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Preparation of insulin nanoparticles and their encapsulation with biodegradable polyelectrolytes via the layer-by-layer adsorption

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Abstract

To develop a new polypeptide delivery system, insulin nano-aggregates with sizes of 100–230 nm were prepared by the salting out method with NaCl and encapsulated via the layer-by-layer (LbL) adsorption to provide the insulin nanoparticles shelled with two oppositely charged polyelectrolytes. Poly(α,β-L-malic acid) (PMA) and water-soluble chitosan (WSC) as the weak polyelectrolytes with good biodegradability and biocompatibility in vivo were chosen to be the encapsulating materials of the LbL adsorption. In the preparation of the insulin nano-aggregates, the NaCl concentration and pH in the medium obviously affected yield and particle size of the insulin nano-aggregates. After eight adsorption cycles of the polyelectrolytes on the insulin nano-aggregates, the insulin–polyelectrolyte nanoparticles with the sizes of 100–250 nm were obtained with about 20% insulin loss. The insulin release from the nanoparticles was mostly pH-dependent owing to sensitivity of the weak polyelectrolytes to pH. Insulin was hardly released from the nanoparticles in a medium at pH 4–5 while it could be released at pH 7.4, corresponding to the pH of the human blood and the body fluid. A burst effect was also observed although it could be reduced via increasing the polyelectrolyte layers of PMA and WSC assembled on insulin nano-aggregates.

Keywords: Nanoparticles; Layer-by-layer adsorption; Insulin; Water-soluble chitosan; Poly(α,β-L-malic acid)

1. Introduction

Micro- and nanoencapsulation are an attractive technique for the drug formulation since they can offer many advantages, such as controlled release, site-specific drug delivery, minimizing side effects, and protecting sensitive drugs (McGee et al., 1995). Several systems, such as microparticles, microcapsules, microemulsion, and liposomes, have been well developed in the past decades. Interfacial polymerization and phase separation were used for the preparation of these particles. However, their application, especially in protein drug delivery systems, was limited by some common problems including polydispersity, uneven shell coverage, core solidification, residual organic solvent, and severe encapsulation conditions (Donath et al., 1998). The restricted application of liposomes largely resulted from their poor stability and low permeability for polar molecules (Donath et al., 1998). In addition, drugs encapsulated by the above methods were usually dissolved in the solvent, and thus these particles had high encapsulation efficiency but low drug loading capacity. To overcome the above obstacles, the layer-by-layer (LbL) adsorption of polyelectrolytes was developed although most of the used polyelectrolytes were not bioabsorbable. The technique was usually performed in the aqueous solution at room temperature, and thus it was suitable to encapsulate polypeptide and protein drugs of a poor stability.

An LbL assembly of two oppositely charged polyelectrolytes at solid surfaces was developed as the alternating adsorption of these polyelectrolytes on a charged substrate due to their electrostatic attraction and the complex formation, resulting in the defined macromolecular layers on the surfaces (Decher, 1997). Formation conditions, thickness determination, a diffusion model, and molecular mechanisms of polyelectrolyte multilayers were studied in the past decade (Klitzing and Möhwald, 1995, 1996; Hoogeveen et al., 1996; Caruso et al., 1997; Lowack and Helm, 1998; Dubas and Schlenoff, 1999). Some micron-sized particles were used as templates instead of macroscopic flat substrates in the LbL adsorption and the microcapsules with polyelectrolyte multilayers as a capsule shell were obtained after

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the colloids were removed (Sukhorukov et al., 1998a). The synthetic polyelectrolytes, biopolymers, liposomes, and inorganic nanoparticles were applied to fabricating capsules by this technique (Caruso et al., 1998; Sukhorukov et al., 1998b; Gao et al., 2000, 2001a; Dudnik et al., 2001). Charged polystyrene particles, slightly cross-linked melamine-formaldehyde particles, dye particles, human erythrocytes and discocytes fixed with glutaraldehyde might be used as the template particles (Dai et al., 2001; Gao et al., 2001b, 2002b; Donath et al., 2002). Generally, such a multilayer should exclude macromolecules but allow ions and small molecules to permeate them (Sukhorukov et al., 1999). Moreover, the capsule permeability might be adjusted by changing the polyelectrolyte structure and environmental conditions, such as pH and the ionic strength (Sukhorukov et al., 2001; Donath et al., 2002; Ibarz et al., 2002). It was also observed that the permeability coefficient of the polyelectrolyte capsules decreased with increasing multilayer thickness (Qiu et al., 2001).

Hollow capsules can be fabricated by removing template particles under a suitable condition from polyelectrolyte template capsules obtained by the LbL adsorption. Some proteins, such as bovine serum albumin and peroxidase, were incorporated into hollow polyelectrolyte capsules by regulating permeability of the multiplayer shell for macromolecules (Sukhorukov et al., 2001; Gao et al., 2002a, 2003). In addition, charged protein particles may be encapsulated directly into polyelectrolyte multilayers by the LbL adsorption. Synthetic polymers including poly(styrene sulfonate) (PSS), poly(allylamine hydrochloride) (PAH), etc., were used as layer constituents to encapsulate directly α-chymotrypsin microaggregates obtained by the salting out method (Balabushevitch et al., 2001). Natural biopolymers such as dextran sulfate and protamine were also employed to encapsulate directly polypeptide particles (Balabushevich and Larionova, 2004). However, the above mentioned synthetic or natural polyelectrolytes are not biodegradable or poorly biodegradable in vivo. It is known that non-biodegradable and poorly biodegradable polymers should not be used as the carriers of protein (or polypeptide) delivery systems for parenteral administrations.

If some biodegradable synthetic polyelectrolytes were applied in the LbL adsorption on the protein or polypeptide nano-aggregates, the useful nano-devices in biomedicine would be afforded. In our laboratory, a new LbL adsorption technique was achieved using a polypeptide drug and two oppositely charged biodegradable polyelectrolytes. Insulin (Mw 5800, p*I* 5.5), an important drug for diabetes, was chosen as a model polypeptide due to its comparative stability and its capability of aggregating and crystallizing under different conditions. Although an attempt in the development of insulin microformulations was carried out (Carino and Mathiowitz, 1999), insulin nano-aggregates were first investigated in detail for the LbL adsorption in our laboratory. Water-soluble chitosan (WSC) was used as the semi-synthetic polycation because of its good biocompatibility, excellent biodegradability in vivo and active pharmacological actions (Begona and Ruth, 1997; Richardson et al., 1999; Kondo et al., 2000; Seo et al., 2000; No et al., 2002; Qin et al., 2002; Kittur et al., 2003; Zheng and Zhu, 2003; Kumar et al., 2004). Synthetic poly(α,β-L-malic acid) (PMA) was chosen as a biodegradable polyanion without toxicity because its degradation product, L-malic acid, could be metabolized in the mammalian tri-carboxylic acid cycle (Braud et al., 1985). In addition, PMA and WSC are weak polyelectrolytes so that their charge density and conformation should be highly pH-dependent. Such a characteristic would be beneficial to adjust the encapsulation and release of a polypeptide. Preparation of the insulin nano-aggregates and their encapsulation with the polyelectrolytes, PMA and WSC, via the LbL adsorption were studied in detail. The properties of the insulin nano-aggregates and the insulin–polyelectrolyte nanoparticles were also investigated.

2. Materials and methods

2.1. Materials

Insulin (potency 30 IU/mg, batch number 0406A08) was the product of Xuzhou Wanbang Biopharmacy Company (PR China). WSC (Mn 8000) with more than 94% deacetylation was prepared by oxidative cleavage of chitosan. PMA (Mn 4500) was synthesized by direct polycondensation of L-malic acid. Coomassie brilliant blue G 250 was obtained from Fluka (USA). All of the other chemicals were of analytical purity and were used without future purification.

2.2. Preparation of insulin nano-aggregates

At 15 °C, a certain amount of insulin was dissolved in dilute HCl solution, respectively, with pH from 1.1 to 3.0. Different amounts of NaCl solid powders or 2.5 M NaCl solutions (pH 1.5) were added to the insulin solution up to a certain concentration. The mixture was stirred 1 h at 15 °C. The resultant suspension was centrifuged (3000 rpm, 3 min) to collect insulin nano-aggregates.

2.3. Fabrication of insulin–polyelectrolyte nanoparticles

A 1 ml of the PMA solution (10 mg/ml) in 0.1 mM HCl containing 0.6 M NaCl was added to the insulin suspension obtained at pH 1.1 and 0.6 M NaCl. The mixture was stirred for 20 min and centrifuged (5000 rpm, 3 min). The precipitates were twice resuspended in 0.1 mM HCl containing 0.6 M NaCl and centrifuged (5000 rpm, 3 min). Then the same operation was preformed for the WSC adsorption process when the adsorption and the washing were preformed under the same conditions but in the presence of 3.0 M NaCl. Two polyelectrolytes were applied alternatively to achieving the required number of adsorption cycles. Finally, the insulin–polyelectrolyte nanoparticles were rinsed three times by 0.1 mM HCl and lyophilized. The lyophilized preparations were stored at 4 °C.

2.4. Characterization of insulin particles and polyelectrolyte particles

The circular dichroism (CD) spectra of the salted out insulin nano-aggregates and the original insulin were measured in a

phosphate buffer solution (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.2–7.4) on a CD spectropolarimeter (J-715, Jasco, Japan) at 25 °C. Each test solution was individually placed in a quartz cuvette having a path length of 0.1 cm and scans were conducted from a wavelength of 300–200 nm using the baseline of a blank buffer solution.

After rinsed repeatedly by 95% ethanol to remove residual NaCl, the salted out insulin nano-aggregates were lyophilized to give the white powder for X-ray powder diffraction (XRD). The experiments were performed on an X-ray powder diffractometer (Rigakudmax, Japan) using Cu $K\alpha_1$ radiation ($\lambda = 1.5406$ Å).

The morphology of the insulin nano-aggregates and insulin–polyelectrolyte nanoparticles was observed on a scanning electron microscope (SEM; PHILIPS XL-30 ESEM, The Netherlands). The insulin nano-aggregates were rinsed repeatedly with 95% ethanol and resuspended in ethanol. The insulin–polyelectrolyte nanoparticles were rinsed repeatedly with 0.1 mM HCl to remove NaCl and resuspended in 0.1 mM HCl. After the samples were ultrasonicated for 2 min at 10 °C, the suspensions were dripped onto a small glass slide and air-dried. After the nano-aggregates and nanoparticles on the glass slides were gold-coated, they were observed under SEM. The average size of the nano-aggregates and nanoparticles was obtained by measuring the size of 100 particles on SEM. The sizes of insulin nano-aggregates and insulin–polyelectrolyte nanoparticles without ultrasonic treatment were determined on a laser particle-size analyzer (BI-90Plus, Brookhaven Instruments, USA).

A drop of the suspension with the insulin–polyelectrolyte nanoparticles was dispersed in twice-distilled water and the pH was adjusted to 4.0 ± 0.2 with 1 mM HCl or 1 mM NaOH. Electrophoretic mobilities of polyelectrolyte particles were determined on a Zetasizer (Zetasizer 3000HSA, Malvern Instruments, USA). The electrophoretic mobility u was converted into the ξ potential by using Smoluchowski relation ($\xi = u\eta/\varepsilon$, where η and ε are the viscosity and the permittivity of the solution, respectively).

2.5. Insulin release from the insulin–polyelectrolyte nanoparticles

To investigate kinetics of insulin release from the insulin–polyelectrolyte nanoparticles, 5 mg of the lyophilized powder was dispersed in 5 ml of the PBS solution (pH 7.4) and incubated under stirring (70 rpm) at 37 °C. Suspension aliquots were collected after 10, 30 min, 1, 2, 4, 8 and 24 h. After the samples were centrifuged (5000 rpm, 3 min), the insulin concentration in the supernatants was determined by Coomassie brilliant blue G 250 staining according to Bradford (1976). Insulin release from the insulin–polyelectrolyte nanoparticles was calculated as the ratio of the insulin contents in the supernatant and in the nanoparticle suspension. The effect of pH on insulin release was investigated at different pH values from 2.0 to 8.0 adjusted by 1 M HCl or 1 M NaOH solution.

The form of insulin released from the insulin–polyelectrolyte nanoparticles was investigated by gel chromatography. The supernatants obtained after the particle incubation (70 rpm, 37 °C) in the PBS solution (pH 7.4) for 1 h were filtered through a cellulose film (pore size 0.45 μm) and separated on a Sephadex G-50 medium column (30 cm × 2.6 cm) in the same buffer. The eluate was detected by an ultraviolet/visible light spectrophotometer (UV-762, Shanghai Precision & Scientific Instrumental Co. Ltd., PR China) at 280 nm.

3. Results and discussion

3.1. Solubility and precipitation of insulin

To prepare the nano-aggregates of insulin, insulin should be dissolved well in a solution. It was found that pH values and ion strength would significantly affect the solubility of insulin. At 15 °C, porcine insulin was dissolved difficultly in the HCl solution at pH 2.3–3.0 and the solution was still turbid overnight, but it was dissolved at pH 2.1 after a while. At pH lower than 2.0, however, it was dissolved instantly. Insulin molecules in the acidic solution bear more electric charges at lower pH farther from its isoelectric point (p*I* 5.5), which leads to easier dissolution of insulin due to the surround by water molecules.

The solubility of insulin was decreased with ion strength because the ions of salts destroy its hydrated shells and screen its electric charges. When the NaCl concentration was lower than 0.5 M NaCl, insulin could be still dissolved. At the concentration higher than 0.55 M NaCl, however, the insulin aggregates appeared in the whole solution and were not dissolved under stirring. Therefore, the suitable NaCl concentration for preparation of the insulin aggregates should be higher than 0.55 M to ensure that insulin is precipitated completely.

3.2. Preparation of insulin nano-aggregates

Addition of NaCl into the insulin solution could result in formation of insulin aggregates. As presented in Table 1, yield of insulin aggregates obtained at different concentrations of NaCl was higher than 90%. More NaCl in the insulin solution could apparently make more insulin precipitated; however, the NaCl concentration might affect the morphology of insulin particles. As shown in Fig. 1, insulin particles, which were obtained at 0.6 M NaCl after ultrasonicated at 10 °C for 2 min, mainly existed as single particles, indicating a good dispersibility of insulin particles. By contraries, a majority of insulin particles was aggregated together at 0.8 M NaCl and further agglomerated at 1.0 M NaCl. In fact, high NaCl concentrations which mean the high ion strength of the insulin solution would decrease the

Table 1
Effect of NaCl concentration on yield of insulin aggregates obtained at pH 1.5

NaCl concentrations (M)	Yield of precipitates (%)
0.55	90
0.6	90
0.8	90
1.0	92
1.2	94
1.4	97

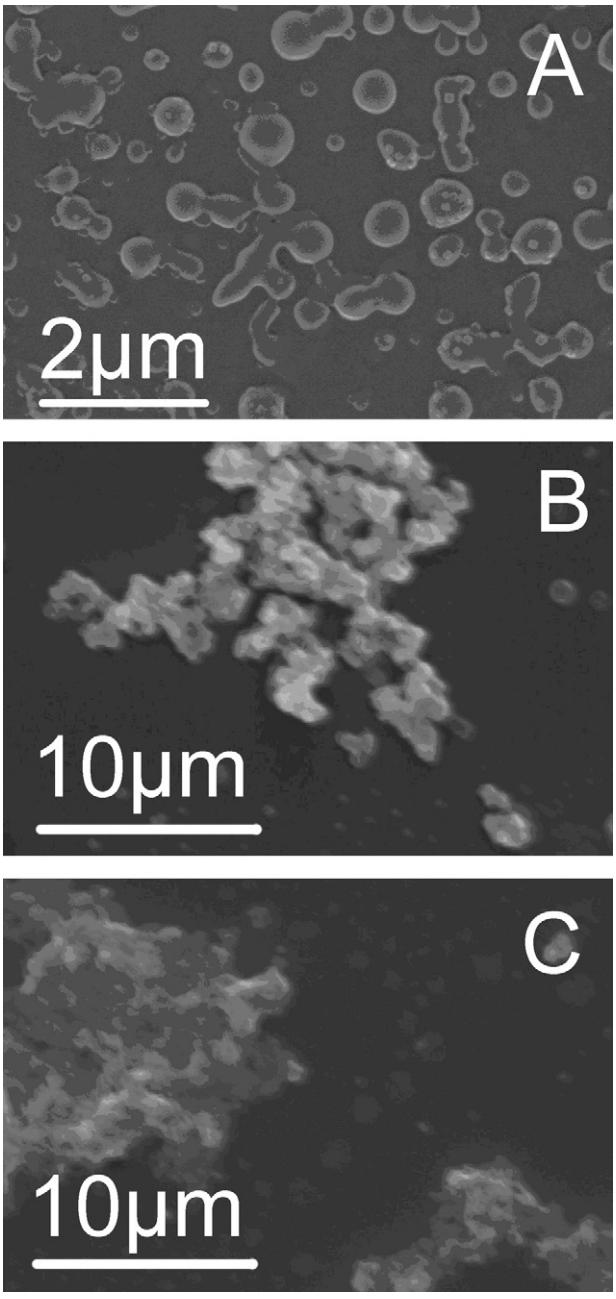

Fig. 1. SEM photographs of insulin particles obtained in different NaCl concentrations at pH 1.5 after ultrasonicated at 10 °C for 2 min. (A) 0.6 M NaCl; (B) 0.8 M NaCl; (C) 1.0 M NaCl. Scale bars represent 2 μm (A) or 10 μm (B and C).

solubility of the nonpolar groups on the surface of insulin particles in water and enhance the hydrophobic interaction between insulin particles, resulting in the aggregation between them (Su, 1986). Hence, the NaCl concentrations for preparation of the insulin nano-aggregates should range from 0.6 to 0.8 M.

The effect of pH value on the yield and size of insulin aggregates was investigated. As seen in Table 2, yield of insulin particles prepared at 0.6 M NaCl in the range of pH 1.1–2.1 was about 90%, and varied irregularly with pH. So the main factor to affect yield of insulin particles is not pH but the NaCl concentration. The size of insulin particles decreased from 900–1600 nm to 100–230 nm as pH decreased from 2.1 to 1.1. At the same time, the dispersity of insulin particles was improved (data not shown). In the salting out method, the activity of water molecules is reduced greatly and a lot of electric charges on the protein surface are neutralized under the influence of neutral salts (for instance, NaCl). As a result, the hydrated shell on the surface of protein molecules is destroyed, which leads to the protein precipitation due to the aggregation between protein molecules. The polar groups in insulin molecules are ionized in the solution with low pH farther from the isoelectric point of insulin (p*I* 5.5), resulting in enhancement of the repulsive interaction between insulin molecules and aggregates. Therefore, the particle size is smaller for insulin particles obtained by salting out at a lower pH. Also, pH affects the dispersion of insulin particles in the same way so that insulin particles disperse better at a lower pH. The pH of the insulin solution would be adjusted to 1.1 in order to make insulin particles smaller and make their dispersibility better.

Table 2
Effect of pH value on yield and particle size of insulin aggregates obtained at the NaCl concentration of 0.6 M

pH	Yield of precipitates (%)	Aggregate size (nm)
1.1	93	100–230
1.3	93	–
1.5	90	400–500
1.7	90	–
1.9	93	–
2.1	90	900–1600

3.3. Insulin stability in its aggregates

According to the previous study in other groups, the bioactivity of insulin were not changed in the acidic aqueous solution with pH higher than 1.2 or the neutral aqueous solution for 3 h (Tang et al., 2004), and insulin was also relatively stable in the basic aqueous solution with pH lower than 10 (Sheng et al., 2000). When insulin solutions were subjected to acid, heat and agitation, however, the normal pattern of insulin assembly (dimers → tetramers → hexamers) was disrupted and the molecule underwent conformational changes allowing it to follow an alternative aggregation pathway (via a monomeric species) leading to the formation of insoluble amyloid fibers (Whittingham et al., 2002). Thus insulin should avoid being heated in the acidic solution with a lower pH in case that its conformation is changed.

In our experiments, white insulin aggregates appeared instantly after NaCl was added into the insulin solution. During the magnetic stirring, the lower temperature (15 ± 1 °C) was kept to ensure the protein activity. The secondary structure of protein molecules can be measured with a CD spectroscopy. The CD spectra of insulin before and after salted out in the PBS solution (pH 7.4) are shown in Fig. 2. The spectrum of control insulin solution prepared freshly in the far UV region (200–250 nm) showed extrema at 209 and 222 nm corresponding to α-helix of insulin molecules. This spectrum is in close agreement with that of insulin obtained previously by other investigators (Ettinger

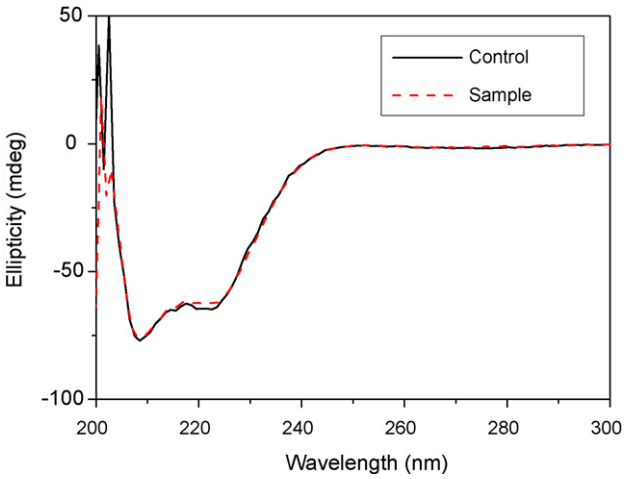

Fig. 2. The CD spectra of insulin aggregates in the PBS solution (pH 7.4). Control is the original insulin; sample is insulin aggregates salted out by NaCl (0.6 M) in the insulin solution (20 mg/ml) with pH 1.1 at 15 °C.

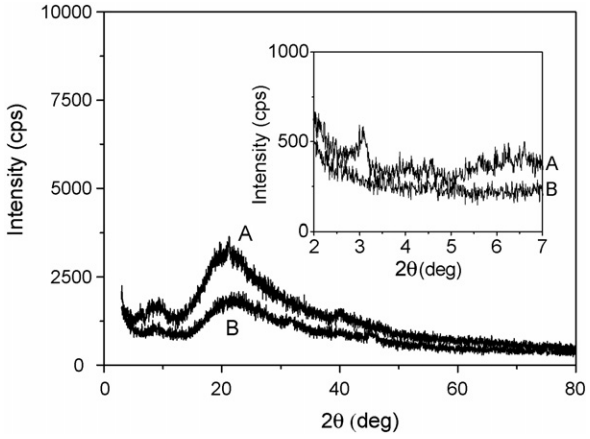

Fig. 3. The XRD spectra of insulin aggregates in the range of 3–80° (2θ). (A) The original insulin (control); (B) insulin particles salted out by NaCl (0.6 M) in the insulin solution (20 mg/ml) with pH 1.1 at 15 °C (sample). The spectra in the inset are those in the range of 0.6–8° (2θ).

and Timasheff, 1971; Wu and Yang, 1981; Wollmer et al., 1987). As compared to the CD spectrum of control insulin, no significant change was observed in that of the insulin aggregates in the PBS solution, indicating that no conformation was changed for insulin while it was salted out by NaCl (0.6 M) in the insulin solution (20 mg/ml) with pH 1.1 at 15 °C.

The XRD spectra of the original insulin and the salted out insulin aggregates were shown in Fig. 3. Some weak diffraction peaks appeared at 9.2°, 19.7°, 21.2° and 40.1° (2θ) for the original insulin in the range of 3–80° (2θ), indicating that a few crystals existed in it. For the salted out insulin aggregates, however, these diffraction peaks almost disappeared, which demonstrated a majority of insulin aggregates obtained by the salting out method were amorphous. It might be also found that the original insulin had a weak diffraction peak at 3° (2θ) in the range of 0.6–8° (2θ), but the salted out insulin aggregates did not show it at the same diffraction angle, further demonstrating that they mainly existed as an amorphous state.

3.4. Fabrication of the insulin–polyelectrolyte nanoparticles

The insulin aggregates as a template of the LbL adsorption might be encapsulated with the charged polyelectrolytes as shown in the Fig. 4. Although some microaggregates would appear during the formation of insulin aggregates, the insulin–polyelectrolyte nanoparticles could be obtained by ultrasonicating these microaggregates at a lower temperature during the encapsulation process. The used polyelectrolyte solution should theoretically have the same pH and ionic strength as those of the precipitating insulin aggregates (pH 1.1, 0.6 M NaCl) to avoid insulin dissolution during the LbL adsorption with polyelectrolytes. However, PMA would have little negative charges at such a low pH, which is not beneficial to the PMA adsorption on bare insulin particles or polyelectrolyte particles with WSC as the outermost layer. The pH of the PMA aqueous solution (10 mg/ml, the same PMA concentration used in the LbL adsorption) is 2.2, the pH of the aqueous WSC solution (10 mg/ml) is higher than 7.0, and the isoelectric point of insulin is about 5.5. To ensure that PMA, WSC, and the insulin particles all bear enough charges, the pH should range from 2.2 to 5.5 in the salt solution during the LbL adsorption. Therefore, PMA and WSC were dissolved, respectively, in the salt solution with pH 4.0 (0.1 mM HCl). Experimental results showed that insulin particles were not dissolved in such a solution with 0.1 mM HCl and 0.6 M NaCl.

The LbL adsorption of PMA and WSC on the insulin particles was performed in 0.1 mM HCl containing 0.6 M NaCl

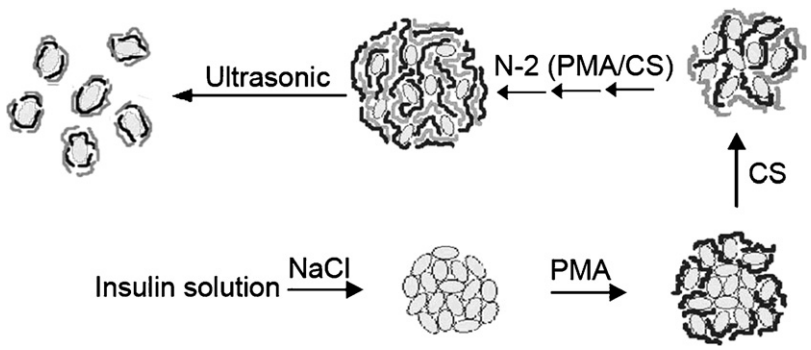

Fig. 4. Scheme of the LbL adsorption of negatively (black) and positively (gray) charged polyelectrolytes on protein particles.

(for PMA) or 3.0 M NaCl (for WSC) until the required number of polyelectrolyte adsorption cycles (N) was obtained. At present, eight cycles has been reached. Total insulin content in the supernatants and in the washing solutions was determined by Coomassie brilliant blue G 250 staining. The results showed about 20% insulin was lost during eight adsorption cycles, of which the encapsulation solution in the LbL adsorption contained about 8% and the washing solution had approximately 12%. The insulin loss is closely related to a slight dissolution of insulin in both solutions. One reason for the insulin loss should be that some insulin molecules are not included in aggregates before adsorption of the first polyelectrolyte layer, leading not to be captured by polyelectrolyte multilayers. Another reason might be that a part of aggregates are dissolved due to their reversibility, followed by the leakage of insulin during the first washing steps. The above two reason can be proved by the experimental results that insulin was lost significantly (about 0.6%) in the first polyelectrolyte adsorption cycle and that the insulin loss decreased in later adsorption cycles up to $N = 5$. Obviously, insulin particles after the preliminary encapsulation are not easily dissolved due to the protection of polyelectrolyte multilayers adsorbed on them. However, any centrifugation step inevitably results in a little insulin loss in the later cycles due to removing some smaller particles. In the experiments, some polyelectrolyte particles with the smaller size were observed clearly in the supernatants of the encapsulation solution and the washing solution after over six cycles.

The alternating adsorption of the oppositely charged polyelectrolytes on insulin aggregates was confirmed by the improved dispersity and microelectrophoresis measurements. Bare insulin aggregates could be dissolved in twice-distilled water at pH 4.0, but the insulin aggregates encapsulated with the polyelectrolyte multilayers might disperse stably in such a solution without dissolution. In Fig. 5, it was demonstrated that the ξ-potential was a function of the number of polyelectrolyte layers coating on insulin aggregates. The ξ-potential alternated between positive and negative values, indicating the successful recharging of the particle with the adsorbed polyelectrolyte multilayer upon each layer deposition (Sukhorukov et al., 1998a). Within the error of the mobility measurements, the ξ-potential approached a constant value as the characteristic for the polyanion and the polycation forming the outermost layer.

3.5. Insulin content and morphology of polyelectrolyte particles

The insulin content in the insulin–polyelectrolyte nanoparticles was determined respectively as 81 ± 4, 69 ± 3, 62 ± 3, and $53 \pm 2\%$ for the 2-, 4-, 6-, and 8-layered insulin–polyelectrolyte nanoparticles. With the increase of adsorption cycles, content of the encapsulated insulin decreased and the adsorbed polyelectrolytes increased accordingly, which corresponded to the results for α-chymotrypsin encapsulated by this method (Balabushevitch et al., 2001; Kittur et al., 2003). Amount of the coated polyelectrolytes ranged from 10 to 20% for each polyelectrolyte pair of PMA and WSC. For the initial polyelectrolyte pair (PMA/WSC), the adsorption amount of the polyelectrolytes on the insulin aggregates was relatively high, more than 15%, which may be attributed to more opportunities to form ion pairs between the polyelectrolytes and bare insulin aggregates. The subsequent adsorption amount for each polyelectrolyte pair was kept approximately at 10%. The prepared nanoparticles were suspended in 0.1 mM HCl and stored at 4 °C. The percentage of insulin leaked from polyelectrolyte particles was less than 5% ($N \geq 6$) or 10% ($N \leq 4$) during 1 month storage of the suspension.

A typical scanning electron microphotograph of the six-layered insulin–polyelectrolyte nanoparticles $(PMA/WSC)_3$ after an ultrasonic treatment is presented in Fig. 6. The insulin–polyelectrolyte nanoparticles were mostly irregular ellipsoids depending on the original shape of the salted out insulin aggregates, and their size was ranged from 100 to 250 nm. The average size of the insulin–polyelectrolyte nanoparticles was about 230 nm while that of the salted out insulin nanoaggregates was approximately 200 nm. It was also observed that the small insulin aggregates or insulin–polyelectrolyte particles could be adhered into large ones. From the measurement on

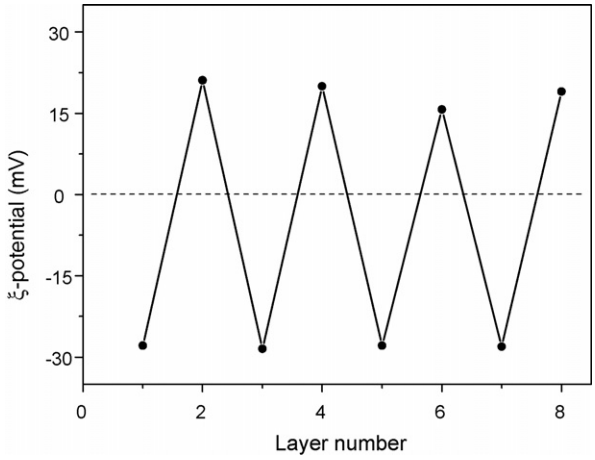

Fig. 5. The ξ-potential change of insulin–polyelectrolyte nanoparticles with polyelectrolyte multilayers, measured at pH 4.0 ± 0.2. Odd layers are PMA and even layers are WSC.

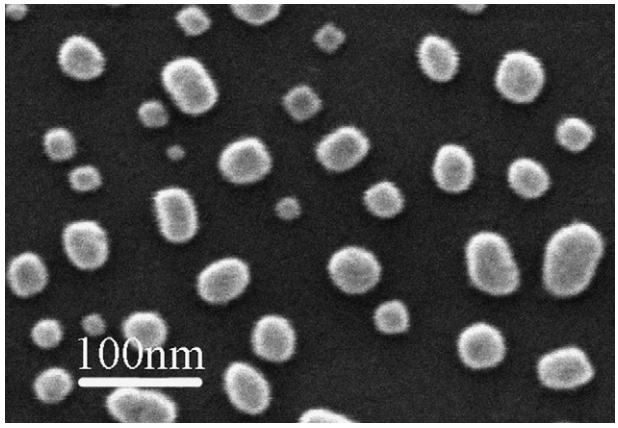

Fig. 6. The SEM photograph of the insulin–polyelectrolyte nanoparticles with six polyelectrolyte adsorption cycles after ultrasonic treating for 2 min at 10 °C. A scale bar represents 100 nm.

a laser particle-size analyzer, an average size of the salted out insulin aggregates without the ultrasonic treatment was 2.4 μm and an average size of eight-layered insulin–polyelectrolyte particles without the ultrasonic treatment increased up to 4.7 μm. Large insulin–polyelectrolyte particles without the ultrasonic treatment existed as microaggregates in the suspension. It may be explained by that weakly charged substrates or polyelectrolyte species facilitated to bridge the flocculation of these particles. Fortunately, the adherence was reversible. Both bare insulin aggregates and the insulin–polyelectrolyte particles were possibly separated into single intact nanoparticles after an ultrasonication at a lower temperature. Such an observation was similar to the experimental results obtained by Balabushevich (Balabushevich and Larionova, 2004).

3.6. Insulin release from the insulin–polyelectrolyte particles

Polyelectrolyte multilayers assembled at certain pH can significantly change their structures and properties when pH has be changed over the p*K* of one polyanion or below that of a polycation used as layer constituents, which may lead to the formation of film pores (Mendelsohn et al., 2000) and dramatically affect the permeability of polyelectrolyte capsules (Sukhorukov et al., 2001). The protein release behaviors from multilayers assembled by the LbL adsorption of polyelectrolytes appeared to be pH-sensitive (Balabushevitch et al., 2001; Balabushevich and Larionova, 2004). The influence of pH on insulin release from the insulin–polyelectrolyte particles was studied and the results were shown in Fig. 7. In the range of pH 2–8, the rate of insulin release from the insulin–polyelectrolyte particles varied obviously with pH. The insulin–polyelectrolyte particles were most stable at pH 4–5 since both PMA and WSC bear enough charges to ensure their complex stability in this situation. When pH was higher than 5, the rate of insulin release increased with the increase of pH up to pH 8. This might be due to the fact that insulin acquired a cumulative negative charge and its complex with the polyanion PMA on the first layer was disrupted at higher than the isoelectric point (p*I* 5.5) (Balabushevich and Larionova, 2004). Another reason should be that the amino groups of WSC became probably uncharged in a basic medium. Accumulation of the negative charges on insulin molecules and polyelectrolyte shell would lead to some distortion in the layers reflecting in the formation of defects in an initially closed structure of polyelectrolyte multilayers, causing the insulin release (Sukhorukov et al., 2001). If pH was lower than 4, the rate of insulin release increased also with the decrease of pH up to pH 2. The main reason for this phenomenon is that negative charges of PMA decreased at a lower pH, which breaks its complex with insulin and simultaneously distorts the PMA/WSC multilayers owing to an accumulation of the positive charges. These results differ from the previous observations where insulin was hardly released from dextran sulfate/protamine multilayers at pH 2–5 (Balabushevich and Larionova, 2004), and where the rate of α-chymotrypsin release from PSS/PAH multilayers increased slightly with the increase of pH at pH 3–5 (Volodkin et al., 2003). The main reason for the difference is the fact that pH affects more dramatically the quantity of electric charge of PMA and WSC since both of them are relatively weak polyelectrolytes.

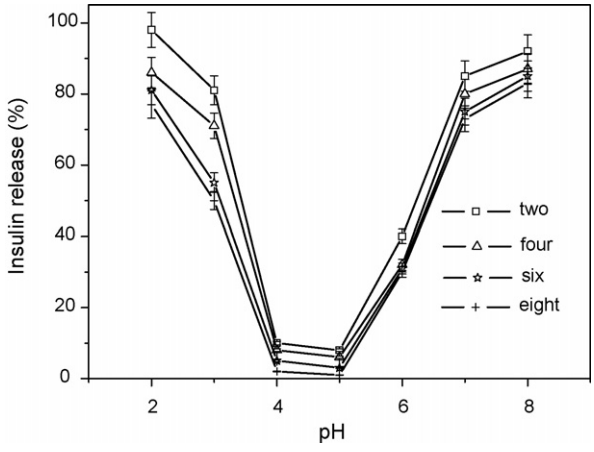

Fig. 7. Influence of pH on insulin release from the insulin–polyelectrolyte particles with different polyelectrolyte adsorption cycles. Incubation time, 1 h.

Besides pH, the rate of insulin release depends on the number of polyelectrolyte layers. At the same pH, the amount of the protein release reduced with the increase of the layer number (Fig. 7). The thickness dependence of permeability can be understood on the basis of film homogenization. As the self-assembled ultrathin multilayers, pores or defects may exist in the multilayers when the adsorbed polyelectrolyte layers are few (Qiu et al., 2001). The permeation through these pores or defects will give rise to the permeability of the multilayers. The pores or defects can be reduced or completely closed with the increase of the deposited layers, resulting in decreasing the multilayer permeability. Furthermore, increase of the layer number would impede the polyelectrolyte multilayers swelling in the dissolution medium as well as the diffusion of insulin, resulting in a low release rate.

3.7. Kinetics of insulin release from the insulin–polyelectrolyte particles

The kinetics of insulin release from the insulin–polyelectrolyte particles in the PBS solution at pH 7.4, corresponding to the human blood and the body fluid, was displayed in Fig. 8. A rapid release of insulin followed by a slowly declining release rate was observed at 8 h. Compared with bare insulin particles, the insulin–polyelectrolyte particles possessed a slow release function of insulin. The initial rate of insulin release (in first 30 min) was reduced gradually as the layer number increased from 2 to 8, indicating that the insulin–polyelectrolyte particles were stabilized with increase of the adsorption cycles. Those particles with defects in the polyelectrolyte multilayers may be responsible for the initial rapid release. The following release rate decreased with the increase of the polyelectrolyte layer number, which might be attributed to an increased steric hindrance.

It is noted worthily that insulin (Mw 5800, p*I* 5.5) release from the polyelectrolyte multilayers assembled by PMA (Mn 4500) and WSC (Mn 8000) is more rapid than α-chymotrypsin

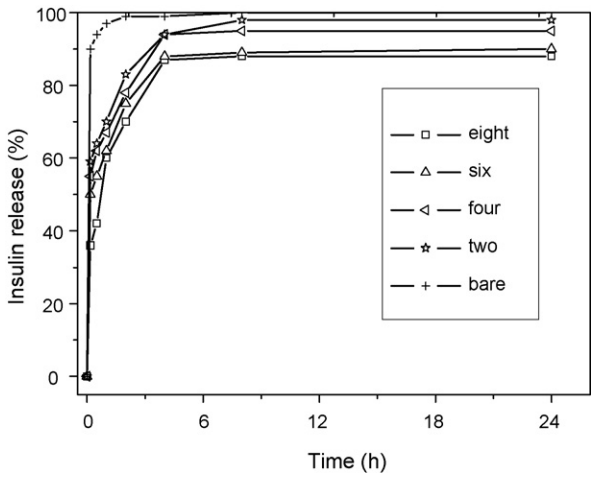

Fig. 8. Kinetics of insulin release from the insulin–polyelectrolyte particles with 2, 4, 6, and 8 adsorption cycles. The particle suspension was incubated in the PBS solution (pH 7.4) under stirring (70 rpm) at 37 °C.

(Mw 24,000, p*I* 8.8) release from the polyelectrolyte multilayers assembled by PSS (Mw 70,000) and PAH (Mw 60,000) (Volodkin et al., 2003). The reasons should be as follows: (1) the smaller molecular weight of encapsulated macromolecules could increase possibility to permeate through the polyelectrolyte multilayers; (2) the high charge density and well-matched charge interactions between the coupling polyions in PSS/PAH multilayers allowed the films to form small or no cavities, resulting in a slower release rate of α-chymotrypsin; (3) insulin had a better solubility than α-chymotrypsin in their respective culture solution because the pH (7.4) of the culture solution was far from the isoelectric point of insulin (p*I* 5.5) but its pH (8.0) was close to that of α-chymotrypsin (p*I* 8.8); (4) insulin bear the negative charges in the culture solution at pH 7.4 disrupting its complex with polyelectrolytes of the first layer, but so does not α-chymotrypsin; (5) the PMA/WSC multilayers may be more easily distorted than PSS/PAH multilayers since both PMA and WSC were the weaker polyelectrolytes and more pH-sensitive than PAH and PSS.

The form of insulin released from the insulin–polyelectrolyte particles in the PBS solution at pH 7.4 was investigated by gel chromatography. As shown in Fig. 9, Similar to the chromatographs of the original insulin and the re-dissolved insulin of the salted out insulin aggregates, only an insulin peak appeared in the sample released from the insulin–polyelectrolyte particles, suggesting that insulin released from PMA/WSC multilayers in the PBS solution at pH 7.4 was free molecule and unbound with the polyelectrolytes (PMA or WSC). It differs from α-chymotrypsin released from PSS/PAH multilayers as a soluble complex with PSS (Volodkin et al., 2003). It should be another reason for the more rapid release rate of insulin.

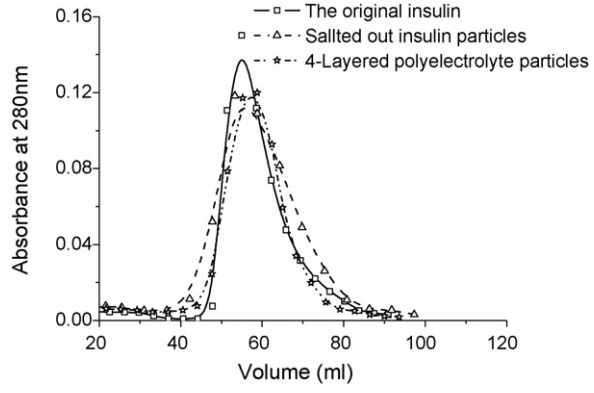

Fig. 9. Gel chromatographs on Sephadex G-50 (pH 7.4) of the original insulin, re-dissolved insulin of the salted out insulin aggregates, and insulin released from the insulin–polyelectrolyte particles with four polyelectrolyte layers during 1 h incubation at pH 7.4.

4. Conclusion

Insulin nano-aggregates with a particle size of 100–230 nm in a good dispersity were obtained with a high yield by salting out from 0.6 M NaCl solution of insulin at pH 1.1 after the ultrasonication at 10 °C for 2 min. With increasing the NaCl concentration of insulin up to 1.4 M, yield of insulin aggregates increased but their agglomeration took place. No conformational change was observed for insulin salted out at pH 1.1 at 15 °C. In addition, the salted out insulin aggregates were mostly amorphous.

Insulin aggregates were encapsulated by the LbL adsorption of PMA and WSC with about 20% insulin loss after eight adsorption cycles of the polyelectrolytes. The insulin–polyelectrolyte particles with were stable under storage conditions as a diluted suspension at pH 4.0 or a lyophilized preparation. The insulin–polyelectrolyte nanoparticles with the particle sizes of 100–250 nm were obtained after an ultrasonication at a low temperature for several minutes.

Insulin release from the PMA/WSC multilayers was pH-dependent. Little insulin was released from the particles at pH 4–5. However, the rate of insulin release increased significantly at the pH values lower than three or higher than six due to a great influence of pH on the electric charges of PMA and WSC as well as insulin molecules. A slow release of insulin from the polyelectrolyte multilayers in the PBS solution at pH 7.4 was observed after a rapid initial release as the burst effect. The rate of insulin release from polyelectrolyte particles was decreased with the number of polyelectrolyte adsorption cycles. Insulin released from the insulin–polyelectrolyte nanoparticles should be free molecule unbound with the polyelectrolytes.

In summary, the insulin–loaded nanoparticles were successfully prepared by the LbL adsorption of the biodegradable polyelectrolytes. In comparison with the other nanoencapsulation techniques, although the encapsulation of polypeptide and protein drugs by the LbL technique is time-consuming due to repeatedly adsorption and centrifugation, it is able to avoid harsh preparative procedures and the use of organic solvent or high temperature so as to minimize the potential loss of protein activity. Also, the release behaviors can be controlled since the shell thickness and its permeability, was readily adjusted on the nanometer scale by polyelectrolyte adsorption cycles. Furthermore, this method can offer high drug loading capacity and high encapsulation efficiency. The insulin-loaded nanoparticles appear to be especially promising for the parenteral administrations of insulin in diabetic patients.

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