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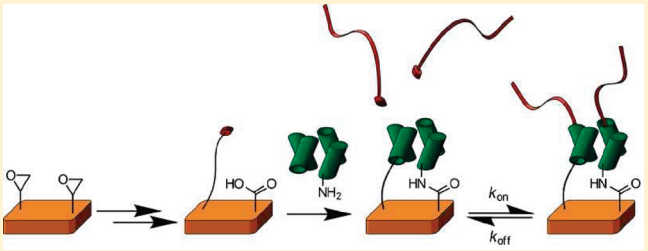
Kinetics and Thermodynamics of Biotinylated Oligonucleotide Probe Binding to Particle-Immobilized Avidin and Implications for Multiplexing Applications

Graham R. Broder,[†] Rohan T. Ranasinghe,[†] Cameron Neylon,[§] Hywel Morgan,[‡] and Peter L. Roach*[,†]

[†]School of Chemistry and [‡]School of Electronics and Computer Science, University of Southampton, Southampton SO17 1BJ, U.K.
[§]STFC Rutherford Appleton Laboratory, Harwell Science and Innovation Campus, Didcot OX11 0QX, U.K.

Supporting Information

ABSTRACT: In this work, the kinetics and dissociation constant for the binding of a biotin-modified oligonucleotide to microparticle-immobilized avidin were measured. Avidin has been immobilized by both covalent coupling and bioaffinity capture to a surface prefunctionalized with biotin. The measured rate and equilibrium dissociation constants of avidin immobilized by these different methods have been compared with those for nonimmobilized avidin. We found that immobilization resulted in both a decrease in the rate of binding and an increase in the rate of dissociation leading to immobilized complexes having equilibrium dissociation constants of $7 \pm 3 \times 10^{-12}$ M, higher than the value measured for the complex between biotin-modified oligonucleotide and nonimmobilized avidin and approximately 4 orders of magnitude larger than values for the wild-type avidin−biotin complex. Immobilized complex half-lives were found to be reduced to 5 days, which resulted in biotin ligands migrating between protein attached to different particles. Different immobilization methods showed little variation in complex stability but differed in total binding and nonspecific biotin-modified oligonucleotide binding. These findings are critical for the design of multiplexed assays where probe molecules are immobilized to biosensors via the avidin−biotin interaction.



The high affinity of the tetrameric proteins avidin and streptavidin for the vitamin biotin, with equilibrium dissociation constants (K_d) of 0.6×10^{-15} and 4×10^{-14} M, respectively, have led to their use as capture elements in a multitude of biotechnological applications.[1,2] Proteins and peptides,[3] nucleic acids[4−6] and aptamers[7] have been modified with biotin and immobilized to a (strept)avidin surface for use in biological sensing and immunoassays.[8−11]

Typically, avidin or streptavidin are immobilized on surfaces via electrostatic interactions,[12,13] covalent coupling[14−17] or bioaffinity capture to a surface prefunctionalized with biotin.[16,17] The most common covalent coupling strategies involve amide bond formation using protein lysine residues, though streptavidin mutants bearing a single cysteine residue have also been developed for attachment to maleimide-functionalized solid surfaces.[18] The wide use of this technology for purification and immobilization of nucleic acids has led to many commercial products including streptavidin-coated magnetic microparticles (Dynabeads, Invitrogen), (strept)avidin and biotin coated polystyrene particles (Spherotech), phosphoramidite derivatives of biotin and desthiobiotin for labeling of oligonucleotides in automated synthesis (Glen Research),[19], and forms of the proteins selected for low nonspecific binding to nucleic acids (avidin DN, Vector Laboratories and NeutrAvidin, Thermo Fisher Scientific).

The widespread use of (strept)avidin for the attachment of biotinylated probes is based upon the great stability of the unmodified complex, with a half-life ($t_{1/2}$) of 200 days for the avidin−biotin complex.[1] However, studies have shown that modification of both the protein and the ligand can reduce this stability. Studies of the dissociation of succinoylavidin with biotinylated insulin have highlighted the importance of a spacer between the biotin and conjugated protein, with $t_{1/2}$ of 2.6 h exhibited without a spacer and 76 days with a six carbon spacer.[20] An increased rate of dissociation was also observed when polyethylene glycol (PEG) (3400/5000) spacers were used in biotin-modified enzymes, reducing the avidin−biotin $t_{1/2}$ to 3.5 days.[21] Of more importance when the system is used in sensors are the kinetics when either the ligand or the protein are immobilized on a surface. Reported rate constants for the binding of fluorescently modified avidin to surface-immobilized biotin vary, though differences in the immobilization strategy used may account for this variation. Zhao and Reichert measured the binding of avidin to biotin-doped lipid films on a fiber-optic sensor observing an association rate constant, k_{on} of 1.2×10^5 M^{-1} s^{-1} and a dissociation rate constant, k_{off} of 3.3×10^{-7} s^{-1} ($t_{1/2} \approx 24$ days).[22] Wayment and Harris studied the binding of avidin to biotin immobilized via an amide linkage to amino-modified glass, where faster kinetics were observed with k_{on} =

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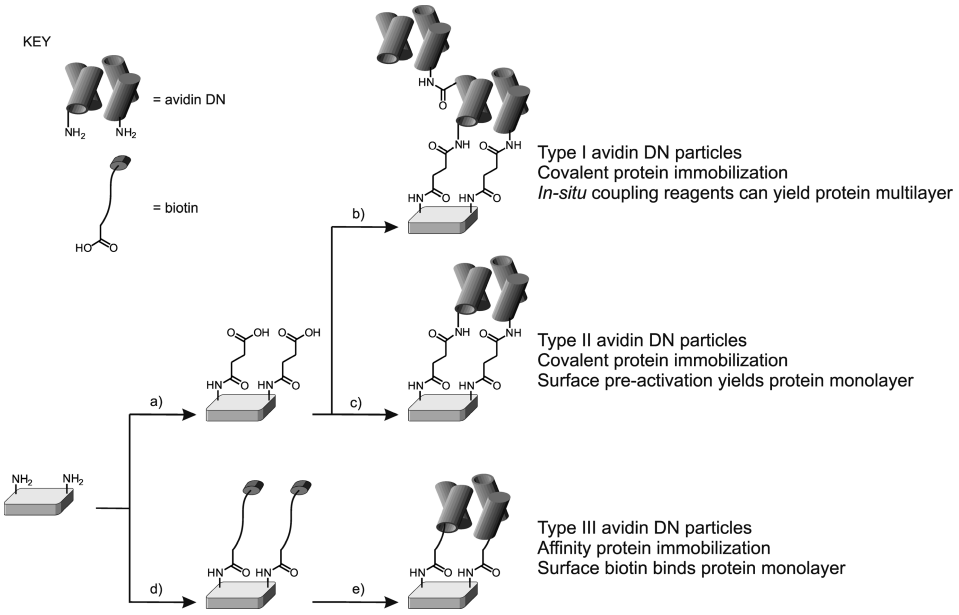

Figure 1. Three different routes to avidin-functionalized particles starting from amine functionalized particles. (a) succinic anhydride, DMAP, DIPEA, DMF, (b) avidin DN, EDC.HCl, pH 7.0, (c) i. EDC.HCl, sulfo-NHS, pH 5.0, ii. avidin DN, pH 7.4, (d) biotin, DIC, DIPEA, DMF, DMSO, (e) avidin DN, pH 7.0. Type 1 avidin DN particles.

$2.1 \times 10^8\ M^{-1}\ s^{-1}$ and $k_{off} = 3.8 \times 10^{-4}\ s^{-1}$ ($t_{1/2} \approx 0.5$ h).[23] Both studies resulted in a similar calculated equilibrium dissociation constant, $K_d \approx 2.3 \times 10^{-12}$ M. A direct measurement of the K_d has been made by Graves and co-workers, using commercial (Spherotech) polystyrene microparticles covalently functionalized with biotin to a much higher density than the previously noted studies, yielding $K_d = 4 \times 10^{-9}$ M.[24] To the best of our knowledge, a detailed kinetic analysis of biotin binding to surface immobilized avidin has not been published, however three previous studies disclose informative kinetic data by studying the binding of various biotinylated proteins. Polzius and co-workers adsorbed avidin onto tantalum oxide sensors and then used this surface to measure the rate of specific biotin binding (using various biotinylated proteins) where $k_{on} = 2.2 \times 10^5\ M^{-1}\ s^{-1}$.[25] Bolivar and co-workers covalently immobilized avidin to carboxymethylated dextran surface plasmon resonance (SPR) sensor chips and determined the K_d to be less than 1×10^{-11} M,[26] while Prosperi and co-workers measured it to be 2×10^{-10} M when studying binding to avidin adsorbed onto nanoparticles.[12] It is important to note that the differing surface materials, immobilization strategies, and modifications of the binding species employed in these studies may significantly affect the kinetics and thermodynamics of binding: the disparity between the reported rate[22,23,25] and equilibrium constants[24,26,12] emphasizes the requirement for a more detailed study of binding in the immobilized system.

In the course of our research on the development of multiplexed hybridization assays on polymer microparticles,[27] we have studied the kinetics of binding between biotinylated oligonucleotide probes and polymer microparticles bearing immobilized avidin DN. In this study, we measured the rates of association and dissociation of biotinylated oligonucleotides with avidin immobilized onto microparticles. The effect on the kinetics of different immobilization chemistries (Figure 1) has been compared with values measured for the nonimmobilized avidin DN control. The immobilization systems studied have all been widely employed to attach protein receptors to surfaces.[28] Measurements were also made of the equilibrium dissociation constant for the immobilized avidin-ligand complex. Furthermore, the differing methods of protein immobilization have been evaluated for surface coverage, activity, and nonspecific binding. In this work, we present the first detailed kinetic and thermodynamic analysis of the binding between immobilized avidin DN and solution phase biotinylated oligonucleotides. The results of this study define the practical limits of stability for the protein:ligand complex, which are important in the design of array based assays that exploit biotin:avidin mediated immobilization.

MATERIALS AND METHODS

Reagents and Apparatus. Labeled and unlabeled biotinylated oligonucleotides FP1: (5′-Cy5-CTAGTTACTCTTGTTC−biotin-3′) and P2 (5′-biotin−TTGTTATAGTTCTCTC) were purchased from ATDBio (Southampton, UK) employing an 8 atom spacer between the biotin and DNA. Epoxy SU-8-5 and microfabrication reagents were purchased from Chestech Ltd. (Rugby, UK). Avidin DN was purchased from Vector laboratories (Burlingame, CA). BCA Assay Kit was obtained from Pierce Biotechnology (Rockford, IL). Other reagents were obtained from Sigma-Aldrich (UK), Alfa Aesar (UK), or ThermoFisher (UK).

Epoxy SU-8 monomer was exposed using an EVG 620 mask aligner with photomask (Compugraphic, UK). Accuspin Micro centrifuges (Fisher Scientific, UK) (r = 8.5 cm) were used for particle sedimentation. Fluorescence and fluorescence anisotropy of FP1 solutions was measured using a SafireII microplate reader (Tecan, Switzerland). Particles were analyzed under a fluorescence microscope (Carl Zeiss AG, Germany) or using a FACSaria flow cytometer (BD Biosciences, U.S.). Kinetic and thermodynamic constants were calculated by fitting data using commercially available software (SigmaPlot 11.0, Systat Inc., Chicago, IL and Origin 8.1, Northampton, MA). Goodness of fit measurements for these analyses is given in Supporting Information (SI) Table S-1.

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Microparticle Fabrication. SU-8 microparticles were fabricated from photoactive epoxy SU-8-5 with dimensions 5 × 10 × 20 μm as reported previously.[27]

Microparticle Functionalization. Amine functionality was introduced to the microparticles by the ring-opening of residual surface epoxide groups of the SU-8 with the *bis*-amine Jeffamine ED-900, as described previously.[29] The loading density of primary amine was quantified by the Kaiser test,[30] and was typically between 25 and 35 μmol g^{-1} (4.4−6.2 nmol cm^{-2}).

Carboxyl groups were introduced by the reaction between succinic anhydride and amino particles. Avidin DN was covalently immobilized to these acid functionalized particles either with the coupling reagent, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), in situ (Type I) or stepwise by first preactivating the acid to the corresponding *N*-hydroxysuccinimidyl (NHS) ester before addition of protein (Type II) (Figure 1).

Biotin functionalization was achieved using carbodiimide-mediated coupling to Jeffamine ED-900-functionalized particles. Avidin DN was immobilized to biotin functionalized particles by affinity capture by allowing both species to mix (in suspension, 2 h) (Type III). Protein functionalized particles required gentle agitation in SSPE (5×) storage buffer (0.75 M NaCl, 50 mM sodium phosphate, 5 mM EDTA, pH 7.0) for 48 h to completely remove any physisorbed protein. The particles were stored as 1 mg mL^{-1} suspensions in SSPE (5×) buffer with 0.02% Tween 20 and 0.01% sodium azide. Immobilized protein was quantified using the BCA assay.[31]

Detailed protocols for all synthetic steps are available as SI.

Binding Studies. The association and dissociation kinetics of the labeled and biotinylated oligonucleotide, FP1, interaction with avidin DN were measured both in solution and for all three modes of protein immobilization to microparticles. The protein-FP1 complex was quantified in solution by measuring the anisotropy of the fluorescence signal from the Cy-5 label on FP1. For solution experiments, it was necessary to use an excess of unlabeled biotin in the reaction mixture, since solutions containing only FP1 and avidin DN were found to undergo self-quenching upon binding, likely due to the proximity of four fluorophores to each other in the protein tetramer (SI Figure S-1).[32] The particle-immobilized complex was quantified using fluorescence microscopy where a linear relationship between fluorescence intensity and amount of bound FP1 was assumed.

The association of biotin and avidin was measured throughout a time course experiment and the resultant data analyzed to determine the association rate constant, k_{on}. For the solution phase reaction this was achieved with real-time monitoring, whereas the binding to particles was terminated at time-points by the addition of an excess of biotin before the measurement of particle fluorescence. Dissociation kinetics were obtained by monitoring the decrease in the fluorescence anisotropy of avidin DN-FP1 complex in solution and the decrease in fluorescence of particles with the complex immobilized when either was exposed to an excess of biotin to impose effectively irreversible complex dissociation.

Equilibrium thermodynamics of binding on microparticles were measured by titrating FP1 with immobilized avidin. The concentration of immobilized complex was determined by measuring particle fluorescence after binding equilibrium had been established. For large sample sizes, particles were analyzed individually using flow cytometry.

RESULTS AND DISCUSSION

Protein Immobilization. The surface densities of immobilized avidin and bound oligonucleotide FP1 were determined by BCA protein-assay and fluorescence microscopy respectively (Table 1). All avidin functionalized particle types were found to have a stable monolayer of protein; repeating the BCA assay after the particles had been suspended in buffer for four weeks indicated no loss of avidin from the surface. Furthermore, the suspension of particles functionalized with fluorescently labeled avidin together with particles functionalized with unlabeled avidin for three days resulted in no change to the fluorescence observed for either particle type. This result confirmed the stability of the immobilized avidin tetramer. Importantly, no protein migration between particles with avidin immobilized by affinity capture (Type III) was detected, likely due to each avidin tetramer being linked to the particle via two biotin ligands.

Initial studies of immobilized complex dissociation using an excess of biotin in a displacement assay format showed biphasic dissociation kinetics made up of two distinguishable unbinding processes (Figure 2). The slow dissociation rate represented the specific protein−ligand complex while the fast rate was assumed to be the release of nonspecifically bound ligand (SI Figure S-2). This nonspecific binding was highest for Type I particles where avidin had been immobilized with EDC in situ. The protein on these particles also displayed the lowest activity with only 1.3% of the total biotin binding sites capturing FP1, compared with 6.5% for Type II particles. It should be noted that greater than 50% binding would not be expected due to the orientation of the avidin tetramer upon immobilization, resulting in ligand access to two of the four binding sites being

Table 1. Coverage and Activity of Avidin DN Immobilized to Particles

			FP1 binding capacity	
avidin DN particle immobilization	avidin loading (pmol cm^{-2})	avidin layers (number)	specific binding (pmol cm^{-2})	nonspecific binding (pmol cm^{-2})
type I	11.6 ± 4.4	1.4 ± 0.5	0.61 ± 0.02	0.95 ± 0.05
type II	6.7 ± 1.0	0.8 ± 0.1	1.73 ± 0.06	1.29 ± 0.06
type III	8.3 ± 2.3	1.0 ± 0.3	0.75 ± 0.02	0.36 ± 0.07

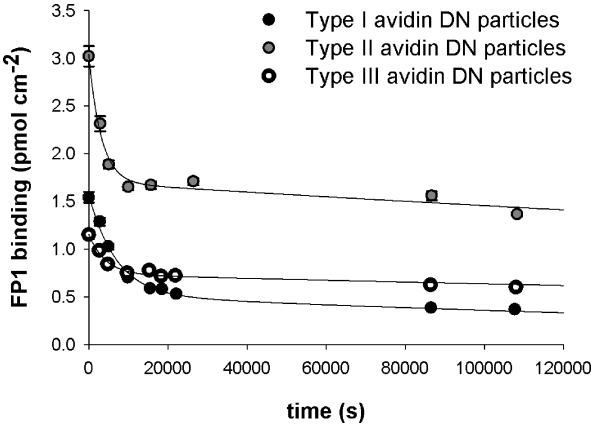

Figure 2. The displacement of biotinylated labeled-oligonucleotide FP1 from immobilized avidin particles in an excess of biotin, displaying biphasic dissociation due to the fast initial loss of nonspecifically bound ligand followed by the loss of complexed ligand. Data are fitted to a double exponential decay. Subsequent experiments used surfactant to eliminate the nonspecific binding (detail in SI Figure S-3).

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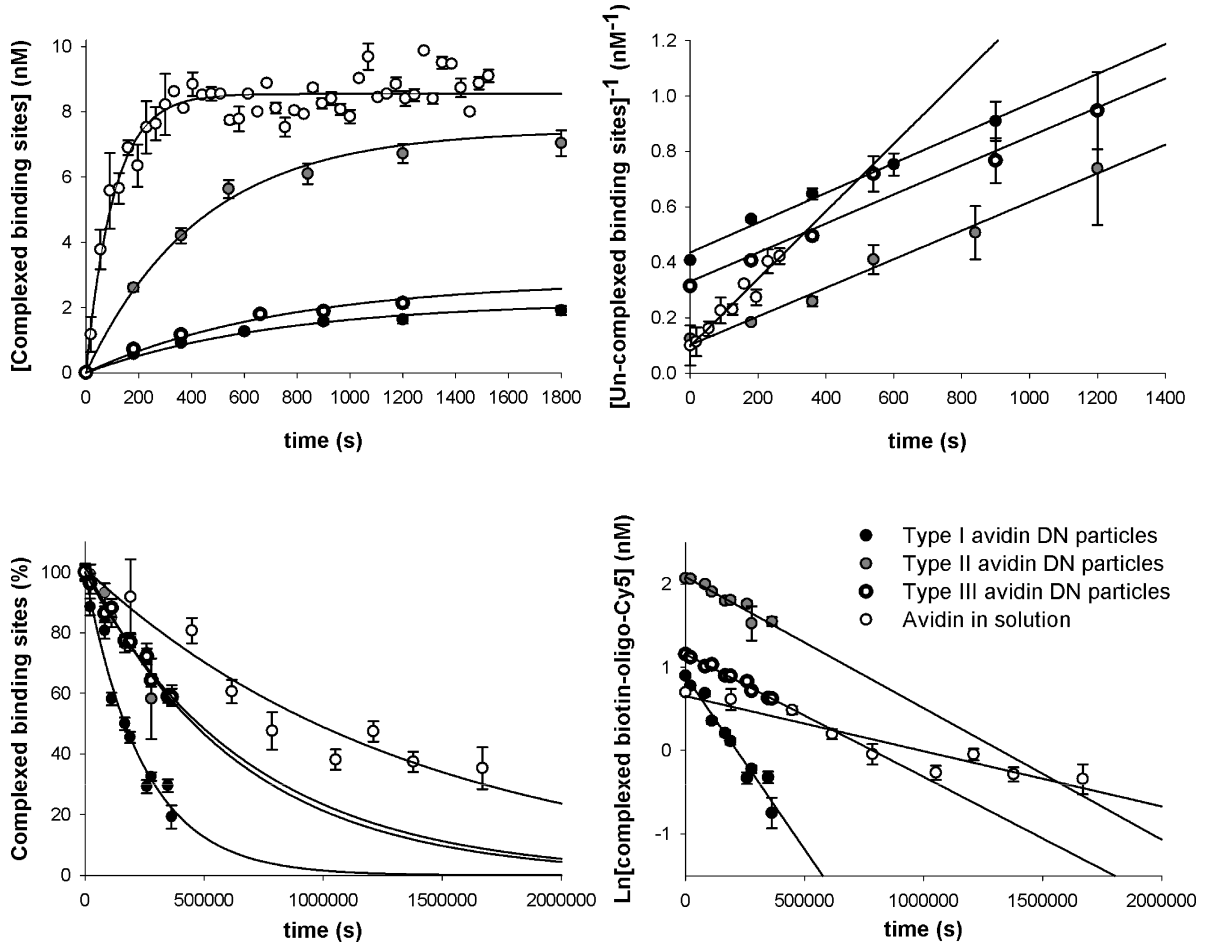

Figure 3. Kinetic studies of the Avidin DN FP1 complex. Association (top); with change in complex concentration with time (left) and linearized data plotted as inverse noncomplexed avidin site concentration versus time (right). Dissociation (bottom); with normalized change in complex concentration versus time when exposed to an excess of biotin, blocking FP1 rebinding (left) and linearized data plotted as the natural Log of concentration versus time (right).

blocked by the particle surface.[14] The lower activity observed for Type I immobilized avidin may be due to cross-linking of the protein in the presence of EDC, which could potentially result in more of the binding sites being rendered inactive. Affinity captured avidin (Type III) was also found to have low activity (2.3% of binding sites active) but did display very little nonspecific binding toward FP1.

Nonspecific binding of FP1 was reduced to below detectable levels with little effect on the specific interaction by using an increased concentration of surfactant in the buffer [Tween-20 (1%) or sodium dodecyl sulfate (SDS, 0.2%)] (SI Figure S-3).

Experimental Determination of Rate Constants for Association and Dissociation of Avidin−biotinylated Oligonucleotide Complex Formation (k_{on} and k_{off}). The capture of biotinylated oligonucleotide FP1 by avidin DN was modeled according to a simple bimolecular association process as shown in eq eq. 1, where [avidin DN], [FP1] and [avidin FP-1] are the concentrations of free avidin, free biotinylated oligonucleotide FP1 and avidin-FP1 complex, respectively.

$$[\text{avidinDN}] + [\text{FP1}] \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} [\text{avidinDN} - \text{FP1}] \qquad \text{(eq. 1)}$$

Data sets measuring the binding in solution and on functionalized microparticles were fitted to eq eq. 2 to determine the rate constant, k_{on} (Figure 3).

$$\frac{\partial[\text{avidinDN} - \text{FP1}]}{\partial\tau} = k_{on}[\text{avidinDN}][\text{FP1}] \qquad \text{(eq. 2)}$$

The solution-phase association rate constant was measured as $1.22 \pm 0.11 \times 10^6$ M^{-1} s^{-1}, whereas this value was halved for all three immobilized systems, being measured as $0.53 \pm 0.06 \times 10^6$ M^{-1} s^{-1}. These values were lower than that exhibited by the wild-type avidin−biotin complex (Table 2). Solution-phase diffusion limited rate constants (k_{diff}) were calculated using the Smoluchowski equation (eq eq. 3, where N_A is Avogadro's constant (mol^{-1}), $R_{\alpha\beta}$ the sum of the (hydrodynamic) radii (m) of avidin DN and the biotin-modified oligonucleotide FP1 and $D_{\alpha\beta}$ the sum of the diffusion coefficients (m^2 s^{-1})). This equation can be modified to allow calculation of the rate constant for the immobilized system (eq eq. 4).[33−35] Diffusion coefficients were based on values reported for similar binding species, wild-type avidin (5.98×10^{-11} m^2 s^{-1})[36] and a 13-mer labeled oligonucleotide similar in size to that used in our study (8.4×10^{-11} m^2 s^{-1}).[37] Hydrodynamic radii were calculated using the Einstein-Stokes equation (eq eq. 5, where k_B is the Boltzmann

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Table 2. Kinetic and Thermodynamic Data Comparing the Stability of the Avidin Biotin Complex with Avidin DN FP1 Complex in Solution and When Particle-Immobilized

protein−ligand complex	k_{on} (M^{-1} s^{-1}) ($\times 10^{6}$)	k_{off} (s^{-1}) ($\times 10^{-6}$)	$t_{1/2}$ (days)	k_{off}/k_{on} (pM)	K_d (pM)
free avidin−biotin (w.t.)[1]	70	0.04	200	0.00057	
free avidin DN-FP1	1.22 ± 0.11	0.73 ± 0.06	11.0	0.60	
type I avidin DN-FP1	0.54 ± 0.04	4.15 ± 0.29	1.9	7.73	10.03 ± 1.72
type II avidin DN-FP1	0.52 ± 0.03	1.57 ± 0.16	5.1	3.04	4.49 ± 0.85
type III avidin DN-FP1	0.52 ± 0.06	1.52 ± 0.08	5.3	2.91	4.05 ± 0.46

constant (J K^{-1}), temperature is 293.15 K, and viscosity (η) is 1.098×10^{-3} Pa s).

$$k_{diff} = 4\pi R_{\alpha\beta} D_{\alpha\beta} N_A 10^3 \quad \text{(eq. 3)}$$

$$k_{diff} = 2\pi R_{\alpha\beta} D_{\beta} N_A 10^3 \quad \text{(eq. 4)}$$

$$D = \frac{k_B T}{6\pi\eta R} \quad \text{(eq. 5)}$$

k_{diff} was thus calculated as 6.09×10^9 and 1.78×10^9 M^{-1} s^{-1} for the solution phase and immobilized systems, respectively. k_{diff} is much greater than the measured rate constant (k_{on}) due to the low steric factor (f) (where only a fraction of the surfaces of avidin and biotin are involved in binding which results in the probability of complex formation from a collision being <1).[38] The steric factor can be described as the ratio between the observed and diffusion limited rate constants and for the solution phase binding discussed herein is calculated as $f = 0.0002$. The difference in the measured association rate as caused by protein immobilization is predicted by these calculations (eqs eq. 3 and eq. 4), supporting our conclusion that binding is limited by diffusion. It is important to note the association rate constant between surface bound avidin DN and oligonucleotide FP1 is decreased by over 2 orders of magnitude compared with the wild-type avidin−biotin binding. This decrease in association rate constant is caused by a composite of two effects: first, conjugation of biotin to the oligonucleotide probe results in a 98% reduction of k_{on} and second, the attachment of avidin to the surface eliminates its diffusion, leading to a further 57% decrease of k_{on}.

Data sets measuring the irreversible dissociation of complex in an excess of biotin were fitted to eq eq. 6 (Figure 3).

$$\frac{\partial[FP1]}{\partial\tau} = k_{off}[\text{avidinDN} - \text{FP1}] \quad \text{(eq. 6)}$$

The avidin DN-FP1 dissociation rate constant in solution was calculated from fluorescence anisotropy measurements as $0.73 \pm 0.06 \times 10^{-6}$ s^{-1}. This value is more than an order of magnitude greater than reported for the wild-type system.[1] Binding studies have identified the urea moiety of biotin as having the strongest interaction with the active-site of avidin and this remains unaltered in our biotinylated probe.[39] However, crystal structures have indicated that additional binding interactions between the carboxylic acid moiety of biotin and residues in the binding site of the protein further stabilize the complex.[40] Other studies using the biotin analogue desthiobiotin found that modification of the acid to the corresponding methyl ester resulted in an 8-fold increase in the rate of dissociation.[41] In many applications of the biotin−avidin system for probe capture including the oligonucleotide-modified ligand FP1 used in this work, biotin is conjugated to the probe via an amide linkage, replacing the terminal carboxylic acid. Interactions of the carboxylic acid functional group of biotin with the binding site are therefore replaced by an amide which has significantly weaker binding and this weakened interaction is observed as an increase in the dissociation rate constant.

The immobilized complexes were found to have a faster rate of dissociation compared with avidin DN-FP1 in solution, regardless of the immobilization method used. FP1 dissociation from Type I immobilized avidin DN was six times faster than the solution system, resulting in a complex half-life of less than two days. Conformational changes in the protein have been shown to occur upon biotin binding, most notably a loop between two β-strands of each avidin tetramer subunit (residues 35−46, avidin isolated from hen egg white) which moves to cap the entrance of the binding pocket when occupied and contributes to the exceptionally high complex stability.[42] The immobilization of avidin and the steric restrictions imposed by close-packing of a surface monolayer may restrict these conformational rearrangements required for maximum complex stability. Avidin immobilized in the presence of coupling reagent (Type I particles) may be further restricted by protein−protein cross-linking which would not form under either Type II/III immobilization conditions or if avidin were spread more sparsely on the surface. Comparing the different values of k_{off} for the three immobilization methods, Type I avidin particles are shown to form the least stable complex with FP1.

$$K_d = \frac{[\text{avidinDN}][\text{FP1}]}{[\text{avidinDN} - \text{FP1}]} = \frac{k_{off}}{k_{on}} \quad \text{(eq. 7)}$$

FP1 titrations against immobilized avidin (SI Figure S-6) resulted in experimentally determined equilibrium dissociation constants when fitted to eq eq. 7, which correlated well with and corroborated the values calculated from the individual kinetic experiments (Table 2).

The Significance of Increased Complex Dissociation on Multiplexed Bioassays. The increased rate of dissociation and resulting decrease in half-life observed for the immobilized avidin DN-FP1 complex has implications for any assay designed to have discrete probes sharing the same analyte solution, such as particle-based multiplexed assays and spotted microarrays. Notably, biotinylated probes could potentially dissociate and reassociate to surface avidin forming a uniform coating of the probe mixture where probe distribution would have previously been patterned. This problem would be magnified over extended assay time scales and was investigated by mixing two discrete populations of avidin-functionalized particles, one complexed with an unlabeled biotinylated oligonucleotide probe (P2) and the other complexed with fluorescently labeled oligonucleotide probe

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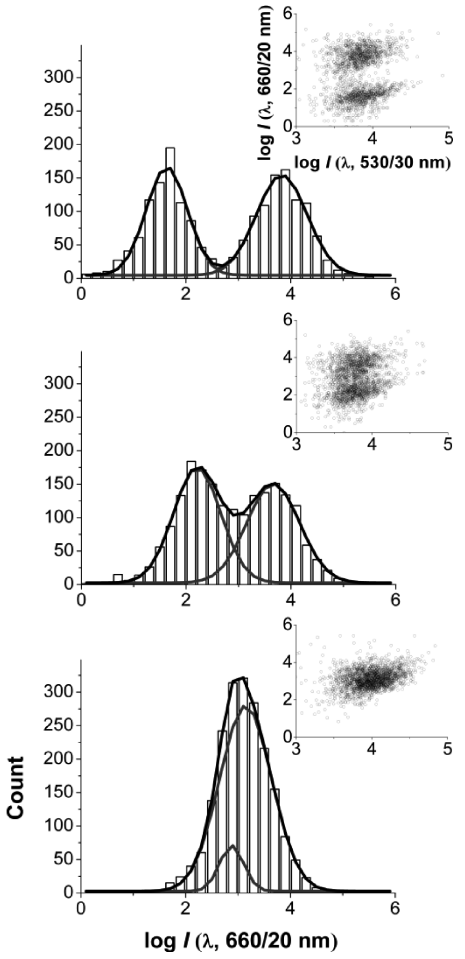

Figure 4. Flow cytometry data highlighting biotinylated-probe migration between Type II immobilized avidin DN particles. A sample containing particles with the unlabeled biotinylated oligonucleotide (P2) bound was mixed with a sample of an equal amount of particles with fluorescently labeled biotinylated oligonucleotide (FP1) attached, the suspension was analyzed by flow cytometry at time points (from top; 0, 143, 1114 min). Initially two discrete fluorescence populations are discernible both visually (histogram and dot-plot) and by Gaussian fitting (Origin 8.1, www.originlab.com). However within 19 h the continued dissociation and reassociation of ligands results in indiscernible populations with the surface of all particles tending toward a mix of both biotinylated-probe types.

(FP1). Particle mixtures were analyzed by flow cytometry and initially two particle populations could be readily distinguished by the difference in their fluorescence intensity (Figure 3). However, over time (up to 19 h), probe dissociation from and reassociation to immobilized avidin merged these into a single fluorescent population, ultimately resulting in a mixture of both fluorescent and nonfluorescent probes on every particle (Figure 4).

The migration of biotinylated probes highlights the importance of minimizing the time between initiation and sample reading in this assay format. An example of this can be found in our previous work where multiplexed encoded-microparticle DNA hybridization assays were completed within 40 min to successfully discriminate between sequences differing by a single base[27] The avidin−biotin capture system may not be ideally suited to assays run over longer time scales, especially where the detection of multiple analytes is required. An example of this may be the immobilization of specific antibodies to quantum dots for use in multiplexed biological imaging, with different cellular targets for each antibody-quantum dot pair.[43] When biotin dissociation is significant, the time scale for any assays would be limited. The rate constants for dissociation and association therefore define the time scale limits for multiplexed experiments. Further problems may be encountered when probes have been immobilized and then stored as a mixture for long periods in advance of their use in a multiplexed assay. Migration problems during long-term storage might be mitigated by storing dry then rehydrating prior to use. This should work well for microarrays, but aggregation may be a problem for some particle-based systems.

SUMMARY

Quantitative comparisons have been made between three popular protein immobilization methods used to attach avidin to microparticles. Stable protein monolayers were formed in each case, however Type I protein coupling (EDC in situ) resulted in both the highest levels of nonspecific and the lowest levels of specific biotinylated-probe binding. Furthermore, complex stability was reduced with a half-life of less than two days. The immobilization of avidin via activation with NHS or affinity capture with immobilized biotin led to identical association and dissociation rate constants and similar levels of nonspecific binding.

The kinetic and thermodynamic properties of biotinylated-oligonucleotide (FP1) binding to avidin DN immobilized on polymer microparticles via different attachment chemistries have been characterized. Immobilization was shown to have no effect on the rate of association beyond that expected from the reduction of the possible approach trajectories of biotin due to surface attachment. Explicitly, the rate of association was decreased for the immobilized system compared with the free system by the proportion predicted using equations derived from collision theory (eqs eq. 3 and eq. 4). Rates of association of FP1 to avidin DN were found to be reduced relative to the binding of free biotin (which is ~60 times faster). This slower binding was due to the decreased rate of diffusion as a consequence of the conjugation of the (relatively) large oligonucleotide to biotin. Rates of immobilized complex dissociation were increased compared with the nonimmobilized avidin system, which in turn displayed faster dissociation than the wild-type avidin−biotin complex. These results showed that both the loss of the carboxylic acid moiety of biotin (in formation of the amide bond to DNA) and the immobilization of avidin have significant effects on complex stability, leading to the system having a complex half-life of only five days. The decreased association rate and increased dissociation rate for modified biotin to avidin increased the equilibrium dissociation constants to 0.6 pM (solution) and 4 pM (immobilized), a significant shift in equilibrium compared with the tighter binding in the wild type system. This is important as when avidin−biotin binding is used to immobilize or link larger species, binding is often assumed to be as stable as that for unmodified binding, which we have demonstrated is not always the case. Furthermore the increased dissociation rate (and reduced half-life) has implications for the use of avidin affinity as a probe immobilization method in multiplexed arrays when different probe types are to share the same sample solution. This was exemplified when different probes were immobilized on

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different particles, then mixed and probe migration was observed with the previously heterogeneous particle sets being indistinguishable within one day.

These results highlight potential problems with the use of the avidin−biotin system for the immobilization of sensor probes in multiplexed biological assays. This need not preclude the use of the system but may be important for researchers to consider. With careful optimization of multiplex assay conditions these problems can be circumvented, most notably by decreasing the storage and assay times over which probes are mixed within the same buffer solution.

■ ASSOCIATED CONTENT

Supporting Information. Figures and discussion regarding fluorescence quenching of complexed avidin DN-FP1 in solution and the determination of nonspecific binding between FP1 and immobilized protein and the blocking of this binding. Also included is a description of the conversion of experimental data into kinetic and thermodynamic constants and equilibrium dissociation constants, K_d, for particle-immobilized avidin DN. Types of line fitting and goodness of fit measurements for graphical data is included and detailed synthetic methods for microparticle functionalization are also documented. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel:+44 2380 595919. E-mail: plr2@soton.ac.uk.

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■ NOTE ADDED AFTER ASAP PUBLICATION

This paper was published on the Web on February 3, 2011. Equations1 and 2 were corrected. The corrected version was reposted on February 15, 2011.