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Controlled release of insulin from PLGA nanoparticles embedded within PVA hydrogels

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Abstract A simple and versatile delivery platform for peptide and protein based on physically cross-linked poly (vinyl alcohol) (PVA) hydrogels containing insulin-loaded poly (lactic-co-glycolic acid) (PLGA) nanoparticles was successfully fabricated. The particle morphology and size were characterized by SEM and laser light scattering method, respectively. Results showed that these particles had a mean diameter of 615 nm with a narrow size distribution and homogeneous particle production. The protein encapsulation efficiency was 72.6%. When insulin-loaded PLGA nanoparticles were administered intraperitoneally as a single dose (20 U/kg) to streptozotocin-induced diabetic mouse, blood glucose levels of these mice decreased and it could be sustained at such levels over 24 h. In vitro release further indicated that entrapment of the nanoparticles into the PVA hydrogels causes a reduction in both the release rate and the total amount of insulin released, which suggesting that PLGA nanoparticles entrapped into the PVA hydrogels showed more suitable controlled release kinetics for protein delivery.

Introduction

The recombinant DNA technology has led to an increase in commercial production of proteins for pharmaceutical use. At the same time, the search for adequate systems for protein and peptide delivery has been intensified [1]. Among therapeutically active peptide molecules, insulin is of great interest for its wide use in the treatment of diabetes mellitus. However, large molecules such as peptides and proteins are not considered feasible candidates for controlled release systems because their physicochemical and biological properties are different from those of conventional drugs, such as molecular size, biological half-life, conformational stability, physicochemical stability, solubility and bioavailability [2, 3], these intrinsic properties severely hinder the approval of new protein-based therapeutics. Thus, one of the most urgent problems at present is to develop suitable protein delivery devices for various pharmaceutical and biomedical applications.

Hydrogels are usually polymers with three-dimensional networks structures that permit a high permeability to drug molecules and have been used for the controlled release of pharmaceutically active compounds [4, 5]. Poly (vinyl alcohol) (PVA) is an example of hydrogels for such applications including implants and artificial organs [6, 7], because it can provide soft, permeable, and hydrophilic interfaces with body tissue. The PVA hydrogels have also gained wide pharmaceutical applications as drug delivery matrices [8–10]. However, drug release rates from PVA hydrogels tend to relatively rapid depending on the pore size, extent of cross-linking, and the nature of the incorporated drug [11]. Theoretically, the release rates can be decreased through a composite drug release platform, which consists of nanoparticles containing drugs embedded within PVA hydrogels. Such a system can be expected for uses in other protein delivery.

In this work, we fabricate a new composite release system based on insulin drug model to improve release kinetics of large molecules drugs. Firstly, insulin-loaded

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nanoparticles were prepared by an improved double-emulsion solvent evaporation technique using a biodegradable copolymer of poly (lactic acid)-poly (glycolic acid) (PLGA), and these particles were then embedded within PVA hydrogels. The effect of several processing parameters on particles properties was investigated. The drug release from free nanoparticles was compared with that from the nanoparticle-embedded PVA hydrogels.

Materials and methods

Materials

PLGA (LA/GA ratio 50:50, average molecular weights M_w 10,000 and 50,000), PVA (99% hydrolysed, M_w 73,500), insulin (from bovine pancreas), Pluronic F-68 were obtained from Sigma. Dichloromethane (DCM), acetone and all the other reagents were of analytical grade and purchased from various suppliers.

Preparation of PVA hydrogels by the freeze-thaw method

The PVA hydrogels were prepared by poured 2 mL PVA (99% hydrolysed, M_w 73,500) aqueous solutions (5% and 10% w/v) into a mold (24-well plate, the cylindrical vessel, 1 cm in height and 0.8 cm in diameter) and freezing at –20 °C for 20 h, followed by thawing for 4 h at room temperature. The hydrogels were subjected to 1–7 freeze-thaw cycles.

Preparation of insulin nanoparticles

PLGA nanoparticles were prepared by an improved double-emulsion (water-in-oil-in-water) solvent evaporation/extraction technique [12]. Briefly, a 0.5 mL internal aqueous phase containing 16 mg insulin was emulsified in a mixed organic solution (DCM and acetone) containing PLGA (200 mg) by homogenization at 10,000 rpm for 1 min. Thereafter, the primary emulsion was poured into 40 mL of the PVA or Pluronic F-68 aqueous solution with a determinated concentration and homogenized at 8,000 rpm for 1 min. The double emulsion was diluted in 200 mL of PVA or Pluronic F-68 aqueous solution with stirring. The resulting suspension was stirred with a magnetic stirrer in moderate speed and the DCM was rapidly eliminated by evaporation under reduced pressure. The nanoparticles were cleaned by repeating a procedure of centrifuging and resuspending in distilled water three times. Finally, the product was dried by lyophilization and stored at 4 °C.

Fabrication of PVA hydrogel composites containing insulin and insulin-loaded PLGA nanoparticles

10 mg of insulin was dissolved in 20 mL of 5% w/v PVA solution, then the mixture was poured it into the molds (same method as described above). PVA hydrogels containing insulin nanoparticles were prepared with 20 mL of 5% w/v PVA solution and 120 mg of insulin-loaded PLGA nanoparticles, followed by homogenization at 3,000 rpm. The molds containing the insulin/PVA solution and insulin nanoparticle/PVA dispersion were sealed and subjected to freeze-thaw cycling, as described above in Section "Preparation of PVA hydrogels by the freeze-thaw method". The resulting PVA hydrogel films containing insulin or insulin-loaded PLGA nanoparticles were air dried after 7 freeze-thaw cycles.

Particle size and morphology

Particle size distribution (mean diameter and polydispersity index) of nanoparticles was determined by dynamic laser light scattering (Malvern Instruments, UK). The morphological characteristics of the nanoparticles were observed using a scanning electron microscope (SEM).

Assay of swelling degree

The swelling experiments were performed in deionised water or in phosphate-buffered saline (PBS) at 37 °C (±1 °C). The percentage of weight changes was calculated as:

$$S_w = \frac{(W - W_0)}{W_0} \times 100\%$$

where S_w is swelling degree and W_0 is the weight of air-dried PVA sample, W is the weight of sample immersed either in deionised water or in PBS buffer for 48 h in a thermostatic water bath.

Determination of protein encapsulation efficiency

The content of insulin in nanoparticles was estimated by an extraction method. The extraction was analysed by UV-spectrophotometer at $\lambda = 276$ nm and the amount of insulin calculated by means of a calibration curve. The protein encapsulation efficiency was calculated by using the following equation:

$$\text{Encapsulation efficiency} = \frac{M_{\text{actual}}}{M_{\text{theoretical}}} \times 100\%$$

where M_{actual} is the actual encapsulation amount of insulin in the nanoparticles determined by the above experiment and $M_{\text{theoretical}}$ is the theoretical encapsulation amount of

insulin calculated from the quantity added during the preparation.

In vitro release study

The releases of insulin from PVA hydrogels, free nanoparticles and nanoparticles embedded within PVA hydrogels were all evaluated.

12 mg of dried insulin-loaded nanoparticles were suspended in 1 mL of 0.01 M PBS (pH 7.4). The samples were placed in a thermostatic water bath at 37 °C, at scheduled times, collected and centrifuged for 5 min at 7,000 rpm. The release medium was completely removed and 1 ml of fresh medium was added. Each sample was analysed by UV-spectrophotometry as described in Section "Determination of protein encapsulation efficiency"

With regard to the release of insulin either from PVA hydrogels or from nanoparticles-embedded PVA hydrogels, they were each placed in 5 mL PBS in individual containers at 37 °C. The release medium was removed at scheduled times, then the fresh PBS was added and the container returned to 37 °C. The insulin contents were analysed using the same method.

Animal experiments

Five-week-old male mice (18–20 g) were housed with free access to a standard diet and water for three days, and then they were fasted overnight before experiment. Animal experiments were conducted using mouse with streptozotocin (STZ)-induced diabetes. Twenty-four hours after mouse received 250 mg/kg of STZ dissolved in 0.1 M citrate–citrate Na buffer (pH 4.5) intraperitoneally, they were given a single intraperitoneal injection of insulin-loaded PLGA nanoparticles (20 U/kg). The same dose of insulin–saline solution was given as control. Blood samples were taken from the tail vein before and after treatment to determine blood glucose levels. These samples were taken at scheduled times. Plasma glucose levels were obtained by using the ACCU-CHEK® Advantage (Roche).

Statistical analysis

Results are presented as the mean ± S.D. Determination of the significance of differences between the two groups was carried out using the Student's *t*-test.

Results and discussion:

Swelling degree of PVA hydrogels

Swelling measurements were performed on pure PVA hydrogels without drug loaded. Figure 1 depicts the degree of swelling of PVA hydrogels, in water and PBS, as a function of freeze-thaw cycling. In both environments the weight change is inversely related to the freeze-thaw cycles. Typically, hydrogels subjected to 1 cycle have water uptake of up to 1,800% of their dry weight, whereas hydrogels with 7 cycles exhibit c.a. 1,100% swelling degree. This differential water uptake is due to the extent of physical cross-linking, which decreases porosity and alters fluid uptake [13]. Although the fluid uptake was lower in high ionic strength media (PBS), the overall trend was similar to that observed in water. This decreased fluid uptake in high ionic strength media may be a result of reinforcement of hydrogen bonding within the gel [14]. The lower cross-linking density results in higher swelling degree. The degree of cross-linking will also influence the drug release rate.

Formulation and character of insulin nanoparticles

Insulin-loaded nanoparticles were prepared by the W/O/W emulsification-solvent evaporation method. The influence of PLGA molecular weight and surfactant concentration on particles properties were evaluated. Table 1 lists the properties of insulin nanoparticles produced using various kinds of PLGA mean M_w and surfactants. Low molecular-weight PLGA shows a high drug loading and encapsulation efficiency. For the nanoparticles made of PLGA with molecular weight of 10,000 (formulation B), the drug loading and encapsulation efficiency are 8.04% and 72.6%, respectively. 0.5% PVA solution and 0.05% Pluronic F68 are used as aqueous surfactant in this experiment, and the results indicate that the surfactants significantly affect particle size. For example, when PVA solution was used as

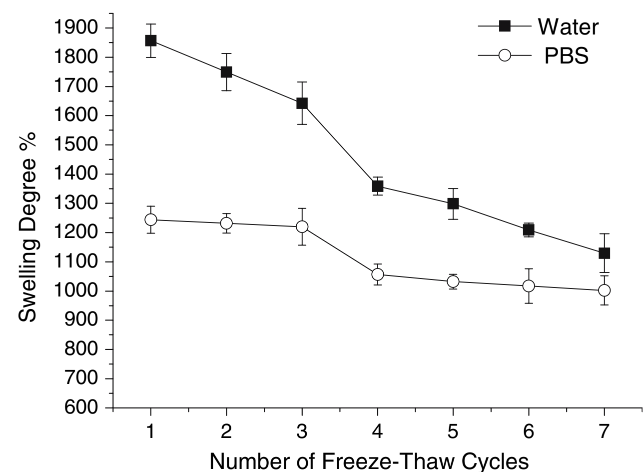

Fig 1 Degree of swelling (% weight change) of PVA gels immersed in water and PBS buffer (pH 7.4) at 37 °C, as a function of the number of freeze-thaw cycles (mean ± S.D., n = 3)

Table 1 PLGA nanoparticle formulations tested in this study

Formulation	Mean M_w	Surfactant	Surfactant concentration (% w/v)	Mean size (nm)	Actual loading (%)	Encapsulation efficiency (%)
A	10,000	Pluronic F68	0.05	531.2	4.59	37.0
B	10,000	PVA	0.5	367.4	8.04	72.6
C	50,000	Pluronic F68	0.05	735.6	3.42	33.4
D	50,000	PVA	0.5	615.1	4.02	58.7

aqueous surfactant, a smaller particle size (formulation A and B) is obtained.

The morphological characteristics of the nanoparticles were observed using SEM. The microphotography in Fig. 2 shows that PLGA particles have a spherical morphology with a mean diameter of 615 nm. There was no significant difference between them. PLGA nanoparticles embedded within a PVA hydrogel are shown in Fig. 3. From the micrograph, it could be seen that the nanoparticles were dispersed homogeneously in PVA hydrogels.

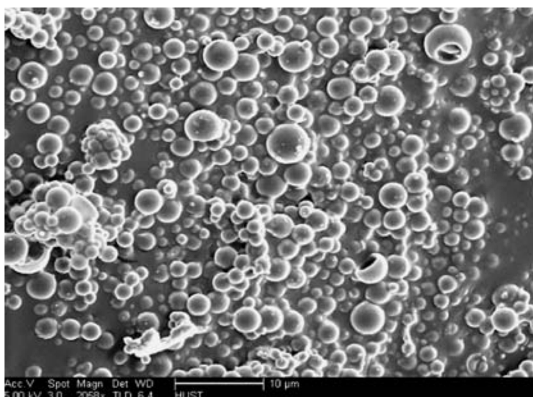

Fig 2 SEM of PLGA nanoparticles loaded with insulin

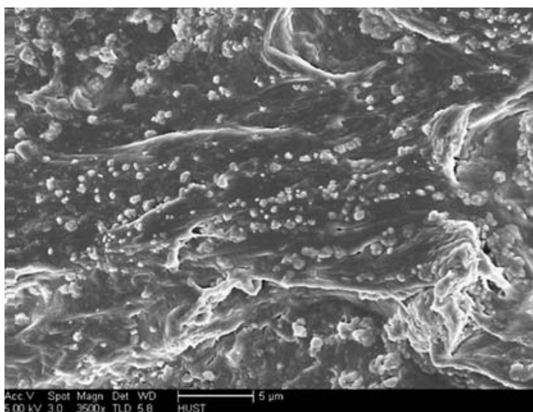

Fig 3 SEM of PLGA nanoparticle loaded with insulin embedded within PVA (5%) hydrogel

Bioactivity of insulin-loaded nanoparticles in vivo

Animal experiments were conducted using mouse with streptozotocin (STZ)—induced diabetes. The blood glucose levels monitored after administration of the insulin-loaded nanoparticles and insulin-saline solution as a reference are shown in Fig. 4, blank nanoparticles as control were also injected to the mouse. When the same dose (20 U/kg) nanoparticles suspension and insulin-saline solution are injected to diabetic mice, blood glucose levels decrease rapidly in two hours, the dispersion of PLGA nanoparticles reduce significantly the blood glucose level over 24 h. In the case of the insulin-saline solution, the basal glucose level shows a minimum at 2 h after administration, and immediately recovers to the initial level. However, the blood glucose level shows a minimum of 10 mmol/L at 1 h after nanoparticles administration, it may be due to the burst release of insulin from nanoparticles. At a later stage, the insulin released from nanoparticles is absorbed, which shows that the hypoglycaemic effect is prolonged.

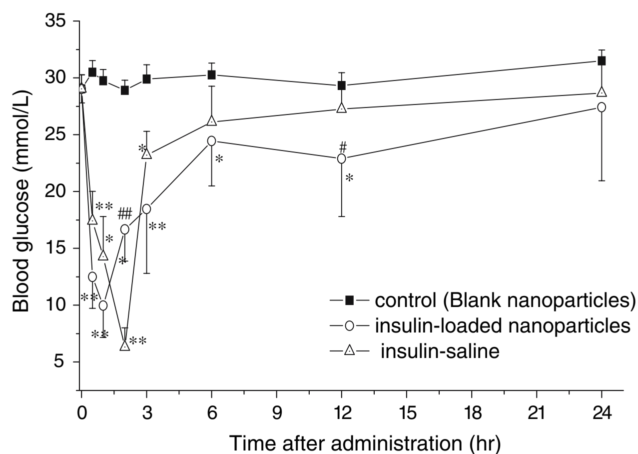

Fig 4 Changes of glucose level in blood of mice as a function of time after the single administration (mean ± S.D., n = 4–6, compared with the control group, ** $p < 0.01$, * $p < 0.05$; compared with the insulin-saline group, ## $p < 0.01$, # $p < 0.05$)

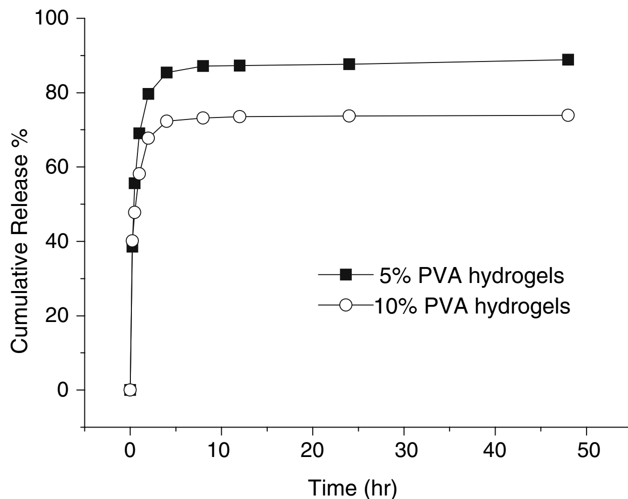

Fig 5 In vitro release of insulin from PVA hydrogels. (PBS buffer, pH 7.4, 37 °C)

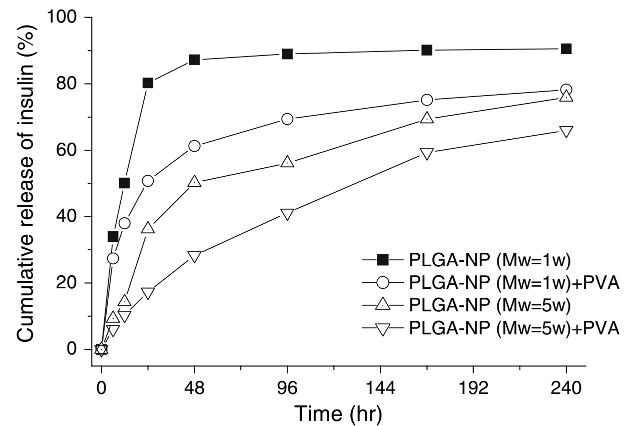

Fig 6 In vitro release of insulin from free PLGA nanoparticles (NP) and PLGA-NP entrapped into PVA hydrogels, prepared starting from PLGA-NP with different molecular weight (10,000, 50,000). (PBS buffer, pH 7.4, 37 °C.)

In vitro release from hydrogels, nanoparticles and hydrogel/nanoparticle composites

Figure 5 shows the release profiles of insulin from the PVA hydrogels prepared with different concentration of PVA solutions. The insulin release from 5% PVA gel is much faster than that from 10% PVA gel. After 12 h, 87.28% insulin have been released from 5% PVA gel while only 73.54% insulin have been released from 10% PVA gel. The concentration of PVA solution has influence on the drug release from the PVA hydrogels. This can be explained that different PVA concentrations might lead to different degree of PVA hydrogel cross-linking and water content in polymeric networks [15, 16].

To observe the effect of PLGA molecular weight on the release profile, two different PLGA with molecular weights of 10,000 and 50,000 were used for preparation of nanoparticles. It can be seen from Fig. 6 that nanoparticles formulated with higher M_w PLGA exhibit a more sustained release pattern than those with lower M_w PLGA. The higher M_w PLGA degrades slower than the lower M_w PLGA, which results in the slower water erosion process [17].

For the purpose of comparison, the release profile of insulin-loaded nanoparticles in the absence of the PVA hydrogels is also shown in Fig. 6. Two curves that nanoparticles formulated with 10,000 M_w PLGA have the same shape. A burst phase is seen during the first 24 h, releasing about 80% of loaded drug. This mass release can be related to the drug adsorbed on the external surface of the particles and the extremely low molecular weights of PLGA [18, 19]. Apparently, the presence of the surrounding PVA hydrogels decreases the burst release of insulin to 50%. As shown in Fig. 6, the insulin release from nanoparticles formulated with 50,000 PLGA shows a biphasic release profile wherein there is an initial burst release followed by a sustained release [20–22]. The initial burst release is observed that 39% of the accumulative amount of insulin releases at the first 24 h, then, the release profile is followed by a constant slow release until to 70% of the accumulative amount of insulin released within 10 days. As compared with it, the insulin release from nanoparticles entrapped into PVA hydrogels shows a nearly zero-order release profile, which indicates that entrapment of the nanoparticles into the PVA hydrogels causes a reduction of the initial release rate. Such phenomena may be attributed to the fact that microcrystalline PVA domains form at the PLGA nanoparticle surfaces, thereby significantly decreasing the ability of water to partition into the nanoparticles, and thus decreasing polymer degradation and drug release [23].

Conclusions

PVA hydrogels embedding the insulin-loaded PLGA nanoparticles were synthesized. This composite system showed more suitable controlled release kinetics and caused a reduction in both the release rate and the total amount of insulin released. It can be expected that this composite release system will show a great potential for controlled release of growth factors and other protein drugs in tissue regeneration.

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