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Tästä kannattaa silmäillä johdanto tuohon materials ja methodsiin asti.

Laboratory Exercises

Determination of the Molecular Size of BSA by Fluorescence Anisotropy*

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This work describes a laboratory experiment to illustrate the usefulness of fluorescence anisotropy in the field of biophysics. Fluorescence anisotropy of dansyl-labeled BSA was determined in media of increasing glycerol concentrations. The Perrin equation was fitted to the experimental data, obtaining the molecular volume of the protein. The simplicity of the experiment and data analysis helped the students to focus on the relationship between probe anisotropy and rotational diffusion. Additionally, this laboratory exercise has the advantage of using a protein and a probe that are inexpensive and very common in many laboratories.

Keywords: BSA, fluorescence anisotropy, molecular size.

Light is an electromagnetic wave that can be described as oscillating electric and magnetic fields perpendicular to each other. The electric field vector can be represented in an X-Y plane perpendicular to the direction of propagation. When the X and Y components oscillate at the same phase, the result is an oscillation in a defined orientation that depends on the relative amplitude of each component. In this condition, the light is called linearly polarized [1].

Molecular electronic transitions are described in quantum mechanics by vectors in the physical space denoted as transition moments. According to this description, the probability of light absorption by molecules irradiated with linearly polarized light will be proportional to the component of the absorption moment parallel to the polarization axis of the incident radiation [2]. This phenomenon is known as photoselection (Scheme I).

As a consequence of photoselection, the resulting emitted light will also be polarized in a plane defined by the emission moment. To characterize the polarized state of the emitted light, it was defined as a parameter called fluorescence anisotropy (r),

$$r = \frac{I_{//} - I_{\perp}}{I_T} = \frac{I_{//} - I_{\perp}}{I_{//} + 2 \cdot I_{\perp}} \qquad \text{(Eq. 1)}$$

where $I_{//}$ and $I_{\perp}$ are the components of the fluorescence intensity that are parallel and perpendicular to the electric vector of the excitation light, and I_T is the total fluorescence intensity [3].

In the absence of diffusion, the anisotropy is given by the relative orientation of the excitation and emission moments of the fluorophore, taking values between −0.2 (perpendicular transition moments) and 0.4 (parallel transition moments). In solution, the excited molecules can rotate, thereby changing their orientation (Scheme II). The angle θ between the average orientation of the molecules at a given time with respect to the original orientation depends on the time, the temperature, the solvent properties, and the size and shape of the molecule. This process, known as rotational diffusion, can be characterized by a parameter called rotational correlational time (ϕ), defined as the time after which $\cos\theta = e^{-1}$ [4].

In 1926, Perrin proposed that rotational diffusion contributes to depolarizing the emitted light and postulated the following relationship to explain the dependence of the anisotropy with the rotational diffusion process [5],

$$r = \frac{r_0}{1 + \tau/\phi} \qquad \text{(Eq. 2)}$$

where r_o is the anisotropy in the absence of diffusion, τ is the fluorescence lifetime, and ϕ is the rotational correlation time of the fluorophore. In a fluorescent conjugate that behaves in solution as a rigid rotator, the parameters r_o and τ depend on the photophysical properties of the fluorophore, whereas ϕ depends mainly on the size and shape of the macromolecule [4].

Equation 2 predicts that, when $\tau \ll \phi$, the particle would almost not rotate at all during the lifetime of the fluorophore, and r approaches the anisotropy measured in the absence of diffusion, *i.e.* r_o. On the other hand, when $\tau \gg \phi$, the emission dipoles of the molecule become randomly distributed as a consequence of the rotational diffusion, and r approaches zero. The anisotropy will be sensitive to factors affecting the rotational motions when $\tau \cong \phi$.

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Considering spherical particles rotating with small angular velocities, the rotational correlation time can be expressed by the Stokes-Einstein-Debye equation [5],

Tätä käytetään

$$\phi = \frac{\eta \cdot V}{k \cdot T} \qquad \text{(Eq. 3)}$$

where η is the solvent viscosity coefficient, k is the Boltzman constant, T is temperature, and V is the volume of the rotating particle. This equation is based on classical hydrodynamics and requires the hypothesis of a continuous and homogeneous solvent. Some doubt may be cast on this assumption in the case of molecules that do not differ greatly in size from the molecules of solvent. However, this approximation appears to be valid for proteins [4].

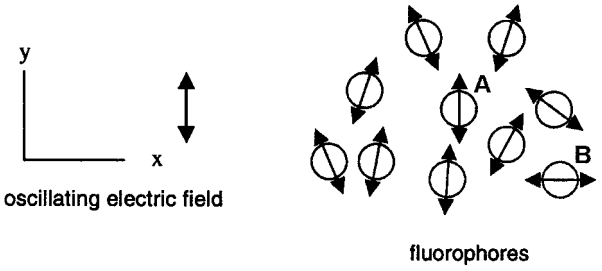
For nonspherical molecules, more than one rotational correlation time is needed to describe their rotational motion. However, if the molecule is slightly asymmetrical, Equation 2 can be applied and ϕ will represent a mean value of the principal relaxation times of the rotation [6]. In this case, the parameter V in Equation 3 will be the volume of a hydrodynamically equivalent sphere.

Combining Equation 3 with Equation 2 results in

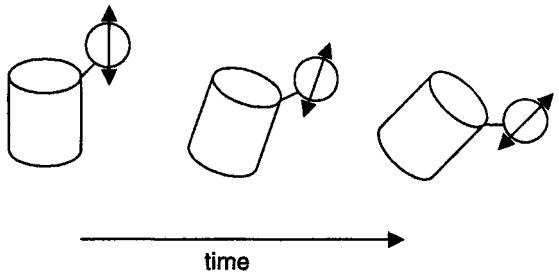
$$r = \frac{r_0}{1 + \frac{\tau \cdot k \cdot T}{\eta \cdot V}} = \frac{\eta \cdot r_0}{\eta + c} \qquad \text{(Eq. 4)}$$

where c is a fitting coefficient that includes the constant terms in the denominator and corresponds to the solvent viscosity at which the steady state anisotropy is reduced at half of the limit value.

Equation 4 shows that fluorescence anisotropy depends on the volume of the rotating particle. This relationship constitutes the basis of many applications of this technique to the study of biological processes involving volume changes (*e.g.* interactions among macromolecules [7], conformational changes [8], etc.). In this work, we show a simple experiment that uses fluorescence anisotropy to determine the molecular size of a protein.



SCHEME 1. **Photoselection of fluorophores.** The electric vector of the excitation light and the absorption dipoles of randomly distributed fluorophores are shown. Molecules A and B exhibit maximal and no absorption of light, respectively.



SCHEME 2. **Rotational diffusion of a labeled molecule.**

MATERIALS AND METHODS

Materials—BSA (catalog no. A-7906) and dansyl-chloride (catalog no. D-2625) were obtained from Sigma Chemical Co. All other chemicals used in this work were of analytical grade.

Labeling with Dansyl-chloride—BSA was labeled by the students in a previous laboratory class [9]. Briefly, they incubated 300 μM dansyl chloride (previously dissolved in a small volume of dimethylformamide) and 300 μM BSA in 10 mM morpholinopropane-sulfonic acid (MOPS)-K (pH 8.4 at 25 °C) for 1.5 h at 25 °C in the dark, with continuous stirring. The reaction was stopped by the addition of 10 mM Tris, and the sample was exhaustively dialyzed against 10 mM Tris-HCl (pH 7.4 at 20 °C). The stoichiometry of labeling was 0.95 [9].

Dansyl-Tris was obtained by incubating, in the dark, 300 μM of the fluorescent probe in 10 mM Tris-HCl (pH 8.4) at 25 °C for 1.5 h. After that, the sample pH was adjusted to 7.4 at 20 °C with 1 M HCl.

Preparation of the Samples—A glycerol solution (25% p/p) was prepared by mixing 10 g of glycerol (87%) with 24.8 g of H_2O. Dansyl-BSA was diluted up to 30 μM in 10 mM Tris-HCl pH 7.4 at 20 °C and increasing quantities of glycerol by following the procedure detailed in Table I. Each sample was prepared in duplicate. Because the protein and buffer concentrations are very low, sample viscosities can be calculated from the glycerol concentration. For this task, students plotted the data of viscosity of glycerol-water solutions as a function of their concentrations (*Handbook of Chemistry and Physics*, www.hbcpnetbase.com) and fitted a second-order polynomial function to these data (Fig. 1). They then calculated the viscosities of the BSA samples using

TABLE I
Preparation of the samples

Sample no.	Final glycerol concentration	BSA (300 μM)	Tris-HCl (1 M, pH 7.4 at 20 °C)	Glycerol (25 % p/p)	H_2O
	% p/p	μl	μl	μl	μl
1	0	300	27	0	2673
2	2	300	27	240	2433
3	4	300	27	480	2193
4	6	300	27	720	1953
5	8	300	27	960	1713
6	10	300	27	1200	1473
7	12	300	27	1440	1233
8	14	300	27	1680	993
9	16	300	27	1920	753
10	18	300	27	2160	513
11	20	300	27	2400	273
12	22	300	27	2640	33

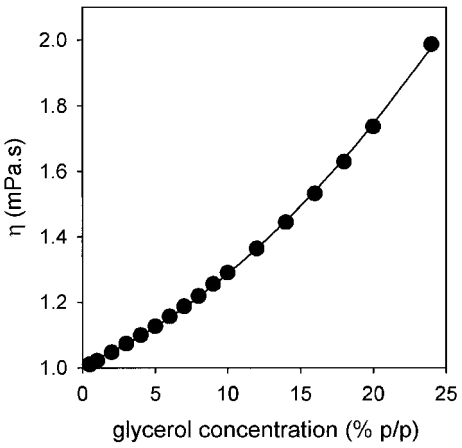


FIG. 1. **Viscosity of aqueous solutions of glycerol.** The viscosity coefficient at 20 °C of glycerol-water solutions are plotted as a function of the glycerol concentration (*c*). A second-degree polynomial was fitted to these data, obtaining the following equation: $\eta = 0.000867\ c^2 + 0.0191\ c + 1.007$. The *continuous line* is the graphical representation of this equation.

the obtained function. Samples were equilibrated at 20 °C for 20 min before measuring the anisotropy.

Spectroscopic Measurements—Fluorescence measurements were performed at 20 °C using a SLM-Aminco Bowman Series 2 (SLM, Urbana, IL) spectrofluorometer equipped with excitation and emission polarizers.

Fluorescence anisotropy was measured, setting the excitation at 340 nm and the emission at 470 nm, with bandwidths of 4 nm. Anisotropy was calculated using Equation 1, including the instrument *G* factor defined as the ratio of sensitivities of the detection system for the vertically and horizontally polarized light [3],

$$r = \frac{I_{VV} - G \cdot I_{VH}}{I_{VV} + 2 \cdot G \cdot I_{VH}}, \text{ with } G = \frac{I_{HV}}{I_{HH}} \qquad \text{(Eq. 5)}$$

where *H* and *V* refer to the horizontal and vertical positions, respectively, of the excitation (first subscript) and emission (second subscript) polarizers.

Data Analysis—Equations were fitted to the experimental data using a nonlinear regression procedure [10]. The dependent variable was assumed to be homoscedastic (constant variance), and the independent variable was considered to have negligible error. Parameters were expressed as the mean ± S.E.

Structure of Class Activities—The laboratory class was organized into three blocks of approximately 90 min each. In the first block, the students (in small groups) discussed the experimental design and prepared the samples to be measured. In the second block, they registered the data. A final discussion was then held in the groups and extended to the whole class.

RESULTS AND DISCUSSION

Measurement of Dansyl Anisotropy—In a previous laboratory class [9], students analyzed the absorption and emission spectra of dansyl-BSA. Based on these data, they chose adequate excitation and emission wavelengths to measure the fluorescence anisotropy of dansyl attached to BSA. After this, they registered the fluorescence intensity of 30 μM dansyl-BSA or dansyl-Tris solutions at the selected wavelengths, after sequential changes of the polarizers positions to horizontal and vertical. The calculated fluorescence anisotropies were 0.219 ± 0.002 (dansyl-BSA) and 0.001 ± 0.003 (dansyl-Tris).

It can be observed that the anisotropy of dansyl-Tris is not significantly different from zero, indicating that the probe reached a random distribution within its lifetime when it is bound to the small Tris molecule. Conversely, the anisotropy of dansyl bound to BSA is significantly higher, suggesting that the mobility of the probe is restricted by attachment to the macromolecule.

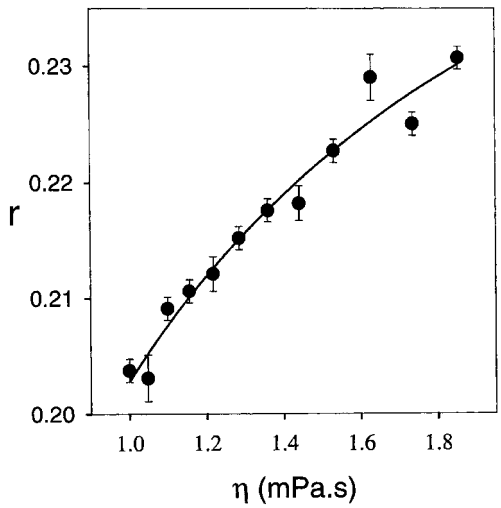


FIG. 2. **Dependence of dansyl-BSA anisotropy on the viscosity of the solution.** Dansyl-BSA anisotropy is represented as a function of the viscosity of the solutions (for experimental details, see *Materials and Methods*). The *continuous line* is the graphical representation of Equation 4 with the best fitting parameter values indicated in the text.

TABLE II
Molecular size and hydration degree of some macromolecules

	M_r	V (nm^3)	R_S (nm)	δ_{max}	δ_{exp}	V_h (nm^3)
BSA	66,430	163	3.4	0.74	0.40	125
Lysozyme	14,100	37	2.1	0.88	0.34	24
Ovalbumin	45,000	88	2.8	0.43	0.33	81
Hemoglobin	68,000	125	3.1	0.36	0.42	132
Chymotrypsinogen	23,200	48	2.2	0.52	0.33	41
DNA	6,000,000	$1.9 \cdot 10^7$	165	1890	0.59	$1.12 \cdot 10^4$

Dependence of Dansyl-BSA Anisotropy on the Viscosity—Figure 2 shows the dansyl-BSA anisotropy values measured after changing the viscosity of the solution by the addition of glycerol. As expected, *r* increased with increasing the viscosity. By fitting Equation 4 to the experimental data, students obtained the following parameter values: $r_o = 0.273 \pm 0.004$ and $c = 0.347 \pm 0.027$ mPa·s.

The limiting anisotropy r_o correspond to the hypothetical situation in which the viscosity of the medium is infinitum, that is, in the absence of rotational motions. Although the r_o value is obtained by extrapolation of the experimental data, its high value suggests a small angle between the absorption and emission moments.

Considering that the lifetime of dansyl attached to BSA is 14 ns [11], students calculated the rotational correlational time of BSA in the absence of glycerol, obtaining $\phi = 40 \pm 3$ ns. This means that, after 40 ns of excitation, dansyl-BSA molecules rotated, on average, 68 degrees with respect to the initial orientation. As a consequence, the fluorescence anisotropy decreased up to $r_o \cdot e^{-1}$ [5].

On the other hand, the volume of the hydrodynamically equivalent sphere can be calculated from the parameter *c*

$$V = \frac{\tau \cdot k \cdot T}{c} = 163 \pm 13 \text{ nm}^3 \qquad \text{(Eq. 6)}$$

From this value, a Stokes radius (R_s) of 3.39 ± 0.27 nm is derived for the BSA molecule. This value is consistant with the value determined by other techniques as traslational diffusion (3.52 nm) [12, 13] and gel filtration (3.48 nm) [14].

Further Discussion— According to a classic hydrodynamic analysis [2, 12, 15], the hydrated volume of a molecule is given by

$$V_h = \frac{Mr}{N} \cdot (v_2 + \delta \cdot v_1^0) \qquad \text{(Eq. 7)}$$

where v_2 is the partial specific volume of the macromolecule, v_1^o is the specific volume of water, and δ is the degree of hydration.

Supposing that the volume calculated according to Equation 6 corresponds to the hydrated volume of BSA (which implies it approximates the BSA shape to a sphere), we can determine the maximum degree of protein hydration from Equation 7. Taking into account that Mr_{BSA} = 66,430; v_1^o = 1.0018 cm^3/g; and v_2 = 0.734 cm^3/g [12]; students solved Equation 7 obtaining δ_{max} = 0.74. This value is consistant with values estimated from measurements of intrinsic viscosity (δ_{max} = 0.75) and translational diffusion (δ_{max} = 1.07) [12]. However, these values are significantly higher than δ_{exp} = 0.4, the degree of hydration experimentally determined by NMR [13].

Equation 7 can also be used to calculate the volume of the hydrated BSA molecule (V_h) from the values of M_r, v_2, δ_{exp}, and v_1^o. This procedure does not assume any specific shape for the BSA molecule. Students performed this calculation, obtaining a value of 125 nm^3. The difference between this value and that previously calculated for the hydrodynamically equivalent sphere (V) can be explained in terms of either a slight molecular asymmetry of BSA [12] or the involvement of dimer monomer equilibrium [16].

Table II summarizes the data of V, R_s and δ_{max} for BSA determined by the students and data corresponding to other macromolecules under the spherical approximation [2, 12]. Table II also includes the experimental degree of hydration (δ_{exp}) of these proteins and the hydrated volumes (V_h) calculated by following a procedure similar to that described above for BSA.

It can be observed that, for globular proteins, the spherical approximation gives volume values close to the hydrated volumes, but, for very asymmetric molecules such as DNA, the difference between both volumes is very large.

CONCLUDING REMARKS

This laboratory class has been successfully implemented during 3 years in undergraduate courses of biophysical chemistry. Students found that the simplicity of the experiment and data analysis allowed them to focus in the relationship between probe anisotropy and rotational diffusion. An excellent review on the principles and applications of fluorescence polarization [17] has been recently published. The authors recommend this review as a complement to the discussion proposed in this article.

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