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Bioconjugation and stabilisation of biomolecules in biosensors

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Suitable bioconjugation strategies and stabilisation of biomolecules on electrodes is essential for the development of novel and commercially viable biosensors. In the present review, the functional groups that comprise the selectable targets for practical bioconjugation methods are discussed. We focus on describing the most common immobilisation techniques used in biosensor construction, which are classified into irreversible and reversible methods. Concerning the stability of proteins, the two main types of stability may be defined as (i) storage or shelf stability, and (ii) operational stability. Both types of stability are explained, as well as the introduction of an electrophoretic technique for predicting protein–polymer interactions. In addition, solution and dry stabilisation as well as stabilisation using the covalent immobilisation of proteins are discussed including possible factors that influence stability. Finally, the integration of nanomaterials, such as magnetic particles, with protein immobilisation is discussed in relation to protein stability studies.

Introduction

Different biosensors rely on different biomolecules such as nucleic acids, antibodies, cells or enzymes, which can work as both bioreceptors and signalling molecules or labels. The immobilisation and stabilisation of the bioreceptor on to the transducer of the biosensor are both of great importance. Biomolecules are immobilised not only on the transducer to act as a bioreceptor, but also on other surfaces such as magnetic, gold or latex particles, and recently in a wide variety of nanomaterials that enhance the analytical performance of the biosensors. Among the wide range of strategies dealing with the immobilisation of biomolecules, in the present review we will focus on the immobilisation of active biological receptors on electrode surfaces for electrochemical biosensors. A detailed discussion of the advantages and disadvantages of the irreversible (covalent binding, cross-linking and entrapment or micro-encapsulation) and reversible (adsorption, bioaffinity, chelation or metal binding and formation of disulfide bonds) immobilisation methods will be presented. Moreover, we will address the importance of the storage and operational stability of the biomolecules involved in the biosensing event and discuss the factors that influence protein stability.

Immobilisation methods on electrode surfaces

New types of transducers have been developed for use as biosensors, the most popular being optical, electrochemical and mass-based transduction methods. As analytical systems, electrochemical-based transduction devices are robust, easy to use, portable and inexpensive [1]. Many electrode

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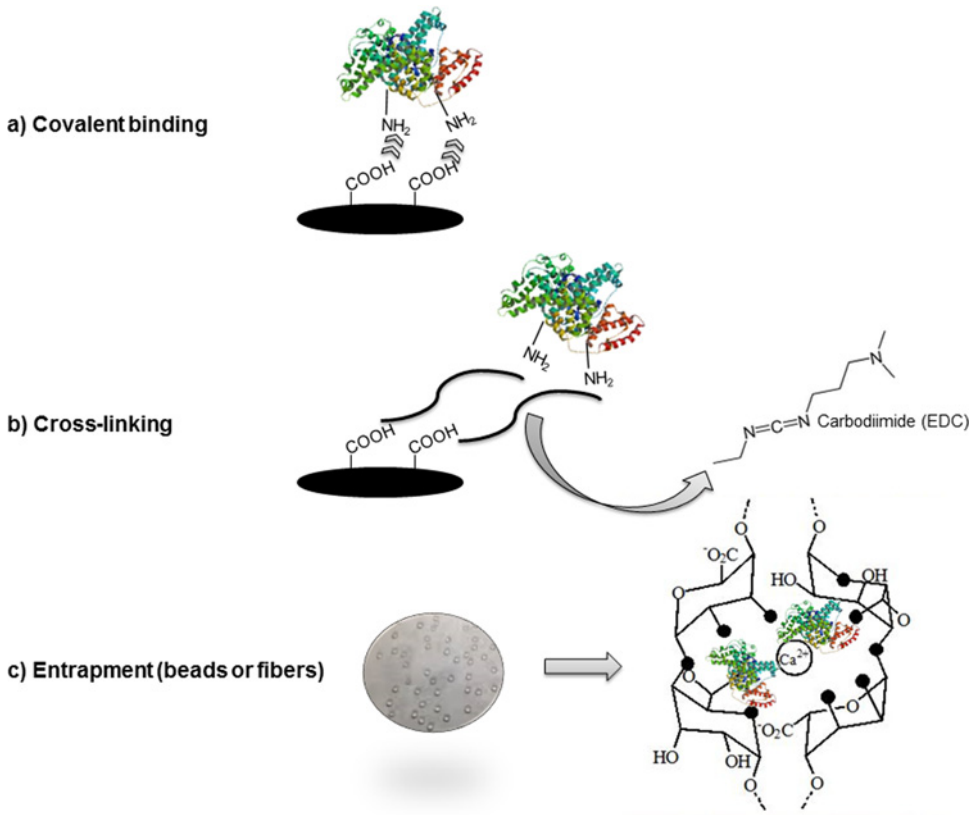

Figure 1. Irreversible immobilisation methods

Schematic representation of some irreversible methods: (**a**) covalent binding through primary amines, (**b**) cross-linking using carbodi-imide, and (**c**) entrapment on beads or fibres and micro-encapsulation.

materials such as glassy carbon, carbon paste, graphite composites, carbon/graphite formulations, carbon nanotubes, graphene and gold among others are used in electrochemical biosensors. Screen-printed electrodes (SPEs) are widely used as the measuring element due to easy and reproducible fabrication at both laboratory scale and in mass production [2,3]. Several types of SPEs, functionalised or not, are now commercially available (e.g. Gwent Group Ltd) and many laboratories have their own facilities for in-house production. However, in addition to the configuration of the electrode and materials being crucial, so is the immobilisation of the bioreceptor on to the electrode surface.

Often when working with biological molecules like proteins, an immobilisation method optimised for one protein may need to be adjusted to take into consideration the unique properties of another protein. For instance, it may be simple to conjugate or modify highly soluble proteins that have a high degree of conformational stability. However, similar reactions carried out on hydrophobic membrane proteins or insoluble peptide sequences will often require changes to the reaction conditions, which will affect the same conjugation process.

The recent advances in bioconjugation techniques are widely described in the literature (reviewed in [4]). The different strategies can be classified according to various levels of selectivity and difficulty, ranging from random methods (e.g. adsorption) to more advanced techniques based on protein engineering used to facilitate directional immobilisation (e.g. bio-orthogonal chemistries and SpyTag/SpyCatcher) [5–7]. In this review, we focus on the most common immobilisation techniques used for biosensor construction, which can be classified into two broad categories: irreversible (Figure 1) and reversible (Figure 2) methods. The advantages and disadvantages of the main methods are summarised in Table 1 [8,9].

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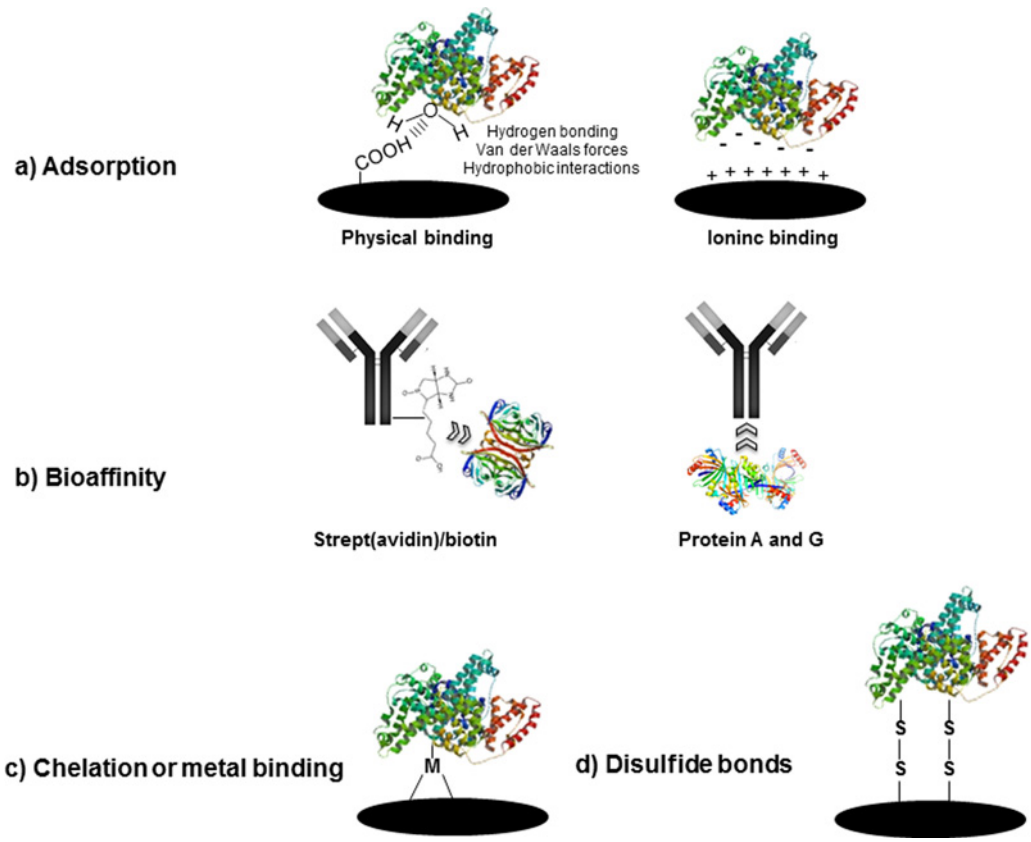

Figure 2. Reversible immobilisation methods

Schematic representation of some reversible methods: (**a**) adsorption, physical and ionic binding, (**b**) bioaffinity with strept(avidin)/biotin and Protein A/G, (**c**) chelation or metal binding, and (**d**) disulfide bonds.

Table 1. Characteristics of different immobilisation methods

Immobilisation method	Interaction	Advantages	Disadvantages
Covalent binding	Irreversible	Stability High binding strength	Cost
Cross-linking	Irreversible	Stability High binding strength	Diffusion limitation Cross-linker toxicity
Entrapment	Irreversible	Stable to changes in pH or ionic strength	Limited by mass transfer
Adsorption	Reversible	Simple Fast Low cost	Less reproducible Random orientation Desorption following change in ionic strength or pH
Bioaffinity	Reversible	Good orientation High specificity High selectivity High functionality Well-controlled	Cost
Chelation or metal binding	Reversible	Simplicity	Less reproducibility
Disulfide bonds	Reversible	Good orientation High sensitivity Well-ordered Stable bond	Cost Need for linkers

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Irreversible immobilisation methods

The immobilisation is irreversible when the bioreceptor attached to the support cannot be detached without destroying either the biological activity of the biomolecule or the support. The most common methods of irreversible immobilisation are covalent binding, cross-linking and entrapment or micro-encapsulation.

Covalent binding

The immobilisation of bioreceptors by methods based on the formation of covalent bonds are among the most widely used. Due to the stable nature of the bonds formed between the biomolecule and support, the bioreceptor is not released into the solution upon use. However, in order to achieve high levels of bound protein activity, the active area of the biomolecule must not be compromised by the covalent linkage chemistry to the support. For instance, the amino acid residues essential for catalytic activity or the recognition area of antibodies must not be hindered or blocked; this may prove a difficult requirement to fulfil in some cases. A wide variety of reactions have been developed depending on the functional groups available on the target. Despite the complexity of the biomolecule structure, only a small number of functional groups comprise selectable targets for practical bioconjugation methods. In fact, just five chemical targets account for the vast majority of chemical modification techniques:

- *Primary amines (–NH_2).* This group exists at the N-terminus of each polypeptide chain and in the side chain of lysine (Lys, K) residues. In physiological conditions, primary amines are positively charged and usually outward-facing on the surface of proteins, thus they are normally accessible for conjugation without denaturing the protein structure. Primary amines can be targeted using several kinds of conjugation chemistries. The most specific and efficient reagents are those that use the N-hydroxysuccinimidyl ester (NHS ester) reactive group.
- *Carboxygroups (–COOH).* This group exists at the C-terminus of each polypeptide chain and in the side chains of aspartic acid (Asp, D) and glutamic acid (Glu, E). Like primary amines, carboxygroups are usually available on the protein surface.
- *Thiols (–SH).* This group exists in the side chain of cysteine (Cys, C). Often, as part of a protein's secondary or tertiary structure, cysteines are joined together between their side chains via disulfide bonds (–S–S–). These must be reduced to thiols to make them available for binding. Reagents that are activated with maleimide or iodoacetyl groups are the most effective for thiol-directed conjugation.
- *Carbonyls (–CHO).* Ketone or aldehyde groups can be created in glycoproteins by oxidising the polysaccharide post-translational modifications (glycosylation) with sodium meta-periodate.
- *Carbohydrates (sugars).* Glycosylation occurs primarily in the constant fragment (Fc) region of antibodies (IgG). Component sugars in these polysaccharide moieties that contain *cis*-diols can be oxidised to create active aldehydes (–CHO) for coupling. Labelling carbohydrates requires more steps than labelling amines because the carbohydrates must first be oxidised to create reactive aldehydes; however, the strategy generally results in antibody conjugates with high activity due to the location of the carbohydrate moieties. Aldehyde-activated (oxidised) sugars can be reacted directly to primary amines through reductive amination (mentioned above) or to reagents that have been activated with hydrazide groups.

Cross-linking

Cross-linking is the process of chemically joining two or more molecules by a covalent bond. Cross-linking reagents (or cross-linkers) are molecules that contain two or more reactive ends capable of chemically attaching to specific functional groups (primary amines, thiols, etc.) on proteins or other biomolecules. Cross-linkers are also commonly used to modify nucleic acids, drugs and solid surfaces. The same chemistry is applied to amino acid and nucleic acid surface modification and labelling. There are several technical handbooks available, which detail the chemical reactivity and the molecular properties of cross-linkers (e.g. [10]).

Entrapment or micro-encapsulation

The entrapment method is based on the occlusion of a biomolecule, mostly enzymes, within a polymeric network that allows the substrate and products to pass through but retains the enzyme. This method differs from the coupling methods described above, in that the enzyme is not bound to the support or membrane. There are different approaches

to entrapping enzymes such as gel or fibre entrapping and micro-encapsulation. The practical use of these methods is limited by mass transfer limitations through membranes or gels.

Reversible immobilisation methods

Reversibly immobilised biomolecules can be detached from the support under gentle conditions. The use of reversible methods for bioreceptor immobilisation is highly attractive, mostly for economic reasons. After using the support, it can be regenerated and re-loaded with fresh bioreceptor. The most common methods of reversible immobilisation are adsorption, bioaffinity, chelation or metal binding and the formation of disulfide bonds.

Adsorption

The simplest immobilisation method is non-specific adsorption, which is mainly based on physical adsorption or ionic binding. In physical adsorption, the bioreceptors are attached to the surface through hydrogen bonding, van der Waals forces or hydrophobic interactions, whereas in ionic binding, the enzymes are bound through salt linkages. The nature of the forces involved in non-covalent immobilisation results in a process that can be reversed by changing the conditions that influence the strength of the interaction (e.g. pH, ionic strength, temperature or polarity of the solvent). Immobilisation by adsorption is a mild easy to perform process and usually preserves the functionality of the biomolecule [11]. The limitations of the adsorption mechanism are the random orientation and weak attachment, which produce desorption and poor reproducibility [12].

Bioaffinity

The principle of affinity between complementary biomolecules such as lectin–sugar, antibody–antigen and biotin–avidin has been applied to biomolecule immobilisation [13,14]. The remarkable selectivity of the interaction is a major benefit of the method. However, the procedure often requires the covalent binding of a costly affinity ligand (e.g. antibody or lectin) to the support. The most established procedures are the (strept)avidin–biotin interaction and the use of Protein A or G for antibody immobilisation:

- *(Strept)avidin–biotin interaction.* Avidin is a glycoprotein found in egg whites that contains four identical subunits. Each subunit contains one binding site for biotin, or vitamin H, and one oligosaccharide modification. The tetrameric protein is highly basic, having an isoelectric point (pI) of about 10. The biotin interaction with avidin is among the strongest noncovalent affinities known, exhibiting a dissociation constant of about 1.3×10^{-15} M. The only disadvantage of using avidin is its tendency to bind non-specifically with components other than biotin due to its high pI and carbohydrate content. Streptavidin is a similar biotin-binding protein to avidin, but it is of bacterial origin and originates from *Streptomyces avidinii*. The primary structure of streptavidin is considerably different from that of avidin. This variation in the amino acid sequence results in a much lower pI for streptavidin (pI 5–6). The strength of the noncovalent (strept)avidin–biotin interaction along with its resistance to break down makes it extraordinarily useful in the bioconjugate chemistry of any biomolecule [15].
- *Protein A and G.* This bioconjugation is mainly used for the immobilisation of antibodies. Protein A is derived from *Staphylococcus aureus* and Protein G is from *Streptococcus* species. Both have binding sites for the Fc of mammalian immunoglobulins (IgG), which ensures good orientation of the antibody after conjugation. The affinity of these proteins for IgG varies with the animal species. Protein G has a higher affinity for rat, goat, sheep and bovine IgG, as well as for mouse IgG1 and human IgG3. Protein A has a higher affinity for cat and guinea pig IgG. In addition to IgG Fc-binding sites, native Protein G contains binding sites for albumin, which can lead to non-specific staining. This problem is addressed by creating recombinant forms of the protein.

Chelation or metal binding

This method is known as 'metal link immobilisation'. The metal salt or hydroxide, mainly titanium and zirconium salt, is precipitated and bound by co-ordination with nucleophilic groups on the surface (e.g. cellulose-, chitin-, alginic acid- and silica-based carriers) by heating or neutralisation. Due to steric factors, not all of the metal binding sites are occupied, therefore some of the positions remain free to interact with groups from the biomolecule. The method is quite simple, but the adsorption sites are not uniform and the metal ion leakage is significant, which leads to a lack of reproducibility. In order to improve the control of the formation of the adsorption sites, chelator ligands such

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as ethylenediaminetetra-acetic acid (EDTA) can be immobilised on the solid supports by means of stable covalent bonds. Elution of the bound proteins can be easily achieved by competition with soluble ligands or by decreasing the pH.

Disulfide bonds

These methods are unique because, even though a stable covalent bond is formed between the support and bioreceptor, it can be broken by reaction with a suitable agent such as dithiothreitol (DTT) under mild conditions. Additionally, because the reactivity of the thiol groups can be modulated via pH alteration, the activity yield of the methods involving disulfide bond formation is usually high, provided that an appropriate thiol-reactive adsorbent with high specificity is used [16].

Protein stabilisation

The stabilisation of proteins is of great importance in many applications, and particularly in biosensor development. Stabilisation is known as the ability of a protein to retain its structural conformation or its activity when subjected to physical or chemical manipulation. The two main types of stability may be defined as (i) storage or shelf stability, and (ii) operational stability. The first relates to the retention of protein activity over time when stored as a dehydrated preparation, a solution or immobilised on to a surface. The second generally relates to the retention of activity when in use. The retention of the biological activity of the biomolecule involved in the biorecognition of an analyte is paramount, and this depends on retention of the biological structure. In most cases, the actual mechanism of stabilisation remains to be fully understood [17]. Protein engineering can be a useful tool for increasing the stability of certain enzymes [18] and antibodies [19]. However, this is only possible so long as structural data are available for the protein under examination. Protein stability is also evaluated using freeze-drying and spray-drying [20–22]. In this section, we will focus on the stabilisation achieved by the use of additives to modify the microenvironment of the protein under investigation and the covalent immobilisation of proteins on to transducers.

Dry and solution stability of proteins

Most of the publications on enzyme stabilisation are focused on the effect of additives on protein stability showing that it has been the most popular method of enzyme stabilisation [23]. The most important parameter in the promotion of structural integrity and thus stability of a protein is to retain the surface water activity. The components of a stabiliser formulation are normally made up of a combination of polyalcohols and polyelectrolytes. The polyalcohols include sugars and sugar alcohols that modify the water environment surrounding a protein, thus replacing and competing with free water within the system. This modified hydration shell confers protection to the protein, maintaining a 3D structure and biological activity, and enables long-term storage of biological materials both in solution and in the dehydrated state. Polyelectrolytes include numerous polymers of different charge and structure that form electrostatic interactions with proteins. As a result, large protein–polyelectrolyte complexes are formed, which retain full biological activity. Where polyelectrolytes and polyalcohols are combined, a synergistic effect is usually observed. Ratios of polyelectrolyte to polyalcohol are extremely important in the overall stabilisation of proteins. The buffer type, pH, ionic strength, concentration and ratio of stabilisers to protein/enzyme all play crucial roles in protein stabilisation both in the dry state and in solution. Some additives such as metal ions are directly related to enzyme structure and as such are not strictly surface interactions. The addition of dilute solutions of metal salts (e.g. magnesium or calcium) often stabilise proteins to a high degree and act synergistically with polyelectrolyte combinations. The addition of polyelectrolytes to solutions of proteins promotes the formation of soluble protein–polyelectrolyte complexes by electrostatic interaction. Polyhydroxyl compounds are then able to penetrate the structure more effectively, leading to enhanced stabilisation.

In most cases, the biological activity of the protein, enzyme or antibody is used as the main parameter to determine the stabilisation effect. When no simple method is available to directly measure biological activity, other techniques can be used to determine any molecular and structural modifications, such as gel electrophoresis, circular dichroism, fluorescence and turbidimetric measurements. Gel electrophoresis is normally used to examine the interactions between proteins and polymers and can be used to predict specific formulations, which lead to improved protein stability. Due to the extremely large size of the polymers, interaction between the polymer and protein is detected as the retardation of the enzyme in the gel matrix. This technique also helps in the determination of the affinity of polymer binding, allowing the prediction of the amount of polymer needed and subsequently reducing the cost of

stabilisation considerably. However, in most cases, simple activity tests or immunoassays are sufficient to evaluate the remaining activity [17].

Stabilisation by protein immobilisation

The covalent immobilisation of proteins onto transducers is considered another way of stabilising the biomolecule involved in the biosensing event. Each protein examined is unique, so in most cases what works for one type of protein rarely works for another. In addition, the problem of stability can be complicated during the immobilisation process, which can result in a stable protein but with vastly reduced residual activity. Of the methods described above, the most common immobilisation method used on a pre-activated carbon transducer utilises covalent coupling to the amino groups. In some enzymes, such as acetylcholinesterase [24], most of the amino groups are situated on the back face opposite to the active site, therefore this methodology ensures retaining the activity after immobilisation. Covalent immobilisation of other enzymes such as glucose oxidase has been described in many cases. The results show that the immobilised complex is more stable than the native immobilised enzyme. Stabilisation of the immobilised enzyme with polyelectrolyte combinations shows a distinct difference from that of the soluble enzyme dehydrated from solution. The orientation of the enzyme on to the surface of the transducer might explain this effect [25,26].

The advantages of having enzymes attached to surfaces have been exploited by living cells for as long as life has existed. In fact, there is experimental evidence to suggest that the immobilised state might be the most common state for enzymes in their natural environment. The attachment of enzymes to an appropriate surface ensures that they remain at the site where their activity is required. This immobilisation enhances the protein concentration at the proper location and it may also protect the enzyme from being destroyed. Multimolecular assembly depends upon the combination of weak non-covalent forces, hydrophobic interactions and covalent bonds (e.g. disulfide bridges) [27,28]. All of these different forces have been exploited in the development of immobilised enzymes.

One of the main problems associated with the use of immobilised enzymes is the loss of catalytic activity. This is probably due to the immobilisation site blocking access to the substrate-binding site of the protein resulting in the observed loss of enzyme activity. There are several strategies to avoid these steric problems. The careful choice of enzyme residues involved in the immobilisation, and the use of hydrophilic and inert spacer arms can reduce steric hindrance dramatically [29].

More recently, other immobilisation strategies have been adopted. These include the use of magnetic particles (MPs), which is attracting interest due to the intrinsic advantages of the material. MPs have been commercially available for many years (e.g. BioMag®, Dynabeads®, Adembeads® and Miltenyi®) and are widely used in laboratories to extract desired biological components, such as cells, organelles or DNA, from a fluid. In recent years, the magnetic properties of MPs have also been used as labels [30] and bioreceptor platforms in biosensing [31]. As shown in Figure 3 (left), they consist of an inorganic core of iron oxide (magnetite (Fe_3O_4), maghemite (Fe_2O_3) or other insoluble ferrites) coated with a polymer to confer stability (e.g. polystyrene, dextran, polyacrylic acid or silica), with added functional groups (e.g. amino and carboxylic acids) to make subsequent conjugations easy. Hence, iron oxide particles can carry diverse ligands, such as peptides, small molecules, proteins, antibodies and nucleic acids. In particular, this material is attractive for its use in immunomagnetic separation where antibodies are conjugated to the particles allowing the capture and orientation of the antigen on the surface of the electrode [32]. An example of solution stability for antibody-modified MPs is showed in Figure 3 (right). The antibody-modified MPs were stored in a number of stabiliser formulations at 32 °C in the solution state. The different MPs were processed over several days by magneto-immunoassay to detect prostate-specific antigen (PSA). Agglomeration of particles in the different solutions caused variability; however, the results showed that the Q2030317P4 stabiliser retained at least 85 % of the original antibody activity after 3 months of storage at this temperature. Meanwhile the unstabilised version lost complete binding ability within 1 month.

Conclusions

The field of bioconjugation has advanced at incredible pace. Tens of thousands of additional publications have appeared in biological, medical, polymer, material science and chemistry journals describing novel reactions and reagents along with their use in a variety of bioconjugation techniques. In this review, we discussed the most typical immobilisation techniques for active biological receptors on electrode surfaces, which could be extended to other transducer systems such as optical, piezoelectric or calorimetric as well as nanomaterials for all types of biosensors. The goal of the immobilisation method is maintaining biological activity while favouring, or at least not altering, the kinetics of the biological reaction.

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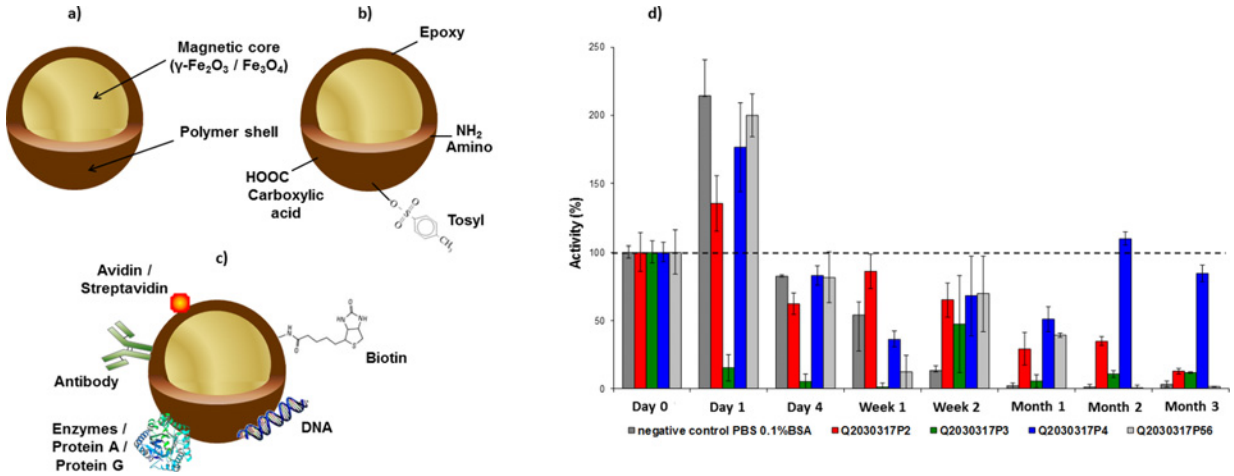

Figure 3. Stabilisation by protein immobilisation

Schematic representation of (**a**) magnetic particles, (**b**) activated with functional groups, and (**c**) conjugated to biological molecules [32]; (**d**) solution stability of antibody-modified magnetic particles over 3 months at 32 °C using stabilisers from Applied Enzyme Technology Ltd. Activity tested by optical magneto-immunoassay using 15 ng/ml PSA, 0.1 mg/ml anti-PSA antibody-modified magnetic particles (Abcam, ab10184) and 1 μg/ml horseradish peroxidase (HRP)-labelled antibody (Abcam, ab24466).

The actual stabilisation factor for most biosensors seems to be a combination of structural stabilisation by immobilisation on to a surface and the addition of specific stabiliser molecules. Both shelf stability and operational stability are improved by using novel polyelectrolyte stabilisers. The methodology is relatively generic and can be adapted for many application areas. The molecular mechanisms of stabilisation are currently under investigation by using more sophisticated techniques such as circular dichroism, fluorescence spectroscopy, differential scanning calorimetry, electrophoretic techniques, analytical centrifugation and electron microscopy. Data accumulated from such experiments will help researchers to understand more about how proteins denature at the molecular level and ultimately enable the stabilisation of proteins in a more predictable fashion.

The combination of immobilising and stabilising biomolecules together with the integration of micro- and nanostructured materials within biosensing devices is providing excellent analytical performances for different applications. The need of more flexible, reliable and sensitive targeting of analytes has promoted research into the potential of nanomaterials and their incorporation into biosensor systems. Most of the immobilisation methods described in this review are used for the bioconjugation of biomolecules into nanomaterials such as carbon nanotubes (CNTs), gold nanoparticles (AuNPs), MPs or quantum dots (QDs).

Research and development into biosensors is focused on designs compatible with technologies, such as screen-printing techniques, which allow the industrial production of low-cost devices. In this context, both suitable bioconjugation strategies and the stabilisation of biomolecules on electrodes are essential for the development of commercially viable biosensors.

Summary

- Biosensors rely on different biomolecules (nucleic acids, antibodies, cells or enzymes) as bioreceptors and signalling molecules or labels. Both immobilisation and stabilisation of the bioreceptor onto the transducer of the biosensor are of great importance.
- Immobilisation of active biological receptors on electrode surfaces for electrochemical biosensors construction. The most common immobilisation techniques used are classified in two broad categories: irreversible methods, when the bioreceptor cannot be detached without destroying either the biological activity of the biomolecule or the support, and reversible methods, when immobilised biomolecules can be detached from the support under gentle conditions.

- Protein stabilisation is known as the ability of a protein to retain its structural conformation or its activity when subjected to physical or chemical manipulations. Stabilisation of proteins is achieved by the use of additives to modify the microenvironment of the protein under investigation.
- Covalent immobilisation of proteins onto transducers is considered another way of stabilising the biomolecule involved in the biosensing event. Immobilisation of proteins onto nanomaterials such as magnetic particles provides analytical advantages.

Abbreviations

MP, magnetic particle; PSA, prostate-specific antigen; SPE, screen-printed electrode.

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Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

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