# Attenuated-Affinity Biotin Analogs for Catch-and-Release with Streptavidin

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## ABSTRACT

The binding between streptavin and biotin, which is one of the strongest non-covalent interactions known in nature, is used ubiquitously in molecular biology. However, when a biotinylated molecule is captured for testing purposes and cannot be easily removed from the streptavidin-biotin complex, the extreme binding strength is a disadvantage. A successfully engineered streptavin-biotin system for capture and release of sensitive biomaterials must have 1) a strong binding interaction, meaning a low equilibrium binding constant, $K_D$; 2) a fast on-rate, $k_a$, permitting formation of a binding complex within short incubation times; 3) a fast off-rate, $k_d$, permitting rapid re-equilibration of the system upon introducing a competitive binder. Here we present several biotin analogs that have attenuated affinities for streptavidin. Using these compounds, we demonstrate several experimental techniques that were not feasible with prior streptavidin or biotin analogs.

***Keywords***: streptavidin, biotin, affinity, kinetics, binding

## 1 INTRODUCTION

Streptavidin and biotin biomolecules are used extensively in biotechnology for their ability to self-assemble. Since streptavidin is a tetrameric protein with four biotin-binding sites, it can be used for homo- or hetero-crosslinking of biotinylated materials. In many cases, the near-irreversibility of the streptavidin-biotin binding interaction is a positive feature, permitting fabrication of semi-permanent nanostructures with biologically compatible building blocks [1]. But for one very important application—enrichment of a biotinylated target for downstream analysis—the irreversibility is a serious hindrance to the workflow.

Several approaches have been used to address the problem of irreversibility of binding, most involving alteration of either the streptavidin protein or the biotin molecule at the sites of interaction. Nitration of tyrosine residues of streptavidin yields a protein with pH-dependent biotin-binding, but this is still problematic when dealing with biomolecules that are only stable around neutral pH. Removal of biotin's sulfur atom yields desthiobiotin, which has a reduced affinity for streptavidin and can be displaced with native D-biotin. In this case, the problem is that the rate of elution of desthiobiotinylated materials is too slow.

What has been mostly overlooked in the engineering of biotin and streptavidin analogs is the importance of the individual rate components, $k_a$ and $k_d$, not just the equilibrium binding constant, $K_D$. Since the $K_D$ is the ratio of $k_d/k_a$, it can be represented by an affinity isotherm.

Behavior of materials on the same affinity isotherms can be very different. For a binding interaction with high $k_a$ and high $k_d$, equilibration is very rapid. This property is useful in dynamic homogeneous systems, such as in-solution sensors. In contrast, if $k_a$ and $k_d$ are both very low, the system takes longer to equilibrate. This property is useful in heterogeneous systems involving changing solutions and washing, such as cell-staining or immunosorbent assays, where a long incubation time is acceptable, but dissociation of the binding complex is not. These two extremes of a 10 n<u>M</u> affinity isotherm are illustrated in simulated binding curves in Figure 1.

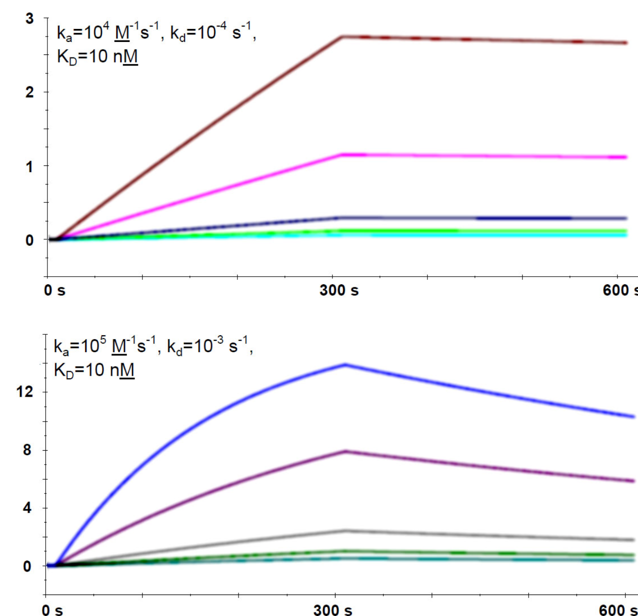


Figure 1. Simulated curves for association and dissociation of 10 n<u>M</u> $K_D$ binding partners, generated with BIAevaluation 4.0.1 software (Biacore / GE Healthcare; binding response in resonance units (RU); first half = association phase; second half = dissociation phase).

Complicating matters for the streptavidin-biotin system, the presence of four biotin-binding sites on streptavidin greatly increases the odds of a biotinylated material rebinding after a dissociation event. This rebinding effect makes the apparent affinity much higher than for monomeric binding partners [5], and poses challenges for analytical kinetic measurements.

Because of the challenges posed in analyzing affinities of biotin analogs, several techniques were employed, including biolayer interferometry kinetic analysis with a FortéBio Octet® RED system, real-time monitoring of dissociation of affinity complexes by homogeneous TR-FRET, and magnetic bead separation followed by elution.

## 2 MATERIALS AND METHODS

Biotin analogs with pendant groups or atom substitutions were synthesized; the moieties responsible for hydrogen bonding to streptavidin amino acid residues were specifically targetted. Structures are shown in Fig. 2.

Biotin analogs were converted to amine-reactive succinimidyl esters with various linkers, which were then reacted with proteins to yield biotin-analog conjugates using typical bioconjugation conditions [6]. Conjugates were purified via size-exclusion chromatography with Bio-Rad P-30 Medium resin.

### 2.1 FortéBio Octet® Kinetic Measurements

Streptavidin-coated sensors were used to measure association and dissociation of biotin-analog Goat anti-Mouse IgG conjugates in a FortéBio Octet® RED biolayer interferometry system. For the dissociation phase, the standard kinetics buffer (Phosphate Buffered Saline (PBS) containing Bovine Serum Albumin (BSA) and Tween®-20) was compared to buffer containing an excess of bis-biotin (a competitive binder) to assess the magnitude of rebinding effects. Curves were analyzed using FortéBio software. Reported rate constants are for dissociation in buffer alone without competitor.

### 2.2 TR-FRET Measurements

A sandwich-style binding complex was made by mixing three conjugates together: 1) streptavidin, 2 µg/mL (labeled with Alexa Fluor® 488 dye), 2) Mouse IgG, 1 µg/mL (labeled with N3'-Ethyl-Biotin, analog 12), and 3) Goat anti-Mouse IgG, 6 µg/mL (labeled with LanthaScreen® Amine Reactive Tb Chelate). All indicated concentrations are for the final mixture in a buffer comprised of PBS pH 7.4, 0.1% BSA, 0.1% Tween-80, and 4 mM sodium azide. Time Resolved Fluorescence Resonance Energy Transfer (TR-FRET) measurements were made with a TECAN Infinite® M1000 plate reader (TECAN Group Ltd.) using an excitation wavelength of 332 nm (slit width = 20 nm), emission wavelength of 520 nm (slit width = 10 nm), a lag time of 50 µs, and an integration time of 2000 µs.

Dilution series of biocytin (Biotinyl-L-Lysine) or bis-biotin were added to a 96-well white, conical-bottom, polypropylene microtiter plate (Greiner Bio-One) at concentrations ranging from 4-256 µM in terms of biotin (each mole of bis-biotin contains two biotin moieties). As biotin binds to streptavidin, blocking binding by the biotin analog, the affinity complex dissociates, increasing the distance between the Terbium-Chelate-labeled TR-FRET donor and the Alexa Fluor® 488 dye labeled acceptor. This causes diminishment of the TR-FRET signal. Relative fluorescence response (RFU) was measured for the first 500 seconds, and these curves were fit to an exponential decay model (Equation 1) using GraphPad Prism 4 software to determine time constants.

$$RFU = Ae^{-t/\tau} + background \quad (1)$$

### 2.3 Magnetic Bead Measurements

R-Phycoerythrin (R-PE) was conjugated to N3'-Ethyl-Biotin-$(Polyethyleneoxide)_4$ using amine-reactive chemistry as described previously. A dilution series of the R-PE conjugate ranging in concentration from 1.6-100 ng/well was added to buffer plus or minus 20 µg/well Dynal My One Streptavidin T1 magnetic beads. The buffer, microtiter plate, and plate reader were the same as used for TR-FRET measurements.

After adding all reagents to the plate, it was incubated overnight on a plate shaker at 850 rpm (Eppendorf Thermomixer R). Following incubation, the magnetic beads were pulled to the sides of the wells using a home-built array of bar magnets, and fluorescence of the supernatants measured using excitation wavelength of 490 nm (slit width = 10 nm) and emission wavelength of 575 nm (slit width = 10 nm).

Following fluorescence measurements, supernatants were removed from the bead-containing wells and replaced with buffer containing 100 µM bis-biotin. Beads were pulled aside and the supernatant fluorescence measured after 40 and 60 minutes of incubation at 850 rpm.

## 3 RESULTS AND DISCUSSION

On- and off-rate constants were determined for biotin analogs using the FortéBio Octet® RED system with dissociation in buffer alone, with no competitive binders present. These results are presented in Figure 3.

The caveat for these rates is that they include the rebinding effect, which greatly increases measured affinity. If rebinding is blocked by a higher-affinity binder (such as biotin or bis-biotin), the measured off-rate, $k_d$, will be much faster. This is shown clearly in Figure 4, where a conjugate of Compound 12 (N3'-Ethyl Biotin) is allowed to dissociate in kinetics buffer with or without bis-biotin.


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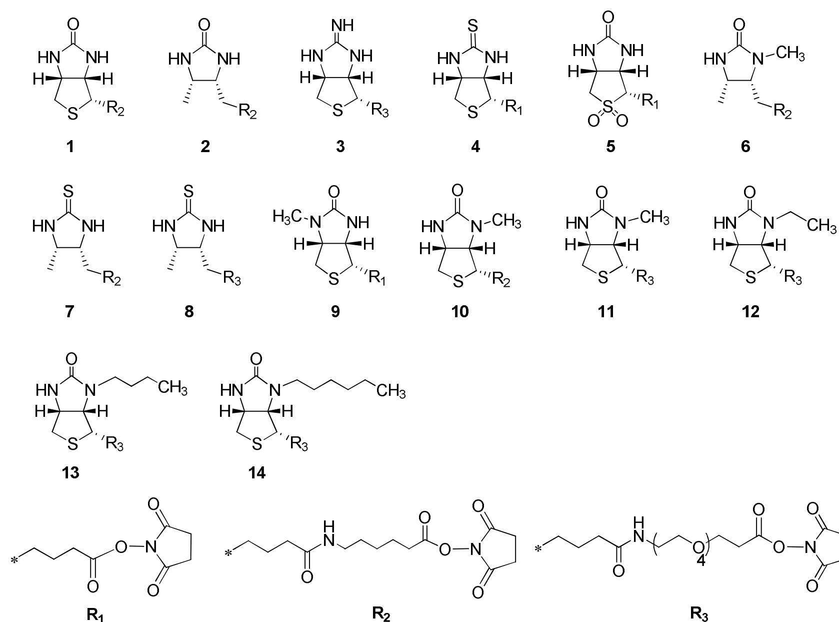


Figure 2. Amine-reactive succinimidyl esters of D-biotin (1) and analogs with various linkers

During the association phase of the binding curve, both association and dissocation occur simultaneously. During the dissociation phase, only dissociation occurs. This implys that determination of the correct on-rate requires an accurate off-rate, which is impossible to determine because of rebinding effects. For affinity measurements of antibody fragments, the way to reduce rebinding effects is to lower the density of surface-bound ligands. Because streptavidin has four biotin-binding sites, both intermolecular and intramolecular rebinding can occur, so lowering surface density of streptavidin has only a limited effect on reducing rebinding artifacts.

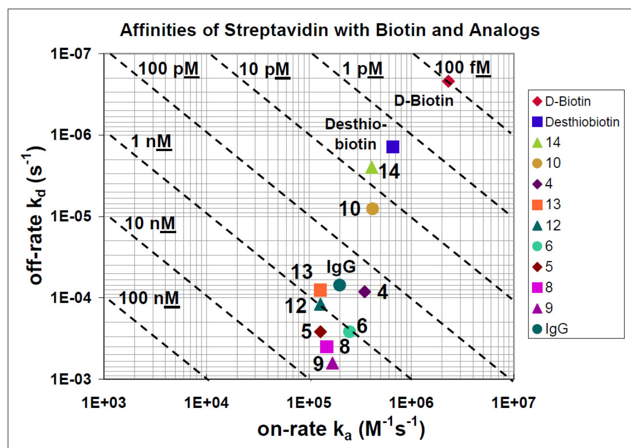


Figure 3. Plot of affinities of D-biotin and analogs with streptavidin. Dashed lines indicate equal affinity ($K_D$) isotherms. A representative antibody (IgG) is included for comparison

Related to the issue of blocking rebinding to streptavidin is the question of what concentration of biotin or bis-biotin to use when eluting biotin analogs from streptavidin. TR-FRET measurements address this issue: as the concentration of biotin in the elution buffer increases, the measured rate of dissociation will approach the true dissociation rate without rebinding.

It is expected that bis-biotin will be more effective than the equivalent concentration of biotin because once one half of the bis-biotin binds to streptavidin, the other half is anchored to the protein in close proximity to the other biotin-binding sites, resulting in an extremely high local concentration of biotin. If this is true, the time constants for dissociation curves for bis-biotin will be systematically lower than for biocytin, which is indeed the case as shown in Figure 5.

Because one of the desired applications of the biotin analogs is enrichment of labeled targets, an R-PE conjugate of Compound 12 was captured on streptavidin magnetic beads, then eluted. Efficiency of capture and release were calculated based on supernatant fluorescence intensity, quantitated using the bead-free R-PE solutions. Figure 6 shows capture of the R-PE conjugate and release by addition of biocytin.

## 4 CONCLUSIONS

The multivalent nature of streptavidin makes rebinding effects impossible to completely eliminate when measuring kinetics of binding. Unlike antibodies, which can be digested into monovalent Fab fragments for kinetic analysis, streptavidin has resisted attempts to be chemically or enzymatically converted into functional monomers. Determination of the "true" affinity may be possible by measuring dissociation in the presence of competitive

binders. However, for practical purposes it may make more sense to think of streptavidin-biotin binding as modal, such that the effective affinity can be switched from nanomolar to micromolar by adding competitive binders. Using biocytin, bis-biotin, or related compounds to elute captured biomolecules is a much gentler method than the current alternatives—for elution of nitrated-streptavidin/biotin, low pH; for unmodified streptavidin/biotin, high concentrations of chaotropes such as urea or guanidine, or detergents and reducing agents.

As was demonstrated with the TR-FRET experiments, a preformed affinity complex using conjugates of biotin analogs can be disrupted with biocytin or bis-biotin competitors. By picking a biotin analog with the desired on- and off-rates, affinity complexes can be made with tunable time-release properties upon addition of eluent. One could imagine drying both affinity complex and eluent components to construct a time-release system activated by water.

Between capture-and-release applications and time-release applications, these attenuated-affinity biotin analogs will add versatility, flexibility, and precision to the molecular biologist's toolbox—a surgeon's scalpel, rather than a sledgehammer. Furthermore, because the components are non-toxic biomolecules, fabrication and consumption of such nanotools will have a low environmental burden.

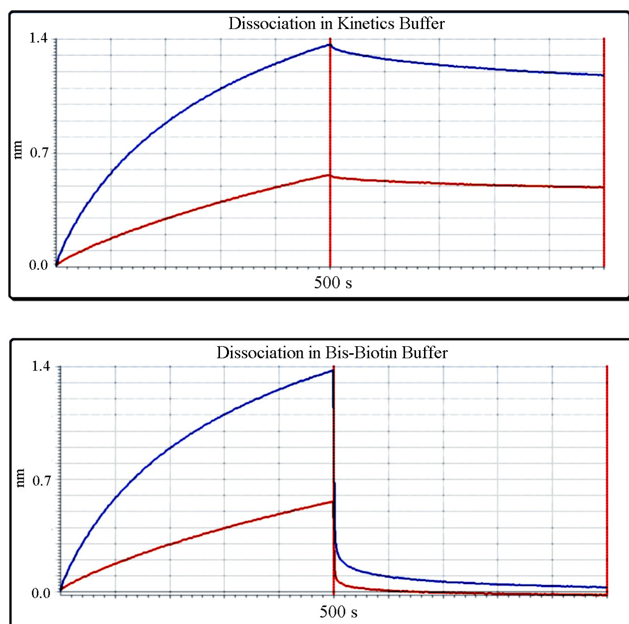


Figure 4. Biolayer interferometry sensorgrams of association and dissociation of a Goat anti-Mouse IgG conjugate of Compound 12 to streptavidin biosensors

## 5 ACKNOWLEDGMENTS

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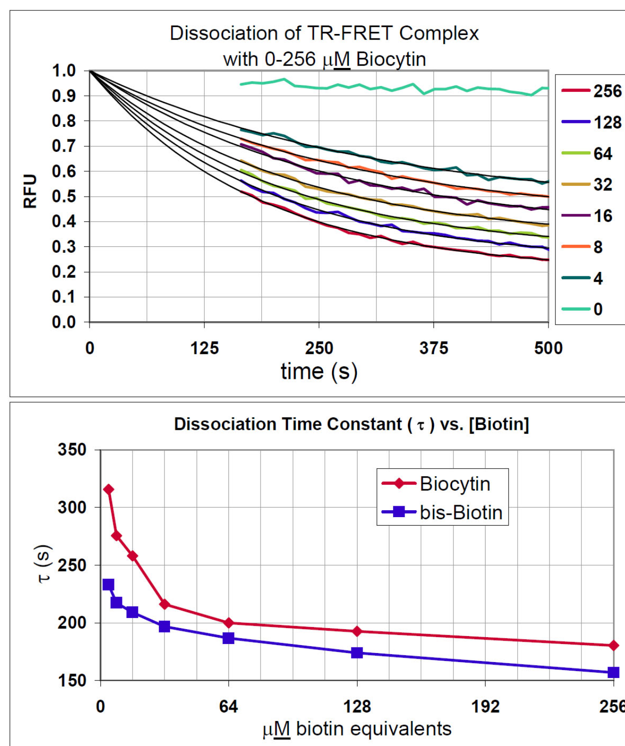


Figure 5. Disruption of an affinity complex by biocytin or bis-biotin as measured by TR-FRET.

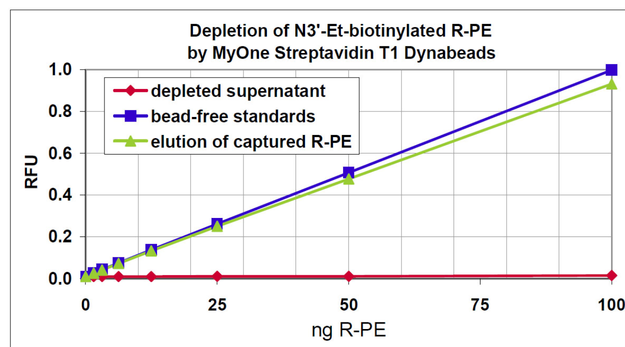


Figure 6. Capture/release of an R-PE conjugate of Compound 12 by streptavidin magnetic beads/biocytin

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