Sensors and Actuators B 234 (2016) 478–484

Contents lists available at ScienceDirect

Sensors and Actuators B: Chemical

journal homepage: www.elsevier.com/locate/snb

The development and optimisation of nanobody based electrochemical immunosensors for IgG

J. Goode[a,b,*], G. Dillon[b], P.A. Millner[a]

[a] School of Biomedical Sciences, Faculty of Biological Sciences, University of Leeds, UK
[b] Abcam Plc., 204 Cambridge Science Park, Cambridge, CB4 0GZ, UK

ARTICLE INFO

Article history:
Received 22 January 2016
Received in revised form 18 April 2016
Accepted 23 April 2016
Available online 30 April 2016

Keywords:
Nanobody
Impedance
Immunosensor
Orientation
Spacer
Steric hindrance

ABSTRACT

Biosensors are increasingly heralded for their potential to create inexpensive diagnostic devices which are sensitive, selective and easy to use. One of the key categories of biosensor are immunosensors, which have historically used antibodies as bioreceptors. Though widely used, antibodies bring inherent limitations such as variability, limited stability and their reliance on animal sources.

This has led to the development of alternative immuno-reagents such as non-antibody binding proteins (NABPs). These are low molecular weight proteins which largely avoid the aforementioned advantages of antibodies. They are commonly produced by bacteria enabling the use of DNA technology to manipulate bioreceptors at the molecular level.

Single chain VHHs (commonly known as nanobodies) are an antibody derived NABP adapted from camelid heavy chain antibodies which are the isolated binding domain. Whilst nanobodies have been used for diagnostic and therapeutic applications, they have limited demonstration in biosensors.

In this study, both antibodies and nanobodies were used to construct a biosensor. In addition nanobody performance was optimised by introducing a novel peptide spacer. The role of nanobody orientation and spacing was thus investigated and spacer length was optimised, leading to an increase in the sensitivity of the biosensor.

1. Introduction

A biosensor may be considered a three component system comprising: (a) the sample or analyte, (b) a transducer mechanism or bioreceptor and (c) a signal output. This report focuses on how altering the bioreceptor may be a crucial step in overcoming the barriers in the production of sensitive, low cost biosensors for both diagnostic and analytical applications. Though many sensors have been developed at the proof -of -concept level, there are only a few examples which have made the leap into the commercial market [1,2].

Most of the binding sensors developed to date rely on antibodies, the primary recognition agent of the immune system [3] and will be referred to as immunosensors throughout. Though antibodies are common reagents with well understood binding physics which allow the use of routine methods, there are a number of major drawbacks. These include batch to batch variability when using polyclonal antibodies, stability of the antibodies and their large size (150 kDa) which precludes the possibility of protein engineering. Antibodies may also bring issues of cross reactivity which cannot be identified until after production. These technical shortcomings are in addition to the ethical issues raised by their reliance on animal use and the financial issues raised with their associated cost [4,5].

For these reasons, there is a movement towards the use of recombinant monoclonal antibodies and the use of non-antibody binding proteins (NABPs), the latter of which may be easily expressed using bacterial techniques which allow the continuous production of a stable protein with no variation.

Whilst this article focuses on the use of nanobodies, the lessons learned may support findings relevant to other types of NABP or engineered Fab fragments or single chain variable fragments (ScFvs). There are currently a number of candidate NABPs for biosensor development such as darpins [6] Adhirons [7] and affybodies [8]. Though the benefits of these receptors have been previously demonstrated using optical biosensor systems [9], there are limited reports on their application in electrochemical sen-

Abbreviations: NABP, non antibody binding protein; V_{HH}, hypervariable region; ScFv, single chain variable fragments; IgNAR, immunoglobulin new antigen receptor.

* Corresponding author at: School of Biomedical Sciences, Faculty of Biological Sciences, University of Leeds, UK.
E-mail address: J.A.Goode@leeds.ac.uk (J. Goode).

http://dx.doi.org/10.1016/j.snb.2016.04.132
0925-4005/

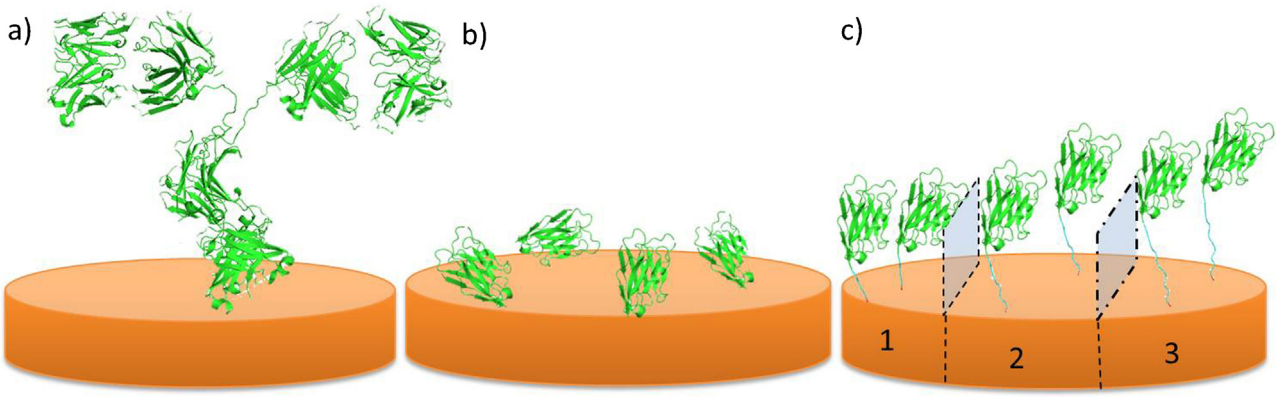

Fig. 1. An Illustration of different bioreceptors used. Antibody based biosensors using full antibodies (a) and non-oriented (b) and oriented nanobody sensors with engineered peptide spacer (c) of 5, 10 and 15 amino acids, (1, 2 and 3 respectively). Figure is illustrative of the size difference between different receptors.

sors and specifically a lack of data reported for impedimetric immunosensors [10]. There is no work to date focussing on both the orientation and inclusion of a peptide spacer to investigate the influence of bioreceptor height above the electrode on detection of analyte and signal generation.

The nanobody binding protein used in this study is an IgG fold domain which has been isolated from cammellid heavy chain only "Immunoglobulin New Antigen Receptors" (IgNARs). cDNA from lymphocytes was taken from immunised *Cammellidae* [11–13]. The cDNA can be sourced from lymphocytes isolated from blood taken from the host, avoiding animal sacrifice and contributing to the 3R's of animal use in research.

The cDNA extracted was isolated using PCR and used to construct a phage display library that could be screened to identify binding candidates for nanobody synthesis before sequencing and subcloning the DNA into a stable plasmid vector [14]. Screening can also be carried out at this stage to check for cross reactivity with candidate biomolecules which may commonly cross react with receptors [15]. This enables the elimination of receptors which cross react before the receptor is generated en-masse.

Once the DNA is ligated into a plasmid, it contains the sequence for a monoclonal-type binder and batch to batch variability is eliminated. This means that only one immunisation is necessary for a potentially endless source of nanobodies, producing a binder that is more reliable when compared to polyclonal antibody production techniques, as well providing the potential for lower cost [16]. These benefits have enabled the development of novel [17–20], as well as showing early promise in the field of diagnostics [20,21].

The nanobody used in this report was engineered to include a His_6-Tag for easy purification. In addition the plasmid was further engineered to create nanobodies with an engineered peptide spacer of 5, 10 or 15 amino acids (a GGGGS motif repeated respectively) with a unique cysteine at the N-terminus to enable oriented conjugation of the receptor using thiol chemistry, the plasmids are summarised in Supplementary Fig. S1 in the online version at DOI: 10.1016/j.snb.2016.04.132. Along with orientation, the use of spacers permitted the optimisation of receptor distance from the transducer surface, a parameter which may greatly impact the electrochemical signal generated.

Whilst the majority of reports aim to attach the bioreceptor intimately at biosensor interface, this may cause undesired effects such as a limitation in analyte binding due to steric hindrance. By providing a spatially coherent peptide spacer, these problems may be overcome as well as an improved degree of receptor orientation. The effect of steric hindrance has been demonstrated as a critical parameter in biosensor signal generation in previous studies [22–25]. It is hypothesised that the use of nanobodies will highlight the importance of orientation of the bioreceptor. As a comparison to the novel nanobody biosensors, data has been collected on antibody based biosensors; the variety of bioreceptors used in this study is illustrated in Fig. 1.

The sensor constructed in the study was developed to specifically detect rabbit IgG, for the commercial demands of the antibody development industry. The sensor developed will provide an accessible method for assaying antibody concentration in both sera and in prepared antibody products. Currently, a multi-step procedure is needed to quantify rabbit IgG accurately, this is inefficient in terms of both time and resources as it often relies on relatively large sample volumes.

2. Materials & methods

2.1. Expression and purification of nanobodies

A series of pHEN6 vectors was generated to create anti-rabbit IgG nanobodies, based on ab191866 (Abcam), the sequence was cloned into each modified pHEN6 vector using PstI and BstEII sites (Supplementary Fig. S2 in the online version at DOI: 10.1016/j.snb.2016.04.132) and modified vectors were generated by ligating in the sequence for include C-terminal spacers with a G4S motif (repeated for 5, 10 and 15 amino acid spacers with a terminal cysteine (Supplementary Fig. S1 in the online version at DOI: 10.1016/j.snb.2016.04.132) followed by a His_6-tag. This was achieved using BstEII and EcoRII sites. Following ligation, plasmids were transformed into WK6 *E. coli* cultures using the heat shock method [26] and cultures were grown on Ampicillin containing TB—Agar plates pre-heated to 37 °C. Individual colonies were then picked and added to 5 ml LB—ampicillin media to form starter cultures. The following day, 2 ml of this culture as added to 500 ml LB—ampicillin and IPTG was used to induce cultures overnight.

Cells were collected by centrifugation at 3000*g* for 30 min. The periplasm was then extracted using TES buffer (0.2 M Tris-HCL 0.5 M Sucrose 0.5 mM EDTA) at 4 °C, shaking on ice for 1 h. Finally, cell debris were collected by centrifugation at 6800*g* for 5 min. The Periplasm extract was then purified on a Ni^{2+} chelating column as previously described [27] and imidazole was removed by dialysing the nanobody against PBS.

2.2. Reduction of nanobody dimers

The purified nanobody at 1 mg ml^{-1} was incubated with 50 mM 2-mercaptoethylamine (2-MEA), in degassed PBS plus 10 mM EDTA pH 7.4 for 90 min at 37 °C. This selectively reduced the disulfide bonds, reducing Nanobody dimers into monomers. Buffer exchange