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Research Paper

Synthesis, Characterization and *In Vivo* Efficacy of PEGylated Insulin for Oral Delivery with Complexation Hydrogels

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Purpose. This work evaluated the feasibility of combining insulin PEGylation with pH responsive hydrogels for oral insulin delivery.
Methods. A mono-substituted PEG–insulin conjugate was synthesized and purified. The site of conjugation was determined by MALDI-TOF MS. Uptake and release of PEGylated insulin was performed in complexation hydrogels to simulate oral dosing. The bioactivity of the conjugate and PK/PD profile was measured *in vivo* in rats.
Results. PEGylation was confirmed to be specifically located at the amino terminus of the B-chain of insulin. Higher loading efficiency was achieved with PEGylated insulin than regular human insulin in pH responsive hydrogels. The release of PEGylated insulin was lower than that of human insulin at all pH levels considered. Full retention of bioactivity of the PEG–insulin conjugate was confirmed by intravenous dosing while subcutaneous dosing exhibited a relative hypoglycemic effect 127.8% that of human insulin.
Conclusions. Polyethylene glycol conjugated specifically to the amino terminus of the B-chain of insulin maintained the bioactivity of the protein and significantly extended the duration of the hypoglycemic effect. Used in combination with pH responsive hydrogels, PEGylated insulin has significant potential for oral delivery.

KEY WORDS: controlled drug delivery; oral protein delivery; PEGylated insulin; pH responsive hydrogel.

INTRODUCTION

One of the most difficult obstacles to oral insulin delivery is maintaining the integrity of the protein against enzymatic degradation in the GI tract. Hydrogels composed of poly (methacrylic acid-g-ethylene glycol) (P(MAA-g-EG)) have exhibited potential to be used for oral insulin delivery. This material forms a copolymer network with water-filled nanopores which exhibits reversible, pH-dependent swelling behavior (1–8). In the acidic environment of the stomach (pH ~1–2) the network collapses, trapping insulin. In the more neutral environment of the lower intestine (pH ~6–7) the network swells, allowing insulin release. In this way, P(MAA-g-EG) can bypass the aggressively degrading environment of the stomach and deliver insulin to the small intestine where there is lower enzymatic activity and increased potential for absorption by the local tissues (6,7,9,10). These hydrogels have also exhibited the ability to inhibit the degradation of insulin in the gastric environment (7,9), bind to the mucus layer of the small intestine (11,12), reduce enzymatic activity in the lumen of the small intestine (6,8), locally and reversibly permeate the tight junctions the epithelial layer (13) and deliver insulin to the bloodstream in a number of *in vitro* and *in vivo* tests (10,14,15). The results of previous work with this delivery design has been somewhat promising with bioavailability relative to subcutaneously administered insulin as high as 9.5% (14). While all of these attributes of P(MAA-g-EG) benefit the design for oral insulin delivery, they are only observed when insulin is in close proximity to the hydrogel, as was the case in earlier studies. The likely scenario of extended exposure to the intestinal lumen could lead to significant enzymatic degradation by lumenal enzymes. In order to improve the design of this delivery system and improve bioavailability of orally delivered insulin, the enzymatic degradation of insulin by GI proteases should me minimized.

Maintaining normal digestion in the stomach and intestine while concomitantly altering the enzymatic degradation of an

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ABBREVIATIONS: ELISA, enzyme-linked immunosorbent assay; FPLC, fast protein liquid chromatography; IEC, ion exchange chromatography; MAA, methacrylic acid; MALDI-TOF MS, matrix-assisted laser desorption ionization time of flight mass spectrometry; PEG, polyethylene glycol; PEGDMA, polyethylene glycol dimethacrylate; PEGMA, polyethylene glycol monomethacrylate monomethyl ether; P(MAA-g-EG), poly(methacrylic acid-graft-ethylene glycol); RP-HPLC, reverse-phase high performance liquid chromatography.

orally delivered drug is a unique challenge; any changes to the proteolytic activity in the intestine must be localized near the protein. One method to do so is PEGylation, the covalent conjugation of polyethylene glycol (PEG) to the protein. PEGylation has been shown to decrease immunogenicity and antigenicity, improve solubility, and increase circulation time for proteins (16–18). However, the most applicable effect to oral protein delivery is the reduced enzymatic degradation of PEGylated proteins (16–19). Steric hindrance of enzymes caused by the presence of PEG decreases the proteolysis of labile peptide residues. By combining the increased enzymatic resistance of PEGylated insulin with P(MAA-g-EG) hydrogels, the bioavailability of the protein may be increased following oral delivery.

There are few potential amino acid targets available for insulin PEGylation. The most commonly investigated residues are the N-termini of the A and B chains, glycine and phenylalanine, respectively, and the lysine at the 29th residue of the B chain as seen in Fig. 1. These sites will henceforth be referred to as GlyA1, PheB1 and LysB29. In 1971, Lindsay and Shall determined that following acetylation of the GlyA1 residue that the bioactivity of insulin was significantly reduced based on its ability to induce a hypoglycemic effect (20). For this reason, the PheB1 and the LysB29 site are generally targeted for modification, though there is still debate as to which location is more suitable. A single PEG modification on a PEG–insulin conjugate is preferred because the bioactivity of insulin was significantly reduced for di-substituted insulin species in which both LysB29 and PheB1 were PEGylated (21–23). In this work the PheB1 was targeted for a mono-substituted PEG–insulin conjugate. Modification at the PheB1 site exhibited a greater reduction in the immunogenicity of the protein and a greater effect on the stability of the protein monomer when compared to LysB29 (22,24,25). Additionally, if any reduction in biological activity occurs due to steric hindrance, the effect of PheB1 PEGylation should be less pronounced than LysB29 due to the relative proximity of LysB29 to the receptor binding site.

The synthesis of PEGylated insulin has been achieved through a variety of methods (18,19,21,25–27). Until recently, however, most of these approaches did not attempt to target a particular amino acid for modification, relying on purification methods to separate the product mixtures. In this work, the site-specific synthesis of PheB1–mPEG–insulin is described and characterized. The loading and release behavior of PEGylated insulin is investigated with three different P(MAA-g-EG) hydrogel formulations and the intravenous and subcutaneous bioactivity of the PEG–insulin conjugate is determined relative to human insulin based on hypoglycemic effect seen in healthy male Sprague Dawley rats.

MATERIALS AND METHODS

Materials

Spectrapor® 6 regenerated cellulose dialysis tubing was purchased from Fisher Scientific (Hampton, NH). Recombinant human insulin was purchased from Seracare Diagnostics (Milford, MA). Methoxy PEG succinimidyl propionate 5,000 (mPEG-SPA) was purchased from Nektar Therapeutics (Huntsville, AL). Immobilized TPCK Trypsin was purchased from Pierce Chemical Company (Rockville, IL). Methacrylate terminated poly(ethylene glycol) monomethacrylate monomethyl ether 1,000 (PEGMA) and polyethylene glycol dimethacrylate 200 (PEGDMA) were purchased from Polysciences Inc. (Warrington, PA). Bicinchoninic acid (BCA) assays were purchased from Pierce Chemical Company (Rockford, IL). Enzyme linked immunosorbent assays were purchased from ALPCO diagnostics (#80-INSHU-E10, Salem, NH). Male Sprague Dawley rats between 280 and 320 g were purchased from Harlan (Indianapolis, IN). Isoflurane, pentobarbital and

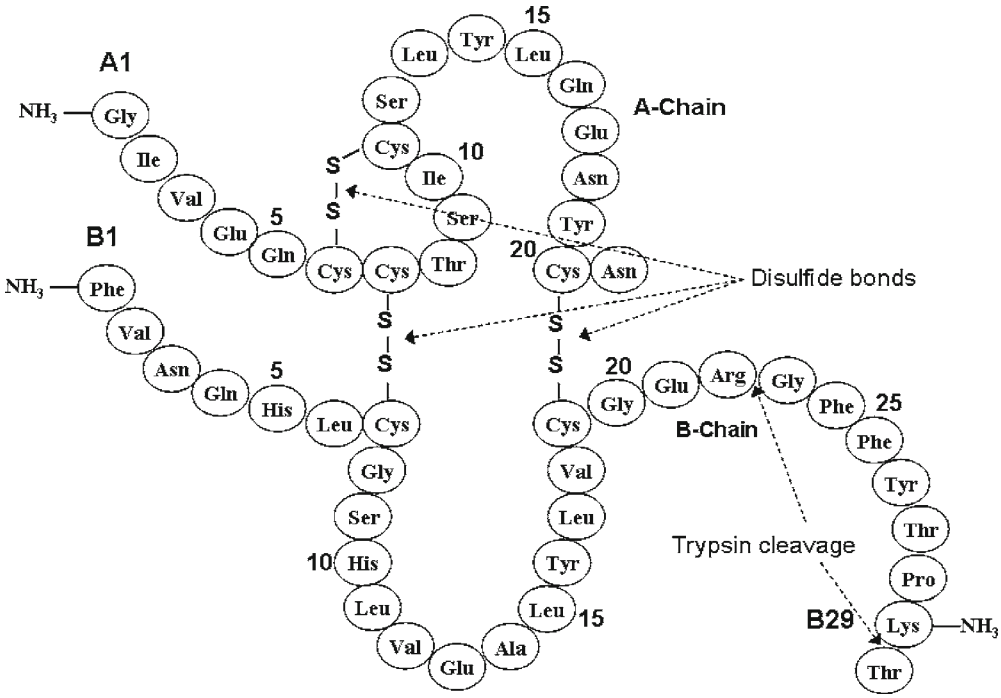

Fig. 1. Amino acid sequence of insulin with disulfide and trypsin cleavage sites indicated.

euthanasia solution were provided by the veterinary staff at Thomas Jefferson University. An insulin pump was used for subcutaneous dosing of insulin (Disetronic Roche Diagnostics, Palo Alto, CA). Blood glucose meters and glucose strips were purchased from Hemocue (Lake Forest, CA). All other chemicals used were purchased from Sigma Aldrich (St. Louis, MO).

Synthesis of Di-BOC Insulin

The reaction of insulin with di-*tert*-butyl dicarbonate (diboc) reversibly alkylates free amine groups with a BOC protecting group. This protection reaction was used to produce an insulin intermediate, as first described by Markussen *et al.* (28), such that the PheB1 residue of insulin was targeted for PEGylation. Di-BOC insulin was synthesized and purified from recombinant human insulin. For clarity, di-$BOC_{LysB29/GlyA1}$, insulin with BOC groups specifically at the LysB29 and GlyA1 residues, will henceforth be referred to as di-BOC insulin. This should be distinguished from diboc treated insulin which refers to insulin which has been reacted with di-*t*-butyl dicarbonate, but not purified.

Briefly, a solution of 500 mg insulin was made in 9.5 mL of dimethyl sulfoxide (DMSO) and 0.5 mL triethylamine (TEA). A second solution with 41 mg diboc was made in 0.5 mL DMSO. The solutions were combined and stirred at 60 rpm for 30 min at room temperature. The reaction was then quenched by the addition of 25 μL of ethanol amine. The reaction mixture was pipetted into 200 mL of acetone and one drop of 6 M HCl was added to initiate precipitation of the protein. The precipitate was removed by vacuum filtration using a 0.22 μm PVDF filter. The precipitate and filter paper were then placed into 30 mL of DI water. After 30 min, the filter paper was removed, rinsed with water, and discarded. The remaining solution was then dialyzed against DI water in a 3,500 MWCO membrane overnight to remove residual acetone, unreacted diboc and ethanol amine.

The crude product of diboc reacted insulin was purified using ion exchange chromatography (IEC) on a Fast Protein Liquid Chromatography unit (FPLC, GE Healthcare, Piscataway, NJ). A gradient elution profile was performed from 0.01 to 0.18 M NaCl over 80 column volumes using a SP FF 16/10 cation exchange column (GE Healthcare, Piscataway, NJ) at a flow rate of 2 mL/min in 7 M urea and 1 M acetic acid. The elution was collected by a Frac-950 fraction collector in 14.5 mL fractions with the elution profile measured by UV absorbance at 280 nm.

Characterization of each fraction was performed using RP-HPLC. The mobile phases were 0.1% trifluoroacetic acid (TFA) in DI water as mobile phase A and 0.1% TFA in acetonitrile as mobile phase B. Injections of 50 μL were run on a Waters Spherisorb® C8 column (5 μm, 4.6×250 mm) using a Waters 2695 separations module equipped with a 996 Photodiode Array detector (Milford, MA) with detection at 214 nm and a flow rate of 1 mL/min. A gradient from 35% to 65% mobile phase B was run for 35 min. Purified fractions with greater than 90% purity of di-BOC insulin were then used for PEGylation. All fractions were dialyzed with 3,500 Da cutoff membrane against 0.01 M NH_4HCO_3 for 3 days, changing the dialysate twice daily. The dialyzed fractions were then frozen and lyophilized.

Di-BOC Insulin PEGylation

To PEGylate the insulin intermediate, purified di-BOC insulin powder was dissolved in 2 mL of DMSO containing 47.5 μL TEA and reacted with a 3:1 molar excess of mPEG-SPA for 24 h at room temperature. The products were then diluted into 100 mL of DI water, dialyzed and lyophilized in the same fashion as the crude diboc treated insulin product. The lyophilized product was dissolved in TFA at 0°C for 90 min to remove the BOC protection groups. The TFA was diluted into ten times the volume of DI water, dialyzed and purified using IEC in the same fashion as di-BOC purification. The major peak corresponding to the PheB1–PEG–insulin conjugate was recovered and stored under nitrogen at −20°C.

PEGylated Insulin Characterization

PEGylated insulin was characterized to confirm that the modification was only present at the PheB1 residue. In one digestion method, the disulfide groups were reduced to separate the A and B chains of insulin (Fig. 1). Two milligrams of PEGylated insulin was dissolved in 5 mL denaturation buffer of 7 M urea, 0.2 M tris and 2 mM EDTA at pH=8.5. The solution was flushed with nitrogen and heated to 50°C for 30 min. A 50-fold molar excess of DTT over the number of moles of disulfide present in insulin was then added and allowed to react for 4 h. A 100-fold molar excess of iodoacetamide over the number of moles of disulfide present in insulin was then added to stabilize the reduced cysteine residues by alkylation. The solution pH was measured following the addition of iodoacetamide and a small amount of ammonium hydroxide was added to maintain the pH of the solution at 8.5. This reaction was allowed to proceed for 20 min at room temperature in the dark to limit the conversion of liberated iodide ion to iodine which would react with tyrosine residues in the protein (29). After treatment with dithiolthreitol, a precipitate formed in the solution which was removed by centrifugation.

An alternative digestion of PEGylated insulin was performed using a gel immobilized trypsin kit. This digestion cleaved two bonds in the amino acid sequence of insulin as seen in Fig. 1. One mg of PEGylated insulin was dissolved in 0.5 mL of 0.1 M NH_4HCO_3 at pH=8.0. A slurry of 0.25 mL of the gel immobilized TPCK trypsin was then added to the solution in a 1.5 mL centrifuge tube. The mixture was flushed with nitrogen, sealed and incubated overnight on a shaking tray at 37°C. The immobilized trypsin was then removed from solution by centrifugation and the supernatant was recovered. Combination of these two digestion techniques allowed for clear identification of modification at any one of the three potential conjugation sites.

MALDI-TOF Mass Spectroscopy

Matrix assisted laser desorption ionization/time of flight mass spectroscopy (MALDI-TOF MS) was used to characterize many of the modified insulin or degraded insulin species throughout this study. Diboc treated insulin and PEGylated di-BOC insulin samples were analyzed on a VG TofSpec E MALDI-TOF (ThermoVG, Beverly, MA) using matrices of 2,5-dihydroxybenzoic acid (DHB) or sinapic acid.

Digested PEGylated insulin samples were analyzed using a Voyager DE Pro MALDI-TOF (Applied Biosystems, Foster City, CA) with α-cyano-4 hydroxycinnamic acid (CHCA) as the matrix. A 2,500:1 ratio of matrix to analyte was used for each run in 50% acetonitrile in DI water with 0.1% TFA. Because this solution did not dry quickly upon spotting on the MALDI-TOF plate, the plate was placed in an oven at 80°C for about 5 min prior to spotting.

Synthesis of P(MAA-g-EG) Hydrogels

Hydrogels of P(MAA-g-EG) were prepared by free-radical photopolymerization of MAA and PEGMA 1,000 according to previously described methods (15,30). The MAA and PEGMA were mixed to yield a 1:1 or 3:2 ratio of MAA/EG units in the gel. The crosslink density of the hydrogels were varied by altering the amount of difunctional PEGDMA along with the weight percent of monomers to create networks with low, medium and high levels of covalent crosslinks. The amount of PEGDMA was defined as a mole percent based on the number of moles of PEGDMA/total moles monomer (MAA and EG) in solution. The formulations considered for low medium and high levels of crosslinking were designated 0.75/33, 1.25/50 and 2.0/66 representing the mole percent of PEGDMA/weight percent of monomer present during polymerization, respectively.

PEGylated Insulin Loading and Release

Loading studies for human insulin and PEGylated insulin were performed in a similar fashion as described in earlier work (30). Release studies were performed in PBS at pH levels of 4.7, 5.6, 6.2, 6.8 and 7.4 to adequately encompass the range in which P(MAA-g-EG) exhibits the largest change in swelling and ionic state. The protein concentrations were determined for the *in vitro* testing using a BCA protein assay according to manufacturer recommendations. Standard curves were established for both human insulin and PEGylated insulin against the supplied bovine serum albumin standards and concentrations were measured by absorbance at 550 nm (BioTek ELX800, Winooski, VT).

In Vivo Determination of PEGylated Insulin Bioactivity

All *in vivo* studies performed in this work were approved by the Institutional Animal Control and Use Committee at Thomas Jefferson University under protocol number 355 version V and adhered to the principles of laboratory animal care. The bioactivity of PEGylated insulin of PEGylated insulin was measured against native human insulin based on its ability to induce a hypoglycemic effect following intravenous (IV) and subcutaneous (SC) injection into healthy rats. Male Sprague Dawley rats between 280 and 320 g were allowed to acclimatize to their environment for a minimum of 3 days before use. The rats were fasted overnight in cages with wire mesh bottoms and provided with unlimited amounts of water during their fast. The following morning, the rats were anesthetized by a SC injection of 40–50 mg/kg of pentobarbital and weighed to determine accurate dosing levels. Rats were restrained in the supine position under a neonatal heating lamp by surgical tape with body temperature maintained at 98–100°F. General anesthesia was maintained throughout the test with 1–2% isoflurane in oxygen administered through a nose cone.

Blood samples were collected directly from the jugular veins which were accessed bilaterally by incisions of about 2 cm along the trachea anterior to the clavicle. Blood samples of 0.2 mL were collected with a 1.0 mL syringe equipped with a 27-gauge needle at 1, 5, 10, 15, 30, 45, 60, 120, 180, 240, 300, and 360 min following IV dosing with additional samples taken at 420 and 480 min following SC dosing. Three baseline samples were also taken following surgery, but prior to dosing, to establish the initial blood glucose levels in each rat. IV administration was delivered directly to the jugular vein while SC doses were administered to the abdomen using an insulin pump. One dosing level of 1.0 IU/kg was considered in this study. Levels for IU of PEGylated insulin were based on the same as that for human insulin of 6 nmol/IU (31). Following testing, the rats were euthanized using a 0.5 mL IV injection of the euthanasia solution according to the Panel on Euthanasia of the American Veterinary Medical Association.

The blood samples from the *in vivo* testing were tested for levels of glucose immediately following sampling by two different blood glucose analyzers in milligrams per deciliter. Both machines were verified daily using a control cuvette to ensure proper operation. The remaining blood was transferred to a serum separator tube and stored on ice for the duration of the test. Within 2 h of sampling, the whole blood samples were centrifuged at 7,500 rpm and the plasma was removed. The plasma samples were centrifuged a second time to eliminate any bubbles and then stored at −80°C until they could be assayed for insulin concentration.

The concentration of human insulin or PEGylated insulin in the rat plasma was determined using enzyme linked immunosorbent assay (ELISA). The kits purchased from ALPCO diagnostics had a cross reactivity with rat insulin of only 0.7%, so any detected changes in insulin concentrations were attributed exclusively to the human insulin which was dosed to the rats. A standard curve of PEGylated insulin was also generated on the ELISA plates and used to adjust the concentrations determined directly from plasma. Any samples which tested high out of range were diluted to 1/100 or 1/1,000 concentration using the zero standard provided with the ELISA kit.

RESULTS

Synthesis and Purification of PEGylated Insulin

In order to target insulin PEGylation to the PheB1 amino acid, the native protein was first modified with di-*t*-butyl dicarbonate (diboc), reversibly alkylating free amine groups with BOC groups. The di-BOC insulin product was synthesized and purified from recombinant human insulin. RP-HPLC spectra for the separated peaks can be seen in Fig. 2. The major peaks corresponding to insulin, mono-BOC insulin, di-BOC insulin and tri-BOC insulin eluted at approximately 10.5, 14, 17, and 22.5 min, respectively. The peak for di-$BOC_{LysB29/GlyA1}$ represented 56.8% of the crude product mixture based on integrated peak area. Fractions of di-BOC insulin with greater than 90% purity were collected and used for PEGylation.

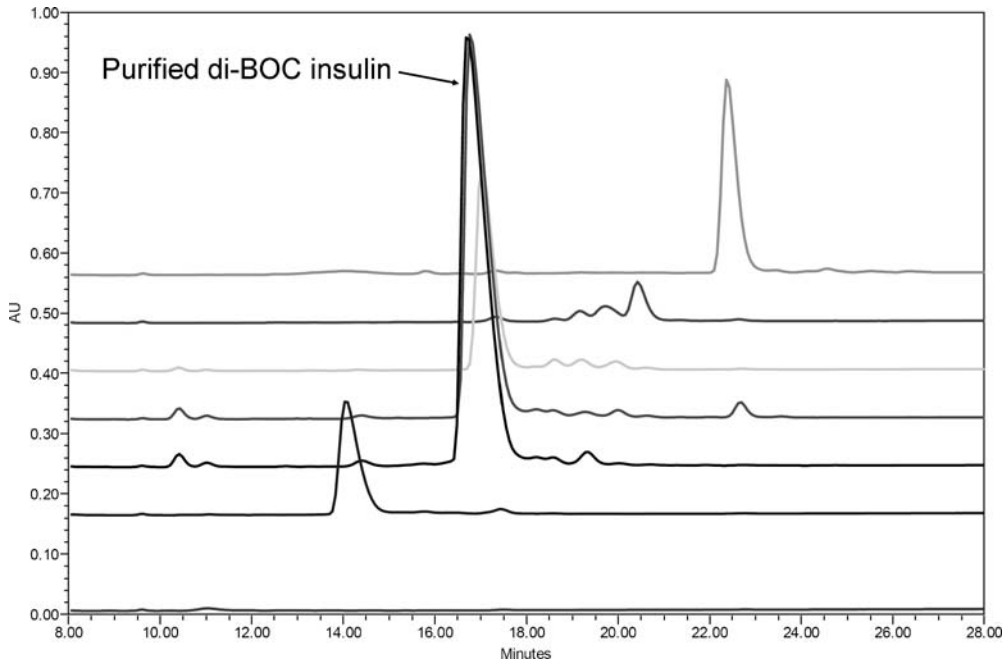

Fig. 2. Reverse phase HPLC chromatograms with absorbance at 214 nm of fractions collected from cation exchange purification.

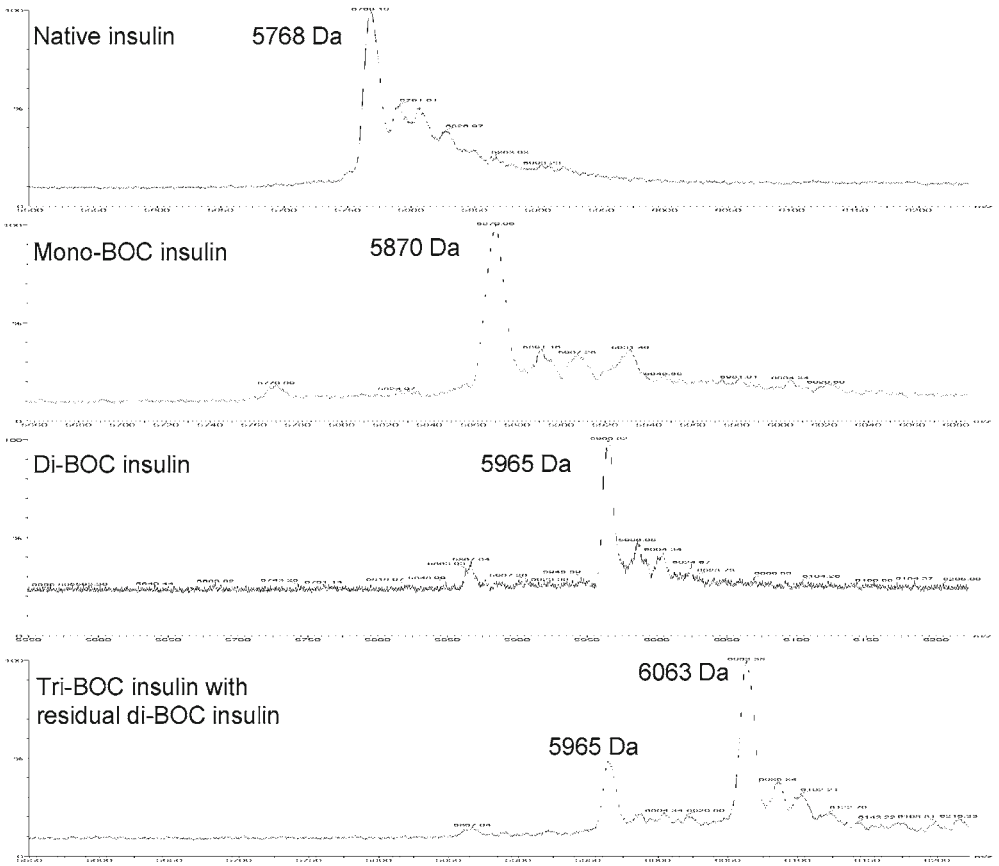

Fig. 3. MALDI-TOF MS of fractions collected from cation exchange purification. Shifts of 100 Da indicate addition of a single BOC group to a free amino group. Additional peaks are due to residual salts associated with the protein.

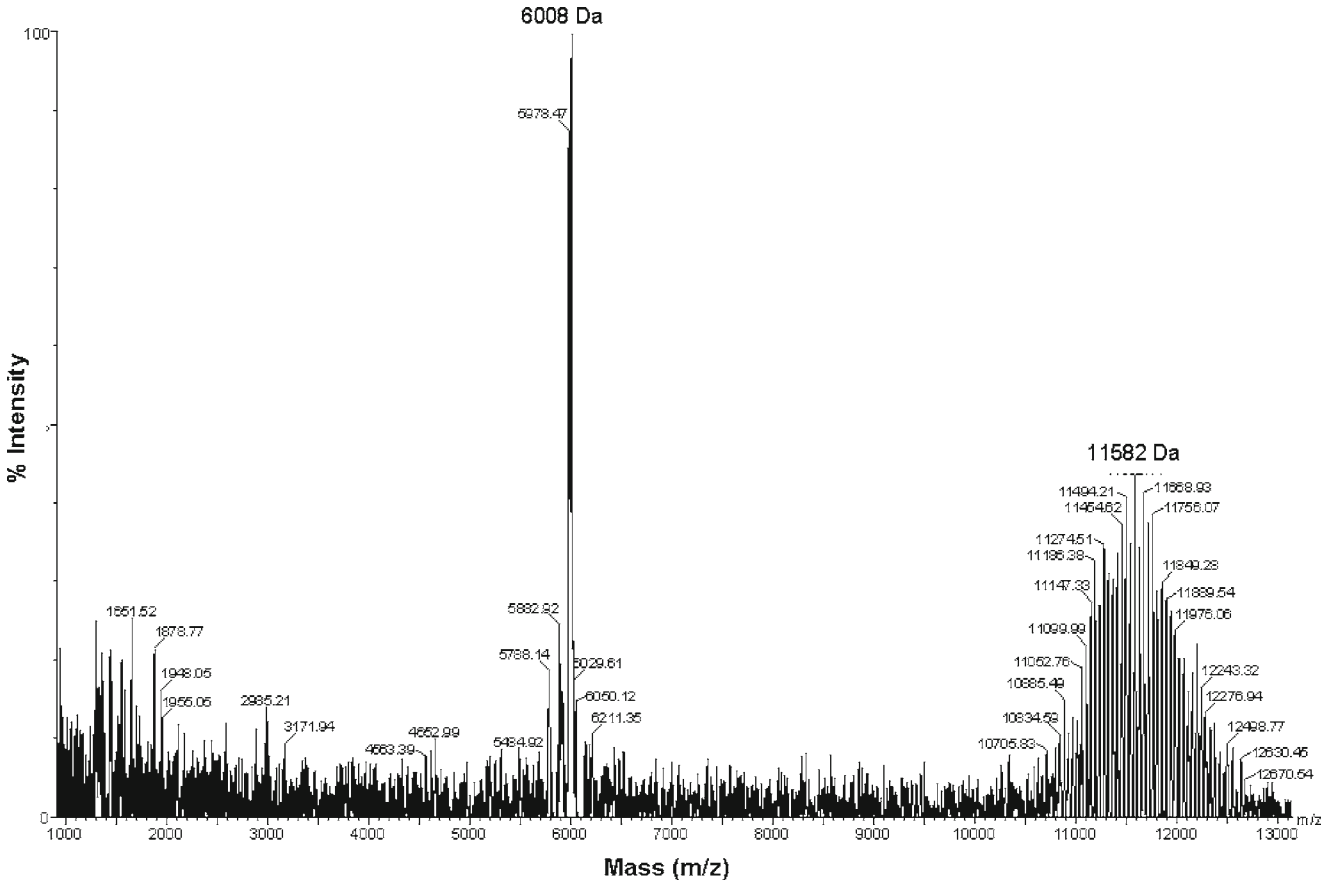

Fig. 4. MALDI-TOF MS of PEGylated diBOC insulin indicated by the molecular weight distribution centered at 11,600 Da. Unreacted diBOC insulin exhibits a peak at 6,008 Da.

Diboc treated insulin products were characterized by MALDI-TOF MS. For diboc treated insulin, the addition of a single BOC modification added 100 Da to the molecular weight of the protein. Fig. 3 shows the molecular weight of the major products of the diboc treated insulin reaction following IEC purification of individual peaks. The molecular weight shifts of 95–102 Da between samples corresponded very well with the addition of a single BOC group. The smaller peaks which had slightly higher molecular weights than the main peaks were due to associated salts which were present in small amounts in the sample solutions.

Insulin PEGylation was confirmed by a series of MALDI-TOF MS analyses as seen in Fig. 4. Unreacted di-BOC insulin had a MW peak at 6,008 Da. The peak distribution centered at ~11,600 Da corresponded to the combination of PEG (MW~5,600) and di-BOC insulin. The presence of PEG was easily distinguished by the Gaussian distribution of its molecular weight with repeated peaks of individual 44 Da monomer units of ethylene glycol.

Characterization of PEGylated Insulin

Identification of the site of PEGylation was determined through targeted degradation of the protein. In a scan on the disulfide reduction reaction retentate between 1,000 and 6,000 Da, a strong peak was evident at 2,634 Da (Fig. 5). This corresponded well to the expected peak at 2,615 Da for the A-Chain with no modification. When the same sample was analyzed between 5,000 and 15,000 Da, a molecular weight distribution was clear with its center at 9,036 Da, corresponding to a PEGylated insulin B-chain with an expected MW centered at 9,174 Da. If PEG were conjugated to the A-chain, a similar distribution would be present centered at 8,127, which was not evident in the MALDI-TOF spectra.

The second method to determine the site of PEGylation for insulin was to digest the modified protein using trypsin. A MALDI-TOF MS spectrum for the mass range of 9,000–13,000 following the trypsin digestion is shown in Fig. 6. There were two distinct peak distributions present with the characteristic Gaussian distribution associated with PEG centered at 10,591 and 11,356 Da. The only other peaks seen in this sample were due to the protein being doubly charged with a molecular weight distribution centered at 5,654 (not shown), roughly half the molecular weight of the major peak at 11,356. These results corresponded very well to expected molecular weights if the site for PEG conjugation was either the GlyA1 or the PheB1, but not on the LysB29.

PEGylated Insulin Loading into P(MAA-g-EG) Hydrogel

The loading for PEGylated insulin was first attempted using P(MAA-g-EG) hydrogels of formulations 0.75/33, 1.25/50 and 2.0/66, representing low, medium and high levels of crosslinking, with a 1:1 ratio of MAA/EG monomers. These hydrogel formulations were able to load native human insulin

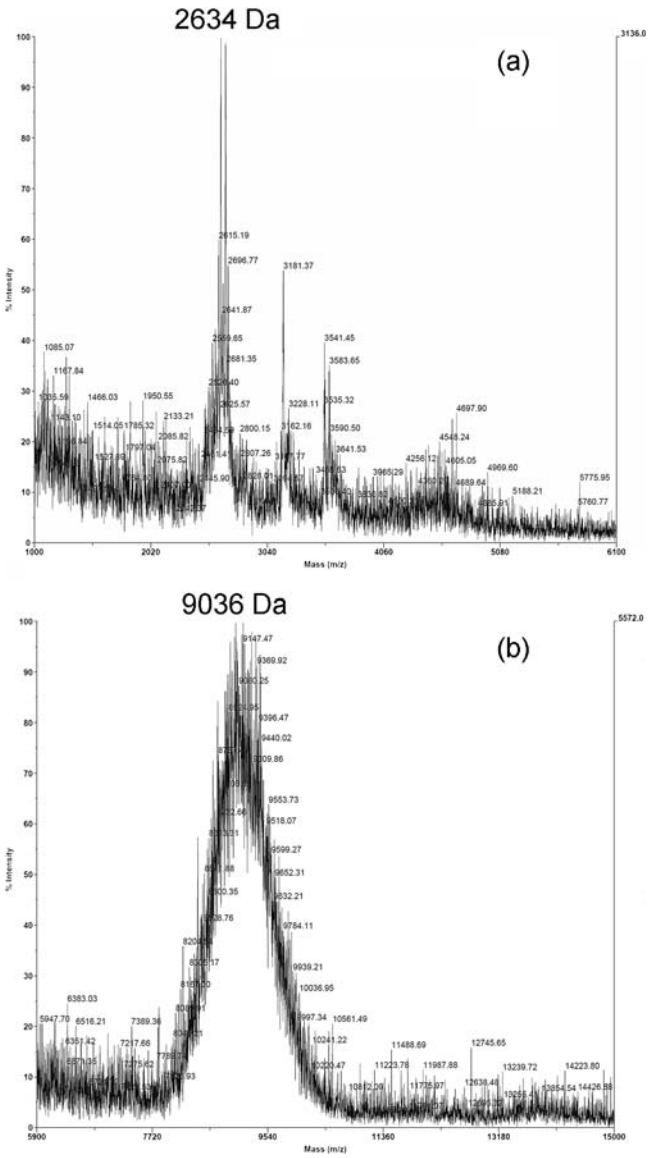

Fig. 5. MALDI-TOF MS of PEGylated insulin following disulfide reduction. **a** The A-chain of PEGylated insulin did not exhibit a change in apparent molecular weight. **b** The unmodified B-chain of insulin was absent with a clear molecular weight distribution centered at 9,036, indicating PEGylation of the B-chain.

to similar level which have been exhibited in previous work (30), but no loading of PEGylated insulin was observed (data not shown). A series of P(MAA-g-EG) hydrogels with the same mole percent of PEGDMA and weight percent of monomers were synthesized with a 3:2 ratio of MAA/EG. The loading efficiencies for insulin and PEGylated insulin are shown in Fig. 7. The loading efficiency for regular insulin increased for formulations 1.25/50 and 2.0/66, but not for formulation 0.75/33 when of monomer ratios were changed from 1:1 to 3:2. The loading efficiencies for PEGylated insulin was higher than native insulin for all formulations considered with a 3:2 monomer molar ratio, and in the formulation 0.75/33 had significantly higher loading efficiency than insulin for either monomer ratio with 90.4% of available PEGylated insulin loaded into the hydrogel.

The release of insulin and PEGylated insulin from P (MAA-g-EG) hydrogels polymerized with a 3:2 ratio of MAA/EG is shown in Fig. 8. Both human insulin and PEGylated insulin release increased with increasing pH. The fractional release for human insulin samples at pH≥6.2 exceeded 80% with insulin for all three formulations except for one sample which still exceeded 70%. Similarly, PEGylated insulin exhibited an increasing release trend as the pH of the release buffer increased from 6.2 to 7.4. However, the relative release amounts of PEGylated insulin were always lower than those seen for native human insulin between pH=6.2–7.4, and the fractional release of PEGylated insulin did not exceed 67% until the pH of the release buffer was pH=7.4.

In Vivo Activity of PEGylated Insulin

A summary of the *in vivo* data for IV insulin and PEGylated insulin administration is given in Table I. The PK profile of insulin and PEGylated insulin following IV administration of 1 IU/kg in healthy male Sprague Dawley rats is shown in Fig. 9. Initial points with concentrations exceeding 500 μIU/mL were omitted from the chart. The concentration of normal human insulin dropped below 500 μIU/mL 10 min after IV administration, but PEGylated insulin required 30 min to drop to the same level. After 60 min the concentration of normal human insulin was 14.6 μIU/mL, and it was almost completely cleared from the circulation after 120 min. PEGylated insulin, on the other hand, sustained plasma concentrations above baseline levels for the duration of the 6-h test and had a concentration of 43.1 μIU/mL after 6 h.

The PD effect of the IV administered insulin and PEGylated insulin, measured by a drop in circulating blood glucose levels, can be seen in Fig. 9. The BG levels were represented as the fraction of the initial values measured by baseline blood samples and were corrected for surgically stimulated glucose changes based on negative controls with PBS. Both regular human insulin and PEGylated insulin induced hypoglycemia in healthy male Sprague Dawley rats following IV administration in a similar fashion. The area above the curve (AAC) values, calculated as the sum of the area of consecutive trapezoids between the curve and 1, for normal human insulin were 61.63 after 4 h and 61.69 after 6 h *versus* 68.42 and 75.43 for PEGylated insulin after 4 and 6 h, respectively. According to these values the level of hypoglycemia induced by PEGylated insulin is 111.0% and 122.3% that of normal human insulin following IV administration.

The PK profile of SC administered insulin and PEGylated insulin at doses of 1.0 IU/kg is given in Fig. 10. The concentrations of both insulin and PEGylated insulin initially increased following dosing as they are absorbed into the bloodstream. Regular human insulin levels reached a maximum concentration (C_{max}) after 30 min at 132.5 μIU/mL. PEGylated insulin levels reached C_{max} 120 min after dosing at 195.0 μIU/mL. The concentration of regular human insulin decreased to near zero after 300 min while PEGylated insulin still had a concentration of 13.5 μIU/mL at the completion of the test at 480 min.

The normalized PD effect of subcutaneously administered insulin and PEGylated insulin is seen in Fig. 10. Again, the circulating glucose levels were corrected for surgically

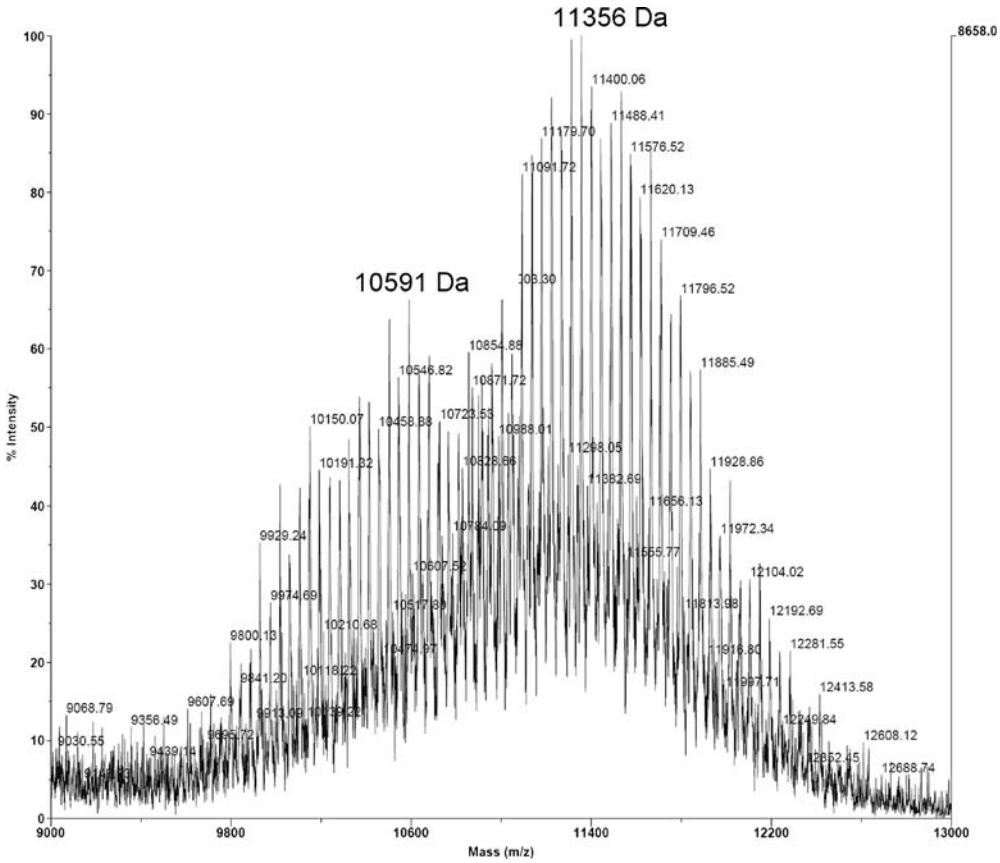

Fig. 6. MALDI-TOF MS of PEGylated insulin following trypsin digestion for 24 h. Two molecular weight distributions are apparent centered at 10,591 and 11,356 Da corresponding to digested and undigested PEGylated insulin, respectively. The absence of a distribution at ~5,600 Da (not shown) indicates that PEG was not conjugated to LysB29.

induced glucose drops. A similar trend was exhibited in the BG levels as was seen for the PK profile in the plasma: a delayed onset of action followed by a sustained effect for PEGylated insulin when compared to regular human insulin. The hypoglycemic effect of regular human insulin was almost instantaneous and was seen after only 5 min. PEGylated insulin did not exhibit a measurable hypoglycemic effect until after 30 min had passed. The maximum hypoglycemic effect for normal insulin was seen after 60 min when BG levels were at 52.9% of initial levels. For PEGylated insulin, the maximum hypoglycemic effect occurred 360 min after SC administration when BG levels were 58.6% that of the initial levels. The AAC values for the normalized curves were 97.3 and 111.8 for normal insulin after 6 and 8 h, respectively. The AAC values for PEGylated insulin were 98.2 and 142.9 for 6 and 8 h, respectively. Using these AAC values, the level of hypoglycemia induced by PEGylated insulin was 100.9% and 127.8% that of regular human insulin.

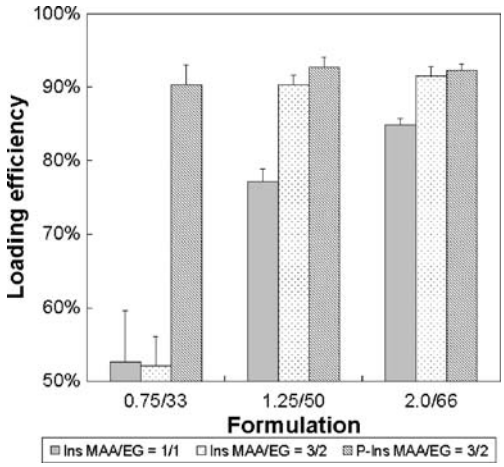

Fig. 7. Loading efficiencies for regular human insulin (*Ins*) and PEGylated insulin (*P-Ins*) in P(MAA-g-EG) hydrogels with MAA/EG monomer ratios of 1/1 or 3/2 (+SD, $^{**}p<0.01$, $^{*}p<0.5$). Groups are designated according to mole percent PEGDMA/weight percent of monomer present during polymerization.

DISCUSSION

The objective of this work was to synthesize a PEGylated insulin species and examine its feasibility for oral administration with P(MAA-g-EG) complexation hydrogels. Insulin PEGylation has the potential to significantly improve the enzymatic resistance of the protein in the GI tract and increase the circulation time of the protein. While these hydrogels have been shown to have potential for oral insulin delivery, they have never been tested with a PEG–insulin conjugate. In this study, the first examination of the interaction between PEGylated insulin and P(MAA-g-EG) hydrogels is performed *in vitro*. Additionally, the site-specific PEGylation of insulin with a PEG chain of ~5,000 Da is

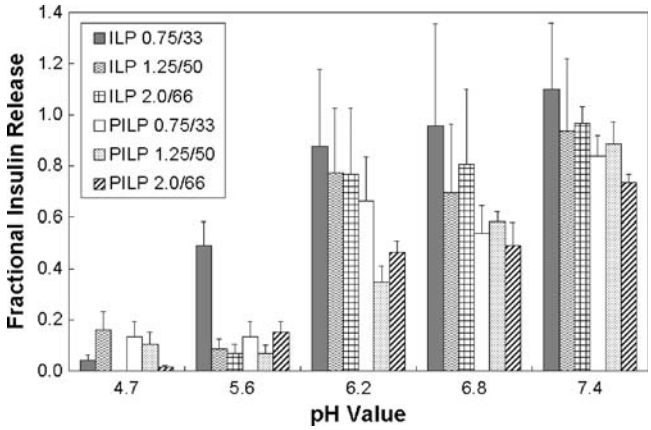

Fig. 8. Insulin loaded polymer (*ILP*) and PEGylated insulin loaded polymer (*PILP*) release from P(MAA-g-EG) hydrogels with a MAA/EG monomer ratio of 3:2 following 3 h of dissolution in PBS at 37°C (+SD, n=3). Series are listed according to mole percent PEGDMA/weight percent of monomer present during polymerization.

performed and measured for its retention of biological activity. This polymer molecular weight is significantly larger than what has been studied in the referenced material which hypothesized that such a high MW would cause a significant drop in the ability of the hormone to induce a hypoglycemic effect.

According to studies by Baudyš *et al.*, the reactivity of the amine groups in insulin proceeds with LysB29≥GlyA1> PheB1 when present in the DMSO/TEA solution used in this study (23). With this in mind, the PEG conjugation was targeted to the PheB1 residue in an attempt to maintain the bioactivity of insulin by pre-treating the protein with di-*t*-butyl dicarbonate (diboc), which can reversibly react with the free amine groups of a protein (28). The diboc reaction created multiple modified insulin products containing zero, one, two, or three BOC species. Careful control of the pre-treatment reaction conditions produced a major intermediate product with BOC groups attached to the LysB29 and GlyA1 residues on insulin. Purification of the di-$BOC_{LysB29/GlyA1}$ allowed for subsequent reaction of the PheB1 by an amine reactive mPEG-SPA. While each individual product of the diboc treated insulin reaction was not characterized, the analysis of the final PEGylated product confirmed that the purified diboc reacted insulin product was, in fact, di-$BOC_{LysB29/GlyA1}$.

The MALDI-TOF MS analysis of the mPEG-SPA/di-BOC insulin reaction product was performed using a matrix, sinapic acid, which produced a signal for proteins, but not on PEG alone. This feature indicated that the signal at ~11,600 Da must have been due to the presence of a protein and could only correspond to a mono-substituted PEG–insulin conjugate. However, the specific site of the modification was unclear without further analysis. In order to distinguish potential modification sites from one another, disulfide reduction and trypsin digestion was used. Disulfide reduction separated the A and B chains of PEGylated insulin producing a peptide fragment with a MW distribution centered at 9,036 Da corresponding to PEGylation of the insulin B chain. No such distribution was observed for the A chain of insulin, thus eliminating GlyA1 as a possible site of PEGylation. Trypsin specifically cleaves the carboxyl side of arginine and lysine residues. The labile peptide bonds on the B-chain of insulin are indicated in Fig. 1. This was relevant for PEGylated insulin because it isolated the LysB29 site from the other two free amine residues. If PEG were conjugated to the PheB1 site as desired, the expected shift in the molecular weight of the PEGylated insulin due to the loss of the eight peptide sequence at the C-terminus of the B-chain (GFFYTPKT) would have been 959 Da. MALDI-TOF analysis of the resulting degradation products exhibited two molecular weight distributions centered at 10,591 and 11,356 Da, a shift in the MW distribution of approximately 765 Da as seen in Fig. 6. This discrepancy was most likely due to the significant amount of crossover of the two distributions, shifting the trypsinized peak to the right. Nonetheless, the peak distributions corresponded well to undigested (11,356 Da) and trypsin digested (10,591 Da) PEGylated insulin. Further, the lack of a similar distribution centered ~6,500 Da indicated that the PEGylation did not occur at the LysB29 residue. Since the site of PEGylation was determined to not be present at the GlyA1 from disulfide digestion, this work confirmed that the mPEG-SPA was conjugated exclusively to the PheB1 residue of insulin. These results are a clear representation of the site-specific, mono-conjugation of PEG to the PheB1 residue of insulin based on the direct analysis of the molecule using MALDI-TOF MS.

One interesting result that was indirectly inferred from the characterization of PEGylated insulin is the fact that less than half of it was degraded by trypsin based on MALDI-TOF MS signal magnitude, even after the digestion was performed for longer than three times the manufacturer recommended duration. Trypsin is a major lumenal enzyme in the intestines, indicating that the covalent attachment of PEG to insulin may significantly increase the residence time of insulin in the intestinal milieu, thus increasing its opportunity for absorption across the intestinal epithelium. It has been reported that increasing the MW of the PEG chain used increases the effects of PEGylation, namely enzymatic resistance (32). In work performed by Hinds *et al.*, PEG lengths of 750 and 2,000 Da were conjugated to insulin (25). These PEGylated insulin species exhibited increased stability

Table I. PK/PD Summary of IV and SC Administration of Insulin and PEGylated Insulin at 1.0 IU/kg to Healthy Male Sprague Dawley Rats

	IV administration		SC administration			
	AAC $\left(\frac{C(t)}{C_o}\right) \times$ min	Relative hypoglycemic effect (%)	AAC $\left(\frac{C(t)}{C_o}\right) \times$ min	Relative hypoglycemic effect (%)	C_{max} (μIU/mL)	t_{max} (min)
Human insulin	61.69	N/A	111.8	N/A	132.5	30
PEGylated insulin	75.43	122.3	142.9	127.8	195.0	120

Hypoglycemic values were determined 6 h after IV dosing and 8 h after SC dosing

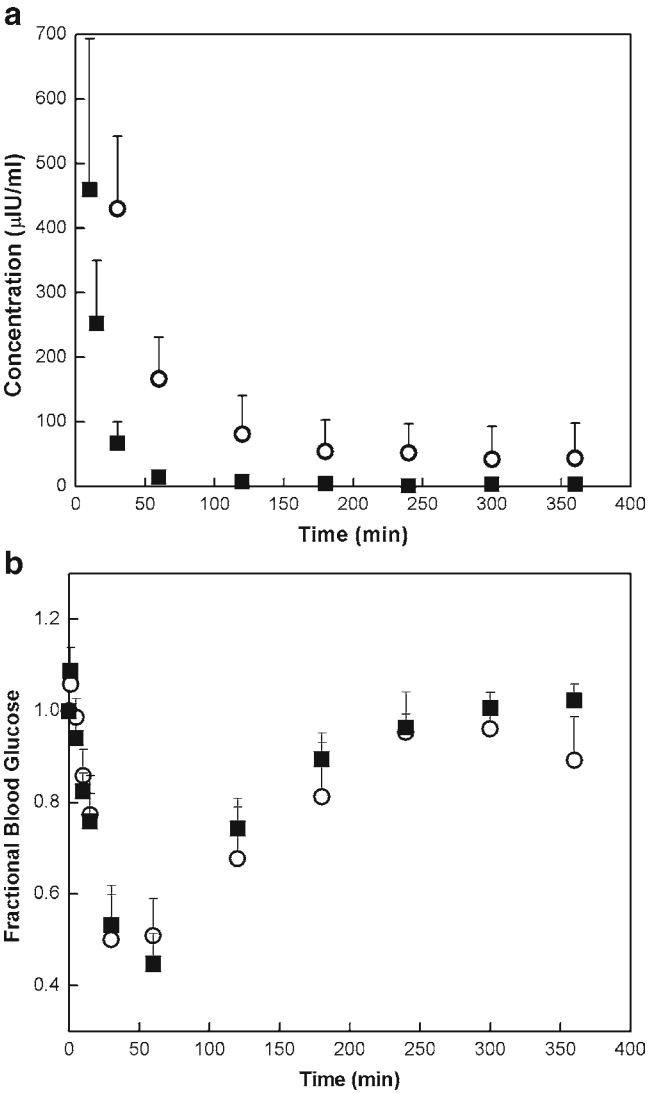

Fig. 9. **a** Pharmacokinetic and **b** pharmacodynamic profiles for insulin (*filled squares*) and PEGylated insulin (*open circles*) following IV injection of 1.0 IU/kg in healthy male Sprague Dawley rats (+SD, n=6).

and reduced immunogenicity with a greater effect observed with increasing MW of the PEG chain. Caliceti *et al.* showed that the enzymatic resistance of PEGylated insulin was significantly higher than native insulin and increased according to the number of PEG substitutions (21). For these reasons, the higher molecular weight of the PEG used in this work should impart more of the beneficial effects of PEGylation on insulin, but it may cause a drop in bioactivity. For this reason, the ability of the high molecular weight PEG–insulin conjugate to induce a hypoglycemic effect was studied. Prior to this investigation, however, the feasibility of the design combining PEGylated insulin with P(MAA-e-EG) had to be established.

Recently, insulin loading and release was shown to depend on both the swelling and pore size of the hydrogel as well as the interaction between the anionic polymer and the protein (30). Initial results indicated that the same interactions which induce the pH responsive behavior, the formation of hydrogen bonds between the MAA and EG groups of the copolymer at low pH and the loss of those bonds and ionic repulsion of anionic MAA groups at neutral pH, may be more important to the performance of P(MAA-g-EG) for insulin loading and release. With that in mind, three hydrogel formulations were synthesized with varying degrees of covalent crosslinks each with two different monomer ratios. At a 1:1 MAA/EG ratio, PEGylated insulin was unable to be loaded into the hydrogel network for all three formulations of the hydrogel. If protein loading was due to hydrogen bonding between the protein and the hydrogel, the additional PEG group on the PEGylated insulin may have made it unable to be incorporated within polymer network. At a 1:1 monomer ratio, the MAA groups preferentially formed hydrogen bonds with PEG chains in the hydrogel backbone. When the monomer ratio of MAA/EG groups was altered to 3:2, PEGylated insulin was loaded with high incorporation efficiencies into all three formulations. Due to the imbalance of monomers, more protonated MAA groups were present than available EG monomers in the PEG grafts

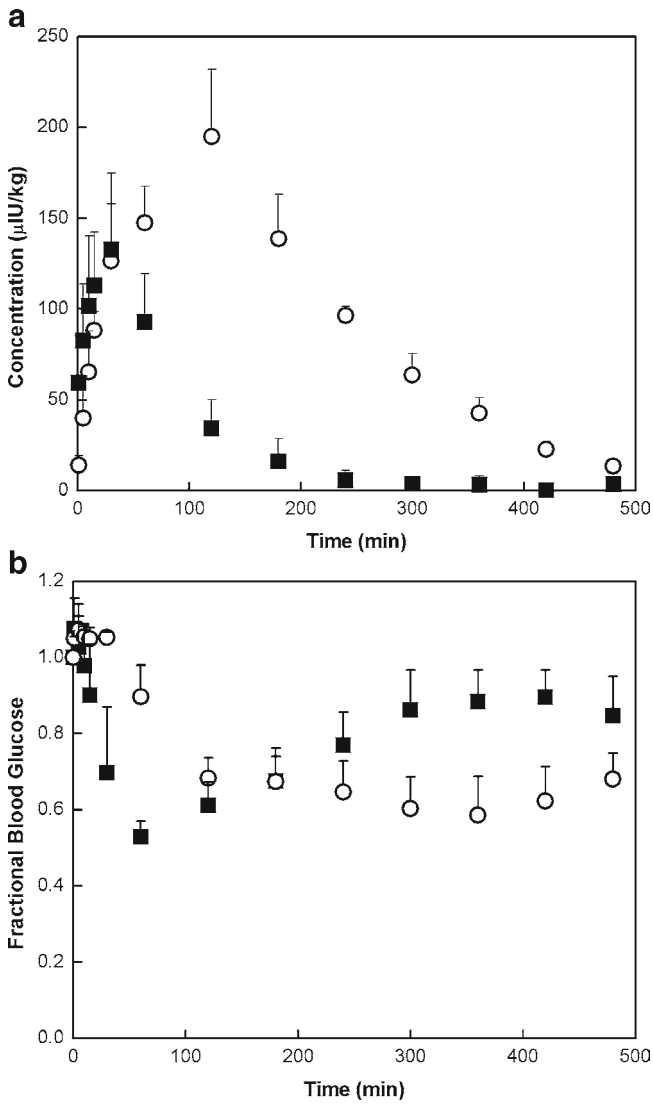

Fig. 10. **a** Pharmacokinetic and **b** pharmacodynamic profiles for insulin (*filled squares*) and PEGylated insulin (*open circles*) following SC injection of 1.0 IU/kg in healthy male Sprague Dawley rats (+SD, n=6).

within the hydrogel. The presence of PEGylated insulin provided additional PEG groups which could bind to the hydrogel backbone, accounting for the dramatic increase in PEGylated insulin loading. Human insulin loading efficiencies were also higher for hydrogels with a 3:2 MAA/EG monomer ratio, indicating that the increase in available hydrogen bonds may have had the same effect on native insulin.

The fractional release of insulin and PEGylated insulin exhibited increasing release with increasing pH, though PEGylated insulin generally was lower than human insulin, suggesting a stronger affinity of PEGylated insulin for the hydrogel. These results offer additional support to earlier findings (30) indicating that the weak bonds formed between the protein and P(MAA-g-EG) are the primary reason for their ability to incorporate insulin with such high efficiencies and release them based on relatively small changes in the size of the network properties. While the kinetics of the protein release are traditionally a meaningful measure for a non-parenteral delivery of a drug, in the case of P(MAA-g-EG) the release is controlled by the pH of the surrounding environment. This behavior has been seen in previous work with this polymer with fractional release occurring in a stepwise fashion based on pH (LOWMAN). The total release of protein from the hydrogel occurred rapidly to the level shown in Fig. 8 (<15 min). The reason each sample was displayed following 3 h of release to ensure that there was, in fact, no increase in release over time. This release behavior could be advantageous for oral insulin delivery because PEGylated insulin would maintain a higher concentration within the hydrogel as it passes through the GI tract. If the release of PEGylated insulin increases proportionally with pH as it passes down the intestines, the possibility for a slow, sustained protein release exists as the polymer moves down the GI tract.

While PEGylated insulin exhibited improved behavior with the P(MAA-g-EG) hydrogels, the true potential of PEGylated insulin for the treatment of diabetes relies on its ability to maintain its ability to bind to the insulin receptor and induce the transport of circulating glucose into cells. In the case of insulin, this can be measured by determining the hypoglycemic effect induced by the introduction of exogenous insulin. A comparison between regular human insulin and PEGylated insulin was measured *in vivo* in rats. It is important to note that the three amino acid difference between murine and human insulin has no effect on the activity of human insulin in rats, but the ELISA assay used in this work was able to distinguish between the two with only 0.7% cross reactivity according to manufacturer specifications. This allowed for measurement of only exogenous insulin and PEGylated insulin levels.

The levels of insulin were measured from plasma samples to monitor the PK profile for both IV and SC administered insulin and PEGylated insulin. The concentrations of both regular insulin and PEGylated insulin were very high following IV administration because of direct delivery to the bloodstream. Compared to regular insulin, PEGylated insulin exhibited a sustained PK profile. The relative extension in circulation indicated that PEGylated insulin had a slower rate of clearance by the reticuloendothelial system (RES) and consumption by the liver, the two major routes of insulin removal from the bloodstream (33). This was expected for PEGylated insulin due to the increase in MW of the protein, and has been seen for other PEGylated proteins. The PK profile for SC administered PEGylated insulin took significantly longer to reach maximum concentration (t_{max}) than that or native insulin, indicating a slower rate of absorption from the SC tissues. This behavior in combination with sustained circulation seen in the PK profile led to a higher maximum concentration (C_{max}) for PEGylated insulin relative to regular insulin. While both sustained circulation and slower absorption were expected to occur for PEGylated insulin, their combination was unexpectedly manifested to give a higher C_{max} for PEGylated insulin.

When insulin concentrations exceeded basal levels (5–15 μU/mL) in the absence of elevated blood glucose levels, a hypoglycemic response occurred (34). In this way, the PD profiles for insulin and PEGylated insulin were monitored according to the drop in blood glucose induced by the exogenous protein. Intravenously dosed insulin and PEGylated insulin had similar PD profiles with onset of hypoglycemia in 5 min, minimum BG levels at 60 min and a return to baseline BG levels in 240 min. This was an important result because it proved that even using a PEG chain of 5,000 Da the PEGylated protein could still effectively bind to the insulin receptor. Pharmacodynamic profiles for subcutaneous administration had more significant differences between human insulin and PEGylated insulin. The onset for human insulin was first observed 10 min after dosing with a maximum hypoglycemic effect in 60 min. PEGylated insulin did not induce hypoglycemia until 60 min after dosing with maximum effect at 360 min. Similar to the PK profile for SC dosed PEGylated insulin, a delayed onset and sustained effect was due to the extended residence time of PEGylated insulin in the bloodstream. This lengthened duration of action was a very interesting result for PEGylated insulin because it proved that it could have a sustained hypoglycemic effect in comparison to regular insulin. If the same behavior can be observed when PEGylated insulin is absorbed across other tissues, such as the intestinal epithelium, then it could have a meaningful impact to diabetic patients following oral delivery. The results also implied that optimization of the MW of PEG could be used to design a new type of long-acting insulin analog.

The level of hypoglycemia induced by PEGylated insulin for both IV and SC administered PEGylated insulin exceeded 100%. While it may seem that modification of insulin would reduce its ability to lower circulating glucose levels, this effect can be explained not by an increased potency of PEGylated insulin, but in its increased residence time in the bloodstream. The reduced rate of clearance causes it to have an extended action and therefore a prolonged hypoglycemic effect. This may also be influenced by a reduced affinity of PEGylated insulin for the insulin receptor such that it is released and binds to subsequent receptors. However, a true measure of the potency of PEGylated insulin *versus* regular insulin requires more information for the binding coefficient and residence time of each species with the insulin receptor which cannot be determined using the methods used in this study. Most importantly, the PK/PD profiles generated by PEGylated insulin following both IV and SC administration provide critical verification that the conjugate maintained its bioactivity in an *in vivo* model.

CONCLUSIONS

In this work, the synthesis and purification of mPEG–PheB1–insulin was clearly demonstrated. The modification was achieved specifically at the PheB1 residue by reacting insulin with diboc prior to conjugation with PEG. The properly protected insulin product, di-$BOC_{LysB29/GlyA1}$, was purified using cation exchange chromatography and PEGylated. The protein conjugation was characterized using a series of protein digestion methods in conjunction with MALDI-TOF MS. By process of elimination, the PheB1 residue was confirmed as the only site of PEG conjugation. Regular human insulin and PEGylated insulin were successfully loaded into P(MAA-g-EG) hydrogels with low, moderate and high levels of crosslinking. PEGylated insulin loading was not observed in the hydrogel formulations with a 1:1 monomer ratio. However, in hydrogels formulated with a MAA/EG ratio of 3:2 PEGylated insulin had the highest loading efficiencies for all three levels of crosslinking. The fractional release of PEGylated insulin increased as the pH increased from 6.2 to 7.4 and was lower than regular human insulin for these pH levels. These results indicated that PEGylated insulin had a greater affinity for the tested formulations of P(MAA-g-EG) hydrogels with a 3:2 MAA/EG monomer ratio than regular human insulin. This could be advantageous in future designs for oral insulin delivery because of the potential for sustained localization of the drug within the hydrogel.

The retention of the bioactivity of PEGylated insulin was confirmed using intravenous (IV) and subcutaneous (SC) dosing at a dosing level of 1.0 IU/kg in healthy male Sprague Dawley rats. The hypoglycemic effect of PEGylated insulin following IV administration was 122.3% that of regular human insulin after 6 h. Subcutaneous administration yielded significantly lengthened PK/PD profiles for PEGylated insulin *versus* that seen for regular human insulin. The hypoglycemic effect of PEGylated insulin following SC administration was 127.8% that of regular human insulin after 8 h. Previous work on PEGylated insulin suggested that the molecular weight of PEG greater that 750 Da would cause a decrease in the biological activity of insulin (35). However, the results of the work presented in the current study indicate that PEGylated insulin in which a 5,000 Da PEG chain conjugated to the PheB1 site of insulin maintained its biological activity *in vivo* based on its ability to induce a drop in circulating blood glucose levels. This was an important result because higher MW PEG species would be able to impart more of the beneficial effects of PEGylation on proteins than lower MW PEG chains. With further studies and optimization, a combination of PEGylated insulin and P(MAA-g-EG) hydrogels have significant promise for oral delivery.

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