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Research paper

Development of acrylic-based copolymers for oral insulin delivery☆

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Abstract

We developed nanospheres of crosslinked networks of methacrylic acid grafted with poly(ethylene glycol), and acrylic acid grafted with poly(ethylene glycol) nanospheres for use as oral insulin delivery devices. The copolymer nanospheres were synthesized via free-radical precipitation/dispersion. The average particle diameter of the copolymer gel nanospheres at various physiologically relevant pH values was characterized using photon correlation spectroscopy. Their size increased dramatically as the surrounding pH rose above the pK_a of the network. The nanospheres ranged in diameters from 200 nm at pH of 2.0 to 2 μm at pH around 6.0. Insulin was loaded into the copolymers at levels of 9.33 and 9.54 mg per 140 mg solid sample, by partitioning from concentrated insulin solutions. In vitro studies were performed to study the passage of the insulin-loaded copolymer samples in the gastrointestinal tract. Insulin was entrapped at low pH (pH = 3.0) but released at more neutral pH (pH = 7.0). Animal studies were performed to investigate the abilities of insulin-loaded copolymer samples to influence the serum glucose levels of rats. In studies with diabetic rats, the serum glucose level was lower than control values for the animals that received the insulin-loaded copolymers and lasted for at least 6 h. The insulin loaded copolymer nanospheres caused a significant reduction of serum glucose with respect to that of a control animal.

Keywords: Oral insulin delivery; Acrylic-based copolymer; Copolymer nanospheres; pH-Sensitive release; Poly(ethylene glycol)

1. Introduction

Combinatorial chemistry and the growing knowledge of the biochemistry of the body have lead to an ever-increasing number of therapeutic proteins in the treatment of diseases. However, these proteins often lack the stability that more traditional small molecular weight drugs possess. Where a simple therapeutic agent, such as aspirin or a simple antibiotic can be taken orally and reach the bloodstream intact, the larger and more delicate protein must often be delivered directly into the blood stream through injection. The harsh conditions of the stomach often destroy most of the protein before it reaches the bloodstream. In the case of insulin, less than 0.1% of the orally dosed insulin reaches the blood stream intact [1,2]. This means that for patients to make use of the ever-expanding protein database, they must receive insulin through injections.

Unfortunately, injections are often painful, and can lead to low patient compliance. Thus, in our laboratory we have tried to find alternate ways of delivering proteins and other unstable therapeutic agents [3–8]. We believe that oral or transmucosal delivery is an improved method of protein delivery and can be much easier than dealing with injections and can lead to improved patient compliance.

Oral administration has numerous barriers to overcome in order to create an effective system for protein delivery [2,5,8]. The greatest barriers are the harsh conditions of the stomach due to the acidic environment and the action of proteolytic enzymes, and the transport barrier of the intestinal wall.

In this study, we developed two sets of copolymers capable of trapping proteins inside the copolymer carriers at pH environments near 3.0, while releasing them at pH values of 7.0. As we will show, these copolymers also increased protein transport across the cellular barrier in

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the upper small intestine. We chose insulin as a model protein because it is a key protein in the treatment of diabetes mellitus and represents a protein drug employed for a disease that affects a large number of people all over the world.

The goal of this research was to study a set of copolymers that were designed for their beneficial properties and to determine which of these copolymers exhibited the greatest efficacy as an oral insulin delivery device. The copolymers comprised either methacrylic acid or acrylic acid for their pH-sensitive nature [1,3,6,8,9] and ability to bind calcium [10], and poly(ethylene glycol) for its ability to stabilize and protect proteins [8,11,12].

2. Experimental part

2.1. Synthesis of P(MAA-g-PEG) and P(AA-g-PEG) nanospheres

Copolymers were prepared by reacting acrylic acid (AA, Sigma, St. Louis, MO), methacrylic acid (MAA, Sigma, St. Louis, MO), methoxy terminated poly(ethylene glycol) monomethacrylate (with PEG molecular weight 1000) (PEGMAA, Polysciences Inc., Warrington, PA) and tetra (ethylene glycol) dimethacrylate (TEGDMA, Polysciences Inc., Warrington, PA).

1-Hydroxylcyclohexyl phenyl ketone (Irgacure-184, Ciba-Geigy Corp., Hawthorne, NY) was used as the photo-initiator in all reactions. Both AA and MAA were vacuum distilled to remove their inhibitors. PEGMAA and TEGDMA were used as received. In all reactions, 1 mol.% of TEGDMA (based on total monomers), was used as a crosslinking agent. In addition, Irgacure-184 was used at a level of 1% weight. Detailed analysis of the kinetics of PEG-tethered monomer reactions has been reported already [13–15].

A 2 vol.% aqueous monomer solution was purged with nitrogen for 20 min and the monomers were polymerized with UV light at 138 mW/cm^2 for 20 min. The resulting nanospheres were subsequently washed until all unreacted monomers were removed, by placing them in sealed dialysis bags and submerging in deionized water.

D(+)-Trehalose was added to the mixture in the amount of 1 g trehalose per g of gel nanospheres. The mixture was freeze-dried to recover the dry nanospheres.

2.2. Characterization by photon correlation spectroscopy

The diameters of the copolymer gel nanospheres of both P(MAA-g-PEG) and P(AA-g-PEG) were measured in triplicate using photon correlation spectroscopy (PCS). A submicron particle sizer (N4, Coulter Corporation, Miami, FL) was used to determine the diameter of the copolymer nanospheres in a range of pH environments. The four different monomer molar feed ratios studied were 4:1, 2:1, 1:1 and 1:2 MAA:EG or AA:EG repeating units. The swelling temperature of the samples was maintained at 37°C and the ionic strength was held constant at 0.1 M.

An autocorrelation function analysis was used to measure the diameter of the copolymer gel nanospheres [16]. The light scattering measurement was performed for 200 s per sample and then the data were analyzed.

2.3. Insulin loading of nanospheres

Insulin was loaded into the copolymeric nanospheres by placing copolymer nanospheres in a concentrated insulin solution. The insulin diffused into the networks and then the pH of the gel samples was lowered to complex the networks and trap the insulin inside.

A 100 ml sample of a 0.5 mg/ml of bovine insulin solution (Sigma, St. Louis, MO) was formed by dissolving insulin in 10 ml of an HCl solution. A pH 7.4 phosphate buffer solution of 80 ml was added to the insulin solution, followed by 10 ml of a NaOH solution. To ensure that insulin did not absorb on the glass surface, 0.02 mg of Tween-80 was added.

Insulin was loaded into both P(MAA-g-PEG) and P(AA-g-PEG) gel nanospheres. The P(MAA-g-PEG) gel nanospheres were prepared with a comonomer molar feed ratio of 1:1 MAA:EG, while for the P(AA-g-PEG) gel nanospheres we used a monomer feed ratio of 2:1 AA:EG. 140 mg of each gel composition were placed in 20 ml of an insulin solution at 25°C for 6 h.

After 6 h of insulin loading, a 0.5 ml sample was taken for loading efficiency studies and analyzed via high-performance liquid chromatography (HPLC). The rest of the insulin loading mixture was sealed in dialysis bags and submerged in an HCl solution to complex the nanospheres and trap insulin. D(+)-Trehalose was added to stabilize the nanospheres [17,18] and the system was freeze-dried.

2.4. Insulin release studies

Release studies were performed by placing 0.03 g samples of insulin-loaded copolymer gel carriers in 50 ml of a pH 3.0 buffer. The low pH was used to simulate the conditions of the stomach. The mixture was then placed in a USP II dissolution apparatus (Distek model 2100B dissolution apparatus, Inc. North Brunswick, NJ) with 100 ml vessels and stirred at 100 rpm. Samples of 0.5 ml were taken at given time intervals to measure the insulin released from the loaded copolymeric gel carriers. To simulate the copolymer materials moving from the stomach into the upper small intestine after 1 h, the pH of the buffer was adjusted to 7.0 by addition of a NaOH solution. Again samples of 0.5 ml were collected at given time intervals and the insulin concentration was measured via reverse phase HPLC.

2.5. Animal studies

Insulin delivery studies of orally administrated insulin-loaded copolymer carriers were conducted in male diabetic Wistar rats (see also ref. [1]) of 200 g average body weight. Diabetic animals were prepared by injecting the rats with streptozotocin (40 mg/kg body weight) once daily for 3 days. After 4 additional days their glucose levels were checked for diabetic conditions. Insulin-loaded nanospheres were fed to the rats using a gelatin capsule. The capsule was administered down the esophagus of the rats and two different insulin-loaded copolymers were tested in the diabetic animal study: P(MAA-g-PEG) nanospheres with a monomer feed ratio of 1:1 MAA:EG repeating units, and P(AA-g-PEG) gel nanospheres with a molar monomer feed ratio of 2:1 AA:EG repeating units. The dosage given to each rat was 50 IU/kg of animal body weight and the corresponding amount of loaded particles was calculated using the results of the loading/release studies.

The animals were allowed to freely move around during the study to simulate natural activity. Blood samples were taken from the jugular vein at specific times after administration of the copolymers to get a serum glucose measurement as a function of time, measured with a glucose oxidase kit.

3. Results and discussion

3.1. Material synthesis and characterization

Uniform nanospheres of the copolymers tested were produced by the UV precipitation polymerization methods used. Photon correlation spectroscopy was used to measure the average diameter of the gel nanospheres in solution.

Fig. 1 shows the results of the swelling behavior of P(AA-g-PEG) gel nanospheres prepared with various monomer feed ratios of 4:1, 2:1, 1:1 and 1:2 AA:EG units. In this graph we plot the mean diameter of a given class of P(AA-g-PEG) gel nanospheres divided by their diameter measured at pH 2.0 versus the pH of the buffer at which the measurement was made. This resulted in curves of normalized gel nanosphere diameters versus the buffer. The base pH of 2.0 was used because at pH 2.0 the copolymer gel nanospheres of P(AA-g-PEG) were in their complexed state. These results should be also compared to our recent studies for optimization of the particle size [4,5].

The results of Fig. 1 show that there was a significant change in equilibrium gel swelling depending on the monomer feed ratio used during preparation of the P(AA-g-PEG). The amount of AA in the monomer feed ratio correlated with the equilibrium swelling volume at the higher pH values. Thus, the amount of AA incorporated into the P(AA-g-PEG) gel nanospheres could be modified by changing the monomer feed ratio. In the case of the highest AA:EG monomer feed ratio, that of 4:1 AA:EG, the greatest normalized diameter at pH = 6.0 was 12.6. The P(AA-g-PEG) gel nanosphere with monomer feed ratios of 1:2 AA:EG exhibited the smallest change in mean diameter at only 3.6 times the mean diameter at pH 2.0. The P(AA-g-PEG) gel nanospheres with monomer feed ratios of 1:1 AA:EG and the 2:1 AA:EG ratios were 4.8 and 7.7 of their pH 2.0 values, respectively. Thus, the higher the AA:EG monomer feed ratio, the more dynamic the change in size on the P(AA-g-PEG) gel nanosphere as the pH was increased.

Fig. 2 shows the size distribution results of the P(MAA-g-PEG) nanospheres with comonomer feed ratios of 4:1, 2:1, 1:1 and 1:2 MAA:EG repeating units. Again, we plotted the mean diameter of a given class of P(MAA-g-PEG) nanospheres normalized with respect to the diameter measured at pH 2.0. Table 1 lists the diameters measured for all of the P(AA-g-PEG) gel nanospheres at

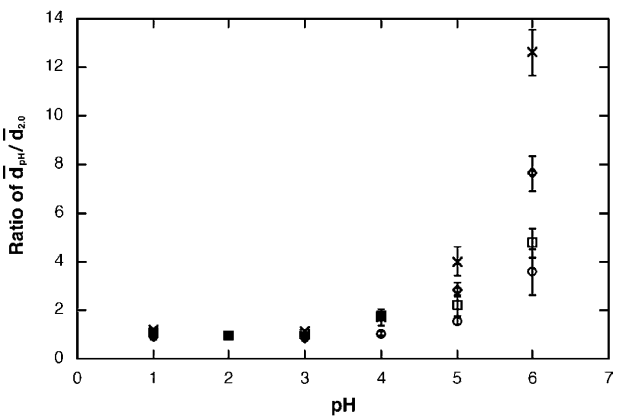

Fig. 1. Normalized diameter (expressed as a ratio of mean diameter at given pH divided by mean diameter at pH 2.0) of gel nanospheres of P(AA-g-PEG) prepared with monomer feed ratios of 4:1 AA:EG (×), 2:1 AA:EG (◇), 1:1 AA:EG (□) and 1:2 AA:EG (○). PCS was used to measure the average diameter. ($n = 3$, error 95% confidence interval).

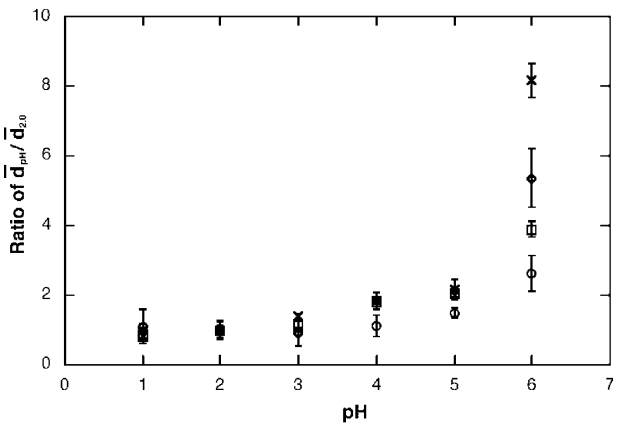

Fig. 2. Normalized diameter (expressed as a ratio of mean diameter at given pH divided by mean diameter at pH 2.0) of gel nanospheres of P(MAA-g-PEG) prepared with monomer feed ratios of 4:1 MAA:EG (×), 2:1 MAA:EG (◇), 1:1 MAA:EG (□) and 1:2 MAA:EG (○). PCS was used to measure the average diameter. ($n = 3$, error 95% confidence interval).

Table 1
Mean diameter of P(AA-g-PEG) gel nanospheres[a]

AA:EG monomer feed ratio	pH 2.0 buffer (nm)	pH 6.0 buffer (nm)
4:1 AA:EG	230 ± 20	2900 ± 200
2:1 AA:EG	250 ± 30	1900 ± 200
1:1 AA:EG	220 ± 20	1100 ± 100
1:2 AA:EG	340 ± 20	1200 ± 300

[a] ($n = 3$, error expressed as 95% confidence intervals).

both the pH 2.0 and 6.0 conditions. It is important to note that in their highly swollen state at high pH values the copolymer gel nanospheres were above 600 nm.

It is well known [2] that hydrophobic particles under 600 nm can be taken up by the Payer's patches. While the copolymers used in this work were all hydrophilic, Florence's work was used as the guideline for this work. Indeed, hydrophilic particles had a drastically reduced uptake by the Payer's patches, which suggests that the copolymer gel nanosphere used in this work would have no uptake by the Payer's patched as long as they were larger than 600 nm in the neutral conditions of the upper small intestine.

Table 2 presents the diameters measured by PCS analysis for all of the P(MAA-g-PEG) gel nanospheres at both the pH 2.0 and pH 6.0 conditions. It was observed that the P(MAA-g-PEG) gel nanospheres at the neutral conditions were larger than 600 nm and therefore probably able to avoid uptake by the Payer's patches, although this study has not been completed yet. The exception was the P(MAA-g-PEG) nanospheres with a monomer feed ratio of 1:2 MAA:EG, which reached a 600 nm diameter at pH 6.0.

3.2. Insulin loading and release

A number of factors affected the loading of insulin into the different gel carriers. An important factor in the insulin loading ability of the copolymer gel carriers was the mesh size of their networks. The three-dimensional structure of insulin was globular in its active state. Thus, orientation of the protein as it enters the network was not a large concern once the mesh size of the network was large enough to accommodate insulin [19–21]. However, as the pH decreased and the network complexed/collapsed, the shrinking mesh size could entrap the insulin inside of the copolymer gel network and hold it [22–26].

Table 2
Mean diameter of P(MAA-g-PEG) gel nanospheres[a]

AA:EG monomer feed ratio	pH 2.0 buffer (nm)	pH 6.0 buffer (nm)
4:1 MAA:EG	260 ± 30	2900 ± 200
2:1 MAA:EG	280 ± 30	1500 ± 200
1:1 MAA:EG	250 ± 30	1120 ± 70
1:2 MAA:EG	240 ± 60	600 ± 100

[a] ($n = 3$, error expressed as 95% confidence intervals).

In the copolymers of P(AA-g-PEG) and P(MAA-g-PEG) the carboxylic acid groups present in the networks complex with the etheric groups of PEG. Thus, at low pH the mesh size was small as the network was in its complexed state while at high pH, the decomplexation of the hydrogen bonding between the ethylene glycol and the carboxylic acid and deprotonation of the carboxylic acid led to an increase of the mesh size.

The hydrogen bonding complexation that occurs in P(MAA-g-PEG) and the P(AA-g-PEG) networks leads to the formation of additional crosslinks in the gel network [23,24]. The degree to which the network complexes and thus forms additional crosslinks in low pH environments was an important reason why Lowman et al. [25] used the P(MAA-g-PEG) gel carriers with monomer feed ratio of 1:1 MAA:EG, as the latter were found to have the highest degree of complexation.

The dependence of insulin loading on the network mesh size was also investigated. At high pH values, where the mesh size was large the insulin could easily diffuse into the network. The network was then collapsed by exposure to low pH environments. This caused the mesh size to decrease, which trapped the insulin in the network. Previously, Lowman and Peppas [1,23–26] found that copolymer gels comprised purely of MAA exhibited very low loading capacity compared to copolymers of P(MAA-g-PEG). These studies gave insight into the phenomenon of PEG shielding occurring in the network. The long neutral PEG chains could interact with the negatively charged carboxylic groups to shield the insulin molecule from the charged forces. Thus, the insulin was able to diffuse in the copolymer. In a similar vein, it has been observed that PEG chains complex with proteins and stabilize them [27,28]. Prestwich et al. [29] showed that insulin has a high affinity for PEG rich environments. The favorable interaction between PEG and insulin would allow the protein to diffuse inside the nanospheres. The importance of these PEG tethered chains in protecting proteins and providing an environment of increased stability has been discussed by us in several previous publications [1,11,15].

The final concern in insulin loading into the copolymer was the concentration gradient or chemical potential gradient present in the loading mixture. Studies were conducted to determine the insulin concentration in the loading solution versus time while in contact with the gel carriers. It was observed that the insulin concentration reached equilibrium after 6 h. The loading efficiency of insulin was calculated using Eq. (1).

$$\text{Loading efficiency} = M_i\left(\frac{C_i - C_o}{C_i}\right) \quad (1)$$

Here C_i was the concentration of insulin in solution before the loading study, C_o was the concentration of insulin in solution after the loading study and M_i was the mass of insulin available.

Table 3
Loading efficiency of insulin into the gel carriers

Copolymer gel carrier	Insulin loading (mg insulin per 140 mg carrier) ($n = 3$)
P(MAA-g-EG) nanospheres 1:1 MAA:EG feed ratio	9.33 ± 0.02
P(AA-g-EG) nanospheres 2:1 AA:EG feed ratio	9.54 ± 0.02

The calculated loading efficiencies of different copolymer gel carriers are listed in Table 3. The loading efficiencies of the copolymer gel carriers were above 9 mg per 140 mg of carrier. The P(AA-g-PEG) gels had loading efficiencies slightly higher than the P(MAA-g-PEG) ones. The P(AA-g-PEG) gel nanospheres, prepared with a monomer feed ratio of 2:1 AA:EG exhibited an insulin loading efficiency of 9.54 ± 0.02 mg insulin per 140 mg copolymer. The P(MAA-g-PEG) gel nanospheres, prepared with a monomer feed ratio of 1:1 MAA:EG, had an insulin loading efficiency of 9.33 ± 0.02 mg insulin per 140 mg copolymer. Thus, the P(AA-g-PEG) gel carriers were able to load more insulin than the P(MAA-g-PEG).

The normalized insulin release was plotted in Fig. 3 from insulin-loaded nanospheres prepared with 1:1 MAA:EG monomer feed ratio. The 1st h of the release study was conducted at pH 3.0 and at the end of that the pH was increased to 7.0 to simulate the insulin-loaded carrier moving from the stomach into the upper small intestine's more neutral pH. Fig. 4 shows the normalized release for insulin-loaded nanospheres prepared with 2:1 AA:EG monomer feed ratio. Unlike the copolymer gel nanospheres of P(MAA-g-PEG), the P(AA-g-PEG) gel nanospheres released little of their loaded insulin while in the low pH environment, only 10 ± 1% was released in

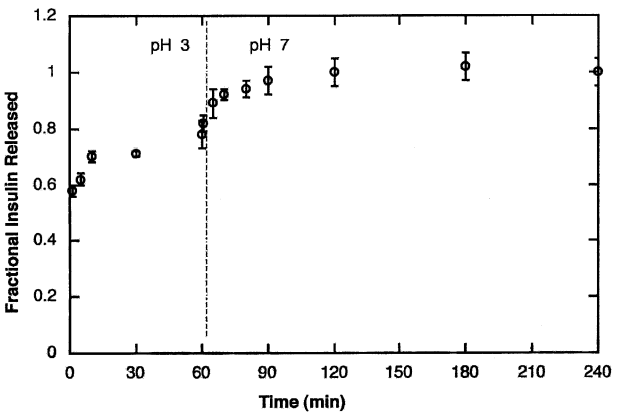

Fig. 3. Fraction of insulin released from P(MAA-g-PEG) gel nanospheres prepared by precipitation/dispersion polymerization of a monomer feed ratio of 1:1 MAA:EG. Release was conducted in a USP II apparatus using a 3,3-dimethylglutaric acid buffer solution at pH 3.0 for the first 60 min, and then at pH 7.0 for the remaining time. Insulin was detected by HPLC with UV detection at 215 nm. ($n = 3$, Error bars were 95% confidence interval).

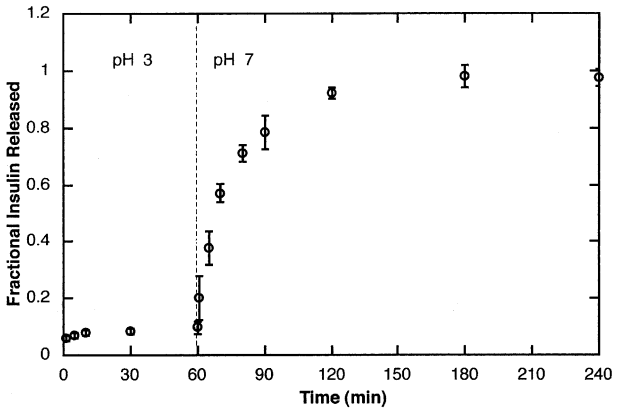

Fig. 4. Fraction of insulin released from P(AA-g-PEG) gel nanospheres prepared by precipitation/dispersion polymerization of a monomer feed ratio of 2:1 AA:EG. Release was conducted in a USP II apparatus using a 3,3-dimethylglutaric acid buffer solution at pH 3.0 for the first 60 min, and then at pH 7.0 for the remaining time. Insulin was detected by HPLC with UV detection at 215 nm. ($n = 3$, Error bars were 95% confidence interval).

the low pH. This difference could possibly be explained by the fact that P(AA-g-PEG) showed a greater volume change due to the pH change, as seen from the PCS experiments. The P(AA-g-PEG) gel nanospheres had a cumulative insulin release of 92 ± 3% after 1 h in the high pH environment.

3.3. In vivo release studies

The serum glucose levels of the healthy animals after administration of the insulin-loaded copolymers are presented in Fig. 5. The effect of the insulin-loaded copolymer on serum glucose levels can be shown by expressing also the reduction capacity of the insulin-loaded copolymers. Thus, we can determine the impact of the insulin-loaded copolymer carrier by relating the serum glucose level of the test animal with that of the control animal under identical conditions.

Diabetic conditions were chemically induced with streptozotocin injections for 3 days, and the serum glucose levels of the animals were monitored for 4 days until their glucose concentrations. The studies monitored the serum glucose levels after administration of insulin-loaded particles. Blood samples were taken from the jugular veins of the animals at 0, 15, 30, 60, 120, 240, 360 and 480 min after administration of the copolymer carrier. Both nanospheres of P(MAA-g-PEG) and P(AA-g-PEG) were investigated for their ability to influence the serum glucose levels of diabetic animals.

Fig. 6 presents the results of the serum glucose levels as a percent of the animal's initial glucose concentration versus time after administration. It is clear that the insulin-loaded copolymer nanospheres had an effect on the serum glucose levels, as the diabetic animals given insulin-loaded copolymeric nanospheres had a significantly reduced

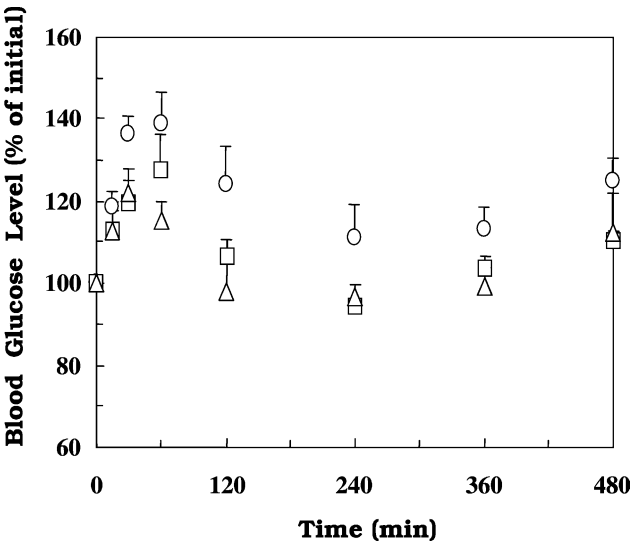

Fig. 5. Serum glucose concentration expressed as a percent of the initial value versus time. Studies were conducted with healthy Wistar rats. Glucose measurements were taken from samples from the jugular vein and analyzed by a glucose oxidase reaction. Animals had been fasted for 48 h previous to the study. Results are presented for the effect of 50 IU/kg body weight insulin-loaded P(AA-g-PEG) gel nanospheres prepared with a molar monomer feed ratios of 2:1 AA:EG (△) ($n = 4$), 50 IU/kg body weight insulin-loaded P(MAA-g-PEG) gel nanospheres prepared with a molar monomer feed ratios of 1:1 MAA:EG (□) ($n = 4$), and from the control experiments (○) ($n = 4$). Error bars represent the 95% confidence intervals of the averaged value.

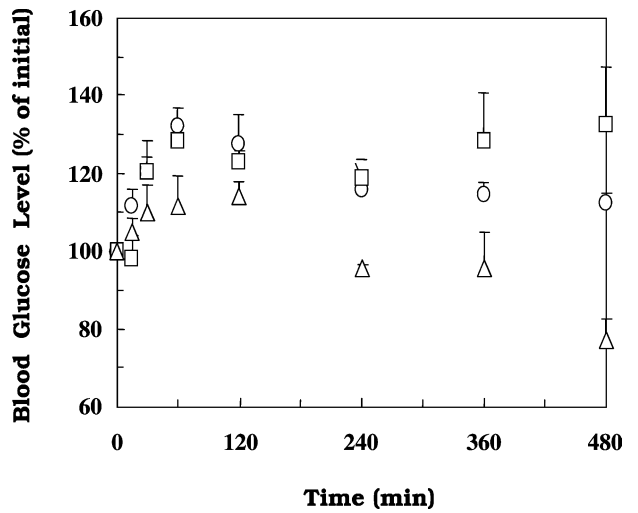

Fig. 6. Serum glucose concentration expressed as a percent of the initial value versus time. Studies were conducted with diabetic Wistar rats. Glucose measurements were taken from samples from the jugular vein and analyzed by a glucose oxidase reaction. Diabetes was induced with injections of streptozotocin. Results are presented for the effect of 50 IU/kg body weight insulin-loaded P(AA-g-PEG) gel nanospheres prepared with a molar monomer feed ratios of 2:1 AA:EG (△) ($n = 4$), 50 IU/kg body weight insulin-loaded P(MAA-g-PEG) gel nanospheres prepared with a molar monomer feed ratios of 1:1 MAA:EG (□) ($n = 4$), and control samples (○) ($n = 7$). Error bars represent the 95% confidence intervals of the averaged value.

serum glucose level over time with respect to the control animals.

Insulin-loaded nanospheres caused decreased serum glucose after dosage. To reflect the ability of the insulin-loaded copolymer nanospheres to influence the serum glucose levels, we examined the reduction of the particles' serum glucose concentration. The insulin-loaded particles had a substantial effect on glucose in 30 min. Insulin-loaded P(AA-g-PEG) nanospheres with a molar monomer feed ratio of 2:1 AA:EG caused a significant serum glucose reduction, nearly 50%, while the insulin-loaded P(MAA-g-PEG) gel nanospheres with a molar monomer feed ratio of 1:1 MAA:EG had a larger experimental error.

4. Conclusions

The best candidates for oral insulin delivery were the P(MAA-g-PEG) gels with comonomer feed ratios of 1:1 MAA:EG and the P(AA-g-PEG) gel carriers prepared with comonomer feed ratios of 2:1 AA:EG. Insulin loading studies showed that these copolymer carriers had a high affinity for loading the insulin. The results of the insulin release studies showed that P(AA-g-PEG) gel nanospheres prepared with a monomer feed ratio of 2:1 AA:EG released a very small percentage of their loaded insulin at low pH, but had an excellent ratio of total insulin released at high pH to the total insulin loaded, estimated at 72 ± 5%.

From the results of insulin delivery in healthy animals, it was concluded that the insulin-loaded copolymers developed in this work affected the serum glucose levels. In diabetic animal studies, the P(AA-g-PEG) gel nanospheres prepared with a molar monomer feed ratio of 2:1 AA:EG had the greatest effect on the serum glucose levels.

Acknowledgements

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