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Review

Nanostructured transducer surfaces for electrochemical biosensor construction—Interfacing the sensing component with the electrode

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ABSTRACT

For fabrication of effective electrochemical biosensors, interfacing the biomolecular receptor with the underlying transducer represents a critical step. The actual approach taken depends on the tethering layer covering the transducer, which is typically either a conducting polymeric matrix, or a thin film, such as an alkanethiol monolayer. Non-specific immobilisation methods can be either covalent, or non-covalent affinity attachment, with multipoint electrostatic attachment of the sensing biomolecule to either a polyanionic or polycationic layer representing the most common approach. Many specific affinity immobilisation strategies exist, but the majority make use of one of two binding systems. The first relies on the specific and strong affinity between biotin and proteins of the avidin family, with both bioreceptor and transducer bearing pendant biotins and avidin used as the crosslinker. The second approach employs a metal chelating group on the transducer to which can be bound a polyhistidine tag present on the N- or C-terminus of the receptor protein and which can be introduced genetically, when the expression sequence for a recombinant proteins is designed.

Contents

1. Introduction

Biosensors rely on the high selectivity provided by the biomolecular receptors, which include antibodies, enzymes, and a range of other binding proteins, plus nucleic acids. These biomolecules are integrated with an electrochemical, optical or piezoelectric transducer. The biological component is usually immobilised on, or in close vicinity to the transducer surface and as a result the immobilisation strategy(s) used is critical in order to maintain biological activity. Once the biological recognition event occurs, i.e. when the immobilised biomolecule and its corresponding ligand interact, a signal relating to the concentration of the ligand present in the sample is produced in a variety of ways, depending on the interrogation method. This review covers the major approaches currently used in the tethering of bioreceptors to the surface of electrochemical biosensors; it is not exhaustive and many other approaches exist outside of those covered.

Abbreviations: 4-ATP, 4-aminothiophenol; AB-NTA, *N*-(5-Amino-1-carboxypentyl)iminodiacetic acid; DOGS-NTA-Ni^{2+}, 1,2-dioleoyl-*sn*-glycero-3-[(*N*-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl]-Ni^{2+}); IMAC, Immobilised Metal Ion Affinity Chromatography; 2-MEA, 2-mercaptoethylamine; PDADM, polydiallyldimethyl ammonium chloride; PSS, polystyrene sulphonate; Sulpho-SMCC, sulphosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate.

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Table 1
Compounds employed for the electrochemical modification of the carbon surface. Surface grafting radicals R• are electrochemically generated from precursors R–X. Substituent X is the leaving group; Ar, aryl.

Method	Compound (R–X)	Radical (R•)	Substituent (X)	Ref.
Amine oxidation	R_1R_2N–H	R_1R_2N•	H^+	[13]
Arylacetate oxidation	$ArCH_2$–COO^-	$ArCH_2$•	CO_2	[14]
Diazonium reduction	Ar–N_2^+	Ar•	N_2	[15–17]
Iodonium reduction	R_1–IAr	R_1•	IAr	[18]
Sulphonium reduction	R_1–S^+(Ar)Ar	R_1•	ArSAr	[19]

2. Derivatisation of carbon surfaces

With the exception of steric confinement and polymer electrodeposition approaches, immobilisation of biomolecules on an electrode surface requires it to bear functional groups to which either covalent or affinity coupling can be carried out. Electrodes for amperometric applications are usually made from inert materials (gold, carbon) which lack these groups. While gold electrodes can be easily functionalised using self-assembled monolayers of thiols [1–4], derivatisation of carbon is much more difficult. Nevertheless, the dramatically lower cost of carbon makes it the leading material for the production of disposable biosensors. Accordingly, many methods have been developed in order to modify carbon surfaces.

Historically, the first procedures for carbon derivatisation included methods based on vigorous, sometimes destructive, oxidation of surface carbon atoms under harsh conditions, e.g. heating in air [5] or in acid solutions [6], electrochemical treatment in acidic [7,8] or alkaline media [9]. These techniques lead to the formation of carboxyl, quinone, ketone, or hydroxyl groups on the surface [10,11] that can be used for further electrode modification. The main disadvantage of this group of approaches is an increase in the background current of modified electrodes [11,12] and high variability of the resulting surface as the amount and nature of the groups obtained is difficult to control. For these reasons carbon surface modification through vigorous oxidation is rarely used nowadays.

More modern approaches for the modification of carbon surface concentrate on the electrochemical synthesis of free radicals, typically from an aryl compound. Generated via redox reactions, these highly reactive molecules are formed close to the electrode surface which enables them to establish covalent bonds with the surface carbon atoms. Several techniques based on this principle have been designed during the last decade. For radical formation they use different classes of organic compounds (Table 1) that provide a simple basis for classification of these techniques. Generally, once the carbon surface has been modified, the other substituent on the aryl ring can then be modified to permit covalent attachment of a range of compounds for immobilisation, e.g. 4-nitro diazonium benzene has been used to introduce nitrobenzene groups onto the carbon surface; the nitro group can then be electrochemically reduced to an amine which is amenable to further chemical reactions ([20], see also Section 4).

3. Applications of conducting polymers

The application of conducting polymers to electrochemical biosensors is mainly based on the idea that they can improve direct electron transport between oxidoreductase enzymes and electrode surface in amperometric biosensors. Conducting polymers support electron flow through the polymer backbone itself which is usually comprised of a π-conjugated double bond system. The exact mechanism of electron conductivity in such polymers is complex and not only depends on their particular structure but also strongly influenced by external conditions. For example, the conductivity of polyacetylene can be increased by 16 orders of magnitude (from 10^{-9} S cm^{-1} to 10^5 S cm^{-1}) via simple doping of

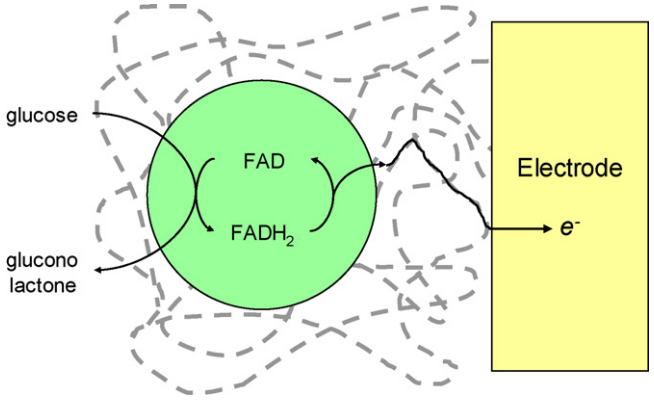

Fig. 1. Electrical communication between enzyme and electrode by means of conducting polymer. Electron flow between the enzyme (here, glucose oxidase) and the electrode is proposed to occur due to the electrical conductivity of the polymeric backbone.

the polymer with the oxidising agent iodine [21]. Despite the lack of complete and exhaustive theory of electric conductivity in this type of macromolecule, amperometric biosensors based on conducting polymers have been intensively developed over the last two decades and a number of comprehensive reviews in this area are available [22–24]. Surprisingly, direct electron transfer between the enzymatic redox centre and the conducting polymer backbone (Fig. 1) has been proposed only in a limited number of studies, e.g. in the glucose oxidase (GOx)-polypyrrole system [25] or horse radish peroxidase (HRP)-polypyrrole [26]. So far, convincing evidence of electron exchange between the enzyme and the electrode through the conducting backbone of the polymeric matrix has been shown for multi-cofactor quinohaemoprotein alcohol dehydrogenase entrapped in poly(pyrrole) [27]. In this enzyme, the haem-c groups act as secondary redox cofactors placed close to the enzyme surface. This enables their direct electron exchange with the surrounding poly(pyrrole) network.

In most instances, however, conducting polymers have been employed to create either a simple carrier-matrix for enzyme immobilisation or a polymeric redox environment via electrochemical (co)polymerisation of appropriate monomers, bearing chemical/affinity groups or redox moieties. This strategy for surface derivatisation is very attractive as it can be performed exclusively on the electrode surface in a highly controllable, automated manner. The resulting polymers possess high adhesion on most surfaces and therefore their deposition is often performed without any surface activation. Three of the most widely applied conducting polymers are presented in Fig. 2.

The application of conducting polymers in biosensors is partially limited since they usually lack suitable reactive groups for biomolecule immobilisation. Amongst the approaches for biomolecule immobilisation four main strategies can be defined. These include conventional covalent immobilisation, entrapment of biomolecules in electrogenerated conductive polymer films, specific non-covalent immobilisation (e.g. the biotin/avidin system—see also Section 4.2) and non-specific non-covalent absorption which is usually based on multiple electrostatic interactions.

The first approach (covalent immobilisation) is typically restricted by the absence of suitable chemical groups (–NH_2, –COOH, –SH) in common conducting polymers. Attempts to introduce such groups in monomers, for example co-incorporation of 4-(2-aminoethylaniline) and aniline to introduce pendant –NH_2 groups, usually brings difficulties such as electropolymerisation problems, changes in conductivity or mechanical properties of the polymer and its water solubility. However, Liu et al. [28] introduced

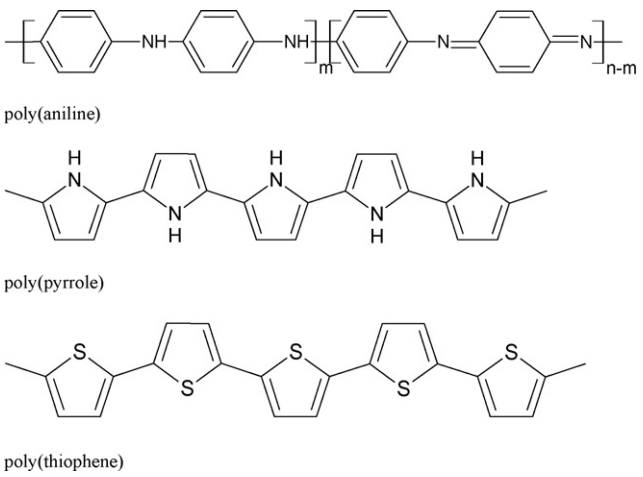

Fig. 2. Structures of the most common conducting polymers. Ideal linear structures of the polymers are shown to give a general overview; in reality some branching of the main chain occurs giving a loose polymeric matrix.

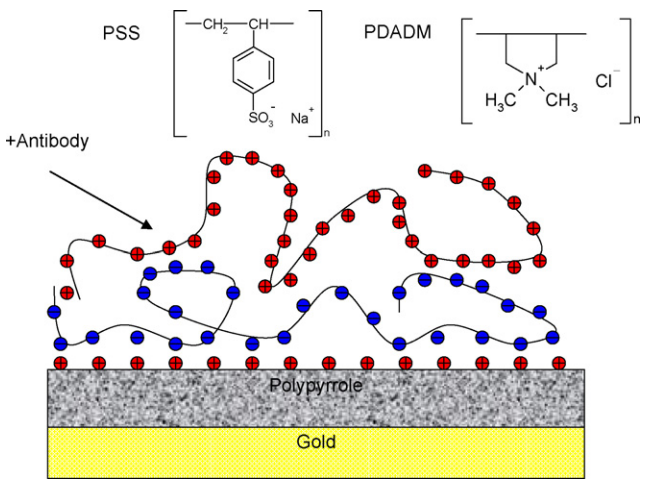

Fig. 3. Electrostatic antibody entrapment using polyanion (PSS) and polycation (PDADM) overlayers onto a polypyrrole base layer. This technique has also been used with a polyaniline base layer.

COOH via copolymerisation of 3-methylthiophene and thiophene-3-acetic acid modified monomers thereby providing a reactive group for covalent coupling of glucose oxidase.

Amongst other immobilization procedures, entrapment of biomolecules in electrogenerated conducting polymer films is the only one which allows a spatially controlled deposition [22,29–31]. However, physical entrapment in polymer films can drastically reduce the catalytic activity of immobilized enzymes [32,33]. This may be ascribed to the hydrophobicity of the organic polymer host, which can induce denaturation processes [34]. In addition, the steric constraints imposed by the surrounding polymer may hinder the conformational flexibility of enzyme molecules, which may be essential for their activity. Moreover, these constraints markedly reduce accessibility of substrates or other analytes to the immobilized biomolecules, thereby causing loss of sensitivity and slow biosensor response to equilibrium. In particular, this constitutes a major handicap for the development of immunosensors.

Specific non-covalent immobilisation of biomolecules, including antibodies, based on an electropolymerisable pyrrole-modified biotin was proposed by Cosnier et al. [35,36]. Their strategy involved the electropolymerisation of biotinylated pyrrole and the subsequent functionalisation of the resulting conducting polypyrrole film by biotinylated enzymes or antibodies using avidin as the crosslinking agent. This method combines the advantages of spatially localized deposition whilst retaining the accessibility of the immobilized biomolecule.

Finally, non-specific electrostatic absorption of biomolecules onto conducting polymeric films provides a gentle and very high avidity systems of immobilising enzymes and antibodies on polymeric films. This approaches can be likened to the interaction between two strips of 'Velcro'; individual interactions between basic and acidic groups on conducting polymer and biomolecule respectively, are of fairly low affinity. However, multiple concerted interactions mean that a high avidity results, with little or no leakage of the sensing biomolecule.

This approach can be limited by insufficient accessibility and density of charged groups on the surface of a conducting polymer film. However, the deposition of multiple alternate layers of the polyanion polystyrene sulphonate (PSS) and the polycation polydiallyldimethyl ammonium chloride (PDADM) (Fig. 3) improve both the kinetics of antibody capture and also antibody loading onto a biosensor surface (Fig. 4.). Digoxin sensors which can be interrogated impedimetrically have been produced in this way.

4. Immobilisation onto self-assembled monolayers

Many metal surfaces, and in particular the noble metals, gold silver and platinum are ideal substrates for the formation of self-assembled monolayers (SAMs) which provide a simple route to functionalise electrode surfaces with a wide range of reactive and affinity groups for the subsequent immobilisation of biomolecules [37].

SAMs are usually organic assemblies formed by the adsorption of molecular constituents from solution or the gas phase onto the solid surface of the substrate; the two-dimensional organisation of the adsorbates occurs spontaneously (and sometimes epitaxially) into crystalline (or semicrystalline) structures. Typically a fast attachment phase is then followed by a slower ordering phase. The self-assembling molecules possess a chemical functionality, having specific affinity for the surface of the substrate [38]. Typical examples are the alkane thiols which chemisorb well to silver or gold, and hydrophobic silanes which attach to platinum; many other examples exist.

As mentioned, sulphur compounds are known for their reactivity towards noble metals such as gold, silver, and platinum. The gold–sulphur interaction is based on the interaction of the outer orbitals of the gold and sulphur atoms whereas most other func-

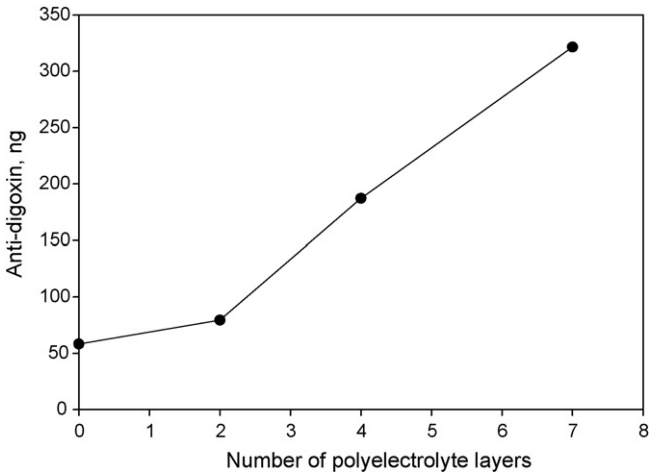

Fig. 4. Dependence of anti-digoxin IgG immobilization on number of polyelectrolyte overlayers.

tional groups are non-reactive to gold. Therefore, the gold–sulphur interaction does not interfere with the functionalisation of the surface with other functional groups. Also, gold is relatively easy to clean, does not oxidise under normal laboratory conditions and weakly adsorbed species on the surface can be easily replaced by adventitious sulphur molecules in the assembly process. Alkyl and arylthiols, and aromatic disulphides can all form SAMs by chemisorption of the sulphur atoms. First, the rapid reaction between the sulphydryl (–SH) group and gold leads to formation of gold–sulphide bond. This initial fast deposition is followed by self-organisation of alkyl or aromatic side chains through Van der Waal's interactions. Longer chain molecules then to form well-packed quasi-crystalline monolayers where as shorter chain molecules give rise to liquid like monolayers [37,38]. A well-defined mixture of molecular structures can give rise to monolayers termed as "mixed" SAMs. It has also been shown possible to incorporate proteins and phospholipids into monolayers during deposition and form mixed self-assembled monolayers (mSAMs). These mSAMs can subsequently be functionalised to act as sensing surfaces for biosensors [39–45].

4.1. Attachment of antibodies via a unique thiol group

Biosensors capable of monitoring antigen-antibody interactions are termed as immunosensors. The sensitivity and specificity of an immunosensor depend on the loading, surface density and orientation of immobilised antibodies. Antibodies can be immobilised onto transducer surfaces through tyrosine residues, lysine ε-amino groups carboxyl groups of aspartic and glutamic acids, thiol groups generated by the reduction of cysteines, and oligosaccharides moieties. Immobilisation via lysine residues, typically using carbodiimide or *N*-hydroxysuccinimide mediated coupling [46], is perhaps most popular as they are abundant on IgGs. However, multiple orientation of the antibody can occur which leads to reduced antibody binding capacity. Again, the four N-terminal amino groups on an antibody molecule have lower pK_a values and therefore, the lysines present in these termini are more reactive than others present on antibody molecules. This sometimes leads to the loss of binding ability because the binding site of the antibody is located towards the N-terminus of the heavy and light chains [47].

To circumvent these limitations, oriented immobilisation of antibodies can be achieved by immobilisation of the antibody through thiol groups. Antibody molecules can be selectively reduced with 2-mercaptoethylamine (2-MEA), which principally cleaves the disulphide bonds linking the heavy chains in the hinge region but leaves the disulphides between the heavy and light chains. This reduction generates two half-antibody fragments (Fab), each containing a single binding site comprising one heavy and one light chain. These sulphydryl groups can be coupled to maleimide-activated surfaces without blocking the antibody binding capacity and serve as an oriented sensing surface for an immunosensor [46]. We have shown that 4-aminothiophenol (4-ATP) is able to form a stable SAM to which the bifunctional crosslinker sulphosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (Sulpho-SMCC) can then be attached via the surface amino groups. Subsequent presentation of the Fab fragment results in their coupling via the now available maleimido groups (Fig. 5) yielding a biosensor that is more

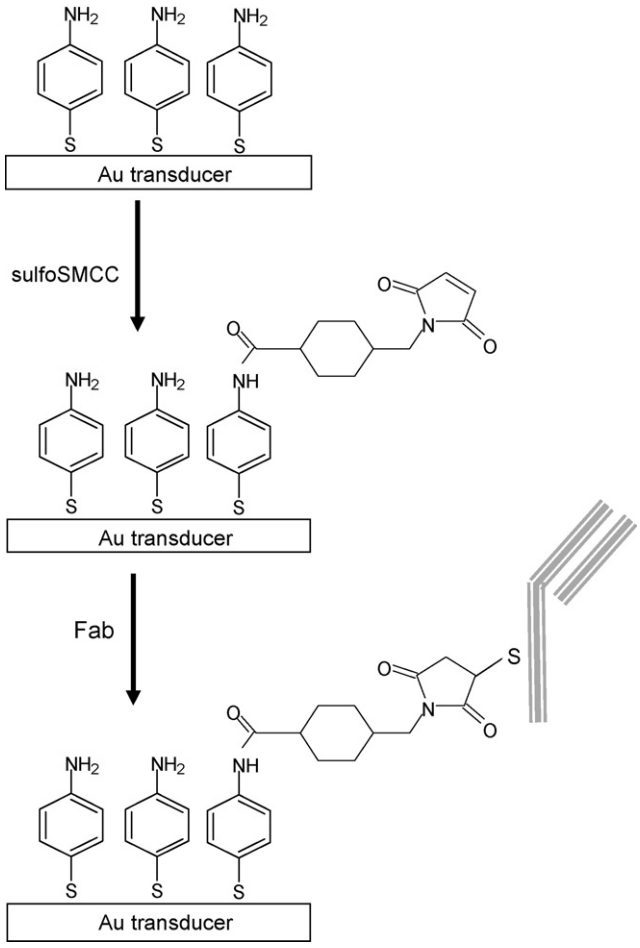

Fig. 5. Scheme showing formation of 4-aminothiothiophenol (4-ATP) SAM on gold and attachment of Fab via by the heterobifunctional crosslinker sulphosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (Sulpho-SMCC).

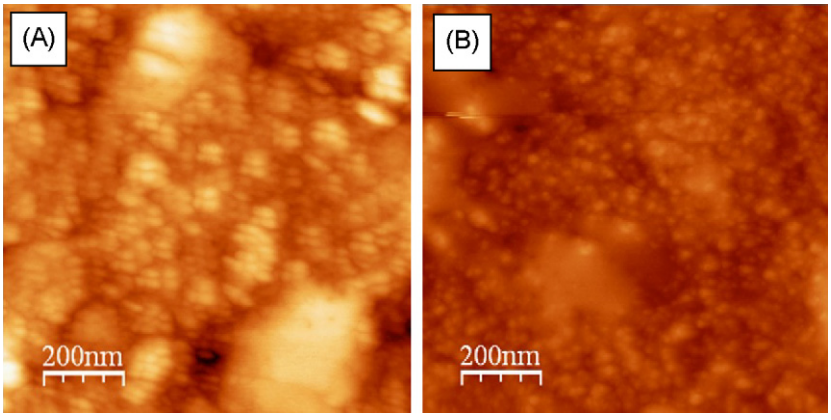

Fig. 6. Atomic force microscope image of (A), anti-myoglobin reduced half-IgG (Fab) tethered to sensor surface and (B), as panel A after binding of the analyte myoglobin.

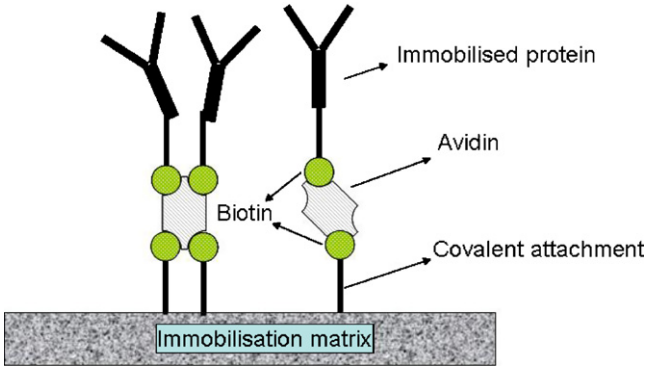

Fig. 7. Biotin mediated coupling of IgG molecules to surface of biosensor.

sensitive than the biotin/avidin based device (data not shown [45] and see Section 4.2). Interestingly, the Fab fragments reassociate on the sensor surface to form clearly visible dimers, but binding of the antigen disrupts this association and larger, non-dimeric complexes are produced (Fig. 6).

4.2. Affinity immobilisation via the biotin/avidin system

Biotin conjugation is a popular method of immobilising biomolecules to surfaces, via the use of avidins, tetrameric proteins which are naturally found in egg albumin and certain bacteria such as *Streptomyces avidinii* (streptavidin). The latter has a lower pI than avidin giving less non-specific binding; an alternative to the relatively expensive streptavidin is Neutravidin, which is essentially non-glycosylated avidin produced by engineering out its glycosylation sites. All avidins have intense affinity towards biotin with a dissociation constant (K_D) of approximately 10^{-15} M. This high affinity constant, which is one of the strongest known in nature, produces very effective immobilisation with saturation of all binding sites [48–50].

Due to its tetrameric nature, each avidin protein contains four biotin binding sites making it an ideal linker between two biotin tagged surfaces (see Fig. 7). Biotin can be incorporated into an mSAM, e.g. of mercaptohexadacanoic acid, using the affinity lipid biotinyl-caproyl-dipalmityl-phosphatidyl ethanolamine, when the lipid tails naturally intercalate into the alkanethiol alkyl chains [43–45], or be coupled to pendant amines or thiols on self-conducting polymeric surfaces using a reactive biotin analog (see below).

Alternatively recombinant proteins have been engineered to express a C or N-terminal strep tag, a 9 amino acid peptide [51,52] which occupies the biotin binding site, allowing direct attachment of the recombinant protein to an avidin linker. Site specific tags also allow all the recombinant proteins to be immobilised in a specific orientation, increasing the effectiveness of the biosensor [53] (or if not positioned favourably, drastically reducing effectiveness). Further advantages of using strep-tags are that they have a lower binding affinity ($2.7 \times 10^4\ M^{-1}$) [52] towards avidins, and can be stripped off using a binding competitor such as biotin or biotin analogs such as 2-iminobiotin or desthiobiotin. Nevertheless, production of recombinant proteins expressing strep tags require a high level of setup, and molecular biological expertise.

Native proteins can be biotinylated using a variety of biotinylation reagents containing reactive groups. These include amine-reactive *N*-hydroxysuccinimide esters or carboxyl terminated biotin analogs which can be coupled to amines via carbodiimide-mediated reaction using (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. While these are not the only reactive methods available, they are the most established methods of biotinylating proteins, and several biotinylation kits are commercially available containing reactive NHS-biotin.

The advantages of biotinylating proteins versus conventional covalent crosslinking methods are that less aggressive reaction conditions can be used, as biotinylation can be performed in an aqueous environment at physiologically acceptable pH and rarely results in aggregates. Furthermore, this method of biotinylation allows the user to add a spacer between the biotinyl group and the reactive group, thus increasing the distance between the biotin tag and the protein surface, reducing any possible steric hindrance effect.

Finally, the activity of the biotinyl-tagged protein may be estimated prior to immobilisation in order to confirm the labelling process has not caused it to lose significant activity. This is of great importance as covalent modification can result in a significant decrease in activity once immobilisation has occurred, whilst biotinyl-tagged proteins usually retain a significant portion of their activity.

4.3. Affinity immobilisation via polyhistidine tags

The affinity of certain amino acids such as histidine and cysteine for heavy metal ions was put forward as a possible protein purification route as early as 1975 [54]. Since few cysteines are present on the surface of most proteins this method, known as Immobilised Metal Ion Affinity Chromatography (IMAC), is usually employed to separate proteins on the basis of the number of histidines present on the protein's surface. This is due to the affinity of intermediate metal ions such as Cu^{2+}, Ni^{2+}, Zn^{2+}, and Co^{2+} for the nitrogen on the histidine side chain. In a flurry of research, the protocols for using an intermediate metal ion immobilised on an agarose or silica matrix to separate proteins using IMAC were developed.

Later, the first papers showing the use of an artificially added histidine-containing sequence were published [55]. These early sequences had been shown to bind to transition metal ions [56], but contained only one or two histidine residues. By the early 1990s, polyhistidine repeat sequences were added to peptides and proteins to facilitate protein purification [57] and histidine tagging became widespread both for this purpose and to immobilise proteins in order to probe their interactions [58].

Metal ions chelaters are now available in numerous forms, including more recent reagents used for immobilisation (rather than purification) of polyhistidine tagged proteins. Two common reagents employed for this purpose in biosensor production are the lipid 1,2-dioleoyl-*sn*-glycero-3-[(*N*-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl]-Ni^{2+}) better known as DOGS-NTA-Ni^{2+} which can be intercalated into long chain alkane thiol SAMs, and *N*-(5-amino-1-carboxypentyl)iminodiacetic acid (AB-NTA) which can be crosslinked to a variety of surfaces. Typically, DOGS-NTA-Ni^{2+} is used at a low mol/mol ratio, of around 5–10%. Histidine tagging is a gentle method of immobilising a protein that requires no chemical modifications, such as biotinylation, that could affect the function of the protein. The modifications are performed on the genetic sequence, typically by inserting the gene of interest into a suitable plasmid. There are many commercially available plasmids that contain the sequences necessary for adding either a His_6 or His_8 both of which show good binding affinities, at the N- or C-terminal end of the protein. Histidine–metal ion immobilisation is not completely devoid of problems however, and proteins immobilised via this route can tend to leach over time, since the linkage is not a permanent covalent one. Small proteins such as lipocalins, chitin binding protein and recombinant Fab have been successfully immobilised using polyhistidine tags in our group (data not shown). We have also found that polyhistidine tagged proteins can often be stripped from a transducer surface using imidazole and the reloaded to regenerate a fresh sensor surface.

5. Conclusions and future perspectives

The most popular covalent and non-covalent approaches to attachment of the sensing component have been considered. However, the methodologies mentioned in no way comprise an exhaustive list and many other approaches can be used, depending on the nature of the sensing molecule and chemical groups on it that might be modified without abrogating its biological activity. For proteins, the useful chemistry that can be employed tends to be limited to amines and thiols (side chains of lysine and cysteine, respectively), either for covalent attachment to the transducer surface or for attachment of some affinity group that can be subsequently employed for immobilisation. However, carboxyl groups (side chains of aspartic and glutamic acids) can be used for peptide bond formation with transducer surface amines, whilst carbohydrates which are often present due to glycosylation of eukaryotic proteins, offer a range of attachment options [46]. Finally, for applications to electrode arrays, the use of complementary oligonucleotide pairs, in which one DNA strand is tethered to a receptor protein whilst its complement is tethered to a unique electrode, offer a neat way of self-assembling a population of biosensor electrodes simultaneously. As ever, each solution to effectively fabricating a biosensor surface remains as much a measure of the researcher's inventiveness as it is of the available chemistries.

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