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Insulin particles as building blocks for controlled insulin release multilayer nano-films

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ABSTRACT

Insulin nanoparticles (NPs) were prepared by pH-shift precipitation and a newly developed disassembly method at room temperature. Then, an electrostatic interaction-based, layer-by-layer (LbL) multilayer film incorporating insulin NPs was fabricated with poly(acrylic acid) (PAA) and poly(allylamine hydrochloride) (PAH), which is described herein as Si/$(PAH/PAA)_5(PAH/PAA\text{-insulin NPs})_n$. The positively charged insulin NPs were introduced into the LbL film in the form of biocompatible PAA-insulin NP aggregates at a pH of 4.5 and were released in phosphate-buffered saline (pH 7.4), triggered by changes in the charges of the insulin molecules. In addition, the insulin-incorporated multilayer was swollen because of the different ionic environment, leading also to insulin release. Eighty percent of the insulin was released from the LBL film in the first stage of 3 h, and sustained release could be maintained in the second stage for up to 7 days *in vitro*, which is very critical for specific diabetic patients. These striking findings could offer novel directions to researchers in establishing insulin delivery systems for diabetic therapy and fabricating other protein nanoparticles applied to various biomedical platforms.

1. Introduction

Diabetes therapy incorporating various nanomaterials and approaches to effectively control glycemic stability has received increased attention over the past decades [1–6]. In implementing new methods, different forms of insulin such as supramolecular insulin assemblies (SIA) [7], insulin analogs [8], insulin nanoparticles (NPs) [9], nanonetworks [10], and nanocomplexes [11] have been prepared and widely employed for insulin delivery, in particular *via* traditional subcutaneous injection. For instance, nano-sized insulin particles could serve as appropriate drug depots at the injection site for long-term release, according to related research by Gupta et al. [7]. A great deal of effort has also been made to establish various insulin delivery systems such as oral administration [12], intranasal therapy [13], gastrointestinal route [14], pulmonary delivery [15], and tablet implantation [16]. However, a number of challenging issues involved in the process of diabetes treatment, including high cost, low compatibility *in vivo*, common infections, patient compliance, and sudden hypoglycemia, have not yet been overcome in a flawless manner.

The layer-by-layer (LbL) technique for multilayer assembly provides a superb route to build up desired films using a range of functional materials, including polymer polyelectrolytes, DNA, proteins, graphene, nanocomplexes, nanoparticles, nanowires, and nanotubes [17–23]. Particular advantages of the LbL method, such as the precise control of film thickness, specific functionality, optional compositions, and versatile morphology, have been investigated and demonstrated in the field of multilayered structures ranging from the nano- to microscale [24–26]. Thus, inorganic NPs, also applicable for nearly any type of charged components, were introduced into desired functional multilayers by virtue of electrostatic interactions [22]. Similarly, LbL multilayered films with building blocks of different drugs can be obtained by taking advantage of the molecular interactions among materials, in particular electrostatic interactions or hydrogen bonding [27,28].

Specifically, researchers have been attracted to the design of insulin delivery systems based on LbL thin films. For example, Chen et al. successfully fabricated a glucose-sensitive multilayer film based on a 21-armstar polymer, showing an on–off switch of insulin release in response to *in vivo* glucose levels [29]. Their group then further developed LbL films constructed from supramolecular insulin assemblies, which were useful for super long-term glycemic control for up to 295 days [30]. Unlike the glucose-sensitive system, insulin release triggered by variations in pH was also observed by Yoshida et al. after exposing a template containing insulin to weakly acidic or neutral solutions [31]. After transcutaneous protein drug delivery was created, we proposed a method for creating insulin-encapsulated nanofilms by LbL, which could be regarded as a nano-container for controlled insulin release [32].

Herein, we aimed to rapidly prepare insulin NPs through pH-shift precipitation and crystal disassembly, which is a novel technique, compared with conventional growth means that can be time-consuming, limited in high temperature or carried out under denaturation condition. Following preparation, insulin NPs in the form of PAA-insulin NP aggregation were assembled into pH-sensitive

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$(PAH/PAA)_n$ multilayered films *via* the LbL method for sustained insulin release by means of transcutaneous administration [33]. In the first place, considering the toxicity of building blocks of PAH, biocompatible PAA was used as an outside coating on insulin NPs while low molecular weight PAH was assembled inside multilayers, which showed extremely low toxicity [34,35]. In our strategy, in order to improve poor compliance in subcutaneous injections, LbL films were packaged in the form of patches, which could also avoid direct contact between insulin and tissues and prevent hypoglycemia. Positively charged insulin microcrystals were prepared in acetic acid at pH 6.5 because of the isoelectric point (pI) of insulin. Negatively charged PAA-insulin NPs were formed in PAA solution at a pH of 4.5 and were then used as building blocks to construct LbL films by alternating deposition with PAH, which is convenient for multilayer assembly. We demonstrate that the resulting films can be partially deconstructed to release insulin into the surrounding medium in pH 7.4, phosphate-buffered saline (PBS) solution, which is largely due to the release of insulin NPs. It is also observed that insulin release from the prepared films in PBS solution could last for up to 7 days, which is very critical for specific diabetic patients.

2. Materials and methods

2.1. Materials

Insulin (from bovine pancreas, $M_w \approx 5{,}733$), poly(acrylic acid) (PAA, $M_w \approx 2{,}000$), poly(allylamine hydrochloride) (PAH, $M_w \approx 15{,}000$), 10 mM phosphate buffered saline (PBS, pH 7.4), and fluorescein isothiocyanate isomer I (FITC, 90%) were purchased from Sigma-Aldrich. All other chemicals and solvents were of analytical grade.

2.2. Preparation and characterization of PAA-insulin NPs

Insulin NPs at 1 mg/mL were prepared according to the modified seed zone method [36]. Briefly, 1 mg/mL bovine insulin was fully dissolved in 0.01 N acetic acid (pH 2.0) under stirring. To the clear solution, 10 N and 1 N NaOH were added in a stepwise manner to increase the pH. Once the pH has reached 10.5 ± 0.5, the aqueous insulin suspension became clear. Subsequently, the pH of the solution was slowly regulated to pH 6.8 by adding 1 N HCl, and the solution became milky. After stirring the solution for 15 min to completely form microcrystals, centrifugation was performed for 10 min at 13,000 rpm. Thereafter, insulin precipitation was obtained, and a 1 mg/mL PAA polyelectrolyte solution at pH 4.5 was added to acquire an insulin suspension. After 15 min of stirring, centrifugation was repeated at 13,000 rpm for 10 min. The supernatant was then separated from the insulin precipitation. Finally, nano-sized insulin NPs in the supernatant were stored at 4 °C in darkness. The morphologies of the insulin NPs and PAA-insulin NPs deposited onto $Si/(PAH/PAA)_5$ and $Si/(PAH/PAA)_5PAH$ substrates were observed by field-emission scanning electron microscopy (FE-SEM, SIGMA) and transmission electron microscopy (TEM, FEI Tecnai G2), respectively.

2.3. Fabrication and characterization of insulin-incorporated LbL films

LbL multilayer films were fabricated on a silicon wafer by hand-dipping the substrate into various aqueous solutions at room temperature. The silicon substrate was pre-treated for 2 min with oxygen plasma to create a negatively charged surface. For the first step in the LbL process, the substrate was dipped into a positively charged 1 mg/mL PAH aqueous solution (pH 7.5) for 10 min. Then, the substrate was rinsed three times in deionized water for 2 min, 1 min, and 1 min. Next, the substrate was immersed in a negatively charged 1 mg/mL PAA aqueous solution (pH 3.5) for 10 min. Similar to the previous washing step, the substrate was rinsed three times in deionized water. $Si/(PAH/PAA)_5$ was obtained as a basic layer after five alternating deposition cycles. Afterwards, the substrate with the basic layer was dipped into a 1 mg/mL PAH aqueous solution (pH 4.5) and washed three times by 0.01 N acetic acid (pH 4.5) for 2 min, 1 min, and 1 min. Subsequently, the substrate was placed into the as-prepared PAA-insulin NP suspension solution for 10 min. Identical rinsing steps were carried out to remove extra PAA-insulin NPs. This dipping process was repeated until the desired number of bilayers was acquired. The morphologies of the $Si/(PAH/PAA)_5(PAH/PAA\text{-insulin NPs})_n$ ($n = 0$, 1, 4, 12, and 20) multilayers were examined *via* FE-SEM. Film thickness growth was monitored by a profilometer (Dektak 150, Veeco) at three different positions on the film surface. Furthermore, the morphologies of $Si/(PAH/PAA)_5(PAH/PAA\text{-insulin NPs})_n$ ($n = 12$ and 20) multilayers were examined using atomic force microscopy (AFM, Park Systems X-10) with a scan area of $2 \times 2\ \mu m^2$.

2.4. Film decomposition triggered by pH

Film decomposition was characterized through profilometry measurement for a $Si/(PAH/PAA)_5(PAH/PAA\text{-insulin NPs})_{20}$ film. Specifically, the thickness variation of the pristine 2145.3 ± 17.2 nm film, dried by N_2 flow, was monitored by profilometry after exposure to a 10 mM PBS solution (37 °C, pH 7.4, 5 mL in a 20-mL vial) for a predetermined period of time.

2.5. Insulin release from LbL film

According to the fabrication of insulin-incorporated films, 0.9 mg/mL bovine insulin and 0.1 mg/mL FITC were completely dissolved in 10 mL of 0.01 N acetic acid (pH 2.0) while stirring in darkness. The pristine 2145.3 ± 17.2 nm $Si/(PAH/PAA)_5(PAH/PAA\text{-insulin NPs-FITC})_{20}$ multilayer films ($1.5 \times 1.5\ cm^2$) were then fabricated by following the assembly process above. After immersing films into 10 mM PBS (pH 7.4), the release of insulin-FITC from multilayers was

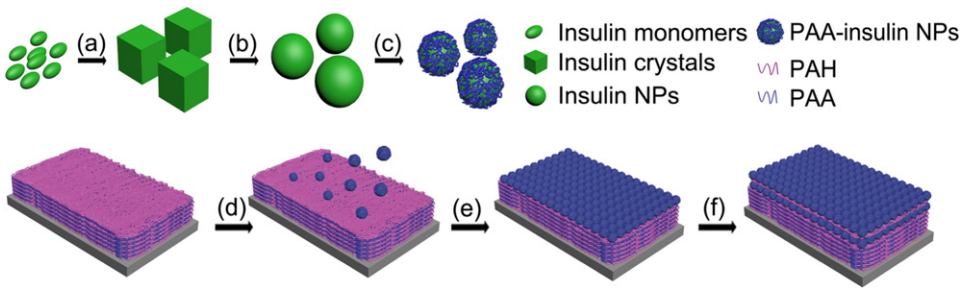

Fig. 1. Schematic presentation of the film assembly of $Si/(PAH/PAA)_5/(PAH/PAA\text{-insulin NPs})_n$ based on layer-by-layer deposition. (a) Formation of insulin crystals by the seed zone method. (b) Fabrication of insulin nanoparticles. (c) Coating insulin NPs with polyelectrolytes of PAA. (d) Dipping $Si/(PAH/PAA)_5/PAH$ substrate in as-prepared PAA-insulin NP solution. (e) Insulin NP deposition process. (f) Layer-by-layer assembly of $Si/(PAH/PAA)_5/(PAH/PAA\text{-insulin NPs})_n$ with repeating PAH and PAA-insulin NP deposition.