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Biotin–Fluorophore Conjugates with Poly(ethylene glycol) Spacers Retain Intense Fluorescence after Binding to Avidin and Streptavidin

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Conventional biotin–fluorophore conjugates with ~14 atom spacers lose most of their fluorescence when binding to avidin or streptavidin, as is demonstrated in the present study. This explains the unusual fact that only biotinylated marker enzymes, but not fluorescent biotins, are regularly used in bioanalytic assays. Novel biotin–spacer–fluorophore conjugates are presented that retain intense fluorescence when binding to avidin or streptavidin. Preservation of fluorescence depends upon the use of poly(ethylene glycol) (PEG) spacers, which are shown not to interfere with biotin function. The observed absence of nonspecific interactions may also be attributed to the PEG chain. These novel fluorescent biotins are expected to be excellent new tools in fluorescence microscopy and related techniques.

INTRODUCTION

Specific detection of immobilized biomolecules is a standard task in modern bioscience. Generally target molecules are recognized by specific probe molecules (antibodies, oligonucleotides, etc.) which are labeled for detection (Wilchek and Bayer, 1990a). Direct labeling of a probe with a marker function (fluorophore, enzyme, etc.) implies an irreversible restriction to a single detection method. Biotinylation of a probe, however, allows the use of the same probe in combination with almost any known detection method because a wide variety of biotinylated markers or (strept)avidin–marker conjugates is commercially available for the postlabeling of biotinylated probes via biotin–(strept)avidin–biotin bridges or via biotin–(strept)avidin bridges, respectively (Wilchek and Bayer, 1988, 1990b).

Fluorescent markers play a pre-eminent role in bioanalytic assayss; therefore, it seems logical to expect extensive use of fluorescent biotins. However, conventional biotin–fluorophore conjugates (with ~14 atom spacers) lose most of their fluorescence when binding to (strept)avidin, as is demonstrated in the present study. This adverse effect explains why only two fluorescent biotins are commercially available—and mostly used for purposes other than postlabeling of biotinlyated probes (Chu et al., 1994; Schray et al., 1988; Shah et al., 1994).

In the present study a series of novel biotin–spacer–fluorophore conjugates is presented that retain intense fluorescence after binding to (strept)avidin. Preservation of fluorescence depends on the use of PEG_{1900}[1] or PEG_{800} as spacer elements: The PEG chains minimize dye–dye and dye–protein interactions, which cause the quenching in complexes of conventional fluorescent biotins with (strept)avidin.

While PEG spacers in biotin–PEG–fluorophore conjugates are beneficial to the fluorophore function, the question remained to be answered whether the PEG spacers would hinder the biotin terminus from binding to (strept)avidin. In the preceding study with nonfluorescent biotin–PEG conjugates (see the first of three papers in this issue) it has been demonstrated that 4:1 complexes with (strept)avidin were indeed formed and that at least three biotin–PEG elements per tetrameric receptor protein were bound on a time scale of hours. The present study shows that nearly four biotin–PEG–fluorophore ligands/protein remain bound on a time scale of minutes, as is desired in bioanalytic fluorescence detections.

EXPERIMENTAL PROCEDURES[2]

Materials. P.a. grade materials were used as far as commercially available. Affinity-purified avidin and streptavidin, *d*-biotin, Boc_2O, and DACA were obtained from Sigma. DMF and Et_3N were purchased from Fluka. NaCl and methanol were obtained from Riedel de Haen. 1,12-diamino-4,9-dioxadodecane was obtained from Aldrich. Acetic acid and chloroform were purchased from Baker. fluorescein–biotin (see footnote 1 for full structure), ANS, 5-(and 6)-carboxyfluorescein succinimidyl ester, and 5-(and 6)-carboxytetramethylrhodamine succinimidyl ester were obtained from Molecular Probes. Cy3

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[1] Abbreviations: ANS, 2-anilinonaphthalene-6-sulfonic acid; biotin, biotinoyl group; biotin–NHS, succinimidyl ester of biotin; biotin–dode–TMR, 5-(and 6)-[[*N*-(12-biotinamido-4,9-dioxadodecyl)]aminocarbonyl]tetramethylrhodamine; biotin–NH-$PEG_{800\ or\ 1900}$-NH_2·HCl, *N*-biotinoyl-*O*,*O*′-bis(2-aminopropyl)poly(ethylene glycol)$_{800\ or\ 1900}$ hydrochloride; biotin–$PEG_{800\ or\ 1900}$–dye, see Scheme 1; biotin–$PEG_{800\ or\ 1900}$–Flu, see Scheme 1; biotin–$PEG_{800\ or\ 1900}$–TMR, see Scheme 1; biotin–$PEG_{800\ or\ 1900}$–Cy3, see Scheme 1; biotin–$PEG_{800\ or\ 1900}$–Cy5, see Scheme 1; Boc_2O, di-*tert*-butyl pyrocarbonate; Boc, *tert*-butyloxycarbonyl group; DACA, *p*-(dimethylamino)cinnamaldehyde; DMF, *N*,*N*-dimethylformamide; EDTA, ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid; Et_3N, *N*,*N*,*N*-triethylamine; Flu, 5-(and 6)-carboxyfluorescein residue; fluorescein–biotin, 5-[[*N*-[5-[*N*-[6-(biotinoyl)amino]hexanoyl]amino]pentyl]thioureidyl]fluorescein; (strept)avidin, streptavidin and/or avidin; FRET, fluorescence resonance energy transfer; NHS, *N*-hydroxysuccinimide; PEG, poly(ethylene glycol); RT, room temperature; TMR, 5-(and

and Cy5 monofunctional dyes (succinimidyl esters) were purchased from Amersham. Sephadex-based gels were obtained from Pharmacia. All other materials were purchased from Merck. Biotin-NHS was prepared as described (Wilchek and Bayer, 1990c). Absolute DMF was prepared according to a standard procedure. Biotin–NH-PEG_{800}-NH_2·HCl and biotin–NH-PEG_{1900}-NH_2·HCl were synthesized as described before (see the first of three papers in this issue).

Buffers. Buffer A contained 100 mM NaCl, 50 mM NaH_2PO_4, and 1 mM EDTA, adjusted to pH 7.5 with NaOH. Buffer B contained 1 mM NaH_2PO_4 (adjusted to pH 7.5 with NaOH) and variable NaCl concentrations (as specified).

Methods. *Synthesis of N-Boc-4,9-dioxa-1,12-diaminododecane·CH_3COOH.* 4,9-Dioxa-1,12-diaminododecane (29.4 mmol) in methanol was reacted with 28.4 mmol of Boc_2O under Ar. After addition of toluene and 5 mL of acetic acid, the mixture was taken to dryness (10.5 g of crude product). Silica chromatography of 3 g of crude product in chloroform/methanol/acetic acid mixtures (90:10:0.1 and 70:30:5) gave 3.20 mmol of product, which was pure by TLC.

Synthesis of N-Boc-N′-biotin-4,9-dioxa-1,12-diaminododecane. *N*-Boc-4,9-dioxa-1,12-diaminododecane·HCl (1.8 mmol) was reacted with 2.7 mmol of biotin-NHS and 240 μL of Et_3N in 10 mL of absolute DMF. Excess biotin-NHS was hydrolyzed with water. After solvent removal, the crude product was purified on silica 60 (eluent chloroform/methanol/acetic acid 120:30:0.5), resulting in 1.11 mmol of product (pure by TLC).

Synthesis of N-Biotin–4,9-dioxa-1,12-diaminododecane·HCl. *N*-Boc-*N*′-biotin-4,9-dioxa-1,12-diaminododecane (1.11 mmol) was deprotected with 98% formic acid, and the crude product was purified by ion exchange chromatography on SP Sephadex C-25. Salt was removed by extracting deprotonated product into chloroform. Drying and lyophilization from dilute HCl gave 0.64 mmol of product (pure by TLC).

Synthesis of Biotin–dode–TMR. *N*-Biotin-4,9-dioxa-1,12-diaminododecane·HCl (19 μmol) was reacted with 30 μmol of 5-(and 6)-carboxytetramethylrhodamine succinimidyl ester and 10 μL of Et_3N in chloroform under Ar. TLC showed quantitative labeling of the primary amine. The mixture was taken to dryness and purified by chromatography on silica 60 (chloroform/methanol/water 70:26:4). In spite of using 100 g of silica 60 it was impossible to remove those two TMR derivatives, which were already present in the commercial TMR reagent and whose R_f values were just below (0.45) or above (0.56) that of the product (0.51). For characterization, TLC spots were harvested quantitatively, extracted with chloroform/ethanol/water (10:15:2), clarified by centrifugation, and checked for TMR contents by their UV–vis spectra. The two byproducts together gave rise to 17% of the absorption at 550 nm, while the correct main product contributed with 83% to A_{550}. From determination of biotin end group contents (by the ANS method, see below) a similar estimate of purity was obtained (87% as compared to the TMR group contents estimated from UV–vis absorption).

Synthesis of Biotin–PEG_{800}–TMR. Biotin–NH-PEG_{800}-NH_2·HCl (8.4 μmol) was quantitatively labeled with 23 μmol of 5-(and 6)-carboxytetramethylrhodamine succinimidyl ester in chloroform/Et_3N under Ar. After evaporation, the crude product was gel filtered on Sephadex G-25 (see above) showed that free TMR gave rise to 2% of the absorption at 550 nm, while the product contributed 98% to A_{550}.

Synthesis of Biotin–PEG_{1900}–TMR. The procedure was the same as for the PEG_{800} derivative. Forty-three milligrams of biotin–NH-PEG_{1900}-NH_2·HCl (nominally 18.7 μmol, ~20% water content) was reacted with 36 μmol of 5-(and 6)-carboxytetramethylrhodamine succinimidyl ester. Yield = 12.8 μmol of biotin–PEG_{1900}–TMR according to biotin end group assay (see below). Quantitative TLC (as performed with biotin–dode–TMR, see above) showed that free TMR gave rise to 3% of the absorption at 550 nm, while the product contributed 97% to A_{550}.

Synthesis of Biotin–PEG_{800}–Flu. Twenty-two micromoles of biotin–NH-PEG_{800}-NH_2·HCl was labeled with 43 μmol of 5-(and 6)-carboxyfluorescein succinimidyl ester in DMF/Et_3N under Ar. After solvent removal, the residue was dissolved in chloroform and successively washed with 200 mM Na_2CO_3 (saturated with NaCl) and with dilute acetic acid (saturated with NaCl). The organic layer was dried, evaporated, redissolved in 2 mL of 3 mM Na_2CO_3, and chromatographed on QAE Sephadex A-25 using buffer B with increasing NaCl concentrations. Salt was removed by extraction into chloroform. Yield = 18 μmol, pure by TLC.

Synthesis of Biotin–PEG_{1900}–Flu. Fifty milligrams of biotin–NH-PEG_{1900}-NH_2·HCl (22 μmol) was reacted with 15.5 mg of 5-(and 6)-carboxyfluorescein succinimidyl ester (33 μmol). The procedure was the same as for the PEG_{800} homologue, except that 50 mM instead of 150 mM NaCl in buffer B was used to elute the product from QAE-Sephadex A-25. Yield = 12 μmol product, pure by TLC.

Synthesis of Biotin–PEG_{800}–Cy3. Biotin–NH-PEG_{800}-NH_2·HCl (1.7 μmol) was reacted with one vial of Cy3 monofunctional dye ("reactive dye to label 1 mg of antibody" according to Amersham) in absolute DMF/Et_3N. After solvent removal, the residue was dissolved in buffer B and chromatographed on QAE Sephadex A-25, using 125 mM NaCl in buffer B for product elution. Yield = 63 nmol determined from A_{550} (ϵ_{550} = 150 000 M^{-1} cm^{-1} for Cy3 according to Amersham) or 54 nmol according to biotin end group assay with ANS (see below). The uncoupled dye was eluted from QAE-Sephadex A-25 with 1 M NaCl and amounted to 74 nmol according to A_{550}.

Synthesis of Biotin–PEG_{1900}–Cy3. The procedure was the same as for the PEG_{800} derivative, except that 0.44 μmol of biotin–NH-PEG_{1900}-NH_2·HCl was reacted, and 75 mM NaCl in buffer B was used for elution from the ion exchange column. Yield = 55 nmol determined from A_{550} or 52 nmol according to biotin end group assay with ANS (see below).

Synthesis of Biotin–PEG_{1900}–Cy5. The procedure was the same as for the corresponding Cy3 analogue, except that 5 mg of biotin–NH-PEG_{1900}-NH_2·HCl (2.2 μmol) was reacted with one vial of Cy5 monofunctional dye. Yield = 55 nmol determined from A_{647} (ϵ_{647} = 250 000 M^{-1} cm^{-1} for Cy5 according to Amersham) or 39 nmol according to biotin end group assay with ANS (see below).

Quantitative Assay for Biotin End Groups and for Biotin Binding Sites. A published fluorescence assay for avidin–biotin interaction (Mock and Horowitz, 1990) was modified as described before (see the first of three papers in this issue). Typically, 2 mL of buffer A containing 1 nmol of avidin was mixed with 20 μL of 5 mM ANS, and ~2 nmol (estimated from UV–vis absorption) of a biotin–

d-biotin in buffer A while the fluorescence of the pseudoligand ANS was monitored at 328 nm excitation (10 nm slit) and 408 nm emission wavelength (10 nm slit). The inflection point in the titration curve indicated saturation of all biotin binding sites (determined by parallel standardization of the avidin stock solution with *d*-biotin alone). Fortunately, none of the biotin–fluorophore conjugates caused significant background fluorescence under these assay conditions.

The ANS assay was also used to determine functional biotin binding sites in avidin stock solutions as described before (see the first of three papers in this issue), whereas streptavidin was functionally characterized by titration with biotin–PEG_{800}–pyrene (see the third of three papers in this issue) because streptavidin is known not to bind the pseudoligand ANS (Mock and Horowitz, 1990).

Gel Filtration Assay for Binding of Biotin–PEG–Dye to Avidin and Streptavidin. Specificity and metastability of biotin–PEG–dye binding to avidin or streptavidin was tested by gel filtration as previously described (see the first of three papers in this issue). Typically, 0.5 mL samples of buffer A containing 50 μM "functional" avidin (see above) or 2 μM "functional" streptavidin (see above) and various amounts of biotin–PEG–dye were incubated for 1 h at 25 °C and subjected to gel filtration on a 1 × 48 cm column of Sephadex G-100 at RT. Elution was at 0.25 mL/min with buffer A while fractions were collected at 5 min intervals. All fractions were assayed for avidin or streptavidin by A_{282} (corrected for $\epsilon_{282} = 0.08\epsilon_{550}$ or $0.20\epsilon_{550}$ or $0.18\epsilon_{496}$ in the case of Cy3–, TMR–, and Flu–PEG conjugates, respectively) and for dye contents by UV–vis absorbance at λ_{max}. Molar extinction coefficients for avidin and streptavidin were taken from the literature (Green, 1990).

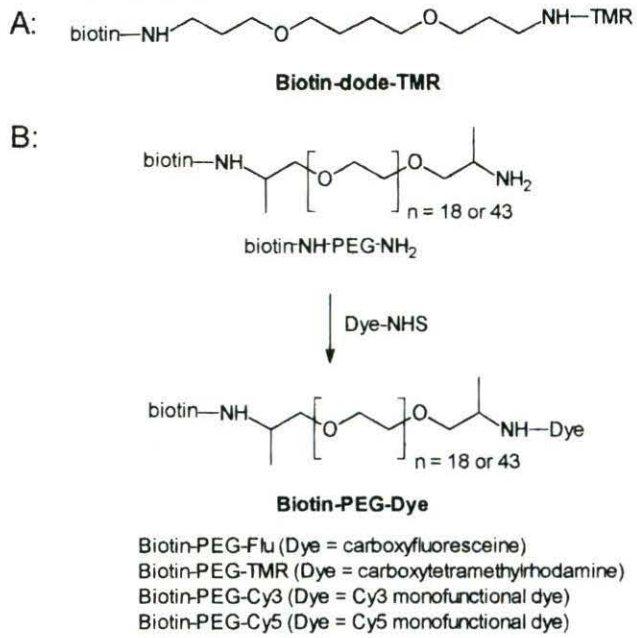
Measurement of Fluorescence in Complexes of Biotin–PEG–Dye with Avidin and Streptavidin. In a "forward titration" 2 mL of receptor protein (≤80 nM) in buffer A was titrated with a stock solution of biotin–PEG–dye (7–15 μM). In the "reverse titration" mode 2 mL of fluorescent ligand (≤320 nM) in buffer A was titrated with stock solutions of avidin or streptavidin (2–4 μM). All fluorescence signals were corrected for the small dilution factors. Time intervals were 3–5 min as required for equilibration at RT (except for titration of streptavidin with fluorescein–biotin, for which 10 min intervals were required). The concentrations of receptor proteins and ligands refer to biotin binding sites and biotin termini, respectively, as determined by functional titrations (see above).

RESULTS

Syntheses of Biotin–Spacer–Dye Conjugates. The goal of the present study was the identification of biotin–spacer–fluorophore structures that retain high fluorescence yield when binding to avidin or streptavidin. PEG_{1900}, PEG_{800}, and a 14-atom homologue were chosen as spacers, and the most popular/promising fluorochrome labels were tested (see Scheme 1).

Biotin–PEG–dyes with anionic fluorophores could be subjected to ion exchange chromatography, resulting in homogeneous products as evidenced by TLC. In contrast, special precautions were necessary to arrive at 97–98% purity with the zwitterionic TMR derivatives (see Experimental Procedures). Purity and 1:1 ratios of biotin/fluorophore end groups were also confirmed by ^{1}H NMR. Virtually noise-free single-pulse spectra were recorded at 500 MHz to obtain correct integrals from unsaturated signals. Moreover, the specific bindability of all biotin–

Scheme 1. Synthesis and Structure of Biotin–Fluorophore Conjugates



further excluded the presence of fluorescent molecules without a biotin terminus.

One short fluorescent biotin (fluorescein–biotin) with a 14-atom spacer was commercially available. For a more systematic study a second example was synthesized in which biotin is linked to TMR via a 14-atom spacer also (see Scheme 1). In spite of moderate purity the product fully served its intended role as a poor fluorescent ligand for avidin and streptavidin (see Figures 2 and 9).

Fluorescence Properties of Biotin–Spacer–Fluorophore Conjugates before/after Binding to Avidin or Streptavidin. The superiority of novel biotin–PEG–dyes over conventional fluorescent biotins is demonstrated in Figures 1 and 2. Short fluorescein–biotin is highly quenched when binding to avidin (Figure 1B, open squares) or streptavidin (Figure 1C, open squares), and the same is true for short biotin–dode–TMR (Figure 2C, circles). Avidin and streptavidin are tetravalent receptor proteins for biotin; therefore, the abrupt rise in fluorescence at ligand/receptor ratios >4:1 indicates stoichiometric binding, and the absence of nonspecific binding is evidenced by the strictly parallel nature of this linear rise with the linear dose response in the absence of receptor protein. Saturation at 4:1 stoichiometry and absence of further binding were also observed with biotin–PEG–dyes (see Figures 1, 2A,B, and 3A) except for the Cy3 derivatives (see Figures 3B,C and 4).

In contrast to short fluorescein–biotin, the long biotin–PEG_{1900}–Flu showed little quenching when bound to avidin (Figure 1A). More quenching was observed with the intermediate conjugate biotin–PEG_{800}–Flu when bound to avidin (Figure 1B, circles) or streptavidin (Figure 1C, circles), but the fluorescence signals in 4:1 complexes with receptor protein were still very intense in comparison to those of short fluorescein–biotin (Figure 1B,C, open squares).

Biotin–PEG–TMR conjugates (Figure 2A,B) differ from corresponding fluorescein analogues in two aspects: The effect of PEG chain length is much less pronounced, and the fluorescence quenching in the bound state is increased, reaching ~50% in the 4:1 complexes with avidin and streptavidin. Taking into account the

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