ELSEVIER

Carbohydrate Polymers 36 (1998) 81–88

Carbohydrate Polymers

Influence of glutaraldehyde on drug release and mucoadhesive properties of chitosan microspheres

Ida Genta[a,*], Monica Costantini[b], Annalia Asti[c], Bice Conti[a], Luisa Montanari[b]

[a]*Department of Pharmaceutical Chemistry, University of Pavia, V. le Taramelli 12, Pavia, Italy*
[b]*Institute of Pharmaceutical and Toxicological Chemistry, University of Milan, V. le Abruzzi 42, Milan, Italy*
[c]*C.G.S., University of Pavia, V. Bassi 21, Pavia, Italy*

Received 18 September 1997; revised 8 January 1998; accepted 13 February 1998

Abstract

Among bioadhesive drug delivery systems, chitosan microspheres can be considered useful formulations for mucosal administration of drugs. The feasibility of modulating drug release from chitosan microparticles is due to polymer cross-linking, i.e. by glutaraldehyde. The aim of this work was to develop a new simple 'in vitro' technique based on electron microscopy in order to study the effect of polymer cross-linking density on mucoadhesive properties of the chitosan microspheres. This technique consists of scanning electron microscopy (SEM) and transmission electron microscopy (TEM) observations on morphological changes of chitosan microspheres with various cross-linking densities in contact with mucin solution. The results of SEM and TEM analyses have permitted to confirm the high affinity for mucin of uncross-linked chitosan microspheres and thus their bioadhesive properties. Moreover, bioadhesive characteristics of the microparticulate drug delivery systems were depressed for glutaraldehyde cross-linked chitosan microspheres.

Keywords: Chitosan; Microspheres; Glutaraldehyde; Mucoadhesiveness

1. Introduction

Chitosan is a natural polymer useful for medical and pharmaceutical applications (Muzzarelli, 1977). Among biocompatible and biodegradable natural polymers it displays interesting biological activities (Akbuga, 1995). Recent studies prove that chitosan has mucoadhesive properties (Lehr et al., 1992), and therefore it seems particularly useful to formulate bioadhesive dosage forms for mucosal administration (ocular, nasal, buccal, gastro-enteric and vaginal-uterine therapy).

'In vitro' tests so far proposed for studying the bioadhesiveness of various materials for pharmaceutical use, were set up mainly for viscous solutions or pharmaceutical dosage forms, such as granules, films, patches or tablets (Duchene et al., 1988). Bioadhesive properties of microparticulate drug delivery systems are poorly studied by these techniques because they do not provide an evaluation of the actual interaction between the mucus layer and the single particle (dimensional range 1–100 μm).

Few 'in vitro' techniques for direct measurements of adhesive force between polymeric microparticles and a mucus layer have been developed (Teng and Ho, 1987; Duchene et al., 1988 and Iida et al., 1993).

Duchene et al. (1988) refers to an apparatus proposed by Peppas and Mikos consisting of a thin channel filled with mucin solution, in which a single spherical particle is placed. Optical microscopical observations permitted to measure the time for detachment, type of motion and distance traveled by the particle submitted to a gas or viscoelastic liquid flow. These parameters were related to mucoadhesive properties of the particles. Teng and Ho (1987) developed the flowing liquid film technique. This consisted of an excised intestinal segment spread on a plastic flute and positioned at an inclined plane; a suspension of polymeric particles flowed down the intestinal strip. The steady-state fraction of particles adsorbed on intestinal tissue was quantified by Coulter counter analyses and related to adhesive properties of the particles. Iida et al. (1993) studied the bioadhesive properties of polymeric particles by the pendulum impact separation method. Particles were attached to different substrates (glass, PVC) and the percentage of particles adhering to the substrate was determined by

* To whom correspondence should be addressed.

0144–8617/98/$19.00
PII S0144-8617(98)00022-8

Table 1
Concentration of glutaraldehyde solutions, theophylline solutions and theophylline/chitosan ratios used in microsphere preparations

Batch	Glutaraldehyde (%, v/v)	Theophylline (%, w/v)	Theophylline/chitosan (w/w)	Microsphere formation
1B	0.60	—	—	No
2B	1.25	—	—	No
3B	2.50	—	—	No
4A (4B)	5.00	2.72 (—)	1:1 (—)	Yes
5A (5B)	7.00	2.72 (—)	1:1 (—)	Yes
6A (6B)	10.0	2.72 (—)	1:1 (—)	Yes
7A (7B)	15.0	2.72 (—)	1:1 (—)	Yes
8A (8B)	25.0	2.72 (—)	1:1 (—)	Yes
9A (9B)	5.00	0.68 (—)	1:4 (—)	Yes
10A (10B)	5.00	0.34 (—)	1:8 (—)	Yes

Keys: A, drug loaded microspheres; B, 'blank' microspheres; No, no formed microspheres; Yes, well-formed microspheres

counting the number of them before and after impact by an image analyzer connected to an optical microscope.

In the present work chitosan was employed for microparticulate drug delivery systems. Chitosan microspheres were prepared by a modified emulsification/separation method (Chithambra Thanoo et al., 1992) and loaded with a hydrophilic model drug, theophylline. A chemical cross-linking agent, glutaraldehyde, was used at different concentrations in order to prepare chitosan microspheres with various cross-linking densities (Jayakrishnan and Jameela, 1996) modulating the rate of theophylline release. Glutaraldehyde reacts with chitosan and it cross-links in an inter- and intramolecular fashion through the formation of covalent bonds mainly with the amino groups of the polysaccharide. Since chitosan mucoadhesive properties seem to be mediated by ionic interactions between positively charged amino groups in polymer and negatively charged sialic acid residues in mucus (Illum et al., 1994), a purpose of the present work was to verify the effect of glutaraldehyde on the chitosan/mucus layer interaction by a new 'in vitro' technique based on electron microscopy.

2. Materials and methods

Chitosan (M_r 750 000; degree of deacetylation, 83.5%; viscosity grade (1% w/v in 1% acetic acid), 200–800 mPa·s (Fluka, Milano, Italy)), theophylline monohydrate (Carlo Erba, Milano, Italy), glutaraldehyde, 25% (v/v) aqueous solution (Fluka), mucin, crude from porcine stomach (Type II) (Sigma, Milano, Italy) and guanidinium chloride (BDH Laboratory Supplies, Poole, UK) were used. All other reagents were of analytical grade.

2.1. Rheological measurements

Viscosimetry was performed with a rotational cup and bob viscometer VT-500 (Haake, Karlsruhe, Germany) equipped with MV1 and SV2 sensor systems.

Analyses were performed on: (a) chitosan solutions at three concentrations (1, 2, 3% (w/v)) in 5% CH_3COOH solution; (b) paraffin oil, 'light' paraffin oil and sunflower oil.

2.2. Microsphere preparation method

Chitosan microspheres were prepared according to an emulsion/separation method (Chithambra Thanoo et al., 1992). The emulsion was constituted by a chitosan solution (dispersed phase) dispersed in an oily phase (continuous phase). Chitosan was solubilized in 5% CH_3COOH: methanol (2:1) at the highest concentration (2.72%) compatible with its siringability. The cosolvent, methanol, was added in order to improve the solubility of theophylline and the formation of spherical chitosan droplets in paraffin oil (Lin and Lin, 1992).

The rheological measurements permitted to choose the most suitable continuous phase to be used in order to obtain a stable emulsion with the chitosan solution employed whose viscosity was <3 mPa·s (at 300 s^{-1}). Viscosity analyses on paraffin oil, 'light' paraffin oil and sunflower oil (216, 38 and 57 mPa·s at 300 s^{-1}) were carried out. The high viscosity of paraffin oil did not permit to obtain microspheres. Sunflower oil was chosen as continuous phase because of its characteristics of viscosity and biocompatibility.

The dispersed phase (15 ml) was injected in a continuous phase made of 225 ml sunflower oil and an emulsifier (2% Span 80) maintained at room temperature, and under continuous stirring at 9500 r.p.m. (Ultraturrax model T25-S25 18G IKA-Labortechnik, Staufen, Germany); the ratio between internal and external phase was kept constant (1:15, v/v).

Fifteen minutes later, 15 ml of glutaraldehyde solution saturated in toluene (Longo et al., 1982) at different concentrations (Table 1) were dropped in the emulsion, agitated by a paddle stirrer at 500 r.p.m. for 1 h at 38°C in order to increase the cross-linking rate (Roberts and Taylor, 1989). The microspheres obtained were washed with petroleum ether and recovered by centrifugation at 10 000 r.p.m. The

spheres were rinsed with citro-phosphate buffer, pH 6.8 (USP XXIII), filtered on 0.45 μm membrane (Millipore S.p.A., Milano, Italy) and dried under vacuum for 48 h (Table 1, batches 4B–10B). Theophylline-loaded microspheres were obtained as described above, by adding different amounts of drug to the polymer solution, as shown in Table 1 (batches 4A–10A), in order to obtain 1:1, 1:4 or 1:8 drug/polymer ratios (w/w).

2.3. Microsphere characterization

2.3.1. Morphology

All batches of microspheres were preliminarily checked for shape and size by optical microscopy. A light transmission microscope model 104 (Nikon, Tokyo, Japan) at 400 × magnification was employed. The samples were prepared by suspending a small amount of microspheres in paraffin oil.

Microsphere shape and surface were evaluated by scanning electron microscopy. The observations were made with a Cambridge Stereoscan (Cambridge Instruments Ltd., Cambridge, UK) operating at 20 kV.

2.3.2. Particle size analysis

Particle size distributions of the batches were determined by the electric sensing zone method (Coulter Counter Multisizer model TA II, Coulter Electronics Ltd., Luton, United Kingdom). Small amounts of microspheres were suspended in a 5% (w/v) NH_4SCN iso-propanol solution and analyzed at 64 size levels, between 1.792 and 58.96 μm. The results were the average of five withdrawals.

2.3.3. Drug content

Theophylline was extracted from microspheres with a mixture of 0.1 N HCl and ethanol (50:50, v/v) for 24 h at room temperature. The samples were filtered and solutions assayed spectrophotometrically at 270 nm wavelength (UV-Vis spectrophotometer model DU-7, Beckman, Geneva, Switzerland).

2.3.4. 'In vitro' dissolution test

'In vitro' dissolution tests were carried out in phosphate buffer pH 7.4 (USP XXIII) at 37°C. A suitable amount of microspheres was added to 400 ml of the dissolution medium in stoppered bottles and shaken at 100 strokes min^{-1} for 48 h. Aliquots of 5 ml were withdrawn at fixed time intervals and assayed for theophylline as explained before.

2.4. Electron microscopy

2.4.1. Scanning electron microscopy (SEM)

2.4.1.1. Sample preparation. Uncross-linked and cross-linked chitosan microspheres were dispersed in distilled water containing mucin (1 mg ml^{-1}) and allowed to stand for 2 min at room temperature.

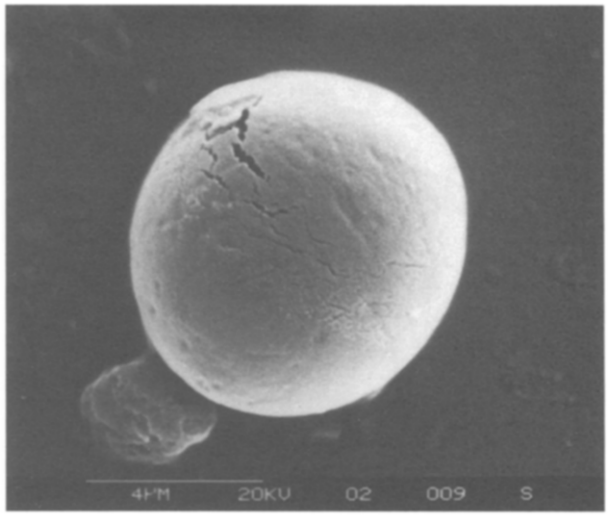


Fig. 1. Scanning electron photomicrograph of chitosan microspheres (batch 5A) (magnification 7000 ×)

2.4.1.2. Analysis. Samples were placed on a support, air dried and fixed on an aluminium stub with a conductive copper tape: then covered with a gold layer (degree of purity, 99.9%) with a Sputter Coater Edwards S 150 A (Edwards High Vacuum International, Crawley, United Kingdom). The observation has been made with a Cambridge Stereoscan (Cambridge Instruments Ltd., Cambridge, United Kingdom) operating at 20 kV.

2.4.2. Transmission electron microscopy (TEM)

2.4.2.1. Sample preparation. Mucin (0.1–0.8 mg ml^{-1}) was solubilized in 6 M guanidinium chloride aqueous solution; uncross-linked and cross-linked chitosan microspheres (1 mg ml^{-1}) were dispersed in 6 M guanidinium chloride aqueous solution containing mucin (0.8 mg ml^{-1}) and allowed to stand for 30 min at room temperature.

Samples were dispersed in 6 M guanidinium chloride aqueous solution in order to avoid a possible tendency for mucin to self-associate (Sheehan and Carlstedt, 1984).

2.4.2.2. Analysis. Samples of porcine mucin and chitosan microspheres, prepared as described above, were placed on

Table 2
Particle size analyses of chitosan microspheres by Coulter counter

Batch	$d^a_{50\%}$ (μm)	$d^b_{90\%}$ (μm)
4A	19.00	28.23
5A	13.01	25.96
6A	14.00	30.33
7A	10.11	19.03
8A	9.66	21.70

[a]Fifty percent of particles has a volume-diameter smaller than that reported in the table

[b]Ninety percent of particles has a volume-diameter smaller than that reported in the table

Table 3
Theophylline encapsulation efficiencies

Batch	Actual drug content (%, w/w)	Encapsulation efficiency[a] (%)
4A	5.62	11.23
5A	6.25	12.50
6A	5.45	10.90
7A	5.75	11.50
8A	4.63	9.26
9A	3.11	12.45
10A	1.19	9.48

[a]Encapsulation efficiency = (actual drug content)/(theoretical drug content) × 100

formvar/carbon-coated grids, air-dried and stained with 2% uranyl acetate, then observed on a Jeol JEM 1200EX electron microscope (Tokyo, Japan) operating at 80 kV.

3. Results and discussion

Chitosan microspheres were prepared by using glutaraldehyde solutions at concentration higher than 5% (Table 1, batches 4A,B–8 A,B) by the emulsion/separation method.

The microspheres obtained were of good morphological characteristics, spherical shape and smooth surface. For

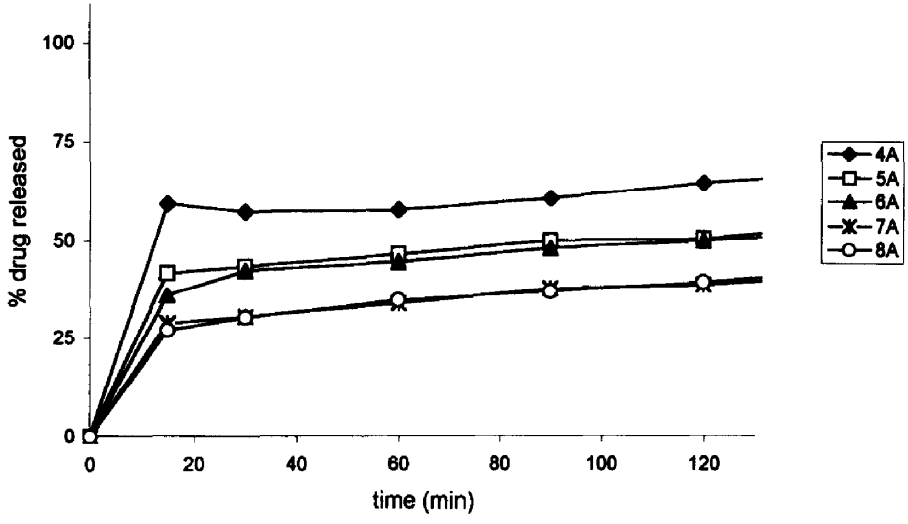


Fig. 2. Theophylline release profiles from chitosan microspheres prepared with glutaraldehyde solutions of different concentrations

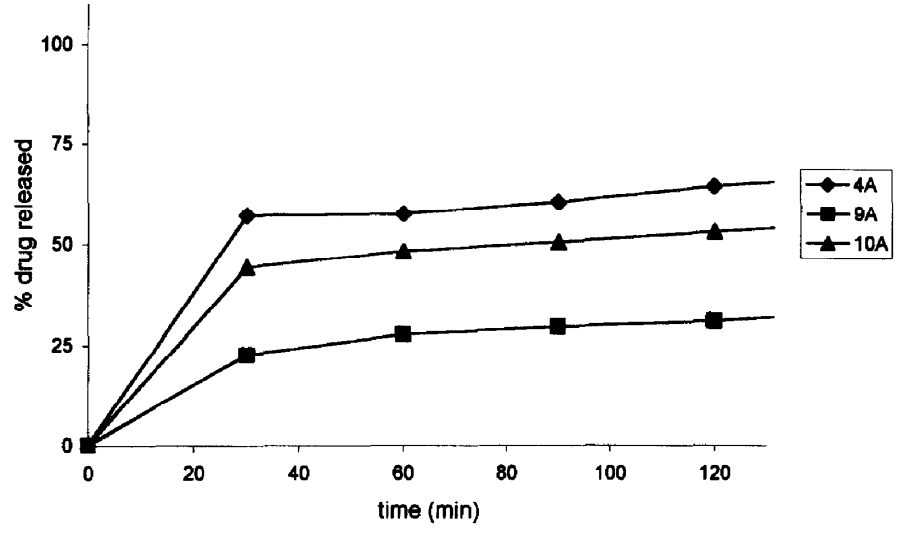
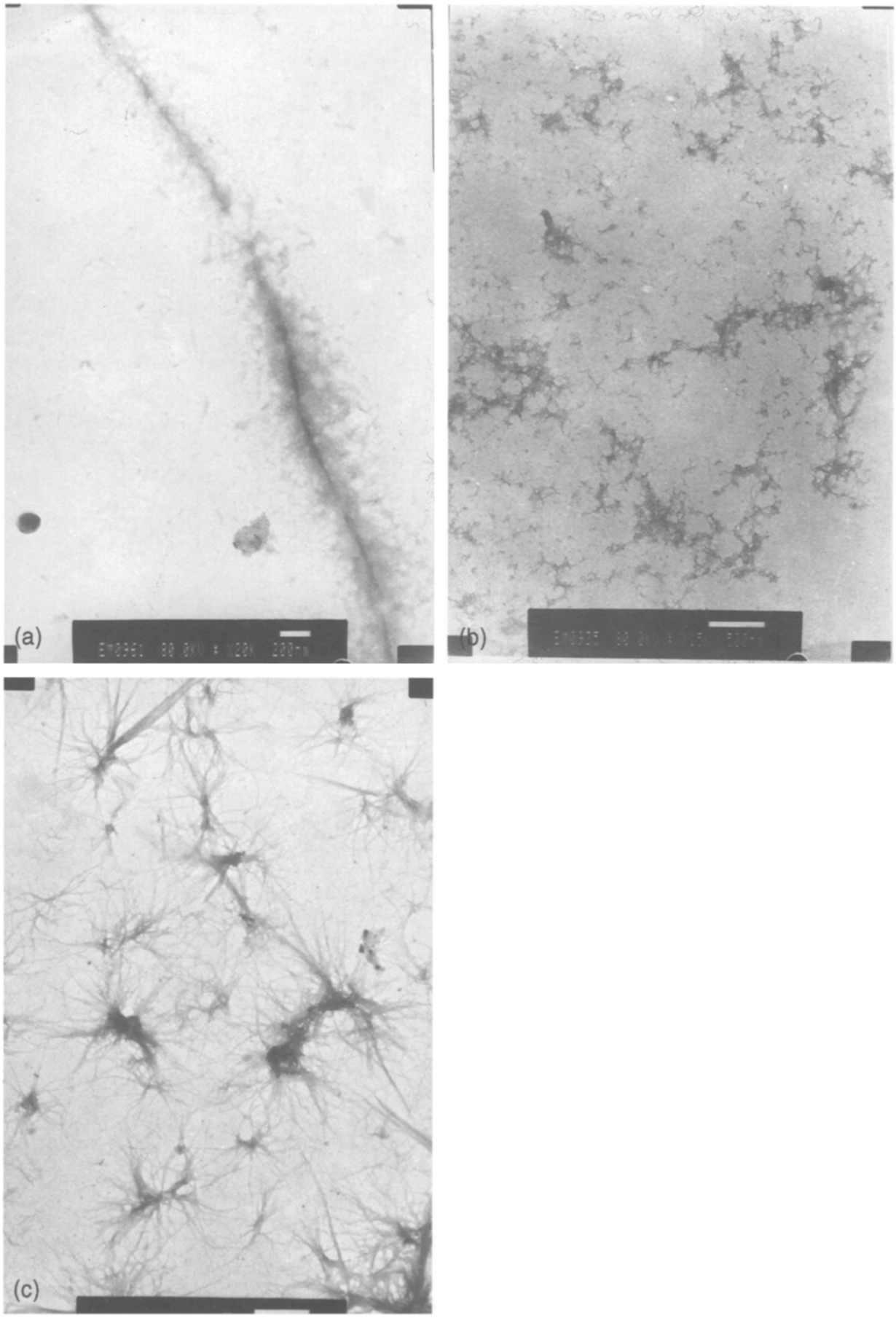


Fig. 3. Theophylline release profiles from chitosan microspheres constituted of different drug/polymer ratios

Fig. 4. Transmission electron micrographs of porcine mucin (100 $\mu g\ ml^{-1}$) in 6 M guanidinium chloride aqueous solution. (*a*) Mucin appears as linear model, with a large number of oligosaccharides covalently linked to a central protein core. Magnification 15 000 × . (*b*) In this picture, the macromolecules appear as aggregated structures. Magnification 15 000 × . (*c*) Another representative form of protein. Magnification 15 000 ×


(a)
(b)
(c)

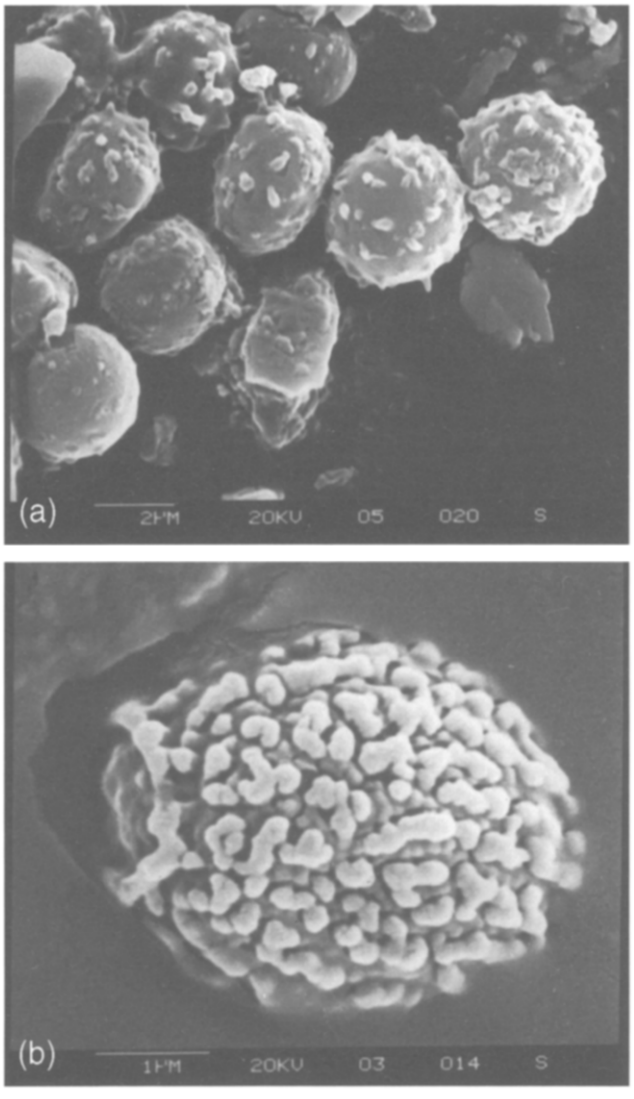


Fig. 5. Scanning electron micrographs of uncross-linked chitosan microspheres with mucin in aqueous solution. (*a*) In this picture it is possible to observe mucin adhering to microparticle surface. Magnification 7000 ×. (*b*) A large quantity of protein completely covers an uncross-linked microsphere. Magnification 18 000 ×

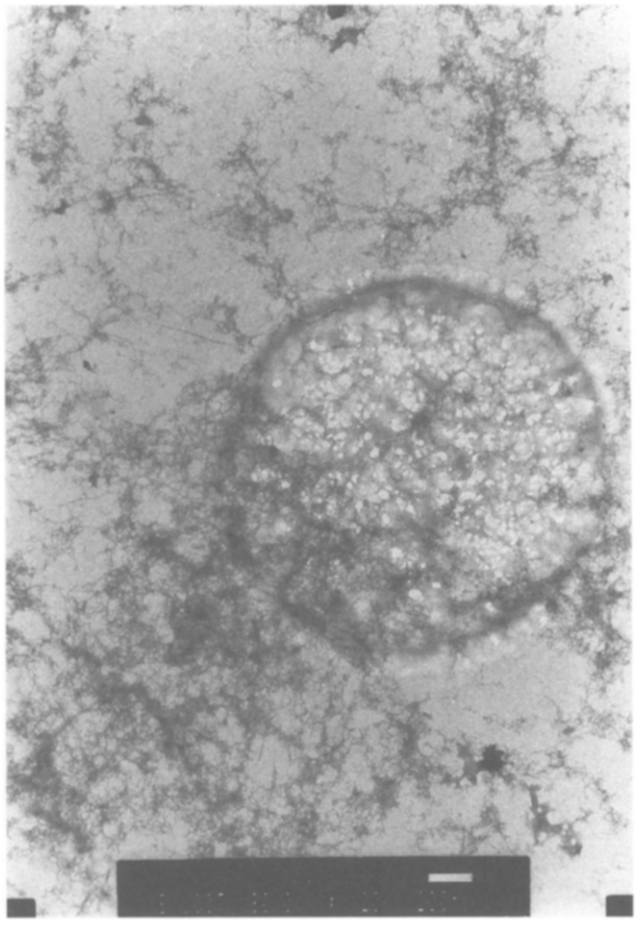


Fig. 6. Transmission electron micrograph of porcine mucin (800 $\mu g\ ml^{-1}$) in 6 M guanidinium chloride aqueous solution with an uncross-linked microsphere. Chitosan microspheres interact with the protein. In this picture mucin appears as aggregate structure. Magnification 20 000 ×

example, Fig. 1 shows a scanning photomicrograph of cross-linked chitosan microspheres (batch 5A). No morphological difference was highlighted for uncross-linked microspheres. Results of particle size analyses are reported in Table 2.

D_{90}% of all microsphere batches ranged between about 20 and 30 μm. The use of glutaraldehyde solutions at concentrations higher than 10% seems to decrease the microsphere sizes (batches 7A–8A). The influence of the cross-linking density on the microsphere sizes can be explained by the cross-linking reaction conditions: the cross-linkage happens during the emulsification phase of the preparation method and for this reason it affects the whole polymeric matrix.

Table 3 lists encapsulation efficiencies of microspheres prepared. The highest encapsulation efficiencies were obtained with the saturated theophylline solution (batches 4A–8A). For these batches, constituted by the same theoretical drug/polymer ratio (1:1, w/w), glutaraldehyde concentration and theoretical drug/polymer ratio did not affect significantly the drug encapsulation efficiency (9.3%–12.5%). Production yields were always higher than 80%.

3.1. 'In vitro' release tests

The 'in vitro' release profiles of theophylline from chitosan microspheres having different cross-linking densities are shown in Fig. 2.

As expected, microspheres prepared using higher glutaraldehyde amount showed slower drug release patterns (Fig. 2).

Microspheres prepared with 7%–10% or 15%–25% (v/v) glutaraldehyde solutions presented no difference in theophylline release profiles. After 90 min, batches 5A,6A and 7A,8A had released, respectively, about 49 and 37% of drug content.

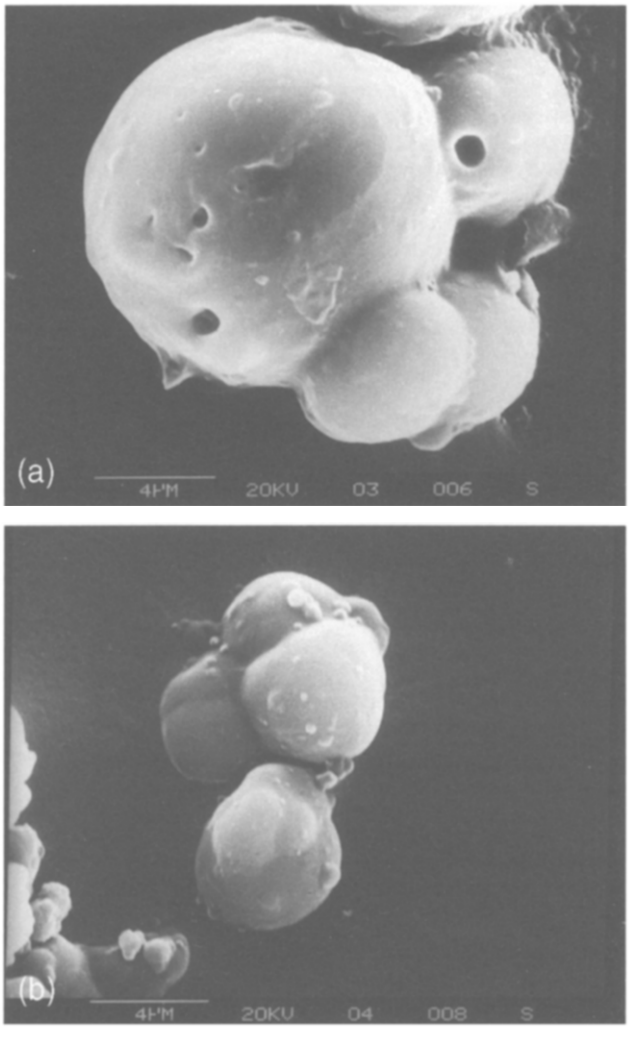


Fig. 7. Scanning electron micrographs of cross-linked chitosan microspheres with mucin in aqueous solution: the smooth microsphere surface in both pictures, seems to be deprived of any interaction with the protein. (*a*) Microspheres cross-linked with 5% Ga (batch 4B); (*b*) microspheres cross-linked with 7% Ga (batch 5B). Magnification for (*a*) and (*b*) 5000 ×

Fig. 3 shows theophylline dissolution profiles from chitosan microspheres (batches 4A, 9A, 1OA) made of different theoretical drug/polymer ratio and with the same cross-linking density. The theophylline release is faster for those microspheres with higher drug content.

3.2. *'In vitro' bioadhesive tests*

Transmission electron microscopy has been employed by Fiebrig et al. (1996) to visualize the structure and organization of pig gastric mucin in a mixture with chitosan dilute solution.

In the present work, electron microscopy techniques have been used with the aim of evaluating the surface interactions between uncross-linked (Genta et al., 1997) and cross-linked chitosan microspheres with mucin. Different structural features of porcine mucin were observed (Fig. 4).

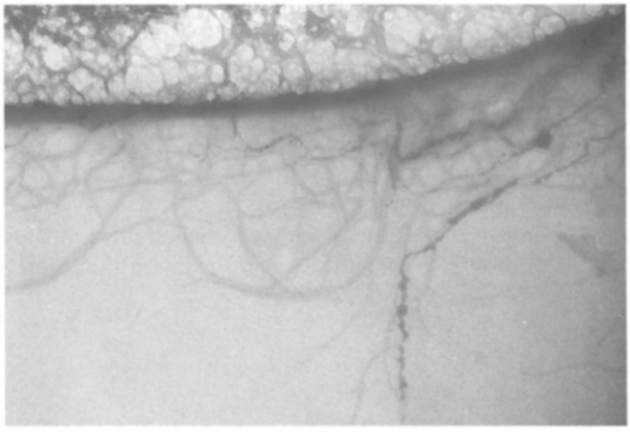


Fig. 8. Transmission electron micrograph of porcine mucin (800 $\mu g\ ml^{-1}$) in 6 M guanidinium chloride aqueous solution and cross-linked chitosan microspheres (batch 4B). The microsphere surface seems to reject the protein probably due to the action of glutaraldehyde. Magnification 25 000 ×

Fig. 5 shows that the uncross-linked microsphere surface is covered by mucin, and this is more evident at higher magnification (Fig. 5*b*); these observations prove the affinity between chitosan and mucin, and confirm the mucoadhesive properties of the polymer, as shown in detail in Fig. 6.

Fig. 7 shows mucin in contact with chitosan microspheres at different cross-linking densities (batches 4A,5A); no interaction seems to take place between protein and microspheres, even for those prepared with the smallest amount of glutaraldehyde (Fig. 7*a*). This behaviour is confirmed by TEM observation (Fig. 8): protein seems to be rejected from the microsphere surface and this indicates that glutaraldehyde, upon reaction with the amino groups responsible for the chitosan/mucin interaction, reduces the affinity of the polymer with mucin and depresses the mucoadhesive properties of chitosan microparticles.

4. Conclusions

Glutaraldehyde cross-linking is useful in order to modulate theophylline release from chitosan microspheres. Microspheres prepared with 7%–10% and 15%–25% glutaraldehyde solution, respectively, show the same drug release profiles: the suitable drug release rate can be obtained with the lowest glutaraldehyde amount (7 or 15%) in order to limit the toxicological effect of the cross-linking agent.

The electron microscopy techniques proposed, are particularly attractive for evaluating the mucoadhesive properties of polymer microparticulate drug delivery systems 'in vitro' in the presence of mucin solution. SEM is a straightforward technique useful to visualize morphological changes on the surface of the microspheres due to mucin; TEM permits to confirm SEM results and to observe the ultrastructural features of the surface interaction between

chitosan microspheres and mucin chains. These microscopic investigations show that the chitosan/mucin chain interaction is related to the presence of glutaraldehyde: uncross-linked chitosan microspheres have high affinity for mucin while cross-linked microspheres loose this property.

Mucoadhesive chitosan microsphere formulations need the optimization of glutaraldehyde concentration in order to control drug release as well as to maintain bioadhesive properties.

Acknowledgements

This work was supported by a grant from FAR (Fondo Ateneo Ricerche).

References

Akbuga, J. (1995). A biopolymer: chitosan. *Int. J. Pharm. Adv.*, *1*, 3–18.

Chithambra Thanoo, B., Sunny, M.C., & Jayakrishnan, A. (1992). Cross-linked chitosan microspheres: preparation and evaluation as a matrix for the controlled release of pharmaceuticals. *J. Pharm. Pharmacol.*, *44*, 283–286.

Duchene, D., Touchard, F., & Peppas, N.A. (1988). Pharmaceutical and medical aspects of bioadhesive systems for drug administration. *Drug Dev. Ind. Pharm.*, *14*, 283–318.

Fiebrig, I., Harding, S.E., Rowe, A.J., Hyman, S.C., & Davis, S.S. (1996). Transmission electron microscopy studies on pig gastric mucin and its interactions with chitosan. *Carbohyd. Polym.*, *28*, 239–244.

Genta, I., Perugini, P., Conti, B., & Pavanetto, F. (1997). 'A multiple emulsion' method to entrap a lipophilic compound into chitosan microspheres. *Int. J. Pharm.*, *152*, 237–246.

Iida, K., Otsuka, A., Danjo, K., & Sunada, H. (1993). Measurement of the adhesive force between particles and a substrate by means of the impact separation method. Effect of the surface roughness and type of material of the substrate. *Chem. Pharm. Bull.*, *41*, 1621–1625.

Illum, L., Farraj, N.F., & Davis, S.S. (1994). Chitosan as a novel nasal delivery system for peptide drugs. *Pharm. Res.*, *11*, 1186–1189.

Jayakrishnan, A., & Jameela, S.R. (1996). Glutaraldehyde as a fixative in bioprostheses and drug delivery matrices. *Biomaterials*, *17*, 471–484.

Lehr, C.M., Bouwstra, J.A., Schacht, E.H., & Junginger, H.E. (1992). In vitro evaluation of mucoadhesive properties of chitosan and some other natural polymers. *Int. J. Pharm.*, *78*, 43–48.

Lin, S.-Y., & Lin, P.-C. (1992). Effect of acid type, acetic acid and sodium carboxymethyl cellulose concentrations on the formation, micromeritic, dissolution and floating properties of theophylline chitosan microcapsules. *Chem. Pharm. Bull.*, *40*, 2491–2497.

Longo, W.E., Iwata, H., Lindheimer, T.A., & Golberg, E.P. (1982). Preparation of hydrophylic albumin microspheres using polymeric dispersion agents. *J. Pharm. Sci.*, *71*, 1323–1328.

Muzzarelli, R.A.A. (Ed.). (1977). *Chitin*. Oxford: Pergamon Press.

Roberts, G.A.F., & Taylor, K.E. (1989). The formation of gels by reaction of chitosan with glutaraldehyde. *Makromol. Chem.*, *190*, 951–960.

Sheehan, J.K., & Carlstedt, I. (1984). Hydrodynamic properties of human cervical-mucus glycoproteins in 6 M-guanidinium chloride. *Biochem. J.*, *217*, 93–101.

Teng, C.L.C., & Ho, N.F.H. (1987). Mechanistic studies in the simultaneous flow and absorption of polymer-coated latex particles on intestinal mucus I: Methods and physical model development. *J. Contr. Rel.*, *6*, 133–149.