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Binding Parameters of Interactions of Monomer–Polymer Systems Based on Quenching of Their Completely Overlapped Fluorescence: A Theory

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Abstract □ A theory is presented which describes the interactions between completely overlapping fluorescing monomer–polymer systems and affords a method to calculate binding parameters. The theory is based on the assumptions that the complex formed from the fluorescing binding site and the monomer molecule does not fluoresce and that the fluorescence intensity is linearly related to concentration. The relationships derived from this theory have provided a sensitive and easy method for calculating the number of binding sites on a polymer molecule from only three emission-intensity values. The theory has been tested on systems containing trimethoprim–serum albumins and found to yield results which are consistent with a curve-fitting approach to the experimental data. This reflects the validity of the theoretical model presented and the various assumptions and approximations made.

Keyphrases □ Monomer–polymer systems—binding parameters, overlapped fluorescence quenching □ Binding parameters—overlapped fluorescence quenching, monomer–polymer system □ Fluorescence quenching—binding parameters, monomer–polymer systems

Fluorescence quenching has been applied to the study of complex formation (1); for example, the interactions of amino acids containing aromatic nuclei and their amines, and oligopeptides containing aromatic amino acids with nucleic acids, were studied by this method (2, 3). Recently, Koumriqian (4) and Borazan and Koumriqian (5) have applied the technique to study interactions between catecholamines and polyadenylic acid. In these studies, the emissions of the polynucleotides were negligible compared with those of the monomers. It was, therefore, easy to derive equations for calculating the binding parameters using the fraction remaining from the fluorescence intensity of the monomers or the observed quantum yield.

However, due to the inherent properties of many systems, both the monomers and polymers in the binary mixtures exhibit almost complete emission overlap over a wide range of excitation and emission wavelengths. Examples for such systems can be found in the fluorescence quenching studies of the interactions of steroids–polynucleotides (6), antineoplastics–polynucleotides (7), and antibacterials–serum albumins (8). Thus, these types of systems impose a difficult problem when analyzing the experimental data to calculate the binding constants. These authors (6–8) have applied an empirical approach involving a curve-fitting technique to analyze the fluorescence data.

The present study is concerned with a theoretical treatment which utilizes the fluorescence technique in situations where complete fluorescence overlap is taking place. This allowed us to determine the number of fluorescing binding sites on a polymer molecule available to the monomer molecules and to calculate the equilibrium constant for the process.

THEORETICAL SECTION

To calculate the binding constants for an interaction between a monomer, M, and a binding site on a polymer, Pb, the following simple mathematical model was assumed:

$$M + Pb = MPb \qquad \text{(Eq. 1)}$$

where MPb, is the complex formed. When using the fluorescence technique to calculate the binding parameters, the solution of the problem is straight-

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forward if one of the reactants does not fluoresce. In the present theoretical treatment, however, one case of concern is when both the monomer and binding site fluoresce at a given set of excitation and emission wavelengths (fluorescence overlap). Another source of concern is the quenching of the emissions of the interacting species when the complex is formed. It is assumed in the following derivations that the polymer molecule contains n fluorescing binding sites and that the emission intensity is linearly related to concentration. When working with dilute solutions:

$$\text{concentration} = \text{constant} \times \text{fluorescence intensity} \qquad \text{(Eq. 2)}$$

Accordingly:

$$I_{obs}^{\lambda} = I_M^{\lambda} + I_{Pb}^{\lambda,\Sigma} \qquad \text{(Eq. 3)}$$

where I_{obs}^{λ} and I_M^{λ} are the observed and free monomer emission intensities and $I_{Pb}^{\lambda,\Sigma}$ is the fluorescence intensity of the polymer sites without a bound substrate. The summation sign, Σ, is used to emphasize that the fluorescence originates from different sites on one polymer molecule.

I_{obs}^{λ} can be related to the total unquenched emission intensity, as:

$$I_{obs}^{\lambda} = \beta_{tot} I_{tot}^{\lambda} = \beta_M I_{M,0}^{\lambda} + \beta_{Pb} I_{Pb_0}^{\lambda,\Sigma} \qquad \text{(Eq. 4)}$$

β_{tot}, β_M, and β_{Pb} are the fractions of remaining fluorescence intensities for the total emission, monomer, and binding sites, and $I_{M,0}^{\lambda}$ and $I_{Pb_0}^{\lambda,\Sigma}$ are the initial emission intensities of the monomer and polymer binding sites. Therefore, from Eqs. 3 and 4:

$$I_M^{\lambda} = \beta_M I_{M,0}^{\lambda} \qquad \text{(Eq. 5)}$$

$$I_{Pb}^{\lambda,\Sigma} = \beta_{Pb} I_{Pb,0}^{\lambda,\Sigma} \qquad \text{(Eq. 6)}$$

According to Eq. 1, the concentration of the complex (MPb) can be expressed in three different ways:

$$(MPb) = (M_0) - (M) \qquad \text{(Eq. 7)}$$

$$(MPb) = (Pb_0) - (Pb) \qquad \text{(Eq. 8)}$$

$$(MPb) = n(P_0) - n(P) \qquad \text{(Eq. 9)}$$

where (M_0) and (M) are the initial and free concentrations of the monomer, respectively; (Pb_0) and (Pb) are the concentrations of the initial and the remaining free polymer binding sites, respectively; and (P_0) and (P) represent the total and free concentrations of the polymer, respectively.

According to the assumption presented in Eq. 2, one can easily verify the following equations:

$$(M) = \beta_M (M_0) \qquad \text{(Eq. 10)}$$

$$(Pb) = \beta_{Pb} (Pb_0) \qquad \text{(Eq. 11)}$$

$$(P) = \beta_{Pb} (P_0) \qquad \text{(Eq. 12)}$$

It is evident from Eqs. 10–12, that β_M and β_{Pb} are essentially equal to the fractions which remain free of interaction. Combining Eqs. 5, 7, and 10:

$$(MPb) = (M_0)\left(1 - \frac{I_M^{\lambda}}{I_{M,0}^{\lambda}}\right) \qquad \text{(Eq. 13)}$$

and Eqs. 6, 9, and 12:

$$(MPb) = n(P_0)\left(1 - \frac{I_{Pb}^{\lambda,\Sigma}}{I_{Pb_0}^{\lambda,\Sigma}}\right) \qquad \text{(Eq. 14)}$$

Equating Eqs. 13 and 14, substituting for $I_{Pb}^{\lambda,\Sigma}$ (Eq. 3), and rearranging equations:

$$I_M^{\lambda} = \frac{(M_0) I_{Pb_0}^{\lambda,\Sigma} I_{M,0}^{\lambda} - n(P_0) I_{M,0}^{\lambda} (I_{Pb_0}^{\lambda,\Sigma} - I_{obs}^{\lambda})}{n(P_0) I_{M,0}^{\lambda} + (M_0) I_{Pb_0}^{\lambda,\Sigma}} \qquad \text{(Eq. 15)}$$

Rearranging Eq. 3 and combining with Eq. 6:

$$\beta_{Pb} = \frac{I_{obs}^{\lambda} - I_M^{\lambda}}{I_{Pb_0}^{\lambda,\Sigma}} \qquad \text{(Eq. 16)}$$

Substituting for I_M^{λ} (Eq. 15) into Eq. 16:

$$\beta_{Pb} = \frac{(M_0) I_{obs}^{\lambda} - (M_0) I_{M,0}^{\lambda} + n(P_0) I_{M,0}^{\lambda}}{n(P_0) I_{M,0}^{\lambda} + (M_0) I_{Pb_0}^{\lambda,\Sigma}} \qquad \text{(Eq. 17)}$$

According to the mathematical model presented in Eq. 1, the equilibrium constant expression, K, can be written as:

$$K = \frac{(MPb)}{(M)(Pb)} \qquad \text{(Eq. 18)}$$

Incorporating Eqs. 9 and 12 into Eq. 7:

$$(M) = (M_0) - n(P_0)(1 - \beta_{Pb}) \qquad \text{(Eq. 19)}$$

The equilibrium constant equation (Eq. 18) can be written in the following form after substituting for (MPb) (Eqs. 9 and 12), M (Eq. 19), and for (Pb) (Eqs. 11 and 12):

$$K = \frac{\left[\frac{1 - \beta_{Pb}}{\beta_{Pb}}\right]}{[(M_0) - n(P_0)(1 - \beta_{Pb})]} \qquad \text{(Eq. 20)}$$

Thus, the value of n must be determined in addition to the other experimental parameters in order to solve for β_{Pb} (Eq. 17) and K (Eq. 20).

In principle, one may be able to arrive at the values of n by performing a curve-fitting technique through assigning values to n and calculating the equilibrium constant, K, at different concentration levels to minimize percent variation (6–8). Thus, the present investigation is undertaken to study the problem from a fundamental point of view, in the hope of arriving at the exact relationships controlling such complicated systems. This can be done through optimizing the K expression (Eq. 20) with respect to n, *i.e.*:

$$\frac{\partial K}{\partial n} = 0 \qquad \text{(Eq. 21)}$$

Therefore, Eq. 20 should lead to the following:

$$[(M_0) - n(P_0) + n(P_0)\beta_{Pb}] \frac{1}{\beta_{Pb}^2} \frac{d\beta_{Pb}}{dn} + \left[\frac{1 - \beta_{Pb}}{\beta_{Pb}}\right]\left[-(P_0) + (P_0)\beta_{Pb} + n(P_0)\frac{d\beta_{Pb}}{dn}\right] = 0 \qquad \text{(Eq. 22)}$$

Rearranging Eq. 22 and setting it up in an integral form, we can write:

$$\int \frac{\gamma d\beta_{Pb}}{(1 - \beta_{Pb})^2 n} - \int \frac{d\beta_{Pb}}{(1 - \beta_{Pb})^2} + \int \frac{2\beta_{Pb} d\beta_{Pb}}{(1 - \beta_{Pb})^2} - \int \frac{\beta_{Pb}^2 d\beta_{Pb}}{(1 - \beta_{Pb})^2} - \int \frac{\beta_{Pb}}{n} dn = 0 \qquad \text{(Eq. 23)}$$

where:

$$\gamma = \frac{(M_0)}{(P_0)} \qquad \text{(Eq. 24)}$$

Eq. 17 can be rearranged to yield n in terms of intensities and concentrations:

$$n = \frac{(M_0)(I_{obs}^{\lambda} - I_{M,0}^{\lambda}) - (M_0)\beta_{Pb} I_{Pb_0}^{\lambda,\Sigma}}{(P_0)\beta_{Pb} I_{M,0}^{\lambda} - (P_0) I_{M,0}^{\lambda}} \qquad \text{(Eq. 25)}$$

To simplify the notations, set:

$$a = I_{obs}^{\lambda} - I_{M,0}^{\lambda} \qquad \text{(Eq. 26)}$$

$$b = I_{Pb_0}^{\lambda,\Sigma} \qquad \text{(Eq. 27)}$$

$$c = I_{M,0}^{\lambda} \qquad \text{(Eq. 28)}$$

$$\beta = \beta_{Pb} \qquad \text{(Eq. 29)}$$

Therefore, Eq. 25 can be written in the following form:

$$n = \frac{\gamma(b\beta - a)}{c(1 - \beta)} \qquad \text{(Eq. 30)}$$

Taking the differential of Eq. 30:

$$dn = \frac{\gamma b d\beta}{c(1 - \beta)} + \frac{\gamma bc\beta d\beta}{c^2(1 - \beta)^2} - \frac{\gamma ac d\beta}{c^2(1 - \beta)^2} \qquad \text{(Eq. 31)}$$

Substituting for n (Eq. 30) and dn (Eq. 31) into Eq. 23:

$$c \cdot \int \frac{d\beta}{(1 - \beta)(b\beta - a)} - \int \frac{d\beta}{(1 - \beta)^2} + 2 \cdot \int \frac{\beta d\beta}{(1 - \beta)^2} - \int \frac{\beta^2 d\beta}{(1 - \beta)^2} - b \cdot \int \frac{\beta d\beta}{(b\beta - a)} - b \cdot \int \frac{\beta^2 d\beta}{(1 - \beta)(b\beta - a)} + a \cdot \int \frac{\beta d\beta}{(1 - \beta)(b\beta - a)} = 0 \qquad \text{(Eq. 32)}$$

The above integrals can be evaluated to yield the following (9, 10):

$$c \cdot \int \frac{d\beta}{(1 - \beta)(b\beta - a)} = \frac{c}{b - a} \cdot \ln \frac{(b\beta - a)}{(1 - \beta)} \qquad \text{(Eq. 33)}$$

$$\int \frac{d\beta}{(1 - \beta)^2} = \frac{1}{1 - \beta} \qquad \text{(Eq. 34)}$$

$$2 \cdot \int \frac{\beta\, d\beta}{(1-\beta)^2} = 2 \cdot \ln(1-\beta) + \frac{2}{1-\beta} \quad \text{(Eq. 35)}$$

$$\int \frac{\beta^2 d\beta}{(1-\beta)^2} = \beta + 2 \cdot \ln(1-\beta) + \frac{1}{1-\beta} \quad \text{(Eq. 36)}$$

$$b \cdot \int \frac{\beta\, d\beta}{(b\beta - a)} = \beta + \frac{a}{b} \cdot \ln(b\beta - a) \quad \text{(Eq. 37)}$$

$$b \cdot \int \frac{\beta^2\, d\beta}{(1-\beta)(b\beta - a)} = -\beta - \frac{a+b}{b} \cdot \ln(b\beta - a) + \frac{b}{b-a} \cdot \ln \frac{(b\beta - a)}{(1-\beta)} \quad \text{(Eq. 38)}$$

$$a \cdot \int \frac{\beta\, d\beta}{(1-\beta)(b\beta - a)} = \frac{a^2}{b(b-a)} \cdot \ln(b\beta - a) - \frac{a}{b-a} \cdot \ln(1-\beta) \quad \text{(Eq. 39)}$$

Substituting for the integral values (Eqs. 33-39) into Eq. 32:

$$\left[\frac{c}{b-a} - \frac{a}{b}\right] \cdot \ln(b\beta - a) - \left[\frac{c}{b-a} - 1\right] \cdot \ln(1-\beta) - \beta = 0 \quad \text{(Eq. 40)}$$

One can easily verify that Eq. 40 holds for $|b\beta - a|$, and when expressing it in its original notations (Eqs. 26-29) it yields the following:

$$\left[\frac{I_{M,0}^{\lambda}}{I_{Pb_0}^{\lambda,\Sigma} + I_{M,0}^{\lambda} - I_{obs}^{\lambda}} - \frac{I_{obs}^{\lambda} - I_{M,0}^{\lambda}}{I_{Pb_0}^{\lambda,\Sigma}}\right] \cdot \ln\left|I_{Pb_0}^{\lambda,\Sigma}\beta_{Pb} + I_{M,0}^{\lambda} - I_{obs}^{\lambda}\right| - \left[\frac{I_{obs}^{\lambda} - I_{Pb_0}^{\lambda,\Sigma}}{I_{Pb_0}^{\lambda,\Sigma} + I_{M,0}^{\lambda} - I_{obs}^{\lambda}}\right] \cdot \ln(1-\beta_{Pb}) - \beta_{Pb} = 0 \quad \text{(Eq. 41)}$$

RESULTS AND DISCUSSION

Solving the nonlinear equation (Eq. 41) is a difficult task using conventional mathematical methods. An approximate solution to the problem is obtained by expanding the logarithmic terms and expressing the equation in terms of the power series of β. This method yields highly approximate results because terms having exponents greater than four are omitted. Higher order equations are almost impossible to solve, however, if one seeks better results. Thus, the most reasonable technique remaining is to solve Eq. 41 numerically with a programmable calculator.

To test the theory, experimental data involving fluorescence quenching studies, where complete emission overlap characterizes the systems, were taken from Saoud (8). His investigation was concerned with interactions between trimethoprim and serum albumins taken from different species. The measured emission intensity data were computed into the coefficients of the natural logarithms of Eq. 41, while the equation is solved by assigning values to β and calculating the value of the equation $[F(\beta)]$. Obviously, the acceptable solution is $F(\beta) = 0$. In all cases, β is solved to 1×10^{-6}. The value of $|F(\beta)|$ that corresponds to this level of accuracy ranged from 3.6×10^{-5} to 6.6×10^{-7}. The results are summarized in Table I.

Applying Eq. 25, the value of β_{Pb}^1 yields unrealistic results, since negative values of the number of binding sites are obtained. Thus the values of β_{Pb}^2 are the ones taken as a base for calculating the binding parameters. It is evident from Table I that as the monomer-polymer ratio is increased by 400%, the equilibrium constant varies only in the range from 8.5 to 19.8%. Furthermore, the estimated difference in the number of binding sites for the same magnitude of increase in the ratio is found to be 20.3–57.8%. These variations reflect the combined effects of the experimental errors, the inherent factors in the systems themselves, and the various assumptions and approximations made. For example, it was assumed in the derivations presented in the theory that all of the binding sites are independent (no site-site interaction took place), equivalent, and fluorescing. Based upon different physical methods, deviations from linearity of the Scatchard plots are reported in the literature. The explanations given include site-site interactions (11, 12), competitive binding between fluorescing and nonfluorescing binding sites, and the presence of nonequivalent binding sites (13).

Saoud (8) has applied Eq. 17 to calculate β_{Pb} by assigning values to the number of binding sites, n. The best n value is the one that generates the least variation in K. Indeed, the n values obtained by the author that induce minimum variation in K were close to the values obtained from Eq. 41. This gives supporting evidence to the validity of the relationships derived in this theory.

It may be concluded that the present theory has provided a powerful tool to calculate the binding parameters for the monomer-polymer interactions

Table I—Fractions Which Remain Free of Binding Interactions on the Polymer and Monomer Molecules Together with Number of Binding Sites and the Equilibrium Constants for the Interactions of Trimethoprim with Serum Albumins [a]

$(M_0)/(P_0) \times 10^{-2}$ [b]	β^1_{Pb}	β^2_{Pb}	β_M	n	K, $M^{-1} \times 10^{-5}$
		Trimethoprim-Human Serum Albumin[c]			
0.69013	0.646359	0.742452	0.952831	12.64	3.64061
1.38026	0.457742	0.580798	0.970633	9.67	3.71803
2.07039	0.323637	0.464287	0.978571	8.28	3.93036
2.76052	0.215785	0.371481	0.982130	7.85	4.30678
Mean				9.61	3.89895
± SD, %				24.92	8.54
		Trimethoprim-Camel Serum Albumin[c]			
0.69013	0.708736	0.783187	0.950220	15.85	2.91337
1.38026	0.530511	0.628165	0.968354	11.75	3.05642
2.07039	0.397371	0.511137	0.975151	10.52	3.26931
2.76052	0.286506	0.413516	0.978418	10.16	3.62393
Mean				12.07	3.21576
± SD, %				23.57	11.05
		Timethoprim-Bovine Serum Albumin[c]			
0.69013	0.735330	0.812953	0.909549	33.37	2.52964
1.38026	0.570280	0.647819	0.958704	16.18	2.83529
2.07039	0.426571	0.514425	0.967674	13.78	3.25150
2.76052	0.313160	0.405888	0.974813	11.70	3.75388
Mean				18.76	3.09258
± SD, %				57.76	19.79
		Trimethoprim-Rat Serum Albumin[c]			
0.69013	0.797670	0.840868	0.963713	15.74	1.96373
1.38026	0.642249	0.709530	0.972037	13.29	2.10580
2.07039	0.488334	0.577188	0.975292	12.10	2.50365
2.76052	0.375465	0.475640	0.980032	10.51	2.81223
Mean				12.91	2.34635
± SD, %				20.26	18.08

[a] In 1 mM aqueous cacodylate buffer, pH 7, based on fluorescence data taken from Ref. 8. [b] The concentration of the albumins was 1.449×10^{-8} M and the measurements were at 25°C. [c] The excitation wavelength was 290 nm and the emission intensities were measured at 335 nm for human serum albumin, 345 nm for camel serum albumin, 352 nm for bovine serum albumin, and 340 nm for rat serum albumin.

from fluorescence quenching, even when a complete overlap in their emission properties is taking place. The method is sensitive and easy, where only three experimental values are necessary to calculate the number of fluorescing binding sites on the polymer: the emissions of the two pure components and the emissions of a mixture containing equimolar concentrations of the components.

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