Polym. Bull. (2012) 69:229–239
DOI 10.1007/s00289-012-0745-7

ORIGINAL PAPER

Use of anionic polysaccharides for the preparation of insulin-containing layer-by-layer films and their pH stability

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Received: 6 September 2011 / Revised: 24 January 2012 / Accepted: 24 March 2012 /
Published online: 30 March 2012

Abstract Insulin-containing layer-by-layer (LbL) thin films were prepared by an alternate deposition of insulin and anionic polysaccharides (heparin, κ-carrageenan, and fucoidan) through an electrostatic force of attraction between positively charged insulin and anionic polysaccharides at pH 3.0. The loading of insulin in the LbL films increased with the increasing number of layers (or the film thickness), depending on the polysaccharide type. LbL films composed of κ-carrageenan contained higher amount of insulin than in heparin- and fucoidan-based films. The LbL films were fairly stable in acidic media, while insulin was released from the films in weakly acidic and neutral solutions as a result of loss of net positive charge in insulin. The released insulin retained its original structure.

Keywords Heparin · Fucoidan · κ-Carrageenan · Insulin · Controlled release · Layer-by-layer film

Introduction

Layer-by-layer (LbL) films can be prepared by an alternate and repeated deposition of oppositely charged polymeric materials on a solid surface through electrostatic and other affinities [1–3]. LbL films have been studied for the development of sensors [4–7], separation membranes [8], molecular recognition [9, 10], and stimuli-sensitive

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systems [11–17]. The materials used for the film construction include polysaccharides as well as synthetic polymers [18–20].

Recently, much attention has been devoted to the development of insulin LbL films for the development of insulin formulations that can be orally administrated [21–26]. It is very clear that oral route for drug delivery is the most convenience and desired as an invasive method of drug delivery compared to injection. For this purpose, insulin formulations have to be stable at acidic environment in the stomach and insulin must be released at the neutral pH in the intestine. In this context, we have recently reported LbL films composed of insulin and poly(anion)s such as poly(vinyl sulfate) (PVS) and poly(acrylic acid) (PAA) [27]. These LbL films were stable at acidic pH while decomposed to release insulin at pH 7.4. For future application of insulin-containing LbL films to insulin delivery, biocompatible polymers should be utilized as material. In this study, therefore, three kinds of polysaccharides have been employed for constructing insulin-containing LbL films. In fact, heparin, fucoidan, and κ-carrageenan were successfully used for preparing insulin films. This paper reports on the preparation of polysaccharide-based LbL films containing insulin and their pH stability.

Experimental section

Materials and apparatus

Insulin (human, recombinant) was purchased from Wako Pure Chemical Ind., Osaka, Japan. Poly(ethyleneimine) (PEI, MW: 60,000–80,000) was obtained from Nakalai Tesque Co. (Kyoto, Japan). Heparin sodium (Wako Pure Chemical Ind.), κ-carrageenan (LKT Laboratories, Inc., Minnesota, USA), and fucoidan (Sigma-Aldrich) are commercial products and used without further purification. The chemical structures of the polysaccharides are shown in Fig. 1. The preparation of LbL films was studied using a quartz crystal microbalance (QCM; 440E QCM, BAS Co., Tokyo, Japan). An 8 MHz AT-cut quartz resonator coated with a thin gold layer (geometric surface area, 0.20 cm^2) was used as a probe, in which the adsorption of 1 ng of substrate induces a ca. -0.75 Hz change in the resonance frequency. For optical evaluation of the films, a UV–Visible absorption spectrometer (UV-3100PC, Shimadzu Co., Kyoto, Japan) was used. An atomic force microscope (AFM; SPM-9600, Shimadzu Co., Kyoto, Japan) was used to estimate the thickness of LbL films. A circular dichroism (CD) spectrometer (J720, Jasco Co., Tokyo, Japan) was used to evaluate the structural integrity of insulin released.

Turbidity of insulin-polycation mixed solutions

The optical density of aqueous mixtures of insulin (0.2 mg/mL) and polysaccharide (0.1 mg/mL) was recorded at 600 nm using a 10 mm pathlength quartz cuvette at pH 2.0–8.0. The pH of the solutions was adjusted with HCl and NaOH.

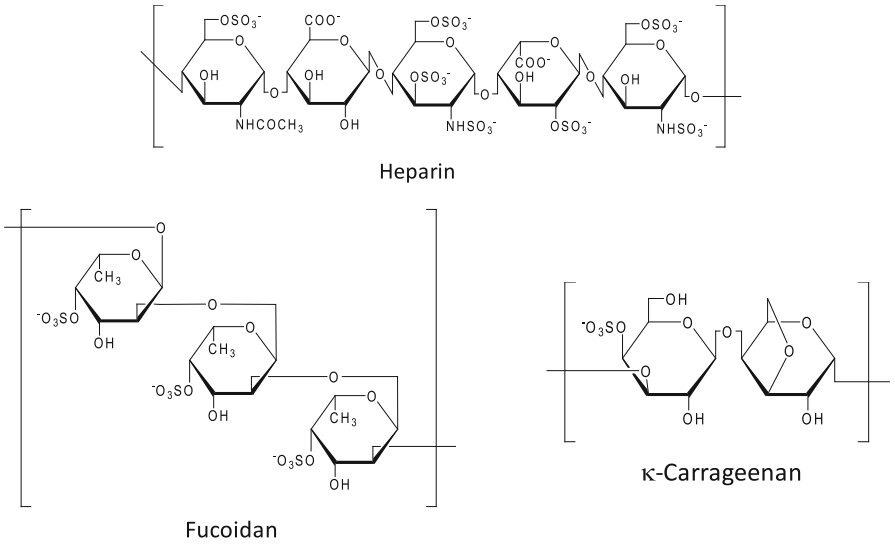

Fig. 1 The chemical structures of polysaccharides used

Preparation of insulin-containing LbL films

The quartz resonator for QCM analysis was mounted in a flow cell and the surface was exposed to 0.5 mg/mL PEI solution in water for 15 min to deposit a first PEI layer. The PEI-modified quartz resonator was exposed to 0.5 mg/mL polysaccharide in 10 mM acetate buffer containing 150 mM NaCl (pH 3.0) for 15 min to deposit the polysaccharide and rinsed with the working buffer for 5 min. The polysaccharide-deposited quartz resonator was then exposed to 0.5 mg/mL insulin solution in the acetate buffer at pH 3.0 for 15 min and rinsed with the buffer for 5 min. The deposition of polysaccharide and insulin was repeated to prepare LbL films. For the evaluation of insulin loading in LbL films from UV-absorption spectrum, the LbL films were prepared on a quartz slide ($10 \times 50 \times 1$ mm^3). However, the insulin-containing LbL films were slightly turbid and not suitable for the quantitative analysis of insulin loading in the films from UV-absorbance of the films. Therefore, the LbL films which had been prepared on the quartz slide were dissolved in 10 mM HEPES buffer at pH 7.4 and the insulin loading was determined by measuring the absorbance of the solution.

pH-sensitive decomposition of insulin-containing films

The surface of the LbL film-coated quartz resonator was exposed to a 10 mM HEPES buffer containing 150 mM NaCl at pH 7.4. For the optical determination of pH stability of the LbL films, the LbL film-coated quartz slide was immersed in the solutions with different pH for 30 min and the absorbance of the solution was recorded at 277 nm to calculate the amount of insulin released. All experiments were carried out at room temperature (ca. 20 °C).

CD spectra

The insulin-containing LbL films were deposited on the surface of quartz slide and then the slide was immersed in a 10 mM HEPES buffer containing 150 mM NaCl at pH 7.4 to release the insulin. The CD spectra of the released insulin were recorded.

Results and discussion

Complexation of insulin and polysaccharide in solutions

The complexation of insulin and polysaccharide in solution was studied by measuring turbidity of insulin–polysaccharide mixtures. The mixed solution of polysaccharide and insulin would become turbid if the intermolecular complexes or aggregates form in the solution. Therefore, the turbidity study affords useful information on the possible use of the polysaccharides to construct LbL films of insulin. Figure 2 shows the optical density of the mixed solutions at 600 nm. The mixed solutions became turbid at pH 5.0 or lower, while remained essentially transparent at neutral pH. Insulin solution without polysaccharide as well as insulin-free polysaccharide solutions remained fully soluble over the pH range tested under the experimental conditions. It is likely that insoluble complexes formed between insulin and the polysaccharides at pH 5.0 and lower. It is probable that positively charged insulin and the anionic polysaccharides formed complexes through electrostatic interactions at pH 5.0 and lower in view of the fact that isoelectric point of insulin is reported to be 5.4 [28]. In contrast, no complex formed in neutral solutions due to the net negative charge on insulin. A higher turbidity observed for the insulin–heparin mixed solution may originate from higher charge density of heparin than those of κ-carrageenan and fucoidan (see Fig. 1). These results suggest that LbL films can be constructed through electrostatic affinity between insulin and polysaccharides in acidic solutions.

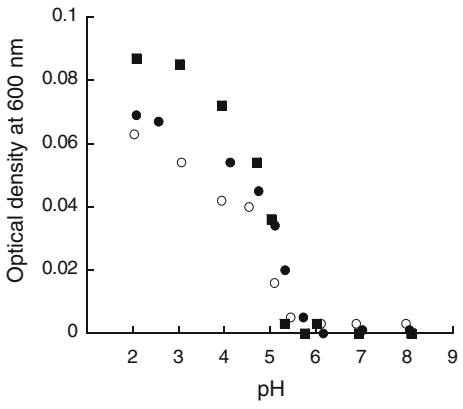

Fig. 2 Turbidity of heparin–insulin (*filled square*), κ-carrageenan–insulin (*filled circle*), and fucoidan–insulin (*open circle*) mixed solutions as a function of pH

Preparation of polysaccharide–insulin films

A typical frequency change in QCM for the alternate deposition of insulin and heparin (A), fucoidan (B), or κ-carrageenan (C) at pH 3.0 and decomposition of the resulting LbL film at pH 7.4 was shown in Fig. 3. The resonance frequency (F) was

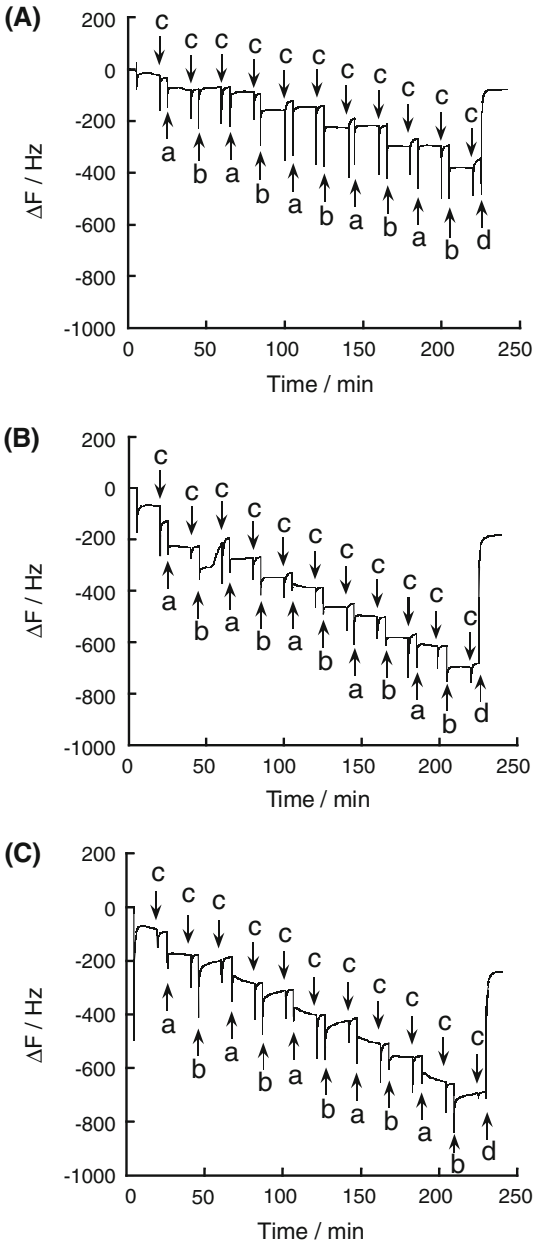

Fig. 3 Typical QCM response for the deposition of insulin and heparin (**A**), fucoidan (**B**), or κ-carrageenan (**C**) at pH 3.0, and the decomposition of the film at pH 7.4. The quartz resonator was exposed to polysaccharide (*a*) and insulin (*b*) solutions. After each deposition, the quartz resonator was rinsed with pH 3.0 buffer (*c*). The LbL film was exposed to pH 7.4 medium (*d*)

decreased when the quartz resonator was exposed to the polysaccharide and insulin solutions at pH 3.0, suggesting a successful deposition of polysaccharide and insulin on the surface of the quartz resonator. Thus, five-layered (polysaccharide–insulin)$_5$ LbL film was constructed through an electrostatic force of attraction. This is reasonable because the isoelectric point of insulin is 5.4 and sulfonate residues in the poloysaccharides are negatively charged at pH 3.0. In contrast, the resonance frequency shifted quickly back to the opposite direction upon exposing the resulting LbL film to a HEPES buffer at pH 7.4, which suggests the LbL film was removed from the surface of the resonator. It is probable that the LbL film was decomposed at pH 7.4 as a result of a loss of the electrostatic force of attraction between insulin and polysaccharide in the film. These results are in accord with the complexation behavior of insulin and the polysaccharides (Fig. 2). Thus, insulin-containing LbL films can be prepared using the anionic polysaccharides.

The film thickness was estimated to be 10 ± 4, 11 ± 2, and 23 ± 2 nm for the (heparin–insulin)$_5$, (fucoidan–insulin)$_5$, and (κ-carrageenan–insulin)$_5$ films in dry state, respectively, based on AFM 3D image of the edge of the LbL films.

The loading of insulin in the films as a function of the number of layers is shown in Fig. 4. The insulin loading was evaluated for insulin- and polysaccharide-terminated films with 5, 10, and 15 insulin layers. The insulin loading in the film increased with increasing the number of layers (or thickness of the films), as expected. The κ-carrageenan-based films exhibited higher loading of insulin than the heparin- and fucoidan-based films. This is probably due to the fact that the content of sulfonate residues along the polysaccharide chain in κ-carrageenan is lower than that in heparin and fucoidan. κ-Carrageenan contains a sulfonate residue in every two saccharide moieties while every saccharide residue in fucoidan is substituted with a sulfonate group. As for heparin, the density of sulfonate groups along the polymer chain is much higher than those in κ-carrageenan and fucoidan. It is envisaged that flexibility in the polysaccharide chains of heparin and fucoidan is rather limited and form a stretched conformation because of the electrostatic repulsion among the sulfonate groups in the polysaccaharide chain. On the other hand, κ-carrageenan may form a coiled conformation to some extent, resulting in higher surface area on the film surface which can accommodate higher amounts of insulin. The insulin loadings in the polysaccharide LbL films were slightly lower than those in previously reported PVS- and PAA-based films [27]. For example, the 10-layer (heparin–insulin)$_{10}$, (fucoidan–insulin)$_{10}$, and (κ-carrageenan–insulin)$_{10}$ films contain 4.7, 7.4, and 15 μg cm^{-2} of insulin, respectively, compared to 14 μg cm^{-2} in (PVS–insulin)$_{10}$ film and 36 μg cm^{-2} in (PAA–insulin)$_{10}$ film. A flexible vinyl backbone of PVS and PAA may be able to form flexible conformations on the film surface to accommodate higher amounts of insulin. Above results explicitly show that the loading of insulin in the film is a function of the number of layers in the film. In other words, insulin loading can be appropriately tuned depending on the purpose of insulin LbL films, which is a clear merit of LbL films.

The effect of the outermost layer on the insulin loading was not significant. For both the insulin- and polysaccharide-terminated films, the loading increased with the increasing number of layers. These results suggest that the loading of insulin can

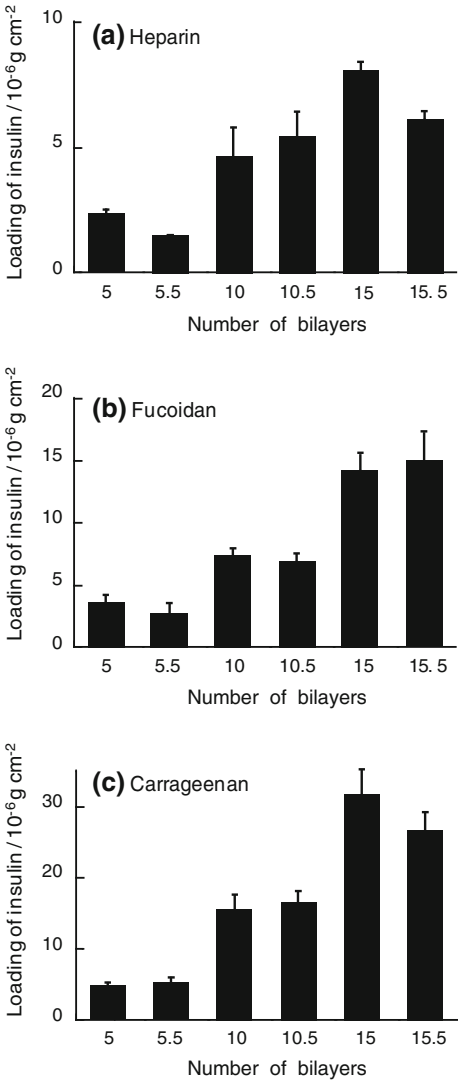

Fig. 4 The loading of insulin in the $(\text{polysaccharide–insulin})_n$ and $(\text{polysaccharide–insulin})_n$ polysaccharide films. The bilayer numbers 5.5, 10.5, and 15.5 show the LbL films whose outermost surface was covered with polysaccharide. The average values of three preparations are shown

be regulated by tuning the number of layers and by a suitable choice of polymer type.

pH-induced decomposition of insulin LbL films

As reported in Fig. 3, the insulin LbL films were decomposed at pH 7.4. We have evaluated pH stability of the insulin-containing LbL films as a function of pH. Figure 5 shows that the LbL films were stable in the media of pH 5.0 or lower, while the films were decomposed at weakly acidic and neutral pH. The pH threshold for

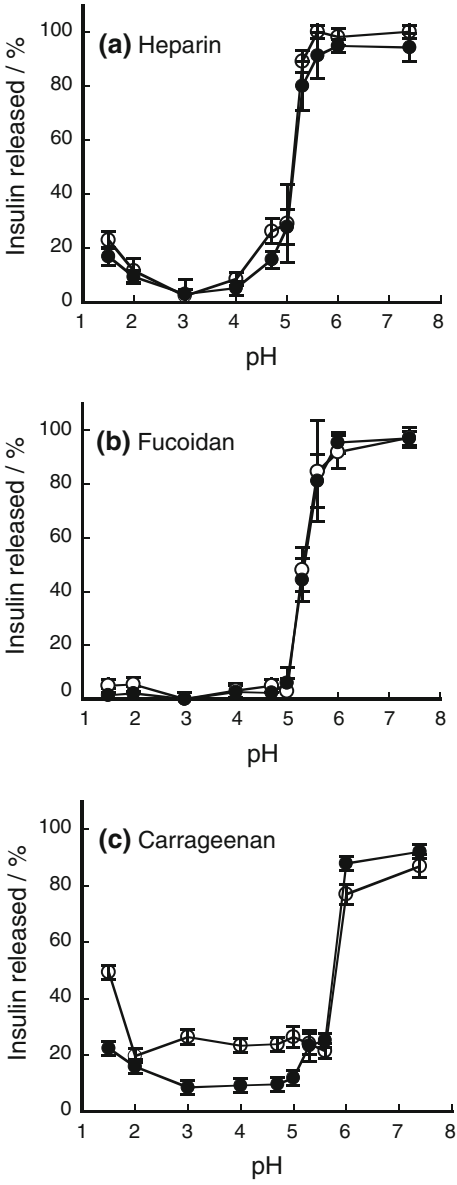

Fig. 5 pH-dependent release of insulin from (polysaccharide–insulin)$_{10}$ (*open circle*) and (polysaccharide–insulin)$_{10}$ polysaccharide (*filled circle*) films at 20 °C. The percent of insulin released after 30-min immersion of the films in the solutions was recorded. The average values for three measurements are plotted

the film decomposition was found at pH 5.0–6.0 for all the films tested, which qualitatively corresponds to isoelectric point of insulin, 5.4. These results support the view that the pH-dependent decomposition of the films is caused by the loss of electrostatic affinity between insulin and polysaccharide in the film, which in turn originates from a shift of the net electrical charge of insulin from positive to negative at pH 5.0–6.0. It is noted that the films were decomposed rapidly upon exposure to weakly acidic or neutral solutions within a few minutes (see Fig. 3).

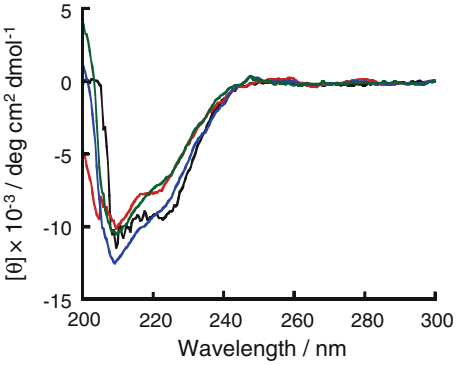

Fig. 6 CD spectra of the insulin released from (heparin–insulin)$_{15}$ (*blue*), (fucoidan–insulin)$_{10}$ (*green*), and (κ-carrageenan–insulin)$_5$ (*red*) films. The insulin was released into a 10 mM HEPES buffer containing 150 mM NaCl at pH 7.4. The spectrum of native insulin recorded under the same conditions was also shown (*black*). (Color figure online)

The rapid response is a merit of LbL films compared with the slower response in the insulin release from hydrogels and microcapsules [21, 29]. We have previously reported that pH stability of insulin-containing LbL films under physiological temperature (37 °C) is nearly comparable to that at 20 °C [27, 30, 31].

pH stability of the films in acidic media was slightly dependent on the type of polysaccharide used. Heparin- and fucoidan-based LbL films are stable at pH 5.0 and lower, while κ-carrageenan-based films were decomposed to some extent even in the acidic media. The instability of (κ-carrageenan–insulin)$_{10}$ film was more significant than that of (κ-carrageenan–insulin)$_{10}\kappa$-carrageenan film. It is most likely that insulin molecules adsorbed on the outermost surface are easily removed. A lower density of negative charge in κ-carrageenan may be responsible for the instability of the (κ-carrageenan–insulin)$_{10}$ film. In contrast, for heparin- and fucoidan-based LbL films, the effect of outermost layer on the pH stability was rather small.

CD spectra of insulin released from LbL films

The structural integrity of insulin released from the LbL films was evaluated using CD spectra. Figure 6 shows the CD spectra of native and released insulin. The CD spectra of the released insulin exhibited similar spectral features to that of native insulin, suggesting that the original conformation is almost preserved in the released insulin.

Conclusion

LbL thin films composed of insulin and polysaccharides are successfully prepared using heparin, fucoidan, and κ-carrageenan. The loading of insulin in the film depended on the type of polysaccharide used and can be controlled by tuning the number of layers in the films. The insulin–polysaccharide films are stable at acidic pH, but insulin can be released in neutral solutions as a result of loss of electrostatic affinity between the polysaccharide and insulin. CD spectra showed that the original

structure is retained in the released insulin. The insulin-containing LbL films may be useful for future development of insulin formulations because of biocompatibility of polysaccharides.

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