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Mucoadhesion on urinary bladder mucosa: the influence of sodium, calcium, and magnesium ions

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The aim of the present work was to establish if different cations present in the lumen of the urinary bladder at the time of application affect the mucoadhesion strength of cationic chitosan, anionic sodium carboxymethyl cellulose (NaCMC), and nonionic hydroxypropyl cellulose (HPC). The mucoadhesion strength of polymeric films was determined on pig urinary bladder mucosa. Sodium, calcium, and magnesium ions decreased the mucoadhesion strength of all three polymers except NaCMC, whose detachment forces were not influenced by the presence of sodium. Lower mucoadhesion strength in the presence of cations should be considered when drug delivery systems, for example microspheres, containing the tested mucoadhesive polymers are applied intravesically. In the majority of the experiments, cations decreased the mucoadhesion strength of the polymers already in concentrations normally present in urine. For stronger mucoadhesion, application of microspheres into the empty urinary bladder would be recommended. Additionally, the mucoadhesion properties of the tested polymers could be controlled by the selection of a proper medium for the suspension of microspheres. Namely, for all three polymers bivalent calcium and magnesium had stronger influence on mucoadhesion compared to univalent sodium, and with increasing concentrations of cations mucoadhesion strength of the polymers decreased.

1. Introduction

Bioadhesion may be defined as a state in which two materials, at least one of which is biological in nature, are held together by interfacial forces for extended periods (Smart 2005). When the adhesive attachment is to mucus or mucosa, the phenomenon is referred to as mucoadhesion. The component of a dosage form that adheres to a tissue is most frequently a mucoadhesive polymer. Requirements for good mucoadhesive polymers include the presence of functional groups that are able to form secondary chemical bonds, suitable wetting and swelling properties as well as sufficient flexibility of the molecules for entanglement with the components of mucus. Several derivatives of cellulose, like sodium carboxymethyl cellulose, hydroxypropyl cellulose, and some other polysaccharides such as chitosan suit the above criteria (Mikos and Peppas 1986; Smart 2005). Mucoadhesion takes place in two stages, the contact or wetting state and consolidation stage in which the adhesive interactions are established (Smart 2005).

Mucoadhesive dosage forms hold great potential in ocular (Ludwig 2005), buccal (Salamat-Miller et al. 2005), nasal (Ugwoke et al. 2005), and vaginal (Valenta 2005) drug delivery. The main advantages of mucoadhesive drug delivery systems are localisation and the prolonged residence time of a dosage form at a specific site resulting in an increased bioavailability of the applied drug (Huang et al. 2000).

The broader aim of our work is to develop mucoadhesive microspheres, which could be advantageous in the local treatment of severe urinary bladder infections or post-operative chemotherapy of superficial bladder cancer. Superficial bladder

cancer accounts for approximately 70% of all bladder cancer cases. The primary mode of its therapy is transurethral resection, while further therapy consists of intravesical chemotherapy and immunotherapy (Malmström 2003). After application through an urethral catheter, suspended microspheres are expected to adhere to the mucosa of the urinary bladder and release the incorporated drug over a certain period of time. Several mucoadhesive drug delivery systems intended for intravesical application have already been developed (Burjak et al. 2001; Eroğlu et al. 2002; Oztürk et al. 2004).

The aim of the present study was to establish whether different cations present in the lumen of urinary bladder at the time of application affect the mucoadhesion strength of selected polymers. Cationic chitosan, anionic sodium carboxymethyl cellulose, and nonionic hydroxypropyl cellulose were tested, all three of them being well-recognized mucoadhesive polymers. The experiments were performed on pig urinary bladder mucosa. Salts of selected cations were added in different concentrations to the buffer used for hydration of the mucosal surface before determination of mucoadhesion strength. Sodium, calcium, and magnesium ions are normal components of humane urine. Besides, microspheres intended for intravesical instillation would be suspended in a suitable buffer before application into the urinary bladder. If cations influence the strength of mucoadhesion, their quantity during application should be controlled. This could be achieved by selecting a proper medium for the suspension of microspheres and by controlling the volume of the urine present in the urinary bladder at the time of application.

2. Investigations, results and discussion

Intravesically instilled suspension of mucoadhesive microspheres could be used in the local treatment of severe urinary bladder infections or superficial bladder cancer. Microspheres with chitosan, NaCMC, HPC, and polycarbophil have already been developed (Bogataj et al. 1999; Burjak et al. 2001). Mucoadhesive polymers incorporated in microspheres are supposed to adhere to a thin layer of glycosaminoglycans which covers the surface of the urothelium. Glycosaminoglycans are highly anionic molecules, present on the bladder luminal surface at very high density. Heparan sulfate, dermatan sulfate, and chondroitin sulfate are the most frequently isolated protein-bound glycosaminoglycans from the luminal surface of the urinary bladder. Additionally, many glycosaminoglycans and glycoprotein molecules are loosely adhered to the bladder surface (Hurst and Zebrowski 1994).

In the present study, the mucoadhesive properties of cationic chitosan, anionic NaCMC, and nonionic HPC were tested on the luminal surface of the urinary bladder wall. For mucoadhesion to occur, the presence of water is very important. The hydration of mucoadhesive polymers results in the relaxation of twisted or entangled molecules, which are then able to interpenetrate and interact with the components of mucus (Mikos and Peppas 1986; Salamat-Miller et al. 2005). There is a critical degree of hydration required for optimum mucoadhesion (Bogataj et al. 1999; Ugwoke et al. 2005). In our experiments the buffer was applied to the mucosal surface before forming a contact with dry polymeric films. The optimal volume of the buffer to obtain the best contact between the polymer and urinary bladder mucosa was preliminarily determined. As seen from Fig. 1, detachment forces of HPC were significantly lower compared to chitosan and NaCMC. This is in accordance with the results obtained on other mucosae, where nonionic polymers appear to have less mucoadhesion strength compared to anionic and cationic polymers (Ludwig 2005; Nafee et al. 2004; Peppas and Buri 1985; Salamat-Miller et al. 2005). On the other hand, there was no significant difference between the detachment forces of chitosan and NaCMC films (Fig. 1). A literature review revealed that in some studies anionic polymers demonstrated greater mucoadhesive strength than cationic polymers (Lehr et al. 1992; Nafee et al. 2004), while in the others the findings were just the opposite (Collaud et al. 2007; Salamat-Miller et al. 2005). In these studies mucoadhesion was measured on intestinal, buccal, and oesophageal mucosa, and the obtained results depend on the polymers tested as well as on the

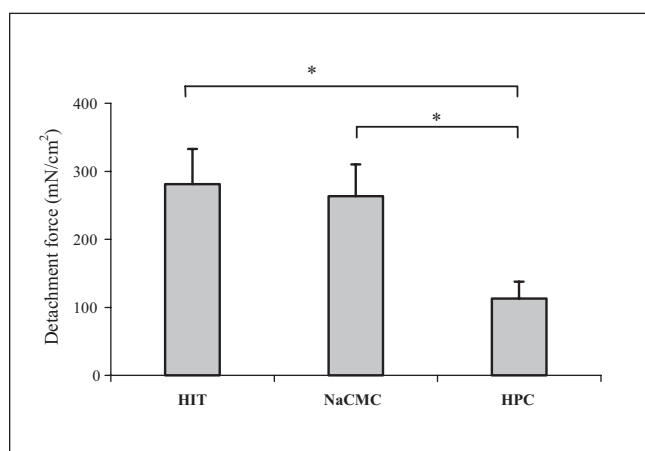


Fig. 1: Detachment forces of chitosan (HIT), sodium carboxymethyl cellulose (NaCMC), and hydroxypropyl cellulose (HPC) films determined on pig urinary bladder mucosa (mean $\pm$ S.D., $n = 18$). Mucosal surface was hydrated with phosphate buffer (PB); * indicates significant differences in the detachment forces

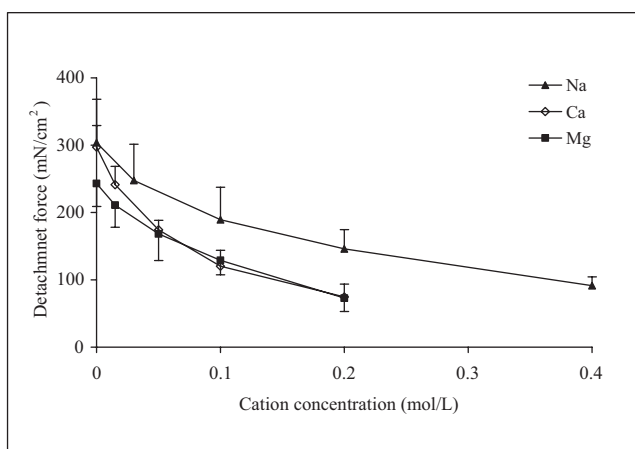


Fig. 2: Detachment forces of chitosan films determined on pig urinary bladder mucosa in the presence of different concentration of sodium, calcium, and magnesium ions, added to the buffer used for hydration of the mucosal surface (mean $\pm$ S.D., $n = 6$)

experimental conditions such as polymer concentration, swelling, and environmental pH. In another study performed on pig urinary bladders (Burjak et al. 2001), chitosan films had significantly higher detachment forces than NaCMC films. However, in that study detachment forces were measured under different experimental conditions. In our study mucosal surface was hydrated with PB adjusted to pH 4.5, while in the other study Tyrode solution with pH 7.4 was used for hydration of polymeric films. Moreover, the volume of buffer used for hydration was different. A pH of 4.5 is within the normal range of urine pH (Lothar 1998), and it was proven in our previous study (Kerec et al. 2005) that a one-hour exposure of luminal urinary bladder surface to PB with pH 4.5 did not affect the morphology of the tissue. The pH of the applied buffer affects the degree of ionisation of mucoadhesive polymers and glycosaminoglycan molecules on the urothelial surface. Besides electrostatic interactions, the contribution of van der Waals forces, hydrogen, and hydrophobic bonds to mucoadhesion strength can vary when hydration buffers with different pH are used (Ugwoke et al. 2005). The volume and the pH of the applied buffer could also affect swelling of the tested polymers. Nevertheless, variability in biological material used in both studies cannot be excluded. In further work, the influence of sodium, calcium, and magnesium ions on mucoadhesion strength of the selected polymers was studied. For determination of detachment forces the selected cations were added in different concentrations to the buffer used for the hydration of the mucosal surface. The lowest tested concentration of each cation was within the physiological range of its urine concentration in a healthy man (Burtis et al. 2006; Lothar 1998). Besides being normally present in urine, certain amounts of sodium, calcium, and magnesium ions might be present in a medium, in which mucoadhesive microspheres would be suspended, before application through a urethral catheter. For this reason, some tested concentrations of cations were higher than physiological ones.

In the presence of added salts, mucoadhesion strength of positively charged chitosan was greatly reduced (Fig. 2). Even the smallest concentration of sodium (0.03 M) and calcium ions (0.015 M) reduced the detachment forces of chitosan films significantly, while for magnesium a significant effect was seen not earlier than at 0.05 M concentration. Therefore, in concentrations normally present in urine the cations significantly affect chitosan mucoadhesion. With increasing concentrations of added cations, the detachment forces decreased for all three cations. Except for the lowest concentration of added calcium and magnesium, both bivalent cations reduced the detachment forces of chitosan to the same values (Fig. 2). On the contrary,

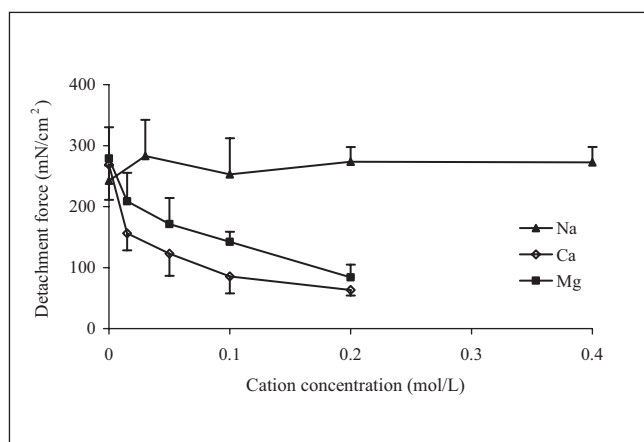


Fig. 3: Detachment forces of sodium carboxymethyl cellulose films determined on pig urinary bladder mucosa in the presence of different concentration of sodium, calcium, and magnesium ions (mean $\pm$ S.D., $n=6$). Cations were added to the buffer used for hydration of the mucosal surface

in the presence of univalent sodium the decrease in mucoadhesion strength was less than for bivalent calcium and magnesium. However, if the detachment forces at two times greater concentration of added sodium than added calcium or magnesium are compared, there are no significant differences in mucoadhesion. It is interesting that not only mucoadhesion strength, but also the ability of chitosan to act as an absorption enhancer is reduced in the presence of cations. Namely, it was revealed in our previous work that with increasing concentrations of added calcium the absorption enhancement effect of 0.5% (w/v) dispersion of chitosan decreased. In one hour a 0.05 M concentration of calcium significantly reduced the permeation of a model drug into the urinary bladder wall and at 0.1 M concentration of calcium the absorption enhancement effect of chitosan could no longer be statistically proven. A 0.5% (w/v) dispersion of chitosan increased the permeability of the bladder wall by causing the desquamation of the urothelium, the extent of which was reduced in the presence of calcium. Calcium obviously interfered in the interactions between chitosan molecules and the surface of urothelium (Kerec et al. 2005). However, mucoadhesion of the chitosan film to the surface of the urothelium less probably resulted in desquamation of urothelial cells. Firstly, for determination of detachment forces chitosan was in contact with the tissue for a much shorter time than during the permeability experiments. Secondly, the polymer was not applied to the mucosal surface as a dispersion, but as a dry polymeric film, hydrated before the contact with the tissue due to the excess of the liquid on the surface of the urothelium. On the other hand, desquamation of the urothelium could occur during the separation of polymeric film and mucosal surface.

The effects of added cations on mucoadhesion strength of NaCMC films are shown in Fig. 3. Calcium and magnesium ions significantly reduced the mucoadhesion of NaCMC, and with an increasing concentration of these cations, detachment forces decreased. Calcium influenced the mucoadhesion strength of NaCMC greater than magnesium did. In contrast to bivalent cations, sodium ions did not affect the detachment forces of NaCMC films significantly. The influence of added cations on mucoadhesion strength of NaCMC is in accordance with the results of our previous study (Kerec et al. 2002), where the mucoadhesion of anionic polymer polycarbophil was studied. Calcium ions significantly decreased the detachment forces of polycarbophil, and their effect was concentration dependent. On the contrary, sodium ions did not influence the mucoadhesive properties of polycarbophil significantly. Moreover, for anionic copolymer Eudragit S100 it was revealed that its mucoadhesion on hydrated mucin from porcine stomach was not significantly

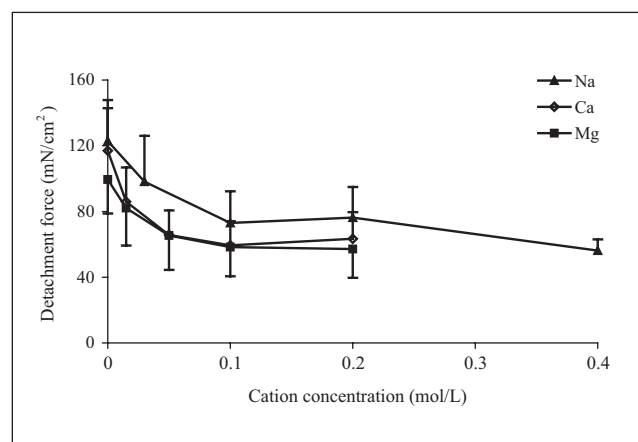


Fig. 4: Detachment forces of hydroxypropyl cellulose films determined on pig urinary bladder mucosa in the presence of different concentration of sodium, calcium, and magnesium ions, which were added to the buffer used for hydration of the mucosal surface (mean $\pm$ S.D., $n=6$)

modified in the presence of calcium or magnesium salts (Cilurzo et al. 2005).

Mucoadhesion strength of HPC films was reduced significantly only at 0.1 M concentration of calcium and 0.4 M concentration of sodium (Fig. 4). However, it is evident from Fig. 4 that with increasing concentrations of all three cations, detachment forces of HPC decreased, and that at the same molar concentration, the influence of bivalent calcium and magnesium was greater than with univalent sodium.

Decreased mucoadhesion strength of the tested polymers determined in the presence of added cations could be a consequence of altered interactions between the polymer and urothelial surface, which are important for mucoadhesion to occur. Chitosan has a very limited affinity for binding alkaline and alkaline-earth metals cations in neutral and weakly acidic media (Bravo-Osuna et al. 2007; Guibal 2004). Therefore, cations most probably interact with negatively charged carboxylic and sulphate groups of glycosaminoglycans on the urothelial surface and prevent their interactions with positively charged amino groups of chitosan. Consequently, chitosan mucoadhesion strength is reduced. Added cations were expected to reduce the repulsion between negatively charged carboxylic groups of NaCMC molecules and negatively charged glycosaminoglycans, which could result in higher mucoadhesion strength. However, the mucoadhesion strength of NaCMC decreased in the presence of calcium and magnesium ions. Altered van der Waals, hydrogen, and hydrophobic bonds are obviously more important for mucoadhesion of NaCMC than electrostatic interactions. No literature data are available regarding the ability of NaCMC to bind different cations. Another anionic polymer, polycarbophil has lower affinity for univalent than for bivalent cations (Kriwet and Kissel 1996). If the same is true for NaCMC, this could explain the stronger influence of calcium and magnesium on the mucoadhesive properties of NaCMC compared to sodium. Moreover, sodium ions were already present in NaCMC films due to the polymer composition. It is possible that due to the plateau effect the added sodium did not further influence the detachment forces of NaCMC. In contrast to the other two polymers, HPC does not have functional groups that would form electrostatic interactions with the urothelium.

Furthermore, cations added to the buffer used for hydration of the mucosal surface increased the buffer's ionic strength. This could affect hydration and swelling of the polymer molecules, which allows uncoiling and spreading of the polymer molecules over the tissue surface. These are important processes for interpenetration of polymer and glycosaminoglycan molecules (Dodou et al. 2005; Peppas and Buri 1985). However, during the

measurements of detachment force the polymeric films were hydrated with the applied buffer less than 5 min. The results of the swelling studies showed that even within 30 min the change of polymer powder volume was for all tested polymers in all tested buffers less than 5%. Therefore, decreased mucoadhesion strength of the tested polymers determined in the presence of added cations is not a consequence of altered hydration and swelling of the polymer molecules. The literature review revealed that an increased ionic strength did not have a significant effect on mucoadhesion of nonionic polymer hydroxypropyl methylcellulose (Lejoyeux et al. 1989), while the reported influence of ionic strength on mucoadhesion of anionic polymers is not uniform. For polycarbophil its apparent volume of equilibrium swelling decreased with increasing ionic strength, although this did not influence the mucoadhesive properties significantly (Park and Robinson 1985). In another study (Lejoyeux et al. 1989) the influence of sodium and calcium ions on mucoadhesion of polyacrylic acid depended on the mucosa tested. Mucosa that was richer in mucus protected the mucoadhesive system from the influence of the surrounding medium.

In our study it was proven that the valence of the added cation affects mucoadhesion of the tested polymers. For all three polymers the mucoadhesion strength was lower in the presence of bivalent calcium and magnesium compared to univalent sodium (Figs. 2–4). Bivalent cations can interact with two binding sites on glycosaminoglycan or polymer molecules. Consequently, it is necessary to double the concentration of sodium ions in order to achieve the same effect as with calcium or magnesium. Moreover, bivalent cations may intra- and inter-molecularly crosslink glycosaminoglycan and polymer molecules. Crosslinked molecules are less flexible, and their interpenetration is more difficult (Park and Robinson 1985). Namely, interpenetration represents the main physical mechanism of mucoadhesion (Peppas and Buri 1985). Sodium as a univalent cation is not able to crosslink the molecules; this could be one of the explanations for its much smaller effect on mucoadhesive properties of the tested polymers.

3. Experimental

3.1. Materials

Chitosan hydrochloride (in the further text referred to as chitosan only) (Protasan UP Cl 213, Novamatrix, Oslo, Norway) with a deacetylation degree of 86% and an apparent viscosity of 1% (w/v) aqueous dispersion 95 mPas was used. Hydroxypropyl cellulose (in the further text referred to as HPC) (Klucel, GXF Pharm) as well as sodium carboxymethyl cellulose (in the further text referred to as NaCMC) (Blanose CMC, 7M8SXF PH) were produced by Aqualon, France. The apparent viscosity of 2% (w/v) aqueous dispersion was 295 mPas for HPC and 655 mPas for NaCMC.

The phosphate buffer saline (PBS) (Ph. Eur. III) consisted of 0.944 g Na_2HPO_4 , 0.19 g KH_2PO_4 , and 8 g NaCl in one litre of deionised water (pH=7.4). The phosphate buffer (PB) consisted of 0.472 g Na_2HPO_4 , 0.095 g KH_2PO_4 , and 1.6 g NaCl in one litre of deionised water. When testing the influence of cations on detachment forces, additional amounts of sodium, calcium, and magnesium ions were added to PB. Buffers with calcium were prepared by adding CaCO_3 and HCl into PB, while in the cases of buffers with magnesium and sodium, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ and NaCl were added, respectively. The amounts of sodium, calcium, and magnesium salts added to PB in order to prepare suitable buffers are shown in the Table. The pH of all the buffers was adjusted to 4.5. All chemicals used were of analytical grade.

3.2. Swelling studies

100 mg of chitosan, NaCMC, or HPC were put into a graduated glass cylinder and 5 mL of either PB or PB with the maximal concentration of added sodium, calcium or magnesium ions were added. The volumes of hydrated polymers were measured at fixed time intervals. Results are means of triplicate experiments.

Table: Amounts of sodium, calcium, and magnesium salts added to the phosphate buffer (PB) in order to prepare 500 mL of the phosphate buffer with a suitable concentration of added cation (PB + cation). The obtained molar concentration of added cation is indicated in the buffer label

Buffer label	Sodium*	Calcium	Magnesium	
	m (NaCl) (g)	m (CaCO_3) (g)	V (1 M HCl) (mL)	m ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$) (g)
PB + 0.015 M cation	/	0.75	15	1.53
PB + 0.03 M cation	0.88	/	/	/
PB + 0.05 M cation	/	2.5	50	5.08
PB + 0.1 M cation	2.93	5.0	100	10.17
PB + 0.2 M cation	5.84	10.0	200	20.33
PB + 0.4 M cation	11.69	/	/	/

* Indicated amounts of NaCl do not include 0.8 g of NaCl present in the PB alone

3.3. Preparation of polymeric films

250 mg of chitosan, HPC, or NaCMC were added to 10 mL of deionised water to obtain dispersions with 2.5% (w/v) concentration of the polymer. The dispersions were stirred overnight. Polymeric films were obtained by pouring 180 mg of chitosan dispersion, 180 mg of NaCMC dispersion, or 205 mg of HPC dispersion on a glass plate with a surface of 2×2 cm and dried overnight at room temperature. Dry films weighed on average 1.25 mg/cm^2 , and 10% deviation from the average mass was allowed.

3.4. Tissue preparation

Pig urinary bladders were obtained from a local slaughterhouse. Until the beginning of the experiment the bladders were kept in carbogen saturated phosphate buffer saline at 5°C . All experiments were performed within 5 h of the animals' deaths, as scanning electron micrographs of urinary bladders revealed that the luminal bladder surface remained intact under the described experimental conditions within the chosen period of time (Burjak et al. 2000). Only the body of the urinary bladder was used for determining detachment forces. At the beginning of the experiments the mucosa was separated from the underlying tissue.

3.5. Determination of detachment forces

The detachment forces between polymeric films and pig urinary bladder mucosa were measured with a modified precision balance. A dry polymeric film was prepared on a glass plate that was mounted to the upper clamp of the apparatus. Before mounting to the lower support of the apparatus, the mucosa was incubated for 30 s in either PB or PB with added cation. As the incubation procedure was the same in all the experiments, we assume that the amount of the buffer that remained on the mucosal surface after incubation of the tissue was approximately equal in each case. One minute before the clamp with the tissue was slowly brought into contact with the polymeric film, 20 μL of the same buffer used for incubation of the tissue was applied to the mucosal surface. To strengthen the contact between the tissue and the polymer an additional weight of 10 g was added immediately after contact was established. Two minutes after the formation of the contact, the upper support of the apparatus was raised, increasing at a constant rate of 0.2 N/min until the detachment force needed for the separation of the two surfaces was determined.

Only one experiment was performed on each mucosal sample, and each polymeric film was used only once. Mucosal samples obtained from the same urinary bladder were used to determine the mucoadhesion strength of one polymer (chitosan, HPC, or NaCMC) at all the selected concentrations of a particular cation. Each experiment was repeated on the mucosa of six different urinary bladders.

3.6. Statistics

For each polymer the influence of a particular tested cation on the detachment forces was evaluated for statistically significant differences by ANOVA for repeated measures using the Bonferroni post hoc test ($\alpha = 0.05$). For statistical evaluation of the differences in detachment forces of a particular polymer in the presence of different cations or differences in mucoadhesion strength of different polymers in the presence of the same cation, the two-tailed Student's unpaired t-test ($\alpha = 0.05$) was applied.

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