Characterization of the Epithelial Permeation Enhancing Effect of Basic Butylated Methacrylate Copolymer—In Vitro Studies

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Membrane destabilizing properties and increased efflux of doxorubicin from liposomes caused by basic butylated methacrylate copolymer (BBMC), better known under its commercial trade name EUDRAGIT E, have been described in the scientific literature. Here, we investigated the effect of BBMC on suspended and filter-grown Caco2 cells with respect to apical-to-basal transport and membrane permeabilization using transport assays, trypan blue exclusion assay, measurements of transepithelial electrical resistance (TEER), confocal laser scanning microscopy, and transmission electron microscopy. The effect of inhibiting protein phosphatase 2A (PP2A) by okadaic acid was investigated by measuring TEER, but a link between PP2A and the observed effects could not be established. Overall, membrane permeabilization of Caco2 cells by BBMC was demonstrated, which went along with increased apical-to-basal transport of the model compounds mannitol, talinolol, and trospium. The effect was concentration- and time-dependent, and reversible. Enhancement occurred at polymer concentrations as low as 20 μg/mL.

Introduction

Formerly, pharmaceutical excipients were considered as relatively inert substances devoid of biological action. However, an increasing number of compounds used as excipients in drug formulation are reported to alter drug absorption on the biological level. For example, sodium dodecyl sulfate (SDS) does not only improve solubility or wettability of a drug, but also alters epithelial permeability.[1] Furthermore, a row of surfactants have been shown to alter absorption by inhibition of P-glycoprotein in vitro or in vivo, as has been reported for tocopheryl polyethylene glycol succinate (TPGS).[2–4] Thus, excipients do not only determine the physicochemical properties of a dosage form, but may also confer it new and unexpected biological properties. Therefore, identification and characterization of such substances is of importance for drug formulation and in drug regulatory issues.

Basic butylated methacrylate copolymer (BBMC) is a cationic polymer at acidic pH, which is used mainly for tablet coatings (Figure 1).

BBMC is better known under its commercial trade name EUDRAGIT E. Alasino et al.[5] showed that it has membrane destabilizing properties and increases efflux of doxorubicin from liposomes. The purpose of the present study was to investigate whether such effects could also play a role in drug absorption. Therefore, the effects of BBMC on membrane permeability and epithelial permeability of mannitol, metoprolol, talinolol, and trospium were studied by using the Caco2 cell model, which is very similar to the intestinal epithelium and is frequently used for studying drug absorption.[6] Mannitol, talinolol, and trospium are low-permeable substances due to their relative hydrophilicity,

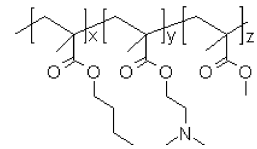

Figure 1. Structure of BBMC; $x/y/z \approx 1:2:1$.

whereas metoprolol is a high-permeable substance. Their structures are shown in Figure 2.

Cellular toxicity and membrane integrity after treatment with BBMC were evaluated by XTT assay and trypan blue exclusion test. Permeabilization of monolayers was also visualized by confocal laser scanning and electron microscopy.

Materials and Methods

Caco2 cells were purchased from the European collection of cell cultures. Dulbecco's modified eagle medium (DMEM) with stable glutamine, penicillin/streptomycin (PEST), nonessential amino acids (NEA), and fetal bovine serum (FBS) were from Biochrom, Berlin. The 12- and 24-well polycarbonate or polyester filter inserts and plates (1.13 cm^2 diameter) were from Corning, Schiphol-Rijk, The Netherlands. Cell viability was tested by an XTT-based in vitro toxicology assay kit purchased from Sigma-Aldrich. The 96-well culture plates were from Corning.

^{14}C-Mannitol (specific activity: 53 mCi/mmol) was from Hartmann Analytics, Braunschweig, and ^{3}H-metoprolol (specific activity: 735 mCi/mmol) was a kind gift of Astra Zeneca, Mölndal, Sweden. ^{3}H-Talinolol (specific activity: 17 Ci/mmol) was from J. Kix, Volxheim, Germany. Mannitol, metoprolol, trypan blue, morpholinoethanesulfonic acid (MES), and fluorescein dextrane (M_r 3300; FD-4) and okadaic acid were from Sigma. Basic butylated methacrylate copolymer was provided by Röhm, Evonik, Darmstadt as colloidal EUDRAGIT E PO.

Cell Culture. Caco2 cells were seeded at a density of 100000/cm^2 on 12- or 24-well polycarbonate transwells (diameter: 1.13 or 0.33 cm^2). Cells were grown in DMEM supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 1% nonessential amino acids, and 10% FBS

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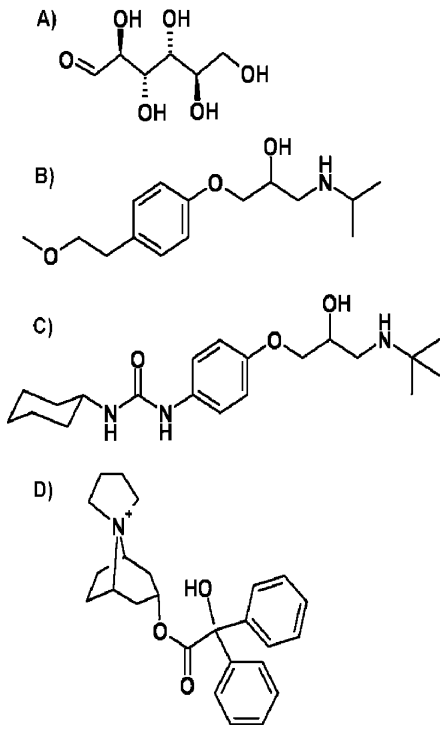

Figure 2. Structures of (A) D-mannitol, (B) metoprolol, (C) talinolol, and (D) trospium.

in an atmosphere of 5% CO_2 and 90% relative humidity. Experiments were performed on days 20–22 postseeding. For maintenance cells were trypsinized with trypsin/EDTA solution (0.25/0.02%) at 80–90% confluence and medium was replaced every other day. For experiments, cells from passages 44–58 were used.

XTT Assay. Cells were seeded into 96-well cell culture plates at a density of about 100000/cm^2 and medium was changed every other day. Experiments were performed on days 20–22 postseeding by incubating cells in Hanks balanced salt solution (HBSS) buffered with 10 mM 2-morpholinoethanesulfonic acid (10 mM MES/HBSS pH 6.5; containing the different concentrations of polymers) for 2 h at 37 °C in an atmosphere of 5% CO_2 and 90% relative humidity. Then the XTT assay was performed according to the manufacturer's protocol. After four hours of incubation with XTT reagent the absorbance was measured at wavelengths of 450 and 690 nm. Absorbance at 690 was subtracted from absorbance at 450 and the resulting values expressed in percent of the according control value.

Trypan Blue Assay. A solution of 0.04% trypan blue was prepared in 10 mM MES/HBSS, pH 6.5. Cells were trypsinized, centrifuged, and resuspended in 10 mM MES/HBSS, pH 6.5. They were incubated for 30 and 60 min in 1.5 mL eppendorf cups with 10 mM MES/HBSS, pH 6.5, containing different polymer concentrations at ambient temperature on a turning wheel. A total of 50 μL of cell suspension were mixed with 50 μL of trypan blue solution. A total of 20 μL of the mixture were given on a hemocytometer and cells were counted under a light optical microscope.

Transport Assay. *Mannitol and Metoprolol.* Solutions of 0.1 mM mannitol and 0.1 mM metoprolol spiked with 18.9 μM ^{14}C-mannitol and 1.36 μM ^{3}H-metoprolol plus the different concentrations of the polymer were prepared in 10 mM MES/HBSS, pH 6.5. A total of 600 μL of the solution were pipetted into the apical chamber, and 1.5 mL of buffer were pipetted into the basal chambers of the transwells. During the experiments cells were kept at 37 °C in an incubator. At the start, 20 μL of buffer were taken from the donor chamber (apical chamber), and the chamber was refilled with 20 μL of fresh buffer afterward. At 2, 15, 30, 60, (90), and 120 min, samples of 250 μL were taken from the acceptor chamber (basal chamber) and replaced with the same volume of fresh buffer.

Talinolol. ^{3}H-Talinolol was used at a concentration of 58.8 nM. The apical side was incubated with polymer solution in 10 mM MES/HBSS buffer, pH 6.5, and the basal side in 10 mM HEPES/HBSS, pH 7.4. At the start, 20 μL samples were drawn from the apical side and the donor compartment was refilled with 20 μL of fresh solution. The 500 μL samples were drawn from the basal compartment after 30, 60, 90, and 120 min, and the volume in the basal compartment was refilled with 500 μL of fresh buffer.

Trospium. Trospium samples were analyzed by HPLC. The apical chamber was incubated with 10 mM MES/HBSS buffer, pH 6.5, containing the polymer and 2 mM trospium and the basal side with 10 mM HEPES/HBSS buffer, pH 7.4. A 100 μL sample was taken from the apical side at the start of the experiment and substituted by 100 μL of fresh buffer. After 120 min, transport was stopped by removing the filters and taking samples from the basal chamber. Samples were stored at –18 °C until HPLC analysis.

Transport Recovery Assay. The assay was performed in 24-well polycarbonate transwells. Cells were incubated for 1 h with a solution of the polymer, 0.1 mM mannitol, and 1 μCi/mL ^{14}C-mannitol in 25 mM MES/HBSS, pH 6.5, on the apical side and with 25 mM HEPES/HBSS, pH 7.4, on the basal side. Apical and basal solutions were carefully removed after 1 h and substituted with 25 mM MES/DMEM, pH 6.5, with 10% FBS containing 0.1 mM mannitol and 1 μCi/mL ^{14}C-mannitol apical and 25 mM HEPES/DMEM, pH 7.4, with 10% FBS basal. Polycarbonate inserts were moved into a new 24-well plate filled with buffer every hour. Aliquots from the basal medium were used for scintillation counting. Aliquots from the apical medium were taken at the start of the experiment and after changing the medium to determine the mannitol concentration in the apical chamber.

Scintillation Counting. Samples were counted in Mini vials A (Carl Roth GmbH+Co, Karlsruhe, Germany) on a liquid scintillation counter LC 6000 (Beckman Coulter, Unterschleissheim, Germany) after thoroughly mixing with 4 mL of scintillation fluid Rotiszint 22 (Carl Roth GmbH+Co, Karlsruhe, Germany). Counting time was set at 5 min for all samples and experiments.

HPLC. Measurements were performed on a Jasco HPLC system composed of a Jasco PU-980 Pump, a Jasco AS-950 autosampler, and a Jasco UV-975 UV/vis detector (Jasco Deutschland GmbH, Groβ-Umstadt, Germany) using amezinium metilsulfate as internal standard. Chromatographic conditions were as follows: column, LiChroCart 125 $\times$ 4 mm, RP-8, Superspher 60, Merck Darmstadt, Germany; mobile phase, HEPES 0.01 M, $K_2HPO_4 \times 3\ H_2O$ 0.003 M, water (bidistilled) 300 mL, acetonitrile 700 mL, phosphoric acid 85% 1.5 mL; temperature, ambient temperature (25 °C); flow rate, 1.2 mL/min; detection, UV absorption 210 nm; injection volume, 50 μL; running time, 7 min. Further details of the method have been previously published[7] and are part of the Supporting Information.

Calculation of P_{app}. P_{app} (apparent permeability) values were calculated from the following equation: $P_{app} = F/(AC_0)$, where F is the flux in dpm/s or mol/s, A is the area of the monolayer (cm^2), and C_0 is the initial concentration of the substance in the apical chamber (dpm/cm^3).

Measurement of Transepithelial Electrical Resistance (TEER). *TEER Measurements.* TEER was measured with a chopstick electrode (Millicell ERS, Millipore, Bedford, U.S.A.). Cell culture conditions were the same as described earlier. Only transwells with an initial TEER higher than 600 Ω/cm^2 were used for experiments. Cells on the transwells were washed and then incubated with 10 mM MES/HBSS buffer, pH 6.5 (600 μL apical, 1.5 mL basal), 20 min at 37 °C. A maximum of 5 μL of a polymer solution with a suitable concentration to give the final concentrations in the apical solution was added to the buffer in the apical chamber. TEER was then measured at 10, 20, 40, 60, 80, 100, and 120 min after addition of the polymer.

TEER Recovery Measurements. TEER recovery experiments were performed in 24-well polycarbonate transwells with 25 mM MES/DMEM, pH 6.5, apical and 25 mM HEPES/DMEM, pH 7.4, with 10% FBS basal. Cells were incubated for 20 min, as described before, and

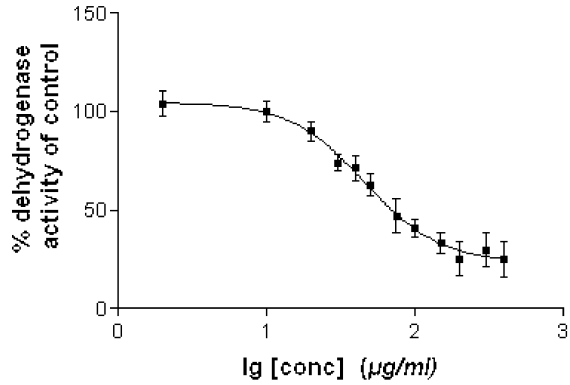

Figure 3. Cell viability as measured by XTT assay. Dehydrogenase activity in percent of control is plotted vs logarithmic concentration [μg/mL] of polymer. Each point represents the mean of four determinations ± SD.

then BBMC solution was added. TEER was followed over 1 h and then the medium in the apical chamber was changed for fresh 25 mM MES/DMEM, pH 6.5, with 10% FBS. Recovery of TEER values was followed over another 6 h period.

Effect of Okadaic Acid. TEER was measured in 10 mM MES/HBSS, pH 6.5, apical and 10 mM HEPES/HBSS, pH 7.4, basal. Experiments with okadaic acid were conducted at a concentration of 100 nM in apical and basal medium.

Tensiometry. Tensiometry was peformed in 10 mM MES/HBSS, pH 6.5, at ambient temperature on a Digital-Tensiometer KIO (Krüss GmbH, Hamburg, Germany) based on the method of Du Nouy. Each measurement was performed in triplicate.

Confocal Laser Scanning Microscopy. CLSM was performed on a Leica TCS SP2 with Caco2 cells grown on polyester membranes for 18 days, as described before. Excitation wavelength was 488 nm and magnification was 20-fold. Cells were incubated in 10 mM MES/HBSS, pH 6.5, with 50 μg/mL BBMC and 1 mg/mL fluorescein dextran (MW 3300) on the apical side and with 10 mM HEPES/HBSS, pH 7.4, on the basal side. Incubation time was 1 h. After incubation, filters were cut off the inserts, carefully moved to a slide, and covered with a coverslip and microscopy was directly performed.

Transmission Electron Microscopy. Cells were grown, as described before, on polycarbonate filters (1.13 cm^2). On day 18 postseeding, cells were incubated 1−2 h with a BBMC solution of 50 μg/mL in 10 mM MES/HBSS. Cells were then washed with 10 mM MES/HBSS and subsequently fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 1 h. The tissue was then postfixed with 2% OsO_4 in cacodylate buffer for 1 h at room temperature, dehydrated in a graded series of ethanol (30–100%), and infiltrated (2×) with propylene oxide and with a 1:1 mixture of propylene oxide and Araldite resin overnight. For embedding, samples were transferred to pure Araldite resin (Plano, Wetzlar, Germany) and polymerized for 48 h at 60 °C. Ultrathin sections were cut with a diamond knife on a Leica Ultracut S. Counterstained ultrathin sections were analyzed with a FEI Technai 12 BioTwin transmission electron microscope and imaged with a SIS MegaView III SCCD camera.

Results

Polymer Toxicity and Membrane Permeabilization. IC_{50} of BBMC was determined by XTT assay as 46 μg/mL with a 95% confidence interval of 40–53 μg/mL by nonlinear regression using the Hill equation (Figure 3).

The absolute decrease in dehydrogenase activity at a concentration of 50 μg/mL, as compared to control, was about 40%; for 20 μg/mL, it amounted to 10%. This indicates that higher concentrations impair vital cellular functions to a certain degree.

Also, the trypan blue assay indicated that membrane integrity was compromised in a concentration-dependent manner at concentrations higher than 20 μg/mL after a one hour incubation with BBMC. More cells were able to exclude trypan blue over the 30 min incubation period than over the 60 min incubation period with BBMC, revealing a time dependence of the effect. At 50 μg/mL, about 50% of the cells were stained blue, that is, were not able to exclude trypan blue dye (Table 1).

Table 1. Percent of Cells Stained after Incubation with BBMC for 30 and 60 Min

BBMC [μg/mL]	% stained cells (after 30 min)	% stained cells (after 60 min)
0	8	7
10	n.d.[a]	10
20	n.d.[a]	9
30	21	26
40	n.d.[a]	40
50	33	51
75	n.d.[a]	70
100	n.d.[a]	78

[a] n.d. = not determined.

Transport Experiments. *Transport.* As can be seen in Figure 4A, absorptive mannitol flux increased continuously when the polymer concentration in the donor solution was augmented, while metoprolol flux did not significantly increase but rather decreased at higher polymer concentrations (Figure 4B).

Mannitol flux was dependent on BBMC concentration and on incubation time as can be seen from the increasing slopes between the different time points of the curves. Talinolol transport showed similar behavior with enhancement in talinolol permeability occurring after 30−60 min at 50 μg/mL BBMC and after 60−90 min at 20 μg/mL (Figure 4C). Table 2 shows P_{app} values of mannitol, metoprolol, talinolol, and trospium.

Transport Recovery. Transport recovery was tested for mannitol, because its permeability seemed to be most sensitive to BBMC treatment. After a 1 h incubation with BBMC, a 2.2- and 5.4-fold increase of apparent mannitol permeability was observed for 30 and 50 μg/mL BBMC (Figure 4D). Subsequently, apparent mannitol permeability rapidly decreased for 2 h and then gradually approached the control level. The transport recovery experiment showed that under certain conditions BBMC can transiently increase permeability of the monolayer without irreversibly impairing its barrier function.

TEER Measurements. The onset of the drop of the TEER is located at 5–10 μg/mL BBMC. The lowest values were found for the highest BBMC concentration used in experiments (50 μg/mL), however, they were only slightly lower compared to the values found for 30 μg/mL BBMC (Figure 5A).

TEER values for 15 and 20 μg/mL were quite similar and only slightly higher than (~5%) those for 30 or 50 μg/mL. A small tendency to recovery of TEER was observed after 60 min for 15, 20, and 30 μg/mL. The onset of transport enhancement of mannitol (20 μg/mL BBMC) does not exactly correlate with the reduction of TEER. Onset occurred at concentrations as low as 5 μg/mL. Enhanced mucosal to serosal transport of mannitol was observed at concentrations higher than 20 μg/mL BBMC. The drop of TEER occurred within 10−15 min after incubation and was, thus, more sensitive to the presence of the polymer than enhancement of mannitol transport. Therefore, decrease in TEER seems to precede enhanced mannitol transport with respect to time and BBMC concentration.

TEER recovery was observed after a 1 h incubation with two different BBMC concentrations (Figure 5B). TEER of BBMC-treated cells initially decreased as expected, however, after changing the medium, TEER of the BBMC-treated cells recovered concentration and time-dependent and approached control level again after about 6 h.

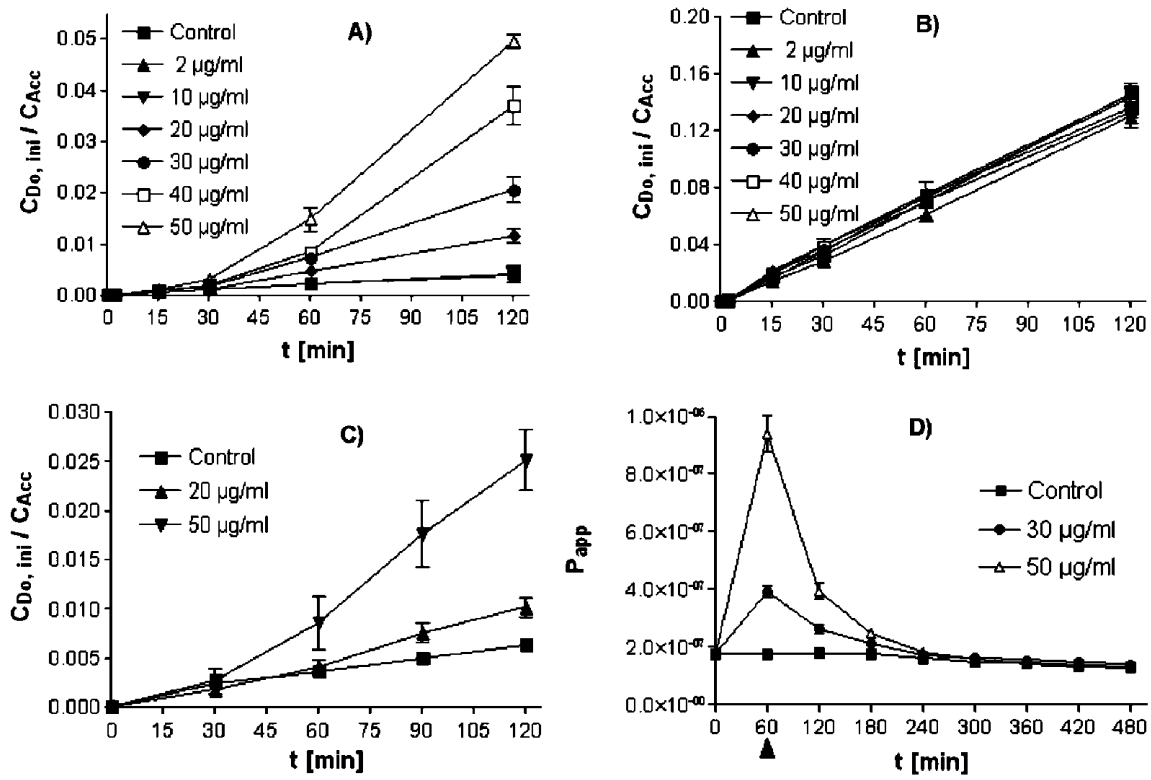

Figure 4. Transport (**A**, **B**, **C**) and transport recovery experiments (**D**). Subsections show transport of mannitol (**A**), metoprolol (**B**), and talinolol (**C**) in the presence of increasing BBMC concentrations. $C_{Do,ini}$ signifies the initial donor concentration and C_{Acc} signifies the acceptor concentration after t minutes. Subsection **D** shows mannitol permeability expressed as P_{app} over 60 min intervals. Cells were incubated with polymer, and after 1 h (indicated by black wedge), the polymer containing medium was replaced with polymer-free medium and P_{app} was monitored for an additional 7 h. Each point represents the mean of three determinations ± SD.

Table 2. P_{app} Values for Mannitol, Metoprolol, Talinolol, and Trospium in the Presence of Different Concentrations of BBMC

BBMC [μg/mL]	P_{app} mannitol [10^{-7} cm/s]	P_{app} metoprolol [10^{-5} cm/s]	P_{app} talinolol [10^{-6} cm/s]	P_{app} trospium [10^{-7} cm/s]
0	5.8 ± 1.2	2.7 ± 0.1	1.0 ± 0.1	4.2 ± 1.1
2	7.5 ± 2.7	2.4 ± 0.2[a]	n.d.[d]	n.d.[d]
10	7.2 ± 2.4	2.4 ± 0.03[b]	n.d.[d]	n.d.[d]
20	22.5 ± 2.7[c]	2.6 ± 0.1	2.1 ± 0.2[c]	5.46 ± 0.6
30	39.9 ± 4.7[c]	2.4 ± 0.1[a]	n.d.[d]	9.66 ± 1.4[c]
40	70.8 ± 6.9[c]	2.6 ± 0.1	n.d.[d]	n.d.[d]
50	96.0 ± 2.8[c]	2.7 ± 0.1	6.0 ± 0.6[c]	25.1 ± 2.1[c]

[a] Significantly different from control, with $p < 0.05$. [b] Significantly different from control, with $p < 0.01$. [c] Significantly different from control, with $p < 0.001$. [d] n.d. = not determined.

Okadaic acid had no marked effect on BBMC-mediated decrease of TEER (Figure 5C).

Tensiometry. Surface tension decreases continuously with BBMC concentration until at about 30 μg/mL a minimum of approximately 40 mN/m is reached (Figure 6).

Visualization of Permeation of Caco2 Monolayer by CLSM. Figure 7A shows untreated Caco2 cells, which exclude FD-4 from cytosol and also from intercellular spaces. Figure 7B shows BBMC-treated cells with fluorescence emitting from the inside of several cells. Fluorescence in these cells was detectable over several planes of the cells, showing its distribution along the z-axis (small annexes below and to the right of xy-plane image).

Transmisson Electron Microscopy Analyses of Treated and Untreated Caco2 Cells. Electron microscopy of untreated control Caco2 cells revealed a monolayer of polarized cells with tightly closed intercellular spaces at the cell contacts (Figure 8A). In addition, on the apical cell membrane of untreated Caco2 cells numerous microvilli are visible (Figures 8A and 9A).

However, in some areas of the cultured monolayer of Caco2 cells, less tightly closed intercellular spaces occur.

After treatment of Caco2 cells with BBMC, obvious cytological changes were observed when compared to untreated cells (Figures 8−10). After 1 h BBMC treatment, overall, less microvilli were present on the apical membrane of Caco2 cells. In addition, enlarged dilated intercellular spaces were found more frequent than in untreated control preparation (Figure 8A,B). Nevertheless, some areas of the Caco2 cell monolayer did not at all show obvious cytological changes after BBMC treatment.

After treatment for 2 h with BBMC, at certain parts of the monolayer, the number of microvilli were strongly reduced at the apical membrane of Caco2 cells. Furthermore, in the monolayer of Caco2 cells treated for 2 h, areas were found where neighboring cells were completely separated by enlargements of the intercellular space (Figure 10A). In addition, abrasions of apical membrane of some cells occurred, indicating severe damage to those cells (Figure 10C). However, most parts

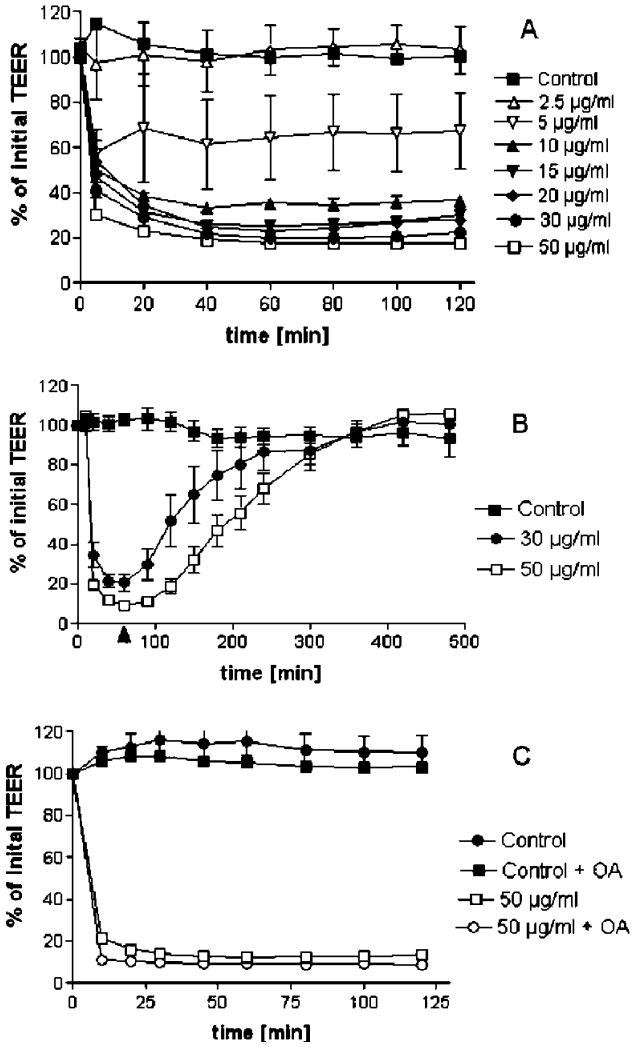

Figure 5. Measurements of transepithelial resistance (TEER). (A) TEER in presence of increasing concentrations of BBMC. (B) Recovery of TEER after an 1 h treatment with the indicated concentrations of BBMC. After the 1 h incubation period (indicated by wedge on the time-axis), medium was changed to polymer-free medium and TEER was measured in intervals for another 7 h. (C) TEER in the presence or absence of BBMC with and without the PP2A inhibitor okadaic acid (OA). OA was used at 100 nM. No substantial effect of OA was observed. Each point represents the mean of three determinations ± SD.

of the 2 h-treated monolayers observed strongly resembled the monolayers treated for 1 h with BBMC (Figures 8B and 10B).

Discussion

In the present study, BBMC enhanced permeation of three low permeable substances. Permeation enhancement went along with a decrease in cell viability as measured by XTT assay (Figure 3) and with increased membrane permeability as measured by trypan blue assay (Table 2) and visualized by CLSM (Figure 7). Electron microscopy of cells treated with 50 μg/mL BBMC revealed dilated intercellular spaces and a reduction of microvilli (Figures 8 and 9) after a 1 h treatment and, additionally, apical membrane wounds and sometimes cells with complete loss of intercellular contact to neighboring cells after a 2 h treatment (Figure 10). While membrane wounds or complete loss of cell−cell contact were not found after a 1 h treatment, membrane and mannitol permeability were neverthe-

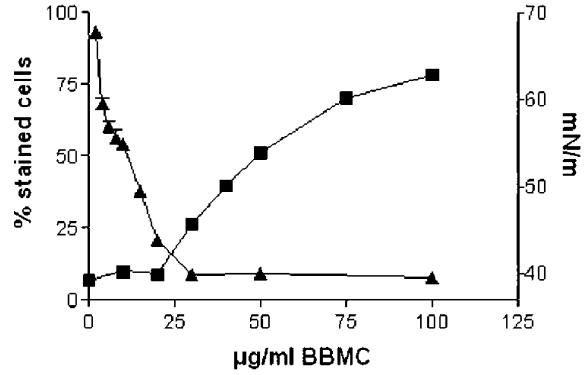

Figure 6. Tensiometry and trypan blue assay. Squares show % of stained cells and triangles surface tension. The rise in the percentage of stained cells coincides with surface tension reaching a minimum.

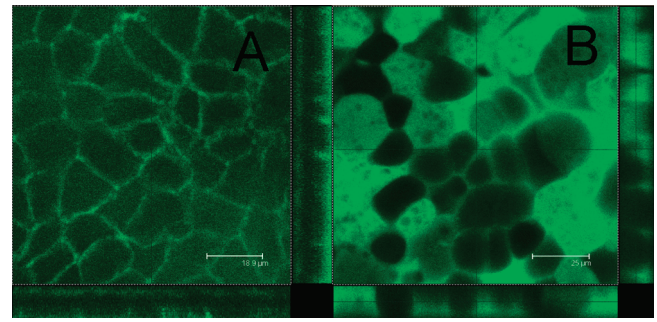

Figure 7. Confocal laser scanning microscopy. (A) A monolayer of untreated Caco2 cells (*xy*-plane). (B) A monolayer of Caco2 cells (*xy*-plane) after a 1 h incubation period with 50 μg/mL BBMC. FD-4 penetrates into cells as observed by fluorescence along the *z*-axis (small annexes below and to the right of the mainframe).

less enhanced as indicated by trypan blue assay, confocal laser scanning microscopy, and increased permeabilities after a 1 h treatment. Furthermore, TEER was decreased within 15 min of incubation and reached a minimum after approximately 30 min (Figure 5A). A link between a decrease in TEER and enhanced paracellular transport was shown by Collares-Buzato et al.[8] Toxicity as estimated by XTT test was rather low (<10%) at BBMC concentrations lower than 20 μg/mL, while a marked decrease in TEER occurred at BBMC concentrations of 5–10 μg/mL already (Figure 5A). Thus, a decrease in TEER does not necessarily imply cellular toxicity. Moreover, the decrease in TEER was reversible (Figure 5B). Mannitol flux, as expected, showed the inverse behavior and was increased after about 30 min of incubation with 50 μg/mL BBMC, but decreased progressively after removal of the polymer (Figure 4D). Surprisingly, reversibility of both TEER and mannitol flux was observed despite toxicity, as determined by XTT assay for the higher BBMC concentrations (>20 μg/mL). Furthermore, the trypan blue assay is often used to evaluate toxicity caused by loss of cell membrane integrity. However, the barrier function of the monolayer was progressively restored within a short period of time despite these findings. This may be due to the presence of FBS in the TEER and transport experiments, while the toxicity tests were carried out in the absence of FBS. FBS, however, contains growth factors, that may be able to trigger cellular repair mechanisms not available in the absence of FBS.

Increased mannitol flux and decreased TEER are parameters rather associated with the paracellular than with the transcellular transport route.[8,9] Furthermore, other cationic polymers known to modify permeability are thought to act via this route.[9,10]

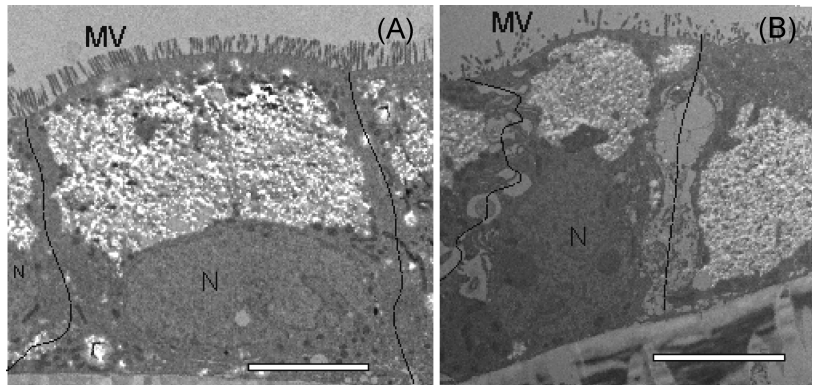

Figure 8. Transmission electron microscopy analysis of Caco2 cells. (A) Untreated Caco2 cells. (B) Caco2 cells treated for 1 h with 50 μg/mL BBMC. After 1 h, BBMC the number of microvilli (MV) on the apical membrane (also see Figure 9) is certainly reduced. Furthermore, the intercellular space between neighboring treated cells is enlarged. Dotted lines indicate symmetrical border lines between neighboring cells. *N* = nucleus. Scale bar: 5 μm.

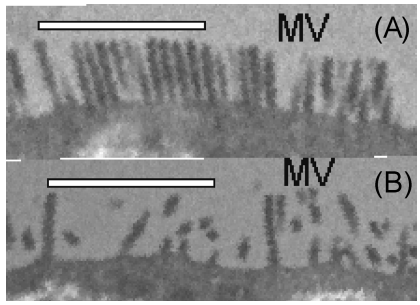

Figure 9. Detailed transmission electron microscopy analysis of apical membranes of untreated (A) and BBMC-treated (B) Caco2 cells. After treatment with 50 μg/mL BBMC for 1 h, the number of microvilli (MV) at the apical membrane is reduced. Quantification of the number of MV: 6 MV/μm in untreated monolayers and 3–4 MV/μm in treated monolayers. Scale bar: 2 μm.

Dilated intercellular spaces seen in electron microscopy seemed more abundant in the treated samples than in control samples, however, normally cell−cell adherence was not fully lost.

Increased membrane permeability for trypan blue and FD-4 were also observed after a 1 h treatment. Therefore, transport may have also been influenced by higher uptake from the apical side and, consequently, a higher gradient or a faster build-up of the gradient between cytosol and basal medium. In contrast, electron microscopy did not reveal damage to the basal membrane, which itself should largely restrict mannitol flux. Permeabilization of red blood cells by EUDRAGIT E above 10 μg/mL has been shown before.[5] This is close to our findings for Caco2 cells, where we found permeabilization between 20 and 30 μg/mL. Hong et al. analyzed different cationic polymers, including polylysine and polyethyleneimine, for their ability to enhance membrane permeability and showed nanoscale hole formation in lipid bilayers by atomic force microscopy after treatment with such polymers, while the tested neutral polymers did not show such properties.[11] Furthermore, membrane-destabilizing properties have been shown for noncationic methacrylic acid copolymers of different composition.[12] Therefore, it seems that methacrylic acid copolymers and especially BBMC have an ability to interact with membranes.

While TEER decreased already at concentrations lower than for which membrane permeabilization or transport enhancement could be observed the onset of transport enhancement and membrane permeabilization occurred at very similar concentrations (20–30 μg/mL). The ability to interact with membranes is also reflected in the results obtained from tensiometry (Figure 6). BBMC was surface active and a minimum of surface tension was reached between 20 and 30 μg/mL. This concentration corresponds to the onset of action with respect to alteration of permeability and could also point toward an involvement of micellar structures, which would be expected to be generated above this concentration.

Electron microscopy revealed rupture of the apical membrane after 2 h treatment with BBMC. This may be an artifact and not indicative of the actual state of the living cell in the presence of the polymer. Nevertheless, it shows that some kind of interaction between the polymer and the membrane constituents takes place, preventing complete fixation as seen for control, for example, reduction in protein content. Furthermore, reduction in the number of microvilli might also be brought about by an interaction between the polymer and Actin, leading to redistribution of Actin as has been shown for chitosan and polyethylenimine by Ranaldi et al.[10]

As a third option, in the case of talinolol and trospium, because membrane permeabilization and surface activity of BBMC were shown, it is also possible that P-glycoprotein activity is influenced by an interaction between the polymer and the membrane, as has been shown for nonionic surfactants as TPGS, Polysorbate 80, or CREMOPHOR EL.[2–4] However, apical to basal transport of talinolol was not increased in the presence of 100 μM verapamil using the present Caco2 cell model (data not shown). Therefore, inhibition of P-glycoprotein is an unlikely explanation for the results of the present study, however, may not be excluded in general.

A time dependence was observed for both membrane permeabilization, as assessed by trypan blue assay and mannitol transport. This might be a coincidence, but could also point toward a causal relationship between membrane permeabilization and increased paracellular transport, as indicated by increased mannitol transport.

Involvement of protein phosphatase 2a in the regulation of tight junctions has been shown for MDCK-cells. Okadaic acid can be used as an inhibitor for PP2A and can suppress its action.[13] Furthermore, polylysine and other polycations have been described as stimulators of certain phosphatases.[14] Therefore, to test whether the effect of BBMC on TEER might be mediated by a stimulation of PP2A we performed an inhibition experiment with okadaic acid. However, addition of okadaic acid at 100 nM rather accelerated or aggravated decrease in TEER. Thus, we could not establish a link between the observed alteration of permeability and PP2A.

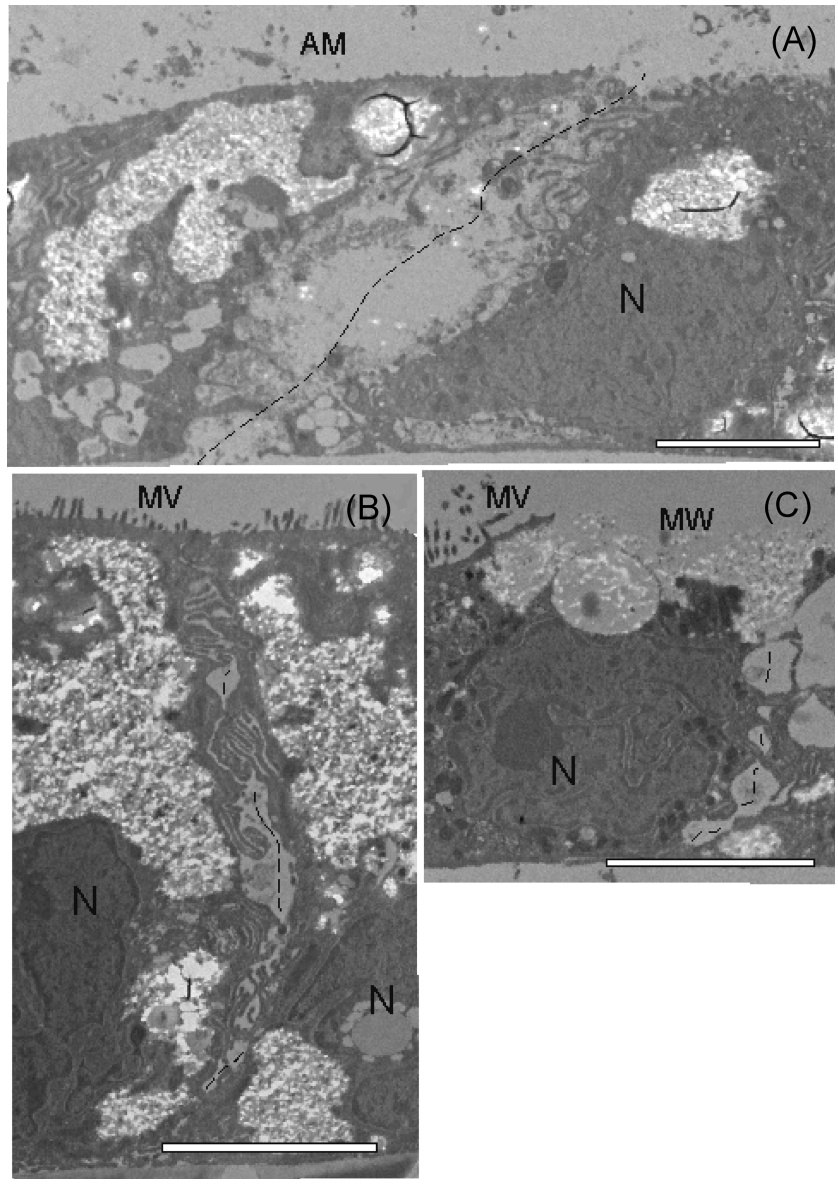

Figure 10. Transmission electron microscopy analysis of Caco2 cells treated for 2 h with 50 μg/mL BBMC. (A) A large intercellular cleft between two neighboring treated cells along the dotted line is visible. In addition, microvilli (MV) are absent from the apical membrane (AM). (B) Prominent dilatation are frequently observed along the cell contacts (dotted line) of treated cells (I). (C) The apical membrane is disrupted and a large membrane wound (MW) is formed. *N* = nucleus. Scale bar: 5 μm.

Overall, our findings are very similar to the effects of sodium dodecyl sulfate on Caco2 monolayers, which is also thought to change membrane and paracellular permeability.[1]

Small but significant decreases in transport were seen for the well-permeable substance metoprolol for some concentrations. A reduction in microvilli and, therefore, surface, as observed by electron microscopy, could well account for these findings. We could, however, show several fold-enhanced permeabilities for the low permeable substances mannitol, talinolol, and trospium. This may imply that use of BBMC will have little consequences for the in vivo absorption of high permeable substances, while permeabilities of low permeable substances may well be increased.

The FDA's (Food and Drug Administration) inactive ingredient database (http://www.accessdata.fda.gov/scripts/cder/iig/index.cfm; April 11, 2007) lists seven different oral dosage forms containing EUDRAGIT E with 1.63–33.39 mg. Dividing by 240 mL (a glass of water) would result in concentrations between 6 and 139 μg/mL. The three dosage forms with most EUDRAGIT E contain 19, 30, and 139 μg/mL. While 19 and 30 μg/mL are close or above the minimal effective concentration for which we observed transport enhancement, 139 μg/mL is almost 7-fold higher than the minimal effective dose of BBMC in our experiments. Certainly, our in vitro results will not translate one-to-one to the in vivo situation, for example, the presence of mucous protein in the intestine might attenuate the permeation enhancement by binding of the polymer. However, we think it is interesting that BBMC shows permeation enhancing effects in the in vitro setting at concentrations, which seem achievable in vivo after administration of marketed dosage forms. Our findings certainly warrant further in vivo investigations of the issue.

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Supporting Information Available. Validation data of HPLC method for trospium determination. This material is available free of charge via the Internet at http://pubs.acs.org.

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