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Developing An Ellipsometry-based Biosensor Platform via Covalent and Oriented Coupling of The Nanobody Targeting The Vascular Cell Adhesion Molecule-1 To A Silicon Wafer

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Abstract

Protein immobilization plays a key role in the development of all protein-based biosensors in order to achieve good performances. Conventional techniques such as physical adsorption or randomly chemical coupling usually lead to low activity and reproducibility. Therefore there is an essential need for uniformly oriented, covalent immobilization approaches. In this study, the nanobody targeting Vascular Cell Adhesion Molecule-1 (NbVCAM1) is engineered with a C-terminal alkyne function via the Intein-mediated Protein Ligation (IPL) technique. The alkynylated nanobody exhibits high binding capacity towards the human VCAM1. Moreover, it can be covalently coupled to an azidified silicon wafer via the Copper(I)-catalyzed azide-alkyne cycloaddition, hyphothetically with a unique orientation. The resulting nanobody-conjugated surfaces exhibit a remarkable increase in binding affinity and sensitivity when monitored by ellipsometry, as compared to those loaded with nanobodies via random couplings. Therefore, the results show an improved performance of the uniformly oriented nanobody-functionalized surfaces as a biosensor platform.

Keywords: biosensor, CuAAC, ellipsometry, VCAM1-targeting nanobody

1. INTRODUCTION

Several approaches exist for immobilizing proteins to surfaces, which is the key step in biosensor development. Non-covalent methods are based on physical adsorption via weak physico-chemical interactions. However, this generally results in a random orientation and weak coupling of the protein to the transducer surface, resulting in a loss of sensitivity and reproducibility since only a fraction of the proteins will have their binding sites accessible for the target molecules (Yoshimura *et al.*, 2012). A more robust way is to immobilize the proteins by means of covalent bonding between the side chain functional groups of the endogenous amino acids and the substrate surface. However, because proteins in general possess multiple copies of the same amino acid, multiple orientations of the protein will also occur on the surface (Steen Redeker *et al.*, 2013). Therefore this project aims for the development of site-specifically functionalized proteins (i.e. nanobodies) towards a covalent and oriented surface coupling for enhancing the performance of the resulting bio-functionalized surfaces.

Nanobodies – the minimized variable domains of single-domain antibodies found in camelidae – have numerous advantages over conventional antibodies: small size (±15 kDa), ease of genetic manipulation and expression in *E. coli*, high stability and strong antigen capacity compared to the full-

length antibodies (Muyldermans *et al.*, 2009). Thus they are promising detecting molecules for the fabrication of biosensor platforms. In this study, the nanobody targeting the vascular cell adhesion molecule-1 – an important marker for atherosclerosis – is site-specifically modified with an alkyne group via the Intein-mediated Protein Ligation (IPL) technique (Muir *et al.*, 1998). The alkynylated nanobody is immobilized via the Copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) (Mendal and Tornoe, 2008) on a silicon wafer. The nanobody-conjugated surface is tested for its binding capacity towards the human VCAM1 antigen using ellipsometry.

2. METHODOLOGY

2.1 Expression and alkynylation of the nanobodies

To produce the site-specifically modified nanobody via IPL, the NbVCAM1 gene is engineered with a LEY spacer peptide, followed by fusing with the *Mxe* intein sequence and a chitin binding domain at its C-terminus. The fusion protein is expressed in *E. coli* SHuffle® T7 cells. The nanobody is cleaved from the intein by passing through a chitin column, followed by adding a cysteine-alkyne linker and incubating overnight at 4°C. The resulting C-terminally alkynylated nanobody is desalted and stored at -20°C for further use. On the other hand, the NbVCAM1-His_6 (expressed and purified as previously described by Broisat *et al*. (2012) is incubated with an NHS-propargylamine linker for 3 hrs to obtain the randomly alkynylated nanobody. The product is then desalted and processed as described above.

2.2 Characterization of the C-terminally alkynylated nanobody

The mass of the resulting nanobody is determined by mass spectrometry to confirm the addition of the alkyne moiety at its C-terminus. Its bioactivity is also determined by a sandwich ELISA test using the NbVCAM1-His_6 and the NbBcII-10-His_6 (a nanobody targeting the bacterial β-lactamase which has a non-specific binding to VCAM1) as positive and negative controls, respectively.

2.3 Immobilization of the alkynylated NbVCAM1-LEY on a silicon wafer via CuAAC

The nanobodies were immobilized on a 0.3 x 3 cm silicon wafer which is pre-functionalized with azide groups. The reaction is performed with 1 μM nanobody in different buffers (PBS pH 7.4 and acetate pH 4.0) with $CuSO_4$, THPTA and sodium ascorbate which are the copper catalyst, the complexing reagent and the reducing reagent, respectively. The immobilization process is monitored in real-time by a precipitate-enhanced ellipsometer (Damen *et al.*, 2009).

2.4 Characterization of the antigen binding by ellipsometry

The nanobody-conjugated silicon wafers are subjected to binding with the recombinant human VCAM1 at different concentrations for 30 min, which is monitored by ellipsometry. The obtained data are used to generate the Scatchard plots from which the kinetic parameters (K_a, K_d and K_D) can be determined. The bound antigen will then be washed away and the surfaces are subjected to the second antigen binding cycle to test for their reproducibility.

3. RESULTS

3.1 The C-terminally monoalkynylated nanobody

The C-terminally alkynylated nanobody (termed NbVCAM1-LEY-alkyne) is obtained in high yield (up to 20 mg per liter of *E. coli* culture). The mass of the monoalkynylated nanobody (Da) is confirmed by mass spectrometry (Figure 1B). The alkynylated nanobodies (both C-terminally and randomly) retain good antigen binding capacity as compared to the unmodified nanobody (NbVCAM1-His_6) (Figure 1).

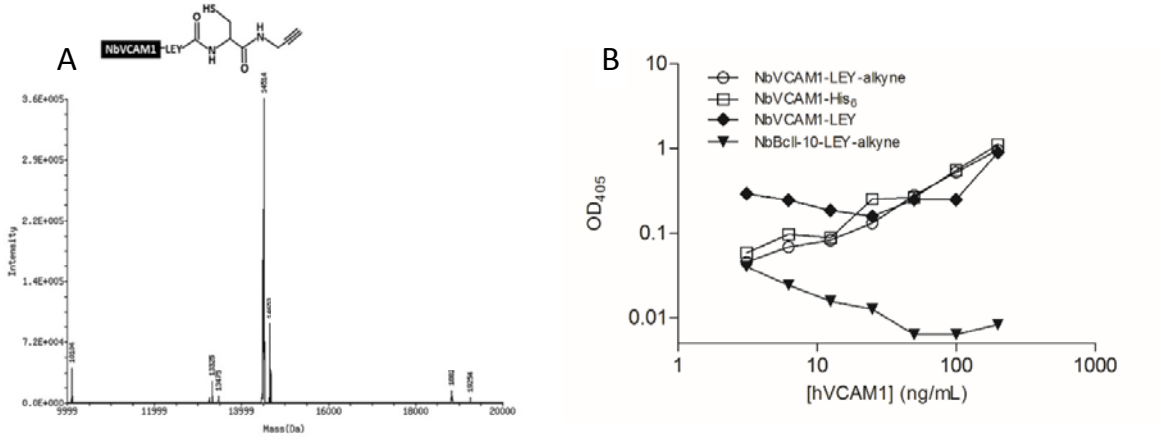

Figure 1. (A) Mass spectrum of the purified NbVCAM1-LEY-alkyne fraction showing the major peak of the monoalkynylated nanobody species. **(B)** Sandwich ELISA test for antigen binding capacity of the produced nanobodies. The experiment was performed in triplicates and the data were plot on a log-log scale.

3.2 Immobilization of the alkynylated nanobodies on silicon wafers

The nanobody is immobilized with the highest coverage (228.8 ng/cm^2) on the silicon surface using acetate buffer pH 4.0. The covalent linkage is stable after stringent washings with SDS, β-mercaptoethanol and PBS. In contrast, the non-alkynylated nanobodies are completely depleted after washing since they are only adsorbed on the surfaces (no covalent coupling) (Figure 2A).

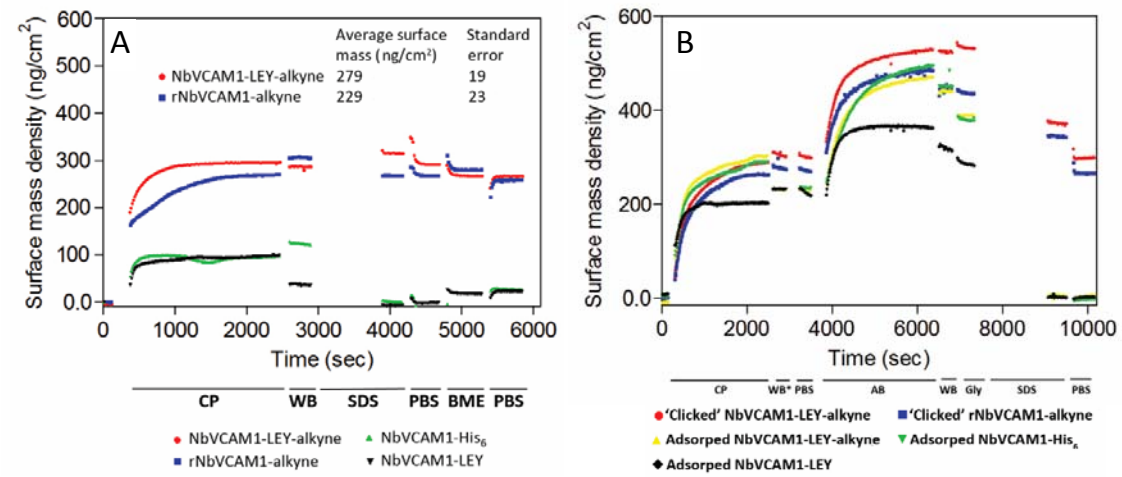

Figure 2. (A) CuAAC-mediated coupling of two nanobodies on azidified silicon wafers (rNbVCAM1-alkyne denotes the randomly alkynylated nanobody). The coupling process (CP) was performed followed by washing with washing buffer (WB), SDS, PBS, BME (β-mercaptoethanol) and PBS. Data was recorded in real-time in four replicates, and one representative curve corresponding to each nanobody species was plotted. **(B)** Antigen binding (AB) of different nanobody-loaded silicon wafers with human VCAM1. The nanobodies are immobilized on azido-silicon surfaces using either CuAAC (for rNbVCAM1-alkyne and NbVCAM1-LEY-alkyne) or adsorption (for NbVCAM1-LEY, NbVCAM1-His$_6$ and NbVCAM1-LEY-alkyne). Antigen binding is followed by a serial washing step WB, Glycine-HCl, SDS and PBS to regenerate the surface.

3.3. Antigen binding

The nanobody-clicked surfaces (both random and uniformly oriented) exhibited better binding capacity as compared to the surfaces adsorbed with the nanobodies (which will be removed after stringent washing) (Figure 2B). On the other hand, the surface with the site-specifically coupled nanobody has a

much higher K_D value (Figure 3) and lower detection range towards the VCAM1, indicating better affinity and sensitivity as compared to the surface with randomly immobilized nanobody.

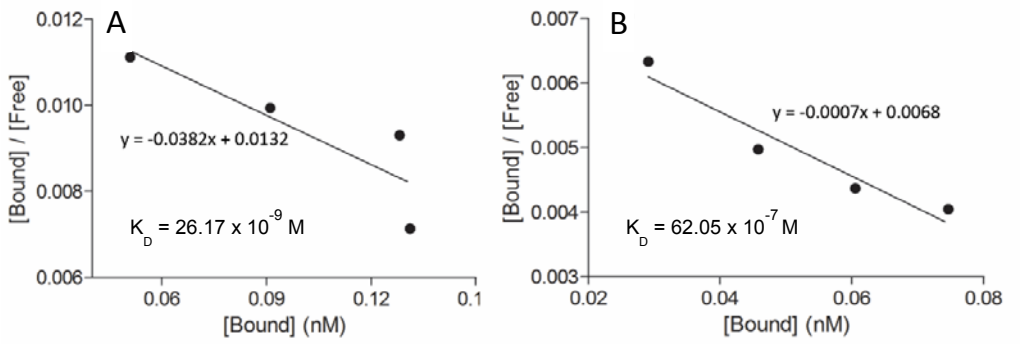

Figure 3. The corresponding Scatchard plots derived from the binding data (i.e. free and bound antigen concentrations) for silicon wafers loaded with **(A)** C-terminally alkynylated nanobody and **(B)** randomly alkynylated nanobody. The association equilibrium constants (K_a) are calculated from the slopes from the linear regression equations, and the dissociation equilibrium constants (K_D) are inversed of the K_a.

4. CONCLUSIONS

The NbVCAM1 is functionally expressed in *E. coli* and is C-terminally alkynylated via IPL while maintaining its binding capacity. The resulting nanobody was covalently and orientedly immobilized onto azidified silicon wafers via the triazole linkage in a CuAAC-mediated coupling reaction. The nanobody-conjugated surface obtained through this strategy exhibits clear advantages in antigen binding affinity, sensitivity and reproducibility compared to the commonly used physisorption or randomly covalent immobilization approaches. These results have paved the way to develop an advanced nanobody-based silicon platform for biosensing purposes.

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