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Research Paper

In Vitro and *ex Vivo* Intestinal Tissue Models to Measure Mucoadhesion of Poly (Methacrylate) and *N*-Trimethylated Chitosan Polymers

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Purpose. The adhesion of a range of polymers based on poly(2-(dimethylamino-ethyl) methacrylate (pDMAEMA) was assessed using human mucus-secreting and non mucus-secreting intestinal cell monolayers, HT29-MTX-E12 (E12) and HT29 monolayers, as well as excised non-everted intestinal sacs from rats. Differentiation of mucoadhesion from bioadhesion was achieved by pre-treatment with the mucolytic agent, *N*-acetyl cysteine (NAC). Adherence of pDMAEMA polymers was compared to that obtained with the mucoadhesive, *N*-trimethylated chitosan (TMC).

Methods. The quantity of adherent coumarin 343-conjugated polymers to HT29, E12, and intestinal sacs was measured by fluorescence. Confocal laser scanning microscopy (CLSM), light microscopy, and fluorescent microscopy were used to provide direct evidence. Measurements of transepithelial electrical resistance (TEER), permeability to FITC-dextran 4000 (FD-4), and the release of lactate dehydrogenase (LDH) were used to assess potential cytotoxicity of polymers.

Results. Adherence of unquaternized and of 10%, 24%, and 32% methyl iodide-quaternized pDMAEMA polymers was measured in E12, HT29, and sacs. All pDMAEMA polymers showed significantly higher levels of adhesion to mucus (mucoadhesion) than to epithelium (bioadhesion). Co-localization of pDMAEMA with mucus was confirmed in E12 by microscopy. TMC showed equally high levels of mucoadhesion as unquaternized and 24% quaternized pDMAEMA, but displayed higher levels of bioadhesion. pDMAEMA-based polymers demonstrated lower levels of adherence to E12 and rat sacs in the presence of NAC, whereas adherence of TMC was unchanged. pDMAEMA significantly decreased the permeability of FD-4 across E12 monolayers and sacs and was less cytotoxic in E12 than in HT29. In contrast, TMC increased the permeability of FD-4 across E12 and sacs and was less cytotoxic in E12 than in HT29.

Conclusions. Human mucus–producing E12 monolayers can be used to assess polymer mucoadhesion and give similar data to isolated rat intestinal sacs. pDMAEMA displayed similar levels of mucoadhesion and lower levels of bioadhesion than a chitosan derivative and it was not cytotoxic. pDMAEMA decreased FD-4 flux in the presence of mucus, whereas TMC increased it. The combination of mucus and methacrylate polymers appears to increase barrier function of the apical membrane.

KEY WORDS: chitosan; HT29 monolayers; living radical polymerization; methacrylate polymers; mucoadhesion.

INTRODUCTION

The use of polymers as bioadhesives (adhering to epithelium) and mucoadhesives (adhering to mucus) offer significant potential for oral drug delivery. Cargos formulated with mucoadhesive polymers may increase gastrointestinal tract residence time leading to improved oral drug bioavailability, as the formulation should allow more opportunity to contact the epithelium (1,2). Adhesive polymeric systems may also be useful for topically coating the damaged intestinal wall in inflammatory bowel disease (3), or for facilitating healing in the oral cavity (4). For these applications, polymers need to be inert, nonabsorbable, stable, and easy to process. A requirement for any adhesive polymer is quantifiable and reproducible adhesion to tissues and/or supramucosal gels. Changes in indices of intestinal barrier structure and function

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ABBREVIATIONS: CLSM, confocal laser scanning microscopy; FD-4, fluoresceinisothiocyanate-dextran (MW 4 kDa); Mn, average molecular weight number; Papp, apparent permeability coefficient; PDi, polydispersity index (molecular weight distribution); pDMAEMA, poly(2-(dimethylamino-ethyl) methacrylate; TEER, transepithelial electrical resistance; TMC, *N*-trimethylated chitosan.

may be caused by adhesion of some polymers. Examples include the opening of tight junctions by chitosan (5) and reduction of bacterial access to the epithelial surface by polyethylene glycol (6).

There are few reliable intestinal models that adequately provide a mucus gel lining in the gastrointestinal tract to which adherence can be measured. Most are based on flow-through systems using isolated mucus-covered mucosae and examples include rat stomach mucosa (7), porcine tissue mucosa (8) and perfused rat intestinal loops (9). Furthermore, attempts to rank polymer adhesion to these preparations maintained under sub-optimal physiologic conditions rely largely on qualitative tensiometer measurements of the force required to separate formulations coated onto solid matrices (10,11). Recently, there has been an attempt to measure particulate adhesion to isolated thawed pig oesophagi through use of a dynamic test system with image analysis (12).

Caco-2 and HT29 monolayers are used as *in vitro* cell culture models of the human intestinal epithelium. Caco-2 cells spontaneously differentiate into monolayers of polarized enterocytes, connected by tight junctions, but they lack mucus-secreting goblet cells (13). In contrast, HT29 monolayers expresses mucin-secreting mature goblet cells in the presence of methotrexate (MTX) selection pressure (14). There are issues for drug delivery studies arising from the variable levels of mucus production and inter-passage inconsistency in early sub-clones (15). Attempts have also been made to create mucus-producing monolayers by co-culturing Caco-2 in specific ratios with the HT29 sub-clones, HT29GlucH (16), and HT29-MTX (17). These mixed co-cultures are, however, difficult to maintain as growth rates of the mixed cell lines vary. This leads to the production of a mucus gel layer that may not be stable and reproducible through successive passages. Stable sub-clones of HT29-MTX have, however, recently been isolated and partially characterized (18). Monolayers of one such subclone, HT29-MTX-E12 (E12), elaborate a 150 μm mucous gel layer that corresponds to *in vivo* measurements of human small intestine. The overlying mucus acts as a significant barrier to the permeation of lipophilic drugs, and also to the uptake of hydrophobic nanoparticles (19). In addition, it expresses the mucins MUC1 and MUC2, which are found in the small intestine and are implicated in host-pathogen relationships (18). Taken together, E12 could be an appropriate *in vitro* model for assessing polymer mucoadhesion.

In studies of mucoadhesive polymers, traditional rat everted intestinal sacs are un-economic due to the large amount of polymer required in the bath. While morphologically intact immediately after inversion, the epithelial surface quickly degrades (20). One aim of the current study was to assess the use of *non-everted* sacs for measuring polymer adherence. We used a rapid dissection method followed by the maintenance of sac viability in a high-nutrient medium (21). In this study E12 and rat intestinal non-everted sacs were compared in the presence and absence of a mucolytic agent, *N*-acetyl cysteine (NAC) (16,22), as it allows differentiation between mucoadhesion and bioadhesion.

The hydrophilic cationic methacrylate-based polymer, poly(2-(dimethylamino-ethyl) methacrylate (pDMAEMA), appears to have potential as a delivery vehicle for gene delivery (23) and tumor targeting (24). The polymer is water soluble at neutral pH and is therefore suitable for adhesion to tissue in physiologic buffer. The surface charge on pDMAEMA can be readily altered by varying the quaternization of the ammonium groups, a process implicated in antimicrobial activity of the polymer *in vitro* (25). In addition, pDMAEMA polymers possess functional side groups in their dimethylamino ethyl moieties, and these may be suitable candidates for glycoprotein interaction with sialic acid residues on mucins (26). pDMAEMA can be synthesized in high yield and purity by the novel and simple process of living radical polymerization (27) and is therefore an appropriate polymer type to assess in adhesion studies. For comparative purposes, we used *N*-trimethylated chitosan (TMC) as a known positive control for adhesion as it is also soluble at pH 7.4 (11).

Our aims were to compare mucus-covered E12 monolayers and non-everted rat intestinal sacs as tissue models to quantitatively assess adhesion of fluorescently labeled pDMAEMA analogues. In addition, light, fluorescent, and confocal laser scanning microscopy were used to visualize polymer binding. Cytotoxic potential and effects on barrier function of pDMAEMA and TMC were also assessed.

MATERIALS AND METHODS

Materials

All tissue culture reagents were from Gibco (Biosciences, Dublin, Ireland). FITC-Dextran (MW 4400) was from Sigma (Sigma-Aldrich, Dublin, Ireland). Tissue culture materials and plates were from Corning Costar (Fannin Healthcare, Dublin, Ireland) and chitosan was obtained from Fluka (Fluka Chemicals, Dorset, UK). Ethidium homodimer-1 was obtained from Molecular Probes (Molecular Probes Europe BV, Leiden, The Netherlands). The Titramax 1000 shaking incubator was from Heidolph Instruments (Germany).

Cell Culture

E12 Cells (passage 50-57) were a generous gift from Professor Per Artursson, Uppsala University, Sweden. HT29 cells were obtained from ATCC (passage 121-128). Cells were grown and subcultured as previously described (18). Both lines were maintained with Dulbecco's Modified Eagles Medium (DMEM) with 10% fetal calf serum (FCS), 1% non-essential amino acids and 1% L-glutamine at 5% CO_2, 95% O_2 at 37°C.

For adhesion and transport studies, HT29 and E12 were subcultured at a density of 2×10^4 cells/filter on 12 mm Transwell polycarbonate membrane inserts (Corning Costar, cat. no. 3401). Monolayers were fed on both sides every two days and differentiated over 21 days. Barrier formation of monolayers was examined by measuring the transepithelial electrical resistance (TEER) of cell monolayers before and after adhesion experiments using an EndOhm electrode system with background correction made for unseeded filters. Monolayers with TEER values below 50 Ω cm^2 were excluded from experiments.

Lactate Dehydrogenase Cytotoxicity Assay

Lactate dehydrogenase (LDH) activity was measured in culture supernatants at 0, 30, and 60 min following polymer exposure to cells grown on Transwell membranes, using a cytotoxicity assay kit (Roche Diagnostics, East Sussex, UK).

LDH concentrations were expressed as percentage LDH release relative to treatment with the detergent Triton-X 100 and the percentage cytotoxicity calculated (28).

Polymer Synthesis

Preparation of Coumarin Initiator

Hydroquinone (110.11 g, 1.0 mol), tetrahydrofuran (800 ml), and triethylamine (15.3 ml, 0.11 M) were placed in a 1 L round-bottom flask equipped with a pressure equilibrating dropping funnel. 2-Bromoisobutyryl bromide (12.4 ml, 0.1 M) and tetrahydrofuran (87.6 ml) were then placed into the dropping funnel. The 2-bromoisobutyryl bromide solution was added dropwise over 2 h with stirring which formed triethylammonium bromide. Upon complete addition, the reaction was stirred for 2 h. The triethylammonium bromide was removed by filtration, and the solvent was removed under reduced pressure. This resulted in a brown white crystalline mixture that was placed into a round bottom flask with chloroform (600 ml) and stirred overnight to separate the unreacted excess hydroquinone. The unreacted hydroquinone was removed from the solution by filtration and the filtrate was concentrated under reduced pressure to give a brown crystalline compound. The resulting brown crystalline solid was purified by column chromatography eluting with 100% dichloromethane followed by 100% methanol to give a pale brown crystalline solid, hydroxyl initiator (Fig. 1A), yield = 18.5 g (71%).

Coumarin 343 (1 g, 3.5 mM, Exciton Inc., Ohio, USA), hydroxyl initiator (0.91g, 3.5 mM), *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (1.3g, 7.0 mM), and 4-(dimethylamino)pyridine (0.043 g, 0.35 mM) were then dissolved in anhydrous dichloromethane (25 ml) under nitrogen and stirred at room temperature for 72 h. The resultant solution was washed with distilled water (2 × 25 ml), 0.5 M HCl (4 × 25 ml), saturated $NaHCO_3$ (4 × 25 ml), distilled water (2 × 25 ml), and then dried over $MgSO_4$. Removal of the solvent *in vacuo* gave an orange product that was purified by column chromatography on silica gel using 9:1 dichloromethane/ethyl acetate, followed by 9:1 dichloromethane/diethyl ether to give the coumarin 343 initiator (Fig. 1B) in 80% yield. This method was adapted from Ref. 29.

Preparation of Coumarin 343-Initiated pDMAEMA Polymers

The methacrylate-based polymers were quaternized derivatives of 2-(dimethylamino- ethyl) methacrylate (DMAEMA) monomer, which were labeled with the fluorescent probe, coumarin 343. Coumarin fluoresces with an excitation wavelength of 400 nm and an emission wavelength of 490 nm. Coumarin 343 initiator 2 (0.50 g, 0.95 mM), Cu(I)Br (0.136 g, 0.95 mM, 1 eq), DMAEMA (14.2 g, 0.090 M), toluene (30 ml), and a magnetic follower were placed in an oven-dried Schlenk tube. The resulting solution was deoxygenated via three freeze-pump-thaw cycles and, following de-gassing, *N*-propyl-2-pyridylmethanimine (0.28 g, 1.9 mM) was added. The reaction was placed in a thermostatically controlled oil bath at 90°C for 4 h. The polymer solution was diluted with toluene (100 ml) and filtered through a column of basic alumina to remove the catalyst. The column was washed with toluene (2 × 100 ml) and the combined filtrate was subjected to rotary evaporation to remove the solvent. The polymer was purified twice by precipitation using dichoromethane/petroleum ether to give coumarin 343-initiated pDMAEMA (M_n 12,300, PDi of 1.08, Fig. 1C).

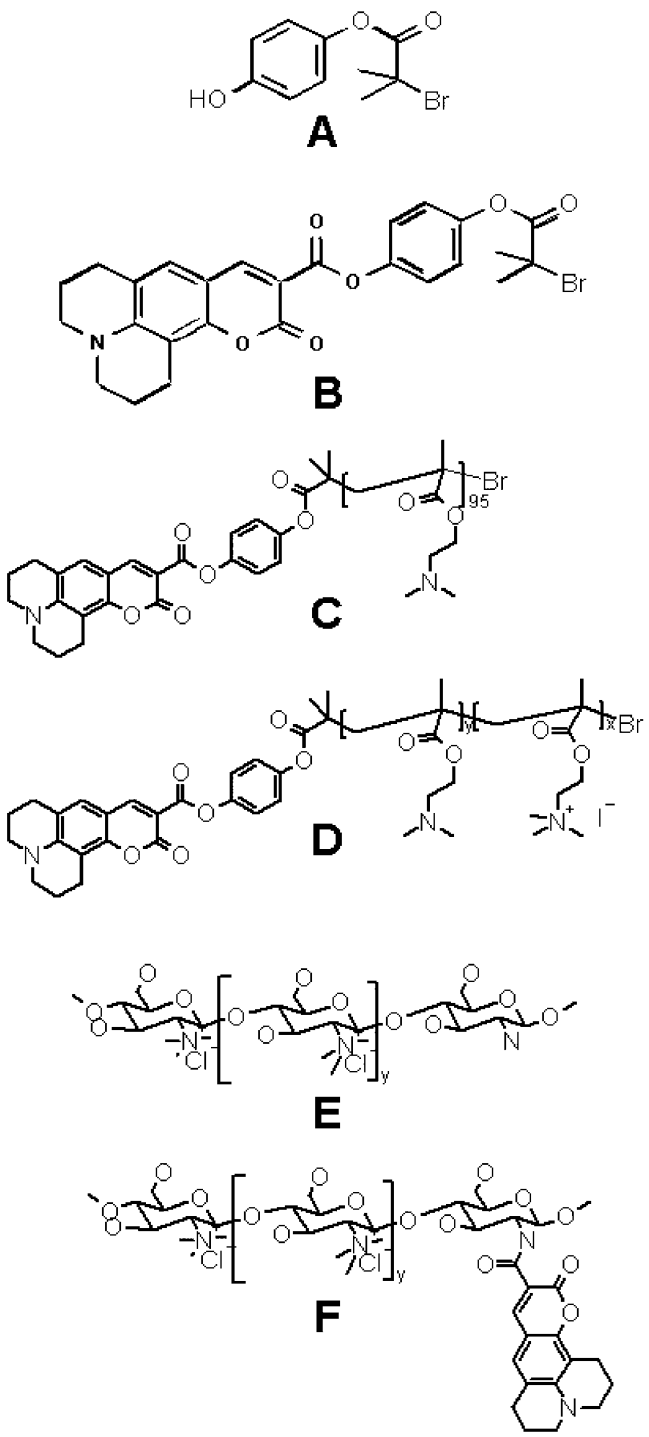

Fig. 1. Structures of compounds. (A) Hydroxyl initiator; (B) coumarin 343 initiator; (C) coumarin 343-initiated pDMAEMA; (D) quaternized coumarin 343-initiated pDMAEMA; where Q10x/(x + y) = 0.1; (E) structure of TMC; (F) coumarin 343-labeled TMC.

Quaternization of Coumarin 343- Initiated pDMAEMA

Coumarin 343-initiated pDMAEMA was quaternized to yield 10% (Q10), 24% (Q24), and 32% (Q32) of the repeat

units. In a typical quaternization, coumarin 343 initiated pDMAEMA (5g, 0.41 mM, Mn 12,300, PDi 1.08) was dissolved in tetrahydrofuran (50 ml) and methyl iodide (0.43g, 3 mM) was added and the solution left stirring for 24 h at ambient temperature. The solvent was removed and the polymer dried under vacuum. The degree of quaternization, as determined by ^{1}H NMR, was 10% of the available amine groups. This gives a polymer with a molecular mass of 13,400 (Fig. 1D).

Trimethylated Chitosan Chloride

TMC was synthesized as previously described, (30). In brief, 1-methyl-2-pyrolidinone (80 ml) was added to a flask containing low-viscosity heat-dissolved chitosan (2 g) and sodium iodide (4.8 g). On addition of NaOH (aq.) (11 ml, 15% w/v) and iodomethane (11 ml), the reaction mixture was condensed for 60 min. The mixture was removed from the oil bath and ethanol (200 ml) was added to precipitate the chitosan derivative. The suspension was centrifuged for 10 min at 4000 × *g*, the supernatant decanted off and the resulting solid was sequentially washed with ethanol and diethyl ether. The product was isolated by centrifugation. The isolated solid was added to 80 ml of 1-methyl-2-pyrolidinone and heated to dissolve. NaI (4.8 g), NaOH (aq) (11 ml, 15% w/v) and iodomethane (11 ml) were added and the mixture condensed for 30 min. Additional iodomethane (2 ml) and NaOH pellets (2 g) were added and stirring continued for 1 h. The product was washed and isolated as described above. It was dissolved in 10% (w/v) aqueous NaCl (80 ml) and stirred for 30 min. The product was precipitated with ethanol and isolated by centrifugation to yield *N*-trimethylated chitosan chloride (TMC, Fig. 1E). The molecular weight of TMC is 85,000 relative to poly(ethylene) glycol narrow molecular weight standards, measured using 2 × 30 cm PL aquagel columns (Polymer Laboratories) at 1 ml/min with differential refractive index detection.

Coumarin-Labeled Trimethylated Chitosan Chloride

A mixture of TMC chloride salt (200 mg) in water (20 ml) was stirred at ambient temperature for 25 min. Coumarin 343 (2 mg, 1% w/w) was added followed by dimethylaminopyridine (DMAP) (1 crystal). Ethyl carbodiimide hydrochloride (EDCI) (6.7 mg) was added and the mixture was stirred at ambient temperature. After 26 h, the reaction mixture was freeze-dried. The solid was dissolved in 25% ethanol in water (40 ml), transferred to a dialysis bag (molecular weight cutoff 12–14,000) and dialyzed against 25% ethanol in water (4 L). The dialysis was undertaken for 7 days, with increasing ethanol concentration to 60% (to remove un-reacted coumarin 343, and then back to 100% water. The contents of the dialysis bag were freeze-dried to yield coumarin 343-labeled TMC (Fig. 1F). Coumarin-labeled TMC has 0.17 molecules of coumarin per TMC chain (i.e., about 1 for every 6 TMC molecules). This was derived from the UV absorbance of coumarin in the visible range calibrated against free coumarin in aqueous solution.

For adhesion assays, polymers were dissolved in HEPES-buffered DMEM (monolayers) and TC-199 medium (sacs), respectively. There was no alteration of fluorescent signal in these media.

Adhesion Assay: Monolayers

HT29 and E12 monolayers were rinsed with HEPES-buffered-DMEM and equilibrated in the same medium at 37°C for 60 min. In the some instances, monolayers were incubated with the mucolytic agent, *N*-acetyl cysteine (10mM, NAC) for 15 min prior to polymer addition. To remove NAC, monolayers were rinsed gently with medium before use. Test polymer (0.5 ml) was added to the apical side of the monolayer at two concentrations (0.1 mg/ml or 0.01 mg/ml). Monolayers were incubated at 37°C in a Titramax 1000 shaking incubator for 60 min at 100 rpm. Polymer concentrations were measured in 50 μl samples taken both sides of the monolayers at 0 and 60 min. Following aspiration of the polymer-loaded donor side, the monolayers were then washed three times in incubation buffer, homogenized, and lysed in 2% SDS and EDTA (50 mM) at pH 8.0. All samples were assayed on an LS 50B Luminescence Fluorimeter as described above and data expressed as μg polymer/cm^2. Negligible amounts of signal were detected in the washes.

Adhesion Assay: Tissue

Rats were starved overnight before euthanasia by cervical dislocation. The intestine was removed after a midline incision, and the jejunum rapidly removed and flushed with oxygenated medium. 6 sacs, each 5 cm long, were cut from the isolated jejunum. Sacs were placed in oxygenated TC199 medium at 37°C, according to the method of Barthe *et al.* (21). The sacs were tied tightly at one end with silk suture and a small animal vascular catheter (Data Sciences International Physiocath 277-1-002) was tied in to the other end. A 1 ml syringe with a sterile 26 gauge micro lance was fixed to the catheter. In some instances, intestinal sacs were pretreated with 10 mM NAC for 15 min, which was flushed out with 20 ml of medium. Sacs were then filled with 0.5 ml polymer solution (1 mg/ml) via the catheter. Each sac was placed in a separate sealed 50 ml flask containing 15 ml of oxygenated TC-199 medium on a shaking water bath for 30 min at 37°C. Duplicate 50 μl samples of incubation medium were removed from the bath after 30 min to assess leakage. Sacs were then removed from the bath and the internal contents recovered using a fresh 1 ml volume syringe. Following aspiration of the polymer-loaded donor compartment, sacs were then washed sequentially four times with a total of 5 ml of medium and the washes collected for assay. Samples were adjusted to pH 7.4 by addition of sodium citrate (10 mM) and assayed by fluorescence as described above. Adhesion to sacs was calculated by subtraction and expressed as μg polymer/cm^2. Polymer mass balance was present in all monolayer and sac experiments (data not shown). None of the washes contained significant concentrations of polymers.

FD-4 Transport Studies

Transport studies were carried out on monolayers and sacs with FITC-dextran 4.4 kDa (FD-4) in order to assess epithelial functional integrity. FD-4 permeability across monolayers was examined according to previous reports (31). In brief, 0.5 ml of FD-4 (250 μg/ml) was added to the apical side of the monolayers. FD-4 flux to the basolateral side was measured over 120 min. Samples (0.1 ml) were collected from the basolateral side every 15 min and subsequently replaced with fresh transport medium. FD-4 was assayed fluorimetrically at excitation wavelength of 490 nm and an emission of

wavelength of 525 nm. In studies using non-everted intestinal sacs, 0.5 ml of FD-4 (1.0 mg/ml) was injected into each sac lumen as described above. Samples (50 μl) were collected from the bath every 15 min and replaced with fresh medium. After 120 min, the sacs were cut open and the contents sampled. The apparent permeability (Papp) for FD-4 was calculated from the following equation: Papp (cm/s) = $(dQ/dt)/(A \cdot C_o)$, where dQ/dt is the transport rate (mol/s), A is the surface area of the monolayer or sac (cm^2), and C_o is the initial concentration in the donor compartment (mol/ml).

Confocal Scanning Laser Microscopy

Polycarbonate membranes with attached monolayers were exposed to 0.1% sodium dodecyl sulfate for 2 min at room temperature to render cell membranes permeable. Membranes were then removed from the insert scaffold and stained with ethidium homodimer-1 (EthD1) (Molecular Probes, Leiden, The Netherlands, cat. no. L-3224). The membranes were placed on glass slides, surrounded by an adhesive compound and covered in 0.2 ml of DMEM-HEPES medium. Glass cover slips were attached, sealed and examined using confocal scanning laser microscopy (CLSM; Leica TCS SL). The excitation/emission wavelengths were 495 nm/635 nm, respectively.

Light and Fluorescent Microscopy

In order to view the mucus gel layer on frozen sections, E12 monolayers were washed in DMEM-HEPES medium, sandwiched between thin strips of chicken liver, and snap-frozen in liquid nitrogen. The tissue was embedded in optimal cutting temperature (OCT) compound, and 20 μm cross-sections were cut with a cryo-microtome and transferred to glass slides for staining. To visualize the mucus gel layer, sections were stained for 10 min at room temperature with alcian blue (1%) in distilled water, adjusted to pH 2.5 with glacial acetic acid (3%). Sections were then washed in a water bath with flowing water. Sections were then counterstained with neutral red (1%) for 5 min at room temperature and washed in a water bath with flowing water. They were viewed by light and fluorescent microscopy using a Nikon Eclipse E400 Fluorescent Microscope with Nikon Digital Camera (model DXM1200). Images were captured using Nikon ACT-1 (version 2.0) imaging software, using alternate transmitted light and fluorescent modes. A field comparison between light and fluorescent images allowed the visualization of fluorochrome-labeled polymers in the mucus gel layer.

Statistics

Statistical analysis for significant differences between groups was calculated using two-tailed student's unpaired *t* tests (GraphPad Instat, version 3.05) unless indicated. p values of <0.05 were considered significant. Values in Figs. 2–5 and Table 1 were expressed as mean ± SEM for each group.

RESULTS

Adhesion Assays

Adhesion of pDMAEMA Derivatives to E12 Monolayers

pDMAEMA, quaternized derivatives (10%, 24%, and 32% levels) and TMC were assessed for their adhesion to E12 and HT29 (Fig. 2). Because E12 monolayers were covered with a mucus layer and HT29 were not, we designate adherence of polymers to these monolayers as "mucoadhesion" and "bioadhesion," respectively. The data showed that all four pDMAEMA derivatives adhered significantly to E12 at both concentrations used (mucoadhesion) and, in each case, they displayed significantly increased mucoadhesion over that seen in HT29 at the matched concentration (bioadhesion). There was no evidence that the coumarin dissociated from the polymer since no fluorescent signal could be detected on the basolateral side of any monolayer exposed to the fluorescent polymers. Furthermore, stability of pDMAEMA formulations were assessed over three weeks in aquous buffer and no diminution of fluorescent signals were detected (results not shown).

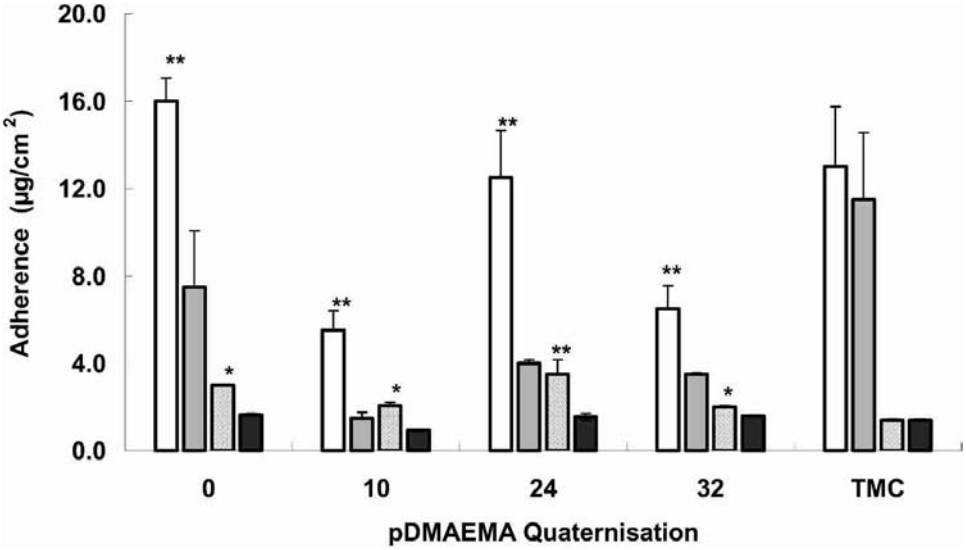

Fig. 2. pDMAEMA adhesion to E12 and HT29 monolayers at four levels of quaternization. TMC was used as a positive control. □ E12 (50 μg/ml); ▨ HT29 (50 μg/ml); ⊡ E12 (5 μg/ml); ■ HT29 (5 μg/ml). n = 12 each group. **p < 0.01; * <0.05, comparing adhesion to E12 to HT29 at two concentrations. Mean ± SEM.

Levels of adhesion were significantly increased for each polymer using a 10-fold higher concentration in each type of culture. In the cases of unquaternized and 24% quaternized pDMAEMA, they were significantly more mucoadhesive than TMC at the 5 μg/ml concentration, while all pDMAEMA polymers displayed similar degrees of bioadhesion to TMC at this concentration, but relatively less bioadhesion at the higher concentration. On the other hand, TMC bound equally well to both E12 and HT29 at both concentrations, suggesting that the presence of a mucus layer had no impact on its binding. There was no evidence that the degree of quaternization of pDMAEMA influenced adhesion to either E12 or HT29. Overall, this data suggested that the presence of a mucus layer contributed significantly to the adhesion of pDMAEMA and its analogues. These polymers retain a significant capacity to bind non-mucus covered epithelia at levels within the range of TMC.

Comparison of Polymer Adherence to E12 and Intestinal Sacs

The adhesion of three polymers (pDMAEMA, 24% quaternized pDMAEMA and TMC) to monolayers and intestinal sacs was compared in the presence and absence of the mucolytic, NAC. Significantly reduced levels of mucoadhesion of both pDMAEMA polymers were present in NAC-treated E12 compared to untreated E12, values being close to those levels detected in HT29. TMC, in contrast displayed similar high levels of adherence to all three monolayer groups (Fig. 3A). In sacs, the same pattern of adhesion was apparent (Fig. 3B), namely, a significant reduction in adherence of both pDMAEMA polymers in the presence of NAC. In contrast, TMC adhered equally well to sacs in the presence and absence of NAC. pDMAEMA was more adherent to sacs than TMC in the presence of mucus, but was less adherent in its absence ($p < 0.05$ in each case). There was no evidence

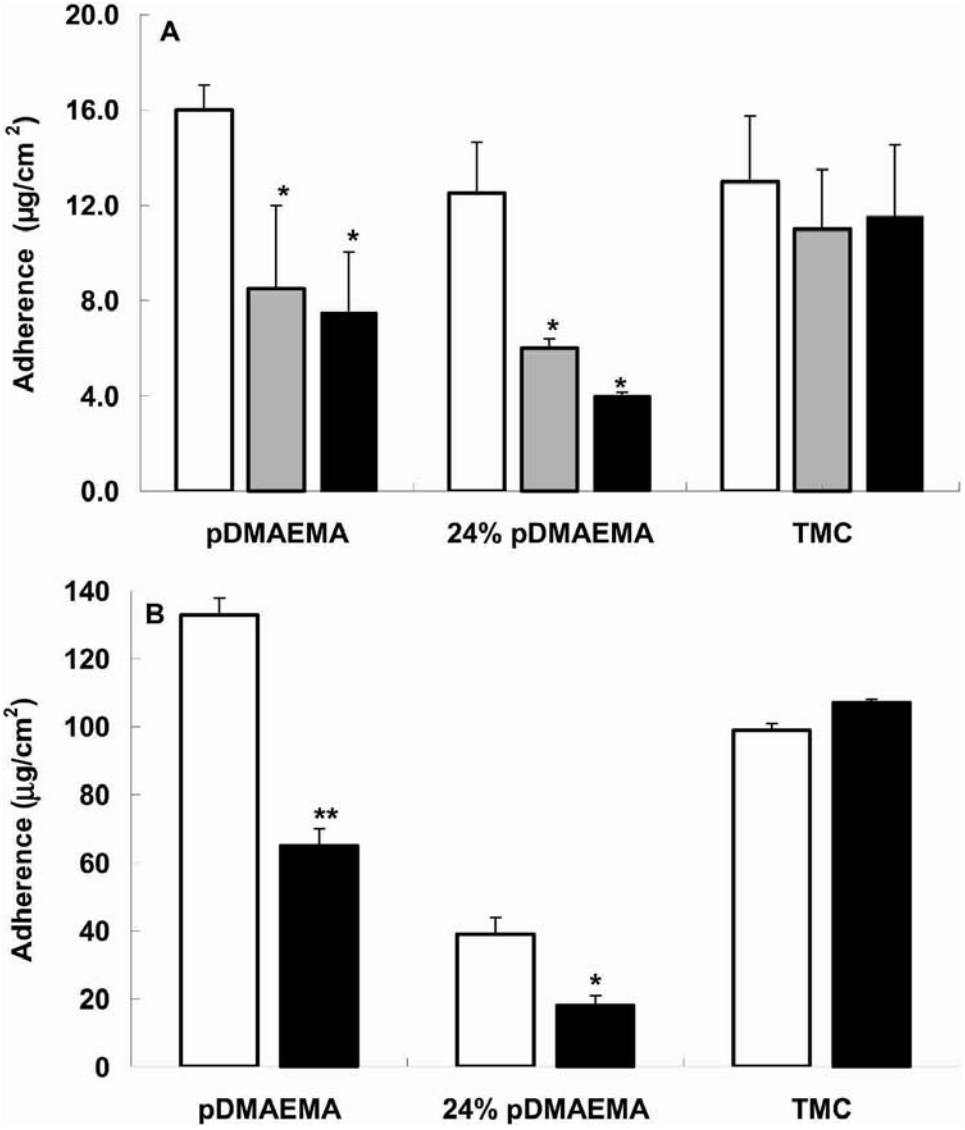

Fig. 3. (A) The effect of NAC on polymer adherence to monolayers. □ E12; ▨NAC-E12; ■ HT-29. 50 μg/ml of each polymer was used. n = 12 for each group. (B) Effect of NAC on polymer adherence to sacs. □ Sac; ■ NAC-Sac. 500 μg/ml of each polymer were used. In (A) and (B), NAC (10 mM) was present for 15 min in advance of polymer before wash-out. (n = 8 for each group). **$p < 0.01$, *<0.05. Mean ± SEM.

that quaternization had any positive effect on adherence to either model. Overall, this data suggests that adhesion of pDMAEMA to both E12 and intestinal sacs is indeed related in large part to the presence of a mucus gel layer and that this class of polymer is therefore predominantly mucoadhesive. TMC does not require mucus in order to be adherent to both cell and tissue models and binds equally well regardless.

Polymer Effects on FD-4 Transport and TEER

The effect of pDMAEMA and TMC on TEER values and permeability to FD-4 was investigated in monolayers and sacs. Over ten passages, E12 monolayers had a mean TEER of 168 ± 29 Ω cm^2 (n = 72), whereas HT29 monolayers had a statistically lower mean TEER of 83 ± 11 Ω cm^2 (n = 72), $p < 0.01$. Basal flux of FD-4 from the apical to the basolateral side of E12 was almost 50% lower than in HT29, data which suggested an inverse relationship of FD-4 flux and TEER. Following exposure of E12 to pDMAEMA (0.01 mg/ml and 0.1 mg/ml) for 60 min, the Papp of FD-4 across E12 was significantly reduced at each concentration, and more so at the higher concentration. In contrast, no statistical difference in the Papp of FD-4 was seen in HT29 exposed to either concentration of the polymer (Fig. 4A), and therefore a physical interaction between pDMAEMA and the paracellular marker appeared unlikely. E12 monolayers pretreated with NAC and then exposed to pDMAEMA also had a statistically reduced Papp for FD-4, but the reduction was far less than in the case of pDMAEMA in the absence of NAC (Fig. 5). Similar to E12, pretreatment of sacs with pDMAEMA (1 mg/ml) also resulted in a significant decrease in the Papp for FD-4 (Fig. 4B). In contrast to pDMAEMA, following exposure of sacs to TMC (0.1 mg/ml) there was a significant increase in the Papp of FD-4 across sacs (Fig. 4b), whereas in E12 the Papp increase was almost significant (Fig. 5). The Papp of FD-4 across E12 exposed to TMC was, on the other hand, significantly greater than that obtained in monolayers treated with pDMAEMA or NAC-pDMAEMA. NAC itself did not impact on FD-4 flux across either E12 or sacs (data not shown). Overall, the FD-4 Papp translated to 0.14% transport across E12; this was decreased to 0.09% with pDMAEMA and increased to 0.19% with TMC. Basal FD-4 flux

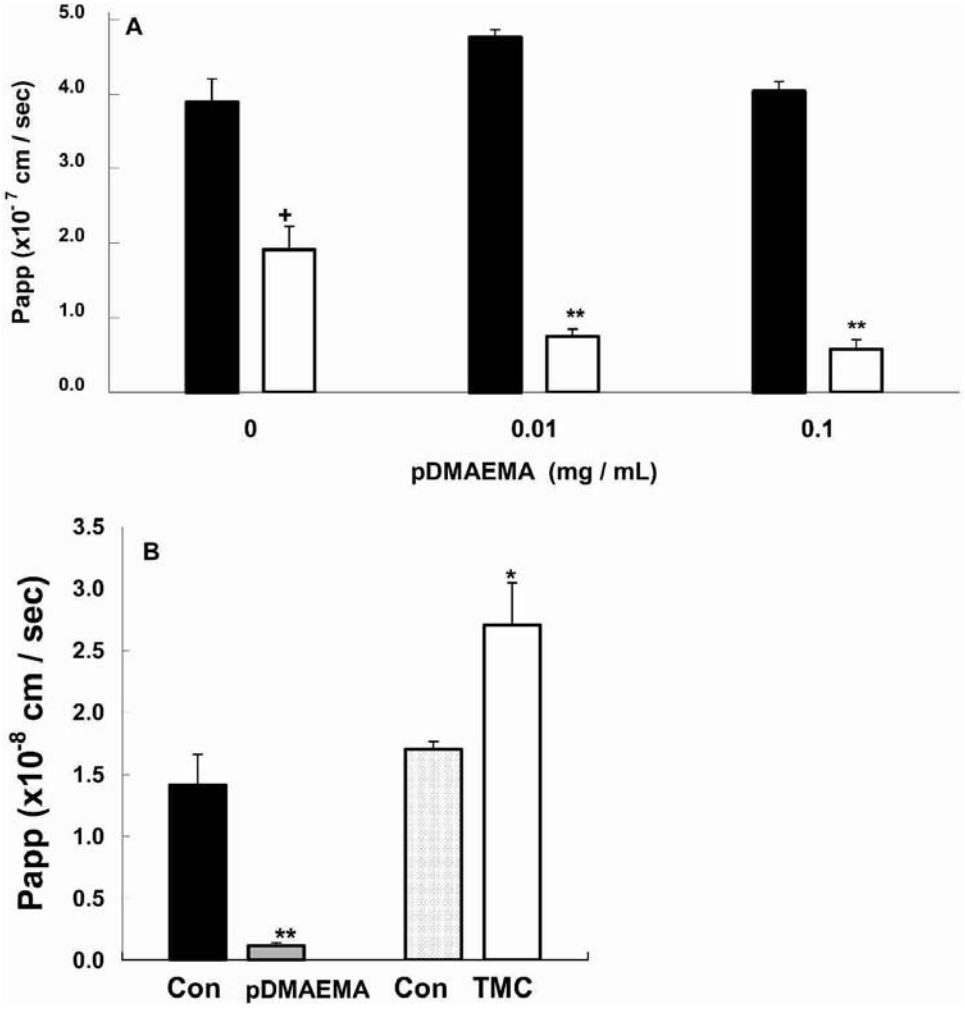

Fig. 4. (A) Papp of FD-4 across E12 and HT29 monolayers exposed to DMAEMA (0.01 and 0.1 mg/ml, 120 min). ■ HT29 and □ E12. + $p < 0.05$, E12 vs. HT29; **$p < 0.01$, polymer-treated E12 vs. untreated E12, unpaired *t* tests. n = 6, each group. (B) Papp of FD-4 across pDMAEMA and TMC-treated sacs (1 mg/ ml, 120 min). ■ Matched untreated controls vs. ▨pDMAEMA, **$p < 0.01$; ⊡Matched untreated controls vs. □ TMC; *$p < 0.05$, n = 5, each group. Mean ± SEM.

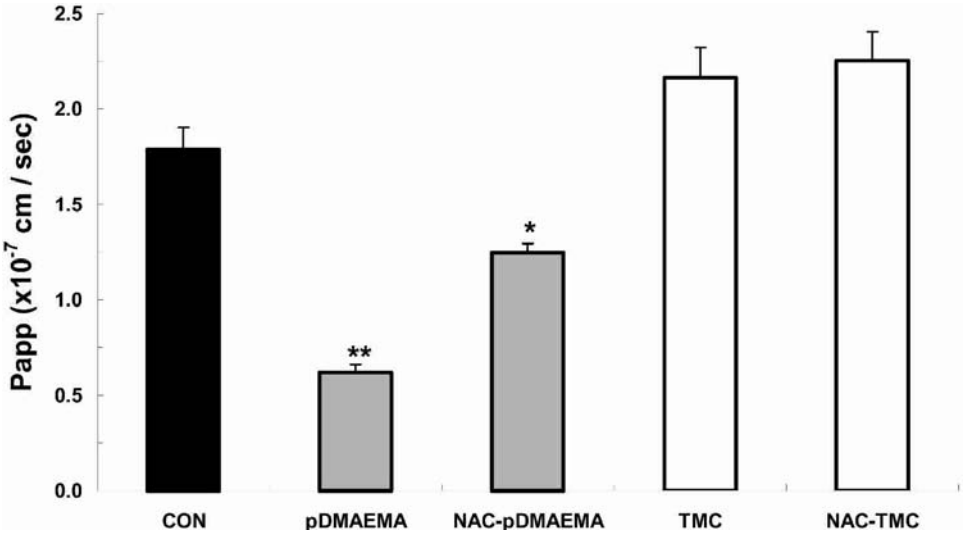

Fig. 5. Papp of FD-4 across E12 and NAC-treated E12 monolayers treated with pDMAEMA and TMC (0.1 mg/ml in each case). **$p < 0.001$, *$p < 0.05$, compared to control flux. One-way ANOVA. $n = 6$, each group. Mean ± SEM.

across sacs was 0.011%; this was decreased to 0.004% with pDMAEMA and increased to 0.019% with TMC. TEER values of E12 and HT29 were unchanged in the presence of either pDMAEMA or TMC at concentrations up to 0.1 mg/ml (data not shown); this is indirect evidence however, that monolayer viability was in large part retained by monolayers following polymer exposure.

Cytotoxicity of pDMAEMA and TMC

The release of LDH was measured following exposure of monolayers to pDMAEMA and TMC in order to assess any *in vitro* cytotoxic effects that might be induced by these two polymers. After 60 min incubation with pDMAEMA (2 mg/ml), LDH release was approximately 8% for E12 and 12% for HT29 (Table I). LDH release was significantly higher in HT29 exposed to pDMAEMA at all concentrations above 0.1 mg/ml in comparison to E12. Cytotoxicity was exacerbated in HT29, presumably because it lacks a mucus gel layer. LDH release increased in a concentration-dependent relationship to pDMAEMA concentration. At 0.1 mg/ml and 0.01 mg/ml concentrations of pDMAEMA, LDH release from E12 and HT29 monolayers was very low. This suggests that pDMAEMA does not cause significant damage to either monolayer type at the concentrations used for adherence. TMC showed similar results to pDMAEMA, with LDH release ranging from <1% at 0.01 mg/ml to ~10% release at 2 mg/ml across both monolayer types. Cytotoxicity was again significantly higher in HT29 than in E12 at the two higher concentrations of TMC. Similar to pDMAEMA, TMC did not cause significant damage to either monolayer type at the concentrations used for adherence. Neither were there any differences in LDH release between TMC and pDMAEMA on either E12 or HT29 monolayers exposed to matched concentrations. In comparison to a positive control, E12 and HT29 monolayers exposed to low concentrations of Triton-x-100 detergent (0.01%) elaborated maximal LDH release over the 60 min period.

Microscopy

E12 and HT29 monolayers stained with EthD1 were examined by CLSM following exposure to pDMAEMA conjugated to coumarin. The mucus gel layer overlying E12 monolayer was calculated to be 100–160 μm thick ($n = 3$). Flouresscently labeled pDMAEMA bound to HT29 in an irregular punctate manner. In contrast, a more continuous layer of polymer was seen over the E12 monolayer (Fig. 6). In corroboration with the adherence studies, more fluorescent pDMAEMA was visualized attaching to E12 than to HT29. By contrasting light and fluorescent images of the same E12 sections it was possible to demonstrate that the majority of the fluorochrome-tagged polymer co-localized with the mucus gel layer in preference to the apical membranes of the epithelia (Fig. 7).

DISCUSSION

Studies on the adhesion of polymers to mucosal surfaces have mainly used tensiometry as a method of choice. This method allows qualitative measurements of the detachment work required to separate polymer formulations from an underlying substrate or tissue (e.g., Refs. 10, 11, 32, 33). It does not however, allow quantitative assessment of the adherence of soluble polymers to fresh viable tissue under physiologic conditions in which epithelial barrier function can be examined in parallel. Neither does the method take into account the presence or impact of overlying mucus. Notable targets for tensile tests have included isolated thawed bovine duodenal (32), porcine esophageal (34), and rat intestinal tissue (35). Recently, atomic force microscopy has been used to quantitatively measure and image polymer interaction with mucin (36), although this technique has yet to be applied to mucus-producing epithelia. In addition, mucoadhesion of microparticles comprising fluorescent polycarbophil to isolated fresh porcine intestinal mucosa has recently been assessed in a flow-through system using phosphate buffer (37). Our aim, therefore, was to assess the suitability of human intestinal E12

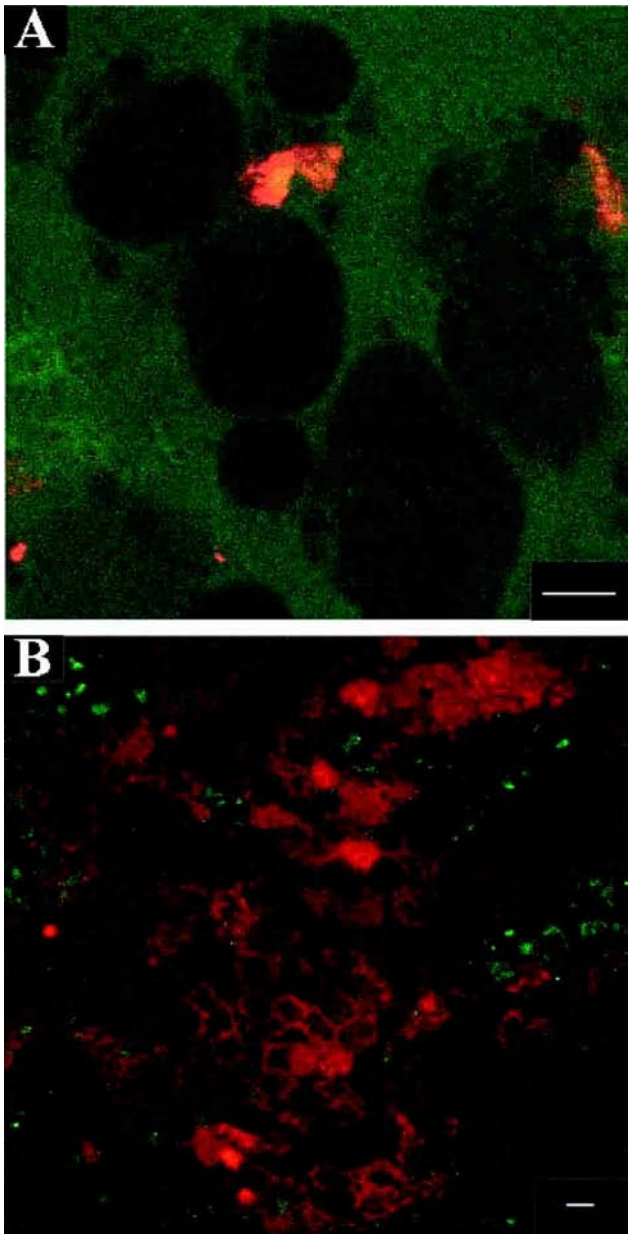

Fig. 6. Confocal images of monolayers stained with EthD1 and exposed to pDMAEMA conjugated to coumarin. (A) pDMAEMA (0.1 mg/ml, green) covers gel layer of E12 but is not associated with epithelia (red). (B) pDMAEMA attaches in punctate fashion to HT29 epithelia. Bar = 10 μm.

monolayers for measuring mucoadhesion of a selection of novel and established soluble fluorescent polymers and to compare data against that obtained in fresh intestinal non-everted sacs from rats. As yet, to our knowledge no stable human mucus-gel forming intestinal epithelial cell line has been used to study polymer mucoadhesion. Non-everted sacs are a useful system to study in parallel, as they offer a rapid and economical method of studying mucoadhesion and tissue viability is maximized in high nutrient medium (21).

The E12 cell line has been partially characterized, but the similarity between its mucus gel and that of the human small intestine *in vivo* remains largely unexplored, although attempts have been made, for example, to measure human mucin gene expression profiles (18). The parental line, HT29,

Table I. LDH Release from Monolayers Following Exposure to pDMAEMA and TMC

[pDMAEMA] (mg/ml)	E12 (%)	HT29 (%)
2	7.8 ± 1.0	11.6 ± 1.8[a]
1	3.2 ± 0.8	5.4 ± 1.0[b]
0.1	1.3 ± 0.2	3.2 ± 0.8[b]
0.01	0.9 ± 0.2	1.8 ± 0.5
[TMC] (mg/ml)		
2	7.1 ± 0.8	0.9 ± 1.0[a]
1	3.4 ± 0.5	5.3 ± 0.8[b]
0.1	1.2 ± 0.3	2.1 ± 0.5
0.01	0.7 ± 0.1	1.6 ± 0.5
Triton-x-100 (0.01%)	100 ± 0.1	100 ± 0.1

[a] $p < 0.01$, [b] $p < 0.05$, E12 versus HT29 at matched concentrations ($n = 6$). Mean ± SEM.

elaborates no mucus and is an appropriate control to assess polymer adherence to the intestinal epithelium. It was also anticipated that data achieved in HT29 would be similar to that demonstrated in E12 exposed to a mucolytic, NAC (16,22). We undertook adhesion experiments with an established adhesive hydrophilic chitosan-derived polymer (TMC) and a group of hydrophilic poly (methacrylate) polymers. Chitosan itself is a known cationic mucoadhesive, but low solubility limits its potential for mucosal drug delivery at physiologic pH values. Trimethylation of chitosan eliminates these solubility issues (30,38,39). Poly(2-(dimethylamino-ethyl) methacrylate (pDMAEMA) is a synthetic polymer that is finding use in gene delivery (23) and cancer targeting studies (24), and was synthesized using the polymerization technique of living radical polymerization (27). The resulting pDMAEMA polymers have tertiary amino side chains that can be conjugated to peptides, stabilizers, and enhancers and may offer potential in non-injected delivery, similar to that recently demonstrated for thiolated poly (acrylic acid)-insulin conjugates (40).

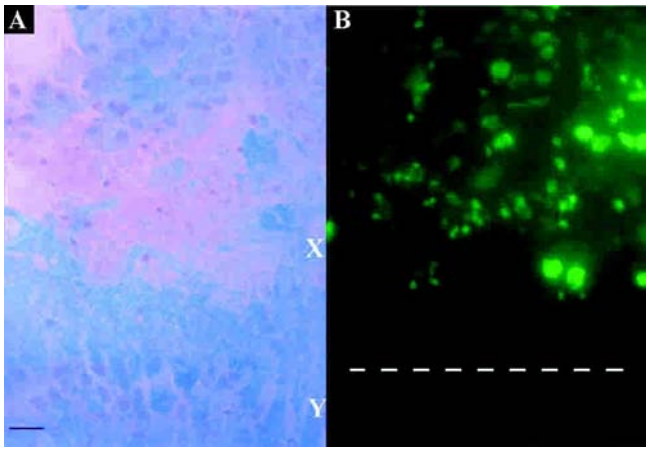

Fig. 7. (A) Light micrograph of alcian blue/neutral red staining of E12 exposed to pDMAEMA-coumarin conjugate. X: Mucus gel layer; Y: Cell monolayer. (B) Fluorescent micrograph of pDMAEMA-coumarin conjugate on same fixed section. Broken white lines denote cell monolayer outline. Co-localization of pDMAEMA with mucus layer is evident by superimposing micrograph (B) on (A). Bars = 25 μm.

When exposed to pDMAEMA polymers (especially the 0 and 24% quaternized forms), significant adhesion levels were obtained in E12 and these were comparable to those achieved in sacs. While pDMAEMA polymers were shown to be as mucoadhesive as TMC, it did not, however, have the same level of adherence as TMC for epithelial membranes *per se,* especially at high concentrations. This difference was evident from NAC-treated E12, HT29 and sac experiments. It was, in addition, clearly visible in the CLSM images of pDMAEMA binding predominantly to the mucus layer of E12 rather than to the epithelium of HT29. TMC was both muco- and bioadhesive to the same extent and it is likely this polymer should retain some mucosal adherence even in the presence of high mucus turnover.

While it has been shown that electrostatic interaction is a major factor in the adhesion of cationic chitosan to purified anionic pig mucin (36), charge-based interactions of polymers with mucus remains poorly understood. The main adhesive interactions between adhesive polymers and mucus occur *via* covalent bonding, hydrogen bonding and electrostatic and hydrophobic interactions or a combination of these (41). A significant contribution to these interactions is made by the negative charge carried by the terminal sialyl- and ester sulfate moieties on mucin glycans (42). A decrease in mucoadhesion with increasing quaternization of TMC has previously been shown, although both quaternized and unquaternized TMC were overall still less mucoadhesive than chitosan (11). Furthermore, it appears that increased quaternization of TMC leads to an associated increase in polymer absorption-enhancing properties (43,44), perhaps indicating that the remaining unassociated free polymer has increased access to the plasma membrane and tight junction proteins. In the case of pDMAEMA, quaternization should theoretically alter the effect of electrostatic interactions between the polymer and glycoproteins due to the increased charged groups on the polymer (45). Of the four levels of quaternization tested however, both the unquaternized and 24% quaternized pDMAEMA showed the highest levels of mucoadhesion to E12 and sacs. Both 10% and 32% quaternized pDMAEMA, however, showed low levels of mucoadhesion. This strongly suggests that, unlike chitosan and TMC, pDMAEMA binding to mucus in monolayers or sacs was not strongly influenced by electrostatic charges arising from fully charged species. This does not rule out the overall importance of subtle hydrogen bonding in mucoadhesion. Other conjugation strategies are likely to further increase pDMAEMA mucoadhesion. A similar type of polymer, poly(acrylic) acid, was thiolated via cysteine conjugation and a significant increase in mucoadhesion was measured, due to the formation of disulphide bridges with mucosal glycoproteins (46).

The adhesion of unquaternized pDMAEMA to E12 monolayers was shown to significantly reduce the apparent permeability of FD-4, whereas HT29 monolayers treated with pDMAEMA showed no decrease in permeability. A significant decrease in FD-4 flux also occurred across intestinal sacs that had been treated with pDMAEMA compared to untreated sacs. The reduction of flux therefore appears to be dependent on the presence of a combination of the mucosal mucus gel and pDMAEMA. Another mucoadhesive polymer, carbopol 934P increases the viscosity of mucins (47) and perhaps this mechanism might explain, at least in part, the mucosal protection by pDMAEMA. The FD-4 Papp was also lower in E12 than HT29 even in the absence of the polymer, although it is not possible to decipher whether this is due to the presence of mucus and/or the higher basal TEER in E12, or to a combination of both. It is worth noting the that the FD-4 Papp values were overall far higher in HT29 and E12 than in tighter epithelia such as Caco-2 and thus reflecting the leakiness of these tissues. Data from E12 (18) and mucus-covered co-cultures (16) suggests that mucus should act as an impediment to the flux of hydrophobic drugs but not to hydrophilic agents such as dextrans. While no significant change in TEER was shown in E12 in the presence of pDMAEMA despite the decrease in FD-4 flux, it is worth noting that paracellular flux and TEER are not always inversely correlated, especially in leaky epithelia such as the small intestine where transcellular resistors contribute significantly to overall resistance (48). The fact that basal FD-4 across both E12 and sacs was detectable confirmed that these are low resistance epithelial models with similar levels of hydrophilic paracellular marker fluxes to those obtained in human jejunum (49) and in recently developed low resistance intestinal cell lines (50).

TMC had opposite effects to pDMAEMA on FD-4 flux in E12 and sacs. It increased epithelial permeability to the paracellular flux marker. This data was in keeping with the well known tight junction opening effects of chitosan and TMC (5,43,44). This property of chitosan(s) may be helpful in enhancing drug delivery across mucosal surfaces such as the cornea (51,52). The capacity of pDMAEMA to reduce mucosal permeability may be especially advantageous for topical localized delivery where a reduction in systemic drug absorption may be desired. Barrier enhancement is a desirable property in adhesive compounds, perhaps offering potential for treatment of inflammatory bowel disease where epithelia and lamnia propria immunocytes are exposed to damaging pathogens (53).

It is known that injected cationic polymers may be cytotoxic (54). Recent data demonstrated that pDMAEMA has a cytotoxic effect on Jurkat and U937 lymphocyte cell lines (55). In contrast, our data showed that LDH release from both types of human intestinal epithelial monolayers exposed to high concentrations of pDMAEMA (and TMC) was low. Furthermore, because LDH release was less in E12 than in NAC-treated E12 or HT29 exposed to the polymer, this suggests that the mucus gel layer overlying E12 cells provides additional cytoprotection. The confocal micrographs further demonstrated that there was little direct interaction between pDMAEMA and E12 epithelial cell membranes, above which mucus is interposed, and this may explain why it caused no cytotoxicity compared to HT29. Moreover, mucus gels also decreased the efficiency of plasmid transfection in an intestinal epithelial co-culture model (56), so it is not unlikely that the mucus gel layer may limit access of pDMAEMA to the epithelial surface of E12. pDMAEMA is unlikely to be absorbed and should be excreted in faeces, thus reducing potential systemic toxicity of this non-biodegradable polymer. Overall, our data suggest that the assumption that pDMAEMA may be cytotoxic is premature, since its disposition depends on the surface characteristics and type of tissue target and as to whether the polymer is internalized.

One of the potential applications of muco-integrated polymer therapeutics is in the area of topical anti-microbial drug delivery. Studies have shown that poly(ethylene) glycol delivered by lavage to the mucus lining of the intestine pre-

vented death in mucosally-compromised mice infected with *Pseudomonas aeruginosa* (6). Chitin and chitosan have also been reported to protect mice against challenge with a range of bacteria (57), while ethyl cellulose appears to prevent bacterial translocation in the liver (58) Recently, quaternized pDMAEMA was directly polymerized onto filter paper and was shown to have antimicrobial action against *Bacillus subtilis* and *Escherichia coli* (25). It seems, therefore, that antibacterial action is present inherently in a number of common polymers, but that the mechanisms remain largely undeciphered. The lack of a reproducible reductionist model should expand investigations into the cellular basis of these effects. E12 may prove a suitable model system for ascertaining the role of human intestinal mucus in pathogen-epithelial interactions and for screening polymers in order to prevent access to the apical membrane. Initial experiments, for example, suggest that cytotoxicity of cholera toxin can be reduced in E12 but not HT29 in the presence of pDMAEMA (Keely and Brayden, unpublished data).

In summary, this study has demonstrated that a mucus-producing sub-clone of HT29 displays adhesive properties similar to an established method, intestinal sacs. It may have useful application in screening mucoadhesives under physiologic conditions. A group of methacrylate-based polymers, pDMAEMA, were made by a novel method of living radical polymerization and was shown to be as mucoadhesive as a soluble chitosan derivative (TMC), and to co-localize in the mucus gel. Furthermore, low level cytoxicity of pDMAEMA and TMC in monolayers was reduced still further in presence of mucus. Finally, this cationic polymer could be differentiated mechanistically from TMC not only on the basis of adhesion, but also by its capability to reduce flux of a paracellular marker in the presence of mucus in the two systems.

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