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Assessment of Poly(Methacrylic Acid-co-N-Vinyl Pyrrolidone) as a Carrier for the Oral Delivery of Therapeutic Proteins Using Caco-2 and HT29-MTX Cell Lines

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Abstract

Hydrogels of poly(methacrylic acid-co-N-vinyl pyrrolidone) were synthesized and evaluated for their use as carriers for oral protein delivery. Insulin loading efficiencies were determined to be near 90% for carriers crosslinked with ethylene glycol dimethacrylate with corresponding weight incorporation levels near 12%. Although no insulin was released in gastric conditions, as desired, near instantaneous release occurred when the pH was raised to values typical of the intestinal area. Cytocompatibility studies with Caco-2 and Caco-2/HT29-MTX cultures demonstrated that microparticles did not elicit toxic effects at concentrations up to 5.0 mg/mL. Insulin transport studies revealed that the carriers did not disrupt the cell layer and thus did not change the insulin permeability in the apical-to-basolateral direction. Therefore, microparticles of this system were best suited for oral delivery of therapeutic agents that do not require transport facilitation.

Keywords

hydrogels; insulin; oral delivery; cytocompatibility; cell culture

INTRODUCTION

The oral delivery of proteins is the preferred route of drug administration because of its avoidance of the fear¹ and pain² associated with injection therapy. In addition to the increase in patient compliance by providing a non-invasive delivery option, oral delivery is the most physiologically relevant delivery route by closely resembling the physiological uptake of other entities.

pH-Sensitive complexation hydrogels have been investigated as potential carriers for oral protein delivery due to their ability to respond to the pH shift between the stomach and small intestine, protecting the protein from release and degradation in the stomach and facilitating release in the small intestine. The protection of the drug in the stomach in these systems is a

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result of the collapsed network and the presence of hydrogen bonding complexes between compatible functional groups.

One of the most extensively investigated hydrogel systems for the oral delivery of proteins is a copolymer of methacrylic acid with grafted poly(ethylene glycol) (PEG) chains, designated as P(MAA-g-PEG) ^{3,4}. The system is an anionic hydrogel and is swollen in intestinal conditions but remains collapsed in the gastric environment. Poly(ethylene glycol) is incorporated for both its high level of biocompatibility and its ability to form hydrogen bonding complexes with methacrylic acid ⁵. The combination of methacrylic acid with PEG tethers has demonstrated mucoadhesive characteristics and improved retention times in the small intestine ⁶.

There are limitations to the P(MAA-g-PEG) system that could be improved upon in order to develop an optimal system for oral protein delivery. The presence of PEG in the hydrogel has been shown to delay protein release upon reaching intestinal pH levels ⁷ and P(MAA-g-PEG) does not provide full protection in gastric pH levels where drug release should be inhibited ^{8,9}. Furthermore, Moriyama et al. ¹⁰ have reported that insulin partitioned into a PEG phase at physiological pH levels in the presence of a negatively charged polyelectrolyte. Although there have been a great number of successes reported with the P(MAA-g-PEG) hydrogel system and others in the literature, these limitations demonstrate that it is imperative to continue the search for materials that complex more effectively, provide better protection from enzymatic degradation and premature release, and improve pharmacological availability so that the development of an optimal carrier for the oral delivery of therapeutic proteins may be achieved.

In this work, hydrogel microparticles of random copolymers of methacrylic acid (MAA) and N-vinyl pyrrolidone (NVP) were studied as potential carriers for use in oral delivery of therapeutic proteins. The strength and stability of complexes between MAA and NVP have been widely reported ^{11,12}, but little work has been done to demonstrate the potential use of these complexes for drug protection. In this work, our hypothesis was that the elimination of PEG and the strength of the hydrogen bonding complexes between MAA and NVP would result in an improved system for oral protein delivery.

The first goal towards proving this theory was to determine the loading capability of therapeutic proteins into the P(MAA-co-NVP) microparticles through equilibrium partitioning by analyzing the loading efficiency and weight incorporation of insulin. The second goal was to analyze the release kinetics of insulin out of the hydrogels in simulated gastric and intestinal conditions. The final goal of this work was to determine the effects of the P(MAA-co-NVP) carriers on the gastrointestinal environment. *In vitro* compatibility studies were performed to ascertain the effects of the microparticles on cell viability. Additionally, *in vitro* transport studies were performed to determine whether or not the presence of the microparticulate systems enhanced insulin permeabilities.

MATERIALS AND METHODS

Hydrogel Synthesis

P(MAA-co-NVP) hydrogels were synthesized using a UV-initiated free radical polymerization. For P(MAA-co-NVP), the monomer mixture included methacrylic acid (MAA) (Sigma-Aldrich, St. Louis, MO) and N-vinyl pyrrolidone (NVP) (Sigma-Aldrich). Two separate crosslinking agents were used: ethylene glycol dimethacrylate (EGDMA) (Acros Organics, Morris Plains, NJ) and tetra(ethylene glycol) dimethacrylate (TEGDMA) (Fluka/Sigma-Aldrich). All components were used as received.

The molar feed ratios of MAA:NVP for the copolymers synthesized were 1:1, 2:1, 4:1, 1:2 and 1:4. The crosslinking ratio, whether using EGDMA or TEGDMA, was 1.0 mol % of the total monomer content. Irgacure[®] 184 (1-hydroxycyclohexyl phenyl ketone, Sigma-Aldrich) was added as a photo-initiator in the amount of 1.0 wt % of the total monomer content. The solvent makeup consisted of a 50:50 w/w water and ethanol mixture and was added to yield a 50:50 w/w total monomer to solvent ratio.

The components were prepared in an amber bottle and briefly sonicated (Bransonic[®] 8510, Branson Ultrasonics Corp., Danbury, CT) to yield homogeneity. The mixture was placed in a sealed glove box and bubbled with nitrogen for 20 min to remove oxygen from the environment. The mixture was then pipetted between two glass plates (150 × 150 mm²) separated by a Teflon spacer (0.7 mm thick) and exposed to UV light (Dymax 2000 Light Curing System, Torrington, CT) at an intensity of 16–17 mW/cm² for 30 min to yield the polymer film. Following synthesis, the film was removed from the glass plates and washed with deionized water (changed daily) for 7 days to remove unreacted components. The washed film was vacuum dried at 30°C for 48 hours and crushed using a mortar and pestle. Sieves were used to yield two size ranges of particles – 90–150 µm and <75 µm. The particles were stored in a desiccator until use.

Insulin Loading

A stock solution of bovine pancreatic insulin (Sigma-Aldrich) was prepared at a concentration of 0.5 mg/mL using 30 mg of insulin and 8 µL 0.5M ethylenediamine tetraacetic acid (EDTA) solution (Fisher Scientific, Fair Lawn, NJ) in 60 mL 1X PBS (diluted from 10X, Fisher Scientific). The solution was kept at 37°C until the solution was clear. The pH of the solution prior to particle addition was approximately 7.4. Sigmacote (Sigma-Aldrich, St. Louis, MO) was used on all glassware to prevent particle and protein adsorption during loading.

P(MAA-co-NVP) microparticles (size range: <75 µm), in the ratio of 3.5 mg particles : 1 mL stock solution, were added to the stock solution and allowed to equilibrate for 4h. After equilibration, 600 µL 1N HCl were added to the solution to lower the pH and collapse the microparticles. The collapsed microparticles were collected by filtration (Whatman Grade 54 filter paper, Whatman International Ltd., Maidstone, England) via water aspiration. The particles were washed with 50 mL 0.1N HCl and 10 mL deionized water, collected into a 50 mL centrifuge tube and frozen at –80°C. The frozen particles were lyophilized to yield a dry powder (Labconco FreeZone 4.5 Liter Benchtop Freeze Dry System, Kansas City, MO). The prepared particles were placed in a desiccator and stored at –20°C until needed.

To determine the amount of protein loaded into the particles, four samples were drawn during the course of the procedure. The first was prior to the addition of particles to the stock solution; the second was following loading but prior to particle collapse; the third was after the particles were collapsed; and the final was from the filtration collection flask following particle washing. For each sample point, 0.2 mL were drawn and collected in HPLC vials (300 µL polypropylene screw neck vials with Lectrabond cap and PTFE/silicone septa, Waters Corp., Milford, MA) following filtration with a 0.22 µm PVDF filter (Millipore Millex-GV filter unit, Bedford, MA). HPLC analysis was performed on a Waters 2695 Alliance separations module (Waters Corp., Milford, MA) equipped with a Waters 2487 Dual λ Absorbance detector (Waters Corp.) with a Symmetry300[™] C4 column (particle size = 5 µm; dimensions = 3.9 mm ID × 150 mm length, Waters Corp.). The mobile phase was 70% water (0.1% trifluoroacetic acid)/30% acetonitrile (0.08% trifluoroacetic acid) with a 20 µL injection volume.

Insulin Release under Dynamic Conditions

Insulin release from the microparticles was evaluated by studies conducted using United States Pharmacopeia (USP) 28 Apparatus 2 (Distek Dissolution System 2100B; North Brunswick, NJ) with overhead impellers operating at 100 rpm and a bath temperature of 37°C.

To simulate the transition between the stomach and small intestine, release studies were performed in a dynamic environment where the pH was raised from 3 to 7 after 1h. 15 mg of microparticles were added to 25 mL 1X PBS at pH 3.0. Samples were drawn over the course of 1h. Immediately after collecting and filtering the samples at 60 min, 80 µL of 5N NaOH were added to each release vessel to raise the pH to approximately 7.0. Samples were then collected for an additional 2h. Buffer for the low pH and high pH conditions was pre-warmed and added following sampling to keep the volume in the release vessels constant. The vials were analyzed via HPLC using the previously described method.

Preparation of Cell Cultures

Caco-2 cells (American Type Culture Collection, Rockwell, MD) and HT29-MTX cells, a kind gift from Dr. Thecla Lesuffleur, INSERM, Paris, France, were utilized in this research. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Mediatech, Herndon, VA) containing 4.5 g/L glucose, L-glutamine and sodium pyruvate. The media was supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Cambrex, East Rutherford, NJ), 1% non-essential amino acids (Mediatech) and an antibiotic/antimycotic solution consisting of 100 U/mL penicillin, 100 µg/mL streptomycin and 25 µg/mL Amphotericin B (Mediatech).

Cells were cultured in T-75 flasks (Corning Inc., Corning, NY) and maintained at 37°C in a humidified environment containing 5% CO₂ in air. Cell media was changed every other day and passage operations were performed once the cells reached 80–90% confluency. Caco-2 cells were seeded at a density of 3.0×10^3 cells/cm² and HT29-MTX cells were seeded at a density of 2.0×10^4 cells/cm². Caco-2 cells were used between passages 60 and 80, and HT29-MTX cells were used between passages 8 and 20.

Microparticle Cytocompatibility

The Caco-2 monoculture and Caco-2/HT29-MTX co-culture were used for cytocompatibility experiments. The cells were seeded on polystyrene 96 well plates (Corning). The Caco-2 cell seeding density was 1.4×10^4 cells/cm² and the HT29-MTX cell seeding density was 2.8×10^4 cells/cm². Media was changed every other day prior to experimentation.

For a given hydrogel composition, microparticles were suspended in Hank's Balanced Salt Solution (HBSS) with Ca²⁺ and Mg²⁺ at a concentration of 5.0 mg/mL. The pH of the suspension was brought to 7.4 by addition of 1N NaOH. Microparticle concentrations of 0.5, 1.0 and 2.5 mg/mL were prepared by serial dilution. For the 4:1 and 1:4 polymers, a suspension was also prepared at 10.0 mg/mL to examine the effect of an abundance of the individual monomers on cell viability. The suspensions were heated to 37°C prior to introduction to the cell culture.

In a sterile environment, growth medium was removed from the 96 well plate and replaced with 200 µL of the suspended particles. Control wells were prepared as described but were not exposed to microparticles. The 96-well plates with cells and microparticle solutions were incubated for 2h at 37°C and 5% CO₂. Following incubation, the microparticle suspensions were removed from each well and the wells were rinsed three times with 200 µL of HBSS.

A colorimetric assay (CellTiter 96[®] AQueous One Solution Cell Proliferation Assay, Promega Corp., Madison, WI) was used to determine cell viability following incubation with the microparticle suspensions. 3.0 mL of the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) solution were added to 15.0 mL of HBSS. 120 µL of this solution were added to each well and incubated for 90 min at 37°C and 5% CO₂. Following incubation, the absorbance of each well was measured at a wavelength of 490 nm using a plate reader (Synergy[™] HT Multi-Mode Microplate Reader, BioTek Instruments, Inc., Winooski, VT). The percentage of viable cells following incubation with microparticles was quantified by Equation (1):

$$\% \text{ Viable Cells} = \frac{A_s}{A_c} \times 100\% \quad (1)$$

where A_s was the absorbance recorded from sample wells and A_c was the absorbance recorded from control wells.

Caco-2 Transwell[®] Culture

Insulin transport studies and transepithelial electrical resistance (TEER) measurements were performed with Caco-2 cells seeded on Costar Transwell[®] plates (Corning) with a polycarbonate membrane and 0.4 µm pore size. Transwell[®] 6-well plates were utilized, with each well possessing a cell growth area of 4.7 cm². Cells were seeded at a density of 6.0×10^4 cells/cm² and cultured for 21–24 days. Cell media was changed every other day and the plates were incubated at 37°C and 5% CO₂.

TEER measurements were taken 1h after the cell media was changed to allow for equilibration of the temperature to 37°C. Prior to cell seeding, a TEER measurement was taken of each well in the presence of media to serve as a blank reading. To determine the resistance across the cell monolayer, R_m , Equation (2) was used:

$$R_m = R_e - R_b \quad (2)$$

where R_e was the experimentally measured resistance and R_b was the resistance of the blank well. TEER measurements were taken with an EVOM volt-ohm meter with a chopstick electrode (World Precision Instruments, Sarasota, FL). All TEER measurements were taken with the cell culture plates placed on a heating mat to maintain the temperature at 37°C.

Insulin Transport

The amount of insulin transported across a cellular monolayer was investigated using Caco-2 Transwell[®] cultures, prepared as previously described. Following 21–24 days of monolayer growth, the media was removed from the apical and basolateral chambers and replaced with HBSS containing Ca²⁺ and Mg²⁺ (1.5 mL in the apical chamber; 2.5 mL in the basolateral chamber). The cells were equilibrated with HBSS for 1h in a 37°C and 5% CO₂ environment with TEER measurements taken at 0, 0.5 and 1h.

Bovine pancreatic insulin (Sigma-Aldrich) solutions were prepared in HBSS at a concentration of 0.2 mg/mL. Bovine serum albumin (Sigma-Aldrich) was added to the insulin/HBSS solution to prevent insulin adsorption to the Transwell[®] plates. The final protein concentration in solution was 0.5 mg/mL. Microparticle suspensions were prepared with P(MAA-co-NVP) microparticles, all sized <75 µm, at a concentration of 1.0 mg/mL. All solutions were preheated to 37°C prior to addition to the apical chamber.

Following the 1h equilibration with HBSS, the contents of the apical chamber were removed and replaced with 1.5 mL of sample. Insulin/BSA/HBSS solution without microparticles was added to control wells. 100 μ L samples were taken from the apical chamber at 0 and 3h and from the basolateral chamber at 0, 1, 2 and 3h. The volumes in each chamber were held constant throughout the experiment by addition of a preheated solution consisting of 0.5 mg/mL BSA in HBSS. To evaluate the effect of the microparticles on the cellular monolayer, TEER measurements were taken at each sample timepoint. After 3h, the chambers were evacuated and washed 2X with HBSS. Cell media was added to the wells and the plates were returned to the incubator for recovery, with TEER readings taken at 24h. All samples were kept at 4°C until evaluated. Insulin transport was evaluated through use of a bovine insulin ELISA kit (Mercodia AB, Uppsala, Sweden).

RESULTS AND DISCUSSION

Insulin Loading

The loading efficiency for insulin was calculated using the following relationship where C_o and C_f are the initial and final protein concentrations in solution (Equation (3)). The weight incorporation was calculated by Equation (4) where m_o is the initial mass of insulin in solution, m_f is the mass remaining in solution following particle washing, and m_p is the mass of microparticles added to the stock solution for loading.

$$\text{Loading Efficiency} = \frac{C_o - C_f}{C_o} \times 100\% \quad (3)$$

$$\text{Weight Incorporation} = \frac{m_o - m_f}{m_o - m_f + m_p} \times 100\% \quad (4)$$

As shown in Figure 1, the loading efficiency for all P(MAA-co-NVP) microparticles that contained 1% EGDMA crosslinking was high and relatively constant in the range of 85–95%. Weight incorporation values were seen in the range of 11–12% for these particles. The system with the highest loading efficiency and weight incorporation was P(MAA-co-NVP) 1:1 1% EGDMA with respective values of $93.1 \pm 2.3\%$ and $11.7 \pm 0.2\%$; the lowest efficiencies were seen with the P(MAA-co-NVP) 1:2 1% EGDMA microparticles where respective values were $87.9 \pm 0.3\%$ and $11.2 \pm 0.04\%$.

Results for the comparison of the loading efficiency and weight incorporation between particles containing 1% EGDMA and 1% TEGDMA are also shown in Figure 1. For the 1:1 MAA:NVP system, loading efficiency decreased from $93.1 \pm 2.3\%$ to $74.7 \pm 0.6\%$ as the length of the crosslinker increased. The weight incorporation also decreased, with values dropping from $11.7 \pm 0.2\%$ to $9.53 \pm 0.1\%$. Opposite trends were seen with the 4:1 MAA:NVP formulation, with loading efficiency increasing from $91.4 \pm 6.3\%$ to $96.65 \pm 0.2\%$ and weight incorporation increasing from $11.5 \pm 0.8\%$ to $12.1 \pm 0.1\%$. This reversal of trends can be attributed to the ionic nature of the materials.

The equimolar ratio formulation has a much lower number of negative charges than the formulation containing more MAA, so the positively charged insulin will be more inclined to bind with the copolymer prepared with the 4:1 monomer molar feed ratio. The negative charges present in the P(MAA-co-NVP) systems are important because they not only affect the loading of charged molecules like insulin, but they are also responsible for the swelling

of the gel above the pK_a of MAA. The loose nature of the network in the TEGDMA-crosslinked materials has an effect on the loading capability of the systems with a more neutral charge as there are fewer attractions between the drug and polymer. As the particles start collapsing due to the addition of HCl, the TEGDMA-crosslinked particles will reach a complexed state slower than the EGDMA-crosslinked particles, so there is more time for the drug to diffuse back out of the network, leading to lower loading efficiencies.

Insulin Release under Dynamic Conditions

It was important to investigate the release behavior of the systems at low pH and high pH as oral delivery systems will pass through both conditions. The transition of the gel from low pH to high pH was analyzed to determine the responsiveness of the gel to a pH shift that would be relevant *in vivo* as the microparticles pass from the stomach to the small intestine.

It must be noted that these studies were a derivation from standard FDA and USP procedures as no protocols specify the detailed change of the pH solution presented here. However, we felt that, for complete understanding and simulation of the conditions in the gastrointestinal tract, our detailed dynamic analysis of protein release was necessary.

Insulin release is reported as the amount released at a given time normalized with respect to the amount released at long times, M_t/M_∞ . As shown in Figure 2, the P(MAA-co-NVP) formulations exhibited an “on-off” release behavior, indicating that minimal to no protein is being released in acidic conditions and rapid release occurs when the pH is increased to neutral and basic levels.

The absence of release at low pH conditions may be attributed to the small mesh size of the network and the complexation effects due to the hydrogen bonding between MAA and NVP. As predicted, these materials inhibited release much better than the P(MAA-g-PEG) system. Wood et al.⁹ have shown release at low pH to be upwards of 10% within the first hour.

The ability to inhibit drug release in gastric conditions is a *crucial component* to the development of an oral delivery system. By maintaining all of the insulin within the network, no drug was being released into the degradative environment of the stomach and more was available for release in the small intestine, which should lead to an increased bioavailability.

Microparticle Cytocompatibility

Studies proved that there was no statistically significant decrease in the viability of the Caco-2 cells when incubated with P(MAA-co-NVP) microparticles. As shown in Figure 3, the percentage of viable cells relative to control wells that did not contain microparticles was near 100% for all formulations tested up to a concentration of 5.0 mg/mL. In the development of this novel biomaterial, the determination that the presence of the microparticles has a limited effect on the cell's metabolic activity is an encouraging result for the applicability of these microparticles in an *in vivo* environment.

The results are similar to the cytocompatibility results for the P(MAA-g-PEG) system at these concentrations by Blanchette and Peppas¹³. At a concentration of 10.0 mg/mL, the P(MAA-co-NVP) 4:1 and 1:4 systems led to a slight decrease in the percentage of viable cells. This result was also seen by Peppas and collaborators^{14,15} when P(MAA-g-PEG) microparticles were incubated at this concentration. This decrease, noted between two different hydrogel systems that are otherwise biocompatible, indicates that there is a maximum particle dose that should be administered orally to not elicit toxic effects.

Cytocompatibility experiments were also performed on the Caco-2/HT29-MTX co-culture to determine whether or not the particles had any effect on a more accurate intestinal model. As shown in Figure 4, the presence of the P(MAA-co-NVP) 1:1 and 1:4 systems did not decrease co-culture cell viability at concentrations up to 5.0 mg/mL. A decrease in cell viability in the presence of the 4:1 MAA:NVP system was detected, however. Foss and Peppas¹⁶ determined that P(MAA-g-PEG) microparticles at a 4:1 ratio also had an effect on cell viability. This was attributed to the increased presence of the deprotonated carboxylic acid group on MAA.

The negative charges on the carboxylic acid group of MAA are capable of binding extracellular Ca^{2+} , an ion that is important for normal cell function as pointed out by Orrenius et al.¹⁷ and Paschen¹⁸. Since the 4:1 system possessed more negative charges, it led to a decrease in cell viability. While long-term exposure of calcium-binding entities could result in cell death, short-term binding of calcium has advantages for oral protein delivery. Calcium binding leads to the opening of tight junctions, which could ultimately lead to an increased protein bioavailability during epithelial transport¹⁹. Madsen and Peppas²⁰ previously showed that Ca^{2+} binding also inhibited the effects of trypsin, which could result in less protein degradation within the gastrointestinal tract. The minimal toxicity of these carriers to both Caco-2 and HT29-MTX cells suggests that these carriers are suitable for oral delivery applications.

Caco-2 Transwell® Culture

The cell culture model utilized in the insulin transport studies was the Caco-2 monoculture. While among the most heavily investigated for epithelial transport experiments²¹, this monoculture has its limitations as an intestinal model. For example, the TEER values for the cultures were anticipated to be greater than those of either the co-culture or human intestinal epithelia. The TEER values of the Caco-2 culture leveled out to approximately $2200 \Omega\text{cm}^2$ by 21 days. These are in line with reported values in the $1400\text{--}2400 \Omega\text{cm}^2$ range by Hilgendorf et al.²² for Caco-2 cells.

The values in this work and those of Hilgendorf et al.²² are higher than TEER values often seen with Caco-2 cultures²³. However, Behrens and Kissell²⁴ have demonstrated that cell culture conditions can lead to changes in expressed features and TEER values from lab to lab. The small intestinal TEER is estimated to be $25\text{--}40 \Omega\text{cm}^2$ ²⁵, indicating that there is significantly more resistance in the cultures used in these experiments than what would be present *in vivo*. An increase in TEER translates to a decrease in overall therapeutic permeability, thus the Caco-2 monoculture should demonstrate lower drug permeabilities than the Caco-2/HT29-MTX co-culture and the intestinal epithelium.

Insulin Transport

Insulin transport studies were conducted to determine if the presence of P(MAA-co-NVP) microparticles had an effect on the intestinal passage of insulin in the apical-to-basolateral direction. A control study was simultaneously performed with an insulin solution that did not contain microparticles in the apical chamber for comparison purposes. Disruption of the epithelial cells was monitored by TEER evaluation and insulin concentrations were determined via a bovine insulin specific ELISA.

Monitoring the TEER values during the transport study provided insight into insulin transport. As shown in Figure 5, there was no significant decrease in TEER values relative to the controls for any of the microparticle systems tested. Lopez and Peppas²⁶ showed that a decrease in TEER values during exposure of Caco-2 cells to P(MAA-g-PEG) microparticles corresponded to an increase in insulin permeability values. Kavimandan et al.

²⁷ confirmed this finding with the delivery of insulin-transferrin conjugates in the presence of P(MAA-g-PEG) microparticles.

ELISA analysis confirmed that insulin transport was not significant in the presence of P(MAA-co-NVP) microparticles. An analysis of the cumulative amount of insulin in the basolateral chamber demonstrated that insulin was being transported in similar quantities as seen with the control wells, or actually decreasing relative to control. The latter could be due to insulin loading into the microparticles in the apical chamber. Blanchette and Peppas ¹⁹ reported on the possibility of drug imbibition under similar transport study conditions to those performed in this project with the delivery of bleomycin, a small molecular weight drug investigated for oral chemotherapy. This effect may be eliminated during *in vitro* and *in vivo* testing by utilizing drug loaded particles instead of drug in the presence of particles as the direction for diffusion will be reversed.

One possible reason that insulin transport was low was that there was no cellular disruption in the presence of the P(MAA-co-NVP) formulations. It was evident through the cytocompatibility studies that formulations containing an increased molar feed amount of MAA disrupted the cells and resulted in a decrease in viability. However, this was only pronounced when the microparticle concentration was increased. It has been reported by Torres-Lugo et al ²⁸, that the P(MAA-g-PEG) system can readily disrupt the epithelial cells and facilitate transport. This was attributed to the PEG, not the MAA, as formulations containing higher amounts of PEG resulted in improved transport.

A promising result from the insulin transport studies was the recovery (return to the original values) of TEER values following incubation with the microparticles. As shown in Figure 6, TEER values of the cells exposed to P(MAA-co-NVP) microparticles returned to similar levels as the cells utilized in the control wells that did not come into contact with the polymers following a 24h recovery period. These results suggest that the system could be utilized for drug delivery applications where an active transport mechanism would be utilized, such as the delivery of insulin-transferrin bioconjugates, or when no transport facilitation was needed without causing any long term damage to the cells.

CONCLUSIONS

Insulin could be readily loaded into P(MAA-co-NVP) microparticles. For systems containing 1% EGDMA crosslinking, the loading efficiency was greater than 85% and the weight incorporation was greater than 10%. By changing the crosslinker, loading efficiencies dropped for the P(MAA-co-NVP) system with a 1:1 monomer molar feed ratio and 1% TEGDMA crosslinking in comparison to the equimolar system with 1% EGDMA crosslinking because of the larger interchain dimensions available for continued diffusion out of the network during particle collapse. This phenomenon did not affect the copolymer with the 4:1 monomer molar feed ratio as the positively charged drug at pH 5.1 was attracted to the presence of negative charges in the network.

At low pH, the formulations did not release any detectable concentration of insulin, allowing for more drug to be available once reaching the higher pH level of the small intestine. The materials responded rapidly to an increase in pH, demonstrating “on-off” release behavior from the networks.

Finally, the effects of P(MAA-co-NVP) microparticles on the cellular environment were studied. It was determined that P(MAA-co-NVP) microparticles had a minimal effect on the viability of the cell cultures up to a concentration of 5.0 mg/mL. However, the cell viability decreased when the concentration was raised to 10.0 mg/mL. These studies led to the

conclusion that the system could be used *in vivo* at lower concentrations without eliciting any long-term damage on the cellular environment.

It was determined by insulin transport studies that the system did not result in cellular disruption and therefore did not facilitate paracellular transport. This finding suggests that the presence of PEG in the P(MAA-g-PEG) system was the key component for disrupting the cells to yield epithelial transport. The transport studies did show that any effects on the cells caused by the presence of P(MAA-co-NVP) were reversible as TEER values recovered following exposure.

In summary, P(MAA-co-NVP) was shown to be an effective system for the delivery of proteins in that there was no release or degradation of the drug in gastric conditions, and release occurred rapidly within a time frame consistent with targeted delivery to the upper small intestine. Cell studies demonstrated that the biomaterial did not disrupt the cells enough to improve protein transport via the paracellular route, but did show that the materials were cytocompatible. These findings suggest that the carrier could be ideal for the oral delivery of an agent that is transported by a transcellular mechanism.

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Figure 1.

Comparison of loading efficiency (left axis and columns) and weight incorporation (right axis and columns) of insulin in P(MAA-co-NVP) microparticles of various monomer molar feed amounts (<75 μm particle size). * 1% EGDMA crosslinking; ** 1% TEGDMA crosslinking (n=3–6 $\pm$ SD).

Figure 2.

Insulin release from P(MAA-co-NVP) microparticles, various monomer molar feed ratios, in 1X PBS in dynamic pH conditions (<75 μm particle size). pH was raised from 3 to 7 after 1h by addition of 5N NaOH ($n=3 \pm \text{SD}$). 1% EGDMA crosslinking: 1:1 (◆), 2:1 (▲), 4:1 (■), 1:2 (●); 1% TEGDMA crosslinking: 1:1 (×), 4:1 (+).

Figure 3. Cytocompatibility of P(MAA-co-NVP) microparticles, 1% EGDMA crosslinking, with Caco-2 monolayers at various monomer molar feed ratios and microparticle concentrations (particle size 90–150 μm). Monomer molar feed ratio: 1:1 (◆), 4:1 (■), 1:4 (▲)

Figure 4. Cytocompatibility of P(MAA-co-NVP) microparticles, 1% EGDMA crosslinking, with Caco-2/HT29-MTX co-culture at various monomer molar feed ratios and microparticle concentrations (particle size 90–150 μm). Monomer molar feed ratio: 1:1 (◆), 4:1 (■), 1:4 (▲).

Figure 5. Percent change from initial TEER value during incubation of P(MAA-co-NVP) microparticles, 1% EGDMA, with Caco-2 cells during insulin transport studies (particle size $<75\ \mu\text{m}$). Control wells (◆); Monomer molar feed ratio: 1:1 (■), 4:1 (▲), 1:4 (×) ($n=3 \pm \text{SD}$)

Figure 6. TEER values after 24h recovery period following insulin transport studies. Values are shown as a percentage of the recovery values seen with control wells.