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The effect of uniform capture molecule orientation on biosensor sensitivity: Dependence on analyte properties

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ABSTRACT

Uniform orientation of capture molecules on biosensors has been reported to increase sensitivity. Here it is investigated which analyte properties contribute to sensitivity by orientation. Orientation of capture molecules on biosensors was investigated using variable domains of llama heavy-chain antibodies (VHHs) as capture molecule, and a surface plasmon resonance (SPR) chip as biosensor. Two VHHs were tested in this study: one recognizing foot-and-mouth disease virus (FMDV) and another recognizing the 16 kDa heat-shock protein of *Mycobacterium tuberculosis*. SPR chips with randomly immobilized biotinylated VHHs were compared to streptavidin-coated SPR chips, on which similar quantities of oriented biotinylated VHHs were non-covalently immobilized. Analytes that differ in molecular weight, epitope number and epitope affinity were compared using the FMDV-recognizing VHH. When binding of intact FMDV particles (146 S; 8200 kDa) or pentameric FMDV coat protein aggregates (12 S; 282 kDa) was detected, a modest (1–2-fold) increase in sensitivity was observed. When a 26-residue peptide (3 kDa) containing the epitope for VHH recognition was tested, much larger effects of capture molecule orientation (14-fold) on signal were observed. A 20–227-fold improvement was also observed when the epitope peptide was covalently linked to bovine serum albumin (67 kDa) or R-phycoerythrin (240 kDa). The results indicate that orientation of the capture molecule hardly affects high-affinity interactions, while it leads to strong improvements in sensitivity for lower-affinity interactions.

1. Introduction

The ongoing need for miniaturization of diagnostic elements such as biosensors implies that space for the actual detection components will be strongly limited. To compensate for this reduction in surface area, the biological detection elements need to be optimized. This optimization may include correct and uniform orientation at high density, in order to enhance analyte binding and to increase sensitivity of the device (Seo et al., 2011; Tajima et al., 2011; Vashist et al., 2011; Zeng et al., 2012).

Sensitivity and specificity of biosensors depend on high-affinity and specific capture molecules. Antibodies are generally used as biological capture molecules in immunoassays such as ELISAs, as they form highly specific and high affinity interactions. Nowadays, many biosensors deploy antibodies as capture molecules (Holford et al., 2012; Zeng et al., 2012). One of the challenges faced in the design of multiplex biosensors is to reach, on a single sensor, a high sensitivity for a large number of targets. One solution may be to achieve a high number of binding units per mm^2, ideally all oriented in the same way. Conventional antibodies, with a typical molecular weight of 150 kDa and a size of $14 \times 9 \times 4\ nm^3$ (Sarma et al., 1971), are big in size. Due to the presence of many exposed reactive residues, site-specific functionalization of these molecules is challenging. Therefore, the truly oriented printing of antibodies on biosensor surfaces is not straightforward. To deploy antibody-like molecules of smaller size, diverse formats were exploited for use in biosensors (Zeng et al., 2012). One such format is the variable domain of heavy-chain antibodies of llamas, which are naturally devoid of light chain (VHH). VHHs offer advantages in terms of size (Dumoulin et al., 2002), stability (Saerens et al., 2008), expression yields (Thomassen et al., 2002) and ease in protein engineering.

Various covalent and non-covalent immobilization strategies for proteins have been employed to attach capture molecules to a surface (Hernandez and Fernandez-Lafuente, 2011). The extraordinarily high affinity of streptavidin to biotin was frequently used to immobilize biotinylated antibodies on streptavidin-modified surfaces (Cho et al., 2007; Saerens et al., 2005). Site-specific in vivo biotinylation at the C-terminus of VHHs can be achieved by introducing a specific tag sequence, which is biotinylated by the BirA enzyme of *Escherichia coli* (Beckett et al., 1999).

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This approach can be used for the orientation of VHH capture molecules onto surfaces. Such orientation provides potential for higher binding capacity of the analyte: whereas in random immobilization, analyte-recognition sites are poorly exposed to the surface, orientation can be tuned for maximal exposure of the capture molecule to the analyte (Fig. 1). Techniques like circular dichroism spectroscopy (Le Brun et al., 2011), total internal reflection ellipsometry (Balevicius et al., 2011), time-of-flight secondary ion mass spectrometry (Baio et al., 2011; Cho et al., 2011; Park et al., 2011) and atomic force microscopy (Farris and McDonald, 2011) were used to display the antibody orientation in a direct manner. As an alternative, and in fact more directly linked to the application, the signal increase of oriented antibodies can be used as indirect method to determine the successful orientation of antibodies. Increased sensitivity after orientation of antibodies is well documented (Cho et al., 2009; Klonisch et al., 1996; Peluso et al., 2003; Shen et al., 2011; Song et al., 2012; Vareiro et al., 2005). However, reported improvements are highly variable, and the impact of analyte properties on the detection limit in uniformly and randomly oriented capture molecules has not been reported.

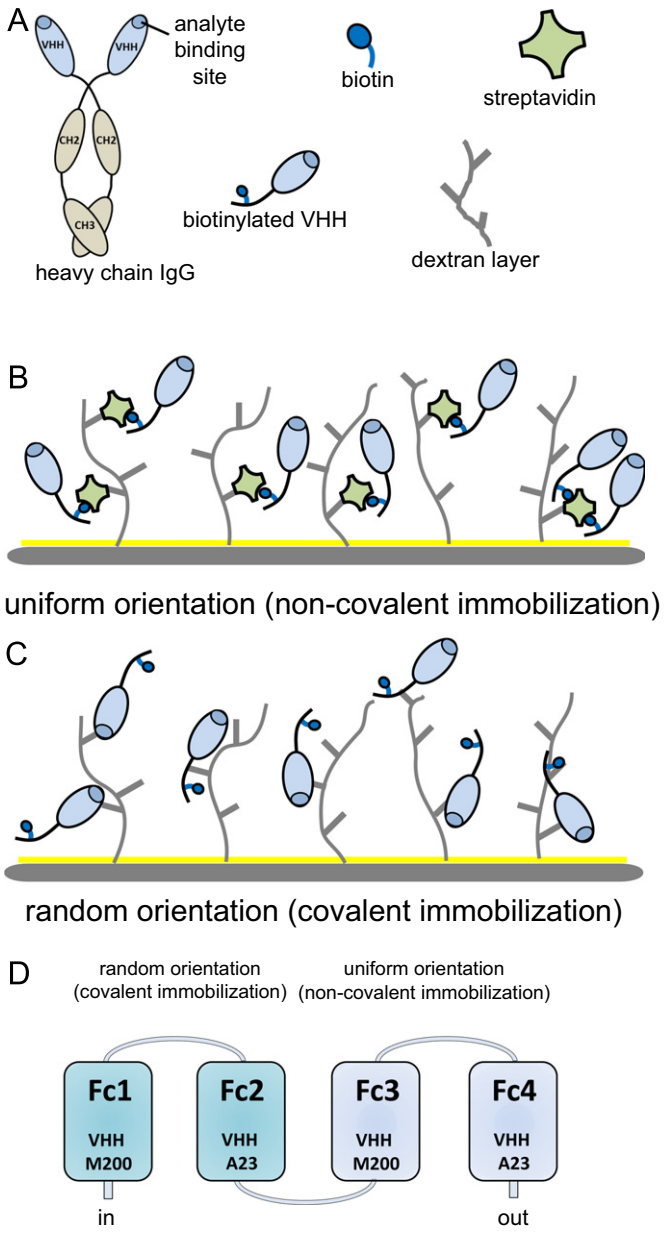

Fig. 1. Schematic illustration of immobilized VHHs on a carboxymethyl dextran sensor chip (CM5). A: heavy-chain antibody IgG. Biotin was introduced at the C-terminus of the VHH, the opposite site of the analyte binding site. B: biotinylated VHHs non-covalently immobilized in a uniform orientation onto streptavidin-coated CM5 sensor chips. C: biotinylated VHHs covalently coupled in random orientation via the amine groups onto CM5 sensor chips. VHHs can be immobilized via the ε-amines, present in lysine side chains, or via the α-amine, present at the N-terminus of the protein. D: set-up of SPR sensor chip with 4 flow channels (Fc).VHH M200 was immobilized randomly oriented in Fc1 and uniformly oriented in Fc3 and VHH A23 was immobilized randomly oriented in Fc2 and uniformly oriented in Fc4. For measurements analyte was injected in series over Fc1, Fc2, Fc3 and Fc4.

In a previous study we showed the use of VHHs as capture molecule using surface plasmon resonance (SPR) (Trilling et al., 2011). In the current study, as a next step, two different VHHs were engineered using a tag sequence to obtain site-specific biotinylation (Beckett et al., 1999). These two tailor-made VHHs were subsequently immobilized on the sensor surface in two ways: in a randomly oriented, covalent manner (via coupling of amine moieties with the activated surface), and in a uniformly oriented, non-covalent way by the use of streptavidin-coated surfaces and the non-covalent biotin-streptavidin interaction. The sensitivity of the resulting surfaces was studied towards a wide range of analytes. This has led to a high sensitivity enhancement upon uniform orientation (up to factor 227). Finally, we will develop a hypothesis to understand which analyte properties seem to govern the effects of uniform capture molecule orientation on the sensitivity of the sensor, with a specific focus on analyte molecular weight, avidity and specifically affinity.

2. Material and methods

2.1. Analytes of VHH

VHH A23 (Genbank accession no. JN234011.1) detects the 16 kDa heat-shock protein of *Mycobacterium tuberculosis*, and was characterized before (Trilling et al., 2011). Mycobacterium lysates (*M. tuberculosis*1, *M. tuberculosis*27 and *M. smegmatis*, Royal Tropical Institute, Amsterdam, The Netherlands) were prepared as described before (Trilling et al., 2011).

VHH M200 is specific for the GH-loop of structural protein VP1 of foot-and-mouth disease virus (FDMV) (Harmsen et al., 2007b). FMDV analyte and 12S particles derived thereof by acidification were prepared as described before (Harmsen et al., 2007a; Harmsen et al., 2011). Peptide PAT49 (acetyl-YGDGTVANVRGDLQVLAQKAAR-ALPC-amide), corresponding to amino acid residues 136–160 of the GH-loop of the FMDV O1 Manisa strain was purchased from Peptide 2.0 (Chantilly, US). Peptide PAT49 was coupled with the additional C-terminal cysteine residue to maleimide-activated bovine serum albumin (BSA) and R-phycoerythrin (Innova Biosciences Ltd., UK). Maleimide-activated protein was reacted overnight at room temperature in phosphate-buffered saline (PBS) pH 7.2 with peptide PAT49 in a 1:20 and 1:5 M ratio, resulting in BSA–PAT49 and R-phycoerythrin–PAT49. To subsequently block unreacted maleimide moieties, cysteine was added in excess. BSA–PAT49 and R-phycoerythrin–PAT49 were purified on Superdex 200 (GE Healthcare, Uppsala, Sweden) and concentrated using filter tubes (Millipore, The Netherlands). Protein concentrations were determined with the Pierce 660 nm Protein assay using NanoDrop 1000 and albumin as reference protein for the standard curve.

2.2. Biotinylation of VHH

VHHs are present in a vector for periplasmic VHH expression under control of the T7 promoter, based on the backbone of the pRSET-A vector (Invitrogen, The Netherlands). The C-terminus of