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Reviews

Layer-by-layer Thin Films and Microcapsules for Biosensors and Controlled Release

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We present an overview of the preparation and properties of layer-by-layer (LbL) deposited thin films and microcapsules in relation to their use in the development of biosensors and controlled release systems. Enzyme biosensors can be constructed by immobilizing enzymes on the surface of electrodes by LbL deposition without loss of their catalytic activity. In addition to synthetic polymers, binding proteins, such as avidin and lectin, are also used for constructing LbL films through avidin-biotin and lectin-sugar interactions. The performance characteristics of LbL film-based biosensors can be tuned by controlling the number of layers and by the choice of film components. The permeability of polyelectrolyte LbL films to ions and molecules is discussed in relation to the use of the films for eliminating interference in biosensors. The possible use of polysaccharide LbL film-coated electrodes for the construction of biosensors is highlighted, and examples of LbL film- and microcapsule-based optical sensors are described. We then focus on the use of LbL films and microcapsules as vehicles for controlled release, in particular on recent progress in the controlled release of insulin from LbL films and microcapsules. LbL films composed of insulin and polymers are sensitive to environmental pH, and release insulin in response to pH changes, suggesting that insulin LbL films can be used in the development of orally administered insulin. The construction of glucose-dependent insulin release systems using LbL insulin microcapsules functionalized with phenylboronic acid, lectin, and glucose oxidase is also examined.

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1 Introduction

Layer-by-layer (LbL) thin films were first developed by Decher and coworkers in the early 1990s.[1-3] They established a protocol for the preparation of thin films based on the alternating adsorption of polymers with opposite charges on the surface of a solid substance through electrostatic attraction. Since then, LbL films have attracted much attention because of their facile preparation and potential use in the development of functional

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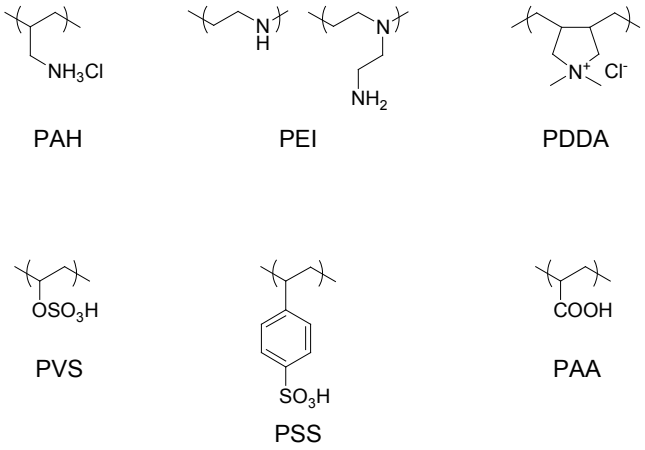

Fig. 1 Chemical structures of cationic and anionic polymers frequently used for constructing LbL films: poly(allylamine hydrochloride) (PAH), poly(ethyleneimine) (PEI), poly(diallyldimethylammonium chloride) (PDDA), poly(vinylsulfonic acid) (PVS), poly(styrenesulfonic acid) (PSS), and poly(acrylic acid) (PAA).

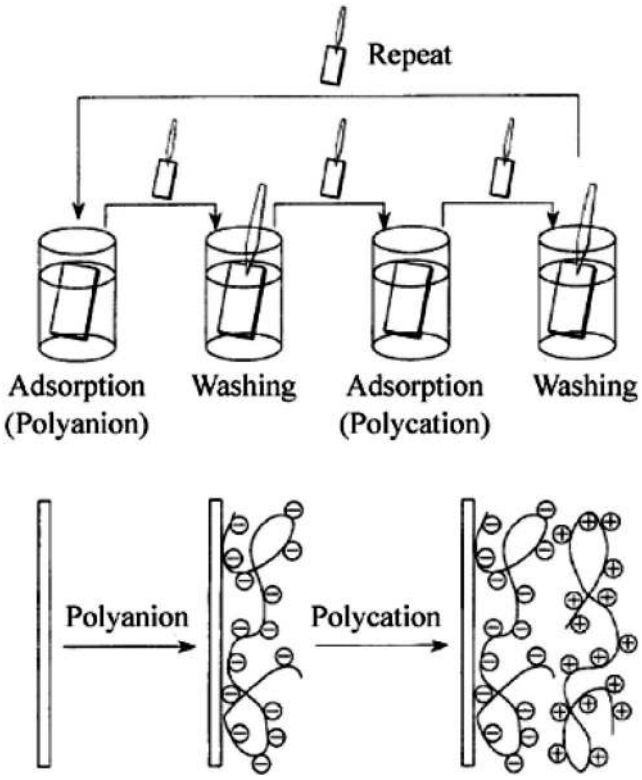

Fig. 2 (Top) Procedure for the LbL deposition of a thin film. (Bottom) A structure of an LbL-deposited polyelectrolyte film on a solid surface. Copyright (2001) The Japan Society for Analytical Chemistry.

devices, including biosensors. Möhwald and coworkers reported the preparation of hollow microcapsules by the LbL deposition of synthetic polyelectrolytes on the surface of microparticles, followed by dissolution of the core particles.[4] They then proposed the possible use of LbL microcapsules for the encapsulation and controlled release of drugs. LbL deposition is a bottom-up nanofabrication technique that relies on the molecular interactions of materials, which is distinct from previous methods for the preparation of polymer films and microcapsules. One of the advantages of LbL films is that the film thickness can be precisely tuned at the nanometer level by changing the number of deposited layers. It is also a merit of the LbL deposition protocol that the process for film preparation is carried out in aqueous media under mild conditions. In addition, one can use a wide variety of synthetic and biological materials for LbL deposition. Thus, the fine structure of LbL films and microcapsules can be tailored by a suitable choice of building block materials.

Significant attention has been devoted to the development of LbL film and microcapsule biosensors and controlled release systems. Therefore, this review focuses on the development of biosensors and controlled release systems based on LbL films and microcapsules. In the following section, we begin with an overview of the preparation of LbL films and microcapsules. The synthesis, structure, and applications of LbL thin films and microcapsules have recently been comprehensively reviewed.[5–15]

2 LbL Films and Microcapsules

2·1 Electrostatic bonding LbL films

LbL films were first prepared by the alternate deposition of polyelectrolytes with opposite charges. The adsorption of polycations or polyanions on a charged surface produces a new surface with the opposite charge under appropriate conditions, because of an over compensation of the surface charge. The most widely used materials for constructing LbL films are positively or negatively charged synthetic polymers (Fig. 1). An advantage of the LbL deposition technique is the wide choice of solid substrates on which LbL film can be deposited, including glass, ceramics, polymers, and metals. Materials with hydrophilic, hydrophobic, charged or uncharged surfaces can be used as solid supports. In a typical protocol, the solid substrate is alternately immersed in aqueous solutions of either positively- or negatively-charged polymers for 15 – 30 min to deposit the polymer on the surface of the substrate (Fig. 2). The substrate surface must be rinsed for 5 – 10 min after each deposition to remove nonspecifically or weakly adsorbed polymers. The thickness of the LbL films can be regulated simply by changing the number of deposited layers, because the film thickness increases with the number of depositions. In addition, the pH and ionic strength of the polymer solutions significantly affects the amount of polymers adsorbed during each deposition, because of the pH- and ionic strength-dependent conformational variation of the polymers in the bathing solution.[16] Highly charged polymers form a stretched conformation, because of the electrostatic repulsion along the polymer chain, whereas weakly charged polymer chains tend to assume a globular form. Salts also affect the conformation of charged polymers by shielding the electrostatic repulsion within the polymer chains. Thus, the effects of the pH and ionic strength on the film thickness are particularly significant for LbL films composed of weak polyelectrolytes such as poly(carboxylic acid) and poly(amine).[16]

The thickness of polysaccharides or polypeptide LbL films often increases exponentially with the number of layers, in contrast to the linear growth of LbL films consisting of synthetic polymers.[17] The exponential increase of the film thickness is thought to originate from the free diffusion of at least one of the polyelectrolytes in the film interior. The polyelectrolyte chains in the film interior are able to diffuse to the film surface and form a complex with the counter polymer, resulting in the formation of a thicker layer.[18] The thickness of LbL films can

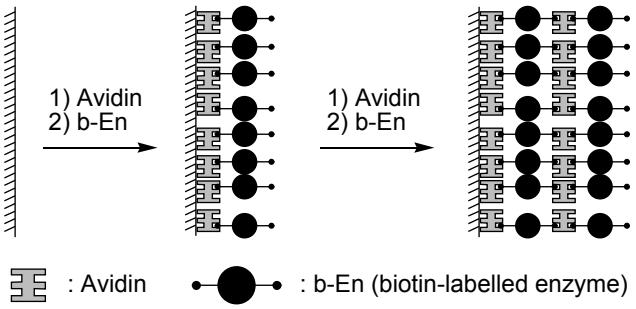

Fig. 3 Structure of an LbL film composed of avidin and biotin-labeled enzyme.

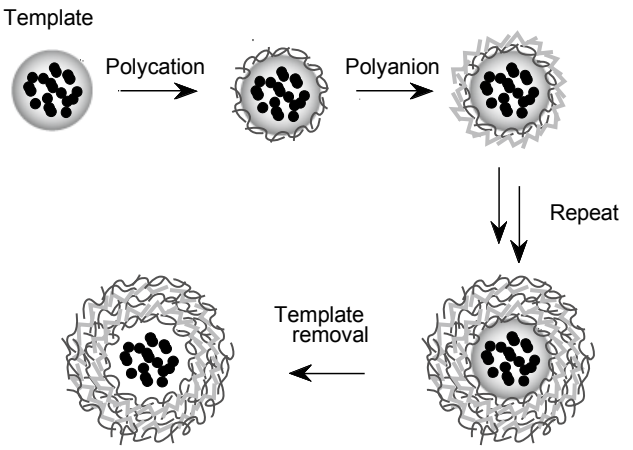

Fig. 4 Procedure for constructing a polyelectrolyte LbL microcapsule.

be controlled on a nanometer scale by tuning these parameters.

Proteins, polysaccharides, and DNA are frequently used as building blocks for constructing LbL films, because these biopolymers contain net electric charges that depend on the pH of the media. The biological activity of the biopolymers can be preserved in the LbL films, and thus biopolymer-based LbL films can be used for the fabrication of biosensors.

2·2 Hydrogen bonding LbL fims

Hydrogen bonding is also a feasible driving force for constructing LbL films. Poly(carboxylic acid)s are often used to construct hydrogen bonding-based LbL films by combining hydrogen bonding acceptor polymers, such as poly(ethyleneglycol) (PEG) and poly(vinylpyrrolidone) (PVP).[19,20] Carboxylated poly(amidoamine) dendrimer and tannic acid have also been used as components of hydrogen-bonding LbL films.[21–23] Hydrogen-bonding LbL films are constructed by an alternate deposition of hydrogen donor and acceptor polymers from aqueous solutions, in a similar manner as that for the preparation of electrostatic bonded LbL films. A unique feature of hydrogen-bonded LbL films is their pH-dependent instability, in contrast to the high stability of electrostatic bonded LbL films. For example, LbL films consisting of poly(acrylic acid) (PAA) or poly(methacrylic acid) (PMAA) are stable in acidic media, whereas they decompose at a neutral pH, because the hydrogen bonds are broken by the deprotonation of the carboxylic acid residues.[24] Therefore, hydrogen-bonding LbL films are usually prepared in acidic solutions. The pH-sensitive nature of hydrogen bonded LbL films can be exploited in pH-triggered release devices.

2·3 Biological affinity-based LbL films

Proteins with multiple binding sites, such as concanavalin A (Con A), avidin, and its homolog protein, streptavidin, are also used for constructing LbL films, in which avidin-biotin and Con A-sugar interactions drive the film formation.[25-27] Avidin and Con A are tetramer proteins, which contain four binding sites for biotin and sugar, respectively.[28–31] Biotin- and sugar-labeled enzymes have been used for preparing enzyme LbL films for biosensors.[32,33] Enzyme molecules labeled with multiple biotin residues can be adsorbed to the avidin layer through avidin-biotin affinity, leaving free biotin residues for further binding of the next avidin layer (Fig. 3). Any type of enzyme could be built into LbL films using this method, because enzymes can be easily labeled with biotin residues using commercially available reagents. Con A and sugar-labeled enzymes can be used in a similar way for LbL film construction. It is an advantage of the Con A-based protocol that glyco-enzymes equipped with intrinsic hydrocarbon chains, such as glucose oxidase (GOx) and horseradish peroxidase (HRP), can be used directly in this protocol without labeling.[34] Thus, a variety of materials are currently being used as building blocks for LbL films, including synthetic polymers and biopolymers, organic dyes,[35] host-guest compounds,[36] metal nanoparticles,[37] and carbon nanotubes.[38]

2·4 LbL microcapsules

In 1998, hollow microcapsules were constructed by the LbL deposition of poly(allylmine hydrochloride) (PAH) and poly(styrene sulfonate) (PSS) on the surface of melamine formaldehyde (MF) colloidal particles, followed by dissolution of the MF core in an acidic solution.[4] This LbL deposition protocol has been widely used for the development of functional microcapsules.[39] Microcapsules are usually prepared by the electrostatic deposition of polyelectrolytes, although LbL films deposited through hydrogen bonding and biological affinity can also be used for microcapsule construction.[40,41] The template materials used include poly(styrene) (PS), MF, $CaCO_3$, and $MnCO_3$ particles a few micrometers in diameter.[42-46] $CaCO_3$ particles are often used for encapsulating proteins, because the synthesis of $CaCO_3$ in the presence of a protein readily affords protein-containing $CaCO_3$ particles.[44,45] The $CaCO_3$ template can be dissolved in mild aqueous solution containing ethylenediamine-*N*,*N*,*N*′-*N*′-tetraacetic acid (EDTA) after it has been coated with the LbL shell, thus producing hollow microcapsules containing the protein (Fig. 4).

The stability and permeability of the shell membrane of the LbL microcapsules is a prime concern for the development of functional microcapsules. LbL microcapsules with a PAH-PSS shell membrane exhibit higher permeability to dextran at low pHs, although the permeability is suppressed at or above pH 8.0.[47] The PAH-PSS microcapsules are stable over a wide pH range, whereas microcapsules consisting of PAH and PMAA decompose at pH 2.5 or lower and at pH 11.5 or higher.[48] The pH-dependent permeability and decomposition of LbL microcapsules depends on the acid-base equilibrium of the weak polyelectrolytes in the LbL films. Therefore, the permeability of LbL microcapsules can be manipulated by changing the environmental pH. Polyelectrolyte microcapsules are permeable to macromolecules, such as proteins, as well as to small molecules.[49] These results demonstrate that the permeability and stability of LbL microcapsules can be controlled by using suitably designed polymers as film components.

3 Electrochemical Biosensors

Electrochemical biosensors are fabricated by immobilizing active components, such as enzymes and antibodies, on the surface of an electrode. It is envisaged that LbL deposition protocol can be employed for immobilizing proteins on electrodes. A significant advantage of the LbL deposition protocol is that LbL films can be prepared in aqueous media under mild conditions without a loss of biological activity of the proteins. In a similar way, electron-transfer mediators can be confined in the LbL films through electrostatic or covalent bonding. In addition, the thickness of LbL films on the electrode surface can be tuned by changing the number of layers. Thus, the electron transfer mechanism and kinetics of biosensors can be regulated systematically. In the following sections, characteristic features of LbL film-based electrochemical biosensors are discussed.

3·1 Electrostatic bonded LbL films

Early publications by Kunitake and coworkers demonstrated that a variety of proteins including enzymes can be built into thin films by electrostatic LbL deposition.[50,51] Positively and negatively charged polymers, such as poly(ethyleneimine) (PEI), poly(diallyldimethylammonium chloride) (PDDA), and PSS, were used, depending on the net electric charge of the proteins. Sun *et al*. constructed electrochemical biosensors sensitive to maltose by depositing GOx-glucoamylase bi-enzyme films on the surface of a gold (Au) electrode.[52] Synthetic polymers which bear electron-transfer mediators in their side chains are widely used for constructing mediator-type enzyme sensors. Mediator-type glucose sensors were constructed using PEI and poly(4-vinylpyridine) (PVPy) derivatives containing ferrocene and an Os-bipyridine complex, respectively, to deposit LbL films containing GOx on the surface of electrode.[53,54] The performance characteristics of the glucose sensors depended on the number of GOx and polymer layers. These studies clearly demonstrated the utility of enzyme-containing LbL films as a catalytic layer for biosensors.

Oxidase-based biosensors often suffer from interference originating from oxidizable contaminants, such as ascorbic acid, uric acid, and acetaminophen in sample solutions. However, LbL films consisting of synthetic vinyl polymers were successfully used to eliminate the interference of enzyme biosensors. The surface of electrodes was coated with LbL films composed of PAH and poly(vinyl sulfonate) (PVS).[55] Figure 5 shows the typical amperometric responses of uncoated and LbL film-coated platinum (Pt) electrodes to hydrogen peroxide (H_2O_2), ascorbic acid, uric acid, and acetaminophen. The uncoated Pt electrode exhibited an oxidation current for all substrates tested at 0.6 V *vs*. Ag/AgCl, indicating that these oxidizable compounds interfere with the detection of enzymatically produced H_2O_2. In contrast, the response of the LbL film-coated electrode to ascorbic acid, uric acid, and acetaminophen was almost completely eliminated, and the response to H_2O_2 was still sufficiently high. The suppressed response of the LbL film-coated electrode did not arise from Donnan exclusion but from size exclusion effects; the oxidation current decreased as the number of layers or the film thickness increased, irrespective of the sign of the LbL film surface charge. Glucose biosensors constructed using PAH-PVS film-coated Pt electrodes satisfactorily eliminated the interference from oxidizable contaminants.[56] Amperometric uric acid sensors were also prepared using a uricase-modified electrode with a PAH-PVS LbL film.[57] The uric acid sensor

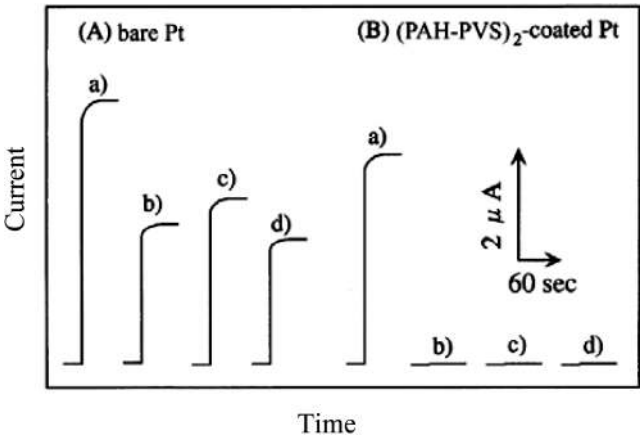

Fig. 5 Typical amperometric responses of (A) unmodified, and (B) $(PAH\text{-}PVS)_2$ film-coated Pt electrodes to (a) 0.1 mM H_2O_2, (b) ascorbic acid, (c) uric acid, and (d) acetaminophen at 0.6 V *vs*. Ag/AgCl. Copyright (2000) The Japan Society for Analytical Chemistry.

exclusively detected H_2O_2 produced by the uricase-catalyzed reaction of uric acid, whereas the direct oxidation of uric acid on the electrode surface, which could produce a bias signal, was prevented by the PAH-PVS film. The size exclusion effects of the LbL films depend on the type of film components; LbL films composed of PEI-PVS and PDDA-PVS pairs were less effective in excluding the oxidizable compounds.[56] Yabuki has recently discussed the size exclusion effects of polyion complex (PIC) films that were prepared by mixing polycation and polyanion solutions.[58] The PIC films composed of poly(L-lysine) and PSS exhibited size exclusion for organic compounds with a molecular weight higher than ~100, and it was H_2O_2-permeable.[59]

Recently, polysaccharide-based LbL films have attracted attention in the development of biosensors and drug-delivery systems because of their biocompatibility and high swellability.[60] Polysaccharide LbL films containing enzymes have been prepared for biosensors and bioreactors.[61-63] We have studied the voltammetric response of a redox species on Au electrodes coated with polysaccharide LbL films.[64,65] Carboxymethyl-cellulose (CMC) and alginic acid (ALG) have been used in combination with PAH, PEI, or PDDA as the cationic counterpart. The redox properties of ferricyanide ions, $Fe(CN)_6^{3-}$, depended strongly on the type of film components and the film thickness (Fig. 6). For thinner film-coated electrodes (n = 1, Figs. 6a and 6b), nearly reversible cyclic voltammograms (CVs) were observed at 0.2 – 0.3 V *vs*. Ag/AgCl, irrespective of the film components, confirming that $Fe(CN)_6^{3-}$ ions freely penetrated the thinner films. For the thicker $(PEI\text{-}ALG)_nPEI$ and $(PEI\text{-}CMC)_nPEI$ film-coated electrodes (n = 2 – 5, Figs. 6a and 6b), the peak currents increased with the film thickness. In addition, the CV peaks broadened, which implied the concurrence of two current maxima. This was more clearly observed for the CVs of the $(PDDA\text{-}ALG)_nPDDA$ and $(PDDA\text{-}CMC)_nPDDA$ film-coated electrodes (Figs. 6c and 6d), in which two redox pairs were visible. These results show that two types of redox reaction occur concurrently in the LbL films (Fig. 7). The two redox pairs in the CVs are caused by diffusing and confined $Fe(CN)_6^{3-}$ ions. The confined $Fe(CN)_6^{3-}$ ions could be used as a catalyst for the electrocatalytic determination of ascorbic acid.[66] In contrast, $Ru(NH_3)_6^{3+}$ ions were not confined in the polysaccharide LbL films, confirming that the positive sites on the poly(amine)s

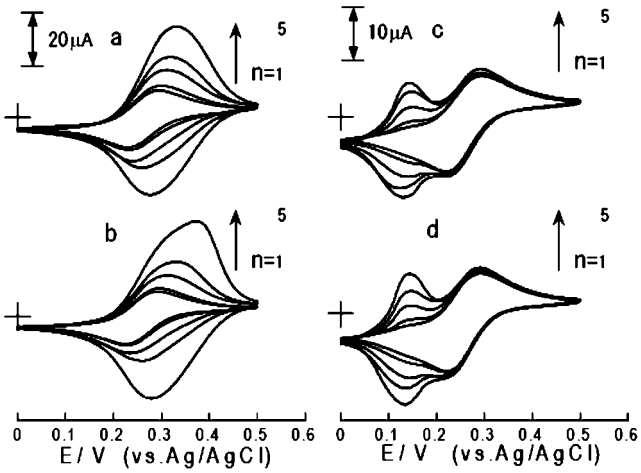

Fig. 6 CVs of 1 mM $Fe(CN)_6^{3-}$ ions on Au electrodes coated with (a) $(PEI\text{-}ALG)_nPEI$, (b) $(PEI\text{-}CMC)_nPEI$, (c) $(PDDA\text{-}ALG)_nPDDA$, and (d) $(PDDA\text{-}CMC)_nPDDA$ films as a function of the number of bilayers in the films. The CVs were recorded at pH 7.4. Copyright (2007) The American Chemical Society.

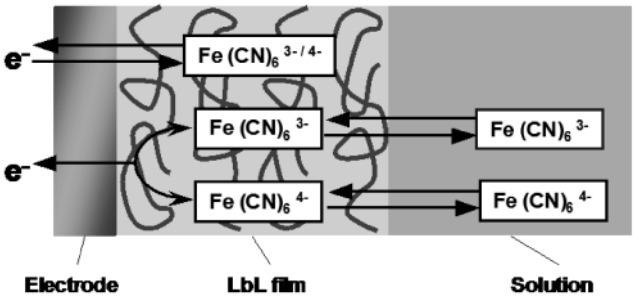

Fig. 7 Redox reactions of diffusing and confined $Fe(CN)_6^{3-}$ ions in a polysaccharide LbL film on the surface of an electrode. Copyright (2007) The American Chemical Society.

confine the $Fe(CN)_6^{3-}$ ions.

A polysaccharide LbL film was employed for fabricating sugar sensors, in which phenylboronic acid-bearing PAH was used as a counter polymer in the LbL film.[67] The $Fe(CN)_6^{3-}$ redox current of the LbL film-coated electrode was decreased in the presence of glucose or fructose, because of the electrostatic exclusion of the $Fe(CN)_6^{3-}$ ions on the surface of the LbL film. The phenylboronic acid formed cyclic boronate esters with diols, which generated a negative charge on the boron atom.[68] Polysaccharide LbL film-coated electrodes may be useful for constructing biosensors by combining electrodes with enzymes. The confinement of $Fe(CN)_6^{3-}$ ions is not always possible in LbL films; only polysaccharide LbL films have been successfully used for this purpose. The LbL films composed of synthetic polymers, such as the PAH-PSS pair, did not confine the $Fe(CN)_6^{3-}$ ions.[64] The high swellability of the polysaccharide LbL films may enable the confinement of $Fe(CN)_6^{3-}$ ions.

LbL film-based electrochemical biosensors, including enzyme sensors, immunosensors, DNA sensors, and enzyme field-effect transistors, have recently been comprehensively reviewed.[69-71]

3·2 Biological affinity-based LbL films

Antibodies, avidin or streptavidin, and lectin have been used for the construction of enzyme LbL films. A significant advantage of using these biological materials for preparing LbL films is that the whole process can be conducted under physiological conditions. Savéant and coworkers constructed antibody-labeled GOx and anti-GOx antibody LbL films on an electrode surface, and studied the catalytic activity of GOx.[72,73] They found that the response current of the electrode to glucose increased with the number of GOx layers, confirming that antibody-antigen pairs can be used for fabricating enzyme LbL films. However, this protocol is complicated because two types of antibodies are involved in the preparation of the enzyme assembly. Simpler routes for the construction of enzyme LbL films were established using avidin or lectin. Avidin is a tetramer protein consisting of four identical polypeptide chains, each of which contains a strong binding site for biotin (binding constant: K_a ~10^{15} M^{-1}).[28,29] Avidin has been widely used for labeling biopolymers and cells because of its strong binding to biotin-tagged molecules, such as fluorophores, DNA, lipids, and proteins.[74] The avidin molecule is roughly cubic (molecular dimensions: ~$4.0 \times 5.0 \times 5.5$ nm) and two pairs of biotin binding sites are located on opposite faces of the molecule.[28,29] It is possible to assemble enzyme architectures through the LbL deposition of avidin and biotin-labeled enzymes (Fig. 3). A variety of enzymes have been built into LbL films for enzyme biosensors, including GOx,[75] alcohol oxidase,[76] HRP,[77] and glutamate oxidase.[78] The amperometric response of all the sensors increased with the number of enzyme layers in the LbL film, confirming that the enzymes retain their catalytic activity and that enzyme substrates penetrate the LbL film. The facile preparation of bi-enzyme biosensors through the avidin-biotin protocol has also been demonstrated; these include enzyme sensors composed of GOx and ascorbate oxidase,[79] GOx and hexokinase,[80] GOx and HRP,[81] choline esterase and choline oxidase,[82] and polyphenol oxidase and alkaline phosphatase.[83] Ferrocene-attached avidin and streptavidin have also been used for the preparation of protein assemblies on an electrode surface to study the electron-transfer mechanism across LbL films.[84,85]

Similarly, LbL films containing antibodies were prepared using a biotinylated antibody and avidin in order to enhance the sensitivity of immunosensors.[86,87] We prepared avidin and anti-fluorescein antibody LbL films, and found that the antibodies located in the outermost four or five layers could bind the antigen, whereas those in the inner layers could not.[86] This is probably caused by the limited permeability of the LbL film. Thus, the use of LbL films for improving the sensitivity of immunosensors was partially successful, although the permeability of the antibody LbL layers must be improved. Dong and coworkers also reported that the binding capacity of the antibody-avidin multilayers increased for layers one to three, and reached saturation after the fourth layer.[87] In addition to biosensors, the avidin-biotin system is also commonly used for the construction of protein nano-assemblies.[88]

The specific affinity of lectin for sugars has also been exploited in the construction of enzyme LbL films.[32-34,89,90] Con A has also been successfully used in LbL films, because it contains four binding sites for D-glucose (K_a, 0.8×10^3 M^{-1}) and D-mannose (K_a, 2.2×10^3 M^{-1}).[30,31] Therefore, the alternate deposition of Con A and a D-glucose- or D-mannose-labeled enzyme affords LbL films. An advantage of using Con A in LbL films is that glyco-enzymes, such as GOx and HRP, can be used without labeling, because these enzymes contain intrinsic polysaccharide chains on the molecular surface.[34,89,90] The Con A-based enzyme sensors were used to optimize the ratio of GOx and HRP,[34] for the determination of phenolic compounds,[89] and for the pH-induced switching of a sensor response.[90] For other enzymes

without an intrinsic polysaccharide chain, the molecular surface must be labeled with saccharide residues before use. Con A-based enzyme assemblies have recently been reported, in which redox active Con A modified with an Os-complex was used instead of native Con A for preparing redox-active protein assemblies.[91,92]

4 Optical Biosensors

Optically-active LbL films have been fabricated by immobilizing dyes on LbL films or by using dye-bearing polymers as film components. LbL films are an excellent scaffold for immobilizing dyes for optical sensors because of their optical transparency, which results from their thin, uniform structure.[71] Brilliant yellow (BY)-doped LbL films were sensitive to changes in the environmental pH.[93,94] The BY was photochemically cross-linked with a diazo-dye-substituted polymer in the LbL film, which prevented the desorption of BY, even at pH 13. The cross-linked BY film showed a rapid response to changes in pH over the pH range 9 – 13. A further pH-sensitive LbL film was constructed using 5,10,15,20-tetrakis(4-sulfonatophenyl)-porphyrin (TPPS) and PAH.[95] TPPS formed J-aggregates in the film at pH 1.5, and H-aggregated TPPS was formed at pH 3.0 or higher. The interconversion between J- and H-aggregates in the film was associated with changes in the absorption spectra; the H-aggregate showed an absorption maximum at 405 nm, whereas absorption maxima at 484 and 691 nm were observed for the J-aggregate. These pH-sensitive LbL films could be used for the construction of biosensors by coupling them with enzymes that catalyze reactions accompanied by pH changes.

A glucose-sensitive system has been constructed by encapsulating fluorescein-modified Con A (F-Con A)-glycogen conjugates in an LbL microcapsule.[96] The fluorescence intensity of the F-Con A-glycogen conjugates in the microcapsules increased in the presence of glucose. The results were explained by the glucose-induced decomposition of the conjugates, which resulted in the dequenching of the F-Con A fluorescence.[97] The response of the microcapsules declined during repeated use after they were washed in buffer solution, which was probably because of the leakage of F-Con A from the microcapsules following the addition of glucose. The leakage of F-Con A could be suppressed by using microcapsules with a lower permeability. LbL film optical sensors have recently been reviewed by Siqueira *et al*.[71]

5 Controlled Release

Proteins and drugs can also be embedded in LbL films as a film component during the film construction. Alternatively, they can be immobilized in as-prepared LbL films as additives. The first strategy allows the amount of protein or drugs to be regulated by changing the number of LbL layers, whereas precise control is difficult using the second route. These routes can also be used to encapsulate compounds in LbL microcapsules. Microcapsule shell membranes are usually very permeable, even to macromolecules under certain conditions. A third route for encapsulation involves the use of porous particles preloaded with proteins or drugs (Fig. 4). The template is coated with LbL films, and then the template material is dissolved away to leave the drugs or proteins in the microcapsules. $MnCO_3$ and $CaCO_3$ are often used as templates, because these materials can be dissolved under mild conditions in water.[45,46] The encapsulation efficiency of this method is high.

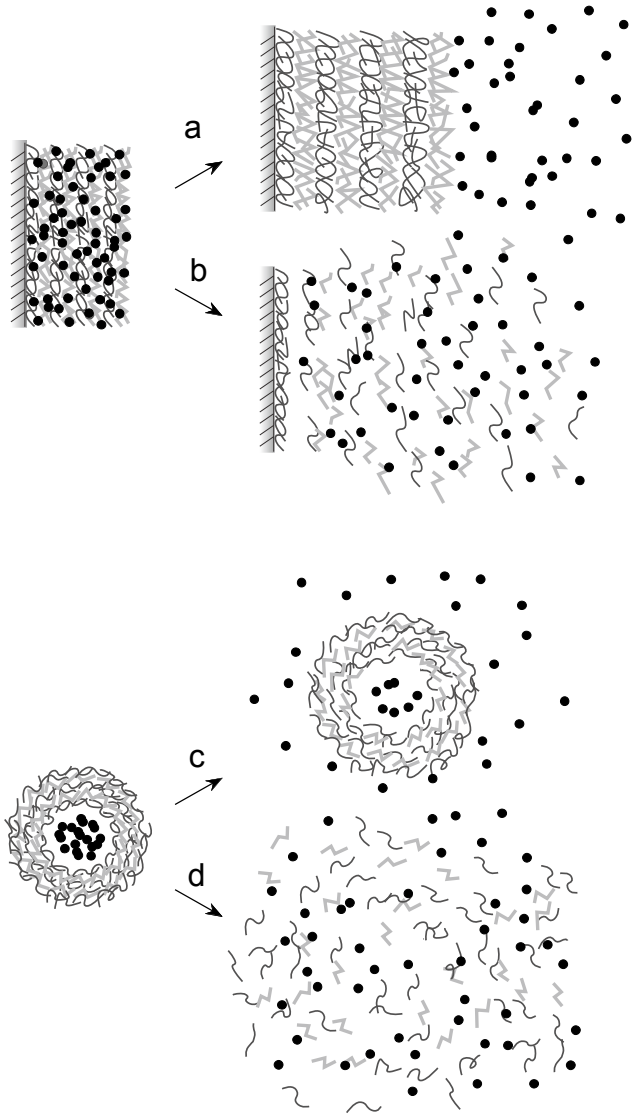

Fig. 8 Possible routes for stimuli-sensitive release of drugs from (a, b) LbL films, and (c, d) microcapsules.

The loaded materials are released from LbL films by external stimuli that increase the permeability of the film (Fig. 8a), or cause the film to decompose (Fig. 8b). Sustained release can be achieved by increasing the permeability of the film. However, decomposition of the film would produce a burst release of the loaded materials. The release rate from LbL microcapsules can be regulated by changes in the permeability of the capsule membrane (Fig. 8c), or by the decomposition of the entire capsule (Fig. 8d). Consequently, a key issue in developing controlled release systems is the design of appropriate stimuli-sensitive polymeric materials and suitable combinations for LbL deposition. A variety of stimuli have been employed for triggering the release of the microcapsule contents, including changes in the pH, ionic strength, temperature, biological molecules, and magnetic and electric fields. In the following section, typical examples of controlled release systems for proteins and drugs are discussed. The use of LbL films and microcapsules for drug-delivery systems has been extensively reviewed by several authors.[14,15,98-101]

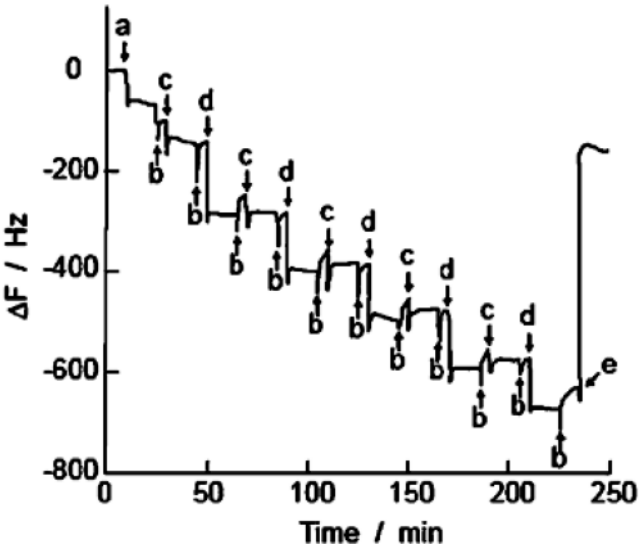

Fig. 9 Preparation of LbL film composed of DS and insulin on the surface of a quartz crystal microbalance (QCM) at pH 3.0 and its decomposition at pH 7.4. The quartz resonator was exposed to (a) PEI, (b) buffer at pH 3.0, (c) DS, (d) insulin, and buffer at pH 7.4 (e). Copyright (2010) The Royal Society of Chemistry.

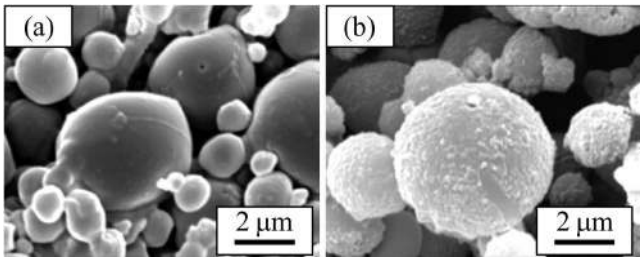

Fig. 10 SEM images of (a) unmodified, and (b) (PVS-insulin)$_5$PVS film-coated PLA microbeads. Copyright (2012) Elsevier.

5·1 Controlled release from LbL films

Electrostatically bonded LbL films composed of synthetic polymers are stable over a wide pH range, and are only unstable under extremely acidic or basic conditions. This is because multiple binding sites exist between the polymer chains in the LbL films, even if the solution pH is more acidic or basic than the apparent pK_a of the polymers. In contrast, the stability of electrostatic LbL films which contain protein film components may be sensitive to environmental pH, because the number of positive and negative sites in proteins is limited. Based on this assumption, we have prepared pH-controlled LbL films for the release of insulin.[102-106] The LbL deposition of anionic polymers and insulin from acidic solutions (pH 3.0) gave thin films containing insulin, because insulin is positively charged under acidic conditions.[102-104] The anionic polymers used were PVS, PAA, dextran sulfate (DS), heparin, fucoidan, and κ-carrageenan. The insulin loadings in the PVS- and PAA-based LbL films were higher than those in the polysaccharide-based films. For example, the 10-layer (PVS-insulin)$_{10}$ and (PAA-insulin)$_{10}$ films contained 14 and 36 μg cm^{-2} of insulin, respectively, compared to the insulin loadings of 4.7 μg cm^{-2} in the (heparin-insulin)$_{10}$ film and 7.4 μg cm^{-2} in (fucoidan-insulin)$_{10}$ film. The insulin LbL films were stable in acidic solutions at pH 5.0 or lower, although they decomposed at neutral pH, and released insulin. Figure 9 shows a quartz crystal microbalance result for the preparation of DS-insulin film and its decomposition.[103] The release of insulin occurred rapidly at pH 7.4. The pH threshold for the film decomposition was at pH 5.0 – 6.0, which corresponds to the isoelectric point of insulin, 5.4. This confirms that the film decomposition was caused by a shift in the net electric charge of insulin from positive to negative. The insulin LbL films may be useful for developing orally administered insulin, because these films would be stable in the acidic environment of the stomach, and the insulin would be released in the intestine at neutral pH. Thus, the polyanion-insulin LbL films can circumvent digestion in the stomach. Polyanion-insulin LbL films have also been used to coat the surface of poly(lactic acid) (PLA) microbeads (Fig. 10).[105] The LbL films were also stable at pHs lower than 5.0, and the insulin was completely released from the PLA microbeads at neutral pHs higher than 6.0. The microbead preparations of insulin would be particularly useful for drug delivery because PLA microbeads can be handled as powder.

In contrast, LbL films prepared using polycations and insulin were stable at neutral pH, because insulin bears a net negative charge at neutral pH. In this protocol, PAH was used as a cationic polymer to construct the insulin LbL films at pH 7.4.[106] The PAH-insulin LbL film was stable at pH 6.0 – 8.0 and decomposed at pH 4.3 or lower, as a result of the change in the net electric charge of insulin from negative to positive. PAH-insulin LbL films may be useful for the future development of pH-triggered delivery systems. These results demonstrate that proteins can be released from LbL films by shifting the solution pH across the pK_a value of the protein. However, in some cases, the stability of polyelectrolyte-protein LbL films depends on both the pH and the salt concentration.[107]

Recently, LbL films have also been used for developing electrochemically-controlled drug-delivery systems. The electric control of drug release is promising, because electric signals are clean and easily controlled.[108-112] The insulin could be released from an electrode coated with an insulin-containing LbL film in response to an applied electric potential, because the pH around the electrode surface would be acidified by the electrolysis of H_2O. However, the operating variables, such as pH, ionic strength, and the magnitude of electrode potential, have to be carefully addressed, because some drugs can be electrochemically oxidized or reduced at an electrode.

5·2 Controlled release from LbL microcapsules

One of the challenges in insulin delivery systems is to develop glucose-sensitive release systems for use in an artificial pancreas.[113-115] Glucose-sensitive LbL microcapsules that decompose in response to glucose have been prepared using phenylboronic acid (PBA)-bearing polymers.[116-118] LbL microcapsules consisting of PBA polymers decomposed in response to glucose at pH 9.0 or higher. However, the glucose sensitivity of these microcapsules was not satisfactory in the physiological pH range, because of the lower affinity of the PBA moiety for glucose. The glucose response of the microcapsules under physiological conditions may be enhanced by PBA derivatives substituted with electron-withdrawing nitro or halogen groups. The substituted PBA derivatives are expected to exhibit higher affinity for glucose in the neutral pH range, because of their lower pK_a values.[119] The use of PBA copolymers containing acrylamide side chains also effectively enhance glucose sensitivity in the physiological pH region.[120]

Con A and GOx have been used to construct glucose-sensitive LbL microcapsules. The LbL assembly of Con A and glycogen

on the surface of an insulin-loaded $CaCO_3$ core afforded insulin-containing microcapsules.[121] The insulin was released slowly from the microcapsules even in the absence of glucose, probably because the capsule shell was porous. The insulin release was accelerated in the presence of 100 mM glucose as a result of the competitive binding of glucose to Con A in the capsule shell. However, the addition of 10 mM glucose only had a small effect on the insulin release. GOx-based LbL microcapsules have also been reported by Li and coworkers. They found that the $(GOx\text{-hemoglobin})_5$ microcapsules became permeable to dextran in the presence of 100 mM glucose, although dextran was impermeable without glucose.[122] They also prepared GOx-catalase LbL layers on the surface of an insulin microcrystal, and found that the dissolution rate of insulin increased in response to glucose.[123] The effects of glucose were explained by the local pH changes induced by the GOx-catalyzed reaction of glucose. For practical applications, the microcapsules should be sensitive to ~10 mM glucose, because a normal glucose blood level is around 5 mM. Therefore, further improvements are required for Con A and GOx microcapsules.

6 Conclusion and Outlook

Enzymes can be immobilized on an electrode surface by the LbL deposition of anionic or cationic polymers through electrostatic binding. Antibody, avidin, and Con A can also be used to prepare enzyme LbL films. Polyelectrolyte LbL films coated on electrode surfaces exhibit size-exclusion effects for oxidizable compounds, such as ascorbic acid, uric acid, and acetaminophen, which contribute to the elimination of interference in biosensors. Electron-transfer mediators can also be immobilized in LbL films through electrostatic confinement, or by covalent modification. Thus, LbL films can be effectively used in the development of high-performance biosensors. In addition, the usefulness of LbL films and microcapsules in the development of controlled release systems has been demonstrated. Insulin-containing LbL films deposited on a flat surface or on PLA microparticles allow for the pH-dependent release of insulin. Insulin microcapsules are also promising for the future development of glucose-dependent insulin release systems for use in an artificial pancreas.

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