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REVIEW

Layer-by-layer construction of protein architectures through avidin–biotin and lectin–sugar interactions for biosensor applications

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Abstract In this review, the preparation and properties of protein architectures constructed by layer-by-layer (LbL) deposition through avidin–biotin and concanavalin A (Con A)–sugar interactions are discussed in relation to their use for optical and electrochemical biosensors. LbL films can be constructed through the alternate deposition of avidin and biotin-labeled enzymes on the surfaces of optical probes and electrodes. The enzymes retain their catalytic activity, resulting in the formation of optical and electrochemical biosensors. Alternatively, Con A can be used to construct enzyme-containing LbL films and microcapsules using sugar-labeled enzymes. Some enzymes such as glucose oxidase and horseradish peroxidase can be used for this purpose without labeling with sugar, because these enzymes contain intrinsic hydrocarbon chains on their molecular surfaces. The Con A/enzyme LbL architectures were successfully used to develop biosensors sensitive to specific substrates of the enzyme. In addition, Con A-based films can be used for the optical and electrochemical detection of sugars.

Keywords Layer-by-layer film · Protein architecture · Microcapsule · Biosensor · Avidin–biotin · Lectin–sugar

Introduction

Layer-by-layer (LbL) thin films have been extensively studied in order to develop surface architectures that act as electrochemical and optical biosensors. The LbL deposition technique was developed by Decher and co-workers around 20 years ago [1–3]. They prepared polyelectrolyte assemblies based on the alternate and repeated deposition of cationic and anionic polymers onto the surface of a solid substrate from solution, as schematically illustrated in Fig. 1. The driving forces for LbL deposition are not only electrostatic forces of attraction but also interactions such as covalent bonding [4], hydrogen bonding [5–7], and biological interactions (including antibody–antigen, avidin–biotin, and lectin–sugar affinities) [8–10]. Various molecules, such as synthetic polymers [11, 12], dyes [13], proteins [14–16], polysaccharides [17–19], DNA [20, 21], dendrimers [22, 23], and inorganic and carbon nanoparticles [24–26] have been employed as building blocks. Consequently, a variety of components and functionality can be incorporated into LbL films to construct functional surface architectures, suggesting a wide variety of options when designing the function of a biosensor. In fact, early studies demonstrated the usefulness of protein-containing LbL films for the construction of electrochemical biosensors [27–30]. The surface of an electrode was coated with an enzyme-containing LbL film, resulting in highly improved performance characteristics of the modified electrode as a biosensor, including the elimination of interference [27], effective electron transfer between the enzyme and electrode [28], amplification of the output signal [29], and the successful use of a bienzyme system [30]. A comprehensive review of LbL film-based electrochemical biosensors was published in 2006 [31].

Hollow polyelectrolyte microcapsules were constructed in 1998 through the LbL deposition of cationic and anionic polymers onto the surface of a colloidal particle, followed by the dissolution of the core material [32]. Organic and inorganic microparticles with diameters of a few micrometers

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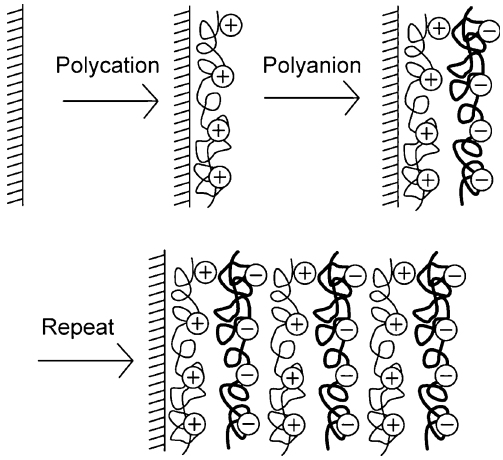

Fig. 1 A schematic illustration of the layer-by-layer deposition of polycation and polyanion on a solid surface

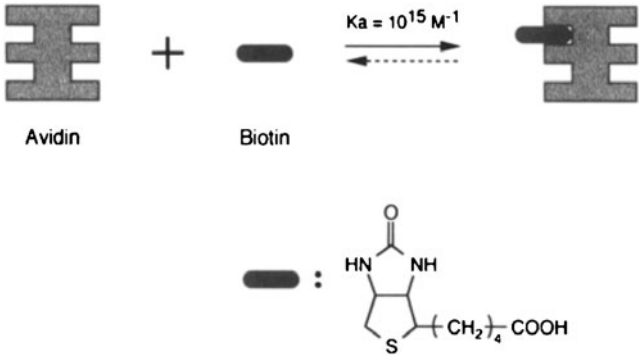

Fig. 2 Binding equilibrium of avidin and biotin. Reprinted with permission from [48] (copyright 1995, The American Chemical Society)

are often employed as the core material. For example, protein-doped $CaCO_3$ particles are often used for the preparation of protein-containing microcapsules [33].

The preparation and properties of LbL-deposited films and microcapsules have recently been comprehensively reviewed by various authors [34–39]. Among the myriad types of LbL films and microcapsules, protein-based LbL architectures have attracted the most attention because of their successful use for the development of high-performance biosensors. Therefore, this review focuses on recent progress in the development of protein architectures constructed by LbL deposition through biological interactions, including avidin–biotin and concanavalin A (Con A)–sugar affinities.

Protein architectures constructed through avidin–biotin interactions

The avidin–biotin system has been widely used in biochemical and biomedical research to label biopolymers and/or the cell surface [40]. This is because avidin binds not only biotin but also biotin-tagged biomolecules such as proteins, lipids, and DNA. A variety of activated biotin derivatives for labeling biomolecules are currently commercially available [41]. Avidin is a glycoprotein (molecular weight: 68,000) isolated from egg white that consists of four identical polypeptide chains, each of which contains a strong binding site for biotin (binding constant; ~10^{15} M^{-1}) (Fig. 2). The strong binding of avidin to biotin relies on multiple interactions; these include a network of hydrogen bridges between the biotin ureido group and amino acid residues in avidin, binding between the biotin sulfur atom and the threonine residue in avidin, and hydrogen bonding of the carboxyl side chain of biotin and the peptide chain in avidin [42]. The avidin molecule is roughly cubic in shape, with molecular dimensions of approximately 4.0×5.0× 5.5 nm^3, and the biotin binding sites are located in two pairs on opposite faces of the molecule [43]. Consequently, LbL architectures may be constructed using avidin and biomolecules labeled with biotin residues.

In fact, avidin and its homolog, streptavidin, have been successfully employed to prepare monomolecular layers of biotin-tagged enzymes on the surfaces of electrodes, in order to generate electrochemical enzyme sensors [44, 45]. These studies prompted researchers to construct enzyme multilayers by the LbL deposition of avidin and biotin-tagged enzymes on the surfaces of electrodes, with the aim of developing high-performance biosensors. In the mid-1990s, Osa's group prepared LbL films composed of avidin and biotin-labeled glucose oxidase (B-GOx) on the surfaces of electrodes to generate glucose sensors (Fig. 3) [46–48]. They found that the amperometric response of the glucose sensors increased with the number of B-GOx layers deposited, confirming that the amount of B-GOx deposited increases with the number of layers. Lactate oxidase and alcohol oxidase were also built into LbL films in order to generate lactate and alcohol biosensors [49, 50]. The B-GOx layer-modified biosensors can be operated at a low electrode potential in the presence of ferrocene derivatives as redox mediators [51]. Later, several groups also reported the LbL deposition of various biotin-labeled proteins and avidin that could potentially be utilized in the development of biosensors and bioreactors; these included hydrogenase [52], horseradish peroxidase (HRP) [9], glutamate oxidase [53], and GOx [54]. Quartz crystal microbalance measurements and ellipsometry were employed to characterize the deposition behavior of streptavidin-based LbL films [55, 56]. One of the merits of the LbL deposition protocol is the ease of preparation of multi-enzyme systems, in which two or more kinds of enzymes can be successively built into the film and the spatial arrangement of the enzyme layers optimized. In this vein, multilayer enzyme films consisting

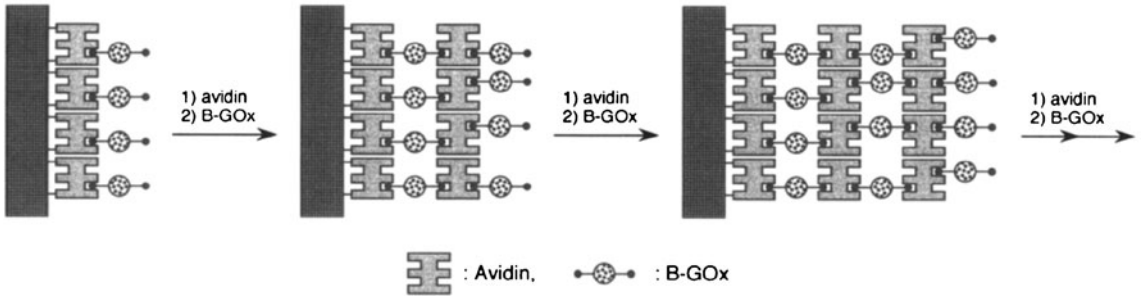

Fig. 3 LbL deposition of biotin-labeled GOx and avidin on the surface of an electrode. Reprinted with permission from [48] (copyright 1995, The American Chemical Society)

of GOx and ascorbate oxidase [57], choline esterase and choline oxidase [58], GOx and hexokinase [59], GOx and β-galactosidase [60, 61], GOx and HRP [61], and polyphenol oxidase and alkaline phosphatase [62] have been reported. B-LOx and B-HRP were immobilized together in different configurations (i.e., avidin/B-LOx/avidin/B-HRP and avidin/B-HRP/avidin/B-LOx films) to exploit the coupled catalytic reactions of the two enzymes to create lactate biosensors. In addition, the catalytic activities of B-HRP immobilized through nonspecific adsorption, avidin-mediated adsorption, and avidin–biotin system-based LbL assembly were compared, and LbL-assembled B-HRP was found to have the highest activity [63].

Padeste and Tiefenauer's group synthesized ferrocene-modified streptavidin (Fc-SAv), which comprises redox-active ferrocene moieties covalently attached to streptavidin via a spacer arm, and prepared an LbL film using a biotin dimer on an electrode surface in order to evaluate the kinetics of electron transfer through the LbL film [64, 65]. Using cyclic voltammetry (CV), they demonstrated that when the scan rate was less than 1 V s^{-1}, electron transfer through the LbL film occurred via the expected mechanism for surface-confined redox species, as evidenced by the linear dependence of the peak current on the scan rate. When the scan rate was higher than 8 V s^{-1}, the peak current was linearly dependent on the square root of the scan rate, suggesting a diffusion-like electron transfer. They suggested that the Fc-Av LbL architecture-modified electrodes could find application as biosensors. The same group also prepared ferrocene-modified avidin in order to construct glucose and lactate sensors [66]. Knoll and coworkers have also used Fc-SAv for the construction of DNA biosensors [67, 68] and biodiodes [69]. Thus, functional groups attached to avidin or streptavidin are useful for improving the efficiency of signal transduction in biosensing.

Recently, well-defined three-dimensional (3D) architectures with active enzymes have been developed based on the LbL deposition of avidin and biotin-labeled enzymes. Kang and coworkers constructed 3D nanostructures containing biotin-labeled lactate oxidase (B-LOx) or B-HRP by surface-templated LbL assembly [70, 71]. Figure 4 illustrates the protocol employed to prepare the 3D architecture. Micro contact printing (μ-CP) was used to pattern a gold surface with a regionally defined self-assembled monolayer (SAM) of a thiol-terminated poly(ethyleneglycol). The unstamped gold surface was filled with a biotin-terminated SAM as a template, onto which avidin–enzyme LbL layers were deposited. The topographic preparation of the enzyme LbL layers was clearly observable by atomic force microscopy (AFM). The 3D assembled enzyme maintained its catalytic activity, and the overall activity increased with the number of enzyme layers. Similarly, Naujoks and Stemmer used electric fields to guide LbL assembly in defined geometric patterns on solid substrates [72]. It was demonstrated that local surface charges served as templates to selectively position proteins on the surface, thereby allowing the site-directed LbL layering of immunoglobulin G (IgG).

LbL films composed of biotin-labeled antibody and avidin were prepared for the construction of immunosensors; the response of the sensors was expected to increase with the number of antibody layers. Anti-fluorescein antibody/avidin [73] and anti-IgG antibody/avidin [74] LbL films were successfully prepared on the surfaces of quartz slides and on the probe for a surface plasmon resonance sensor, respectively, for the optical sensing of corresponding antigens. In both cases, the optical signal representing the detection of antigen increased with the number of antibody layers up to three or four layers, after which the signal became saturated. These results can be explained by the limited diffusion of the antigens through the antibody LbL layers. In other words, only a few outer layers are available for the binding of antigen. An extreme case was reported by Ngundi and Anderson, who found that virtually all of the binding activity of LbL-deposited antibody (anti-staphylococcal enterotoxin B) could be attributed to the antibody in the outermost layer [75]. Thus, the use of LbL films for immunosensor development is somewhat troublesome, especially for the detection of

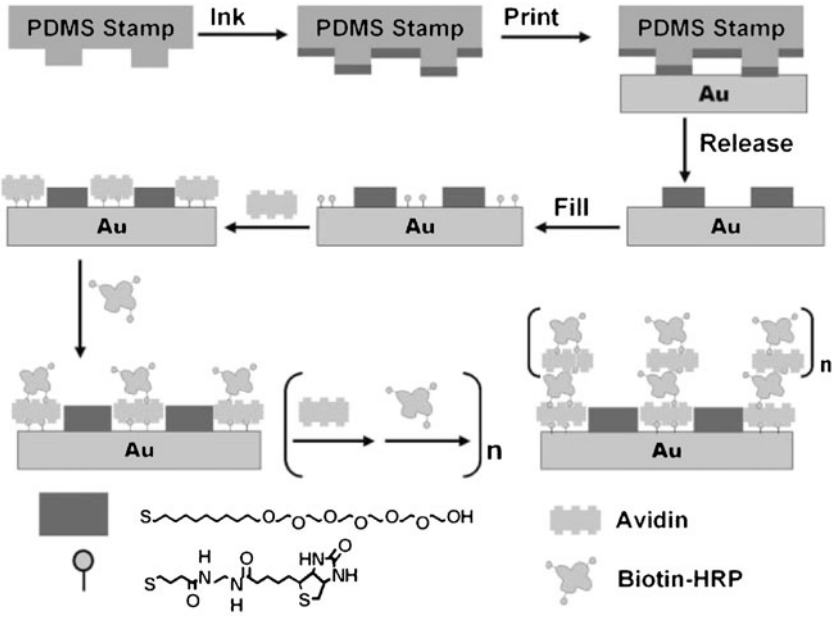

Fig. 4 A protocol for the preparation of 3D architectures of enzyme and avidin through micro contact printing and LbL deposition. Reprinted with permission from [71] (copyright 2010, The American Chemical Society)

relatively large molecules or proteins. The further development of LbL architectures with higher permeability to large molecules or proteins is required.

Carbon nanotubes (CNTs) have been used extensively for the development of electrochemical biosensors because of their high conductivity, mechanical stability, and amenability to chemical modification with biomolecules [76]. CNT-containing layered assemblies have been constructed, based on the avidin–biotin system, on the surfaces of electrodes to develop enzyme biosensors. The surfaces of single-walled CNTs were modified with biotin-appended pyrenes or polypyrroles with biotin side chains in order to construct avidin/GOx assemblies on the CNTs [77, 78]. The modified CNTs were then deposited on the surface of a platinum (Pt) electrode, and the amperometric response of the sensors to glucose was evaluated. Both the pyrene- and the polypyrrole-based sensors exhibited satisfactory responses to glucose in the concentration range of 1–10 mM, confirming the usefulness of the avidin/GOx layered architecture on CNTs in the construction of biosensors. An alternative biosensor was proposed by Doorn and coworkers, who reported a model that utilized reversible fluorescence quenching in CNTs in response to avidin/biotin binding [79]. It was found that the fluorescence of CNTs was severely quenched by the adsorption of biotin derivatives bearing anthracene or azobenzene, and the fluorescence intensity was recovered upon the addition of avidin as a result of the formation of avidin–biotin complexes on the CNT surface, resulting in the possible detection of avidin at the nanomolar level. The CNT-based protocol may be highly versatile for the optical detection of a wide range of biological analytes. In addition, carbon nano-onions (CNOs) and carbon biocomposites have recently been functionalized for biosensing applications using avidin/biotin layering [80, 81].

It has been shown that avidin can be built into LbL architectures through electrostatic binding with oppositely charged silver (Ag) nanoparticles [82] and GOx [83]. Aroca and coworkers described LbL films composed of Ag nanoparticles and avidin for surface-enhanced resonance Raman scattering (SERS) [82]. The LbL deposition of avidin and Ag nanoparticles was possible because the isoelectric point of avidin is 10.5 and the colloidal Ag nanoparticles are negatively charged at neutral pH. The avidin/Ag nanoparticle LbL film was successfully used as a SERS substrate for the selective detection of biotin-labeled fluorescein with a lower detection limit of 10^{-7} M. Another example of the electrostatic LbL deposition of avidin was reported by Liang and coworkers in a study on the preparation of protein nanotubes [83]. Nanoporous membranes of anodized aluminum oxide (AAO) were used as a template in which the walls of the nanopores (average diameter; 200 nm) were coated alternately with avidin and GOx at neutral pH, followed by the dissolution of the template to form avidin/GOx LbL nanotubes. Electron microscopy revealed the formation of a nanotube with a 55 nm thick LbL-deposited wall (Fig. 5). The avidin/GOx nanotube may find applications in biosensing.

The construction of LbL film-based microcapsules may be also interesting from the viewpoint of their applications for biosensing. Chaikof and coworkers developed microcapsules through the LbL deposition of SAv and biotin-bearing poly(lysine) with a polyethylene glycol spacer on the surface of melamine

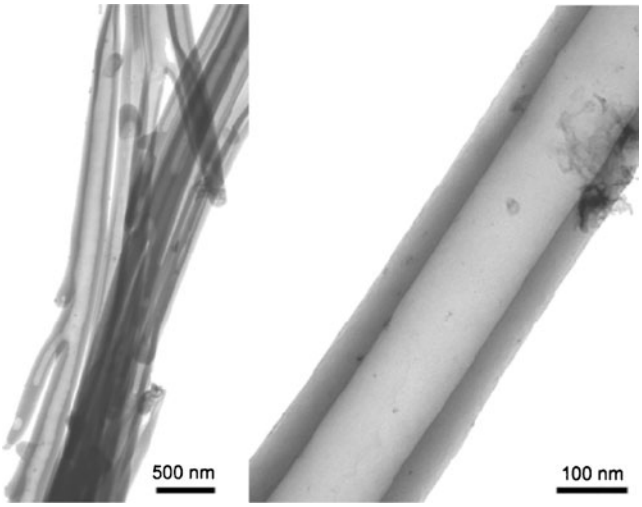

Fig. 5 Transmission electron microscope (TEM) images of protein nanotubes composed of avidin and GOx. Reprinted with permission from Ref [83] (copyright 2009, The American Chemical Society)

formaldehyde (MF) microparticles, followed by the dissolution of the MF core with 0.1 M HCl [84]. The hollow microcapsules thus prepared contain avidin in the capsule wall. In contrast, a polyelectrolyte microcapsule containing avidin in the cavity was also prepared by the alternate deposition of cationic and anionic polymers on the surfaces of $CaCO_3$ microparticles doped with avidin [85]. The avidin-containing microcapsule exhibited uptake and release of biotin derivatives.

LbL architectures sensitive to external stimuli can be constructed through avidin–biotin binding. 2-Iminobiotin-labeled poly(ethyleneimine) (ib-PEI) has been used to construct an LbL architecture consisting of avidin and ib-PEI (Fig. 6) [86]. It is known that avidin binds 2-iminobiotin less strongly than biotin and that the binding constant is pH dependent; the binding constant is 2.9×10^{10} M^{-1} in basic media, while it decreases to 10^3 M^{-1} in acidic solutions as a result of protonation to 2-iminobiotin. Indeed, the avidin/ib-PEI architecture was decomposed by changing the environmental pH from basic to weakly acidic, or by adding biotin to the solution in which the LbL architecture was immersed, as illustrated in Fig. 6. In addition, an avidin/ib-PEI architecture prepared on a Pt electrode can be disintegrated in response to the application of electrode potential [87]. This LbL architecture was shown to decompose upon the application of +0.9 or +1.0 V of electrode potential in 1 mM buffer at pH 9.0, probably due to a local pH change induced by the electrolysis of water in the vicinity of the electrode surface. The electrochemical decomposition was highly accelerated in the presence of hydrogen peroxide (H_2O_2) as a result of the electrolysis of H_2O_2 at a lower electrode potential, +0.6 V, according to Eq. 1 below [88]. It may be possible to couple the avidin/ib-PEI system with oxidase enzymes that produce H_2O_2 through the catalytic reaction, such as GOx, to develop combined biosensing and delivery systems.

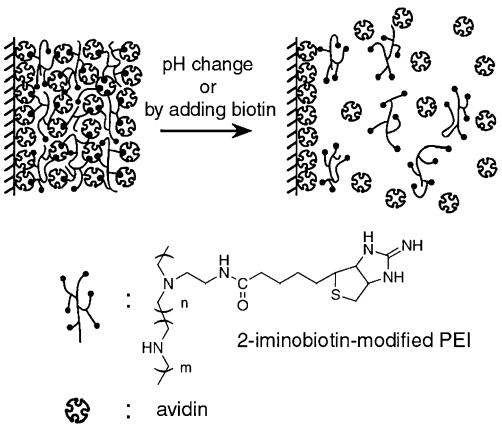

Fig. 6 The disintegration of the avidin/ib-PEI assembly upon changing the pH or adding biotin. Reprinted with permission from [86] (copyright 1995, The American Chemical Society)

$$H_2O_2 \rightarrow O_2+2H^+ +2e^-. \quad (1)$$

Protein architectures constructed through Con A–sugar interactions

Con A is a member of the lectin protein family, and is known to contain four binding sites to sugars such as D-glucose and D-mannose [89]. The binding constants of Con A to D-glucose and D-mannose are reported to be 0.8×10^3 and 2.2×10^3 M^{-1}, respectively [90]. Because of the specific binding of Con A to sugars, Con A has been utilized to immobilize sugar-labeled proteins onto solid supports in order to construct biosensing devices [91]. Kunitake and coworkers first used Con A to develop LbL films in combination with glycogen, a polysaccharide composed of D-glucose units [92, 93]. Then, in 1997, Scheper and coworkers first reported LbL-deposited enzyme films composed of Con A and glycoenzymes [94]. Later, our own group prepared LbL architectures composed of Con A and sugar-labeled enzymes on the surface of an electrode in order to develop an enzyme biosensor [30, 95]. One of the benefits of using Con A to construct LbL films is that certain kinds of enzymes, such as GOx and HRP, can be used without labeling because such enzymes intrinsically contain polysaccharide chains on their molecular surfaces [94, 96]. This clearly contrasts with avidin–biotin systems, in which enzymes have to be labeled with biotin before use. Zeng and coworkers constructed Con A/HRP multilayer films on the surface of a glassy carbon electrode in order to

generate an electrochemical sensor that is sensitive to phenylhydrazine [97].

Lin's group has studied enzyme biosensors prepared using Con A-containing LbL films (Fig. 7) [98–102]. They prepared Con A/HRP films on the surface of an Au electrode and evaluated the amperometric response of the sensor to phenols [98], thiols [99], and sulfide ion [100]. For the latter two cases, the measurements were based on the inhibition of the electron mediation reaction by quinine/hydroquinone. For all sensors tested, four bilayer (Con A/HRP)$_4$ film-modified electrodes afforded the highest response due to enhanced loading of HRP, while the responses of sensors modified with thicker films reached a plateau, probably due to limiting diffusion of the substrates. These sensors are sufficiently sensitive to the substrates; the lower detection limits were in the submicromolar range. The same group successfully constructed bienzyme sensors based on a combination of Con A, HRP, and GOx for the determination of phenols and aromatic amines [101]. The HRP/GOx bienzyme system was further coupled with CNTs to enhance the response [102]. These CNT-based bienzyme sensors could be used to determine glucose in diluted serum without pre-processing.

Recently, Hu and coworkers reported pH-dependent electrocatalysis on electrodes modified with LbL films composed of Con A and enzymes [103–106]. A Con A/dextran LbL film-coated electrode was further modified with myoglobin (Mb), and the catalytic reductions of O_2 and H_2O_2 at the electrode were studied [103]. The pH-dependent electrochemical reactions of $Fe(CN)_6^{3-}$, $Ru(NH_3)_6^{3+}$, and ferrocene carboxylate ions on the Con A/dextran LbL film-coated electrode were studied using cyclic voltammetry [104]. The modified electrode exhibited a well-defined reversible voltammogram for the $Fe(CN)_6^{3-}$ ion at pH 4.0, while the redox reaction was fully suppressed at pH 9.0 due to the electrostatic repulsion between the $Fe(CN)_6^{3-}$ ion and the negatively charged Con A in the LbL film (Fig. 8A). The redox current of the modified electrode was repeatedly switched on and off (Fig. 8B). Interestingly, the pH dependence of the redox reaction was reversed when the $Ru(NH_3)_6^{3+}$ ion was used in place of the $Fe(CN)_6^{3-}$ ion; the electrochemical reaction was suppressed at pH 4.0 due to the electrostatic repulsion between the $Ru(NH_3)_6^{3+}$ cation and positive charges in the Con A/dextran film. The same group also studied pH-switchable catalytic reactions of electrodes modified with Con A/HRP and Con A/GOx films [105, 106]. For both systems, the enzyme-coupled electrode reactions were regulated as a function of pH and the number of enzyme layers in the LbL film. These findings support the possible use of the Con A/enzyme LbL films for the development of biosensor interfaces that can be switched in response to pH.

LbL films constructed using Con A may be sensitive to sugars because these films rely on the reversible binding

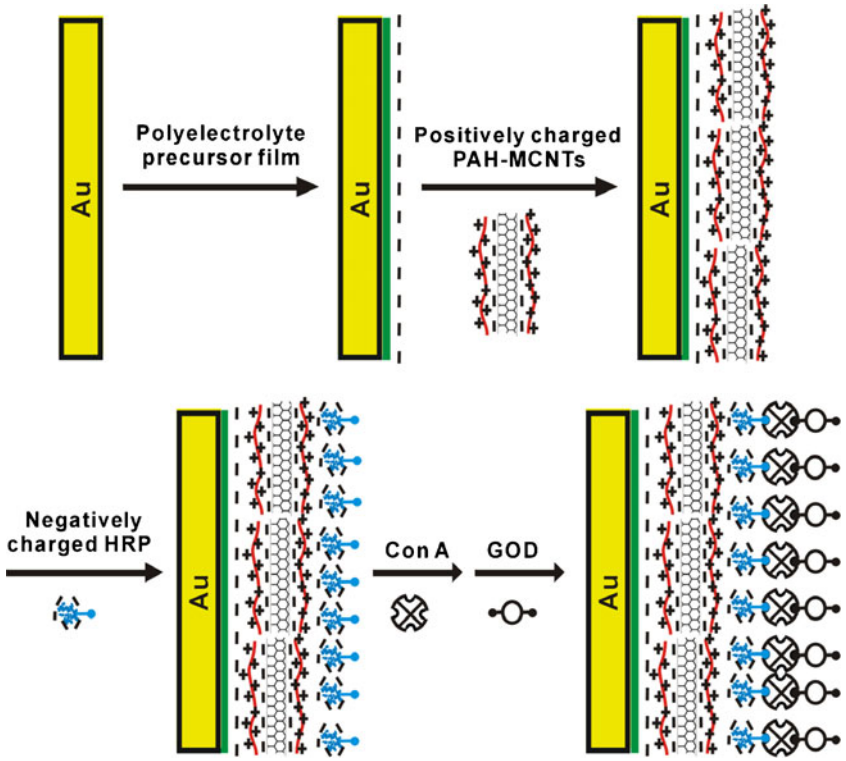

Fig. 7 Construction of an enzyme LbL film through Con A/sugar interactions on the surface of an electrode. Reprinted with permission from [102] (copyright 2010, Elsevier)

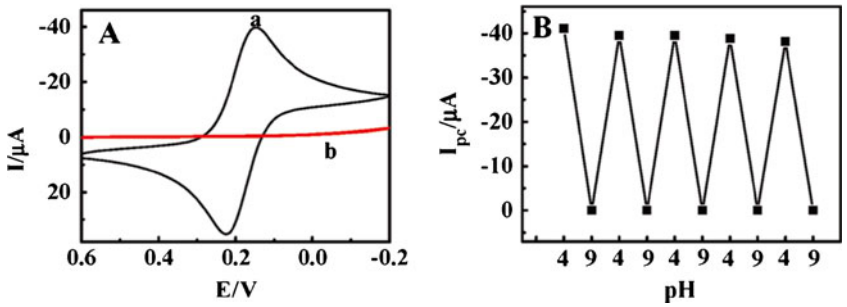

Fig. 8 **A** Cyclic voltammograms of the $Fe(CN)_6^{3-}$ ion on the Con A/dextran LbL film-modified electrode at pH 4.0 (*a*) and pH 9.0 (*b*). **B** pH-dependent switching of the reduction current. Reprinted with permission from [104] (copyright 2009, The American Chemical Society)

between Con A and sugar residues. In fact, Chinnayelka and McShane investigated fluorescence sensors for glucose that were based on microcapsules constructed through the LbL deposition of a fluorescence-labeled dextran and Con A [107]. The fluorescence resonance energy transfer (FRET) in the microcapsules was dependent on the concentration of glucose. These results can be explained by changes in the geometry of dextran and Con A originating from the displacement of dextran from Con A by the added sugar, demonstrating the feasibility of using Con A-based LbL microcapsules as optical biosensors. Our group has studied the sugar-induced disintegration of Con A/glycogen LbL films as a basis for electrochemical and optical sugar sensors, and they found that the Con A-based LbL films are decomposed in the presence of various sugars [10]. For example, the ten-bilayer (Con A/glycogen)$_{10}$ film was almost completely decomposed in 10–20 min in the presence of 10 mM methyl-α-mannose (Me-man) or methyl-α-glucose (Me-glu). Glucose also induced the decomposition of the LbL film depending on its concentration; 70–80% of the LbL film remained intact in the presence of 5 mM glucose, while 50 mM glucose induced nearly full decomposition. Based on these findings, LbL films composed of ferrocene-appended glycogen (Fc-glycogen) and Con A were used for the construction of electrochemical sensors for glucose (Fig. 9) [108]. The Con A/Fc-glycogen film-coated electrode exhibited a voltammetric signal originating from the redox reactions of Fc-glycogen in the film at ~0.3 V, and the peak height increased with the number of Con A/Fc-glycogen layers. On the other hand, the redox signal decreased upon exposure to 5–20 mM mannose and 10–100 mM glucose. Subsequently, we demonstrated that Con A/glycogen conjugates can be used for the optical sensing of sugars [109, 110] and for sugar-induced delivery of insulin [111].

Conclusion and outlook

Protein architectures including thin films and microcapsules can be successfully constructed through LbL deposition on the surfaces of optical probes and electrodes in order to generate biosensors. The usefulness of the avidin–biotin and Con A–sugar affinities for constructing these protein architectures has been demonstrated. The avidin–biotin systems are very advantageous because the binding between the molecules is so strong that it can be regarded as a covalent one. Another advantage of avidin-biotin systems is that biological materials such as proteins and DNA can easily be labeled with the biotin tag using activated biotin derivatives, which are currently commercially available. On the other hand, the binding affinity between Con A and sugars is not as high as that in avidin–biotin systems. Therefore, in some cases, one must pay attention to its stability, especially in acidic and basic environments. An advantage of Con A-sugar systems is that glycoproteins can be used to construct architectures directly without labeling. Protein-containing LbL architectures have found application in the development of enzyme sensors, immunosensors, and other biosensor interfaces. It has also been demonstrated that combining avidin–biotin and Con A–sugar systems with nanomaterials such as CNTs and metal particles can further improve biosensor performance.

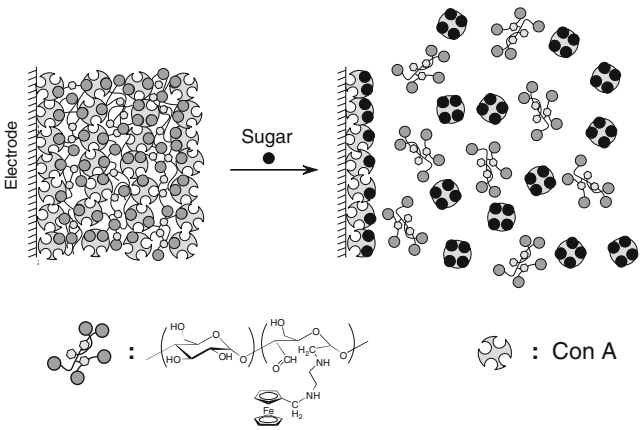

Fig. 9 Sugar-induced decomposition of Con A/Fc-glycogen LbL film for the electrochemical determination of sugars

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