Characterizing Transport Enhancement by P(MAA-g-EG) Drug Carriers in the Presence of Mucus

Omar Z. Fisher[1, 2] , Ming Lin[2], and Nicholas A. Peppas[1, 2, 3, 4]

[1]Biomaterials, Drug Delivery, Bionanotechnology and Molecular Recognition Laboratories,
[2]Department of Biomedical Engineering, [3]Department of Chemical Engineering,
[4]Division of Pharmaceutics, The University of Texas at Austin, Austin, TX, 78712, U.S.A.

Abstract

Previous attempts to evaluate the transport of proteins across the intestinal mucosa using the caco-2 cell line have neglected the mucus layer as a barrier to drug absorption. To achieve a more accurate assessment of the absorption a coculture can be used that combines the enterocyte-like transport properties of the caco-2 cell line with the gastric mucin secretion of the HT29-MTX cell line. Understanding the degree to which micro- and nanoparticulate poly(methacrylic acid-g-ethylene glycol) (P(MAA-g-EG)) drug carriers may act as paracellular permeation enhancers may also require the presence of the mucus barrier. In this study we utilize quantitative fluorescence microscopy and transepithelial resistance to analyze the effect of drug carrier size on the tight junction integrity. In this study Caco-2 and HT29-MTX monolayers were grown on polycarbonate Costar® transwell membranes for 21 days until confluent. Monolayers were exposed to P(MAA-g-EG) microparticles or nanoparticles in the apical chamber. Methanol fixed memebranes were immunolabeled for E-cadherin and ZO-1 and imaged using laser scanning confocal microscopy. The mucus secretion of the monolayers was confirmed by transmission electron microscopy and alcian blue staining. Of the junctional complex molecules chosen, E-cadherin appears to be the most advantageous as a marker for the integrity of the epithelial. Besides for providing the strongest signal, the calcium dependent of binding of E-cadherins has been shown to be coupled with the disruption of both claudin1 and ZO-1. The inhomogeneous staining of claudin-1 suggests that expression is different between the Caco-2 and HT29-MTX cell lines.

Introduction

pH-Sensitive hydrogels consisting of poly(ethylene glycol) (PEG) grafted on poly(methacrylic acid) (PMAA) are a promising vehicle for the oral delivery of biopharmaceuticals. A major reason they have yet to reach therapeutic efficacy is low absorption of macromolecules in the small intestine. The important characteristic of these hydrogels is the ability to protect susceptible proteins from degradation in the stomach (Blanchette et al., 2004). A secondary characteristic is the ability to modulate paracytosis in the small intestine. The mechanism behind this activity deserves further investigation due to the relationship between paracellular permeation enhancement and epithelial pathology (Mullin et al., 2005). It has been suggested that the ability of these biopolymers to chelate calcium leads to disruption in the integrity of the tight junctions (Madsen & Peppas, 1999; Torres-Lugo et al., 2002; Ichikawa & Peppas, 2003).

The transport properties of the intestinal mucosa can be modeled in vitro with the use of the enterocyte-like caco-2 cell line. These cells are of colonic origin and will form

a confluent polarized monolayer with tight junction strength equal to or higher than that of human intestinal mucosa (Grasset et al., 1984 ; Hilgendorf et al., 1999). The tight junction itself is a network of non-covalently crosslinked transmembrane proteins. The transepithelial resistance of caco-2 monolayers is mostly due to the integrity of the tight junction (Grasset et al., 1984). In transport studies the resistance across a monolayer is commonly used as the primary quantitative measure of paracellular coherence. A common problem with resistance measurements is the large sample variability. Also, the resistance measurement is associated with macroscopic changes in the membrane. Markers for macromolecular permeability through tight junctions include nonpolar tracers such as fluorescent or radioisotope labeled dextran and or poly(ethylene glycol) (Torres-Lugo et al., 2002; Kondoh et al., 2005; Lane & Corrigan, 2006).

The various cytoskeletal and transmembrane protein constituents of the tight junction that can be imaged include occludin (Sheth et al., 2004), the claudins ZO-1 and junctional adhesion molecule (JAM) (Johnson, 2004), actin (Behrens & Kissel, 2003), E-cadherin, β-catenin (Seth et al., 2004; Sheth et al., 2004) and desmoplakin (Wan et al., 1999) all successful imaging agents to detect disruption. To further investigate the type of permeation enhancement associated with P(MAA-*g*-EG) drug carriers, claudin-1 and E-cadherin were chosen to use in this study. The former was chosen as a marker for the biochemical stability of caco-2 monolayers, the latter for biophysical stability. These states were assessed by analyzing changes in cell-cell adhesion using conventional laser scanning confocal microscopy combined with a simple quantification algorithm.

Materials and Methods

Preparation Methacrylic acid (MAA), tetraethylene glycol dimethacrylate (TEGDMA) and 1-hydroxycyclohexyl phenyl ketone (Irgacure 184™) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Poly(ethylene glycol) monomethylether monomethacrylate (PEGMA) (PEG molecular weight of 1000, corresponding to 23 repeating units) was purchased from Polysciences Inc. (Warrington, PA, USA). Fetal bovine serum (FBS), monoclonal mouse anti-E-cadherin antibody, polyclonal rabbit anti-claudin-1 (strong cross reactivity for claudin-3), polyclonal FITC labeled goat anti-mouse and TRITC labeled goat anti-rabbit antibodies were all obtained from Invitrogen Corp. (Carlsbad, California). Dulbecco's modified eagle's media (DMEM), Dulbecco's phosphate buffered saline (DPBS), Hank's balanced salt solution (HBSS), non-essential amino acids, and pencillin/streptomycin were all obtained from Mediatech Inc. (Herndon, VA).

Microparticulate drug carriers were synthesized as previously described (Ichikawa & Peppas, 2003). Briefly, P(MAA-g-EG) films were prepared by a UV-initiated free radical solution polymerization of methacrylic acid MAA and PEGMA. MAA was vacuum distilled prior to use to remove the inhibitor hydroquinone. PEGMA was used as received. The two monomers were mixed together at a 1:1 ratio of MAA and ethylene glycol repeats. TEGDMA was added at 1 mol% as a crosslinker. Irgacure 184™ was added at 0.1 wt%. The monomer solution solution was diluted in a 1:1 water/ethanol solution. Nitrogen gas was bubbled through the mixture for 20 minutes to remove dissolved oxygen. The mixture was then pipetted between two glass slides separated by a 0.7 mm Teflon spacer and exposed to UV light (Dymax R 2000EC Light Curing System) at an intensity of 16 mW/cm^2 for 30 minutes. Following polymerization the resultant

films were washed in distilled deionized water for 7 days, with water being changed twice daily. The films were then dried in a vacuum oven for 48 hours, crushed with a mortar and pestle and sieved to particles sized <150 μm. Prior to use the particles were suspended in HBSS without calcium or magnesium and homogenized using an ultrasonic homogenizer (Misonix, Inc). Lyophilized particles were imaged with a scanning electron microscope (LEO 1530. P(MAA-*g*-EG) microparticles were placed onto double sided conductive tape attached to an aluminum SEM stage and then gold coated.

Caco-2 cells (American Tissue Culture Collection; Rockville, MD) were grown on 75-cm^2 tissue culture flasks in DMEM with 4.5 g/L glucose & L-glutamine, without sodium pyruvate, , 1% non-essential amino acids, 100 U/mL penicillin, 100 μg/mL streptomycin and 10% fetal bovine serum with the media replacement every second day. The cells were kept at 37^{o}C in humidified incubator with 5% CO_2. Cells passaged between 60-80 weeks were grown for 21-23 days, until confluent, on polycarbonate transwell inserts. The monolayers were incubated with HBSS without Ca^{2+} and Mg^{2+} an hour before the start of transport experiments. At t=0 the experimental group of membranes was exposed to 10mg/ml of microparticles in the apical chamber. A positive control group was exposed 2 μM EDTA (Fisher Scientific International Inc., Hampton, NH) in the apical chamber. Transepithelial resistance was monitored until a 40% resistance drop was observed in the positive control group. For immunolabeling the membranes were washed twice with ice cold phosphate buffered saline (PBS), fixed in methanol at -20^o C for 5 minutes then dried overnight at room temperature.

Immunolabeling of Monolayers All the following steps were conducted at room temperature. Membranes were rehydrated with DPBS for 10 minutes followed blocking in tris buffered saline with 0.05% Tween 20 (Sigma-Aldrich, St. Louis, MO) and 1% bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO) for 30 minutes. The blocking solution was removed and each membrane was exposed to 3.25 μg of mouse anti-E-cadherin antibody and rabbit anti-claudin-1 in 650 μl of PBS with 1% BSA for an hour. They were each then washed 3x with PBS and exposed to 9.75 μg of both FITC labeled goat anti-mouse antibody and TRITC labeled goat anti-rabbit in 650 μl of PBS with 1% BSA for 1 hour. After this they were washed 3x with PBS and placed for a glass slide and covered with mounting media (Biomeda Corp., Foster City, CA) and coverslip. Slides were stored in the dark at 4^o C.

Confocal Microscopy and Image Processing Images were acquired using a Leica SP2 AOBS confocal microscope. Z-stacks through the thickness of each membrane were acquired at ΔZ = 289 nm, with sequential excitation at 488 nm and 543 nm, 3 frame averaging, constant laser power and photomultiplier (PMT) gain at 512 x 512 or 1024 x 1024 pixel resolutions. Maximum projection images of each z-stack were used for quantification of immunolabeled protein spread. Each image was converted to a binary image at a set threshold.

Results and Discussion

The size effects of P(MAA-g-EG) microparticles on insulin absorption have been analyzed with control for mass but not for surface area (Morishita, et al., 2004; Lopéz & Peppas, 2004). The enhanced contact area between carriers and intestinal tissue may account for the increased insulin transport with smaller sized particles. Despite the lack of cytotoxicity (Foss & Peppas, 2003), these drug carriers may have a pronounced affect on paracytosis due to surface contact between the carriers and enterocytes. The preparation method for the microparticulate drug carriers leads to a non-spherical morphology as shown on electron micrographs (Figure 1).

To rule out any affect of polymer swelling on cell-cell adhesion integrity, polymers were pre-swollen in HBSS w/o Ca^{2+}/Mg^{2+}. They tended to form aggregates if added dry to the apical chamber of polarized caco-2 cells. Because their morphology and swelling characteristics it is difficult to measure the available contact area between the carriers and monolayers using this fabrication method.

Figure 2 shows the drop in transepithelial resistance measurements over time. The first reading was taken 10 minutes after the addition of P(MAA-g-EG) microparticles and EDTA to apical chambers. The microparticles precipitated as a suspension soon after being applied as monolayers, providing intimate contact with the membranes. Subsequent readings were taken every 30 minutes thereafter. The EDTA treated group achieved a 40% drop in resistance by the 40 minute time point. This drop was sustained throughout the course of the experiment. Both the control and microparticle treated group showed a slight jump in resistance followed by a slow decline over the course of the experiment.

Upon viewing the projection images of immunolabeled control and microparticle exposed monolayers show the similar cobblestone morphology characteristic of Caco-2 monolayers (see Fig. 2). In the EDTA group the network of intercellular junctions was almost completely disrupted, resulting in the rounded out appearance of individual cells. When the projection images are juxtaposed the staining for both claudin and E-cadherin appears more diffuse in the both the microparticle and EDTA treated group. The claudin-1 staining pattern makes the tight junctions appear thinner. This is due to claudin-1 restriction to the apical most portions of the junctions. E-cadherin staining was more diffuse along the thickness of the membrane, causing the junctions to appear thicker. The images quantified were obtained at 512 x512 pixel resolutions. Larger pixel resolutions and a smaller ΔZ required much longer acquisition times for each z-stack. The increased time was at the expense of ease-of-use. Plots of image fraction shows different trends for the effect of both microparticles and EDTA on the spread of claudin-1 and EDTA (see Fig. 3). The image fraction of E-cadherin was greater in all groups but greatest in microparticle treated monolayers. Claudin spread was greatest in membranes exposed to EDTA. Image fractions were significantly different from control for both experimental group. But they were not significantly different from each other.

What is most notable about these results compared to the TEER values is the much higher significance in the differences in image fraction. Also, the sample sizes used correspond to the number images obtained from an experimental group. The membranes used for imaging were the same as those used for TEER measurements. The central idea behind this attempt to quantify paracellular disruption is that a disruption off tight junction integrity shows as a lateral displacement of fluorescently tagged TJ molecules.

This two dimensional approach simplifies other techniques which have tried to reconstruct the three dimensional paracellular space from fluorescent image stacks and account for volume fraction of tagged proteins (Wan et al., 1999). These results support the efficacy of the 2-dimensional approach.

The usefulness of the drug carriers evaluated here has been proven for delivering proteins orally to the small intestine. Their permeation enhancement properties may result from more than just calcium chelation. Although not significantly higher than that of the EDTA treated group, E-cadherin disruption was slightly higher in the microparticle treated group. The tight junctions, more specifically the claudins, are known have both a 'fence' and 'gate' function (Johnson, 2005). The highly pegylated of P(MAA-*g*-EG) microparticles could have an effect on membrane fluidity. Poly(ethylene glycol) is commonly used as a fusogen for protoplasts in agricultural biology (Assani et al., 2005), and has been shown to 'seal' axotomized nerve endings (Shi & Borgens, 1999, 2000; Shi et al., 1999) because of its effects of membrane fluidity. Also, the effect has been show to occur without direct contact between PEG and cell membranes (Yamazaki et al., 1989). These microparticles may be enhancing the migration of transmembrane membranes, such as E-cadherin, by affecting membrane fluidity. This would be occur synergistically with the abatement of the calcium dependent gate function of tight junctions.

Further investigations would be required to distinguish these processes. Also, the local affect of cell contact carrier bound PEG grafts can not be ruled out. Permeation enhancement in an excess of calcium is the most reasonable method for these carriers in vivo. Calcium is at near saturation at the serosal side of the intestinal mucosa, negating the affects of any agent that would require calcium chelation to increase drug transport. Given this fact, the benefit these particles have shown on insulin transport in preclinical studies must result from other factors such as protease inhibition by grafted PEG chains, mechanical effects, or changes in enterocyte plasma membrane fluidity.

Time is among the imaging parameters that should be varied in future work. The intercellular homophilic interaction between tight junction proteins is a dynamic process. Recovery of transepithelial resistance has been shown after administration and removal of these drug carriers in vitro (Ichikawa & Peppas, 2003; Lopéz & Peppas, 2004). It would be important to consider the state of tight junction integrity with a quantitative imaging approach after TEER recovery. Also, oral drug delivery can be a chronic or acute therapy. Looking at the effects of repeated carrier administration on the fatigability of tight junction contacts would be an important starting point for future work.

Conclusions

In this work we have initiated the first steps in adding another useful technique for the study or oral protein delivery. The lateral displacement of tight junctional proteins was more indicative of permeation enhancement than measurements of transepithelial resistance. By quantifying this displacement, the effects the effect of pH-sensitive P(MAA-*g*-EG) drug carriers on intestinal mucosa become clearer. The long term goal is to use these systems for the control of biomacromolecular transport in the intestine, not just enhancement. In order to achieve this, all the factors involved in how these systems affect absorption must be identified. This work is a step in that direction.

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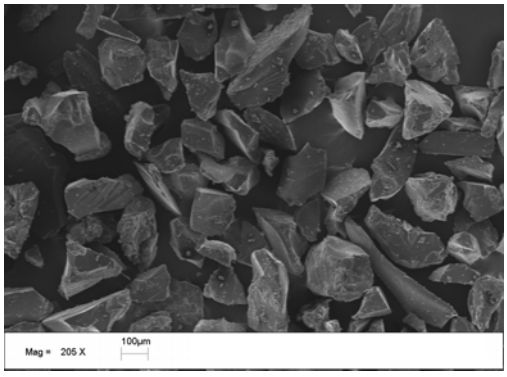

Figure 1: Scanning electron micrograph of P(MAA-g-EG) microparticles crushed and passed through a 150 µm sieve.

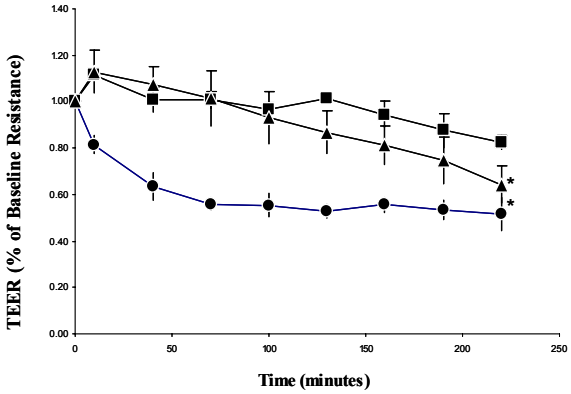

Figure 2: TEER change over time in Caco-2 monolayers exposed to 10 mg/ml P(MAA-*g*-EG) microparticles in the apical chamber (n=6) (▲), 2 µM EDTA in the apical chamber(n=3) (■) and a control group (n=3) (●). Monolayers were grown on 4.71 cm^2 membranes with 3 µm pore sizes. All membranes contained HBSS without calcium or magnesium in both the apical and basolateral chambers. Each data point represents ±SD. *$p < 0.05$, significantly different from control group at all time points.

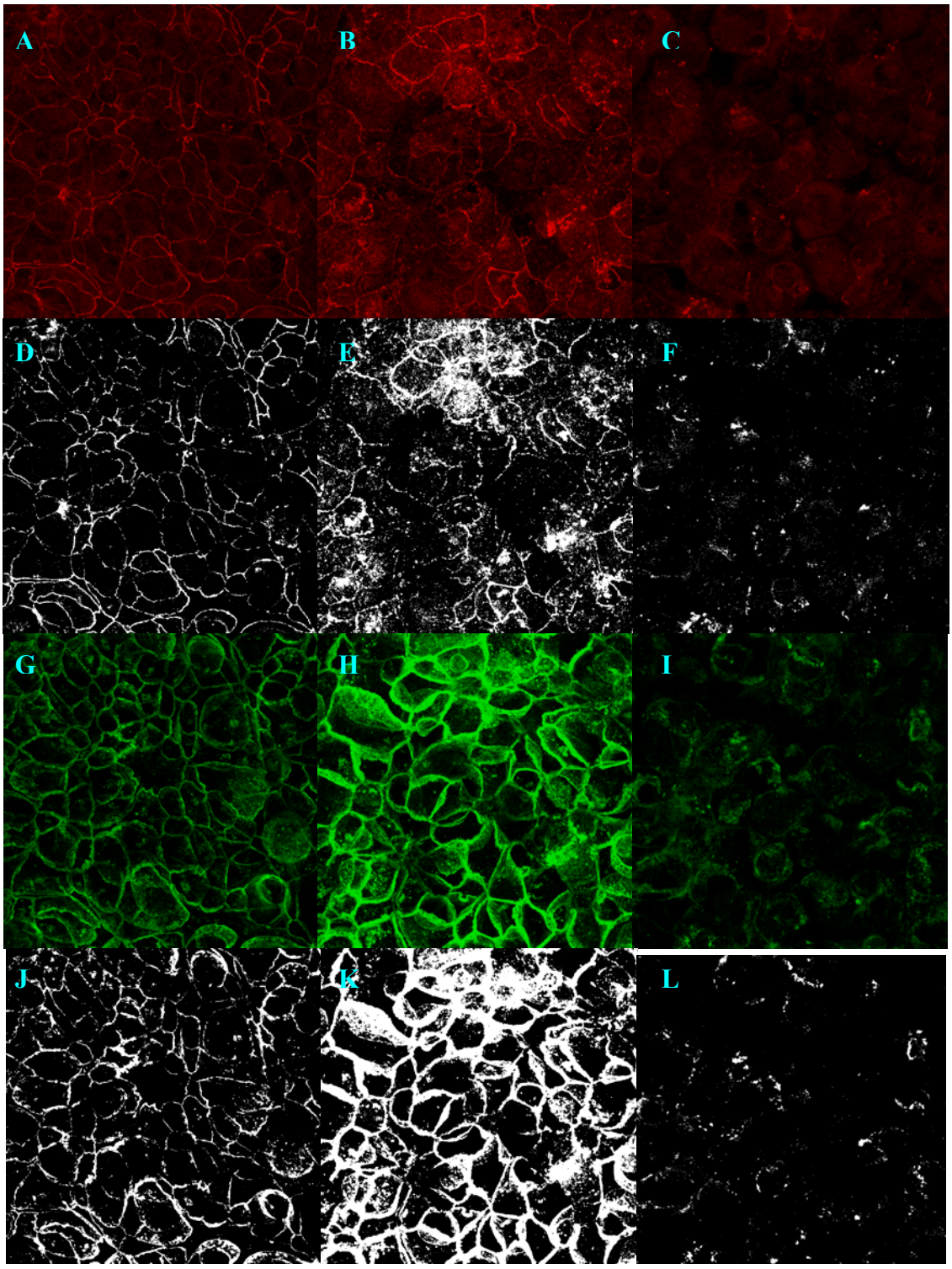

Figure 3: Maximum Z-stack projection images of polarized Caco-2 monolayers double immunolabeled for claudin-1 (a-f) and E-cadherin (g-l) and imaged with 63x objective power at 1024x1024 pixel resolutions. The first column (a, d ,g, j) shows a control monoloyar, the second column (b, e, h, k) shows a monolayer exposed to 10mg/ml P(MAA-*g*-EG) microparticles and the third (c, f, I, l) shows a monolayer exposed to 2 µm EDTA, both in the apical chamber. The images that result from binary conversion at a 50% intensity threshold (d-f, j-l) are show just below the original.