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Cellular evaluation of insulin transmucosal delivery

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Abstract—P(MAA-g-EG) microparticles have been extensively investigated as carriers for oral delivery of proteins such as insulin. In this study, we investigated the effect of the molecular weight of the PEG tethered chains in the copolymer network and of the microparticle size on the transepithelial electrical resistance (TEER) and insulin epithelial permeability, using monolayers of human intestinal epithelial Caco-2 cells. Two molecular weights of the PEG chains, 400 and 1000, were investigated, as well as three different dry microparticle sizes: 25–90, 90–150 and 150–212 μm . Their effect on the cell monolayer integrity was studied by monitoring TEER as a fraction of time and determining insulin permeability. The presence of insulin-loaded P(MAA-g-EG) microparticles decreases the TEERs value by 50% with respect to the control. This disruption of the cell monolayer was recovered in 3 h after the removal of the polymer microparticles. Within the range of PEG molecular weights studied, there was no significant change of the TEER values. However, decreased microparticle sizes and short PEG chains systems led to higher permeability values. Insulin-loaded P(MAA-g-EG) microparticles enhanced the transport of insulin through the Caco-2 cell monolayers.

Key words: Oral delivery; insulin; Caco-2 cells; transmucosal permeability.

INTRODUCTION

Noninvasive methods of insulin delivery are highly desirable in diabetes treatment, with the oral route clearly being the most convenient [1]. The oral route of administration faces several barriers such as the presence of proteolytic enzymes in the gastrointestinal tract, chemical instability and poor permeability of proteins across biological membranes.

In our laboratory, we have successfully developed microparticulate and nanoparticulate systems based on unique crosslinked copolymers that can be used as carri-

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ers for oral delivery. These systems consist of hydrogels of poly(methacrylic acid) (PMAA) or poly(acrylic acid) (PAA) grafted with long chains of poly(ethylene glycol) (PEG). These networks will be henceforth designated as P(MAA-g-EG) and P(AA-g-EG). These hydrogels exhibit complexation characteristics as well as pH sensitivity. Thus, they can protect the protein from the harsh environment of the stomach and release it in the small intestine when needed [2–4]. These copolymer systems exhibit good mucoadhesive properties due to the presence of the PEG tethers [5]. In addition, we have shown that they can protect and stabilize proteins and inhibit proteolytic enzymes [6].

In previous studies, cultured epithelial cells have been successfully used as tools for the screening of drug permeation and transport [7]. In this study, we used a CaCo-2-cell line as an intestinal epithelium model, which can be used as an intestinal model for drug absorption, transport and permeability studies. CaCo-2 cells are derived from human colorectal adenocarcinoma. They undergo spontaneous differentiation in culture and form monolayers that exhibit morphological and functional similarities to the small intestinal epithelium [8, 9].

The absorption of large hydrophilic molecules is limited by paracellular and transcellular barriers. Specifically, the paracellular pathway is restricted because of the presence of the tight junctions between cells. Different approaches have been used to induce paracellular transport of large molecules, including proteins, by disrupting the tight junctions. Some of these approaches include the use of surfactants [10], H₂-antagonists [11], polysaccharides such as chitosans [12, 13] and poly(acrylic acid) derivatives [13–16]. However, some of these approaches compromise the integrity of the cell monolayer in order to enhance permeability [17].

Previous studies in our laboratory have shown that of P(MAA-g-EG) microparticles and nanospheres are not cytotoxic [18, 19] when in contact with CaCo-2 cell monolayers. We have also proven the ability of P(MAA-g-EG) microparticles to improve the transport of proteins such as insulin [18] and calcitonin [20] through the cell monolayer. Our studies have shown beyond doubt that protein transport is due to the ability of this copolymer system to bind calcium causing disruption of the tight junctions and facilitating the paracellular transport [7].

In our present studies with insulin-loaded P(MAA-g-EG) microparticles we investigated the molecular weight of the PEG tethered chains and the importance of the microparticle size on protein transport. We focused on the effect of PEG molecular weight on transport resistance and permeability because we have previously shown that tethered PEG chains act as mucoadhesion promoters [1, 6]. We wanted to examine if this mucoadhesive process could have an adverse effect on cellular transport. Studies of effect of microparticle size on cellular transport became necessary because of previous studies on the influence of particle transport on bioavailability [1].

The first studies involved the measurement of the transepithelial electrical resistance (TEER) in order to monitor the integrity of the tight junctions in the presence

of the protein-loaded microparticles. The second set of studies was done to evaluate the effect of these variables on the insulin permeability through the cell monolayer.

MATERIALS AND METHODS

Materials

Methacrylic acid (MAA) and poly(ethylene glycol) ether monomethacrylate (PEGMA) with PEG molecular weights of 400 and 1000 were purchased from Aldrich (Milwaukee, WI, USA) and used to synthesize P(MAA-g-EG) microparticles. Tetra(ethylene glycol) dimethacrylate (TEGDMA) was purchased from Polysciences (Warrington, PA, USA) and used as the crosslinking agent in the amount of 0.75% mol of the total monomers. Irgacure[®] 184 was employed as the photoinitiator and obtained from Ciba-Geigy (Hawthorne, NY, USA); it was incorporated in 0.1% (w/w) of the monomer mixture. Bovine insulin was purchased from Sigma (St. Louis, MO, USA).

Preparation of P(MAA-g-EG) microparticles

P(MAA-g-EG) hydrogel films were prepared by free radical solution UV-polymerization of MAA and PEGMA in the presence of TEGDMA. The monomers were mixed in 1:1 molar ratio of methacrylic acid-ethylene glycol units. PEGMAs with molecular weights of 400 and 1000 of the PEG graft chains were used in the synthesis. TEGDMA was used as the crosslinking agent and was added in the amount of 0.75 mol% of the total amount of monomers. The photoinitiator, Irgacure 184 was added in the amount of 0.1 wt% of the total amount of monomers. The monomer mixture was diluted with a mixture of 50% (v/v) ethanol and deionized water. Nitrogen was bubbled through the well-mixed solution for 15 min.

This mixture was poured between microscope slides (75 × 50 × 1 mm; Fisher, Pittsburgh PA, USA) separated by Teflon spacers with a thickness of 0.9 mm. The microscope slides were then placed in a nitrogen atmosphere under a UV light source of 11 mW/cm² at 365 nm for 30 min. The ensuing polymer films were washed in deionized water for 7 days in order to remove unreacted monomers, crosslinking agent, initiator and sol fraction. The polymer films were then dried in a vacuum oven at 27°C for 3 days, crushed by using a mortar and pestle and then sieved to the desired particle size range (25–90, 90–150, or 150–212 μm).

Insulin loading

Insulin loading was accomplished by equilibrium partitioning of insulin into the P(MAA-g-EG) microparticles. Insulin stock solutions with a concentration of bovine insulin of 0.5 mg/ml and ionic strength of 0.1 M were prepared. In a typical experiment, 7 mg of polymer/ml of insulin stock solution was mixed at a pH of 7.4 for 6 h. Due to the hydrogen bonding decomplexation, the particles swelled, thus

absorbing insulin. Then, the system was rendered acidic by adding 0.1 M HCl. The protein-loaded microparticles were vacuum-filtered with a 0.45 μm Millipore filter paper and dried by freeze drying.

Caco-2 cell culture

Caco-2 cells were cultured in 75 cm^2 culturing flasks (VWR Scientific, West Chester, PA, USA) with 10 ml of Dulbecco's Modified Medium, (DMEM, Bio Fluids, Biosource International, Camarillo, CA, USA), at a seeding density for cultivation of 2.5×10^5 cells/flask. Cells were maintained in an incubator at a controlled atmosphere of 37°C, 95% relative humidity and 5% CO_2 . The culture medium was replaced with fresh medium every other day for 6 days, until the cells reached 60–80% confluence. At this stage, a passage operation was performed. In this process, cells were detached from the culturing flask by trypsinization and resuspended in fresh culture medium. Then, the cells were counted and transferred with the desired seeding density to a new culturing flask or experimental wells. In these cells studies, cells with passage numbers between 60 and 80 were used.

Transepithelial electrical resistance studies

Caco-2 cell cultures were grown in 96-well plates (4.71 cm^2/well) (Costar, Corning, NY, USA) with DMEM for 21 to 24 days until they achieved a constant transepithelial electrical resistance. The culturing cell density was 2.35×10^5 cells/well. Transwell cells were used for the transport studies. Polycarbonate membranes with pore size of 0.45 μm separated the two half cells. The medium used for these studies was Hanks' balanced salt solution (HBSS) containing Ca^{2+} .

Cell membranes were allowed to equilibrate with the experimental medium, HBSS with Ca^{2+} , for 1 h prior to the initiation of the experiment. The transepithelial electrical resistance (TEER) was monitored using a voltmeter with a chopstick electrode (World Precision Instrument, Sarasota, FL, USA). After the equilibration period, the apical chamber medium was removed, replaced with fresh medium containing the P(MAA-g-EG) microparticles and TEER of the cell monolayer was monitored as a function of time at 37°C.

The reduction in the transepithelial electrical resistance was determined by calculating the degree of TEER and reporting it as a percent of the total: TEER%: $((R_t - R_{w/o}) / (R_0 - R_{w/o})) \times 100\%$, where R_t is the resistance value at time t , R_0 is the initial resistance value and $R_{w/o}$ is the resistance value in the absence of the cell monolayer.

Insulin transport studies

Caco-2 cell cultures were grown in 96-well plates with DMEM for 21 to 24 days. DMEM was removed from both the apical and basolateral chamber and then replaced with fresh HBSS with Ca^{2+} . The experimental plates were incubated for

1 h before the experiment. The donor chamber medium was removed and replaced with fresh medium containing the insulin-loaded P(MAA-g-EG) microparticles. Samples were withdrawn from the receiver chamber at different time intervals for 3 h using a micropipette. The amount withdrawn from each chamber depended on the amount of insulin loaded into the P(MAA-g-EG) microparticles. The same percentage (v/v) was taken from both the apical and the basolateral chamber. Once the experiment was completed, the samples were analyzed using a bovine insulin ELISA kit (Alpco Diagnostics, Windham, NH, USA). In all cases triplicates were used.

The apparent permeability coefficient was used to quantify transport in a two-chamber diffusion system. The apparent insulin permeability coefficient, P_{app} , was calculated using $P_{app} = (dQ/dt)/(A \cdot C_0)$, in which dQ/dt (mol/s) was the rate of insulin transport in the receiver chamber, C_0 (mol/ml) was the initial insulin concentration in the donor chamber and A (cm²) was the surface area of the cell monolayer.

RESULTS

As we have indicated in previous studies [1, 6] the presence of PEG grafted chains in the polymer network of the carrier particles contributes to a mucoadhesion promotion mechanism [27–32]. For this purpose, we studied the effect of the molecular weight of the PEG tethered chains on the TEER values of the cell monolayer. As shown in Fig. 1, insulin-loaded P(MAA-g-EG) microparticles in contact with the cell monolayer led to a significant decrease of the TEER, of the order 40%. Each datum point represents an average of three wells and one

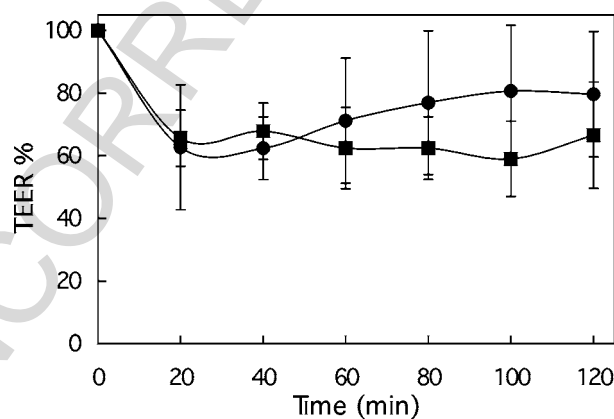


Figure 1. Effect of the molecular weight of the PEG tethered chains on the TEER of the cell monolayer. Insulin-loaded P(MAA-g-EG) microparticles with an initial monomer feed ratio MAA : EG of 1 : 1 and a PEG molecular weight of (●) 400 and (■) 1000 were used in this experiment. 10 mg of microparticles were in contact with the cell monolayers in each well. Caco-2 cell monolayers with a passage number of 62 were used at 37°C. Error bars represent one standard deviation, $n = 3$.

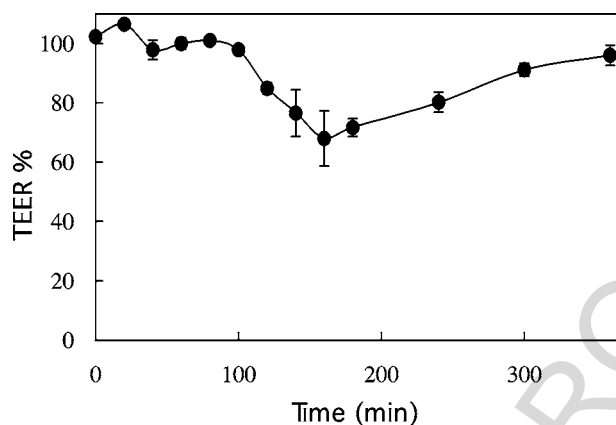


Figure 2. Reversible effect of the reduction of TEER. Insulin-loaded P(MAA-g-EG) microparticles with an initial monomer feed ratio MAA : EG of 1 : 1 and a PEG molecular weight 1000 were used in this experiment. 10 mg of microparticles were in contact with the cell monolayers in each well. Caco-2 cell monolayers with a passage number of 75 were used at 37°C. Error bars represent one standard deviation, $n = 3$.

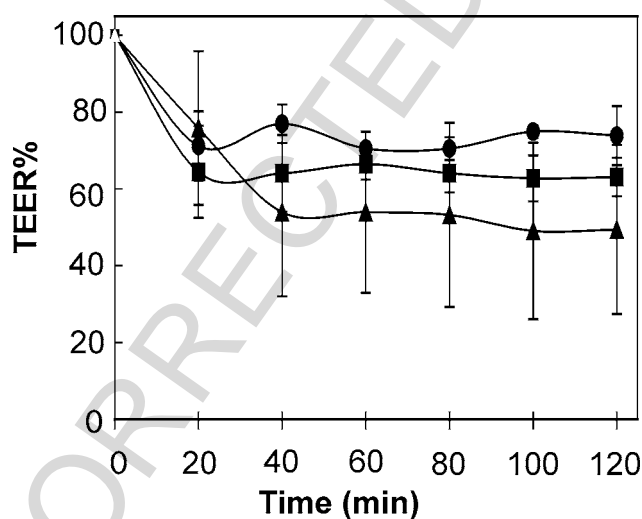


Figure 3. Effect of the amount of P(MAA-g-EG) microparticles on the cell monolayer TEER. Insulin-loaded P(MAA-g-EG) microparticles with an initial monomer feed ratio MAA : EG of 1 : 1 and PEG tethered chains of molecular weight of 1000 were used in this experiment. Different amounts of loaded microparticles, (●) 5 mg/well, (■) 10 mg/well and (▲) 20 mg/well, were in contact with the cell monolayer. Caco-2 cell monolayers with a passage number of 62 were used at 37°C. Error bars represent one standard deviation, $n = 3$.

standard deviation. However, the size of the PEG molecular weight did not affect the TEER values measured. Consequently, while the presence of PEG chains contributed to mucoadhesion, as shown by Madsen and Peppas [6], its size did not affect paracellular transport, at least not in the range of molecular weights studied.

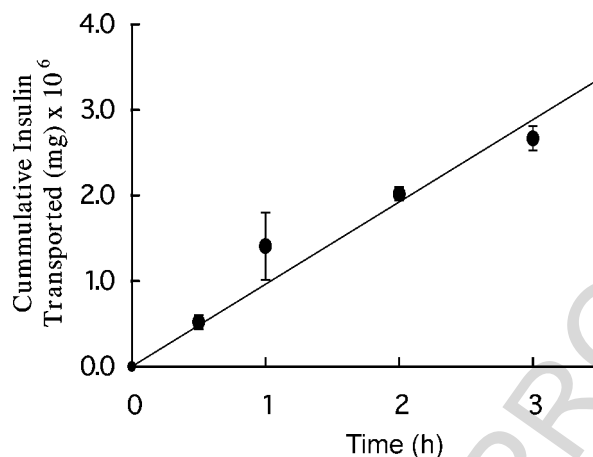


Figure 4. Transport of insulin from apical to basolateral chamber through Caco-2 cell monolayers in the presence of P(MAA-g-EG) microparticles. Microparticles with an initial monomer feed ratio MAA:EG of 1:1 and a PEG molecular weight of 1000 were used in this experiment. 20 mg of microparticles with a size of 150–212 μm were in contact with Caco-2 cell monolayers with a passage number of 67 at 37°C. Error bars represent one standard deviation, $n = 3$.

Figure 2 indicates that the effect of the insulin-loaded microparticles in the TEER reduction was reversible. During the first hour equilibration occurred after DMEM was replaced by the HBSS experimental medium. After this period, 10 mg of loaded microparticles were placed in contact with the monolayer for 3 h. At this point reduction in the TEER value of approx. 35% was observed. Then the microparticles were removed and the experimental medium was replaced with DMEM. Complete and reversible recovery of the TEER was seen within 3 h.

The effect of the amount of particles in contact with the cell monolayer was studied at 5, 10 and 20 mg/well. In all cases, no cytotoxicity was observed when exposed to the Caco-2 cell monolayers. As shown in Fig. 3, the TEER values decreased by up to 50%.

We also studied the difference in insulin transport from the apical to the basolateral side between insulin-loaded P(MAA-g-EG) microparticles and mixtures of insulin-free particles with insulin. In both systems, the initial monomer ratio was MAA:EG 1:1, the molecular weight of the PEG tethered chains was 1000 and the microparticle size was 150–212 μm . Figure 4 shows the cumulative amount of insulin transported from the loaded microparticles through the cell monolayer as a function of time. Table 1 shows that the insulin permeability coefficient was much higher for insulin/particle mixtures than for insulin-loaded microparticles.

The cumulative amounts of insulin from the apical to the basolateral as well as from the basolateral to the apical chamber were monitored as a function of time. Insulin-loaded P(MAA-g-EG) microparticles with an initial monomer ratio of MAA:EG 1:1, PEG tethered chains with molecular weight of 1000 and microparticle size of 150–212 μm were used in this experiment. The insulin permeability

Table 1.

Insulin permeability coefficient across Caco-2 cell monolayers in contact with two P(MAA-g-EG) microparticle systems

P(MAA-g-EG) microparticle system	Permeability $\times 10^6$ (cm/s)
Mixture of insulin/particles	12.7 ± 1.73
Insulin-loaded particles	4.69 ± 0.03

Transport from apical chamber to basolateral chamber was studied. P(MAA-g-EG) microparticles with an initial monomer feed ratio MAA : EG of 1 : 1 and PEG molecular weight of 1000 were used. 20 mg of microparticles with a size of 150–212 μm were in contact with Caco-2 cell monolayers with a passage number of 75. The experiment was carried out at a constant temperature of 37°C. Error bars represent one standard deviation, $n = 3$.

Table 2.

Effect of transport direction on the permeation of insulin across Caco-2 cell monolayers

Transport	Permeability $\times 10^6$ (cm/s)
Apical to basolateral side	4.69 ± 0.03
Basolateral to apical side	4.94 ± 0.02

Insulin-loaded P(MAA-g-EG) microparticles with an initial monomer feed ratio MAA : EG of 1 : 1 and PEG molecular weight of 1000 were used in this experiment. 20 mg of microparticles with a size of 150–212 μm were in contact with Caco-2 cell monolayers with a passage number of 75. The experiment was carried out at a constant temperature of 37°C. Error bars represent one standard deviation, $n = 3$.

Table 3.

Effect of the molecular weight of the PEG tethered chains of grafted P(MAA-g-EG) microparticles on the permeability coefficient of insulin across Caco-2 cell monolayers

Molecular weight of PEG tethered chains	Permeability $\times 10^6$ (cm/s)
400	6.37 ± 0.02
1000	4.65 ± 0.03

Transport was from the apical chamber to the basolateral chamber. Insulin-loaded P(MAA-g-EG) microparticles with an initial monomer feed ratio MAA : EG of 1 : 1 were used in this experiment. 20 mg of microparticles with a size of 150–212 μm were in contact with Caco-2 cell monolayers with a passage number of 75. The experiment was carried out at a constant temperature of 37°C. Error bars represent one standard deviation, $n = 3$.

coefficients were virtually the same under these experimental conditions as seen in Table 2.

We also studied the effect of the molecular weight of the PEG tethered chains on the insulin transport from the apical to the basolateral chamber. Results from Table 3 show that the insulin permeability values for the P(MAA-g-EG) microparticles containing PEG tethered chains with molecular weight of 400 were higher than for those with molecular weight of 1000.

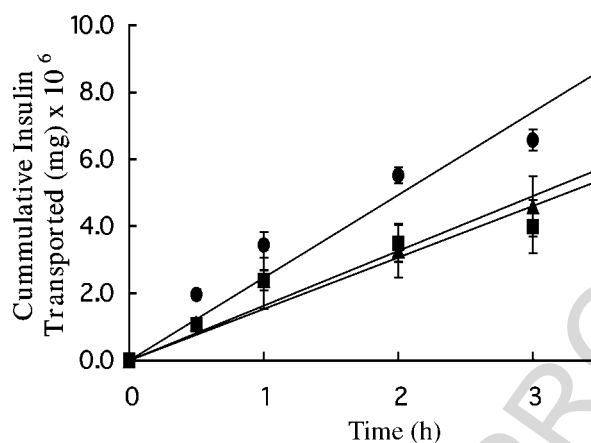


Figure 5. Effect of particle size on insulin transport through Caco-2 cell monolayers. Transport from the apical to the basolateral chamber was studied in this experiment. Loaded P(MAA-g-EG) microparticles with an initial monomer feed ratio MAA:EG of 1:1 and a PEG molecular weight of 1000 were used in this experiment. 20 mg of microparticles with a size of (●) 25–90 μm , (■) 90–150 μm and (▲) 150–212 μm were in contact with Caco-2 cell monolayers with a passage number of 72 at 37°C. Error bars represent one standard deviation, $n = 3$.

Table 4.

Effect of P(MAA-g-EG) carrier particle size on insulin permeability through Caco-2 cell monolayers

P(MAA-g-EG) microparticle size (μm)	Permeability $\times 10^6$ (cm/s)
25–90	3.32 ± 0.03
90–150	2.30 ± 0.02
150–212	2.48 ± 0.01

Transport was from the apical chamber to the basolateral chamber. Loaded P(MAA-g-EG) microparticles with an initial monomer feed ratio MAA:EG of 1:1 and a PEG molecular weight of 1000 were used in this experiment. 20 mg of microparticles were in contact with Caco-2 cell monolayers with a passage number of 78, at 37°C. Error bars represent one standard deviation, $n = 3$.

P(MAA-g-EG) microparticle on the insulin transport through the Caco-2 cell monolayer. Figure 5 shows the cumulative amount of insulin released from P(MAA-g-EG) microparticles of size (25–90, 90–150 and 150–212 μm). There were no statistical differences on the cumulative insulin increase between the various insulin-loaded microparticles studied. However, the 25–90 μm insulin-loaded microparticles showed a statistically higher cumulative insulin increase. Table 4 shows the insulin permeability coefficients from the different P(MAA-g-EG) microparticle sizes.

DISCUSSION

Cell tight junctions are dynamic protein complexes that hold adjacent cells together and impede transepithelial diffusion of solutes between cells. Different approaches have been investigated in order to induce paracellular transport of large molecules, such as proteins, by disrupting the tight junctions [10–16]. As tight junctions are calcium dependent, in the absence of Ca^{2+} these complexes are disrupted. Peppas and Madsen [6] have shown the ability of P(MAA-g-EG) microparticles to bind Ca^{2+} , inhibiting the proteolytic activity of calcium-dependent enzymes such as trypsin. Clearly, Ca^{2+} is bound to P(MAA-g-EG) microparticles because of the presence of negatively charged carboxylic acids on the MAA repeating units at physiological conditions.

The integrity of tight junctions can be monitored by measuring the transepithelial electrical resistance. A decrease in this value indicates a disruption in the tight junctions, which would allow the paracellular transport of the drug. In the present study insulin-loaded P(MAA-g-EG) microparticles were kept in contact with the Caco-2 cell monolayers. Figures 1 and 2 show that the effect of the insulin-loaded microparticles in the TEER reduction is reversible. The first hour shows the equilibration period, after the DMEM media was replaced with the HBSS experimental medium. Torres-Lugo *et al.* [19] previously reported the existence of a second steady state because of an initial drop on the TEER value during the equilibration period. After this period, the microparticles were placed in contact with the monolayer for 3 h. Then the microparticles were removed the experimental medium was replaced with DMEM and the TEER values were recovered in a period of 3 h. These results indicate that the insulin-loaded microparticles causes a reversible change in the integrity of the cell monolayer, probably by opening tight junctions and creating conditions of paracellular transport.

The decrease in the TEER value was dependent on the amount of P(MAA-g-EG) microparticles in contact with the Caco-2 cell monolayer. Decreases in TEER of up to 50% are attributed to the ability of the MAA carboxylic acids to bind Ca^{2+} causing the opening of the tight junctions. However, the molecular weight of the PEG tethered chains did not have an effect on the extent to which the TEER values were decreased [33–38].

The insulin released from the polymer carrier and transported across the Caco-2 cell monolayer was monitored as a function of time. The permeability values for the insulin transport from the apical to the basolateral chamber were similar to the ones from the basolateral to the apical chamber and in general agreement with previous studies of our lab [37, 38]. Thus, we believe that active carriers or efflux transporters were not involved in insulin transport through the Caco-2 cell monolayer.

P(MAA-g-EG) microparticles were able to enhance the transport of insulin through the Caco-2 cell monolayers. Clearly, our studies indicate that these systems are a good candidate for oral delivery of insulin. However, for optimal P(MAA-g-EG) characteristics for the oral delivery of insulin, both moderate mucoadhesion

by PEG tethers and low hindrance effects them so their transport barriers should be optimized.

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