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Note

Preparation of stable insulin-loaded nanospheres of poly(ethylene glycol) macromers and *N*-isopropyl acrylamide

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Abstract

A series of nanospheres composed of temperature-sensitive poly(*N*-isopropylacrylamide), poly(ethylene glycol) 400 dimethacrylate, and poly(ethylene glycol) 1000 methacrylate was prepared by a thermally-initiated free radical dispersion polymerization method. Insulin was loaded into the nanoparticles by equilibrium partitioning. The loading capacity of insulin into the nanoparticles was 2.1% (2.1 mg insulin/100 mg nanoparticles). The stability of the loaded insulin at elevated temperatures was investigated by reverse phase high pressure liquid chromatography. The nanoparticles were able to protect the loaded insulin, as more than 80% of the loaded insulin could still be detected compared to 0% for the control (0.1% insulin solution in PBS) when heated to 80 °C for 5 h. The stability of the loaded insulin at high shear stress (289 1/s) was also investigated. No significant loss of insulin was detected both from nanoparticles loaded with insulin sample and the control (0.1% insulin solution in PBS). The results showed that shear stress alone did not have a major effect on insulin denaturation. The ability of the nanoparticles to protect the insulin from high temperature and high shear stress made the system a good candidate as a carrier for insulin for fluidized bed coating technology. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Nanoparticles; Hydrogels; Poly(ethylene glycol); Temperature sensitive; Insulin

1. Introduction

The stability characteristics of proteins and peptides are important in the formulation of protein delivery products. Chemical stability refers to re-forming and breaking of covalent bonds due to deamidation, oxidation, and disulfide exchange. The sensitivity of particular amino acid residues towards deamidation and oxidation is affected by the accessibility of a protein domain to exogenous oxidants, presence of metal binding sites and potential neighboring group effects [1]. The ability to create a system that will be able to provide an extra barrier in order to limit the accessibility of denaturing agents to sensitive sequence of amino acids of a protein or

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peptide is very important to increase the chemical stability.

Numerous researchers have reported studies on the damaging effects of shear stresses in fluid flow on globular proteins [2–10]. For example, Alder and Lee [4] studied the stability of lactate dehydrogenase (LDH) during the spray drying process and also on subsequent dry storage. They found that the process temperature has a measurable effect on LDH inactivation. At a high operating temperature (T_{out}= 95 °C), 25% of the LDH was lost due to denaturation. At a lower operating temperature (T_{out}=70 °C), 11% of the LDH was lost. However, the storage stability was a problem for the system prepared at a lower operating temperature. At a lower operating temperature, the system could not dry effectively, and hence, resulted in high moisture content in the system. This remaining moisture content caused the protein to denature during the storage period. They also found out that the air–liquid interface had an adverse effect on LDH stability. LDH is surface active which causes the LDH to assemble on the interface. This caused the LDH to be shear-denatured during the spray-drying process.

Clarkson et al. [5] tried to elucidate the mechanism by which protein molecules became denatured during foaming. They found that the damage to the protein was due to surface denaturation at the air–liquid interface. They hypothesized that a fraction of the proteins absorbed to the interface did not refold to their native state when they desorbed. They found a direct correlation between the degree of denaturation and the interfacial exposure. Oxidation was eliminated as the major cause of denaturation because the same degree of denaturation was observed when the air was replaced with nitrogen.

Maa and co-workers [6–10] investigated the effect of shear and air–liquid interface on protein denaturation in detail. They found no major loss of anti Ig-E antibody, rhGH and rhDNase due to shear stress alone. However a significant aggregation was observed for rhGH when the protein was exposed to shear stress combined with high air–liquid interface. They found that the degree of aggregation was proportional to the total air–liquid interface and was independent of the concentration of the rhGH.

The work of Alder [4], Clarkson et al. [5], and Maa and colleagues [6–10] clearly substantiated the significance of the protein stability problem posed by elevated temperature, air–liquid interface and shear stress. In our work, these three problems were addressed by the synthesis of poly(ethylene glycol)-containing nanoparticles.

With respect to the stability of proteins and peptides, the major challenges of a microencapsulation process with a fluidized bed coating are the stability against elevated temperatures and high shear stresses encountered during the coating process. In this study, we designed two sets of experimental set-ups that allowed these harsh conditions of the real coating process to be studied independently of each other. The protective effect of the synthesized nanoparticles on the thermal and mechanical stability of insulin was then assessed.

2. Methods

2.1. Nanoparticle preparation

A free radical dispersion polymerization was used to prepare nanoparticles of poly(*N*-isopropyl acrylamide-co-poly(ethylene glycol) 1000 methacrylate) (P(NIPAAm-co-PEGMA)). The crosslinker used was poly(ethylene glycol) 400 dimethacrylate (PEGDMA). Prior to the reaction, NIPAAm (Fischer Scientific, Pittsburgh, PA) was recrystallized in benzene/hexane. PEGDMA and PEGMA (Polysciences, Warrington, PA) were used as received. In a typical experiment, NIPAAm, PEGDMA, and PEGMA (70:20:10 w/w%) were dissolved in deionized-distilled water to form 2% aqueous solution. The mixture was bubbled with argon for 30 min to remove any dissolved oxygen. The monomer mixture was then heated to 85 °C using silicon oil temperature bath. Ammonium persulfate (Aldrich, Milwaukee, WI) was added into the system as the thermal initiator and the polymerization process was allowed to continue for 45 min. The resulting dispersion was washed using the regenerated cellulose dialysis membrane with 14 kDa molecular weight cut-off (Spectrum Laboratories, Rancho Dominquez, CA) for 5 days with water changed daily.

2.2. Insulin loading into the nanoparticles

Insulin loading was accomplished by equilibrium partitioning of insulin into the nanoparticles. Bovine pancreatic insulin (27 IU/mg, Sigma, St Louis, MO) was added to the nanoparticle dispersion in the amount of 3.2% w/w of the polymer content. In a typical loading study, 80 mg of insulin were dissolved in 5 ml of phosphate buffer solution (pH 7.4) followed by the addition of a small amount of 0.1 N HCl. The solution was added to 2.5 g of nanoparticles dispersed in an appropriate volume of water to form a dispersion containing 5% w/v polymer content and 0.15% w/v drug content. The dispersion was cooled to 4 °C, stored for a certain time period and then heated quickly to 37 °C.

Loading efficiency studies were conducted as a function of time. One gram of the nanoparticulate dispersion containing insulin was added into a 1.5-ml poly(propylene) micro-centrifuge tube. After a certain time period at 4 °C, the dispersion was annealed to 37 °C. The dispersion was then centrifuged at 13 500 rpm for 20 min to separate the nanoparticles from the supernatant. The supernatant was collected, filtered through a low protein binding polysulfone syringe filter (Whatman®, Clifton, NJ) and analyzed with RP-HPLC (Waters 2690 Separation Module equipped with Waters 2487 Dual λ Absorbance Detector) to determine the insulin that was not loaded into the nanoparticles. The column used was a Vydac C18 Reversed-Phase Column (218TP54) with 5-μm particle size, 300 Å pore diameter. The mobile phase in this analysis consisted of aqueous phase (Solvent A) and organic phase (Solvent B). Solvent A was 0.1% v/v trifluoroacetic acid (TFA) in deionized distilled water. Solvent B was 0.085% v/v TFA in acetonitrile. The solvent gradient used was 72.5% Solvent A to 60% Solvent A in 15 min. The insulin was detected with UV detector at the wavelength of 215 nm.

2.3. Insulin temperature stability studies

In a typical study, 1 g of the insulin loaded nanoparticulate dispersion (as described in the previous section) was filled into five micro-centrifuge tubes. The tubes were centrifuged at 13 500 rpm for 20 min and the solid was collected. The solid samples were then placed inside a laminar flow oven, which had been preset at 60 °C. At time intervals of 1, 2, 4 and 8 h, one tube was removed from the oven. One milliliter of PBS was added to all samples and the samples were then stored at 4 °C for 24 h to extract the insulin from the nanoparticles. A preliminary set of experiments revealed that 1 ml of PBS was a sufficient volume as an extracting media. The samples were then centrifuged as before, and the supernatants from each sample were filtered and analyzed with RP-HPLC to determine the concentration of insulin that remained in the sample. These studies were conducted at temperatures of 40, 50, 60, 70 and 80 °C.

2.4. Insulin shear stress stability studies

A rheometer (Brookfield®, Model DV-III, Chicago, IL) with small sample adapter was used to provide the required shear rate on the dispersion. In a typical study, 4 g of the insulin-preloaded nanoparticle dispersion (as described above) was added into the sample chamber. The spindle was immersed into the sample chamber, and then rotated by increasing the rotational speed slowly until it reached the maximum shear rate of 289 1/s. The dispersion was sheared for 15 min at 25 °C before it was collected. The sheared dispersion was centrifuged to separate the solids, and the supernatant was analyzed to determine the remaining intact insulin using RP-HPLC.

3. Results and discussion

3.1. Insulin loading into the nanoparticles

The temperature sensitivity of the nanoparticles was used for the loading of the insulin. The loading process was conducted at 4 °C for 24 h and then the nanoparticles were collapsed at 37 °C. The temperatures of 4 and 37 °C were selected because they were the lowest temperatures providing maximum volume swelling. Indeed we wanted to avoid higher collapsing temperature and avoid induction of a possible thermal denaturation of insulin in the loading process

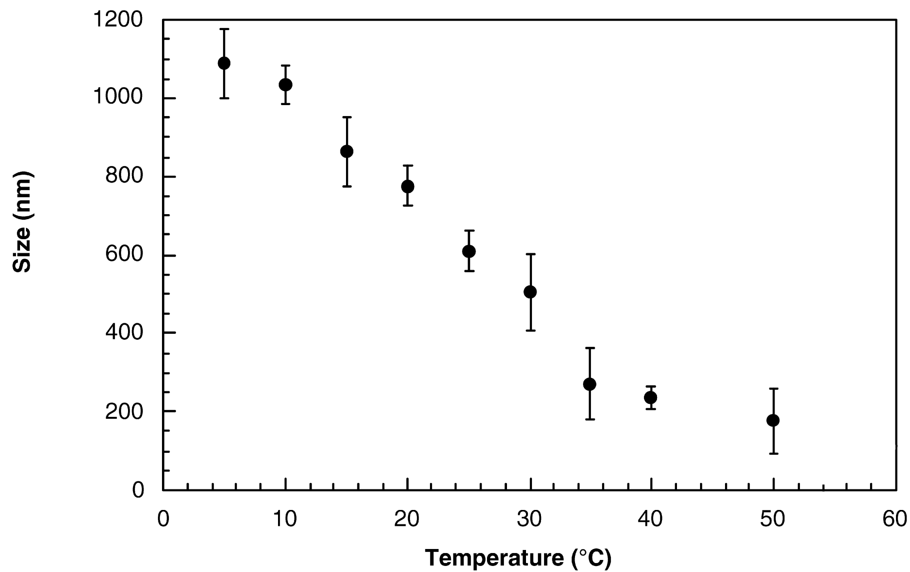

Fig. 1. Particle size measured using photon correlation spectroscopy of the 70:20:10 (NIPAAm/PEGDMA/PEGMA) nanoparticle formulation as a function of the temperature of the swelling media. The nanoparticle dispersion is prepared by the thermal initiated free radical dispersion polymerization at 85 °C for 45 min.

(Fig. 1). The loading efficiency increased rapidly until about 12 h when it leveled off as shown in Fig. 2. The maximum loading efficiency attainable after loading for 24 h was 65%.

3.2. Temperature stability of insulin loaded into the PEG-nanoparticles

The thermal stability of the insulin at 60 °C loaded

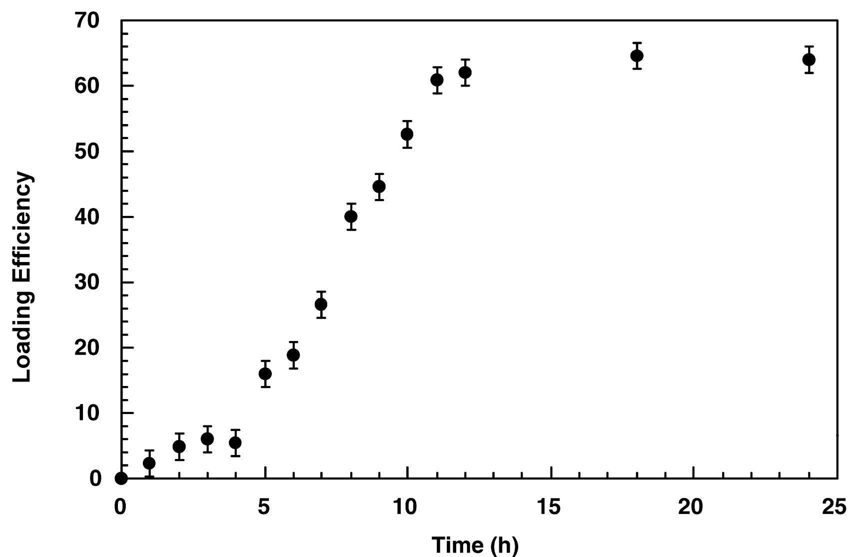

Fig. 2. Insulin loading efficiency into the nanoparticles of (70:20:10) (NIPAAm/PEGDMA/PEGMA) as a function of time. A maximum loading efficiency of 65% was achieved after 24 h.

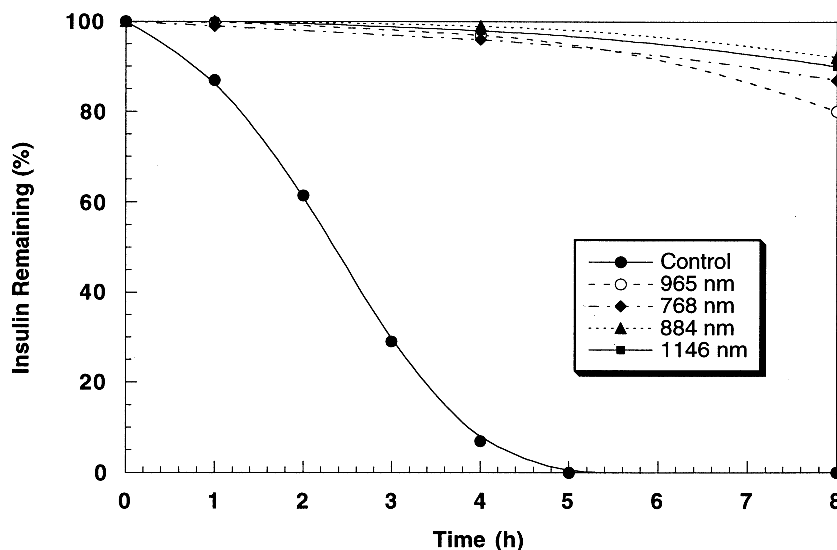

Fig. 3. Thermal stability of the insulin at 60 °C loaded into nanoparticles of (70:20:10) (NIPAAm/PEGDMA/PEGMA) with different sizes was compared with control (0.1% insulin solution in PBS).

into PEG-nanoparticles was compared with insulin alone as a control (0.1% insulin solution in PBS) as shown in Fig. 3. The control degraded immediately, approximately 40% of the insulin was degraded in 2 h, and almost no insulin could be detected after 5 h. On the other hand, the insulin loaded into the nanoparticles remained relatively stable and 80% or more could still be detected after 8 h. This remarkable performance would be ascribed from the presence of PEG in the system and the temperature sensitivity of the nanoparticles. As the temperature of the system was increased, the nanoparticles collapsed and were most probably frozen around the insulin, thus, securing the tertiary structure of the insulin from unfolding.

The effect of nanoparticle size on the ability to protect the protein was also studied. The nanoparticles sizes studied were 765, 884, 975 and 1146 nm. There was no significant effect of the nanoparticle size on the protein protecting ability. All the nanoparticle sizes studied showed a good insulin stabilization property. These results suggested that most of the insulin was incorporated inside the nanoparticles rather than being absorbed on the surface of the nanoparticles. If a significant portion of the insulin loaded was absorbed on the surface of the nanoparticles, the change in the surface area by changing the particle size would affect the protein stability.

The thermal stability of the insulin loaded into the PEG-nanoparticles as a function of temperature is shown in Fig. 4. After 12 h of heating at 80 °C, 25% of the insulin could still be detected from the samples compared with the insulin control (0.1% insulin solution in PBS) that was all degraded after 5 h of heating at 60 °C. These results showed the very good insulin protecting properties of the PEG-nanoparticles from an extreme temperature.

3.3. Shear stability of insulin loaded into the PEG-nanoparticles

The shear stress stability of the insulin loaded into PEG-nanoparticles compared with insulin alone as a control (0.1% insulin solution in PBS) is shown in Fig. 5. After 15 min of shearing at shear stress of 289 1/s, most of the insulin could still be detected from the insulin loaded into the nanoparticle sample. The same observation was obtained from the control. There were no significant differences between the control and the insulin loaded into the PEG-nanoparticles; both showed no significant denaturation of insulin by the shear stress. This result is in

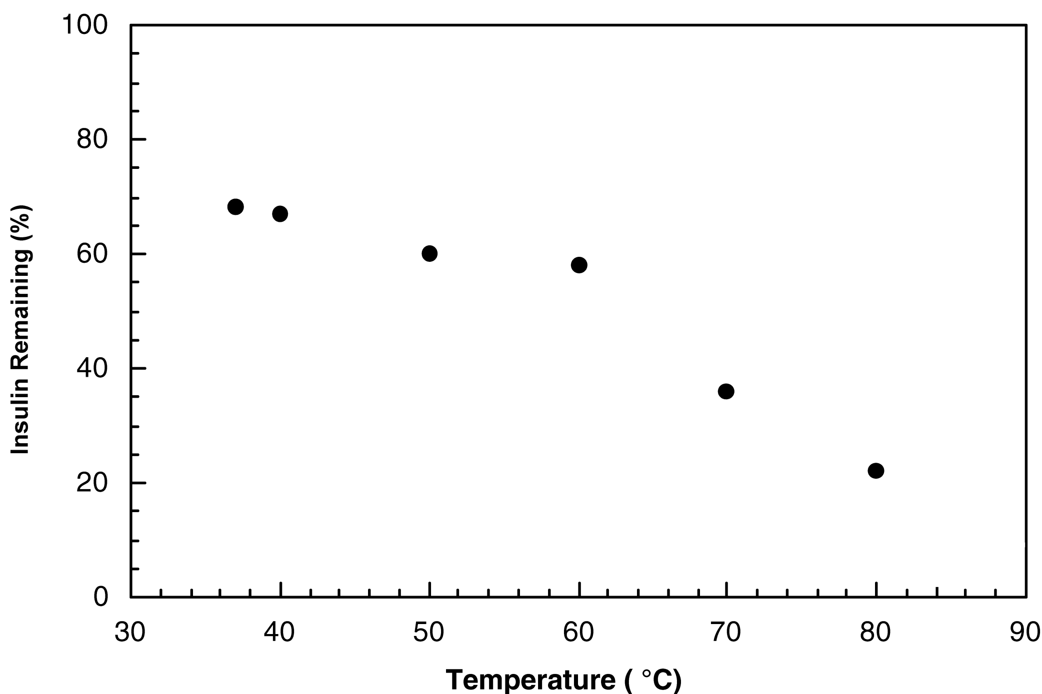

Fig. 4. Thermal stabilities of the insulin loaded into the nanoparticles of (70:20:10) (NIPAAm/PEGDMA/PEGMA) as a function of temperature. After heating for 12 h at 80 °C, 25% of the insulin could still be detected.

agreement with what has been reported in the literature [2–10].

4. Conclusions

The PEG-nanoparticles showed good insulin protecting properties from high temperature and high shear stress and could be used as a carrier for sensitive proteins and peptides during fluidized bed coating process. The PEG-nanoparticles could be loaded with insulin with a high loading efficiency at 65%. The loading capacity was 2.1% (w insulin/w nanoparticles). The PEG-nanoparticles also showed remarkable results in protecting the insulin from elevated temperature. After 8 h of heating at 60 °C, 80% of the insulin could still be detected, while all the insulin was degraded after 5 h without the presence of the PEG-nanoparticles. Various sizes of nanoparticles showed no significant effect on the ability of the nanoparticles on protecting the insulin. The shear stress did not significantly affect the stability of insulin. The insulin loaded into the PEG-nanoparticles showed almost no effect of shear stress on its stability. The same observation was also found from the insulin without the PEG-nanoparticle system. Thus, shear stress alone did not have a detrimental effect on the protein. A major problem appeared when the shear stress was combined with the effect of air–liquid interface. Incorporating the insulin into the PEG-nanoparticles eliminated the exposure of insulin to the air–liquid interface, and hence, allowed the PEG-nanoparticles to be applied in the fluidized bed coating process.

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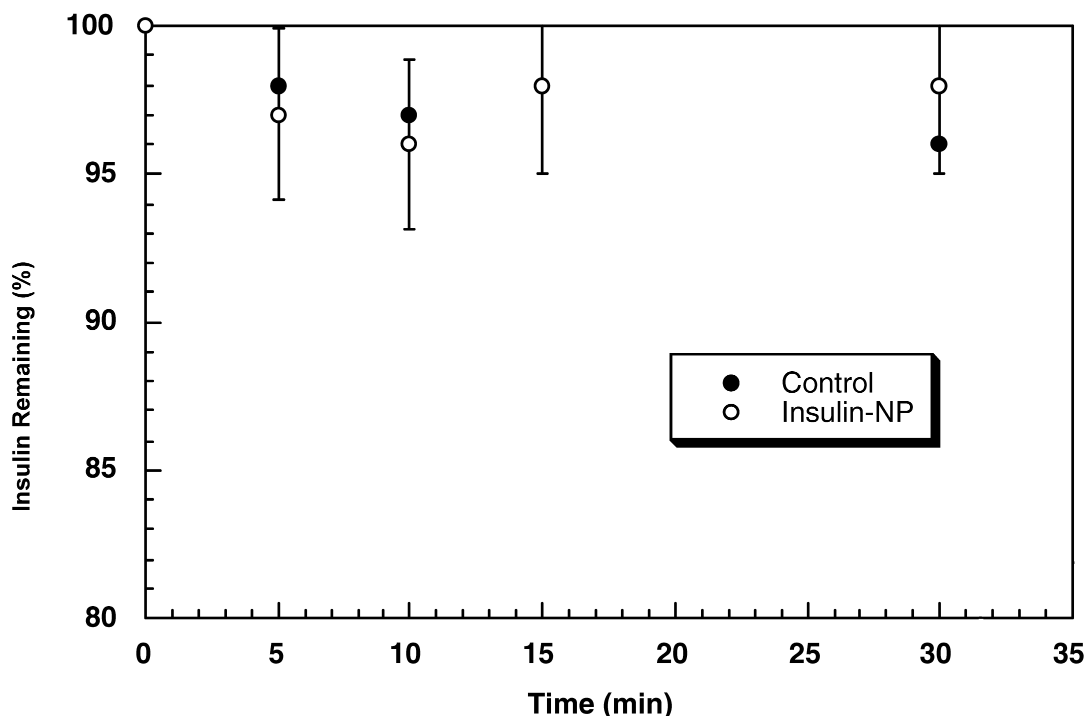

Fig. 5. Shear stress stability of the insulin loaded into nanoparticles of (70:20:10) (NIPAAm/PEGDMA/PEGMA) compared with insulin alone as a control. There were no significant differences between the insulin loaded into the PEG-nanoparticles and the control (0.1% insulin solution in PBS). Insulin was not affected very much by the shear stress.

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