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Review Article
Perspectives and challenges for self-assembled layer-by-layer biosensor and biomaterial architectures

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This review focusses on challenges for biosensor platforms with self-assembled layer-by-layer (LbL) architectures. Inherent questions involving construction and characterisation of these assemblies, self-assembled monolayers and multilayer LbL structures, are discussed. The preparation of electrochemical biosensors is then addressed in more detail, especially regarding incorporation of nanomaterials. Future perspectives for LbL assemblies for electrochemical biosensing are indicated.

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Introduction and principles

The morphology and structure of electrode-solution and modified electrode-solution interfaces and within modifier films at the molecular and nanometric level influence the rates of electrode reactions. Hence, control and manipulation of the surfaces of modified and unmodified electrodes is an important objective which, if it can be achieved, is an important step towards the production of sensors and biosensors with identical characteristics. The concept of layer-by-layer (LbL) arose with precisely this aim of better controlling the modification of electrodes in order to give structurally well-defined material surfaces, prepared by spontaneous interactions (self-assembly) between a modifier or modifiers and an electrode substrate, which itself may also have been previously modified [1,2]. Many of the most important applications using this concept are in sensing and biosensing. If LbL construction is carried out correctly, then there can be expected to be an enhancement of biosensor performance in terms of higher sensitivity and lower detection limit as well as repeatability, reproducibility and sensor stability over time. In addition, the quantities of the needed chemical reagents that are immobilized are usually much less. Hundreds of articles are published each year involving the LbL process, not all electrochemical.

Self-assembly implies that links are formed between adjacent layers whether it be by electrostatic interactions or the formation of covalent bonds. It is expected, in all cases, that successive layers will have alternate charges. Thus, a multilayer structure constructed on top of a suitably-prepared electrode substrate will normally consist of bilayers, each bilayer consisting of a positively and negatively charged component, see Figure 1. The uppermost layer will normally contain the recognition element that reacts with the desired species in solution, in the case of biosensors the biological recognition element.

A recent review [3] has described many of the principles of LbL and applications in bioelectrochemical systems focusing on the previous decade. Here we concentrate on some of the fundamental concepts behind self-assembled monolayers (SAM) and LbL structures, the conceptual advantages of the approach and future perspectives for application in electrochemical biosensors.

Investigating the formation of SAM and LbL structures

Optimisation of SAM and LbL structures requires understanding the formation process at the molecular level. Tools are available for monitoring the formation of SAM or LbL structures before and after each construction step or in real time. The voltammetry of immobilised electroactive species can be recorded after formation of each layer and, if the layer structure is constructed on semi-transparent electrodes, such as indium-tin oxide, ITO, it is possible that UV-vis spectra can be recorded, as with iron-substituted α-Keggin polyoxotungstates [4]. Electrochemical impedance spectra recorded at the same time can also supply valuable information since the spectra can be fitted to an electrical equivalent circuit, which should reflect the physical model of the multilayer structure. Electrical elements should include, at the very least, charge transfer resistance, interfacial capacitance and the electrical resistance of the structure [5].

A powerful technique to monitor the formation of LbL structures *in situ*, in real time, is the quartz crystal

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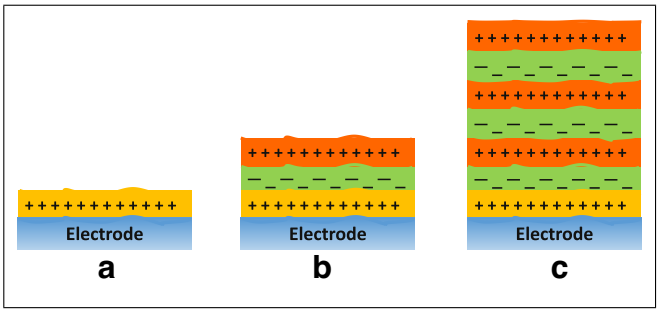
Figure 1



LbL self-assembly scheme, illustrated for an electrode, not smooth at the molecular level, which is given a positive charge to allow assembly (a) Initial surface functionalization (b) adsorption/deposition of the first two layers to give the first bilayer (c) with 3 bilayers. It is assumed that the negative layer is a polyelectrolyte that is thicker than the positively charged layer.

microbalance (QCM). The QCM can probe the mass increase as well as rate of deposition during each deposition step, and can take into account any viscoelastic effects. Used as an electrochemical QCM, it can also be used to examine the voltammetry of the structures and any accompanying mass changes due to ion or solvent insertion/expulsion on varying the applied potential after each assembly step. Surface plasmon resonance can also be applied successfully to monitor the LbL structures and biological molecule immobilisation.

Surface analysis techniques are nearly all *ex situ* and often carried out in vacuum (e.g. scanning electron microscopy), which leads to a change in the surface morphology due to dehydration, possibly cracking, and probably does not indicate the real surface roughness. An exception is atomic force microscopy, but it is experimentally difficult to examine the morphology of soft structures in solution with high resolution and how they change on building up the multilayers.

Self-assembled monolayers

The construction of LbL structures usually starts with the formation of a self-assembled monolayer. A good SAM-forming molecule should have one end that attaches easily to the surface and the other end have functional groups that interact with species present in the LbL component solution as part of the LbL process. Large self-assembly molecules can be synthesised before assembly or produced in a step-wise fashion on the surface by strategies such as cross-linking. In addition, and for maximum effect, the molecule geometry should be such that packing with a high density is possible, in order that all of the surface is covered.

The most widely formed SAMs are organosilane based monolayers on silicon dioxide and hydroxylated surfaces. However, these are not usually appropriate for electrochemical sensors and biosensors, although the results reported in [6] suggest that there may be some possible applications in the future.

SAMs for electrochemical applications are normally prepared with organosulfur compounds, often alkanethiols, because they spontaneously adsorb onto gold, silver, platinum and copper. Such alkanethiols can be functionalized at the outer end of the molecule, the functionalization chosen so as to promote interaction with further layers of biomolecules or other species. Examples from the recent literature include lipid bilayers [7], oligo(ethylene glycol) alkanethiols for tethering pre-functionalized neurotransmitter precursors [8] and thiol-modified dopamine aptamers [9]. Another important point is chain length. It is clear that increasing alkanethiol chain length increases the electrical resistance of the LbL assembly that can compromise its functioning; these criteria were investigated extensively in the past e.g. [10]; in practice it is not normally a problem up to at least dodecanethiols.

Gold is the most frequently used substrate for alkanethiol SAMs because it is the easiest for enabling adsorption on an oxide-free surface and, additionally, it is the preferred metal deposited on piezoelectric crystals in QCM, which can be used to monitor the mass changes on build-up of the LbL assembly. The gold-thiolate bonds that are formed are expected to lead to a well-defined monolayer with complete coverage, and this has been recently investigated in detail [11]. Such monolayers are stable at positive potentials, but it is possible to remove the alkanethiols at a sufficiently negative potential. Although this could be an advantage for regenerating the electrode substrate surface, a possible disadvantage would be limiting use in applications involving probing electrochemical reduction of an electroactive species [12].

Schemes that depict SAM formation nearly always ignore the possible complexity caused by surface roughness and non-uniformity. Surfaces are not smooth at the nanometric or atomic level, except at specially-prepared monocrystal surfaces. On polycrystalline surfaces, which have different crystallographic faces exposed as well as grain boundaries, the forces of attraction can vary over the surface.

A simple electrochemical impedance spectroscopy experiment with analysis of phase angles gives evidence, at least in the case of dodecanethiols, that surface non-uniformities are suppressed by the formation of the alkanethiol layer. In a zone of potential without faradaic reactions, the substrate should behave like a pure capacitor, with a phase angle of 90° in complex plane plots. In fact it is less, denoting non-uniformity, but on adsorption of dodecanethiol becomes 90° whether the SAM is formed at open circuit or is formed faster when

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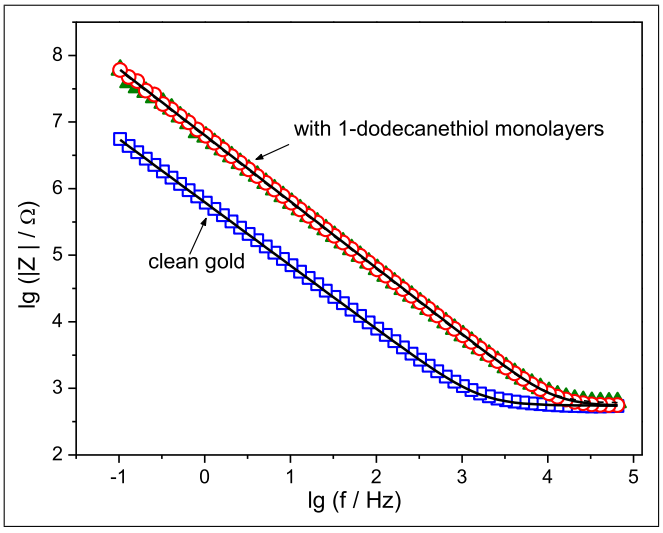
Figure 2



Impedance magnitude Bode plots of spectra of bare, clean polycrystalline gold (□) and of gold-covered 1-dodecanethiol monolayers formed at (▲) a fixed potential of +0.9 V vs. Ag/AgCl (3 M KCl) during 1000 s and (○) at open circuit potential during 14 h. Solid lines show fitting of spectra. The slope changes from 0.85 to 1.0 on monolayer formation. Adapted from [12], with permission of John Wiley and Sons.

assisted by an applied potential, see Figure 2. This points to complete coverage and forming a hydrophobic region that is able to compensate for the surface roughness of the gold surface [12].

A second way of probing complete coverage is to determine the response of the modified electrode to an electroactive marker, usually hexacyanoferrate (II). This methodology is also used for complete LbL structures. The disadvantage of such an approach is that hexacyanoferrate (II) has a negative charge, so that if the upper layer in an LbL structure also has a negative charge then repulsion effects may mean that no response is recorded, even though there are pinholes in the structure. In this sense, impedance measurements may be more powerful because they are not dependent on using an electroactive marker in solution.

An alternative approach is to form covalent bonds, exemplified by aryl diazonium salts. Their reduction to give a radical species on the electrode substrate (which can be prepared to have exposed functional groups) gives a film with the aromatic part directed towards solution, and which can then be further modified. Such an approach can be used for carbon electrodes, gold, platinum as well as other metals, ITO and unoxidized silicon. ITO is of great interest for spectroelectrochemical studies and the effect of its surface properties on SAM modification has been recently reviewed [13].

The LbL self-assembly strategy

Well-defined materials must be used to modify electrode surfaces in the LbL strategy. Interconnection can be through weak interactions, such as hydrogen bonding, through biospecific recognition, coordination, electrostatic, hydrophobic and dipole–dipole interactions. Since formation is spontaneous, the native structure of biological molecules, and thence their activity, should be preserved during immobilization. Smaller quantities of reagents are required than in more conventional methods such as coating and there should be better access to active sites of biological molecules such as proteins.

After initial modification, as discussed above, the LbL structure is built up by successive exposure to components of opposite charges with rinsing in between. Normally only two components are used which are adsorbed/deposited alternately on the surface, as in Figure 1. Often simple immersion in a solution containing the SAM-forming molecule is done, or assistance is given from, for example, a light source, increased temperature or applied potential. The LbL process is carried out until the required number of layers/bilayers has been achieved.

In most cases, only one of the components contains electroactive/active species. The other layer is present to hold the assembly together, and for electrostatic adsorption it is usually a polyelectrolyte. Examples of common positively-charged polyelectrolytes are poly(ethyleneimine) and poly(allylamine hydrochloride) and of negatively-charged polyelectrolytes poly(vinyl sulfonate) and poly(styrene sulfonate). Control of pH ensures that the polyelectrolytes have the required charge, since otherwise alterations in pH could lead to charge disappearance and to the structure falling apart. It is often polyelectrolyte conductivity that limits the number of bilayers owing to the influence of increasing polyelectrolyte electrical resistance effects in the assembly. Finally, access of the electroactive and/or biological species to the biological recognition element in the inner layers of the multilayer structures in a biosensor will become less and less as the number of bilayers is increased.

In principle, the LbL strategy should lead to modified electrodes with identical responses, owing to the rationally-designed LbL nanoarchitectures [14•]. The term nanoarchitectonics has been coined to refer to a fully-organised assembly of LbL structure controlled at the nanometric and molecular level. An excellent review describes many electrochemical devices based on LbL assemblies, focusing on supercapacitors and electroresponsive LbL as well as biosensors [15•], and recent work has

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explored this in terms of stratified supramolecular structures [16].

Morphology and organization of LbL assemblies

The expression "LbL" indicates that each layer is deposited directly on top of the smooth layer beneath, as is usually shown in schemes of LbL construction. Apart from a general lack of smoothness, as indicated in Figure 1, and non-uniform interactions at the nanometric level on the substrate, this is not likely to be the case when one of the self-assembly components is a polyelectrolyte. Even when neither component is polymeric, effects can be predicted.

These effects, plus those of reorganization were illustrated by a study of the immobilisation of myoglobin on polycrystalline gold, the other component being hyaluronate [17]. The first SAM layer was 3-mercapto-1-propanesulfonate followed by adsorption of poly(diallyldimethylammonium chloride), and a positive charge exposed to solution. There are positively-charged amino acid residues on the surface of myoglobin at pH 5.0 (pI of myoglobin is 6.8) and at this pH hyaluronate has negatively charged carboxylate groups. Multilayer build-up can be followed with a QCM, the decrease in frequency being associated with an increase in mass.

A large variation in the initial surface preparation steps between different experiments and in the deposition of the first two bilayers can be seen. However, above 2 bilayers the uncertainty in frequency change becomes much smaller and viscoelastic effects tend to disappear. This suggests non-homogeneous distribution of the adsorbates as well as different masses adsorbed due to non-uniformity effects.

Further information can be obtained from analysis of electrochemical impedance spectra recorded before and after deposition of the bilayers. The semicircular complex plane spectra can be analysed in terms of an electrical equivalent circuit of a charge transfer resistance in parallel with an interfacial capacitance, which is an ideal capacitance (C) or a non-ideal capacitance. A non-ideal capacitance reflects surface/interface non-uniformity and/or roughness and is represented by a constant phase element CPE. Electrical models can be developed in which a constant value of the capacitance for each bilayer is added to that already existing for the structure with one less bilayer, taking into account dielectric constant and assuming equal bilayer thickness. It can be deduced that the layers are not of equal thickness and that the inner ones change their thickness during the assembly construction. Above two bilayers, there is no more reorganization so that a minimum of 3 bilayers is needed in this example. This deduction is in agreement with voltammetric results and atomic force microscopy.

This illustrative example shows that there is a minimum number of bilayers needed but also there is no point in increasing the number of bilayers above what is necessary to get maximum efficiency. Few (electro)active species will be able to access the inner layers to react and the electrical resistance of the assembly will increase with more layers. The problem of electrical resistance can be addressed by incorporation of nanomaterials such as functionalised carbon nanotubes or graphene that themselves can act as the joining materials in the assembly as well as electrical bridges through the electrode substrate support. These may even be necessary for structures with two, or even one, bilayer. Many studies in the literature find that the number of bilayers for maximum response is 3 or 4, above which there is a decrease in response.

Enzyme biosensors

Electrochemical enzyme biosensors using LbL are usually second-generation biosensors, in which a mediator mediates the shuttling of electrons between enzyme redox centres and the electrode substrate surface or a third-generation biosensor where there is direct electron transfer between the enzyme and the electrode surface.

Concerning second-generation enzyme biosensors, conventional assembly methods, such as deposition of a layer containing the mediator or its formation through electrodeposition, followed by drop casting of an enzyme layer, leads to rather thick layers. Analyte molecules will react with the outermost active enzymes in the enzyme layer and most of it will not be used. Products of the enzyme reaction will have to diffuse through to the mediator layer in order to give the electrochemical response, and some will be lost to solution. These are excellent reasons for reducing the thickness of both layers, especially the enzyme layer, to the minimum possible. It can thus be readily seen how the LbL technique can aid in furnishing increased efficiency. Approaches can include adsorption of enzymes on SAM/LbL structures, so long as the enzyme has an overall surface charge opposite to that of the outer layer of the SAM/LbL structure, or cross-linking covalent attachment of redox enzymes.

A typical sort of assembly that can be made is described in [18]. The poly(amidoamine) (PAMAM) dendrimer inside which are deposited gold nanoparticles, is the positively-charged component. The negatively-charged component is poly(vinyl sulphonate). For a second-generation electrochemical biosensor, the metal hexacyanoferrate mediator is deposited on the gold nanoparticles following normal procedures. The enzyme is finally attached to the outer layer of PAMAM.

Carbon nanomaterials have found much use in the preparation of LbL assembled modified electrodes. For example, carbon nanotubes functionalised with carboxylate in strongly acidic media, have a negative charge. Besides

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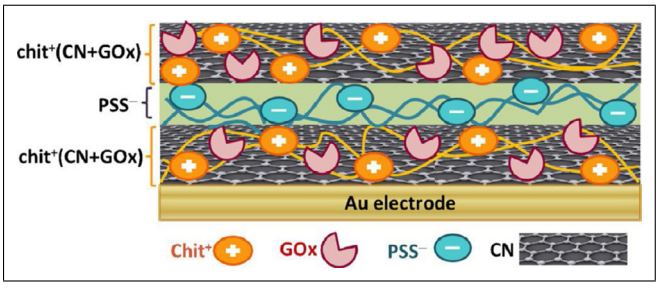
Figure 3



LbL assembly on a gold electrode surface showing sequential layers of $\{chit^{+}(CN+GOx)/PSS^{-}\}_{n}$. where "chit" is a chitosan matrix, "CN" is carbon nanomaterial and PSS- is poly(styrene sulfonate). From [19] with permission of John Wiley and Sons.

this, carbon nanomaterials can act as electrical bridges, reducing the electrical resistance of the assembly [19,20], see Figure 3.

Further modification of the ends of the tubes and of defects in the walls can be done; the same is true for graphene. Such negative charges promote the adsorption of metal nanoparticles, particularly gold, which have a positive charge. It has been shown that the use of metal nanoparticles within self-assembled LbL architectures leads to more efficient biosensor platforms with enhanced performance [21]. There is an overall increase in electronic conductivity and electroactive surface area of the modified electrodes, enabling an improvement in analytical performance.

Immunosensors and DNA sensors and biomaterials

An immunosensor employs an antibody, as biorecognition element, to detect and quantify an antigen. The antibodies are proteins, immunoglobulins, generated by the immune system as a response to foreign species such as bacteria, viruses or parasites. Fragments of antibodies or aptamers can also be used as recognition elements so long as they provide the same specificity. Heterogeneous immunosensors involve the immobilisation of Ab or Ag so that the biosensing inherently consists in LbL sensing with just one layer. These could be built up into multilayer systems. The alternate adsorption of polyelectrolytes of different charges, one of which contain the antibody, can lead to an increase in efficiency. The same sort of strategy can be used with DNA sensors and aptasensors, since DNA has a negative charge, as described in a recent review [22]. Another recent paper illustrates the increased corrosion resistance of LbL assembled DNA coatings on magnesium alloys as a method for increasing biocompatibility [23], and a microRNA LbL structure has also been suggested built onto gold-coated silica nanoparticles for the intracellular delivery of miRNA [24].

More complex LbL biosensor structures

More than two components can be used in LbL assembly, so long as the integrity of each monolayer can be ensured. Different positively charged and negatively charged components can be assembled stepwise on the electrode substrate. However, examples are few. In [25] two enzymes, glucose oxidase and horseradish peroxidase are mixed together and used in a biofuel cell, with the purpose of increasing the efficiency and catalytic activity of the anode. In [26], carbon nanotubes are functionalised with different chemical and biological species, having a positive or negative charge. They are then assembled and permit the catalysis of the oxidation of sucrose in an enzyme cascade bioanode for a biofuel cell. Since the power associated with biofuel cells is very small, they are amenable to LbL modified electrode assembly.

Final remarks

Research into the LbL construction of modified electrodes has opened up the possibility of more effective sensing platforms as well as probing the voltammetry of immobilised species. Since biomaterials, such as proteins, lipids, and nucleic acids, can self-assemble, their use together with the strategies outlined above with nanomaterials, should lead to many opportunities for innovation in LbL assembly strategies [27•], as well as the development of reproducible biosensors with well-controlled and stable nanostructures.

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