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In vitro evaluation of the mucoadhesive properties of chitosan microspheres

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Abstract

The mucoadhesive properties of chitosan and chitosan microspheres were evaluated by studying the interaction between mucin and chitosan in aqueous solution by turbidimetric measurements and the measurement of mucin adsorbed on the microspheres. A strong interaction between chitosan microspheres and mucin was detected. Adsorption studies were carried out for the adsorption of mucin to chitosan microspheres with different crosslinking levels. The adsorption of type III mucin (1% sialic acid content), to chitosan microspheres followed Freundlich or Langmuir adsorption isotherms. When the contents of sialic acid was increased (i.e. type I-S mucin, 12% sialic acid content), the adsorption type followed more closely an electrostatic attraction type of isotherm. The heat of the adsorption was found to be 13–23 kJ/mol. A salt-bridge effect has been proposed for the interaction of the positively charged mucoadhesive chitosan microspheres with the negatively charged mucus glycoprotein. The extent of mucus adsorption was proportional to the absolute values of the positive zeta potential of chitosan microspheres and negative 'zeta potential' of mucus glycoprotein. Factors leading to a reduction or a reversal of these absolute values (e.g. different crosslinking levels of chitosan microspheres, different types of mucin, different pH, or ionic strength of the medium used) led to a reduction in the amount adsorbed. The extent of this reduction depended upon the decreasing extent of the repective zeta potentials. Biological studies showed that chitosan microspheres were retained by a biological tissue; rat small intestine.

Keywords: Chitosan microspheres; Mucoadhesion; Adsorption isotherm; Thermodynamics; Biological studies

1. Introduction

Chitosan (poly[β-(1-4)-2-amino-2-deoxy-D-glucopyranose]) is a cationic polysaccharide, derived by the deacetylation of chitin, which is the most

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abundant polysaccharide in the world, next to cellulose. As early as the 1980's, chitosan was evaluated as a matrix for sustained release granules (Miyazaki et al., 1981; Hou et al., 1985), tablets (Kawashima et al., 1985; Akbuga, 1993), and more recently as a component of gels (Knapczyk, 1993; Kristl et al., 1993), and membranes (Thacharodi and Rao, 1993) as well as a film matrix (Chandy and Sharma, 1991; Angelova et al., 1995).

Pharmaceutical applications of chitosan in the form of beads, microspheres and microcapsules were developed in the early of 1990's. Large chitosan microspheres and beads (up to thousands of microns) have typically been used for the prolonged release of drugs (Hou et al., 1994; Wan et al., 1994; Sezer and Akbuga, 1995; Acikgoz et al., 1996) and proteins such as bovine serum albumin (Jameela et al., 1994), DNA (Alaxakis et al., 1995), and brain derived neurotrophic factor (Mittal et al., 1994). Small particle size ($< 5\ \mu$m) chitosan microspheres, containing anticancer agents such as 5-fluorouracil (5-FU) (Ohya et al., 1993), and magnetic microspheres (Hassan et al., 1992) have been described for site specific delivery.

Chitosan is a hydrophilic, biocompatible and biodegradable polymer of low toxicity. It is commercially available in a range of molecular weights, degrees of deacetylation and types of salts such as glutamate, hydrochloride and lactate. Chitosan possesses OH and NH_2 groups that can give rise to hydrogen bonding and the linear molecule expresses a sufficient chain flexibility, the conformation of which is highly dependent on ionic strength. These properties are considered essential for mucoadhesion (Smart et al., 1984; Peppas and Buri, 1985; Robinson et al., 1987; Robinson and Mlynek, 1995). Furthermore, the cationic polyelectrolyte nature of chitosan could provide a strong electrostatic interaction with mucus or a negatively charged mucosal surface. The importance of the mucoadhesive properties of chitosan has been demonstrated in earlier work by Lehr et al. (1992), Illum et al. (1994), Lueßen et al. (1994), Fiebrig et al. (1995) and Aspden et al. (1996). More specifically, chitosan has been used as a delivery vehicle for the nasal and peroral delivery of peptide drugs, in order to improve drug absorption (Illum et al., 1994; Lueßen et al., 1996, 1997). Mucoadhesive tablets, containing chitosan, have been developed by Takayama et al. (1990), Miyazaki et al. (1994) and Nakayama et al. (1994). These tablets can be used for oral, intraoral or sublingual drug delivery. They have both adhesive and sustained release characters. It was also shown that the coating of liposomes with chitosan improved their adsorption to mucosal surfaces (Takeuchi et al., 1996). The application of chitosan in ocular mucoadhesive drug delivery systems has been reviewed by Greaves and Wilson (1993).

The in vitro evaluation of the mucoadhesive properties of a polymer or polymeric microsphere is a basic step in the development of a mucoadhesive drug delivery system. The aim of the present study has been to evaluate the mucoadhesive properties of chitosan, particularly, of the chitosan microspheres. Previously the aspect has been little discussed. Based on the fundamental principles of physical chemistry, these properties have been assessed by evaluation of the interaction between chitosan and mucin in aqueous solution, adsorption isotherms of mucus glycoprotein to chitosan microspheres, and the corresponding heat of the adsorption. Furthermore, using a biological approach, the adhesion of chitosan microspheres to mucosal tissue (rat small intestine) has also been evaluated.

2. Materials and methods

2.1. Materials

Chitosan (Seacure CL 210) was obtained from Pronova A/S. Norway. Ethyl cellulose (EC) was purchased from Sigma (Dorset, UK). Mucin, Type III, and Mucin Type I-S, which contain approximately 1% and 12% sialic acid, respectively, were purchased from Sigma. Glutaraldehyde (50% aqueous solution) and poly(vinyl alcohol) (PVA, Mwt 50000) were obtained from Aldrich (Dorset, UK). All chemicals, reagents and solvents used were of the highest grade available and used as provided.

2.2. Preparation of chitosan microspheres

Chitosan microspheres were produced by a spray drying method. Briefly, 200 ml of 0.5% of chitosan aqueous solution were prepared by dissolving overnight 5 mg of chitosan per ml of distilled water under stirring and adding 0.25, 0.5, 1, 2 or 4 ml of 4% glutaraldehyde aqueous solution to the chitosan solution before spray drying, corresponding to batch no. 5, 4, 3, 2, and 1, respectively. Co-current spray drying was performed using a SD-04 spray drier (Lab Plant, England), with a standard 0.5 mm nozzle. The inlet temperature and spray flow rate were set at 160°C and 6 ml/min, respectively.

As a comparison, ethyl cellulose (EC) microspheres were prepared from 2% EC in dichloromethane by a spray drying method. The spray drying conditions were set as the following: inlet temperature: 35°C; spray flow rate: 6 ml/min.

2.3. Characterization of the chitosan and EC microspheres

Microspheres were sized using a Malvern MasterSizer (model MS 1002). The volume mean diameter (VMD) and distribution of particle size were measured. Zeta potentials were measured by electrophoresis, which was performed with a Malvern Zetasizer 4 apparatus. pH 7.0 phosphate buffers (0.005 M, 0.0005 M and 0.0001 M), pH 5.5, 4.0, 3.5 acetate buffers (0.001 M) and simulated gastric fluid (2.0 g of sodium chloride and 7.0 ml of hydrochloric acid in 1 l of distilled water, pH 1.2) were used as different environments.

2.4. Interaction between mucin and chitosan in aqueous solution – turbidimetric measurement

Stock solutions (2 mg/ml) of polymer (chitosan and PVA as a control) and mucin (Type III) were prepared with pH 4.5 acetate buffer ($I = 0.1$). They were then filtered with a Whatman No.1 filter paper, stored in amber glass bottles at 4°C. The samples were prepared by mixing different ratios (1:3; 1:1; 3:1; 9:1) of mucin stock solution with chitosan or PVA stock solution. The absorbancies of these samples were recorded at 500 nm with a UV spectrophotometer (UVIKON 860 spectrophotometer, Kontron Instruments, Switzerland) after 30 min of mixing. The absorbancies of the individual polymers and mucin in acetate buffer were measured as controls. The absorbancies at 500 nm were used to give the theoretical values for a non-interacting system.

2.5. Mucus glycoprotein assay

A colorimetric method, Periodic acid/Schiff (PAS) colorimetric method (Mantle and Allen, 1978) was used for the determination of the free mucin concentration for the studies on the adsorption of mucin on the chitosan microspheres. Two reagents were prepared. Schiff reagent contains 100 ml of 1% basic Fuchsin (Pararosaniline) aqueous solution and 20 ml of 1 M HCl. Sodium metabisulphite (0.1 g) was added to every 6 ml of Schiff reagent before use and the resultant solution was incubated at 37°C until it became colourless or pale yellow. Periodic acid reagent was freshly prepared by adding 10 μl of 50% of periodic acid solution to 7 ml of 7% acetic acid solution.

Standard calibration curves were prepared from 2 ml of mucin standard solutions (0.25, 0.5, 0.75 and 1 mg/2 ml). After adding 0.2 ml of periodic acid reagent, the samples were incubated at 37°C, for 2 h, in a water bath. Then, 0.2 ml of Schiff reagent was added at room temperature. Thirty minutes later, the absorbance of the solution was recorded at 555 nm in a UV spectrophotometer. Duplicate samples were run. Samples were determined with the same procedure. The mucin content was calculated from the standard calibration curve.

2.6. Adsorption of mucin on chitosan microspheres

Mucin aqueous solution with different concentrations (0.025, 0.05, 0.1, 0.2 and 0.5 mg/ml) were prepared. Chitosan microspheres (10 mg) with different crosslinking level (batches no. 1–5) were dispersed in the above mucin solutions, vortexed, and shaken at 5°C, 20°C and 37°C for 5 to 120

Table 1
Physicochemical characteristics of chitosan microspheres

Batch no.	Amount of glutaraldehyde (ml)	Size (μm)	Zeta potential (mV)			
			pH 7 phosphate buffer (M)			pH 4 acetate buffer
			0.005	0.0005	0.0001	
1	4	3.3	3.5	12.6	17.6	20.5
2	2	4.9	4.6	13.0	20.0	21.3
3	1	7.9	5.7	14.6	21.1	22.6
4	0.5	9.0	6.6	17.7	22.6	23.5
5	0.25	12.0	7.5	20.1	23.7	26.3
EC microspheres		5.1	−15.4	−14.5	−15.0	−5.2

min, separately. Then the dispersions were centrifuged at 4000 rpm for 2 min and the supernatant was used for the measurement of the free mucin content. The influence of pH, ionic strength and mucin type on the adsorption were studied by adjusting the medium pH (5.5, 3.5, 1.5), ionic strength ($I = 0.005$, 0.02, 0.2) and using the two types of mucin with different sialic acid content, respectively.

2.7. Rat gut loop studies of mucoadhesion

Male Wistar rats, with a mean weight about 300 g, were anesthetized and killed with an overdose of barbiturate. The small intestine was removed and washed with physiological saline using the following procedure (Ranga Rao and Buri, 1989), with a syringe: 5–10 ml/min for 10 min, then 20–30 ml/min for about 20 min. At least 500 ml of the saline was used for cleaning the intestine. The cleaned tissues were used immediately or kept at −15°C until use, which was within 2 days.

A required amount of chitosan microspheres (batch no. 1–5) or EC microspheres were suspended in physiological saline and sonicated. The microsphere suspension was filled into lengths of small intestine (about 15 cm in length) and sealed. These tubes were incubated in saline at 37°C for 60 min. The microsphere suspension was then removed and the number of microspheres present in the suspension before and after the adhesion study was counted using a Coulter Counter (Coulter® MULTISIZER II, Coulter Electronics Limited, England). The percentage of microspheres adhered to the tissue was calculated from the difference of the counts. The validity of the counting technique was proven by the existence of a linear relationship between the amount of the chitosan microspheres suspension added to a fixed 100 ml of electrolyte, and the resultant counts. At least five measurements were made for each sample.

3. Results and discussion

3.1. Physicochemical characteristics of the microspheres

The physicochemical characteristics of the five different chitosan microspheres with different levels of cross-linking (batches no. 1–5) and the EC microspheres are shown in Table 1. The characteristics of chitosan microspheres are closely associated with the level of the cross-linking. The particle size of the chitosan microspheres ranged from 3–12 μm. The more of the cross-linking agent added, the less irregular were the microspheres formed, and the smaller the particle size of the microspheres. The zeta potential of the microspheres was measured in different media. Phosphate buffer influenced the measurement of zeta potential, due to the effect of the counterions on the positively charged chitosan microspheres. The positive zeta potential in phosphate buffer

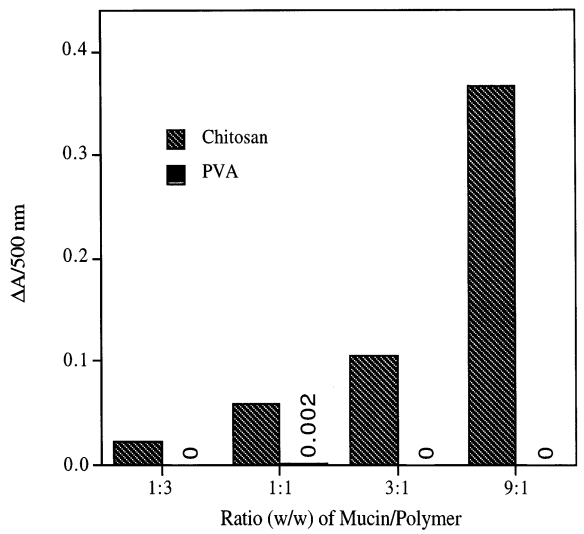


Fig. 1. Turbidimetric measurement of the interaction between polymer and mucin.

decreased with an increasing amount of cross-linking agent. The zeta potential in very dilute phosphate buffer (0.0001 M) was almost the same as that in acetate buffer solution. In contrast, the negative zeta potential for EC microspheres was nearly the same in the different concentrations of phosphate buffer. The particle size of EC microspheres was about 5 μm.

3.2. Interaction between mucin and chitosan in aqueous solution – turbidimetric measurement

The results of the turbidimetric studies on the mixed systems of mucin/chitosan and mucin/PVA are summarized in Table 2. The absorbance (A) of the mixture of mucin/polymer and absorbancies of the individual mucin and the individual polymer sample at 500 nm were measured. The theoretical absorbance (A_{Theor}) for the mixture of mucin/polymer system was calculated from the individual absorbancies. The absorbance difference (ΔA) between the measured and theoretical values for the mixture of mucin/polymer system was also calculated. If no interaction took place, the value of ΔA should be zero. The absorbance differences, measured for the mucin/chitosan systems were much higher than those calculated for the individual solutions, $\Delta A >> 0$. This result suggests that there was a strong interaction between chitosan and mucin. In contrast, the absorbance differences obtained from the mucin/PVA system were basically the same as the algebraic sum of the value for the individual solutions, $\Delta A \approx 0$. Therefore, there was little interaction between mucin and the control polymer PVA (Fig. 1).

Electrostatic attraction is thought to play an important role, because chitosan and mucin are an attraction pair with different charges, whereas PVA/mucin are a repulse pair with the same charges.

3.3. Adsorption kinetics

In order to determine the time for the equilibrium of adsorption to be reached, the free concentration of mucin in the suspension of the mixture of chitosan microspheres at different temperatures (5°C, 20°C, and 37°C) and at different times was measured. The amount of mucin adsorbed on the microspheres was calculated. The results are shown in Table 3. Adsorption equi-

Table 2
Turbidimetric measurement of the bioadhesion between polymer and mucin

Mucin:Polymer	1:3		1:1		3:1		9:1		Mucin	Individual polymer	
Polymer used	Chitosan	PVA	Chitosan	PVA	Chitosan	PVA	Chitosan	PVA		Chitosan	PVA
A_{500}	0.144	0.100	0.272	0.205	0.413	0.298	0.729	0.357	0.200	0.014	0.003
A_{Theor}	0.121	0.100	0.214	0.203	0.307	0.300	0.363	0.361			
$\Delta A = A_{500} - A_{Theor}$	0.023	0	0.058	0.002	0.106	−0.002	0.366	−0.004			

Table 3
Amount of adsorption of mucin at different times for chitosan microspheres batches no. 1 and 5 (concentration of mucin: 0.1 mg/ml)

Temperature (°C)	Chitosan microspheres	Amount of adsorption ($mg/cm^2 \times 10^3$) at different times (min)					
		5	10	30	60	90	120
5	No. 5		7.2	8.32	9.36	11.06	11.16
	No. 1		0.57	0.7	0.75	0.87	0.88
20	No. 5	10.56	10.92	12.16	13.36	13.36	
	No. 1	0.35	0.7	1.35	1.37	1.38	
37	No. 5	11.36	11.64	12.4	14.96	14.92	
	No. 1	1.16	1.32	1.74	1.83	1.88	

librium was reached at about 1 h and 1.5 h at temperatures of 20°C and 37°C, and 5°C, respectively. Therefore, the adsorption amount and the free equilibrium concentration of mucin in solution for the adsorption isotherms could be measured after 1 h (20°C, and 37°C) or 1.5 h (5°C).

3.4. Assessment of the mucoadhesive behaviour of chitosan microspheres

Since a strong interaction exists between mucin and chitosan, mucin should also be spontaneously adsorbed to the surface of the chitosan microspheres. For this reason, the mucoadhesive behaviour of chitosan microspheres was assessed by the suspension of chitosan microspheres in different amounts of mucin (Type III) in aqueous solutions at room temperature. As comparison, the mucoadhesive potential of EC microspheres, was also assessed with the same procedure. The free mucin concentration was determined after 1 h incubation. The amount of mucin adsorbed was determined from the free concentration of mucin in the reaction vessel, before and after adsorption. The results showed that a relatively large amount of mucin was adsorbed on the various batches of the chitosan microspheres (Fig. 2). The amount of mucin adsorbed increased with the increasing mucin concentration. In contrast, little mucin was adsorbed on the negatively charged EC microspheres. These results confirm that chitosan microspheres had the ability to adsorb mucin.

3.5. Adsorption isotherms

The adsorption of mucin on chitosan microspheres was measured by the determination of the equilibrium free concentration of mucin in solution at different temperatures for the different batches (No. 1–5). Two different types of mucin, with the different sialic acid contents (Type III, 1%; and Type I-S, 12%), were used in the present study. The adsorption isotherms for Type III mucin on chitosan microspheres were smooth curves (Fig. 3). These adsorption isotherms were fitted with Freundlich and Langmuir equations. Straight lines were obtained (Figs. 4 and 5). The constants from these lines are listed in Table 4. In

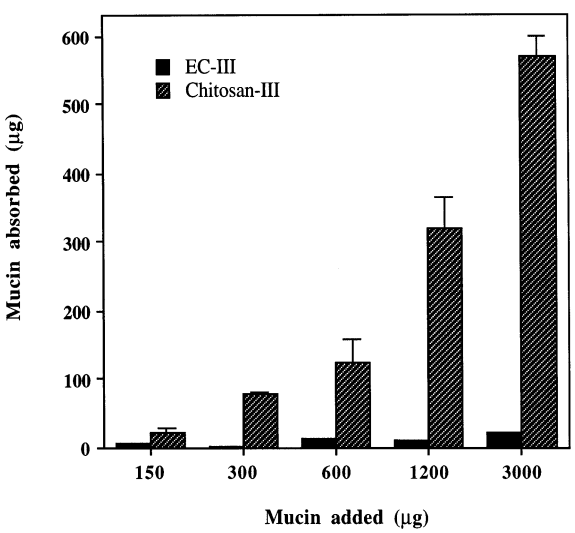


Fig. 2. Adsorption of mucin (type III) on different microspheres (chitosan or EC).

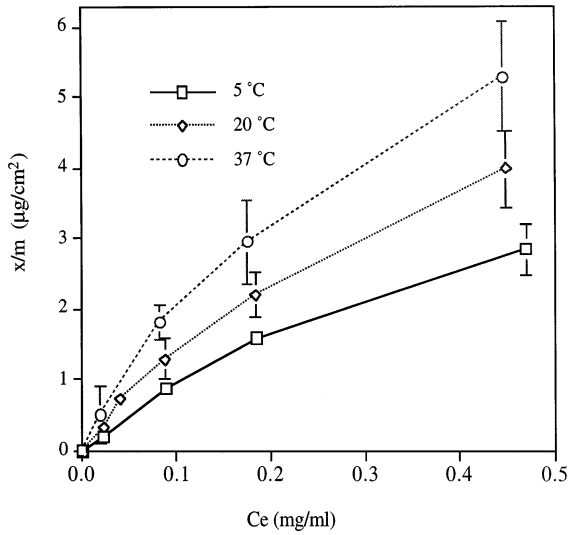


Fig. 3. Adsorption isotherms of mucin (type III) on chitosan microspheres (batch no. 2).

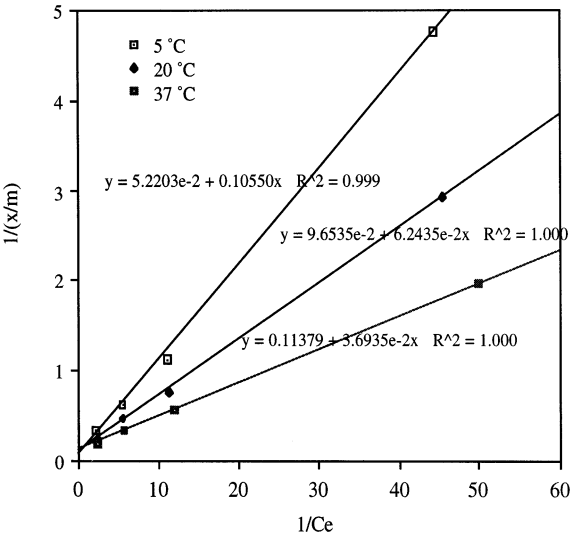


Fig. 5. Langmuir adsorption isotherms for mucin adsorbed on chitosan microsheres (batch no. 2).

contrast, the adsorption isotherm of Type I-S mucin on chitosan microspheres (batch no. 1) increased rapidly in the first phase and more slowly in the second phase (Fig. 6). This is indicative of a more specific adsorption process where electrostatic interaction is involved. The difference in the adsorption of the different mucins to chitosan microspheres is due to the difference in the sialic acid contents. The adsorption of mucin to chitosan is expected to be dominated by the electrostatic attraction between the positively charged chitosan and negatively charged mucin. Therefore, the surface charges of chitosan microspheres and mucin represented by zeta potential, would influence the amount adsorbed. It was shown in the present work that, the amount of mucin adsorbed increased with increasing mucin concentration. Secondly, in conformity with the electrostatic attraction theory, the amount of the adsorption decreased with an increasing amount of the cross-linking agent used in particle production (which led to decreased zeta potential of the particles). The microspheres of batch no. 5 (which had the lowest extent of cross-linking by glutaraldehyde and the highest zeta potential), had the largest amount of adsorbed mucin.

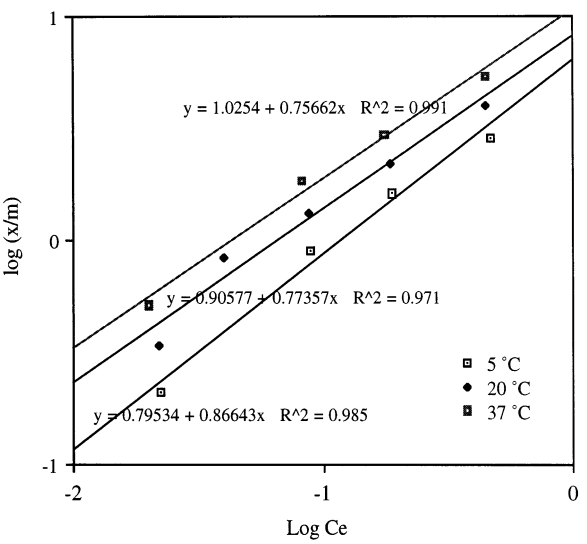


Fig. 4. Freundlich adsorption isotherms of mucin adsorbed on chitosan microspheres (batch no. 2).

The negative charge of mucin is due to the ionization of sialic acid. If the sialic acid content is low (e.g. mucin (Type III), 1%), the electrostatic attraction between the mucin (Type III) and chitosan will be relatively weak. When the contents of sialic acid is increased (such as the mucin (Type I-S)), the adsorption type would follow more closely an electrostatic attraction type of isotherm.

Table 4
The isotherms fitted with Freudlich and Langmuir equations

Temperature	Chitosan MS	Freudlich isotherm[a]			Langmuir isotherm[b]		
		K	n	r^2	a	b	r^2
5°C	No. 1	8.327	1.807	0.958	2.376e-2	8.466e-2	0.977
	No. 2	6.24	1.154	0.985	5.220e-2	0.10550	0.999
	No. 3	6.718	1.227	0.986	7.846e-2	8.121e-2	0.997
	No. 4	7.665	1.951	0.983	0.13182	2.651e-2	0.989
	No. 5	172	1.025	0.960	2.530e-2	3.478e-3	0.992
20°C	No. 1	14.72	1.082	0.957	7.634e-2	6.065e-2	0.998
	No. 2	8.050	1.293	0.971	9.654e-2	6.244e-2	1.000
	No. 3	10.05	1.276	0.962	0.12133	3.996e-2	0.997
	No. 4	19.2	1.43	0.982	5.772e-2	1.536e-2	0.980
	No. 5	193.2	1.055	0.973	2.359e-2	2.738e-3	0.995
37°C	No. 1	26.18	0.9808	0.995	5.371e-2	3.588e-2	1.000
	No. 2	10.60	1.322	0.991	0.1138	3.694e-2	1.000
	No. 3	11.38	1.34	0.977	8.803e-2	3.472e-2	1.000
	No. 4	16.69	1.98	0.947	6.731e-2	1.040e-2	0.997
	No. 5	226.6	1.076	0.972	2.353e-2	1.945e-3	0.999

[a] $x/m = \mathrm{K} \cdot \mathrm{Ce}^{1/n}$.
[b] $1/(x/m) = \mathrm{a} + \mathrm{b} \cdot 1/\mathrm{Ce}$.

3.6. Factors that influence bioadhesion

3.6.1. pH

The influence of pH on the adsorption of mucin (Type III and Type I-S) on chitosan microspheres was studied at pH 1.5, 3.5 and 5.5 at 20°C. The results are shown in Fig. 7. With an increasing pH value, the amount of mucin adsorbed increased within the pH range studied.

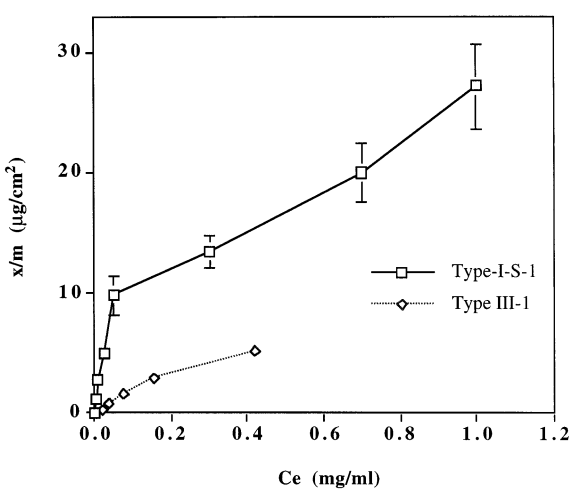


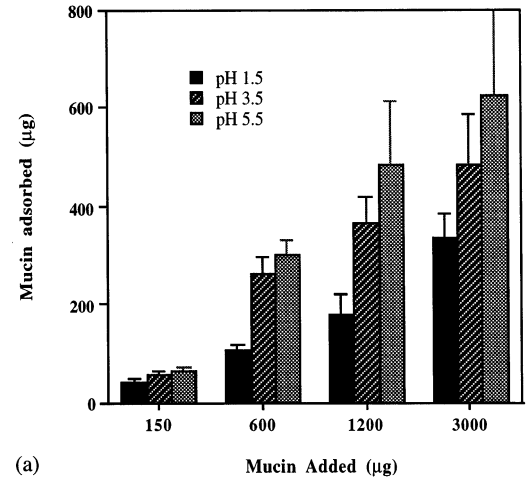
Fig. 6. The influence of mucin type on the adsorption of mucin on chitosan microspheres.

3.6.2. Ionic strength

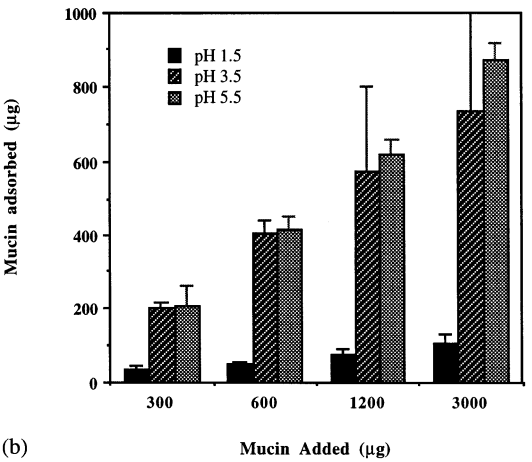
The influence of ionic strength was tested by adjusting the ionic strength ($I = 0.2$, $I = 0.02$ and $I \approx 0.005$) with sodium chloride at 20°C. The amount of mucin adsorbed on chitosan microspheres decreased with an increasing ionic strength (Fig. 8). At the highest ionic strength condition ($I = 0.2$), the adsorption amount was reduced dramatically. This effect was larger for mucin Type I-S than for mucin Type III.

3.6.3. Type of mucin

The mucins with the different sialic acid contents (Type III, 1%, and Type I-S, 12%) were used to study the adsorption of mucin on chitosan microspheres. The results are shown in Fig. 9. The amount of sialic acid groups on the mucin clearly influenced the electrostatic interaction between mucin and chitosan. A greater amount of adsorption was found with a high sialic acid content in the mucin (Type I-S) used, as compared to the

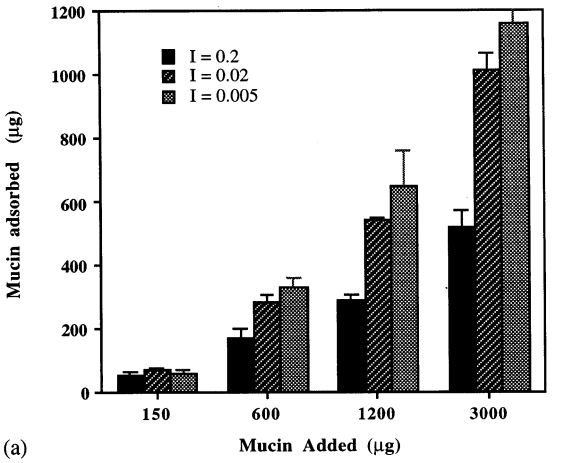


(a)

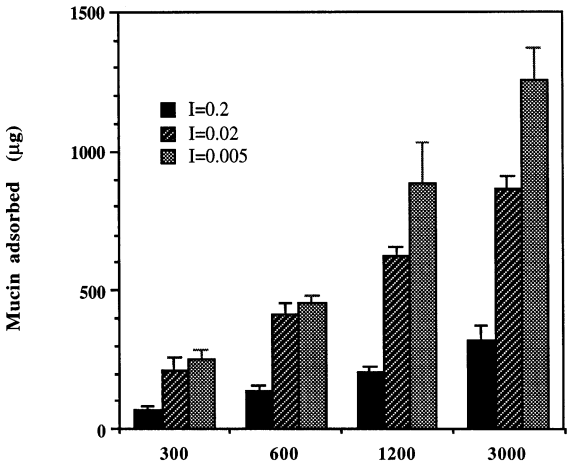


(b)

Fig. 7. The influence of pH on mucin adsorption on chitosan microspheres (10 mg). (a) Mucin type III, CMS, batch no. 5; (b) mucin type I-S, CMS, batch no. 1.



(a)



(b)

Fig. 8. The influence of ionic strength on mucin adsorption on chitosan microspheres (10 mg). (a) Mucin type III, Chitosan microspheres (CMS), batch no. 5; (b) mucin type I-S, CMS (batch no. 1).

low sialic acid content in the mucin (Type III). This was especially noticeable in batches no. 2–5, where a large amount of mucin (Type I-S) was adsorbed on the surface of the chitosan microspheres.

The studies of the factors that influenced the bioadhesion are further evidence that the interaction between mucin and chitosan microspheres is dominated by electrostatic attraction, which can be related to the effective surface charge. The 'zeta potentials[1]' of the two different mucins and chitosan microspheres were measured in various

[1] Mucin is a macromolecule. It will ionize in an aqueous environment. The ionized molecule will move under an electric field. Therefore, the mobility of the molecule can be measured by electrophoresis. Assuming that a macromolecule is a microparticle, "zeta potential" of the microparticle (although it is not a real zeta potential) can be calculated according to Smoluchowski equation, just for the purpose of data comparison.

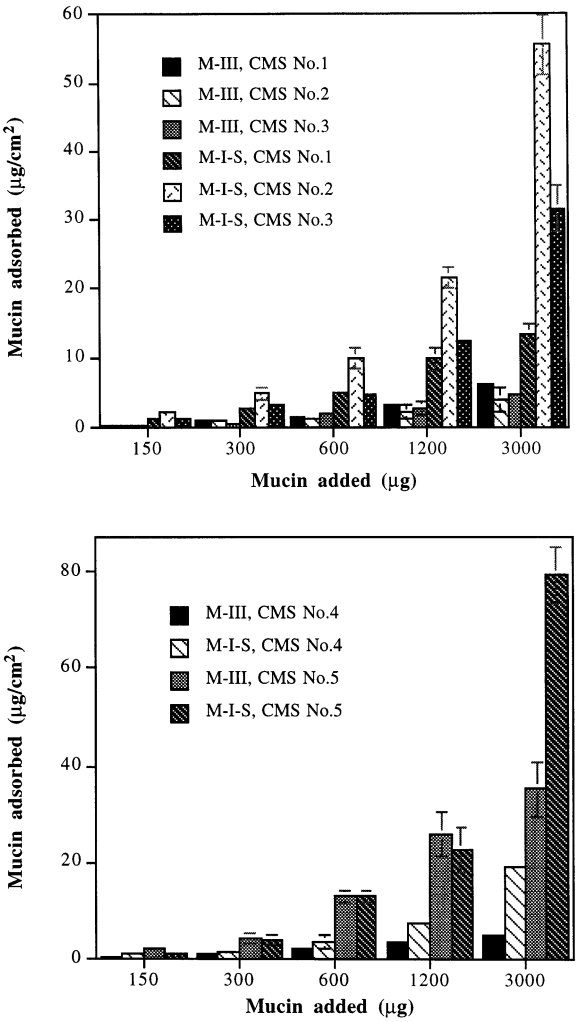


Fig. 9. The influence of mucin type on the adsorption, Chitosan microspheres (CMS) (batches no. 1–5).

solutions of different ionic strength and pH values. The results are shown in Table 5. These results support the theory that adsorption is dominated by an electrostatic attraction. For example, the amount of adsorption for the mucin (Type I-S) was much greater than that for mucin (Type III), which can be explained by the fact that the negative 'zeta potential' of mucin (Type I-S) was much greater than for mucin (Type III). In the pH 5.5 environment, the 'zeta potential' for mucin (Type III) was -5.9 mV, but the 'zeta potential' for mucin (Type I-S) reached -25.5 mV.

The effect of ionic strength can also be related to the measured zeta potentials. Chitosan microspheres (batch no. 5 and no. 1) were used to adsorb the two different types of mucin. In the case, where the ionic strength increased from $I \approx 0.005$ to 0.02, the zeta potentials of chitosan microspheres (No. 5) and mucin (Type I-S) decreased from 22.7 to 19.9 and from -25.5 to -20.3, respectively. When the ionic strength changed from $I = 0.02$ to 0.2, the zeta potentials of chitosan microspheres and mucin fell rapidly from 19.9 to 12.0 and from -20.3 to -10.7, respectively, corresponding to a large decrease in the adsorption amount (Fig. 8). The same kind of phenomena, but less pronounced, was observed in the case of mucin (Type III) adsorbed on chitosan microspheres of batch no. 1.

The pH value influenced the adsorption in the acidic environment, especially in the strong acidic environment (e.g. in pH 1.2 simulated gastric fluid), because the degree of the ionization of sialic acid or the different forms of the glycoprotein will be influenced by the pH value of the environment. Chitosan is a cationic polyelectrolyte, whose pK_a is about 6.5 (Roberts, 1992). In the acidic environment (e.g. $pH < 5.5$), a large amount of the amino groups ($> 90\%$) in chitosan exists as the ionic form. In this condition, there is little influence of pH on the degree of the ionization of the amino groups in chitosan. Sialic acid is a saccharic acid, and mucin is a glycoprotein. The values of pK_a and pI for sialic acid and mucin are 2.6 (Johnson and Rainsford, 1972) and about 3–5, respectively (Merck Index, 1989). Hence, the ionization of the sialic acid and the glycoprotein will be more sensitive to pH, in the acidic environment. As the pH value decrease, the amount of the ionized sialic acid will decrease, and 'zeta potential' of mucin will decrease. Hence, when the pH value changed from pH 5.5 to pH 3.5, the zeta potentials measured for the chitosan microspheres were nearly the same value but in contrast the negative value for 'zeta potential' of mucin decreased. This result also indicates that the number of the ionized sialic acid decreased. Therefore, the amount of adsorption of mucin on chitosan microspheres decreased. Furthermore, as the surrounding pH value changed from 3.5 to 1.2, the

Table 5
Zeta potentials of mucin and chitosan microspheres at various conditions

	pH 1.2 Simulated gastric fluid	pH 3.5 Acetate buffer	pH 5.5 Acetate buffer 0.001 M		
			$I \approx 0.005$	$I = 0.02$	$I = 0.2$
Mucin III	+2.6	−1.7	−5.9	−3.2	−1.4
Mucin I-S	+3.8	−20.8	−25.5	−20.3	−10.7
CMS-No. 1	+12.0	+23.3	+24.2	+19.4	+14.9[a]
CMS-No. 5	+10.3	+19.2	+22.7	+19.9	+12.0[a]

[a] $I = 0.1$; CMS = Chitosan microspheres.

'zeta potential' of mucin reversed from negative to positive. This corresponded to a dramatic fall in the adsorption amount (Fig. 7).

3.7. The heat of the adsorption

The heat of adsorption was calculated according to the van't Hoff isochore (Hunter, 1993).

$$(\partial \ln K/\partial T)_\theta = \Delta H_{ads}/RT^2$$

integrated at constant coverage θ

$$\log K = -\frac{\Delta H_{ads}}{2.303RT} + \text{Constant}$$

where ΔH_{ads} is the heat of adsorption, R is the gas constant ($R = 8.314$ J/mol per K), T is the absolute temperature. K is the equilibrium constant of the adsorption, which can be substituted by the amount of adsorption at constant coverage or derived from the Langmuir isotherm (Hunter, 1993). The van't Hoff isochore, the semilogarithmic plots of K versus the reciprocal of the absolute temperature are shown in Fig. 10. These plots were used to calculate the adsorption enthalpy. The heats calculated from these lines are listed in Table 6. The values of the heat of adsorption were about 20 kJ/mol, except for batch no. 5, where the heat of adsorption was about 13 kJ/mol. The positive enthalpy of adsorption of mucin on chitosan microspheres indicates an endothermic process. The adsorption amount increased with increasing temperature.

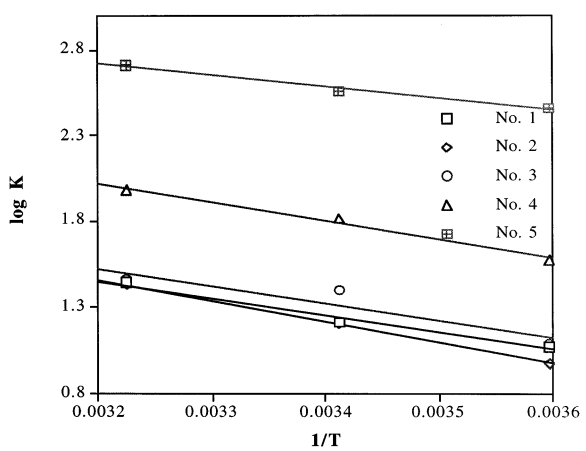


Fig. 10. van't Hoff isochores for the adsorption of mucin on chitosan microspheres (batches no. 1–5).

The interaction between the negatively charged mucin and the positively charged chitosan is an ionic neutralization process. This neutralization process should be exothermic. On the other hand, in an aqueous environment, the charged mucin and chitosan were hydrated.

$$CHI^{z+} + m \cdot H_2O\ (l) \rightarrow CHI(H_2O)_m^{z+}$$

$$M^{z-} + n \cdot H_2O\ (l) \rightarrow M(H_2O)_n^{z-}$$

where CHI^{z+} and M^{z-} are the forms of the charged chitosan and charged mucin, $CHI(H_2O)_m^{z+}$ and $M(H_2O)_n^{z-}$ are the forms of the hydrated chitosan and hydrated mucin, respectively. Usually, a hydration of a charged macromolecule is an exothermic process with reduction of entropy (Robbin, 1967). Therefore, when chitosan and mucin were brought into contact, in the aqueous environment, some of the hydrated water will be lost (dehydrated).

$$CHI(H_2O)_m^{z+} + M(H_2O)_n^{z-}$$

$$\rightarrow CHI \cdot M(H_2O)_q^{z(m-n)+} + (m + n - q) \cdot (H_2O)$$

Table 6
Enthalpy calculation by van't Hoff isochore for chitosan microspheres batches no. 1–5

Chitosan microspheres	Batch no.				
	1	2	3	4	5
ΔH_{ads} (kJ/mol)	19.24	23.84	19.00	20.98	13.00

The overall heat for the process of the interaction between mucin and chitosan should be the combination of exothermic charge neutralization and endothermic dehydration processes and the total process has been observed to be endothermic. Therefore, dehydration is the dominant process. According to the Flory, Krigbaum and Prigogine theories of a dilute macromolecular solution (van Oss, 1994), the enthalpy and entropy for the hydration of macromolecules are both negative. For the reverse dehydration process, both enthalpy and entropy will be positive, and the positive entropy will drive the process.

3.8. Mucoadhesion of chitosan microspheres in rat small intestine

The adsorption of chitosan microspheres (batches no. 1–5) on rat small intestine was tested by counting the number of the particles adsorbed to the tissue. The results are shown in Table 7. EC microspheres were assessed by the same procedure, as a comparison. More than 50% of chitosan microspheres was adsorbed on the tissue, whereas but few of the EC microspheres were adsorbed to the tissue. This is the further evidence for the strong interaction between chitosan microspheres and mucus glycoprotein and/or mucosal surfaces. Not only can mucin be adsorbed on chitosan microspheres, but more importantly from the standpoint of bioadhesion, chitosan microspheres can be adsorbed onto mucosal tissue.

Electrostatic interaction apparently plays an important role in the mucoadhesion of chitosan microspheres onto rat small intestine tissue. The amount of chitosan adsorbed on the tissue increased with the decreasing crosslinking level (which leds to increase in the positive zeta potential of chitosan microspheres). The negatively charged EC microspheres, on the other hand, could not be adsorbed on the rat small intestine.

The application of chitosan as a mucoadhesive delivery vehicle has attracted an increased attention. Junginger's group has recently studied the mucoadhesive properties of chitosan for peroral peptide drug delivery (Borchard et al., 1996; Lueßen et al., 1996, 1997). They used a solution of chitosan and other polymers as mucoadhesive delivery vehicles, to improve the bioavailabilities of peptide drugs. However, the duration of the effect, was relatively short. From the present studies it can be seen that chitosan microspheres were adsorbed onto the rat small intestine tissue and hence might be utilised as a bioadhesive intestinal delivery system.

Coating chitosan onto other materials might be a strategy to improve mucoadhesive properties of a delivery system. Takeuchi et al. (1996) developed a system of chitosan coated liposomes, loaded with insulin. This system could be adsorbed on the rat small intestine. The blood glucose level was reported to be significantly decreased after oral administration of this formulation to rats.

4. Conclusions

A strong interaction between chitosan and mucus glycoprotein in aqueous solution was measured. Positively charged chitosan microspheres, prepared by a spray drying method, had the ability to adsorb mucus glycoprotein.

Adsorption studies (adsorption kinetics, adsorption isotherms and heat of adsorption) were carried out for the adsorption of different types of mucin on chitosan microspheres with different crosslinking levels. The adsorption of type III

Table 7
Mucoadhesive measurement of chitosan microspheres on rat small intestine by particle counting technique

Chitosan microspheres	Counts of chitosan suspension		Adhered %
	Before incubation	After incubation	
No. 1	10 916 ± 3812	5560 ± 2424	49.1
No. 2	5773 ± 1315	2710 ± 1607	53.1
No. 3	7604 ± 1487	2634 ± 963	65.4
No. 4	7934 ± 2238	1737 ± 211	78.1
No. 5	11 670 ± 1686	3489 ± 953	70.1
EC-MS	3983 ± 597	4037 ± 845	0

mucin followed Freundlich or Langmuir adsorption isotherms. The heat of the adsorption was calculated to be 13–23 kJ/mol, according to van't Hoff isochore.

A relationship is proposed between the positive zeta potential of mucoadhesive chitosan microspheres, and the negative 'zeta potential' of mucus glycoprotein and adsorbed amount. The extent of adsorption was proportional to the absolute values of the positive zeta potential of chitosan microspheres and negative 'zeta potential' of the mucus glycoprotein. Any factor leading to reduced absolute values (e.g. different crosslinking level, different pH, or ionic strength of the medium used) will reduce the adsorption amount.

A biological study was performed by measuring the adsorption of a suspension of chitosan microspheres in the rat small intestine. This showed that more than 50% of chitosan microspheres were adsorbed on the tissue as compared to EC microspheres which are negatively charged.

In conclusion, chitosan and chitosan microspheres have excellent mucoadhesive properties. The electrostatic attraction between the positively charged mucoadhesive chitosan microspheres and negatively charged mucus glycoprotein plays an important role in the adsorption of mucin on chitosan microspheres and vice versa. Factors causing the reduction of this attraction would lead to a reduction in the adsorption.

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References

Acikgoz, M., Kas, H.S., Orman, M., Hincal, A.A., 1996. Chitosan microspheres of diclofenac sodium: I. application of factorial design and evaluation of release kinetics. J. Microencapsulation 13, 141–160.

Akbuga, J., 1993. Use of chitosonium malate as a matrix in sustained-release tablets. Int. J. Pharm. 89, 19–24.

Alaxakis, T., Boadi, D.K., Quong, D., Groboillot, A., O'Neill, I., Poncelet, D., Neufeld, R.J., 1995. Microencapsulation of DNA within alginate microspheres and crosslinked chitosan microspheres for in vivo application. Applied Biochem. Biotech. 50, 93–106.

Angelova, N., Manolova, N., Rashkov, I., Maximova, V., Bogdanova, S., Domard, A., 1995. Preparation and properties of modified chitosan films for drug release. J. Bioact. Comp. Poly. 10, 285–298.

Aspden, T.J., Illum, L., Skaugrud, O., 1996. Chitosan as a nasal delivery system-evaluation of insulin absorption enhancement and effect on nasal membrane integrity using rat models. Eur. J. Pharm. Sci. 4, 23–31.

Borchard, G., Lueben, H.L., de Boer, A.G., Verhoef, J.C., Lehr, C.M., Junginger, H.E., 1996. The potential of mucoadhesive polymers in enhancing intestinal peptide drug adsorption. III: Effects of chitosan-glutamate and carbomer on epithelial tight junctions in vitro. J. Control. Rel. 39, 131–138.

Chandy, T., Sharma, C.P., 1991. Biodegradable chitosan matrix for the controlled release of steroids. Biomater. Artificial Cells and Immobilization Biotech. 19, 745–760.

Fiebrig, I., Harding, S.E., Rowe, A.J., Hyman, S.C., Davis, S.S., 1995. Transmission electron-microscopy studies on pig gastric mucin and its interactions with chitosan. Carbohydrate polymers 28, 239–244.

Greaves, J.L., Wilson, C.G., 1993. Treatment of diseases of eye with mucoadhesive delivery system. Adv. Drug Del. Rev. 11, 349–383.

Hassan, E.E., Parish, R.C., Gallo, J.M., 1992. Optimized formulation of magnetic chitosan microspheres containing the anticancer agent, oxantrazole. Pharm. Res. 9, 390–397.

Hou, W.M., Miyazaki, S., Takada, M., 1994. Preparation and evaluation of ion-exchange cross-linked chitosan microspheres. Yakuzaigaku 54, 10–18.

Hou, W.M., Miyazaki, S., Takada, M., Komai, T., 1985. Sustained release of indomethacin from chitosan granules. Chem. Pharm. Bull. 33, 3986–3992.

Hunter, R.J. (Ed.) 1993. Introduction to modern colloid science. Oxford University Press Inc., New York, pp. 168-175.

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

Jameela, S.R., Misra, A., Jayakrishnan, A., 1994. Cross-linked chitosan microspheres as carriers for prolonged delivery of macromolecular drugs. J. Biomater. Sci. Polymer. 6, 621–632.

Johnson, P.M., Rainsford, K.D., 1972. The physical properties of mucus: Preliminary observations on the sedimentation behavior of porcine gastric mucus. Biochim. Biophys. Acta 286, 72.

Kawashima, Y., Lin, S.Y., Kasai, A., Handa, T., Takenaka, H., 1985. Preparation of a prolonged release tablet of aspirin with chitosan. Chem. Pharm. Bull. 33, 2107–2113.

Knapczyk, J., 1993. Chitosan hydrogel as a base for semisolid drug forms. Int. J. Pharm. 93, 233–237.

Kristl, J., Smid-Korbar, J., Struc, E., Schara, M., Ruppecht, H., 1993. Hydrocolloids and gels of chitosan as drug carriers. Int. J. Pharm. 99, 13–19.

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.

Lueßen, H.L., Rentel, C.O., Kotzé, A.F., Lehr, C.M., de Boer, A.G., Verhoef, J.C., Junginger, H.E., 1997. Mucoadhesive polymers in peroral peptide drug delivery. IV. Polycarbophil and chitosan are potent enhancers of peptide transport across intestinal mucosae in vitro. J. Contr. Rel. 45, 15–23.

Lueßen, H.L., de Leeuw, B.J., Langemeÿer, M.W.E., de Boer, A.G., Verhoef, J.C., Junginger, H.E., 1996. Mucoadhesive polymers in peroral peptide drug delivery. VI. Carbomer and chitosan improve the intestinal absorption of the peptide drug Buserelin in vitro. Pharm. Res. 13, 1668–1672.

Lueßen, H.L., Lehr, C.M., Rental, C.O., Noach, A.B.J., de Boer, A.G., Verhoef, J.C., Junginger, H.E., 1994. Bioadhesive polymers for the peroral delivery of peptide drugs. J. Contr. Rel. 29, 329–338.

Mantle, M., Allen, A., 1978. A colorimetric assay for glycoproteins based on the periodic acid/schiff stain. Biochem. Soc. Trans. 6, 607–609.

Mittal, S., Cohen, A., Maysinger, D., 1994. In vitro effects of brain derived neurotrophic factor released from microspheres. Neuroreport 5, 2577–2582.

Miyazaki, S., Nakayama, A., Oda, M., Takada, M., Attwood, D., 1994. Chitosan and sodium alginate based bioadhesive tablets for intraoral drug delivery. Bio. Pharm. Bull. 17, 745–747.

Miyazaki, S., Ishii, K., Nadai, T., 1981. The use of chitin and chitosan as drug carriers. Chem. Pharm. Bull. 29, 3067–3069.

Nakayama, A., Oda, M., Miyazaki, S., Takada, M., 1994. Oral mucosal adhesive tablets of indomethacin using chitosan and sodium alginate. Yakuzaigaku 54, 185–190.

Ohya, Y., Takei, T., Kobayashi, H., Ouchi, T., 1993. Release behaviour of 5-fluorouracil from chitosan-gel microspheres immobilizing 5-fluorouracil derivative coated with polysaccharides and their cell specific recognition J. Microencapsulation 10, 1–9.

Peppas, N.A., Buri, P.A., 1985. Surface, interfacial and molecular aspects of polymer bioadhesion on soft tissues. J. Contr. Rel. 2, 257–275.

Ranga Rao, K.V., Buri, P., 1989. A novel situ method to test polymer and coated microparticles for bioadhesion. Int. J. Pharm. 52, 265–270.

Robbin, O. (Ed.) 1967. Ionic reaction and equilibria, The Macmillan Company, New York.

Roberts, G.A.F. (Ed.) 1992. Chitin chemistry, Macmillan, Hampshire, pp. 274-280.

Robinson, J.R., Mlynek, G.M., 1995. Bioadhesive and phase-change polymers for ocular drug delivery. Adv. Drug Del. Rev. 16, 45–50.

Robinson, J.R., Longer, M.A., Veillard, M., 1987. Bioadhesive polymers for controlled drug delivery. Ann. New York Acad. Sci. 507, 307–314.

Sezer, A.D., Akbuga, J., 1995. Controlled release of piroxicam from chitosan beads. Int. J. Pharm. 121, 113–116.

Smart, J.D., Kellaway, I.W., Worthington, H.E.C., 1984. An in vitro investigation of mucosa-adhesive materials for use in controlled drug delivery. J. Pharm. Pharmacol. 36, 295–299.

Takayama, K., Hirata, M., Machida, Y., Masada, T., Sannan, T., Nagai, T., 1990. Effect of interpolymer complex formation on bioadhesive property and drug release phenomenon of compressed tablets consisting of chitosan and sodium hyaluronate. Chem. Pharm. Bull. 38, 1993–1997.

Takeuchi, H., Yamamoto, H., Niwa, T., Hino, T., Kawashima, Y., 1996. Enteral absorption of insulin in rats from mucoadhesive chitosan-coated liposomes. Pharm. Res. 13, 896–901.

Thacharodi, D., Rao, P., 1993. Propranolol hydrochloride release behaviour of crosslinked chitosan membranes. J. Chem. Tech. Biotech. 58, 177–181.

van Oss, C.J. (Ed.) 1994. Interfacial forces in aqueous media. Marcel Dekker, Inc., New York.

Wan, L.S.C., Lim, L.Y., Soh, B.L., 1994. Drug release from chitosan beads. S.T.P. Pharma. Sci. 4, 195–200.