | 90,0 | 13,5 |
| 7 | 10 | 50 | 38,1 | 76,2 | 19,1 |

molar ratio Glu:$NH_2$-chitosan on the entrapment efficiency and the percent of loading is investigated. The highest values of EE and PL are estimated in case of a large molar excess of Glu and large initial amounts of Dau used. Generally, the determined entrapment efficiencies were more than 70 % (Table 1).

The morphology of Glu cross-linked chitosan microparticles is examined by scanning electron microscopy. The microparticles show nearly spherical shape, smooth surface and diameter of ca 300 μm that is consistent with the observations reported by other authors [13]. The SEM image of a chitosan microsphere is shown in Fig. 2.

FT-IR studies are performed to confirm the drug entrapment into the microparticles. The FT-IR spectrum of non-crosslinked chitosan microparticles (Fig. 3) shows typical bands at: 1654 $cm^{-1}$ for amide-I, which indicates that chitosan is not fully deacetylated and a less intense primary amine band at 1596 $cm^{-1}$. The presence of –OH and $–NH_2$ is confirmed by stretching vibration at 3434 $cm^{-1}$ and the polysaccharide structure between 1155 $cm^{-1}$ - 1031 $cm^{-1}$. The C–H stretching of methyl or methylene group of chitosan is observed at 2876 $cm^{-1}$. The FT-IR spectrum of Dau (Fig. 3) shows a band at 3390 $cm^{-1}$ (O–H stretching) and two strong bands at 1616 $cm^{-1}$ (stretching vibration of quinone carbonyl groups) and 1579 $cm^{-1}$ (aromatic cycle C=C stretching) are observed. The intense characteristic band of the carbonyl group (see Fig. 1a) appears at 1712 $cm^{-1}$.

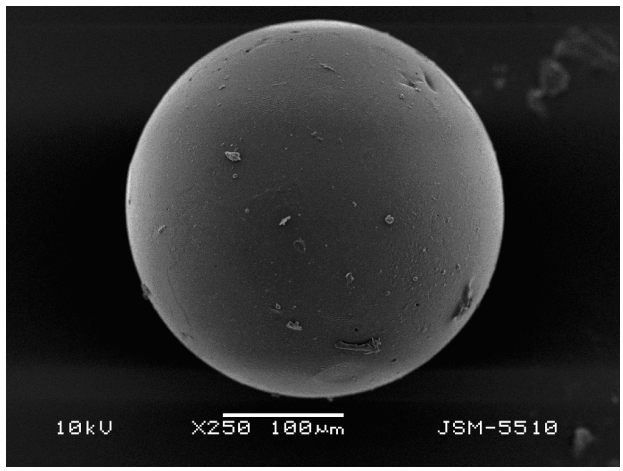


Fig. 2. SEM micrograph of chitosan microparticle crosslinked with glutaraldehyde.

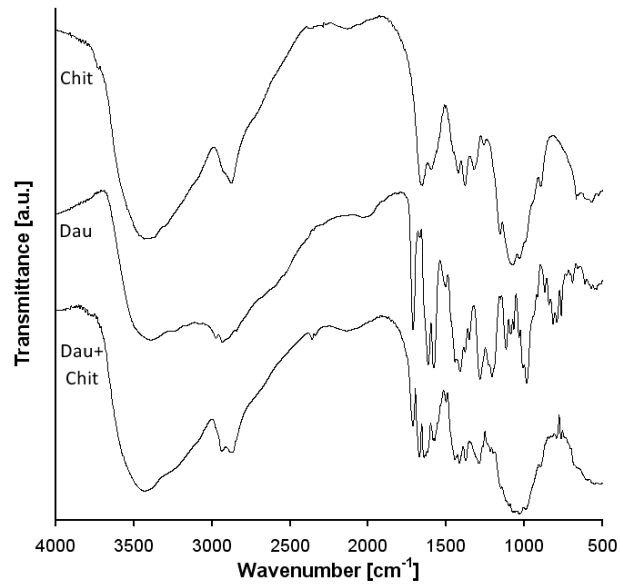


Fig. 3. FT-IR spectra of chitosan, Dau and Dau-loaded chitosan microparticles recorded in KBr disks.

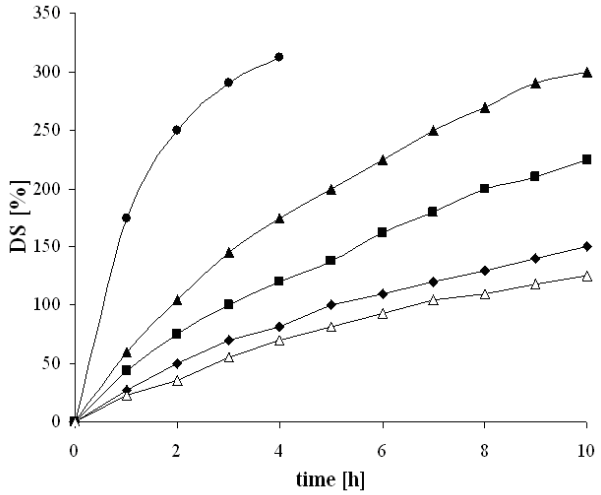


Fig. 4. Dependence of the DS of chitosan microparticles on the molar excess of Glu: (0) —●—; (0.5) —▲—; (1.0) —■—; (5.0) —◆—; (10.0) —△—.

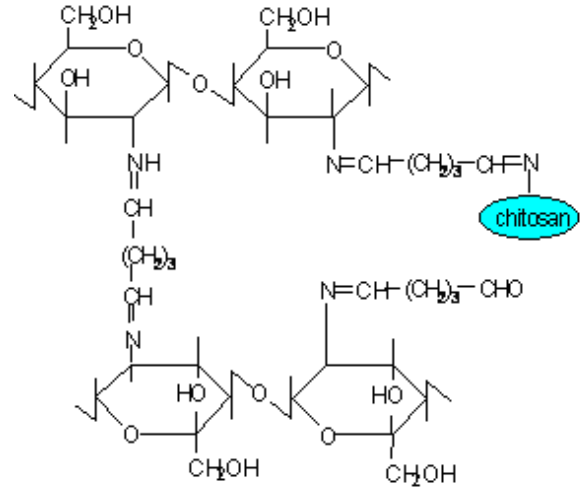


Fig. 5. Crosslinking process of chitosan with monomeric glutaraldehyde.

The FTIR spectrum of Dau loaded crosslinked and activated chitosan microparticles (Fig. 3) shows a band at 1654 $cm^{-1}$. It refers to the amide-I aldehyde groups which is shifted from 1641 $cm^{-1}$. A quite strong peak observed at 1671 $cm^{-1}$ is assigned to the imines. All other characteristic bands of chitosan remain unchanged. The two characteristic bands assigned to the stretching of the quinone carbonyl groups at 1616 $cm^{-1}$ (less intense) and to the aromatic cycle (C=C) stretching at 1579 $cm^{-1}$ of DAU molecule are also observed. The band at 1712 $cm^{-1}$ assigned to the carbonyl group appears with reduced intensity. These findings evidence the presence of drug molecules within the chitosan microparticles.

The *in vitro* swelling studies are carried out at pH 1.2 and the degree of swelling is determined. Plots of crosslinked microparticles swelling are given in Fig. 4. The swelling mechanism at pH 1.2 in case of control (none crosslinked, GLu = 0) microparticles (see Table 1, sample 1) involves the protonation of the free chitosan amine groups. It results in dissolution after 4 h. The degree of microparticles crosslinking is another factor that influences the swelling as it is essential for preserving the integrity of the microparticles in the crosslinked samples after 10 h. It is found that the degree of crosslinking depends on the molar excess of Glu. A large excess leads to a smaller degree of swelling when compared to that of the microparticles without Glu (no crosslinking).

Although Glu is widely used in different fields there is no agreement concerning the mechanism of the interaction of its molecules with substances containing amino groups. Most often the mechanism of crosslinking of chitosan is associated with formation of covalent bonds with the amino groups via a Schiff reaction (Fig. 5).

Monteiro and Airoldi [13] evidence the formation of an ethylene double bond as a result of the chitosan–glutaraldehyde interaction. Thus, the free pendant amine groups of the chitosan polymer interact with the aldehyde group of the glutaraldehyde forming stable imine bonds (HN=CHCH=C) due to the resonance established with adjacent double ethylene bonds.

We assume the mixed (complex) mechanism of crosslinking of chitosan chains in microparticles proposed by Kildeeva et al. [14] Chitosan catalyzes the aldol reaction of glutaraldehyde which is then followed by dehydration (aldol condensation) to form α,β unsaturated polymeric glutaraldehyde (Fig. 6a). These reactions strongly depend on the concentration of Glu and pH of the reaction medium. The possible reactions involved in the crosslinking of chitosan with polymeric glutaraldehyde are outlined in Fig. 6b,c,d,e.

The free amine groups of chitosan can interact with the aldehyde group of the polymeric glutaraldehyde to form some of the following products [15]: (*i*) *unstable imine bonds* (Fig. 6b) - a reaction of the carbonyl and the amino groups proceeds with the formation of Schiff bases which are unstable under acidic conditions and break down to regenerate the aldehyde and the amine; (*ii*) *stable imine bonds* (Fig.6c) – the proceeding conjugation

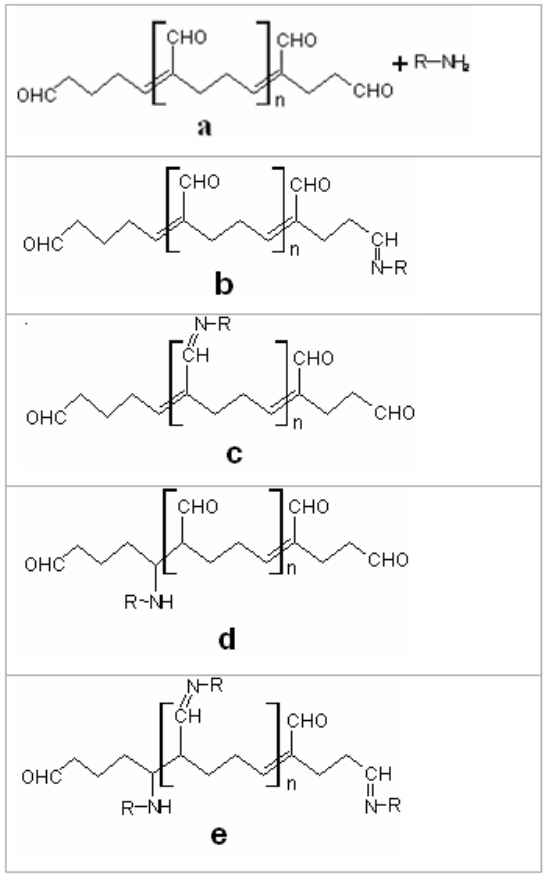


Fig. 6. Possible mechanisms of interaction of chitosan (R-$NH_2$) (b,c,d,e) with polymeric glutaraldehyde (a).

of the double bond of the >C=N with the double bond at α,β position stabilizes the formation of Schiff bases; (*iii*) *secondary amines* (Fig. 6d) - a stable bond of the type of that of the secondary amines is formed by the coupling reaction (Michael addition) of the chitosan amino groups to the double bonds of the polymeric glutaraldehyde (it is considered that the stable imine bonds might be dominating in this case) and *(iiii*) *a combination of the three mechanisms* (Fig.6e).

The mechanisms pointed above are taken into consideration when crosslinked and activated by Glu chitosan microparticles are used for Dau loading. Indeed, both aldehyde chitosan groups and the double bonds of the polymeric glutaraldehyde that are not involved in the crosslinking process are able to react with the amino groups of Dau resulting in covalent bonding by a mechanism shown in Fig. 6b,c,d,e. The interaction of Dau with the chitosan microparticles obtained leads to two types of immobilization of the drug molecules. Part of them are physically entrapped in the gel structure of the microparticles, while the other part is covalently bonded following the same mechanism (Fig. 6b,c,d,e).

It is known that the release rate of drugs from chitosan microparticles is strongly affected by pH [16]. That is why the *in vitro* release tests are performed at pH of 1.2 (0 – 10 h) and pH of 7.5 (10 – 20 h). The release profiles obtained are presented in Fig. 7. At acidic pH the microparticles tested swell and allow the complete release of the physicaly entrapped Dau by diffusion. This process is time-dependent, which can be explained by the different swelling rate. 100 % release is observed in none-crosslinked (control) chitosan microparticles (sample 1, Table 1) after 1 h, since the chitosan microparticles dissolve rapidly at pH of 1.2 (Fig. 7). In the crosslinked and activated by Glu samples the entrapped drug is released more slowly, from 2 to 10 h (see Fig. 7).

The *in vitro* release of the covalently bounded Dau can be associated with the erosion of the chitosan microparticles through enzyme hydrolysis of the polysaccharide chains. Among the enzymes with different specificities able to hydrolyze chitosan [17, 18], lysozyme is the most popularly used, since it is found in various human body fluids. However, Lim et al. [19] show that the degree of acetylation of chitosans is responsible for the completeness of their degradation by lysozyme. The reason is the different enzyme accessibility to the acetylated segments. The efficiency of Dau release from chitosan microparticles in simulated intestinal fluids (phosphate buffer, pH 7.5) containing lysozyme is presented in Fig. 7. In general, the released amount of Dau decreases with the increase of the molar excess of the crosslinking agent. At a lower crosslinking degree, the

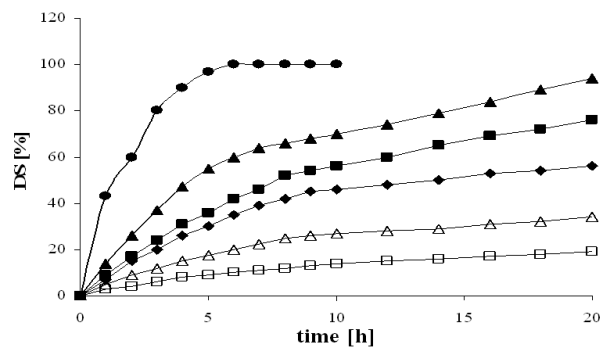


Fig. 7. Release behavior of Dau from chitosan microparticles at: pH 1,2 (0 - 10 h) and pH 7,5 (10 - 20 h). (—●—) none crosslinked (control) chitosan microparticles and crosslinked with GLU used in molar excess of 0.5 ( —▲— ), 1.0 (—■—), 5.0 ( —◆— ), 10.0 (—△—), 10.0 (—□—).

hydrolysis leads to erosion of the microspheres causing a drug release. The high crosslinking density results in lower swelling ability, therefore the drug release is decreased. These findings of the drug release dependence on the extent of crosslinking are consistent with those of Yao et al. [15] and Jameela et al. [20, 21]. The authors reveal that the crosslinking density has a remarkable effect on the release of progesterone and mitoxantrone from the chitosan microspheres. On the other hand, as previously mentioned, the enzyme hydrolysis with lysozyme depends on the degree of chitosan acetilation. Evidently, the post-treatment (crosslinking and activation) of the microparticles with Glu hampers the enzyme erosion of chitosan which in turn results in decreased release of Dau.

## CONCLUSIONS

The present study demonstrates the efficient immobilization of Dau by chitosan microparticles both through physical entrapment and chemical bonding. It is found that the release rate of Dau can be properly controlled by varying several parameters during microparticles preparation. The most important parameters appear to be the molar excess of Glu and the initial amount of Dau used. Chitosan microparticles with no crosslinking (prepared without Glu) release quickly the drug molecules. With the increase of the molar excess of Glu a delayed drug release is observed. It is also shown that the release rate of the physically entrapped Dau is controlled by the degree of swelling of the microparticles matrix. The entrapped Dau can be completely released at acid pH for 10 h due to the microparticles swelling. The sustained release of the covalently linked Dau can be accelerated after hydrolysis by lysozymal enzymes in the digestive tract or by microparticles erosion with the participation of the microbial exoenzymes in the colon. So, the investigated samples revealing different release rates and profiles are able to meet the specific applications requirements. The results from the *in vitro* release tests are very promising regarding the use of Dau loaded chitosan microparticles in the pharmaceutical area.

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