

# In Vitro Investigation of Oral Insulin Delivery Systems Using Lectin Functionalized Complexation Hydrogels

*Kristy M. Wood, Gregory M. Stone and Nicholas A. Peppas*

Departments of Chemical and Biomedical Engineering, University of Texas at Austin, Austin, TX 78712,

## Abstract

The main advantage of oral protein delivery is that it improves patient compliance and comfort over other routes of administration (i.e. injection), thus leading to a more effective treatment regimen. Although oral protein delivery could benefit many individuals, maintaining the functionality of the protein and low bioavailability of the delivered drug have prevented it from becoming a successful therapy. We have developed a class of environmentally responsive complexation hydrogels composed of methacrylic acid and grafted ethylene glycol chains (P(MAA-g-EG)) functionalized with wheat germ agglutinin (WGA) to overcome these challenges. The drug carriers were designed to (1) minimize the effects of the harsh environment of the gastrointestinal tract and (2) target delivery of the protein drug to the upper small intestine by exploiting the pH shift between the stomach and the small intestine. In addition, functionalization of PEG chains with WGA will allow for specific binding to carbohydrate moieties present in the intestinal mucosa to improve residence time of the carrier at the delivery site. Hydrogel microparticles were prepared by UV-initiated free radical solution polymerization. PEG chains were then functionalized with WGA through a biotin-avidin interaction. Insulin, a model protein, was used to determine if the functionalization process affected the loading and release behavior of the microparticles. Insulin entrapment in the polymer network was unaffected by the WGA functionalization and loading efficiency was determined to be 75% in both functionalized and unfunctionalized microparticles. A release study was done to mimic the conditions of the pH shift between the stomach and the small intestine. The hydrogel carriers prevented release at a low pH (3.2) and rapidly released insulin when the pH was increased to 7.0. In vitro mucoadhesive characteristics of the functionalized polymer were evaluated using a mucin coated 96-well plate. As expected, WGA functionalized microparticles displayed a higher adhesion to the mucin than non-functionalized microparticles. In addition, a competitive carbohydrate assay was used to demonstrate that there was a specific interaction between the WGA and the carbohydrate groups present within the mucus layer. Insulin transport studies were carried out in both the presence and absence of microparticles by using a mucus secreting co-culture of Caco-2 and HT29-MTX cells seeded on a permeable Transwell® plate. The cellular model was designed to more accurately mimic the small intestinal epithelia by inclusion of a mucus secreting cell. Apparent permeability ( $P_{app}$ ) increased for wells with P(MAA-g-EG) ( $15.01 \pm 0.65 \times 10^{-9}$  cm/s) and P(MAA-g-EG) WGA ( $15.20 \pm 1.43 \times 10^{-9}$  cm/s) as compared to an insulin only solution ( $2.98 \pm 0.27 \times 10^{-9}$  cm/s) ( $p < 0.01$ ), which correlates to approximately a 5-fold increase in  $P_{app}$ . Functionalizing complexation hydrogels with WGA improved the mucoadhesive properties of this polymer. In addition, the presence of the microparticles increased the amount of insulin transported across a cellular monolayer. Future work is focused on elucidating the exact mechanism by which insulin transport is increased across the cellular monolayer.

## Introduction

Hydrogels are three dimensional polymer networks that are insoluble due to the presence of physical and/or chemical crosslinks<sup>1</sup>. In addition, hydrogels are often used in biomedical applications due to their biocompatibility and ability to imbibe large amounts of water. Complexation hydrogels are a specific class of hydrogels that form physical crosslinks through non-covalent interactions, such as hydrogen bonding<sup>2</sup>. Our lab has successfully developed a class of environmentally sensitive complexation hydrogels containing MAA and PEG tethers (designated as P(MAA-g-EG))<sup>3,4</sup>. More specifically, P(MAA-g-EG) is a pH responsive hydrogel that is capable of swelling and deswelling due to the formation of temporary physical crosslinks, or interpolymer complexes, between the PMAA pendant groups and the tethered PEG chains.

Previous research in our lab has investigated and characterized P(MAA-g-EG) with a 1:1 molar feed ratio and shown this system to be the most promising for oral protein delivery<sup>3-6</sup>. Lowman et al.<sup>5</sup> determined that polymers using a 1:1 molar feed ratio of MAA:EG and PEG chains of MW 1000 had the highest degree of complexation and swelled to the highest degree in the uncomplexed state. Kim et al.<sup>6</sup> further verified the complexation/decomplexation behavior of P(MAA-g-EG) at low and high pH by use of ATR-FTIR. The goal of this work was to synthesize the previously established system of P(MAA-g-EG) with a 1:1 molar feed ratio of MAA:EG and then functionalize these carriers with WGA.

## Materials and Methods

**Hydrogel Synthesis.** Hydrogels were prepared by UV-initiated free radical solution polymerization. The monomer mixture contained methacrylic acid (MAA) (Sigma-Aldrich, St. Louis, MO), poly(ethylene glycol) monomethylether monomethacrylate (PEGMMA, molecular weight 1000) (Polysciences, Warrington, PA), tetraethylene glycol dimethacrylate (TEGDMA) (Sigma-Aldrich), Irgacure 184® (1-hydroxycyclohexyl phenyl ketone) (Sigma-Aldrich), water, and ethanol. MAA was vacuum distilled at 54 °C and 25 mm Hg prior to use in order to remove the inhibitor (hydroquinone). All other components were used as received.

The monomers were mixed in a 1:1 molar feed ratio of MAA:ethylene glycol units, thus in a typical reaction 3.6 g of MAA and 2.0 g of PEGMMA were used. The crosslinker, TEGDMA, was added in the amount of 1.0 mol% of total monomers and Irgacure® 184 was added in the amount of 1.0 wt% of total monomers to initiate the reaction. The solvent was a 50:50 w/w mixture of water and ethanol and was added in a 50:50 w/w ratio of total monomer to solvent.

Monomers, crosslinker, initiator, and solvent were added to an amber bottle and sonicated for 15 minutes prior to polymerization. The monomer solution was then placed in a sealed glove box environment and purged with nitrogen for 20 minutes to remove oxygen, which is a free radical scavenger. After purging, the glove box remained sealed to ensure a nitrogen environment during polymerization. Two glass plates (15 cm x 15 cm x .3 cm) were separated by a 0.7 mm Teflon spacer and the monomer solution was carefully pipetted between the glass slides. The glass plates were exposed to UV light (Dymax 2000-EC Light Curing System, Torrington, CT) for 30 minutes at an intensity of 17.0 mW/cm<sup>2</sup>. The resulting polymer was removed from the glass plates and washed in deionized water for 7 days to remove any unreacted monomer. Drying of the polymer was done in a vacuum oven at 35 °C for 2 days. The polymer was then crushed and sieved into microparticles of various sizes. If polymer disks were desired, disks (1 cm diameter) were cut prior to drying the polymer. The subsequent polymer was composed of a MAA backbone with grafted PEG chains (P(MAA-g-EG)).

**Carrier Functionalization.** Hydrogel synthesis remained the same as previously described while the protocol for carrier functionalization took place in two distinct steps. P(MAA-g-EG) was functionalized with biotinylated-WGA (B-WGA) through a biotin-avidin linkage. The first step of the functionalization occurred before the polymerization, while the second step was performed after the gel was crushed into microparticles.

The first step of the reaction was to synthesize acryloyl-poly(ethylene glycol)-biotin (ACR-PEG-B) by previously established protocols<sup>7,8</sup>. Acryloyl-poly(ethylene glycol)-N-hydroxy succinimide (ACR-PEG-NHS, Molecular weight 3400) (Nektar Therapeutics, Huntsville, AL) was dissolved in 50 mM sodium bicarbonate buffer, pH 8.2, at a concentration of 86 mg/ml and reacted with EZ-Link® biotin-PEO-Amine (Pierce, Rockford, IL), which was separately dissolved in 50 mM sodium bicarbonate buffer, pH 8.2, at a concentration of 1 mg/ml. NHS esters react readily with primary amines, thus the reason that biotin functionalized with a single primary amine was selected.

ACR-PEG-NHS was added dropwise to the biotin solution to yield a 2:1 molar ratio of ACR-PEG-NHS:biotin-PEO-amine. The reaction was allowed to proceed for 2 h at room temperature, after which time the resulting product was placed in a -80 °C freezer for 30 minutes, lyophilized at -50 °C under vacuum (LabConco Model 77500, Kansas City, MO) for 2 days, and stored at -20 °C until use. The resulting dried product was ACR-PEG-B, which could then be added to the monomer solution before polymerization. The double bond functionality of the acryloyl group allows the functionalized

PEG chain to be incorporated in the polymer through free radical polymerization. A fluorescamine assay<sup>9</sup> was used to determine the efficiency of PEG functionalization with the biotin, which confirmed 90% of the PEG was functionalized with biotin.

During the course of the experiment, samples were taken of the supernatant and analyzed by HPLC (Waters 2695 Separations Module, Milford, MA) to determine avidin and B-WGA concentration. Decreases in the concentrations of avidin and B-WGA helped confirm that functionalization was occurring. Briefly, a mixture of 80% water/20% acetonitrile with 0.1% trifluoroacetic acid was used as the mobile phase in a Symmetry300<sup>TM</sup> C4 column. The 20  $\mu$ L samples were injected and a flow rate of 1 mL/min was used.

**Insulin Loading.** Insulin loading was done by equilibrium partitioning, consisting of a 0.5 mg/ml bovine pancreatic insulin (Sigma-Aldrich, St. Louis, MO) solution and 140 mg of polymer microparticles sized 90-150  $\mu$ m. A detailed protocol can be found in Appendix 1.4. P(MAA-g-EG) and P(MAA-g-EG) WGA were prepared as previously described. All glassware was siliconized by Sigmacote (Sigma-Aldrich) prior to use to minimize protein and particle adsorption to the glassware.

An insulin stock solution was made with 80% v/v 1X phosphate buffered saline (PBS), pH 7.4, 10% v/v 0.1 N HCl, and 10% v/v 0.1 N NaOH. The insulin was dissolved in the acidified buffer and the NaOH was then added to return the pH of the solution to 7.4. A 140 mg sample of polymer microparticles was added to 20 ml of the insulin stock solution and stirred for 2 hours at room temperature. Before the addition of microparticles, a 200  $\mu$ l sample was taken and filtered using a low protein binding 0.22  $\mu$ m PVDF filter (Millipore, Bedford, MA) and then replaced with equal amounts of 1X PBS, pH 7.4. An additional sample was taken in the same manner after loading. The particles were collapsed using 10 ml 0.1 N HCl, filtered with Whatman grade 4 filter paper, and washed with 20 ml of deionized water. After filtering, particles were frozen in a -80 °C freezer and lyophilized at -50 °C under vacuum (LabConco Model 77500) for 24 hours. Particles were stored at -20 °C until use.

Insulin concentration was determined using HPLC (Waters 2695 Separations Module, Milford, MA) analysis to calculate the loading efficiency. Briefly, a mixture of 70% water/30% acetonitrile with 0.1% trifluoroacetic acid was used as the mobile phase in a Symmetry300<sup>TM</sup> C4 column. The 20  $\mu$ l samples were injected and a flow rate of 1 ml/min. was used.

**Insulin Release.** Release studies were performed using a dissolution apparatus (Distek model 2100B, North Brunswick, NJ). A 10 mg sample of insulin-loaded microparticles was added to a siliconized vessel containing 50 ml of 0.1 M dimethylglutaric acid buffer (DMGA), pH 3.2. The solution was stirred at 100 rpm and maintained at 37 °C. After 60 minutes, pH was raised to 7.0 by the addition of 5 N NaOH. 0.5 ml samples were taken over the course of 3 hours and filtered using a 0.22  $\mu$ m PVDF filter. Samples were replaced with an equal volume of the appropriate pre-warmed buffer. Determination of insulin concentration was done using HPLC. Appendix 1.5 contains a detailed protocol.

**Coating Microplates with PGM.** Black, polystyrene 96-well plates (high protein binding capacity) (Thermo Electron, Milford, MA) were coated with pig gastric mucin Type II (PGM) (Sigma, St. Louis, MO). A 100  $\mu$ l sample of 0.1%, 1%, or 10% of a PGM-solution in PBS, pH 7.4, was added to each well of the 96-well plate and incubated for 24 hours at 4 °C. The PGM solution was removed and the plate was washed 3 times with 100  $\mu$ l of PBS. A 100  $\mu$ l sample of a 1% bovine serum albumin solution (Fraction V) (Sigma) in PBS was incubated with each well for 2 hours at 37 °C to block free binding sites. The BSA solution was removed and the plate was washed 3 times with 100  $\mu$ l of PBS. Plates were stored at 4 °C with 100  $\mu$ l of PBS in each well until use. Protocols for this study were similar to protocols established by Wirth et al.<sup>10</sup> for examining binding capacity of a variety of fluorescent lectins using PGM-treated microplates.

**PGM Binding Capacity of P(MAA-g-EG) and P(MAA-g-EG) WGA.** P(MAA-g-EG)-PolyFluor<sup>TM</sup> 407 and P(MAA-g-EG)-PolyFluor<sup>TM</sup> 407-WGA were synthesized as previously described. Microparticles were suspended in PBS, pH 7.4, at concentrations of 1.0 and 0.5 mg/ml. A 50  $\mu$ l sample of either 1.0 mg/ml or 0.5 mg/ml microparticles was incubated with a BSA-blocked 1% PGM plate for 2 hours at 37 °C. The initial fluorescent intensity of each well was determined by a microplate reader (Bio-Tek Synergy HT, Winooski, VT) at an excitation of 362 nm and an emission of 407 nm. The

microplate was then washed with 100  $\mu$ l of PBS to remove unbound microparticles. Fluorescent intensity of each well was again determined after washing at an excitation of 362 nm and an emission of 407 nm. Control wells were incubated with 50  $\mu$ l of PBS containing no microparticles. Autofluorescence of the coated wells was subtracted from each well by determining fluorescent intensity of the control wells.

The percentage of bound particles was determined as follows:

$$\% \text{ Particles Bound} = \frac{RFI_{AW}}{RFI_{BW}} \cdot 100$$

where  $RFI_{AW}$  is the fluorescent intensity after washing each well and  $RFI_{BW}$  is the fluorescent intensity before washing. Calculating the percentage of bound particles by this method eliminated any potential differences in the pipetting of microparticles into each well.

**Specificity of P(MAA-g-EG) and P(MAA-g-EG) WGA Binding.** P(MAA-g-EG)-PolyFluor<sup>TM</sup> 407 and P(MAA-g-EG)-PolyFluor<sup>TM</sup> 407-WGA were synthesized as previously described. Microparticles were suspended in PBS, pH 7.4, at concentrations of 1.0 and 0.5 mg/ml. A 50  $\mu$ l sample of either 1.0 mg/ml or 0.5 mg/ml microparticles was incubated with a BSA-blocked 1% PGM plate for 1 hour at 37  $^{\circ}$ C. After 1 hour, 50  $\mu$ l of a serial dilution of N,N',N''-triacetyl-chitotriose (1.0 – 0.0625 mg/ml) was added to each well and incubated for 1 hour at 37  $^{\circ}$ C. The microplate was then washed 2 times with 100  $\mu$ l PBS to remove N,N',N''-triacetyl-chitotriose and unbound microparticles. Control wells were incubated with 50  $\mu$ l of PBS containing no microparticles. The fluorescent intensity of each well was determined by a microplate reader (Bio-Tek Synergy HT, Winooski, VT) at an excitation of 362 nm and an emission of 407 nm. Autofluorescence of the coated wells was subtracted from each well by determining fluorescent intensity of the control wells.

**Adhesion of P(MAA-g-EG) and P(MAA-g-EG) WGA to Caco-2 Cells.** Mucoadhesion of the WGA carrier was evaluated using human colon adenocarcinoma (Caco-2) cells. Cells were cultured in T-75 flasks (Corning, Corning, NY) as described in Sec 8.2.1 and then seeded in 12 well plates (Corning). Cells were seeded at a density of  $5.0 \times 10^4$  cells/cm<sup>2</sup> and media was changed every other day until the cells reached confluency.

P(MAA-g-EG)-PolyFluor<sup>TM</sup> 407 and P(MAA-g-EG)-PolyFluor<sup>TM</sup> 407-WGA were synthesized as previously described. The fluorescently tagged microparticles were suspended in DPBS, pH 7.4 (Mediatech, Herndon, VA), pre-warmed to 37  $^{\circ}$ C at a concentration of 1 mg/ml. The medium was aspirated from the cells and each well was washed with DPBS, pH 7.4, pre-warmed to 37  $^{\circ}$ C. A 1 ml sample of the microparticle suspension was incubated with the cellular monolayer for 30 minutes at 37  $^{\circ}$ C on a shaker plate. After incubation, non-adherent microparticles were removed by washing two times with 1 ml of Hank's balanced salt solution (HBSS) (Mediatech). Control wells contained cells that were incubated with microparticles, but did not undergo a wash step. The fluorescent intensity of each well was determined by a microplate reader (Bio-Tek Synergy HT, Winooski, VT) at an excitation of 362 nm and an emission of 407 nm.

## Results and Discussion

**Insulin Loading.** The first step in insulin loading was to perform a loading profile to determine the appropriate amount of time for loading of insulin into the microparticles. From the loading profile, it was determined that loading for longer than two hours does not significantly increase the loading efficiency. For our purposes, loading efficiency was defined as follows:

$$\text{Loading Efficiency} = \frac{C_o - C_f}{C_o} \cdot 100$$

where  $C_o$  is the initial insulin concentration and  $C_f$  is the final insulin concentration remaining in solution.

Loading efficiencies were determined both before and after the additional of HCl, which is added to collapse the microparticles and entrap insulin within the network (Table 1). Before the addition of HCl, P(MAA-g-EG) had a loading efficiency of  $87.80 \pm 1.31$  and P(MAA-g-EG) WGA had a loading efficiency of  $97.66 \pm 0.19$ . It should be noted that loading efficiencies are always lower after the addition of the HCl because the collapse of the network forces some of the insulin out of the polymer.

P(MAA-g-EG) had a final loading efficiency of  $84.00 \pm 0.90$ , whereas P(MAA-g-EG) WGA had a slightly lower final loading efficiency of  $73.66 \pm 1.12$ . Both of these loading efficiencies corresponded to about 5 wt % loading of insulin, which is defined as mg of insulin per mg of polymer.

Functionalizing the microparticles with WGA, a 36 kDa protein, leads to some interference in the loading process. Most likely, the avidin-WGA complex present on the surface of the microparticle interacts with the insulin while being loaded, which resulted in a higher loading efficiency before collapse of the network. The interaction of the avidin-WGA complex with the insulin would have then prevented diffusion of a certain percentage of the insulin into the polymer network, thus keeping more insulin at the surface of the microparticle. This insulin would therefore be washed off during the collapse of the microparticles, which would account for the lower loading efficiency in the WGA functionalized microparticles. It is possible that a longer loading time would compensate for this difference.

**Insulin Release.** The previous release studies looked at insulin release from microparticles at pH 1.2 and pH 6.8 in two separate experiments. In this release study, we wanted to simulate the pH changes the microparticle would experience *in vivo* when passing through the stomach and into the small intestine all in one experiment. DMGA was chosen as the buffer because it is capable of buffering from pH 3.2 to pH 7.0 shows the results of insulin release from P(MAA-g-EG) and P(MAA-g-EG) WGA. The functionalization process does slightly reduce the amount of insulin released from the carrier.

At a low pH, both P(MAA-g-EG) and P(MAA-g-EG) WGA limited release of insulin from the microparticles, with both releasing less than 10% of the loaded insulin. After 60 minutes, the pH was increased and insulin was rapidly released from the both of the carriers. Functionalizing the microparticles with WGA decreased the amount of insulin released at pH 7.0, which is in agreement with the previous experiments. Both carriers reached the maximum amount of insulin release after 60 minutes in the pH 7.0 buffer, with P(MAA-g-EG) releasing 80% of the loaded insulin and P(MAA-g-EG) WGA releasing 70% of the loaded insulin.

This study demonstrated that insulin is quickly released from the carriers after the pH is increased above the pKa of MAA. In addition, results of this experiment show that the change in pH between the stomach and the small intestine can be used as a physiologic trigger to release insulin from the microparticles.

**PGM Binding Capacity of P(MAA-g-EG) and P(MAA-g-EG) WGA.** All microparticle and PGM-binding studies were conducted with the 1% PGM pre-treated microplates, as these microplates gave the best results after incubation with the F-WGA.

P(MAA-g-EG) and P(MAA-g-EG) WGA were suspended in PBS, pH 7.4 at concentrations of 1.0 mg/ml and 0.5 mg/ml and then incubated with the pre-treated microplate. The percentage of microparticles bound was determined by measuring fluorescent intensity in the wells before and after washing. Figure 2 shows the results of the adhesion experiment.

At a concentration of 1 mg/ml, P(MAA-g-EG) WGA significantly improved adhesion ( $p < 0.01$ ) with 33.49% of the initial microparticles bound as compared to only 15.55% of the initial amount of P(MAA-g-EG) microparticles bound. P(MAA-g-EG) WGA (30.01%) also improved adhesion at a concentration of 0.5 mg/ml, with a 10% increase in binding over P(MAA-g-EG) (20.67%). These results were not statistically significant, but did show a trend of increased binding of P(MAA-g-EG) WGA to the PGM-treated microplates.

From this study, we did not see a concentration dependent increase in binding. This might be explained by some of the difficulty in consistently pipetting the same concentration of microparticles into each well. From analyzing the fluorescent intensity results before washing, there was a variation in the amount of microparticles in each well. To eliminate this variation, the percentage of bound microparticles was determined for each individual well as a function of the fluorescent intensity after washing divided by the fluorescent intensity before washing.

**Specificity of P(MAA-g-EG) and P(MAA-g-EG) WGA Binding.** PGM-treated microplates were incubated with 1 mg/ml of P(MAA-g-EG) and P(MAA-g-EG) WGA microparticles for 1 hour at

37 °C. After 1 hour, a competitive carbohydrate was added to demonstrate that the binding of functionalized microparticles was specific and reversible. Figure 3 shows this behavior.

The addition of the competitive carbohydrate had no effect on the wells containing P(MAA-g-EG). Since there is no specific interaction between P(MAA-g-EG) and the PGM, it was not expected that the competitive carbohydrate would decrease the amount of P(MAA-g-EG) binding. In contrast, adding the competitive carbohydrate to the wells containing P(MAA-g-EG) WGA did decrease binding of the microparticles. A concentration dependent decrease in P(MAA-g-EG) WGA binding with an increase in the concentration of competitive carbohydrate was not seen as with the F-WGA binding to PGM, but instead the competitive carbohydrate reduced the amount of binding almost equally in all wells as compared to wells that were not incubated with the competitive carbohydrate.

It is possible that the range of concentrations used for the competitive carbohydrate were too high to see a concentration dependent reduction in binding and that at all concentrations used the specific binding was completely inhibited. Using a lower range of concentrations might give us similar results as were seen with the F-WGA and the competitive carbohydrate. From this study, we can say that P(MAA-g-EG) WGA binding to PGM-treated microplates is specific and reversible, while any binding of P(MAA-g-EG) to the microplates appears to be non-specific.

**Adhesion of P(MAA-g-EG) and P(MAA-g-EG) WGA to Caco-2 Cells.** Caco-2 cells, which possess a glycocalyx layer containing carbohydrate moieties that WGA can bind, was used to confirm that the P(MAA-g-EG) WGA can create an improved adhesion between the polymer and the mucosal layer. Specifically, WGA binds to N-acetyl-glucosamine and sialic acid, which are expressed by Caco-2 cells. Previous studies have shown that Caco-2 cells bind WGA and WGA-conjugates<sup>11-15</sup>. This experiment was an attempt to look at adhesion of functionalized microparticles in an *in vitro* setting.

In comparison to unwashed control wells, P(MAA-g-EG) WGA adhesion ( $39.1 \pm 5.3\%$ ) was significantly higher than P(MAA-g-EG) adhesion ( $22.2 \pm 7.7\%$ ) ( $p < 0.05$ ) as shown in **Error! Reference source not found.** Adhesion was improved by 17% in wells containing the functionalized microparticles. This information suggests that WGA is able to improve the mucoadhesive characteristics of this delivery system.

In contrast to the adhesion study with PGM-treated microplates, we did not show a specific interaction between the functionalized microparticles and the cellular monolayer by using a competitive carbohydrate. For this reason, we relied on this study in conjunction with the PGM adhesion study to confirm the improved mucoadhesive properties of the functionalized microparticles. While this experiment did show improved adhesion of the functionalized microparticles, it was somewhat difficult to conduct due to a shearing of the cellular monolayer after incubation on a shaker plate with the microparticles. Wells where the cellular monolayer was clearly detached from the 12-well plate were not included in the results. For this reason, we did not conduct this study with a mucus-secreting co-culture of Caco-2 and HT29-MTX cells, which was more susceptible to a shearing of the cellular monolayer.

## Conclusions

Insulin was loaded into the hydrogel microparticles after two hours with loading efficiencies greater than 70% for both P(MAA-g-EG) and P(MAA-g-EG) WGA. The pH-responsive release results demonstrate that the pH shift from the stomach to the small intestine can be used as a physiologic trigger to release insulin from P(MAA-g-EG) and P(MAA-g-EG) WGA microparticles, thus limiting release of insulin into the acidic environment of the stomach.

Microplates were successfully treated with PGM to create a surface that allowed for specific binding between mucins and lectins. The 1% PGM treatment followed by a 2 hour BSA blocking step gave the most consistent results when incubated with F-WGA. In addition, the PGM-treated microplates were shown to create specific interactions between F-WGA and the PGM by use of a competitive carbohydrate.

The 1% PGM treated microplates were also used to show that adhesion was improved in the P(MAA-g-EG) WGA microparticles over the P(MAA-g-EG) microparticles. The interaction between

the PGM-treated microplate and P(MAA-g-EG) WGA was again shown to be specific by adding a competitive carbohydrate, while the interaction between P(MAA-g-EG) was non-specific.

The final study used a cellular monolayer as another method for showing that the functionalized microparticles increase adhesion over the non-functionalized microparticles. From all of these studies, we can conclude that the addition of the WGA on the microparticles improves adhesion to carbohydrate-containing surfaces.

## Acknowledgements

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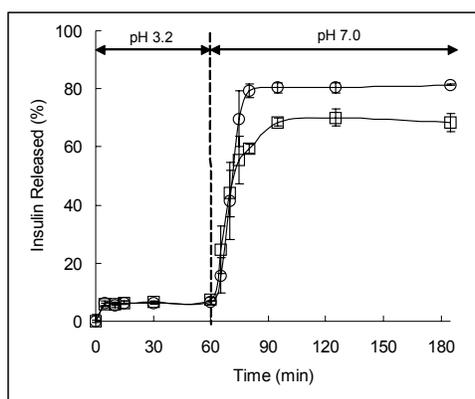
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**Table 1: Insulin Loading Efficiencies for P(MAA-g-EG) and P(MAA-g-EG) WGA**

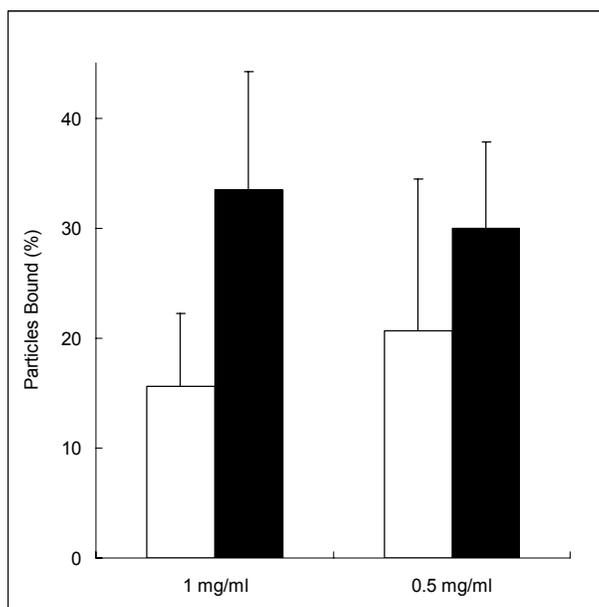
Polymer microparticles were loaded with insulin for two hours. After which time, 10 ml 0.1 N HCl was added to collapse the microparticles and entrap insulin in the network. Insulin concentration was determined by HPLC analysis of the supernatant. Wt % loaded is defined as mg of insulin per mg of polymer.

Polymer Sample	Loading Efficiency Before HCl (%)	Loading Efficiency After HCl (%)	Wt % loaded
P(MAA-g-EG)	87.80 ± 1.31	84.00 ± 0.90	5.38 ± 0.38
P(MAA-g-EG) WGA	97.66 ± 0.19	73.66 ± 1.12	5.03 ± 0.11



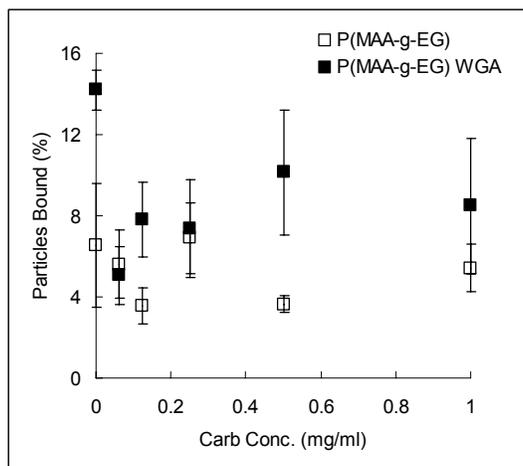
**Figure 1: pH Responsive Insulin Release from P(MAA-g-EG) and P(MAA-g-EG) WGA**

A 10 mg sample of insulin-loaded P(MAA-g-EG) (○) or insulin-loaded P(MAA-g-EG) WGA (□) microparticles were placed in 50 ml of DMGA buffer, pH 3.2. After 60 minutes, the pH of the solution was raised to 7.0 by the addition of 5 N NaOH. Solutions were stirred at 100 rpm and maintained at 37 °C. Samples were taken over the course of 3 hours and insulin concentration was determined by HPLC.



**Figure 2: P(MAA-g-EG) and P(MAA-g-EG) WGA Binding to PGM Microplates**

Pre-treated microplates were incubated with 1 mg/ml or 0.5 mg/ml of P(MAA-g-EG) (□) and P(MAA-g-EG) WGA (■) for 2 hours at 37 °C. Wells were washed with 100 μl of PBS to remove unbound microparticles. The percentage of bound particles was calculated by dividing the fluorescent intensity of each well after washing by the fluorescent intensity of each well before washing. n=6 ± SD



**Figure 3: Specificity of P(MAA-g-EG) and P(MAA-g-EG) WGA Binding to PGM Microplates**

Pre-treated microplates were incubated with 1 mg/ml of P(MAA-g-EG) (□) and P(MAA-g-EG) WGA (■) for 1 hour at 37 °C. A competitive carbohydrate, N,N',N''-triacetyl-chitotriose, was then added at various concentrations to each well and incubated for 1 hour at 37 °C. A wash step was performed to remove the competitive carbohydrate and unbound microparticles. The percentage of bound particles was calculated by dividing the fluorescent intensity of each well after washing by the fluorescent intensity of each well before washing. n=3 ± SD