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Investigation of drug loading and *in vitro* release mechanisms of insulin-lauryl sulfate complex loaded PLGA nanoparticles

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Insulin, a water soluble peptide hormone, was hydrophobically ion-paired with sodium lauryl sulfate (SDS) at the stoichiometric molar ratio of 6 : 1. The obtained insulin-SDS complex precipitation was subsequently formulated in biodegradable poly (D,L-lactic-co-glycolic acid) (PLGA) nanoparticles by a modified spontaneous emulsion solvent diffusion method. Compared with a conventional method for free insulin encapsulation, direct dissolution of SDS-paired insulin in the non-aqueous organic phase led to an increase in drug recovery from 42.5% to 89.6%. The more hydrophobic complex contributes to the improved affinity of insulin to the polymer matrix, resulting in a higher drug content in the nanoparticles. The drug loading was investigated by determining initial burst release at the first 30 min. The results showed that 64.8% of recovered drug were preferentially surface bound on complex loaded nanoparticles. The *in vitro* drug release was characterized by an initial burst and subsequent delayed release in dissolution media of deionized water and phosphate buffer saline (PBS). Compared with that in PBS, nanoparticles in deionized water medium presented very low initial burst release (15% vs. 65%) and incomplete cumulative release (25% vs. 90%) of the drug. In addition, dialysis experiments were performed to clarify the form of the released insulin in the dissolution media. The results suggested that the ion-pair complex was sensitive to ionic strength, insulin was released from the particular matrix as complex form and subsequently suffered dissociation from SDS in buffer saline. Moreover, the *in vivo* bioactivity of the SDS-paired insulin and nanoparticulate formulations were evaluated in mice by estimation of their blood sugar levels. The results showed that the bioactivity of insulin was unaltered after the ion-pairing process.

1. Introduction

Because of their desirable biocompatible and biodegradable properties, colloidal carriers made of poly (lactic acid-co-glycolic acid) (PLGA) have arisen as a promising alternative for controlled release of pharmacologically active substances (Bala et al. 2004; Avgoustakis 2004). Among the methods available for preparing nanoparticles, the spontaneous emulsion solvent diffusion (SESD) method presents distinctive advantages (Niwa et al. 2001; Murakami et al. 1999) in that it enables the production of nanoscale particulates (100–300 nm) without extended energy input such as shearing, sonication or high temperatures. So this method is very suitable for loading bioactive protein and peptide drugs due to their instability. The spontaneous formation of nanoparticles is governed by the so-called Marangoni effect (Quintanar-Guerrero et al. 1998), under which an interfacial turbulence was generated at the interface of the water miscible solvent and the aqueous phase and resulted from diffusion and surface tension variations.

Nevertheless, it has been documented that the encapsulation of hydrophilic drug substances inside the polymer matrix always suffers from problems of unsatisfactory loading capacity using the SESD method, which is basically applicable to lipophilic drugs (Barichello et al. 1999; Govender et al. 1999). If there is a poor affinity between hydrophilic substances and polymers, the drug encapsulated will tend to move from the organic phase to the outer aqueous medium during the spontaneous emulsification process, which induces drugs leaking from the precipitating matrix and leads to very low drug recovery.

In order to minimize even more drug leakage, it is possible to modify protein lipophilicity by a hydrophobic ion-pairing (HIP) technique (Meyer and Manning 1998; Quintanar-Guerrero et al. 1997). In our previous studies, complex containing insulin-sodium lauryl sulphate (Shi et al. 2008) have been developed and subsequently formulated into PLGA nanoparticles with satisfactory drug loading capacity. With HIP, the complex formed is much more hydrophobic than the corresponding free hydrophilic protein drugs, which permits large amounts of proteins to be dissolved in nonaqueous organic solvents. In addition, the affinity of proteins to hydrophilic polymers was improved after ion-pairing. Therefore, satisfactory drug recovery was

expected to be achieved with a combination of the HIP and the SESD technique.

On the basis of previous work, the main aim of this study was to investigate the mechanisms of drug loading and in vitro release of protein-amphiphilic surfactant complex loaded polymeric nanoparticles. In all investigations, bovine insulin was used as a model drug due to its water solubility, ease of analysis and ready availability. PLGA was used as biodegradable polymer. The nanoparticles loaded with insulin-lauryl sulfate complex were prepared by the spontaneous emulsion solvent diffusion method. The *in vitro* release behavior of the nanoparticles was investigated in various dissolution media. Meantime, the drug loading was characterized by 0.5 h of initial burst release assay. The existing forms of released insulin from complex loaded nanoparticles were also investigated using the dialysis method. Moreover, the *in vivo* bioactivities of SDS-paired insulin and nanoparticulate formulations were evaluated in mice by estimation of their blood sugar levels.

2. Investigations, results and discussions

2.1. Complex formation

The complex formation between insulin and sodium lauryl sulphate (SDS) was performed in 0.01 M dilute hydrochloric acid (pH 2), in which the medium pH value was below the insulin isoelectric point (pI) (pI = 5.5) and the basic amino groups of insulin become completely protonated. The precipitation behavior of the insulin-SDS complex is shown in Fig. 1. At molar ratios of less than 6 : 1, an increase of the addition of SDS into insulin solution led to an increase of complex precipitate recovery, which indicates the decrease of insulin aqueous solubility. Beyond this latter ratio, the addition of excessive amount of SDS tends to solubilize the surfactant-protein complex precipitate. This phenomenon is in good agreement with findings reported previously that excess surfactant can dissociate the complex precipitate by surfactant micelle solubilization (Turro et al. 1995).

Insulin is a peptide hormone composed of 51 amino acid residues and has six basic groups in the polypeptide chain, to which a negatively charged sulfate group of SDS was bound in a stoichiometric manner of six SDS molecules per insulin (Matsuura et al. 1993). At low SDS concentration employed for ion-pairing with insulin, the types of interactions are mostly ionic in nature, whereby one single positive charge on the protein interacts with one molecular SDS. Before reaching the stoichiometric ratio of 6 : 1 (SDS/INS molar ratio), the aqueous solubility of complex was gradually reduced as addition of SDS in insulin solution. As a result, water insoluble complexes tended to aggregate with each other and form white precipitates. A further addition of excessive amounts of SDS over the maximum binding point dissociate the complex precipitating into surfactant micelles.

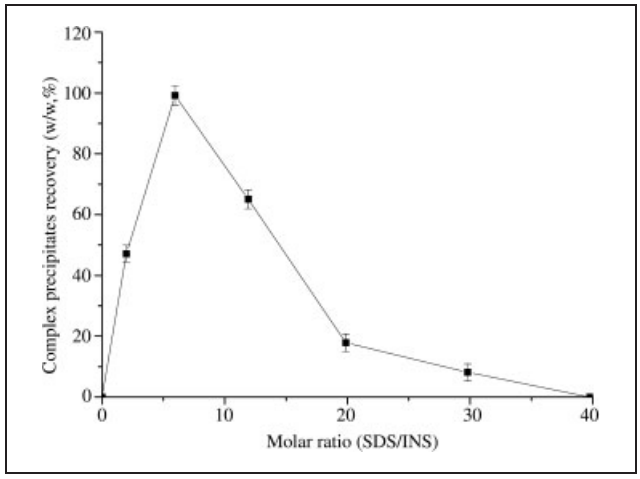

Fig. 1: Complex precipitates recovery upon addition of SDS to insulin solution

2.2. Preparation of nanoparticles

The loading capacity of insulin in PLGA nanoparticles is shown in Table 1. Compared with the conventional method for free insulin encapsulation, direct dissolution of SDS-paired insulin in the non-aqueous organic phase led to an increase in drug recovery from 42.5% to 89.6%. The results demonstrated that a more hydrophobic complex would not suffer from the problems of leakage of drug into the external aqueous phase, resulting in improved drug content of the nanoparticles. For free insulin, the very low affinity to PLGA polymer was suggested as possible reason for the low encapsulation.

It was surprising to observe that neither free nor SDS-paired insulin can be encapsulated in PLGA nanoparticles using phosphate buffer saline (10 mM, pH 5.5) as external aqueous phase. Both the two kinds of nanoparticles displayed approximately zero drug recovery in the particulate matrix (0.9–1.1%). Although some previous reports suggested that a pH value of the external aqueous phase approaching protein pI was favourable for an improvement of drug recovery (Barichello et al. 1999), the effect of ionic strength on drug binding on PLGA nanoparticles during the SESD process seems more prevailing. These results indicate that ionic interactions between insulin and polymer play an important role in drug loading. Within insulin-free PLGA nanoparticles, this affinity mainly comes from ionic interactions between the negatively charged terminal carboxyl groups of PLGA and the positively charged ammonium groups of the drug (Blanco and Alonso 1997). This interaction seems rather weak due to the limited number of terminal carboxyl groups in PLGA polymers, resulting low drug loading. Whereas for the complex, the hydrophobicity of SDS-paired insulin was largely improved because most of ionogenic groups were masked by SDS through ionic interactions between the

Table 1: Properties of nanoparticles containing free and SDS-paired insulin

NP type	N.Y (%)	D.R (%)	D.C (%)	Size (nm)	PDI	Zeta (mv)
Free INS NP (aq.)	86.9	42.5	2.0	242	0.121	−24.8
SDS-paired INS NP (aq.)	85.3	89.6	4.5	238	0.093	−28.6
Free INS NP (PBS)	70.1	0.9	<0.1	236	0.105	−24.2
SDS-paired INS NP (PBS)	68.2	1.1	<0.1	232	0.116	−24.9

negatively charged sulfate groups of SDS and the positively charged ammonium groups of insulin. Consequently, the hydrophobic complex prevents insulin from leaking into the external aqueous phase the during solvent diffusion process, resulting in enhanced drug recovery. The existence of extrinsic salts will interfere these ionic interactions via ion exchange.

The mean particle sizes and the polydispersity index (PDI) of PLGA nanoparticles containing free or SDS-paired insulin are also shown in Table 1. As estimated by laser light scattering, the mean particle size of nanoparticles was in the range of 230–250 nm, and with a relatively narrow particle size distribution for all formulations. Compared with that of insulin-free PLGA nanoparticles, the smaller size of the nanoparticles containing SDS-paired insulin may have resulted from more sulfate groups of SDS, which stabilized the first emulsion and prevented fast coalescence of the droplets during the SESD process. This explanation is also supported by the fact that the absolute surface potential value of SDS-paired loaded nanoparticles was higher than that of nanoparticles containing free insulin, owing to the large number of negative sulfate groups covering the surface of PLGA nanoparticles.

In addition, no clear relationship has been established between particle size and drug loading in PLGA nanoparticles, which suggested that drug loading with the SESD method may mainly be surface bound. This hypothesis will be further discussed in section 2.3.

2.3. In vitro release studies

Figures 2–4 illustrate the release behaviors of free and SDS-paired insulin loaded PLGA nanoparticles in various dissolution media. All the Figures indicate a biphasic release pattern characterized by an initial burst release and subsequent delayed release for each dosage form. It can be observed that both nanoparticles show very low cumulative drug release in deionized water medium (as shown in Fig. 2). Indeed, after an initial burst stage during which small amounts of insulin were released rapidly over 0.5 h (less than 15%), the drug release profiles displayed a plateau for an extended period over 6 h characterized by a very slow and incomplete subsequent release (<30%) resulting from the only diffusion of the drug dispersed into the polymeric matrices. The rapid initial release of insulin was probably due to drug which was adsorbed or close to the surface of nanoparticles and the large surface to volume ratio of the nanoparticle geometry because of their size (Barichello et al. 1999). This effect has been reported by other research groups as well (Song et al. 1997; Peracchia et al. 1997). The delayed release may be attributed to diffusion of the drug within the PLGA core of the nanoparticle into the dissolution medium. However, nanoparticles in PBS medium exhibited higher cumulative drug release compared with those in deionized water (>85%). The release profiles in Fig. 3 and Fig. 4 show that 60–65% of the drug was immediately released at the first sampling time of 0.5 h and 80–88% after 2 h. The marked difference of release profiles, especially the immediate burst release in deionized water and PBS, suggested that ionic strength rather than pH has a very important influence on *in vitro* drug release behavior. As mentioned above, the higher drug recovery in PLGA nanoparticles resulted from ionic interactions between insulin and SDS. When SDS-paired insulin loaded nanoparticles were exposed in PBS enriching salt ions, various cations can replace the amino group of insulin from the carboxyl group

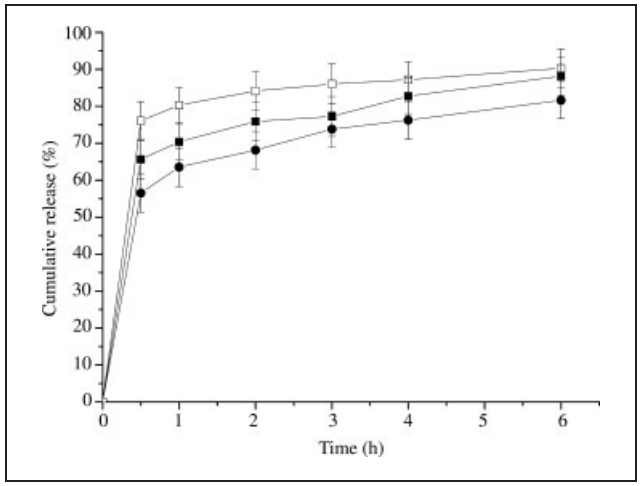

Fig. 3: *In vitro* release profiles of insulin and SDS from nanoparticles in PBS, pH 7.4: insulin-free nanoparticulate (●); SDS-paired insulin loaded nanoparticulate (■); SDS (□)

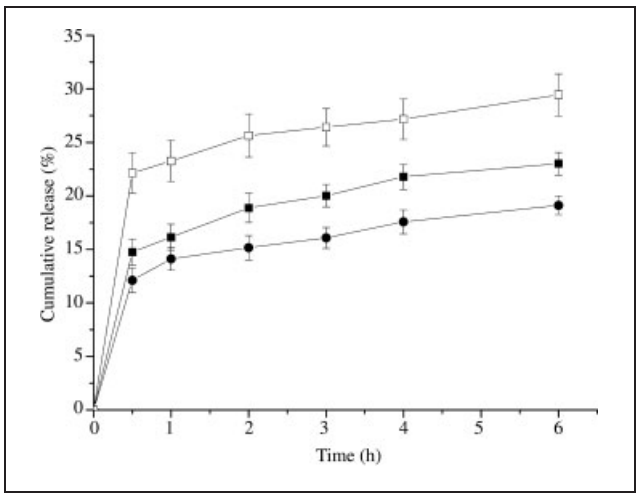

Fig. 2: *In vitro* release profiles of insulin and SDS from nanoparticles in deionized water: insulin-free nanoparticulate (●); SDS-paired insulin loaded nanoparticulate (■); SDS (□)

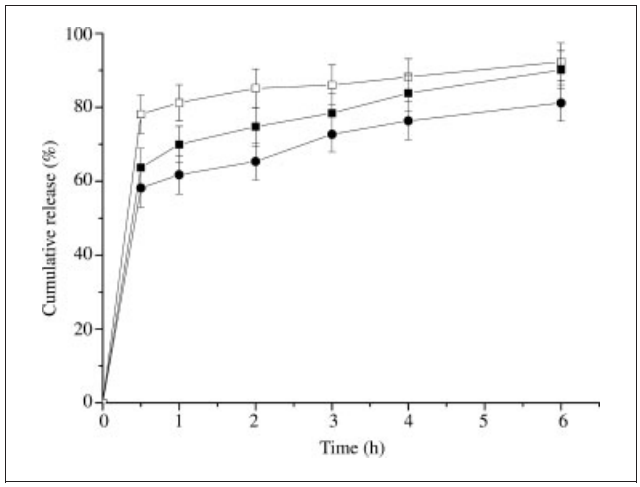

Fig. 4: *In vitro* release profiles of insulin and SDS from nanoparticles in PBS, pH 2.0: insulin-free nanoparticulate (●); SDS-paired insulin loaded nanoparticulate (■); SDS (□)

Table 2: Drug loading in PLGA nanoparticles

NP type	Drug encapsulated, DE (%)	Drug surface bound, DSB (%)	DSB/DR (%)
Free INS NP	18.9	23.6	55.5
SDS-paired INS NP	31.5	58.1	64.8

of SDS, which leads to dissociation of insulin from nanoparticles and immediate release into dissociation media. On contrary, the release of SDS-paired insulin from nanoparticles was not affected by the medium of deionized water because of insufficient amount of external cations, while release kinetics mainly depended on the slow diffusion of the drug from the superficial layer of the nanoparticle into the dissolution medium.

Similar to that of SDS-paired insulin loaded nanoparticles, the release profile of nanoparticles containing free insulin displayed large immediate burst release (55–60%), which indicated that most of drug was associated with PLGA matrix through ionic interaction. The results are in accordance with those observed by other authors, who reported that the ionic interaction between amino group of insulin and terminal carboxyl group contributed most to drug payload (Blanco and Alonso 1997). In addition, an isochronous release behavior of SDS with drug indicated that insulin was released from a particular matrix as complex form and subsequently suffered dissociation from SDS when encountered with salts in PBS. The existing form of insulin within the dissolution medium will be discussed in Section 2.4.

According to *in vitro* drug release studies, it is suggested that shown drug loading could be characterized by initial burst release of 0.5 h. As in Table 2, 64.8% of recovered drug were preferentially surface bound on SDS-paired insulin loaded PLGA nanoparticles. Just 35% of the insulin was encapsulated into the nanoparticles. The results were similar to those with insulin-free PLGA nanoparticles which have 55% of insulin surface bound and 45% encapsulated. Thus we can describe the payload in the nanoparticulate matrix as presented in Fig. 5.

Fig. 5: Schematic representation of drug loading in PLGA nanoparticulate matrix

2.4. Investigation of released drug forms

According to previous studies, SDS-paired insulin presents distinctive properties such as improved hydrophobicity, which is favorable for loading with hydrophobic polymers such as PLGA. Moreover, known as anionic surfactant, SDS may facilitate absorption of insulin across the mucosa membrane. However the fate of SDS-paired insulin being released from the particulate matrix is still unknown after. Therefore it is necessary to clarify the form of released insulin in the dissolution media, free or ion-paired with SDS.

This investigation was carried out by a designed dialysis experiment. Because the molecular cut off of dialysis bag was selected as 2KD, which permits substances with a molecular weight of less than 2KD to transit freely, free SDS will permeate across the dialysis-membrane and separate from insulin-paired SDS. An apparent dissociation constant was developed to evaluate the stability of SDS-insulin complex within the dissolution medium. The dissociation behavior of the complex can be described by Eq. (1):

$$[\mathrm{SDS-INS}]_c \overset{K_d}{\rightleftarrows} [\mathrm{SDS}]_f + [\mathrm{INS}]_f \tag{1}$$

where K_d is the apparent dissociation constant of the SDS-insulin complex, $[\mathrm{SDS\text{-}INS}]_c$ is the equilibrium concentration of the complex, $[\mathrm{SDS}]_f$ and $[\mathrm{INS}]_f$ are the equilibrium concentrations of free SDS and insulin, respectively. According to the model (1), K_d can be expressed as the Eq. (2):

$$K_d = \frac{[\mathrm{SDS}]_f \cdot [\mathrm{INS}]_f}{[\mathrm{SDS-INS}]_c} \tag{2}$$

In view of difficulties to determine the equilibrium concentration of ion-paired insulin, Eqs. (3) and (4) were employed since SDS is bound to the insulin in a stoichiometric ratio.

$$[\mathrm{SDS-INS}]_c = [\mathrm{INS}]_c = [\mathrm{SDS}]_c/6 \tag{3}$$

$$[\mathrm{INS}]_f = [\mathrm{INS}]_T - [\mathrm{INS}]_c \tag{4}$$

Therefore Eq. (2) can be simplified as:

$$K_d = [\mathrm{SDS}]_f \left(\frac{[\mathrm{INS}]_T}{[\mathrm{INS}]_c} - 1 \right) \tag{5}$$

Where $[\mathrm{SDS}]_c$ is the equilibrium concentration of ion-paired SDS, $[\mathrm{Ins}]_T$ is the equilibrium concentration of total insulin.

The results are presented in Table 3. It can be seen that complexes were not stable in buffers, almost all of insulin was dissociated into free form. In addition, there seems little difference of K_d in pH 7.4 and pH 2.0 media, which indicated that a medium pH value does not play a principal role in the dissociation process of the complex.

Table 3: Dialysis results for investigation of released insulin forms

	pH 7.4	pH 2.0
$[\mathrm{INS}]_T \times 10^6$ (mol/L)	5.39	5.76
$[\mathrm{SDS}]_c \times 10^6$ (mol/L)	2.81	3.36
$[\mathrm{SDS}]_f \times 10^6$ (mol/L)	10.26	9.95
$[\mathrm{INS}]_c/[\mathrm{INS}]_T$	0.087	0.097
$K_d \times 10^5$	10.78	9.24

2.5. Bioactivity assay

It was necessary to verify whether insulin retained its bioactivity after the encapsulation process that involves steps of interface contact with the organic solvent. In addition, known as an ionic detergent usually used in gel electrophoresis, SDS may have protein denaturing potency when ion-paired with protein. Therefore the bioassay of insulin within complex and nanoparticles was carried out by the estimation of blood sugar in mice.

In view of fact that the noticeable insulin response to blood sugar appears 45–90 min after subcutaneous (s.c.) administration of insulin (Eneroth and Ahlund 1968), the plasma glucose levels of mice after s.c. administration of various samples were determined after 60 min (Fig. 6). The bioactivity of ion-paired insulin relative to free insulin was calculated according to the following expression:

$$\mathrm{R.B}\% = 100^{*}\left(\frac{100-\mathrm{A_{TL}}}{100-\mathrm{A_{SL}}}+\frac{100-\mathrm{A_{TH}}}{100-\mathrm{A_{SH}}}\right)\Big/2 \qquad (6)$$

where A_{TL}, A_{TH} are plasma glucose levels after s.c. low or high dose of test samples; A_{SL}, A_{SH} is plasma glucose levels after s.c. low or high dose of free insulin solution.

The results in Table 4 show that the bioactivity of insulin was unaltered after ion-paired with SDS and the emulsion-solvent evaporation process since it preserved its hypoglycemic activity. As reported, the reason for preservation of bioactivity was governed by the aggregation state of the detergent (Reynolds and Tanford 1970; Bordbar et al. 1997). Monomeric detergents bind to the native state as conventional ligands, that is, they bind to a small number of sites in a saturable manner. However micelles act as denaturants, which attack and distort the native state of proteins. Because a low concentration of SDS was used in this study, the critical micelle concentration was not satisfied to form micelles (Otzen and Oliveberg 2002).

In addition, it was interesting to observe that, the hypoglycemic activity of SDS-paired insulin was slightly higher than that of free insulin. This could be explained by the avoidance of insulin hexamer formation when ion-paired with SDS (Polaschegg 1998). Normally regular insulin exists as hexamer, which is required to disassociate into the dimer and further monomer before being absorbed. Relative to the ion-paired form, the disassociation of regular insulin hindered its pharmacodynamic action within the initial first hour.

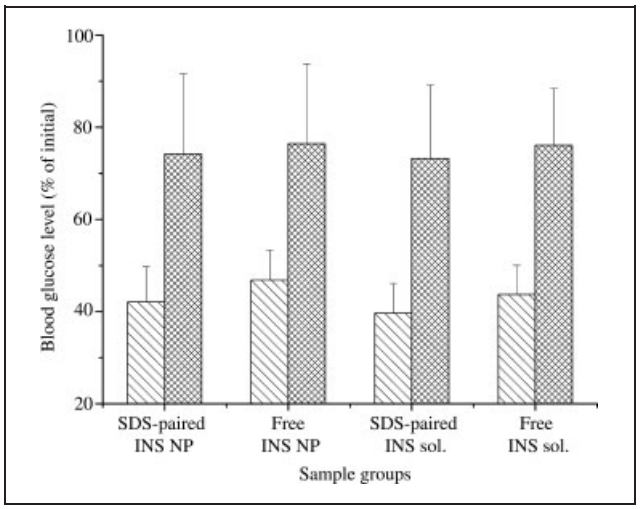

Fig. 6: The blood glucose levels after s.c administration of test solutions in normal mice

Table 4: Relative bioactivity of various insulin samples

Samples	Relative bioactivity (%)
SDS-paired INS NP	105.43 ± 12.50
Free INS NP	96.45 ± 11.90
SDS-paired INS sol.	109.64 ± 11.21
Free INS sol.	100.00

2.6. Conclusion

In this paper, we have shown that a hydrophilic peptide, such as insulin, can be successfully formulated into PLGA nanoparticles by formation of a hydrophobic HIP complex. Particles with a small size and high drug recovery can be obtained by the SESD method. *In vitro* release studies suggested that binding on the nanoparticle surface was the main drug loading mode both for free and ion-paired insulin. Inorganic salt ions have strong influence on the stability of the complex. The results of the dialysis study show that insulin was released from the particular matrix in complex form and subsequently suffered dissociation from SDS when encountered with counterions in PBS. In addition, results of an *in vivo* evaluation allow us to conclude that the bioactivity of insulin was unaltered after ion-pairing and the subsequent emulsion-solvent evaporation process.

3. Experimental

3.1. Materials

Bovine insulin (INS, 29 IU/mg) was purchased from SIGMA Chemical Co., Ltd., USA. Sodium lauryl sulfate (SDS) was supplied by Nacalai Tesque Inc, Japan. PLGA 7520 (75 : 25, Av.M_W 20000) was obtained from the Wako Pure Chemical Ind. Ltd., Japan). Poly (vinyl alcohol) (PVA-403) was supplied by Kuraray Co., Ltd., Japan. All other reagents were of analytical grade.

3.2. Preparation of insulin-lauryl sulfate complex

Insulin powder and SDS were co-dissolved in 0.01 mol/L dilute hydrochloric acid at molar ratio of 6 : 1. The resulted cloudy suspension was centrifuged at 5000 rpm for 5 min. The white precipitates recovered were rinsed with distilled water, lyophilized, and stored at −20 °C before further use.

3.3. Preparation of nanoparticles

PLGA nanoparticles were prepared according to a modified spontaneous emulsion solvent diffusion method (Kawashima et al. 1999). Briefly, PLGA, insulin -SDS complex were co-dissolved completely in the mixture of acetone and ethanol. The resultant polymer-drug solution was poured into 10 ml PVA aqueous solution (1.0%, w/v). The emulsified system was stirred at 400 rpm for 1 h using a propeller type agitator with three blades (Heidon 600G, Shinto Scientific Co., Ltd., Japan). The entire dispersed system was then subjected to centrifugation (40,000 g for 15 min; Kubota 7780, Kubota Co., Ltd., Japan). The nanoparticles were washed two times with distilled water to remove free drug and PVA before freeze-drying. The nanoparticle yield (N.Y) was calculated from Eq. (7).

$$\mathrm{N.Y}\% = \frac{\text{Mass of nanoparticle recovered}}{\text{Mass of (PLGA + INS + SDS) used in formulation}} \times 100 \qquad (7)$$

3.4. Determination of drug loading capacity

The freeze-dried nanoparticles were dissolved in acetonitrile, to which 0.01 M hydrochloric acid was added to preferentially precipitate the polymer. The drug content in the supernatant after centrifugation (40,000 g for 15 min, Kubota 7780, Kubota Co., Ltd., Japan) was measured spectrophotometrically at 214 nm by means of an HPLC method (LC-20AD pump, SPD-20A detector, CTO-910AS column oven, Crestpak C18S column, Shimadzu Co., Ltd., Japan). Drug loading capacity was expressed both as drug recovery (D.R, %) and drug content (D.C, % w/w); represented by Eqs. (8) and (9), respectively.

$$\mathrm{D.R}\% = \frac{\text{Mass of drug in NPs}}{\text{Mass of drug used in formulation}} \times 100 \qquad (8)$$

$$\mathrm{D.C}\% = \frac{\text{Mass of drug in NPs}}{\text{Mass of NPs recovered}} \times 100 \qquad (9)$$

3.5. Particle size analysis

The dried nanoparticle samples were suspended in distilled water and were sonicated before measurement. The obtained homogeneous suspensions were subjected to examination. The particle size distribution, expressed as mean diameter and polydispersity index, was determined by photon correlation spectroscopy (PCS) using Zetasizer Nano-ZS90 (Malvern Instruments, UK).

3.6. In vitro release studies

In vitro drug release from the nanoparticles was performed in various dissolution media at 37 °C with continuous orbital mixing (50 r/min). At appropriate intervals, the entire suspensions were subjected to ultracentrifugation at 40 000 rpm for 10 min. The pellets were re-suspended in equal volume of fresh release medium. The amount of insulin and SDS released in the supernatant was evaluated by RP-HPLC analysis and methylene blue method (Hayashi 1975), respectively. The cumulative drug or SDS percentage released from the matrix was calculated as the ratio of the amount of drug or SDS released at time (t) to the initial amount used.

3.7. Dialysis of complex

The existence form of released insulin in the dissolution media was evaluated using a dialysis method. Briefly, insulin-SDS complex loaded PLGA nanoparticles were allowed to be released in various dissolution media for 6 h. Then the suspensions were ultracentrifuged to separate the supernatant. The collected suspensions were subjected to dialysis for 3 days until an equilibrium was reached, the SDS concentration within inner and outer phase was determined by the methylene blue method (Hayashi 1975).

3.8. In vivo bioactivity

The *in vivo* bioactivity of insulin in the complex was evaluated in ddY mice weighing 27 ± 2 g. Test solutions were prepared as follows: for group 1 and 2, insulin-SDS complex loaded PLGA nanoparticles and insulin-free PLGA nanoparticles were introduced in phosphate buffer saline (PBS, pH 7.4) to let insulin release (as seen in section 3.6). After 6 h, the suspensions were subjected to ultracentrifugation at 4 °C ($40\,000 \times$ g, 10 min) and the supernatants were collected as test samples. For group 3 and 4, insulin-SDS complex and free insulin were dissolved in PBS (pH 7.4) respectively as control. All of the four solutions were diluted to an insulin-equivalent concentration of 80 mIU/ml by PBS before administration.

The biological activity of the insulin recovered in the supernatant was evaluated by the measurement of hypoglycemic activity in mice based on a blood glucose assay. Briefly, the animals were fasted for 12 h before experiments. The above test solutions were administered to them by subcutaneous injection (s.c) with dosage of 0.4 IU/kg and 0.8 IU/kg, respectively. Blood samples were collected from the retroorbital plexus of the mouse prior to s.c to establish baseline glucose levels. At 60 min after dosing, blood samples were collected in the same way. Glycemia was determined by the glucose-oxidase method (Glucose GOD-PAD kit, Wako Pure Chemical Industries, Ltd. Osaka, Japan). Data represents the mean ± SD., $n = 5$ per group. The study protocol was reviewed and approved by the Institutional Animal Care and Use Committee, Aichi Gakuin University, School of Pharmacy, Japan.

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