In Vitro Evaluation of Microparticles and Polymer Gels for Use as Nasal Platforms for Protein Delivery

Claudia Witschi[1,2] and Randall J. Mrsny[1,3]

Received September 11, 1998; accepted December 7, 1998

Purpose. Nasal delivery of protein therapeutics can be compromised by the brief residence time at this mucosal surface. Some bioadhesive polymers have been suggested to extend residence time and improve protein uptake across the nasal mucosa. We examined several potential polymer platforms for their *in vitro* protein release, relative bioadhesive properties and induction of cytokine release from respiratory epithelium.

Methods. Starch, alginate, chitosan or Carbopol® microparticles, containing the test protein bovine serum albumin (BSA), were prepared by spray-drying and characterized by laser diffraction and scanning electron microscopy. An open-membrane system was used to determine protein release profiles and confluent, polarized Calu-3 cell sheets were used to evaluate relative bioadhesion, enhancement of protein transport and induction of cytokine release *in vitro*.

Results. All spray-dried microparticles averaged 2–4 μm in diameter. Loaded BSA was not covalently aggregated or degraded. Starch and alginate microparticles released protein more rapidly but were less adhesive to polarized Calu-3 cells than chitosan and Carbopol® microparticles. Protein transport across polarized Calu-3 cells was enhanced from Carbopol® gels and chitosan microparticles. A mixture of chitosan microparticles with lysophosphatidylcholine increased protein transport further. Microparticles prepared from either chitosan or starch microparticles, applied apically, induced the basolateral release of IL-6 and IL-8 from polarized Calu-3 cells. Release of other cytokines, such as IL-1β, TNF-α, GM-CSF and TGF-β, were not affected by an apical exposure to polymer formulations.

Conclusions. We have described two systems for the *in vitro* assessment of potential nasal platforms for protein delivery. Based upon these assessments, Carbopol® gels and chitosan microparticles provided the most desirable characteristics for protein therapeutic and protein antigen delivery, respectively, of the formulations examined.

KEY WORDS: microparticles; *in vitro* release; nasal delivery; bioadhesive polymers; Calu-3 cells.

INTRODUCTION

The nasal mucosa is an attractive site for the non-parenteral delivery for protein therapeutics (1) and for the uptake of antigens for the induction of mucosal immunization (2). While nasal delivery has a number of clear advantages including ease of administration and patient acceptability, there are a few obvious drawbacks and obstacles to this approach. First, similar to other mucosa, the nasal mucosa provides a substantial barrier to the free diffusion of macromolecules (3). Second, enzymatic activities present in nasal secretions can limit protein delivery (4). Third, surface area of the human adult nasal mucosa is only ~ 150 cm^2 (1), limiting the amount of protein which can be absorbed from any one application of a dry-powder or liquid formulation. Finally, the typical residence time of a protein delivered to the nasal mucosa is only 15–30 minutes due to rapid ciliary clearance (5).

A number of approaches have been described to overcome the low inherent bioavailability of macromolecules across nasal mucosa which either increase the residence time of the administered protein at the mucosal surface and/or increase epithelial permeability and/or decrease degradation rates of the applied protein. Some bioadhesive platforms not only increase residence time at the nasal mucosa (6) but may also increase protein solutions stability (7) and/or enhance protein uptake (8). These bioadhesives are typically high molecular weight polymers with flexible chains which can interact with mucin through hydrogen bonding, electrostatic, hydrophobic or van der Waals interactions (9). The potential of various bioadhesive polymers as nasal delivery platform can be assessed by determining their ability to retain and release a specific protein, capacity to interact with mucosal surfaces following the incorporation of protein and biocompatibility with tissue at the site of administration.

One aspect of polymer biocompatibility is the potential effect for it to induce a cytokine response. Although it is well established that implanted polymers can induce the release of inflammatory cytokines from adhering cells, such as monocytes and macrophages (10), information is lacking on whether a similar modification of the biological properties of epithelial cells can occur following exposure to polymer particles. Since epithelial cells have the ability to secrete a number of cytokines (11) and polymers such as chitin/chitosan can induce the release of cytokines from spleen and fibroblast cells (12,13) the induction of cytokine responses in epithelial cells may represent a previously unappreciated issue of biocompatibility of polymer delivery platforms applied to a mucosal surface.

Presently, we have performed a systematic *in vitro* comparison of the bioadhesive polymers alginate, starch, chitosan and Carbopol® as potential platforms for nasal protein delivery. A number of parameters associated with protein release, relative particle adherence and biocompatibility were investigated using spray-dried polymer microparticles loaded with BSA as a test protein. Protein release was examined using an open membrane system and parameters of particle adherence and biocompatibility were addressed using a human airway epithelial cell line, Calu-3. Our results using these *in vitro* systems demonstrate distinct properties of relative bioadhesiveness as well as differences in protein transport and biocompatibility, which may provide distinct application advantages for future *in vivo* studies requiring a platform for nasal delivery of therapeutic proteins or nasal immunization.

MATERIALS AND METHODS

Materials

Fraction V bovine serum albumin (BSA), Tween® 20 and low viscosity alginic acid sodium salt from *Macrocystis pyrifera* were purchased from Sigma Chemical Co. (St. Louis, MO,

[1] Drug Delivery Biology Group, Department of Pharmaceutical Research and Development, Genentech Inc., South San Francisco, California.

[2] Reprint requests: FibroGen, 225 Gateway Blvd., South San Francisco, California.

[3] To whom correspondence should be addressed. (e-mail: mrsny@gene.com)

0724-8741/99/0300-0382$16.00/0

USA). Texas Red®-conjugated BSA (TR-BSA) was from Molecular Probes (Eugene, Oregon, USA). Soluble starch powder (amylodextrin prepared by dilute HCl treatment of potato starch) was obtained from Mallinckrodt, Inc. (Paris, KY, USA). Carbopol® 971P-NF was a gift of BFGoodrich Co. (Cleveland, OH, USA). High molecular weight chitosan [poly(D-glucosamine)] coarse ground (300,000 Da average molecular weight; 83–86% deacetylated) from crab shells was purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI, USA). Trappsol® methyl(-di)(random) β-cyclodextrin (RM-β-CD) was a gift of CTD, Inc. (Gainesville, FL,USA). L-α-lysophosphatidylcholine (LPC) prepared from egg lecithin was purchased from US Biochemicals (Cleveland, OH, USA).

Preparation of Spray-Dried Microparticles

BSA or a 9:1 (w/w) mixture of TR-BSA was dissolved in water purified through a MilliQ apparatus (Millipore, Bedford, MA, USA) and mixed with aqueous solutions of starch, alginic acid sodium salt, chitosan or Carbopol® 971P-NF. Chitosan was first dissolved in 1% HPLC-grade acetic acid while the others were dissolved directly in purified water. Polymer solution concentrations were kept constant at 2% (w/v) for starch and alginate and, due to polymer viscosity, at 0.5% (w/v) for chitosan and Carbopol®. Solutions, formulated to yield protein-loaded microparticles (mi.) of either 9, 20 or 33% (w/w), were spray-dried through a 0.5 mm nozzle using a modified laboratory Büchi® 190 spray dryer (Büchi, Flawil, Switzerland) (14) with an inlet temperature of 80°C, a pump flow of 5 mL/min, an air flow of 912 L/h and an outlet temperature of 57°C. Following spray-drying, one formulation of chitosan microparticles was prepared by dry-powder mixing with 8% (w/w) LPC.

Characterization of Spray-Dried Microparticles

The protein content of BSA-loaded alginate and starch microparticles was determined by BCA (Pierce, Rockford. IL, USA) assay following dissolution in water for 24 h. In the case of chitosan microparticles, samples were dissolved in 0.1% acetic acid, precipitated with 0.4 M NaOH and then centrifuged to remove chitosan prior to BCA analysis. Aqueous solutions prepared from Carbopol® microparticles were dissolved as described for starch and alginate, but the BSA released was determined by measurement optical absorbance at 280 nm. Determinations were performed in triplicate.

Microparticles were sized by a Malvern laser diffraction analyzer (Mastersizer®-X, Malvern, England) following suspension in HPLC-grade isopropanol containing 1:10,000 (v/v) Tween® 20 and bath sonication for one min. Morphological assessment of microparticles was performed using a Philips scanning electron microscope (Model 525 M) after adhering preparations to mounting studs and sputter-coating with 10 nm platinum using a Hummer XP (Anatech, Ltd.; Alexandria, VA, USA).

In Vitro Protein Release from Microparticles

An open membrane system, similar to one previously described (15), consisting of a release chamber covered horizontally with a 1 cm^2 (available surface area) Durapore® filter (Millipore Co.; Bedford, MA, USA) with a 0.45 μm pore diameter was used to monitor *in vitro* protein release. Microparticles (5 mg) were deposited on the membrane at the air-liquid interface established by the filter. At selected times 90 μL of the 1.8 mL of phosphate buffered saline (pH 6.5) was sampled and the volume replaced. Protein in the collected release media was quantitated from triplicate samples by BCA assay. Protein released from microparticles during assessment of loading percentages was analyzed by SDS-PAGE after mixing with a dissociation buffer and electrophoresis through a 8–16% polyacrylamide gradient gel using non-reducing conditions (16). Gels were stained with Coomassie Brilliant Blue-R.

Polymer Gel Formulations

BSA:TR-BSA (9:1 w/w) was dissolved in water and added to solutions of either Carbopol® or chitosan to produce a final polymer concentration of 0.5% w/v and a protein concentration of 0.25%. Carbopol® was dissolved in water while chitosan was solubilized in 1% acetic acid. Other formulations were prepared similarly to obtain gels with a final polymer and protein concentration of 0.5 and 0.25% (w/v), respectively. The pH of all Carbopol® gels was adjusted to 6.0 and the pH of chitosan gels was adjusted to 5.0 with NaOH.

Adhesion of Microparticles to Calu-3 Cells

Calu-3 cells, obtained from ATCC (Rockville, MD, USA), were grown as confluent sheets at an air-liquid interface on Transwell® filters (Costar, Cambridge, MA, USA) as described previously (16,17). On day 12 after filter seeding cultures demonstrated fluid absorption from the apical surface which was covered with a uniform layer of mucus. Transepithelial electrical resistance (TEER) values measured with a Millicell®-ERS (Millipore, Bedford, MA, USA) were between 300–400 $\Omega \cdot cm^2$. These confluent, polarized Calu-3 cell sheets were used to assess microparticle adhesion, protein transport and cytokine release at that time (n = 3–6). For bioadhesion studies, dry-powder microparticles (200 μg) or liquid aqueous or gel formulations (20 μL) were applied to the apical surface of cell sheets. PBS containing 0.25% BSA:TR-BSA in a 9:1 (w/w) ratio was applied as a control. In the case of the 0.5% BSA-loaded Carbopol® gel, 10 μL volumes were applied to maintain comparable quantities of total added protein. At 30 min, 2 h or 6 h time points, 250 μL PBS was added to the apical surface of the cell sheets. The filter was swirled gently for 2 min and the PBS collected to remove poorly adhering microparticles and non-absorbed BSA. Next, 500 μL of cell culture media was added to the apical chamber, the TEER was measured and the media collected. Microparticles not removed from the cell surface by this protocol performed at each time point were considered to be adhering. At that time, the cell media (1 mL) in the basolateral compartment was collected and the cell sheets were lysed in 0.5 mL of water. Samples of each collected fraction were placed in opaque 96-well micro-titer plates and the TR-BSA content was determined using a Series 4000 CytoFluor® fluorescence reader from PerSeptive Biosystems (Framingham, MA, USA) using 590 nm excitation and 645 nm emission settings.

Cytokine Release from Calu-3 Cells

Basolateral media collected after 6 h of exposure to microparticle and aqueous formulations were analyzed in triplicate

for cytokine levels using Predicta® ELISA kits (Genzyme Diagnostics, Cambridge, MA, USA). The cytokines and their (assay detection limits) were as follows: IL (interleukin)-1β (3 pg/mL); IL-6 (18 pg/mL), IL-8 (1 pg/mL), TNF (tumor necrosis factor)-α (3 pg/mL); TGF (transforming growth factor)-β1 (50 pg/mL); and GM-CSF (granulocyte macrophage colony-stimulating factor) (2.5 pg/mL). Collected media from different studies (n = 2–3) was analyzed without further dilution, following protocols enclosed in the kits except in the case of IL-8 measurements where samples were diluted with the sample diluent provided in the kit.

RESULTS

Characterization of BSA-Loaded Microparticles

Some properties of the spray-dried microparticles, such as BSA loading, yield, and particle size are summarized in Table 1. The protein concentration of spray drying solutions containing starch, chitosan or Carbopol® did not influence the yield of microparticles. Microparticle size was similar although decreasing slightly depending on the polymer used in the order of starch > alginate > Carbopol® > chitosan. Microparticle size also tended to decrease slightly with decreasing BSA loading, with the exception of starch (Table 1). Consistent with particle sizing data, scanning electron micrographs demonstrated structures with a large size range (Fig. 1). While chitosan and alginate microparticles containing BSA were smooth and spherical, starch and Carbopol® microparticles were much more irregular in shape and surface characteristics. BSA loading did not affect microparticle morphology (data not shown).

Release of BSA from Microparticles

Cumulative BSA release curves of triplicate samplings obtained from microparticle batches with different protein loadings were obtained using an *in vitro* release system (Fig. 2). As a control for the rates of protein dissolution and membrane transit, the release rate obtained for powdered BSA is plotted for comparison (Fig. 2). BSA release from starch and alginate microparticles had no initial lag phase and was not affected appreciably by protein loading. The initial rate of protein release from starch was slightly faster than that observed for alginate, with a 50% release being achieved by approximately 50 and 80 min, respectively. BSA released from 33% nominal loaded chitosan and Carbopol® microparticles was slower (nearly 200 min for 50% release) than that observed for starch and alginate microparticles and the release rate decreased with decreasing protein loading. Although slight lags in the initial release of BSA from alginate and Carbopol® may have occurred, the only prominent lag phase of BSA release was observed from chitosan microparticles. This lag phase increased as protein loading decreased. Complete release of BSA within 4 h was only obtained for starch and alginate microparticles. At 33% nominal protein loading chitosan and Carbopol® microparticles released about 60% of their protein within this time.

BSA can form oligomers, particularly when spray-dried at slightly elevated temperatures (we used an outlet temperature of 57°C) and exposed to the increased surface area of this procedure. Since the release (and transport) properties of BSA are dependent upon its molecular size, we performed SDS-PAGE analysis of protein released from microparticles. We could not detect any appreciable covalent BSA aggregates or degraded protein fragments in any of the formulations used in these studies (Fig. 3).

Table 1. Physical Properties of Spray Dried Microparticles

Polymer	Nominal BSA loading (%)	Actual BSA loading (%)	Calculated yield (%)	Particle size d_{50} (μm)[a]
Starch	33	32.3 ± 3.3	48.7	3.27
	20	22.0 ± 1.2	42.0	3.25
	9	9.4 ± 0.4	45.5	3.38
	—	—	45.7	3.81
	33 with TR-BSA[b]	36.6 ± 0.7	25.2	3.24
Alginate	33	25.9 ± 1.9	23.6	2.47
	20	16.4 ± 0.8	37.6	2.33
	9	8.1 ± 0.3	52.3	2.36
	—	—	37.0	2.20
	33 with TR-BSA[b]	32.0 ± 3.3	16.0	2.63
Chitosan	33	28.2 ± 0.9	20.6	2.13
	20	15.9 ± 0.8	26.0	2.02
	9	7.3 ± 1.8	27.0	1.81
	—	—	28.2	1.95
	33 with TR-BSA[b]	23.6 ± 1.2	21.52	2.04
Carbopol®	33	29.4 ± 2.3	31.1	2.34
	20	20.9 ± 2.4	29.2	2.26
	9	7.5 ± 0.6	15.7	2.17
	—	—	28.0	2.02
	33 with TR-BSA[b]	28.7 ± 0.8	14.0	2.24

[a] Mean volume diameter.
[b] TR-BSA = (BSA:Texas-red® BSA) in a 9:1 (w/w) ratio.

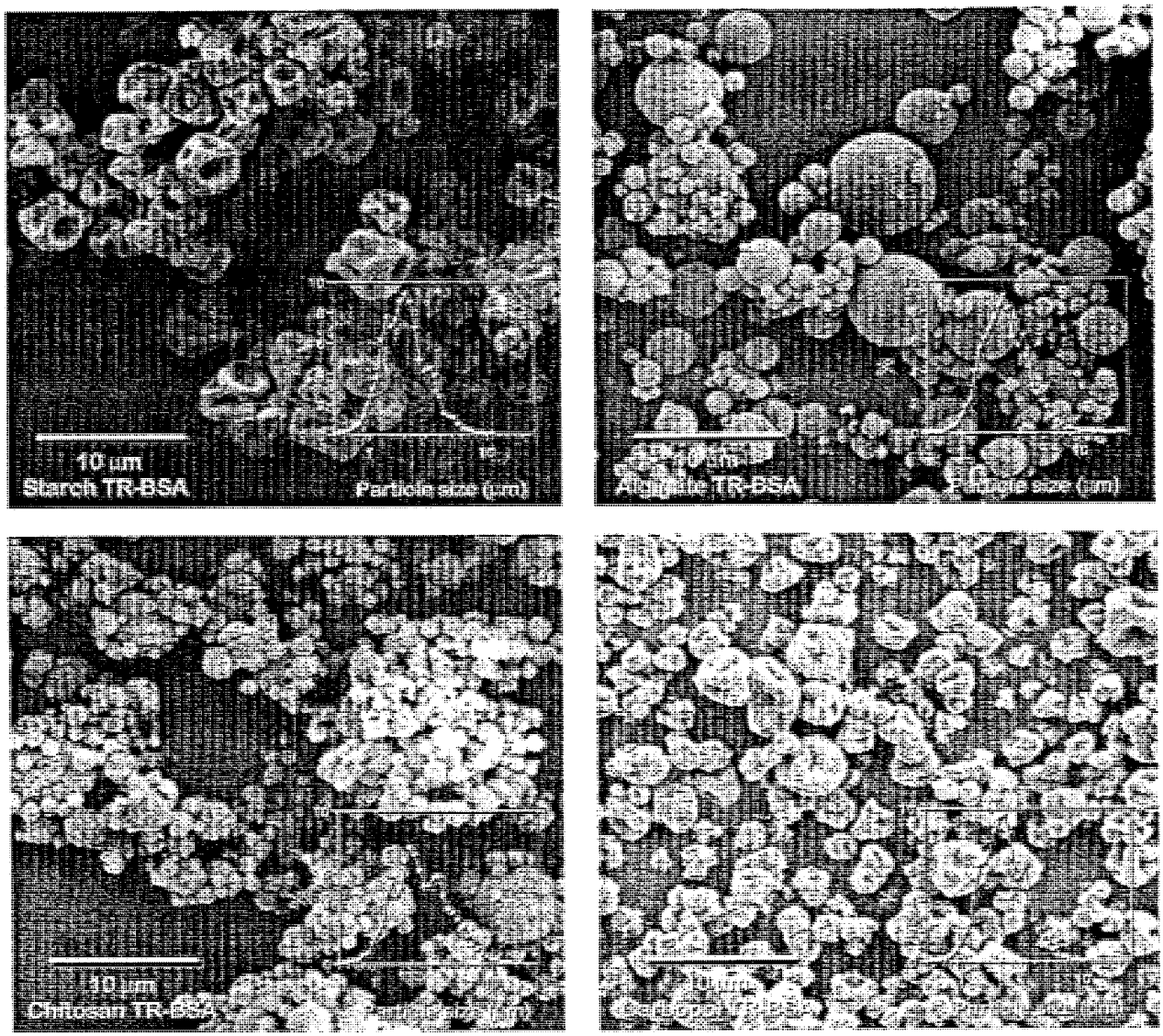

Fig. 1. Spray-dried microparticle morphology is polymer-dependent. Scanning electron micrographs of Texas-red® BSA/BSA (1:9) loaded microparticles of 33% (w/w) nominal loading are shown with measured particle size distributions.

Adhesive Properties of Microparticle Preparations

Based upon the release profiles of the microparticles loaded with different amounts of BSA, microparticles with 33% nominal protein loading were selected for further study. Since the efficacy of a nasal delivery platform results from a combination of factors, including residence time on the mucosal surface, we used an *in vitro* method to simultaneously assess several parameters including the relative bioadhesiveness of the various microparticle preparations containing fluorescent BSA (Fig. 4). As anticipated, BSA in PBS showed no adhesion when applied to the apical surface of Calu-3 cell sheets and examined after washing 30 min later (Table 2). Under the conditions used, alginate and starch microparticles lost their adhesive properties between the 30 min and 2 h time points. Chitosan microparticles were just starting to lose adhesion at 6 h while Carbopol® microparticles were still adhering at 6 h. Incorporation of 8% LPC with chitosan microparticles (w/w) by dry powder mixing slightly reduced the adhesive properties of this material to the surface of Calu-3 cells. Gels prepared from chitosan or Carbopol® were not as adhesive as microparticle counterparts. In our model system, BSA was not more bioadhesive when dissolved in 5% (w/v) methyl(-di)(random) β-cyclodextrin (RM-β-CD). Although these results suggest that some of the adhesive properties of the microparticles may have been due to hydration, bioadhesion appeared to be dictated primarily by the nature and properties of the polymer.

Transport of BSA Across Epithelia

Over the 6 h study a low level of Texas Red® fluorescent signal could be measured in the basolateral media of Calu-3 cells exposed to BSA in PBS (Fig. 5). Application of starch or alginate microparticles with 33% nominal BSA loading to the apical surface of Calu-3 cell sheets resulted in a slight enhancement in fluorescent signal transport. Chitosan microparticles with 33% nominal BSA loading provided a significant increase in the transport of signal. Incorporation of 8% LPC (w/w) in

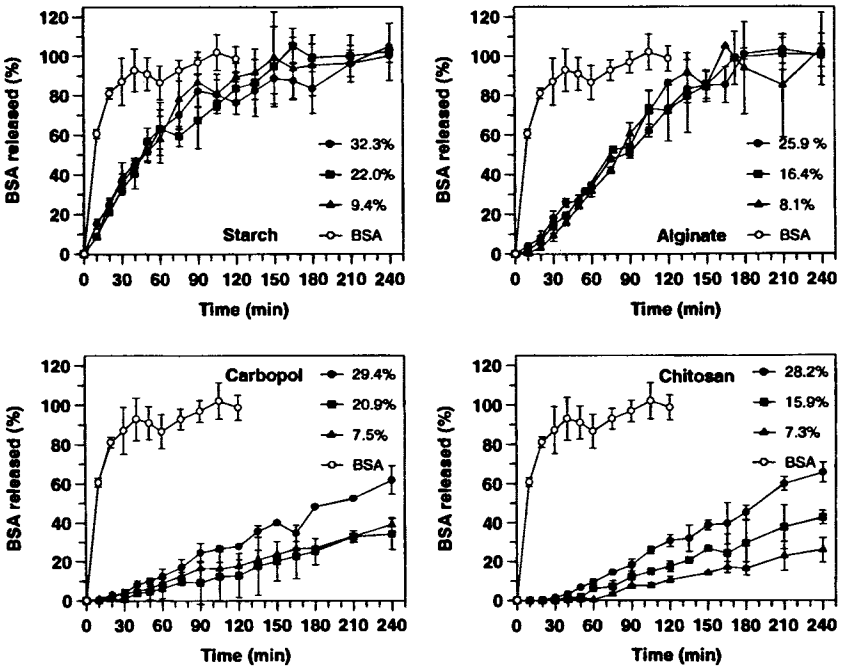

Fig. 2. Protein release profiles from spray-dried microparticles is polymer-dependent. Cumulative BSA release curves for microparticles with different protein loadings are shown. BSA powder is included as control for the rate of protein solubilization and transport across the filter to the sampling reservoir. Error bars represent standard deviations.

a dry powder mixture with these chitosan microparticles further enhanced this transport. Interestingly, Carbopol® microparticles, which were the most adhesive of the solid formulations tested, did not facilitate an increase in transport. Gel formulations composed of either chitosan or Carbopol®, which showed intermediate bioadhesive properties also significantly enhanced transport of fluorescent signal. Carbopol® gel formulations showed increased protein transport when the protein concentration was doubled from 0.25% to 0.5%. Formulations with a lower Carbopol® concentration (0.25% compared to 0.5%) also showed an increase in transport, suggesting a complex association between the polymer and transport of this protein across Calu-3 cells exists in this *in vitro* system. Presentation of BSA in a solution with 5% (w/v) RM-β-CD failed to enhance signal transport.

Cytokine Release by Calu-3 Cell Sheets

The basolateral media from Calu-3 cell sheets collected following 6 h of exposure to formulations being assessed for adhesion (Table 2) and facilitation of protein transport (Fig. 5), were assayed for cytokine levels. Epithelial cells can secrete a wide range of cytokines and we examined the release of IL-1β, IL-6, IL-8, TNF-α, TGF-β1 and GM-CSF. Polarized Calu-3 cell constitutively released high levels of IL-8 and TGF-β1 as well as low levels of IL-6. No release of either IL-1β, TNF-α or GM-CSF was detected by the assays employed. Following apical exposure to chitosan microparticles, increased levels of both IL-6 and IL-8 could be detected in the basolateral media bathing polarized Calu-3 cells (Fig. 6). Starch microparticles produced a similar result, but to a lesser degree than chitosan microparticles. Due to the low background levels of expressed IL-6, the induction of this cytokine is described in absolute values while IL-8 levels are described as a response relative to control values due to inter-experiment variability of constitutively released IL-8 (Fig. 5). There was no detectable induction of any of the other cytokines assayed by either chitosan or starch microparticles. Exposure of polarized Calu-3 cells to Carbopol®, alginate, RM β-CD or LPC failed to induce any striking cytokine releases.

We observed that the incorporation of BSA (in combination with TR-BSA) into starch and chitosan microparticles increased the release of IL-6 and IL-8 compared to protein-free formulations. To examine the possibility that this was due to endotoxin contamination in these albumins, we repeated these studies using formulations loaded with low endotoxin BSA (Fraction V, Sigma, St. Louis, MO). The lack of interleukin release with these low endotoxin formulations, however, could not be recovered to induce IL-6 and IL-8 release by the intentional re-addition of an endotoxin (*E. coli* lipopolysaccharide; Serotype 055:B5, Sigma, St. Louis, MO). Thus, if endotoxin is involved in the induction of IL-6 and IL-8 release from polarized Calu-3 cells, it appears that endotoxin derived from a bacterium commonly present in human flora does not stimulate this induction. Further, alginate and Carbopol® microparticles, which failed to induce IL-6 and IL-8 release, were loaded with the same BSA as the starch and chitosan microparticles which did enhance the release of these interleukins. Examination of endogenous endotoxin content of the polymers used in these studies demonstrated low amounts in the naturally-derived polymers alginate, starch and chitosan and less than detectable

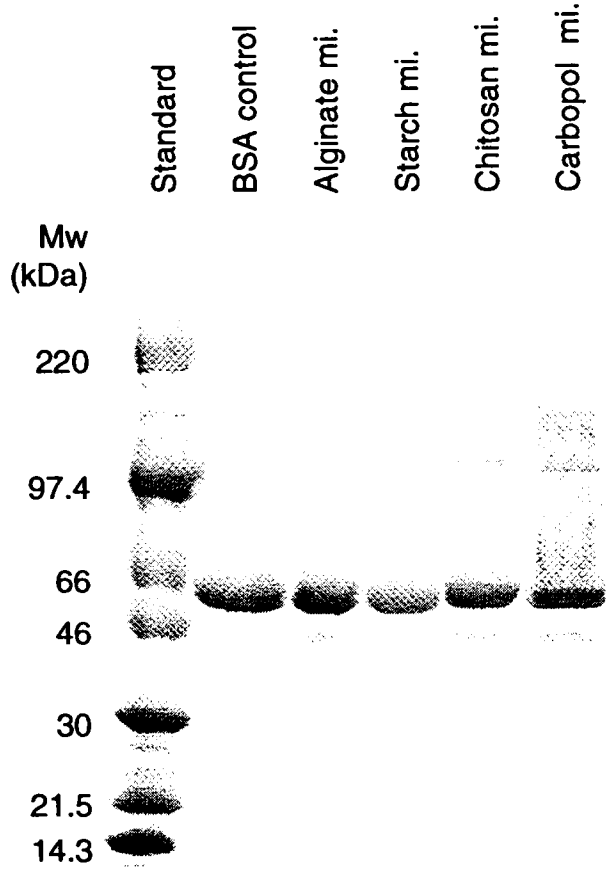

Fig. 3. Microencapsulation of BSA by spray-drying does not induce covalent protein aggregation or cleavage. SDS-PAGE analysis of BSA released by dissolution from starch, alginate, chitosan and Carbopol® microparticles (mi.) showed no detectable differences from the control sample prepared with BSA which was not spray-dried. Samples shown are from 33% (w/w) nominal loaded microparticles which were representative of other protein loadings.

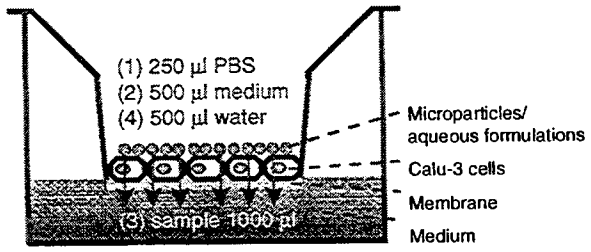

Fig. 4. Diagram of Calu-3 cell system used to assess bioadhesion, transepithelial protein transport and epithelial cell cytokine release. Microparticle and aqueous formulations containing 50 µg BSA were added to the apical surface of Calu-3 cells cultured at an air-liquid interface. After 30 min, 2 h or 6 h 250 µL PBS (1) was added and removed from the apical surface. 500 µL of cell culture media (2) was then added to the apical chamber and transepithelial resistance (TEER) was measured. After collection of this apically added media, the media (1 mL) in the basolateral compartment (3) was collected. Finally, Calu-3 cells were lysed in 500 µL of water (4).

Table 2. Relative Adhesiveness of Polymer Microparticle Formulations Applied to the Apical Surface of Polarized Calu-3 Cells *In Vitro*

Formulation	Relative adhesiveness[a]		
	30 minutes	2 hours	6 hours
BSA in PBS	−	−	−
Starch microparticles[b]	+	−	−
Alginate microparticles	+	−	−
Chitosan microparticles	+	+	+/−[c]
Chitosan microparticles plus LPC[d]	+	+	−
Carbopol® microparticles	+	+	+
0.5% BSA / 0.25% Chitosan gel	+	+	−
0.5% BSA / 0.25% Carbopol® gel	+	+	−
0.5% BSA / 0.5% Carbopol® gel	+	−	−
0.25% BSA / 0.25% Carbopol® gel	+	−	−
RM-β-CD[e]	−	−	−

[a] Bioadhesion to Calu-3 cells was monitored (n = 3) by visually assessing the relative retention of formulations containing Texas-red® BSA to the surface of confluent, polarized Calu-3 cell cultures. (+) = adhesion; (−) = no adhesion; (+/−) = variable adhesion.
[b] Microparticle formulations contained 33% nominal BSA loading.
[c] N = 2.
[d] LPC is L-α-lysophosphatidylcholine.
[e] RM-β-CD is Trappsol® methyl(-di)(random) β-cyclodextrin.

amounts in the Carbopol®. These results do not support the induction of IL-6 and IL-8 release by starch and chitosan microparticles through an endotoxin-related process.

DISCUSSION

For a bioadhesive platform to be useful for nasal protein delivery it must have a desired release profile, have a sufficient residence time at the mucosal surface and be biocompatible. We have prepared and characterized spray-dried protein-microparticle preparations of starch, alginate, chitosan and Carbopol® and assessed them for these criteria using two *in vitro* systems. The test protein, BSA, incorporated into all four of these microparticle preparations did not undergo striking covalent aggregation or cleavage during spray-drying or release. Using an open-membrane system, we compared the protein release profiles from these microparticle preparations *in vitro* and noted slower release kinetics from chitosan and Carbopol® compared to those obtained with starch and alginate. Calu-3 cell cultures were then used simultaneously to not only assess relative bioadhesion but also protein transport and the induction of cytokine release (a measure of biocompatibility) following the application of protein-loaded microparticles.

Optimally, these *in vitro* studies would have used primary cultures of human nasal epithelia. Considerations of human tissue availability and a desire to utilize a human model system we selected Calu-3 cells for these studies as a surrogate for human nasal epithelia. Calu-3 cells are a well-characterized human respiratory tract epithelial cell line which can be grown as confluent sheets at an air-media interface (and in fact fail to differentiate when submersed in media). Once confluent, Calu-3 cultures polarize to establish a mixed phenotype including ciliated and secreting cells with physical and electrical properties comparable to nasal mucosa (17,18) and secrete mucins to produce a complete sheet of mucus at their apical

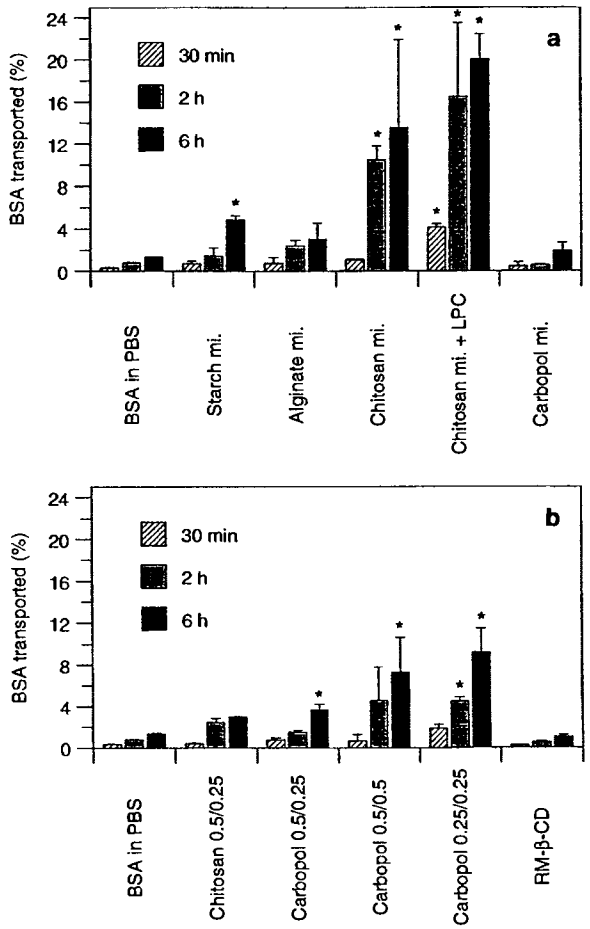

Fig. 5. Transepithelial protein transport *in vitro* can be augmented by polymer formulations. Apical to basolateral transport of Texas-red® BSA across Calu-3 cells was assessed by florescence. a) Microparticles (mi.) with 33% (w/w) nominal protein loading and b) aqueous formulations corresponding to 50 μg total BSA were added to the apical surface. Aqueous gel formulations of chitosan and Carbopol® are expressed as percentages (w/v) for polymer/protein ratios. RM-β-CD was 5% in PBS. Chitosan microparticles (mi.) plus LPC describes dry powder mixing of chitosan microparticles with 8% LPC (w/w). *Values were statistically different from control ($P < 0.05$) by Student's t test.

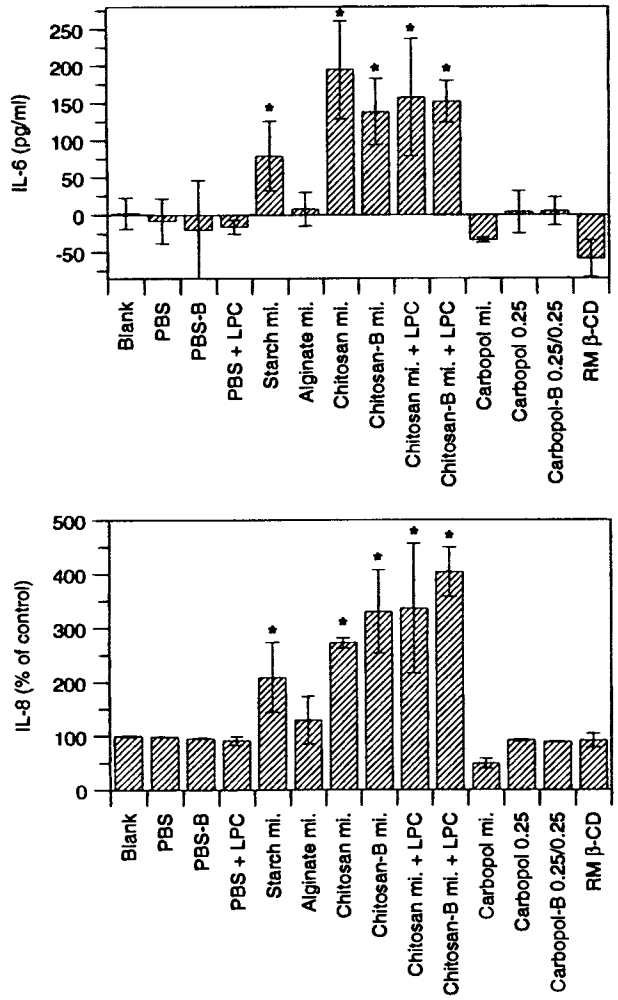

Fig. 6. Apical application of chitosan and starch microparticle (mi.) formulations induce the basolateral release of IL-6 and IL-8 from polarized Calu-3 cells. Polarized Calu-3 cells secreted IL-8 constitutively at levels from 1,000 to 10,000 pg/mL. Due to this interexperimental variation, values for IL-8 are expressed as % of the corresponding control value. IL-6 values correspond to basolateral concentrations directly detected by ELISA. Blank values were obtained from untreated Calu-3 cells. B: low endotoxin BSA; mi.: microparticles. LPC: lysophosphatidylcholine; 0.25: concentration (% m/m) of the polymer and protein in the Carbopol® gel. RM-β-CD: methyl(-di)(random) β-cyclodextrin. *Values were statistically different from control ($P < 0.05$) by Student's t test.

surface. Additionally, these cells are capable of cellular crosstalk with non-epithelial cells through paracrine cytokine signaling (B.-Q. Shen, A.L. Daugherty and R.J. Mrsny, unpublished results).

The differences observed in protein release *in vitro* from the various microparticle preparations may reflect the relative bioadhesive characteristics observed for the polymers examine in our studies using confluent cultures of polarized Calu-3 cells *in vitro*. In our studies, the assessment of relative bioadhesion to the apical surface of Calu-3 cell sheets probably represented the association with both a mucus sheet and the microvilli of the apical plasma membrane of these cells. It is unknown if the absolute microclimate of ions, pH, charge and visco-elasticity of these Calu-3 cell cultures were identical to the human nasal mucosa *in vivo*, but we assume substantial similarities based upon previous characterizations (17,19–21). Unlike the apparent correlation between release rates and relative bioadhesion, differences in the enhancement of protein uptake from the various microparticle preparations did not appear to be correlated with relative bioadhesiveness. Of the two strongly adhering polymers, chitosan microparticles but not Carbopol® microparticles significantly enhanced the transport of BSA. Both of the two less adhesive polymers, starch and alginate microparticles, produced a slight increase in protein transport.

Our observation that chitosan microparticles enhanced the uptake of BSA is consistent with previous studies showing chitosan can increase epithelial permeability (22). We also observed that LPC, although slightly decreasing its relative bioadhesion, further augmented the protein uptake by chitosan microparticles, similar to previous studies where the addition of LPC facilitated nasal insulin absorption from a microparticle

platform (23). Since LPC has been suggested to enhance protein transport by increasing cell membrane permeability (23), we carefully examined the effect of LPC on TEER values of polarized Calu-3 cells. In an average experiment involving LPC the TEER values of polarized Calu-3 cells, which started at 300–400 $\Omega \cdot cm^2$, dropped by approximately 100 $\Omega \cdot cm^2$ by the end of the 6 h experiment whereas control cells dropped by approximately 50 $\Omega \cdot cm^2$. Thus, although there was no dramatic drop in epithelial resistance, the additional decrease induced by LPC could have helped mediate the increased permeability to BSA. We also examined the effects of RM-β-CD in our systems since this compound has been identified as a promising absorption enhancer in the nasal delivery of peptide and protein drugs (24). RM-β-CD, which forms guest-host associations with molecules, failed to affect the adherence or transport of BSA in our systems. Further, it did not affect any measurable cytokine response, suggesting that the mechanism(s) of protein transport enhancement by the RM-β-CD are unrelated to the parameters evaluated in these two *in vitro* systems.

To our knowledge, the potential for a microparticle preparation used for protein delivery to induce the release of cytokines from an epithelium as an index of biocompatibility has not been described. The rationale to assess induction of cytokine release by polymer microparticles is based upon several previous observations: 1) The respiratory mucosa can release cytokines (11,2) Polymers have been shown to induce cytokine release (23) exposure of airway cells to airborne particulates can stimulate the release of cytokines (26). We focused our examination of cytokine release on a series of paracrine signaling proteins which play important roles in inflammation, growth/repair and the immune response at mucosal surfaces. In our studies, the only formulations which induce a significant cytokine release were that of chitosan and starch microparticles. The induction of IL-6 is a particularly promising finding which may be useful for mucosal vaccination since this cytokine has the ability to stimulate both the humoral immune system (production of antibodies) and the cellular immune system (production of cytotoxic T cell populations) through activation of B cells (27) and CD4 expressing T cells (28), respectively. Further, IL-6 is expressed locally in mucosal immune tissue (29), making it a central player in the paracrine signaling events at the site of antigen presentation.

In contrast, IL-8 is a chemotactic agent for neutrophils, eosinophils, basophils and T-lymphocytes which can result in the stimulation of undesired inflammatory reactions. Inflammatory cytokines also increase the permeability of epithelial cell barriers (30). It is therefore possible that the enhanced protein absorption observed across polarized Calu-3 cells induced by chitosan was partially the result of increased inflammatory cytokine release although we did not observe significant decreases in TEER to correlate with this possibility. Although none of the polymers used in the present studies were of pharmaceutical grade, the results presented highlight the possibility that some polymers may have the potential to induce a cytokine release following application as a microparticle preparation to mucosal surfaces *in vivo*. Previous studies have suggested microparticle uptake to be involved in the cytokine response of cells such as macrophage. Since epithelial cells are not professional phagocytic cells, like macrophage, it is unclear how application of microparticles could stimulate cytokine release. It is also presently unclear what role potential endotoxin contamination of a polymer microparticle or its contents may play in the enhanced protein transport and cytokine induction. While chitosan, starch and alginate, all natural polymer products, had measurable levels of endotoxin, Carbopol®, a synthetic polymer, appeared to be free of endotoxin.

Based upon our results using the polymers described, chitosan microparticles and Carbopol® gels to be more promising bioadhesive platforms than starch or alginate microparticles for the delivery of proteins to the respiratory mucosa such as in the nose and upper airway. Additionally, chitosan (and to a lesser extent starch) induced the release of IL-6 and the inflammatory cytokine IL-8. Thus, chitosan microparticles may be particularly promising for the mucosal administration of antigens since this formulation facilitates protein uptake and may enhance a desired mucosal immune response through the local release of IL-6, although the influence of increased IL-8 has to be investigated further. Ultimately, studies using polymer preparations and incorporated proteins of pharmaceutical grade to advance the application of these generic observations toward a product application.

ACKNOWLEDGMENTS

The authors thank Yuh-Fun Maa and Phuong-Anh Nguyen for their assistance in spray-drying and particle size measurements. Linda Rengall is acknowledged for assistance in obtaining the scanning electron micrographs. We thank Ann Daugherty for the suggestion of using Calu-3 cells as an *in vitro* model and critical reading of the manuscript. This study was supported by a grant of the "Schweizerischer Nationalfonds zur Förderung der Forschung," Switzerland.

REFERENCES

1. L. Illum and A. N. Fisher. Intranasal delivery of peptides and proteins. *In*: A. L. Adjei and P. K. Gupta (eds.), Inhalation delivery of therapeutic peptides and proteins, pp. 135–184, New York: Marcel Dekker, 1997.
2. A. J. Almeida and H. O. Alpar. Nasal delivery of vaccines. *J. Drug Target.* **3**:455–467 (1996).
3. M. D. Donovan, G. L. Flynn, and G. L. Amidon. Absorption of polyethylene glycols 600 through 2000: The molecular weight dependence of gastrointestinal and nasal absorption. *Pharm. Res.* **7**:863–868 (1990).
4. M. A. Sarkar. Drug metabolism in the nasal mucosa. *Pharm. Res.* **9**:1–9 (1992).
5. N. G. M. Schipper, J. C. Verhoef, and F. W. H. M. Merkus. The nasal mucociliary clearance: Relevance to nasal drug delivery. *Pharm. Res.* **8**:807–814 (1991).
6. L. Illum, H. Jorgensen, H. Bisgaard, O. Krogsgaard, and N. Rossing. Bioadhesive microspheres as a potential nasal drug delivery system. *Int. J. Pharm.* **39**:189–199 (1987).
7. H. L. Lueben, J. C. Verhoef, G. Borchard, C.-M. Lehr, A. B. G. de Boer, and H. E. Junginger. Mucoadhesive polymers in peroral peptide drug delivery. II. Carbomer and polycarbophil are potent inhibitors of the intestinal proteolytic enzyme trypsin. *Pharm. Res.* **12**:1293–1298 (1995).
8. L. Ryden and P. Edman. Effect of polymers and microspheres on the nasal absorption of insulin in rats. *Int. J. Pharm.* **83**:1–10 (1992).
9. C.-M. Lehr. From sticky stuff to sweet receptors-achievements, limits and novel approaches to bioadhesion. *Eur. J. Drug Metabol. Pharmacokin.* **21**:139–148 (1996).
10. T. L. Bonfield, E. Colton, R. E. Marchant, and J. M. Anderson. Cytokine and growth factor production by monocytes/macro-

phages on protein preabsorbed polymers. *J. Biomed. Mater. Res.* **26**:837–850 (1992).
11. L. D. Martin, L. G. Rochelle, B. M. Fischer, T. M. Krunkosky, and K. B. Adler. Airway epithelium as an effector of inflammation: molecular regulation of secondary mediators. *Eur. Respir. J.* **10**:2139–2146 (1997).
12. Y. Shibata, L. A. Foster, W. J. Metzger, and Q. N. Myrvik. Alveolar macrophage priming by intravenous administration of chitin particles, polymers of *N*-acetyl-D-glucosamine, in mice. *Infect. Immun.* **65**:1734–1741 (1997).
13. T. Mori, M. Okumura, M. Matsuura, K. Ueno, S. Tokura, Y. Okamoto, S. Minami, and T. Fujinaga. Effects of chitin and its derivatives on the proliferation and cytokine production of fibroblasts *in vitro*. *Biomaterials* **18**:947–951 (1997).
14. Y.-F. Maa, H. R. Costantino, P.-A. Nguyen, and H. C. C. The effect of operating and formulation variables on the morphology of spray-dried protein particles. *Pharm. Develop. Technol.* **2**:213–223 (1997).
15. A.-L. Cornaz, A. De Ascentis, P. Colombo, and P. Buri. In vitro characteristics of nicotine microspheres for transnasal delivery. *Int. J. Pharm.* **129**:175–183 (1996).
16. B.-Q. Shen, J. H. Widdicombe, and R. J. Mrsny. Effects of lovastatin on trafficking of CFTR in human tracheal epithelium. *J. Biol. Chem.* **270**:25102–25106. (1995).
17. B.-Q. Shen, W. E. Finkbeiner, J. J. Wine, R. J. Mrsny, and J. H. Widdicombe. Calu-3: a human airway epithelium cell line that shows cAMP-dependent Cl^- secretion. *Am. J. Physiol.* **266**:L493–L501 (1994).
18. D. Cremaschi, C. Porta, and R. Ghiradelli. Endocytosis of polypeptides in rabbit nasal respiratory mucosa. *News Physiol. Sci.* **12**:219–225 (1997).
19. R. C. Boucher and E. H. C. Cheng. Human nasal epithelial cultures. *In*: G. Wilson, S. S. Davis, L. Illum, and A. Zweibaum (eds.), Pharmaceutical applications of cell and tissue culture to drug transport, Vol. 218, pp. 249–258. New York: Plenum Press, 1991.
20. A. R. Tweedie. The reaction of the nasal mucus. *Acta Oto-laryngologica* 151–153 (1936).
21. R. T. Jackson and J. S. Turner Jr. Some observations on nasal pH. *Arch. Otolaryng.* **84**:446–450 (1966).
22. P. Artursson, T. Lindmark, S. S. Davis, and L. Illum. Effect of chitosan on the permeability of monolayers of intestinal epithelial cells (Caco-2). *Pharm. Res.* **11**:1358–1361 (1994).
23. L. Illum, N. F. Farraj, H. Critchley, B. R. Johansen, and S. S. Davis. Enhanced nasal absorption of insulin in rats using lysophosphotidylcholine. *Int. J. Pharm.* **57**:49–54 (1989).
24. E. Marttin, J. C. Verhoef, and F. W. H. M. Merkus. Efficacy, safety and mechanism of cyclodextrins as absorption enhancers in nasal delivery of peptide and protein drugs. *J. Drug Target.* **6**:17–36 (1998).
25. J. A. Hunt, B. F. Flanagan, P. J. McLaughlin, I. Strickland, and D. F. Williams. Effect of biomaterial surface charge on the inflammatory response: evaluation of cellular infiltration and TNF alpha production. *J. Biomed. Mater. Res.* **31**:139–144 (1996).
26. K. E. Driscoll, J. M. Carter, D. G. Hassenbein, and B. Howard. Cytokines and particle-induced inflammatory cell recruitment. *Environ. Health Perspectives* **105**:1159–1164 (1997).
27. T. Hirano. Interleukin 6 and its receptor: ten years later. *Inter. Rev. Immunol.* **16**:249–284 (1998).
28. S. B. Joseph, K. T. Miner, and M. Croft. Augmentation of naive, Th1 and Th2 effector CD4 responses by IL-6, IL-1 and TNF. *Eur. J. Immunol.* **28**:277–289 (1998).
29. P. T. Odinot, J. H. A. J. Curfs, J. F. G. M. Meis, W. J. G. Melchers, and J. A. A. Hoogkamp-Korstanje. Local expression of cytokine mRNA in spleen and Peyer's patches of rats is involved in resistance against infection with *Yersinia enterocolitica*. *Cytokine* **10**:206–212 (1998).
30. S. E. Sanders, J. L. Madara, D. K. McGuirk, D. S. Gelman, and S. P. Colgan. Assessment of inflammatory events in epithelial permeability: a rapid screening method using fluorescein dextrans. *Epithelial Cell Biology* **4**:25–34 (1995).