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Orientation of llama antibodies strongly increases sensitivity of biosensors

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ABSTRACT

Sensitivity of biosensors depends on the orientation of bio-receptors on the sensor surface. The objective of this study was to organize bio-receptors on surfaces in a way that their analyte binding site is exposed to the analyte solution. VHH proteins recognizing foot-and-mouth disease virus (FMDV) were used for making biosensors, and azides were introduced in the VHH to function as bioorthogonal reactive groups. The importance of the orientation of bio-receptors was addressed by comparing sensors with randomly oriented VHH (with multiple exposed azide groups) to sensors with uniformly oriented VHH (with only a single azide group). A surface plasmon resonance (SPR) chip exposing cyclooctyne was reacted to azide functionalized VHH domains, using click chemistry. Comparison between randomly and uniformly oriented bio-receptors showed up to 800-fold increase in biosensor sensitivity. This technique may increase the containment of infectious diseases such as FMDV as its strongly enhanced sensitivity may facilitate early diagnostics.

1. Introduction

Biosensors combine analytical devices with bio-receptors to sense the presence of analytes. Typical bio-receptors are antibody proteins which are widely used in biosensors due to their high specificity. In addition to full-sized antibodies (e.g. Immunglobulin G [IgG]), fragments of antibodies, such as Fabs (fragment, antigen binding) have been employed in biosensors (Chen et al., 2010; Yoshimoto et al., 2010). These fragments contain the analyte recognition site, but can be designed and produced as recombinant proteins for applications in biosensors (Conroy et al., 2009; Zeng et al., 2012). The smallest natural antibody-based binding unit is the variable domain of llama heavy-chain antibodies (VHH), which has a diameter of a few nm (Hamers-Casterman et al., 1993; Deffar et al., 2009). Such binding fragments are attractive for nanotechnology, since they can be easily tailored by protein engineering and included in biosensors (Saerens et al., 2005; Trilling et al., 2011).

Immobilization methods have an impact on the analytical performance in biosensors, as they affect the lifetime and orientation of the antibodies. Amine groups located at the surface of the antibody are often used to attach the protein covalently to activated carboxyl groups on sensor materials (Trilling et al., 2013a; Vashist et al., 2011). This method results in a random orientation, which reduces the efficiency of the sensor, as a large portion of the antibodies will not expose the analyte binding site to the analyte solution. Several techniques to position the antibodies in a uniform orientation on the surface have been explored, such as protein A or G for immunoglobulin or the biotin-streptavidin system (Bereli et al., 2011; Trilling et al., 2013a, 2013b; Huy et al., 2011; Park et al., 2011a, 2011b; Song et al., 2011). A common drawback of these methods is that they require an intermediate protein and rely on non-covalent protein–protein interactions. Inevitably this will negatively affect the lifetime of the biosensor and increase the distance between capture component and sensor surface (Trilling et al., 2013a). To establish stable covalent links to proteins in an aqueous environment, biocompatible click reactions, such as the 1,3-dipolar cycloaddition between an azide and an alkyne, have recently been developed (Kolb et al., 2001). Such reactions can be catalyzed by copper(I), or may occur in the absence of a catalyst, when the ligand contains a highly strained cyclic alkyne (e.g. cyclooctyne) with a low activation energy (so-called copper(I)-catalyzed azide-alkyne cycloaddition [CuAAC] and strain-promoted alkyne-azide cycloadditions [SPAAC] reactions, respectively) (Debets

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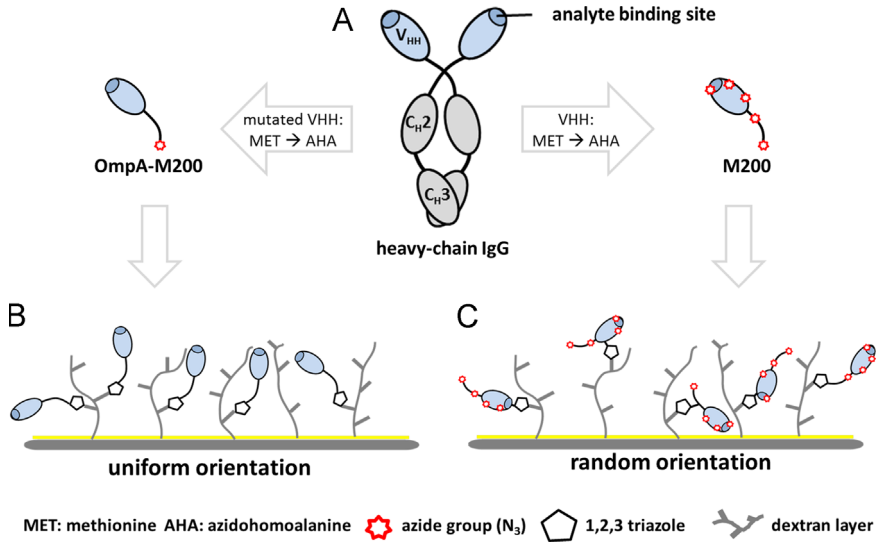

Fig. 1. Schematic illustration of the orientation of immobilized VHHs with tailor-made modifications on a sensor chip. (A) Azide-functionalized VHH receptors M200 and engineered VHH OmpA-M200 are smaller analogues of full llama heavy-chain IgG. (B) Azide-functionalized VHH OmpA-M200 covalently coupled in a uniform orientation onto a cyclooctyne-functionalized CM5 sensor. (C) Azide-functionalized VHH M200 covalently coupled in a random orientation via one of 5 available azides onto a cyclooctyne-tailored CM5 sensor.

et al., 2011; Jewett and Bertozzi, 2010). Azides may be incorporated into proteins as the non-natural amino-acid azidohomoalanine (AHA), an analogue which replaces methionine (Kiick et al., 2002). In this way, click chemistry has been deployed for the labelling of cells by dye conjugation, or for establishing enzyme complexes (Hong et al., 2010; Schoffelen et al., 2010). Here we explore both the CuAAC and SPAAC reactions to finally obtain a uniform orientation of VHH antibodies onto a surface plasmon resonance (SPR) biosensor surface (Fig. 1). This biosensor detects epitopes from foot-and-mouth disease virus (FMDV), and the virus itself.

2. Material and methods

2.1. Variable domain of llama heavy-chain antibodies (VHH)

VHH M200 (GenBank accession no: AJ811563.1) is specific for peptide PAT49 (acetyl-YGDGTVANVRGDLQVLAQKAARALPC-amide), corresponding to amino acid residues 136-160 of the GH-loop of the foot-and-mouth disease virus (FDMV) of the O1 Mansia strain (Harmsen et al., 2007). Native VHH M200-encoding DNA is present in the PRI-VSV expression vector (Trilling et al., 2013a). A variant (OmpA-M200) of this plasmid was made which expresses a protein with only a single methionine. To this end two internal methionine codons (coding for M83 and M88: Fig. 2) were altered to alanines by overlap extension PCR using oligonucleotides M200-M83A-M88A-F and M200-M83A-M88A-R (ESI, Table S1). Subsequently, the methionine residue (M153) in the C-terminal part of the protein, just before the His_6 tag was altered to glycine using the same strategy and oligonucleotides M200-M153G-F and M200-M153G-R. A methionine was introduced at the C-terminus by introducing six additional nucleotides (GGGATG) before the stop codon, encoding glycine and methionine, by amplifying the engineered M200 fragment using oligonucleotides M200-F and M200-R, and recloning the engineered fragment in the PstI/NotI restriction sites of the PRI-VSV expression vector. Lastly, an OmpA signal sequence was introduce at the N-terminus of engineered M200 using oligonucleotides OmpA-F and OmpA-R. Oligonucleotides were hybridized and ligated into PstI/NdeI digested PRI-VSV expression vector. Introduction of OmpA signal sequence results in loss of N-terminal start methionine in the mature protein. The entire VHH fragment of the resulting plasmid PRI-VHH-OmpA-M200 (Fig. 2) was analyzed by DNA sequencing using oligonucleotides M200-F and M200-R.

2.2. Expression and purification of recombinant VHHs

Plasmids PRI-VHH-M200 and PRI-VHH-OmpA-M200 were introduced into *Escherichia coli* B834 (DE3) pLysS bacteria (Novagen). Bacteria were used for protein expression as previous described (Trilling et al., 2011). AHA synthesis and expression of AHA containing VHHs (VHH-AHA) were performed as described before (Schoffelen et al., 2008). To obtain recombinant protein, bacteria were lysed by sonication. In short, pellets were dissolved in 1/10 volume lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, adjusted to pH 6.0), complemented with 1/100 volume protease inhibitor cocktail (Sigma, St Louis) and 1 mg mL^{-1} lysozyme. Lysozyme digestion was allowed for 30 min at 4 °C before cells were sonicated with a Soniprep150 (MSE, London, United Kingdom) in 5 mL batches for 10 cycles á 10 s with a break of 10 s. Cell lysate was then centrifuged for 30 min at 13.000 rpm and 4 °C. Supernatant was used for VHH purification using Ni-NTA Superflow resin (QIAGEN, Germany) as reported before (van Houwelingen et al., 2008). Eluates were concentrated to 200 μL using Amicon ultra-15 centrifugal filter units (cut-off 10K, Millipore, the Netherlands). Further purification was performed by size exclusion chromatography using a Superdex 200 GL 10/300 column on an ÄKTA FPLC™. VHHs were stored with a final concentration of 15% glycerol at −20 °C. Protein concentration was determined using Bradford test (Bradford, 1976) while successful expression and purification was verified by 15% sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) stained with SYPRO ruby (BioRad, the Netherlands).

2.3. Investigation of engineered VHH by enzyme linked immunosorbent assay (ELISA)

Wells of ELISA plates (Maxisorb, NUNC, the Netherlands) were coated with 5 μg mL^{-1} peptide PAT49 in 50 mM carbonate buffer pH 9.6 and incubated at 4 °C overnight. Antigen-coated wells were emptied and washed three times with 200 μL 10 mM phosphate-buffered saline pH 7.4 (PBS) with 0.05% Tween 20 (PBST) and then blocked with 200 μL PBS containing 3% nonfat powdered milk (w/v)