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Optical microchips based on high-affinity recombinant protein binders—Human serum albumin detection in urine

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ABSTRACT

Recent developments in molecular evolution technologies have led to novel types of high-affinity recombinant protein binders (PB) able to substitute antibodies in many diagnostic and therapeutic applications. Despite almost a decade of research, they have so far only been sporadically used for biosensor construction. Here, we present a proof-of-principle comparative study focused on the application of three types of PB recognizing human serum albumin (HSA) in the fabrication of diagnostic optical microchips detecting clinically relevant HSA levels in urine. The PB tested were: (i) biotinylated anti-HSA Affibody (AF) (IgG binding domain of protein A, *Staphylococcus aureus*); (ii) biotinylated protein construct based on albumin-binding domain (ABD) of protein G (*Streptococcus* G148) fused with long TolA spacer (6xHis-WT-ABD-TolA-AviTag) and (iii) WT-ABD-Trp leader-streptavidin tetrameric fusion protein (SA-ABD-WT). Open glass microchips with 24 independent microwells (volume 8 µL) and micropatterned detection zones were prepared and used for oriented binding of proteins through the biotin/streptavidin chemistry. The analytical performance of the optical microchips was tested by performing direct specific detection of fluorescently labelled HSA in various environments. Results show that the length of peptide spacer present between the binding protein domain and sensor surface is a key factor influencing biosensor performance. The biosensor based on SA-ABD-WT reached the limit of detection (LOD) for HSA in urine (LOD = 0.65 µg/ml) sufficient to identify the chronic kidney disease caused by high blood pressure or diabetes. Furthermore, it offers the highest signal intensity, low noise and significant simplification of microchip preparation due to a simple one-step immobilization procedure. Our results may be further exploited in development of diagnostic microchips dedicated to the detection of a wide range of molecular targets recognized by specific ABD protein binders.

1. Introduction

Design of the affinity binder molecules and their surface immobilization is still the crucial issue of successful introduction of new sensors into diagnostic medicine [1]. The field has been dominated by monoclonal antibodies (mAb), which are the most common biorecognizing component of the immunosensors. Despite their usefulness and widespread applicability, mAbs suffer from several drawbacks, including high molecular weight, instability and high production cost [2]. Other kinds of proteins have been tested as backbones for affinity molecules by converting them to a library of PB following randomization of amino acids at the binding surface. From this complex library of PB, a specific binder can be selected using phage, bacterial, ribosome, yeast display or other similar approaches [3]. Such novel strategies have been shown to be effective for production of various PBs with affinities comparable or even better than mAbs. High affinity binders such as ABD, Adnectin, Affibody and Preonectin are based on simple low MW structures and share favourable structural and biochemical properties that support their use in a vast range of applications [1]. Affibodies, PBs derived from the IgG binding domain of protein A (*Staphylococcus aureus),* being also commercially available, have attracted increased attention in diagnostic and therapeutic fields during the past few years [4].

ABD is a small, three-helix bundle domain (46 amino-acid residues) which is a part of protein G (*Streptococcus* G148, PDB ID: 1GJT, residues 20–65). It naturally binds HSA with nM affinity [5]. To date, more than 100 engineered variants with altered specificity, improved affinity or stability and even new binding specificities have been reported [6].

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Their application has been demonstrated in peptide pharmacology [7], nanoparticle labelling [8] and even biosensing [9].

The immobilization of proteins on the surface of solid materials is a key step in the production of protein arrays, biosensors and microfluidic devices. For these applications, it is necessary to arrange the orientation of immobilized proteins as well as their density and surface distribution so that their function will be maintained [10]. Proteins may be immobilized by physical adsorption [11], covalent coupling [10], as a self-assembled monolayer (SAM) [12] or through affinity interactions (e.g., avidin/biotin [10], Ni-NTA (nickel-nitrilotriacetic acid)/His-tagged protein [13–15]). For practical use, the immobilization should be based on the minimized number of steps to enable the scale-up of the device production and to decrease the complexity of sensor surface preparation.

Despite their large application potential, there are only few examples of Affibody use in biosensor fabrication [16,17] and even less in the case of ABD proteins [9]. The question of an effective way of protein binder immobilization with the respect to signal intensity, sensitivity and limit of detection has not been studied in more details. In this study, we target such issues by comparison of the analytical performances of the optical microchips, based on three different recombinant PBs, all recognizing HSA as the target analyte: (i) commercially available anti-HSA Affibody; (ii) 6xHis-WT-ABD-TolA-AviTag construct and (iii) WT-ABD fusion protein with streptavidin (SA-ABD-WT).

2. Materials and methods

2.1. Chemicals and reagents

Chemical reagents obtained from commercial suppliers were used without further purification. More details about the chemicals used are available in SI.

2.2. Design and production of HSA protein binders

Anti-HSA Affibody® Molecule (Biotin) (ab31898) was purchased from Abcam (UK). The *in vivo* biotinylated ABD protein was designed according to Refs. [18,19], produced in the *E. Coli* BL21 (DE3) BirA strain and purified from cell extracts on Ni-NTA agarose columns according to Ref. [20]. The process of designing and construction of the SA-ABDwt protein has been described recently[8]. Detailed description of production of those proteins is available in Supplementary information (SI).

2.3. Fabrication of microchips

The microchip for assay (open glass microchip) was propounded as follows: microchips should contain 6 × 4 microwells (volume 8 µL) with detection zones for biosensing located on their bottom, each zone should consist of 4 × 4 squares, 150 × 150 µm wide with 150 µm distance between the squares. For detailed description of the whole fabrication process, please see SI.

2.4. Fluorescence-based direct assay of labelled HSA

High-affinity PBs based on biotinylated molecules (6xHis-WT-ABD-TolA-AviTag and AF) were attached to the microchip surface via streptavidin − biotin interaction. Streptavidin-fusion based variants (SA-ABD-WT) were attached directly to the biotinylated surface of the microchip via the same chemistry. Based on previous knowledge from optimization experiments, the concentration of proteins immobilized to the microchip surface was 10 µg/ml for streptavidin and SA-ABD-WT and 40 µg/ml for biotinylated proteins (6xHis-WT-ABD-TolA-AviTag and AF); incubation time was one hour at 4 °C. After incubation, the microchips were rinsed several times with water. Then, fluorescently labelled HSA analyte (0–30 µg/ml) in different media (B-PBS or F-PBS buffer) was loaded into the microwells and incubated for one hour at 4 °C. Microchips were then rinsed with water and used for fluorescence measurements (please see SI for more details).

2.5. Competition assay for estimation of HSA concentration

For the competition assay, the immobilization of PBs (AF, 6xHis-WT-ABD-TolA-AviTag, SA-ABD-WT) was performed in the same way as in the previous case. For all PBs, the concentration of fluorescently labelled HSA was initially set to be 10 µg/ml to reach the saturation of the surface; for SA-ABD-WT, the concentration was then decreased to 2 µg/ml. The concentration range of unlabelled HSA was kept the same as in the direct assay (i.e. 0–30 µg/ml). Evaluation of the data was performed in a similar manner as in the previous case.

2.6. Data analysis and statistics

Data are presented as the mean value ± standard deviation (error bars, S.D.). Each experiment was conducted in at least three independent runs. The statistical and *data* analysis was performed with GraphPad Prism 7 (GraphPad Software, Inc, USA) software. The experimental data from dose-response curves were background subtracted and normalized before non-linear regression fitting. A one-site total binding model with linear component characterizing non-specific binding was used for data fitting:

$$Y = Bmax*X/(Kd + X) + NS*X + Background \quad (1)$$

where X = log of analyte concentration, Y = normalized signal response, Kd = equilibrium binding constant, NS is the slope of non-specific binding in Y units divided by X units and Background is the amount of nonspecific binding with no added analyte.

For more details about the calculation of sensitivity, limit of detection (LOD) and limit of quantitation (LOQ) used in this work, please see SI.

3. Results and discussion

3.1. Design and production of protein binders

To develop an affinity assay for HSA detection, specific PB should be immobilized on the detection platform. Here, three different PBs − anti-HSA Affibody (AF), 6xHis-WT-ABD-TolA-AviTag and SA-ABD-WT − were used in biosensor construction and their analytical performance was compared. Fig. 1 illustrates a schematic molecular design of each PB, tested in our experiments. In general, AF comprises one recognition part with a zero-length peptide spacer based on the IgG-binding domain of protein A (58 amino acids); the molecular weight is 14 kDa. On the C-terminus, the AF is chemically biotinylated through the sulfhydryl group present on the terminal cystein. The albumin-binding domain of streptococcal protein G (wild-type G148-GA3, pdb id 1gjt), lacking disulfide bridges and naturally recognizing HSA with high affinity (K_D = 1.2 nM)[5]), was used in the form of an N-terminally polyhistidinylated tag (12 amino acids) fused with the ABD (46 amino acids), a helper TolA helical protein spacer (305 amino acid residues) and C-terminal AVItag sequence consensus (15 amino acids), forming a fusion protein of 378 amino acids. This 38 kD ABD protein has recently been characterized for its stability (melting temperature Tm = 58 °C) and binding specificity [7,18–20].

The third protein (SA-ABDwt) contains a natural core of streptavidin (amino acid residues 13–139 [21]. Its C-terminal part is genetically attached to the ABD via a short linker (Trp leader; Fig. 1**B**) [8]. The SA-ABDwt protein was produced and isolated as a denatured monomeric form (22 kDa) and the functional tetrameric form (88 kDa), which enables high-affinity binding to biotin. The tetramer formation and its stability were verified by SDS PAGE electrophoresis.