

A Rhizavidin Monomer with Nearly Multimeric Avidin-Like Binding Stability Against Biotin Conjugates

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Abstract: Developing a monomeric form of an avidin-like protein with highly stable biotin binding properties has been a major challenge in biotin-avidin linking technology. Here we report a monomeric avidin-like protein—enhanced monoavidin (eMA)—with off-rates almost comparable to those of multimeric avidin proteins against various biotin conjugates. Enhanced monoavidin (eMA) was developed from naturally dimeric rhizavidin by optimally maintaining protein rigidity during monomerization and additionally shielding the bound biotin by diverse engineering of the surface residues. eMA allowed the monovalent and nonperturbing labeling of head-group-biotinylated lipids in bilayer membranes. In addition, we fabricated an unprecedented 24-meric avidin probe by fusing eMA to a multimeric cage protein. The 24-meric avidin and eMA were utilized to demonstrate how artificial clustering of cell-surface proteins greatly enhances the internalization rates of assembled proteins on live cells.

Streptavidin (STA) and avidin proteins are homotetramers that have extraordinarily high binding affinities for the small molecule biotin ($K_d \approx 10^{-14}$ M).^[1] This remarkably strong and specific interaction between (strept)avidin and biotin has been exploited in a wide range of applications from molecular labeling and bio-interfaces to nanostructure assembly.^[2] The tetrameric nature of avidin proteins, however, can lead to unwanted cross-linking of the biotin conjugates of interest. For example, streptavidin-based labeling of biotinylated cell-surface receptors disrupted the normal function of the receptors through oligomerization.^[3] However, developing monomeric (strept)avidin without a dramatic decrease ($K_d \approx 10^{-7}$ – 10^{-9} M) in biotin affinity has not been successful, because a tryptophan residue from an adjacent subunit is critical for strong biotin binding.^[4] A tetrameric but mono-

valent streptavidin with a single biotin binding site was developed as an alternative approach.^[5] Although this monovalent streptavidin is very effective for biotin labeling without cross-linking,^[5] a truly monomeric (strept)avidin protein is still highly desired for minimal perturbation of biotin-labeled targets and even more diverse applications of avidin proteins.^[6] For example, monomeric avidin can be genetically fused with other proteins of interest without oligomerization, and this fusion will also allow the creation of new forms of avidin proteins with different spatial organization and valency of the biotin binding sites.

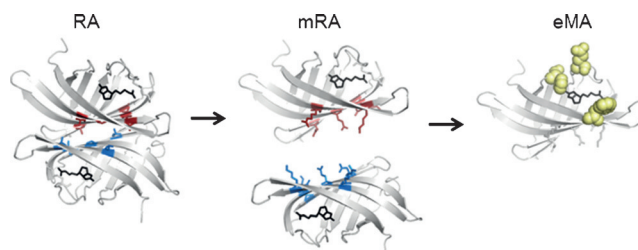
Rhizavidin (RA) from *Rhizobium etli* is the first natural dimer in the avidin protein family with a high binding affinity for biotin.^[7] Interestingly, the biotin binding pocket of rhizavidin consists of residues from a single monomer subunit without the critical inter-subunit tryptophan.^[8] Dimeric rhizavidin, however, contains a characteristic disulfide bond in the biotin binding site, which restrains the protein and leads to a rigid and preformed binding pocket for tight biotin binding without the tryptophan residue.^[8] We, therefore, envisioned that a monomeric avidin protein with a high binding affinity for biotin could be derived from rhizavidin by monomerizing the dimer while minimally altering its natural structure, especially the rigid binding pocket. To monomerize rhizavidin, we introduced various numbers (one, six, and ten mutations) of charged and hydrophilic amino acids at the dimeric interface (Scheme 1; see also Figure S1 in the Supporting Information). The biotin binding properties of the rhizavidin variants were investigated by a gel mobility shift assay with a biotinylated green fluorescent protein (biotin-GFP; Figures 1 a,b; see also Figure S2). Divalent wild-type rhizavidin formed two separated complexes, whereas all the rhizavidin mutants formed a single complex with biotin-GFP, thus indicating successful monomerization of the protein. The monomerized protein with a single mutation (S69R), however, readily aggregated in buffered solutions,

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Scheme 1. Schematic representation of the engineering of monomeric rhizavidin (mRA) and enhanced monoavidin (eMA) from dimeric rhizavidin (RA).

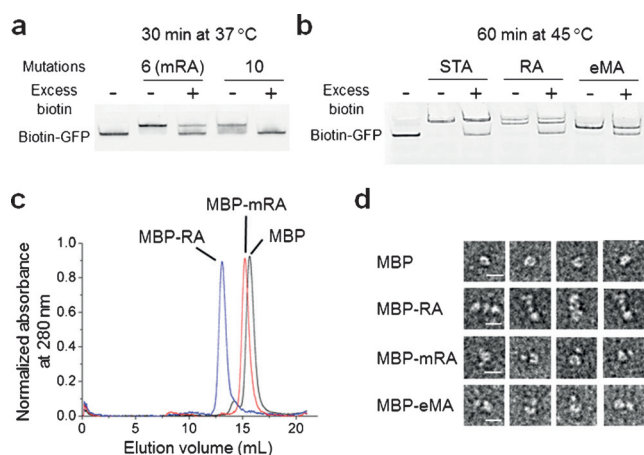


Figure 1. Construction of the monomeric avidin-like protein. a) Native-gel analysis of the biotin binding stability of monomerized rhizavidin variants with six (mRA) or ten mutated residues. The rhizavidin variants were treated with biotin-GFP. The mixtures were incubated with excess biotin for 30 min at 37°C and then analyzed on a 15% native polyacrylamide gel. b) Binding stability of streptavidin (STA), rhizavidin (RA), and eMA toward biotin-GFP upon incubation with excess biotin for 60 min at 45°C. c) Size-exclusion chromatography and d) representative TEM images of MBP and MBP-fused avidin proteins. Scale bars: 5 nm.

likely because many hydrophobic residues at the dimeric interface are exposed to the solution. On the other hand, more heavily mutated monomers (six or ten mutations) were stable without any aggregation (up to ca. 1 mg mL⁻¹). Upon incubation with excess free biotin, however, the monomeric variant with ten mutated residues underwent visibly faster dissociation from biotin-GFP than did the monomer with six mutations (Figure 1a). The more crowded environment of the long hydrophilic residues on the dimeric interface may disrupt the rhizavidin structure and thereby also the preformed biotin binding pocket. The monomeric rhizavidin with six interfacial mutations, which we termed mRA, contains optimal modifications to stabilize the exposed dimeric interfaces as well as to maintain the rigid structure of rhizavidin for tight biotin binding.

The dissociation of mRA from biotin-GFP by excess free biotin was still, however, much faster than that of dimeric rhizavidin (Figure S3), likely because of the inevitable loss of the overall protein rigidity by monomerization. Therefore, we further modified mRA with the aim of shielding the bound biotin from solvent and slow the biotin dissociation.^[9] Two amino acids near the entrance of the biotin binding pocket (E115 and S23) were mutated into various large (often hydrophobic) residues for biotin shielding (Scheme 1). A residue Q46 near E115 and S23 was additionally mutated to a charged residue to maintain protein solubility by compensating for the enhanced hydrophobicity. Among many constructed mRA variants, the E115W/S23H/Q46E mutant showed a highly slowed off-rate from biotin-GFP with preserved protein solubility (Figure S4). Impressively, the binding complexes between the mRA E115W/S23H/Q46E variant and biotin-GFP were fairly stable during incubation at

high temperature (60 min at 45°C), where dissociation was even similar to that of the original dimeric rhizavidin (Figure 1b; Figure S4). We termed this optimized monomeric rhizavidin, with a biotin binding stability significantly improved over previous monomeric avidin proteins,^[4a,b,d] as enhanced monomeric (eMA).

The monomeric structure of mRA and eMA was further confirmed by size-exclusion chromatography (SEC), dynamic light scattering (DLS), and transmission electron microscopy (TEM). The relatively large maltose binding protein (MBP, ca. 44 kDa) was fused to both monomeric and dimeric rhizavidin proteins to enhance the differences in the size as well as the shape of these proteins. Monodisperse and size-dependent SEC elution profiles of free MBP and MBP-fused proteins support the highly homogeneous monomeric structure of monomerized rhizavidin proteins (Figure 1c; Figure S5), which is also strongly validated by DLS analysis (Figure S6). TEM images of MBP-fused proteins visualized the spatial arrangements of the large MBP as well as the small avidin subunits (Figure 1d; Figure S7). A single MBP is linked to monomeric mRA as well as eMA, whereas MBP is clearly dimerized by genetic fusion to dimeric rhizavidin. The thermal stability of eMA was examined by circular dichroism spectroscopy. The melting temperatures of eMA without and with biotin was 41.2°C and 56.2°C, respectively (Figure S8). In addition, eMA was much more susceptible to digestion by proteinase K than tetrameric avidins, as reported with previous monomeric streptavidin proteins (Figure S9).^[10] The eMA protein is soluble in phosphate-buffered saline (PBS, up to ca. 0.5 mg mL⁻¹) and can be stored at -20°C without visible aggregation or loss of binding function.

The stability of eMA binding to various biotin conjugates was evaluated by measuring the off-rate constants under excess biotin at diverse temperatures. At 45°C, the dissociation rate constant of eMA from biotin-GFP was $6.4 \pm 0.2 \times 10^{-5} \text{ s}^{-1}$, which is nearly identical to that of rhizavidin, and only 3.4-fold faster than that of tetrameric streptavidin ($1.9 \pm 0.2 \times 10^{-5} \text{ s}^{-1}$; Figure 2a; Table S1). At 50°C, the off-rate of eMA from biotin-GFP ($2.3 \pm 0.4 \times 10^{-4} \text{ s}^{-1}$) is 2.3-fold faster than that of streptavidin ($9.9 \pm 0.3 \times 10^{-5} \text{ s}^{-1}$), while the off-rates are nearly identical at 55°C (Figure S10). At 37°C, the measured off-rate for eMA ($2.3 \pm 0.1 \times 10^{-5} \text{ s}^{-1}$) indicated a dissociation half-life of 8.3 h, which is even slower than that of rhizavidin (3.8 h). A similar biotin binding stability of eMA was also observed against another biotinylated protein as well as a peptide (Figure S11). We also examined the binding of biotinylated DNA to the avidin proteins. Interestingly, dissociation of wild-type rhizavidin from biotin-DNA ($23 \pm 1.0 \times 10^{-5} \text{ s}^{-1}$) was considerably faster than that of eMA ($2.7 \pm 0.3 \times 10^{-5} \text{ s}^{-1}$) at 37°C (Figure 2b; Figure S12). In the cases of tetrameric streptavidin and neutravidin, only about 20% of the avidins were dissociated from biotin-DNA, even after incubation for 10 h at 37°C (Table S1). Impressively, at 23°C, eMA also showed only 22% dissociation from biotin-DNA after incubation for 10 h (Figure 2c; Figure S13). Moreover, eMA dissociation from biotin-GFP at 23°C was similar to that of neutravidin and of streptavidin (less than 7% dissociation after 10 h), which is significantly slower than that of the most recently developed monomeric streptavidin^[4d] (mSA, $t_{1/2} =$

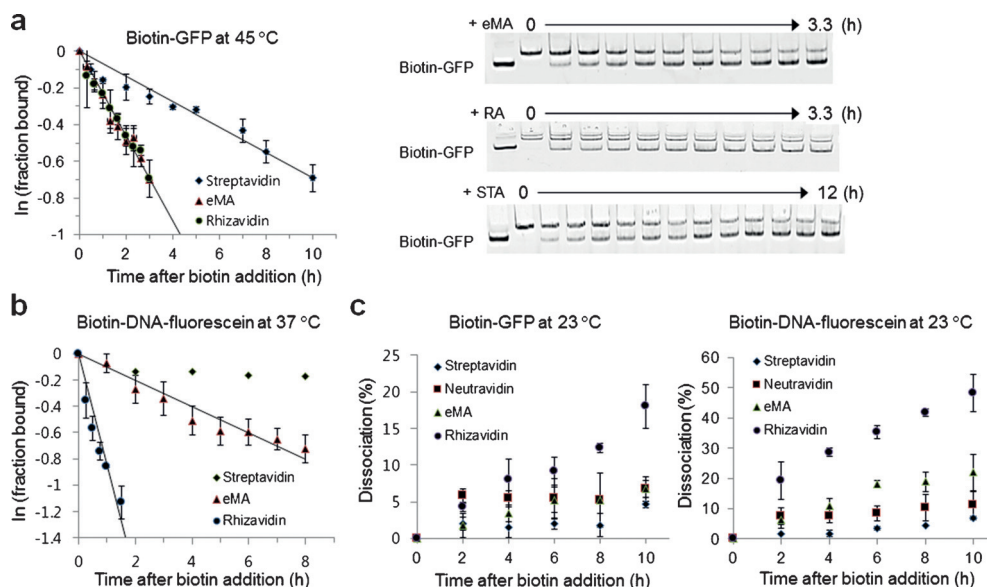


Figure 2. Dissociation rates of avidin proteins from biotin conjugates. a) Off-rates from biotin-GFP at 45 °C. Biotin-GFP was mixed with streptavidin (STA), rhizavidin (RA), or eMA. After various incubation time with excess biotin at 45 °C, the dissociated biotin-GFP was separated and measured on a 15% native polyacrylamide gel (right). b) Off-rates from biotin-DNA-fluorescein at 37 °C. c) Dissociation rates of avidin proteins from biotin-GFP (left) and biotin-DNA-fluorescein (right) at 23 °C. Error bars: 1 standard deviation ($n=3$).

52 min; Figure S14). In addition, half-life times of mSA binding to biotin conjugates at 37 °C were less than 10 min. Dissociation of eMA from surface-bound biotin conjugates was not observed by surface plasmon resonance (SPR) analysis (Figure S15). Against free biotin, eMA shows a faster off-rate ($1.2 \pm 0.3 \times 10^{-3} \text{ s}^{-1}$) with a relative K_d value of $31 \pm 1.0 \text{ pM}$ at 37 °C, which was estimated by analyzing the competition with streptavidin ($K_d \approx 40 \text{ fM}$) for [^3H]biotin (Figures S16 and S17).^[3] Although the binding affinity of eMA toward free biotin is visibly weaker than that of streptavidin, eMA has almost multimeric avidin-like binding stability toward diverse biotin conjugates, and thus the protein can be applicable practically as a monomeric avidin probe against biotinylated targets.

To investigate the monovalent (and thereby nonperturbing) biotin labeling by eMA, we measured the lateral diffusion dynamics of head-group-biotinylated lipids on supported bilayer membranes labeled with dye-conjugated eMA (Figure 3a). Diffusion trajectories of single-dye-conjugated eMAs labeled on the biotinylated lipids were directly observed under a fluorescence microscope (see Video S1), from which the diffusion constants were calculated. The lipids labeled with dye-conjugated eMA showed similar diffusion constants as the lipids labeled with small atto-dye molecules (Figure 3a), which indicates that the labeling of eMA is monovalent and does not modify the mobility of single lipids in the bilayer membranes. Biotin-lipid diffusion constants were previously found to be significantly reduced (more than twice) by multivalent labeling with streptavidin-conjugated quantum dots but not by monovalent quantum dots.^[11]

In addition to monovalent biotin labeling, monomeric eMA can be fused to diverse multimeric proteins to create

new multivalent avidin probes. Here we fused eMA to 24-meric human ferritin, which is a cage-like protein with a diameter of about 12 nm.^[12] Monoavidin-fused ferritin (eMA-Ft) has 24 biotin binding sites displayed symmetrically on the protein cage surface (Figure 3b). Native gel analyses confirmed that fabricated eMA-Ft has a monodisperse protein structure with a high molecular weight as well as eMA having biotin binding ability (Figure S18). In addition, TEM images clearly revealed that eMA-Ft was assembled into the cage-like structure and eMA proteins were well displayed on the exterior surface of the ferritin cage (Figure 3b; Figure S19). A binding titration of eMA-Ft with a biotinylated

DNA probe strongly indicated that all 24 eMA subunits of eMA-Ft are able to bind the biotin-conjugated ligand (Figure S20).

We next applied eMA and eMA-Ft to examine how forced and stable cross-linking of cell-surface proteins affects the internalization rates of these proteins in live cells. Asymmetric protein crowding on a lipid bilayer and subsequent membrane bending is one of the proposed mechanisms for the generation of biomembrane curvatures, which also likely contribute to membrane internalization.^[13] However, little is known regarding how cell-surface membranes react to the artificial clustering of membrane-bound proteins. To generate various degrees of stable protein clusters on a live cell surface, membrane proteins were randomly biotinylated and subsequently treated with dye-conjugated avidin proteins from monomeric eMA, tetrameric STA, to 24-meric eMA-Ft (Figure 3c; Figure S21). Following the avidin binding at 4 °C, plasma membrane activities were initiated by a temperature change to 37 °C. eMA proteins on HeLa cells were slowly internalized after 30 min, whereas streptavidin proteins were visibly internalized after only 10 min. Interestingly, 24-valent eMA-Ft was nearly immediately internalized, with the protein signals being fully inside the cytosols after incubation for 5 min at 37 °C. For comparison, stable monitoring of biotinylated proteins with previous mSA^[4d] was not possible, since most mSA was dissociated from the cell (from biotin conjugates) before 10 min (Figures S14 and S22). The fluorescence signals of avidin proteins are biotin-specific, with no signals being detected without biotinylation (Figure S23). Rapid internalization by eMA-Ft clustering was also observed in other cell lines (Figure S24). Internalized proteins appeared to remain clustered inside cells during incubation

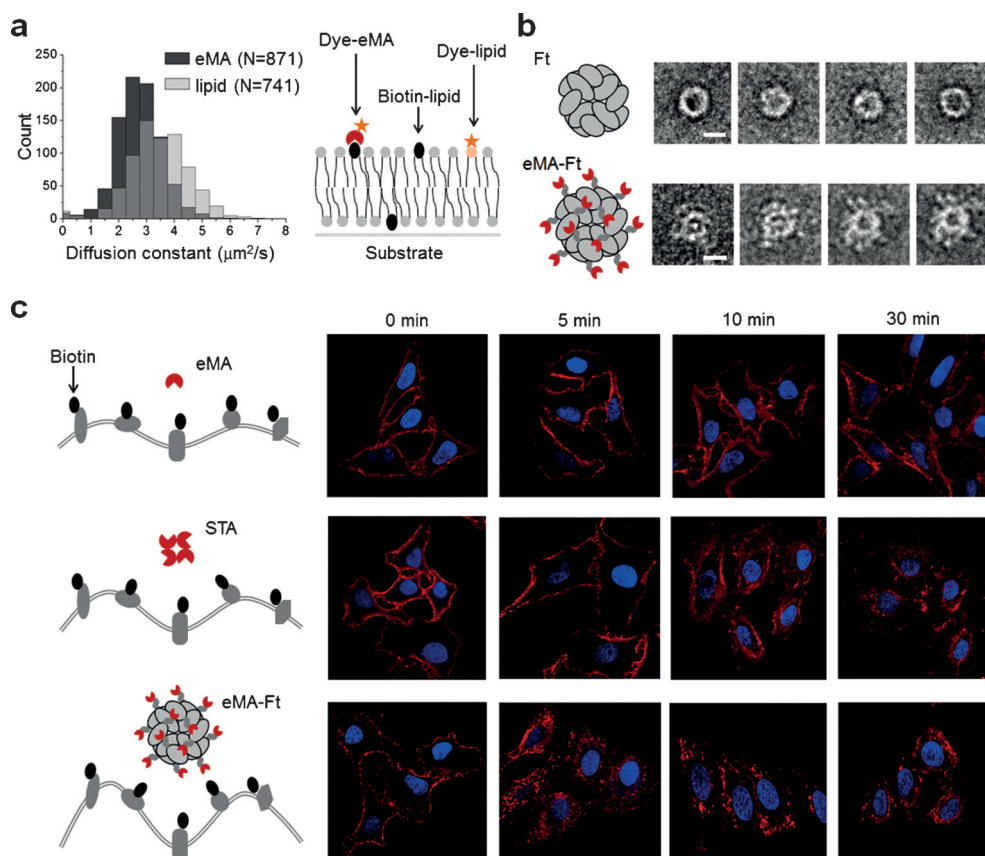


Figure 3. New mono- and high-valent eMA probes. a) Histograms (left) of diffusion constants of single-dye-labeled lipids and eMA-labeled lipids in supported lipid bilayers with a schematic representation of the experiment (right). b) Representative TEM images of 24-meric human ferritin (Ft) and eMA-fused ferritin (eMA-Ft). Scale bars: 10 nm. c) Artificial clustering of cell-surface proteins by mono- (eMA), tetra- (STA), and 24-meric (eMA-Ft) avidin proteins. Cell-surface proteins were randomly biotinylated and treated with Cy5-labeled avidin proteins (red) at 4 °C. Cell-surface labeling and subsequent internalization of avidin proteins were monitored at various time points during incubation for 30 min at 37 °C.

for 30 min without specific targeting to certain organelles (Figure S25). Although more studies are needed to elucidate the physical or cellular mechanism of clustered protein internalization, the data show that the tight and high-valent cross-linking of membrane proteins leads to unusually fast internalization of protein.

In conclusion, we reported a monomeric avidin-like protein that showed almost multimeric avidin-like binding stability against various biotin conjugates. Off-rates of eMA are often slower than those of dimeric rhizavidin, and even comparable to those of tetrameric avidin proteins at room temperature. Our eMA offers the first practically applicable monomeric avidin linker, which allows truly monomeric biotin labeling with minimal perturbation. In addition, the possibility of fabricating new high-valent avidin probes with designed orientations and valencies (such as 24-meric eMA-Ft) will greatly diversify avidin/biotin linking strategies to build new bio- and nanostructures.^[14] For example, high-valent avidin probes can be utilized effectively to assemble highly potent multivalent vaccines.^[15] Further efforts will be directed toward improving the rather limited solubility of eMA and expressing soluble eMA in cells.

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