Comparative Uptake Studies of Bioadhesive and Non-Bioadhesive Nanoparticles in Human Intestinal Cell Lines and Rats: The Effect of Mucus on Particle Adsorption and Transport

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Purpose. The interaction of nanoparticles (NP), consisting of hydrophobic polystyrene, bioadhesive chitosan, and stealth PLA-PEG with two human intestinal cell lines, the enterocyte-like Caco-2 and mucus-secreting MTX-E12, was investigated and compared to the *in vivo* NP uptake in rats.

Methods. The extent and mechanism of cellular association of different NP with Caco-2 and MTX-E12 was investigated using confocal laser scanning microscopy (CLSM) and a cellular association assay. *In vitro* results were compared to gastrointestinal distribution of chitosan NP in rats after intra-duodenal injection.

Results. Cellular association of NP with Caco-2 cell monolayers showed the following rank order: polystyrene > chitosan >> PLA-PEG. Mucus (MTX-E12) significantly decreased the association of hydrophobic polystyrene NP. While no mucus binding was observed for PLA-PEG, association of chitosan NP with mucus strongly increased. Intra-duodenal administration of chitosan NP in rats confirmed these *in vitro* results, demonstrating that NP could be detected in both epithelial cells and Peyer's patches. Chitosan NP internalization was saturable, as well as energy- and temperature-dependent. It could be inhibited by an excess of protamine and by removal of anionic sites of the apical membrane. By contrast, polystyrene NP uptake was found to be largely independent of these factors, except for a temperature-dependency.

Conclusions. In contrast to Caco-2 cells, the presence of mucus presented a major barrier for the uptake of hydrophobic polystyrene NP and showed an even more profound effect upon the uptake of chitosan NP. A correlation between the uptake in cell culture models and *in vivo* rat epithelial cells was confirmed for chitosan NP. Moreover, chitosan NP seemed to be taken up and transported by adsorptive transcytosis, while polystyrene NP uptake was probably mediated by non-adsorptive transcytosis.

KEY WORDS: nanoparticles, Caco-2, mucus, endocytosis, peroral drug delivery, oral vaccines.

INTRODUCTION

Through recent advances in biotechnology a large number of hydrophilic macromolecular drug candidates, such as peptides, proteins, oligonucleotides, and plasmids, are under consideration as potentially useful therapeutic approaches. These molecules share high enzymatic degradation in the gut lumen and poor transport properties across mucosal barriers as common features. Therefore, they usually require frequent parenteral administrations inconvenient to the patient. Because peroral delivery is by far the most popular route of application, colloidal drug carriers, such as nanoparticles and liposomes, have been intensively investigated as oral drug delivery systems able to overcome the above mentioned bioavailability problems (1). Nanoparticles (NP) are of particular interest, as they provide protection of sensitive molecules against enzymatic and hydrolytic degradation (2). However, their transport across biologic and mucosal barriers is somewhat controversially discussed in the literature (2). Uptake can occur not only via micro-fold (M)-cells, highly specialized epithelial cells in the Peyer's patches and isolated follicles of the gut associated lymphoid tissue (GALT), but also across the apical membrane of enterocytes (1). The relative importance of the Peyer's patches as the principle site of particulate absorption may have been overemphasized, because M-cells represent less than 1% of the human intestinal epithelial cell population, in contrast to the rodent animal models frequently used for NP uptake investigations (3). For instance, a significant uptake of polystyrene NP in non-lymphoid intestinal tissue has been reported by a number of different authors (4–8). From a mechanistic point of view, it would be desirable to establish an *in vitro* cell culture model that reflects the main barrier properties of the GIT mucosa with respect to NP transport.

Recently, the widely used *in vitro* cell culture model of the small intestine, Caco-2, was used for the characterization of NP uptake. Desai *et al.* (9) found a size-, concentration-, and temperature-dependent uptake of poly(lactide-co-glycolide) (PLGA) NP (100 nm) in Caco-2 cells, which are compatible with an active internalization process. Uptake of poly(DL-lactide), PLA NP, on the other hand, seemed to be low capacity, size-dependent and compatible with a transcellular transport mechanism (10).

The contribution of the gastrointestinal mucus layer on NP uptake, however, has not been studied in detail. The main component of GIT mucus is the glycoprotein mucin that has been reported to considerably decrease the diffusion of drug molecules according to their hydrodynamic radius (11). Moreover, mucus also provides a physical/mechanical barrier for NP transport through the GIT mucosa. It entraps NP causing their agglomeration, which results in an increase in net size and a decreased diffusion. Norris and Sinko, for instance, found a sharp decrease in the transport of polystyrene NP transport of increasing diameter in purified pig mucin with a cut-off at 500 nm (12).

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ABBREVIATIONS: BSA, bovine serum albumin; CLSM, confocal laser scanning microscopy; FD-4, fluorescein isothiocyanate-dextran Molecular weight 4400; FITC, fluorescein isothiocyanate; GALT, gut associated lymphoid tissue; LDH, lactate dehydrogenase; PBS, phosphate buffered saline; PEG , poly(ethylene glycol); PLA, poly(lactic acid); SDS, sodium dodecyl sulfate; TEER , transepithelial electrical resistance; TRITC, tetra-methyl-rhodamine isothiocyanate; TPP, penta-sodium-tri-poly-phosphate; WGA, wheat germ agglutinin.

The aim of this study was to examine the effect of mucus on the cellular association of NP with different physicochemical surface properties. Therefore, we investigated NP interaction with two *in vitro* cell culture models, enterocyte-like Caco-2 cells, and mucus-secreting MTX-E12 cells, using a cellular association assay and confocal laser scanning microscopy (CLSM). Moreover, to validate our cell culture models, we compared the uptake and the intracellular distribution of chitosan NP after intra-duodenal injection in rats to *in vitro* results. Since chitosan and PLA-PEG NP have been found to facilitate insulin and tetanus toxoid transport over the nasal mucosa (13,14), the exact mechanism of uptake on a cellular level was explored.

MATERIALS AND METHODS

Materials

FITC-BSA, cholic acid sodium salt, fetal calf serum (FCS), lactate dehydrogenase assay, acetylcystein, and TRITC-WGA were obtained from Sigma (Taufkirchen, Germany). Tissue culture reagents were purchased from Gibco (Eggstein, Germany) and tissue culture articles from Nunc (Wiesbaden, Germany). Protamine sulfate was obtained from Roth (Karlsruhe, Germany). Polystyrene yellow green labeled NP (200 nm) were purchased from Polyscience Inc. (Eppelheim, Germany). PLA-PEG (PLA molecular weight 45,000 and PEG molecular weight 5000) were obtained from Alkermes (Boston, USA). Chitosan (Protasan™Cl 110) was purchased from Pronova Biopolymer A.S. (Norway). All other chemicals were obtained from Merck (Darmstadt, Germany) in analytical quality.

Preparation and Characterization of NP

Chitosan NP were prepared by ionotropic gelation of chitosan with TPP anions according to the procedure described by Fernández-Urrusuno *et al.* (13). For loading FITC-albumin into chitosan NP, the label was added to the TPP solution (0.725 mg/mL). NP were isolated by centrifugation at 11,000 *g* on a glycerol bed, for 45 min. PLA-PEG NP were prepared using the double emulsion technique conveniently modified to reduce the size to the submicron range by Blanco and Alonso (15). Briefly, a solution of FITC-albumin was emulsified in a solution of PEG-PLA in ethyl acetate by sonication (Branson, Sonifier 250, UK) for 15s (15 W). Then an aqueous sodium cholate solution (1% w/v) was added to this emulsion and the resulting (w/o)/w emulsion was formed by sonication for 15 s (15 W). The double emulsion was diluted in 100 mL sodium cholate solution (0.3% w/v) and the solvent was eliminated by evaporation. Particle sizes and ζ-potentials were determined by photon correlation spectroscopy (PCS) and Laser Doppler Anemometry (LDA), respectively, using a Zetasizer™ 3000 HS (Malvern Instruments, Malvern, UK). The release of FITC-BSA from NP was checked after 4-h incubation in transport buffer at 37°C.

Cell Culture

Mycoplasma-free Caco-2 cells were used at passage numbers 42-50 (HD, DKFZ, German Cancer Research Institute, Heidelberg, Germany) under conditions described earlier (16) and HT29-MTX clone E12 at passage numbers 35–37 (17). Cells were seeded at a density of $6*10^4$ cells/cm^2 either on cell culture dishes or on uncoated polycarbonate Transwell™ filter inserts (Costar, Bodenheim, Germany, 0.4 μm pore size, area: 4.71 cm^2) and cultivated over 21 days. Medium was changed every second day.

In Vitro Cytotoxicity Studies

Release of Lactate Dehydrogenase (LDH)

Caco-2 monolayers grown on 12 well dishes (polystyrene) were incubated with the NP suspensions (250 μg/mL) in transport buffer (PBS 0.1 M, pH 7.2 supplemented with Ca^{2+} and Mg^{2+} and 15 mM glucose), 0.1% Triton-X 100 in transport buffer (positive control) or pure transport buffer (negative control). After 60 and 120 min of exposure 100 μL samples were withdrawn and processed according to the manufacturer's instructions (Testkit: DG 1340-K, Sigma Diagnostics). All LDH concentrations were normalized relative to Triton-X 100 and expressed as relative LDH release. Each experiment was run in triplicate.

Transepithelial Electrical Resistance

The integrity of the monolayer was checked at 21 days post seeding by measuring TEER before and after CLSM experiment (17).

Uptake of NP by Caco-2 and MTX-E12 Cells

Binding and uptake studies were performed 21 days post seeding on polystyrene 12 wells. Cells were rinsed with transport buffer, allowed to equilibrate at 37°C for 15 min, and were incubated with 500 μL of a 250 μg/mL NP suspension in transport buffer under different conditions. (i) To investigate time-dependent uptake, cells were incubated for 5, 15, 30, 60, 90, and 120 min at 37°C. (ii) To study the effect of incubation temperature on cellular uptake, cells were incubated for 60 min at 4°C. (iii) To study the effect of sodium azide treatment, 100 mM sodium azide was dissolved in the NP solution and cells were incubated for 60 min at 37°C. (iv) To study the effect of protamine treatment, 1 mM protamine sulfate was dissolved in the NP solution and cells were treated for 60 min at 37°C. (v) To investigate the effect of desulfurization, cells were preincubated with 35 mM sodium chlorate in cell culture medium for 48 h according to Mislick *et al.* (18), rinsed twice with transport buffer and incubated with the NP suspension. (6) To achieve lysis of glycosaminoglycans from the surface, cells were pretreated for 1 h at 22°C with 10 U/mL heparinase II (18), rinsed twice with transport buffer, and incubated with the NP suspension for 60 min (7). To evaluate the effect of different concentrations of NP on the uptake, Caco-2 cells were incubated with NP in the concentration range of 31.25–1000 μg/mL for 60 min at 37°C. After incubation supernatants were removed, the cells were then washed five times with ice-cold transport buffer pH 5.0, once with 5 mM EDTA pH 5.0 for 15 min to remove adhering NP ("acid wash"), and dried overnight at room temperature under light exclusion. Following cell lysis via incubation with a solution of 2% sodium dodecylsulfate (SDS) and 50 mM EDTA pH 8.0 at room temperature (19), cell associated fluorescence was determined by fluorescence spectroscopy using a 96 well plate reader, LS 50B (Perkin Elmer, Überlingen, Germany) (exci-

tation: 497 nm, emission: 521 nm). The quantity of associated fluorescence, which represents the associated dose of NP, was calculated from standard curves prepared under the same conditions and is reported as the amount of NP associated (μg) per area of the cell monolayer (mm^2). The efficiency of NP uptake was calculated from the theoretical dose of NP exposed to Caco-2 cells (0.71 $\mu g/mm^2$ per monolayer) and the actual uptake, as described earlier (9).

Removal of the Mucus Layer of MTX-E12 Monolayers

A removal of the mucus layer prior to uptake experiments was performed according to (20). Briefly, MTX-E12 monolayers were washed with 2.0 mL of a solution consisting of 10 mM acetylcysteine in transport buffer and incubated under agitation (plate-shaker at 135 rpm) for 60 min at 37°C. Subsequently, cells were washed twice with transport buffer and treated with the NP solution as described above. To remove the mucus layer after uptake experiments, monolayers were washed two times with a 3% (v/v) formalin solution in PBS (0.1 M, pH 7.4) following the usual washing procedure.

Confocal Laser Scanning Microscopy

Cells grown on Transwell™ filter inserts for 21 days were treated as described above. After washing three times with ice-cold transport buffer, they were fixed with 3% (v/v) formalin in PBS at room temperature for 30 min, counter-stained with TRITC-labeled wheat germ agglutinin (WGA) (0.01 μg/mL) for 30 min, and DAPI (4′6-diamidino-2-phenylindole) (1 μg/mL) for 20 min, both under light exclusion. Samples were embedded in PBS/glycerol (2:1) and examined under confocal laser scanning microscope (Axiovert™, Zeiss CLSM 501, Jena, Germany) equipped with a Zeiss Neofluor 40*/1.3 objective. Excitation wavelengths were 364 nm (long pass filter (LP) 385 nm) for DAPI, 488 nm (LP 505 nm) for FITC, and 543 nm (LP 560) for TRITC. A gallery of 80 optical sections (0.4 μm) was collected and xz and yz composites were processed using Zeiss LSM 510™ software. Sensitivity was kept constant during different experiments.

Intra-Duodenal Administration of Chitosan NP to Rats

Sprague-Dawley rats (250 g) were provided with laboratory diet *ad libitum* and fasted 18 h before experiments. Animals were given general anesthesia with an intra-peritoneal injection of sodium pentobarbital. A volume of 1 mL chitosan NP suspension (1 mg/mL) was administered by intra-duodenal injection. After 1 h the animals were sacrificed, samples of the intestine (duodenum, jejunum and ileum) with and without Peyer's patches were removed, and fixed in 4% (v/v) formalin in PBS. Tissues were dehydrated by a graded series of ethanol, embedded in paraffin and sections of 5 μm were cut using a microtome (Frigocut Mod. 2700, Reichert-Jung, Germany). The sections were stained with TRITC-WGA, as well as DAPI, and viewed under CLSM as described above.

Calculations and Statistics

Results were depicted as mean value ±SD from three experiments. Significance between the mean values was calculated using ANOVA one way analysis (GraphPad InStat version 3.00 for Windows 95, GraphPad Software, San Diego, CA, USA). Probability values $p > 0.05$ were considered significant.

RESULTS AND DISCUSSION

The aim of the present study was to investigate the influence of mucus on the cellular association of NP by comparing the interaction of hydrophobic polystyrene, mucoadhesive chitosan and stealth-like PLA-PEG NP with cellular membranes in two intestinal cell culture models: Caco-2 and MTX-E12. While Caco-2 cells are generally regarded as an adequate model for enterocytes in the small intestine, they lack a mucus layer. MTX-E12 cells represented the second major cell type in the small intestine, namely mucus-secreting goblet cells. MTX-E12 cell monolayers are covered by a continuous sheet of mucus, allowing studies of transport and mucus interaction (17). To validate our cell culture results, we compared the *in vitro* data from the cell culture studies to the *in vivo* uptake of chitosan NP in rats. Although chitosan and PLA-PEG NP have been previously shown to transport insulin and tetanus toxoid over the nasal mucosa and consequently increase antibody titers in rats (13,14), their mechanism of uptake on a cellular level has not been investigated.

Characterization of NP

NP in the size range of 200–300 nm were produced under optimized formulation conditions. Their physico-chemical properties are summarized in Table I. As a function of polymer properties, NP showed differences in ζ-potential, but not in the particle size. While PLA-PEG NP were characterized by a negative surface charge, probably due to carboxylic-end groups at the particle surface, chitosan and polystyrene NP exhibited a positive and neutral ζ-potential, respectively. In contrast to polystyrene NP with a covalently bound yellow green fluorescence label, FITC-BSA was encapsulated into chitosan and PLA-PEG NP. Differences in the encapsulation efficiency of the label may be caused by different encapsulation techniques. To ensure that the fluorescence determined and observed in the cell layers was due to FITC-BSA associ-

Table I. Physico-Chemical Properties, TEER-Values (Determined During CLSM Studies), and LDH-Release of Polystyrene, Chitosan, and PLA-PEG NP

	Polystyrene	Chitosan	PLA-PEG
Size (nm)	213 ± 8	290 ± 7	196 ± 20
ζ-potenial (mV) in PBS	+1.1 ± 1	+17.5 ± 3.6	–23.9 ± 1.2
Encapsulation efficiency (%)	covalently bound	92.3 ± 0.7	56.0 ± 2.07
TEER (% of initial)	110 ± 0.6	119 ± 4.7	121 ± 1.6
LDH-release after 120 min (%)	2.01 ± 0.5	2.86 ± 0.85	1.97 ± 0.52

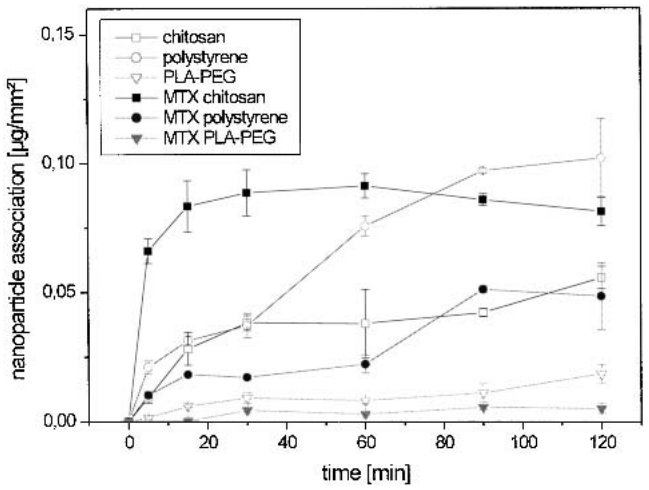

Fig. 1 Time course of chitosan (●), polystyrene (■), and PLA-PEG (▼) NP association in enterocyte-like Caco-2 (open symbols) and mucus-secreting MTX-E12 cells (closed symbols). (n = 3)

ated with NP, the release of hydrophilic FITC-BSA from NP was studied over 4 h in transport buffer. Since the release was below 1%, it can be assumed that the fluorescence measured was mainly due to fluorescent-labeled nanoparticles.

According to data from the literature, polystyrene NP are uncharged and very hydrophobic in nature. Therefore, interactions with cell membranes are thought to occur through hydrophobic interactions. By contrast, the hydrophilic polycation chitosan showed strong interactions with negatively charged mucosal surfaces, due to electrostatic interactions (21). PLA-PEG NP displaying a hydrophilic PEG-coat on their surface are thought to interact only weakly with mucosal surfaces. To explore these hypothesis, we investigated the cellular association and uptake of these NP with different *in vitro* cell culture models of the small intestine.

Cytotoxicity Studies

To rule out cytotoxic effects causing cell damage and uptake of NP, transepithelial electrical resistance (TEER) and the release of lactate dehydrogenase (LDH) were monitored as indicators for tight junction functionality and membrane disruption, respectively. As shown in Table I, TEER values remained in the range of 110–121% of the initial value and the LDH-release was below 10%, suggesting that association of the NP with the cell culture model did not cause significant changes in tight junction permeability or membrane integrity.

Cellular Association of NP with Enterocyte-Like Caco-2 and Goblet-Cell-Like MTX-E12 Cells

The cellular association of NP was assessed under *in vitro* conditions and represents both the bound and internalized fraction of NP. Two different techniques were utilized: An assay determining fluorescent label associated with NP after cell lysis according to Russell-Jones *et al.* (19) and CLSM. The quantity of associated fluorescence summarizes the amount of NP internalized and to a minor extent bound to the cell surface of Caco-2 cells, as confirmed by CLSM (data not shown). Because the mucus layer of MTX-E12 cells could not be removed by a simple washing procedure, in MTX-E12 cells associated fluorescence represents NP entrapped in the mucus and internalized in the cells (Fig. 1). To further characterize the barrier properties of mucus, we compared cellular association of NP before and after the removal of the mucus layer (Fig. 2). Therefore, a mild washing procedure, using acetylcysteine to cleave sulfide-bonds of mucin was applied. The washing procedure had no effect on the integrity of the cell layers, as TEER-values remained unchanged (data not shown). The amount of NP entrapped in the mucus layer was determined by removing the mucus layer with formalin solution in a final washing step, as described elsewhere (22). To corroborate the results from the cellular association assay and to visualize the internalization of NP in Caco-2 and MTX-E12 monolayers, confocal laser scanning microscopy (CLSM) was used (Fig. 3). Cells were double-stained with TRITC-WGA (membrane, red) and DAPI (blue, nuclei) to distinguish between different cellular structures. The lectin WGA strongly binds to the tetra-saccharide, N-acetylglucosamine, which is

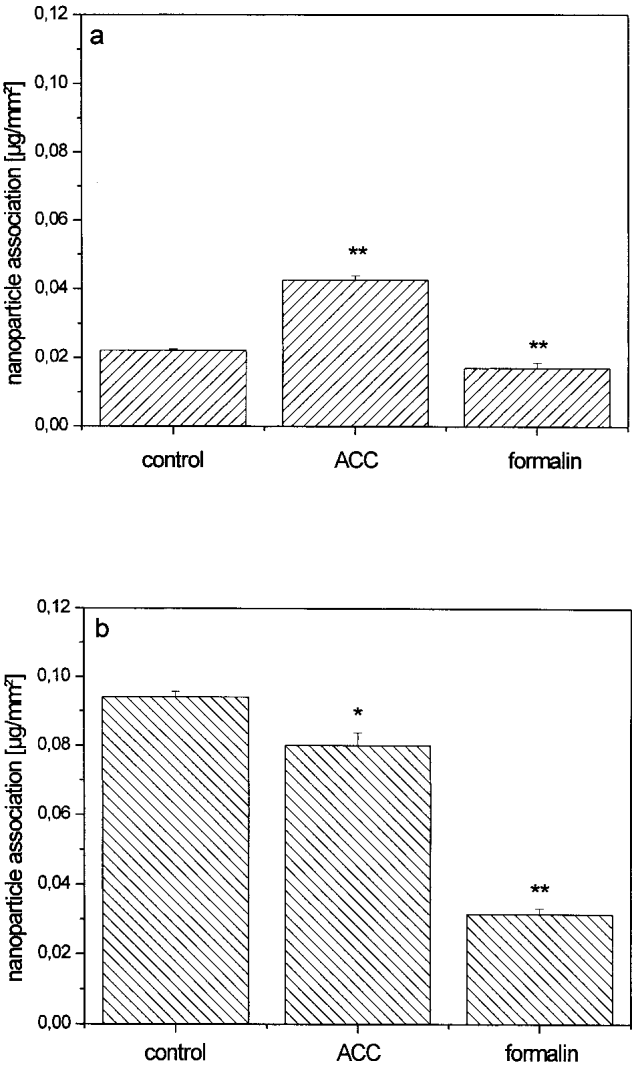

Fig. 2. Cellular association of fluorescence of polystyrene (a) and chitosan (b) NP after 60 min with untreated mucus-secreting MTX-E12 (control) and after the removal of the mucus layer before the experiment by acetylcysteine treatment (ACC) and after the experiment by formalin treatment. (n = 3)

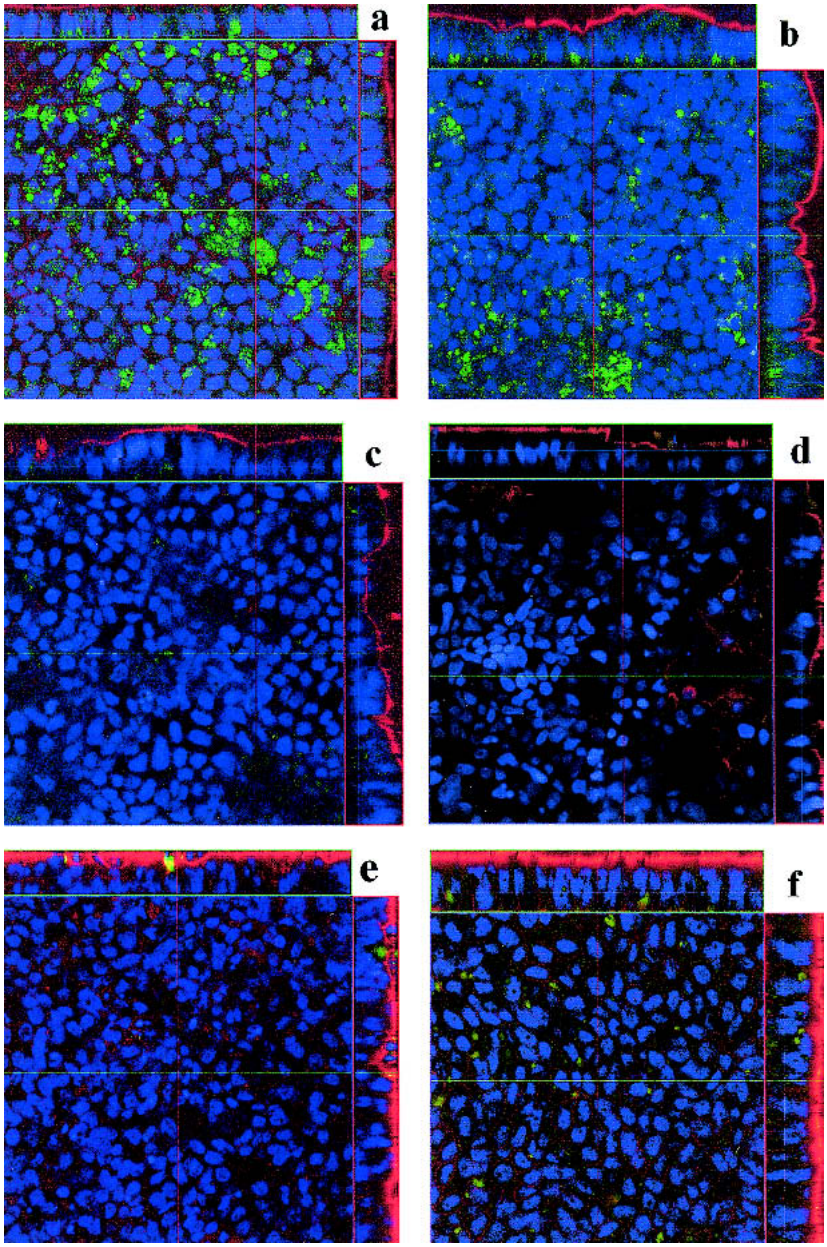

Fig. 3. Confocal laser scanning images: Uptake of polystyrene (a), chitosan (b), PLA-PEG (c) NP and FITC-BSA in solution (d) in Caco-2 (a–d) and MTX-E12 (e–f) after 120 min. Formalin fixed monolayers were double-stained with DAPI (nuclei, blue) and TRITC-WGA (membrane, red); NP were green. Confocal data sets are presented as xy-, xz- and yz-projections. xy-sections were taken from the basolateral part of the monolayer.

highly present at the apical membrane of epithelial cells in the small intestine and Caco-2 cells (16). DAPI intercalates DNA and, therefore, was used as a marker for cell nuclei. Fluorescence signals were collected using appropriate filters allowing separate analysis.

The cellular association of chitosan, PLA-PEG, and polystyrene NP varied strongly as a function of their physicochemical properties and the cell line. After 120 min, the following rank order was observed in Caco-2 cells: Polystyrene > chitosan >> PLA-PEG (Fig. 1, open symbols) and in MTX-E12: Chitosan > polystyrene >> PLA-PEG (Fig. 1, closed symbols).

In comparison to the more hydrophilic chitosan and PLA-PEG NP, hydrophobic polystyrene NP showed the strongest interaction with Caco-2 cell monolayers reaching a maximum of 0.102 ± 0.015 μg/mm^2 (14.3%). This is consistent with Hillery *et al.* (23), who observed that a poloxamer coating of polystyrene NP, leading to a higher degree of hydrophilicity, decreased the gastrointestinal uptake in rats. Similar to Norris and Sinko (12), polystyrene NP also showed the strongest decrease of association with mucus-secreting MTX-E12 cells (−60%) suggesting that NP hydrophobicity could be a major obstacle for mucus penetration.

This was also supported by the twofold increase of polystyrene NP association after the removal of the mucus layer prior to incubation, reaching approximately Caco-2 cell val-

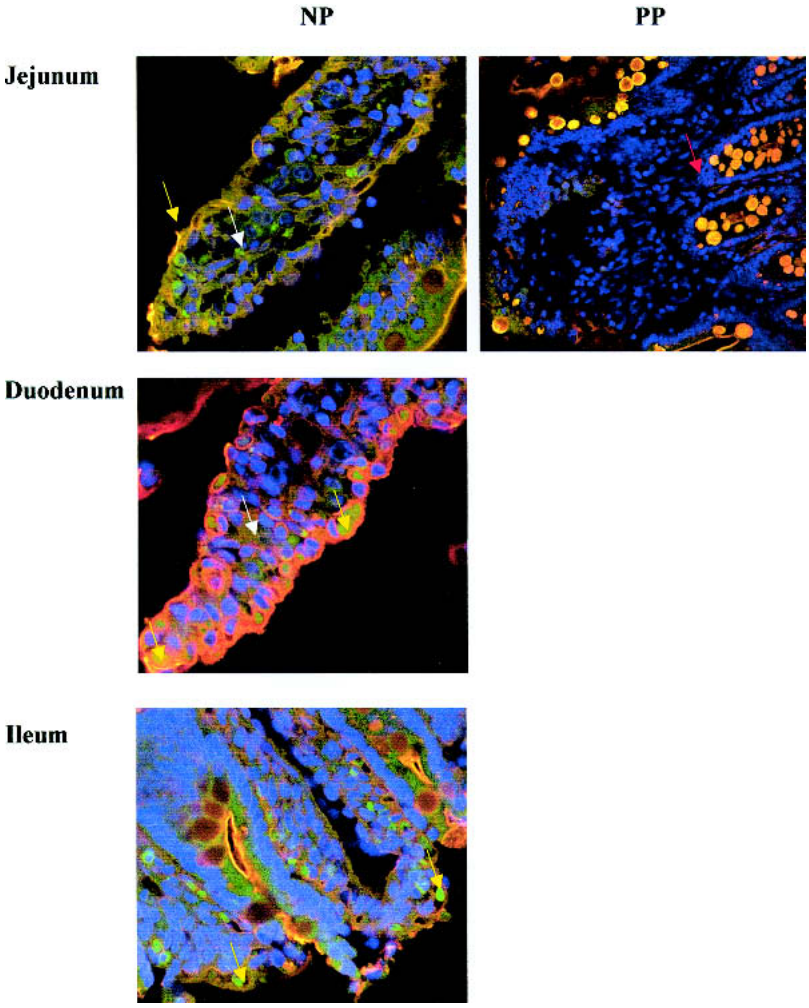

Fig. 4. Histologic analysis of Peyer's patches (PP) and non-patch tissue (NP) in different parts of the small intestine: jejunum, duodenum and ileum exposed to chitosan NP in rats after 60 min. Tissue sections were double-stained with DAPI (nuclei, blue) and TRITC-WGA (membrane, red); NP were green. Yellow arrows indicate the internalization of NP in epithelial cells, red arrows in lymphocytes in the Peyer's patches and white arrows in the lamina propria of the villi.

ues (Fig. 2a). A removal of mucus after the experiment decreased cellular association by 23% indicating that the internalized amount of the initial concentration was only 0.8% of the initial concentration (fluorescence of control—fluorescence after formalin treatment).

CLSM micrographs confirmed these data from the cellular association assay. After 120 min internalized green/yellowish fluorescence (NP uptake) was highest for polystyrene NP in Caco-2 cells (Fig. 3a) in comparison to chitosan and PLA-PEG NP. Moreover, hardly any fluorescence was detectable in MTX-E12 cells (Fig. 3e).

Polycationic, mucoadhesive chitosan NP also revealed a strong association with Caco-2 cell monolayers (0.056 ± 0.004 $\mu g/mm^2$, 7.8%). Again, a large number of green fluorescent vesicles could be seen in the CLSM micrographs (Fig. 3b). However, incubation with mucus-secreting MTX-E12 cells led to a higher association of chitosan NP (+58%) (Fig. 2b). Moreover, the steady-state level of association was already reached after 15 min, in contrast to 30 min in Caco-2 cells (Fig. 1). This points to a very strong interaction with mucus, probably due to an intense electrostatic interaction between positively charged chitosan NP and negatively charged mucin, containing sulfate and sialic acid-groups (24). A 14% decrease of association following the removal of the mucus layer prior to incubation (Fig. 2b) confirmed these findings. However, CLSM and the cellular association of NP after a post-experiment removal of the mucus layer revealed that the largest fraction of NP was bound to mucus (67%). The internalized fraction (4.6%) of chitosan NP was still larger compared to polystyrene NP, demonstrating that chitosan NP can penetrate the mucus layer (Fig. 2b).

Hydrophilic PLA-PEG NP showed almost no association with Caco-2 or MTX-E12 cells. After 120 min only 0.018 ± 0.004 $\mu g/mm^2$ (2.6%) were taken up into Caco-2 cells, no significant decrease was found in MTX-E12 cells (Fig. 1) and no fluorescence was detected in CLSM micrographs (Fig. 3c). PEG-chains on the NP surface probably inhibit interaction with cell surfaces (25).

Furthermore two additional control experiments were carried out, using FITC-BSA, in an equivalent concentration

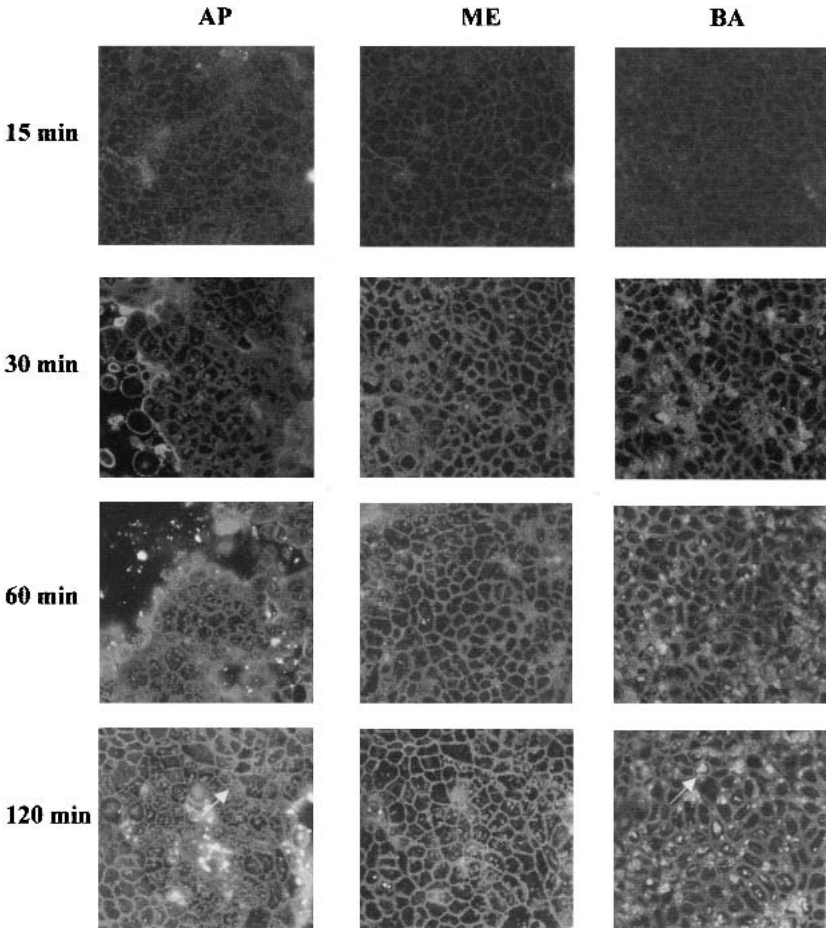

Fig. 5. CLSM images: Exemplary for all investigated NP, uptake of polystyrene NP in Caco-2 cells is depicted after 15, 30, 60 and 120 min. Optical sections were taken from the apical (AP), medium (ME) and basolateral (BA) part of the cell layers. White arrows indicate the granular sequestration of NP in endocytotic vesicles.

as in chitosan NP (0.011 ± 0.001 $\mu g/mm^2$) confirming that the fluorescence determined in cell monolayers was due to the uptake of NP and not due to released fluorescent dye. Moreover, uptake of chitosan NP due to interaction of chitosan with tight junctions was ruled out. Fluorescence after incubation of Caco-2 cells with a solution of FITC-BSA mixed with chitosan was significantly lower (0.018 ± 0.002 $\mu g/mm^2$) than that of chitosan NP (0.055 ± 0.004 $\mu g/mm^2$).

Uptake of Chitosan NP into Rat Mucosa after Intra-Duodenal Administration

To validate our *in vitro* results, we determined the interaction of chitosan NP in different parts of the small intestine after intra-duodenal injection in rats, because chitosan NP are unstable at acidic pH in the stomach. Cross-sections of the jejunum, the duodenum and the ileum were prepared and stained with TRITC-WGA and DAPI. In Fig. 4, green chitosan NP can be easily distinguished from the red membrane and the blue nuclei. As found by others (5,6), a significant amount of NP was taken up by epithelial cells (yellow arrow) and Peyer's patches (red arrow). Green fluorescence in the lamina propria suggests that some nanoparticles were shuttled through the epithelial cells (white arrow). The yellow fluorescence results from the red membrane- and green NP-label. It seems that a large number of NP were attached to the apical surface of the GIT, especially in the jejunum. Fluorescence was most prominent in lymphocytes of the Peyer's patches (red arrow). Similar to Desai *et al.* (7), chitosan NP internalization was found to be higher in the jejunum and ileum than in the duodenum. These findings confirm our results from *in vitro* cell culture models and are indicative of a comparable uptake of NP in rat enterocytes and goblet cells.

Characterization of the Transport Mechanism for Chitosan and Polystyrene NP in Caco-2 Cells

After having shown that NP of different polymers interacted with Caco-2 and MTX-E12 cells, the mechanism of polystyrene and chitosan NP uptake was now examined in more detail on a function and morphologic level in Caco-2 cells. NP can be transported by different routes across the intestinal epithelium, namely by the paracellular pathway, by endo- or transcytosis or by phagocytosis via M-cells (2). Exemplary for all investigated NP, Fig. 5 shows the time-dependent internalization of polystyrene NP-bound in three different compartments of Caco-2 cell monolayers: apical (AP), medium (ME) and basolateral (BA). Fluorescence appeared in the cytoplasm just below the apical plasma membrane 15 min after the application, seemed to move to the

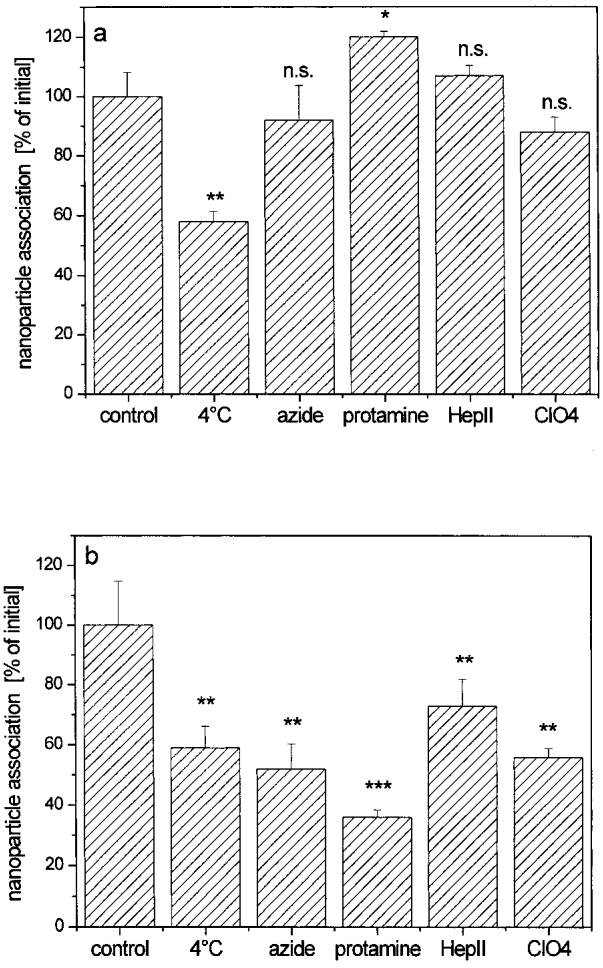

Fig. 6. Cellular association of polystyrene (a) and chitosan (b) NP with enterocyte-like Caco-2 cells after 60 min. Untreated monolayers (control) were compared to the incubation at 4°C, co-incubation with 100 mM sodium azide or 1 mM protamine and a desulfurization of the membrane prior to incubation using heparinase II (Hep II) or sodium chlorate (ClO4) (n = 3).

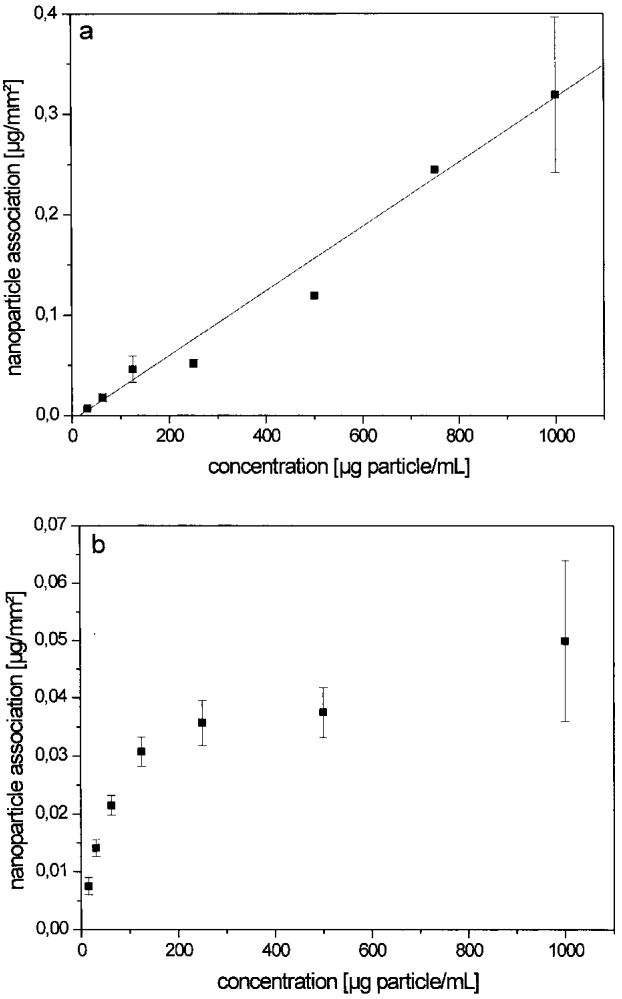

Fig. 7. Cellular association of polystyrene (a) and chitosan (b) NP with enterocyte-like Caco-2 cells after 60 min in a concentration range of 31.25–1000 µg/mL (n = 3).

basolateral section, where it became visible after 30 min and increased up to 120 min. The granular pattern of the staining (white arrow) and its absence in of NP in endo- or transcytotic vesicles. nuclei (Fig. 3) are compatible with sequestration.

To further elucidate the transcytotic mechanism for chitosan and polystyrene NP, their uptake was investigated under different conditions using the cellular association assay (Fig. 6 and Fig. 7). Results are given as % of control (incubation under standard conditions).

Chitosan NP were transported through Caco-2 cells most likely by adsorptive transcytosis for several reasons. First, the cationic charge of chitosan is thought to be a major determinant for adsorptive transcytosis (26). Uptake was significantly reduced (–41 ± 7.2%) under conditions that blocked active transport processes, in our case a temperature decrease to 4°C and the addition of sodium azide (Fig. 6b). Protamine, which has been previously found to undergo adsorptive endocytosis (26), led to the strongest decrease of uptake (–64%) by competition. Moreover, the protamine effect was concentration dependent. It was lowest at 0.25 mM (78% if the initial amount) and highest at 2.5 mM (24%) protamine (data not shown). Electrostatic interactions between positively charged chitosan and the negatively charged glycocalyx were involved, because NP uptake decreased following the removal of anionic sites on the apical membrane. Such groups were removed by pre-digestion with heparinase II or by incubation with sodium chlorate, an inhibitor of glycosaminoglycan sulfatation prior to the experiments. Incubation of Caco-2 monolayers with NP suspensions in a concentration range of 31.25 to 1000 µg/mL resulted in a typical saturation curve (Fig. 7b).

A different mechanism, possibly nonadsorptive endocytosis, is likely for polystyrene NP (Figs. 6 and 7a). In agreement with the literature (9), NP uptake was significantly decreased upon incubating at 4°C, but no effects were observed with protamine or removal of anionic binding sites. Moreover, NP uptake was independent from the concentration and showed non-saturable linear profile (r = 0.993) (Fig. 7a).

In summary, using two different cell culture models, we found that for effective endocytotic uptake in nonmucus covered intestinal cells, e.g., M-cells, apart from size hydrophobicity seemed to be the most critical surface property. This is in agreement with Jani *et al.* (27), who also observed that most hydrophobic polystyrene NP showed the strongest affinity to M-cells. We also noted that a lack of hydrophobicity can be

compensated by electrostatic interactions with the negatively charged glycocalyx on the apical membrane of GIT cells as shown for chitosan NP strongly interacting with Caco-2 cells. The mucus layer covering enterocytes and goblet cells, was found to be a major barrier for NP absorption. In this case, chitosan NP showed a higher association and internalization with GIT cells due to electrostatic interactions. In contrast, hydrophobic polystyrene NP exhibited the lowest degree of mucus penetration. Tissue distribution of chitosan NP after intra-duodenal injection revealed an extensive absorption in Peyer's patches and non-patch tissue corroborating the relevance of our results from *in vitro* cell culture models. Finally, the endo- and transcytosis of chitosan and polystyrene NP through Caco-2 cell monolayers was confirmed by CLSM. Chitosan NP were taken up by adsorptive endocytosis, while the exact mechanism of polystyrene NP uptake requires further investigations.

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