

## Permeability of Pig Urinary Bladder Wall: Time and Concentration Dependent Effect of Chitosan

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**Chitosan in 0.5% w/v concentration enhanced the permeability of the isolated pig urinary bladder wall by desquamation of the urothelium as ascertained in our previous study. The aim of the present work was to determine the time and concentration dependence of chitosan's effect on the permeation of a model drug into the bladder wall and to establish if the mechanism of permeation enhancement depends on the concentration of chitosan used. In the permeability studies performed by the use of diffusion cells, transport of a model drug moxifloxacin into the isolated pig urinary bladder wall was determined. For morphological observations of the urothelium in response to chitosan treatment scanning and transmission electron microscopy were applied. Within 90 min the effect of chitosan on the tissue amounts of moxifloxacin gradually increased and approached its plateau. In one hour even 0.0005% w/v dispersion of chitosan significantly enhanced the permeability of the pig urinary bladder wall for the model drug and at 0.001% w/v concentration the maximal effect on the tissue permeability was achieved. All concentrations of chitosan that significantly enhanced the permeability of the bladder wall triggered necrosis of superficial cells or desquamation of the urothelium. However, at lower concentrations and shorter exposure times the damage of the urothelium was limited to the changes in tight junctions. Chitosan was ascertained to increase the permeation of moxifloxacin into the urinary bladder wall in a time and concentration dependent manner.**

**Key words** urinary bladder; chitosan; permeability enhancement; desquamation

Chitosan is a linear copolymer of *N*-acetyl-D-glucosamine and D-glucosamine. This biocompatible and biodegradable polymer is obtained by partial *N*-deacetylation of chitin, one of the most abundant natural polysaccharides. Chitosan hydrochloride is often used in permeability studies as an absorption enhancer. The molecular weight, degree of deacetylation as well as the positive charge of chitosan determine many properties of this polymer, including its effect on tissue permeability.<sup>1–4</sup> Chitosan acts as an absorption enhancer only when its amino groups are protonated. This enables the polymer to interact with a negatively charged mucosal surface.<sup>5</sup> High charge density is not obtained at neutral and alkaline pH values, as a  $pK_a$  value of the D-glucosamine residue of chitosan is about 6.2–7.0.<sup>6</sup>

It was reported that in *in vitro* experiments as well as in some *in vivo* studies chitosan increased the transport of substances across intestinal,<sup>7</sup> ocular,<sup>8</sup> nasal,<sup>9,10</sup> buccal<sup>11,12</sup> and vaginal<sup>12</sup> mucosae, across Caco-2 cells<sup>5,13–16</sup> as well as into the urinary bladder wall.<sup>17</sup>

The mechanism by which chitosan affects epithelial permeability was studied mostly on Caco-2 cells. It was shown that chitosan increased the permeability of Caco-2 cells in a dose and time dependent way.<sup>14,15</sup> The mechanism of absorption enhancement caused by chitosan was suggested to be a combination of mucoadhesion and chitosan's effect on the proteins of tight junctions (ZO-1, occludin) as well as F-actin.<sup>13,14,16</sup>

In our previous study<sup>17</sup> it was ascertained that in contrast to Caco-2 cells, 0.5% w/v chitosan increased the permeability of the pig urinary bladder wall by desquamation of the urothelium. Application of calcium ions to the luminal surface of the urinary bladder together with chitosan reduced the permeation of the model drug into the bladder wall. However, the desquamation of the urothelium caused by chitosan

was not reduced to such an extent as would be expected from the permeability studies. Calcium obviously interferes in the interactions between chitosan and the surface of the urothelium.

The objective of the present work was to determine the time and concentration dependence of chitosan's effect on the permeability of the isolated pig urinary bladder wall. Moreover, we aimed to establish whether the mechanism of permeability enhancement caused by chitosan depends on the concentration of the polymer. To achieve these goals, permeability studies were performed where the influence of different concentrations of chitosan on transport of a model drug moxifloxacin into the bladder wall was assessed. For time-dependence studies the urothelium was incubated in dispersions of chitosan for different time periods. Scanning and transmission electron microscopy were applied to determine the morphological properties of the tissue exposed to various concentrations of chitosan for different exposure times.

### MATERIALS AND METHODS

**Materials** Chitosan hydrochloride (in the further text indicated as chitosan) (Protasan CI 213, degree of deacetylation 86%, apparent viscosity of 1% w/v aqueous dispersion 95 mPa·s) was purchased from Pronova Biopolymer, Oslo, Norway.

Moxifloxacin was kindly provided by Bayer AG, Leverkusen, Germany. Moxifloxacin is a fourth-generation fluoroquinolone with a true partition coefficient  $P=0.832$  and ionisation constants  $pK_1=6.25$  and  $pK_2=9.29$ .<sup>18</sup>

For chromatographic determination of moxifloxacin in the tissue samples methanol and acetonitrile for preparative liquid chromatography (PChromasolv, Sigma-Aldrich Labor-

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chemikalien) were used as well as analytical grade trichloroacetic acid (Merck, Darmstadt, Germany).

Phosphate buffer saline (PBS) (Ph. Eur. IV) consisted of 0.944 g  $\text{Na}_2\text{HPO}_4$ , 0.19 g  $\text{KH}_2\text{PO}_4$  and 8 g NaCl in 1 l of deionised water (pH=7.4). Phosphate buffer (PB) consisted of 0.472 g  $\text{Na}_2\text{HPO}_4$ , 0.095 g  $\text{KH}_2\text{PO}_4$  and 1.6 g NaCl in 1 l of deionised water (pH=7.4). 0.1 M cacodylate buffer consisted of 21.4 g of sodium cacodylate and 6.74 ml of 0.2 M HCl in one litre of deionised water (pH=7.2–7.4).

**Tissue Preparation** Pig urinary bladders were obtained from a local slaughterhouse (Meso Kamnik, Kamnik, Slovenia). Until used, the bladders were kept at 5 °C in PBS saturated with carbogen (95%  $\text{O}_2$ , 5%  $\text{CO}_2$ ). The corpus of the urinary bladder was cut into pieces with an approximate size of 25×25 mm. Each piece was mounted into a diffusion cell, developed at the Faculty of Pharmacy, Ljubljana, Slovenia.<sup>17)</sup> The donor chamber of the diffusion cell had a volume of 10 ml and the tissue exposure area was 4.5 cm<sup>2</sup>. In the diffusion cell the luminal side of the urinary bladder wall was exposed to a suitable solution or dispersion.

**Permeability Studies** In the permeability studies a quinolone moxifloxacin was used as a model drug. The solutions of moxifloxacin and the dispersions of chitosan were prepared in PB and their pH was adjusted to 4.5. In all experiments the concentration of moxifloxacin was 0.16% w/v. Permeability studies were performed at room temperature within 4 h after the pigs were sacrificed.

When the concentration dependence of permeation enhancement caused by chitosan was studied, the luminal side of the urinary bladder wall was exposed in the diffusion cells for 60 min to 0.0001, 0.0005, 0.001, 0.005, 0.05, 0.1 or 0.5% w/v dispersion of chitosan with moxifloxacin. Each concentration was tested on at least six different urinary bladders. A 60-min exposure of the tissue to the solution of moxifloxacin served as a control.

To establish the time dependence of chitosan's effect the tissue was exposed in the diffusion cells to 0.0005, 0.005 or 0.5% w/v dispersion of chitosan with moxifloxacin for 15, 30, 60 or 90 min. At each concentration of chitosan the time dependence was tested on urinary bladders of at least six different animals. Additionally, the permeation of the drug into the bladder wall from the solution of moxifloxacin was determined within the same period of time.

At the end of the experiments in diffusion cells the tissue was washed three times with PB. The tissue was then placed between two parallel stainless steel plates and the distance between them was regulated regarding the tissue thickness. Afterwards, the tissue was rapidly frozen with liquid nitrogen to obtain tissue pieces with flat surfaces, which were sectioned by cryostat (Leica CM 1850, Nussloch, Germany) in sections of 20  $\mu\text{m}$  thickness parallel to the luminal surface up to 1.2 mm of the tissue depth. Three consecutive sections were pooled into one sample, whose weight was determined. The samples were kept at –20 °C until the analysis.

For morphological studies with scanning and transmission electron microscopy the luminal side of the bladder wall was exposed in the diffusion cells either for 15 min to 0.0005, 0.005 or 0.5% w/v dispersion of chitosan with moxifloxacin or for 60 min to 0.0001, 0.0005, 0.001 or 0.5% w/v dispersion of chitosan with moxifloxacin. At each tested time period experiments were performed on the urinary bladders of

two different animals. As a control the tissue of the same animals was exposed for 60 min to the solution of moxifloxacin.

**Determination of Moxifloxacin in the Tissue** For chromatographic detection of moxifloxacin the mobile phase was made of 0.2% trichloroacetic acid, methanol and acetonitrile with a volume ratio of 67/4/29. At the beginning of the analysis the tissue was unfrozen and 200–600  $\mu\text{l}$  of mobile phase was added to each sample dependent on the expected amounts of moxifloxacin in the tissue samples. To ensure complete extraction, the samples were first vortexed until all the tissue sections were sunken into the mobile phase and then shaken (2 h at 225 cpm, room temperature). After centrifugation of the samples (10 min at 25000 rpm, room temperature), the concentration of moxifloxacin in 20  $\mu\text{l}$  of the supernatant was determined by high performance liquid chromatography. A PRP-1 column (150×4.1 mm) with 5  $\mu\text{m}$  particles (Hamilton, Reno, U.S.A.) and a precolumn of the same type were used. The flow of the mobile phase was 1 ml/min. Fluorescence detection was applied (LC 540, Perkin Elmer, Beaconsfield, U.K.). The retention time of moxifloxacin was approximately 2.5 min. Concentrations of the model drug in the supernatant were determined using external standards. Cumulative amounts of moxifloxacin that permeated into each piece of the tissue were also calculated ( $c_{\text{tot}}$ ) and expressed as the amounts of moxifloxacin (ng) per mg of the tissue.

**Statistics** The data obtained on the urinary bladders of the same animals were evaluated for statistically significant differences by ANOVA for repeated measures with the Bonferroni *post hoc* test ( $\alpha=0.05$ ). For statistic evaluation of the data obtained on the urinary bladders of different animals the two-tailed Student's unpaired *t*-test ( $\alpha=0.05$ ) was applied.

**Scanning Electron Microscopy** At the end of the permeability experiments the tissue was washed with PB. Afterwards the main portion of the muscles was removed and the urothelium with remaining connective tissue was flooded with a solution of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 3 h at 4 °C. Following the rinse during the night in 0.1 M cacodylate buffer the tissue samples were postfixed in buffered 1% osmium tetroxide (Serva, Heidelberg, Germany) for 1 h at 4 °C. After the tissue was dehydrated in acetone solutions with increasing concentrations (30–100%), critical point drying was performed. Before the tissue was examined at 15 kV with a scanning electron microscope (Jeol JSM 840A, Japan), the dried samples were sputter-coated with gold (Balzers Union AG, Liechtenstein).

**Transmission Electron Microscopy** When the permeability experiments were finished, the tissue was washed with PB. A part of the muscles was removed from the tissue samples and the epithelium was fixed in a solution of 4.5% paraformaldehyde and 2% glutaraldehyde in 0.1 M cacodylate buffer for 3 h at 4 °C. After rinsing in 0.33 M sucrose solution in 0.1 M cacodylate buffer at 4 °C, the tissue samples were postfixed in 1% osmium tetroxide (Serva, Heidelberg, Germany) in 0.1 M cacodylate buffer for 1 h at 4 °C. The tissue was then dehydrated in a graded ethanol (30–100%) and embedded in Epon (Serva, Heidelberg, Germany). Ultrathin sections were stained with uranyl acetate (Merck, Darmstadt, Germany) and lead citrate (Merck, Darmstadt, Germany). The sections were examined with a Philips CM100 transmis-

sion electron microscope.

## RESULTS

**Concentration Dependence** The influence of chitosan concentrations on the permeation of moxifloxacin into the urinary bladder wall is shown in Figs. 1 and 2. The tissue amounts of moxifloxacin under all the tested conditions decreased with increasing tissue depth (Fig. 1). In contrast to other concentrations of chitosan used in these experiments, 0.0001% w/v chitosan did not significantly increase the permeability of the urinary bladder wall for moxifloxacin (Fig. 2). Although 0.0005% w/v chitosan significantly increased the tissue amounts of moxifloxacin compared to the tissue that was exposed to the solution of moxifloxacin only, the effect of permeation enhancement was significantly lower than at higher concentrations of chitosan. Moreover, there were no significant differences in the cumulative amounts of moxifloxacin, which permeated into the tissue that was exposed to dispersions of chitosan in a concentration range from 0.001 to 0.5% w/v.

**Time Dependence** The cumulative amounts of moxifloxacin that permeated into the bladder wall from the solution of moxifloxacin in 15, 30, 60 or 90 min slightly increased with increasing exposure time. However, the tissue amounts of moxifloxacin did not differ significantly (Fig. 3). In the presence of 0.0005, 0.005 or 0.5% w/v chitosan the permeation of moxifloxacin into the bladder wall increased more evidently with longer exposure of the tissue to the dispersions of the polymer with moxifloxacin (Fig. 3). Chitosan increased the permeability of the bladder wall rapidly. Already a 15-min exposure of the tissue to 0.005 or 0.5% w/v dispersion of chitosan resulted in significantly higher tissue amounts of moxifloxacin compared to the amounts in the tissue that was exposed to the solution of moxifloxacin only. However, in the case of 0.0005% w/v chitosan, a significant permeation enhancement was achieved only after 60 min. In the presence of 0.5% w/v chitosan, the amounts of moxifloxacin that permeated into the bladder wall in 30, 60 or 90 min were no longer significantly different. When the tissue was incubated with 0.005 or 0.0005% w/v dispersion of chitosan, the insignificantly different tissue amounts of moxifloxacin were determined only in 60 and 90 min of the tissue exposure.

**Scanning Electron Microscopy** The results of scanning electron microscopy revealed that a 1 h exposure of the urothelium to the solution of moxifloxacin with pH 4.5 (a control) did not affect the morphology of the urothelium (Fig. 5A). The superficial cells on the surface of the urothelium were well differentiated, tight junctions between the cells were intact and no visible desquamation of the urothelium occurred.

After a 15-min treatment with 0.0005% w/v chitosan (Fig. 4A) or 0.005% w/v chitosan (Fig. 4B) the urothelium was covered with a layer of chitosan that was thinner at a lower concentration of the polymer. In both cases no desquamation of the urothelium occurred. However, at 0.005% w/v chitosan the superficial cells appear to be slightly inflated. A 15-min incubation of the tissue in 0.5% w/v dispersion of chitosan already resulted in occasional desquamation of the urothelial cells (Fig. 4C).

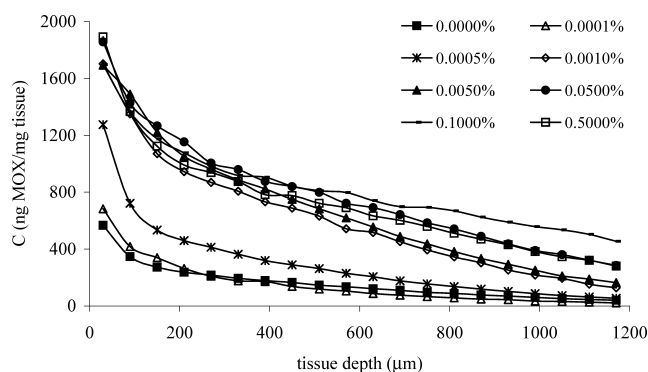


Fig. 1. The Amounts of Moxifloxacin (MOX) That Permeated into the Urinary Bladder Wall as a Function of the Tissue Depth

The tissue was exposed for 60 min to the solution of moxifloxacin or the dispersions of chitosan with moxifloxacin (mean,  $n=6-22$ ). Tested concentrations of chitosan are labelled.

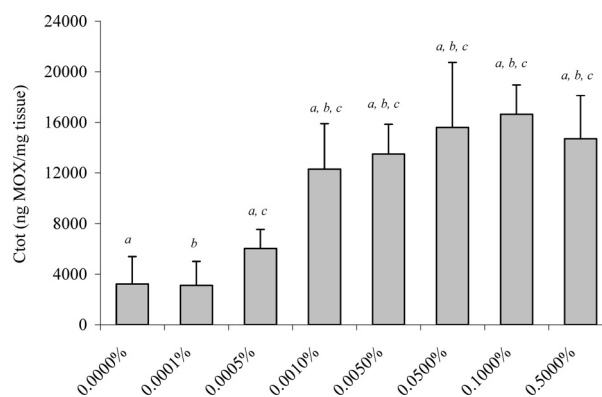


Fig. 2. The Cumulative Amounts of Moxifloxacin (MOX) That Permeated into the Urinary Bladder Wall When the Tissue Was Exposed for 60 min to the Solution of Moxifloxacin or the Dispersions of Chitosan with Moxifloxacin (Mean  $\pm$  S.D.,  $n=6-22$ )

Tested concentrations of chitosan are labelled. *a*, *b* and *c* indicate the significant differences in the cumulative amounts of moxifloxacin compared to the tissue exposed to the solution of moxifloxacin (*a*), to 0.0001% w/v dispersion of chitosan (*b*) or to 0.0005% w/v dispersion of chitosan (*c*).

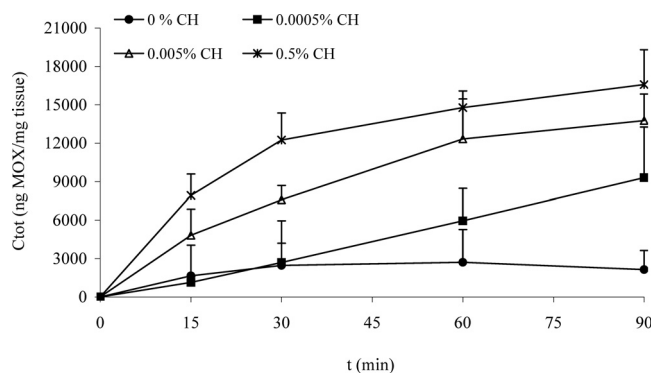


Fig. 3. The Cumulative Amounts of Moxifloxacin (MOX) That Permeated into the Urinary Bladder Wall That Was for 15, 30, 60 or 90 min Exposed to the Solution of Moxifloxacin or to 0.0005, 0.005 or 0.5% w/v Dispersion of Chitosan with Moxifloxacin (Means,  $n=6$ )

After incubation of the tissue for 60 min with 0.0001% w/v chitosan the morphology of the urothelium was similar to the control, even though the urothelium was covered with a thin layer of chitosan (Fig. 5B). In the tissue exposed for 60 min to 0.001 or 0.0005% w/v chitosan large areas of the

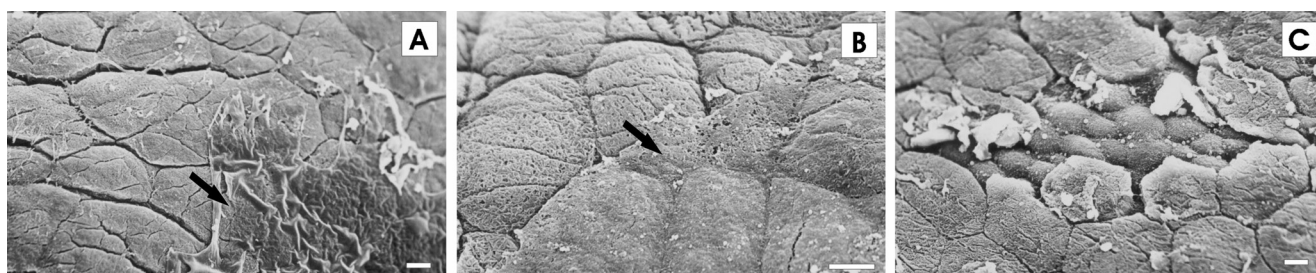


Fig. 4. Scanning Electron Microscopy of Urothelial Surface Exposed for 15 min to 0.0005% w/v Dispersion of Chitosan with Moxifloxacin (A), 0.005% w/v Dispersion of Chitosan with Moxifloxacin (B) or to 0.5% w/v Dispersion of Chitosan with Moxifloxacin (C)

Arrows indicate the layer of chitosan on the urothelium. Bars: 10  $\mu$ m.

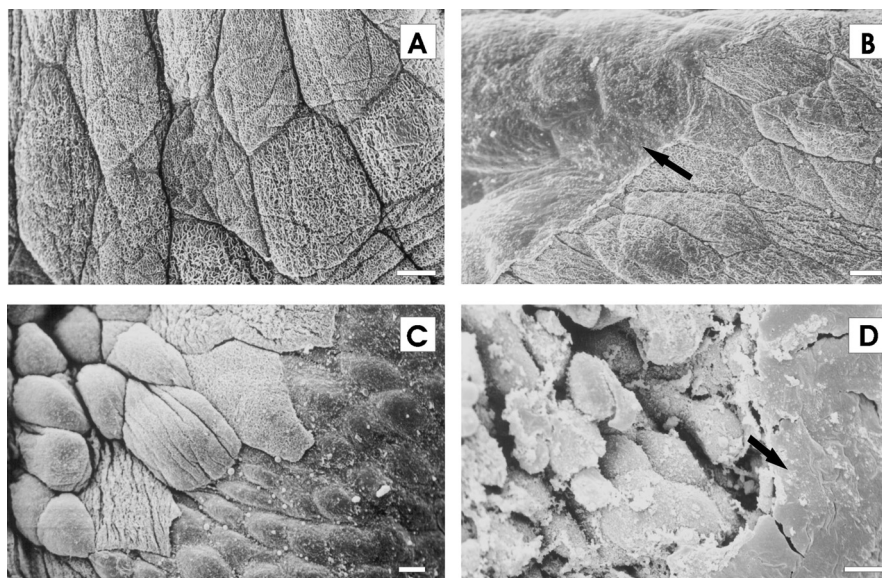


Fig. 5. Scanning Electron Microscopy of Urothelial Surface

Urinary bladders were exposed for 60 min to the solution of moxifloxacin (A), 0.0001% w/v dispersion of chitosan with moxifloxacin (B), 0.001% w/v dispersion of chitosan with moxifloxacin (C) or to 0.5% w/v dispersion of chitosan with moxifloxacin (D). Arrows indicate the layer of chitosan on the urothelium. Bars: 10  $\mu$ m.

urothelium became desquamated (Fig. 5C). Most of the surface was covered with intermediate cells and only a few superficial cells were detected on the surface of the urothelium. After a 60-min treatment with 0.5% w/v chitosan a massive desquamation of the urothelium occurred. In the areas where the urothelium was not covered with a layer of chitosan, intermediate or even basal cells were seen on the urothelial surface (Fig. 5D). The desquamation was much more intense in comparison with the tissue that was exposed for 15 min to the same concentration of chitosan (Fig. 4C).

**Transmission Electron Microscopy** In the tissue incubated for 1 h with the solution of moxifloxacin without chitosan (a control), the intercellular junctions of superficial cells were well preserved (Fig. 7A). The cytoplasm of superficial cells was filled with fusiform vesicles characteristic for differentiated cells. The ultrastructural appearance of the junctional complex, composed of tight and adherence junctions, was characteristic of urothelial cells in forming a prominent ridge, which is bounded by a groove on both sides. Parallel membranes of neighbouring cells within the tight junctions indicate an effective barrier function. Moreover, the urothelial cells did not show any signs of necrosis.

After a 15-min exposure of the bladder wall to 0.0005% w/v chitosan the superficial cells showed no signs of necro-

sis. However, the tight junctions between them were partly opened (Fig. 6A). Incubation of the tissue for 15 min in 0.005% w/v dispersion of chitosan already resulted in necrosis of the cells, containing damaged mitochondria and vesiculated organelles (Fig. 6B). Intermediate cells were often seen on the surface of the urothelium. Tight junctions between necrotic cells were mainly destructed, but some of them appear morphologically normal.

After a 60-min incubation of the tissue in 0.0001% w/v dispersion of chitosan, the urothelium was morphologically similar to the control tissue, although occasional changes in the structure of tight junctions were observed (Fig. 7B). When 0.001% or 0.0005% w/v chitosan was applied to the tissue, necrosis of the urothelial cells could be observed (Fig. 7C). Due to the similar effect of 0.001% and 0.0005% w/v chitosan on the tissue morphology, only the tissue exposed to 0.001% concentration is shown in Fig. 7. After a 60-min incubation of the tissue in 0.5% w/v dispersion of chitosan drastic changes in the urothelial morphology occurred (Fig. 7D). Intermediate or even basal cells were found on the surface of the urothelium. They showed clear signs of necrosis with damaged plasma membrane and swollen organelles, but there were no noticeable signs of apoptosis. No intercellular junctions could be identified.

## DISCUSSION

In our previous studies<sup>17)</sup> it was already shown that 0.5% w/v chitosan increased the permeability of the urinary bladder wall by causing desquamation of the urothelium. This is not in accordance with the results obtained with Caco-2 cells where chitosan increased the permeability of the cells by opening tight junctions.<sup>14–16)</sup> In contrast to Caco-2 cells,

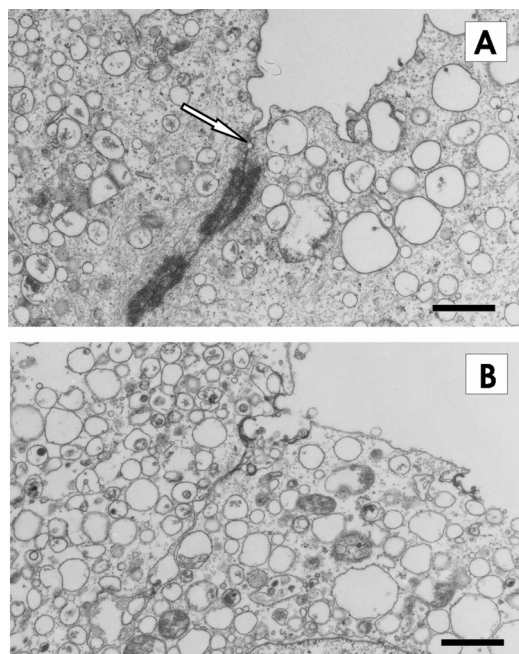


Fig. 6. Transmission Electron Microscopy of Urothelium Exposed for 15 min to 0.0005% w/v Dispersion of Chitosan with Moxifloxacin (A) or 0.005% w/v Dispersion of Chitosan with Moxifloxacin (B)

Arrow indicates a partly opened tight junction. Bars: 1  $\mu$ m.

which are a monolayer cell culture, the urothelium is composed of a layer of superficial cells, several layers of intermediate cells and a layer of basal cells. Differentiation of the cells increases from the basal to the superficial layer.<sup>19,20)</sup> However, also in the urothelium mostly one cell layer is important in the regulation of the bladder wall permeability. The permeability barrier of the urothelium that prevents the diffusion of substances from urine into the bladder wall is composed of tight junctions between superficial cells as well as membrane plaques and a layer of glycosaminoglycans on the apical surface of superficial cells.<sup>20–22)</sup> The purpose of the present work was to establish whether chitosan affects the permeability of the urinary bladder wall in a time and concentration dependent manner and to determine whether the mechanism of permeation enhancement depends on the chitosan concentration since it is possible that lower concentrations of chitosan do not cause desquamation of the urothelium, but only alter the integrity of tight junctions.

Chitosan affects the permeability of the pig urinary bladder wall in a concentration dependent manner (Fig. 1). When the tissue was exposed for 60 min to the dispersions of chitosan, at least 0.0005% w/v concentration of the polymer was needed to significantly increase the permeation of moxifloxacin into the bladder wall. Moreover, at a concentration of chitosan as low as 0.001% w/v the plateau of permeation enhancement into the urinary bladder wall was achieved. Also for Caco-2 cells it was reported that the influence of chitosan on TEER as well as the passage of model substances across the cells is concentration dependent.<sup>14–16)</sup> Smith *et al.*<sup>16)</sup> reported that 0.1% chitosan significantly decreased TEER of Caco-2 cells and increased the transport of horseradish peroxidase compared to the control. Moreover, by measuring TEER and the permeability coefficient of mannitol Dodane *et al.*<sup>14)</sup> established that 0.01% w/v chitosan was effective as an absorption enhancer and at 0.1% w/v

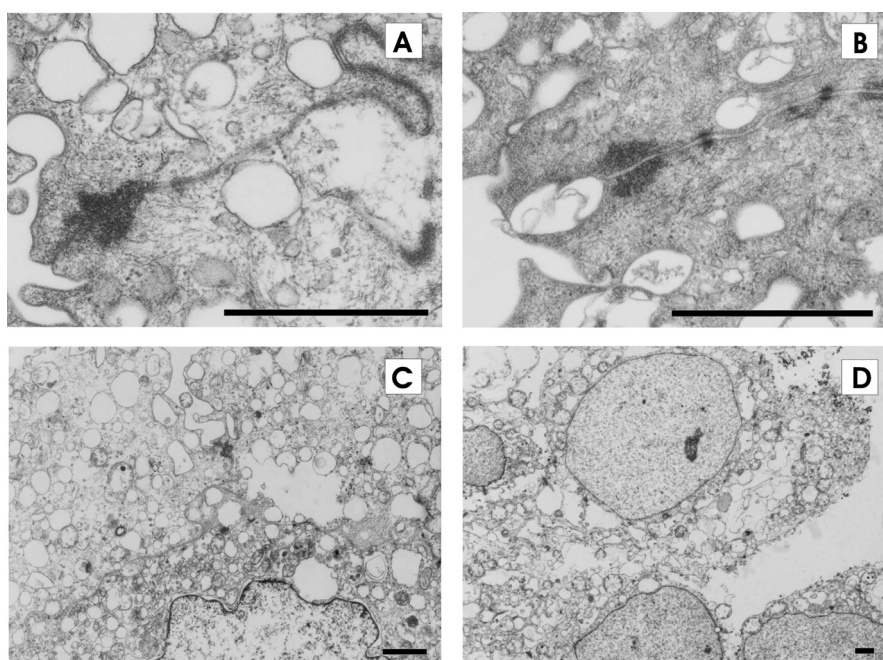


Fig. 7. Transmission Electron Microscopy of Urothelium

Urinary bladders were exposed for 60 min to the solution of moxifloxacin (A), 0.0001% w/v dispersion of chitosan with moxifloxacin (B), 0.001% w/v dispersion of chitosan with moxifloxacin (C) or 0.5% w/v dispersion of chitosan with moxifloxacin (D). Bars: 1  $\mu$ m.

concentration of the polymer the plateau of absorption enhancement across Caco-2 cells was obtained. However, in another study it was revealed that even concentrations as low as 0.002% were effective in the reduction of TEER and enhancement of inulin passage.<sup>15)</sup> However, these concentrations of chitosan are still higher than the ones that were effective in our experiments.

In the presence of chitosan moxifloxacin permeated into the isolated pig urinary bladder wall in a time dependent way (Fig. 2). The time dependence of chitosan's effect on epithelial permeability was studied mostly by measuring the TEER of Caco-2 cells and by this method the effects of chitosan are seen earlier than by measuring the transport of model substances across epithelia.<sup>15)</sup> For Caco-2 cells it was reported that in the presence of 0.01% w/v concentration of chitosan TEER declined in 15 min by 50% and after 1 h by 75%.<sup>14)</sup> Similar results were obtained by Ranaldi *et al.*<sup>15)</sup> However, a 30-min incubation of Caco-2 cells with 0.002% chitosan did not significantly increase the transport of inulin across the cells.<sup>15)</sup> On the other hand, the results obtained in our study show that 0.005 and 0.5% w/v dispersions of chitosan had been effective as absorption enhancers in as little as 15 min (Fig. 2). From our results we can also ascertain that the permeation rate of moxifloxacin into the bladder wall slowly decreased with time. Therefore, the mechanism by which chitosan increases the permeability of the pig urinary bladder wall is obviously triggered quite early, but it needs some time to be completely established. Moreover, at lower concentrations of chitosan a longer period of time is needed to approach the maximal effect of chitosan on tissue permeability.

Compared to Caco-2 cells the permeability of the urinary bladder wall seems to be enhanced earlier and at lower concentrations of chitosan. This finding was not expected especially because a physiological function of the urothelium is to prevent substances from permeating from the urine into the urinary bladder wall. However, it is difficult to directly compare the results obtained in our study with the results on Caco-2 cells. First of all the model substances used in the studies were different. Moxifloxacin used as a model drug in our experiments is a small molecule compared to inulin and horseradish peroxidase used on Caco-2 cells.<sup>15,16)</sup> Secondly, the urothelium is morphologically different compared to Caco-2 cells. Moreover, the luminal surface of the urothelium is covered with a layer of glycosaminoglycans that carries a strongly negative charge and influences the interactions of chitosan with the urothelium.

When the tissue was exposed only to the solution of moxifloxacin with pH 4.5 for 60 min no morphological changes of the urothelium were observed by scanning and transmission electron microscopy (Figs. 5A, 7A). After a 60-min incubation of the tissue in 0.0001% w/v dispersion of chitosan (Figs. 5B, 7B) or after a 15-min incubation in 0.0005% w/v chitosan (Figs. 4A, 6A) no necrosis or desquamation of the urothelium occurred, but the morphology of tight junctions was changed in comparison to the control tissue. It could be expected that modified tight junctions enable moxifloxacin to permeate into the bladder wall to a greater extent compared to the control. However, under the above-mentioned conditions chitosan did not significantly increase the bladder wall permeability for moxifloxacin. The remaining permeability barriers obviously still prevent an increased diffusion of

moxifloxacin into the bladder wall. In concentrations that significantly increased the permeability of the bladder wall after a 15- or 60-min exposure to chitosan dispersions, necrosis of superficial cells and desquamation of the urothelium occurred. Necrotic cells have seriously damaged plasma membranes, while desquamation of the urothelium removes all three types of permeability barriers. In both cases an enhanced permeation of a model drug into the bladder wall is enabled.

The extent of desquamation depends on the concentration of chitosan as well as on the time of the tissue exposure to the tested polymer. When the bladder wall was exposed to 0.5% w/v chitosan for 15 min desquamation of the urothelium occurred to a lower extent compared to a 60-min exposure. Moreover, after a 1 h exposure to 0.5% w/v concentration of chitosan the damage of the urothelium was much more severe than in the case where the tissue was exposed for the same time to 0.001% w/v chitosan. However, also at a 0.001% concentration the layer of superficial cells was mostly damaged. It is interesting that the permeation of moxifloxacin into the bladder wall was not significantly different in both concentrations of chitosan. This indicates that the removal of the superficial layer is crucial for enhanced permeation of substances into the bladder wall. The intermediate and basal cells obviously do not offer a significant permeability barrier, as the permeation of moxifloxacin was the same although these cells were present in the urothelium to a different extent. Therefore, in concentrations of chitosan as high as 0.5% w/v the permeation enhancement is the same as at lower concentrations, but the damage of the urothelium is greater. As confirmed in the morphological studies, a higher concentration of chitosan earlier triggers the degree of urothelial damage, which enables an increased permeation of moxifloxacin into the bladder wall. After a 15-min exposure to 0.0005% w/v concentration only opening of tight junctions occurred, 0.005% w/v chitosan already caused the necrosis of urothelial cells, while at 0.5% w/v concentration desquamation of the urothelium was evident.

Therefore, concentrations of chitosan that significantly increased the permeability of the pig urinary bladder wall caused necrosis of superficial cells as well as desquamation of the urothelium and did not affect tight junctions only as proposed for Caco-2 cells. The time and concentration dependence of chitosan's effect on the permeability of the bladder wall determined in our study could be different for model substances with physico-chemical properties diverse from those of moxifloxacin. However, the mechanism of permeation enhancement established in our work is valid irrespective of the model substance used. The difference in the mechanism of absorption enhancement could be explained by the specific structure of the urothelium compared to Caco-2 cells. Morphological characteristics typical of the urothelium are membrane plaques and the layer of glycosaminoglycans on the apical side of urothelial superficial cells. Moreover, the urothelium belongs to tight epithelia as its TEER is reported to be  $10000 \Omega \cdot \text{cm}^2$  or even higher, which is a very high value compared to Caco-2 cells, where TEER is usually smaller than  $1000 \Omega \cdot \text{cm}^2$ .<sup>23)</sup> Most of all, desquamation is a common reaction of urinary bladder mucosa as it is known from the literature that urothelial cells normally respond to bacterial infection with desquamation and consecutive excre-

tion of infected superficial cells together with attached bacteria.<sup>24)</sup> Thus, it is not surprising that the attachment of a chitosan layer provoked desquamation of the urothelial cells.

## CONCLUSION

Chitosan increased the permeability of the isolated pig urinary bladder wall in a time and concentration dependent manner. At lower concentrations and with shorter exposure times chitosan did not significantly increase the permeation of the model drug into the pig urinary bladder wall, although the structure of tight junctions was affected. With prolonged time and higher concentrations of the polymer a necrosis and desquamation of the urothelial cells was found to be the main reason for significantly increased permeability of the urothelium. It has also been ascertained that with higher concentration of chitosan the deterioration effect on the urothelium was dramatically increased, even though the permeability remained constant.

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