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Design of a reversible biotin analog and applications in protein labeling, detection, and isolation†

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To expand the applicability of the biotin–(strept)avidin system, a biotin analog with reversible binding under non-denaturing conditions has been designed, and its applications in protein labeling, detection, and isolation have been evaluated.

The interaction between biotin and avidin or streptavidin is the strongest non-covalent interaction known in nature.[1] The biotin–(strept)avidin system has become a standard platform for many applications in biotechnology and nanotechnology.[2–4] One of the major limitations of the biotin–(strept)avidin system is the almost irreversible binding under physiological conditions.[5] To disrupt a biotin–(strept)avidin complex, it is usually necessary to denature the avidin or streptavidin proteins using harsh conditions.[6]

In order to make the binding of biotin with (strept)avidin more easily reversible under mild conditions, much work has been done to modify avidin or streptavidin by chemical modification[7] and genetic mutations.[8] Another approach is to design biotin analogs with reduced affinity to avidin and streptavidin.[9–11] The reported biotin analogs such as biotin sulfone, 2′-thiobiotin, and desthiobiotin provide partial reversibility, but there is still a need for better biotin analogs that permit truly reversible binding, both (1) fast and complete binding, and (2) fast and complete release, under mild, physiological conditions.

The crystal structures of biotin–avidin and biotin–streptavidin complexes provide valuable information for designing new biotin analogs.[12,13] The biotin-binding pocket of streptavidin, shown in Fig. 1, displays a virtually perfect fit with biotin, and the ureido-ring of biotin provides the most important hydrogen bonding interactions at the streptavidin-binding sites. By detailed analysis of the biotin-binding pocket of streptavidin, the position of 3′-*NH* of biotin has open space available for modification. Thus, we decided to focus on the modification at 3′-*NH* of biotin to make lower affinity biotin analogs by blocking the hydrogen bonding interaction with Ser45.

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† Electronic supplementary information (ESI) available: Details of experiments for synthesis, characterization, capture and release assay, kinetic measurement, and protein labeling, detection and isolation, additional cell images. See DOI: 10.1039/c1cc12738a

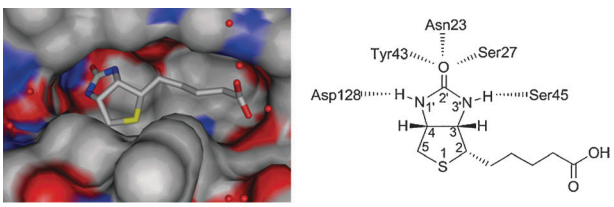
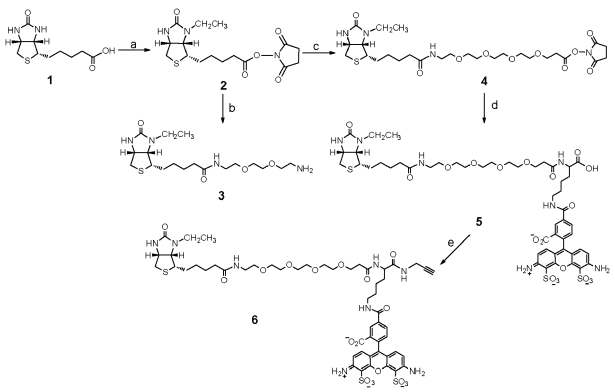


Fig. 1 Illustrations of the biotin-binding pocket and key H-bonding interactions between biotin and streptavidin.



Scheme 1 Synthesis of *N*3′-ethyl biotin derivatives. (a) HCl, MeOH; DMTrCl, DMAP, pyridine; CH_3CH_2I, NaH, DMF; 80% HOAc; 1 M NaOH, MeOH; TSTU, TEA, DMF; (b) 1,8-diaminotriethyleneglycol, DMF; (c) amino-dPEG$_4$-acid, TEA, DMF, H_2O; TSTU, TEA, DMF; (d) H-Lys(Boc)-OBu^t, TEA, DMF; TFA, DCM; AF488-5TFP, TEA, DMF; (e) TSTU, DMAP, DMF; propargylamine, DMF.

Scheme 1 shows the synthesis of the new biotin analog, *N*3′-ethyl biotin. Biotin **1** was converted to biotin methylester, followed by selective protection with the 4,4′-dimethoxytrityl (DMTr) group at the *N*1′ position, and alkylation at the *N*3′ position with iodoethane. The DMTr protecting group was removed by 80% HOAc, and the methylester was hydrolyzed to the free acid by NaOH, then was converted to succinimidylester **2** using *O*-(*N*-succinimidyl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TSTU). The *N*3′-ethyl biotin succinimidylester **2** was converted to **3** using 1,8-diaminotriethyleneglycol. Compound **2** was also reacted with amino-dPEG$_4$-acid, and then converted to succinimidylester **4** with TSTU. Compound **4** was conjugated with H-Lys(Boc)-OBu^t, followed by deprotection with TFA and labeling with Alexa Fluor 488 to give **5**.

Table 1 The capture and release efficiency of biocytin and *N*3′-ethyl biotin with streptavidin

Compound	Capture efficiency (%)	Release efficiency (%)
Biocytin	96 ± 2	<5
*N*3′-Ethyl biotin **5**	94 ± 3	95 ± 4

Compound **5** was converted to succinimidylester, then reacted with propargylamine to afford **6**.

In order to evaluate the binding affinity and reversibility of *N*3′-ethyl biotin with streptavidin, a simple fluorescent binding and release assay was designed. Briefly, *N*3′-ethyl biotin-AF488 conjugate **5** was incubated with M-280 Streptavidin Dynabeads in pH 7.4 PBS buffer. After 25 min incubation at room temperature, the magnetic beads were separated from the supernatant, which was used to measure the capture efficiency, by measuring the fluorescent intensity of the supernatant solution to determine the amount that was not captured. Then, the *N*3′-ethyl biotin **5** bound magnetic beads were washed with PBS buffer to remove weak or non-specific binding. The reversibility was tested by incubating the *N*3′-ethyl biotin **5** bound magnetic beads with 2 mM biotin in PBS buffer at room temperature for 5 min, then the magnetic beads were concentrated by a magnet, and the fluorescent intensity in the solution was measured to determine the amount that was released. As a control, the biocytin-AF488 conjugate was also tested in the same assay format. As shown in Table 1, the *N*3′-ethyl biotin displays high binding efficiency and the binding is fully reversible with a biotin competing reagent under non-denaturing conditions.

The binding constant and on-and-off rate constant of *N*3′-ethyl biotin with streptavidin were tested using a label-free ForteBio system.[14] The *N*3′-ethyl biotin **3** was covalently immobilized to amine reactive sensor tips from ForteBio. To generate the association curve, the *N*3′-ethyl biotin labeled biosensor tips were incubated with streptavidin at 25 °C. The dissociation curve was determined by incubating the streptavidin bound sensor tips in PBS buffer described above, as well as in PBS buffer containing 2 mM biotin at 25 °C. As shown in Fig. 2, the *N*3′-ethyl biotin shows a fast on-rate with streptavidin ($k_{on} \approx 1.5 \times 10^5$ M^{-1} S^{-1}), and a relative slow off-rate from streptavidin in PBS buffer ($k_{off} \approx 1.2 \times 10^{-4}$ S^{-1}). The dissociation constant K_D of *N*3′-ethyl biotin is about 0.8 nM. In the presence of 2 mM biotin, the *N*3′-ethyl biotin displays fast off-rate ($k_{off} \approx 5.8 \times 10^{-2}$ S^{-1}), thus leading to complete dissociation from streptavidin.

The reversibility of *N*3′-ethyl biotin with streptavidin provides valuable applications in protein labeling, detection, and isolation. To evaluate the efficiency using *N*3′-ethyl biotin, the *N*3′-ethyl biotin alkyne **6** was prepared for labeling and isolation of azide-containing proteins *via* click chemistry.[15] Recently, the bio-orthogonal labeling of glycoproteins in cells incubated with azido sugars or alkynyl sugars has been developed for imaging the localization, trafficking, and dynamics of glycans.[16–18] We have adapted the azido sugar labeling system to demonstrate the ability of *N*3′-ethyl biotin for efficient labeling, detection, and isolation in a biological system. The HeLa cells were first incubated with azido sugars (Ac_4GlcNAz, Ac_4GalNAz, Ac_4ManNAz) containing media for 24 h, respectively. The fixed HeLa cells were labeled with *N*3′-ethyl biotin alkyne **6** in the presence of $CuSO_4$, tris(3-hydroxypropyltriazolylmethyl)amine (THPTA), and sodium ascorbate.[19] The cells incubated with azido sugars show a increased fluorescence signal when compared to control cells that were not incubated with azido sugars, as shown in Fig. 3 and ESI†, indicating the labeling of cells with *N*3′-ethyl biotin alkyne **6** *via* formation of a triazole linkage. The enhancement of the fluorescence signal is only observed in the presence of both catalyst Cu(I) and azido glycans (ESI†). The Cu(I) chelating ligand, THPTA, facilitates the reaction and protects protein damage,[19] but is not necessary for labeling of cells. These results demonstrate *N*3′-ethyl biotin alkyne **6** is an efficient reagent for labeling and detection in cells.

In order to isolate azido sugar containing glycoproteins from a cell mixture, the Jurkat cells were cultured in Ac_4GlcNAz supplemented media for 24 h. The cell lysate was prepared at ~3 mg mL^{-1} in 0.5% SDS/PBS. *N*3′-Ethyl biotin alkyne **6** and biotin alkyne **7** (Invitrogen) were used to perform parallel protein enrichment experiments. Briefly, cell lysate was incubated with alkyne **6** or **7** in the presence of $CuSO_4$, THPTA, and sodium ascorbate at room temperature. After click labeling, the proteins were enriched with streptavidin agarose at room

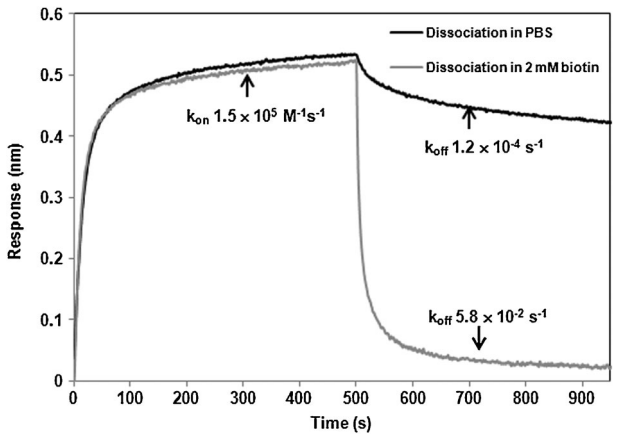


Fig. 2 Association and dissociation curves between *N*3′-ethyl biotin and streptavidin on the ForteBio system.

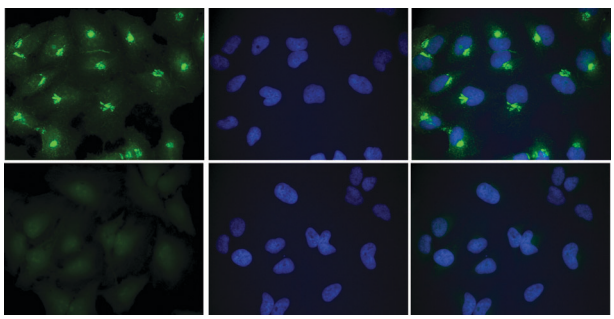


Fig. 3 Fluorescence microscopy of HeLa cells labeled with *N*3′-ethyl biotin alkyne **6**. Cells incubated with Ac_4ManNAz supplemented media (top) or unsupplemented media (bottom) were labeled with *N*3′-ethyl biotin alkyne **6** in the presence of Cu(I)/THPTA as a catalyst. Fluorescence signal from AF488 colored green (left), the nuclear stain, Hoechst 33342, colored blue (center), and the merged images (right) are shown.

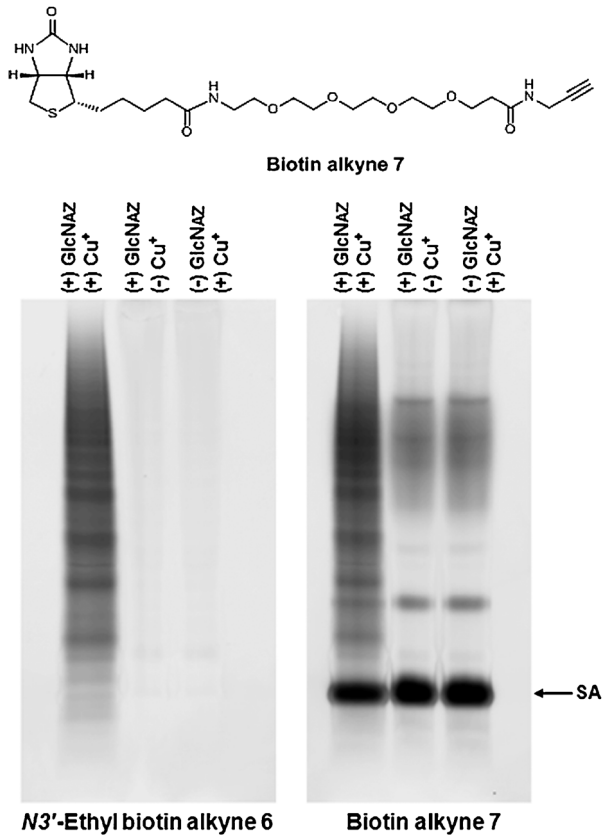


Fig. 4 Isolation of glycoproteins using click chemistry. Cell lysate was incubated with *N*3′-ethyl biotin alkyne **6** or biotin alkyne **7** in the presence of Cu(I)/THPTA, captured by streptavidin agarose, washed, and eluted with either 10 mM HCl or 1% SDS in H_2O at 95 °C, respectively. The purified fractions were analyzed by a reducing 4–12% SDS-PAGE gel and stained with SYPRO-Ruby.

temperature. After the washing step, the proteins labeled with *N*3′-ethyl biotin alkyne **6** were eluted out from streptavidin agarose using 2 mM biotin or 10 mM HCl in H_2O; the proteins labeled with biotin alkyne **7** were eluted out by heating protein-bound streptavidin agarose in 1% SDS in H_2O at 95 °C for 5 min. As a control, cell lysate was also incubated with alkyne **6** or **7** without the Cu(I) catalyst. Another control is using untreated cell lysate to perform the same isolation procedure. As shown in Fig. 4, both control experiments without the Cu(I) catalyst, and without Ac_4GlcNAz treatment, show a very low background using *N*3′-ethyl biotin alkyne **6**, indicating the azido containing glycoproteins were selectively isolated by labeling with *N*3′-ethyl biotin alkyne **6** *via* formation of a triazole linkage, followed by capture with streptavidin agarose *via* interaction of *N*3′-ethyl biotin with streptavidin. One major problem using biotin alkyne **7** is the isolated proteins are contaminated with streptavidin (SA), which is leached out from agarose beads by the harsh, denaturing condition. These results demonstrate using *N*3′-ethyl biotin has great advantage in gentle release to isolate clean proteins for downstream analysis.

In summary, we have developed a new biotin analog, *N*3′-ethyl biotin, with reversible binding affinity with streptavidin ($K_D \approx 0.8$ nM), and with excellent capture and release efficiency with streptavidin under gentle non-denaturing conditions. Applications in glycoprotein labeling, detection, and isolation using *N*3′-ethyl biotin alkyne **6** *via* click chemistry have been demonstrated. This new biotin analog should be useful to expand the new applications of standard biotin–streptavidin technology.

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