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The enhancement of the bioadhesive properties of calcium alginate gel beads by coating with chitosan

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Abstract

Alginate-chitosan microcapsules have been investigated as a bioadhesive drug delivery system. Calcium alginate gel beads uncoated and coated with chitosan were tested for adhesive properties, using novel techniques, with negatively charged chromatography particles and in vitro with pig oesophagus and stomach mucosa. The addition of a chitosan coating increased the adhesive properties significantly. The adherence of both coated and uncoated beads was much greater to the stomach mucosa than to oesophageal mucosa. The difference in adhesive properties between the coated and uncoated microcapsules was also found considerably larger for the stomach mucosa. The homogeneity of the alginate gel core as measured by the alginate concentration gradient through the cross section of the beads also influenced the adhesive properties with homogeneous capsules adhering better than the inhomogeneous capsules.

Keywords: Alginate; Bioadhesion; Chitosan; Microcapsules

1. Introduction

There has been growing interest in polymer based bioadhesive drug delivery systems. One of the goals of such systems is to prolong the residence time of a drug carrier in the gastrointestinal (GI) tract (Lehr, 1996; Chickering et al., 1997). The bioadhesive bond can be of a covalent, electrostatic, hydrophobic or hydrogen bond nature (Peppas and Buri, 1985; Leung and Robinson, 1992). Ionic polymers are reported to be more adhesive than neutral polymers, and an increased charge density will also give better adhesion (Pep-

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pas and Buri, 1985; Jiménez-Castellanos et al., 1993), suggesting that the electrostatic interactions are of great importance.

Except for the oesophagus, the entire GI tract including the stomach, is covered with a continuous layer of insoluble mucus gel (Allen et al., 1993; Dixon et al., 1996). The mucus gel mainly consists of glycoproteins, and due to their content of ester sulphate and sialic acid groups, the mucus layer has an overall strong net negative charge (Allen et al., 1993). The mucus layer has been considered as a possible site for bioadhesion and drug delivery by several groups (Longer et al., 1985; Miyazaki et al., 1995; Takeuchi et al., 1996; Chickering et al., 1997).

Alginate is a negatively charged polysaccharide with high charge density and has been reported to be bioadhesive (Chickering et al., 1997; Craig and Tamburic, 1997; Gomotz and Wee, 1998).

Unlike most known bioadhesive polymers, chitosan is positively charged through its free amino groups in the deacetylated glucosamine residues. The polycationic nature is also unusual among biopolymers in general. Chitosan has been shown to interact with mucin (Fiebrig et al., 1994, 1995), and liposomes coated with chitosan have also been shown in vivo to have a prolonged residence time in the GI tract of rats relative to uncoated liposomes (Takeuchi et al., 1996).

The immobilisation of cells, enzymes and also drugs in calcium alginate polycation microcapsules is a simple and well known technique (Kokufuta et al., 1988; Smidsrød and Skjåk-Bræk, 1990; Hari et al., 1996; Rilling et al., 1997). We have recently characterised alginate-chitosan microcapsules regarding the quantitative binding of chitosan, the capsule stability and permeability as function of two capsule generation procedures and polymers different in composition and average molecular weight (Gåserød et al., 1998a,b). In the present paper such capsules have been quantitatively analysed in vitro for adhesive properties towards negatively charged microparticles and the non-mucous and mucous tissue samples of porcine oesophagus and stomach, respectively.

2. Materials and methods

2.1. Materials

The alginate used in all microcapsules was from the stipe of the brown algae *Laminaria hyperborea* (LF 10/60, Pronova Biopolymer, Drammen, Norway). The content and distribution of β-D-mannuronic acid (M) and α-L-guluronic acid (G) residues units was determined by ^{1}H NMR spectroscopy. The fraction of G units (F_G) was 0.70, and the fractions of the dimers F_{GG}, F_{MG} and F_{MM} were 0.59, 0.11 and 0.20, respectively. The intrinsic viscosity, $[\eta]$, was measured to 640 ml/g in 0.1 M NaCl at 20°C in a capillary viscometer (Schott-Geräte). Chitosan chlorides of different molecular weights were prepared by degradation with sodium nitrite. The number average molecular weights, M_n were determined from the intrinsic viscosity, $[\eta]$, measured in 0.1 M NaCl at pH 4.5 and 20°C in a capillary viscometer (Schott-Geräte), using the Mark–Houwink–Sakurada–Kuhn equation and the parameters obtained by Anthonsen et al. (1993). The fraction of acetyl groups was determined by ^{1}H NMR spectroscopy (Vårum et al., 1991).

2.2. Capsule formation

The alginate-chitosan capsules were made by different techniques and with chitosans of different composition, summarised in Table 1 and described in detail below. The solutions of alginate were made by dissolving the sodium alginate in deionized water to a concentration of 2% (w/v). To provide the beads with colour, 3% (v/v) of a suspension of yellow-green FluoSpheres (1 μm) (F-8823 Molecular Probes, Oregon, USA) was added to the alginate solution. The formed coloured beads was visible and could be counted manually. The chitosan solutions were made by dissolving the polymer in 0.02 M sodium acetate/acetic acid buffer, pH 5.0, to a final concentration of 0.3% (w/v).

2.2.1. One-stage process

In a one-stage procedure, the alginate solution was dripped into the chitosan solution and then

Table 1
Presentation of the various capsule fractions

Capsule fraction	One-stage/two-stage	Homogeneous/inhomogeneous	Chitosan M_n (Da)	Chitosan F_A
A	—	Inhom	Uncoated	—
B	—	Hom	Uncoated	—
C	One	Inhom	16 000	0.11
D	One	Hom	16 000	0.11
E	Two	Inhom	17 000	<0.01
F	Two	Hom	17 000	<0.01
G	Two	Inhom	16 000	0.11
H	Two	Hom	16 000	0.11
I	Two	Inhom	62 000	<0.01
J	Two	Hom	62 000	<0.01
K	Two	Inhom	62 000	0.11
L	Two	Hom	62 000	0.11
M	Two	Inhom	210 000	0.09
N	Two	Hom	210 000	0.09
O	Two[a]	Hom	16 000	0.11

One-stage/two-stage is referring to the capsule generation procedure.
[a] Capsules were made without calcium chloride in the chitosan solution.

the chitosan solution was replaced with an aqueous solution of 50 mM $CaCl_2$.

2.2.2. Two-stage process

When a two-stage procedure was used, calcium alginate beads were formed by dropping the alginate solution into a solution of 50 mM $CaCl_2$. After at least 30 min of gelation, the beads were transferred to a chitosan solution containing 300 mM of $CaCl_2$, and reacted for 5 h on a shaker. The capsules were washed in 0.02 M sodium acetate/acetic acid buffer, pH 5.0 and stored refrigerated in the buffer solution containing 2 mM sodium azide and 5 mM $CaCl_2$ to prevent microbial growth and capsule swelling, respectively.

2.2.3. Homogeneity of alginate core

The alginate gel core was made more or less inhomogeneous, with respect to the alginate concentration gradient through the cross-section of the bead, by adjusting the concentration of NaCl in the gelling bath (Skjåk-Bræk et al., 1989). Inhomogeneous beads were made with only 50 mM of $CaCl_2$ in the gelling solution, and for the homogeneous beads the gelling solution contained 50 mM of $CaCl_2$ and 200 mM of NaCl. The alginate distribution in such beads is determined by Thu et al. (1997) and Skjåk-Bræk et al. (1998).

2.2.4. Capsule size and size distribution

The capsule fractions were made with average diameters ranging from 490 to 526 μm for two-stage capsules and from 581 to 621 μm for one-stage capsules, by using an electrostatic bead generator (Kulseng et al., 1998). For each fraction the standard deviation for the capsule diameters were always lass than 5% of the average diameter.

2.3. Particle adhesion assay

Adhesive properties in suspension were monitored as the ability for the beads to bind negatively charged chromatographic particles, which have an average diameter of 34 μm and are charged with SO^{3-}- groups (Pharmacia HiTrap SP). Ten capsules and 0.5 ml of a suspension of the particles (10 μl sedimented particles/ml) in 0.02 M Na-acetate/acetic acid buffer, pH 5.0, were mixed on a shaker for 10 min. The capsules were allowed to sediment, and the number of particles adhered to each capsule surface facing up from the bottom of the container were determined in a microscope (Nikon Inverted Microscope, Diaphot-TMD). The diameter of each capsule was measured, and the number of particles were related to the surface area of the half of

the bead sphere. The average value and standard deviation were calculated from at least 30 capsules.

2.4. Tissue adhesion assays

2.4.1. Artificial saliva and gastric juice

Artificial saliva was produced according to the Documenta Geigy Scientific Tables (Diery and Lentner, 1970) of natural saliva contents, and was prepared as a solution of 0.21 g/l of $NaHCO_3$, 0.43 g/l NaCl, 0.75 g/l KCl, 0.22 g/l $CaCl_2 \cdot 2H_2O$, 0.91 g/l $NaH_2PO_4 \cdot H_2O$ and 2.7 g/l porcine gastric mucin, type II crude (Sigma, St. Louis, MO) in deionized water. The solution was stored refrigerated and used within 2 days after preparation. Artificial gastric juice, without pepsin, was made by mixing 2.0 g/l NaCl and 7.0 ml/l of concentrated HCl in deionized water (US Pharmacopeia Convention, 1994).

2.4.2. Tissue preparation

Fresh pig oesophagus and stomachs were obtained from the local abattoir within 1 h of killing the animal and then placed on ice. The muscle layer of the oesophagus was stripped off, and the epithelial layer was frozen immediately after stripping. The stomachs were opened, and the insides were rinsed with cold tap water removing stomach contents, loose mucus and mucus degrading enzymes. The mucosa with the adherent mucus was separated from the underlying muscle layers and then frozen. Prior to the experiments, the tissues were thawed in a refrigerator overnight.

2.4.3. Adhesion testing

2.4.3.1. Oesophagus. The oesophagus was cut open, and about 8 cm of the middle section was cut out for use. The tissue was then placed on a aluminium plate (3×7 cm) with the epithelial surface of the oesophagus facing up from the plate, and orientated so that the upper part of the oesophagus was at the top of the slope, i.e. the flow over the tissue is in the same direction as it occurs down the oesophagus. The tissue was sewed to the plate and stretched around all the four sides of the plate to make the tissue surface as planar as possible. During this preparation, the tissue was wetted with water to prevent drying. The plate with the tissue was fixed in an angle of 40° relative to the horizontal plane. Artificial saliva was pumped at a rate of 2 ml/min over the tissue for 10 min before the capsules were added. Both the oesophagus and stomach experiments were performed over a water bath and under a humidity hood at 37°C, and the artificial saliva and gastric solutions were kept at the same temperature (Fig. 1).

Prior to depositing the microcapsules onto the tissue, a frame leaving an area of about 1×2 cm was placed 2 cm from the top of the plate on the oesophagus (Fig. 2A). About 0.05 ml of capsules measured in a 1 ml syringe were suspended in 1 ml of water and transferred to the frame. The beads were then allowed to sediment onto the tissue, and the water slowly flowing out under the frame was sucked up until the water inside the frame was removed. The frame was taken away, and the flow of artificial saliva turned on. The apparatus was designed so that the artificial saliva spread across the tissue such that all of the capsules were exposed to the liquid flow. The capsules washed off the tissue at 5, 10, 15, 20, 30 and 45 min were collected and counted. At the end of each run, the residual capsules left on the tissue were washed off and counted. The same piece of tissue was used for up to six runs. The reproducibility was tested by running the same capsule fraction six times on the same piece of tissue with consistent result. The tissue to tissue variation was found larger than the variation on the same tissue represented by standard deviations of 11.3% ($N = 10$) and 5.5% ($N = 6$), respectively, for the proportion of adhered samples after 45 min. When comparing different capsule fractions, each tissue was tested with a chosen standard microcapsule sample, and the resulting adhesion for each sample was compared to this particular sample.

2.4.3.2. Stomach. For stomach tissue adhesion experiments, a similar procedure to that for the oesophagus experiments was used, but the following modifications were made to promote removal as early tests showed that the capsules had a much higher ability to adhere to this tissue.

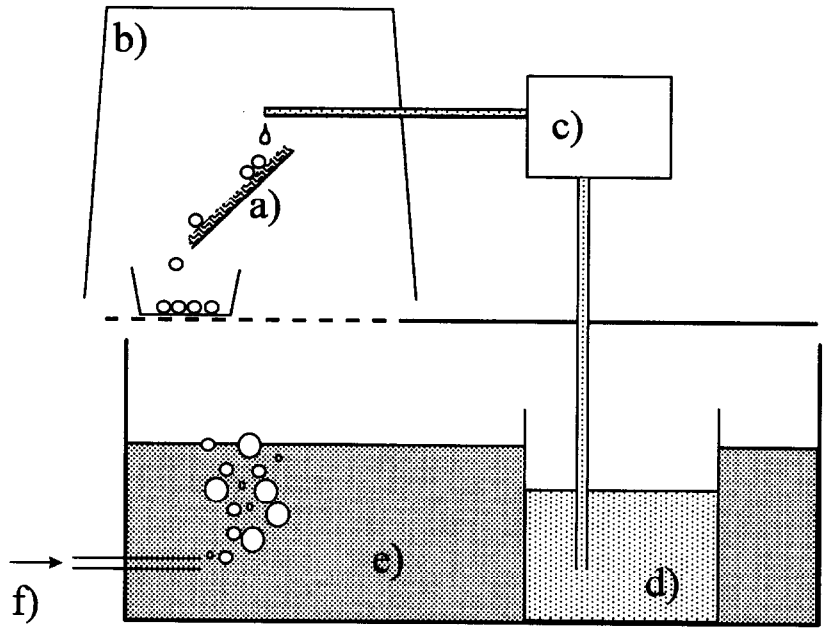

Fig. 1. Schematic representation of the experimental set-up for the mucosal tissue adhesion assay. (a) tissue, (b) humidity hood, (c) pump, (d) artificial saliva or gastric juice, (e) waterbath at 37°C and (f) air flow to promote humidification.

Known number of beads (150–220) were deposited in the initial positioning frame which was placed only 5 mm from the bottom edge of the slope (Fig. 2B). The flow of artificial gastric juice was increased to 4.5 ml/min, and the flow was from a greater height of 5.7 cm above the tissue so that it hit the tissue directly above the deposited capsules (Fig. 2B). The angle of the slope of tissue was increased to 50° relative to the horizontal plane. Only the capsules washed off the tissue were collected and counted and each piece of stomach mucosa was used only once.

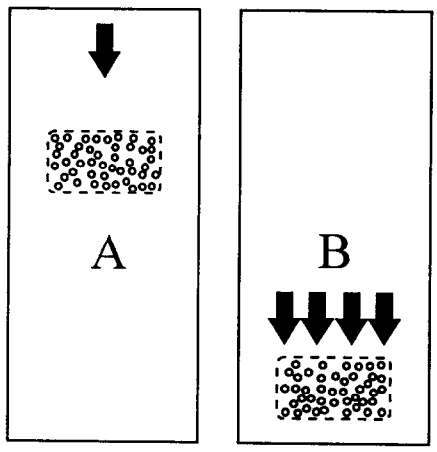

Fig. 2. Illustration of the starting position of the beads on the mucosa for A: oesophagus and B: stomach. The arrows mark flow outlet and direction of the flow of artificial saliva and gastric juice.

3. Results

Two principally different assays were developed for testing the adhesive properties of the alginate-chitosan capsules. The particle adhesion assay was a very simple and rapid assay.

3.1. Particle adhesion assay

There was a large variation in the number of adhered particles on the surface of the capsules of the same capsule sample fraction, which is seen on the micrograph in Fig. 3. However, despite this variability, strongly enhanced adhesive properties were observed when a chitosan coating was added

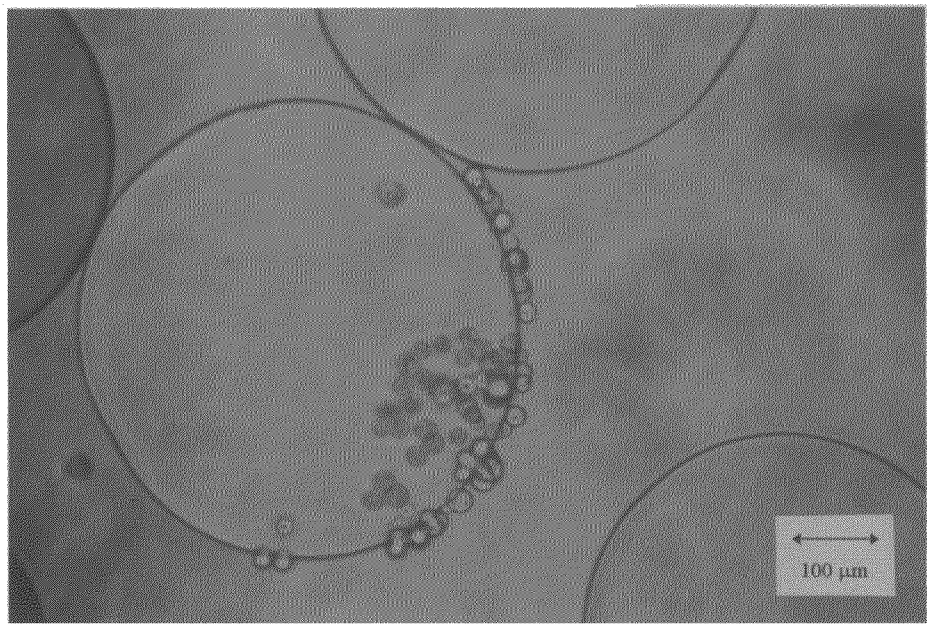

Fig. 3. Micrograph of negatively charged chromatography particles adhering to the surface of chitosan capsules.

to the alginate beads relative to the uncoated beads (Fig. 4). All of the chitosan capsule fractions had remarkably higher average numbers of particles on the surface than the uncoated beads. Due to the high variations, it was difficult to distinguish between the adhesiveness for the different capsule fractions. However, an obvious conclusion was that the homogeneous capsules are more adhesive than the inhomogeneous and this was independent of the type of chitosan or capsule generation procedure. The average values for homogeneous and inhomogeneous capsules were 25.1 ± 8 and 12.7 ± 8 particles/mm^2, respectively.

3.2. Tissue adhesion

3.2.1. Oesophagus tissue

For testing the significance of the chitosan coating on the adhesion both to oesophagus and stomach mucosa, two bead fractions were chosen. The uncoated alginate beads were homogeneous and the same beads were coated with a chitosan with M_n of 17000 and $F_A < 0.01$ according to the two-stage procedure (Fraction B and F, Table 1). These capsules are known to have a high chitosan content (Gåserød et al., 1998a). Again, the difference in adhesiveness for uncoated relative to coated beads was significant on pig oesophageal tissue (Fig. 5). The adhesion differences between the various chitosan coated capsules are shown in Fig. 6. Relative adhesion is calculated by the fraction of adhered beads after 45 min related to the result for the standard capsule fraction run on each tissue. The overall trends in the results from the particle and oesophagus adhesion assays are very comparable, including the effects of alginate gel homogeneity (Fig. 6).

3.2.2. Stomach tissue

The adhesion of beads to pig gastric mucosa was in general considerably stronger than to the oesophageal mucosa. When the experimental conditions from the oesophagus assay were copied, 100% of both coated and uncoated beads remained on the tissue after 1 h. A difference in adhesive properties could still be seen, because some the uncoated beads had migrated a shorter distance down from where they were deposited before settling again, whereas the coated beads

did not move at all. After increasing the angle of the tissue, increasing the flow rate, adjusting the flow positioning and initial bead positioning as described above, a major difference in adhesive properties for coated and uncoated beads was detected (Fig. 7). However, the chitosan coated capsules still showed little movement, while about 60% of the uncoated beads were washed off the tissue during 45 min. Since the adherence of coated beads was not affected by the high flow, the difference in the adhesion to mucus is probably even larger than demonstrated in Fig. 7. Further adjustments to achieve even more rigorous test conditions could not be readily achieved, because a high flow of liquid with a hard impact on the mucosa resulted in major degradation of the mucus and the underlying epithelial tissue during the time of the experiment. Interestingly, the chitosan capsules managed to stick to the mucosa even if the underlying tissue became eroded. With this assay, it was not possible to distinguish between the different chitosan coated capsule fractions with adhesion being independent of homogeneity, the average molecular weight of chitosan or whether the one-stage or two-stage generation procedure was used. In each case the capsules stuck practically 100% to the mucous surface. When 2.7% purified gastric mucin was included in the artificial gastric juice, still no loss of coated beads from the tissue occurred (data not shown). In this case, it should be stressed that contact between the capsules and the tissue was achieved before washed with the mucin containing solution. However, an interaction with mucin was demonstrated when capsules were incubated in a solution of 2.7 g/l of purified gastric mucin in artificial gastric juice prior to depositing on the stomach tissue. The adhesive properties were then reduced with increasing time in the mucin solution (Fig. 8).

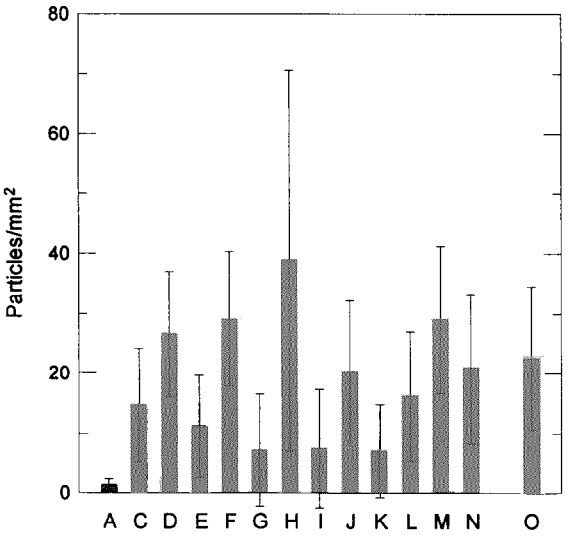

Fig. 4. The adhesion of negatively charged chromatography particles to the surface of uncoated and chitosan coated calcium alginate beads. The characteristics of the various capsule fractions are given in Table 1. The standard deviation is represented by error bars. For each bar at least 30 capsules have been analysed.

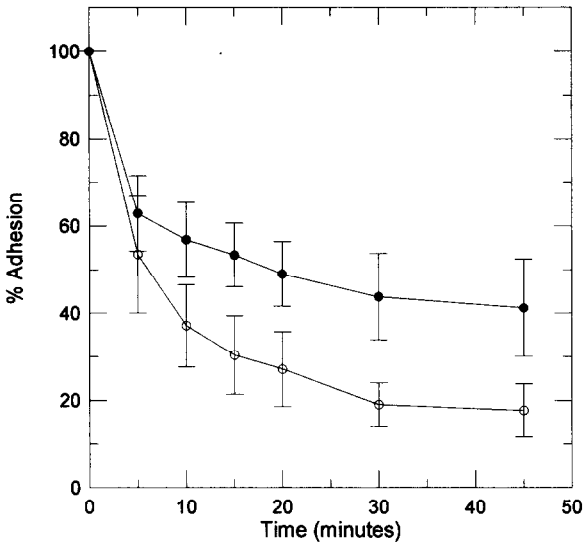

Fig. 5. The adhesion to pig oesophageal tissue of homogeneous alginate beads uncoated (○) and coated (●) with chitosan (Fraction B and F, respectively). The data were obtained from three different tissues, and both bead fractions were run eight times in total. The standard deviations are represented by the error bars.

4. Discussion

In this work frozen tissues were used after slowly thawing over night. The reason for this was to obtain many tissues from the same stock of animals to minimise the tissue to tissue varia-

tion. It is further shown that the mucus layer structure is recovered with respect to reological properties (Bell et al., 1984). The appearance of the mucosal surfaces was neither not changed during the freezing and thawing.

When beads are present on a tissue surface, several factors other than molecular interactions may assist the beads in remaining adhered. Physical hindrances like ridges combined with gravitational forces and surface tensions are probably playing a role, equally for all types of capsules of the same size. However, as the same bead fraction uncoated and coated with chitosan shows major differences in adhesive properties (Figs. 5 and 7), it can be concluded that the observed effect actually is due to the presence of a chitosan coating. The increased adhesive forces are probably mainly electrostatic, located between the positive charges of chitosan and negative charges of sulphate and sialic acid groups on mucin in the stomach or non-mucous negative charges on the epithelial cell surface of the oesophagus.

In the particle adhesion assay there is only the intermolecular force which makes the capsules and

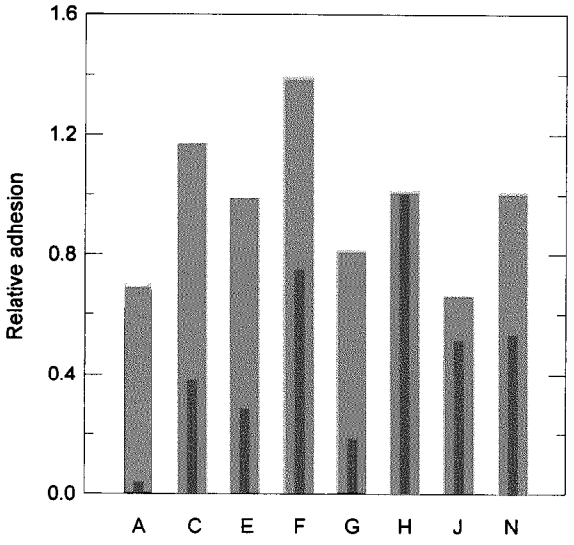

Fig. 6. The adhesion to pig oesophageal tissue of alginate beads uncoated and coated with chitosan. Details of the various capsule sample fractions are given in Table 1. For comparison, the black bars show the relative adhesion results from the particle adhesion assay taken from (Fig. 4) for the same capsule fractions.

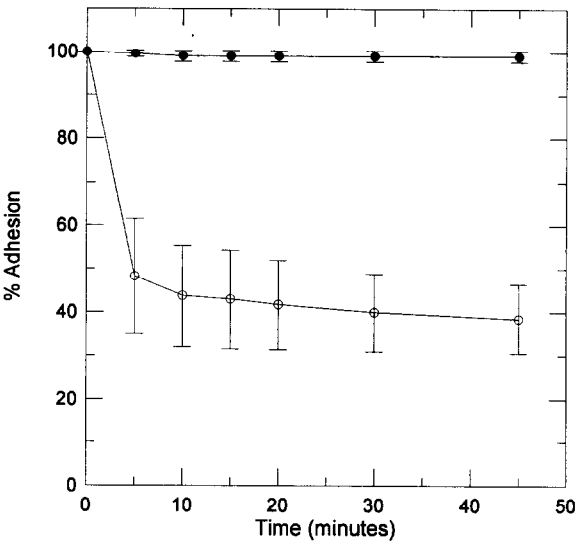

Fig. 7. The adhesion to pig stomach tissue of homogeneous alginate beads uncoated (○) and coated (●) with chitosan (Fraction B and F, respectively). Each fraction was run four times on separate tissues, and the standard deviations are represented by the error bars.

particles adhere. The absence of other factors like physical hindrances and surface tensions, which act independently of intermolecular forces, is probably the reason why larger differences are seen in this assay compared to the tissue adhesion assay. The large variability in the number of adhered particles on different beads of the same capsule fraction, may be explained by a particle being more likely to settle on a capsule which already is occupied by one or more particles, since the particles may protect each other from shear forces in the suspension. Since the electrostatic interaction seems important for bioadhesion, this is a very rapid and useful test in scanning large amounts of materials for large differences in adhesive properties.

Both the chitosan coated and uncoated beads adhered much better to the stomach mucosa than to the oesophagus mucosa. The presence of the mucus gel layer on the stomach tissue is likely to be the reason for this. The mucin in the adherent layer probably has a higher negative charge density than the epithelial cell surface of the oesophagus, and may also be softer than the oesophageal cell surface yielding a larger contact area between the tissue and the beads.

Even if the capsules seem to adhere well to the stomach mucosa through the period of the experiment, the fate of the capsules in vivo may be different. The presence of pepsins in the gastric lumen continuously degrading the mucus layer at the surface into soluble degraded mucin (Allen et al., 1993), may remove the site of adhesion and thus remove the capsules themselves. In our in vitro stomach experiment the capsules did, however, remain adhered even if the mucosa was considerably degraded around and under the capsules, which is a promising result. Another important factor which may affect the adhesion, is the exposure to soluble mucins and other material in the stomach lumen before contact with the adherent mucus is achieved. In Fig. 8 the exposure to dissolved mucin was shown to decrease the adhesive potential for the chitosan capsules. If the time prior to tissue contact in vivo is below 5 min, which is reasonable, the adhesive potential is still high. The dissolved mucin protein is probably slowly bound to the chitosan, again through electrostatic interactions, gradually disabling the capsule from binding to the adherent mucus gel.

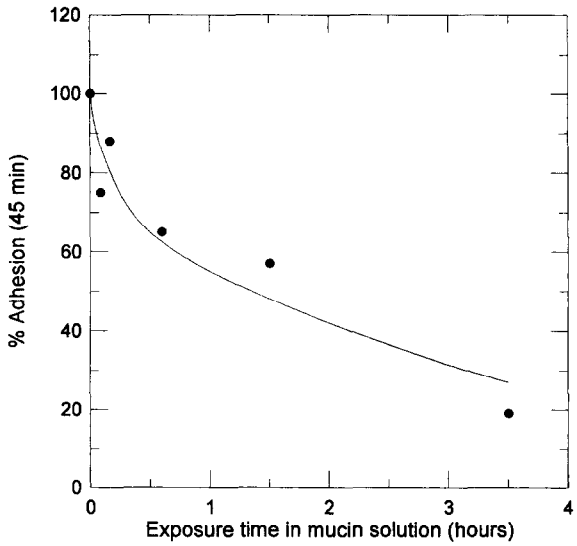

Fig. 8. The adhesion of coated beads (Fraction F) to pig stomach tissue after exposure to solution of artificial gastric juice solution containing dissolved pig gastric mucin.

The adhesion to oesophageal tissue is probably increased in the in vivo situation when considering the fact that the oesophagus here is more folded than in our experiments. The contact areas between tissue and capsules may then be increased. Whether the action of peristalsis will increase or decrease the capsule adhesion, is hard to predict from this work.

The gel homogeneity was also of importance for the adhesion properties. The homogeneous capsules were considerably more adhesive than the inhomogeneous (Fig. 4). There are two probable explanations for this. Firstly, capsules made from homogeneous beads bind more chitosan than those from inhomogeneous bead cores (Gåserød et al., 1998a), probably yielding more chitosan charges on the capsule surface. Secondly, the lower alginate concentration at the bead surface in a homogeneous capsule may reduce eventual repulsive forces between alginate carboxyl groups and the negatively charged surfaces. The latter argument is further supported by the results from the homogeneous and inhomogeneous one-stage capsules (Fig. 4). Here, the alginate-chitosan membrane is formed before gelling of the core, and should therefore be exactly the same, independent on the later settled homogeneity. However, the results in Fig. 4 suggest that the homogeneous one-stage capsules adhere somewhat better than the inhomogeneous.

Except for adhesive differences due to varied homogeneity, capsules produced by different procedures or with chitosans of different average molecular weight or degree of acetylation showed little difference in adhesiveness (Figs. 4 and 6). It is known that the total amount of chitosan in the capsules tested differed considerably. But in the case of high chitosan contents, it is also known that the chitosan is not only located near the capsule surface, but is then bound through the entire gel matrix (Gåserød et al., 1998a,b). This is because the low molecular size of these chitosans allows for diffusion into the gel network. This suggests that the chitosan present in the capsule core has a minor effect on the adhesion, and that the amount of chitosan on the capsule surface of the different capsules is similar.

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