Polyclonal Antibodies Elicited via Immunization with a $Ru(bpy)_3^{2+}$-Methyl Viologen Conjugate: Is a Polyclonal Antibody Immune Response Always Heterogeneous?

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It is a commonly held belief that polyclonal immune responses are highly heterogeneous. In contrast to this view, we wish to report a photophysical investigation of polyclonal IgG antibodies elicited via immunization with a tris(2,2′-bipyridine)ruthenium(II)–methyl viologen hapten (**1**) (Figure 1).[1] Tris(2,2′-bipyridine)ruthenium(II) (**2**) ($Ru(bpy)_3^{2+}$), when bound by the *entire* hapten-specific polyclonal IgG sample, exhibits surprisingly homogeneous photophysical behavior and an excited-state lifetime of 1500 ns in N_2-saturated buffer.[2] This first report of polyclonal antibodies that bind $Ru(bpy)_3^{2+}$ also showcases the use of time-resolved luminescence as a tool for the investigation of polyclonal immune responses.

Antigen-specific polyclonal antibodies are produced when the immune system is challenged by infection or immunization. A complete characterization of the functional distribution of antigen-specific antibodies in a polyclonal immune response is of fundamental importance to our understanding of how the immune system operates, having application to numerous fields ranging from medicine[3] to catalytic antibodies.[4] Unfortunately, few techniques are available for the quantitative functional analysis of the *distribution* of antibodies present in a polyclonal sample. Antibodies to luminescent molecules,[5–7] most notably fluorescein,[8,9] have led to important advances in our understanding of antibody–antigen interactions. Because the photophysical properties of $Ru(bpy)_3^{2+}$ have been shown to be sensitive to local environmental factors,[10–13] it can be considered a well-suited luminescent probe of antibody binding pockets.[14] In particular, as demonstrated in this Communication, the use of time-resolved luminescence spectroscopy to probe the antibody-bound $Ru(bpy)_3^{2+}$ provides a powerful method for analysis of the functional distribution of antibodies in a polyclonal IgG immune response.

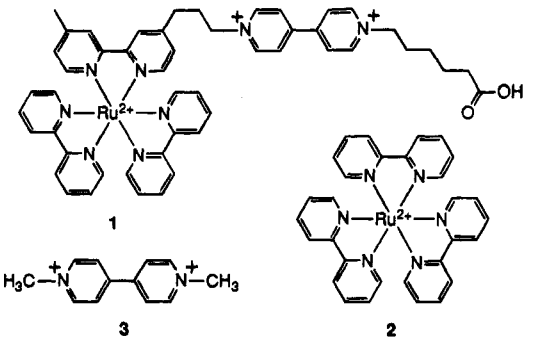

Figure 1. Structures of the hapten (**1**), $Ru(bpy)_3^{2+}$ (**2**), and methyl viologen (**3**).

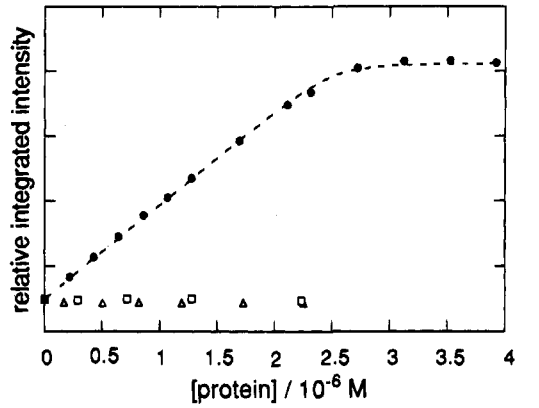

Figure 2. Steady-state luminescence titration of 5×10^{-7} M $Ru(bpy)_3^{2+}$ with anti-**1** polyclonal IgG antibodies (●), pre-immunization polyclonal IgG antibodies (□), and BSA (△) in air-saturated buffer, pH 7.4. Shown is an interpolative fit (– –) for the K_a of the hapten-specific anti-**1** polyclonal antibodies binding to $Ru(bpy)_3^{2+}$ that yields a value of 5×10^8 M^{-1}.

Adding increasing amounts of the anti-**1** polyclonal IgG antibodies to a fixed concentration of $Ru(bpy)_3^{2+}$ resulted in the enhanced steady-state emission of the bound $Ru(bpy)_3^{2+}$. As important controls, $Ru(bpy)_3^{2+}$ was titrated with either nonspecific, "pre-immunization" IgG antibodies or bovine serum albumin, neither of which resulted in a significant change in the emission yield (Figure 2). These controls, therefore, confirm that the enhanced emission results from specific binding with the hapten-induced antibodies.

Over the course of the titration with anti-**1** polyclonal antibodies (Figure 2), $Ru(bpy)_3^{2+}$ emission increases linearly with increasing antibody concentration. This allows us to assign a lower limit to the association constant for $Ru(bpy)_3^{2+}$ binding to the polyclonal sample of 5×10^8 M^{-1}. Assuming that one

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(1) The hapten **1** gave satisfactory ^1H NMR, ^{13}C NMR, HRMS (FAB), and elemental analyses. A male New Zealand white rabbit was immunized five times at 21 day intervals with a keyhole limpet hemocyanin conjugate of the hapten **1**. Ten days following the fifth and final injection, serum was removed from the rabbit, and the polyclonal IgG antibodies were isolated according to ref 4b. This protein G-based isolation technique, unlike methods utilizing hapten-affinity chromatography, produces polyclonal samples that are presumably not biased with respect to binding selectivity or affinity. Thus, the antibody sample used in this study is truly representative of the *entire* distribution of polyclonal IgG antibodies present in the serum of the immunized rabbit. For more information on the production and isolation of antibodies, see: Harlow, E.; Lane, D. *Antibodies: A Laboratory Manual*; Cold Spring Harbor Laboratory: Cold Spring Harbor, New York.

(2) All measurements were conducted in 10 mM sodium phosphate, 120 mM NaCl, pH 7.4. All reported kinetic values were reproducible to within 5%. Because the hapten is internally quenched and relatively nonluminescent, $Ru(bpy)_3^{2+}$ was used to obtain the maximum amount of luminescence for this study. See: Yonemoto, E. H.; Saupe, G. B.; Schmehl, R. H.; Hubig, S. M.; Riley, R. L.; Iverson, B. L.; Mallouk, T. E. *J. Am. Chem. Soc.* **1994**, *116*, 4786–4795.

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(14) Complex luminescence decay kinetics have been observed for $Ru(DIP)_3^{2+}$, $Ru(phen)_3^{2+}$, and $Ru(bpy)_3^{2+}$ in the presence of DNA and have been attributed to the ability of these metal complexes to sense different, localized binding environments, see: (a) Kumar, C. V.; Barton, J. K.; Turro, N. J. *J. Am. Chem. Soc.* **1985**, *107*, 5518–5523. (b) Tossi, A. B.; Kelly, J. M. *Photochem. Photobiol.* **1989**, *49*, 545–556.

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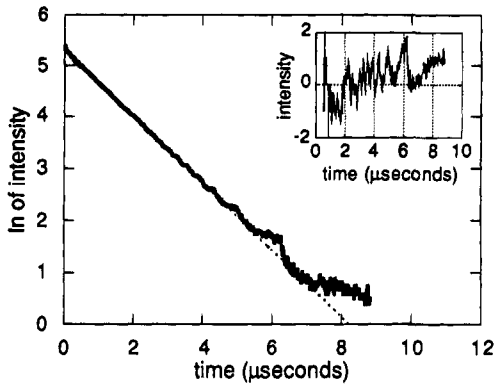

Figure 3. Semilogarithmic plot of the luminescent decay trace for Ru(bpy)$_3^{2+}$ (1×10^{-5} M) in the presence of anti-**1** polyclonal IgG antibodies (1.4×10^{-4} M in antibody binding sites) in N_2-saturated PBS. The accompanying inset shows the residual after the decay trace was fit to a lifetime of 1500 ns (– –).

Ru(bpy)$_3^{2+}$ binds per antibody binding site, these data indicate that 9% of the polyclonal antibodies bind Ru(bpy)$_3^{2+}$. The emission spectra from the beginning to the end of this titration exhibit a 3.3-fold increase in the integrated emission and blue-shift in the emission spectrum upon binding Ru(bpy)$_3^{2+}$ to the polyclonal antibodies.[15]

A titration of Ru(bpy)$_3^{2+}$ with anti-**1** polyclonal antibodies was carried out in air-saturated buffer. At low concentrations of Ru(bpy)$_3^{2+}$, only a single species with a lifetime of 1300 ns, assigned as the bound Ru(bpy)$_3^{2+}$, was observed.[16] At concentrations of Ru(bpy)$_3^{2+}$ sufficient to fill over 8% of the total binding pockets in the polyclonal sample, a second component appeared with a lifetime of 380 ns. This latter lifetime agreed with that for Ru(bpy)$_3^{2+}$ in air-saturated buffer (380 ns) and therefore was assigned to Ru(bpy)$_3^{2+}$ free in solution. Increasing the concentration of Ru(bpy)$_3^{2+}$ further served only to increase the amount of the free species. Throughout the titration, the antibody-bound Ru(bpy)$_3^{2+}$ exhibited a lifetime of 1300 ns, indicating that the entire hapten-specific polyclonal sample was functioning in a homogeneous manner.

At relatively high concentrations of polyclonal antibodies, the luminescent signal for the antibody-bound Ru(bpy)$_3^{2+}$ could be fit to a single-exponential decay.[17] Shown in Figure 3 is the semilogarithmic plot of the emission decay trace for this species in N_2-saturated buffer, and accompanying this plot is the resulting residual after the decay trace was fit to a lifetime of 1500 ns. This clean, single-exponential decay for the bound Ru(bpy)$_3^{2+}$ demonstrates that the elicited polyclonal antibodies change the photophysical properties of the bound Ru(bpy)$_3^{2+}$ in a homogeneous way. Interestingly, because **1** was injected as the racemate, the resulting pool of polyclonal antibodies would be expected to have at least two kinds of shape-selective pockets to accommodate the two enantiomers. However, the observed single-exponential decay implies that the immune system of the rabbit has taken some kind of convergent approach to the problem of creating a Ru(bpy)$_3^{2+}$ binding motif in response to **1**, at least in terms of the factors responsible for modulating the extended excited-state lifetime of the antibody-bound Ru(bpy)$_3^{2+}$.[10-13]

Two pieces of evidence rule out the possibility that any significant fraction of the polyclonal antibodies that bind Ru(bpy)$_3^{2+}$ results in a nonluminescent complex. First, in air-saturated buffer, an identical increase was observed for antibody-bound Ru(bpy)$_3^{2+}$ to free Ru(bpy)$_3^{2+}$ in both the steady-state emission yield and excited-state lifetime measurements. Second, the value of 8% obtained from the time-resolved titrations for the amount of hapten-specific antibody in the polyclonal sample agrees well with the 9% value obtained with the steady-state titration (Figure 2).

In air-saturated buffer, an excited-state lifetime of 1300 ns was observed for the antibody-bound Ru(bpy)$_3^{2+}$, and the resulting luminescence decay trace also fit to a single-exponential decay process. Because varying degrees of protection from oxygen, a known quencher of the excited state of Ru(bpy)$_3^{2+}$,[18] would result in a distribution of excited-state lifetimes, the absence of any more than one component in the presence of oxygen indicates that Ru(bpy)$_3^{2+}$ is shielded by protein to the same extent throughout this polyclonal sample.[19] Bimolecular quenching constants of 2.9×10^8 and 3.6×10^9 M^{-1} s^{-1} were measured for the quenching by O_2 of the antibody-bound Ru(bpy)$_3^{2+}$ and free Ru(bpy)$_3^{2+}$, respectively.

In addition, a N_2-saturated solution containing both free and antibody-bound Ru(bpy)$_3^{2+}$ was titrated with methyl viologen (3),[20] and the luminescence lifetimes of both these species were monitored as a function of methyl viologen concentration. The resulting Stern–Volmer plots showed no evidence for a static quenching component and, within error, were both linear. Bimolecular quenching constants of 2.6×10^8 and 1.7×10^9 M^{-1} s^{-1} were measured for the antibody-bound Ru(bpy)$_3^{2+}$ and free Ru(bpy)$_3^{2+}$, respectively. As in the case when O_2 served as a quencher, the antibody-bound Ru(bpy)$_3^{2+}$ is shielded from quenching by methyl viologen.

At least two limiting explanations, or a combination thereof, could explain the single exponential decay shown in Figure 3. First, the entire polyclonal response could be essentially monoclonal, with all of the hapten-specific antibodies being genetically and therefore functionally related. Alternatively, the different antibodies could be genetically unrelated, but simply recognize the hapten in a similar fashion. Studies are currently underway to distinguish between these possibilities.

Carrying out time-resolved studies with luminescent haptens should provide a general way of probing the distribution of polyclonal antibodies in an immune response. For example, auramine O[21] or a merocyanine dye[22] could be used as other luminescent haptens to probe polyclonal antibody binding site flexibility or polarity, respectively, through the course of an immunization. The results, in combination with a detailed genetic analysis, will provide a much clearer picture of the functional composition of a polyclonal response.

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(15) There is also a slight splitting in the emission spectrum of the bound Ru(bpy)$_3^{2+}$. The effect is similiar to what is observed in matrix isolation studies. See: Elfring, W. H., Jr.; Crosby, G. A. *J. Am. Chem. Soc.* **1981**, *103*, 2683–2687.

(16) As a control, the excited-state lifetime of Ru(bpy)$_3^{2+}$ in the presence of BSA (10 mg/mL) in 5 mM HEPES, pH 8.5, was measured as 420 ns. Ru(bpy)$_3^{2+}$ has been reported to bind to proteins under similar conditions of low ionic strength and high pH. See: Nilsson, T. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 6497–6501.

(17) The observed decay profile gave a satisfactory fit to a single-exponential process over 4τ. Attempting to fit it to a sum of two exponentials had no statistical meaning. Alternatively, the decay profile can be fit as a Gaussian distribution of first-order rate constants around some mean value, but in this case the width of the distribution is so narrow that it is equivalent to a first-order process. The semilogarithmic plot for a biexponential process or for a process composed of a Gaussian distribution of first-order rate constants would be curved, not linear. For an example of how time-resolved luminescence can be used in the analysis and resolution of a multicomponent system, see: Harriman, A.; Novak, A. K. *Pure Appl. Chem.* **1990**, *62*, 1107–1110.

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