

TECHNICAL NOTES

Seta-633 - A NIR Fluorescence Lifetime Label for Low-Molecular-Weight Analytes

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We describe the photophysical properties of Seta-633, a commercially available near-infrared (NIR) dye, and its use as a fluorescent label to study the interaction between low-molecular-weight analytes and proteins using fluorescence lifetime as the readout parameter. In a model assay, we demonstrate that a biotinylated Seta-633 tracer binds to antibiotin with high specificity. Importantly, the lifetime of Seta-633–biotin increases about 1.8-fold upon binding to a specific antibody (antibiotin, MW = 160 kDa), while the titration with bovine serum albumin (BSA) or nonspecific antibody does not result in a noticeable change in lifetime. This behavior is contrary to that of fluorescent tracers like Cy5 or Alexa 647, which typically exhibit much smaller lifetime changes upon binding to antibodies.

INTRODUCTION

Fluorescence detection is widely used in biomedical research applications (1–3). A majority of these applications are based on the use of extrinsic labels (4–8). Red and near-infrared (NIR) dyes exhibit advantages over short-wavelength absorbing and emitting dyes due to reduced autofluorescence from biological samples beyond 600 nm. Among these labels, the cyanine-based labels of the Cy, Alexa, Atto, and Dy series are the most common ones used for bioanalytical applications (5–13).

Seta dyes are another group of commercially available dyes based on squaraines (14, 15). Squaraines are 1,3-disubstituted squaric acid derivatives. They show high extinction coefficients ($\epsilon > 200\,000\text{ L mol}^{-1}\text{ cm}^{-1}$) in the red region, display high photostability, and allow the introduction of reactive functional groups such as *N*-hydroxysuccinimide (NHS) esters (16). As previously reported (14–18), these dyes exhibit shorter lifetime in aqueous solutions but increased lifetimes upon covalent or noncovalent binding to proteins (BSA, antibodies, and avidin). The phenomena of quantum yield and lifetime increases of squaraine dyes upon noncovalent binding to BSA and covalent binding to an antibody were thoroughly investigated and described in detail earlier (14, 17). Briefly, squaraines are considered more polar molecules as compared to open-chain cationic cyanines (19) (e.g., Cy5 or Alexa 647), and therefore, the quantum yields and fluorescence lifetimes of squaraines are significantly decreased in polar, protic solvents such as water or aqueous buffers (15) and substantially increase in hydropho-

bic microenvironments (e.g., upon binding to proteins (BSA, IgG)) (16, 17) or in aprotic solvents (15).

Fluorescence lifetime is one of the most robust readout parameters available for fluorescence detection (20). It is mostly independent of the fluorophore concentration, and therefore to some degree unaffected by changes in the probe volume and photobleaching effects (20).

In frequency domain (FD) measurements, the intensity of the excitation light is sinusoidally modulated at a specific frequency (21). Upon excitation of a fluorescent molecule with modulated light, the emitted fluorescence will also be modulated with the same frequency. However, depending on the frequency of the excitation and the lifetime of the fluorescent molecule, the modulation depth of the emitted light vs the excitation light will be decreased. In addition, the phase will be shifted in the emitted light. This phase and modulation change is then used to calculate the lifetime based on ref 21

$$\omega\tau = \tan \Phi = (1/M^2 - 1)^{1/2} \quad (1)$$

where ω is the angular modulation frequency (2π times modulation frequency); τ is the lifetime; θ is the phase angle; and M is the modulation. To recover the fluorescence decay parameters from the signal response of the emission, a nonlinear least-squares fitting procedure is performed (21).

Here, we discuss the use of Seta-633 as a lifetime label for tracing the interaction of small molecules with proteins, wherein the interaction is accompanied by a change in the fluorescence lifetime. We use the biotin/antibiotin interaction as a model system and demonstrate that binding of proteins to the Seta-633–biotin is accompanied by a distinct increase in lifetime that could be easily measured with inexpensive instrumentation such as a light source modulated at a fixed wavelength and a long-in-amplifier. Single label assays that are based on a lifetime readout

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are preferred, as they are not limited by factors such as size or distance of binding partners.

EXPERIMENTAL PROCEDURES

Chemicals, Proteins, and Media. Seta-633 (catalog no: K8-1663), a reactive dye containing one NHS ester group, is commercially available from SETA Biomedicals, Urbana, IL (<http://www.setabiomedicals.com>) and was used as is. Cy5 and Alexa Fluor 647 are commercially available from GE Healthcare and Invitrogen.

Bovine serum albumin (BSA, essentially fatty acid free), immunoglobulin G (IgG) from bovine serum (reagent grade, $\geq 95\%$), biotin, approximately 99% TLC, biotin-NHS, anti-biotin antibody produced in goat, affinity isolated antibody, Sephadex G25, and Sephadex G50 are commercially available from Sigma; all other chemicals and solvents were from Aldrich. All chemicals were used without further purification.

Phosphate buffer pH 7.4 (PB) (67 mM) was prepared by mixing of 808 mL of $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ (11.876 g L^{-1}) and 192 mL of KH_2PO_4 (9.078 g L^{-1}). Bicarbonate buffer of pH 9.0 (50 mM) was obtained by dissolving 2.1 g of NaHCO_3 in 500 mL of distilled water.

General Protein Labeling Procedures and Dye-to-Protein Ratios. Protein labeling reactions were carried out using 50 mM bicarbonate buffer (pH 9.0). A stock solution of 1 mg of dye in 200 μL of anhydrous DMF was prepared; 5 mg of BSA or 3 mg of immunoglobulin G (IgG) protein were dissolved in 1 mL of a 50 mM bicarbonate buffer pH 9.0, and various amounts of dye from the stock solution were added, and the mixture was allowed to stir for 2 h at room temperature.

Unconjugated dye was separated from the labeled proteins using gel permeation chromatography with Sephadex G25 for BSA conjugates or Sephadex G50 for IgG (1.5 cm \times 20 cm column) and a 67 mM phosphate buffer solution of pH 7.4 as the eluent. The first colored fraction containing the dye-protein conjugate is isolated, while the blue or bluish-green band with a much higher retention time (free label) is discarded. A series of labeling reactions as described above were set up to obtain different dye-to-protein ratios.

The molar dye-to-protein ratio (degree of labeling) (22) for each purified dye conjugate was calculated as the molarity of dye divided by the molarity of protein. The dye concentration of the conjugate was determined according to Lambert-Beer's law from the absorbance of the dye at the absorption maximum. The protein concentration was assessed by measurement of the absorption of the protein at 278 nm. The dye-to-protein ratios (D/P) were calculated using the following formula (with the assumption that the extinction coefficients for the free and conjugated dyes are about the same) (22-24)

$$D/P = \frac{A_{\text{conj}(\lambda_{\text{max}})}\epsilon_{\text{p}}}{(A_{\text{conj}(278)} - xA_{\text{conj}(\lambda_{\text{max}})})\epsilon_{\text{dye}}} \quad (2)$$

where $A_{\text{conj}(\lambda_{\text{max}})}$ and $A_{\text{conj}(278)}$ are the absorbances at absorption maxima and at 278 nm of the dye-protein conjugate, respectively; ϵ_{dye} is the extinction coefficient of the dye at λ_{max} , ϵ_{p} is the extinction coefficient of the protein at 278 nm: for BSA, $\epsilon_{\text{p}} = 45\,540 \text{ M}^{-1} \text{ cm}^{-1}$, and for IgG, $\epsilon_{\text{p}} = 201\,700 \text{ M}^{-1} \text{ cm}^{-1}$. The factor x in the denominator accounts for dye absorption at 278 nm ($A_{\text{dye}(278)}$), which is a percent of the absorption of the dye at its maximum absorption ($A_{\text{dye}(\lambda_{\text{max}})}$) ($x = A_{\text{dye}(278)}/A_{\text{dye}(\lambda_{\text{max}})}$).

Absorption Spectra and Extinction Coefficients. Absorption spectra were measured at room temperature on a Perkin-Elmer Lambda 35 UV/vis spectrophotometer. Absorption maxima were recorded with an accuracy of 0.5 nm.

For determination of the extinction coefficients, each dye (7-10 mg) was dissolved in 50 mL of phosphate buffer pH

7.4 (PB). The stock solution was diluted (1:2000) and the absorbance (A) was measured in a 5 cm standard quartz cell. All dye concentrations were between 0.1 and 0.2 μM . The extinction coefficients were calculated according to Lambert-Beer's law. The reproducibility for determining the extinction coefficients was $1000 \text{ M}^{-1} \text{ cm}^{-1}$.

Emission Spectra and Quantum Yields. Emission spectra and quantum yields of the dyes, dye-BSA, and dye-IgG conjugates were measured in PB at room temperature on a Varian Cary Eclipse spectrofluorometer in a standard 1 cm quartz cell. All dyes were excited with an excitation slit width of 5 nm and emission slit width of 2.5 nm. The spectra were corrected. All concentrations of the fluorophores were chosen to be between 0.1 and 0.2 μM .

For the determination of the quantum yields (QY), the integrated relative intensities of the dyes or dye-protein conjugates were measured against Cy5 as the reference. All absorbances at the excitation wavelength (λ_{exc}) were in the range 0.12-0.18 (when measured in a 5 cm cell). The fluorescence spectra of the solutions were measured and the QYs were determined relative to Cy5 (QY = 27% (25)) using the formula (26)

$$\text{QY} = \text{QY}_{\text{Cy5}} \left(\frac{F}{F_{\text{Cy5}}} \right) \left(\frac{A_{\text{Cy5}}}{A} \right) \quad (3)$$

where QY_{Cy5} is the quantum yield of Cy5, F_{Cy5} and F are the integrated areas of the fluorescence spectra ($F = \int I(\lambda) d\lambda$), and A_{Cy5} and A are the absorbencies at the excitation wavelength of Cy5 and the fluorophore under examination, respectively.

The QY of each sample was independently measured 3-4 times, and the average value was calculated. The QYs of the dye-BSA conjugates were measured for various D/P ratios, and then, the QYs for $D/P = 1$ were determined by a nonlinear interpolation.

Fluorescence Lifetime Measurements. Fluorescence lifetime measurements were acquired on ChronosFD - the laser-diode based frequency domain instrument produced by ISS Inc., Champaign, IL, USA. A 635 nm laser diode was used as the excitation source. Two synthesizers (model IFR2023) providing modulation frequencies in the range from 9 kHz to 1.2 GHz were utilized for the measurements. Data analysis was done with Vinci - Multidimensional Fluorescence Spectroscopy - a comprehensive software package for the analysis of time-resolved data from ISS. The analysis of the time-resolved fluorescence data was carried out using the traditional nonlinear least-squares method. This method evaluates how close a model selected by the user matches the data acquired with the instrument; the Marquardt-Levenberg algorithm was utilized for the minimization routine of the χ^2 -function that compares the selected model with the experimental data. In all lifetime measurements, the dye concentration in PB (pH 7.4) was between 0.1 and 1 μM .

Mass Spectrum. The mass spectrum of *N*-[2-(D-biotinylamino)ethyl]amine was recorded on a Varian 1200 L mass spectrometer system using the electron ionization (EI) method.

Photostability. Photostability measurements were performed in aqueous solutions with Cy5, Alexa Fluor 647, and Seta-633 and their IgG conjugates. The optical density of the long-wavelength maximum was controlled to be between 0.09 and 0.10, and all measurements were carried out in 1 cm cells. These solutions were placed at the distance of approximately 30 cm from a 500 W lamp and irradiated with occasional stirring. The absorption and emission spectra of the solutions were measured before irradiation and during light exposure. The relative photostabilities were calculated as the ratio between (i) the measured absorbances at the longwavelength maximum before

Table 1. Spectral Properties for Seta-633 and Seta-633 Conjugates

dye or dye conjugate	λ_{\max} (abs) (nm)	ϵ ($M^{-1}\cdot cm^{-1}$)	λ_{\max} (em) (nm)	QY (%)	Stokes' shift ($\Delta\nu$) (cm^{-1})
Seta-633	633	250 000	644	7	270
Seta-633-BSA	646		656	53	240
Seta-633-IgG	637		647	26	240

and after exposure (A/A_0) and (ii) the relative fluorescence intensities before and after exposure (I/I_0), and the corresponding plots were generated.

Biotin Conjugation. *N*-[2-(*D*-Biotinylamino)ethyl]amine. To a stirred solution of 63 mg (0.18 mmol) of *D*-biotin-NHS in 1.5 mL DMF were added 500 μ L (7.48 mmol) of ethylenediamine, and stirring was continued for 1 h at room temperature. The product was precipitated with the addition of 50 mL diethyl ether, filtered, and dried under vacuum. Yield: 49 mg (93%).

1H NMR (200 MHz, $DMSO-d_6$), δ , ppm: 8.00 (1H, s), 7.91–7.67 (2H, m), 6.40 (2H, d, 13.3 Hz), 4.36–4.24 (1H, m), 4.18–4.06 (1H, m), 3.17–2.92 (5H, m), 2.82 (1H, dd, 5.0, 12.6 Hz), 2.57 (1H, d, 12.2 Hz), 2.06 (2H, t, 7.2 Hz), 1.69–1.15 (6H, m). MS-EI spectrum (m/z 40–600): calcd mass using formula $C_{12}H_{22}N_4O_2S$, $[M+H]^+$ 287.15; found m/z 287.

Seta-633-CONH-(CH₂)₂NH-biotinyl. A mixture of 20 mg (19.8 mmol) of Seta-633, 8.1 mg (28.3 mmol) of *N*-[2-(*D*-biotinylamino)ethyl]amine, and 5 μ L (28.7 mmol) of DIPEA in 1 mL of DMF was stirred for 2 h at room temperature. The reaction was monitored by HPLC (Discovery Bio Wide Pore C18, gradient from 0.05% trifluoroacetic acid (TFA) in water to 50:50 (0.05% TFA in water:0.05% TFA in acetonitrile). The reaction mixture was diluted with 20 mL of water and then purified by column chromatography (Lichroprep RP-18, MeOH–water (1:9, v/v) as eluent) to give Seta-633-mono-CONH-(CH₂)₂NH-biotinyl as a dark-blue solid with yield 18.7 mg (79%). The purity of Seta-633-CONH-(CH₂)₂NH-biotinyl was 89.3% by HPLC. The structure of Seta-633-CONH-(CH₂)₂NH-biotinyl was confirmed by 1H NMR (200 MHz, $DMSO-d_6$) spectroscopy. The corresponding signals of the biotin moiety which can be found at 7.88 (2H, broad s), 6.41 (2H, d, 11.8 Hz), 4.37–4.23 (1H, m), 2.82 (1H, dd, 5.0, 12.6 Hz), 2.57 (1H, d, 12.1 Hz) are visible. All other proton signals of the biotin moiety overlap with dye proton signals.

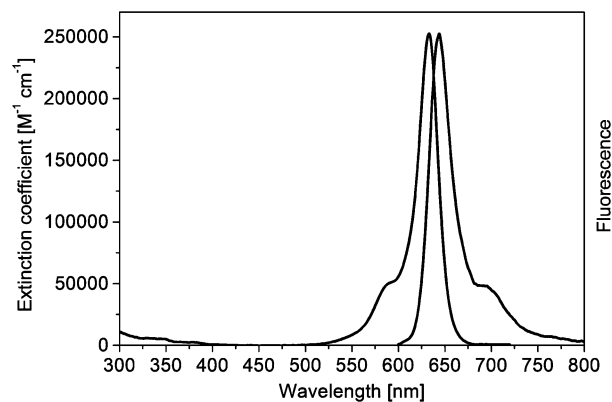
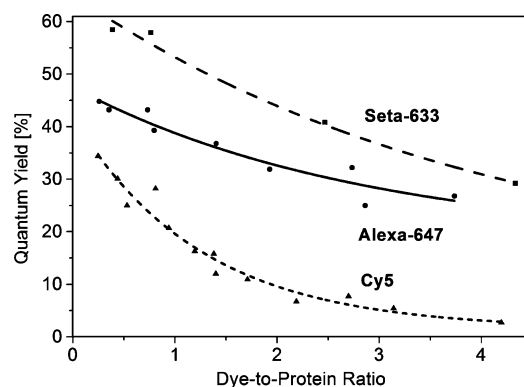
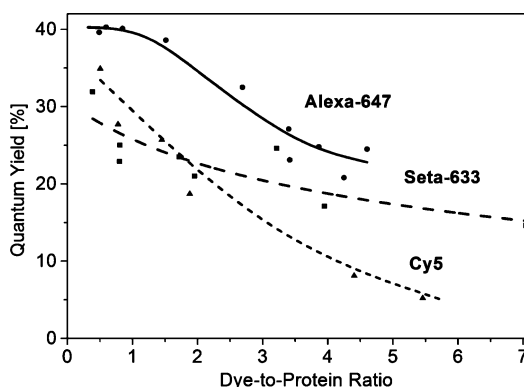
Immunoassay. The immunoassay experiments included a titration of Seta-633–biotin with specific (antibiotin antibody produced in goat, affinity isolated antibody, Sigma) and nonspecific (IgG from bovine serum (reagent grade, $\geq 95\%$, Sigma) antibodies. A 0.6 μ M solution of Seta-633–biotin was prepared in PB pH 7.4.

During each titration step, 0.06 μ M of the antibody was added to the labeled biotin solution. After each addition of the antibodies, the solution containing the labeled antigen and antibodies was incubated at 37 $^\circ$ C for 5 min in the dark, followed by measurement of the fluorescence lifetime.

RESULTS AND DISCUSSION

Absorption, Emission Spectra, and Stokes' Shifts. The spectral characteristics of Seta-633 and their BSA- and IgG-conjugates in PB pH 7.4 are given in Table 1; a representative absorption and emission spectrum of Seta-633 is shown in Figure 1.

The absorption and emission maximum of Seta-633 (Table 1) makes it perfectly suited for use with the 635 nm diode laser. Covalent binding of Seta-633 to BSA produces a red-shift of both the absorption (13 nm) and emission (12 nm) maxima, while the red-shift for the IgG conjugates is substantially smaller (4 and 3 nm, respectively).

**Figure 1.** Absorption and emission spectrum of Seta-633.**Figure 2.** Quantum yield vs dye-to-protein ratio of dye–BSA conjugates in PB pH 7.4.**Figure 3.** Quantum yield vs dye-to-protein ratio of dye–IgG conjugates in PB pH 7.4.

Seta-633 exhibits a lower quantum yield in aqueous solutions, but these quantum yields (QYs) substantially increase after binding to proteins. The QYs of the Seta-633-BSA conjugates are as high as 50% and generally exceed those of Cy5-BSA and Alexa Fluor 647-BSA conjugates for the same dye-to-protein ratios (Figure 2). The QY increase is less pronounced upon binding to proteins such as IgG (Figure 3, Table 1).

Photostability. The photostability of dyes plays important role for their practical use. We investigated the photostabilities of Seta-633 and its IgG conjugates and compared them to Cy5 and Alexa Fluor 647. These measurements were carried out in PB pH 7.4. The relative photostabilities for the free dyes and the IgG conjugates are presented in Figure 4a and b. Figure 4b clearly demonstrates reduced photobleaching for Seta-633-IgG conjugates upon prolonged exposure to light.

Fluorescence Lifetimes. As expected, the mean fluorescence lifetime (τ_{mean}) of Seta-633 measured in PB 7.4 is shorter than

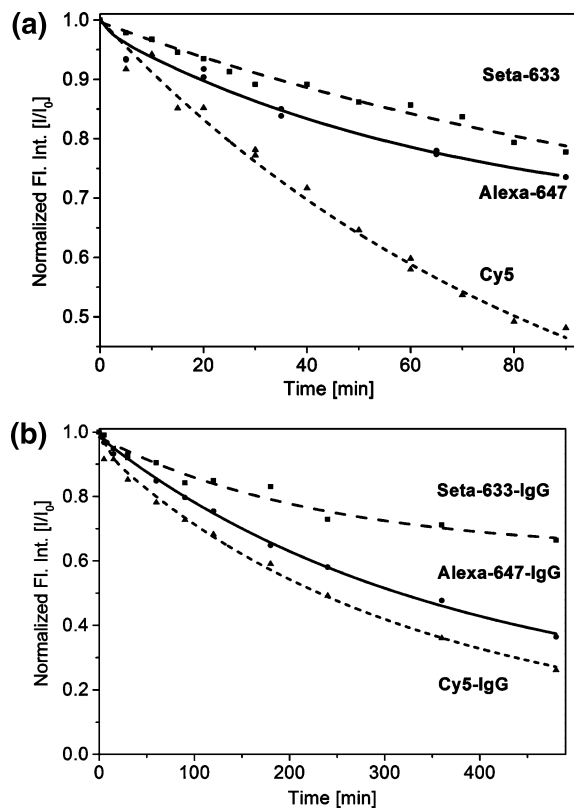


Figure 4. a. Normalized fluorescence intensity changes for free dyes upon exposure to light from a halogen lamp (500 W). b. Normalized fluorescence intensity changes for dye-conjugates upon exposure to light from a halogen lamp (500 W).

Table 2. Dye-to-Protein Ratios, Lifetimes, and Fractional Contributions for Selected Dyes and Dye-Conjugates

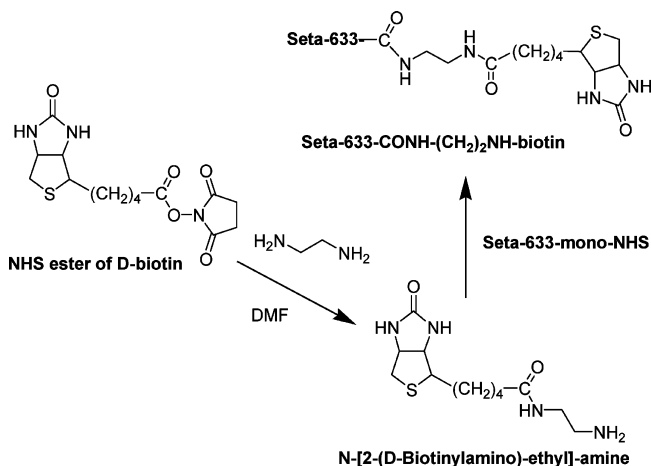
dye or dye conjugate	D/P	τ_1 (ns)	f_1	τ_2 (ns)	f_2	χ^2	τ_{mean} (ns)
Cy5	—	1.00	1.00	—	—	1.20	1.00
Cy5-BSA	0.94	0.28	0.10	1.88	0.90	1.49	1.72
Cy5-IgG	1.90	0.28	0.23	1.47	0.77	1.95	1.20
Alexa 647	—	1.17	1.00	—	—	1.99	1.17
Alexa 647-BSA	0.90	0.33	0.07	1.91	0.93	0.63	1.80
Alexa 647-IgG	1.83	0.31	0.20	1.29	0.80	1.87	1.09
Seta-633	—	0.12	0.61	0.46	0.39	0.58	0.25
Seta-633-biotin	—	0.27	0.33	0.87	0.67	1.22	0.66
Seta-633-BSA	0.90	0.56	0.05	2.93	0.95	0.94	2.81
Seta-633-IgG	1.10	0.40	0.26	1.88	0.74	1.19	1.49

those for Cy5 and Alexa Fluor 647 (Table 2), which is because polar compounds are quenched in polar solvents and squaraine dyes can be considered more polar than cyanine dyes (17). While the intensity decays for Cy5 and Alexa Fluor 647 are well-fitted with monoexponential curves, the decays for Seta-633 are better fitted with a biexponential decay curve (15). The observed biexponential decay times for Seta-633 and Seta-633-biotin are most probably due to dye-solvent effects (27).

The fluorescence lifetime of Seta-633 as well as Seta-633-biotin increase several-fold after binding to proteins. Covalent binding of Seta-633 to BSA leads to an 10-fold lifetime increase from 250 ps to 2.81 ns, while the average lifetime increase between the free and IgG-bound Seta-633 is about 6-fold. This is in contrast to cyanine-based labels, which show less pronounced fluorescence lifetime changes upon labeling to proteins—especially antibodies (Table 2).

The observed large fluorescence lifetime changes between Seta-633 free in solution and coupled to IgG were instrumental to our decision to evaluate Seta-633 in a lifetime-based immunoassay. We intentionally chose the immunoassay format

Scheme 1. Synthesis of Seta-633-Biotin



over the biotin-avidin/streptavidin interaction because of its broader applicability. In general, biotin could be replaced by any other low-molecular-weight antigen.

Model Immunoassay. For the model immunoassay, a labeled biotin-conjugate of Seta-633 as shown in Scheme 1 was synthesized. Coupling between D-biotin and Seta-633 was facilitated by introducing an ethylenediamine linker into biotin (28). N-[2-(D-Biotinylamino)ethyl]amine was synthesized analogously to a literature procedure but by using biotin-NHS instead of biotin and CDI (1,1'-carbonyldiimidazole) (29). Dimer formation was prohibited by using an excess of ethylenediamine. Subsequently, the amino-group of N-[2-(D-biotinylamino)ethyl]amine was conjugated to Seta-633 to yield Seta-633-CONH-(CH₂)₂NH-biotin (see Biotin Conjugation).

The introduction of the biotin-ethylenediamine moiety into Seta-633 causes an increase in the average lifetime from 0.25 to 0.66 ns (Table 2). Titration of the small-molecular-weight tracer with its specific antibody (antibiotin, MW = 160 kDa) leads to a steady increase of the fluorescence lifetime to a maximum value of 1.82 ns, a 176% increase compared to the unbound tracer. Importantly, no change is observed when the tracer was titrated with nonspecific antibody or BSA, indicating that the nonspecific interactions due to hydrophobic binding of are minimal (Figures 5 and 6).

We also made an attempt to demonstrate a competitive lifetime-based phase-modulation assay with the biotin/antibiotin model system. In this competitive assay, fixed concentrations of 0.65 μM Seta-633-biotin tracer and antibiotin (ratio = 1:1) were incubated with increasing concentrations of free biotin.

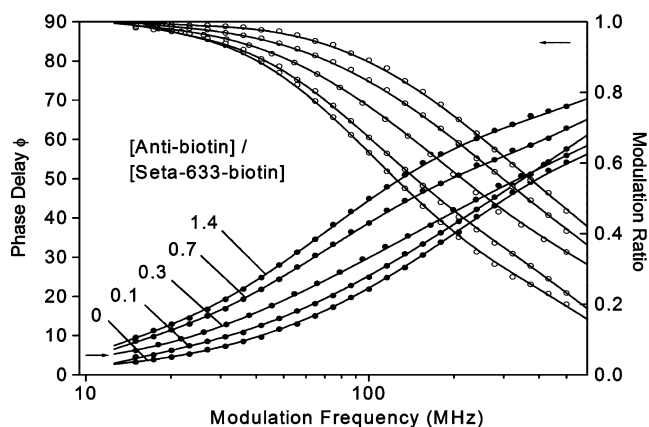


Figure 5. Frequency response curves (phase angle and modulation ratio vs modulation frequency) of Seta-633-biotin (0.65 μM) upon titration with antibiotin (specific antibody).

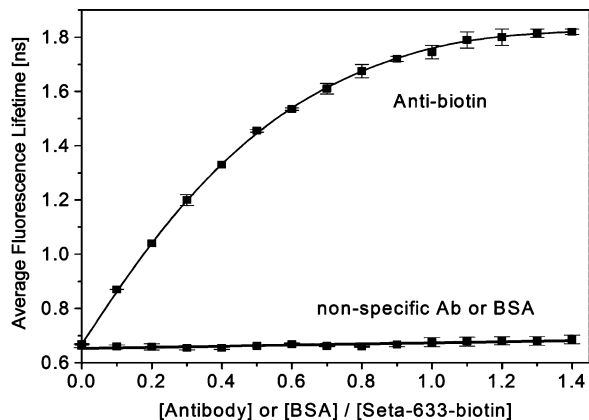


Figure 6. Average fluorescence lifetime changes of Seta-633-biotin (0.65 μM) upon titration with antibody (specific), (nonspecific) human IgG, or BSA.

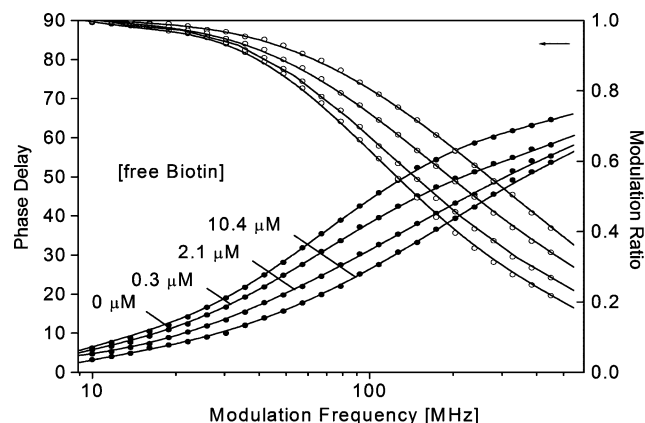


Figure 7. Frequency response curves for the titration of the Seta-633-biotin-antibiotin complex with free biotin (competitive reaction).

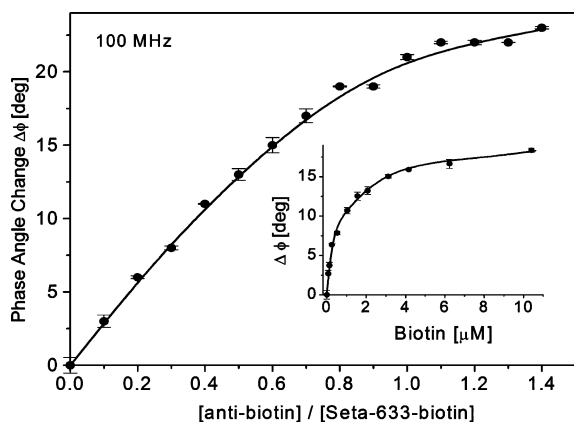


Figure 8. Changes in phase angle upon titration of Seta-633-biotin (0.65 μM) with antibody (specific antibody), measured at 100 MHz. The inset shows phase angle changes of a mixture of 0.65 μM Seta-633-biotin and 0.65 μM antibody (ratio of 1) upon titration with free biotin, measured at 100 MHz.

Replacement of the labeled biotin tracer by free biotin leads to a shift of the frequency response curves to higher frequencies (Figures 7, 8) and thus shortening of the fluorescence lifetime. Addition of 0 to 10 μM concentrations of biotin leads to a total change of 18° in phase angle. Present instrumentation allows the phase angle to be measured accurately to 0.1° and modulation with an accuracy of 0.005 (30).

Homogeneous immunoassays can be performed even in whole blood, because red and NIR dyes allow excitation beyond the absorptive bands of hemoglobin and tissue.

CONCLUSIONS

The hydrophilic, amine-reactive NIR fluorescent label Seta-633 exhibits favorable properties as a lifetime label for the measurement of small-molecular-weight analytes. The label is compatible with readily available light sources (635 nm or 640 nm diode lasers) and filter sets (as for Cy5 or Alexa Fluor 647). Seta-633 exhibits lower quantum yields and shorter fluorescence lifetimes when free in solution, but these values substantially increase upon interaction with proteins, which is contrary to fluorescent tracers like Cy5 or Alexa 647, which typically exhibit much smaller lifetime changes in particular upon binding to antibodies. Importantly, Seta-633 and its conjugates are also more photostable as compared to Cy5 and Alexa Fluor 647.

ACKNOWLEDGMENT

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Supporting Information Available: Mass spectrum of *N*-[2-(*D*-biotinylamino)ethyl]amine. This material is available free of charge via the Internet at <http://pubs.acs.org>.

LITERATURE CITED

- (1) Daehne, S., Resch-Genger, U., and Wolfbeis, O. S., Eds. (1998) *Near-Infrared Dyes for High Technology Applications, NATO ASI Ser. 3 (High Technology) 53*, Kluwer Academic Publishers, Dordrecht, The Netherlands.
- (2) Van Dyke, K., Ed. (1990) *Luminescence Immunoassay and Molecular Applications*, CRC Press, Boca Raton, FL.
- (3) Lakowicz, J. R., Ed. (1992) *Topics in Fluorescence Spectroscopy Volume 3, Biochemical Applications*, Plenum Press, New York.
- (4) Wolfbeis, O. S., Ed. (2008) *Fluorescence Methods and Applications, Spectroscopy, Imaging, and Probes*, Wiley-Blackwell, New York.
- (5) Panchuk-Voloshina, N., Haugland, R. P., Bishop-Stewart, J., Bhalgat, M. K., Millard, P. J., Mao, F., Leung, W.-Y., and Haugland, R. P. (1999) Alexa dyes, a series of new fluorescent dyes that yield exceptionally bright, photostable conjugates. *J. Histochem. Cytochem.* 47, 1179–1188.
- (6) Chen, F. T., and Evangelista, R. A. (1994) Feasibility studies for simultaneous immunochemical multianalyte drug assay by capillary electrophoresis with laser-induced fluorescence. *Clin. Chem.* 40, 1819–1822.
- (7) Lay, M. J., and Wittwer, C. T. (1997) Real-time fluorescence genotyping of factor V Leiden during rapid-cycle PCR. *Clin. Chem.* 43, 2262–2267.
- (8) Qin, Q.-P., Peltola, O., and Pettersson, K. (2003) Time-resolved fluorescence resonance energy transfer assay for point-of-care testing of urinary albumin. *Clin. Chem.* 49, 1105–1113.
- (9) Mezzasoma, L., Bacarese-Hamilton, T., Di Cristina, M., Rossi, R., Bistoni, F., and Crisanti, A. (2002) Antigen microarrays for serodiagnosis of infectious diseases. *Clin. Chem.* 48, 121–130.
- (10) Berlier, J. E., Rothe, A., Buller, G., Bradford, J., Gray, D. R., Filanoski, B. J., Telford, W. G., Yue, S., Liu, J., Cheung, C.-Y., Chang, W., Hirsch, J. D., Beechem, J. M., Haugland, R. P., and Haugland, R. P. (2003) Quantitative comparison of long-wavelength Alexa Fluor dyes to Cy dyes: Fluorescence of the dyes and their bioconjugates. *J. Histochem. Cytochem.* 51, 1699–1712.
- (11) Huss, K. L., Blonigen, P. E., and Campbell, R. M. (2007) Development of a Transcreeper kinase assay for protein kinase A and demonstration of concordance of data with a filter-binding assay format. *J. Biomol. Screen.* 12, 578–584.

- (12) Miller, A. E., Fischer, A. J., Laurence, T., Hollars, C. W., Saykally, R. J., Lagarias, J. C., and Huser, T. (2006) Single-molecule dynamics of phytochrome-bound fluorophores probed by fluorescence correlation spectroscopy. *Proc. Natl. Acad. Sci. U.S.A.* 103, 11136–11141.
- (13) Bruckbauer, A., James, P., Zhou, D., Yoon, J. W., Excell, J. D., Korchev, Y., Jones, R., and Klenerman, D. (2007) Nanopipette delivery of individual molecules to cellular compartments for single-molecule fluorescence tracking. *Biophys. J.* 93, 3120–3131.
- (14) Terpetschnig, E., Szmecinski, H., Ozinskas, A., and Lakowicz, J. R. (1994) Synthesis of squaraine-N-hydroxysuccinimide esters and their biological application as long-wavelength fluorescent labels. *Anal. Biochem.* 217, 197–204.
- (15) Terpetschnig, E., Szmecinski, H., and Lakowicz, J. R. (1993) Synthesis, spectral properties and photostabilities of symmetrical and unsymmetrical squaraines; a new class of fluorophores with long-wavelength excitation and emission. *Anal. Chim. Acta* 282, 633–641.
- (16) Oswald, B., Patsenker, L., Duschl, J., Szmecinski, H., Wolfbeis, O. S., and Terpetschnig, E. (1999) Synthesis, spectral properties, and detection limits of reactive squaraine dyes, a new class of diode laser compatible fluorescent protein labels. *Bioconjugate Chem.* 10, 925–931.
- (17) Tatarts, A. L., Fedyunyayeva, I. A., Dyubko, T. S., Povrozin, Y. A., Doroshenko, A. O., Terpetschnig, E. A., and Patsenker, L. D. (2006) Synthesis of water-soluble, ring-substituted squaraine dyes and their evaluation as fluorescent probes and labels. *Anal. Chim. Acta* 570, 214–223.
- (18) Patsenker, L., Tatarts, A., Kolosova, O., Obukhova, O., Povrozin, Y., Fedyunyayeva, I., Yermolenko, I., and Terpetschnig, E. (2008) Fluorescent probes and labels for biomedical applications. *Ann. N.Y. Acad. Sci.* 1130, 179–187.
- (19) Yasui, S., Matsuoka, M., and Kitao, T. (1988) Synthesis and some properties of infrared-absorbing croconium and related dyes. *Dyes Pigm.* 10, 13–22.
- (20) Turconi, S., Bingham, R. P., Haupts, U., and Pope, A. J. (2001) Developments in fluorescence lifetime-based analysis for ultrafast. *DDT* 6, 27–39.
- (21) Lakowicz, J. R. (2006) *Principles of Fluorescence Spectroscopy*, 3rd ed., Springer, Berlin/Heidelberg.
- (22) Mujumdar, S. R., Mujumdar, R. B., Grant, C. M., and Waggoner, A. S. (1996) Cyanine-labeling reagents: Sulfoindocyanine succinimidyl esters. *Bioconjugate Chem.* 7, 356–362.
- (23) Povrozin, Y. A., Markova, L. I., Tatarts, A. L., Sidorov, V. I., Terpetschnig, E. A., and Patsenker, L. D. (2009) Near-infrared, dual-ratiometric fluorescent label for measurement of pH. *Anal. Biochem.* 390 (2), 136–140.
- (24) Schobel, U., Egelhaaf, H.-J., Brecht, A., Oelkrug, D., and Gauglitz, G. (1999) New donor-acceptor pair for fluorescent immunoassay by energy transfer. *Bioconjugate Chem.* 10, 1107–1114.
- (25) Mujumdar, R. B., Ernst, L. A., Mujumdar, S. R., Lewis, C. J., and Waggoner, A. S. (1993) Cyanine dye labeling reagents: Sulfoindocyanine succinimidyl esters. *Bioconjugate Chem.* 4, 105–111.
- (26) Parker, C. A. (1968) *Photoluminescence of Solutions*, Elsevier Publishing Company, Amsterdam.
- (27) Law, C.-Y. (1987) Squaraine Chemistry. Effects of structural changes in the absorption and multiple fluorescence emission of bis[4-(dimethylamino)phenyl]squaraine and its derivatives. *J. Phys. Chem.* 91, 5184–5193.
- (28) Patnaik, S., Swami, A., Sethi, D., Pathak, A., Garg, B. S., Gupta, K. C., and Kumar, P. (2007) .N-(Iodoacetyl)-Nε-(anthraquinon-2-oyl)-ethylenediamine (IAED): A new heterobifunctional reagent for the preparation of biochips. *Bioconjugate Chem.* 18, 8–12.
- (29) Li, Z., Ortega-Vilain, A.-C., Patil, G. S., Chu, D.-L., Foreman, J. E., Eveleth, D. D., and Powers, J. C. (1996) Novel peptidyl r-keto amide inhibitors of calpains and other cysteine proteases. *J. Med. Chem.* 39, 4089–4098.
- (30) Murtaza, Z., Chang, Q., Rao, G., Lin, H., and Lakowicz, J. R. (1997) Long-lifetime metal-ligand pH probe. *Anal. Biochem.* 247, 216–222.

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