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Highly efficient fluorescent label unquenched by protein interaction to probe the avidin rotational motion

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Abstract

Fluorescent dye alexa-fluor-594 was fixed to biotin with a spacer chain of four atoms. The static, dynamic fluorescence and fluorescence polarization properties of the avidin/labeled-biotin conjugate were investigated and compared with those of free fluorescent dye and directly labeled avidin. With 0.8 label on the average per avidin, one single fluorescence lifetime and one single correlation time related to the actual protein motion were recorded. Moreover, the excited state fluorescent probe is stabilized in the conjugate, with a quantum yield of 0.87, by slowing down the non-radiative processes, while fluorescence lifetime and fluorescence quantum yield increase by 10%, with respect to free alexa-fluor-594.

Keywords: Fluorescent label; Avidin; Alexa-fluor-594; Avidin alexa fluor conjugate; Biotin

1. Introduction

The understanding of protein–protein and protein–surface interactions at interfaces is relevant to many topics: studies on transfer through biological bilayer membranes as well as industrial synthetic membranes [1], manufacturing biosensors like microchips [2,3] devoted to protein identification, health and environmental sciences for prion adsorption in soils [4]. The analysis of interaction processes requires data on interfacial concentration, protein conformation [5–7] and dynamic behavior of proteins at interfaces [8]. Dynamic aspects which give access to protein motion during and after the adsorption process have been much less studied [9,10]. Time resolved fluorescence and steady state fluorescence polarization can give important information on molecular environment and motion of molecules [11,12]. Generally however, intrinsic or extrinsic fluorescence of protein does not permit an easy access to protein rotational motion and environment. The analysis of the intrinsic fluorescence properties in molecular dynamics studies is not easy because the protein involves many tryptophans with low fluorescence quantum yield [13], and most of them with multiple fluorescence lifetimes [14] depending on the environment. Fluorescence anisotropy of tryptophans generally relates to the polypeptide chains motion rather than to the global protein motion. Such kind of study however was carried out with enough accuracy to determine the conformational state of the inhibited form of Subtilisin 309 adsorbed on particles of hydrophilic silica and hydrophobic Teflon [15]. Previous works analyzing fluorescence properties at flat interfaces considered the Total Internal Reflection Fluorescence (TIRF) technique with polarized beam to determine the orientation of molecules. Care was taken to be sure that the fluorescent probe orientation with respect to the molecule was constant to make conclusions on the molecule orientation with respect to the adsorption surface [16,17]. Slow rotational motion was assumed in the interpretation of the data. Fluorescence anisotropy technique is a useful tool to check this assumption. In addition, since it is a goal to study protein interactions at the microscopic level using confocal microscopy, where the volume under examination is of the order of 1 fl (1 μm^3), a labeled protein whose photophysics is simple and well defined with a high quantum yield is required to get a significant signal. Future work about its behavior in interaction with surfaces is

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considered. The most convenient protein should have one single fluorescent lifetime, a high fluorescence quantum yield and one single correlation time.

To study the rotational Brownian motion of protein, the direct labeling commonly used in fluorescence microscopy is not judicious, because the probe is located outside the protein and generally rotates freely, which means that it is no longer correlated to the protein movement. During the past decade the development of fluorescent proteins as molecular tags [18] like green fluorescent protein [19] (GFP) or dsRed [20] were not ideal as GFP has two fluorescence lifetimes [21] and dsRed two correlation times [22]. Finally, we considered to locate the probe as close as possible to the protein surface. For this purpose, we used the high affinity of a protein for its substrate, the latter bearing the fluorescent label.

The avidin–biotin molecular recognition system has many uses in both research and biotechnology [23]. Avidin is a glycoprotein [24] having a molecular weight 66,000 g mol^{-1}, of dimensions [25] 5.6 nm × 5.0 nm × 4.6 nm, composed by 4 identical 128 amino-acids subunits [26]. The very important peculiar feature of avidin is its high affinity for biotin [27] ($K_A = 10^{15}\ M^{-1}$) which permits many kinds of labeling via biotin modifications. The four avidin sites of biotin fixation are equivalent, so the fluorescence of the labels should be the same whichever its location on the tetramer, and chemical routes for biotin modifications much simpler than those linked to genetic protein modification [19].

Nevertheless, prior to use the avidin–biotin system, we have to consider the possible fluorescence quenching by avidin [28,29] as it occurred for many dyes such as fluorescein or Cy5 with 4 or 14 atoms spacers [28,30,31]. On the contrary, the alexa fluor dye [32] (Molecular Probes) is not quenched by the formation of biotin fluorescent dye avidin complex with a 14 atoms long spacer [33]. Choosing a shorter spacer than 14 atoms, it is supposed that the movement of the protein will be probed by the chromophore, hopefully without quenching.

Thus, the aim of this work was to find out with alexa fluor dye a simple convenient avidin labeling route leading to high fluorescence quantum yield, single fluorescence lifetime and single correlation time related to the protein motion. Moreover, the labeling should provide information on the change in the local polarity of the medium surrounding the protein and its motion. Knowledge of the photophysical properties of the conjugate is obviously essential to evaluate the labeling route. In this paper, we present three different labeling procedures of avidin. The first one is the direct labeling of avidin by alexa-fluor-594 (A-al). The two others correspond to labeling by formation of avidin–biotin conjugates, via biotin ethylenediamine (A-B4al) and biocytin (A-B7al).

2. Experimental

We used purified avidin (Sigma, Fluka No. 11368), biotin ethylenediamine (Sigma A5223, as *N*-(2-aminoethyl)biotinamide hydrobromide), biocytin (Sigma, B4261), Na_2HPO_4 and NaH_2PO_4 (Sigma) and alexa-fluor-594 succinimidyl ester (Labeling kit A10239, Molecular Probes, Eugene, OR) as received. Buffer PBS_A pH 7.4 contained 100 mM sodium phosphate and 1.5 M NaCl in de-ionized water (MilliQ system, Millipore). Buffer PBS_B was buffer PBS_A diluted 10 times.

2.1. Direct labeling of avidin (A) with alexa (al): A-al

The labeling reaction of avidin (A) with alexa-fluor-594 (al) succinimidyl ester was achieved according to Molecular Probes recipe provided in the labeling kit. Avidin (0.18 μmol) was dissolved in 1 mL PBS_A (pH 7.4) and mixed with 150 μL 1 M bicarbonate solution. Bicarbonate, pH 8.3, is added to raise the pH of the reaction mixture, since succinimidyl esters react efficiently at pH 7.5–8.5 (Molecular Probes notes). This solution was poured on lyophilized alexa-fluor-594 succinimidyl ester (0.37 μmol), and the mixture stirred at room temperature 1 h to get the product A-al. To separate some alexa not bearing the succinimidyl group (10% mol/mol according to the company), which did not react with avidin, and alexa-labeled avidin, all mixture components were separated by chromatography on resin provided in the same kit. The maximal final mean number of labels per avidin molecule is 1.8. Assuming the loss by hydrolysis to 30% as for reaction with biotin ethylenediamine (see below), the mean number is more likely close to 1.3

2.2. Labeling of avidin(A) via labeled biotin ethylenediamine (B4al) and labeled biocytine (B7al): A-B4al, A-B7al

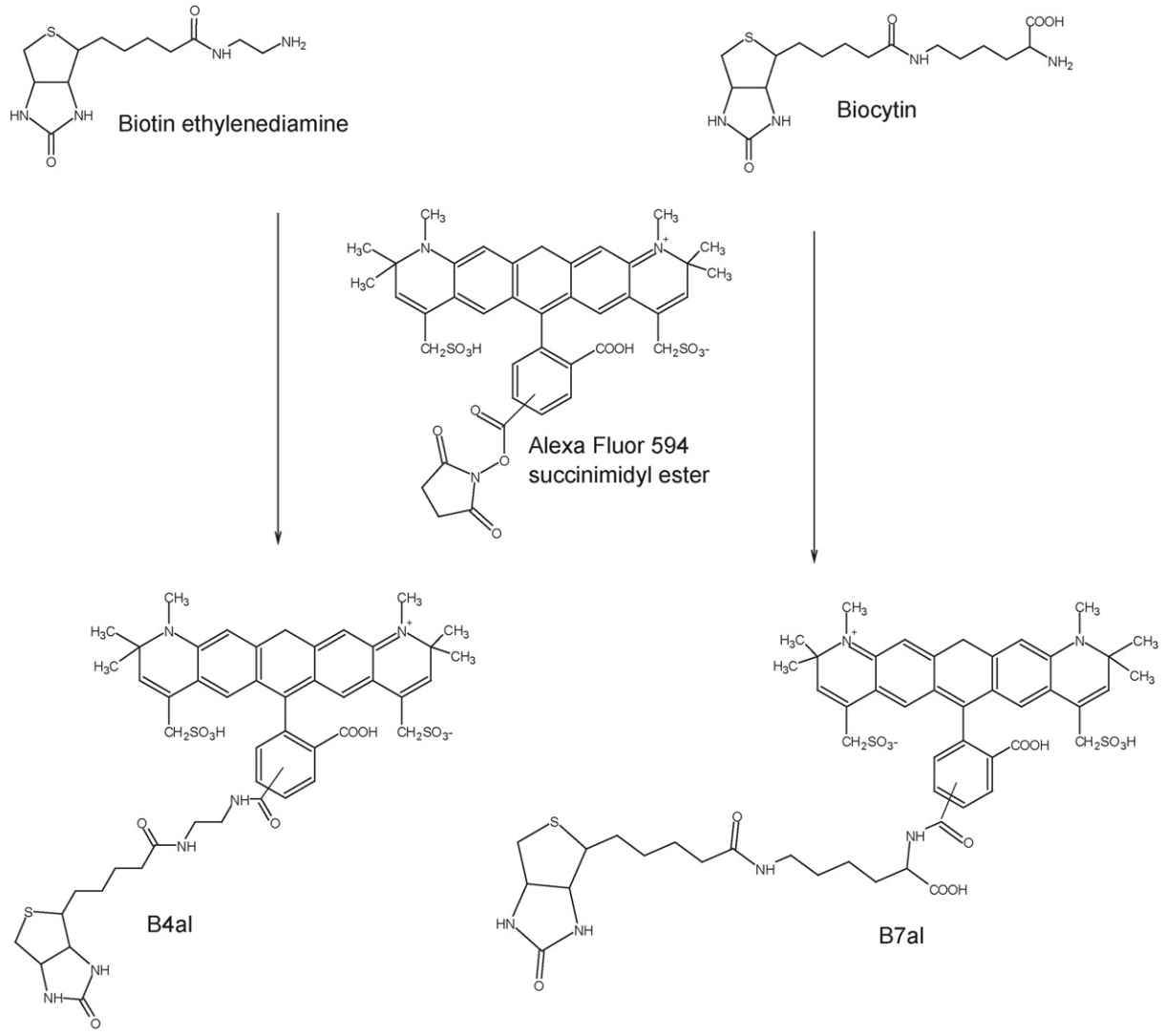
The reaction routes for attachment of alexa-fluor-594 to biotin are represented in Scheme 1. Preliminary experiments focused on the experimental conditions to minimize the percentage of the hydrolysis. We will present successively (i) the optimized procedure for the reaction of biotin ethylenediamine with succinimidyl ester to limit hydrolysis to 30%, (ii) the labelings of avidin with the fluorescently labeled biotin and biocytin and finally and (iii) the yield of the reaction of the labeled biotin and biocytin with avidin.

2.2.1. Determination of the percentage of hydrolysis

Biotin ethylenediamine (0.16 μmol) was dissolved in 100 μL PBS_A (pH 7.4) and mixed with 10 μL 1 M bicarbonate solution, as recommended by the provider (see above). This solution was poured on lyophilized alexa-fluor-594 succinimidyl ester (0.145 μmol), and the mixture stirred at room temperature 1 h to get the product B4al. Then the mixture was analyzed by HPLC on μbondpack C 18 (Waters) reversed phase chromatography column, with water/ethanol (1/1) as eluent (flow 0.6 ml min^{-1}) and fluorescence at 610 nm as means of detection (Waters 474). In such conditions, we obtained (Millennium 32 waters software) 30% (mol/mol) hydrolysis (Fig. 1). No changes in the figure occurred when using 50 μL instead of 10 μL of bicarbonate solution.

2.2.2. Labeling with biotin–ethylenediamine alexa-fluor-594 (B4al)

The previously determined conditions were repeated to get B4al. Biotin ethylenediamine (0.16 μmol) was dissolved in



Scheme 1. Reaction of the two biotin derivatives with alexa-fluor-594 to get biotinylated fluorophores.

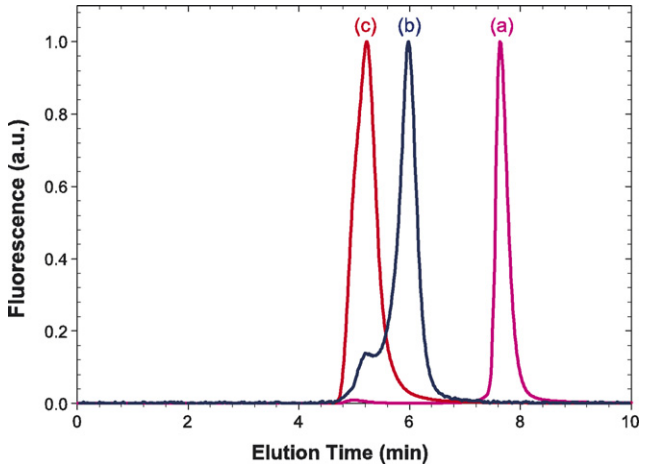


Fig. 1. Chromatograms for analysis of the succinimidyl ester hydrolysis: (a) ester at time 0; (b) after 1 h reaction with biotin ethylenediamine; (c) after complete hydrolysis (3 h), without biotin ethylenediamine.

100 μL PBS_A (pH 7.4) and mixed with 10 μL 1 M bicarbonate solution. This solution was poured on lyophilized alexa-fluor-594 succinimidyl ester (0.145 μmol), and the mixture stirred at room temperature 3 h to get the product B4al. The molar excess of biotin ethylenediamine (B4) with respect to alexa was chosen to avoid the subsequent possibility of direct labeling of avidin. Taking into account the hydrolyzed fraction, the amount of B4al is $0.7 \times 0.145 = 0.10$ μmol. Then, the solution was added to an avidin solution (270 μL at 0.18 mM, 0.05 μmol) in phosphate buffer (PBS_A), corresponding to a theoretical molar ratio fluorescent biotin/avidin of 2. The mixture was stirred during 1 h. We can expect the biotin ethylenediamine excess to be complexed with avidin in the second step. All mixture components were separated by chromatography on resin provided in the kit (BioGel P-30 Fine size, Bio-Rad). The chromatographic column separates the protein (avidin, avidin with B4al or B4) from the other smaller compounds present in the mixture. The fluorescence analysis by excitation at 584 nm is relative only to A-B4al (see Fig. 2, below).

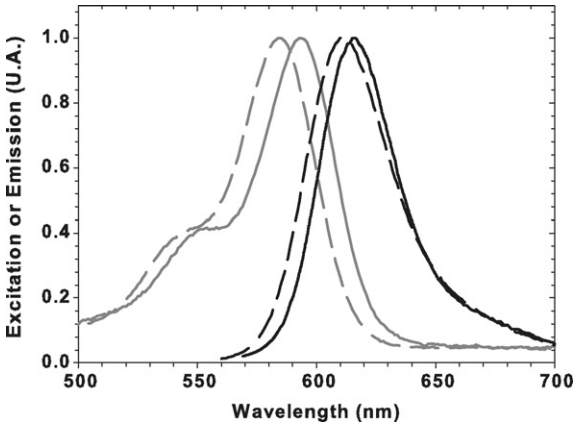


Fig. 2. Excitation spectrum with emitted fluorescence measured at 620 nm (gray) and fluorescence emission measured with excitation at 590 nm (black). Spectra of alexa (▬ ▬) and A-B4al (▬). Spectra of A-al, B4al and A-B7al are superposed to the alexa spectra.

2.2.3. Labeling with biocytine–alexa-fluor-594 (B7al)

The previously described procedure for labeling via biotin ethylenediamine was also applied for labeling via biocytin.

2.2.4. Yield examination of the coupling between avidin and fluorescently labeled biotin

The fraction of B4al (and B7al) coupled with avidin were determined from the absorbances at 584 and 282 nm. Alexa absorbs at 584 nm ($\varepsilon = 78000\,M^{-1}\,cm^{-1}$) and 282 nm ($\varepsilon = 42126\,M^{-1}\,cm^{-1}$). Avidin absorbs at 282 nm ($\varepsilon = 96000\,M^{-1}\,cm^{-1}$). Taking into account the hydrolyzed fraction, the yield of the coupling reaction between avidin and B4al was only 65%, instead of an expected value near 100% as indeed observed with B7al. It corresponds anyway to the fraction of the 1–3 isomer in the mixture of the two isomers 1–3 and 1–4 and impurity, according to the provider. This would suggest that the 1–3 isomer might be the only one involved in the conjugate with the protein. In the case of labeling with B7al, the 100% yield of attachment is probably due to the longer chain which avoids too strong steric constraints. Working with relative molar amount alexa/avidin of 2, with 10% impurity and 30% hydrolysis of succinimidyl ester, the mean number of labels per avidin molecule is 0.8 for A-B4al and 1.3 for A-B7al.

2.3. Absorbance and fluorescence analysis

Absorption spectra were recorded on a Kontron (Uvikon 940) UV–vis spectrophotometer. All buffers used for absorption measurements were filtered on microfiltration membranes (Millex 0.1 μm, Millipore). Fluorescence spectra were obtained on a Spex (Fluorolog 1681) spectrometer at a scanning rate of $2\,nm\,s^{-1}$. For quantum yield measurements, a solution of rhodamine B in ethanol was used as reference (fluorescence quantum yield 0.49) [34]. All experiments were carried out at 19 °C. The time resolved fluorescence decays were obtained by the technique of time correlated single photon counting [35]. The excitation wavelength was provided by a cavity dumped (Spectra Physics 344) rhodamine 6G dye laser (Spectra Physics 375) that was synchronously pumped by the 82 MHz output of a mode locked argon ion laser (Spectra Physics 2030). The excitation pulse duration is estimated for such a device at about 20 ps (FWHM). For UV excitation, we used for second harmonic generation a frequency doubler (Spectra Physics 390).

Fluorescence emission was collected through a chain of optical components: polarizer, half-wave plate to get similar sensitivities for normal and parallel polarized emission detections, double monochromator (Jobin-Yvon DH10) and photomutiplier tube (PM) (Hamamatsu H7313) optimized for photon counting. The measured instrument response time (160 ps) is limited by the PM tube FWHM. The excitation frequency was set to 800 kHz and the count rate limited to 4 kHz. In such conditions, mono-exponential decays were collected when using rhodamine 6G as standard.

3. Theory and calculations

Lifetimes and correlation times were extracted from the experimental decay curves by the use of a program using the Levenberg–Marquardt algorithm [36].

Assuming a linear response of the apparatus, the experimental decay curves $F(t)$ were fitted by convolution [37] of a calculated fluorescence decay $f(t)$ with the excitation pump profile $P(t)$ obtained from a scattering sample.

The fluorescence decay law $s(t)$ and the anisotropy decay law $r(t)$ were analysed as sums of exponentials [38]:

$$s(t) = \sum_{i=1}^{n} \alpha_i \exp\left(\frac{-t}{\tau_{Fi}}\right) \tag{1}$$

$$r(t) = r_0 \sum_{j=1}^{m} \beta_j \exp\left(\frac{-t}{\theta_j}\right) \tag{2}$$

where τ_{Fi} is the lifetime of fluorescence, α_i the pre-exponential factor related to the contribution of the compound i to the total fluorescence, r_0 the limiting anisotropy and β_j the fractional contribution to the total depolarisation attributed to species j having a rotational correlation time θ_j.

For a spherical molecule, $r(t)$ decays mono-exponentially with one correlation time θ directly proportional to the viscosity η of the medium and the volume V of the rotating molecule according to the Stokes–Einstein [39] relationship:

$$\theta = \frac{\eta V}{k_B T} \tag{3}$$

where k_B is the Boltzmann constant and T the absolute temperature; $s(t)$ and $r(t)$ are related to the emission by the relations:

$$i_{//}(t) = \frac{1}{3} s(t)[1 + 2r(t)] \tag{4}$$

$$i_{\perp}(t) = \frac{1}{3} s(t)[1 - r(t)] \tag{5}$$

or,

$$s(t) = i_M(t) = i_{//}(t) + 2i_{\perp}(t) \tag{6}$$

$$r(t) = \frac{i_{//}(t) - i_{\perp}(t)}{i_{//}(t) + 2i_{\perp}(t)} \quad (7)$$

where, $i_{//}(t)$, $i_{\perp}(t)$ and $i_M(t)$ means the intensity of fluorescence of light polarised parallel, perpendicular and at magic angle (54.73°) with respect to the excitation polarisation, respectively.

As depolarisation effects [14] are avoided at magic angle, the determination of τ_{Fi} values in such condition is straightforward.

The corresponding experimental decays are expressed as:

$$I_{//}(t) = P(t) * \left\{ \frac{1}{3} s(t)[1 + 2r(t)] \right\} \quad (8)$$

$$I_{\perp}(t) = P(t) * \left\{ \frac{1}{3} s(t)[1 - r(t)] \right\} \quad (9)$$

$$I_M(t) = P(t) * s(t) \quad (10)$$

where '*' represents the operator of the convolution product.

A correction factor has been introduced in order to take into account the difference in the detection sensitivity between parallel and perpendicular polarized emissions through the monochromator. Since the great difference between the lifetime and the correlation time of the Alexa in water solution (ratio of about 10), this factor can be easily determined using this solution as a reference.

Lifetimes and correlation times were calculated by iterative adjustment after convolution of the pump profile with a sum of exponentials [37,38]. The analysis is performed on both decays $I_{//}(t)$, $I_{\perp}(t)$ simultaneously by a global analysis algorithm. We assumed a Poisson distribution for counts in the calculation of the χ^2 criterion used to estimate the quality of the adjustment [35,40]. Analysis of weighted residuals, as well as calculation of the autocorrelation function of the residuals, were also always performed, especially to check the occurrence of aberrant points which can lead to high χ^2 values.

4. Results and discussion

4.1. Fluorescence lifetime

We compare in Table 1 the maximum wavelength values of steady state absorption and fluorescence emission spectra of the different synthesized conjugates. No significant differences are observed between alexa, B4al, A-al and A-B7al. However, for A-B4al conjugate we observe a red-shift of 6 nm in the absorption as well as in the emission spectra (Fig. 2).

Table 1
Spectroscopic characteristics: wavelengths of absorption λB_{Abs} (nm) and emission λB_{Emi} (nm) maxima

	λB_{Abs} (nm)	λB_{Emi} (nm)	τB_{FB} (ns)	χP^{2P}
Alexa	585	610	3.82	1.06
B4al	585	609	3.77	1.13
A-al	585	609	3.79	1.14
A-B7al	585	610	3.70	0.94
A-B4al	593	616	4.13	1.05

Fluorescence lifetime τB_{FB} (ns) and statistical adjustment parameter χ^2 of alexa, B4al, A-al, A-B7al and A-B4al in solution PBS_B pH 7.4.

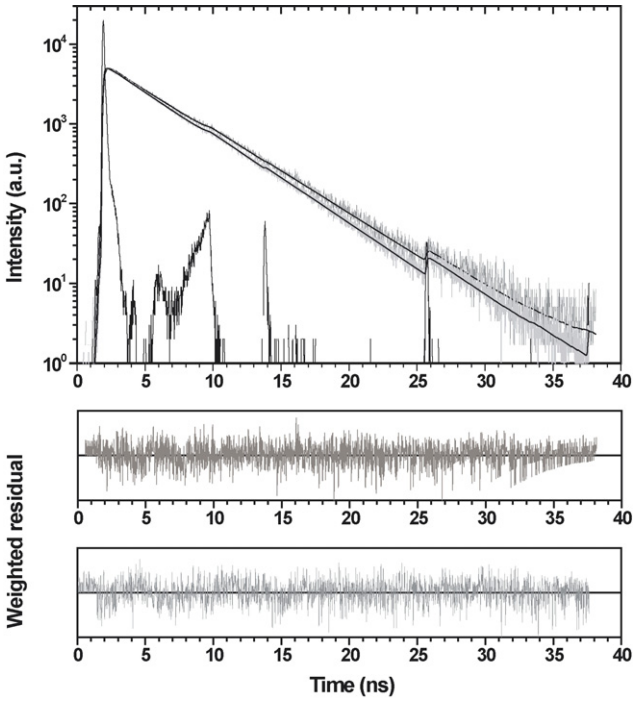


Fig. 3. Fluorescence decays $I_M(t)$ and residual function of alexa (gray) below A-B4al (black) accumulated at magic angle. The bumps in the decays are due to re-excitation of the fluorophore by secondary pulses (bottom peaks).

The fluorescence lifetime (Table 1, Fig. 3) of A-B4al (4.13 ns) is 10% larger than those of alexa, B4al, A-al and A-B7al (3.8 ns).

These results show that dye linked directly or linked via biocytin have the same fluorescence properties than alexa-fluor-594 free in solution. The fluorescence lifetime τ_F is related to the kinetic constants k_f and k_{nr} of radiative and non-radiative processes, respectively, by $\tau_F = (k_f + k_{nr})^{-1}$ and the quantum yield is $Q = \tau_F k_f$. From the measurements of τ_F and Q (Table 2) are deduced k_f and k_{nr}. With respect to alexa or B4al, both A-B4al quantum yield and fluorescence lifetime increases are 10%, thus leading to no variation in the radiative constant k_f and to decrease of k_{nr}.

Globally these results emphasize the stabilization, with respect to free alexa, of the excited state of alexa-fluor-594 when linked to avidin via biotin ethylenediamine, as the radiative process keeps the same rate while the non-radiative processes are slower. We can infer that this stabilization is induced by the localization of alexa-fluor-594 close to the protein surface with the four atoms spacer of B4al, which is almost lost in the case of the seven atoms spacer of B7al. This will be confirmed below by the fluorescence anisotropy study. It may be useful to point out

Table 2
Photophysical characteristics: fluorescence quantum yield Q, radiative k_f and non-radiative k_{nr} rate constants of alexa, B4al and A-B4al

	Q	k_f (ns^{-1})	k_{nr} (ns^{-1})
Alexa	0.79	0.208	0.053
B4al	0.79	0.208	0.053
A-B4al	0.87	0.210	0.031

The quantum yield was measured with rhodamine 6G as a reference.

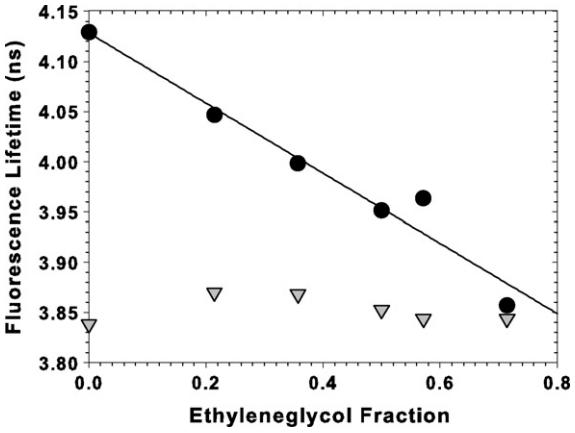


Fig. 4. Fluorescence lifetime of alexa fluor (▼) and A-B4al (●) as a function of ethylene glycol (EG) volume fraction in the mixtures with PBS_B solutions. Viscosities (mPa s) are 1.03; 1.86; 2.77; 4.17; 5.12 and 7.57 for ethylene glycol volume fractions 0.00; 0.214; 0.357; 0.50; 0.57 and 0.71, respectively.

that the number of fluorophores per avidin is smaller than two. The same kind of results was found with B4-Cy3 which was exceptionally fluorescent in the avidin-bound state [28] at least if no more than two ligands were bound per avidin tetramer. We did not study avidin–ligand complexes with three or four bound biotin-fluorophore conjugates which could show quite different photophysical properties than the studied complexes with less than two fluorescent biotins per avidin tetramer.

Fig. 4 illustrates the variation of fluorescence lifetime of alexa, free and in the conjugate A-B4al, as a function of ethylene glycol volume fraction *f*. A decrease of the A-B4al fluorescence lifetime with molecular environment *via* the addition of ethylene glycol was observed. The variation is linear τ (ns) (±S.E.) = 4.13 (±0.02) −0.35 (±0.04)*f*. Conversely, the fluorescence lifetime of the isolated alexa was constant, as expected. The same was observed with A-al. Thus, the change in the solvent composition has an effect on the interaction between avidin and the chromophore in A-B4al. The fluorescence lifetimes tend to reach the same value at high ethylene glycol fraction while the correlation time of the complex remain that of the conjugate showing that the alexa is not released by the change in solvent composition. Hence, a slight change of the local environment of the chromophore in the complex presumably explains the lifetime decrease evoked above. If this is indeed the case, then the conjugate might be used as a probe for changes in the interfacial environment resulting from protein/material interactions. The question whether the photophysical properties of the conjugates are related to the tryptophans interactions with alexa chromophores can be arisen: a short limited study is provided in Appendix A.

4.2. Fluorescence anisotropy: rotational correlation time

We will now compare the correlation times and depolarizations of A-al, A-B4al and A-B7al (Table 3).

When alexa-fluor-594 is directly linked to avidin, the correlation time is the same than that of the dye in solution: the chromophore rotates freely and this type of labeling does not provide any information concerning the protein motion. Two correlation times were determined for A-B7al: one (∼410 ps) corresponds to the dye free rotation, the other (39 ns) to the avidin motion, according to theoretical estimation model [14] (Eq. (3)). For A-B7al the depolarization contribution is much smaller for the long correlation time than for the short one, which shows that the greatest part of the signal is due to the protein-linked dye free rotation. For A-B4al the spacer is shorter and a single correlation time of 33.5 ns was obtained (Fig. 5).

Table 3
Fluorescence anisotropy characteristics in PBS_B solution pH 7.4: correlation time θ (ns), global depolarization factor r_0 and fit statistical adjustment parameters $\chi^2_{//}$ and $\chi^2_{\perp}$ for parallel and perpendicular polarization decays, respectively

	θ (ns)	r_0	$\chi^2_{//}$	$\chi^2_{\perp}$
Alexa	0.37	0.40	1.03	1.03
A-al	0.31	0.39	1.24	1.1
A-B7al	0.41	0.33	1.03	0.99
	39	0.04		
A-B4al	33.5	0.36	1.18	1.0

Two components of depolarization were found for A-B7al (see text).

This correlation time corresponds to the motion of avidin in solution. In this case, the attached chromophore cannot rotate freely. The fact that a single correlation time is deduced relates well with the spherical shape of avidin and the equivalence of the four sites of fixation for biotin linkage. From the correlation time the hydrodynamic diameter of avidin is estimated via Eq. (3) as 6.1 nm, which corresponds to the dimensions of the avidin tetramer [25]. The conjugate correlation time is much larger than the correlation time of free alexa; it suggests a tight attachment of the fluorophore to the protein. To check the sensitivity of the correlation time with the environment, measurements were performed at different viscosities, by mixing labeled aqueous PBS_B solutions and ethylene glycol in various proportions (Fig. 6). The viscosity variation with ethylene glycol volume fraction at 19 °C was approximated, after conversion of measured volume fraction to weight fraction (http://coe.ou.edu/cems/ulab/CHE4262/sec002/experiments/experiment1/Evaporator/UC-2167 ethylene glycol), by data at 20 °C [41]. The figures were majored by 2.5%, the viscosity relative varia-

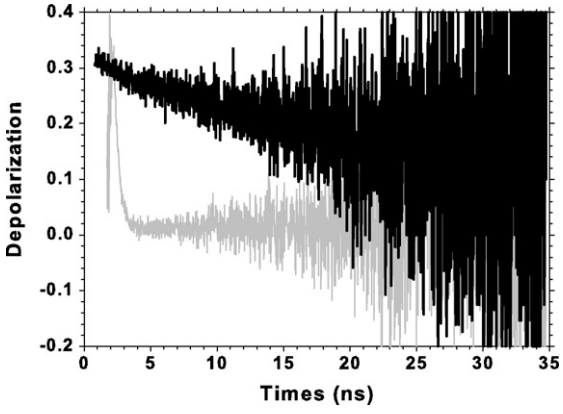


Fig. 5. Depolarization $r(t)$ for alexa (gray) and A-B4al (black) in PBS_B pH 7.4 (10 mM sodium phosphate, 0.15 M NaCl).

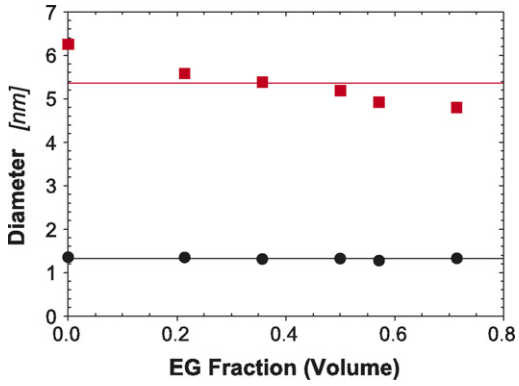


Fig. 6. Sphere equivalent hydrodynamic diameter of A-B4al (■) and B4al (●) as a function of ethylene glycol volume fraction in buffer PBS_B.

tion of the pure components per °C around 20 °C. We observed within experimental error a linear variation of θ as a function of viscosity, still compatible with the size of the protein. However, many additional effects could exist, because with such a mixture of solvents the variation of viscosity is associated especially with changes in protein ionization and dielectric constant of the medium. For instance, the Debye screening length of 0.73 nm in aqueous buffer will vary in the presence of ethyleneglycol. Such effects might be responsible for the observed variation of the florescence lifetime of A-B4al (Fig. 4). The same study for alexa and A-al gave the hydrodynamic volume of 1.18 nm^3 (Eq. (3)), thus confirming the independent rotation of the label when directly linked to avidin. No size variation of B4al was observed in mixtures of buffer and ethylene glycol (Fig. 6).

This variation is a piece of evidence that these experiments actually relate well with the measurement of correlation times linked to the motion of the protein and to that of the free chromophore. These measurements point also to the fact that correlation times till 120 ns could be measured with satisfying accuracy with a label having a fluorescence lifetime of only 4 ns.

Avidin is a very stable protein which resists any conformational effects induced by up to 8 M urea [42] or 3 M guanidine [43]. We observed the same stability for A-B4al. It was necessary to use 9 M urea conditions to observe the denaturation of the complex. Three correlation times were obtained: The smaller one (∼0.32 ns) is presumably due to B4al released from avidin. The native value of 32 ns is between the two others (∼126 and ∼6.5 ns) which could reflect the deformation of the spherical complex to an ellipsoid shape and/or aggregation. The value of 6.5 ns could also be attributed to the monomer unit produced by dissociation of the tetramer avidin: as a first approximation indeed, the correlation time is proportional to the mass, or to the degree of oligomerization ($\theta_{monomer} \approx 1/4\ \theta_{tetramer}$). Anyway, whatever the real state of the solution in 9 M urea, the anisotropy measurements showed clearly the denaturation of the complex A-B4al. Furthermore, we recovered in these denaturating conditions the fluorescence lifetime (3.8 ns) and the spectral properties of the free fluorophore: the stabilizing interactions between avidin and biotin–alexa present in the native conjugate form are there lost.

5. Conclusions

Taking advantage of the avidin biotin affinity, we labeled avidin with a fluorescent probe imaging the protein rotational motion. Using the same route than previously described by Gruber et al. [28] with a four atoms spacer (biotin ethylene diamine), we chose as fluorescent probe alexa-fluor-594 instead of fluorescein. The labeled avidin A-B4al has the fluorescence emission parameters of alexa-fluor-594 and the correlation time of avidin. In A-B4al, we noticed (i) an increase of the dye fluorescence lifetime and quantum yield, thus a stabilization of the excited state of alexa in the complex by slowing down the non-radiative processes, and (ii) a larger mean fluorescence lifetime of the avidin tryptophans, compared to those measured for the avidin–biotin complex. It should be kept in mind however that the present results were obtained with a number of fluorophores per avidin smaller than two and could be different for higher numbers, as observed with other chromophores [28]. The correlation time of avidin could not be obtained with A-B7al, where the spacer was seven atoms long (biocytin).

The synthesized A-B4al conjugate opens few perspectives for an approach of protein interactions with substrates. The occurrence of several correlation time components of the anisotropy decay and their respective values in the denaturating conditions correlate well the avidin conformational changes. We showed that the fluorescence lifetime also depends on the liquid phase composition. All these observations indicate that A-B4al conjugate is a potential useful model for tracking the rotational dynamics and motion of proteins interacting physically and chemically with interfaces, where local environment, restricted motion and/or partial unfolding of proteins can occur.

As an example, for A-B4al adsorbed at high interfacial concentration on mica, we measured by confocal fluorescence dynamics [44] a correlation time larger than 300 ns and a fluorescence life time of 3.2 ns. The large correlation time shows the highly reduced rotational freedom at the interface, as assumed in interpretation of TIRF data [16,17], while the change in fluorescence lifetime is even outside the range observed in solution in presence of ethylene glycol and is probably linked to the protein–protein and/or protein–surface interactions.

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Appendix A

The fluorescence lifetimes of the tryptophans (Table 4) are modified by complexation of the protein with biotin. Three fluorescence lifetimes were observed for avidin, avidin–biotin (4 biotins per avidin) and A-B4al* (3.2 biotins and 0.8 alexa–biotin on the average per avidin). As already described by other authors [45,46] fluorescence quenching of avidin was observed in the presence of biotin. However, the complex of avidin with B4al led

Table 4
Average fluorescence lifetime τB_{ave}, fluorescence lifetimes (τB_1, τB_2, τB_3) and pre-exponential factors (A_1–A_3) of tryptophans measured in solutions of avidin, avidin biotin complex and A-B4al*

	$\tau B_{ave\ B}$(ns)	$\tau B_{1\ B}$(ns)	$\tau B_{2\ B}$(ns)	$\tau B_{3\ B}$(ns)	AB_{1B}	AB_{2B}	AB_{3B}	χP^{2P}
Avidin	1.69	3.12	1.36	0.372	0.095	0.435	0.471	1.08
Avidin–biotin	0.96	2.65	1.04	0.439	0.011	0.565	0.424	1.11
A-B4al*	1.2	4.17	1.31	0.43	0.01	0.43	0.56	1.07

Biotin in excess ensured the occupation of all the sites of avidin in the complexes Avidin–biotin and A-B4al*.

to a higher mean fluorescence lifetime than for the avidin–biotin complex, and we recovered for A-B4al the same second fluorescence time of the non-complexed avidin with the same pre-exponential factor. This presumably indicates that a specific interaction exists between alexa fluor and at least one of the tryptophans, and/or that the fluorophore linked to biotin modifies indirectly the avidin–biotin interaction.

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