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10-(2-Biotinyloxyethyl)-9-acridone A novel fluorescent label for (strept)avidin–biotin based assays

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Abstract

A novel biotinylated fluorophore, 10-(2-biotinyloxyethyl)-9-acridone **3** has been synthesized and its fluorescent properties were examined in the presence and absence of avidin or streptavidin. In aqueous solutions the novel fluorophore **3**, as well as its precursor 10-(2-hydroxyethyl)-9-acridone **2** exhibit intense fluorescence and can be detected down to 8.62×10^{-10} and 1.90×10^{-10} M, respectively. A short spacer was chosen in order to minimize steric repulsion between the adjacently bound to avidin or streptavidin residues of acridone. The novel biotinylated fluorophore **3** exhibits rapid, specific and stoichiometric binding to the four biotin binding sites in avidin or streptavidin tetramers, even at low concentrations (20 nM). Preliminary measurements showed that the new conjugate can be applied for the fluorimetric determination of solid-phase immobilized mouse IgG with detection limits down to 1.3 ng per assay.

Keywords: Acridone; Biotin; Avidin; Streptavidin; Fluorescence

1. Introduction

Biotin-fluorophores have often been used for the detection of biotinylated biomolecules, such as proteins, peptides or DNA via biotin–(strept)avidin–biotin bridges but most of them lose part of their fluorescence when binding to (strept)avidin. Preservation of high fluorescence upon binding to (strept)avidin is a necessary but insufficient criterion for biotinylated fluorophores if they are to be used as labels. Most important is that they must show (a) high values of fluorescence efficiency in aqueous solutions; (b) absence of non-specific binding and (c) high affinity to avidin or streptavidin.

In this paper, we present a novel biotin-fluorophore based on acridone which fulfil all the above mentioned criteria and retains about 60% (or 25%) of its fluorescence after binding to avidin (or streptavidin). A short three-atom spacer was chosen between the fluorescent molecule and biotin in order to minimize steric repulsion between the adjacently bound to avidin or streptavidin residues of acridone which comes in agreement with corresponding studies reported in literature [1–3]. Further, encouraged by our very promising published results in which acridinium–biotin conjugates comprising short spacer moieties can be applied for the detection of biotinylated proteins [4], we also decided to test the novel conjugate **3** for the detection of immobilized biotinylated mouse IgG.

2. Experimental

2.1. Equipment/reagents

^{1}H- and ^{13}C nmr spectra were measured on a Brucker AC 250 spectrometer. ESI Mass spectra were recorded on a Finnigan spectrometer, AQA Navigator at a flow rate of 0.1 ml min^{-1} using a Harvant Syringe pump. Hot nitrogen gas was used for desolvation (Dominic-Hunter UHPLC MS-10). Infrared spectra were obtained using a Perkin-Elmer 283 FT-IR spectrometer. Elemental analyses were obtained with a Perkin-Elmer CHN 2004 instrument. The melting point was recorded on a Gallenkamp apparatus and is uncorrected. Absorption spectra were run on a JASCO V-560 spectrophotometer while fluorescence spectra run on a JASCO FP-777 spectrofluorimeter (Scan speed 200 nm min^{-1}, emission band 5 nm). The solid-phase fluores-

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cence measurements were performed on a Perkin-Elmer LS-50B luminescence spectrometer.

2.2. Reagents

All solvents used in the present work [*N*,*N*-dimethylformamide (DMF), dimethylsulfoxide (DMSO), chloroform] were dried and distilled prior to their use. Affinity purified avidin, streptavidin and biotin were purchased from Sigma. Acridone, 10-methyl-9-acridone, ethylene carbonate and *N*,*N*-carbonyldiimidazole were purchashed from Aldrich and used without any further purification. Phosphate buffer, stock solution (PBS) was prepared from Na_2HPO_4 (10 mM), KH_2PO_4 (1.8 mM), NaCl (140 mM), KCl (2.7 mM) and maintained in refrigerator at 4 °C after filtering over 0.45 μM Whatman paper. Working solutions of PBS were daily prepared by 1:10 dilution of the stock solution and adjusted to pH 7.4.

2.3. Syntheses

2.3.1. 10-(2-Hydroxyethyl)-9-acridone, **2**

For the synthesis of this compound a known procedure was followed [5]. In a 100 ml round flask containing 4 ml DMF, acridone (1.0 g, 5.12 mmol), ethylene carbonate (0.8 g, 9.1 mmol) and KOH (11 mg, 0.196 mmol) were added successively and the mixture was stirred under reflux for 6 h. Then, the solvents were evaporated under reduced pressure chloroform was added to the residue and then extracted with water. The product precipitated as a yellow solid in the aqueous layer and was filtered. Chemical yield (400 mg, 33%); m.p.: 192–193 °C; UV–vis (H_2O): λ = 393, 410 nm; fluorescence (H_2O): λ_{em} = 427, 450 nm, λ_{exc} = 393 nm; IR (KBr): ν_{max} 3321, 2972, 2922, 2905, 2874, 1610 (C=O), 1596, 1589, 1498, 1460, 1379, 1350, 1290, 1269, 1182, 1177, 1088, 756, 673 cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6): δ 8.34 (dd, 2H, *J* = 7.79, 1.38 Hz), 7.88 (brd, 2H, *J* = 8.71 Hz), 7.80 (ddd, 2H, *J* = 8.71, 7.33, 1.38 Hz), 7.32 (ddd, 2H, *J* = 7.79, 7.33, 0.46 Hz), 5.09 (t, 1H, *J* = 5.5 Hz), 4.59 (t, 2H, *J* = 5.96 Hz), 3.89 (dt, 2H, *J* = 5.96, 5.5 Hz); ^{13}C NMR (125 MHz, DMSO-d_6): δ 176.45 (C=O), 142.08, 133.87, 126.50, 121.54, 121.15, 116.34, 58.27, 47.54; MS (*m*/*z*, %): 240.2 (M^++1, 67), 196.2 (M^+-43, 100), 167.2 (14); Anal. Calc. for $C_{15}H_{13}NO_2$ (239.26): C 75.30, H 5.47, N 5.85; found C 74.89, H 5.71, N 5.68.

2.3.2. 10-(2-Biotinyloxyethyl)-9-acridone, **3**

Biotin (244 mg, 1 mmol) was added under argon to 5 ml DMF containing 50 mg molecular sieves (4 Å) and was heated at 80 °C until biotin was dissolved. *N*,*N*′-Carbonyldiimidazole (162 mg, 1 mmol) was added and the mixture was heated at 80 °C for another 15 min until CO_2 evolution ceased. The mixture was then stirred for 2 h at room temperature during which time the intermediate biotinylimidazolide came out of solution as a flocculent white precipitate. Then, a DMF solution of 10-(2-hydroxyethyl)-9-acridone (240 mg, 1 mmol) was added at room temperature and stirred at 110 °C for 7 h. The solvents were evaporated under reduced pressure and the product was purified by flash chromatography (silica gel, firstly with chloroform/diethylether/acetone 9:3:1 in order to remove the residual reactant **2** R_f = 0.41, and then chloroform/methanol 3:1 in order to obtain the product). Chemical yield: 230 mg (50%); UV–vis (H_2O): λ = 391, 408 nm; Fluorescence (H_2O): λ_{em} = 425, 448 nm, λ_{exc} = 393 nm; IR (KBr): ν_{max} 3391, 3271, 3074, 2926, 2856, 1732, 1701, 1630, 1607, 1597, 1493, 1462, 1375, 1292, 1265, 1180, 756, 675 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 8.42 (dd, 2H, *J* = 7.79, 1.38 Hz), 7.62 (ddd, 2H, *J* = 8.71, 7.33, 1.38 Hz), 7.51 (brd, 2H, *J* = 8.71 Hz), 7.18 (ddd, 2H, *J* = 7.79, 7.33, 0.46 Hz), 6.30 (s, NH), 5.96 (s, NH), 4.53 (t, 2H, *J* = 6.87 Hz), 4.43 (t, 2H, *J* = 6.87 Hz), 4.40 (m, 1H), 4.18 (m, 1H), 3.03–2.63 (complex area, 3H), 2.20 (t, 2H, *J* = 7.33 Hz), 1.62–1.32 (complex area, 6H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 177.61 (C=O), 173.29 (C=O), 163.89 (C=O), 141.68, 133.90, 127.50, 122.17, 121.41, 114.51, 61.81, 60.02, 59.99, 55.32, 43.71, 40.35, 33.48, 28.14, 28.03, 24.34; MS (*m*/*z*, %): 467.2 (M^+ +2, 31), 466.2 (M^+ + 1, 100), 227 (26); Anal. Calc. for $C_{25}H_{27}N_3O_4S$ (465.57): C 64.50 H 5.84, N 9.03, found: C 64.82 H 6.03, N 8.87.

2.4. Fluorescence measurements

2.4.1. Fluorescence quantum yields and detection limits

The fluorescence quantum yields of the compounds **2** and **3** were determined according to a known procedure [6]. 10-Methyl-9-acridone was employed as reference compound considering that its fluorescence spectra are similar to those of the compounds **2** and **3**. Its fluorescence quantum yield in water is equal to 0.82 [7]. The fluorescence quantum yields were calculated by the equation: $\Phi_u = (F_u A_s n_u^2 / F_s A_u n_s^2)\Phi_s$ where Φ_u and Φ_s are the fluorescence quantum yields of unknown and reference compound; F_u and F_s the integrated emission area of the unknown and reference compound between 400 nm and 550 nm; A_u and A_s the absorbance of the unknown and standard compound at the excitation wavelength; n_u and n_s the refractive indexes of the solvents containing the unknown and reference compound. For the determination of the detection limits of the compounds **2** and **3** aqueous solutions of 10^{-4} to 10^{-11} M were prepared by sequential dilutions of a stock solution (10^{-3} M) in anhydrous DMSO with distilled water. The fluorescence measurements were performed at λ_{exc} = 393 nm, λ_{em} = 425 nm and excitation/emission slits equal to 5 nm.

2.4.2. Determination of functional concentrations of avidin/streptavidin and biotinylated compound **3**

The functional concentration of (strept)avidin was determined by titration with D-biotin by monitoring its fluorescence quenching at 350 nm (excitation at 290 nm) [8]. In a typical experiment, avidin or streptavidin was dissolved in PBS buffer at ∼3 μM (stock solution, nominal concentration by weight). Three millilitres of (strept)avidin solution in PBS (100 nM, nominal concentration) was pipetted into a 4 ml fluorimeter cell and titrated with a 10 μM biotin standard solution by successive additions of 10 μl increments at 3-min intervals. The breakpoint between the progressive quenching and the subsequent plateau indicates the amount of D-biotin needed for satura-

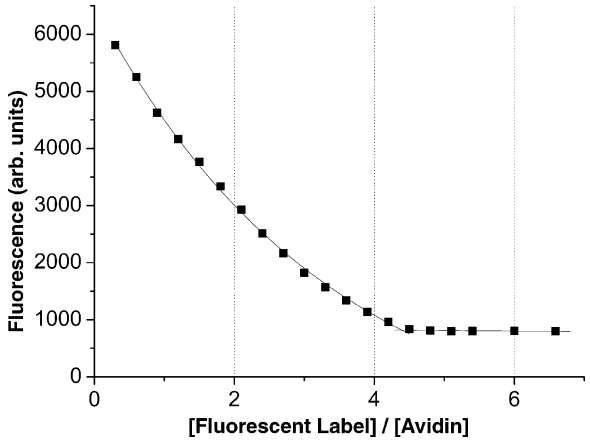


Fig. 1. Dependence of avidin fluorescence in the presence of various amounts of biotin–acridone **3**.

tion of all biotin-binding sites in (strept)avidin. The functional concentration of (strept)avidin was defined as [biotin-binding sites]/4.

The functional concentration of the conjugate **3** was determined by monitoring the decrease of tryptophan fluorescence at 350 nm as follows: 3 ml of avidin solution in PBS (111.2 nM functional concentration) was titrated with a PBS solution of compound **3** (10 μM nominal concentration) by successive additions of 10 μl increments at 5-min intervals. As shown in Fig. 1, the decrease in tryptophan fluorescence at 350 nm continued until the fluorescent label/avidin concentration ratio attained the value of nearly 4/1. Aqueous stock solution of the biotin–acridone conjugate was stable for at least 3 days at room temperature.

*2.4.3. Fluorescence measurements of the conjugate **3** in the presence of (strept)avidin*

The effect of (stept)avidin on the fluorescence of the novel conjugate **3** was determined by monitoring the fluorescence quenching at 425 nm (excitation at 393 nm). In a typical "forward titration", 3 ml of a PBS avidin solution (40 nM) (Fig. 2, solid squares) or 3 ml of a PBS buffer solution (solid triangles) was titrated with a 3.24 μM standardized conjugate **3** solution in PBS buffer by successive additions of 10 μl increments. Further, in parallel control experiments, 3 ml pre-saturated avidin (40 nM) with a 80-fold excess of D-biotin was titrated by successive additions of 10 μl aliquots of a 10 μM biotin–acridone derivative **3** solution (open circles). Initially, preliminary fluorescence experiments were performed to determine the time required for equilibration at room temperature between the conjugate **3** and avidin. Five minutes was the time required for the equilibrium to be reached, thus this time interval between successive additions of biotin–acridone conjugate was used in all further experiments. Similar experiments were performed with avidin or streptavidin (Fig. 3) at 20 nM concentrations. In the "reverse titration" mode, 3 ml of standardized fluorescent label **3** (90 nM) was titrated with a 700 nM standardized (strept)avidin solution in PBS by successive additions of 10 μl increments at 5-min intervals (Fig. 4).

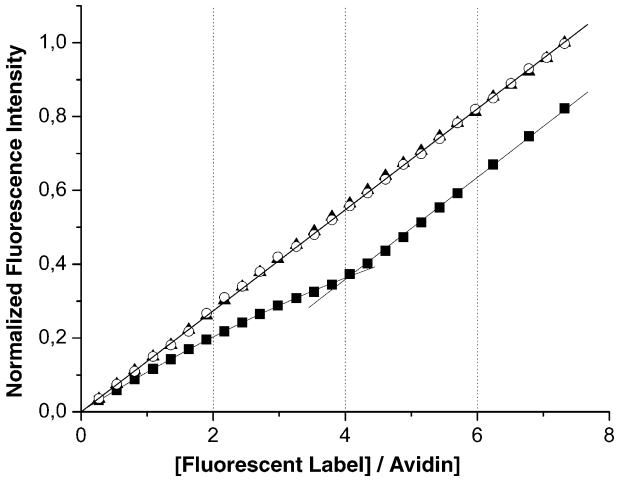


Fig. 2. Normalized fluorescence intensities of biotin–acridone conjugate **3** in the presence (■) and absence of avidin (▲). Avidin has been blocked with excess of biotin before titration with fluorescent ligand (◯). Normalized fluorescence values are obtained by dividing the fluorescence intensity of each point in a curve with the highest one.

2.4.4. Fluorimetric determination of solid-phase immobilized biotinylated mouse IgG

The critical step by this assay is to find the optimized ratio of streptavidin to biotin–acridone conjugate. If the latter reagent is in excess, the streptavidin will be present in less than optimal concentration and the sensitivity of the assay will not be optimal.

The exact amount of the streptavidin–biotin–acridone complex was found empirically by titration. Generally, controlled amounts of streptavidin were mixed with appropriate amounts of conjugate **3** as follows. To 1 ml solution of a 60 g/l bovine serum albumin in 0.1 M Tris buffer, pH 7.80 containing 10 μg (0.166 nmol) of streptavidin were added various amounts of biotin–acridone label **3** 40–232 ng (0.086–0.498 nmol). The optimal mass of biotin–acridone conjugate was found to be 65 ng (0.14 nmol). The optimized mixture is then incubated for 1 h at 55 °C and used directly for the determination of immo-

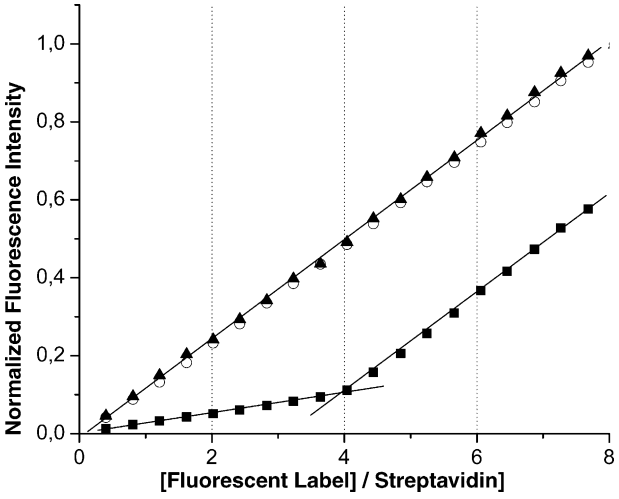


Fig. 3. Normalized fluorescence intensities of biotin–acridone conjugate **3** in the presence (■) and absence of streptavidin (▲). Streptavidin has been blocked with excess of biotin before titration with fluorescent ligand (◯).

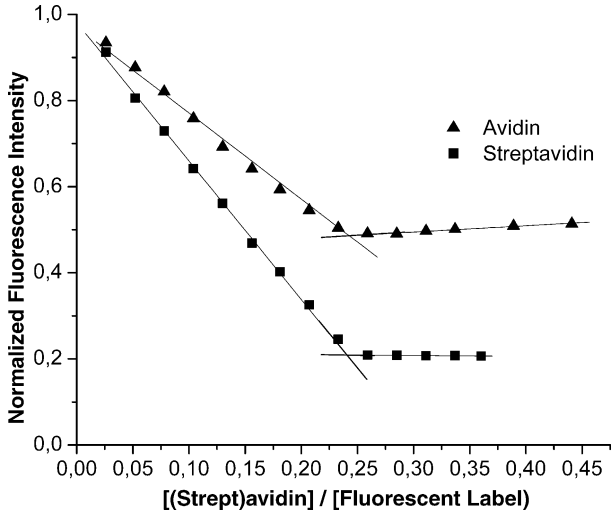


Fig. 4. Inverse titration curves produced by cumulative additions of 10 μl (700 nM) avidin or streptavidin solutions to a standardized biotin–acridone conjugate **3** solution (3 ml, 90 nM).

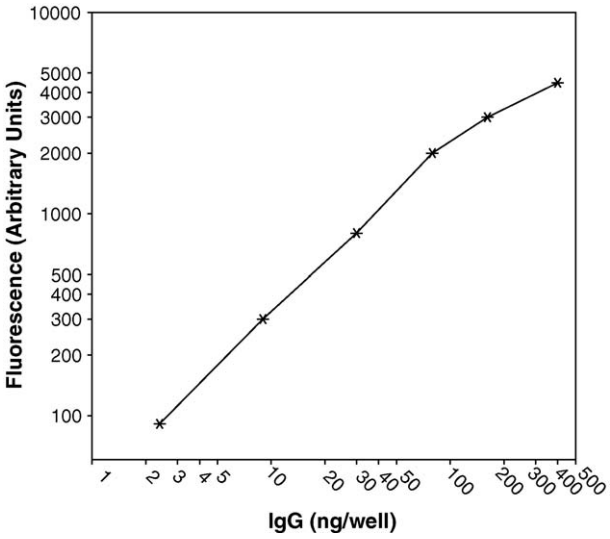


Fig. 5. Calibration curve for quantifying immobilized biotinylated mouse IgG.

bilized biotinylated mouse IgG. By these experiments, high capacity, flat bottomed, white 96-well microtiter plates (Greiner labortechnik) were directly coated overnight at room temperature with varying amounts of a buffered (0.05 M Tris buffer, pH 7.80) biotinylated mouse IgG solution (0–500 ng/well, 100 μl/well). After washing, 100 μl of buffered optimized streptavidin–biotin–acridone complex containing bovine serum albumin was added. After incubation for 25 min, the plate was washed and dried in a forced air microplate dryer and the solid-phase fluorescence was measured. The sensitivity and linearity of measured mouse IgG are presented in Fig. 5.

3. Results and discussion

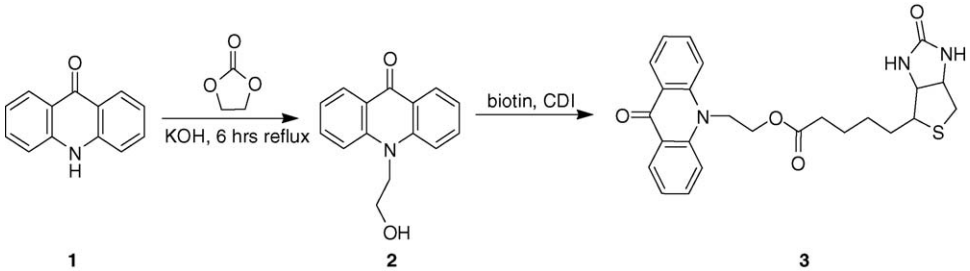
As mentioned in the introduction, we showed in a previous work that biotin–acridine conjugates with short spacers (four-atom spacer) lead to specific, rapid and stoichiometric binding to (strept)avidin [3] and retained most of their fluorescence. Encouraged by these results we desired to extend this work by synthesizing a novel biotin-fluorophore based on acridone with a three-atom spacer between biotin and fluorophore. Acridone was chosen as the light emitting moiety due to the relative high fluorescence efficiency in aqueous solutions ($\Phi_{Fl} = 0.97$) [9]. The primary aim of this work was to determine the fluorescent properties of compound **3** before and after binding to avidin or streptavidin as well as to show its potential application for the fluorimetric determination of solid-phase immobilized biotinylated mouse IgG. It was expected that the sensitivity of this label would be much better than that of biotinylated acridines [3].

3.1. Synthesis of the novel biotin–acridone conjugate 3

The synthesis of the novel biotin–acridone conjugate **3** was carried out in two steps (Scheme 1). In the first step, 9-acridone was converted to 10-(2-hydroxyethyl)-9-acridone, **2** by its reaction with ethylene carbonate according to a known procedure [5]. For the biotinylation of the compound **2**, two different methods were tested, that of the chloroformate [10] and the other of the CDI [11] with the latter one leading to higher chemical yields.

3.2. Fluorescent properties

The biotin–acridone conjugate **3** as well as its precursor **2** could be detected down to subnanomolar concentrations in aqueous solutions (Table 1). Neither the change of solvent nor the biotinylation of the compound **2** does substantially affect the fluorescence efficiency of compounds **2** and **3** (Table 2). Generally, the fluorescence quantum yields of both derivatives are higher in water than in the other solvents. This observation is important since these molecules are intended for analytical applications in aqueous solutions. Furthermore, the fluorescence



Scheme 1. Synthetic route of the biotinylated acridone label **3**.

Table 1
Analytical characteristics of the biotinylated acridone **3** and its precursor **2** associated with fluorescence measurements

Compound	Fluorescence[a] (arbitary units)	Linear range[b] (M)	Detection limit[c] (M)	*A*	*B*	S.D.	*r*
2	5078	10^{-7} to 10^{-9}	1.908×10^{-10}	7.950	0.620	0.049	0.9984
3	4589	10^{-7} to 10^{-9}	8.62×10^{-10}	9.522	0.842	0.082	0.9976

[a] Referred to sample concentration of 10^{-7} M in water; $\lambda_{em} = 425$ nm, $\lambda_{ex} = 393$ nm.
[b] Calculated from the equation: $\log I_{FL} = A + B \log C$.
[c] As detection limit was considered the analyte concentration which gives signals three times greater than blank sample (pure solvent).

quantum yields of the acridone derivatives **2** and **3** are much higher than these of the corresponding acridine derivatives [3]. Biotin–acridone **3** is 10-fold more fluorescent than the previously published biotin–acridine conjugate [3] and can thus be detected at much lower concentrations, exhibiting a linear concentration range over two orders of magnitude (0.1 μM to 1.0 nM).

*3.3. Fluorescence properties of biotin–acridone conjugate **3** before/after binding to (Strept)avidin*

The 10-fold increase in sensitivity observed in the case of the biotin–acridone **3** was encouraging and prompted us to test this compound in a fluorimetric immunoassay. Considering this result, it was of our interest to test the fluorescent properties of the conjugate **3** at low concentrations in the presence of (strept)avidin. For this purpose, we initially investigated the behaviour of the label **3** in a standardized 40 nM avidin solution in PBS. To our satisfaction the novel fluorophore showed fast (within 5 min), specific, and stoichiometric binding to avidin. As shown in Fig. 2, the fluorescence of the acridone derivative **3** is progressively quenched, exhibiting the highest reduction at a molar ratio of label to avidin equal to 4:1 (solid squares). The linear rise in fluorescence at >4 ligands per avidin tetramer was always strictly parallel to the titration profile in the absence of avidin (solid triangles), as expected in the absence of non-specific interaction. Furthermore, the titration profile of blocked avidin with excess of D-biotin (open circles) was indistinguishable from the corresponding control experiment without avidin (solid triangles) confirming the absence of non-specific binding of the biotinylated compound **3** to avidin.

Despite the partial fluorescence quenching that occurred upon binding of the label **3** to avidin, it was of interest to investigate the affinity of the label towards even lower concentrations of avidin or streptavidin. For this purpose, 3 ml of avidin or streptavidin (20 nM) were titrated with 1.62 μM solution of label **3**. It is remarkable that even at this low concentration, the fluorescence quenching of biotin–acridone with avidin in the bound state (data not shown) is similar to this of Fig. 2, while this with streptavidin is more pronounced (Fig. 3). At this point it is important to note that the 4:1 ligand/receptor protein complexes remained stable in buffered solutions for at least 1 h which is within the time scale of a typical bioanalytical application.

The extent of partial fluorescence quenching of the novel conjugate **3** is more obvious in a "reverse titration" mode (Fig. 4). As expected, the highest reduction of the fluorescence intensity of the label **3** occurs at a molar ratio of receptor protein/label equal to 0.25 indicating stoichiometric binding of the conjugate **3** to the four binding sites of (strept)avidin.

Comparing the properties of the novel biotin–acridone **3** to known biotin-fluorophores with short spacer, like biotin-4-fluorescein, biotin-4-FITC, biotin-4-acridines and biotin-4-cyanines [1,3,12] it could be said that all of them present stoichiometric and specific binding to (strept)avidin. Among them, biotin-4-fluorescein and biotin-4-FITC have shown the highest affinity (stoichiometric binding even at 0.2 nM (strept)avidin) and the fastest binding at nanomolar concentrations of (strept)avidin (<1 min at 8 nM (strept)avidin), while biotin-4-Cy3 is the first small fluorescent biotin with intense fluorescence in the (strept)avidin-bound state. The novel three-atom spacer biotin–acridone exhibits high fluorescence yields in aqueous solutions, retains satisfactory fluorescence after binding to (strept)avidin like the above mentioned labels and can be useful in both homogeneous and heterogeneous fluorescence assays. Furthermore, the novel label can mimic D-biotin in terms of high affinity and rapid association and is unique for its exceptional small size, low cost and spectral properties not covered by other dyes. The spectral properties of biotin–acridone conjugate would be even better for excitation with a standard UV-transilluminator than those of biotin-4-fluorescein used in the detection of new (strept)avidin mutants in SDS-PAGE environment [13].

Table 2
Fluorescence quantum yields of biotinylated acridone **3** and its precursor **2** in various solvents

Compound	Fluorescent quantum yield[a]			
	H_2O	DMSO	DMF	$CHCl_3$
2	0.51	0.45	0.37	0.40
3	0.45	0.38	0.29	0.31

[a] Calculated by the equation $\Phi_u = (F_u A_s n_u^2 / F_s A_u n_s^2)\Phi_s$ [8].

*3.4. Application of conjugate **3** in the fluorimetric determination of solid-phase immobilized biotinylated mouse IgG*

Highly sensitive detection technologies based on enzymatically-amplified time-resolved fluorescence, using europium chelates [14–16] as labels to detect several tumor markers [17–21] have been recently described. In this paper, a novel biotin–acridone fluorophore is presented which can be used for the fluorimetric determination of solid-phase biotinylated mouse IgG. As shown in Fig. 5, the sensitivity and linearity of the graph obtained by these measurements

is adequate and may have diverse bioanalytical applications in immunoassays, microarrays and proteomics. At the level of 2.2 ng/well (15.4 fmol/assay), the signal to background ratio of fluorescence is about 10-fold. The detection limit is ~1.3 ng/well (9 fmol/well).

4. Conclusions

In this paper, we presented a novel biotin–acridone fluorophore with favourable properties for bioanalytical applications. The new biotin–acridone conjugate has high fluorescence yields in aqueous solutions and retains about 60% or 25% of its fluorescence after binding to avidin or streptavidin, respectively. Moreover, the novel conjugate presents some advantages, like fast and stoichiometric binding to the four binding sites in (strept)avidin even at 20 nM concentrations and non-specific binding is absent. The adequate remaining fluorescence on solid phase after binding to (strept)avidin could be used for the detection of immobilized mouse IgG at detection limits 9 fmol/assay and may have diverse bioanalytical applications in biosciences.

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