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Cross-Linked Starch Microspheres: Effect of Cross-Linking Condition on the Microsphere Characteristics

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Cross-linked starch microspheres were prepared using different kinds of cross-linking agents. The influence of several parameters on morphology, size, swelling ratio and drug release rate from these microspheres were evaluated. These parameters included cross-linker type, concentration and the duration of cross-linking reaction. Microspheres cross-linked with glutaraldehyde had smooth surface compared with those prepared with epichlorhydrine or formaldehyde. The particle size increased with increasing the cross-linking time and increasing the drug loading. Swelling ratio of the particles was a function of cross-linker type but not the concentration or time of cross-linking. Drug release from starch microspheres was measured in phosphate buffer and also in phosphate buffer containing a-amylase. Results showed that microspheres cross-linked with epichlorhydrine released all their drug content in the first 30 minutes. However, cross-linking of the starch microspheres with glutaraldehyde or formaldehyde decreased drug release rate. SEM and drug release studies showed that cross-linked starch microspheres were susceptible to the enzymatic degradation under the influence of alpha-amylase. Changing the enzyme concentration from 5000 to 10,000 IU/L, increased drug release rate but higher concentration of enzyme (20,000 IU/L) caused no more acceleration.

Key words: Starch, Microspheres, Cross linking, Enzymatic degradation

INTRODUCTION

The controlled release of bioactive molecules, e.g., pharmaceutical agents, has been the subject of extensive research over the last half of the twentieth century. The controlled release of pharmaceutical agents is of high importance for biopharmaceutical applications. Long acting doses of a variety of drugs are now available, allowing once or twice-a-day dosage regimens where immediate release forms called for multiple and sometimes impractical administrations. Effective slow-release dosage regimens have demonstrated superior patient compliance and hence improved efficacy over multiple immediate release forms (Vincent *et al.*, 2000). Furthermore, there is currently considerable interest in the development of selective delivery of drugs into the colon. Site specific delivery to the colon can have major advantages for both treatment of local conditions such as irritable bowel syndrome (anti-inflammatory drugs) and improved absorption of sensitive drugs such as peptides and proteins (Watts, 2001).

Polysaccharides have been used widely in pharmaceutical, chemical and biochemical drug delivery. This family of natural polymers has been applied to the area of controlled release coatings, matrices, macromolecular carriers and biodegradable carriers (Azevedo *et al.*, 2003; Atyabi *et al.*, 2005a, 2005b). Starch is one of the most interesting polymers used in this field. As a natural carbohydrate, starch is considered to be one of the major constituent of human diet. It is biodegradable and is naturally metabolized by the human body (Kost and Shefer, 1990). Adding the possibility of mass production with a high purity at a very economical price, makes this polysaccharide to be considered as one of the most attractive biopolymers for drug delivery purposes.

Starch can be modified through physical, chemical or enzymatic processes (Dumoulin *et al.*, 1998). Cross-linked starches have long been used as food additives because of their safety and low cost. Very low levels are used and these are approved by the FDA (CFR 172.892). Different covalent cross-linking agents have been used

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like: 2,3-dibromoropanol, epichlorhydrine, sodium trimetaphosphate, tripolyphosphate, mixed carbonic carboxylic acid anhydrides (Lenaerts *et al.*, 2001). A few years ago it was discovered that they also possess unique features that suggest their use as an excipient for the manufacturing of controlled release solid oral dosage forms of drugs. Among these properties is the possibility to obtain quasi zero order release profiles with a very simple and cost-effective manufacturing process and very low sensitivity of release profiles to manufacturing conditions such as i.e. tableting pressure (Lenaerts *et al.*, 1998). Usually, starch is cross-linked to increase its resistance to shear or to prevent gelatinization when heated. The preparation of modified and/or cross-linked starch is described in different articles (Lenaerts *et al.*, 1998; Hamdi and Ponchel, 1999; Hamdi *et al.*, 2001; Mulhbacher *et al.*, 2001, 2004; Rahmouni *et al.*, 2001, 2003; Mulhbacher and Mateescu, 2005).

In this study, cross-linked starch has been used as a possible carrier for enzymatic controlled colonic drug delivery. Cross-linked starch is starch usually treated with a bi-functional reagent so that a small number of the starch polymer chains are chemically linked by the cross linking reagent. Cross-linking inhibits granule swelling on gelatinization and gives increased stability to acid, heat treatment and shear forces (Dumoulin *et al.*, 1998).

Except for some routes of administration, degradability within the body is generally requested for microspheres. Moreover, degradability can be a convenient means for triggering the drug release process at the site of action of the drug and further controlling the release process. Mechanisms of degradation which can be applied are mainly hydrolysis and enzymatic degradation. For many applications, enzymatic degradation is advantageous because of the presence of enzymes in the body.

In this study, cross-linked starch microspheres were prepared and the effect of different cross-linking agents on microspheres properties were investigate. The potential of the use of cross-linked starch as an enzymatic degradation dependent polysaccharide for colonic delivery was investigated.

MATERIALS AND METHODS

Materials

Soluble starch, cyclohexane, span 80, α-amylase (from Bacillus subtillis), formaldehyde (FOR) and glutaraldehyde (GLU) were purchased from Merck, (Germany). Epichlorhydrine (ECH) was supplied from Fluka (Germany), and sodium diclofenac was donated as gift by Sobhan Pharmaceutical Company (Iran). All other materials were of analytical grade and used as received.

Preparation of starch microspheres

For preparation of microspheres an inverse emulsion polymerization technique was applied (Hamdi *et al.*, 2001). First, aqueous phase was prepared by dissolving different amounts of sodium diclofenac (3, 6, or 12% w/w) in 12 mL of 2M sodium hydroxide solution. Subsequently, 8 g of starch was added to this solution while the mixture was stirred to dissolve completely. The aqueous phase was added to an organic phase consisting of 80:20 cyclohexane : chloroform mixture containing 2% w/w span 80 while stirring with an overhead mixer at 1300 rpm to form water-in-oil emulsion. After 2 minutes, 4 or 20% v/v epichlorhydrine was added to the above emulsion as a cross-linking agent. In this stage speed of the stirrer was set on 1000 rpm and the temperature of the reaction medium was kept at 40°C. The reaction was left to continue for 4, 12, or 18 h. Resulted microspheres were separated by filtration, washed with excess of cyclohexane and dried at room temperature.

In some formulations, these microspheres were subjected to further cross-linking by dispersing in either formaldehyde or glutaraldehyde solution. Concentrations of 5, 20, 50 or 100 percent of secondary cross-linking agents for 1, 2.5 or 4 h at room temperature were applied. Microspheres were then separated by filtration, washed with acetone and dried at room temperature.

Morphology of microspheres

The effect of formulation variables on particle morphology was examined by scanning electron microscope (SEM) (DSM 960A, Zeniss, Germany). Samples were mounted on metal stubs and sputter-coated with gold for 4 minutes prior to examination under SEM. For cross-section studies, microspheres were cut with a razor blade manually.

Particle size determination

Particle size of the microspheres was determined with a series of standard sieves with the mesh size of 212, 300, 420, 500 and 1000 mm. 1000 μg microspheres were located on the top of the sieve series. The system was shaken for 10 minutes. Then the amount of microspheres remained on each sieve was determined and particle size distribution curve was obtained by plotting the weight percentage of the microspheres on each sieve against sieve mesh size.

Drug loading measurement

Drug content of the microspheres was determined by incubating 10 mg microspheres in 10 mL phosphate buffer pH 7.4 under vigorous stirring for 48 hours. Then the sample was centrifuged for 10min at 5000 rpm and the amount of drug in the supernatant was determined spectrophotometrically at 275 nm using a UV-visible apparatus (S3600, Scinco, Korea).

Drug release study

Drug release profile of prepared microspheres was studied using USP dissolution apparatus I. A known amount of microspheres was placed in the basket rotating at 50 rpm. Dissolution vessel was filled with 900ml phosphate buffer solution pH 7.4 at 37±0.5°C. At appropriate time intervals 5ml sample was withdrawn while replaced by the same volume of fresh buffer. Amount of released drug was determined spectrophotometrically at 275 nm. For studying the effect of the presence of α-amylase enzyme (which is naturally present in the colon) on drug release profile, after 5 h of drug release study in the above condition, microspheres were transferred to another medium composed of phosphate buffer pH 6 containing 5000, 10000 or 20000 unit α-amylase per liter and 0.132 g/lit of calcium chloride which is necessary for α-amylase activity (Brena *et al.*, 1996). Withdrawn samples were analyzed for the amount of released drug during the next 5 h. The drug release profile was obtained by plotting the percentage of drug released from the microspheres against time.

Swelling ratio

The swelling ratio of the starch microspheres was determined as the percentage of particle size change after incubation in the phosphate buffer. A number of beads with similar diameter were chosen. Diameter of the beads was measured before and after incubating in phosphate buffer pH 7.4 for 12 h under optical microscope.

The swelling percent was calculated as follow:

(Particle diameter in swollen state – particle diameter in dry state) / Particle diameter in dry state × 100

RESULTS AND DISCUSSION

Cross-linked starch microspheres were prepared using epichlorhydrine as the cross-linking agent. As can be seen in Fig. 1a, the microspheres were of regular and spherical shape. Higher magnification revealed that microspheres cross-linked only with ECH had a rather rough surface compared with those cross-linked with a secondary agent. Also the surface of the microspheres cross-linked with glutaraldehyde was smoother and more homogenous than those cross-linked with formaldehyde (Fig. 1b-1d).

Effect of the concentration of cross-linking agent (ECH), cross-linking time and the amount of loaded drug on the particle size distribution of microspheres were investigated. It was found that changing the amount of ECH from 4 to 20% slightly decreased the particle size of microspheres as 74 and 62% of the particles were between 425-500 μm when cross-linked with 4 and 20% ECH, respectively (Fig.

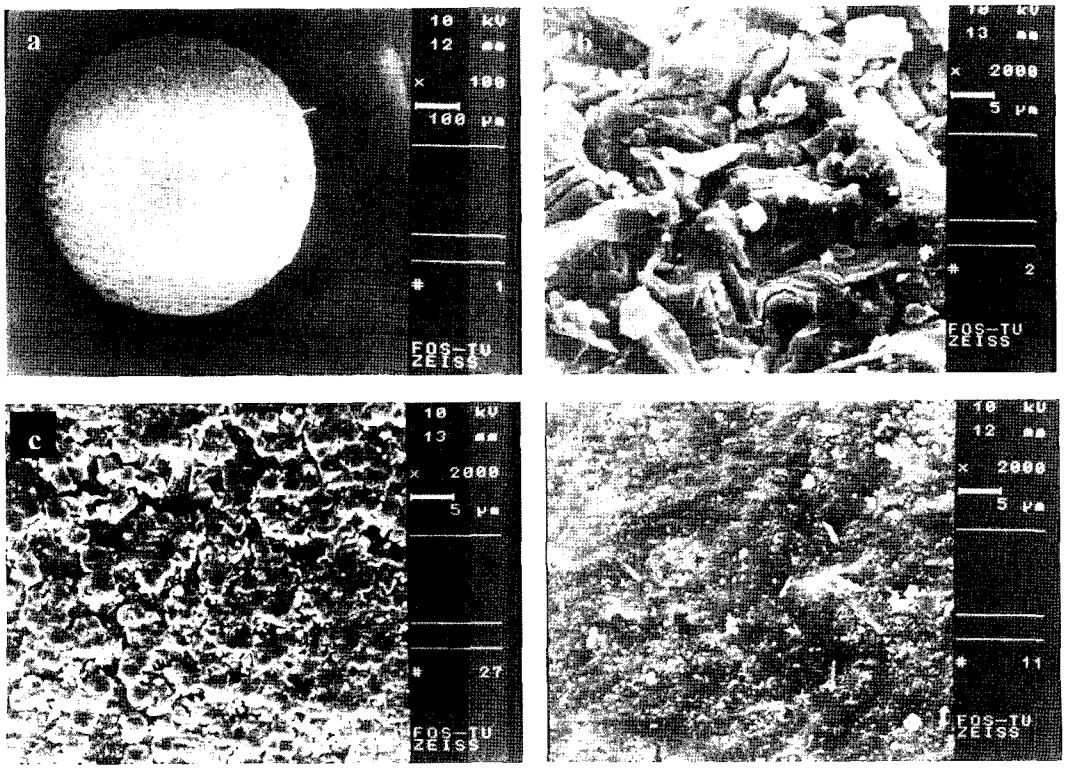

Fig. 1. SEM picture of a starch microsphere (a) and the surface of microsphere cross-linked with (b) epichlorhydrine, (c) formaldehyde and (d) glutaraldehyde

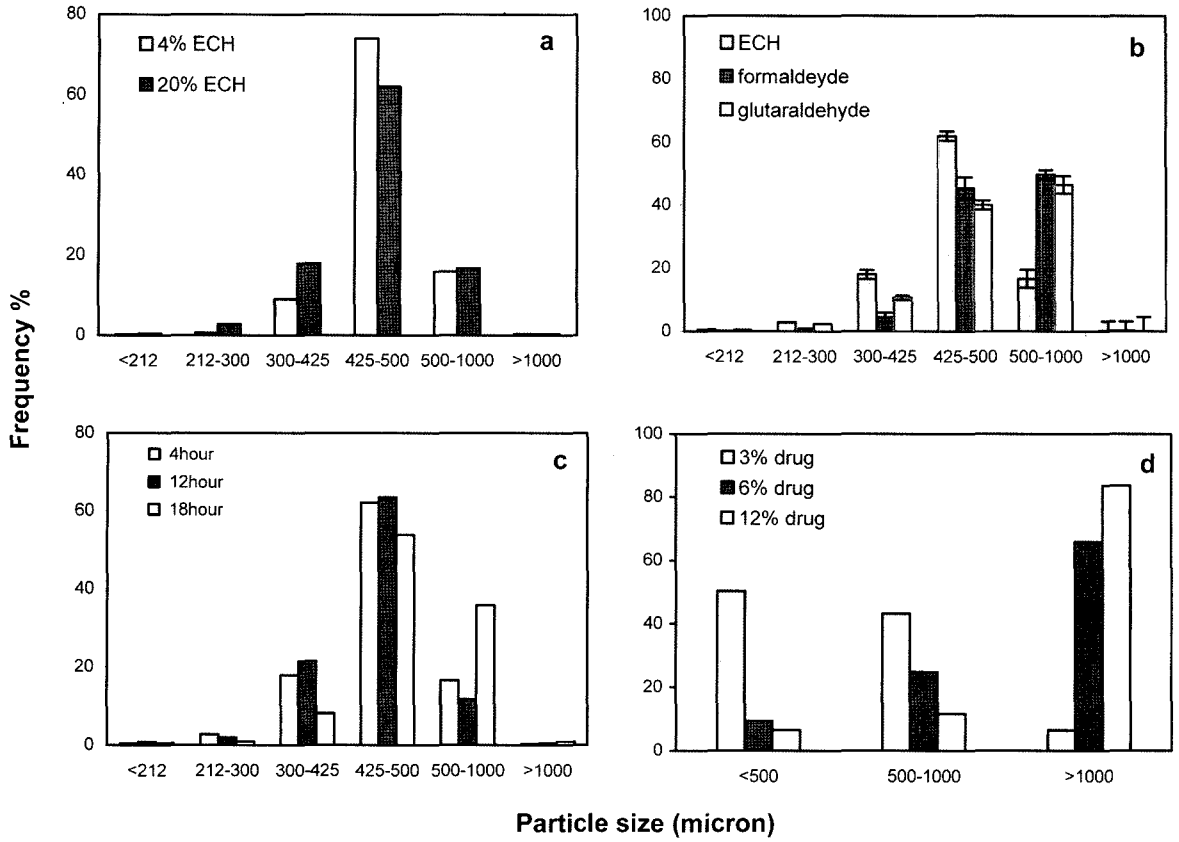

Fig. 2. Effect of different parameters on particle size distribution of microspheres. (a) ECH concentration, (b) cross-linker type, (c) cross-linking time, and (d) amount of drug loading

2a). However further cross-linking with formaldehyde and glutaraldehyde moved the mode of particle size toward producing larger particles. About 48% of microspheres had diameter of 500-1000 μm when cross-linked with formaldehyde and glutaraldehyde while only 16% of the microspheres cross-linked with ECH were laid between this ranges (Fig. 2b). The effect of cross-linking time on the particle size of microspheres was assessed as well. Increasing the reaction time from 4 h to 12 or 18 h when ECH was used as cross-linking agent resulted in larger particles. As it has been shown in Fig. 2c, after 4 h cross-linking only 16% of the particles were between 500-1000 μm while this amount increased to 35% when cross-linking time was set to 18 h. Fig. 2d shows the effect of drug loading on the particle size distribution of starch microspheres: the more drug loading, the greater size of resulted particles. Formulations containing 3% drug are mostly under 500 μm while those having 6 and 12% drug content had modes greater than 1000 μm. This phenomenon was due to the increase of the viscosity of the emulsion droplets in high drug concentration which makes more resistance against the droplets breakdown to the smaller ones (Lee and Rosenberg, 1999; Soppirnath and Aminabhavi, 2002).

When placed in the dissolution medium, various microsphere formulations swelled to different degrees. Swelling ratio of the prepared microspheres was determined as the percentage of particle size change after incubation in phosphate buffer for 12 h (Table I). The results indicated that there was no significant difference between the swelling

Table I. Effect of the type and concentration of cross-linking agent and cross-linking time on the swelling percent of starch microspheres in phosphate buffer

	Swelling ratio(%)		
	cross-linking time		
Cross-linker	4 h	12 h	18 h
EPH-4%	64	63	64
EPH-20%	62	59	58
Second Cross-linker	1 h	2.5 h	4 h
FOR-20%	28	29	29
FOR-50%	28	29	29
FOR-100%	28	29	29
GLU-20%	38	37	38
GLU-50%	38	40	37
GLU-100%	36	38	37

ratios of the microspheres cross-linked with ether 4 or 20% of ECH for 4, 8 or 12 h ($p>0.05$). These factors (time and concentration of the cross-linker) were considered for the secondary cross-linkers as well. As it is noticeable in Table I, treatment of microspheres with a secondary cross-linker affects the swelling behaviour. Microspheres which were brought in contact with either glutaraldehyde or formaldehyde as secondery cross-linker swelled significantly less than those samples which were cross-linked only with ECH, with more considerable effect for formaldehyde. This difference could be attributed to the proportion of the resulted cross-linking that forms a complex network in the matrix system during gelatinization, restricting rehydration of starch matrices. But the time and concentration of second cross-linker did not change the swelling ratio inside each group. This obsevation argues with the finding of some other researchs which stated reverse relation between concentration of cross-linker and swelling ratio of particles (Chawla *et al.*, 2000; Azevedo *et al.*, 2003). In summary it seems that the type of cross-linker is the main factor in determining the degree of swelling.

Drug release experiment was performed in phosphate buffer medium pH 7.4. Effect of the amount of drug loading on diclofenac release profile is shown in Fig. 3. There was no significant difference in drug release rate between formulations with 3, 6 or 12% w/w drug loading. This was surprising, because usually it is expected that higher drug loading increases the release rate. This result could be attributed to the effect of drug loading on microsphere size. As it was discussed earlier, microspheres with higher level of drug content were larger and thus had lower surface area which could be responsible for slower drug release.

Microspheres prepared with epichlorhydrine as the cross-linking agent after subjecting to the dissolution medium, were not able to retain their drug content for a long period of time as 90% of drug was released after 30 minutes incubating in the dissolution medium. Increasing the cross-linking time and concentration of ECH (4, 12 and 18 h and concentrations of 4 and 20%) could not suppress drug release rate from microspheres (data are not shown).

It was thought that applying another cross-linker at the end of the microspheres preparation makes them harder and microspheres could retain drug molecules more tightly. Therefore, in another attempt prepared microspheres were subjected to a secondary cross-linking agent to make the particles harder and less susceptible to the dissolution medium. Glutaraldehyde or formaldehyde was used for this reason. Applying supplementary cross-linking changed the pattern of drug release. As it is shown in Fig. 4, only 40% of the drug content of microspheres is released in the first 30 minutes and approximately 50% within 2 h in the case of applying glutaraldehyde as secondary cross-linker. When formaldehyde was used, about 60% of the microspheres drug content was released within the first 30 minutes. This difference in cross-linking property could be attributed to the chemical structure of formaldehyde and glutaraldehyde. Glutaraldehyde has 2 potential sites for cross-linking action. So in the same concentration, glutaraldehyde could create more links between starch chains than formaldehyde. The SEM pictures (Fig 1c and 1d) support this hypothesis.

Concentration of the applied solution of glutaraldehyde was another parameter which was studied to evaluate the effect of the secondary cross-linking on drug release pattern from microspheres. The cross-linking time of 4 hours as an optimum time and concentrations of 5, 20, 50 and 100% of glutaraldehyde in acetone was chosen (Fig. 5). The pattern of drug release from these concentrations

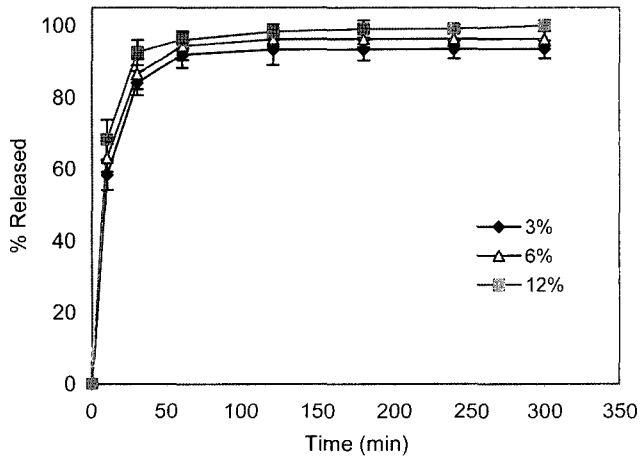

Fig. 3. Effect of amount of diclofenac loading on the drug release rate from cross-linked starch microspheres

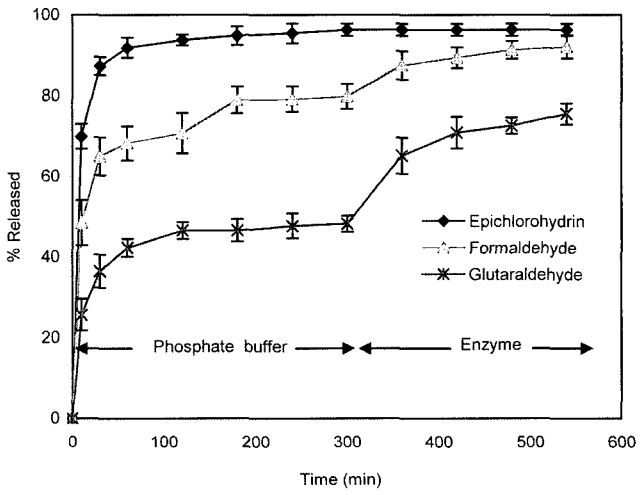

Fig. 4. Effect of different cross-linking agents on drug release pattern from starch microspheres

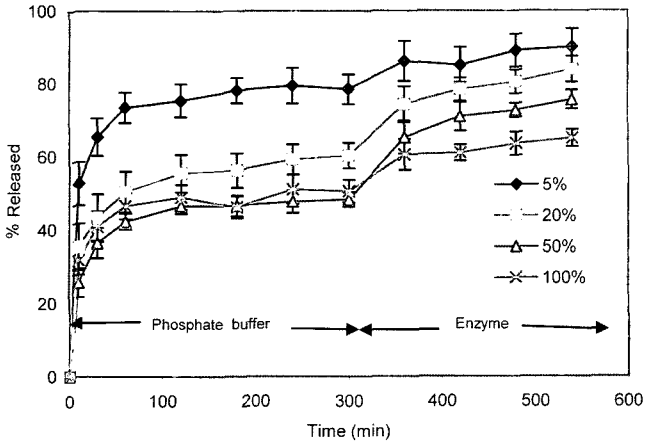

Fig. 5. Effect of concentration of glutaraldehyde on drug release from starch microspheres in phosphate buffer and buffer + enzyme medium

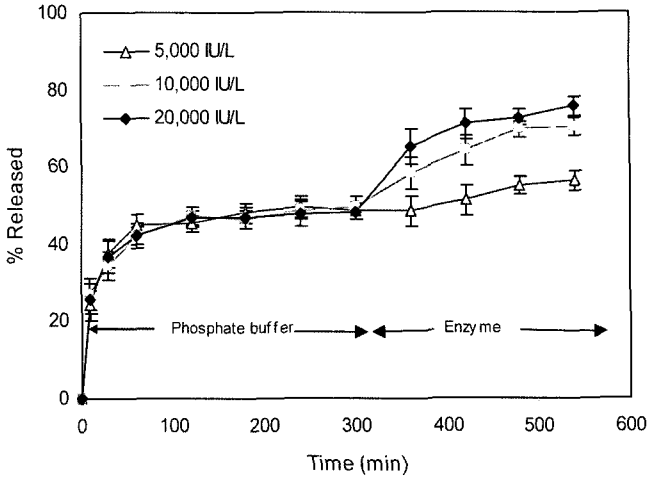

Fig. 6. Effect of concentration of a-amylase on drug release rate from starch microspheres prepared with 50% glutaraldehyde as the secondary cross-linker

shows that applying 5% glutaraldehyde could slow down the rate of drug release. Better results could be obtained with even more concentration of glutaraldehyde up to 20% and 50%. However increasing the amount of cross-linker to 100% could not diminish any more drug rate.

Amylose and amylopectin (two main components of starch) are degraded by colonic bacteria and are resistant to, among other things, pancreatic enzymes. Alpha-amylase is the key enzyme involved in the starch degradation, contributing changes on the physicochemical properties of starch as a result of breakage of glycosidic linkage (C-O-C) and production of glucose, the major product of the enzymatic degradation of starch. Nevertheless, starch can also be degraded by other amylolytic enzymes but in a smaller extent. Susceptibility of the starch-based microspheres to enzymatic degradation by amylolytic enzymes (alpha-amylase) and their colon specificity were investigated by means of incubating the microspheres in a buffer solution containing alpha-amylase enzyme for 4 h (simulated colonic fluid) after 5 h incubation in phosphate buffer (simulated small intestine fluid). The effect of the presence of enzyme in the dissolution medium on drug release from samples prepared with different types and concentrations of cross-linker is shown in Figs. 4 and 5. A sudden increase in drug release occurred with altering the medium from buffer pH 7.4 to the medium containing α-amylase. This augmentation is desirable in colonic delivery in which drug should release from an intact delivery system and absorbed in the first 30 minutes of entry to the colon otherwise the possibility of the absorption is lessen due to reduction of the water content of the region. This phenomenon was observed with different samples prepared with various concentrations of glutaraldehyde. As can be seen in Fig. 5 the variance between the patterns of drug release from these four is significant even concerning concentrations of 50% and 100% which their distinction was not noticeable in phosphate buffer in first 5 hours in buffer solution.

The effect of the concentration of α-amylase on drug release studied assuming that increasing the amount of the enzyme would cause faster drug release. Three concentrations of the enzyme were applied for this reason (5000, 10000 and 20000 IU/L). As expected an obvious increase in drug release rate was observed upon increasing the amount of the enzyme from 5000 to 10000 IU/L (Fig. 6). Whereas higher enzyme concentration (20000 IU/L) had no significant increasing effect on drug release rate.

SEM pictures of microspheres after incubating in dissolution media with and without enzyme are shown in Fig. 7. It is apparent that microspheres were not dissolving and remained even spherical after hours placing in the medium but surface erosion occurred. Enzymatic degradation of microspheres in presence of the enzyme is clear as a lot of fractures and holes appeared on the surface of microspheres and no internal rupture of the microspheres occurred during the degradation process. In contrast, the incubation of the polymers in buffer only, did not cause significant changes on the material's properties and morphology and only very fine porous texture is present at the surface of the microspheres after the water penetration which accounts for the swelling and the observed sponge-like behavior. This is in agreement with previous observation that shows degradation of starch microspheres is a surface-controlled mechanism (Hamdi *et al.*, 1998).

CONCLUSION

The results presented in this study demonstrate that the type of cross-linking agent has a significant impact on starch microspheres. Using glutaraldehyde as the cross-

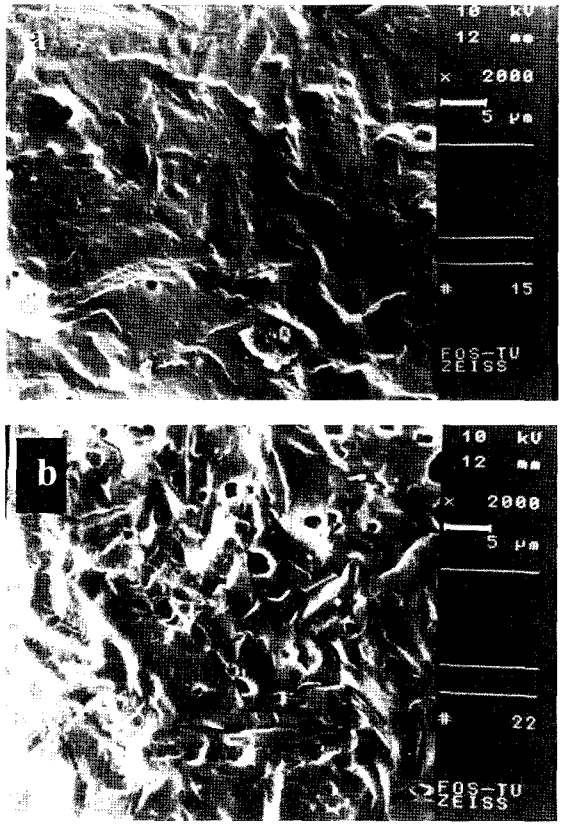

Fig. 7. SEM picture of the surface of microspheres cross-linked with glutaraldehyde after 8 h incubation in the dissolution medium: (a) phosphate buffer (b) phosphate buffer containing α-amylase enzyme.

linker resulted in microspheres with smoother surface, less swelling ratio and slower drug release rate. Particle size, swelling ratio and release characteristics of the microspheres could be controlled by changing the type and concentration of cross-linking agent as well as cross-linking time. SEM and drug release studies showed that cross-linked starch microspheres were susceptible to the enzymatic degradation under the influence of alpha-amylase and have the potential to be used as a carrier for colonic drug delivery.

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