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Engineering Camel Single-Domain Antibodies and Immobilization Chemistry for Human Prostate-Specific Antigen Sensing

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The specificity and affinity characteristics of antibodies make them excellent probes in biosensor applications. Unfortunately, their large size, unstable behavior, and random immobilization properties create numerous problems. The single-domain antigen-binding fragment derived from heavy-chain antibodies of camelids (termed VHH) offers special advantages in terms of size, stability, and ease of generating different antibody constructs. In this study, we show the potential of those VHHs in sensing human prostate-specific antigen (hPSA) by SPR technology. Different VHH constructs were immobilized onto commercial and custom-built sensor surfaces by metal chelation, biotin−streptavidin interaction, or covalent coupling. The detection of subnanogram per milliliter hPSA concentrations could be attained on a covalently coupled three-dimensional dextran surface. Moreover, the ratio of different hPSA isoform concentrations could be assessed via a sandwich assay and resulted in the detection of clinically significant antigen concentrations within 15 min. In addition, for the first time, the intrinsic protein stability is presented as an important probe design factor, since our results reveal that higher intrinsic stability offers higher resistance to harsh regeneration conditions. In conclusion, we present VHHs as a novel class of biosensor probes rivaling conventional antibodies and their derived antibody fragments.

Current bioanalytical research aims at ameliorating the detection of important analytes. This raises a growing request for more diverse and better performing probes.[1,2] Antibodies offer in many respects an attractive solution for probe development as they recognize their cognate antigen with high affinity and specificity.[3] Monoclonal antibodies are often preferred over polyclonal serum antibodies for immunosensor developments because of their specificity.[4] Unfortunately, monoclonal antibodies against predefined epitopes are often difficult to retrieve, especially when the antigen is hard to purify, sterically shielded, or less immunogenic. Yet, antibody engineering techniques allow the production of a huge number of recombinant antibodies in bacterial cells and the selection for antibodies that bind to unique or nonimmunogenic epitopes.[5,6] Occasionally, those recombinant antibodies provide an additional source of high-quality detection reagents to identify pathogens in environmental samples.[7] Moreover, to extend the application range or to tailor the antibodies to particular needs, several antibody miniaturization strategies have been introduced. Some of these lead to diverse antigen-binding formats (e.g., scFv, Fab, diabodies), possibly outperforming the intact monoclonal antibodies.[8] One of the areas where antibody engineering might have significant advantages is the directional probe immobilization, since this is important to achieve a maximal analyte binding activity.[9−11] Many approaches used to fabricate biosensors and microarrays fail to immobilize the antibodies at a uniform density or in a single orientation. Consequently, the immobilized antibodies recognize their antigens with variable efficiency and functionality.[12,13] This limitation in probe attachment

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and stability are likely reasons for the fact that only 5% of the commercially available antibodies were found to be suitable for microarray-based analysis.[14]

The variable domain of the heavy chain of the heavy-chain only antibody (VHH), a single-domain antigen-binding fragment derived from heavy-chain antibodies of camelids occupies a particular and undervalued position within these possible formats. This monomeric antibody fragment offers special advantages in terms of expression yield,[15] size, stability,[16] and ease of generating bivalent, bispecific constructs, or immunoconjugates.[17,18] Antigen-specific VHHs regularly target epitopes that are less accessible for conventional antibodies (e.g., enzymatic clefts or phage receptors[19,20]). In addition, their small size permits their coupling to a higher density on the biosensor surfaces, which will ultimately lead to faster and more sensitive analyte detection. Hence, the VHHs create a number of opportunities that might be trickier to accomplish with antigen-binding fragments from classical antibodies.

Switching to VHHs as the probe would circumvent some of the above problems observed with conventional antibody fragments. First, the single-domain character of the VHH could facilitate tagging with single chemical reactive groups for directional coupling. Second, their elevated conformational stability[16] compared to conventional antibody fragments could lead to higher resistance to surface regeneration conditions. Moreover, the recent identification of a scaffold for VHHs that acts as a generic recipient for antigen-binding loop grafts with transfer of the antigen-binding capacity of the loop donor[21] generates probes of nearly identical biochemical and biophysical properties. These chimeric VHHs based on the same scaffold could be more uniformly conjugated to the sensor, which is a significant advantage for multiple probe immobilizations as required for biosensors and protein chip applications.[22] In addition, they often exhibit an increased conformational stability compared to loop-donor VHHs and are therefore expected to be more resistant to regeneration conditions.

In a previous study, we identified several different VHHs, binding to human prostate-specific antigen (hPSA).[23] These high-affinity anti-hPSA VHHs might be suitable probes for detection of hPSA, the most widely used target for early prostate cancer (PCa) screening.[24] In this study, the performance of our VHHs as a hPSA probe was compared with that of conventional monoclonal antibodies and their derived fragments. Different immobilization strategies were evaluated using Surface plasmon resonance (SPR) to detect clinical significant hPSA concentrations. These immobilization schemes comprise the use of dextran-coated surfaces and mixed self-assembled monolayers (SAMs) of poly(ethylene oxide) (PEO) containing thiols in combination with direct or indirect coupling procedures. Furthermore, by detecting several molecular forms of hPSA[25] with engineered VHHs, a more specific PCa diagnostic assay was designed to discriminate between benign or malignant diseases. Finally, the effect of the probe's intrinsic stability on the sensor layer stability was examined.

Abbreviations: ACT, alpha-1-antichymotrypsin; BAD, biotin activation domain; cAb, camel single-domain antibody; EDC, *N*-ethyl-N′-(3-dimethyl aminopropyl)-carbodiimide hydrochloride; Fab, fragment of antibody; Fc, constant part of immunoglobulin; HBS, HEPES buffered saline; hPSA, human prostate-specific antigen; LOD, level of detection; mAb, monoclonal antibody; ND, Not Determined; NHS, N-hydroxysuccinimide; NTA, nitrilotriacetic acid; PBS, phosphate buffered saline; PCa, prostate cancer; RU, Resonance Units; SA, streptavidin; SAM, Self-Assembled Monolayer; PEO, poly(ethylene oxide); scFv, single-chain Fv; SD, standard deviation; SPR, surface plasmon resonance; VH, variable domain of the immunoglobulin heavy-chain; VHH, variable domain of the heavy-chain of the heavy-chain only antibody; VL, variable domain of the immunoglobulin light-chain.

EXPERIMENTAL SECTION

Reagents. Human prostate-specific antigen in free form ($hPSA_{free}$) or complexed to α-1-antichymotrypsin (hPSA−ACT) was obtained from Scipac Ltd. Biotinylated $hPSA_{free}$, mouse monoclonal antibodies (3E6 and 1A7) against human prostate-specific antigen and VL/VH genes of 3E6 were from Epsilon-Biotech, while mouse monoclonal antibodies (PSA10 No. 304 and PSA66 No. 310) were obtained from CanAg, Sweden. The 2-(2-(2-(2-(2-(11-Mercaptoundecyloxy)ethoxy)ethoxy)ethoxy)ethoxy)ethoxy)acetic acid (11-PEO-COOH) was synthesized according to previous publications.[26−28] The synthesis of 2-(2-(2-(11-mercaptoundecyloxy)ethoxy)ethoxy)ethanol (11-PEO) was described by Lahiri et al.[26] The composition was verified using ^{1}H NMR and mass spectroscopy and compared to earlier reports.[26−28]

Antibody Engineering, Expression, Purification, and Affinity Measurement. The cAbPSA-N7, binding both $hPSA_{free}$ and hPSA−ACT, and cAbPSA-N50, specific for $hPSA_{free}$, were previously isolated, cloned into pHEN6, expressed, and purified.[23]

The human IgA-1 hinge (Ser-Pro-Ser-Thr-Pro-Pro-Thr-Pro-Ser-Pro-Ser-Thr-Pro-Pro) and the biotin activation domain (BAD; Gly-Gly-Leu-Asn-Asp-Ile-Phe-Glu-Ala-Gln-Lys-Ile-Glu-Trp-His) were inserted via PCR in the pHEN6 vector at the C-terminal end of the VHH, i.e., between the framework-4 and a His_6-tag. This VHH−BAD construct was transformed into *Escherichia coli* AVB101

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Table 1. Properties of Different Immobilization Strategies[a]

cAbPSA-N7	surface	coupling strategy	coupling density (RU)	coupling resistance	SD (RU)	LOD (ng mL^{-1})	paragraph label
His_6-tagged	NTA	metal chelating	200	nd	0.44	1.4 ± 0.2	a
in vivo biotinylated	SA	biotin−streptavidin	2000	0.1%	0.31	0.31 ± 0.01	b
His_6-tagged	CM5	covalent via primary amines	2000	0.01%	0.20	0.190 ± 0.003	c/d
His_6-tagged	PEO SAM	covalent via primary amines	1800	0.08%	1.4	3.5 ± 0.3	e
in vivo biotinylated	SA PEO SAM	biotin−streptavidin	1200	nd	1.3	2.2 ± 0.1	e

[a] His_6-tagged and in vivo biotinylated cAbPSA-N7 was immobilized onto different sensor surfaces for subsequent detection of $hPSA_{free}$. The coupling density on every sensor surface was recorded in RU. The coupling resistance was defined as the amount of coupled antibody construct lost after each detection cycle compared to the original coupling density depicted in percent (nd, not determined). The standard deviation on buffer injection (SD) was calculated for 10 buffer injections on each sensor surface. Corresponding LOD values were calculated according to Experimental Section.

strain (Avidity). Expression and purification was performed according to Cloutier et al.[29]

The pHEN6 vector encoding a His_6-tag at the C-terminal of the VHH was modified to encode a Strep-tag II (Trp-Ser-His-Pro-Gln-Phe-Glu-Lys) instead of the His_6-tag. An additional construct, harboring two more Lys residues behind the Strep-tag II, was prepared. Both Strep-tag II constructs were expressed and purified on Strep-Tactin resin according to manufacturer's instructions (IBA GmbH).

The cAbPSA-N7 and cAbPSA-N50 were cloned into the pcDNA3.1 vector harboring the gene fragments encoding the hinge, CH2, and CH3 domains (i.e., the Fc part) of human IgG1 and transfected into NSO cells. Subsequent selection of individual clones was performed by ELISA. The supernatant was loaded onto $hPSA_{free}$ captured by the hPSA-specific monoclonal antibody 3E6. Bound VHH−Fc was detected using an anti-human IgG1 alkaline phosphatase conjugate (Sigma). Positive clones were expanded and grown in RPMI medium supplemented with 1% IgG-depleted fetal calf serum. Supernatant containing VHH−Fc was loaded onto a protein-A column, and unbound proteins were removed with 100 mM NaCl, 10 mM Tris-HCl (pH 7.5). Pure VHH−Fc was eluted with 10 mM glycine-HCl (pH 2.5), and dialyzed against phosphate-buffered saline (PBS).

A single-chain Fv fragment (scFv) of the monoclonal antibody 3E6 was constructed. The VL gene was linked to the N-terminus of the VH gene, using a $(Gly_4-Ser)_3$ linker via PCR, and cloned into the pHEN6 vector.[23] Expression and purification was performed similar to His_6-tagged VHH constructs. Kinetic constants of the scFv for $hPSA_{free}$ and hPSA−ACT were measured using SPR according to Saerens et al.[23] Details of antibody constructs can be found in Table S-1 (Supporting Information).

Detection of hPSA in ELISA. Purified proteins were coated on Maxisorp ELISA plates (Nunc) at 20 μg mL^{-1} in PBS overnight at 4 °C. Next, the plates were blocked with PBS + 1% milk (Nestle). Biotinylated $hPSA_{free}$ was diluted from 20 to 0.2 ng mL^{-1} and captured by the coated probe. Bound biotinylated $hPSA_{free}$ was detected via extravidin alkaline phosphatase conjugate (Sigma). The OD at 405 nm was measured 30 min after adding alkaline phosphatase substrate (Sigma).

Surface Plasmon Resonance Instrumentation. The Biacore 3000 SPR instrument operated at a constant temperature of 25 °C was equipped with different sensor chips, i.e., SIA kit, CM5 chip, Ni−NTA chip, and SA chip. The SIA kit contains bare gold substrates that can be covered with in-house surface chemistry. The CM5 chip consists of a carboxymethyl-terminated dextran layer, while the SA chips are covered with streptavidin. The Ni−NTA chip uses the Ni−His coupling to immobilize receptors on the sensor surface. Antibody constructs were immobilized onto flow cell 2 of the chip, while the surface of flow cell 1 was used as a control. Flow data were recorded as the signal in flow cell 2 subtracted with the signal in flow cell 1. In the case of the mixed SAM surfaces, flow cells 1−3 were used for antibody construct immobilization, while flow cell 4 was used as a control. The control lane was coupled with a control antibody. The amount of protein captured on the surface was measured after the injection phase and given in relative resonance units (RU), as run without analyte (i.e., just buffer) was subtracted from each run with analyte. One RU corresponds to a shift in resonance angle of ~0.1 mdeg[30] and 1 pg of protein/mm^2. Recorded data runs were analyzed with the BIAcontrol and BIAevaluation software (Biacore). The quantitative level of detection (LOD) in nanogram per milliliter was calculated as $10 \times SD \times S^{-1}$, whereby SD is the standard deviation of 10 buffer injections (Table 1) and S denotes the slope of the linear regression on the detection data.[31] The error on the LOD value was calculated on the basis of the errors on SD and S values.

VHH Immobilization and hPSA Detection. Immobilization on Ni−NTA biochip was performed according to manufacturer's instructions (Biacore). Otherwise, purified VHHs were covalently coupled onto CM5 chip according to the manufacturer's instructions (Biacore). Alternatively, purified VHH−BAD construct was injected onto the SA chip (Biacore) at a concentration of 25 μg mL^{-1}. Different concentrations of $hPSA_{free}$ spiked in 1% bovine serum albumin were injected for 10 min at 30 μL min^{-1}. The amount of bound $hPSA_{free}$ (expressed as relative RU) was monitored at the end of the injection phase. The Ni−NTA surface was regenerated with a pulse of Ni−NTA regeneration buffer (Biacore). The SA and the CM5 surfaces were regenerated using 10 mM glycine hydrochloride (pH 2.2−2.5).

Gold substrates provided with the SIA kit were cleaned with a homemade UV−O_3 cleaning apparatus containing an ozone-producing mercury grid lamp (BHK Inc.). The UV−O_3 cleaning

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is know to be very adequate in removing organic contamination of gold substrates prior to SAM deposition.[26,27] The realization of mixed SAM surfaces was similar to that described in Frederix et al.[32] The SAM deposition time was varied between 3 min and 24 h. No significant difference concerning their performance in biochemical interaction analyses could be distinguished. Therefore, the deposition time was fixed to 3 h. The mixed PEO SAM surfaces were deposited from a 1 mM thiol−ethanol solution, containing 5% (v/v) 1 mM 11-PEO-COOH/1 mM 11-PEO in ethanol. Antibody constructs were directly immobilized via their primary amines onto these in-house-made PEO SAM surfaces or via an indirect coupling procedure comprising a streptavidin−biotin coupling. A continuous flow of HBS at 5 μL min^{-1} was maintained during the immobilization period. In detail, the carboxylic groups of the mixed SAM surfaces were activated by EDC/NHS according to manufacturer's procedures (Biacore). Next, 132 μL of the probe solution (500 μg mL^{-1} in 10 mM acetate buffer, pH 5.0 for conventional antibody and pH 5.5 for cAb) was injected, followed by injection of 50 μL of 1.0 M ethanolamine in order to block remaining NHS-ester groups and by two 10-μL injections of 10 mM glycine (pH 2.2) in order to remove nonspecifically bound molecules from the surface. A similar protocol was applied for the streptavidin-coated PEO SAM. The 132 μL of streptavidin (500 μg mL^{-1} in 10 mM acetate buffer pH 5.0, Sigma) was injected followed by the ethanolamine and glycine injection. Next, the biotinylated antibody constructs were immobilized by a manual injection of undiluted VHH−BAD construct and 160 μg mL^{-1} biotinylated 1A7 antibody until saturation occurred.

Sandwich Assay Detection. Concentrations of $hPSA_{total}$ were prepared as 90% hPSA−ACT and 10% $hPSA_{free}$ (gold standard of PSA testing[33]). Both cAbPSA-N7 and cAbPSA-N50 were coupled onto the CM5 chip. Different dilutions of $hPSA_{free}$ and $hPSA_{total}$ were injected for 10 min at a flow rate of 30 μL min^{-1}, and subsequent secondary detection antibody was loaded at 10 μg mL^{-1} for 2 min. Bound secondary antibody (expressed in relative RU) was monitored at the end of the injection phase.

RESULTS AND DISCUSSION

hPSA Detection Performance of Different Antibody Formats. Current available hPSA detection assays rely on monoclonal antibodies as primary probes to capture the target from the patients' sample and the subsequent detection of the retained hPSA by a secondary labeled monoclonal antibody.[34] Obviously, the sensitivity and speed of analyte detection in complex samples depends critically on the performance of the primary antibody. We first evaluated the capturing performance of different antibody formats in an ELISA assay. For conventional probes, the monoclonal antibody 3E6 and its scFv derivative constructed by fusing the VL to the VH via a $(Gly_4-Ser)_3$ linker were chosen. Conversely, the probes based on camel single-domain antibodies consisted of the VHH and VHH−Fc construct of cAbPSA-N7, which has already been used successfully in hPSA detection assays.[35,36] The cAbPSA-N7 and the scFv-3E6 were expressed in

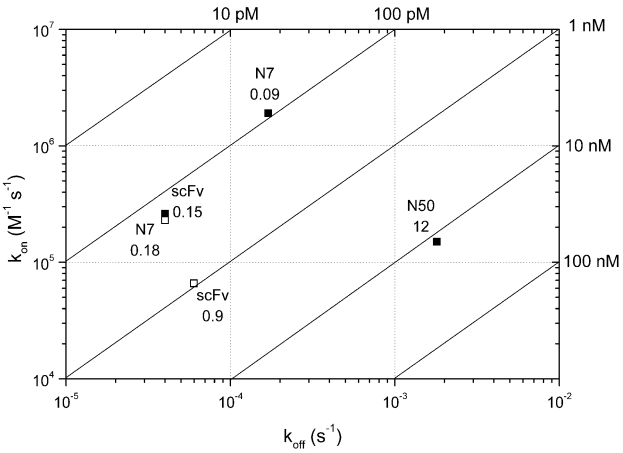

Figure 1. Rate plane with isoaffinity diagonals plot. The kinetic rate values k_{on} and k_{off} were determined by SPR and plotted on a two-dimensional diagram so that data points located on the same diagonal line have identical K_D values. The equilibrium dissociation constant (in 10^{-9} M) for $hPSA_{free}$ (■) and hPSA−ACT (□) is given below the identifier. Values for cAbPSA-N7 (N7) and cAbPSA-N50 (N50) were taken from Saerens et al.,[23] and the affinity of scFv (3E6) was measured according to Saerens et al.[23]

bacteria and had similar equilibrium dissociation constants for $hPSA_{free}$, although their kinetic on rates were 10-fold different, as well as their off rates (Figure 1). The VHH, VHH−Fc (i.e., a heavy-chain antibody format), monoclonal antibody, and scFv-3E6 were adsorbed onto microtiter plates and subsequently used to capture biotinylated $hPSA_{free}$ at a concentration between 0 and 20 ng mL^{-1}, which was detected by the streptavidin conjugate. The $hPSA_{free}$ capturing ability of both the VHH−Fc and the monoclonal antibody are comparable, as well as the capturing ability of VHH and scFv (Figure 2A). However, the classical and heavy-chain antibody formats (i.e., monoclonal and VHH−Fc antibody) surpass their smaller antigen-binding fragments (i.e., scFv and VHH) in $hPSA_{free}$ capturing. Several explanations can be proposed to clarify this observation. A lower accessibility of the paratope from the monomeric fragments compared to that of the VHH−Fc and monoclonal antibody due to the proximity between adsorptive surface and target's epitope could influence the antigen binding.[37] Another explanation can be found in a preferable adsorption of the Fc domain onto the hydrophobic Maxisorp ELISA plates generating an "end-on" orientation of the probes. The Fc domains of IgGs are known to be more hydrophobic than the Fab domains.[38,39] In addition, the heavy chain and conventional antibodies might exhibit higher antigen capturing values due to avidity effects resulting from their bivalency. Indeed, a 10-fold reduction in affinity for a scFv relative to its progenitor monoclonal antibody is frequently observed.[7] Hence, the presence of the Fc part generates better antigen capturing agents in ELISA than smaller, monomeric antibody fragments devoid of the Fc.

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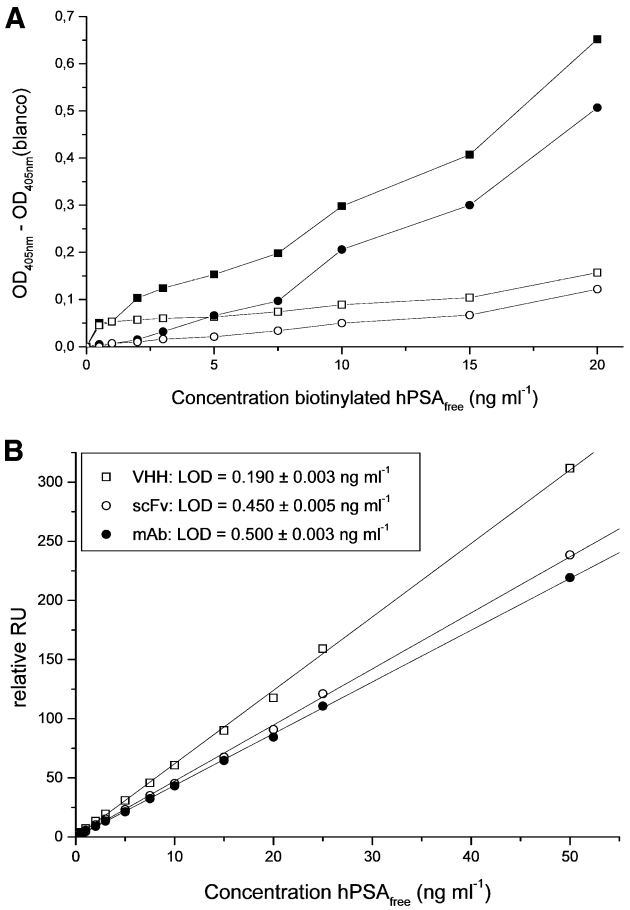

Figure 2. hPSA detection performance of different antibody formats. Purified cAbPSA-N7−Fc (■), monoclonal antibody 3E6 (●), cAbPSA-N7 (□), and scFv-3E6 (○) were coated on an ELISA microwell plates; biotinylated $hPSA_{free}$ was captured and detected by an extravidin alkaline phosphatase conjugate. The OD at 405 nm was measured 30 min after addition of substrate (A). Purified monoclonal antibody 3E6 (●), cAbPSA-N7 (□), and scFv-3E6 (○) were immobilized onto CM5 chip, and different $hPSA_{free}$ concentrations were injected. After injection phase, the bound protein was assessed and corresponding LOD values were calculated (B).

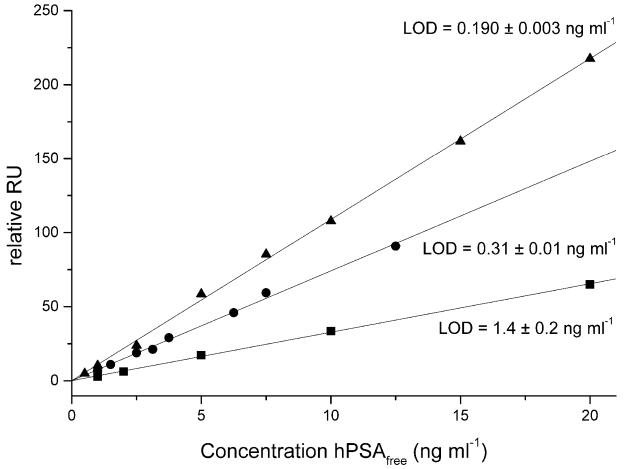

Figure 3. Detection of $hPSA_{free}$ by cAbPSA-N7 immobilized onto different surfaces. The His_6-tagged cAbPSA-N7 was immobilized via His_6-tag onto an Ni−NTA chip (■). The in vivo biotinylated cAbPSA-N7 was loaded on an SA chip (●). The His_6-tagged cAbPSA-N7 was covalently coupled on a CM5 chip (▲). Bound $hPSA_{free}$ protein was assessed 10 min after the start of the injection phase, and corresponding LOD values were calculated.

However, the premises for ELISA diverge from those of biosensor-based assays. The IgG macromolecules might lose their biological activity upon immobilization due to their asymmetrical properties.[40] Therefore, the VHH cAbPSA-N7, scFv-3E6, and monoclonal antibody 3E6 were immobilized on a CM5 surface (Biacore), and their subsequent $hPSA_{free}$ capturing capacity was tested by SPR (Figure 2B). Although similar amounts of VHH, scFv, and monoclonal antibody were immobilized on the surface (between 150 and 200 fmol mm^{-2}), the VHH detects lower $hPSA_{free}$ concentrations than the scFv and monoclonal antibody with LOD values at 0.190 ± 0.003, 0.450 ± 0.005, and 0.500 ± 0.003 ng mL^{-1}, respectively. Since both VHH and scFv have equivalent equilibrium dissociation constants, the VHH appears to be immobilized in a more functional state than the scFv. The scFv might be inappropriately presented upon immobilization due to partial unfolding or improperly packed VH and VL domains.

In summary, the analyte detection sensitivity by large, bivalent antibodies and their smaller, monovalent antigen-binding fragments is highest for the latter probe in biosensors, in contrast to the ELISA-based detection technique. The difference in sensor surface area, inherent assay sensitivity, probe immobilization chemistries, and the functional probe density between ELISA and biosensors are likely at the origin of this reversal.

Sensitivity of $hPSA_{free}$ Detection by VHH Immobilized through Various Strategies. The effect of different VHH immobilization approaches onto distinct biosensor surfaces on the subsequent detection of low $hPSA_{free}$ concentrations was evaluated (Table 1).

(a) Ni−NTA Coupling to His_6-Tagged Antibody Constructs. Recently, Wingren et al.[41] showed that scFv immobilization through its C-terminal tag resulted in very specific and sensitive antibody microarrays. Recombinant VHHs, like our cAbPSA-N7, generally carry a C-terminal His_6-tag for purification. This tag is also adapted for noncovalent immobilization onto Ni−NTA chips. Furthermore, the His_6-tag and the paratope of the VHH, i.e., antigen-binding site, are located at opposite ends of the particle so that Ni−NTA attachment results in an optimally oriented cAbPSA-N7 to capture antigen from the solute. Different concentrations of $hPSA_{free}$ between 1 and 20 ng mL^{-1} flowed at 30 μL min^{-1} over the Ni−NTA biosensor surface that was previously loaded with cAbPSA-N7 to the extent of 200 RU. The amount of $hPSA_{free}$ captured by the cAbPSA-N7 was plotted versus the $hPSA_{free}$ concentration (Figure 3). The detected signal was direct proportional to the analyte concentration in the range up to 20 ng mL^{-1}, and a LOD value of 1.4 ± 0.2 ng mL^{-1} $hPSA_{free}$ was obtained. It was impractical to load larger VHH levels on the Ni−NTA chip as these were washed out progressively during analyte measuring, resulting in a drifting baseline, and leading to difficult interpretation of data. Another drawback encountered with this chelating chip is the complete removal of Ni^{2+} and VHH from the surface during regeneration with EDTA or glycine hydrochlo-

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ride solutions. This implies that new VHH immobilizations are necessary for every detection cycle, rendering this method cumbersome. Clearly, although the Ni−NTA chip is convenient for an initial screening of multiple candidate probes (with a His_6-tag), the sensor surface stability and a high LOD value at low probe loadings prevent its use for optimal prostate cancer diagnosis.[42]

(b) Streptavidin Coupling to Biotinylated Antibody Constructs. Higher probe densities and a more permanent probe attachment could improve the detection sensitivity of $hPSA_{free}$ toward the subnanogram per milliliter concentration range. Hence, alternative probe immobilization methods were introduced. A first approach relies on the strong interaction between biotin and streptavidin. The in vitro biotinylation of proteins occurs at random on the chemical reactive groups (i.e., mostly free amino groups of lysine) spread over the probe surface. Consequently, the streptavidin-captured probe will be fixed in various orientations, of which only a fraction is active for analyte recognition. In contrast, in vivo biotinylation accomplished by the BirA enzyme, which recognizes the BAD sequence, adds one biotin molecule to a specific Lys residue in the BAD sequence.[29,43] The attachment of the BAD tag at the C-terminal end of the VHH, at the antipode of the paratope, ensures a unique probe biotinylation. On streptavidin-coated surfaces, this posttranslationally modified probe will occupy an optimal orientation with its paratope directed toward the solution, as for the His_6-tagged VHH immobilization on the Ni−NTA chip, albeit of higher stability. The biotinylated VHH−BAD construct was expressed, purified, and immobilized on a SA chip (Biacore). About 2000 RU could be immobilized onto the streptavidin surface, which leads to a LOD value of 0.31 ± 0.01 ng mL^{-1} $hPSA_{free}$ (Figure 3). Thus, this 10-fold increase of immobilized cAbPSA-N7 on the SA chip compared to the Ni−NTA chip (Table 1), leads to a nearly 5-fold improvement in antigen detection. Additionally, each regeneration cycle on the SA chip with acid between two measurements removes only 0.1% of the immobilized probe from the surface, another net improvement compared to the Ni−NTA chip (Table 1).

(c) Chemical Coupling to Antibody Fragments. The carbodiimide-mediated covalent cross-links between the VHH and the CM5 chip constituted another effort to reach higher immobilization levels. The covalent bond is formed between the carboxyl groups on the CM5 surface and the free amino groups accessible on the surface of the VHH. The amounts of VHH coupled onto the CM5 chip were similar to those obtained via the biotin−streptavidin coupling onto the SA chip (Table 1). Different concentrations of $hPSA_{free}$ were injected; the captured amount was recorded and plotted versus the concentration (Figure 3). The LOD of 0.190 ± 0.003 ng mL^{-1} for $hPSA_{free}$ was better than the value for the SA chip. The regeneration of the cAbPSA-N7-coupled CM5 chip removed less than 0.01% immobilized protein from the surface per cycle, which is a 10-fold improvement compared to the results obtained with the SA chip (Table 1).

(d) Chemical Coupling at Extra Lys Residues. Obviously the chemical coupling leads to the highest sensitivity (and a more permanent tethering), which might be further enhanced by a more directional probe immobilization. This was attempted by adding extra Lys residues at the C-terminal end of the cAbPSA-N7. In the same effort, we also replaced the His_6-tag by the Strep-tag II, harboring two additional Lys residues (Trp-Ser-His-Pro-Gln-Phe-Glu-Lys-Lys-Lys). The higher density of Lys residues, the reactive groups for covalent coupling to CM5 chips, at the C-end of the VHH was expected to lead to a more uniform orientation of the paratope toward the solute, hence toward more functional VHHs immobilized on the sensor surface. However, the clustering of the Lys residues at the tail increased the pI of the VHH, prohibiting a high-level probe coupling onto the CM5 chip. This suboptimal level of sensor-attached VHH resulted in less favorable LOD values.

(e) Coupling of Antibody Fragments to Self-Assembled Monolayers. The detection sensitivity might benefit from the incorporation of an interface between the sensor surface and the proteinaceous probe. This was shown previously with antibody fusion proteins based on S-layer[36] and cutinase.[44] However, a more flexible setup was proposed by Huang et al.[35] Here a mixed SAM of alkanethiols on a gold substrate was used for the covalent carbodiimide coupling of the cAbPSA-N7 into a homogeneous array. This linkage strategy resulted in 30% active VHHs on the sensor surface and a considerable amount of nonspecific adsorption. Expanding the strategy by a first coupling of streptavidin on a PEO SAM followed by loading with the in vivo biotinylated cAbPSA-N7−BAD construct or the in vitro biotinylated monoclonal antibody 1A7 was now assessed. The comparison of the data is clearly in favor of the VHH−BAD construct as its LOD value surpasses by 15-fold those of the in vitro biotinylated monoclonal antibody 1A7 (Figure 4A). Most likely the large size, random biotinylation, and possible loss of functionality due to biotinylation of the monoclonal antibody 1A7 result in the high LOD value. Apparently, the 10-fold smaller size and site-specific biotinylation lead to a maximally active immobilized probe. In addition, the VHH−BAD construct attached onto the streptavidin detects $hPSA_{free}$ more sensitively than the VHH covalently coupled onto the PEO SAM (Figure 4B). Conversely, the capturing of $hPSA_{free}$ from solution by the monovalent cAbPSA-N7 seems to be slightly more efficient than that by the bivalent, monoclonal antibody 310 when coupled directly onto the PEO SAM.

In summary, the VHH−BAD construct performs better on a two-dimensional PEO SAM equipped with streptavidin, whereas the analyte detection achievements with the His_6-tagged VHH probes occur best via direct chemical coupling on a three-dimensional CM5 dextran chip. This indicates that the positive effects arising from a directional probe attachment critically depend on the actual surface.[37] Moreover, from the different sensor surfaces and immobilization strategies, the CM5 direct chemical coupled biosensor has the additional advantages in that the best LOD values come together with the most resistant sensor surface (Table 1). This experimental setup was used in further assay development to detect low $hPSA_{free}$ and $hPSA_{total}$ concentrations.

Assay Development: $hPSA_{free}$ versus $hPSA_{total}$ Measurements. Measurement of the $hPSA_{total}$ concentration in serum is a well-established diagnostic technique to screen for prostate cancer. Current cutoff values are set at 4 ng mL^{-1} $hPSA_{total}$,[45]

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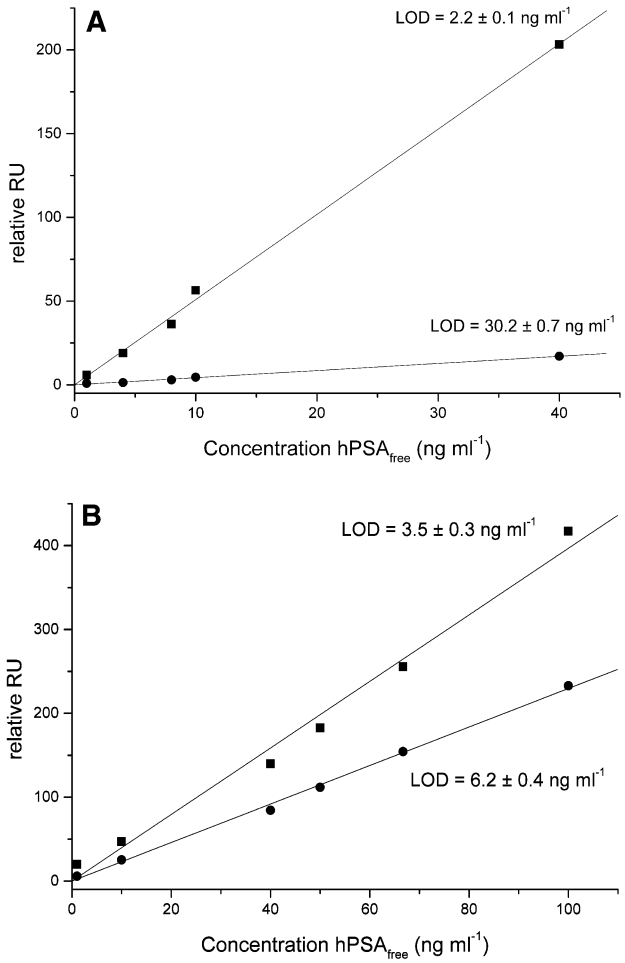

Figure 4. Comparison of $hPSA_{free}$ detection on custom SAM surfaces. The in vivo biotinylated cAbPSA-N7 VHH (■) and in vitro biotinylated monoclonal antibody 1A7 (●) were coupled onto a streptavidin-coated PEO SAM layer. Detection of different $hPSA_{free}$ concentrations was monitored, and corresponding LOD values were calculated (A). Both the His_6-tagged cAbPSA-N7 (■) and monoclonal antibody 310 (●) were immobilized onto a PEO SAM layer, with subsequent detection of different $hPSA_{free}$ concentrations. Corresponding LOD values were calculated (B).

although several studies have argued to lower this cutoff value to 2.5 ng mL^{-1} to improve diagnosis.[46] Moreover, this method to detect prostate cancer based on $hPSA_{total}$ suffers from low specificity, especially in the "gray" zone between 4 and 10 ng mL^{-1} $hPSA_{total}$.[47] About 10−30% of patients with such $hPSA_{total}$ levels have prostate cancer.[48] The hPSA circulates in blood in different isoforms, and $hPSA_{total}$ is in fact a mixture of $hPSA_{free}$ and hPSA in complex with several inhibitors,[49] of which hPSA−ACT predominates. The discrimination between the benign prostatic hyperplasia and prostate cancer is more reliable by determining the ratio of $hPSA_{free}$ to $hPSA_{total}$.[50]

A $hPSA_{total}$ sample was prepared as a 90% hPSA−ACT and 10% $hPSA_{free}$ mixture[33] that mimics the natural ratio. Concentrations $hPSA_{total}$ between 0 and 25 ng mL^{-1} were detected with our cAbPSA-N7 coupled CM5 biosensor (Figure 5B). A satisfactorily LOD value of 0.200 ± 0.002 ng mL^{-1} could be obtained, corroborating the suitability of the cAbPSA-N7 surface to detect clinically relevant $hPSA_{total}$ concentrations.[51]

The cAbPSA-N7 recognizing both $hPSA_{free}$ and hPSA−ACT (hence $hPSA_{total}$) requires the inclusion of an $hPSA_{free}$-specific probe to develop a PCa diagnostic test. This specificity can be provided by the cAbPSA-N50, a VHH with lowest K_D value for $hPSA_{free}$ and which does not interact with hPSA complexed to inhibitors such as α-1-antichymotrypsin.[23] The employment of a biosensor CM5 surface containing the covalently immobilized cAbPSA-N50 to monitor various $hPSA_{free}$ dilutions resulted in a LOD value of 1.80 ± 0.09 ng mL^{-1} $hPSA_{free}$ (Figure 5C). This value is clinically insufficient for hPSA biosensing as $hPSA_{free}$ needs to be scored in the subnanogram per milliliter range.

A sandwich assay constitutes a solution to obtain improved LOD values for the specific $hPSA_{free}$ detection.[35] Evidently, the signal of biosensors depends on the molecular mass of the captured target biomolecules. Therefore, the larger molecular mass of a VHH−Fc construct should generate higher RU values. Two options could be envisaged to design a sensitive $hPSA_{free}$ test involving a sandwich biosensing assay with our VHH probes (Figure 5A).

In the first option, the cAbPSA-N50 coupled to the sensing surface would be used to capture specifically the $hPSA_{free}$ out of the $hPSA_{total}$ pool containing a secondary antibody to increase the mass of the target. The cAbPSA-N7 reconstituted in a heavy-chain antibody or any other hPSA-specific monoclonal antibody binding to a nonoverlapping epitope with that of cAbPSA-N50 is a likely candidate. However, the sandwich step with the cAbPSA-N7 reconstituted in a heavy-chain antibody showed only moderate improvement of $hPSA_{free}$ detection (Figure 5C). This could be explained by the low number of $hPSA_{free}$ molecules captured on the cAbPSA-N50 surface, as the kinetic rate properties might be insufficient for fast capturing of subnanogram per milliliter $hPSA_{free}$ (Figure 1).

In the alternative and preferred option for fast $hPSA_{free}$ quantification in an hPSA mixture, the cAbPSA-N7 coupled surface would first capture the $hPSA_{total}$. In a subsequent step, the $hPSA_{free}$ fraction will be assessed with a target-specific larger antibody, such as the cAbPSA-N50 reconstituted in a heavy-chain antibody. Concentrations of $hPSA_{total}$ between 0 and 80 ng mL^{-1} were injected over the cAbPSA-N7 surface, and subsequently, an $hPSA_{free}$ specific signal was generated by the cAbPSA-N50−Fc construct (Figure 5B). In the clinically significant $hPSA_{free}$ concentration range (below 1 ng mL^{-1}), a satisfactory LOD value of 0.30 ± 0.02 ng mL^{-1} was obtained (Figure 5D).

In summary, these results support the utility of the VHH, cAbPSA-N7 attached to the CM5 chip as a bioprobe to detect clinically relevant concentrations of $hPSA_{total}$ (Table S-2). Moreover, inclusion of the cAbPSA-N50−Fc construct drastically enhances the $hPSA_{free}$-specific detection sensitivity within an

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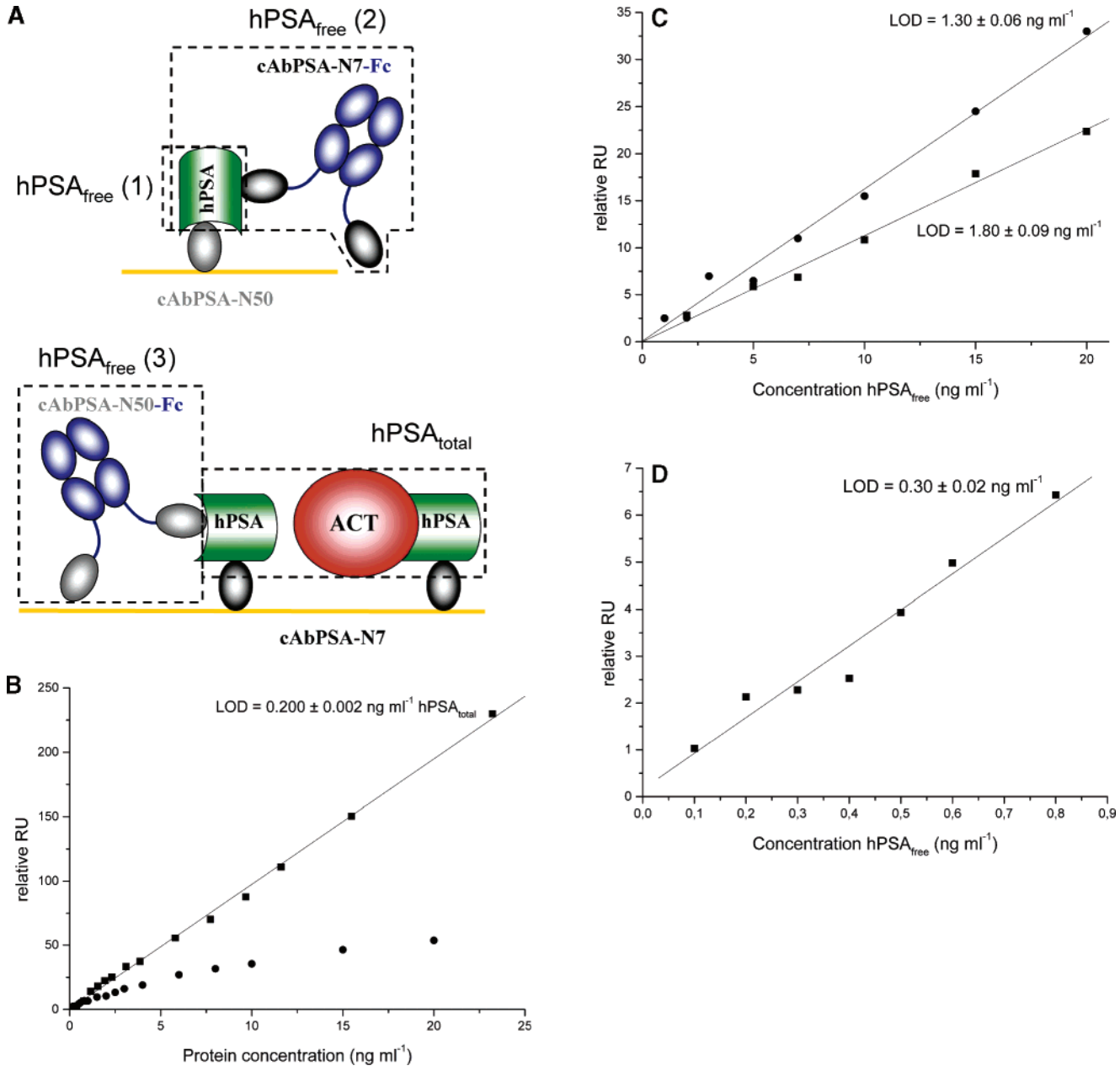

Figure 5. $hPSA_{free}$ versus $hPSA_{total}$ assay development. Sandwich assay detection is depicted in a two-option scheme. On the cAbPSA-N50 surface, $hPSA_{free}$ detection is performed either directly ($hPSA_{free}$ (1)) or enhanced by the cAbPSA-N7−Fc construct ($hPSA_{free}$ (2)). On the cAbPSA-N7 surface, $hPSA_{total}$ concentrations can be detected, with subsequent enhancement of the $hPSA_{free}$ specific signal ($hPSA_{free}$ (3)) by the cAbPSA-N50−Fc construct (A). Different concentrations of $hPSA_{total}$ (90% hPSA−ACT and 10% $hPSA_{free}$) were captured by cAbPSA-N7 coupled on CM5 chip ($hPSA_{total}$ = ■). In addition, the cAbPSA-N50−Fc construct was injected for 2 min at 30 μL min^{-1}. The signal corresponding to the $hPSA_{free}$ within the captured $hPSA_{total}$, i.e., the $hPSA_{free}$-specific signal ($hPSA_{free}$(3) = ●) was plotted against the original $hPSA_{free}$ concentration within the sample (B). Capturing of different $hPSA_{free}$ concentrations was performed on a CM5 chip coupled with cAbPSA-N50 ($hPSA_{free}$(1) = ■). In addition, the cAbPSA-N7−Fc construct was injected for 2 min at 30 μL min^{-1}. The $hPSA_{free}$-specific signal ($hPSA_{free}$(2) = ●) was plotted against the $hPSA_{free}$ concentration, and corresponding LOD values were calculated (C). On the cAbPSA-N7 surface, a linear signal upon cAbPSA-N50−Fc injection below 1 ng mL^{-1} $hPSA_{free}$ was obtained and the corresponding LOD value was calculated (D).

$hPSA_{total}$ mixture. This sandwich assay manages, within 15 min, to determine the $hPSA_{total}$ concentration and to verify the $hPSA_{free}$ to $hPSA_{total}$ ratio.

Regeneration Resistance Engineering. Multiple regenerations and re-usage of the probe is often a necessity to arrive at an economically practical biosensor. Recently, it was shown that increased stability of the probe after protein engineering resulted in a net reduction of irreversible and nonspecific protein adsorption.[52] Hence, the intrinsic stability of the proteinaceous probe has an important influence on adsorption phenomena and release from the sensor surfaces. As explained above, covalent coupling of the VHH onto the sensor surface results in the most permanent fixation of the bioprobe. However, the intrinsic stability of the probe itself is also an important player in the regeneration of a stable sensor. Recently, we noticed that the cAbBCII10-N-N-N (i.e., a stable VHH scaffold with grafted antigen-binding loops of

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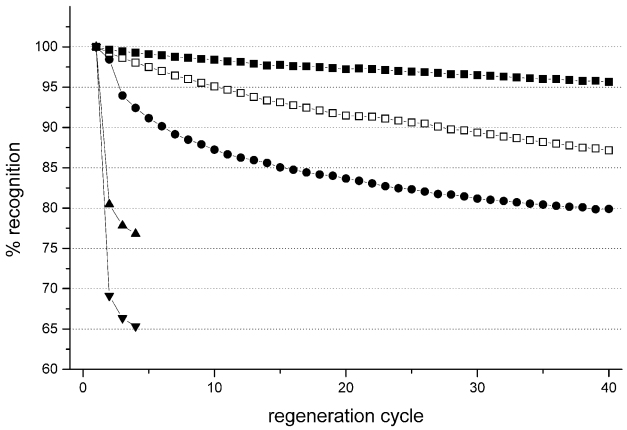

Figure 6. Regeneration resistance of different antibody constructs. The cAbPSA-N7 (□), cAbBCII10-N-N-N (■×ad) and monoclonal antibodies (310, ●; 304, ▼; and 1A7, ▲) were coupled onto PEO SAM surface and subjected to 40 cycles of 1 μg mL^{-1} hPSA$_{free}$ detection and regeneration with 10 mM glycine hydrochloride (pH 2.5).

cAbPSA-N7) was 18 kJ mol^{-1} more stable than the wild-type cAbPSA-N7, while the equilibrium dissociation constants for hPSA differed only marginal.[21] Both, the parental cAbPSA-N7 and the cAbBCII10-N-N-N graft were coupled onto the PEO SAM surface and subjected to 40 detection and regeneration cycles (Figure 6). The more stable cAbBCII10-N-N-N was revealed to be 10-fold more resistant to regeneration cycles with 10 mM glycine hydrochloride. While the cAbBCII10-N-N-N lost only 0.025% per regeneration cycle, the cAbPSA-N7 lost 0.225% of its target binding capacity. This might reflect a more explicit reversibility of VHH folding from the acid-denatured state.[53]

Many applications with antibody fragments in the cosmetic, chemical, or environmental sectors will depend critically on the retention of the proper antigen-binding properties in adverse environments, e.g., polar or nonpolar solvents, surfactants, proteases, etc. Classical antibody fragments are prone to denaturation and degradation under these circumstances, which render them less adequate for operating technologies.[7] This was witnessed with anti-hPSA monoclonal antibodies 304, 310, and 1A7 coupled onto the PEO SAM and subjected to sequential antigen-binding and regeneration cycles. Whereas the monoclonal antibody 310 lost 2% of its antigen-binding capacity per cycle, the monoclonal antibodies 304 and 1A7 had already lost 35 and 23%, respectively, of the original binding activity after only five regeneration cycles. This concurs with a documented activity loss of 40% upon antibody modification.[54] Recent reports suggest that heavy-chain antibody-derived fragments can overcome these limitations. It has been shown that VHHs retain their full antigen-binding capacity when exposed to elevated temperatures or to hostile surfactants.[53,55] The observed robustness of our biosensor coupled VHH upon multiple exposures to regeneration adds another example of VHH advantages to the long list.

CONCLUSION

Recently, several studies exploited genetic engineering techniques to adapt antibodies either by fusion to a carrier protein[44] or by introducing cysteine residues[40,56] with the aim to improve their immobilization yield onto biosensor surfaces. Here we instigate VHHs, the strict monomeric antigen-binding entities of the heavy-chain IgGs found exclusively in *Camelidae*. Robust VHHs with high antigen affinity and specificity can be obtained readily against a myriad of targets. The cAbPSA-N7, a VHH against hPSA, was extended with a His$_6$-tag, a BAD-tag, or extra Lys residues at its C-end to achieve highest possible density and uniformity after direct immobilization on Ni−NTA, SA chips, or after chemical tethering to CM5 or SAM chips. The benefits and limitations of all these strategies to detect antigen with a VHH biosensor were evaluated. It was shown that VHHs outperform the classical antibodies (and their scFv derivatives) in the detection of clinically significant concentrations of hPSA (below 1 ng mL^{-1}) within 15 min. The VHH could also be enlarged by the Fc part of an immunoglobulin to generate bivalent antigen binders with higher molecular mass that detects analyte with still higher sensitivity in biosensors. In addition, the VHHs with an elevated intrinsic stability resist repeatedly the harsh regeneration conditions, where monoclonal antibodies rapidly lose their activity. In conjunction, the VHHs combine all favorable properties to develop into a versatile, valuable, and innovative tool with great opportunities in diagnostic biosensor applications.

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SUPPORTING INFORMATION AVAILABLE

Table S-1 (containing the details of the antibodies and antibody constructs) and Table S-2 (summarizing the LOD values for assay development). This material is available free of charge via the Internet at http://pubs.acs.org.

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