Elucidation of the Mechanism of Enhanced Insulin Uptake and Release from pH Responsive Hydrogels

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Summary: Environmentally responsive hydrogels composed of poly(methacrylic acid-g-ethylene glycol) (P(MAA-g-EG)) have shown promise for oral insulin delivery due to their pH responsive complexation behavior. A series of hydrogel formulations were polymerized with varying amounts of crosslinker and varying monomer volume fraction. The mesh size of the network depended primarily on pH, varying from 8.0 to 27.2 nm. Insulin loading efficiency varied directly with crosslink density, ranging from 42.7 to 84.9% of available insulin loaded into the hydrogels. The release of insulin was performed with each polymer formulation at 5 pH levels ranging from 2.7 to 6.8. Insulin release was less than 20% for all formulations tested with insulin for the duration of the 3 hour release study for all pH levels considered except when the pH was 6.8, at which point the release occurred as a burst. Loading studies performed with insulin glargine, an insulin analog with an increased pI, showed the same trends as native insulin. However, the release of insulin glargine only occurred at a pH level above that of the pI of the protein. These results indicate that hydrogen bonds and ionic interactions between the protein and P(MAA-g-EG) may strongly influence its loading and release behavior *in vitro*.

Keywords: drug delivery systems; hydrogels; insulin; oral protein delivery; pH responsive

Introduction

Oral insulin delivery continues to be an elusive goal due to the enzymatic degradation and low macromolecular absorption of the protein in the gastrointestinal tract. Complexation hydrogels composed of poly(methacrylic acid-g-ethylene glycol), henceforth designated P(MAA-g-EG), have shown promise as an oral delivery device for insulin due to their pH responsive swelling, mucoadhesive and calcium binding behavior.[1–7] This hydrogel has been shown to protect insulin from enzymatic degradation in the stomach and allow it to reach the small intestines where enzymatic activity is significantly lower.[1,8,9] To date, the delivery of insulin is assumed to be directly attributable to the physical size of the nanoporous hydrogel and the uptake and release of insulin has been assumed to be controlled primarily by diffusion. However, a study with P(MAA-g-EG) loaded with calcitonin suggested that hydrogen bonding may also play an important role for drug loading and release.[10] The purpose of the current study is to examine the *in vitro* behavior of P(MAA-g-EG) hydrogels with insulin with respect to the ionic states of the hydrogel and protein. A series of hydrogel formulations were polymerized and the network mesh size for each formulation was determined at 10 pH levels between 2.2 and 6.8. Insulin was incorporated into each formulation to examine the effect of crosslink density and network mesh size on the loading efficiency. Because hydrogen bonding in P(MAA-g-EG) is dependent on the pH of the surrounding medium, insulin glargine was also studied as a model drug.

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Insulin glargine is an insulin analog which has two arginie residues added to the C-terminus of the B chain. These two residues cause the isoelectric point (pI) of the protein to shift from 5.2 to 6.7.[11] By using a model protein with modified ionic characteristics but similar size and chemistry, changes in the *in vitro* behavior can be attributed to the differences in the interaction between the protein and the hydrogel. Release studies were performed with human insulin and insulin glargine at 5 pH levels between 4.6 and 6.8, encompassing the ionic transition points for both insulin species as well as P(MAA-g-EG).

Materials and Methods

Synthesis and Characterization of P(MAA-g-EG) Hydrogels

Hydrogels of P(MAA-g-EG) were prepared by free-radical photopolymerization of methacyrlic acid (MAA) and poly(ethylene glycol) monomethcarylate monomethyl ether (PEGMA 1000) as described previously.[12] The hydrogel formulas were altered from previous methods by varying the mole fraction of poly(ethylene glycol) dimethacrylate (PEGDMA 200) and the monomer weight fraction present during polymerization. Mole fractions of PEGDMA 200 were 0.375, 0.75, 1.25 and 2.0% (mole PEGDMA 200/total moles monomer). Monomer weight fractions were 33.3, 50.0 and 66.7% (weight monomers/total weight of reaction solution). The samples were designated according to the mol% of PEGDMA and the monomer weight fraction present during polymerization (e.g. 0.75/50). Hydrogel swelling and tensile mechanical experiments were performed to characterize the mesh size of the network copolymer as described previously.[12]

Insulin Loading and Release Studies

Insulin loading and release was performed according to previously reported methods.[12] Solutions with 0.5 mg/ml of insulin in phosphate buffered saline (PBS) at pH = 7.4 were used for loading. The P(MAA-g-EG) polymer samples were stirred in insulin solutions for 4 hours at 37 °C. For insulin glargine loading all techniques were the same as that of human insulin except the loading solutions were made at pH = 9.5 to ensure full dissolution of the protein. A test study was performed with human insulin which confirmed that the higher pH did not affect the efficiency of protein loading (data not shown). Insulin release was performed with 10 mg of insulin or insulin glargine loaded hydrogels dispersed into 20 ml of PBS at the desired pH stirred at 300 rpm at 37 °C. Insulin concentrations were determined using RP-HPLC (Waters Sperisorb® column, 5 μm, 4.6 × 300 mm). A gradient using from 35–65% acetonitrile with 0.1% trifluoroacetic acid (TFA) for 30 minutes in DI water with 0.1% TFA. Concentrations were compared to insulin standards with detection at 214 nm on a Waters 996 Photodiode detector (Milford, MA). Insulin incorporation efficiency was determined based on the amount of insulin remaining in solution following removal of P(MAA-g-EG) by filtration.

Results and Discussion

Characterization of P(MAA-g-EG) Hydrogels

The properties of the hydrogel network were based on equations derived by Peppas and Merrell[13] based on Flory's theory of rubber elasticity.[14] The network was characterized by the distance between crosslinks, or the network mesh size (ξ). These values were determined using the swelling and tensile data for each polymer formulation according to the following equations

$$\frac{\tau}{(\alpha - 1/\alpha^2)} = RT\rho_{2,r}\left(\frac{1}{\overline{M}_e} - \frac{2}{\overline{M}_n}\right)\left(\frac{\upsilon_{2,s}}{\upsilon_{2,r}}\right)^{1/3}$$

$$\xi = \left(\frac{2C_n\overline{M}_e}{M_o}\right)^{1/2} l\upsilon_{2,s}^{-1/3}$$

where $\upsilon_{2,r}$ and $\rho_{2,r}$ are the polymer volume fraction and density of the hydrogel in the

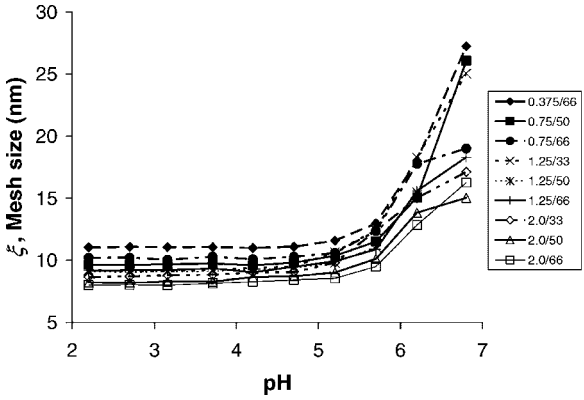

Figure 1.
Hydrogel network mesh size for 9 different polymer formulations of P(MAA-g-EG). The values for ξ increase with increasing pH from 7 nm – 28 nm. The apparent pKa of the hydrogel is 5.8-6.0. Samples are identified as mol% PEGDMA 200/wt% monomer.

relaxed state, $\overline{M}_e$ is the effective molecular weight between crosslinks, and M_o is the monomer molecular weight. Elongation, α, was the length at any time divided by the initial sample length. Tensile stress, τ, was the average force per cross-sectional area of the unstretched hydrogel strips between 0–5% strain. The polymer volume fraction of the swollen hydrogel, $\upsilon_{2,s}$, was determined by the buoyancy technique[6]. The number average molecular weight of the linear polymer chains in the absence of crosslinks, $\overline{M}_n$, as determined by GPC, were 24930 ± 1160, 19370 ± 330 and 16150 ± 750 g/mol for sample hydrogel formulations made with 66.7, 50.0 and 33.3% (w/w) polymer, respectively. Three of the hydrogel samples could not be tested for network characteristics due to their low physical integrity. As expected, the network mesh size exhibited increasing values with increasing pH. Interestingly, increased covalent crosslink density of the formulations, due to increased PEGDMA 200 present or higher monomer weight fraction during polymerization, had very little effect on the overall crosslink density. This is especially true when in the complexed state at low pH levels.

In vitro Human Insulin Loading and Release Studies

Insulin was incorporated into each formulation to examine the effect of crosslink density and network mesh size on the loading efficiency (Table 1). In general, the insulin incorporation efficiency exhibited a direct correlation with the amount of covalent crosslinks associated to changes in PEGDMA amounts. Insulin incorporation efficiency also varied directly with the

Table 1.
Insulin incorporation efficiencies for native human insulin loading into P(MAA-g-EG) hydrogel formulations. Values represent the percentage of available insulin in solution which is loaded into the hydrogel based.

PEGDMA 200 (mol%)	Monomer fraction (wt%)		
n = 3	33 wt%	50 wt%	66 wt%
0.375%	N/A	42.7 ± 25.3	76.6 ± 4.7
0.75%	52.7 ± 7.0	76.7 ± 2.2	77.8 ± 0.3
1.25%	67.3 ± 3.4	77.1 ± 1.8	81.9 ± 1.4
2.00%	75.2 ± 3.6	82.0 ± 2.1	84.9 ± 0.9

monomer fraction present during polymerization.

Release studies were performed for each insulin loaded hydrogel formulation at pH values of 2.7, 3.7, 4.7, 5.6 and 6.8. All formulations exhibited the same behavior as those displayed by the examples represented in Figure 2. The values represent the fractional insulin release during a 3 hour release study. Overall the release of insulin did not exceed 20% until the pH of the release buffer was at 6.8, at which point the release occurred as a burst for all formulations and exceeded 80% after only 5 minutes. These results did not demonstrate a release curve which would be expected from diffusion. Instead they suggest that insulin release from P(MAA-g-EG) may be influenced by more than diffusion alone.

In vitro Insulin Glargine Uptake and Release Studies

The similarities between insulin glargine and human insulin makes it an ideal candidate to examine its *in vitro* behavior with P(MAA-g-EG) when compared to insulin. Because it is derived from human insulin, the amino acid sequence, tertiary structure, and molecular size and weight are nearly identical to that of human insulin.[11] The critical difference for the purpose of this study is that the pI for insulin glargine is above the pKa of P(MAA-g-EG), whereas human insulin is below. Therefore, any changes in the uptake or release behavior can then be attributed to the ionic differences between human insulin and insulin glargine. The loading efficiencies for insulin glargine are listed in Table 2. Due to limited

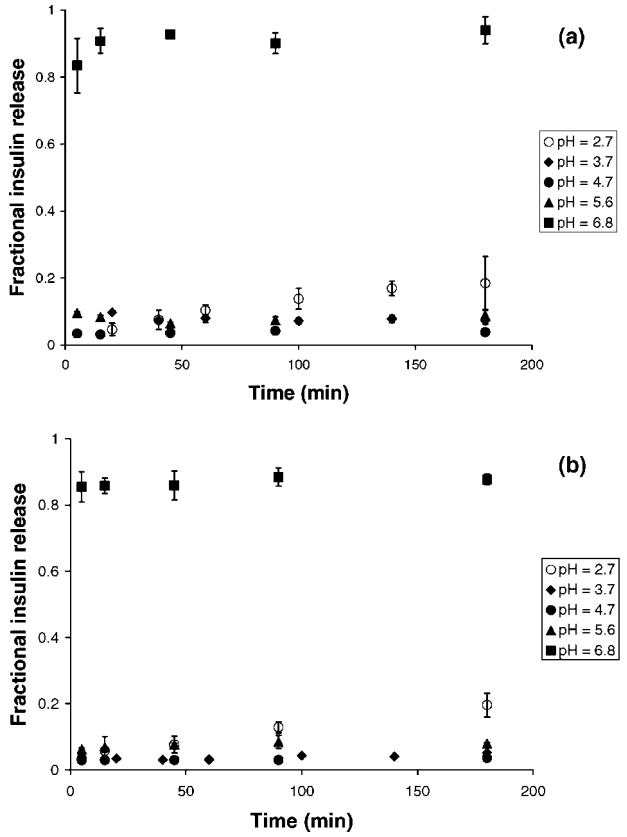

Figure 2.
Fractional insulin release from P(MAA-g-EG) hydrogel formulations (a) 0.75/66 and (b) 2.0/66. Insulin fractional release only exceeded 20% in hydrogels in PBS at pH = 6.8.

Table 2.
Insulin glargine incorporation efficiencies into P(MAA-g-EG) hydrogel formulations. Values represent the percentage of available insulin glargine in solution which is loaded into the hydrogel based.

PEGDMA 200 (mol%)	Monomer fraction (wt%)	
	33 wt%	66 wt%
0.75%	45.7	64.7
2.00%	61.9	83.2

amounts of the insulin analog, only four formulations were considered. The direct correlation between crosslink density and incorporation efficiency are the same as that of human insulin and the overall values are near that of human insulin. Because the solubility of insulin glargine was lowest at a pH value used for human insulin loading, the loading studies were performed from solutions which started at pH = 9.5. Sample loading studies were performed with human insulin from similar pH levels with no significant change in loading efficiency (data not shown).

Release studies with insulin glargine were performed using PBS at pH levels of 4.6, 5.6, 6.2, 6.8, and 7.4. The pH values considered were used because they provided a greater focus on the pH range encompassing the pI for both insulin species as well as that of P(MAA-g-EG) (pKa ~ 5.8–6.0). The release for insulin glargine is shown in Figure 3. The points represent the total release of the protein after a 3 hour study. In general, the release

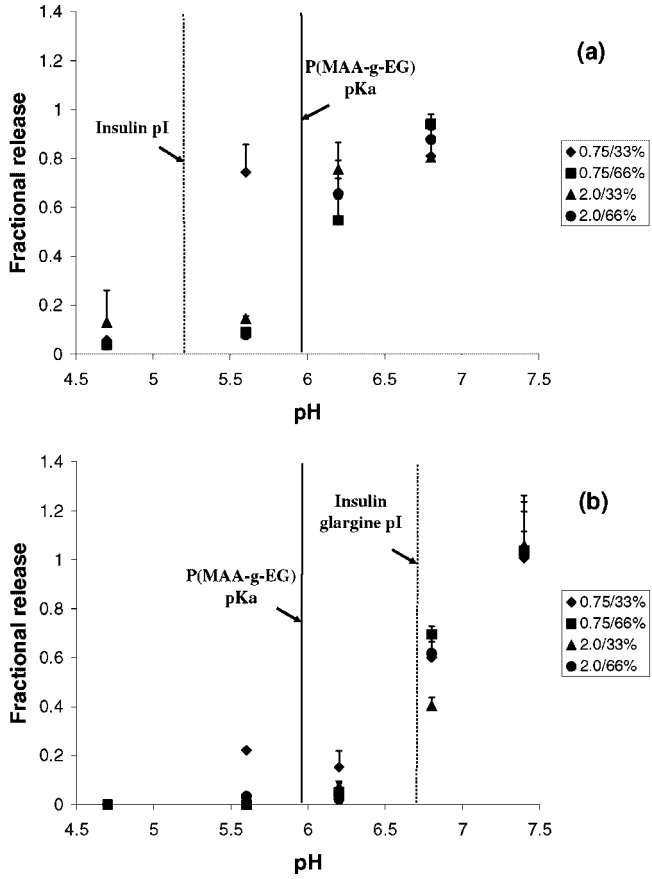

Figure 3.
Total release of (a) human insulin and (b) insulin glargine from P(MAA-g-EG) formulations following 3 hour release studies at various pH levels.

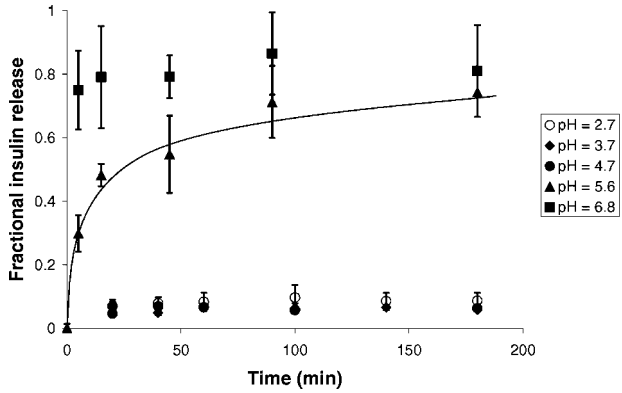

Figure 4.
Fractional release of human insulin from formulation 0.75/33 over a 3 hour release study at various pH levels.

from each formulation only occurred in PBS at pH levels above the both the pKa of the hydrogel as well as the pI of the incorporated insulin species. There was one exception to this trend exhibited by the formulation 0.75/33% with human insulin at pH = 5.6. This hydrogel formulation was the most loosely crosslinked of the four considered for this study. Examination of the release of insulin with respect to time (Figure 4), reveals a fractional release curve representative of traditional diffusion kinetics for this sample. These results suggest that only under certain conditions that diffusion is the mode for insulin release. For the P(MAA-g-EG) formulations considered in this study, most release profiles indicate that the incorporated insulin species is driven from the hydrogel network due to the negative charges of the MAA backbone of the hydrogel. These results corroborate those found by Sipahigil et al.[10] with calcitonin as the incorporated protein.

Conclusions

A series of P(MAA-g-EG) hydrogels were polymerized and characterized. The network mesh size increased with increasing pH due to hydrogen bonding between the MAA backbone and PEG at low pH levels (<4) and ionic repulsion of the negatively charged MAA in higher pH levels (>6). Increasing the amount of covalent crosslinks in the network had only a small effect on the network mesh size. Increased crosslink density did cause increased loading efficiency for both human insulin and insulin glargine. In general, the release of the incorporated insulin species did not occur until the pH of the release buffer was above both the pKa of P(MAA-g-EG) and the pI of the protein. These results suggest that ionic repulsion play an important role in the release of a protein from P(MAA-g-EG) hydrogels.

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