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Reversible Biofunctionalization of Surfaces with a Switchable Mutant of Avidin

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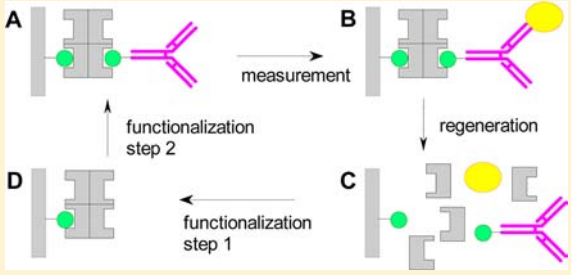
Supporting Information

ABSTRACT: Label-free biosensors detect binding of prey molecules ("analytes") to immobile bait molecules on the sensing surface. Numerous methods are available for immobilization of bait molecules. A convenient option is binding of biotinylated bait molecules to streptavidin-functionalized surfaces, or to biotinylated surfaces via biotin−avidin−biotin bridges. The goal of this study was to find a rapid method for reversible immobilization of biotinylated bait molecules on biotinylated sensor chips. The task was to establish a biotin−avidin−biotin bridge which was easily cleaved when desired, yet perfectly stable under a wide range of measurement conditions. The problem was solved with the avidin mutant M96H which contains extra histidine residues at the subunit−subunit interfaces. This mutant was bound to a mixed self-assembled monolayer (SAM) containing biotin residues on 20% of the oligo(ethylene glycol)-terminated SAM components. Various biotinylated bait molecules were bound on top of the immobilized avidin mutant. The biotin−avidin−biotin bridge was stable at pH ≥3, and it was insensitive to sodium dodecyl sulfate (SDS) at neutral pH. Only the combination of citric acid (2.5%, pH 2) and SDS (0.25%) caused instantaneous cleavage of the biotin−avidin−biotin bridge. As a consequence, the biotinylated bait molecules could be immobilized and removed as often as desired, the only limit being the time span for reproducible chip function when kept in buffer (2−3 weeks at 25 °C). As expected, the high isolectric pH (p*I*) of the avidin mutant caused nonspecific adsorption of proteins. This problem was solved by acetylation of avidin (to p*I* < 5), or by optimization of SAM formation and passivation with biotin-BSA and BSA.



INTRODUCTION

Affinity biosensors are prepared by immobilization of specific bait molecules (nucleic acids, proteins, carbohydrates, etc.) on the surfaces of suitable transducers.[1] Label-free biosensors have the additional requirement that the sensor surface must be resistant to nonspecific adsorption.[1] This is achieved either by dense coverage of the sensor surface with bait molecules[2] or by coating of the sensor surface with a "protein-resistant" monolayer of short or long polymer chains[3−5] whereby the bait molecules are attached to the inert polymer chains.

Conventionally, the bait molecules are immobilized via covalent bonds, e.g., between the amino groups of proteins and aldehyde or carboxyl groups on the sensor surface. The reason for the popularity of these methods is the high abundance of amino groups on most proteins (exemplified by 80−90 lysine residues per antibody molecule[6]). Covalent coupling of amines works well on flat chip surfaces which are densely covered with amino-reactive groups.[2,7] On protein-resistant surfaces, however, covalent coupling is retarded by steric repulsion between the protein-resistant polymer layer and the bait molecules. As a consequence, aldehydes are inapplicable on protein-resistant sensor surfaces[8] and activated carboxyls can only be used with

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special precautions.[9,10] Similar problems as with proteins were encountered when trying to immobilize oligonucleotides with aminohexyl groups on chip surfaces with activated COOH groups.[11]

A widely used alternative to covalent coupling is immobilization of biotinylated bait molecules on preimmobilized (strept)avidin as shown in Figure 1. Immobilization of (strept)avidin

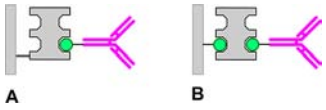

Figure 1. Two alternative strategies of chip functionalization with biotinylated bait molecules. (A) Direct attachment of (strept)avidin to the chip surface. (B) Formation of a biotin–avidin–biotin bridge.

can be achieved by covalent bonds to the chip surface (Figure 1A) or by attachment to surface-bound biotin groups (Figure 1B). The latter scheme is based on the fact that the four biotin-binding sites in (strept)avidin are grouped in two pairs on opposite sides of the streptavidin tetramer.[12] Consequently, (strept)avidin can be used as a mediator between the biotinylated sensor surface and biotinylated bait molecules.[13]

The advantages of biotinylated bait molecules are numerous: (i) Due to the robustness of streptavidin, sensor surfaces prefunctionalized with streptavidin are commercially available. (ii) Most bait molecules are commercially available with a biotin label or can be biotinylated on the time scale of an hour. (iii) No chemical skill is required for immobilization of a biotinylated bait molecule on a streptavidin surface. (iv) Due to the high affinity and fast association kinetics between streptavidin and biotin,[12,14] low concentrations (<1 μM) of bait molecules are sufficient for dense functionalization of streptavidin surfaces on a short time scale. (v) Streptavidin chips proved ideal for immobilization of single-stranded DNA (ssDNA) or double-stranded DNA (dsDNA) at the optimal density for hybridization or for interaction studies with DNA-binding proteins, respectively.[11,15] (vi) The streptavidin–biotin bond is insensitive to short pulses (30 s) of 10 mM HCl or NaOH, as frequently used to regenerate the biosensor after binding of prey molecules to the immobile bait molecules. (vii) The streptavidin–biotin bond is stable on the time scale of days at/near neutral pH; therefore, the chip can be reused for many measurements.

The high stability of the streptavidin–biotin bond has its downside also. At the end of a measurement series it would often be desirable to cleave the streptavidin–biotin bond for subsequent functionalization of the same chip with a new biotinylated bait molecule. The motivation for exchange of the biotinylated bait is as follows: (i) It eliminates human intervention for chip exchange and concomitant interruption of the work flow. (ii) It eliminates the deviation between different sensor chips, which is a problem of some product lines. (iii) It allows for extended use of valuable sensor chips which contain complex instrumentation or complex nanostructures.[16,17] (iv) The most urgent need for in situ exchange of bait molecules is encountered in those cases when the prey molecule cannot be removed from the biotinylated bait molecule without denaturing the latter. In such a case it is necessary to discard the whole sensor chip after each single measurement.

A viable solution to these problems has been found by inserting a DNA double helix between a dextran-coated chip surface and streptavidin. For this purpose, both the chip surface and streptavidin have been derivatized by attachment of single-stranded DNA (Biotin CAPture Kit, GE Healthcare product no. 28920233, GE data file 28–9577–47 AA, available at https://www.gelifesciences.com/gehcls_images/GELS/Related%20Content/Files/1314787424814/litdoc28957747AA1_20110831132219.pdf). The two ssDNA molecules have a complementary sequence (undisclosed, possibly poly dT and poly dA)[18] which allows for stable immobilization of streptavidin by double helix formation. Subsequently, a biotinylated bait molecule is attached for a series of measurements with cognate prey molecules. The series is ended by injection of 100 mM HCl which dissociates the double helix whereupon new modified streptavidin molecules are immobilized for a new series of measurements. The drawbacks of this method are that it is not applicable to the analysis of DNA-binding proteins and that it is only available for the surface plasmon resonance (SPR) biosensors of one company.

An alternative strategy for reversible anchorage of streptavidin on surfaces relies on a biotinylated phospholipid monolayer (Figure 2A). Such monolayers are formed if a hydrophobic self-assembled monolayer (SAM) is incubated with lipid vesicles.[19,20] Lipid monolayers can be used to present specific lipid components for binding of bacterial toxins or blood-clotting factors.[21,22] After completion of a measurement

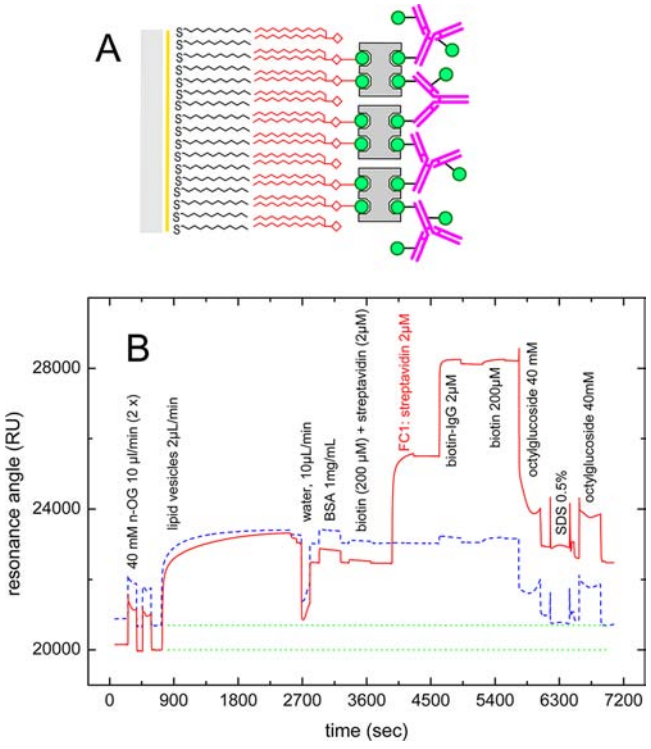

Figure 2. Test for reversible HPA chip functionalization with a phospholipid monolayer, streptavidin, and biotinylated IgG. In both flow cells, a lipid monolayer was formed by slow injection of sonicated lipid vesicles (2 mg/mL, 90% DOPC and 10% biotin-cap-DOPE). In FC1 (solid red line), streptavidin was bound to the biotin residues of the lipid. All subsequent injections were simultaneously applied to both flow cells: biotin-IgG (2 μM), free D-biotin (200 μM), octyl glucoside (40 mM), SDS (0.5%), and again octyl glucoside (40 mM). PBS 7.3 was used as running buffer and for preparation of the lipid vesicles. Unless stated otherwise, the flow was 20 μL/min.

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series, the lipid monolayer is removed with 40 mM *n*-octylglucoside or 20 mM CHAPS (http://www.sprpages.nl/Index.php), thereby regenerating the hydrophobic SAM on which a new lipid monolayer can be formed.[23] Monolayers with 10−50% biotinylated lipid have been used for immobilization of streptavidin and biotinylated bait molecules.[24−26] One of these studies reports on repeated functionalization of the identical chip with different biotinylated peptides.[24] It was not clear, however, whether this method is also applicable to statistically biotinylated proteins; therefore, this important question was examined in the present study (see Results section).

Reversible immobilization of (strept)avidin on biotinylated surfaces (Figure 1B) can also be achieved by derivatives of biotin which exhibit a reduced (or pH-dependent) affinity for (strept)avidin. Three derivatives of biotin have been reported to bind (strept)avidin with lower affinity than biotin itself: iminobiotin,[27−29] desthiobiotin,[30−32] and N_3-ethylbiotin.[33] Iminobiotin binds well at pH 11,[27] whereas at neutral pH its rapid dissociation from (strept)avidin[34] prohibits its use for immobilization of bait molecules on sensor surfaces. Unstable binding at neutral pH is also seen with N_3-ethylbiotin.[33] The opposite problem is encountered with desthiobiotin. Due to its high affinity, complete dissociation of streptavidin from a desthiobiotinylated sensor chip takes ∼1 day,[31] and imperfect chip regeneration is seen on a shorter time scale.[30] The same problems are expected if the bait molecules carry modified biotin residues. Moreover, a wealth of biotinylated bait molecules is commercially available, while none are available with iminobiotin, desthiobiotin, or N_3-ethylbiotin as labels. In conclusion, biotin derivatives are useful for affinity chromatography[27−30,32−36] but not for reversible biosensor functionalization.

The obvious alternative to modified biotin is the use of modified (strept)avidin with reduced or switchable affinity. In this respect, the design shown in Figure 1A has widely been used for affinity chromatography (or affinity capture on latex beads). The first version was "monomeric avidin".[37−39] The method has been improved by a double mutant of streptavidin which is also monomeric but exhibits less leaching of biotinylated ligands.[35] Reduced affinity for biotinylated target molecules was also achieved by nitration of a tyrosine residue in avidin ("captavidin") and this protein has largely been used for affinity chromatography.[40,41] Recently an attempt has been published in which captavidin was irreversibly attached to the sensor chip (as in Figure 1A) and the pH was switched between pH 7.4 and pH 10 to achieve specific binding to, or efficient release of biotinylated proteins from, the immobilized captavidin.[42] Unfortunately, the reproducibility of the on/off switch was insufficient for practical application in biosensing. As an alternative, streptavidin has been derivatized with "smart polymers" next to the entrance of each biotin-binding site,[43] thereby introducing a pH- and temperature-dependent switch. This would mean that all measurements have to be performed at 4 °C and the sensor must be heated to 37 °C in order to remove the biotinylated bait molecule from the sensor surface. Such temperature cycling, however, is incompatible with label-free biosensors which must be operated at a very constant temperature. Moreover, the switch is not responding with 100% efficiency. In conclusion, the use of modified (strept)avidin as depicted in Figure 1A is suitable for affinity chromatography but not for reversible biosensor functionalization.

In biosensing, the strategy in Figure 1B has a big advantage over that in Figure 1A if switchable variants of (strept)avidin are to be employed: In the case of the biotin−avidin−biotin bridge it is not a problem if the (strept)avidin molecules are irreversibly destroyed during "rigorous regeneration" of the chip surface because they are completely removed anyway (see Figure 3). With this in mind, we screened the literature for a good candidate among the published mutants of (strept)avidin. Proper pH dependence of protein stability and of biotin binding was the most interesting parameter. Among a list of pH-dependent avidin mutants,[44] the single mutant M96H of avidin looked most promising because it shows high stability at neutral pH, while a severe drop in pH is required to abolish biotin binding and to dissociate the native tetramer into nonfunctional monomers. As shown in the present study, avidin M96H is a valuable tool for implementation of reversible chip functionalization (Figure 3) if the nonspecific adsorption of proteins toward avidin is suppressed by proper precautions.

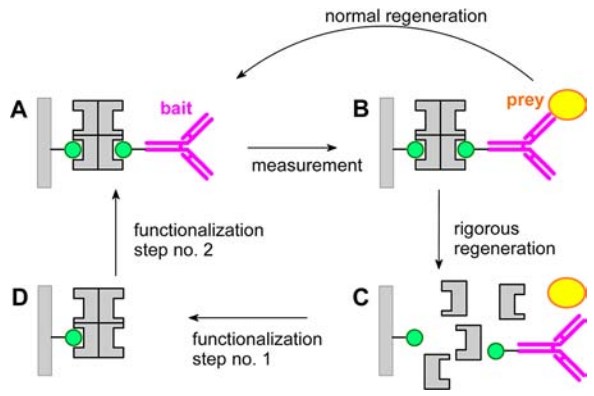

Figure 3. Reversible chip functionalization by denaturation of mutated avidin. (A) A biotinylated chip with bound avidin and biotinylated bait is ready for the measurement. (B) Prey molecules are bound during the measurement. If possible, the prey molecules are removed by "normal regeneration" after the measurement. (C) Rigorous regeneration of the sensing surface is achieved by denaturation of avidin. (D) A new cycle of chip functionalization starts with binding of mutated avidin.

EXPERIMENTAL PROCEDURES

Materials. Captavidin was purchased from Invitrogen. Bovine serum albumin (BSA, fatty acid-free) was obtained from Roche Applied Science. BSA with covalently attached hexahistidine was prepared as described.[45] The avidin mutant M96H[44] was constructed for bacterial expression in *E. coli* by introducing mutation M96H to OmpA chicken avidin in pET101/D (Hytönen, V. P., Laitinen, O. H., Airenne, T. T., Kidron, H., Meltola, N. J., Porkka, E. J., Hörhä, J., Paldanius, T., Määttä, J. A., Nordlund, H. R., Johnson, M. S., Salminen, T. A., Airenne, K. J., Ylä-Herttuala, S., and Kulomaa, M. S. (2004) Efficient production of active chicken avidin using a bacterial signal peptide in Escherichia coli. *Biochem. J. 384*, (Part 2), 385−390, Määttä, J. A., Eisenberg-Domovich, Y., Nordlund, H. R., Hayouka, R., Kulomaa, M. S., Livnah, O., and Hytönen, V. P. (2011) Chimeric avidin shows stability against harsh chemical conditions−biochemical analysis and 3D structure. *Biotechnol. Bioeng. 108*, 481−490), by QuikChange mutagenesis according to manufacturer's instructions (Stratagene, La Jolla, CA, USA). The protein was produced and purified as described

in (Määttä, J. A., Eisenberg-Domovich, Y., Nordlund, H. R., Hayouka, R., Kulomaa, M. S., Livnah, O., and Hytönen, V. P. (2011) Chimeric avidin shows stability against harsh chemical conditions—biochemical analysis and 3D structure. *Biotechnol. Bioeng. 108*, 481—490). Maltose-binding protein (MBP) with a C-terminal His_6 tag (MBP-His_6) was the kind gift of Jacob Piehler, University of Osnabrück, Germany). Anti-MBP antibody, avidin, biotin-protein G, D-biotin, lysozyme, immunoglobulin G (IgG) from goat, and streptavidin were purchased from Sigma-Aldrich. Sulfo-NHS acetate and sulfo-NHS-SS-biotin were obtained from Pierce. The IgG-binding peptide acetyl-HWRGWVC-NH_2 (>95% purity) was custom-synthesized by Peptide 2.0 (www.peptide2.com). The conjugate of this peptide and biotin, with a poly(ethylene glycol) linker (PEG) in between (termed HWRGWVC-PEG_{18}-biotin), as well as the components of the mixed self-assembled monolayer (SAM) shown in Figure 4, were synthesized as described in the

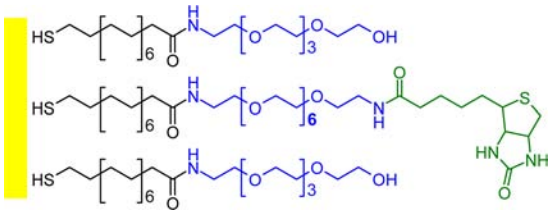

Figure 4. Composition of the mixed SAM used in this study. Two kinds of 16-mercaptohexadecanoic acid derivatives were mixed at the ratio 80/20 (mol/mol). The "matrix alkane thiol" (MAT) carried an OH-terminated penta(ethylene glycol) chain which provided for high protein resistance[51] and the "biotin-terminated alkane thiol" (BAT) contained a longer poly(ethylene glycol) chain ($n = 6$) with a terminal biotin, as required for optimal binding of streptavidin.[52]

Supporting Information. Biotin-tris-NTA was prepared as described.[46,47] Biotin-cap-NHS, biotin-IgG, and biotin-S-S-lysozyme were prepared as published.[48,49] 1,2-Dioleoylphosphatidylcholine (DOPC) and *N*-[6-(biotinamido)hexanoyl]-1,2-dioleoylphosphatidylethanolamine (biotin-cap-DOPE) were obtained from Avanti Polar Lipids. Sonicated vesicles with the desired molar ratio of these phospholipids (90/10) were prepared as described.[50]

Buffers. Phosphate-buffered saline (PBS 7.3) contained 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4. Buffer 11.0 was prepared from PBS 7.3 by first adjusting pH 10.0 with Na_2CO_3 (7.8 mg/mL, same refractory index as PBS 7.3) and then to pH 11.0 with NaOH (6.1 mg/mL, same refractory index as PBS 7.3). HEPES-buffered saline (HBS) contained 150 mM NaCl and 10 mM HEPES, adjusted to pH 7.4 with NaOH. Immediately before the corresponding BIAcore experiments, this buffer was supplemented with a final concentration of 10 μM $NiSO_4$ and sterile-filtered (0.22 μm) with strong aspirator suction to degas the buffer. The same procedure was applied to PBS 7.3 before BIAcore experiments, except that $NiSO_4$ was omitted.

Surface Plasmon Resonance Experiments. Unless stated otherwise in the figure legend, cleaning of bare glass chips, evaporation of chromium (3 nm) and gold (41 nm), as well as cleaning of the gold surface and coating with a mixed SAM of the alkanethiol derivatives shown in Figure 4 was performed as described,[51] using a total thiol concentration of 50 μM. However, the SAM components were different from those in the cited publication. Most SAMs were formed with the OH-terminated matrix component and the biotin-terminated anchor component, the syntheses of which are described in the Supporting Information. SAMs of 1-octadecanethiol were formed in the same way, except that ethanol (analytical grade) was used in place of acetonitrile. This chip is equivalent to the commercial HPA chip (BIAcore). The chips were mounted on the chip holders with double-sided adhesive tape (nonpermanent) and inserted in a BIAcore X device for measurement of binding by SPR. Degassed buffer (PBS 7.3 or HBS) was constantly run over the chip surface, typically at 10—20 μL/min, as stated in the figure legends. Unless stated otherwise, all injection volumes were 100 μL.

RESULTS AND DISCUSSION

Reversible Immobilization of Streptavidin on Monolayers of Biotinylated Phospholipid. In a preceding study, the scheme in Figure 2A was successfully used for reversible immobilization of peptides which had a single biotin residue on their C-terminus.[24] Here the biotinylated peptide was replaced by IgG carrying ~5 biotin labels on average.[48] The results are shown in Figure 2B. First, the monolayer of gold-bound octadecyl chains was washed by two short injections of octyl glucoside. Second, a lipid monolayer was formed during slow injection of lipid vesicles containing 10% of biotinylated lipid. Third, water was applied to dissociate the adsorbed lipid vesicles. Fourth, BSA was injected to cover empty patches void of lipid, but evidently there were none. The transient rise of the resonance angle during the BSA injection was due to the high concentration of BSA in solution, but after the injection there was no net change of the resonance angle. Absence of binding was also seen when streptavidin (2 μM) was blocked with a large excess of biotin (200 μM) before the injection (FC2, blue dashed trace). In contrast, extensive binding was seen in flow cell 1 (FC1, red solid trace) when streptavidin was injected in the absence of biotin. Subsequently, biotinylated IgG was applied in both flow cells, leading to pronounced binding on top of streptavidin (FC1, red solid trace) and no binding on top of the phospholipid monolayer (FC2, blue dashed trace). The protein double layer in FC1 (as illustrated in Figure 2A) was completely resistant to free biotin (200 μM). Octyl glucoside was able to remove all of the lipid monolayer in FC2 (blue dashed trace) but only part of the material in FC1 (red solid trace). Further injections of SDS and octyl glucoside did not cause much improvement either.

The partial resistance of the protein double layer in FC1 to detergent was interpreted by formation of patches where biotinylated IgG was bound to several streptavidin molecules and the latter was simultaneously connected to two adjacent IgG molecules (as illustrated in Figure 2A). This model predicts that complete chip regeneration with detergent should be achieved if molecules with a single biotin group bind on top of streptavidin, as actually observed for biotinylated peptides.[24]

Switchable Binding of the Avidin Mutant M96H to Biotin Residues on a Sensor Chip. The above results indicate that reversible immobilization of (strept)avidin alone does not solve the problem of reversible chip functionalization with biotinylated bait molecules. At the same time it is necessary to prevent the formation of large aggregates of (strept)avidin and biotinylated bait molecules (see Figure 2), or to find an efficient way for the dispersion of such aggregates. As depicted in Figure 3, denaturation of (strept)avidin appears ideal for the latter strategy. Denaturation is accompanied by dissociation of (strept)avidin into its four subunits;[12] therefore,

clusters of (strept)avidin and biotinylated proteins (as in Figure 2) are dissociated into soluble proteins which can be washed away.

We first tested this strategy with wild-type streptavidin on a mixed SAM on gold (see Figure 4). The long 16-mercaptohexadecanoic acid linker and was chosen because it provides for long-term stability of the SAM, and the penta(ethylene glycol) chain on the matrix molecule was selected for minimization of nonspecific protein adsorption.[51] The difference in length between the OH-terminated matrix alkanethiol (MAT) and the biotin-terminated alkanethiol (BAT) was adjusted for optimal binding[52] and the molar ratio (80:20) for particularly slow dissociation of streptavidin.[52] We tried to find a denaturant which was compatible with the microfluidic flow cell of the BIAcore instrument and at the same time strong enough to remove streptavidin at 25 °C within several minutes. Unfortunately, no such reagent could be found.

The next step was a literature search for a derivative or a mutant of (strept)avidin with distinct switching behavior: Binding of biotin was desired to be strong over a wide range of measurement conditions (e.g., 4 < pH < 10). At the same time the protein was to be easily denatured with a buffer that was compatible with a microfluidic flow cell. No derivative/mutant of streptavidin was found that meets these criteria. In contrast, a number of pH-sensitive avidin mutants are known, in which hydrophobic amino acids at the subunit interfaces are replaced by histidine residues.[44] Protonation of these histidines at low pH causes subunit dissociation and denaturation.[44]

From a condensed overview of the literature data (see Table S1 in the Supporting Information) it was concluded that the avidin mutant M96H was the most promising candidate for successful implementation of the scheme in Figure 3. The first test of avidin M96H is shown in Figure 5A. After a short wash with SDS, the mixed SAM did not adsorb any BSA, indicating perfect protein resistance. The avidin mutant M96H, however, was instantaneously bound to the biotin-terminated SAM. At the end of the avidin injection, the resonance angle shows a sudden jump to a higher value. This was caused by the fact that the buffer salt concentration of the avidin sample was slightly lower than of the running buffer. The level of avidin binding (determined after the end of the avidin injection) was 2300 RU, which is similar to the "thickness" of a streptavidin monolayer.[7] The subsequent injection of citric acid (1%, pH 2.2) removed only a small fraction of bound avidin M96H. In contrast, a 1:1 mixture of 0.5% SDS and 5% citric acid (final concentrations 0.25% SDS and 2.5% citric acid, pH 2.0) caused almost instantaneous removal of avidin M96H from the biotinylated chip surface. This can be seen from the short spike at the beginning of the corresponding injection. The spike reflects two processes with opposite effects upon the resonance angle: rapid replacement of running buffer (PBS 7.3) by SDS/citric acid which has a higher bulk refractory index, and rapid dissociation of avidin M96H.

After the SDS/citric acid injection, the resonance angle was the same as before binding of avidin M96H (see green dotted line). The same observation was made in two further cycles of binding and subsequent desorption by SDS/citric acid. Interestingly, the application of SDS alone (0.25%) had a minor effect on chip-bound avidin M96H, as shown in the second cycle of avidin binding and desorption (Figure 5A).

In conclusion, a 1:1 mixture (v/v) of 0.5% SDS and 5% citric acid is the optimal reagent for rapid dissociation of avidin

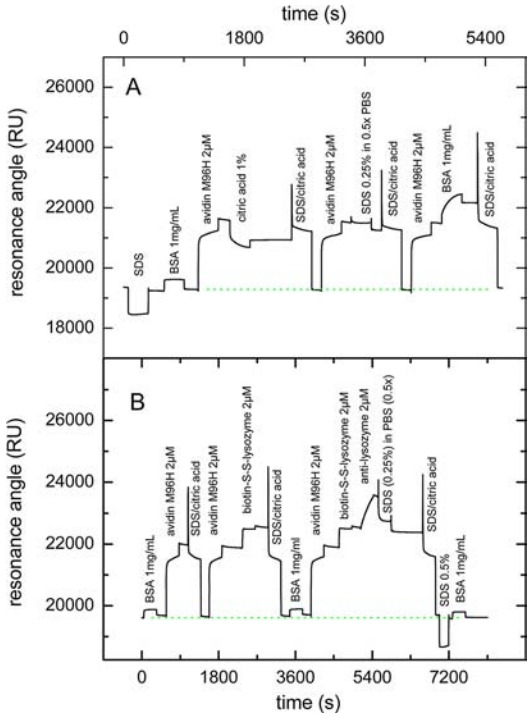

Figure 5. Test for reversible binding of the avidin mutant M96H to the mixed SAM shown in Figure 4. The running buffer was PBS 7.3 and the flow was 20 μL/min. 100 μL volumes of the samples noted in the above figure were injected, corresponding to 300 s injection times. (A) Search for the optimal reagent for desorption of avidin M96H. (B) Test for dissociation of a network consisting of avidin M96H, biotin-S-S-lysozyme, and antilysozyme, without cleavage of the disulfide bonds.

M96H from the biotin-terminated SAM shown in Figure 4. The logic next step was to examine whether this reagent can dissolve the networks between avidin and a biotinylated protein (Figure 3A and 3B) by dissociation of avidin M96H into four denatured subunits (Figure 3C). The corresponding data are shown in Figure 5B.

The first segment of Figure 5B was a mere repetition of the tests in panel A, i.e., only avidin M96H alone was bound to the chip and subsequently removed with SDS/citric acid. At the end of the SDS/citric acid injection, the baseline was the same as at the beginning, providing further support for complete removability of avidin M96H from the biotinylated SAM.

In the second segment of Figure 5B, biotinylated lysozyme was bound on top of avidin M96H. The small response was due to the small molecular mass of lysozyme (M_r = 14000) as compared to avidin M96H (M_r = 58000). Thus, the signal indicates full coverage of avidin M96H with biotinylated lysozyme. In spite of cross-linking of avidin M96H with statistically biotinylated lysozyme, the subsequent injection of SDS/citric acid could instantaneously remove all proteins from the chip surface.

In the third segment of Figure 5B, a trilayer of three cognate proteins was formed: avidin M96H, biotin-S-S-lysozyme, and anti-lysozyme. Subsequently, a 1:1 mixture of SDS (0.5%) and PBS 7.3 (final SDS concentration 0.25%) was injected which obviously removed the antibody only. In contrast, the mixture of SDS and citric acid caused complete regeneration of the chip surface. A tiny remnant was removed by an injection of pure SDS (0.5%) in water.

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From Figure 5 it is concluded that avidin M96H fulfills important criteria for reversible functionalization of biotinylated chip surfaces: (i) This avidin mutant rapidly binds to the biotinylated SAM and remains firmly attached. (ii) Biotinylated proteins quickly bind on top of immobilized avidin M96H and remain firmly bound. (iii) The biotin−avidin−biotin bridge is completely resistant to SDS (0.5%) and surprisingly resistant to acid, even at pH 2.2. (iv) When desired, all biotin−avidin−biotin bridges can instantaneously be broken by the combination of SDS with citric acid, even in the case of a multiply biotinylated protein!

However, the third cycle in Figure 5A gives a first indication of the downside of avidin, as compared to streptavidin: Due to its high isoelectric point (pI ∼ 10−10.5)[12,53] it is prone to adsorb nucleic acids, as well as most proteins which are usually negatively charged at neutral pH. Here, BSA was adsorbed on top of immobilized avidin M96H. Fortunately, both proteins were completely removed by SDS/citric acid at the end of the third cycle in Figure 5A.

The problem of nonspecific adsorption was studied more closely in Figure 6A. Lysozyme has a high pI value (∼11.2);[54] therefore, it has a net positive charge at neutral pH, just as avidin M96H. This explains why lysozyme did not adsorb on top of avidin M96H, in contrast to BSA and IgG which were significantly bound in the subsequent injections (Figure 6A, FC2, blue dashed line). Unfortunately, the problem of nonspecific protein adsorption could not be solved by "saturation of the adsorptive sites" with BSA, as is common practice in many solid phase assays such as ELISA:[55] Two successive injections of BSA and the subsequent injection of IgG caused a progressive rise of the resonance angle in each step (Figure 6A, FC2, blue dashed line). A similar overall rise of the resonance angle was also obtained when injecting IgG alone in flow cell 1 (Figure 6A, FC1, red solid line).

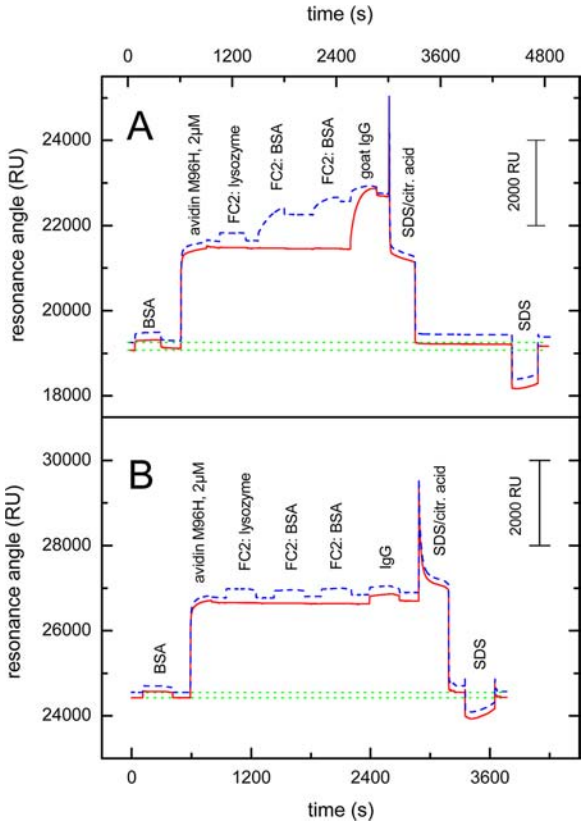

Figure 6. Test for nonspecific adsorption of lysozyme, BSA, and goat IgG (blue dashed trace, FC2) on top of the monolayer of avidin M96H. In FC1 (red solid trace), only goat IgG was applied to the layer of avidin M96H. Protein concentrations were 1 mg/mL, except for avidin M96H (2 μM). All other conditions were as in Figure 5. In panel A, the mixed SAM shown in Figure 2 had been prepared by the usual procedure.[8] In panel B, the SAM components had been mixed and subsequently reduced with zinc/acetic acid to ensure a molar ratio of 8/2 (MAT/BAT) not only in solution but also on the gold surface (for details, see text and Supporting Information). Solid traces and red color refer to flow cell 1 (FC1), dashed traces and blue color to flow cell 2 (FC2).

Reversible Sensor Chip Functionalization with Acetylated Avidin M96H. Since the undesired nonspecific adsorption of proteins on top of immobilized avidin M96H is caused by the excess of positive charges, the obvious solution to this problem is modification of the surface charges, either by suitable chemical reagents or by genetic engineering. In principle, genetic engineering is to be preferred because it results in a homogeneous sample in which all avidin molecules have exactly the same modifications. The alternative was modification of lysine residues with acetyl groups[56] or with succinic anhydride.[57] The disadvantage of chemical modification is its statistical mechanism, which yields a range of pI values. Its advantage over genetic engineering is simplicity and a shorter time scale. We therefore decided to perform a preliminary study with chemical modification in order to find out whether genetic engineering is worth the effort. As shown below, the answer was clearly yes: the problem of nonspecific adsorption was largely reduced by chemical modification of avidin M96H while retaining its favorable switching behavior. Even better results are to be expected from genetic engineering which has the advantage of producing a homogeneous population of modified avidin molecules.

First, it was necessary to determine the optimal degree of acetylation that caused suppression of nonspecific adsorption, without impeding specific binding of biotin. Given the close similarity between wild-type avidin and avidin M96H, the test series with different degrees of acetylation was performed with the wild-type protein. As described in the Supporting Information, suppression of nonspecific adsorption was maximal when avidin was treated with a 40-fold excess of sulfo-NHS acetate under carefully controlled conditions. The same procedure was then applied to avidin M96H, yielding so-called "avidin M96H-40" which had a narrow pI distribution between 4.5 and 4.8 (see Supporting Information). After acetylation, avidin M96H-40 was tested for specific binding to biotin and for nonspecific adsorption of proteins (Figures 7−9).

The most important finding in Figure 7 is that the avidin mutant M96H-40 has fully retained its switching behavior, in spite of acetylation. After some initial tests of protein adsorption (BSA, IgG) to the bare SAM, two cycles of specific binding and subsequent desorption were performed with avidin

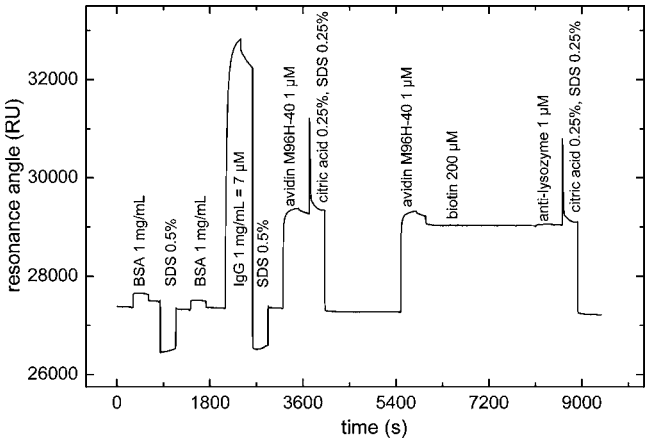

Figure 7. Reversible binding of acetylated avidin M96H-40 to the mixed SAM shown in Figure 4 and suppression of nonspecific adsorption by acetylation. The operating conditions were the same as in Figure 5

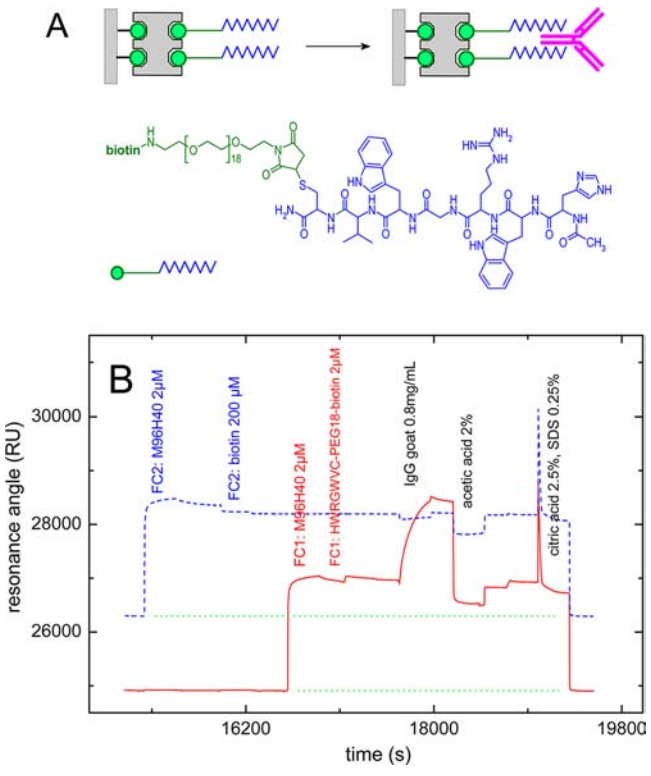

Figure 8. Functionalization of chip-bound avidin M96H-40 with an IgG-binding heptapeptide. The peptide HWRGWVC had been linked to biotin via a PEG_{18} chain with 18 ethylene glycol units (for details see Supporting Information). The operating conditions were the same as in Figure 5. Solid traces and red color refer to flow cell 1 (FC1), dashed traces and blue color to flow cell 2 (FC2).

M96H-40. In both cycles, a similar level of binding was achieved. Moreover, injection of SDS/citric acid always resulted in perfect regeneration of the chip surface by complete removal of bound avidin M96H-40.

In the second cycle, free D-biotin was applied on top of avidin M96H-40, with two effects: (i) About 10% of surface-bound avidin M96H-40 were dissociated. (ii) The remaining 90% of avidin M96H-40 were perfectly retained on the chip surface,

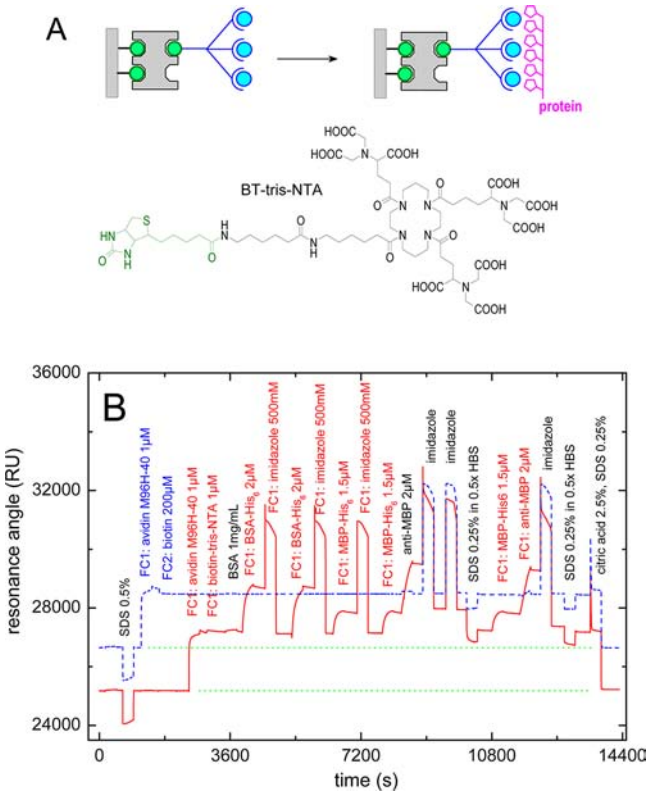

Figure 9. Application of acetylated avidin M96H-40 for immobilization of biotin-tris-NTA, thereby creating a chip surface with high affinity for His_6-tagged bait molecules. The latter were repeatedly bound to immobilized biotin-tris-NTA in the presence of 200 μM Ni^{2+} and released by 500 mM imidazole. The operating conditions were the same as in Figure 5, except that HBS (supplemented with 10 μM Ni^{2+}) was used as running buffer in place of PBS 7.3. Solid traces and red color refer to flow cell 1 (FC1), dashed traces and blue color to flow cell 2 (FC2).

while before the addition of free D-biotin, some dissociation was visible from the SPR trace.

Biotin-induced dissociation from the mixed SAM was not unique for acetylated avidin M96H-40. It was also seen with nonacetylated avidin M96H (6 ± 2%, data not shown). The most probable explanation for the distinction between stable and unstable binding is that firmly bound avidin molecules simultaneously bind to two adjacent biotin groups on the chip surface, while the small fraction of loosely bound avidin "sits" on a single surface-bound biotin (as stated in ref 52). In the case of acetylated avidin M96H-40, the loosely bound fraction shows spontaneous dissociation even in the absence of biotin and it is instantaneously released upon addition of free biotin. In the case of nonacetylated avidin M96H, the "loosely bound fraction" shows no spontaneous dissociation and can only be released by addition of free biotin.

Before the last injection of SDS/citric acid in Figure 7, an antibody (antifluorescein antibody, 1 μM) was injected. Fortunately, no nonspecific adsorption was seen at this concentration which is typical for biosensing experiments. This finding is particularly significant because in the same figure the bare SAM had shown a high level of nonspecific adsorption of IgG (at $t \sim 2400$ s).

Figures 8 and 9 exemplify the reversible immobilization of biotinylated bait molecules on top of acetylated avidin M96H-40. In Figure 8, the hexapeptide HWRGWV was applied for

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reversible capture of IgG molecules on a sensor surface. Among all cysteine-free hexapeptides, this peptide sequence exhibits the highest affinity for IgG molecules from various mammalian species.[58,59] The binding site for the peptide is known to be located in the Fc domain of IgG molecules.[58] Here, we added an extra cysteine (HWRGWVC) for subsequent linking to maleimide-PEG_{18}-biotin (see Supporting Information). The resulting conjugate HWRGWVC-PEG_{18}-biotin was bound on top of a monolayer of avidin M96H-40 (Figure 8, FC1, red solid trace). It was not possible to actually see the binding of the peptide-PEG-biotin conjugate in the SPR trace, for two reasons: (i) The small molecular mass of this molecule leads to a small SPR signal. (ii) As explained above, a small fraction of avidin M96H-40 dissociates when challenged with biotin (or a small, flexible derivative of biotin). These processes have opposite effects on the SPR trace. Nevertheless, it is obvious that peptide-PEG-biotin was bound because the subsequent injection of IgG (1 mg/mL) resulted in pronounced binding of IgG (Figure 8, FC1, red solid trace), followed by slow dissociation after the end of injection. No binding of IgG was seen in the control cell (Figure 8, FC2, blue dashed trace) where the monolayer of avidin M96H-40 had been blocked with D-biotin before the injection of IgG.

Interestingly, all bound IgG could be dissociated from the immobilized peptide with 2% acetic acid (pH 2.6), without much effect on the avidin M96H-40 monolayer (see solid and dashed trace in Figure 8). The latter could only be removed by a solution containing 2.5% citric acid (pH 2.0) and 0.25% SDS. These findings emphasize the robustness of immobilized M96H-40 over a wide range of measurement conditions which nicely contrasts the instantaneous response to SDS/citric acid when its removal is actually desired.

In Figure 9, the biotinylated bait molecule was biotin-tris-NTA. It is known to function as a general adaptor for His_6-tagged proteins.[46,47] When occupied with Ni^{2+} ions, each of the three NTA groups in biotin-tris-NTA binds two histidine residues, with the effect that a His_6 tag is tightly bound on the time scale of hours.[46,60] Here, we combined biotin-tris-NTA with acetylated avidin M96H-40, resulting in a new chip surface which holds significant potential for practical application (see sketch on top of Figure 9). After immobilization of avidin M96H-40 on the biotinylated SAM, biotin-tris-NTA was bound on top of avidin (red solid trace in Figure 9). The next injection was a test for nonspecific adsorption. In spite of the high concentration of BSA (1 mg/mL), nonspecific adsorption was absent, both in the measuring cell with the immobilized biotin-tris-NTA (red solid line) and in the control cell with biotin-blocked avidin M96H-40 (blue dashed line).

Then, many cycles were performed in which a His_6-tagged protein was specifically bound to biotin-tris-NTA in the presence of 200 μM Ni^{2+} and subsequently released with 500 mM imidazole. As can be seen from the data, imidazole caused perfect release of the His_6-tagged proteins, without affecting the sublayer of avidin M96H-40. In the fourth cycle, anti-MBP antibody was bound on top of MBP-His_6, thereby demonstrating the practical purpose of this design: The His_6-tagged protein serves as bait molecule and the interaction with a complementary molecule is to be monitored by SPR. While the antibody showed intense binding in the measuring cell (red solid line), no binding was seen in the control cell (blue dashed line). The only imperfection in this fourth cycle was that imidazole could not fully release the antigen−antibody complex from the chip surface, in spite of two successive injections. The problem was solved by injecting a 1:1 mixture of the running buffer with 0.5% SDS. The resulting baseline was identical with the baseline before the first injection of a His_6-tagged protein. Moreover, the remaining layer of avidin M96H-40 was still able to bind His_6-tagged maltose-binding protein in the next cycle. These observations in Figure 9 confirm that SDS alone does not harm avidin M96H-40—just like acetic acid alone did not hurt avidin M96H-40 in Figure 8.

Suppression of Nonspecific Protein Adsorption without Acetylation. Acetylation of avidin M96H-40 allowed us to prove that nonspecific adsorption of proteins can be eliminated by lowering of the p*I* of the avidin mutant. The logic next step was to combine the mutation M96H with other mutations of avidin which lower the p*I* and abolish nonspecific adsorption.[61] This study is under way in our laboratories.

Fortuitously we discovered a simple alternative for complete suppression of nonspecific adsorption which works well with unmodified avidin M96H. The trick was to improve the quality of the biotin-terminated SAM. So far, the mixed SAMs were formed by combining stock solutions of the individual SAM components (1 mM in ethanol) at the desired molar ratio before diluting with acetonitrile and starting the incubation with the cleaned gold surface,[8,51] following an earlier example.[62]

In the course of this study we remembered an important report about the kinetics of mixed SAM formation.[63] It was shown that, in mixtures of thiols and disulfides, the rate of chemisorption to the gold surface is >10 times faster for the thiol form than for the corresponding disulfide (even when the disulfide concentration was counted twice). This made us worry about the oxidation state of MAT and BAT (Figure 4) in the 1 mM stock solutions. If disulfide formation of MAT was more extensive than of BAT, then BAT would be greatly overrepresented in the SAM (and *vice versa*). Deviation of the MAT/BAT ratio in the SAM from the optimal value of 80/20 was expected to have very adverse effects on binding of (strept)avidin.[52]

In order to ensure the identical oxidation state of MAT and BAT, we mixed solid MAT and BAT at the desired molar ratio and treated the mixture in THF with zinc/acetic acid which is known to ensure complete reduction of dialkyldisulfides.[64] The resulting thiol mixture was completely reoxidized to the disulfidic state, containing a statistical mixture of disulfidic MAT−MAT, MAT−BAT, and BAT−BAT adducts (see Supporting Information). The mixture was dissolved in chloroform, dried down in aliquots, and stored under argon at −25 °C where no further chemical changes are to be expected. One 0.44 mg aliquot was used to prepare the mixed SAMs on five gold chips with a simplified procedure (see Supporting Information).

The big surprise with these newly prepared SAMs was a greatly reduced level of nonspecific protein adsorption. The dramatic effect of the SAM preparation method is exemplified in Figure 6 where panel A shows pronounced nonspecific adsorption for the old SAM type and panel B near absence of protein adsorption on the new SAM type. The beneficial effect of the new SAM preparation method was consistently seen on all (seven) chips tested from two different batches of chip formation, in many cycles of functionalization with avidin M96H and subsequent removal with SDS/citric acid. In a series of experiments, the extent of BSA adsorption (from 1 mg/mL BSA) was 26 ± 23 RU and the extent of IgG adsorption (from 2 μM IgG, applied after BSA) was only 12 ± 4 RU.

Based on the above findings, an optimized protocol for the differential functionalization of sample cell and control cell was established, as depicted in Figure 10A. Biotin−BSA was used to block the control cell (FC2, blue dashed line). Subsequently, biotin−protein G was immobilized in the sample cell (FC1, red solid line). The integrated flow cell (IFC) was washed (2×) and both flow cells were treated with biotin−BSA and also with BSA to further minimize nonspecific adsorption of proteins. Finally, free IgG (2 μM) was injected, resulting in extensive specific binding (2600 RU) on top of immobilized protein G (FC1, red solid line), while very little IgG (30 RU) was bound in the control cell (FC2, blue dashed line).

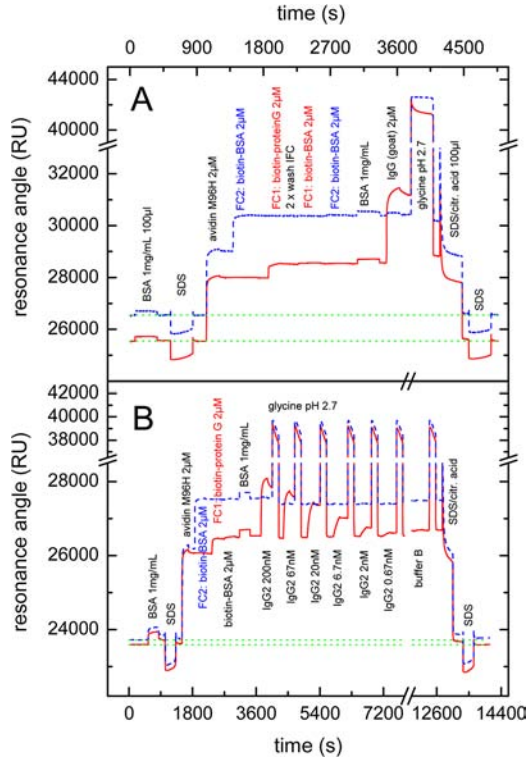

Figure 10. Selective binding of IgG to immobilized biotin−protein G after inertization of avidin M96H with biotin−BSA and free BSA. (A) Protocol for the preparation of an inert control cell (FC2, blue dashed trace) and a sample cell with high specificity for goat IgG (FC1, red solid trace). (B) Reversible association and dissociation of human IgG2κ on immobilized biotin−protein G, using 50 mM glycine (pH 2.7) for selective removal of IgG from avidin-bound biotin−protein G. The different concentrations of IgG2κ were prepared by serial dilution of a 7 μM stock solution of IgG2κ (in PBS 7.3) with buffer B (1 μM BSA in PBS 7.3). In all experiments of this figure, the SAM had been prepared by the improved procedure which is described in the Supporting Information. Solid traces and red color refer to flow cell 1 (FC1), dashed traces and blue color to flow cell 2 (FC2).

The time course of IgG binding and dissociation in Figure 10A looked inviting for kinetic analysis by the BIAcore evaluation software. This IgG, however, was a mixture of all IgG subtypes from goat, thus no meaningful data were to be gained from such analysis. In Figure 10B, the heterogeneity problem was solved by injecting different concentrations of human IgG2κ, always removing bound IgG2κ after a short dissociation period with 50 mM glycine (pH 2.7). IgG2κ was diluted with 1 μM BSA (in the same buffer) in order to prevent loss of IgG2κ in dilute solutions by denaturation or adsorption at the solid/liquid or air/liquid interface.

The association/dissociation periods at different concentrations of IgG2κ were evaluated by the double referencing method,[65] as shown in Figure 11. The Langmuir model (1:1 binding between immobile protein G and IgG2κ) was clearly inadequate (Figure 11B) while the "bivalent analyte model" proved to be well suited to describe both association and dissociation. Given the C_2 symmetric nature of IgG, it makes sense that the two identical heavy chains in the Fc portion are bound by two adjacent biotin−protein G molecules on the avidin surface.

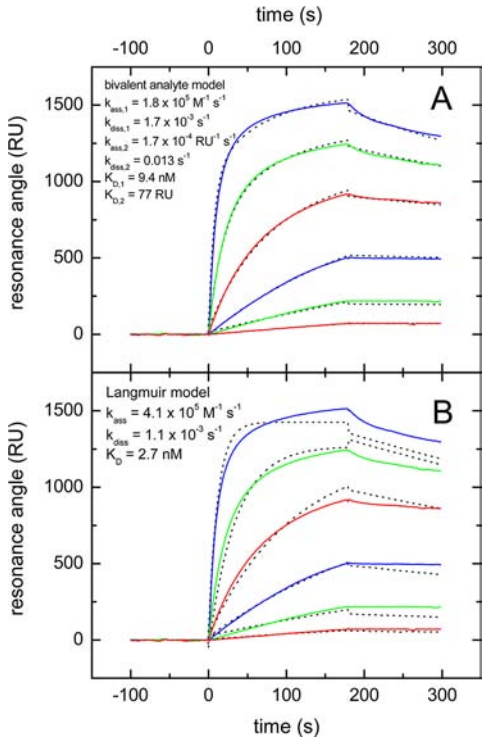

Figure 11. Kinetic analysis of reversible binding of human IgG2κ to immobilized biotin−protein G. The data in Figure 10B were analyzed according the double referencing method[65] using the program BIAevaluation 3.2 RC1. The results of global fits are shown for the "bivalent analyte model" (A) and for the conventional Langmuir model (B). The measured traces are shown in color (solid lines), the calculated traces in black (dotted lines).

Figures 10 and 11 show that avidin M96H can well be used for sophisticated biosensing, in spite of its high pI value, if proper precautions are taken. One important aspect is the new method of mixed SAM preparation and the other is passivation with biotin−BSA and BSA.

Examination of Captavidin as Potential Alternative to Avidin M96H. Many years ago we tested whether switchable biotin−avidin−biotin bridges can be formed on biotin-terminated SAM with the help of captavidin. We saw pronounced leaching of captavidin from the biotinylated surface. At that time, however, it was not clear whether this was due to captavidin or to insufficient SAM quality. Having optimized the preparation of biotin-terminated SAMs, as described above, we now repeated the test with newly purchased captavidin. The data (in Figure 12) show that captavidin cannot compete with avidin M96H in case of biotin−avidin−biotin bridges (Figure 1B): (i) The layer

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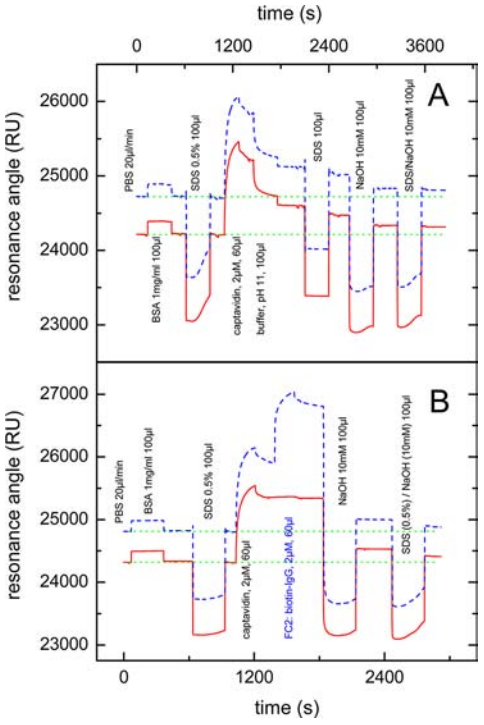

Figure 12. Test for reversible binding of captavidin the mixed SAM shown in Figure 4. (A) Binding of captavidin (2 μM) and subsequent desorption by buffer 11.0 (see Experimental Procedures). (B) Successive binding of captavidin (2 μM) and biotin-IgG (2 μM), followed by an attempt of complete chip regeneration with NaOH and NaOH/SDS. The SAM had been prepared by the improved procedure which is described in the Supporting Information. The running buffer was PBS 7.3 and the flow was 20 μL/min. Solid traces and red color refer to flow cell 1 (FC1), dashed traces and blue color to flow cell 2 (FC2).

thickness of captavidin is a little over 1000 RU, as compared to >2000 RU in the case of avidin M96H. (ii) Bound captavidin shows rapid dissociation from the biotin-terminated SAM. Progressive dissociation was also seen after binding of biotin-IgG (Figure 12B) which had been expected to stabilize captavidin on the chip by cross-linking of adjacent molecules. (iii) In spite of weak binding to the sensor surface, it was not possible to remove all bound captavidin (±biotin-IgG) on the same time scale as shown above for avidin M96H.

This comparison with captavidin demonstrates that the avidin mutant M96H is uniquely suited for reversible biofunctionalization of biotinylated surfaces. It shows very stable biotin-binding over a wide range of measurement conditions, even in SDS, and yet its function can be switched off within minutes when desired.

Hypothesis for the Synergy between SDS and Citric Acid in Dissociating Avidin M96H. It is easy to rationalize the combined effect of acid (pH 2.0) and SDS upon biotin-bound avidin M96H (see Figure 13). This avidin mutant has four additional histidines at the subunit−subunit interfaces which are accessible to protonation at low pH.[44] If protonated, these cationic sites should exhibit an enhanced affinity for negatively charged SDS molecules, thereby facilitating intrusion of hydrophobic SDS tails between the subunit interfaces. Together with the global destabilization by other protonated sites, the additionally bound SDS may be responsible for the unusually quick denaturation and subunit dissociation from the chip surface caused by SDS at pH 2.0.

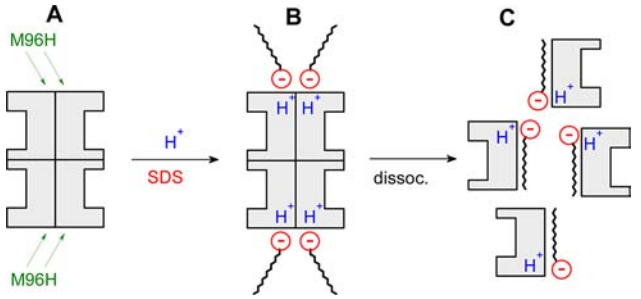

Figure 13. Hypothesis for the synergy between H^+ and SDS with respect to the denaturation of avidin M96H. Position 96 of each monomer is located at a subunit−subunit interface[44] (A). Protonation of the artificially introduced histidine residues is thought to promote binding of the negatively charged SDS molecules (B) which cause dissociation and denaturation of the subunits (C).

CONCLUSIONS

The goal of this study was to identify a variant of (strept)avidin which functions as a reversible bridge between surface-bound biotin residues on one hand and biotinylated bait molecules on the other hand. This goal has successfully been reached by use of the avidin mutant M96H. The latter proved to be very robust, retaining full function at neutral pH, even in the presence of SDS, or at low pH (>3) in the absence of SDS. Only the combination of SDS and low pH (2.0) was able to denature avidin M96H, causing instantaneous dissociation from a biotinylated chip surface.

The problem of nonspecific protein adsorption on top of chip-bound avidin M96H was solved by simple measures: (i) an improved method of mixed SAM formation and (ii) passivation with biotin−BSA and free BSA. The alternative method (acetylation of avidin M96H) proved equally suited to suppress nonspecific adsorption. The adverse side effect of acetylation was the appearance of a small fraction of avidin derivatives with reduced binding strength. Nevertheless, the goals of the acetylation studies were fully reached because its main purpose was to test whether lowering of the p*I* value was sufficient to abolish nonspecific protein adsorption on top of avidin M96H. The method of choice for this purpose is of course genetic engineering of the surface charges on avidin M96H and the corresponding study is under way.

ASSOCIATED CONTENT

Supporting Information

Screening of published avidin mutants for optimal pH dependence of biotin binding. Acetylation of avidin and avidin M96H and characterization of the products. Syntheses of the OH-terminated matrix alkanethiol (MAT) and of the biotin-terminated alkanethiol (BAT). Synthesis of the peptide-PEG_{18}-biotin conjugate with the peptide sequence HWRGWVC. Optimized version of mixed SAM formation. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

Avidin M96H-40, avidin mutant M96H derivatized with a 40-fold excess of sulfo-NHS acetate; BAT, biotin-terminated alkanethiol (see Figure 4); biotin-cap-DOPE, *N*-(6-(*N*-biotinyl)aminocaproyl-1,2-dioleoyl-phophatidylethanolamine; biotin-S-S-lysozyme, lysozyme derivatized with sulfo-NHS-SS-biotin; biotin-tris-NTA, see Figure 11; BSA, bovine serum albumin; DOPC, 1,2-dioleoylphosphatidylcholine; dsDNA, double-stranded DNA; HBS, HEPES-buffered saline; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-ethanesulfonic acid; MBP-His_6, maltose-binding protein with a C-terminal His_6 tag; HPA chip, BIAcore chip with a hydrophobic SAM of long alkyl chains; HWRGWVC-PEG_{18}-biotin, see Figure 8; IgG, immunoglobulin G; MAT, matrix alkanethiol (see Figure 4); MBP, maltose-binding protein; MBP-His_6, maltose-binding protein with a C-terminal His_6 tag; NHS, *N*-hydroxysuccinimide; NTA, nitrilotriacetate; PBS, phosphate-buffered saline; PEG, poly(ethylene glycol); p*I*, isoelectric point; RU, resonance units (1 RU = 0.0001° change of the resonance angle); SAM, self-assembled monolayer; SDS, sodium dodecyl sulfate; SPR, surface plasmon resonance; ssDNA, single-stranded DNA; (strept)avidin, streptavidin or avidin; sulfo-NHS acetate, *N*-2-sulfosuccinimidyl acetate; sulfo-NHS-SS-biotin, *N*-2-sulfosuccinimidyl 3-[2-(biotinamido)ethyl-dithio]-propionate sodium salt

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