Chem Soc Rev

Cite this: *Chem. Soc. Rev.*, 2011, **40**, 2146–2156

www.rsc.org/csr

TUTORIAL REVIEW

Electrosynthesized polymers for biosensing

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Received 27th August 2010
DOI: 10.1039/c0cs00090f

This *tutorial review* briefly surveys the chronological evolution of biosensor concepts based on electrogenerated polymers. The most common procedures of biomolecule immobilization are classified as direct electropolymerization, physical entrapment, covalent linkage, and anchoring by affinity interactions *via* electropolymerized films. These are discussed, and recent bioanalytical applications are described. The discussion emphasizes the use of templates for controlling the formation of nanowires and composite polymers. Recent advances in the design of three-dimensional biological architectures are also highlighted.

1. Introduction

Rapid detection and monitoring in clinical and food diagnostics and in environmental and bio-defense have paved the way for the elaboration of alternative bioanalytical devices: biosensors (a generic term that encompasses enzyme, DNA, and protein sensors; immunosensors, and biochips). More than four decades ago, Clark & Lyons[1] reported the ingenious concept of intimately combining the recognition properties of biological macromolecules with the sensitivity of electrochemical, optical, thermal or gravimetric sensors. This pioneering work led to the evolution of biosensors as faster and more economical tools for clinical, chemical, and pharmaceutical laboratories. In a time when dangerous viruses and diseases such as acquired immunodeficiency syndrome, severe acute respiratory syndrome, Ebola, and West Nile, are afflicting more and more humans, early detection and real-time, on-site monitoring of pathogenic germs are essential tools with which to fight such viruses. Thanks to the adaptability of biosensors, their ease of use in relatively complex samples and their portability, interest in biosensors is growing exponentially and constitutes one of the mainstays of analytical chemistry.[2]

A biosensor, by definition requires stable and reproducible immobilization of a biological entity (*e.g.* vitamins, coenzymes, proteins, DNA, polypeptides, cells, microorganisms) on a transducer surface, with complete retention of its biological activity. Immobilization of biological macromolecules has been achieved through physical adsorption, cross-linking, covalent binding and entrapment in organic or inorganic gels, membranes, sol–gels or microcapsules,

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Dr Serge Cosnier is First Class Director of Research at CNRS and head of the Analytical and Electrochemical Biosystems team at Grenoble university (France) where he began his research career in 1983. Since 2001, he has been the President of the French Group of Bioelectrochemistry and in 2007 was elected President of the Rhône-Alpes section of the Chemical Society of France. In 2009 he received the Katsumi Niki Prize for Bioelectrochemistry of the International Society of Electrochemistry. Dr Cosnier's activity is focused on molecular electrochemistry and bioelectrochemistry in particular biosensors, biofuel cells, conducting polymers, modified electrodes and bioconversion.

Michael Holzinger

Dr Michael Holzinger has worked since the late nineties in the field of functionalization of single-walled carbon nanotubes and developed several new organic chemical reactions for the exohedral modification of nanotubes like the attachment of radicals, carbenes or nitrenes. He has worked in different groups in Germany and France on the fabrication of reinforced carbon nanotube composites and the production, purification and functionalization of nitrogen-doped carbon nanotubes. Since 2006 he has worked as a CNRS researcher in the BEA group of Serge Cosnier where his main research is focused on the development of biosensors and biofuel cells based on functionalized carbon nanotubes and other nanostructures.

Langmuir–Blodgett deposition and formation of self-assembled biomembranes.

Most of the transducers used for biosensor construction have conductive surfaces. An innovation in this regard is the use of electropolymerized polymers, which have aroused widespread attention in the design of biomimetic sensors, enzyme sensors, biochips and DNA sensors.[3] Indeed, the electropolymerization process leads to the simple and reproducible formation of organic films with precise spatial resolution over surfaces, whatever their size and geometry. Also, electropolymerization allows easy control over the properties of the polymeric coating such as morphology and thickness. Moreover, the electrogeneration of polymer films, known to be compatible with bulk manufacturing procedures, leads to polymeric coatings that are stable in organic and aqueous solvents. Consequently, immobilization of biomolecules by electrochemically generated polymers can be performed and is compatible with biological functions and easily applicable to a wide variety of proteins and oligonucleotides. Thanks to the synthetic access to a wide variety of monomers, such films have become a powerful platform for the development of biosensors. In particular, the electrical conductivity of electrogenerated polymers, their reversible doping process, the incorporation of stabilizers and their chemical functionalization have been widely exploited to provide a better microenvironment and electrochemical transduction of biological events.

Owing to the intense research activity in this field, we aim not to give a complete coverage of the biosensor literature involving electrogenerated films, but rather to cover the main strategies employed for biomolecule immobilization. Namely entrapment within the polymer matrix during its electrochemical growth, covalent binding between the biomolecule and functionalized polymers and anchoring of the biomolecule by affinity interactions with the underlying film (Fig. 1). We also briefly review the direct electropolymerization of biomolecules, the use of template procedures and the design of three-dimensional biological architectures. The easiest method for biomolecule immobilization consists of their adsorption on electropolymerized films. Initiated two decades ago, this immobilization method includes simple adsorption, electrostatic interactions, electrochemical polymer doping and the chemical cross-linking of biomolecules onto electrogenerated polymers. The latter approach was recently used to combine redox polymers and enzyme reactions but the resulting biosensors present a weak sensitivity. For instance, a glucose biosensor based on poly(brilliant cresyl blue) and chemically cross-linked glucose oxidase exhibited a glucose sensitivity of 917 μA M^{-1} cm^{-2}.[4] Despite the steady and successful use of these approaches for the immobilization of DNA, enzymes, and antibodies they have been discounted because the polymer acts only as a permselective inner layer.

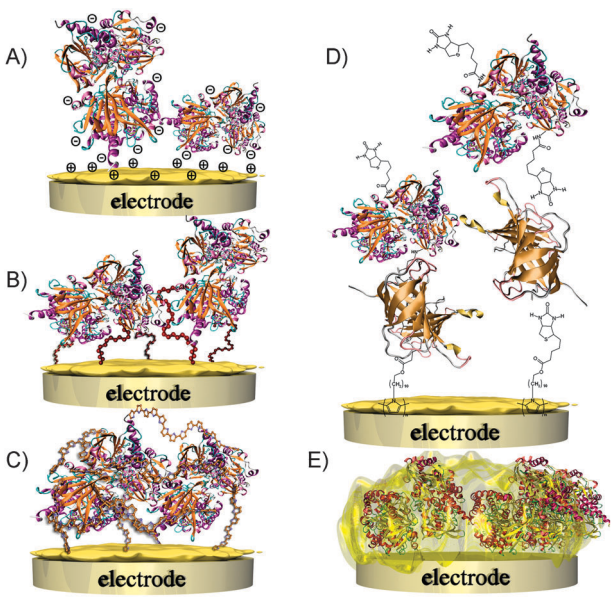

Fig. 1 Schematic presentation of developed immobilization methods. (A) Physical adsorption of biomolecules onto polymer films represented by electrostatic interactions. (B) Covalent binding between biomolecules and functionalized polymers. (C) Cross linking and direct electropolymerization of biomolecules. (D) Anchoring of biomolecule by affinity interactions with the underlying film represented by the biotin/avidin affinity system. (E) Entrapment within a polymer matrix during its electrochemical growth.

2. Historical evolution of the initial concept and appearance of new directions

The concept of biosensors based on electropolymerized films was first developed in 1986 with the fabrication of an amperometric enzyme electrode.[5,6] This concept consists of mixing a biomolecule and a soluble monomer in aqueous medium and trapping enzymes in a polymer film during its electrochemically induced growth on an electrode surface. The idea of biomolecule entrapment was initially applied to enzymes, in particular glucose oxidase (GOx), and to polypyrrole films. Although initially based on conductive polymers, this concept rapidly extended to non-conducting polymers such as polyphenols and poly(*o*-phenylenediamine) with permselective capacities.[7]

Researchers were initially interested in the electrical connection of the immobilized enzymes and within two years passed from redox mediators in solution, over incorporated mediators in the film to mediators chemically bound to the polymer or the enzyme.[8] Thereafter, the conductivity of the polymers was also explored to establish the now famous direct electrical wiring between the enzyme and the films.[9] Since 1987, theoretical investigations of bio-analytical mechanisms have garnered a great deal of interest.[10]

In 1990, a few years after the first model based on polymer entrapment was built, other concepts of immobilization such as adsorption or covalent grafting of biomolecules on electropolymerized films, were reported.[11] Contrary to the initial concepts, these approaches allowed investigators to generate polymers in organic or aqueous media with a pH incompatible for most biomolecules. However, this possibility was not exploited, mostly because the films were generally used to develop amperometric biosensors. Consequently, these films must remain permeable in the aqueous support and must therefore not be too hydrophobic. As an extension of the earlier concept of covalent binding, an innovative photoelectrochemical procedure was developed, allowing the

Table 1 Timeline of principal achievements in biomolecule immobilization

Year	Main technical and conceptual advances
1986	First biosensor based on electropolymerized film: Entrapment of glucose oxidase in conductive polypyrrole film.[5,6]
1988	Entrapment and electrical wiring of glucose oxidase by a ferrocene-modified polypyrrole film.[8]
1990	Ultra-thin enzyme film based on an insulating film.[7]
	Covalent binding of enzymes onto functionalized polypyrrole films.[11]
1992	Direct electropolymerization of an enzyme bearing pyrrole groups.[13]
1994	Electropolymerization of pyrrole-DNA derivatives.[14]
1998	Biotinylated films (polyphenol and polypyrrole) for the protein immobilization *via* biotin-avidin bridges.[15–17]
1999	Reversible anchoring of histidine-tagged proteins on a poly(pyrrole)-Ni(II) film.[18]
2003	Affinity binding of DNA on poly(dithienylpyrrole-phophonic acid) *via* Mg^{2+} bridge.[52]
	Photografting of enzymes on a photoactivatable polypyrrole film.[12]
2005	Use of negatively charged carbon nanotubes as dopants during the electrogeneration of polypyrrole enzyme films.[21]
2009	Anchoring of GOx modified by β-cyclodextrin on poly(pyrrole-adamantane) *via* host–guest interactions.[19]
2010	Anchoring of biotinylated biomolecules by coordination of the biotin group onto an electropolymerized poly(pyrrole-nitrilotriacetic acid)-Cu^{2+} film.[20]

direct covalent binding of protein *via* irradiation of polypyrrole films bearing photo-active benzophenone entities.[12]

In 1992, direct electrochemical polymerization of proteins (one of the so-called 'holy grails' of bioelectrochemistry) was attempted by modifying enzyme shells using electropolymerizable pyrrole groups.[13] Although the electropolymerization of proteins remained questionable, the electropolymerization of DNA and peptides, functionalized by pyrrole groups, led to a new avenue for the easy fabrication of DNA and protein chips.[14] The majority of the immobilization procedures that use electropolymerized films (*e.g.*, entrapment, adsorption, chemical grafting and electropolymerization) were described in 1992.

A dozen years passed until the anchoring of biomolecules by affinity interactions between the polymer and the biomolecule was performed. This concept consists of taking the advantage of the high affinity of avidin to biotin, whereby avidin forms a connecting bridge between biotinylated polymers and biotin-labeled biomolecules.[15–17] The success of this approach lies mainly in the vast range of commercially available biotinylated biomolecules necessary for already developed enzyme-linked immunosorbent assay (ELISA) tests. In 1999, a second affinity system was adopted, involving immobilization on metal ions initially developed for chromatography. This approach involves the formation of specific interactions between a chelated Ni(II) ion and histidine tags. The metal ions were immobilized in polymers, enabling the anchoring of histidine-tagged proteins.[18] In 2009, a further specific affinity system based on electropolymerizable adamantane derivatives and their inclusion in β-cyclodextrins, attached to biomolecules, was reported.[19] Finally, a novel and simple immobilization strategy for biotinylated biomolecules onto electropolymerized poly(pyrrole-nitrilotriacetic acid) (NTA)-Cu^{2+} films without avidin as a connecting bridge was reported in 2010.[20] Thanks to the polymerized chelating NTA group, the polymer enables the conventional coordination of Cu^{2+} and then the coordination of the biotin group onto the chelated Cu^{2+} centers. This system combines the advantages of the affinity systems biotin/(strept)avidin and NTA/Ni^{2+}/oligo histidine by eliminating the detriments of each system.

Concerning the different possibilities of transduction in the field of biosensors, amperometric and potentiometric methods were introduced to bio-analytics. Then, in addition to optical and gravimetric transduction, impedimetric transduction gained popularity for the detection of DNA, antigens, antibodies, and proteins without supplementary labeling. The search for higher sensitivities and better accessibility to biomolecules launched the development of three-dimensional structures by, for instance, incorporating carbon nanotubes (CNTs) in polymer-biomolecule coatings.[21,22] The chronological evolution of biosensor concepts based on electropolymerized films is summarized in Table 1.

3. Physical entrapment into electrogenerated polymers

More than 20 years ago, an ingenious procedure involving biomolecule immobilization, based on the entrapment of biomolecules in polymer films during their electrogeneration on an electrode surface, was published by Foulds & Lowe[5] and Umaña & Waller.[6] This method entails the application of an appropriate potential to a transducer (electrode, quartz microbalance, *etc.*) soaked in aqueous solution containing both, biomolecules and monomers. The biological macromolecules in the immediate vicinity of the electrode surface are thus incorporated into the growing polymer. Because this entrapment process does not allow chemical reactions between the "*in situ*" formed polymers, the biomolecules thus preserve their biological activity. The advantage of electrochemical entrapment lies in the simplicity and speed of the one-step procedure. Various parameters of the entrapment by the electropolymerization procedure such as: chemical structure of the monomer, nature of the supporting electrolyte, pH, concentration of the monomer and biomolecule, polymerization potential, polymerization charge and polymerization methods (potentiostatic, galvanostatic or pulse techniques) were investigated to optimize the biosensor performance.[23] For instance, the electropolymerization of *m*-phenylenediamine under optimum conditions led to a glucose biosensor based on a non-conducting film, exhibiting anti-interference ability towards ascorbate and L-dopamine with a good glucose sensitivity, namely 29.4 mA M^{-1} cm^{-2}.[24] Moreover, the incorporation of aptamers during the polypyrrole formation provided a markedly rougher film surface than that of regular polypyrrole. The aptamer-doped polymer was successfully applied to label-free monitoring by impedance measurements

of the platelet-derived growth factor, an important cytokine involved in neural inflammation.[25] Also, the electrochemical control of the polymer growth allowed the formation of ultrathin polymers. For instance, the entrapment of sulfite oxidase in a 54 nm thick polypyrrole film led to the determination of sulfite in beer and wine samples with better performance than that recorded with thicker polypyrrole-sulfite oxidase electrodes.[26] In particular, the ultrathin polypyrrole-sulfite oxidase biosensor enabled a 50-fold improvement in detection limit (0.9 μM). A particular case for enzyme entrapment was realized by Moretto *et al.* in order to construct a nitrate sensor.[27] A thin anion-permselective coating of 1-methyl-3-(pyrrol-1-ylmethyl)pyridinium was electropolymerized on the surface of a microporous support membrane retaining a sensing solution which contains the enzyme nitrate reductase and the mediator methyl viologen within an electrode. The ultrathin film prevents loss of these components by enabling the diffusion of the analyte NO_3^-. The generation of ultrathin conducting or non-conducting polymers also constitutes a convenient tool for improving the selectivity of the electrochemical detection. Beside steric and/or electrostatic interactions, the appearance of the film may change from a globular structure to a uniformly smooth morphology with the thickness increase. Recently, the selective determination of folic acid in presence of the archetypal interferences represented by ascorbic acid and uric acid was successively performed at glassy carbon electrodes covered by electropolymerized ultrathin film of 5-amino-2-mercapto-1,3,4-thiadiazole.[28] In contrast to bare electrodes, the modified electrode allows discrimination of each voltammetric signal arising from not only uric acid, folic acid, ascorbic acid but also dopamine. More recently, a thin layer (10 nm) of polypyrrole film functionalized by an affinity system composed of a *N*-alpha bis(carboxymethyl)-l-lysine chelating a copper cation itself anchoring a histidine-tagged anti-D-dimer antibody, was used as an electrochemical immunosensor for a D-dimer.[29] Thanks to its thin thickness, the film can promote an easy electron transfer between the electropolymerized copper complex and the electrode surface. This process was elegantly investigated and used to quantify the D-dimer, a biomarker of deep vein thrombosis, in human plasma by differential pulse voltammetry.

Entrapment in electrogenerated polymers is easily applicable to a wide variety of biological macromolecules and the monomers are often commercially available. Most of the electropolymerized films used for biomolecule immobilization are conducting polymers such as polyacetylene, polythiophene, polyaniline, polyindole and polypyrrole. Thanks to the polymer conductivity, this entrapment method enables exact control of the polymer thickness and hence modulation of the amount of immobilized of biomolecules. Also, several polymer-biomolecule layers can be successively electrodeposited providing multienzyme configurations. This approach constitutes an elegant tool for the functionalization of microelectrochemical systems such as biochips and interdigitated ultramicroelectrodes. The electropolymerization of poly(3,4-ethylenedioxythiophene) was thus used for the entrapment of GOx onto microelectrodes combined with a microchannel.[30] The resulting microfluidic system allowed the interference-free detection of glucose through use of an integrated prereactor. Another advantage of this approach is the possibility of simultaneously entrapping biomolecules together with redox mediators, coenzymes, or soluble nanoparticles. Mauko *et al.* studied different carbon support electrodes (glassy carbon, screen-printed carbon, and carbon fibers) for mediated detection of glucose, entrapped in a poly(1,2-phenylenediamine) membrane to reduce interferences.[31] However, due to the different surface chemistry and geometry of the investigated carbon based electrodes, the permeability of the electrogenerated poly(1,2-phenylenediamine) membrane was affected leading to different types of calibration curves for glucose. Entrapment of biomolecules was widely exploited for improving biocompatibility, enzyme wiring, electronic properties and enzyme loading. This method continues to be used as the foundation for many biosensor designs. For example, the co-immobilization of nitrate reductase and β-NADH in polypyrrole films was designed for the development of a new potentiometric nitrate biosensor measuring the potential change induced by the charge transfer related to the enzymatic conversion of nitrate to nitrite and β-NADH to NAD^+. In addition, the replacement of incorporated NADH by redox mediators such as thionin acetate, safranin, azure A or viologen within the polymer provided a wider linear concentration range (50–5000 μM) than those previously reported for amperometric nitrate biosensors.[32] Moreover, this strategy constitutes a cheaper alternative for the development of biosensors based on dehydrogenases or reductases.

Owing to the hydrophobic characteristic of electropolymerized organic film, research has been focused on the improvement of the biocompatibility of the host polymers by functionalization of pyrrole, carbazole and thiophene monomers by hydrophilic groups such as ethoxy, hydroxy, glucosyl or lactobioamide groups.[3] Recently, the incorporation of a polysaccharide and chondroitin-4-sulfate A as the polyanion during the polypyrrole growth, was proposed to promote cell adhesion and tissue formation onto the polypyrrole biomaterial.[33] A slightly different procedure of biomolecule entrapment by electropolymerization consists in a biochemical preoxidation of the monomer *via* enzymatically generated hydrogen peroxide. This one-pot strategy leads first to oligomer-enzyme composites that were then electropolymerized on the electrode surface by electro-oxidation of the remaining monomers. This procedure was illustrated with 2,4-dimercapto-1,3,4-thiadiazole and GOx as monomer and biomolecule models. The comparison with the conventional electropolymerization process demonstrates a marked improvement in biosensor performances that was ascribed to a larger amount of immobilized enzyme. For instance, the sensitivity to glucose increased from 0.43 to 51 mA M^{-1} cm^{-2} and the detection limit enhanced from 10 to 0.2 μM.[34] It should be noted that this biosensor sensitivity is within the range of the best glucose sensitivities (47–83 mA M^{-1} cm^{-2}) reached for various approaches of enzyme entrapment based on organic gels, nanosized calcium carbonate aggregates or layered inorganic clays.

4. Formation of covalent linkage between biomolecules and polymers

With the aim of reducing the disadvantage related to the weak accessibility of entrapped biomolecules, investigators

attempted to immobilize biomolecules by covalent linkage between biomolecules and polymers. Schuhmann *et al.*[11] initiated this procedure in 1990 by chemically grafting GOx onto amino group functionalized polypyrrole electrodes in the presence of water soluble carbodiimide. Covalent binding of DNA and antibodies was reported in 1997 and 2002.[3] The main advantage of this sequential procedure of electropolymerization and covalent binding is the possibility of satisfying optimal conditions for each step. In particular, the electropolymerization process can be carried out in organic solvents and/or at high potential values, both of which are harmful to biomolecules. This procedure is currently applied to the development of enzyme electrodes, immunosensors, and DNA sensors. The films are mainly based on polypyrrole, polyaniline, and polythiophene bearing amines, carboxyls, aldehydes, or activated carboxylic acid esters with *N*-hydroxysuccinimide (NHS), *N*-hydroxyphthalimide, or pentafluorophenyl (Fig. 2). Covalent binding is among the most popular electrochemical procedures for biomolecule immobilization.[35–37] Recently, a glycidyl epoxide functionalized polythiophene copolymer was electrochemically synthesized.[37] The epoxide allowed the acid induced chemical grafting of peroxidase for the conventional amperometric detection of H_2O_2.

Along with enzyme wiring, the conductivity of such films is an important factor. Direct electron transfer (DET) between conducting poly(terthiophene-3-carboxylic acid) (poly-TTCA) and covalently grafted cytochrome c_3, cytochrome *c*, and xanthine oxidase provided efficient amperometric biosensors for superoxide radicals, NO and xanthine, respectively.[35] Cyt *c*-bonded poly-TTCA based microbiosensors could even be tested *in vivo* due to fluctuations of NO concentrations in an intact rat brain evoked by the drug cocaine [35] where the xanthine biosensors showed in real blood serum and urine samples detection limits in the micromolar and nanomolar range with anodic and cathodic processes, respectively. Clear improvements in DET could recently be achieved by electropolymerization of poly(1,8-diaminonaphthalene) (polyDAN) on a electrodeposited gold nanoparticle layer. Subsequent covalent grafting of horseradish peroxidase (HRP) *via* glutaraldehyde crosslinking resulted in a hydrogen peroxide biosensor with direct electrochemical regeneration of the HRP.[38] This setup allowed the determination of H_2O_2 within a wide linear concentration range: 10 μM–25 mM.

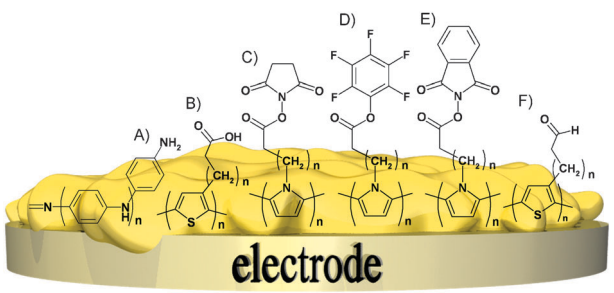

Fig. 2 Examples of functionalized polymer films bearing reactive groups for covalent anchoring of biomolecules. (A) amines (polyamines), (B) carboxyls (polythiophenes), (C) N-hydroxysuccinimide esters (polypyrroles), (D) pentafluorophenyl esters (polypyrroles), (E) N-hydroxyphthalimide esters (polypyrroles), (F) aldehydes (polythiophenes).

An alternative approach for improved DET using gold nanoparticles is based on the simultaneous immobilisation of the gold nanoparticles and the biomolecules *via* glutathione modified conductive polymers (here, poly-3′,4′-diamine-2,2′,5′,2″-terthiophene (PDATT)). Glutathione is a tri-peptide of amino acids glutamic acid, cysteine, and glycine. The carboxylic acid groups in glutathione can serve for the covalent grafting to the amine groups of PDATT, and hence the glutathione modified PDATT can be used as a matrix for the simultaneous capture of AuNPs *via* S–Au bond formation and biomolecules (here, cholesterol oxidase) *via* amide coupling. A stable amperometric cholesterol biosensor was obtained with a sensitivity (850 mA M^{-1} cm^{-2} at −0.3 V) higher than those (13.2–450 mA M^{-1} cm^{-2}) previously reported for amperometric cholesterol biosensors.[39]

Following the chemical grafting of DNA onto a polypyrrole film, this conductivity was employed for detecting hybridization through the decrease of the polypyrrole oxidation wave.[3] According to the same logic, investigators prepared redox polymers in order to apply their redox properties to the transduction of molecular recognition events. Polymers functionalized with ferrocene or naphthoquinone groups were thus used for the chemical binding of amino-terminated DNA probes. Recording the evolution of the signal of the polymerized redox groups enabled the label-free detection of a DNA target. The duplex formation on the polymer surface sterically changed the permeation of counter ions in the polymer films thus, both a decrease (ferrocene) and an increase (naphthoquinone) in current intensity were reported. An efficient alternative for amine terminated DNA immobilization is again the use of a glutathione modified polymer poly(5-hydroxy-1,4-naphthoquinone) (poly(HNQ-*co*-HNQ–Glu)) as anchor to bind the DNA.[40] The hybridization with the complement DNA strand led to change of the quinone signal in differential square wave voltammograms which enabled target detection in the picomolar range.

Concerning the recent immunosensor field, Dong *et al.*[36] created an immunosensor for mouse immunoglobin G (IgG) by electropolymerizing of pyrrole propylic acid [conducted by cyclic voltammetry and controlled by surface plasmon resonance (SPR)] by chemically binding sheep anti mouse IgG. In their original approach, the authors compared the electrochemical signal and the optical (SPR) signal for protein detection, demonstrating similar sensitivities in both transductions. In addition, their comparison of the immobilized amount of goat IgG obtained in the absence of an activation step by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)/NHS or with a self-assembled monolayer coating confirmed the superiority of the activated polypyrrole propylic acid. Particularly interesting results were obtained by using the co-polymer poly(5-hydroxy-1,4-naphthoquinone-*co*-hydroxy-2-thioacetic acid-1,4-naphthoquinone) (poly(HNQ-*co*-HNQA)) as both, immobilization and transducer matrix for label free immunodetection.[41] This copolymer enabled spatially controlled immobilization of Ovalbumin (OVA) IgG antibodies with sufficient clearances for unhindered ion transport into the polymer film. After the immuno-reaction with anti-OVA IgG this ion transport is blocked leading to a signal decrease in square wave voltammograms.

The construction of impedimetric immunosensors for the label-free detection of ciprofloxacin, an antibiotic belonging to synthetic fluoroquinolones, was performed by chemical binding of anti-ciprofloxacin antibody or a fluoroquinolone model onto an electrogenerated poly(pyrrole-NHS) film.[42] The latter was applied to the immobilization of a layer of anti-ciprofloxacin antibody onto the polymer surface by immunoreaction. The detection of ciprofloxacin by immunoreaction with the grafted antibody or by displacement in solution of the antibody immobilized by the hapten provided detection limits (1–10 pg mL^{-1}) which are more sensitive than those (100 pg mL^{-1}–2 ng mL^{-1}) described for HPLC or spectrofluorometry methods.

In an extension of the earlier concept of covalent binding, Cosnier *et al.*[12] reported an innovative photoelectrochemical procedure inspired by photopatterning. This elegant approach provides the direct covalent binding of protein by the irradiation of polypyrrole films bearing photo-active benzophenone units. This immobilization method combines the advantages of the electrochemical addressing of the polymer with the spatial localization of protein by light. This approach was successfully applied to the elaboration of electrochemical and optical immunosensors. In particular, the electrogeneration of poly (pyrrole-benzophenone) films at the end-face of an optical fiber previously modified with a conductive and transparent indium tin oxide (ITO) layer allowed the grafting of antigens by internal light irradiation at 345 nm. Following this process, the hepatitis C virus as antigen was photochemically attached to the optical fiber and applied to the detection of the corresponding antibody in real blood samples through a chemiluminescence reaction. This methodology has now been extended to the bio-functionalization of ITO coated glass chips modified by a poly(pyrrole–benzophenone) film. This film served as a substrate platform for patterned photo-immobilization of bioreceptor reagents used for the simultaneous analyte detection of anti-cholera toxin B, anti-hepatitis B virus surface, and core protein antibodies.[43] The use of conductive optical fibers opened the possibility to compare optical and electrochemical detection with the same biosensor setup. It has been shown that the detection limit of anti-cholera toxin antibodies of such modified optical fibers was 80 ng mL^{-1} using electrochemical methods which is almost comparable to the detection limit of 40 ng mL^{-1} obtained with optical detection.

5. Attachment by affinity interactions between biomolecules and polymers

The chemical grafting of biomolecules onto electrogenerated films involves the irreversible formation of several covalent linkages per biomolecule that imply a potential loss of catalytic activity or molecular recognition properties. Also, the biomolecule is linked to the surface in random orientations. However, the concept of biomolecule immobilization by affinity interactions should preserve the biological activity by formation of a single attachment point enabling a specific orientation. Generally, the films present molecular groups that can selectively interact with tags on the biomolecules *via* reversible association.

Of the different affinity systems displaying binding interactions between biomolecules and electropolymerized films, the principal approach involves the immobilization of biotinylated biomolecules on biotinylated polymers *via* avidin bridges between the two biotinylated components. The strong associations between biotin (a vitamin) and avidin (a protein that comprises four identical subunits with a very strong affinity for biotin) have been widely used in ELISA tests. The electrogeneration of biotinylated polymer films therefore enabled the successive attachment of avidin and biotinylated proteins, oligonucleotides or bacteria on the transducer surface.[3,15–17] In contrast to conventional chemical grafting, this step-by-step approach can also be applied to the preparation of multilayered biotinylated biological assemblies. Another advantage of this approach is the ability to regenerate the transducer surface through denaturation of avidin by surfactants, high pH or protease attack. In addition, specific properties (such as adsorption, chirality, redox activity, and photosensitivity) can be conferred to the biotinylated polymers, by modulating the chemical structure of biotinylated compounds such as chiral dicarbazole, pyrene, viologen or metal complexes (tris(bipyridyl) iron(II) or ruthenium(II) complexes).[44,45]

Although various electropolymerizable biotins such as phenol and carbazole derivatives have been investigated, an overwhelming majority of biotinylated films are based on polypyrrole backbones. They have mainly been exploited for the design of DNA chips, immunosensors, and protein sensors.

Along with the electropolymerization of biotin derivatives, a parallel strategy lies in the covalent binding of avidin or biotin-NHS onto polyaniline films.[46] For instance, Higson and coworkers[47] developed immunosensors by covalent binding of biotin for affinity binding of biotinylated antibodies. This development allowed the authors to use impedance measurements for the label-free detection of prostate-specific antigen, fluoroquinolone antibiotics and neuron-specific enolase, a marker of cerebral injury. It appears thus that the detection limit for the prostate cancer marker antigen (PSA) was 1000 times lower (1 pg mL^{-1}) with the affinity binding construction than those recorded with immunosensors fabricated by entrapment method in polyaniline.

However, the weak point of this immobilization strategy is the size of compact avidin bridge layers. The presence of an intermediate layer has a detrimental effect on the biosensor sensitivity for immunoreaction or hybridization detection. An alternative to this approach is the use of polymeric affinity systems, which mimic the biological avidin-biotin interactions in order to replace the protein bridge by smaller metal ions. The first system described by the Cooper group[18] involved the immobilization of Ni^{2+}, Zn^{2+} or Cu^{2+} coordinated within electrogenerated polypyrrole films containing carboxylate or imidazole groups. Histidine-tagged proteins were then anchored by coordination to the metal centers. More recently, the chelating ligand, nitrilotriacetic acid (NTA), modified with a pyrrole group, was efficiently electropolymerized, enabling the successive coordination of Cu^{2+} and histidine-tagged GOx as a close-packed enzyme monolayer.[48] As these results suggest, the

anchoring of histidine-tagged anti-atrazine antibodies on such films provided an impedimetric immunosensor for atrazine without reagent and labeling step.[49] The immunoreaction of atrazine on the attached anti-atrazine antibody directly triggers an increase in the charge transfer resistance. This system led to the detection of extremely low atrazine concentration (10 pg mL^{-1}) compared to the European Union Drinking Water Directive limit for triazine related compounds in drinking water (0.1 μg L^{-1}). In the same vein, this approach was extended to the fabrication of DNA sensors by the immobilization of ssDNA modified by a polyhistidine tag. After the coordination of ssDNA from the human immunodeficiency virus (HIV), the impedance transduction of the hybridization without a labeling step was investigated with hydroquinone as the redox probe.[50] The impedimetric measurement indicated an increase in charge transfer resistance due to the duplex formation that provided a detection limit (1×10^{-15} mol L^{-1}) better than those currently recorded for impedimetric DNA sensors (1×10^{-10}–5×10^{-15} mol L^{-1}). Another possibility to exploit this strategy that simplifies the monomer synthesis, involved the electropolymerization of a poly(pyrrole-NHS) and the covalent binding of a NTA group. Recently, this approach was applied to the development of an immunosensor and its characterization by electrochemical impedance spectroscopy.[51] Furthermore, this biosensor construction is renewable because the addition of EDTA (ethylenediaminetetraacetic acid) or imidazole leads to competitive ligand exchange and therefore to the release of histidine-tagged biomolecules. Thompson *et al.*[52] described a second system involving Mg^{2+} ions as the connecting element between a phosphonic acid-modified poly(2,5-dithienylpyrrole) film and the phosphonate groups of DNA. In an extension of earlier work, the binding of DNA probes *via* Mg^{2+} linkers was accomplished on microelectrodes previously modified by regular polypyrrole and poly(2,5-dithienylpyrrole-phosphonic acid). The electrostatic modulation of the chloride ion-exchange properties of the copolymer enabled extremely sensitive label-free detection of DNA hybridization (1.82×10^{-21} mol L^{-1}) *via* cyclic voltammetry.[53]

Another affinity system without intermediate connectors such as avidin and metal ions was recently proposed on the basis of the host–guest interactions between adamantane and β-cyclodextrin. The electropolymerization of an adamantane-pyrrole derivative thus provided a new affinity-binding polymer.[19] This polymer was successfully applied to the direct attachment of β-cyclodextrin modified GOx and the attachment of adamantane tagged GOx on an amplifying layer of β-cyclodextrin modified gold nanoparticles. The electropolymerizable affinity systems described in this section are summarized in Fig. 3.

6. Direct electropolymerization of biomolecules

An alternative to the entrapment of biomolecules in polymers and the post-biofunctionalization of affinity films was to give electropolymerizable properties to the biomolecule itself. The advantages of this approach are the simplicity and rapidity of the one-step procedure and the theoretically high biomolecule density of the resulting polymer film. In their pioneering 1992 paper, Lowe *et al.*[13] described the electropolymerization of glucose oxidase that was chemically modified with 30 pyrrole groups. The covalent modification of GOx was carried out by coupling its amine-terminated protein shell with carboxyethyl pyrrole in the presence of carbodiimide. However, this concept of polypyrrole formation, also developed by Schuhmann *et al.*,[3] was required to undergo a copolymerization

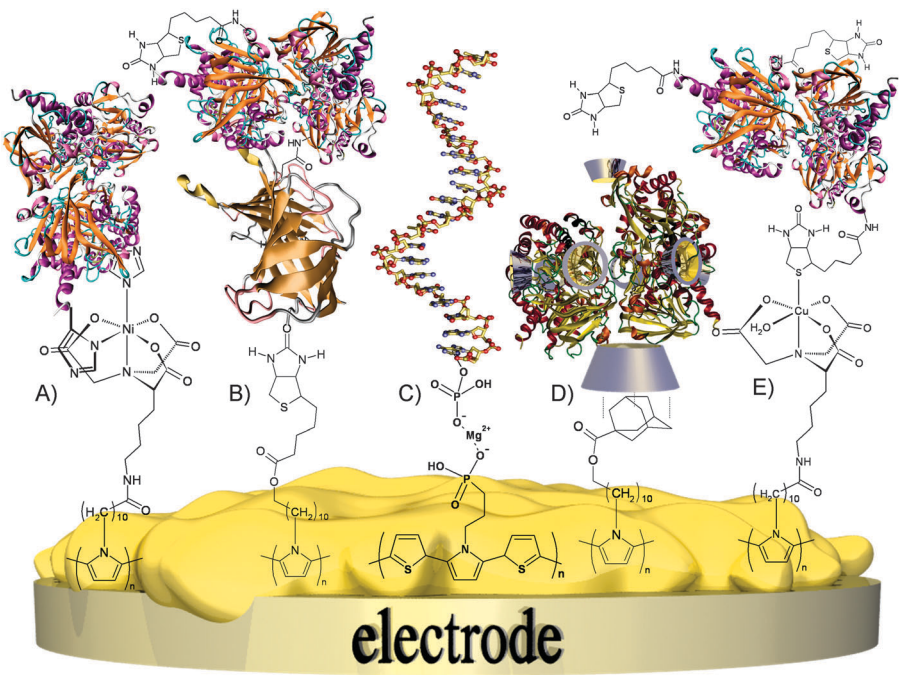

Fig. 3 Common affinity systems for biomolecules immobilization utilizing specific supramolecular interactions. (A) Nitrilotriacetic acid/Ni^{2+}/histidine; (B) Biotin/avidin/biotin; (C) Phosphonate/Mg^{2+}/phosphate; (D) Adamantane/β-cyclodextrin; (E) Nitrilotriacetic acid/Cu^{2+}/biotin.

process with regular pyrrole. As a consequence, the enzyme immobilization resulted in a combination of entrapment and electrochemical polymerization rendering the enzyme less accessible.

Such immobilization concepts are more applicable to small biomolecules such as peptides, short oligonucleotides, oligosaccharides, vitamins, and coenzymes.[14,54,55] The electropolymerization of biomolecules was initially focused on the fabrication of polypeptide and poly(pyrrole-DNA) films on biochips.[14] For instance, the polymerization of pyrrole-terminated DNA enabled the electrochemical construction of DNA matrix on microelectrode arrays. These arrays were then applied to the genotyping of hepatitis C virus in blood samples.[54] Localized electropolymerization of micrometer-sized poly(pyrrole-DNA) spots was also achieved through the use of mobile cantilevers carrying electrochemical microcells. In an effort to develop label-free detection of protein or DNA, surface plasmon resonance (SPR) imaging was applied to electrogenerated poly(pyrrole-biomolecule). Simple measurement of the angle changes in SPR reflectivity as a function of time allowed mutation-screening assays and real time protein-interaction studies that used poly(pyrrole-DNA) and poly(pyrrole-oligosaccharides).[54,55]

Although a discussion of conventional biosensors is beyond the scope of this review, we briefly mention an innovative approach to elaborating a new type of electrogenerated imprinted polymer. In this method the co-electropolymerization of phenol and 3-hydroxyphenyl boronic acid complexed with D-glucose or D-mannose led, after chemical release of the monosaccharide, to polymers that exhibited electrochemical enantioselective recognition properties for mono-saccharides.[56]

7. Polymer-biomolecule-carbon nanotube composites

One of the limitations of common biosensors lies in the surface detection of the target. The improvement of this sensitivity is at the origin of the significant efforts focused on the development of three-dimensional biomaterials dedicated to improve the detection limit. Among the various possibilities envisioned for such a task, the use of carbon nanotubes (CNTs) aroused increasing interest owing to their geometry and conductivity. Nanotubes have an impressively high specific surface of approximately 1000 $m^2\ g^{-1}$, making them promising candidates for the construction of highly porous three-dimensional nanostructured frameworks. In particular, the ingenious combination of CNTs and electrogenerated polymers, through the use of conventional procedures of biomolecule immobilization *via* electropolymerized films (adsorption, entrapment, chemical grafting and affinity interactions), has led to a new generation of biomaterials.[21,22,57–62]

The main procedure comprises two steps: (*a*) the formation of CNT coating on the electrode surface, followed by (*b*) the electropolymerization step. The second step is used either for biomolecule immobilization by entrapment during polymer growth or by subsequent covalent binding to the polymer or to confer redox properties to the resulting coating. In the latter case, biomolecule immobilization occurs by adsorption onto the polymer (often by electrostatic interactions) or by chemical cross-linking. For instance, a single-walled carbon nanotube (SWCNT) deposit was modified by electropolymerization of a poly(nil blue) film exhibiting electrocatalytic properties toward NADH oxidation. The subsequent adsorption of glucose dehydrogenase and its cross-linking by glutaraldehyde led to an interference-free glucose biosensor at 0.05 V exhibiting a sensitivity (15 mA $M^{-1}\ cm^{-2}$) which is lower than those reported for the entrapment procedure (51 mA $M^{-1}\ cm^{-2}$).[59] Shirsat *et al.*[58] recently used SWCNTs and polypyrrole to create such a sensor. A cross-linked GOx layer modified the resulting SWCNT-polypyrrole multilayer providing a glucose sensor with an excellent linearity in the range of 1 to 50 mM. More recently, researchers have developed attractive designs of biological architectures based on the electropolymerization of affinity binding polymers based on avidin-biotin or adamantane-cyclodextrin interactions on CNT coatings (Fig. 4).[19,45] The performance of such designs was investigated *via* the anchoring of GOx as a biomolecule model. The best configuration provided a glucose sensitivity of 31.02 mA $M^{-1}\ cm^{-2}$ at 0.7 V whereas the similar configuration without SWCNT led only to 0.98 mA $M^{-1}\ cm^{-2}$.

Another method consists of producing CNTs that were soluble in water and to electropolymerize a monomer in the presence of CNTs and biomolecules. In addition, Wang & Musameh[21] demonstrated in 2005 that negatively charged CNTs can act as polymer dopants during the electrogeneration of conducting polypyrrole enzyme electrodes. This approach was mainly developed with oxidized CNTs bearing carboxylic groups. An easier method of CNT solubilization involved the adsorption of a protein such as bovine serum albumin or peroxidase onto SWCNTs during sonication. The SWCNT–protein conjugates were then used as dopants for the elaboration of the H_2O_2 polypyrrole biosensor.[57] An original approach for the fabrication of homogeneous SWCNT–polypyrrole composites consists of dispersing SWCNTs in water by ultrasound in the presence of the electropolymerizable (11-pyrrolyl-1-yl-undecyl) triethylammonium tetrafluoro-borate as a surfactant.[61] Highly homogeneous biocomposite material can be electrogenerated by adding enzymes to this stable dispersion. GOx and polyphenol oxidase have thus been entrapped in this SWCNT-polymer, leading to glucose sensitivities of up to 130 mA $M^{-1}\ cm^{-2}$. Compared to the maximum glucose sensitivity (51 mA $M^{-1}\ cm^{-2}$) reported for biosensors based on enzyme entrapment in polymers, this value (130 mA $M^{-1}\ cm^{-2}$) illustrates clearly the beneficial geometrical role played by the nanotubes. More recently, the co-entrapment of peroxidase and GOx in poly(toluidine blue O) film electropolymerized on MWCNT allowed the determination of glucose at −0.1 V *via* the peroxidase wiring with an excellent sensitivity, namely 113 mA $M^{-1}\ cm^{-2}$.[63]

A less common approach involves with the electrogeneration of the polymer and the successive adsorption of CNTs and biomolecules. For instance, the Pingarrón group[60] demonstrated the advantage offered by the conductive hybrid material (polymer–MWCNTs) for the electrochemical oxidation of NADH at low potential value (0.3 V). This method made it easy to design dehydrogenase based biosensors. Because some attempts showed the possibility of electropolymerizing CNTs functionalized by

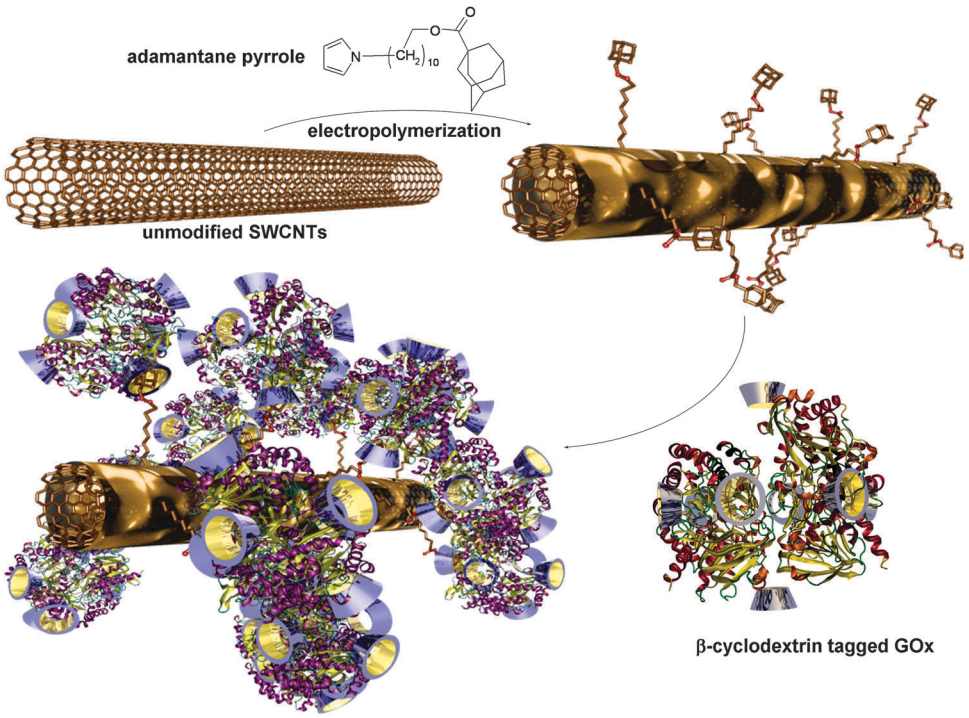

Fig. 4 Reaction scheme of the functionalization of single-walled carbon nanotubes by electropolymerization of adamantane-pyrrole derivatives around the sidewalls of the tubes. β-cyclodextrin tagged biomolecules can be immobilized through the formation of supramoleculear host–guest interactions.

amine, aniline or pyrrole groups,[62,64] future developments will include polymerization of such CNTs in water in the presence of biomolecules. In particular, Willner and coworkers initiated recently this direction by electropolymerizing aniline-functionalized CNT and thioaniline-modified GOx.[62] Another main advantage of CNT lies in their conductivity. As previously reported by this group,[65] the resulting three-dimensional CNT configuration allowed an efficient GOx wiring with a turnover rate of electrons (1025 s^{-1}) that exceeds the conventional turnover recorded with O_2 (700 s^{-1}). Besides the construction of biosensors, such strategy thus seems promising for the development of biofuel cells and bioreactors.

In parallel to the use of CNTs, the design of composite biosensors based on electrogenerated polymer-nanoparticles, and biomolecules has attracted much attention, particularly for enhancing the transduction properties of the biosensor. For instance, a label-less impedimetric immunosensor exhibited an excellent detection limit for interleukin (10 fg mL^{-1}) thanks to the combined conductivity of polypyrrole and Au nanoparticles.[66] Nanobiocomposite polymers were prepared *via* electropolymerization of pyrrole in the presence of an enzyme (GOx) and poly(amidoamine) dendrimers containing Pt nanoparticles (with a diameter of 3 nm). The nanoparticles amplified the electrocatalytic properties (H_2O_2 oxidation) of the biosensor leading to a glucose sensitivity of 164 mA M^{-1} cm^{-2}.[67]

8. Template-based synthesis of nanostructured biomolecule-polymers

As nicely illustrated by Parthasarathy & Martin,[68] there is considerable interest in the idea of using a template-based electropolymerization method for the electrogeneration of nanowires. To develop miniaturized biosensors, researchers are focusing on obtaining biofunctionalized polymeric nanowires. Some work is dedicated to the fabrication of polymeric nanowires through use of a well-established template-directed electropolymerization based on porous alumina, whose pore sizes are between 100 and 200 nm. For instance, poly(pyrrole)-NTA nanotubes have been synthesized by electrochemical polymerization using such nanoporous alumina templates. These nanotubes were employed to develop a chemi-resistive sensor for detection and quantification of heavy metal ions and histidine-tagged proteins.[69] Anodic aluminium oxide membranes have also been used as template for the fabrication of polyaniline nanotubes, with GOx immobilized into their inner walls, that provide direct electron transfer.[70] A brush made of polypyrrole nanotubes has also been generated *via* alumina disc templates. The resultant increase in the specific polymer surface enhanced the amount of immobilized peroxidase and facilitated direct electron transfer between the enzyme and the polypyrrole. An impressive sensitivity for H_2O_2 reduction (1 A M^{-1} cm^{-2}) was recorded with a detection limit of 5 nM.[71] In similar work, electrogeneration of poly(pyrrolepropylic acid) nanowires was accomplished *via* an alumina template. The resulting nanowires were covalently modified with antibodies, which allowed the construction of an immunoreactive nanosensor where the resistance of the nanowire was affected by the binding of the antigen.[72]

Other original approaches for nanostructuring conducting polymer were developed with polystyrene particles and onion-type multilamellar vesicles.[73] For instance, polystyrene particles were self assembled by electrostatic interactions

onto a polyaniline film. The electrochemical growth of polyaniline then occurred in the interstitial spaces between the spheres. After the polystyrene spheres dissolved, the peroxidase was loaded into the empty sites and blocked by a thin chitosan membrane. The resulting H_2O_2 biosensor displayed direct electron transfer between peroxidase and polyaniline, but it had a less satisfying sensitivity and a detection limit than those observed with the biosensor based on the polypyrrole brush, namely 615 mA M^{-1} cm^{-2} and 0.36 μM.[73] Nevertheless, this strategy was extended to the formation of peroxide sensors composed of hollow polyaniline spheres and porous polyaniline films combined with peroxidase.[74] The latter were fabricated from structurally multiordered polyaniline films electropolymerized on screen-printed carbon electrodes modified by polystyrene nanoparticles. Nevertheless, the H_2O_2 sensitivity (410 mA M^{-1} cm^{-2}) remains lower than that observed with the biosensor based on the polypyrrole brush.[71]

9. Conclusion

Among the numerous procedures employed for biomolecule immobilization, the "older" electrochemical concepts, based on electropolymerized films combined with nanostructured materials or templates, continue to yield an extremely diverse array of electrochemical biosensors. Even now, these concepts are contributing to the development of biofuel cells. The vast success of this approach lies in the modularity of the conductivity and chemical composition of polymers coupled with the electrochemical addressing of films.

A significant number of emerging directions, such as magnetic modulation of the permeability of the biopolymer *via* entrapment of magnetic microbeads and the use of biological complexes (for instance DNA duplexes) or gas bubbles as templates, make this research field quite exciting. The promises and challenges of these new technologies require truly interdisciplinary research efforts. Complex nanobiostructures on interfaces are steadily helping to develop real-world applications.

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