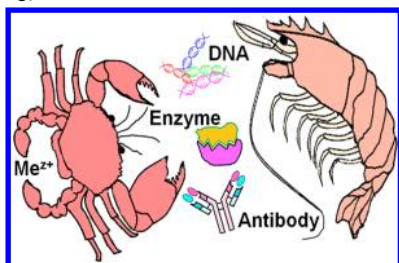


Electrochemical Biosensor Applications of Polysaccharides Chitin and Chitosan

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1. INTRODUCTION

Because of their remarkable structural and functional properties biopolymers chitin and chitosan (Figure 1) have received much attention in fundamental science, applied research, and industrial biotechnology.^{1–10} Chitin is a long-chain polymer of *N*-acetyl glucosamine and the structural material of, for instance, fungal cell walls, insect and crustacean exoskeletons, mollusk radulas, and cephalopod beaks. Natural marine chitin or chitin residues are the exclusive nutrient source for many marine bacteria, which use them efficiently as metabolic fuels. Microbial chitin utilization involves enzymatic breakdown of the biopolymer into short-chain chito-oligomers by secreted chitinases^{11–15} and subsequent efficient internalization of the resultant chitosugars through specialized bacterial outer

membrane proteins ("chitoporins").^{16–18} Man-made chitin waste from seafood processing, on the other hand, is a common starting material for production of commercial purified chitin by processes involving harsh chemical treatment. Though not yet developed on an industrial scale, biowaste from the silk, mushroom, and honey-harvesting industries has been proposed as an alternative source for chitin production, and this idea has already delivered promising results in the laboratory.^{19–23} Chitosan is the synthetic product of controlled chitin deacetylation and, by varying the chain length of the precursor material or the degree of chemical depolymerization and the extent of deacetylation, is available with a broad range of chemical and physical attributes.

The two materials and their derivatives have practical applications in the form of films, gels, suspensions, microscopic threads, fibers, and spheres in many fields: biotechnology,^{24–28} human^{29–42} and veterinary^{43,44} medicine, pharmacy,^{24,45–51} agriculture,^{52–55} food engineering,^{56–61} environmental technology,^{29,62–67} and textile^{68,69} and paper⁷⁰ industries. An indication of the widespread exploitation and constantly growing importance of chitinous resources is the total of over 10 000 scientific articles and 240 reviews published between January 1, 2005 and July 28, 2012 that have chitin or chitosan in their title. A number of books also include these two particular biomaterials as a key topic.

In addition to the applications already mentioned, chitin and especially chitosan have found widespread use as advanced biofabrication materials. In 2005 Payne's group described the potential of native or chemically modified chitosan in the surface adaptation of cell/protein-integrating biological systems and functional components of diagnostic devices and sensors.⁷¹ About the same time Krajewska discussed the advantages of chitinous polymers for enzyme surface immobilization in preparation of medical sensing devices.²⁷ The large number of published studies on analytical applications of all sorts that followed these two key reviews and which are reviewed in this review helped raise awareness of the importance and competitiveness of the two materials, and chitosan is now accepted as an important material in the field of advanced sensor technology.

Biosensors incorporate functional proteins, nucleic acids, cell organelles, or even whole living cells, which are fixed ("immobilized") on a physicochemical transducer surface that is able to translate specific interactions of the immobilized bioentity with its corresponding binding partner (analyte) into measurable, concentration-dependent electrical signals.^{72,73} The

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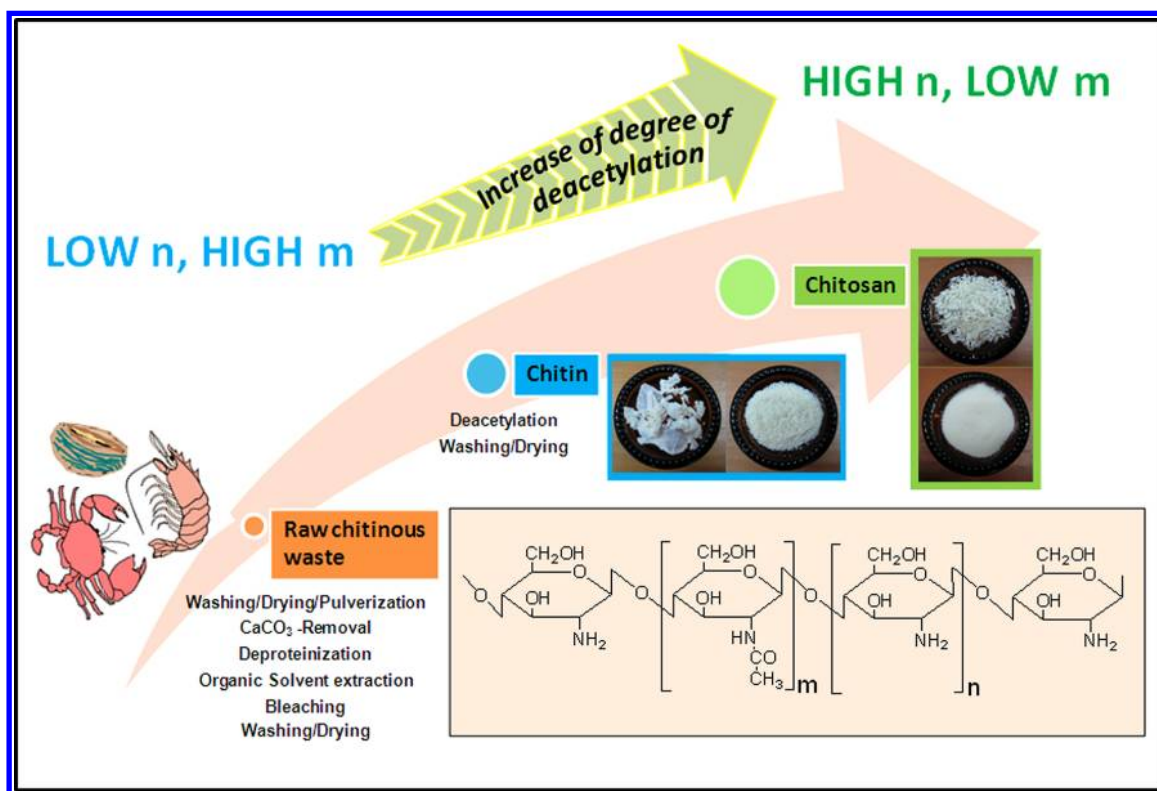


Figure 1. Preparation and chemical structures of chitin and chitosan. For chitin m , the number of acetamido groups, is larger than n , the number of free amino groups, while for chitosan $n > m$. Degrees of deacetylation and acetylation are $(n/(n + m)) \times 100$ and $(m/(n + m)) \times 100$, respectively.

quality of gentle but firm immobilization of the analyte-selective biological component is a determining factor of biosensor performance, and chitin or chitosan films have proved to be useful transducer surface modifiers, compatible with advanced sensor properties, at least under certain circumstances. The properties that make chitin and chitosan valuable for analytical sensor R&D are summarized in Figure 2. The primary advantage of the two chitosugar-based natural polymer

materials is their combination of ready availability as highly developed industrial products in a variety of grades with remarkable biocompatibility and pronounced film-forming capability.

Another valuable feature of chitin/chitosan is a chemical structure that includes many intrinsic oxygen- and nitrogen-based functional groups that can serve as the starting points for covalent modification and/or chitosugar chain cross-linking. In addition to these common benefits chitosan has some other important advantages over chitin, namely, its solubility in mildly acidic aqueous solution, an ability to form tough hydrogel-like deposits, and the possibility of being electrodeposited on electrode surfaces as a thin (hydrogel) film in a potential-controlled manner, which is invaluable in miniaturized applications such as micro- and nanobiosensor fabrication. With these additional benefits, chitosan is, unsurprisingly, more widely employed than chitin in the field of sensor technology.

Despite its topicality, the subject chitin/chitosan in biosensors has not yet been specifically reviewed. The aim of this review is thus to overview existing options for electrochemical (EC) biosensors that work with chitin/chitosan-based electrode modifications and offer guidelines for related research and development activities. Basic principles of electrochemical biosensing are considered first to introduce inexperienced but interested readers to the topic of biosensors; then relevant aspects of chitin/chitosan chemistry, biochemistry, and structure are described and discussed with respect to feasible sensor configurations. Finally, we include descriptions of specific applications and give examples of enzyme-based, antibody-based, and single-stranded DNA-based biosensors involving chitin/chitosan as part of the detector architecture.

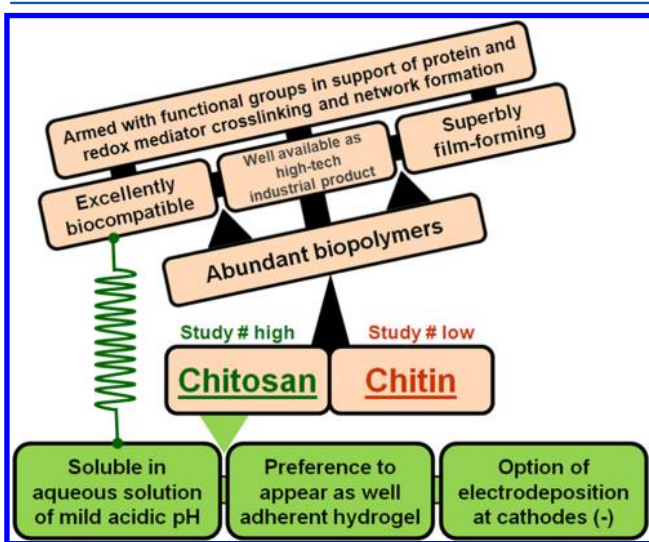


Figure 2. Advantages of chitin and chitosan for biosensor applications. Chitosan, with its higher solubility and its hydrogel-forming⁷⁴ characteristics, is by far the more widely used material in this field; balance between the number of published studies with the two materials is thus firmly on the side of chitosan.

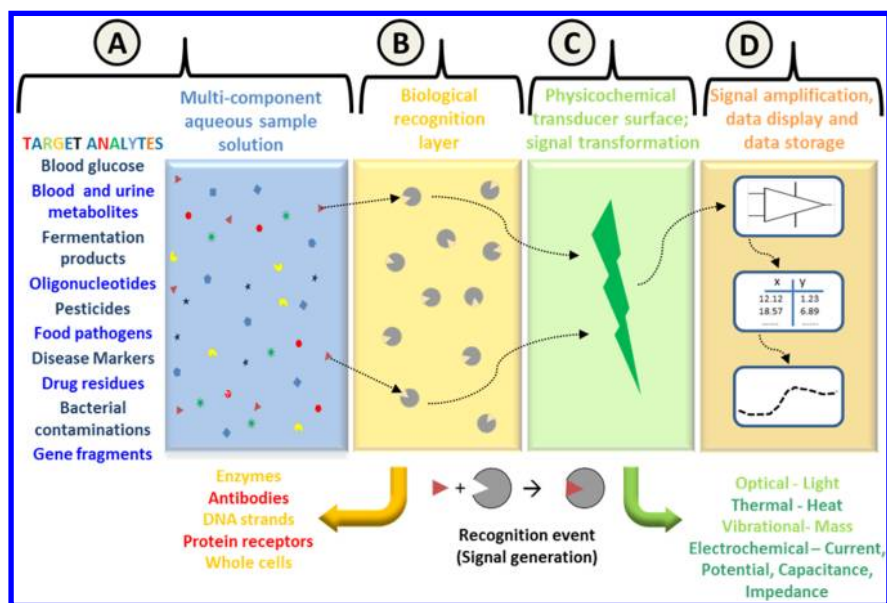


Figure 3. General scheme of a typical biosensor architecture, showing potential analyte species in sample solution (A), immobilization layer for biological recognition elements (B), physicochemical platform (transducer) that generates measurable electrical signal upon interaction of the biological recognition element with the corresponding substrate (C), and electronic module for signal amplification, display, and storage (D).

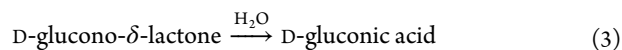
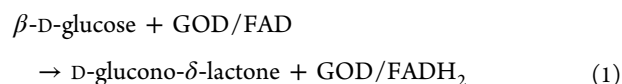
2. PRINCIPLES OF ELECTROCHEMICAL BIOSENSORS

Enzymes, receptors, antibodies, and single-stranded (ss) DNA are molecular biological recognition elements (BREs) that display high specificity and high affinity for their substrates or binding partners. Figure 3 is a representation of the architecture of biosensors, showing how these tools make use of BREs for analyte detection in a broad range of applications.

An absolute requisite for a well-functioning biosensor is the firm, durable, and nondestructive fixation (“immobilization”) of the BRE of choice onto the transducing platform, which can be an optical (spectroscopic) sensing device, the top of a quartz crystal microbalance, the tip of a thermocouple, or the liquid/solid interface of an electrochemical sensor, e.g., the disk face of a gold (Au), carbon (C), or platinum (Pt) electrode or the surface of an ion-selective electrochemical sensor. Immobilization of the BRE locates the molecular recognition event close to or even directly upon the physicochemical detector surface, and concentration-dependent signaling of the process is then possible through conversion of associated light, mass, heat, or impedance/potential/redox changes into a measurable and storable electrical property.

This review focuses on electrochemical biosensing in which signals are generated and transduced by amperometry (current, I , measured over time, t , at fixed working electrode potential, E), voltammetry (I measured as a function of E), potentiometry (equilibrium working electrode potential measured as a function of t at $I = 0$), and potential step and alternating current impedance measurements. The wide range of sensing opportunities that an electrochemical signal transduction offers for analyte detection is exemplified by glucose quantification with glucose oxidase (GOD) enzyme biosensors.^{75–80} GOD catalyzes oxidation of α -D-glucose to D-glucono- δ -lactone with concomitant reduction of its flavin adenine dinucleotide (FAD) prosthetic group (reaction 1). Reoxidation of the reduced FAD by dissolved oxygen, the natural electron acceptor, regenerates the enzyme’s original state (reaction 2), forming hydrogen peroxide as an electroactive byproduct. In aqueous media the

primary product D-glucono- δ -lactone is eventually hydrolyzed to gluconic acid (reaction 3).



Fixation of GOD onto a detector spatially limits the enzyme activity to a zone close to the surface, and the related changes in $[\text{O}_2]$, $[\text{H}_2\text{O}_2]$, and pH occur thus within the entrapping polymer matrix. With traditional electrochemical glucose biosensors the local chemical changes occurring at the sensor/sample solution interface are turned into a quantifiable signal by immobilizing GOD on an electrode that measures oxygen (e.g., a Pt or C disk electrode held at sufficiently negative potential for diffusion-limited oxygen reduction), hydrogen peroxide (e.g., a Pt or C disk electrode held at sufficient positive potential for diffusion-limited peroxide oxidation), or pH (e.g., a glass electrode). In more highly developed versions of amperometric EC glucose biosensors the natural electron acceptor, oxygen, is replaced by dissolved artificial electron acceptors, preferably with an oxidation potential favorably below that of H_2O_2 . This pioneering adaptation allows glucose quantification independent of the aeration state of the measuring solution, making analysis possible in the presence of redox contaminants that would interfere at the peroxide oxidation potential. The most advanced glucose sensor designs currently available make use of sophisticated redox polymers/hydrogels as a tailored immobilizing matrix in which interaction between randomly distributed redox relays in the polymer chains establish a nondiffusive flow of electrons (“hopping”) between GOD and the electrode in the presence of analyte; otherwise, biosensors may be designed to promote direct electron transfer between oriented GOD molecules and the sensor surface. Establishment of enzyme biosensors that use molecular biocatalysts other than

GOD offers similar challenges: proper BRE immobilization, identification of an electrochemically detectable product, and eventually adaptation of amperometric, voltammetric, or potentiometric detection schemes for analyte (substrate) quantification. Examples of successful applications include enzyme biosensors using other oxidases (e.g., galactose, lactate, glutamate, cholesterol, choline, monoamine, and alcohol oxidases), dehydrogenases (e.g., fructose, glucose, glutamate, lactate, cellobiose, formaldehyde, and alcohol dehydrogenases), uricase, penicillinase, and urease to name just a few. A description of all these variants is beyond the scope of this review; however, detailed Supporting Information on the construction and performance of modern enzyme-based biosensors is available in recent review articles^{81–85} and books or book chapters.^{86–89}

Single-stranded deoxyribonucleic acid (DNA) fragments and antibodies, in contrast to enzymes, do not catalyze chemical reactions and so do not change the concentrations of possibly electroactive substances, such as substrates and cofactors, but instead interact with their substrates simply through high-affinity binding. Design of EC detectors in DNA and immunosensors must take this major distinction into account. One commonly used option is to track changes in relevant electric features of the modified sensor surface. It is, for instance, possible to exploit electrochemical impedance spectroscopy, alternating current voltammetry, or potential step measurements of impedance properties such as the sensor's double-layer capacitance and charge transfer resistance for hybridization recognition and antigen quantification, respectively. Alternatively, synthetic inorganic, organic, or organo-metallic molecular reporters with a reversible redox activity or enzymes that produce electrochemically detectable species upon exposure to substrate may be used to detect conjugate formation. Several types of redox indicator-supported analysis have been reported: signaling molecules may be diffusible components of the measuring buffer or redox labels covalently bound to the probe or target DNA strands in DNA sensors or to the antibody or antigen in immunosensing. Some recent review articles cover electrochemical detection of DNA hybridization^{90–98} and antibody/antigen conjugation.^{99–108}

The choice of the BRE and selection of the electroanalytical detection scheme are early steps in EC bio- and immunosensor design; however, the strategy for fixation of the BRE onto the detector must also be decided, and its quality is a main determinant for later sensor performance. Options for BRE immobilization include (A) simple retention of the functional molecular entities behind a thin semipermeable membrane, (B) covalent BRE bonding to reactive functional groups that either are intrinsic to the sensor surface itself or have been introduced through predeposited thin-film coatings, (C) adsorption, (D) covalent cross-linking of the BRE to form a network, and (E) placement of the BRE within a polymer or hydrogel layer.

A variety of synthetic (e.g., polyacrylamides, polyacrylates, and polyvinyl alcohols) and natural (e.g., cellulose, agarose, and collagen) polymers have proved suitable for biosensor assembly: whichever polymer is used, it is essential that the immobilization process does not adversely affect the biomolecules' molecular accessibility and function. With this stipulation in mind chitin and to a considerably greater extent chitosan, a good hydrogel-former, gained favor as immobilizing materials for biosensors as they were well documented in other applications because of their biocompatibility and chemically adaptability through reactions of intrinsic functional groups.

The following section is a description of the derivation of usable purified chitin and chitosan from their natural sources and also summarizes their chemical and functional properties that are relevant to biosensor construction and performance.

3. RELEVANT CHEMICAL AND FUNCTIONAL PROPERTIES OF CHITIN AND CHITOSAN

Citations 1–74 broadly link the chemical and physical properties of chitin and chitosan with their potential applications and are suggested as complementary sources of information on uses of these compounds. Chitin and chitosan are both aminoglucopyrans: extended linear chains of (β 1–4)-linked *N*-acetylglucosamine and glucosamine residues, randomly distributed. The chemical difference between purified natural chitin and synthetic chitosan is the degree of acetylation of the 2-amino groups (Figure 1). In chitin more than 50% of these residues are acetylated, while in chitosan they are predominantly deacetylated. More specifically, levels of *N*-acetylation may be >90% in biologically derived chitin and, if controlled during production, low in special technical chitosan variants.

Well-developed industrial chemical processes are used for production of purified chitin and then chitosan, either from crustacean shell waste from marine food production or from the waste material of the silk, mushroom, and honey-harvesting industries. Acidic carbonate removal, mild alkaline deproteinization, and decoloring and bleaching with organic solvents and hypochlorite makes pure crystalline chitin out of, for instance, finely ground shrimp, crab, and lobster residues, silkworm cuticles, or bee exoskeletons. If desired, the chitin can be converted into chitosan through *N*-deacetylation by hot and strong alkali. The particle size of the starting material and intensity of later chemical treatments (temperature, duration, and solution concentrations) affect the degree of polymerization and acetylation and the crystallinity and purity of the final products. Enzymatic degradation of chitin with purified deacetylases offers a gentler pathway to chitosan with a controlled ratio of acetylated to deacetylated amino groups;^{109–111} however, this process has not yet reached a level of development suitable for generation of larger industrial-scale quantities of chitosan.

Natural chitin has an average molecular weight in the MDa range; however, this is not maintained in industrial purified chitin because of random breakdown of carbohydrate chains during the chemical fabrication procedure, and a few hundreds of kDa are more likely. Commercial chitin comes as whitish powder, flakes, beads, or nanoscale whiskers,¹¹² typically with 75–95% acetylation. It does not dissolve in simple aqueous media, but dilute solutions can be made in, for instance, alcohol saturated with CaCl_2 or *N,N*-dimethylacetamide containing LiCl . Like chitin, chitosan is marketed in the physical forms of white powders, flakes, and small beads but with high (>~500 kDa), medium (~50–500 kDa), and low (<~50 kDa) molecular weights and a range of degrees of deacetylation. The plentiful amino functionalities throughout the chitosan polymer can be protonated, so under acidic conditions chitosan is able to dissolve reasonably well in water as an induced polycation, the water solubility depending on the degree of deacetylation. The solubility of chitosan depends on the pH and ionic strength of the aqueous medium and is influenced by the proportion and distribution of acetylated and deacetylated residues along the backbone. The availability of aqueous chitosan solutions under near-physiological conditions con-

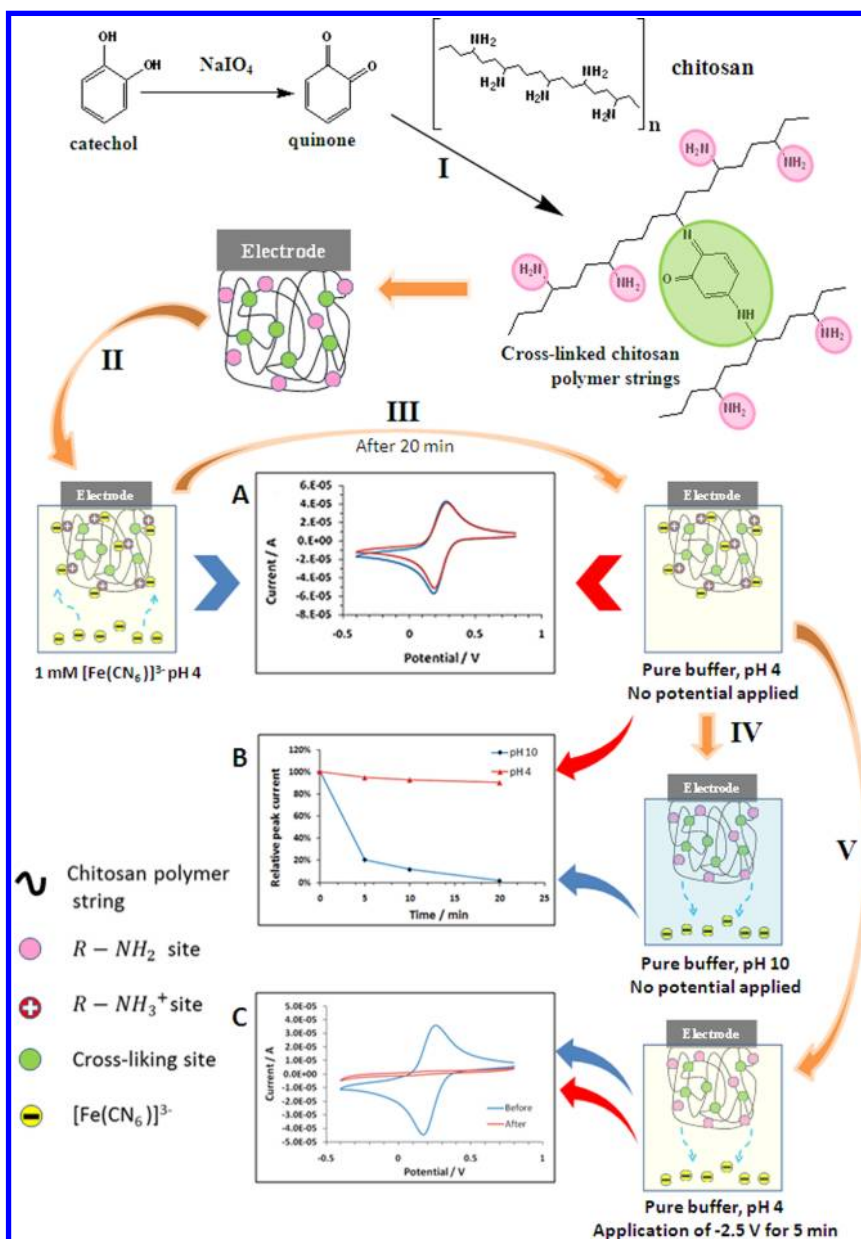


Figure 4. Catechol-induced cross-linking of chitosan polymer strings and pH-responsive release of electrostatically entrapped ferricyanide from a catechol-linked chitosan electrode coating. (I) Principle of the cross-linking procedure. (II) Immersion of modified electrode in $1.0 \text{ mM } [\text{Fe}(\text{CN})_6]^{3-}$ solution at pH 4; cyclic voltammogram (CV) measured 20 min after immersion; anodic and cathodic current peaks (A, blue) represent the reversible electron transfer reaction of the iron species at the working electrode. (III) Removal of the chitosan-modified working electrode, water rinsing, and subsequent immersion in a buffer solution, still pH 4 but free of $[\text{Fe}(\text{CN})_6]^{3-}$; though no dissolved $[\text{Fe}(\text{CN})_6]^{3-}$ is present, the CV displays the typical voltammetric ferricyanide redox wave (A, red) with the cathodic peak current remaining stable over time (B, red). Apparently a steady load of ferricyanide in the chitosan matrix exists at pH 4 through charge attraction between $[\text{Fe}(\text{CN})_6]^{3-}$ and chitosan's ammonium groups. (IV) $[\text{Fe}(\text{CN})_6]^{3-}$ -loaded electrode now measured in alkaline buffer solutions, pH 10. Cathodic peak current decays rapidly to zero after 20 min (B, blue). (V) Immersion of a ferricyanide-loaded chitosan-modified electrode into a mediator buffer of pH 4. CVs were recorded before (C, blue) and after (C, red) 5 min application of a cathodic potential of -2.5 V vs reference electrode. Initially observed ferricyanide redox peak disappeared after this treatment, because potential-induced OH^- generation and consequent deprotonation of amino groups in the chitinous electrode coating caused loss of ferricyanide anions into the bulk solution. Scan rate for all cyclic voltammograms was 50 mV s^{-1} .¹⁴²

trasts with the parent material, chitin; for this reason organic solvents can be avoided in the preparation of casting or spinning solutions, which makes exploitation of chitosan more biocompatible and thus widens the range of its applications. Nonetheless, well-adherent thin films of both materials can be simply prepared using their dilute solutions in casting, spinning, or dip-coating procedures, resulting in structures of density and porosity that are adjustable by the number of casting/dipping/

spinning repetitions and by the specific composition of the casting/dipping/spinning solutions. It is also noteworthy that dissolution of chitosan salts in NH_4HCO_3 solutions with pH as high as 9.6 is possible and can be used to prepare solutions of chitosan as carbamate ammonium salts for spray-drying applications.¹¹³

Chitin and chitosan possess good biocompatibility, have potential to form uniform films and hydrogels, and contain

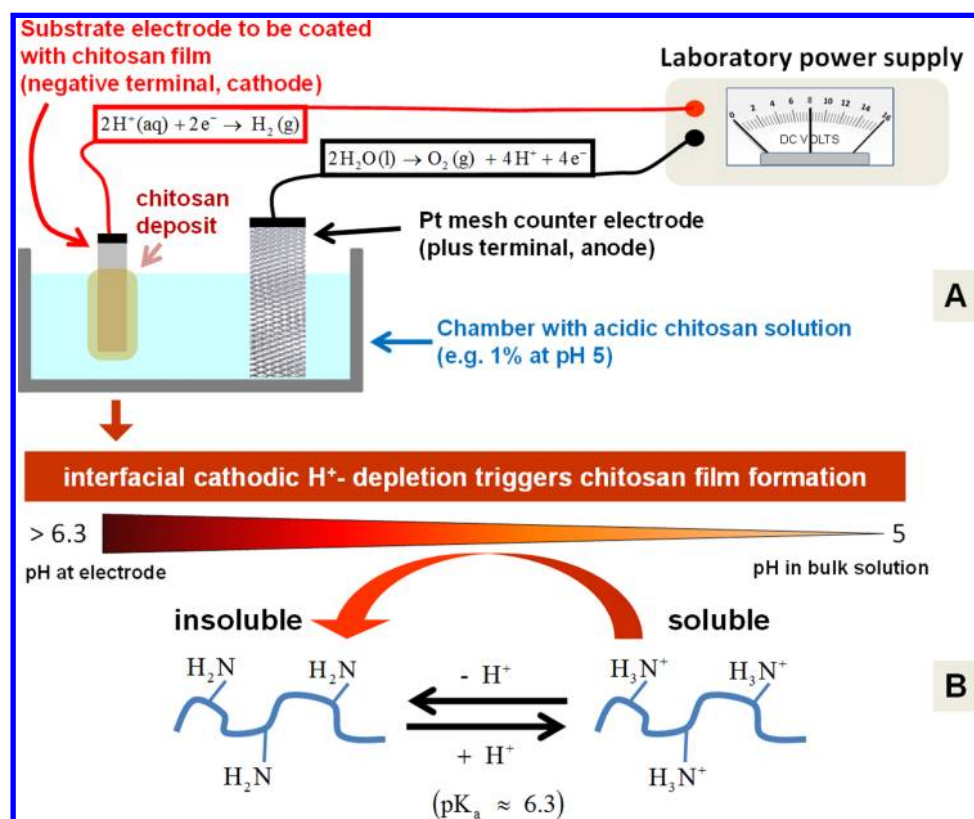


Figure 5. (A) Instrumental setup for electrodeposition of thin chitosan films on substrate electrodes, and (B) mechanism of chitosan precipitation onto cathode surfaces.

multiple oxygen- and nitrogen-based functional groups that can be chemically modified. For *in vitro* EC work the biocompatibility of the immobilization matrix and its functional constituents determines the integrity and lifetime of the entrapped macromolecules that enable analyte recognition and quantification. A stable and reproducible long-term response is achieved only when the matrix-induced degradation of the immobilized BRE occurs at a negligible rate. The situation is more complex when EC biosensors are used for *in vivo* measurements, e.g., for metabolite diagnostics in hospitals or in medical or pharmaceutical research with laboratory animals. In these cases the sensing tip of implantable versions of a biosensor is placed in the tissue surrounding the target sites in the bodies of patients or animal models. The compatibility of the immobilizing layer with the BRE is still crucial for durable sensor performance, but in addition, the sensor's composition and tip design should not trigger a local inflammatory host response, as there is a risk of sensor fouling by cellular release of absorbable proteins and lipids or immunoprotective fibrous tip encapsulation. For *in vivo* applications the aim must therefore be to construct the biosensor from materials that are as nontoxic as possible for both the BRE and the tissue under study. As expected for biological polymers, the tissue compatibility and low immunogenicity of chitin and chitosan have been confirmed in many clinical trials. Laboratory and pilot studies have also indicated their value in mild and curative wound coating,^{114–116} as supportive scaffolds for tissue and bone regeneration and engineering,^{117–122} and in drug^{123–128} and vaccine^{129–131} delivery systems, suggesting the possibility of using these biomaterials for improved biosensor design, as explored in this review.

As shown in Figure 1, the structures of chitin and chitosan contain abundant $-\text{OH}$, $-\text{NHCOCH}_3$, and $-\text{NH}_2$ functional groups throughout the polymer strands. These well-distributed reactive entities permit derivatization of the biopolymers if chemical tailoring would benefit sensor performance or is required for realizing a particular sensing scheme. Chitin and chitosan derivatives that have been synthesized for various applications by covalent modification of the backbone include sugar-modified, phosphorylated, quaternized, cyclodextrin-linked, thiolated, sulfated, azidated, ferrocene-branched, and crown ether-bound versions.^{29,34,132–140} An important advantage of chitosan is that modification of its primary amino groups with chosen electron-donating or -withdrawing functional groups alters the charge on the biomaterial, allowing fine tuning of the electrostatic interaction of chitosan immobilization matrices with negatively charged biomolecules and/or electrochemical redox mediators. Introduction of bulky substituents, on the other hand, is another option for regulating the level of conjugate formation with oppositely charged binding partners by controlled steric hindrance. These strategies were exploited in a recent study that reported addition of $-\text{CH}_3$, $-\text{Cl}$, $-\text{OH}$, cyclohexane, benzene, or phthalate entities to the $-\text{NH}_2$ groups in chitosan, resulting in clear differences in the efficiency of chitosan variants in binding single-stranded DNA.¹⁴¹ A more recent study utilized *o*-quinones as bifunctional modifiers of the amino groups of chitosan and produced promising hydrogels of the material.¹⁴² The *in situ*, metaperiodate-induced oxidation of a dissolved precursor, catechol, generated the reactive quinone that then produced covalent cross-linking of individual chitosan strings by Michael addition and Schiff's base formation (Figure 4).

When applied to predeposited chitosan electrode coatings this procedure produced adherent hydrogels that in low-pH solutions could entrap the redox probe $[\text{Fe}(\text{CN})_6]^{3-/4-}$ by electrostatic interaction with residual amino groups (Figure 4A). The redox probe was, however, readily released when the protonated groups within the catechol-cross-linked chitosan electrode layers were neutralized by exposure to high-pH bulk solutions or application of potentials negative enough to produce cathodic water splitting and hence generation of OH^- . The pH- or voltage-driven switch between capture and release of $[\text{Fe}(\text{CN})_6]^{3-/4-}$ was demonstrated by cyclic voltammetry in mediator-containing and mediator-free supporting electrolytes of low and high pH and thus under loading and discharge conditions, respectively (Figure 4B and 4C). Successful establishment of stimulus-responsive chitosan through quinone-based cross-linking is a promising example of the power of material design and an important step toward accomplishment of smart chemically modified systems for sensor and other biomedical devices.

The chitin/chitosan modifications so far cited in this section are representative successful cases and should provide examples for future developments. The accessibility of the entire complement of hydroxyl and acetylamido (for chitin) or amino (for chitosan) groups suggests, moreover, the feasibility of future biosensor advancement through appropriate modification of these functional groups. Before or after formation of chitin or chitosan films on an electrochemical detector surface, endogenous or introduced reactive groups may be used for cross-linking individual strands of the immobilizing polymer, for example, with glutaraldehyde, for linking other functional components in the layer or for extra modification of the chemical sensor after immobilization. Another interesting option is to exploit the free electron pairs on the nitrogen and oxygen atoms and, in the case of chitosan, the charges on protonated C2 amino groups, for internal complex formation, metal ligation, and ionic binding. Metal fixation by chitosan-based composite materials has actually been developed into an efficient procedure for removal of heavy metal^{143–147} and dye^{148,149} contaminants of industrial wastewater.

In addition to the wide range of adaptations of properties that can be achieved through synthetic chemistry, the feasibility of electrochemical deposition as a thin electrode covering from diluted aqueous solutions is another major asset of chitosan for advanced biosensor fabrication. Thin film chitosan electrodeposition was first reported by Wu et al.¹⁵⁰ In contrast to the common electrodeposition of, for instance, metal coatings from their hydrated ions in solution, chitosan “electrodeposition” does not involve direct or indirect redox conversion of the film-forming material itself. Instead, deposition of chitosan on an electrode is brought about by cathodic hydrogen evolution from water electrolysis, which consumes protons and generates hydroxide ions at the interface between the negatively polarized electrode and the electrolyte. As depicted in Figure 5, the corresponding local increase in interfacial pH neutralizes the positive charges on the ammonium groups in chitosan chains reaching the cathode through electrostatic attraction. Continuous electrophoretic delivery and concomitant removal of positive charges produces chitosan thin film formation on the sensor surface, as the material’s solubility falls to the point of precipitation (“sol–gel transition”).

Apart from the nature of the waterborne polyelectrolyte, chitosan electrodeposition is mechanistically comparable to the familiar industrial process of electrodeposition of paint (EDP),

based on water-dispersible anionic polyacrylic or cationic polyepoxy resins. Among customers the practice of EDP is also known as electrocoating, electropainting, e-coating, electrophoretic coating, or electrophoretic painting and is a proven method for applying corrosion-protective paint layers to automobile bodies and the interior of food tins.^{151–155} However, in a pioneering step in sensor fabrication, EDP was established in the 1990s as a convenient, nonmanual approach for effectively insulating etched Pt/Ir and W scanning tunneling microscopy (STM) tips^{156,157} and carbon fibers^{158,159} in order to produce electrochemical STM probes and carbon disk microelectrodes, respectively. A subsequent study by Kurzawa et al. confirmed the suitability of commercial EDP formulations for formation of enzyme biosensor immobilization matrices when the final heat-curing step was omitted so as to preserve electrical paint conductivity.¹⁶⁰

To optimize deposition, industrial EDP baths are supplemented with pigments, surfactants, antifouling agents, or (electro-)catalysts, and these additives are not intentionally optimized for biocompatibility as the end use of the paint formulations is corrosion protection of metallic products rather than biosensor applications. On the other hand, chitosan electrodeposition solutions are freshly prepared, and at least in sensor fabrication and work with sensitive biological recognition elements, use of toxic chemical additions can easily be avoided with critical awareness and proper choice of materials. This experimental freedom is one advantage of chitosan over anodic or cathodic EDP as long as commercial EDP systems are the alternative. Another is that the operational pH is closer to physiological values than that used with anodic and cathodic paints based on micellar acrylic and epoxy polyelectrolytes.

Electrodeposition of chitosan is particularly important in miniaturization of electrochemical biosensor devices and in selective placement of immobilizing biocompatible polymer deposits on the active sites of individually addressable micro- and nanoelectrode arrays that are not easily reached with other (manual) film-forming procedures. Figure 6 is a representative

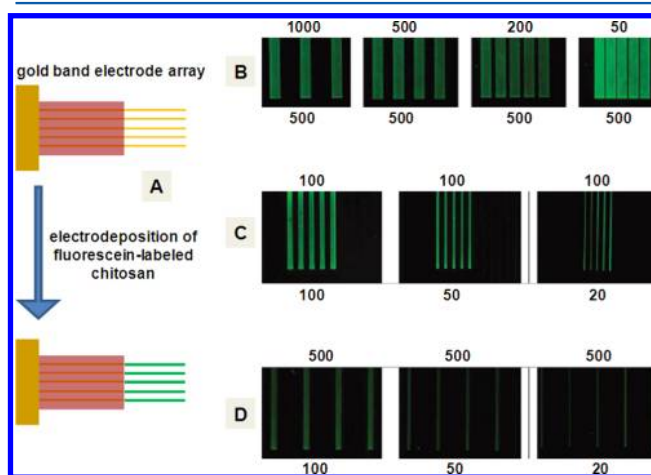


Figure 6. (A) Scheme showing electrochemically driven, pH-induced deposition of fluorescein-labeled chitosan onto the individual conductive entities of gold band electrode arrays of various band widths and separations. (B–D) Photomicrographs taken with an optical fluorescence microscope after the spatially selective chitosan electrodeposition on the patterned gold template was performed. Band widths and separations of the studied electrode test structures, in micrometers, are shown below and above the images, respectively.¹⁶¹

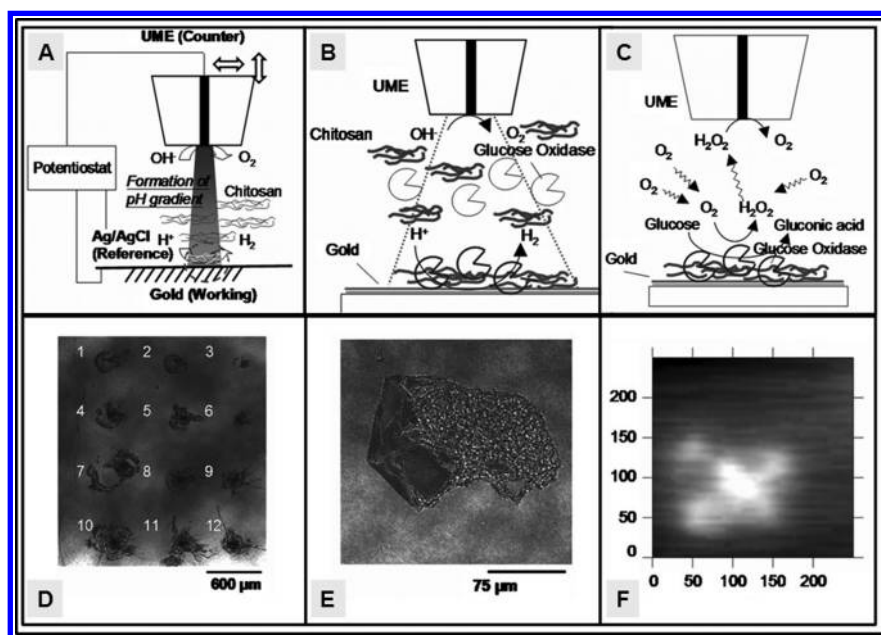


Figure 7. Generation of chitosan micropatterns in a scanning electrochemical microscope (SECM). (A) SECM arrangement for probe tip-controlled local pH elevation on a flat gold substrate electrode to be chitosan decorated. (B) Entrapment of glucose oxidase (GOD) into chitosan electrodeposits that grow in front of a positioned SECM probe tip. (C) Detection of hydrogen peroxide production in a GOD–chitosan spot upon provision of the enzyme’s substrate, glucose. H_2O_2 produced in the spot diffuses to the anodic disc of the probe and there generates current through electrooxidation. Two-dimensional (x,y) plot of the SECM tip current provides a map of the spatial distribution of H_2O_2 , which reflects the localization and dimension of the immobilized spot. (D) Confocal laser scanning microscope (CLSM) reflectance image of chitosan spots that have been electrodeposited by the procedure depicted in A through application of potential pulses ($E_{\text{substrate}} = -1.0$ V vs Ag/AgCl, pulse length = 1 s) between the SECM tip (Pt microdisk electrode; radius (r) = 25.9 μm ; RG factor ($\text{radius}_{\text{glass insulation}}/\text{radius}_{\text{Pt}}$) = 4; tip-to-substrate separation (d) = 10 μm) and the gold substrate. One (spots 1, 2, and 3), two (spots 4, 5, and 6), three (spots 7, 8, and 9), and four (spots 10, 11, and 12) potential pulses were applied for spot generation. Deposition solution contained 1.5% (w/v) chitosan, pH 5.7. (E) CLSM reflectance image of GOD–chitosan spot obtained by a single localized pulse deposition from a solution containing 5 mg/mL of the enzyme and 1.5% chitosan. Other experimental conditions were as listed in D. (F) Generator-collector type of SECM image that visualizes H_2O_2 production in a tiny GOD–chitosan spot prepared by the procedure described in C on exposure to 50 mM glucose in the air-saturated surrounding electrolyte (0.1 M phosphate buffer, 0.1 M KCl, pH 7.0). Parameters for SECM image acquisition: $E_{\text{Pt-ECM tip}} = +0.6$ V vs Ag/AgCl; $r_{\text{Pt-SECM tip}} = 13.2$ μm , RG = 25; $d = 10$ μm , tip scan speed = 10 $\mu\text{m/s}$. Reprinted with permission from ref 167. Copyright 2009 John Wiley and Sons.

selection from one of the earlier studies on chitosan electrodeposition on gold band electrode arrays,¹⁶¹ and the images of the chitosan-coated gold strips confirm the excellent spatial selectivity of the stimulus-responsive deposition method. As can clearly be seen, individual members of the five-band array structure could be chitosan coated with precision and neither a band separation as narrow as 20 μm nor a bandwidth as thin as 20 μm led to visible failures. The excellent precision of the chitosan electrodeposition was confirmed by the demonstration that substrate electrode edges could be constructed from electrodeposited chitosan scaffolds with an accuracy of 0.5–1 μm ¹⁶² and compared very well with that of an EDP modification of a gold microdisk structure.¹⁶⁰ A more recent study demonstrated that defined redox activity could be established in thin electrodeposited chitosan films through their reaction with the products of anodic catechol oxidation at the covered electrode.¹⁶³

Many original research articles have addressed the topic of voltage-induced chitosan deposition. In the context of sensor studies, immobilization of oxidases, horseradish peroxidase, acetylcholine esterase, gelatin, albumin, and silk fibroin has been achieved using entrapment into chitosan electrode layers formed from solution by an electrochemically produced pH shift. Incorporation of nanoscale immobilization matrix modifiers such as CNTs, metal, or metal oxide nanoparticles by coentrapment into nascent chitosan electrodeposits or

through chemical modifications of freshly electrodeposited chitosan films by postdeposition protein cross-linking was also successful. Space limitations preclude detailed description of individual procedures here, but many of the examples have been discussed in a set of recent comprehensive reviews on site-directed voltage-dependent protein assembly,^{164–166} and these are recommended as sources of further information.

Electrodeposition of chitosan on the active sites of prefabricated electrode microarrays can generate microscopic chitosan patterns that can be further processed into spatially confined sensor entities incorporating biomolecules such as enzymes, DNA strands, antibodies, or antigens. Recently, chitosan-based BRE micropatterning was also achieved using the surface alteration capability of the tiny microelectrode tips of a scanning electrochemical microscope (SECM) for pattern formation.¹⁶⁷ The principle of the proposed scheme is illustrated in Figure 7. Briefly, a gold-covered microscope slide was connected in an SECM electrochemical cell as a large-area, plate-like cathode above which a disk-shaped Pt microelectrode (the “SECM tip”; diameter 10–50 μm) was positioned as a static counter-electrode (anode) at a working distance about the diameter of the insulated microelectrode metal disk. At a potential carefully adjusted to produce a steady rate of water electrolysis, cathodic proton reduction was spatially restricted to the area of the gold plate directly opposite the counter-electrode and only at this specific location

was the pH increased enough to neutralize dissolved chitosan, triggering surface precipitation and forming round patches of the material (Figure 7A). Repeated SECM tip-directed local electrodepositions at different x, y grid points on the substrate gold electrode formed regular arrays of microscopic chitosan sediments (Figure 7D). Addition of GOD to the chitosan electrolyte solution resulted in coprecipitation (Figure 7B), producing chitosan spots with firmly entrapped, fully functional enzyme (Figure 7C, E and F). Though further work is needed, this first successful demonstration of SECM-based chitosan pattern generation illustrates the power of the scanned probe technique in the fabrication of miniaturized biomimetic (sensor) devices. A future development may be extension of the methodology to the various types of noble metal and carbon nanoelectrodes that are now standard tools in electrochemical science, so as to move from the micro- to the nanoscale. Meanwhile, pattern generation with probe microscopes other than the SECM instrument remains a task for the future.

Chitin and chitosan are not the only film-forming biopolymers that may be used as natural surface modifiers of electrochemical (bio-)sensors. Polymeric arginine, lysine, glutamic acid, hyaluronic acid, and alginic acid, for instance, all have possible sensor applications, but among the many possibilities chitin and chitosan are attractive options as they are highly developed, nonhazardous, and cheaply available from commercial sources in various forms. Furthermore, the critical pH for chitosan's soluble–insoluble transition is about 6.3. Chitosan deposition on electrode surfaces can thus, in contrast to other natural polymers with similar behavior, occur under mild conditions through chemically or electrochemically induced pH changes close to physiological normality, ensuring suitably gentle conditions for immobilization of enzymes, antibodies, or nucleic acids. The amino groups in poly-L-lysine, for instance, have a pK_a of about 10.5 and transition between the protonated soluble and the neutral insoluble form of the polymer strings occurs at a highly alkaline pH, which is likely to cause denaturation of protein-based biological recognition elements and is therefore unfavorable to sensor assembly.

Recently, efficient anodic (instead of the usual cathodic) electrodeposition of chitosan hydrogels was reported.¹⁶⁸ The novel scheme starts with anodic oxidation of chloride ions to chlorine, which forms reactive HOCl through reaction with water. This can oxidize alcohol groups within chitosan to aldehydes, which then covalently cross-link to amine groups in other strands through Schiff base formation, creating chitosan hydrogel networks. An attractive feature of the anodic chitosan hydrogels is that they can repeatedly swell and shrink, with up to 3-fold volume changes in response to cyclic pH changes, an effect that has the potential to establish actuation functions such as valve controls in miniaturized fluidic devices. Furthermore, anodically deposited chitosan offers intrinsic aldehyde groups for covalent fixation of enzymes, antibodies, and oligonucleotides, avoiding the need for cross-linkers such as glutaraldehyde. The strategy was validated by construction of functional glucose biosensors using immobilized glucose oxidase.

4. SURVEY OF ELECTROCHEMICAL (BIO-)SENSOR DESIGNS WITH CHITIN OR CHITOSAN AS SURFACE MODIFIER

4.1. Chitin as Electrochemical (Bio-)Sensor Component

Chitosan is somewhat soluble in mildly acidic aqueous media and can thus be handled under conditions that are close to physiological, while chitin needs harsh organic solvents for dissolution and processing. Because of this difference chitosan is more frequently used than chitin in constructing electrochemical (bio-)sensors. However, chitin has been used as a tunable electrode modifier in several studies, and the following examples illustrate some possible options. Thin and flexible cast chitin membranes^{169–171} or chitin dispersed in carbon/platinum pastes^{172,173} proved to be suitable matrices for electrostatic immobilization of enzymes. On the surface of Clark-type oxygen or noble metal hydrogen peroxide electrodes GOD-loaded chitin films performed well as glucose biosensors, as did GOD/chitin/carbon/Pt pastes. Two other studies used the amine and hydroxyl functionalities of partially deacetylated chitin, with glyoxal, carbodiimide, or epichlorohydrin being used both to cross-link individual chitin chains and to attach enzymes to the chitin networks.^{174,175} Corn or pea peroxidase was bound to the chitin matrix and the chitin added to carbon pastes to construct functional biosensors for screening for adrenaline and rosmarinic acid, respectively, in pharmaceutical formulations. Alternatively, the amino-reactive cross-linker glutaraldehyde can be used for covalent linkage of enzyme molecules to the surface of a chitin membrane. This strategy was used successfully for choline oxidase, with choline oxidase-modified chitin films placed on a Pt disk electrode as part of an electrochemical flow cell to create a choline-sensing flow-injection analysis system for measuring the cholinesterase inhibitory activities of synthetic chemicals or natural products.¹⁷⁶ Furthermore, chitin films can be used as immobilizing platforms not only for enzymes but also for other protein-based BRE's. This was shown by a study in which streptavidin was attached to chitin electrode coatings through electrostatic interactions; successful fixation of the biomolecule was demonstrated by pulse voltammetry measurements in solutions of biotin with daunomycin attached as an electroactive redox indicator.¹⁷⁷ More recently, smooth, homogeneous, ultrathin chitin films have been reported as suitable platforms for biosensor architectures.¹⁷⁸

Though not directly related to biosensors it is worth mentioning that electrostatic binding of inorganic anions by protonated residual amino groups in chitin electrode coatings permits applications in an accumulation scheme in stripping voltammetry (SV), for instance, chitin films on glassy carbon disk electrodes allowed voltammetric detection of molybdate (MoO_4^{2-}) in seawater at concentrations in the nanomolar range.¹⁷⁹ Also, a conductometric humidity sensor was devised based on tailored chitin–polyaniline blends formed into films that changed their internal electrical resistance as a function of the water vapor content in their gas-phase environment.¹⁸⁰

4.2. Chitosan-Based Electrochemical Nucleic Acid Biosensors

Biosensing with DNA chips or microarrays is used to screen biological samples for the presence of fragments of single-stranded target cDNA or short-chain oligonucleotide (OND) ladders that have been preidentified in clinical studies as markers for the onset or manifestation of a variety of common

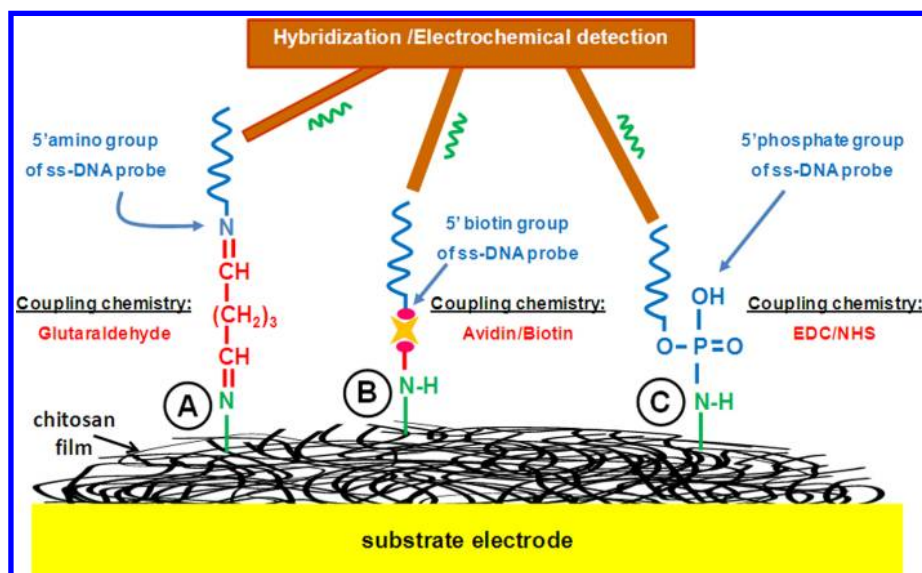


Figure 8. Chitosan-supported DNA immobilization on electrode surfaces. (A) Utilization of glutaraldehyde-based coupling chemistry. (B) Exploitation of biotin-modified chitosan and DNA and use of avidin as a high-affinity bridging molecule. (C) Coupling the phosphate groups of the DNA to the amino groups of chitosan with EDC/NHS.

diseases, indicators of infection with bacterial pathogens or as signs of contamination with other toxic biological matter.^{181–190} The key issues for solving diagnostic tasks with blood, urine, or tissue extracts are production of genomic nucleotide probes (i.e., synthesis of sections of end-modified single-stranded cDNA, oligonucleotides (ONs), or peptide nucleic acids (PNAs)), their precise microstructured assembly (immobilization) on the analytical platform, typically a solid transducer onto which high- or low-density arrangements of microscopic spots of probe DNA are placed in a regular pattern, and, finally, sensitive detection of hybridization between immobilized probe and dissolved complementary target material in some of the spots. The most widely used probe spot carrier and electrode material in electrochemical DNA chip technology is gold, although carbon and metal oxide surfaces have also been used. DNA probe immobilization on Au substrates exploits the strong affinity between Au and sulfur atoms, which produces rapid self-assembly of covalently anchored two-dimensional monolayers upon exposure of the surface to the thiol-containing organic modifier molecules. This scheme can be applied to ss-DNA probes with sulfhydryl groups at one end, and this chemistry has become routine for immobilizing nucleic acid strands to Au slides by computerized DNA microspotting procedures. Since thiol-based immobilization is usually limited to gold substrates, other methods are needed for attaching DNA probes to the surfaces of transducers such as glassy carbon or screen-printed carbon and platinum electrodes. For example, covalent coupling through oxygen atoms on preactivated carbon electrodes or fixation of biotinylated DNA probes through stable complex formation, with avidin attached to a covalently premodified sensor surface, may be used. Several studies have reported the potential of chitosan for DNA probe immobilization and even hybridization detection, used either in unmodified form as a coating system or as a composite material with, for instance, carbon nanotubes (CNTs) or metal/metal oxide nanoparticles. Chitosan-based DNA surface fixation works with both covalent and electrostatic bonding to the biopolymer, the latter depending on attraction between protonated and thus cationic amino groups in the

chitosan sensor coating and anionic phosphate groups in the backbone of the DNA chains.

Different schemes for DNA immobilization through attachment to chitosan are shown in Figure 8. They exploit chitosan's reactive amino groups which with a proper choice of reagents allow covalent bond formation or affinity conjugation to functional groups at, for instance, the 5' terminals of chemically adapted probe DNA. Linkage through glutaraldehyde to 5' amino entities,^{191,192} for instance, and EDC/NHS-directed chemical cross-linking to 5' phosphate groups^{193–197} are feasible strategies for linking DNA to preformed chitosan sensor surface coatings, but other coupling procedures may also be used. Biotinylated probe DNA can be bound to chitosan on electrode surfaces by biotin–(strept)avidin coupling, which allows noncovalent but still very tight binding of the two components. An electrochemical platform for detection of gonorrhea, a widespread sexually transmitted disease, has been established using this methodology.¹⁹⁸

Although not focused on biosensors, a study dealing with pH-induced capture and release of DNA by chitosan-coated polymer particles demonstrated the potency of chitosan surface modifications for controlled electrostatic DNA immobilization.¹⁹⁹ At slightly acidic pH values, polycationic chitosan scaffolds attracted and bound anionic nucleic acid strands; however, a change to alkaline conditions with consequent deprotonation of the chitosan allowed effective elution of the DNA from the beads. This type of ionic interaction can be used for immobilizing nucleic acid fragments on biosensor surfaces. Fish sperm ds-DNA, for instance, was immobilized electrostatically on chitosan/CNT-modified screen-printed carbon (SPCE)^{200,201} and graphite²⁰² electrodes, and with the aid of a redox marker the arrangement allowed detection of deep DNA damage.²⁰⁰ The ds-DNA capturing layer was created by dropping CNT, dispersed in a chitosan solution, onto the active discs of the SPCEs and allowing the solvent to evaporate. Condensation of chitosan with DNA segments was then achieved by exposure of the freshly formed sensor coatings to a stock solution of the target material. Likewise, cross-linked CNT–chitosan coatings were used to entrap calf thymus ds-

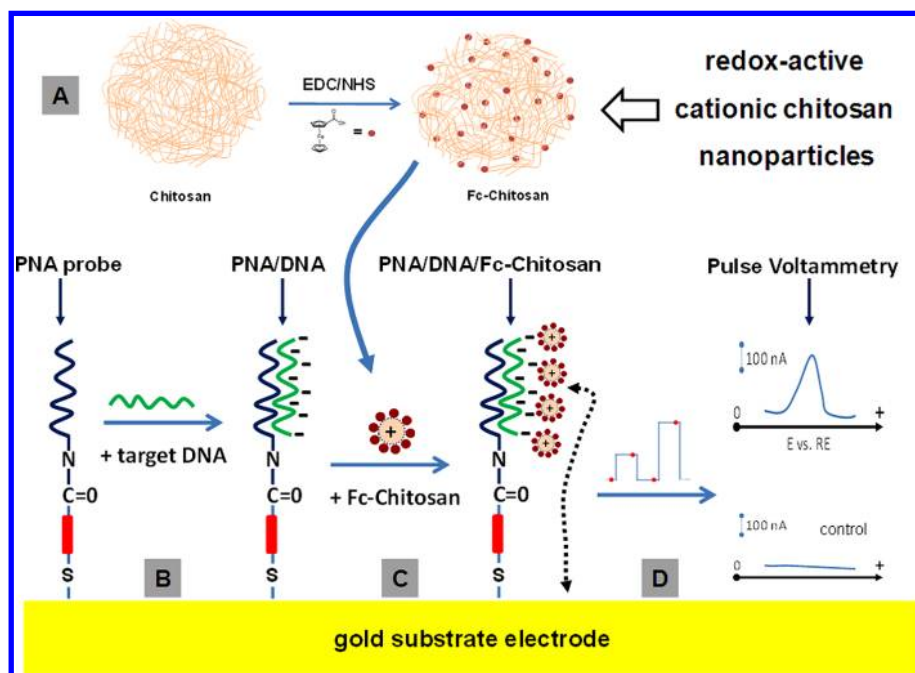


Figure 9. Chitosan-supported hybridization detection scheme for PNA/DNA biosensors. (A) Preparation of ferrocene-conjugated chitosan nanoparticles as the electroactive label. Under slightly acidic solutions amino groups are protonated, rendering the polymeric chitosan–Fc nanoparticles cationic. (B) PNA probe immobilization as self-assembled monolayers and through thiol coupling and binding of the complementary target DNA in a hybridization test. (C) Exposure of the PNA/DNA hybrid to a solution containing chitosan–Fc nanoparticles and electrostatic conjugation of the chitosan label to the nucleic acid double helix. (D) Detection of the presence of the chitosan–Fc label in particular PNA probe spots by pulse voltammetry.

DNA,²⁰³ a synthetic 20-bp oligonucleotide sequence related to the dengue virus genome²⁰⁴ was bound to drop-coated chitosan films, and the complementary oligonucleotides for DNA sequences associated with colorectal cancer were bound by a CNT–zirconium oxide (ZrO₂) nanoparticle–chitosan matrix at the appropriate pH.²⁰⁵

These examples illustrate the practicability of using DNA/chitosan ionic interactions for probe immobilization. However, as recently shown by Kerman et al., the effect can also be used to establish an elegant chitosan-supported hybridization detection scheme.²⁰⁶ For this purpose, electroactive chitosan nanoparticles were synthesized through exposure of finely dispersed nanometer chitosan beads to ferrocene carboxylic acid (Fc–COOH) and EDC/NHS as reactive chemical coupling agents (Figure 9A). Amide bond formation linked ferrocene (Fc) molecules covalently to some of the internal and external N functionalities in the chitosan nanoparticles, loading the structure with an oxidizable mediator for electroanalysis. The principle of the final hybridization detection scheme is illustrated in Figure 9B–D. Briefly, PNA probe strands, carrying no net charge, were immobilized on the surface of a gold electrode through their thiol groups. Incubation with the complementary target DNA formed a negatively charged duplex structure which, in the labeling step, bound prefabricated Fc–chitosan nanoparticles electrostatically. Anodic oxidation of surface-proximal Fc labels during differential pulse voltammetry produced typical bell-shaped current peaks, indicating the presence of PNA/DNA hybrids. As expected, no current signals were obtained when in control experiments the PNA probes were hybridized with complementary target PNA strands, because the Fc–chitosan nanoparticles had no affinity for the neutral PNA/PNA conjugates.

Ideally, signal generation with DNA biosensors should be closely coupled to the hybridization between surface-bound and dissolved target DNA strands and consequent double-helix formation or the binding of short reporter DNA strands. However, nonspecific adsorption of the target or reporter DNA strands to the sensor surface may be unavoidable and, depending on the electrochemical scheme in use for transduction, will compromise the analytical performance to a greater or lesser extent. With a chitosan-based immobilization matrix for DNA sensors there is a risk of nonspecific binding to the functional surface coating, stemming from electrostatic interaction between the DNA backbone and the residual protonated amino groups in the chitosan network. Decreasing the number of amino functionalities within the chitosan thin film sensor coating, during or after probe strand attachment, minimizes nonspecific DNA binding, and their reaction with glutaraldehyde to form imino groups is a feasible approach. Alternatively, the surface of chitosan-based DNA sensors could be treated after the hybridization step with solutions of Mg²⁺ or Ca²⁺ salts and urea. Divalent cations tend to neutralize the negative charges on DNA strands and inhibit their electrostatic binding to chitosan, while urea weakens hydrogen bonding between DNA and chitosan. A thorough wash with MgCl₂ and/or urea-containing buffer should therefore remove most or all of the nonspecifically bound molecules from the sensor surface and improve the quality of quantitative applications.

4.3. Chitosan-Based Electrochemical Immunosensors

Immunosensors, whether employing electrochemical or other detection schemes, take advantage of the specific recognition of antigen molecules by complementary antibodies (immunoglobulins). The basis of the outstanding analytical performance of existing immunological assays is the strong Ab–Ag binding (dissociation constants, K_D , of $\leq 10^{-7}$ M)²⁰⁷ and the high

Table 1. Chitosan-Based Electrochemical Immunosensors: Target Analytes and Performance

analyte	electrode modification ^a	detection method	linear response range (ng/mL)	detection limit (ng/mL)	ref
α -1-fetoprotein (AFP)	BSA/anti-AFP/AuNPs/Thi/CS-AuNPs	CV	0.4–200.0	0.24	232
	anti-AFP/AuNP/CNT/CS	EIS and CV	1.0–55.0	0.6	233
	ITO/TiO ₂ /CdS/CS/anti-AFP/BSA	EIS	0.05–50	0.04	234
carcinoembryonic antigen (CEA)	anti-CEA/AuNPs/MnO ₂ and CS/PB	CV	0.25–8.0; 8.0–100.0	0.083	235
	anti-CEA/AuNPs/CNT/CS	CV and EIS	0.3–2.5; 2.5–20.0	0.01	236
	Anti CEA/AuNPs/CS	CV	0.2–120.0	0.06	237
	CS/AuNPs/anti-CEA	CV and EIS	0.1–2.0; 2.0–200.0	0.04	238
	BSA/anti-CEA/CS-CNTs-AuNPs	CV	0.01–80.0	0.0034	239
	anti-CEA/Au-Gra/CS-Fc and TiO ₂ NPs	CV and EIS	0.2–10.0; 10.0–160	0.08	240
	BSA/anti-CEA/AuNPs/Thi/CS				
	anti-CEA/AuNPs-Nafion/Fc-CS/GCE	CV and SWV	0.01–150.0	0.003	241
	HRP-anti-CEA/CEA/Au-CS	CV	2.0–20	1.0	242
human immunoglobulin G (huIgG)	QDs/CNTs-PDDA/AuNPs-CS	ECL	0.006–150	0.001	243
	huIgG/anti-huIgG/AuNPs/	EIS	0.3–120.0	0.1	244
	CNCPE Anti-huIgG/coral-shaped AuNPs-CS	CV EIS	0.05–50.0	0.005	245
human chorionic gonadotrophin ^b (hCG)	BSA/anti-hCG/AuNPs-TiO ₂ /Thi/GA/MWCNTs-CS/GCE	DPV	0.2–300.0	0.08	246
	HRP-anti-hCG/hCG/AuNPs-CS/GCE	CA	0.2–100.0	0.1	247
ochratoxin A	r-IgGs/PANI-CS	EIS	up to 10.0	0.1	248
	BSA/r-IgGs/CS-SiO ₂	DPV	0.005–0.06	0.003	249
	IgGs/CS-Fe ₃ O ₄	DPV	0.005–0.06	0.005	250
	IgGs/CS/TiO ₂	EIS	up to 10	NI	251
	r-IgGs/BSA/CNT/CS		0.25–6	0.25	252
hepatitis B surface antigen (HBsAg)	CS-Fc/AuNPs/anti-HBs	DPV	0.05–305	0.016	253
	anti-HBs/CS-SiO ₂ NP	CV and EIS	6.85–708	3.89	254
ferritin	antiferritin/Fe ₃ O ₄ magnetic NPs/CS	DPV	20.0–500.0	7.0	255
prostate-specific antigen (PSA)	anti/PSAAu-hydroxyapatite nanocomposite/CS	potentiometry	3.5–30	2.6	256
<i>Shigella flexneri</i> ^c	HRP-anti- <i>S. flexneri</i> /CNT/CS	CV	10 ⁴ –10 ¹⁰	2.3 × 10 ⁰³	257
dengue virus envelope protein (DENV)	anti-DENV/CS	CA	1.0–175.0	0.94	258

^aAbbreviations: CS, chitosan; PB, prussian blue; NPs, nanoparticles; CNT, carbon nanotube; GCE, glassy carbon electrode; Gra, graphene; QDs, quantum dots; PDDA, poly(diallyldimethylammonium chloride); CsNPCE, chitosan nanoparticle entrapped-carbon paste electrode; Thi, Thionine; GA, glutaraldehyde; PANI, polyaniline; Fc, ferrocene; HRP, horse radish peroxidase; r-IgG, rabbit immunoglobulin G; NI, no information; HBsAb, hepatitis B surface antibody; CV, cyclic voltammetry; DPV, differential pulse voltammetry; SWV, square wave voltammetry; EIS, electrochemical impedance spectroscopy; ECL, electrochemiluminescence; CA, chronoamperometry. ^bUnits of the linear range and detection limit are mIU/mL. mIU: milli international units. ^cUnits of the linear range and detection limit are cfu/mL. cfu: colony forming units.

specificity of protein complex formation. When applied in advanced configurations with optimized experimental parameter sets, detection limits of attomolar^{208–210} and even subattomolar²¹¹ concentration are possible with immunosensors. Comparable sensitivity is likewise available with DNA biosensors^{212–214} as these also are affinity-based tools with responses governed by strong binding characteristics. Enzyme biosensors, on the other hand, usually do not exceed femtomolar²¹⁵ detection limits if native rather than genetically engineered²¹⁶ proteins with enhanced turnover rates are employed for signal generation. This analytical quality has led various types of immunosensing to be used not only in medical diagnostics²¹⁷ but also in food screening^{218,219} and environmental analysis.^{220–224} A crucial step in construction of efficient electrochemical immunosensors is electrode surface immobilization of the antibody or antigen, and many strategies have been proposed for this kind of sensor modification. As stressed in representative original^{225–227} and review^{228–231} articles, one condition for achievement of good sensitivity is to achieve a high loading of the active electrode area with the capturing immunochemical component, since the surface density of receptors on the transducer governs the magnitude of the electrical signal generated on analyte binding. Accordingly,

strategies for simple but effective, reproducible, and stable antibody or antigen loading of electrodes at high density are intensely sought after, and among other surface preparations, various forms of chitosan films have been used as immunosensor platforms. In this context, a virtue of chitosan is that the number of amino sites available for covalent protein attachment on chitosan materials is flexible within a wide range, simply through a variation of the degree of deacetylation of the chosen variant.

Thus far, chitosan-supported EC immunosensors have been used for detection of biomarkers for hepatitis B, various cancers, pregnancy, the iron content of blood, the food-contaminant ochratoxin A, and the diarrhea-triggering bacteria *Shigella flexneri* (see Table 1). The table shows representative recent work on chitosan-supported electrochemical immunosensors with their performance characteristics (detection limits and dynamic linear ranges). In the successful cases drop-coated or electrodeposited chitosan films provided the elementary stage for surface immobilization either of antibody or of antigen and the supplementary components that with the film-forming biopolymer collectively formed the functional electrode coating.

Table 1 shows that the supplements in chitosan-based electrochemical immunosensors can be redox-active mediators

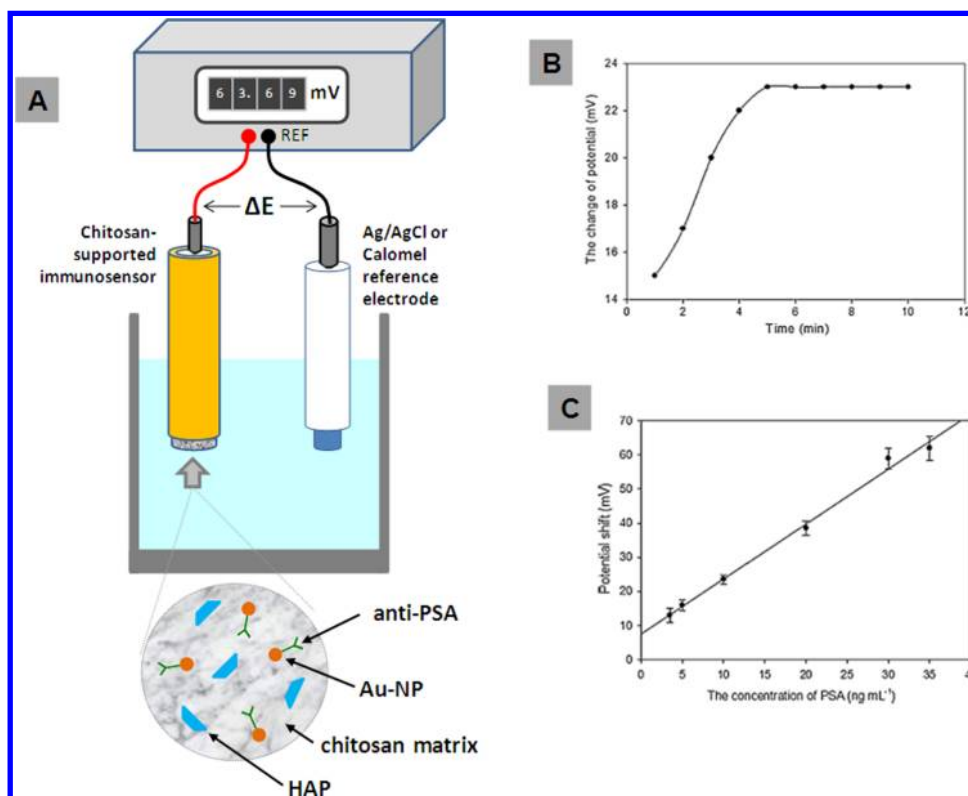


Figure 10. Potentiometric antigen quantification with chitosan-supported immunosensors. (A) Schematic representation of the experimental arrangement and sensor design. (B) Potentiometric time-dependent response of the immunosensor in phosphate buffer, pH 7.4, to addition of 10 ng/mL prostate-specific antigen. (C) Calibration plot for immunosensor. Individual data points are averages of five measurements. Abbreviations: anti-PSA, antiprostata-specific antigen (antibody); Au-NP, gold nanoparticles (anchor for antibody); HAP, biocompatible hydroxyapatite nanocrystals (porous high surface area adsorbent). Curves in B and C are reprinted with permission from ref 256. Copyright 2011 Elsevier Limited.

such as ferrocene and thionine or carbon nanotubes, graphene, conductive polymers, nanohydroxyapatite, gold nanoparticles, metal oxide, and metal sulfides. An option for antibody fixation in the chitosan matrix is covalent binding between amino groups in the biopolymer and the immunoglobins employing, for example, glutaraldehyde as cross-linking reagent. Alternatively, immobilization may be achieved with coimmobilized gold nanoparticles, which tightly adsorb antibody molecules without involvement of a covalent coupling agent. Normally the antigen/antibody interaction on chitosan-modified electrochemical platforms is translated into a signal that is detected by amperometry, voltammetry, or electrochemical impedance spectroscopy. However, as confirmed for detection of the hepatitis B surface antigen (HBsAg)²⁵⁴ and prostate-specific antigens, potentiometric measurements are also practicable.

Figure 10 shows the design and outcome of a typical potentiometric immunosensing procedure using a chitosan/antibody-modified gold electrode. The time course of the immunosensor potential, E_{IS} , is measured with a common reference electrode. The interaction of immobilized antibody with antigen in the measuring buffer solution and formation of the antibody/antigen conjugate affect the net electrical charge of the immunosensor/electrolyte interface, and a measurable shift in the sensor potentials is observed when the antigen concentration is raised from zero to a new level. In calibration, plots of E_{IS} against antigen concentration are linear over a certain range (see, for instance, Figure 10C) and can be used for antigen quantification.

As with DNA- and enzyme-biosensors, chitosan is an attractive immobilization material also for immunosensors

because of its abundant chemically modifiable functional groups and the feasibility of nonmanual, voltage-driven electrochemical deposition. Chitosan for use in immunosensor construction may usefully be covalently modified with synthetic functional groups. For instance, in order to minimize leakage of incorporated electroactive molecules, an initial covalent coupling of the redox mediator ferrocene to chitosan chains was carried out.²⁵³ On the other hand, prior covalent attachment of thionine to chitosan was used to increase the polymer's chemisorption affinity toward gold nanoparticles, on the electrode surface of the immunosensor.²³² Other studies have explored electrodeposition of unmodified or modified chitosan as a simple and easily controllable alternative to drop- or spin-coating procedures for creation of merged multielement immunosensor coatings.^{232,238,246,247,253,254}

Most successful electrochemical immunosensors using chitosan-supported immobilization matrices avoided the problem of nonspecific adsorption by blockage of nonspecific surface binding with bovine serum albumin (BSA). Though BSA treatment successfully maintains analyte sensitivity, other blocking agents may occasionally be a better choice for priming the sensor surface, and options to try include, for instance, ovalbumin, preimmune serum, skimmed milk proteins, and surfactants/detergents such as Tween 20, all of which have previously been used in immunoassay systems to minimize the nonspecific, background response.

4.4. Chitosan-Based Electrochemical Enzyme Biosensors

The number of reports on biosensors employing chitosan/enzyme complexes is far greater than for chitosan/DNA or

chitosan/antibody systems and increased continuously during the period 2005–2011. Summarizing the almost 300 publications on this subject is beyond the scope of this review. Instead, some representative studies will be described in order to demonstrate the potential of chitosan in enzyme biosensor construction. Most of the recently proposed designs are complex multicomponent systems that combine plain or chemically premodified chitosan variants with a particular enzyme and extra functional materials such as carbon nanotubes, graphene sheets, metal/metal oxide nanoparticles, ionic liquids, and clays, to name just a few. The required analyte specificity obviously determines selection of the enzyme, and oxidases, peroxidases, acetylcholine esterases, laccases, dehydrogenases, tyrosinases, reductases, hydrolases, and phosphatases have all been used. The functional supplements mentioned above have been incorporated individually or in combination, for instance, to improve the conductivity of the immobilization matrix or create redox pathways that connect the entrapped enzyme's active sites electrically with the electrode surface.

Chitosan itself is valued as a tightly adhering and biocompatible porous polymer that provides secure fixation of the enzyme to the biosensor surface while allowing substrate and cofactor mobility for their continuous access to the enzyme. Physical entrapment in the pores and channels of the chitosan matrix and electrostatic and covalent enzyme bonding to the polycationic chitosan chains have all been shown to be feasible means of immobilizing enzymes and avoiding leakage. As described above for chitosan-based DNA immobilization on electrode surfaces, covalent coupling of enzymes to chitosan electrode coatings can be achieved using chemically reactive groups within the two sensor elements. Most published strategies for enzyme-chitosan linking have used common cross-linking reagents such as glutaraldehyde, carbodiimides, *N*-hydroxysuccinimide, epichlorohydrin, or glyoxal, but some specialized cross-linkers such as cyanuric chloride²⁵⁹ have also been used successfully. There is an indication that the choice of cross-linker may affect the performance of the sensor in a recent study that used electrochemical impedance spectroscopy to evaluate the electrical properties of chitosan films prepared with different cross-linking chemistry.²⁶⁰ A procedure that avoids chemical modification of the enzyme is physical entrapment within a network of chitosan cross-linked by electrostatic interaction between protonated amino groups in the chains and a multivalent anion, tripolyphosphate.²⁶¹

Abundant functional groups and pH-dependent solubility are the most important attributes of chitosan in enzyme biosensor fabrication as they, respectively, facilitate chemical adaptation of the material and its cross-linking with other sensor components and electrochemically induced, spatially confined deposition onto a miniaturized/arrayed electrode surface. In attempts to develop advanced biosensors with interaction between the enzyme and the electrode catalyzed by a redox mediator, composites of chitosan covalently modified with redox mediators were constructed. For instance, synthesis of a special soluble Fc-modified polyaminosiloxane (Fc-PAS) was described, which after purification was covalently linked to dissolved chitosan polymer with glutaraldehyde.²⁶² Sensor coatings of the resulting Fc-PAS/chitosan composite and, for comparative voltammetric measurements, pure Fc-PAS, were applied to disks of glassy carbon or printed carbon electrodes by a drop-and-dry procedure. Cyclic voltammetry in phosphate buffer showed that the presence of chitosan in the immobilized

Fc-labeled polymer coating significantly altered the shape of the reversible Fc redox wave and shifted the anodic and cathodic peaks toward more negative values. This improvement was attributed to an increase in the hydrophilicity and therefore in ion mobility within the polymeric Fc environment, produced by the chitosan. Loading Fc-polysiloxane/chitosan networks with glucose oxidase molecules in an additional drop-and-dry step, followed by anchoring the enzyme with glutaraldehyde, produced biosensors that were responsive to glucose, with a linear range, sensitivity, and apparent Michaelis–Menten constant (K_m) value of 0–6 mM, $0.9 \mu\text{A mM}^{-1} \text{cm}^{-2}$, and 2.2 mM, respectively. The rather low K_m value suggests a relatively high enzyme/substrate affinity produced by the biocompatible chitosan-containing immobilization matrix. Another study reported that after reductive *N*-alkylation of chitosan with 4-pyridinecarboxaldehyde the anionic redox mediator pentacyanoferrate (PCF, $[\text{Fe}(\text{CN})_5(\text{NH}_3)]^{3-}$) can be bound through a ligand-exchange reaction.²⁶³ Immobilizing the PCF-modified chitosan in the absence or presence of supplementary carbon nanotubes together with glucose oxidase onto GC electrodes formed glucose-biosensing platforms that detected glucose at a working potential of 0.35 V vs Ag/AgCl, as expected for the mediator-supported transduction process. For sensors without and with the CNT addition the detection limits were relatively low: 110 and $30 \mu\text{M}$, respectively. Linear ranges, however, were only 0.8–4 (without CNT) and 0.1–1.0 μM (with CNT) and need improvement before this type of sensor is suitable for analytical use. Construction of a sensitive mediator-based lactate electrode became possible by merging a network of cross-linked chitosan with polyvinylimidazole (PVI)-Os, a redox polymer familiar from its many applications in glucose sensors.²⁶⁴ On the sensor surface, the positively charged biological and synthetic polymers jointly produce electrostatic attachment of negatively charged lactate oxidase (LOD). To increase the strength of the porous chitosan/PVI-Os/LOD matrix, oxidized carbon nanotubes with terminal $-\text{COO}^-$ groups have been included as tubular linkers between the two constituent polymers with the negative point charges spread along their chains. Because of the presence of the polymeric redox mediator in the immobilization layer, the system exhibited a low working electrode potential of 300 mV vs Ag/AgCl for amperometric measurements of lactate. Calibration measurements with lactate revealed fast response times (<7 s), good sensitivity ($\sim 20 \mu\text{A mM}^{-1} \text{cm}^{-2}$), a $5 \mu\text{M}$ detection limit, and a linear response up to 1 mM. An example of the application of chemically modified chitosan variants is in a report of nitrite biosensors with good storage stability, a low detection limit (40 nM), and a linearity of response up to 11 μM .²⁶⁵ These were made by immobilizing nitrite reductase into the chitosan layer with covalently attached methyl-viologen, which was trapped on the surface of a glassy carbon electrode behind a thin hydrophilic polyurethane membrane.

The principles of cathode-specific chitosan electrodeposition were introduced in an earlier section, and this nonmanual procedure for attachment of a biocompatible immobilization matrix is particularly attractive for enzyme biosensor preparation when sensor miniaturization, simplification, or automation of sensor manufacture and realization of high-density multiple-analyte micro- and nanosensor arrays are the goals. Dispersed nanoparticles can be incorporated through coentrapment in electrochemically grown chitosan films,²⁶⁶ and in similar fashion, simultaneous electrodeposition of chitosan and enzyme from a solution of the two was reported as the simplest form of

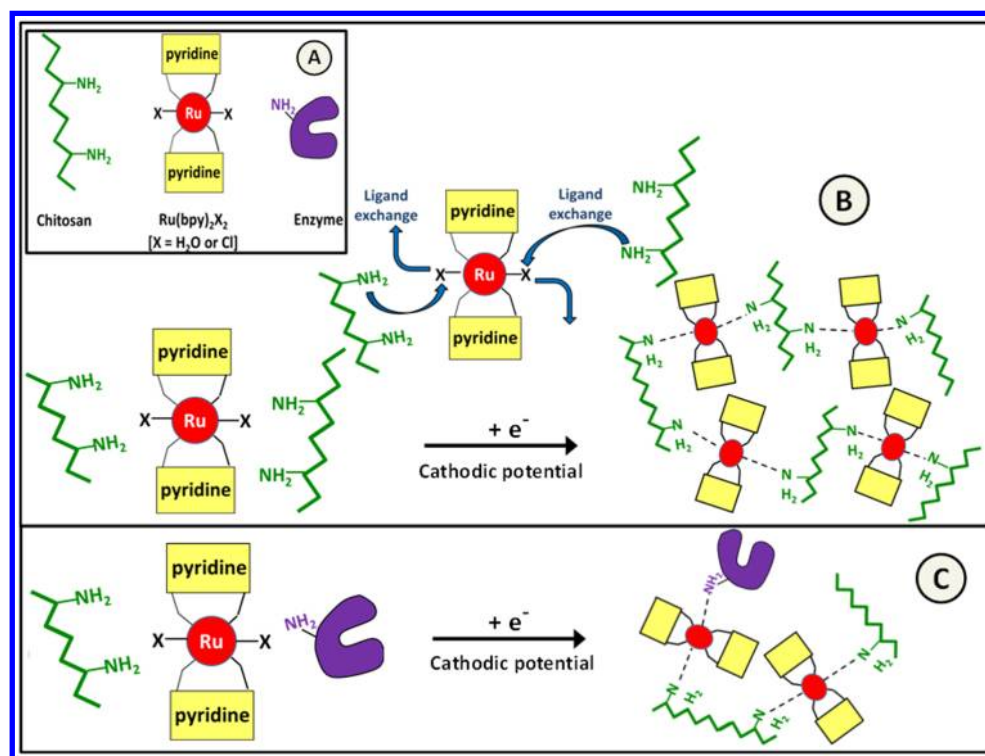


Figure 11. Enzyme immobilization on electrode surfaces through an electrochemically triggered covalent bond formation between dissolved, freely diffusible chitosan chains and between chitosan chains and macromolecules such as enzymes. (A) Simplified representations of the two components to be cross-linked, i.e., chitosan and an enzyme of choice, and the ruthenium complex that is used as molecular linker. (B) Principle of the ligand-exchange reaction that is triggered at low cathodic potentials (about -1.5 V vs reference electrode), leading to coordination of the ruthenium metal center with the nitrogen atoms from the chitosan structure. Replacement of the initial weak ruthenium ligands (water or chloride) by nitrogen from two different chitosan polymer chains forms covalent bridges and, in the course of the reaction of many chains, leads to formation of a three-dimensional chitosan network and biopolymer precipitation onto the electrode. (C) Process described in B with inclusion of enzymes in the reaction mixture forms 3D chitosan networks with covalently attached biocatalyst molecules.²⁸⁰

chitosan-mediated enzyme electroimmobilization on biosensor facades.^{267–274} Alternatively, pH-shift-induced cathodic deposition of a simple chitosan sensor coating can be followed by covalent coupling of enzyme molecules to complete the biosensor configuration.^{275–277} Another option is to prefabricate an enzyme-conjugated chitosan derivative, for instance, with a carbodiimide cross-linker,²⁷⁸ and then perform electro-deposition of the enzyme-modified biopolymer.²⁷⁹

The local pH increase at cathodes and consequent localized precipitation of chitosan polymer through neutralization is not the only strategy for a nonmanual, site-directed deposition of chitosan immobilization matrices on sensors. In fact, the electrochemically triggered covalent cross-linking of chitosan to the surface has recently been proposed as another option for electrochemical enzyme immobilization.²⁸⁰ The method exploits the change in the coordination sphere of the Ru atom within the complex $\text{Ru}(\text{bpy})_2\text{X}_2$ (where bpy = bipyridine, $\text{X} = \text{Cl}, \text{H}_2\text{O}$) that occurs upon cathodic reduction, causing replacement of the weak ligands Cl or H_2O by stronger ligands, i.e., primary amino groups in chitosan and proteins. Figure 11 shows the potential-induced coordinative interaction between freely diffusing ruthenium centers and amino groups of chitosan and enzymes and exploitation of this effect for formation of a 3-dimensional chitosan network with tethered enzyme molecules.

In the original study tyrosinase was used as a model and attached to chitosan to make a sensitive phenol biosensor with a broad dynamic range; however, the methodology is easily

adaptable for other enzymes used in sensing applications. Ruthenium complex-supported chitosan electrodeposition is irreversible, and the resultant electrode coatings are therefore more robust than those originating from reversible neutralization of charged amino entities. Other advantages are the lower working potential and excellent retention of the enzymes in the chitosan matrix, as a consequence of covalent bonding rather than physical or electrostatic entrapment.

A recent study, which explores in detail the behavior of *Agrocye aegerita* peroxxygenase at the surface of a glassy carbon electrode modified with chitosan-capped gold nanoparticles, nicely illustrates direct electron transport between chitosan-tailored electrodes and enzymes.²⁸¹

The tips of chitosan-based enzyme biosensors normally have macroscopic dimensions and are used for substrate (analyte) determinations in the bulk phase of solutions in conventional beaker-type electrochemical cells. A case of a biosensor with a miniaturized, needle-like tip is a dopamine biosensor used for amperometric detection of localized dopamine release in the brains of rats.²⁸² The microbiosensor for *in vivo* neurotransmitter measurements was obtained by immobilizing tyrosinase in a chitosan/cerium oxide/titanium oxide composite on a $100\ \mu\text{m}$ diameter carbon filament protruding from the tip of a pulled glass micropipet (see Figure 12 A). A detection limit of $1\ \text{nM}$, a linear range of >5 orders of magnitude ($10\ \text{nM}$ to $220\ \mu\text{M}$), and a sensitivity of about $14\ \text{nA}/\mu\text{M}$ together with a good selectivity against possible interference by ascorbic acid, uric acid, 5-hydroxytryptamine, norepinephrine, and 3,4-

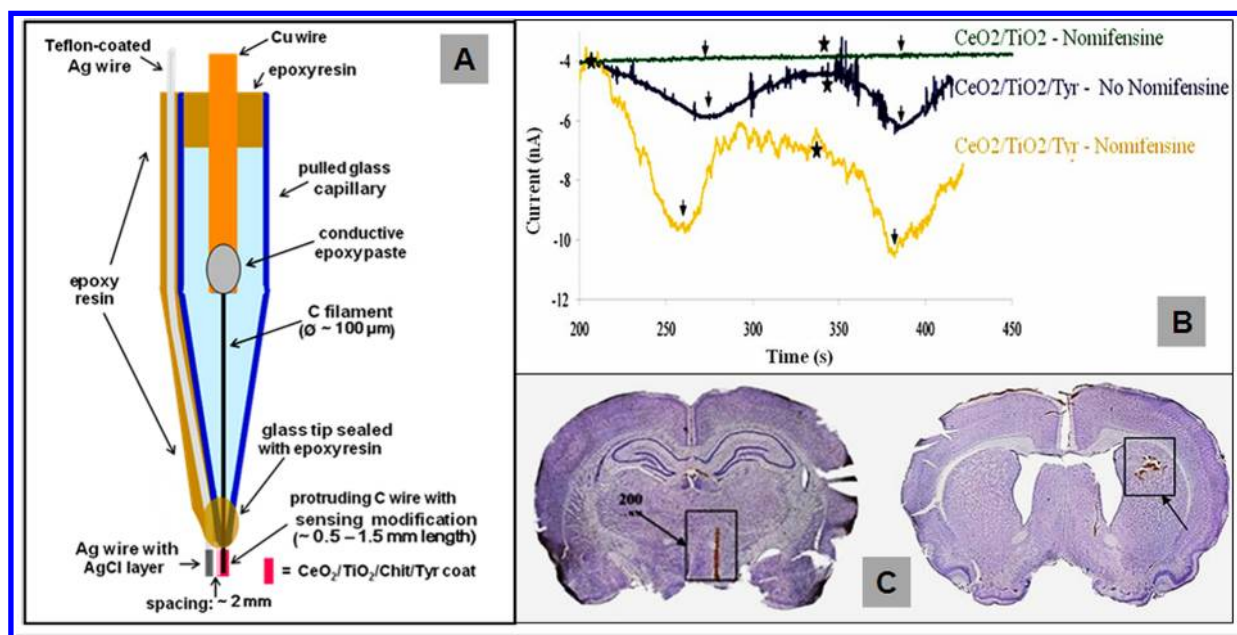


Figure 12. (A) Structural design of chitosan/metal oxide/tyrosinase-based dopamine microbiosensors developed for *in vivo* measurements of dopamine levels in the rat brain. (B) Histochemical images of two brain slices, showing the sites of stimulation and recording in neurochemical studies with microbiosensors as shown in A. Stimulatory electrode was placed near the median forebrain bundle (left-hand image), while dopamine release was measured with the chitosan/tyrosinase microbiosensors in the striatum (right-hand image). (C) *In vivo* amperometric responses of the dopamine microbiosensors in the presence (yellow trace) and absence (blue trace) of the dopamine reuptake inhibitor nomifensine. Dopamine was detected at an applied potential of -150 mV vs Ag/AgCl during electrical stimulation of the median forebrain bundle. Stars indicate the onset of electrical stimulation, while black arrows indicate when stimulation was stopped. As expected, the sensor tip reports higher levels of neurotransmitter in the presence of nomifensine because the rate of cellular reuptake is reduced. Green trace shows a control experiment with a microelectrode that was coated with immobilized chitosan and the metal oxides without tyrosinase and so was 'blind' to dopamine.²⁸²

dihydroxy-L-phenylalanine (L-Dopa) enabled continuous, real-time detection of dopamine release in the striatum of the rat brain, triggered by electrical stimulation of neurons near the median forebrain bundle (see Figure 12B and 12C).

More recently, a glucose microbiosensor small enough for measurements in the openings of the discharge tubing of a capillary electrophoresis device was constructed by immobilization of GOD in chitosan/CNT composite films electrodeposited on platinized Au microelectrodes.²⁸³ Test trials with human serum were successful and confirmed the practicability of glucose analysis in clinical samples.

4.5. Chitosan-Modified Voltammetric Electrodes for Trace Analysis

Potential chelation by intrinsic oxygen and nitrogen atoms with free electron pairs makes natural or chemically modified chitosan an efficient sorbent material for heavy metals, pesticides, and dyes. In the removal of environmental pollutants from contaminated wastewater, analyte species are drawn out of solution into top layers of the material on voltammetric electrodes and analysis can be accomplished without the electrodeposition step that is used in anodic stripping voltammetry of heavy metals such as lead, cadmium, and zinc using mercury or bismuth film electrodes. Examples of trace metal electroanalysis by chitosan-supported adsorptive stripping voltammetry are the quantitation of Hg(II) ²⁸⁴ and Cu(II) ²⁸⁵ by carbon paste electrodes with chitosan additives, determination of Cd(II) using a glassy carbon electrode modified with a drop-coated nano- TiO_2 /chitosan composite film,²⁸⁶ determination of Hg(II) ²⁸⁷ and copper(II)²⁸⁸ with chitosan/carbon nanotube paste electrodes, and quantification of Cd(II) , Cu(II) , Pb(II) , and Hg(II) with chitosan-modified

screen-printed carbon electrodes.²⁸⁹ The feasibility of using chitosan for trace analysis of organic species is evident from a series of published studies reporting the use of glassy carbon electrodes coated with carbon nanotube/chitosan composites for detection of polyphenols,²⁹⁰ paracetamol and uric acid,²⁹¹ and L-dopa and 5-hydroxytryptamine.²⁹² Moreover, chitosan–calcium carbonate sensor arrays were used for voltammetric organophosphate pesticide quantification,²⁹³ an acetylene black/chitosan film electrode for methimazole voltammetry,²⁹⁴ a carbon nanoparticle/chitosan-modified glassy carbon electrode for niclosamide,²⁹⁵ and a multiwalled carbon nanotube/chitosan for trace bromide measurements.²⁹⁶

5. CONCLUDING REMARKS AND PERSPECTIVES

Chitin is the remarkable outcome of long evolutionary optimization of a biomaterial and occurs throughout the biosphere as an important structural component of many living species. Chitosan, on the other hand, is an industrial derivative of chitin that has been developed to high quality standards for applications in such areas as medicine, agriculture, food, and environmental technology. As abundant polysaccharides, chitin and chitosan combine the benefits of natural availability and inherent biocompatibility. Additional advantages of the two materials in their many proposed medical and technical applications are their adhesive film-forming properties and the numerous oxygen and nitrogen residues that can be used to fine tune the materials' properties to the needs of a particular application through chemical modification. A drawback of chitin in biosensor applications is its intrinsic insolubility in aqueous media, under conditions mild enough not to cause inactivation of biological recognition elements as desired; a possible way out of this problem might be suspension of

colloidal chitin into the preferred buffers for biomatrix formation on sensor surfaces. For chitosan, the chemical reversibility of hydrogel film formation may be problematic during exposure to media of low enough pH for extensive reprotonation of the amine groups, which may cause adverse internal matrix conversion and/or loss of surface adhesion and exfoliation.

In this review, attention was drawn to the dynamic field of chitin and chitosan utilization in electrochemical sensor design. The reported success cases of chitin- and chitosan-supported DNA-, enzyme-, and antibody/antigen-based biosensors are an inspiring indication of the potent role that these biopolymers can play as thin film surface modifiers of electrodes of all sorts. In particular, stable immobilization of biological recognition elements in chitosan matrices on transducer electrodes under mild conditions, such as near-physiological pH values, has proved effective and many of the early problems in chitosan electrode fabrication have been solved. The outcome of recent research has been valuable manual and nonmanual (electrochemical) procedures for placement of the functional sensor coatings, either as simple or as composite chitosan films, with additional components ranging from redox-active compounds and conductive nanoparticles to metal oxide catalysts. However, until now only the basic feasibility of chitin/chitosan biosensors has been demonstrated and their performance confirmed in proof-of-principle studies. The obvious and potentially difficult next step is extension of this work to creation of competitive chitin or chitosan biosensors that are commercially available and routinely used analytical tools for analysis of everyday samples, as handled on a daily basis in point-of-care clinical, biotechnological, and environmental control laboratories or personal health care. As the potential for this certainly exists, it will be interesting to see whether chitin- or chitosan-based biosensors will progress in a reasonable time from their current laboratory setting to claim a market share among other biosensor devices for medical diagnostics, process control, and pollutant screening and how long realization of the first viable chitin/chitosan sensor products will take. The way forward may be through a more systematic optimization and utilization of the strategy of chemical chitin/chitosan modification in advance of sensor immobilization than has so far been undertaken. Issues to be tackled in the course of design improvements include the fine details of the polymers' chemical and physical microstructure in the immobilized state, in particular their molecular attributes such as the type²⁹⁷ and degree of chain cross-linking and related material characteristics such as porosity, density, rigidity, and morphology of the final chitinous surface layers. A good example of such an approach is a recently published report on the influence of salt addition to chitosan solutions on the electrodeposition of hydrogels of the material.²⁹⁸ An important observation was that salt supplementation led to chitosan films that grew faster in thickness and had a greater surface roughness. Furthermore, the mechanical properties of electrodeposited hydrophilic chitosan networks varied with salt concentration: electrolysis at elevated concentrations resulted in soft hydrogel coatings, while at lower concentrations more inflexible deposits were created. This approach, it was suggested, could be used to tailor functional chitosan electrodes to the particular requirements of a given application in the life sciences or the analytical sector.

Bearing in mind trends in micro- and nanotechnology and -fabrication, there is a clear need to advance smart electrodeposition of chitosan and its derivatives from its current proof-

of-principle stage to a point where prefabricated electrode micro- and even nanoarrays can reproducibly be transformed into marketable, multiple-analyte biosensor platforms for reliable and fast real sample bioanalytics. The strategies of some recent studies on production of anodic and cathodic electrodeposition paints for use as enzyme immobilization matrices^{299–301} and/or the backbone structure of redox polymers for horseradish peroxidase-based biosensors³⁰² may also be relevant to the fine tuning of chitosan electrodeposition for (bio-)sensor applications. As for the paints, libraries of assorted chitin/chitosan derivatives of different properties might be generated and then systematically screened in a combinatorial approach for their quality as sensor components after immobilization. The near future will be an exciting period in the chitin/chitosan/biosensor research area, with novel sensor architectures to be sought and, it is hoped, many unforeseen developments to come.

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Notes

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Wipa Suginta received her B.Sc. degree in Genetics from Chulalongkorn University, Bangkok, Thailand, in 1990 and M.Sc. degree in Biochemistry from Mahidol University, Bangkok, in 1993. After her Master's thesis (1995), which involved isolation and characterization of carbohydrate-degrading enzymes and was carried out under the supervision of Professor Dr. M. R. Jisnuson Svasti, she was granted a Royal Thai Government scholarship for doctoral studies in the Department of Biochemistry, School of Medicine, The University of Edinburgh, Scotland, under the supervision of Dr. Linda Gilmore. Her Ph.D. thesis was entitled "Structural and functional characterization of *Vibrio harveyi* chitinase". After obtaining her Ph.D. degree in 1999, she joined Dr. Richard Ashley's laboratory in the Membrane Biology Group at Edinburgh University. Her postdoctoral research was funded by the Wellcome Trust and involved functional characterization of rat brain chloride intracellular ion channels (CLICs). In January 2001, she returned to her Lectureship in the School of Biochemistry, Suranaree University of Technology, Thailand. A particular theme of her past and present research is structural and functional characterization of bacterial outer membrane proteins (porins) and chitinases and chitobioses from marine bacteria to humans. Apart from promotions to Assistant (2004) and then Associate (2007) Professor, she received for her scientific achieve-

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Panida Khunkaewla received her B.Sc. degree in Biochemistry and Biochemical Technology in 1997 and M.Sc. degree in Biochemistry in 2000 from Chiang Mai University, Thailand. In 2005 she received her doctoral degree in Dr. Scient. Med. (Immunology) from the Medical University of Vienna, Austria, for a thesis on identification and characterization of functional partners of the integral membrane glycoprotein molecule CD147, under the supervision of Professor Dr. Hannes Stockinger. From 2006 to 2007 she worked as a postdoctoral fellow for The National Science and Technology Development Agency, National Center for Genetic Engineering and Biotechnology (BIOTEC) at the Biomedical Research Unit, Faculty of Associated Medical Sciences, Chiang Mai University, Thailand, under the supervision of Professor Dr. Watchara Kasinrerker. In 2008 she became a lecturer at the School of Biochemistry, Institute of Science, Suranaree University of Technology. Her current research interests are focused on production of monoclonal antibodies and functional analysis of leukocytes surface molecules that are involved in regulation of the immune system.



Albert Schulte studied Chemistry at the University of Münster, Germany, and received his doctoral degree in Natural Science in 1994 for a thesis on preparation and characterization of ultramicroelectrodes and probes for electrochemical scanning tunneling microscopy under the supervision of Professor Dr. Jürgen Otto Besenhard. During postdoctoral appointments in the Department of the Molecular Biology of Neuronal Signals at the Max Planck Institute for Experimental Medicine in Göttingen, Germany, and the Department of Physiology at the University of Edinburgh, Scotland, he worked on joint patch clamp electrophysiological and electrochemical measure-

ments of the process of vesicular chemical release from single secretory cells. A research position in the Department of Physics of Edinburgh University with scanning probe microscopy work was then followed in 2000 by employment as Senior Research Officer in Professor Dr. Wolfgang Schuhmann's electroanalysis group at Ruhr University in Bochum, Germany. His scientific activities in Bochum included development of novel modes of scanning electrochemical microscopy, biosensor miniaturization, detection of cellular action, and inspection of the local corrosion of shape memory alloys. In January 2006, he became Senior Lecturer in Physical Chemistry at the University of the West Indies in Trinidad and Tobago, and in June 2007 he was appointed Associate Professor in Physical and Analytical Chemistry at the School of Chemistry, Faculty of Science, Suranaree University of Technology in Nakhon Ratchasima, Thailand. His current research is directed toward various aspects of micro-, nano-, and bioelectrochemistry, and ongoing projects are focused on development of probes for electrochemical tunneling and scanning electrochemical microscopy, an application of microtiter plate-based robotic electroanalysis of drugs, environmental pollutants and food content, voltammetry in microliter-volume electrochemical cells, and finally enzyme biosensor advancements and electrochemical immunosensing.

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