

# Enzyme-Encapsulated Layer-by-Layer Assemblies: Current Status and Challenges Toward Ultimate Nanodevices

Katsuhiko Ariga, Qingmin Ji, and Jonathan P. Hill

**Abstract** Alternate layer-by-layer (LbL) adsorption has received much attention as an emerging methodology. Biocompatibility is the most prominent advantage of the LbL assembly process because the technique employs mild conditions for film construction. Most enzymes, especially water-soluble ones, have charged sites at their surfaces so that electrostatic LbL adsorption is suitable for construction of various protein organizations. In this review chapter, we summarize recent developments on enzyme-encapsulated LbL devices and their related functions where “encapsulated” does not always entail entrapment within spherical structures but generally includes immobilization of enzymes within the LbL structures. Recent examples, with various functions such as reactor sensors and medical applications, are described within a classification of structural types, i.e., thin films and spherical capsules. In addition to conventional applications, advanced systems including integration of LbL structures into advanced devices such as microchannels, field effect transistors, and flow injection amperometric sensors are introduced as well as recent developments in hybridization of LbL assemblies with functional nanomaterials such as carbon nanotube, dendrimers, nanoparticles, lipid assemblies, and mesoporous materials, all of which can enhance bio-related functions of LbL assemblies.

**Keywords** Devices · Enzymes · Hollow capsules · Layer-by-layer assemblies · Thin films

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K. Ariga (✉), Q. Ji, and J.P. Hill  
World Premier International (WPI) Research Center for Materials Nanoarchitectonics (MANA),  
National Institute for Materials Science (NIMS), 1–1 Namiki, Tsukuba, Ibaraki 305-0044, Japan  
and  
Core Research of Evolutional Science and Technology (CREST), Japan Science and Technology  
Agency (JST), Tokyo, Japan  
e-mail: [ARIGA.Katsuhiko@nims.go.jp](mailto:ARIGA.Katsuhiko@nims.go.jp)

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## Abbreviation

AChE	Acetylcholinesterase
ADP	Adenosine diphosphate
AFP	$\alpha$ -1-Fetoprotein antigen
ALP	Alkaline phosphatase
ATP	Adenosine triphosphate
BMS	Bimodal mesoporous silica
BSA	Bovine serum albumin
ChO	Choline oxidase
CNT	Carbon nanotube
Cyt P450 <sub>cam</sub>	Cytochrome P450 <sub>cam</sub>
DEAE	Diethylaminoethyl
DL-1	Delta-like 1
DMPC	Dimyristoyl phosphatidylcholine
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DPPC	Dipalmitoyl phosphatidylcholine
dsDNA	Double-stranded DNA
ENFET	Enzyme field-effect transistor
FAD	Flavin adenine dinucleotide
GA	Glucoamylase
GCE	Glassy carbon electrode
GDH	Glucose dehydrogenase
GOD	Glucose oxidase
HA	Hyaluronic acid
Hb	Hemoglobin
HRP	Horseradish peroxidase
HSA	Human serum albumin
IgG	Immunoglobulin G

ISFET	Ion-sensitive field-effect transistor
ITO	Indium tin oxide
LB	LangmuirBlodgett
LbL	Layer-by-layer
LDH	Lactate dehydrogenase
LOD	Lactate oxidase
Mb	Myoglobin
MWNT	Multi-wall carbon nanotube
OPH	Organophosphorus hydrolase
PAA	Poly(acrylic acid)
PAH	Poly(allylamine hydrochloride)
PAMAM	Poly(amidoamine)
PDDA	Poly(diallyldimethylammonium chloride)
PEI	Poly(ethyleneimine)
PET	Poly(ethylene terephthalate)
PGA	Poly(L-glutamic acid)
PLA2	Phospholipase A2
PLL	Poly(L-lysine)
PMA	Poly(methacrylic acid)
PMMA	Poly(methyl methacrylate)
POD	Peroxidase
PSS	Poly(styrenesulfonate)
PVP	Poly(vinylpyridine)
QCM	Quartz crystal microbalance
SAM	Self-assembled monolayer
SWNT	Single-walled carbon nanotube

## 1 Introduction

Miniaturization of functional structures into small areas or volumes as ultrasmall devices and machines has been extensively demonstrated in recent nanotechnology. However, more complex microdevices can be found in biological systems. Biomaterials such as proteins, polysaccharides, and nucleic acids have sophisticated structures which express highly specific and efficient processes of molecular recognition, materials conversion, and information transduction. In particular, enzymes that can modify chemical structure might be regarded as nanosized fabrication facilities. Higher functions of enzymes have been optimized over geological time periods during biological evolution. Following considerable efforts over the development of science and technology, we have now obtained knowledge and technology sufficient for mimicking and utilizing enzyme functions as seen in the recent progress in biotechnology, biomimicry, and supramolecular chemistry [1–20].

Research efforts have been made to synthesize artificial chemical structures that can promote enzyme-like reactions and to create various artificial enzymes

[21–24]. Molecular cavities of artificial enzymes are designed to recognize specific substrate molecules, stabilize reaction intermediates and/or their transition state, and provide reactive groups in appropriate proximity to the trapped substrate molecules. Supramolecular chemistry based on host-guest interaction is often used for molecular design of artificial enzymes. For example, imidazole-functionalized cyclodextrins [25, 26] and guanidinium-bearing molecular clefts [27–29] can promote hydrolysis of phosphate esters as a mimic of nuclease. Instead of synthesizing enzyme function purely by using organic chemistry, integration of natural enzymes into artificial structures can also be useful for exploiting enzyme function in artificial systems. Because enzymes have high affinities for biomolecules such as lipids and oligosaccharides, immobilization of enzymes at a biomolecular interface such as a lipid assembly is regarded as an effective strategy. For example, immobilization on an electrode of glucose oxidase (GOD) using the lipid-assisted Langmuir-Blodgett (LB) technique resulted in a glucose-sensitive real-time amperometric sensing device [30, 31]. Co-immobilization of enzyme (lactate dehydrogenase, LDH) and artificial receptors onto bilayer lipid membrane enabled control of the enzyme activity by chemical and photonic stimuli, resulting in a biological AND-type logic gate device [32–35]. Such interfaces can also be used as working media appropriate for recognition and accommodation of many different biological components [36–51].

Device preparation requires use of facilitative methodologies for the organization of biological components in a particular configuration. Self-assembled monolayer (SAM) [52–56] and LB methods [57–65] have been used for organization of functional elements in two-dimensional or layered structures, respectively. These methods offer opportunities for immobilization of functional components into well-organized structures. However, tight packing of the resulting formations sometimes obstructs intra-structural substrate/product transport that is often required for operation of biological devices. In addition, applicability to a wide range of materials is not always guaranteed for these methods (i.e., they are *too* specific). As a novel methodology that can compensate for deficiencies of the former techniques, alternate layer-by-layer (LbL) adsorption has been paid much attention as an emerging methodology [66–73]. As described in the following section, LbL provides a simple and inexpensive method to create layered structures of requisite thicknesses and layer sequences from a wide variety of components. Packing densities of components within LbL films is not always high, and this feature is advantageous for material transport across and along layers. Actually, these characteristics have been apparently keenly awaited in the construction of biological nanodevices. Recent research has proved the great applicability of the LbL technique not only for preparation of bio-related devices but also for producing various device structures, including sensors [74–76], photovoltaic devices [77–81], electrochromic devices [82–85], fuel cells [86–88], and rugate filters [89].

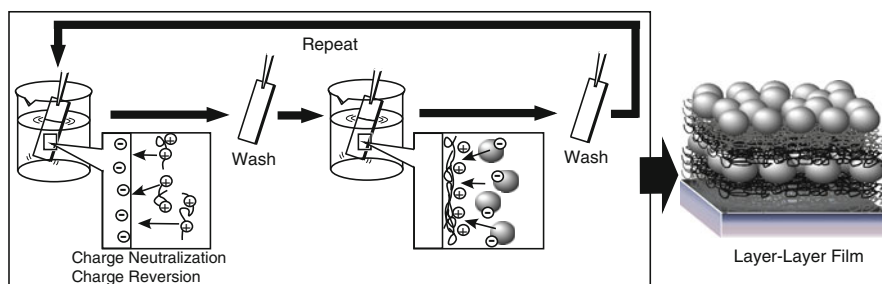
As one can easily imagine from the above-mentioned examples, it is high time to develop biological nanodevices based on LbL techniques. We here summarize recent developments on enzyme-encapsulated LbL devices and related functions where “encapsulated” does not always entail entrapment within spherical structures but generally includes immobilization of enzymes within LbL structures.

This review chapter describes both thin film and capsule type enzyme-encapsulated LbL devices. Initially, a brief explanation is given of the merits of immobilization of enzymes by using the LbL method, and this is followed by a description of recent advanced techniques and future perspectives.

## 2 LbL as Bio-Friendly Methods for Nanofabrication

The concept of LbL assembly was first proposed for charged colloidal particles in 1966 by Iler [90] who suggested that alternating adsorption of anionic and cationic species would result in the formation of regular multilayered assemblies. Decher and co-workers later realized this concept using mainly linear polyelectrolytes or bolaamphiphiles [91–94]. Concurrently, Mallouk and coworkers developed LbL-type assembly through interaction between  $\text{Zr}^{4+}$  ions and diphosphonic acids [95–97]. Subsequently, over the course of the 1990s the applicability of the LbL technique to a wide range of materials was extensively studied. Materials that can be used in LbL methods cover a wide range including conventional polyelectrolytes [98–100], conductive polymers [101–104], dendrimers [105–107], proteins [108–111], nucleic acids [112, 113], saccharides [114–116], virus particles [117], inorganic colloidal particles [118–120], quantum dots [121, 122], clay plates [123], nanosheets [124–129], nanorods [130–132], nanowires [133, 134], nanotubes [135, 136], dye aggregates [137–139], micelles [140–142], vesicles [143, 144], LB films [145], and lipid membranes [146, 147]. The LbL technique has now become one of the most powerful methods for preparation of nanostructured supermolecules.

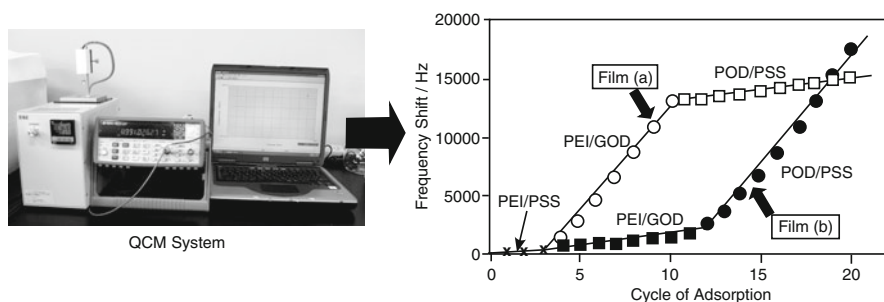
In most cases, the LbL assembly is carried out based on electrostatic interactions. As illustrated in Fig. 1, adsorption of counterionic species at relatively high concentrations leads to excess adsorption of the substances, as a result of charge neutralization and resaturation, finally resulting in charge reversal. This process was proved by Berndt et al. through a direct surface force measurement [148]. This simple mechanism allows us to assemble required components in various sequences using a low-cost experimental set-up. In fact, well-controlled nano-sized films can be fabricated only using beakers and tweezers. Driving forces for



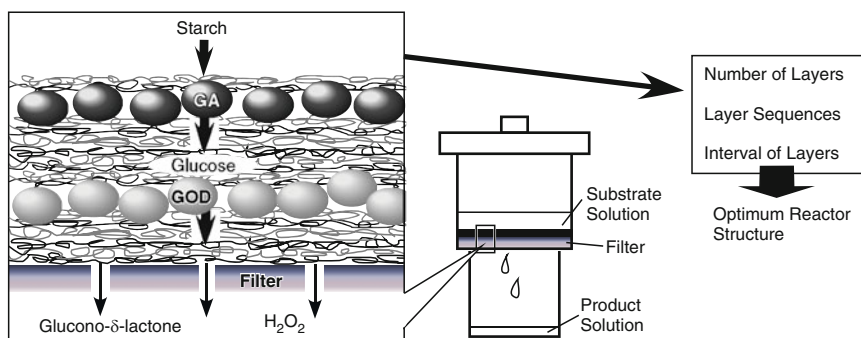
**Fig. 1** LbL assembly based on electrostatic interaction

LbL assembly are not limited to electrostatic interactions alone. Various interactions including metal-ligand interaction [149–152], hydrogen-bonding [153–155], charge transfer [156–158], supramolecular inclusion [159], bio-specific recognition [160–162], and stereo-complex formation [163, 164] can be used for LbL assembly. Wide freedom in assembling techniques is also guaranteed. For example, combination of spin-coating with LbL methods provides well-organized multilayer films within a very short time [165, 166]. LbL assembly using a spraying process can be easily controlled and is very reliable, permitting regular multilayer growth even under conditions where dipping fails to produce homogeneous films [167]. Automatic machines for film preparation have also been developed [168–171].

Biocompatibility is the most prominent advantage of the LbL assembly process because this technique requires mild conditions for film construction. Most proteins, especially those soluble in water, have charged sites on their surface, and so the electrostatic LbL adsorption is useful for the construction of various protein organizations. As has already been demonstrated, a large variety of proteins have been assembled in combination with oppositely charged polyelectrolytes [172]. Unlike other methods for protein immobilization, various types of layered structures can be prepared, where the number of layers and the layering sequence can be easily modified. Figure 2 illustrates primitive examples of LbL assemblies between enzymes and polyelectrolytes. The LbL films were prepared on the surface of a quartz crystal microbalance (QCM). Frequency shifts represent weight of adsorbed proteins and polyelectrolytes with nanogram precision [173–177]. Alternate assemblies of poly(ethyleneimine) (PEI) and GOD were assembled on the precursor film (four layers of PEI and PSS poly(sodium styrenesulfonate) (PSS) film) (Fig. 2a) [178]. Film growth was constant with an average frequency change of approximately 2,000 Hz for a single GOD-PEI layer. This assembly process was stopped at the PEI adsorption step of the 10th cycle followed by adsorption of PSS for conversion of the surface charge to negative. Then LbL assembly between peroxidase (POD) and PSS was carried out, where constant film growth was repeatedly observed with an average frequency change of approximately 180 Hz per a single POD-PSS layer. LbL assembly with a reversed protein sequence was also examined (Fig. 2b); that is, the POD-PSS film was initially assembled, and then the PEI-GOD film was assembled.



**Fig. 2** QCM evaluation of enzyme-polyelectrolyte LbL films. The QCM frequencies change in proportion to weight of adsorbed layers



**Fig. 3** Multi-enzyme reactor of LbL films containing GA and GOD on ultrafilter

The observed frequency changes of each assembly were essentially identical to those of the former example. These two kinds of protein-polyelectrolyte assembly processes are essentially independent and inner layers do not interfere with assembly of the outer layers.

These attractive features of the LbL assembly in protein adsorption allow us to construct multi-enzyme reactors with optimized layered sequences. Figure 3 illustrates examples of multi-enzyme reactors containing GOD and glucoamylase (GA) immobilized on an ultrafilter through which a substrate solution was passed [179]. The starting material of the reaction sequence is starch, which does not rapidly diffuse through the film because of its high molecular weight. Hydrolysis of the glycoside bond in starch by GA produces glucose. Glucose is converted to glucono-lactone by GOD with H<sub>2</sub>O<sub>2</sub> as a co-product. The effect of film organization on the reaction efficiency was investigated using the multi-enzyme films with varied assembling sequences. A film with the best efficiency satisfies two essential conditions: (1) matching of enzyme sequence with reaction scheme and (2) appropriate interval between two enzyme layers. Because the sequential reaction requires that the reaction with GA proceeds before the oxidation of glucose catalyzed by GOD, the order of the enzyme layers in these films agrees with the order of the sequential enzymatic reactions. Correct intervals between GA and GOD layers are necessary for efficient reaction. This is related to possible inhibition of GA activity by glucono-lactone that is one of the products in the second reaction. These examples strikingly demonstrate the importance of well-organized layered structures in design of multi-enzyme reactors.

LbL assembly improves the stability of immobilized proteins. For example, GOD immobilized in the LbL films keeps its high activity for more than 14 weeks at 4°C [180]. Most enzymes are denaturated and lose their activity at high temperature, but immobilization of GOD in the LbL films drastically enhances thermostability. A significant decrease in activity was not detected even after incubation at 50°C. Immobilization of protein molecules in films through interaction with a polyelectrolyte matrix effectively prevents denaturation of protein structures. An interesting advantage of the LbL assembly over the LB technique was found in

the effect of film thickness on reaction efficiency. Reaction efficiency per enzyme in GOD-immobilized LB films decreases as the number of layers increases. Difficulties of substrate diffusion cause deterioration of enzyme activity in well-packed thick LB films. Conversely, GOD activity in LbL assembly did not decrease even when the numbers of layers increased. Loosely assembled LbL structures are advantageous in reactor applications that require easy diffusion of substrates and products.

### 3 Enzyme-Embedded LbL Devices

#### 3.1 *Thin Film Type*

As described above, the LbL technique has a wide applicability in available components and variations of layered structures, and various kinds of LbL films composed of proteins and polyelectrolytes have been explored. Typical recent examples concerned with flat films are described here.

##### 3.1.1 Progress on Fundamental Aspects

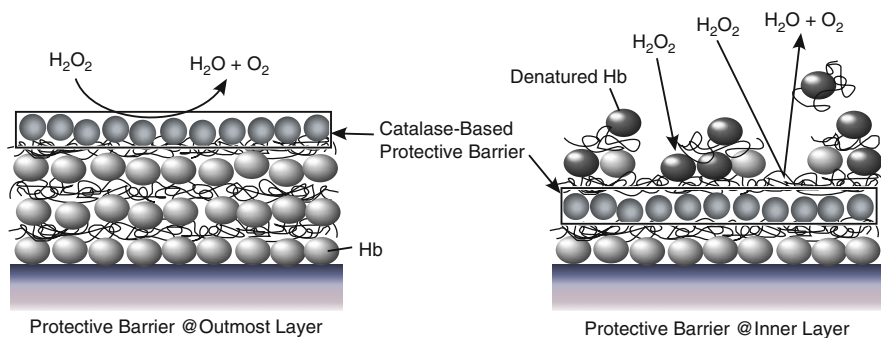
Although electrostatic interaction often stabilizes protein structures, it may actually be detrimental in cases of structurally less rigid species. Biospecific interaction such as recognition between lectin and saccharides would provide an ideal situation for protein assembly. For example, Lvov et al. demonstrated assembly of Concanavaline A with glycogen through biospecific recognition and with PEI through electrostatic interaction [181]. Biospecific interactions usually possess very high binding constants and are more effective than electrostatic interaction. Anzai et al. assembled avidin with biotin-attached poly(amine)s under conditions where electrostatic repulsions work between both positive species [182]. The deposition behavior and the structure of the avidin/poly(amine) multilayer films depend significantly on the molecular geometry of the poly(amine) components. The poly(amidoamine) (PAMAM) dendrimer with globular structure resulted in a monomolecular deposition of avidin. In contrast, multilayer deposition of avidin was observed for LbL assembly with randomly branched and linear PEI. Because biotinavidin interaction is highly specific, binding structures should be strongly influenced by the shape of poly(amine) components. This in turn means that the loading of biomaterials in the LbL thin films can be regulated by a suitable choice of polymeric materials.

Control of structures including conformation of proteins adsorbed on the surfaces of thin films is a target of some practical importance. Fibronectin is a highly flexible glycoprotein and plays essential roles in numerous biological phenomena including cell adhesion and spreading, wound healing, phagocytosis, and differentiation. These biological activities are known to be sensitive to conformation of fibronectin. Van Tassel and coworkers investigated the adsorption of fibronectin onto LbL films



of poly(allylamine hydrochloride) (PAH) and PSS [183]. Deposition density and thickness of fibronectin on a PAH-terminated positive surface were estimated as roughly double those on a negatively charged PSS-terminated film. Considerable enhancement of binding of monoclonal antibodies specific to the protein's cell binding site to fibronectin adsorbed on the PSS-layer was confirmed, which indicates that fibronectin on the PSS-layer is more accessible to incoming species. Fibronectin molecules individually adsorb onto a PSS-terminated film primarily in side-on-orientation, while those on a PAH-terminated film form clusters in end-on-oriented monolayers.

Various types of bioreactors have been fabricated based on the LbL technique. For this purpose, the surface modification of substrates for physical protein immobilization is also an important factor. Serizawa and coworkers proposed controlling the activity of  $\beta$ -galactosidase for hydrolysis of *p*-nitrophenyl- $\beta$ -D-galactopyranoside immobilized on supports coated with structurally regular poly(methyl methacrylate) (PMMA) using the stereocomplex, isotactic-PMMA and atactic-PMMA films [184]. They demonstrated that a slight difference of polymer surface structure strongly affects activities of immobilized enzymes, even though polymers have the same chemical component. Some enzymes can digest harmful chemicals and form as protective layers for inside weaker functional layers. Shutava et al. demonstrated catalase-based protective barriers to oxidative environments (Fig. 4) [185]. They examined the influence of a catalase layer located at different depths in hemoglobin/PSS LbL films on the kinetics of hemoglobin degradation under hydrogen peroxide treatment with respect to internal (film thickness and architecture) and external (hydrogen peroxide concentration) conditions. The highest activity in hydrogen peroxide decomposition was observed when the catalase layer was situated on top of the LbL multilayers. Hemoglobin (Hb) in such films was demonstrated to retain its activity for a longer period of time. Hu and coworkers also investigated the protective effect of exterior catalase layers using electroactive myoglobin (Mb) or horseradish peroxidase (HRP) in these films as a probe to monitor the extent of protein damage by  $H_2O_2$  [186]. The results similarly demonstrated that the outer catalase layers controlled the rate of  $H_2O_2$  entry into inner regions of the film.



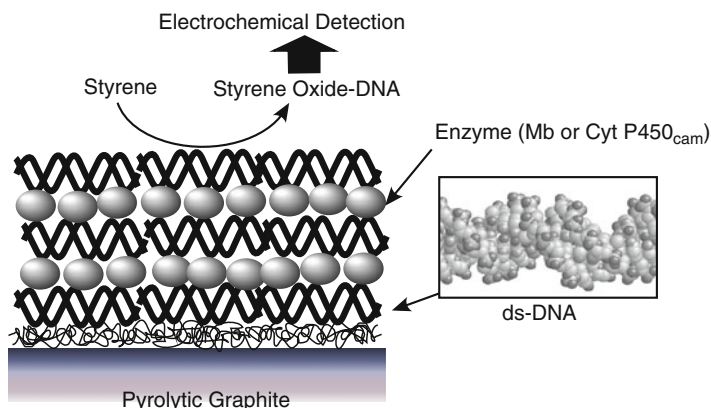
**Fig. 4** Function of catalase-based protective barrier to suppress destruction of enzyme layers

### 3.1.2 Sensor Applications

The LbL technique is undoubtedly one of the best methods to incorporate biological components into man-made devices. Therefore, sensor applications must be one of the most promising subjects for LbL assemblies of biomaterials. For example, Leblanc and coworkers used several bilayers of chitosan and poly(thiophene-3-acetic acid) as cushion layers for stable enzyme films [187]. The first five bilayers of the cushion layer allowed for better adsorption of organophosphorus hydrolase than the corresponding adsorption on a quartz slide. The immobilized enzyme becomes more stable and can be used under harsher conditions. The assembled LbL films can be used for spectroscopic detection of paraoxon, an organophosphorus compound. This cushion layer strategy provides a well-defined substrate-independent interface for enzyme immobilization, in which the bioactivity of the enzyme is not compromised. This leads to fast detection of paraoxon and quick recovery times.

Because immobilization of protein LbL films on various electrodes is a big trend of LbL-related applications, electrochemical analyses on the bio-active LbL films are important research targets. Rusling and coworkers are extensively developing LbL-based electrochemical sensors. For example, Lvov, Rusling, and coworkers assembled on gold electrodes LbL films of Mb or cytochrome P450<sub>cam</sub> (CytP450<sub>cam</sub>) with oppositely charged polyions, including DNA, acting as “electrostatic glue” [188]. Direct, reversible electron transfer between electrodes and proteins involved the heme Fe<sup>III</sup>/Fe<sup>II</sup> redox couple. The presence of oxygen induced electron transfer to the Fe<sup>II</sup>–O<sub>2</sub> complexes of these proteins resulting in production of hydrogen peroxide. Consequently, these LbL films may be used for electrode-driven enzyme-like catalysis, for example, for the epoxidation of styrene. They also pioneered DNA damage analyses using the LbL constructions. They assembled LbL films of Mb or Cyt P450<sub>cam</sub> and DNA on electrodes, which were activated by hydrogen peroxide, and the enzyme in the film generated metabolite styrene oxide from styrene metabolism. DNA damage in the human liver could then be mimicked through reaction between the resulting styrene oxide with double-stranded DNA (dsDNA) (Fig. 5) [189]. DNA damage was detected by square wave voltammetry by using catalytic oxidation with Ru(bpy)<sub>3</sub><sup>2+</sup> and by monitoring the binding of Co(bpy)<sub>3</sub><sup>3+</sup> (bpy = 2,2'-bipyridine). They proposed that their method could be suitable for in vitro screening of toxicity of organic metabolites and their parent compounds. Guo and coworkers also demonstrated that the GOD-incorporated LbL multilayer film could be used as a photoelectrochemical sensor for the detection of in situ oxidative DNA damage through the metal-induced Fenton reaction [190]. DNA damage was detected by monitoring the change of photocurrent of an indicator such as a Ru complex. Differences in affinity with the sensor films between intact and damaged DNAs induced changes in photocurrent signals.

Ram et al. reported electrochemical properties of GOD assembled with a conductive polypyrrole through LbL techniques [191]. The results obtained provided information, with respect to the electron transfer processes, on the spatial arrangement of GOD molecules on the polypyrrole surfaces, which might give crucial and important insights for construction of glucose-responsive biosensors. Wollenberger



**Fig. 5** LbL sensor for DNA damage detection

and coworkers prepared LbL films of cytochrome c and sulfite oxidase with the aid of poly(aniline sulfonate) [192], which were co-immobilized on the surface of a gold electrode. Electro-catalytic properties upon sulfite addition to the electrodes were characterized and the role of the different components in the electron-transport chain was clarified. They suggested that this multilayer electrode could act as an anode in a bio-fuel cell or could be exploited as a biosensor for the detection of sulfite, which is used as a preservative in wine and other foodstuffs. Balkenhohl et al. prepared multi-enzyme LbL films of cytochrome c (inner layer) and laccase (outer layer) on a gold electrode using poly(anilinesulfonic acid) as a polyelectrolyte component [193]. The catalytic oxygen reduction current was linearly dependent on the oxygen concentration in solution indicating that cytochrome c molecules in the inner layer of the LbL multilayer film function as biological redox carriers effectively transferring electrons from the gold electrode through the multilayer film towards the immobilized laccase molecules at the outer layer. Chen and coworkers fabricated  $\text{Fe}_3\text{O}_4$  multilayer film and reported its application in promoting direct electron transfer of hemoglobin [194]. Characteristic superparamagnetic properties of  $\text{Fe}_3\text{O}_4$  nanoparticles persisted in the film and could be controlled simply by changing the assembly layers. In addition, the LbL multilayer film exhibited good compatibility with proteins, the catalytic activity of the immobilized hemoglobin was retained, and the direct electron transfer with the underlying glassy carbon electrode (GCE) could be realized.

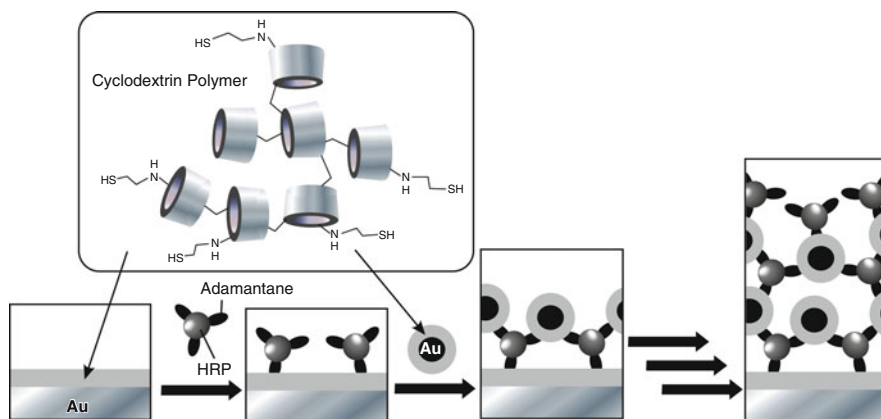
### 3.1.3 Inclusion of Functional Nanomaterials

One of the most outstanding features of LbL assemblies lies in the great freedom in selection of assembled components, which permits us to introduce various components in the LbL structures to improve sensor performance. Incorporation of nanomaterials such as gold nanoparticles was demonstrated to enhance

sensitivity of the LbL sensors. Zhang et al. used adsorption processes of enzymes with the non-enzymatic LbL layers [195]. They first prepared LbL films using poly(diallyldimethylammonium chloride) (PDDA) and gold nanoparticles on a gold electrode, and incubation of this electrode resulted in absorption of GOD into the LbL films. Electrochemical impedance measurements revealed that these LbL films have lower electron transfer resistance, due to the presence of gold nanoparticles, than that of a conventional assembly of PDDA and GOD. Chen and coworkers fabricated LbL assembled films composed of chitosan, gold nanoparticles, and GOD onto the surface of the Pt electrode [196]. GOD immobilized in the film showed excellent catalytic properties for glucose substrate, and the gold nanoparticles not only provided a suitable environment for stable immobilization of GOD but also effectively improved the electron transfer between analyte and electrode surface. Hu and coworkers reported enhanced formation of gold nanoparticles through in situ electrodeposition in Mb-loaded LbL films of chitosan and silica nanoparticles [197]. Positively charged chitosan and negatively charged silica nanoparticles were alternately assembled on the graphite electrode which was then immersed in Mb and  $\text{HAuCl}_4$  solution. The  $\text{Au}^{\text{III}}$  loaded in the films was subsequently electrochemically reduced, giving Au nanoparticles and providing nanocomposite films. Compared with films without Au nanoparticles inside, the composite films exhibited improved electrochemical and electrocatalytic behavior of Mb, because Au nanoparticles formed inside the films were located in proximity to Mb and acted as electron bridges between Mb molecules, inducing activity in more Mb molecules in the films to become electroactive. Furthermore, the permeability or porosity of the films played an important role in realizing the direct electrochemistry of Mb. Yuan and coworkers reported an amperometric enzyme immunosensor for  $\alpha$ -1-fetoprotein antigen (AFP) using gold electrodes modified with LbL films containing HRP, anti-AFP, gold nanoparticles, and thionine on Nafion [198]. Sun and coworkers fabricated a bienzymatic sensor by covalently attachment of periodate-oxidized GOD and HRP on controlled multilayer films of sulfonate-capped gold nanoparticles/thionine as a mediator of electron transfer [199]. The resulting biosensor film exhibited good electrocatalytic response toward glucose and the electrocatalytic response increased with the number of thionine layers.

Villalonga and coworkers used specific inclusion at the cyclodextrin cavity as a supramolecular concept for preparation of an enzyme-immobilized LbL sensor [200]. This strategy was based on the supramolecular immobilization of alternating layers of HRP (either modified with 1-adamantane or  $\beta$ -cyclodextrin-branched carboxymethylcellulose residues) on Au electrodes coated with polythiolated  $\beta$ -cyclodextrin polymer. Frago et al. adopted a similar strategy by the immobilization of a first layer of thiolated cyclodextrin polymer on a gold electrode followed by the supramolecular capture of adamantane-modified HRP (Fig. 6) [201]. Successive enzyme layers were then attached using cyclodextrin-modified gold nanoparticles as linkers. This method provides a regenerable surface that can be further used for the attachment of new enzyme layers.

Dendrimers are branched polymers of controlled structures where functional groups can be immobilized in a predesignated manner [202–209]. Therefore, use



**Fig. 6** Regenerative LbL-based sensor with concept of supramolecular inclusion by cyclodextrin conjugate

of dendrimers instead of the conventional polyelectrolytes usually used for the LbL processes would be attractive for construction of functional films. For example, Kim and coworkers developed a reagentless biosensor using the surface-functionalized fourth generation PAMAM dendrimers partially modified with redox-active ferrocenyl groups [210]. The modified dendrimers were used for the construction of LbL films with periodate-oxidized GOD on a gold electrode. By taking into consideration the surface concentration of deposited ferrocenyls, active enzyme coverage, and electrode sensitivity, the degree of ferrocenyl functionalization could be optimized. Zucolotto et al. assembled LbL films of Cl-catechol 1,2-dioxygenase with PAMAM dendrimers [211]. The Cl-catechol 1,2-dioxygenase kept its activity in the LbL films, which can be used for detecting catechol in solutions at very low concentrations ( $10^{-7}$  to  $10^{-10}$  M) by employing optical and electrical measurements. The same research group recently reported an LbL method for dendrimer-assisted immobilization of alcohol dehydrogenase (ADH) onto Au-interdigitated electrodes for ethanol detection using electrical capacitance measurements [212]. By combining the electrical capacitance data from the three electrodes using principal component analyses, the system was able to detect and distinguish between ethanol solutions with concentrations as low as 1 ppmv (parts per million by volume).

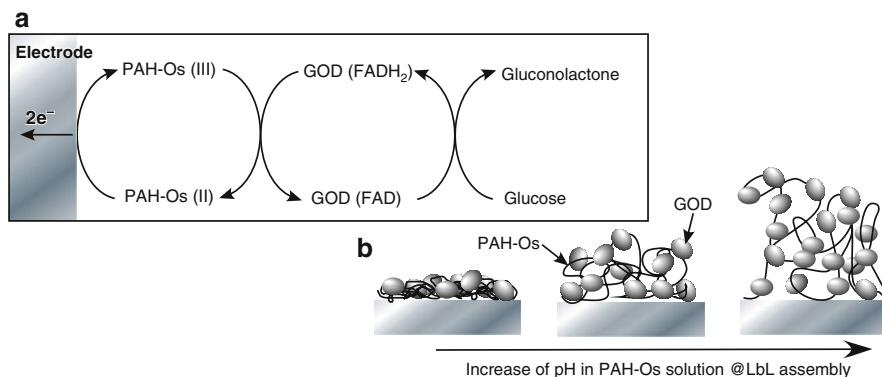
### 3.1.4 With Electroactive Mediators and Wires

Incorporation of electroactive components often improves performance of electrochemical sensors. Ferreira et al. exploited the LbL technique to produce sensitive and stable glucose biosensors by the immobilization of GOD onto an indium tin oxide (ITO) substrate modified with a Prussian Blue layer [213]. The high sensitivity was attributed to the ultrathin nature of the LbL film in addition to the low operating potentials that could be used due to the efficient catalysis of  $\text{H}_2\text{O}_2$  produced in the

enzymatic reaction in the presence of Prussian Blue. These LbL biosensors showed high stability, as demonstrated in tests performed for approximately 20 days with a reliable amperometric response in almost constant sensitivity for the whole period of experiments. Tight linking of GOD to the electrode may result in such high stability. Lin and coworkers prepared LbL films of HRP with PSS in which low molecular dye methylene blue was pre-adsorbed [214]. Methylene blue can act as an electron mediator to promote the electrochemical reaction between the immobilized HRP and the electrode surface. The prepared LbL films are useful for the sensing layer of reagentless  $\text{H}_2\text{O}_2$  biosensor.

Electroactive carbon nanotubes are especially attractive components for LbL-based electrochemical sensors. Yan et al. reported fabrication of a transparent and flexible glucose biosensor where multi-wall carbon nanotubes (MWNTs) and GOD were assembled on a polymer substrate [215]. Negatively charged MWNTs and GOD were LbL assembled on the polymer substrate modified by Au. The film showed a porous structure where the assembled MWNTs were mainly in the form of individual tubes or small bundles. Electrochemical studies demonstrated that the multilayer membrane exhibits remarkable electrocatalytic activity to detect glucose molecule. Schmidtke and coworkers prepared LbL-based biosensors from enzyme-coated single-walled carbon nanotubes (SWNTs) and redox polymers [216]. Gold electrodes were first functionalized with negatively charged 11-mercaptoundecanoic acid followed by alternate assembly in solutions of a positively charged redox polymer, poly(vinylpyridine) (PVP) having Os complex and a negatively charged GOD containing SWNTs. The oxidation peak currents of this film during cyclic voltammetry increased 1.4–4.0 times as compared to films without SWNTs. Similarly the glucose electro-oxidation current also increased (6–17 times) when SWNTs were present. By varying the number of multilayers, the sensitivity of the sensors could be controlled.

If polyelectrolytes carry redox-active side chains, they can work as electroactive wires to bridge proteins and electrode. Calvo et al. demonstrated that only a small fraction of active GOD molecules electrostatically adsorbed onto PAH with Os complex on the electrode are actually wired (Fig. 7a) [217], even though there is an excess of osmium groups in the polymer with respect to the enzyme concentration. This electrochemically wired enzyme was analyzed with the catalytic current dependence on glucose concentrations for the ping-pong mechanism of glucose oxidation. The catalytic current increased with the number of LbL layers because of the increase in the enzyme loading while the efficiency of enzyme  $\text{FADH}_2$  oxidation by the Os redox polymer remained almost constant. They also made a systematic study of the structure, thickness, and electrocatalysis for the oxidation of  $\beta$ -D-glucose by GOD, mediated by an Os bipyridine complex modified PAH, in LbL multilayer structures obtained at different pH of the dipping redox polyelectrolyte solution (Fig. 7b) [218]. A systematic change in the pH of the PAH-Os dipping solution leads to a variation in the linear charge carried by the redox polyelectrolyte and strongly affects the polyelectrolyte-enzyme multilayered structure and catalytic properties. Sun and coworkers assembled LbL films through alternate deposition of negatively charged HRP and positively charged quaternized PVP with Os complex



**Fig. 7** (a) Glucose sensing by electrically wired LbL assembly. (b) pH dependence of amount of immobilized GOD

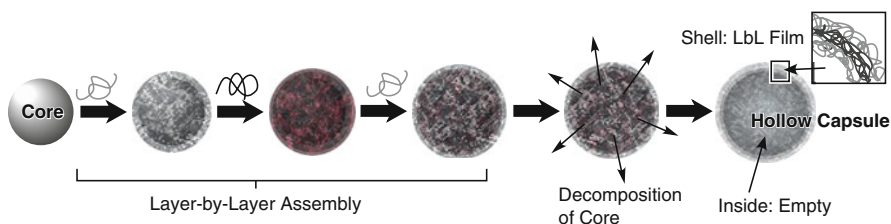
on the negatively-charged alkanethiol-modified gold electrode surface [219]. This enzyme electrode of multilayer films was sensitive for the electrocatalytic reduction of hydrogen peroxide and can be used as amperometric sensors for hydrogen peroxide.

### 3.2 Particle and Capsule Type

One of the most influential innovations in the history of LbL technology so far must be LbL assembly on a colloidal particle with subsequent hollow capsule preparation [220–223]. In the first decade of LbL technology, researchers regularly assembled films on a flat solid support of visible dimensions. However, the mechanism of the LbL assembly does not exert any limitations on the size of supports or their shape. Therefore, LbL assembly on microscopic solid surfaces dispersed in solution is reasonable, opening the way to fabrication of both three-dimensional structures and nano/micro-sized objects through the LbL process. As shown in Fig. 8, the concept of the assembly is simple. LbL films are assembled sequentially on a colloidal core in a similar way to conventional LbL assembly on a flat plate. Dissolution of the central particle core upon exposure of the particles to appropriate solvents then results in hollow capsules.

The construction of LbL multilayer films of biomaterials on colloid particles is of particular interest in applications where a microscopic contact is essential, such as protein interaction and cell communication, and where high surface area is desirable, such as in catalysis. For example, enzyme LbLs on particle surfaces are useful for biorelated catalysis since microscopic objects of higher surface area can potentially yield higher enzymatic reaction efficiencies than their planar film counterparts. If the LbL assembly is conducted on microparticles with certain functions, we can





**Fig. 8** LbL assembly on colloidal particle and subsequent preparation of hollow capsule

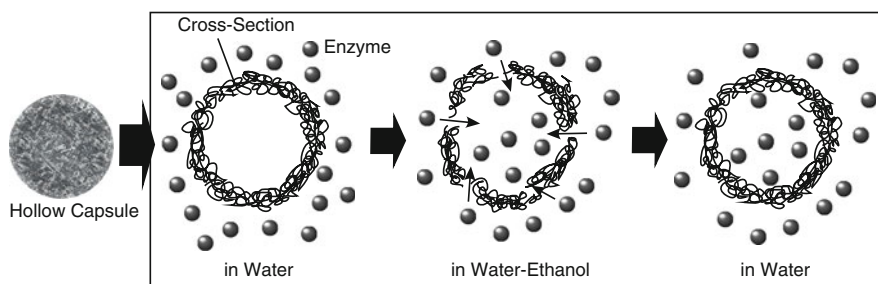
combine properties from both LbL films and core materials. For example, the LbL assembly of enzymes on magnetic particle enables us to use biocatalytic activities and magnetic properties, leading to freely collectable biocatalytic microparticles.

### 3.2.1 Progress on Fundamental Aspects

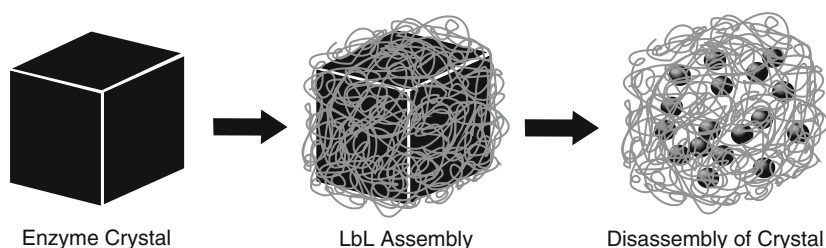
LbL hollow capsules have excellent potential in important biological applications, such as drug delivery. Biocompatible polymers such as chitosan and chondroitin sulfate can be used as polyelectrolyte components. Storage and controlled release of small drug molecules using LbL hollow capsules are both possible and even large proteins can be entrapped in the LbL hollow capsules. For example, the capsule can be used as a nano-container for enzymes (Fig. 9) [224]. Stable hollow polyelectrolyte capsules were first produced by LbL assembly between PAH and PSS on melamine formaldehyde particles followed by particle decomposition at low pH. Capsules prepared in this way and suspended in water do not allow the enzymes to permeate to the interior. However, enzyme permeability can be induced by exposing the capsules to a water–ethanol mixture. Resuspension in water closes the capsule pores, leading to entrapment of the enzyme. The hollow capsules are permeable by small molecular species, and the entrapped enzyme then exhibits biocatalytic activity. Greater loading of enzymes into the LbL hollow capsules can be performed using enzyme crystals as core material (Fig. 10) [225]. This approach leads to extremely high enzyme loading in a nano-sized capsule. Although the enzymes are coated with very thin polyelectrolyte films, the entrapped enzymes are quite stable against protease degradation.

If template synthesis is united with LbL techniques, as depicted in Fig. 11, then formation of self-assembled microtubes can be achieved if the template contains regular pores. Sequential assembly of polymers within a controlled pore followed by template removal can result in self-standing tubular structures. Li et al. reported fabrication of microtubes based on hydrogen-bonding LbL self-assembly from poly(acrylic acid) (PAA) and PVP [226]. They also demonstrated successful removal of the PAA and the resulting porous walled tubes could also be useful as carriers in drug delivery or as catalyst supports. The same research group applied this strategy to fabrication of microtubes from biocomponents. Human serum albumin (HSA) is structurally stable under acidic or basic conditions because of strong

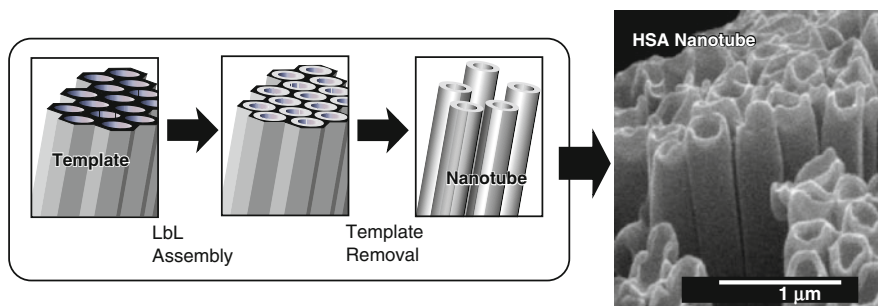




**Fig. 9** Entrapment of enzymes into hollow capsules through solvent treatment



**Fig. 10** Hollow capsule formation using enzyme crystal as a core material for high loading of enzyme



**Fig. 11** Template synthesis of protein nanotube through LbL assembly. Micrographic image of HSA nanotubes were kindly provided by Prof. Junbai Li

binding sites within its subdomains [227, 228]. The surface charge of human serum albumin can be modified to be either more positive or more negative by varying pH, making it possible to form an LbL assembly only from HSA that has smooth and clean surfaces with a wall thickness of around 30 nm and a length of 60 μm.

LbL capsules and related objects have great potential for biomaterial fabrication and applications. Research activity on LbL capsules is becoming extensive. Parts of recent examples are described below.

### 3.2.2 LbL Assembly on Colloidal Particles

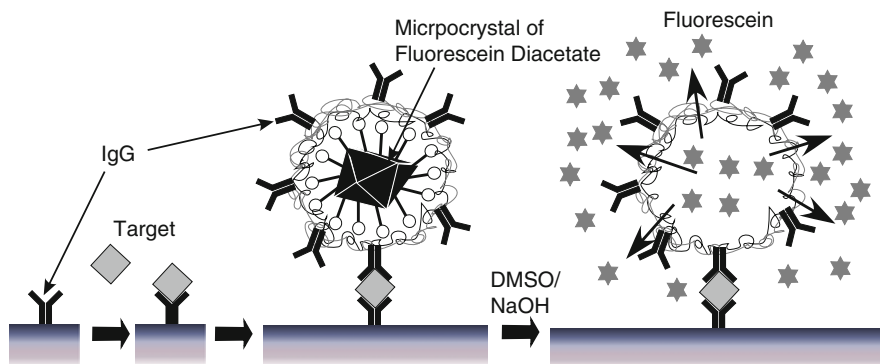
In the one of the simplest yet pioneering examples, Schüler and Caruso assembled GOD on submicrometer-sized polystyrene spheres through LbL adsorption with PEI [229]. The high surface area afforded by colloids can be exploited to minimize substrate diffusion effects that limit the use of enzyme multilayer films assembled on a planar surface. Therefore, the high surface area of the bio-multilayer coated particles formed were efficiently utilized in enzymatic catalysis. The same researchers similarly investigated bicomponent colloidal reactors [230]. The coupled enzymatic reaction between GOD and POD assembled on the same poly(styrene) particle (bicomponent enzyme film) was demonstrated. Enzyme multilayers constructed by the alternating adsorption of polyelectrolyte and premixed enzyme/polyelectrolyte complexes exhibited a lower activity than the assemblies prepared from the uncomplexed species. The successful recovery and reuse of magnetic-functionalized particles coated with enzyme layers with a magnet was also demonstrated. Ball and coworkers investigated stability of immobilization of alkaline phosphatase (ALP) as a model enzyme on the surface of Affi-gel heparin beads functionalized through LbL assembly between biopolyelectrolytes, poly(L-glutamic acid) (PGA) and poly(L-lysine) (PLL) [231]. The enzyme was adsorbed either on the top of the LbL film or embedded under five polyelectrolyte layers. When ALP was adsorbed on the top of the LbL architecture, initial detachment was detected but no further desorption was observed over storage times larger than 3 months. In the case of ALP embedding under two PLL-PGA bilayers, no enzyme was released and the embedded enzyme retained about 30% of its initial activity after 3 months of storage.

On the other hand, reevaluation of the stability of enzymes entrapped in LbL layers on the colloidal core has now become an important subject. Ansorge-Schumacher and coworkers very recently pointed out the importance of the combination of capsules' polyelectrolyte, reactants, and enzyme [232]. The biocatalysts (lipase B) were adsorbed to  $\text{CaCO}_3$  or DEAE-cellulose colloidal cores on which LbL multilayers of PAH and PSS had been coated. Residual activity of the enzymes investigated gradually decreased as the number of LbL films increased. The activity decrease was attributed to mass transfer restrictions as well as direct interactions between polyelectrolytes and catalytically active molecules. It was proposed that preselection should be performed based on all available knowledge on the complex interactions between polyelectrolytes, biocatalyst, and reactants with regard to activity/productivity and stability. Zhu et al. used alginate microspheres for combined physical and chemical immobilization in order to improve stability of encapsulation and activity [233]. GOD was successfully encapsulated into calcium-cross-linked alginate hydrogel microspheres. LbL coatings were applied on alginate/GOD microspheres. The technique presented was shown to be a practical and effective way to fabricate GOD-encapsulating alginate microspheres for future use as implantable glucose biosensors.

Sensor applications are also a big target for LbL-coated colloidal systems. Rusling and coworkers used enzyme-DNA biocolloid systems for detection of DNA adducts and reactive metabolites using chromatography-mass spectrometry

analyses [234]. In their approach, silica microbead bioreactors coated with DNA and enzymes such as  $\text{cyt P450}_{\text{cam}}$  and Mb were fabricated to measure reactive metabolites and DNA-adduct formation rates relevant to genotoxicity screening using the LbL technique. The LbL-coated colloid particles formed were tested through oxidation of guaiacol, styrene, and (4-methylnitrosoamino)-1-(3-pyridyl)-1-butanone. Enzyme turnover rates for formation of reactive metabolites were monitored using gas chromatography/mass spectrometry and liquid chromatography-mass spectrometry. Dramatic improvements in surface area to volume ratio over similar films on macroscopic surfaces were confirmed. They claimed that the proposed method would be particularly useful as a chemical complement to established microbiological assays for in vitro toxicological screening, enzyme kinetic, inhibition analysis, and drug discovery. Viswanathan and Ho reported dual electrochemical determination of glucose and insulin [235]. The LbL films on the ferrocene microcrystal followed by anti-insulin antibody sensitization were employed for the biolabeled ferrocene microcapsules. The prepared microcapsules were used to perform a sandwich immunoassay for the detection of insulin. Addition of releasing-reagent such as DMSO resulted in the release of a large amount of electrochemical signal-generating ferrocene molecules into the outer medium through the permeable capsules' wall. The released ferrocene molecules were then measured amperometrically. Simultaneously, glucose was also determined amperometrically using GOD immobilized on the electrode.

Trau et al. proposed an efficient immunoassay using LbL capsules assembled on fluorescent microcrystals (Fig. 12) [236]. Their strategy starts with encapsulation of microcrystalline fluorescein diacetate with an average size of 500 nm with the LbL multilayers of PAH and PSS. Molecules for biorecognition, (immunoglobulin G, IgG) were then attached at the surface of the LbL layer for sandwich immuno-assay. Following the immuno-reaction, the fluorescein diacetate core was dissolved by exposure to organic solvent, leading to the release of the fluorescein diacetate molecules into the surrounding medium. Amplification rates between  $70\times$



**Fig. 12** Highly sensitive immunoassay through sandwich assay using LbL capsule coated with IgG

and  $2,000\times$  of this microcrystal label-based assay compared with the corresponding immunoassay performed with direct fluorescently labeled antibodies were reported. The proposed technique has the potential to compete with enzyme-based labels since long incubation times are not required, thus speeding up bioaffinity tests. Tong and coworkers prepared LbL multilayers on drug crystals that could be used as a drug delivery system [237]. The LbL wall made of sodium alginate/chitosan multilayer film was directly deposited on indomethacin microcrystals. Erosion by pepsin induced release of indomethacin from the LbL composite. The release rate could be controlled by several factors. Increasing temperature of the LbL assembly effectively reduced the release rate of the drug because of improvements in the structure of the multilayer film. Cross-linking the neighboring layers of sodium alginate and chitosan with 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide also significantly reduced the enzymatic desorption and release rate. These factors are important for protection of the multilayer film from enzymatic erosion and for prolonged release intervals of the encapsulated drug.

### 3.2.3 Hollow Capsules

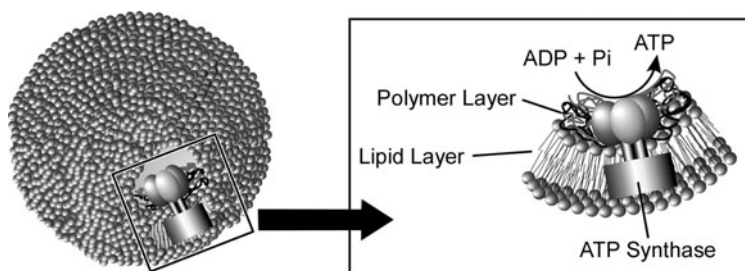
Hollow capsule structures are also useful in various applications. Lvov and coworkers reported enzyme-catalyzed polymerization of phenols using LbL capsules [238]. Their approach is based on selective permeability of capsule walls to monomer molecules and their impermeability to the resulting polymeric products. Biocatalysts and polymeric products cannot leave the capsules' interior because of their high molecular weight. HRP was encapsulated within PSS/PAH LbL capsule using a pH-driven pore opening mechanism. In this case, 4-(2-aminoethyl)phenol hydrochloride (tyramine) was used as a monomer giving easily detectable fluorescent polymeric products after addition of hydrogen peroxide into the system. A wide variety of enzymes and monomers can be used to provide new types of micro- and nanocomposites with desirable properties in a biologically friendly environment. It should also be possible to synthesize functional materials in the microcapsule such as light-emitting polymers and to modify permeation properties of the microcapsule walls by depositing polymers in their walls.

McShane and coworkers demonstrated real-time evaluation of enzymatic catalysis in LbL microcapsule systems [239]. Capsules containing entrapped enzymes, GOD and HRP, were produced through polyelectrolyte LbL assembly on enzyme-doped  $\text{CaCO}_3$  microparticles. Upon template dissolution, the adsorbed GOD and HRP were released and subsequently bound to the interpenetrating polyelectrolyte matrix by electrostatic interactions, thus resulting in microcapsules containing GOD and HRP. Catalytic reactions were colorized using indicating dye, Amplex Red. Real-time observation by confocal laser scanning microscopy revealed that the encapsulated enzymes were active, and readily catalyze glucose oxidation at the capsule interior and within the capsule walls. It also indicated that the majority of glucose catalysis occurred within the capsule walls as compared with that of the capsule interior. Zhu and McShane proposed a method for enzyme-entrapment

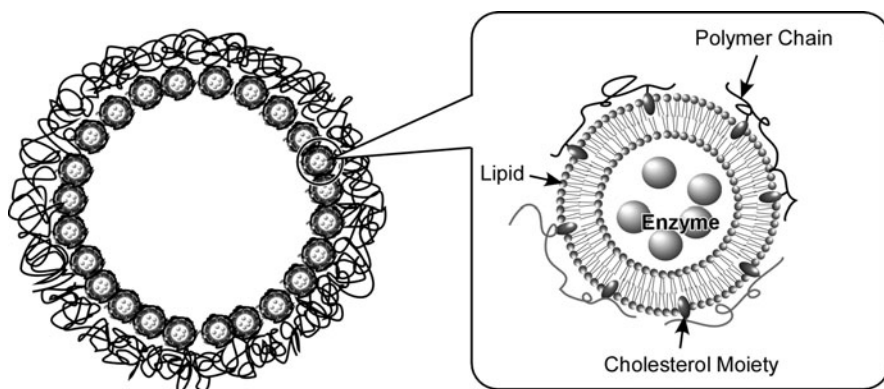
into LbL capsules that had been prepared with photosensitive diazoresin-based polyelectrolyte on  $\text{MnCO}_3$  template [240]. The change of the multilayer wall permeability before and after UV irradiation was explored for stable enzyme (such as GOD and POD) encapsulation in diazoresin-based microcapsules. The loading procedure is simple. The permeability of the diazoresin-based multilayer wall changed substantially due to cross-linking by exposure to ultraviolet light. Akashi and coworkers reported time-modulated release of multiple proteins from the LbL capsules [241]. They prepared enzymatically degradable capsules composed of chitosan and dextran sulfate multilayers as a drug carrier. The release of encapsulated FITC-labeled albumin could be controlled by the enzymatic degradation of the chitosan membranes with chitosanase. When two kinds of guests are separately incorporated inside the capsule and into the capsule membrane, independent release of the trapped guests by exploiting the enzymatic degradation of the capsule membrane becomes possible.

LbL assemblies including lipid vesicles, which can be regarded as models of cell membrane, should provide unique opportunities for creating novel bio-assemblies. Katagiri et al. pioneered LbL assembly using a superstable organic-inorganic vesicle cerasome with a silica network at the surface [242–244]. LbL assemblies between cationic polyelectrolyte and anionic cerasome [245] as well as the LbL assemblies between the anionic cerasome and the cationic cerasome [246, 247] were demonstrated without rupture of the vesicular structures. The latter assemblies are expected to be used as multi-cellular mimics. Schaaf and coworkers demonstrated incorporation of phospholipid vesicles in the LbL films composed of PGA and PAH [248]. Composite capsules between lipid components and polyelectrolytes have also been investigated. The same research group reported liposome-embedded LbL films, where enzymes included in the vesicles showed reactor activity [249]. Shutava et al. demonstrated LbL assembly between anionic phospholipids such as phosphatidic acid and phosphatidylglycerol and polycation on poly(methacrylic acid) microspheres [250]. The composite microcapsules obtained composed of lipid and polyelectrolyte were subjected to research of intramembrane diffusion of fluorescent dye.

Katagiri and Caruso applied step-by-step LbL assembly to preparation of lipid bilayer vesicles with asymmetric structures between inner and outer shells [251]. They first assembled polyelectrolyte multilayers on melamine-formaldehyde particles and terminated the assembly with negative outermost surfaces. Monolayer of cationic lipid was then adsorbed on the anion-covered particle to give surfaces coated with hydrophobic alkyl chains. Contacting the particles formed with vesicles of an anionic lipid in aqueous solution induced monolayer coverage of the anionic lipid. The outermost layers of the composite particles formed were then coated with polycation. Removal of the core material provided asymmetric bilayer vesicles. Although asymmetry plays a very important role in naturally occurring cell membranes, conventional processes on lipid assemblies cannot give such asymmetric structures. Therefore, LbL methods should give new opportunities in model studies on biological membranes. Lipid/polyelectrolyte composite LbL capsules provide an appropriate medium for bioactive materials. Li and coworkers immobilized ATP synthase into composite capsules (Fig. 13) [252, 253]. They prepared



**Fig. 13** Incorporation of ATP synthase into lipid/polyelectrolyte hollow capsule and production of ATP from ADP



**Fig. 14** Structure of capsosome

polyelectrolyte LbL films on melamine-formaldehyde cores that were destroyed giving hollow capsules. Contact of the hollow capsules formed with vesicles incorporating ATP synthase resulted in hollow microcapsules covered with lipid bilayer and including ATP synthase. Production of ATP from ADP was realized when a proton gradient through the membrane was applied.

Caruso and coworkers invented capsosome as a microreactor with thousands of subcompartments (Fig. 14) [254]. Phospholipid liposomes (DMPC/DPPC 80:20 wt%) including model enzyme,  $\beta$ -lactamase was first stabilized on a template particle by sandwiching the liposomes between a cholesterolmodified poly(L-lysine) (PLL<sub>c</sub>) precursor layer and a poly(methacrylic acid)-*co*-(cholesteryl methacrylate) (PMA<sub>c</sub>) capping layer. The template particles were suspended in a solution of PLL<sub>c</sub>, washed three times, suspended in the phospholipid liposome solution, washed three times, and a capping layer of PMA<sub>c</sub> was adsorbed. Next, five bilayers of PVP and thiol-modified PMA (PMA<sub>SH</sub>) were sequentially adsorbed, which were then cross-linked using and the template core was removed. Destruction of liposome structures by certain surfactants such as Triton X induced release of entrapped enzyme within polyelectrolyte capsule. As the number of subcompartments and the active cargo (drugs and/or reagents) within each liposome are effectively controlled

by the assembly protocol, capsosomes are particularly attractive as novel systems for a range of biomedical applications, including drug and gene delivery, and as microreactors.

In most of the research, LbL capsules of 4–5  $\mu\text{m}$  diameters are used but they are too large for injection into the human body. For example, US FDI requirement of injections is set to the limitation that particles have to be less than 200 nm. In order to improve such practical difficulties, Lvov and coworkers developed a novel technique, sonicated LbL assembly, in which LbL polyelectrolyte shell is prepared under powerful ultrasonication [255,256]. This approach allows us to produce stable drug nanocolloids of high concentration and with good colloid stability. In contrast to common micelle delivery systems, where 3–5% of drugs are included, their LbL nanocapsules carried up to 90% of drug and its release can be adjusted within 5–50 h with appropriate designs of capsular shell architecture. Use of polycations co-polymerized with poly(ethylene glycol) at the external layer of the capsule leads to longer circulation of the capsules in blood. Introduction of antibody at the exterior layer enables us to use these LbL capsules for the targeting of cancer cells. This approach was especially successful when applied to low solubility cancer drugs. Nanoencapsulation of poorly soluble cancer drugs was achieved through powerful ultrasonication of the drug powder and simultaneous sequential polyelectrolyte deposition. Specific targeted ligands could be absorbed on the surface of nanocolloidal particles of the poorly soluble drugs by using a polymer with free reactive groups as the outer coating. In their system, drugs are not modified during the process of solubilization and are subsequently released as free drug molecules. In addition, a very small quantity of a polymer is required compared with other protocols used at present for administration of poorly soluble pharmaceuticals. Using gelatin 100-nm diameter core, LbL capsules for sustained release of polyphenols were also developed by the same research group [257].

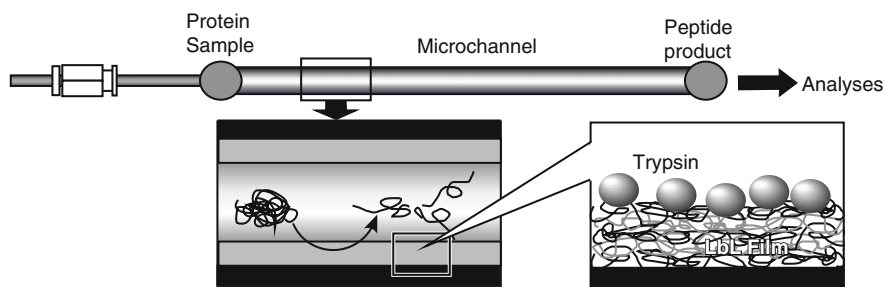
## 4 Advanced Techniques

Because the LbL method has great flexibility in selection of support materials, various advanced uses have been considered. Recently, LbL methods have been used for various advanced purposes including integration with device structures, advanced biomedical applications, hybridization of nanomaterials, and construction of hierarchic structures. In this section, these aspects are introduced with recent enzyme-related examples.

### 4.1 *Integration into Device Structures*

Integration of LbL structures into man-made microdevices such as microchips has recently been investigated. Yang, Liu, and coworkers demonstrated modification



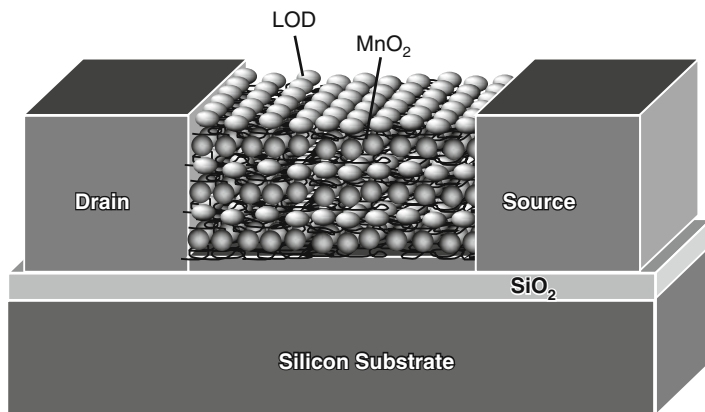


**Fig. 15** Microchip channel modified by the LbL film for detection of protein digestion

of microchip channels by using the LbL technique (Fig. 15) [258]. The microchip reactor modified with the LbL films were used for sensitive detection of protein digestion. Natural polysaccharides, positively charged chitosan and negatively charged hyaluronic acid (HA), were assembled into multi-layers onto the surface of a poly(ethylene terephthalate) (PET) microfluidic chip in order to form a microstructured and biocompatible network for enzyme immobilization. The controlled adsorption of trypsin in the multilayer membrane was monitored using a QCM technique and an enzymatic activity assay. The peptide resulting from digestion was analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. The maximum proteolytic velocity of the adsorbed trypsin was thousands of times faster than that in solution. BSA, myoglobin, and cytochrome *c* were used as model substrates for the tryptic digestion. This simple technique may offer a potential solution for low-level protein analysis. They also used a similar system for biological samples such as casein extracted from bovine milk and attenuated hepatitis A virus vaccine [259]. The LbL-modified microreactor provides a large surface-area-to-volume ratio and a confined microenvironment, resulting in an increased reaction rate. Within a few seconds, the peptides derived from digestion of proteins are detected with high sequence coverage.

Palmer and coworkers immobilized organophosphorus hydrolase (OPH) enzyme onto the walls of silicon microchannels manufactured by the LbL technique [260]. Enzyme microreactors were constructed with one and two layers of enzyme to compare activity performance where hydrolysis of methyl-parathion by the OPH enzyme into *p*-nitrophenol was investigated. The microreactors with two channel dimensions were tested, one with 98 parallel channels 60  $\mu\text{m}$  wide and another with 1,000 parallel channels 5  $\mu\text{m}$  wide. The microreactor with the smaller channel width demonstrated superior performance that was proportional to the increase in available surface area. Xu et al. developed an enzyme field-effect transistor (ENFET) through immobilizing lactate oxidase (LOD) and  $\text{MnO}_2$  nanoparticles in PDDA films using LbL assembly to construct  $(\text{PDDA}/\text{MnO}_2/\text{PDDA}/\text{LOD})_n$  multilayer films on the surface of ion-sensitive field-effect transistors (ISFET) (Fig. 16) [261].  $\text{MnO}_2$  nanoparticles were introduced as an oxidant to react with  $\text{H}_2\text{O}_2$ , resulting in a pH change in the sensing membrane of the ISFET with the addition of lactate with high sensitivity even to low lactate concentration. Dong and coworkers reported a





**Fig. 16** Enzyme field-effect transistor (ENFET) based on LbL assembly of enzymes with MnO<sub>2</sub> nanoparticle and polyelectrolyte on the surface of ion-sensitive field-effect transistor (ISFET)

one-compartment glucose/O<sub>2</sub> biofuel cell based on the LbL technique on 3D ordered macroporous gold electrodes [262]. The macroporous gold electrodes were functionalized with Au nanoparticles and enzyme, glucose dehydrogenase (GDH), or laccase. The macroporous gold electrode modified with LbL multilayer of gold nanoparticle showed excellent bioelectrocatalytic activity towards glucose where the direct electroreduction towards oxygen was realized. The maximum power output of this biofuel cell was almost 16 times larger than that based on the flat electrode. The performance can be greatly enhanced by the integration of 3D ordered macroporous electrode and LbL technique. De Smedt and coworkers reported a unique labeling system of LbL-based drug delivery [263]. They introduced the barcode concept to LbL drug delivery by using encoded fluorescent polystyrene with captured antibodies. With this system, quantification of proteins in serum plasma samples should become possible.

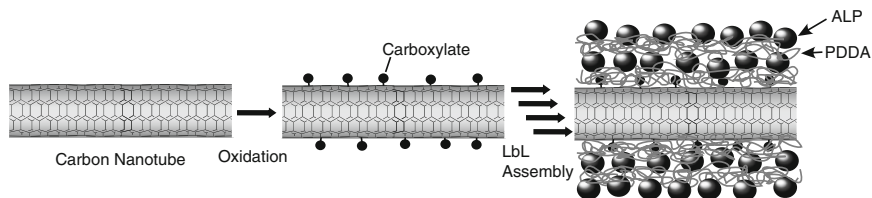
## 4.2 Advanced Medical Applications

Advanced medical uses are attractive targets for forefront applications of LbL films. Lee and Kotov used the LbL technique for stem cell culture [264]. Delta-like1 (DL-1) notch ligand was immobilized through the LbL assembly on the surface of 3D inverted colloidal crystal pores since the solution-based LbL coating procedure can be readily applied to the surface coating of complex 3D porous substrates while minimizing distortion of protein structures. The effectiveness of topology of D inverted colloidal crystal pores and the bioactivity of LbL-immobilized DL-1 notch ligands in ex vivo T-cell development of human hematopoietic stem cells. The LbL technique showed substantial advantages for creating an artificial 3D thymic stromal cell layer such as convenience, versatility, high controllability with nanoscale

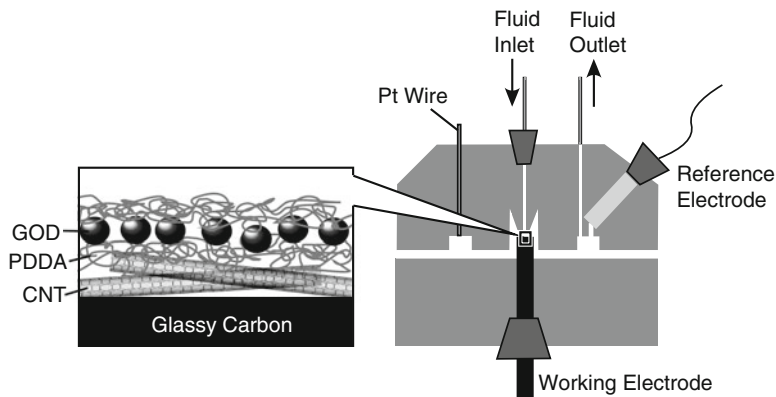
resolution, and capacity to build a multifunctional complex structure. McShane and coworkers constructed micropatterned structures for neuronal cell cultures [265]. On combining photolithography and LbL self-assembly, precise construction of nanocomposite films of potentially complex architectures and patterning of these films on substrates becomes possible using a modified lift-off procedure. Using neuronal cells as a model biological system, comparison chips were produced with secreted phospholipase A2 (sPLA2), a known membrane-active enzyme for neurons, for direct comparison with gelatin, PLL, or bovine serum albumin (BSA). Cell culture studies showed that neurons respond and bind specifically to the sPLA2 enzyme embedded in the polyelectrolyte LbL films. These findings point to the potential of this method to be applied in developing test substrates for a broad array of studies aimed at identifying important biological structure-function relationships. Patterned co-cultures of primary neurons and astrocytes on LbL films of conventional polyelectrolytes without the aid of adhesive proteins/ligands were demonstrated by Kidambi et al. [266]. De Smedt and coworkers conducted in vivo studies on cellular uptake, degradation, and biocompatibility of LbL polyelectrolyte microcapsules that were fabricated from dextran sulfate and poly(L-arginine) layers [267]. Control of cellular apoptosis by bone morphogenetic protein and its antagonist, noggin, embedded in LbL films was examined by Benkirane-Jessel and coworkers [268]. Ogier and coworkers investigated doping of biologically active adenoviral vector in various LbL films [269]. LbL coating of biological cells, such as blood platelets and stem cells, which have dimensions of tens of micrometers, and of much smaller microbes of around 1–2  $\mu\text{m}$  was realized by Lvov and coworkers [270, 271]. Such coating allows varying of cell surface charge, preserving them alive and regulation of cell nutrition uptake. Interestingly, cell division was possible, and both resulting cells possessed LbL shells.

### **4.3 Hybridization with Nanomaterials Toward Hierarchic Assemblies**

LbL assemblies on functional nanomaterials should create novel possibilities in nanotechnology and nano-bio fields. In particular, LbL assembly on carbon nanotubes (CNT) is an attractive research target. Wang and coworkers utilized the concept of signal amplification to achieve a remarkably sensitive electrochemical detection of biomaterials using enzyme LbL multilayers on CNT [272]. As shown in Fig. 17, the LbL multilayer films of ALP were prepared by alternate electrostatic deposition of oppositely charged PDDA onto oxidized and shortened single-wall CNT (SWNT). Because the sonication-induced acid oxidation process resulted in negatively charged carboxylate groups on the CNT surface, adsorption of cationic PDDA becomes possible, which was followed by sequential adsorption of ALP and PDDA. The unique properties of CNT, particularly their huge surface area-to-weight ratio, make them extremely attractive amplification platforms. Such amplified bioelectronic assays allow detection of DNA and proteins down to 80 copies (5.4 aM) and



**Fig. 17** LbL assembly of enzyme and polyelectrolyte on oxidized carbon nanotube



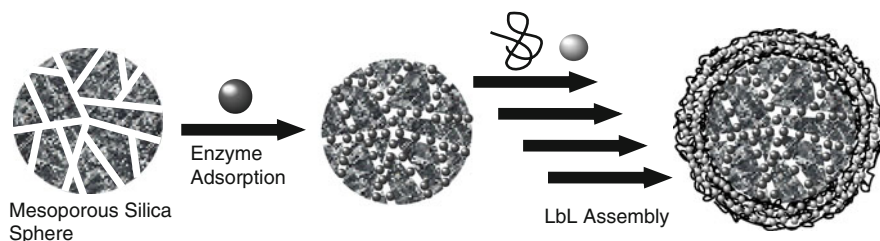
**Fig. 18** Flow injection amperometric glucose biosensor based on LbL assembly of GOD on a CNT-modified glassy carbon transducer

2,000 protein molecules (67 aM), respectively. Liu and Lin prepared a flow injection amperometric glucose biosensor based on electrostatic self-assembling GOD on a CNT-modified glassy carbon transducer (Fig. 18) [273]. The mechanical and electrical properties of CNTs enable sensitive determination of glucose concentration. The sensor showed an excellent detection limit, wide linear range, good precision, and operational stability as well as being free of interference from co-existing electroactive species. The same research team made similar amperometric sensors using LbL assemblies with CNT and appropriate enzymes such as choline oxidase (ChO), and acetylcholinesterase (AChE) as well as a bi-enzyme system of ChO and HRP [274].

Coating of various materials with enzymes by the LbL method has been widely investigated. Coating of useful and conventional materials with LbL enzyme films often creates practical applications. Lvov and coworkers immobilized laccase and urease on cellulose fibers through electrostatic LbL assembly to fabricate functional biocomposites [275]. With laccase-fiber composites, around 50% of the initial enzyme activity was preserved after 14 days of storage in water. Urease-fiber composites were successfully applied for biomineralization to grow calcium carbonate microparticles needed for paper whitening. The strategy presented could be used for the creation of cellulose fiber-based biocomposites with various functions that can be precisely controlled by the film nanoarchitecture. Bhattacharyya and

coworkers immobilized enzymes on microfiltration membranes through the LbL assembly technique. Either PLGA or PLL was covalently attached to the membrane surface [276]. Subsequently, PAH and PSS were alternately assembled then protein adsorbed depending on surface charge. Accessibility to the active site in the immobilized proteins was comparable to that obtained in the homogeneous phase, which was confirmed by affinity interaction (avidinbiotin) and two enzymatic reactions (catalyzed by GOD and ALP). The results obtained suggest that only minor conformational changes occur after biomolecule immobilization as well as some advantages such as reusability and the ease of regeneration. Shi et al. reported composite layered films of Hb and SiO<sub>2</sub> prepared by combined use of LbL assembly and vapor-surface sol-gel deposition [277]. The vapor-surface deposition technique was employed to develop an SiO<sub>2</sub> sol-gel matrix on GCE. The modified GCE was soaked in Hb solution, allowing the adsorption of the protein to form a SiO<sub>2</sub>/Hb bilayer. Through repetition of these processes, controlled thickness multilayer films of SiO<sub>2</sub>/Hb were fabricated. Catalytic activity for H<sub>2</sub>O<sub>2</sub> reduction was observed for the SiO<sub>2</sub>/Hb LbL film modified electrode with an apparent heterogeneous electron-transfer rate constant of 1.02 s<sup>-1</sup> and a Michaelis-Menten constant of 0.155 mM.

Mesoporous materials can be attractive materials for many kinds of applications [278–280], because these materials provide huge surface areas and pore volumes as well as well-defined pore geometries [281–288]. Research on mesoporous materials is currently extremely popular and detailed descriptions can be found in several reviews [289–295] and reports on advanced functions [296–299]. Such mesoporous materials provide excellent media for LbL assembly in the preparation of hierarchic nanostructures [300–302]. Several modifications of the preparation procedures have permitted syntheses of mesoporous silicates with designed pore geometries in various forms including powders and films [303–306]. Mesoporous materials are known as effective media to adsorb biomaterials such as proteins [307–312] and amino acids [313–316]. Therefore, combination of two powerful concepts of mesoporous materials and LbL assembly is anticipated to result in bio-functional nanostructures. Wang and Caruso demonstrated coating of mesoporous microspheres by using LbL assembly for effective biocomponent immobilization (Fig. 19) [317,318]. Densely enzyme-loaded particles were prepared through loading of the enzymes into mesoporous silica spheres followed by coating of the spheres with LbL multilayers. Following enzyme loading, a polyelectrolyte/nanoparticle LbL shell was assembled on the surface of porous spheres, thus preventing enzyme leakage. The encapsulated catalase can be recycled 25 times with an associated loss of activity of 30%, as compared to the 65% loss in activity for catalase immobilized in mesoporous silica spheres lacking the LbL coating. The same research team demonstrated immobilization of enzymes in interconnected macropores of three-dimensional zeolite membranes [319]. The composite membranes were amenable to surface modification with a variety of silane-coupling reagents for various application purposes. Yu and coworkers developed an immuno-labeling system with a



**Fig. 19** Entrapment of enzymes into mesoporous silica sphere covered with LbL films of polyelectrolyte and silica nanoparticle

high fluorescence/protein ratio through loading the organic dye fluorescein diacetate into hollow periodic mesoporous organosilica particles followed by polyelectrolyte LbL encapsulation and antibody attachment [320].

Composites of mesoporous materials and LbL films can be converted to novel nanostructured materials based on concepts of template synthesis [321–327]. For example, Caruso and coworkers demonstrated that the LbL coating of mesoporous silica spheres with polyelectrolytes and the subsequent removal of the silica templates results in formation of micrometre-sized nanoporous polyelectrolyte spheres [328, 329]. Wang and Caruso further applied this strategy to protein-based LbL films [330]. Proteins such as lysozymes were first adsorbed into mesopores of silica spheres, followed by several washing cycles to remove loosely adsorbed protein. The protein-loaded mesoporous silica spheres were dispersed in an aqueous solution of PAA, which most likely associates with the proteins through electrostatic interactions. This assembly was further stabilized through cross-linking using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride. Exposure of the composite to an HF/NH<sub>4</sub>F buffer induced selective removal of the silica component, finally resulting in nanoporous protein spheres. The spheres obtained have a large surface area and provide opportunities for easy approach of substrate molecules to proteins including enzymes. For example, Caruso and coworkers also demonstrated preparation of capsule-type reactors, similarly by LbL assembly on mesoporous silica spheres and subsequent template removal, in which a triggered enzymatic reaction with a nucleic acid substrate was realized [331]. In their system, bimodal mesoporous silica (BMS) particles were used as template for LbL assembly, where primary amine groups introduced at the silica surface provided positive charges for efficient electrostatic attraction with fluorescently labeled dsDNA. Infiltration of deoxyribonuclease (DNase) proceeded for 1 h following charge reversal by PMA. Coencapsulation of DNase I and dsDNA enabled the degradation of the DNA within LbL-assembled polymer capsules under the control of an external stimulus. High-throughput monitoring of the encapsulated, fluorescently labeled DNA provided a novel means of measuring the kinetics of the reaction.

## 5 Perspectives

Because conditions required for LbL assembly are very mild, this method is suitable for immobilization of biomaterials. Among biomaterials, enzymes have high-level functions, and can perform highly specific material conversion with incredible efficiency. Enzymes and their clusters can be regarded as ultrasmall machines and factories, respectively. Therefore, immobilizing them in organized nanostructures should lead to preparation of highly functional devices. As described above, enzyme-embedded and/or enzyme-encapsulated LbL structures provide various opportunities for device applications such as sensors and reactors. Most published research trials appear very successful. However, we would like to pose some questions here. *Have we reached the limits of the potential of LbL assemblies? Do the current functions represent the pinnacle of LbL technology? Are you satisfied with the current level of research?* We must answer *no* to these questions. Functions developed using LbL technology, such as sensing and reactor applications, lag far behind similar functions seen in the biological world. Functionality in naturally occurring systems is autonomous, self-regulated, and multi-responsive. One might say that biological systems contain the ultimate functional devices. A significant difference between biological systems and LbL assemblies lies in the complexity of their structures. In nature, several functional units are organized in sophisticated and/or hierarchic structures in which several functions are integrated, as seen in photosynthetic and signal transduction systems. If we construct such well-developed structures then we might realize the ultimate nanodevices. The LbL technique permits various creative possibilities in preparation of nanostructures. It is also flexible in terms of materials' selection, widely applicable to various structures, and enables coupling with man-made devices such as electrodes and transistors. These features are not available in most other fabrication techniques in either top-down or bottom-up disciplines. Therefore, we believe that LbL assembly is the most promising method for creation of the ultimate nanodevices.

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