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A Multifunctional Single-Attachment-Point Reagent for Controlled Protein Biotinylation

Elisabeth Garanger[†], Ralph Weissleder^{†,‡}, and Lee Josephson^{†,*}

[†]Center for Molecular Imaging Research, Massachusetts General Hospital and Harvard Medical School, Building 149, 13th Street, Boston, MA 02129 (USA)

[‡]Center for Systems Biology, Massachusetts General Hospital and Harvard Medical School, 185 Cambridge Street, Boston, MA 02114 (USA)

Abstract

The biotin/avidin system is one of the most widely used affinity detection and affinity capture systems in biology. However, the determination of the exact number of biotin tags attached onto a substrate is complicated by the fact that biotin does not present any light-absorbing or -emitting properties. Here we describe a fluorescent biotinylation reagent designed from the general Multifunctional Single-Attachment-Point (MSAP) reagent concept. A Lys-Cys dipeptide scaffold was used to display a biotin functional group and a fluorescein functional group along with a *N*-Hydroxysuccinimide ester reactive group. The resulting bifunctional MSAP reagent, FI-Biotin-NHS, was used to prepare a monobiotinylated version of cetuximab, which was further reacted with avidin to obtain a soluble avidin-based cetuximab oligomer. The MSAP peptide-scaffold approach allows fluorophores, chromophores or reactive groups to be combined with biotin and provides a broad approach to obtain multifunctional biotin-based reagents.

INTRODUCTION

The recognition of biotinylated molecules by avidin (or streptavidin) is one of the most widely exploited affinity capture systems in immunohistochemistry, biochemical purification, and in a variety of assay systems.(1) In many systems, where either avidin or biotin is immobilized, the capture is not highly sensitive to the number of biotins per mole of biotinylated protein. However, when the reaction between avidin and a biotinylated protein occurs in solution, the degree of biotinylation of the protein is of considerable importance. The self-assembly of biotinylated proteins into avidin-mediated protein oligomers can produce profound changes in bioactivity, such as those which occur when biotinylated MHC molecules form streptavidin-based tetramers.(2)

Reacting avidin with biotinylated proteins containing more than one biotin per mole can lead, not to tetramers, but to extended complexes of avidin and biotinylated proteins, which eventually precipitate (Figure 1A). Reacting avidin with biotinylated proteins containing an average of less than one biotin per mole, leads to mixtures of avidin-mediated oligomers and non-biotinylated proteins (Figure 1B). Reacting avidin with proteins containing an average of one biotin per mole maximizes the formation of soluble avidin-mediated oligomers (Figure

*Corresponding author: Center for Molecular Imaging Research, Massachusetts General Hospital and Harvard Medical School, Building 149, 13th Street, Boston, MA 02129 (USA), Phone: 617-726-6478, Fax: 617-726-5708, E-mail: ljosephson@mgh.harvard.edu

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1C). Thus when synthesizing soluble avidin-mediated protein oligomers, there is a need to obtain proteins that have (on average) a single biotin per mole. This in turn requires a simple, sensitive and accurate method for determining the numbers of biotins.

We describe here a *N*-Hydroxysuccinimide (NHS) ester activated version of biotin and fluorescein, which can be used to determine the degree of biotinylation of proteins based on fluorescein's absorbance or fluorescence. The reagent, termed "FI-Biotin-NHS", employed our multifunctional single-attachment-point (MSAP) technology(3), where fluorescein, biotin and the NHS ester are attached to a peptide scaffold. The FI-Biotin-NHS reagent was used to obtain a monobiotinylated cetuximab that was further reacted with avidin to prepare a soluble avidin-based cetuximab oligomer. To demonstrate the flexibility of the MSAP peptide scaffold approach to reagent design, a second fluorescent biotin reacting with thiol groups, "FI-Biotin-MAL" is also presented.

EXPERIMENTAL PROCEDURE

Synthesis of Biotin-Lys(monosuccinimidylsuberate)-Cys(5-acetamidofluorescein)-NH₂, FI-Biotin-NHS

The linear Biotin-Lys(Boc)-Cys(Trt) sequence was elongated onto the Rink Amide MBHA resin using standard Fmoc/*t*Bu solid phase peptide synthesis procedures described earlier.(3) Peptide full deprotection and cleavage from the resin with TFA/TIS/H₂O/EDT (95:2:2:1) afforded the biotin-Lys(H)-Cys(H)-NH₂ peptide as a white powder after lyophilization. (C₁₉H₃₄N₆O₄S₂) Calc. exact mass: 474.2; found m/z: [M+H]⁺=475.1. Solutions of Biotin-Lys(H)-Cys(H)-NH₂ (12.1 mg, 25.5 μmol) in 510 mL anhydrous DMF, fluorescein-5-iodoacetamide (8 mg, 15.5 μmol, 0.6 equiv.) in 155 μL anhydrous DMF and DIPEA (5 μL, 28.7 μmol, 1.1 equiv.) were mixed together overnight at room temperature. The reaction mixture was purified by RP-HPLC and the fraction collected lyophilized. Biotin-Lys(H)-Cys(5-acetamidofluorescein)-NH₂ (8.4 mg, 9.7 μmol, 63% yield) was obtained as a yellow powder. (C₄₁H₄₇N₇O₁₀S₂) Calc. exact mass: 861.3; found m/z: [M+H]⁺=862.0. A solution of Biotin-Lys(H)-Cys(5-acetamidofluorescein)-NH₂ (5.9 mg, 6.8 μmol) in 274 μL anhydrous DMF containing DIPEA (1.3 μL, 7.5 μmol, 1.1 equiv.) was added slowly to a solution of DSS (50 mg, 135.7 μmol, 19.8 equiv.) in 1360 μL anhydrous DMF. The reaction mixture was stirred for 48 h at room temperature. The reaction mixture was purified by RP-HPLC and the fraction collected lyophilized. Biotin-Lys(monosuccinimidylsuberate)-Cys(5-acetamidofluorescein)-NH₂ (5.2 mg, 4.7 μmol, 68% yield) was obtained as a yellow powder. (C₅₃H₆₂N₈O₁₅S₂) Calc. exact mass: 1,114.4; found m/z: [M+H]⁺=1,115.0, [M+Na]⁺=1,137.0.

Synthesis of Biotin-Lys(g-maleimidobutyryloxy)-Cys(5-acetamidofluorescein)-NH₂, FI-Biotin-MAL

A solution of Biotin-Lys(H)-Cys(5-acetamidofluorescein)-NH₂ (4.2 mg, 4.9 μmol) in 240 μL anhydrous DMF containing DIPEA (1.0 μL, 5.7 μmol, 1.2 equiv.) was added to a solution of GMBS (6.9 mg, 24.6 μmol, 5.1 equiv.) in 246 μL anhydrous DMF. The reaction mixture was stirred overnight at room temperature. The reaction mixture was purified by RP-HPLC and the fraction collected lyophilized. Biotin-Lys(g-maleimidobutyryloxy)-Cys(5-acetamidofluorescein)-NH₂ (3.3 mg, 3.2 μmol, 66% yield) was obtained as a yellow powder. (C₄₉H₅₄N₈O₁₃S₂) Calc. exact mass: 1,026.3; found m/z: [M+H]⁺=1,027.0, [M+Na]⁺=1,149.9.

Biotinylation of cetuximab with FI-Biotin-NHS

Various amounts of a solution of FI-Biotin-NHS in DMSO (10 mM) were added to Erbitux (2 mg/mL). Typically, 1.35 equiv. of reagent for a final degree of biotinylation of 1 were used. The reaction was left 1 h at room temperature and purified by size-exclusion chromatography

using a Sephadex PD-10 column. Immunoconjugates were prepared in phosphate buffer pH 8 at 0.8 mg.mL⁻¹.

Absorbance measurements

Absorption spectra were measured on a Varian Cary 50 Bio UV-Visible spectrophotometer. Solutions of increasing concentrations of FI-Biotin-NHS in phosphate buffer pH 8 were used to obtain a standard curve of optical density versus FI-Biotin concentration. The absorbance was measured at the 493 nm absorbance maximum of FI-Biotin-NHS. Solutions of cetuximab-(FI-Biotin)_n immunoconjugates were diluted two times. Absorbance was measured at 496 nm, absorbance maximum of cetuximab-(FI-Biotin)_n. Each measurement was repeated three times. The number of biotin tags for each immunoconjugate was obtained from the standard curve.

Fluorescence measurements

Fluorescence emission spectra were recorded on a Varian Cary Eclipse fluorescence spectrophotometer. Solutions of increasing concentrations of FI-Biotin-NHS in phosphate buffer pH 8 were used to obtain the standard curve of fluorescence intensity at 519 nm versus FI-Biotin concentration. Solutions of cetuximab-(FI-Biotin)_n immunoconjugates were diluted two times. Each measurement was repeated three times. The number of biotin tags for each immunoconjugate was obtained from the standard curve.

Preparation of the cetuximab:avidin oligomer

A solution of Avidin-Cy3.5_{1.4} (0.8 mg/mL ; 800µL, 10 nmol) in phosphate buffer pH 7.2 was added to a solution of cetuximab-FI-Biotin_{1.1} (3.1 mg/mL ; 2.75 mL, 57 nmol). The mixture was stirred overnight at 4°C and purified by size exclusion chromatography using a Sephacryl 300-HR column volume=59.2 mL). The high valency was eluted in 34-40 mL phosphate buffer pH 7.2.

Determination of the number of cetuximab in the cetuximab:avidin oligomer by absorbance

Absorption spectrum (200-800 nm) of the cetuximab:avidin oligomer was measured in phosphate buffer pH 7.2. Absorbance at 583 nm gave the concentration of Cy3.5 in the complex (150,000 M⁻¹.cm⁻¹), without interference from fluorescein. Absorbance at 496 nm, corrected for the absorption of the Cy3.5 fluorochrome at this wavelength (18,000 M⁻¹.cm⁻¹), gave the concentration of fluorescein in the complex (74,000 M⁻¹.cm⁻¹). The ratio between fluorescein and Cy3.5 concentrations enabled an estimation of the number of cetuximab per avidin based on 1.1 FI-Biotin per cetuximab and 1.4 Cy3.5 per avidin.

Photon Correlation Spectroscopy

Volume weighted average diameters were obtained by light scattering using a Nano-2S Zetasizer (Malvern Instruments). Solutions of (a) ferritin (1 mg/mL), (b) cetuximab (1 mg/mL), (c) avidin (1 mg/mL), (d) avidin (1 mg/mL)+ cetuximab (1 mg/mL), (e) cetuximab:avidin oligomer, were measured.

“Native” gel electrophoresis

“Native” SDS-PAGE was performed using a Tris-HCl gel from 4 to 15% polyacrylamide. Samples were prepared by using 20 µL of protein solution and 5 µL of a buffer containing Tris (25 mM), Glycine (192 mM), pH 8.3, 10% glycerol and bromophenol. Electrophoresis was run (from the anode to the cathode) with a buffer containing SDS (3.5 mM), Tris (25 mM) and Glycine (192 mM) at pH 8.3. No reducing Agent (β-mercaptoethanol) was used.

RESULTS AND DISCUSSION

The principles behind the MSAP reagent concept are shown in Figure 2. As shown in Figure 2A, the MSAP reagent, “Fl-Biotin-NHS” had two functional groups (F^1 = biotin, F^2 = fluorescein) and a reactive group (RG) as an NHS ester. The chemical structure of Fl-Biotin-NHS is further clarified in Figure 2B. A Lys-Cys dipeptide scaffold was employed for the attachment of biotin (F^1), fluorescein (F^2) and a reactive *N*-hydroxysuccinimide ester reactive group (RG).

To demonstrate some of the flexibility possible with the peptide scaffold-based MSAP strategy, we prepared “Fl-Biotin-MAL”, where a maleimide RG replaced the NHS ester. Fl-Biotin-MAL becomes a site specific labeling reagent when reacted with bioengineered proteins containing a single thiol. The properties of Fl-Biotin-NHS and Fl-Biotin-MAL are given in Table 1. All subsequent experiments employed Fl-Biotin-NHS.

The Fl-Biotin-NHS MSAP, formally Biotin-Lys(monosuccinimidylsuberate)-Cys(5-acetamidofluorescein)-NH₂, was synthesized as shown in Figure 3. Biotin was attached to the N-terminus of the protected Lys(Boc)-Cys(Trt) peptide on the solid phase. After deprotection and release from the resin, the thiol and amine groups were selectively reacted with fluorescein-5-iodoacetamide and with the cross-linker dissuccinimidyl suberate (DSS), respectively. Full characterization of Fl-Biotin-NHS is provided in the supplementary information accompanying this manuscript. Fl-Biotin-MAL was prepared similarly by using an amine to thiol heterobifunctional cross-linker (N-[*g*-maleimidobutyryloxy]succinimide, GMBS).

Fl-Biotin-NHS was employed to prepare biotinylated versions of the monoclonal antibody cetuximab where the number of biotins varied from 0.1 to 2.5 per mole of antibody. Absorbance and fluorescence standard curves acquired with Fl-Biotin-NHS were used to determine the number of Fl-Biotin moieties per antibody. A perfect fit between the absorbance of the immunoconjugates and the amount of Fl-Biotin-NHS reagent used per cetuximab was obtained. The fluorescein absorption signal (496 nm) enabled to directly quantify a wide range of biotinylation levels. (Figure 4, A and C) Samples containing from 0.5 up to 25 biotins per antibody could be measured from a single standard curve. Biotinylation levels obtained from fluorescence and absorbance signals were in very good concordance until the number of biotin reached a value of 15 (Figure 4, C). Indeed, fluorescence quenching was noticed to occur when the conjugates contained more than 15 Fl-Biotin motifs. However, in the case of low degrees of biotinylation, the exact number of biotin per antibody can be more accurately determined by fluorescence than by absorbance. (Figure 4, B) Indeed, when the Fl-Biotin content of the immunoconjugate is low, the fluorescence peak can still be distinguished from the background conversely to the absorbance signal.

In every case, we found both absorbance and fluorescence methods of Fl-Biotin more sensitive than the HABA method. (Figure 4, A and B) The commonly used method for estimating the extent of biotinylation of proteins, *i.e.* the molar ratio of biotin to protein, is based on the displacement of the HABA dye (4'-hydroxyazobenzene-2-carboxylic acid) from an HABA:Avidin complex absorbing visible light at 500 nm. Replacement of HABA by biotin in the complex translates into a decrease in the absorption intensity at 500 nm proportionally to the number of bound biotins. In the microplate assay format suggested in the “EZ™ Biotin Quantitation Kit” (Pierce), the decrease in absorbance for a regular antibody (MW 150 kDa, at 1 mg.mL⁻¹) containing an average of five biotins per protein is around 0.057, corresponding to a 10% decrease of the initial absorbance value. For the same antibody modified with only one biotin, the decrease is 0.011, corresponding to a 2% decrease of the initial absorbance value. This method lacks accuracy for low biotinylation levels.

We then considered the preparation of an avidin-based cetuximab oligomer from a monobiotinylated cetuximab and a tetrameric avidin. FI-Biotin-NHS was thus used to modify cetuximab with 1.1 FI-Biotin motifs per antibody. The latter was further incubated with avidin modified with the Cy3.5 fluorochrome. (Figure 5) Modification of the antibody and of the avidin with fluorescein and Cy3.5 fluorochromes, respectively, facilitated (i) the separation of the complex by gel filtration (Sephacryl 300-HR) and (ii) the determination of the ratio of cetuximab to avidin in the cetuximab:avidin oligomer. Absorbance measurements concluded to 2.3 cetuximab per avidin (data not shown). The oligomer was characterized by dynamic laser light scattering and gel electrophoresis. The size of the oligomer was 17 nm (volume-weighted) as measured by light scattering. (Figure 5, B) The size of the oligomer was also assessed by “Native” (no reducing agent) SDS-PAGE and compared to ferritin, a globular protein, presenting two major bands, p210 and p440, at 210 and 440 kDa respectively. The oligomer presents two species. The most prominent species is slightly smaller than 440 kDa ferritin. Altogether, the results of the three characterization methods converge to the conclusion that the cetuximab oligomer obtained is a mixture of the dimer, 359 kDa, and of the trimer, 506 kDa.

An advantage of the MSAP synthetic strategy shown in Figure 3 is its ability to allow substitutions of the functional groups or reactive groups on the biotin-Lys-Cys dipeptide. Since the dipeptide is made by manual solid phase synthesis, sufficient dipeptide was obtained to allow it to be split into portions for reactions with different functional groups. In particular, the single amine and single thiol of the dipeptide can be reacted in a chemoselective manner with a variety of reagents under mild conditions, to generate a variety of biotinylated molecules. Since fluorescein was not exposed to the harsh conditions of deprotection, any thiol reactive fluorochrome can be substituted for fluorescein-5-iodoacetamide, including near infrared fluorochromes, some of which do not survive the harsh conditions of deprotection. Thus MSAP chemistry provides a broad new strategy for designing biotinylated reagents that can feature a variety of possible reporter groups (e.g. chromophores, fluorochromes, chelators) and, as we have shown, N-hydroxysuccinimide ester or maleimide reactive groups.

CONCLUSION

A peptide scaffold based, multifunctional, single-attachment-point reagent (MSAP) was used to obtain the fluorescent biotin, FI-Biotin-NHS. FI-Biotin-NHS was then used to attach 1.1 biotins per mole to cetuximab and the biotinylated antibody reacted with avidin to obtain an avidin-cetuximab oligomer. The FI-Biotin-NHS can be used to biotinylate protein substrates with the degree of biotinylation determined by absorbance or fluorescence. In addition, MSAP reagents provide a broad new approach to the design of multifunctional biotins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

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Figure 1.

Reactions of biotinylated proteins with a tetrameric avidin. (A) When biotinylated proteins are overbiotinylated, with multiple biotins per mole ($n > 1$), large soluble aggregates or precipitates form, due to avidin tetravalency. (B) When biotinylated proteins are underbiotinylated, $n < 1$, non-biotinylated protein is present. (C) Monobiotinylated proteins, $n \sim 1$, maximize the formation of soluble avidin-based protein oligomers.

Figure 2.

Multifunctional Single-Attachment-Point (MSAP) reagents for the synthesis of biotinylated proteins. (A) A protein substrate, such as a monoclonal antibody, reacts with the MSAP reagent "FI-Biotin-NHS" to yield a fluorescent (F²), biotinylated (F¹) probe in one step. (B) Design of the "FI-Biotin-NHS" and "FI-Biotin-MAL" MSAP reagents. To the Lys-Cys dipeptide scaffold are attached the F¹ functional group Biotin, the F² functional group Fluorescein-acetamide and either a *N*-Hydroxysuccinimide (NHS) ester or a Maleimide (MAL) reactive group RG.

Figure 3.

Synthesis of the "Fl-Biotin-NHS" MSAP or Biotin-Lys(monosuccinimidylsuberate)-Cys(5-acetamidofluorescein)-NH₂ (C₅₃H₆₂N₈O₁₅S₂; MW=1,115.23 g.mol⁻¹). Biotin was attached to the N-terminus of the protected Lys-Cys peptide on the solid phase. After deprotection and release from the resin, the thiol and amine groups are selectively reacted with fluorescein-5-iodoacetamide and the crosslinker dissuccinimidyl suberate, respectively. The high flexibility of the synthetic strategy allows various substitutions of the Lys-Cys peptide scaffold and thus various MSAPs compositions.

Figure 4.

Comparison of the sensitivity of FI-Biotin-NHS with the HABA dye displacement-based method for the determination of biotinylation of a monoclonal antibody (cetuximab). (A) Biotin per protein determined by FI-Biotin's absorbance (496 nm, ●) versus the HABA method (500 nm, □). FI-Biotin is more sensitive than the HABA method, based on the displacement of the HABA dye from the HABA:Avidin complex by Biotin ($A_{500} = A_{500}\text{HABA:Avidin} - A_{500}\text{HABA:Avidin:Biotin}$).

(B) Biotin per protein at low biotinylation levels determined by FI-Biotin's absorbance (496 nm, ●), the HABA method (500 nm, □) and by FI-Biotin's fluorescence (519 nm, ○).

(C) Quenching of FI-Biotin's fluorescence with high numbers of FI-Biotin per mole of protein. At high levels of FI-Biotin ($n > 15$), fluorescence quenching causes an underestimation of the number of FI-Biotin per protein.

Note that each measurement is the average of three replicates, error bars are included.

Figure 5.

Synthesis and characterization of a cetuximab-avidin oligomer. (A) Synthesis of the oligomer. Six molar equivalents of a monobiotinylated cetuximab was reacted with avidin(Cy3.5). The reaction mixture was purified by gel filtration and the oligomer was isolated. (B) Characterization of the cetuximab oligomer by laser light scattering. The volume-weighted diameter was 17 nm. (C) Characterization of the cetuximab oligomer by SDS-PAGE (no mercaptoethanol). Lane “MW Std” shows various molecular weight standards. Lane “a” is a ferritin standard which gave species of 210 and 440 kDa. Lane “e” was the cetuximab-avidin oligomer. The most prominent species was slightly smaller than 440 kDa ferritin. The cetuximab-avidin oligomer contains a mixture of the dimer, 359 kDa, and of the trimer, 506 kDa.

Table 1

Fluorescent Biotin MSAPs

Peptide Scaffold	F ¹	F ²	RG	MW (g.mol ⁻¹)	Designation
F ¹ -Lys(RG)-Cys(F ²)-NH ₂	Biotin	Fluorescein-5-acetamide	N-Hydroxy succinimide	1,115.2	Fl-Biotin-NHS
F ¹ -Lys(RG)-Cys(F ²)-NH ₂	Biotin	Fluorescein-5-acetamide	Maleimide	1,027.1	Fl-Biotin-MAL