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Luminescence and Anisotropy Decays of *N*-3-Pyrene Maleimide Labeling IgG Proteins and Cells

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The Py.M (N-3-Pyrene Maleimide) is a dye that covalently binds to reactive amino or sulfhydryl groups to give highly fluorescent protein conjugates. Measurements of luminescence lifetimes and anisotropy decays have been performed with a Phase and Modulation Fluorometer. Complexes of Py.M-antibody (IgG antimouse) and tumoral cells C6 labeled with Py.M have been investigated. The Py.M fluorescence in buffer solution and the protein and cells natural fluorescence have been checked. For Py.M-IgG and labeled cells, the fluorescence decays present interesting behaviours. The least-squares analysis of the experimental results on Py.M-IgG complex points out two lorentzian distributions centered at 74 ns and 11 ns, on the contrary, for the labeled cells, a discrete component at 100 ns and a lorentzian distribution centered at 5 ns are shown. In both systems a weak component lower than 1 ns is observed. The fluorescence decays, mainly the long lifetime one, are very sensitive to oxygen quenching, showing the high efficiency of O_2 quenching. For samples N_2 bubbled, the lifetime experimental results show a decrease of the oxygen accessibility from free probe in solution to Py.M-IgG complex and to labeled cells, compatible with a more compact packing of the probe binding site. The experimental results of anisotropy decays of degassed samples show for Py.M-IgG complexes a long rotation correlation time of about 200 ns at T = 5°C, assigned to overall rotation of the protein, besides shorter correlation times attributable to inner protein motions. For labeled cells, the long rotation correlation time becomes of the order of 580 ns confirming a progressive increase of the stabilization of the binding site.

KEY WORDS: Luminescence; anisotropy decays; *N*-3-pyrene maleimide; IgG proteins.

INTRODUCTION

The drawbacks arising from unspecific fluorescence put some limitations on immunocytofluorometric techniques. Suitable long-lived fluorochromes allow, in principle, discrimination of the background signal and acquisition of more information on labeling specificity in immunocytofluorometric analysis.

The pyrene derivatives have been described as probes with a long fluorescence lifetime that bind covalently to monoclonal antibodies without significantly affecting their ability to react with antigens. To separate the unwanted contributions from specific fluorescence, the fluorescence of the fluorochrome, free, conjugated to antibody, and bound to cells, was carefully analyzed, so as to identify physical parameters suitable for evaluating correctly the degree of specificity of the label.

EXPERIMENTAL

Samples were free *N*-3-pyrene maleimide (Py.M) in buffer solution (phosphate buffer), Py.M–IgG antimouse adduct, and labeled C6 glioma cells.

The antibody conjugation and cell labeling proce-

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dure is described in Ref. 1. The binding of Py.M to antimouse IgG was carried out at a pH value that favors the formation of the Py.M covalent adduct with the amino groups of the protein (7 < pH < 8) [2,3]. C6 glioma cells were purchased and incubated in the presence of 5-bromo-2′-deoxyuridine (BrdU).

Fluorescence lifetime and anisotropy decay were measured with a ISS multifrequency phase fluorometer [4,5]. The light sources were a 300-W xenon arc lamp and a dye laser tuned at 340 nm. Excitation wavelengths were selected by a Jobin–Yvon monochromator, while emissions were observed through cutoff filters. For luminescence lifetime measurements 2-2′-*p*-phenylenebis(5-phenyl)oxazole (POPOP) in ethanol (τ = 1.35 ns) was used as reference. The lifetime results were analyzed assuming multiple noninteracting fluorescent species by means of both a sum of exponentials and continuous lifetime distributions.

The anisotropy decay results were analyzed assuming two noninteracting fluorescent species either as anisotropic rotators or as hindered rotators.

Data analysis was carried out by minimizing the reduced chi-square with a routine based on the Marquardt algorithm using ISS 187 Decay Analysis and Global Unlimited software. Analyses were performed by using a constant frequency-independent standard deviation of 0.2° for the phase and of 0.004 for the modulation.

RESULTS AND DISCUSSION

Fluorescence Lifetimes. Typical experimental curves for multifrequency phase and modulation are shown in Figs. 1 and 2. Py.M in buffer solution shows a very weak signal characterized by three exponential lifetimes. The shortest component can be attributed either to scattered light or to Raman contribution. The experimental data on fluorescence decays obtained at 20°C for different samples are shown in Table I.

The data presented in Table I show that the longer lifetime increases significantly on passing from free Py.M to Py.M–IgG adducts and then to labeled cells and its value discriminates the natural fluorescence ($\tau \leq 10$ ns) and gives information on the labeling specificity. According to these results, the long lifetime is the most significant parameter in characterizing the specific fluorescence signal of the labeling and provides a powerful tool for evaluating the specific antigen–antibody binding in the cells.

The observed multiplicity of the fluorescence lifetime (Lorentzian distribution) for the protein may be due both to different labeling sites, so that the probe experiences a large number of heterogeneous local environments, and to local fluctuations among similar conformational substates [6], while the association to the cells may result in immobilization of the probe binding site in a more protected environment (discrete lifetime).

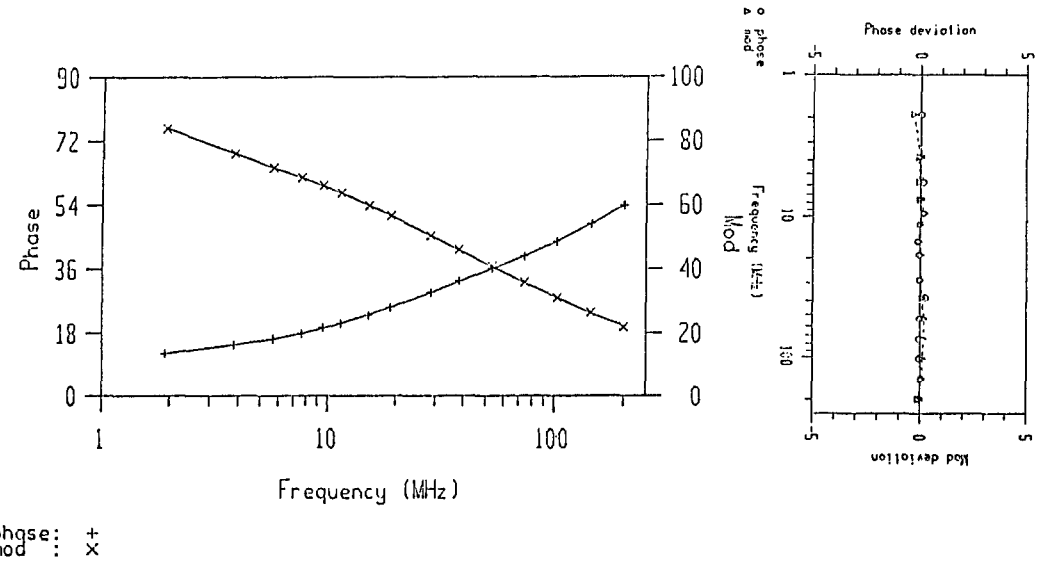

Fig. 1. Differential phase (degrees) and modulation ratio (%) for N_2-bubbled Py.M–IgG anti-mouse at 5°C. λ_{exc} = 340 nm; $\lambda_{em} \geq 370$ nm. The best fit (solid line, χ^2 = 0.182) gives two lifetime distributions (Lorentzian), centered at 101 and 2.6 ns, respectively; and a discrete component of 0.7 ns.

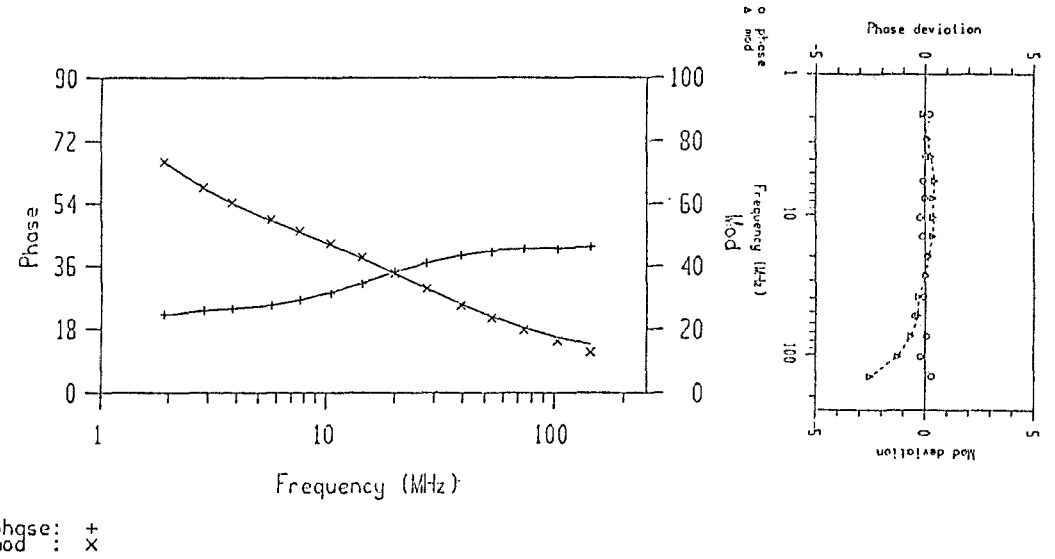

Fig. 2. Differential phase (degrees) and modulation ratio (%) for N_2-bubbled labeled cells at 20°C. λ_{exc} = 340 nm; $\lambda_{em} \geq 370$ nm. The best fit (solid line, $\chi^2 = 3.253$) gives a long discrete lifetime (τ = 117 ns), a Lorentzial distribution centered at 6.6 ns, and a short component of 0.3 ns.

Table I. Fluorescence Lifetimes (τ) and Fractional Intensities (f) Measured in Air-Satured Solutions of Free Py.M, Py.M–IgG Conjugate, and labeled C6 Cells After BrdU Incorporation: λ_{exc} = 340 nm, $\lambda_{em} \geq 370$ nm, T = 20°C

Sample	τ_1 (ns)	f_1	τ_2 (ns)	f_2	τ_3 (ns)	f_3
Py.M (in buffer solution)	38±2	0.35±0.10	5.1±1.5	0.50±12	0.15±0.05	0.15±0.08
Py.M–IgG anti-mouse	74±5	0.55±0.15	11±4	0.30±0.15	0.55±0.08	0.15±0.10
Labeled cells	100±7	0.41±0.12	5.7±3	0.34±0.10	0.22±0.03	0.25±0.10

The further increase in the lifetime value in the labeled cells suggests that the antigen–antibody interaction increases the stability of the excited state of the probe at the binding site. For such long-lived species, both the large influence of the microenvironment and oxygen interaction have been considered to perturb the excited state of the probe [7].

To clarify this point, we estimated the accessibility to oxygen of fluorescent probe in different situations performing measurements on samples after N_2 bubbling to reduce the oxygen concentration. The lifetime values of the long-lived component for samples degassed with N_2 bubbling for 15 min at 20°C increase up to 70 ns for free Py.M in buffer solution, to 100 ns for Py.M–IgG anti-mouse, and to 127 ns for labeled cells, pointing out the high efficiency of O_2 quenching, as expected for a long-lived excited state [8].

Assuming that the reduction in the oxygen concentration is the same for all samples (N_2 bubbling was performed with the same procedure), the apparent quenching rate (k_q) is reduced to about one-third when the probe is conjugated with IgG anti-mouse and to about one-fifth for the labeled cells. These variations suggest that the accessibility to oxygen of the fluorescent probe decreases after conjugation with the antibody and cell labeling, confirming a more compact packing of the binding site [9].

Anisotropy decays. Measurements of the emission anisotropy of Py.M–IgG complexes and labeled cells (Figs. 3 and 4) give additional information on the antigen–antibody interaction of the selective protein association to the cellular system, in particular, on the mobility of the probe at the binding site.

Analysis of the emission anisotropy is complicated

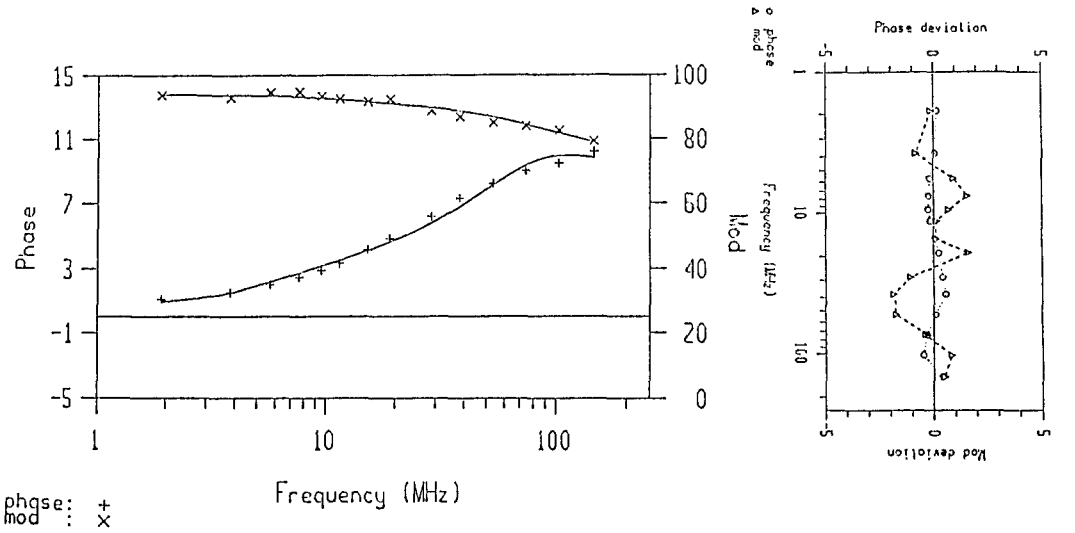

Fig. 3. Differential polarized phase (degrees) and modulation ratio (%) measurements for N_2-bubbled Py.M–IgG anti-mouse adduct at 5°C. The best fit (solid line, $\chi^2 = 5.6$) was obtained considering two-molecular species associated with the two lifetime distributions, assigning one rotational motion to the long-lived molecular species and two rotational motions to the other species. The rotational correlation times are 260 and 2.61 ns and 0.30 ns, respectively.

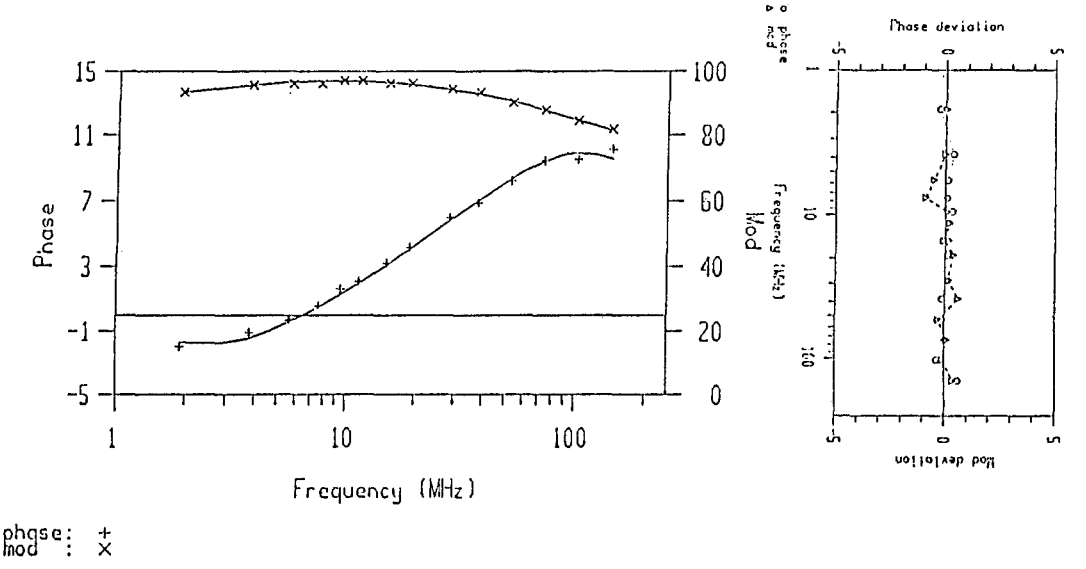

Fig. 4. Differential polarized phase (degrees) and modulation ratio (%) measurements for N_2-bubbled labeled cells at 20°C. The best fit (solid line, $\chi^2 = 1.49$) was obtained by the same model used for the Py.M–IgG adduct. The rotational correlation times are 580 and 16.8 ns and 0.37 ns.

owing to the presence of an amount of not-well-defined shorter components in the fluorescence decay of the Py.M adduct [3,7,10], by the presence of inhomogeneites in the cell population, by the contribution of debris, and so on.

Anisotropy decays were analyzed assuming the presence of several species with more than one rotational correlation time. For the conjugates, the anisotropy decays show, besides short correlation times attributable to inner protein motion, a long correlation time, Φ, of about 220 ± 50 ns at 5°C and about 50 ± 15 ns at 20°C, which can be assigned to overall rotation of the protein.

For the labeled cells, besides short rotational components of various origins, a correlation time of about 580 ± 50 ns at 20°C was measured. This result, together with fluorescence decays, where a discrete long lifetime is evident, suggests that only restricted motions about the main conformation are possible, in agreement with a greater stabilization of the probe binding site [3,10].

In conclusion, when Py.M is bound in the combining site of IgG anti-mouse and linked to cells, its fluorescence lifetime does progressively increase, indicating that the labeling has taken place in a more oxygen-protected environment for the ligand, with increased stability of the probe at the binding site. This kind of measurement promises to be useful in characterizing the different binding sites in cellular systems.

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