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Non-antibody protein-based biosensors

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Biosensors that depend on a physical or chemical measurement can be adversely affected by non-specific interactions. For example, a biosensor designed to measure specifically the levels of a rare analyte can give false positive results if there is even a small amount of interaction with a highly abundant but irrelevant molecule. To overcome this limitation, the biosensor community has frequently turned to antibody molecules as recognition elements because they are renowned for their exquisite specificity. Unfortunately antibodies can often fail when immobilised on inorganic surfaces, and alternative biological recognition elements are needed. This article reviews the available non-antibody-binding proteins that have been successfully used in electrical and micro-mechanical biosensor platforms.

Affimer binders and peptide aptamers in biosensing

Biosensors typically involve a molecular recognition element to detect the analyte, a transducer to convert detection into a signal and an amplifier that converts the signal into a measurement. In the biosensors considered here, the measurement is of an electrical characteristic, and the transducer is an electrode that can be affected by changes to the molecular recognition element, such as the binding of an analyte. Because non-specific binding of abundant molecules to the recognition element or indeed to the electrode itself may also lead to changes in electrical characteristics, it is crucial that: (i) the electrode be modified in such a way as to minimise signals from non-specific binding; (ii) the recognition element itself is exquisitely specific for its analyte; and (iii) the system utilises recognition elements whose properties can be measured by the adjacent electrode.

Proteins fulfil many of these requirements. At the molecular level, much of the work of life is mediated by proteins through their interactions with each other, with other biomolecules, and with inorganic elements and compounds. Proteins are composed of amino acids whose chemical nature makes them hydrophobic, polar or charged in aqueous solutions. It is the combination and the order of the amino acids in a protein that confers its ability to recognise its biologically relevant partners in the largely aqueous environment of the cell.

The most well-studied and widely used proteins for molecular recognition are antibodies (Figure 1A). These are naturally occurring proteins of the mammalian immune system whose role is to detect changes that may be dangerous, such as invading viruses or other pathogens, or changes in the animal's proteins that are the result of potentially cancer-causing mutations in the animal's genes. The ability of antibodies to mediate such exquisitely specific molecular recognition, combined with technological advances that have made them readily available [1], has made them a tool of choice in diagnostics. However, there are many cases where an antibody may not be available, cannot be made or, if available, may not possess the required specificity or affinity performance characteristics. In particular, it is not generally possible to immunise an animal with a toxic protein, or the animal may not be able to produce an antibody against a discontinuous epitope or one that carries a particular chemical modification. In addition, commercial antibodies are known to suffer from poor characterisation and batch-to-batch variability [2], which can make finding the right tool difficult, and no guarantee of long-term availability. In terms of
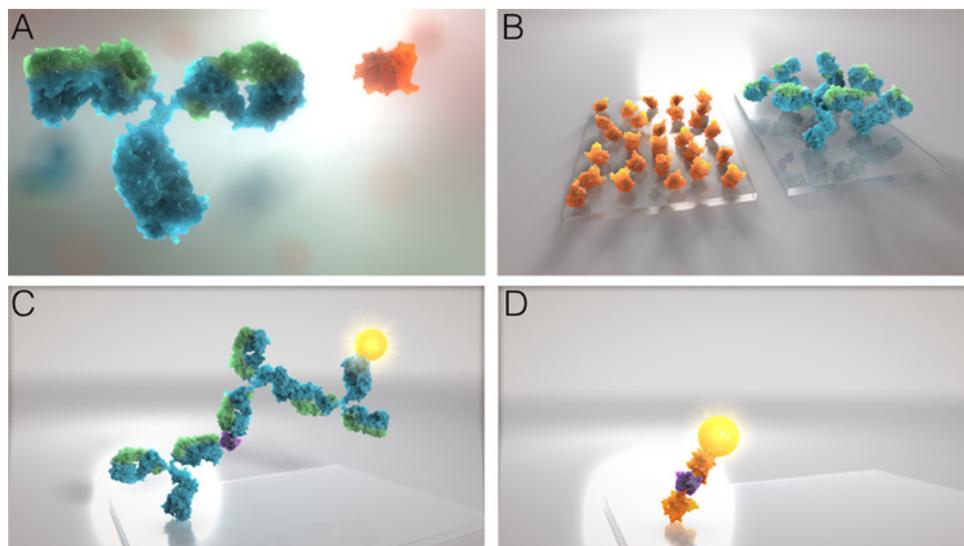
Figure 1. Schematic comparison of antibody and non-antibody affinity molecules

(**A**) Antibody molecules (shown on the left in blue and green) are significantly larger than non-antibody affinity molecules (represented by an Affimer protein, shown in orange on the right). (**B**) The small size of non-antibody-binding proteins means that more molecules can be immobilised per unit of surface area, meaning that the sensitivity of assays can be improved, at least for molecules that are smaller than the size of the antibody molecule. (**C**) In some biosensor formats, multiple antibodies may be used. In this illustration, an immobilised antibody (blue/green) binds to the target (purple), allowing the recruitment of a second antibody that is in turn recognised by a third antibody carrying a label (shown in yellow) for detection. (**D**) If non-antibody-binding proteins are used instead of antibodies, the whole assay can be moved closer to the surface, which is a major advantage for electrical or electrochemical detection if the surface is the working electrode. In addition, the recombinant nature of non-antibody-ending proteins means that they can be chemically modified in rational ways, increasing the efficiency of production and the efficacy of the ultimate measurement.

performance characteristics, a key feature for electrical and other biosensors is the need to immobilise the recognition element on a surface (the electrode; Figure 1B). Antibodies, however, generally function in solution (in the blood) and only become immobilised when they bind to a pathogen, when their conformation signals that the pathogen–antibody complex needs to be degraded. This means that a large number of antibodies raised against the same target may need to be screened to be able to identify one that retains its affinity and binding specificity when immobilised on a surface, and may also mean that the gradual loss of performance of the immobilised antibody will be the rate-limiting factor determining the shelf life of the resulting biosensor. Recombinant antibodies may address most of these issues and are the focus of Chapter 2. This review considers the use of engineered proteins as recognition elements for molecular detection in electrical biosensors.

Recognising these and other problems with the use of antibodies, multiple groups have sought to develop alternative non-antibody affinity reagents that can be used as recognition elements (Figure 1A). The first of these were nucleic acid aptamers, first described in 1990 [3,4], which are addressed in Chapter 4 and will not be further discussed here. Other groups have focused on non-antibody-binding proteins, including Affibodies (reviewed in [5]), Darpins [6], Anticalins (reviewed in [7]) and peptide aptamers [8]. The common feature of all of these technologies is that a constant engineered core protein, called the scaffold, is used to present a variable recognition/binding surface, mimicking the function of antibodies (reviewed in [9]). One advantage of all of these technologies is that the design of the scaffold protein usually results in a chemically uniform protein that may be tuned to recognise different analytes without grossly affecting biosensor set-up, as well as providing an increase in packing density of the recognition element on the biosensor surface (Figure 1B). In addition, the smaller size of both nucleic acid aptamers and most non-antibody-binding proteins means that any label (e.g. a redox-active small molecule) can be brought closer to the sensor or electrode, increasing the sensitivity of the biosensor (compare Figures 1C and 1D). More recently, attention has turned to the need for a biophysically robust biologically neutral protein scaffold with our own development of the
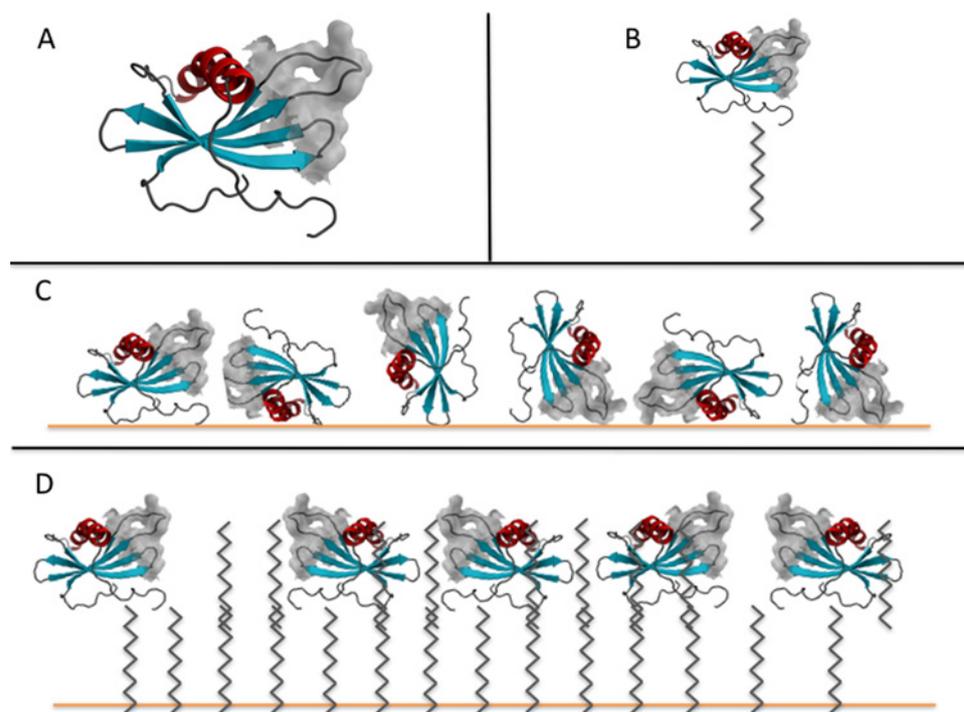
Figure 2. The Affimer scaffold and its utility for biosensing

Affimer binders are derived from a small stable human protein called Stefin A (**A**). Stefin A lacks a cysteine residue, which can be added and then chemically modified through thiol or maleimide chemistry, for example, to add a poly(ethylene glycol) chain (**B**). Although the Affimer proteins can be directly adsorbed on a surface, this will usually result in a mixture of proteins whose binding surface is masked by the surface or by neighbouring Affimer proteins (**C**). The use of a modified cysteine residue allows for the controlled immobilisation and orientation of the Affimer binders on the surface, maximising the efficacy and sensitivity of the ensuing biosensing device (**D**). See [21–30] for details.

Affimer® technology [10–12] and the emergence of the related Adhiron scaffold* [13]. In this review, we will focus on non-antibody platform technologies, although it has been shown that antibody domains can be used (nanobodies) [14], and that bespoke biosensors can be fabricated if a biological protein–protein interaction can be mimicked on the electronic surface (see, for example, [15,16]).

Non-antibody protein-based biosensors

Of the various non-antibody-binding protein scaffolds described in the literature, only three (thioredoxin, Affibodies and Affimer binders) have been used in biosensing. However, the range of applications has been broad and considerable success has been achieved.

In all three cases, the scaffold proteins were initially assessed for their ability to recognise fluorescently labelled target proteins in solution, when the scaffold proteins had been immobilised on glass slides (as illustrated in Figures 1C and 1D; and Figure 2). A major advantage of recombinant non-antibody-binding proteins is that it is possible to engineer them for controlled and oriented immobilisation on surfaces (Figures 1B and 1D) while their small size means that many more of these proteins can be packed into a biosensor compared with antibodies (Figure 1B; for a visualisation of the effects of the relative size differences between non-antibody-binding proteins and antibodies, see Figures 1C and 1D, which also show that the smaller size of non-antibody-binding proteins can be used to increase the signal by bringing reporters and labels closer to the sensing surface). For example, Renberg et al. [17,18] used

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*Affimer® and Adhiron proteins are both commercialised by Avacta Life Sciences under the Affimer brand.

Table 1. Performance of protein biosensors using engineered proteins

Method	Analyte/ligand	Matrix	Sensitivity	Reference
Electrochemical impedance spectroscopy	CDK2, CDK4	Yeast cell lysates diluted in phosphate buffer	Detects human protein expressed in yeast cells	[21]
SPR on a gold electrode	CDK2/Affimer	Buffer	2 nM	[22]
Electrochemical impedance, open circuit potential	CDK2, CDK4/multiple Affimer binders	Yeast cell lysates diluted in phosphate buffer	100 fM	[23]
Electrochemical impedance spectroscopy and cyclic voltammetry	C-reactive protein/Affimer binder	Buffer	Better than 10 nM	[24]
Electrochemical impedance spectroscopy	Anti-Myc/Affimer binder	Buffer	6.7 pM	[25]
Impedance	HER2/Affibody	Buffer and serum	6 ng/ml in buffer; serum also used	[26]

Affibodies to detect Taq polymerase, IgA, IgE, IgG, tumour necrosis factor α (TNF-α) and insulin with the most sensitive assay having a limit of detection of 70 fM for IgG in buffer. Laurenson et al. [19] used thioredoxin-based peptide aptamer microarrays to detect both endogenous cellular proteins (cyclin-dependent kinases 2 and 4 (CDK2 and CDK4)) and virally encoded E6 and E7 proteins in cells infected with human papilloma virus (HPV). Using Affimer reagents, we were able to both quantify CDK2 in human cell lysates and detect new human proteins in a discovery experiment both in human cells and in extracts of human placenta, used as a model human tissue. Song et al. [20] calculated that they could detect as little as 7×10^{-18} moles/cell (420 000 molecules) of human CDK2 per cell, using a sample comprising approximately 8000 lysed cells [20]. At least part of the reason for this success was the use of controlled and oriented immobilisation of the binding proteins on the surface (Figure 2 and [29]).

Although promising, the use of fluorescent labelling of the sample has two major drawbacks. The first of these is that the target proteins are chemically modified by the addition of the largely hydrophobic fluorescent dye. Because chemical modifications (such as protein phosphorylation) are known to affect the behaviour of many proteins, fluorescent labelling may potentially alter the biology of each protein, thereby clouding any biological interpretation. The second drawback is that some proteins may not be amenable to labelling, whereas other, perhaps larger, proteins may be labelled with multiple molecules of fluorophore, making quantification difficult.

Electrical biosensors offer the prospect of 'label-free' protein detection, and may also be able to benefit from massive parallelisation in their fabrication, borrowing technologies from the semiconductor industry. Marrying these with an engineered protein technology that is itself amenable to scaled manufacturing should open the way to the widespread development of biosensors with applications in fields as diverse as environmental sensing and personalised medicine. Table 1 shows several examples of electrical protein detection, largely using Affimer binders as few other scaffold proteins have been tested. In all cases, the authors also confirmed the electrical detection using another method, usually surface plasmon resonance (SPR) or quartz crystal microbalance (QCM). Key to the success of these approaches is the ability to chemically modify the non-antibody recognition elements (Figures 2A and 2B), allowing for the controlled orientation of the recognition element on the surface (compare Figures 2C and 2D) which results in greater signal-to-noise performance [21].

Although Affimer proteins have been used on bare gold in both electrical [21] and optical [27–29] settings, the bare gold surface represents a plane of infinite charge that will attract many proteins and other molecules present in a biological sample. In the case of electrical detection, this will result in significant but false positive signals. Significant effort has therefore been put into the development of self-assembling monolayers (SAMs) that will prevent non-specific binding to gold electrodes (Figure 2) [21–24,27,29] and this has also been shown to work in micro-mechanical biosensors [28]. The most effective SAM used with Affimer proteins has proven to be thiolated alkane polyethylene glycol, $HS(CH_2)_{11}(OCH_2CH_2)_3OH$ [23] with the thiol being used to drive immobilisation on the gold electrode through either co-ordination (an unstable bond where the lone pair of sulphur electrons interacts with the gold layer) or, more probably, through covalent bonding, after deprotonation of the thiol group and formation of a strong Au–S bond (the mechanisms of thiol-mediated immobilisation on gold are reviewed in [30]). Ravalli et al. [26] also used a

thiol-driven assembly of a blocking layer of 6-mercaptohexanol around immobilised anti-human epidermal growth factor receptor 2 (HER2) Affibodies, with the formed layer then further blocked using fat-free milk [26]. Typically, the use of these engineered proteins involves thiol-stabilised and -oriented immobilisation of the protein on a gold electrode, followed by an organic protective layer to prevent non-specific adsorption of sample proteins on to the electrode in a procedure commonly called back-filling. It would be interesting to compare the use of a simple chemical layer [22–24] with the use of fat-free milk [26], which may lead to the formation of a thicker layer. The latter may itself lead to higher background noise, but by raising the inactive surface away from the electrode, it may also mitigate the effects of non-specific protein binding by removing the event from the electrode.

Having addressed the coating of the electrode, let us now consider the engineered proteins that have been used in label-free biosensing. Affibodies are derived from a short (58-amino acid) engineered fragment of Protein A, a bacterial protein that itself has evolved to bind to immunoglobulins. Until now, their use has largely been limited to imaging and therapeutic applications [5]. Nonetheless, Ravalli et al. [26] were able to demonstrate a sensitive assay capable of detecting HER2 spiked in serum at 6 ng/ml.

Affimer proteins are approximately twice the size of Affibodies, at 120 amino acids. This confers on them a diameter of roughly 2.7 nm [29] and allows them to form uniform monolayers that have been imaged using atomic force microscopy [22,27]. Affimer binders to CDK2 and CDK4 that had been well-characterised in the fluorescent assays were used in the early development of Affimer protein-based biosensors, whereas more recent work has used an anti-C-reactive protein (CRP) Affimer binder [24], a model Affimer protein that recognises the anti-Myc antibody [25], and an anti-HER2 Affibody [26]. The low height of the Affimer layer from the electrode surface (visualised in Figures 1C and 1D) as well as their controlled orientation (compare Figures 2C and 2D) has been proposed to confer superior detection properties on Affimer proteins compared with antibodies that are proposed to form a layer 15 nm high from the electrode [24,29]; it will be interesting to see whether even smaller proteins such as Affibodies confer similar advantages, or even further improve the sensing properties.

Conclusion

Although the immobilisation of proteins on electrodes is not a new idea, the extension of the success of glucose oxidase-based glucose sensors to other proteins, particularly antibodies derived from an animal host, has been problematic. The advent of engineered alternatives to such antibodies, including recombinant antibody technologies as described elsewhere in this volume and the engineered non-antibody proteins discussed here, may lead to a step change in biosensor research by enabling the identification of structurally, biophysically, chemically robust yet biologically versatile recognition elements. The biological versatility is important because a platform technology that enables the detection of a wide range of analytes would streamline industrial as well as academic research and development. Chemical robustness will be important in ensuring that biosensors can be used with a range of biological samples, for example, urine in human diagnostics or milk in farm animal surveillance. Biophysical robustness will be important in determining product shelf life and applicability in a range of climates around the world. Finally, the platform nature of the technology should also lend itself to multiplexing recognition elements with similar performance characteristics within a single device, greatly extending the power and specificity of biosensor assays.

Summary

- Affimer proteins, peptide aptamers and other engineered proteins offer significant advantages over animal-derived antibodies in electrical biosensors.
- Blocking the electrode surface is key to lowering background.
- Highly sensitive assays have been devised.
- Further work on multiplexing will yield even more useful assays.

Abbreviations

CDK, cyclin-dependent kinase; HER2, human epidermal growth factor receptor 2; SAM, self-assembling monolayer; SPR, surface plasmon resonance.

Competing interests

PKF is employed by and owns shares in Avacta Life Sciences Ltd.

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