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Technical Note

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Fluorometric Titration Approach for Calibration of Quantity of Binding Site of Purified Monoclonal Antibody Recognizing Epitope/Hapten Nonfluorescent at 340 nm

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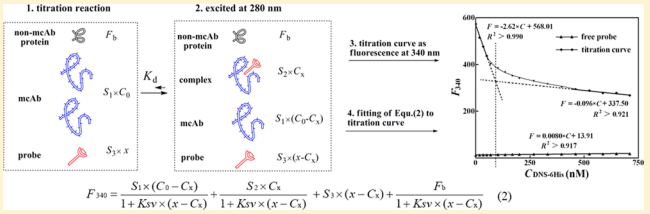
Supporting Information

ABSTRACT: A fluorometric titration approach was proposed for the calibration of the quantity of monoclonal antibody (mcAb) via the quench of fluorescence of tryptophan residues. It applied to purified mcAbs recognizing tryptophan-deficient epitopes, haptens nonfluorescent at 340 nm under the excitation at 280 nm, or fluorescent haptens bearing excitation valleys nearby 280 nm and excitation peaks nearby 340 nm to serve as Förster-resonance-energy-transfer (FRET) acceptors of tryptophan. Titration probes were epitopes/haptens themselves or conjugates of nonfluorescent haptens or tryptophan-deficient epitopes with FRET acceptors of tryptophan. Under the excitation at 280 nm, titration curves were recorded as fluorescence specific for the FRET acceptors or for mcAbs at 340 nm. To quantify the binding site of a mcAb, a universal model considering both static and dynamic quench by either type of probes was proposed for fitting to the titration curve. This was easy for fitting to fluorescence specific for the FRET acceptors but encountered nonconvergence for fitting to fluorescence of mcAbs at 340 nm. As a solution, (a) the maximum of the absolute values of first-order derivatives of a titration curve as fluorescence at 340 nm was estimated from the best-fit model for a probe level of zero, and (b) molar quantity of the binding site of the mcAb was estimated via consecutive fitting to the same titration curve by utilizing such a maximum as an approximate of the slope for linear response of fluorescence at 340 nm to quantities of the mcAb. This fluorometric titration approach was proved effective with one mcAb for six-histidine and another for penicillin G.



Monoclonal antibodies (mcAbs) are indispensable biomaterials for bioanalysis, and their quantities should be determined accurately for standardizing their applications. To date, quantities of mcAbs for bioanalysis are mostly indexed by mass weights derived from absorbance at 280 nm and a putative absorptivity, which is an average of absorptivity of a population of mcAbs and depends on the abundance of aromatic amino acid residues in those mcAbs surveyed (Note S1, Supporting Information). This method has satisfactory precision and efficiency but suffers errors due to deviations of abundance of aromatic amino acid residues in the mcAbs of interest from the average of those mcAbs surveyed. For an accurate assay of a mcAb by absorbance at 280 nm, its absorptivity should be estimated and a preparation of the purified mcAb of calibrated quantity is mandatory. The largest dilution ratio of a preparation of a mcAb with any probe suffers too low precision for calibration.[1,2] IgGs/IgMs in human sera are biomarkers for some diseases. For quantifying IgGs/IgMs in human sera, IgGs/IgMs of calibrated quantity are mandatory reference materials but usually inaccessible.[3−5] Hence, new methods are desired for calibration of quantities of mcAbs.

Under the excitation at 280 nm, most mcAbs produce fluorescence with peaks nearby 340 nm due primarily to emission of tryptophan residues. Interestingly, fluorescence of common mcAbs at 340 nm can be easily quenched by the binding of nonfluorescent haptens/epitopes, supporting such haptens/epitopes can be nonfluorescent probes of mcAbs.[6] Moreover, conjugates of nonfluorescent haptens/epitopes and fluorophores that bear excitation valleys nearby 280 nm and excitation peaks nearby 340 nm to serve as Förster-resonance-energy-transfer (FRET) acceptors of tryptophan, or fluorescent haptens themselves bearing excitation valleys nearby 280 nm and excitation peaks nearby 340 nm, can be FRET probes of mcAbs.[6−9] Specially, under the excitation at 280 nm, the binding of FRET probes to mcAbs quenches fluorescence of mcAbs at 340 nm and enhances fluorescence of the FRET probes.[6−9] Those two types of probes can have high purity. Hence, the binding site of mcAbs can be titrated with either type of probe under the excitation at 280 nm, by recording fluorescence of mcAbs at 340 nm or of the FRET probes applied. This fluorometric titration approach should be resistant to variations of abundance of aromatic amino acid residues in

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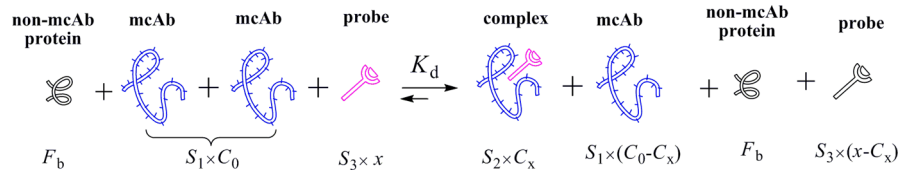
Scheme 1. Components and Parameters Involved in a Generic Model



mcAbs and affinities of probes and may work for *de novo* calibration of quantities of mcAbs.

This fluorometric titration approach should be applicable to purified mcAbs recognizing continuous epitopes deficient in tryptophan residues, haptens nonfluorescent at 340 nm under the excitation at 280 nm, and fluorescent haptens bearing excitation peaks nearby 340 nm and excitation valleys nearby 280 nm. Haptens/epitopes of applicable mcAbs are favorable titration probes since the recording of titration curves by fluorescence at 340 nm is always practical. Six-histidine (6His) is widely used for fusion expression of proteins, and its mcAb (mcAb-6His) is useful for detection of 6His-tagged proteins.[10] Dansylamide is an acceptor of tryptophan and has specific emission at 540 nm. Thus, 6His is a nonfluorescent probe while dansylated 6His (DNS-6His) is a FRET probe of mcAb-6His.[7−9,11,12] To estimate molar quantity of binding site of mcAb-6His, a universal model considering both static and dynamic quenching of fluorescence of mcAb-6His was derived for fitting to titration curves with either probe. This fitting was easy when titration curves were recorded as fluorescence specific for DNS-6His (the FRET probe) but encountered nonconvergence when titration curves were recorded as fluorescence of mcAb-6His at 340 nm with either type of probes. Herein, we proposed a novel strategy for fitting of the universal model to titration curves recorded as fluorescence at 340 nm or specific for FRET probes; the fluorometric titration approach was thus proved universally effective for calibrating quantities of binding site of mcAb-6His with either type of probes and of mcAb for penicillin G with penicillin G as the probe.

EXPERIMENTAL SECTION

For cross-validation, both DNS-6His and 6His were used as probes and were prepared with purity over 98% via solid synthesis by Beijing Sci-light Biotechnology Co. Ltd. (China; http://www.scilight-peptide.com/pro.aspx?CateId=58&ProductsCateId=58&FCateId=12). Mouse mcAb-6His (cat no. Abm-00114) was from Nanjing Zhongding Biotechnology Co., Ltd. (Nanjing, China; http://www.zoonbio.com/). Mouse mcAb against penicillin G (mcAb-PNG) was from Abcam (ab15070, Cambridge, England). By the providers, quantities of mcAbs were determined by absorbance at 280 nm (Note S1, Supporting Information).

Molecular weight of binding site of mcAb-6His was 80.0 kDa to derive its molar quantities from protein contents as stated by the provider. For comparison, protein content was also determined by the Bradford method with bovine serum albumin (BSA) as the reference.[13] To record titration curve, reaction buffer was 10.0 mM sodium phosphate at pH 7.0 and filtered with 0.20 μm membrane. With reaction buffer, mcAb-6His was diluted to 0.1 g/L according to protein content stated by the provider and kept at 2−6 °C during analysis in 2 days. Each probe was dissolved in reaction buffer at no less than 25 μM (content of DNS-6His was calibrated by absorbance at 330 nm[14]) and kept in dark prior to use.

Agilent Cary Eclipse fluorescence spectrophotometer was utilized with slit widths of 10 nm for excitation and emission, unless otherwise stated. The initial volume of reaction mixture was 2.0 mL in a 4.0 mL quartz cell containing mcAb-6His at an indicated level below 0.23 μM. The concentration of each probe was 10-fold of that of mcAb-6His calculated from protein content as stated by the provider. Each time, 5 to 10 μL of solution of a titration probe was added to solutions of a mcAb in the cell and mixed gently. Under the excitation at 280 nm, fluorescence of each solution was recorded at 340 and 540 nm concomitantly each time with DNS-6His as the probe, while at 340 nm alone with 6His or penicillin G as the probe, after reaction for 5.0 min since the addition of a probe. Effects of dilution and signals of the buffer were corrected. Titration curves were determined in duplicate at least. Curve-fitting-tool in Matlab 7.11 was used to fit titration curves (Note S2, Supporting Information). Coefficient of variation (CV) was derived from mean and standard deviation.

RESULTS AND DISCUSSION

Theoretical Consideration. For a titration curve of a mcAb with any probe, it is common to estimate the intersection point of one asymptote for probe levels (x) approaching zero and the other for x approaching infinite as an approximate of x for 1:1 binding of the probe to the mcAb. Inevitably, this approximation approach requires tiny differences in x at the beginning, saturation binding at the largest x, steady signals at probe levels in great excess, strong affinity, and high solubility of the probe. Unfortunately, the following factors devalue the approximation approach. The asymptote for x approaching zero usually suffers larger uncertainty. A probe in great excess may cause a dynamic quench. Limited affinity and/or solubility of a probe prevent saturation binding at the achievable largest x. A probe may have some signals under the excitation at 280 nm. Contaminated proteins/substances in a mcAb may give background fluorescence. Therefore, fitting of a rational model to a titration curve is much favored for the estimation of molar quantity of binding site of a mcAb recognizing the titration probe.

Considering all the factors mentioned above, components involved in titration and their fluorescence signals are presented in Scheme 1. Assign (a) background fluorescence of contaminated nonantibody proteins, unwanted antibodies, and other substances to F_b, (b) the initial concentration of a mcAb in a titration mixture to C_0, (c) the total concentration of a titration probe to x, (d) the dissociation constant of their complexes to K_d, (e) the concentration of the complex to C_x, (f) the slopes for linear responses of fluorescence to molar quantities to S_1, S_2, and S_3 for the free mcAb, its complexes with the probe, and the free probe, respectively, (g) K_{sv} to Stern−Volmer constant for dynamic quench. Accordingly, there are eq 1 and thus eq 2 based on 1:1 binding of a titration probe to

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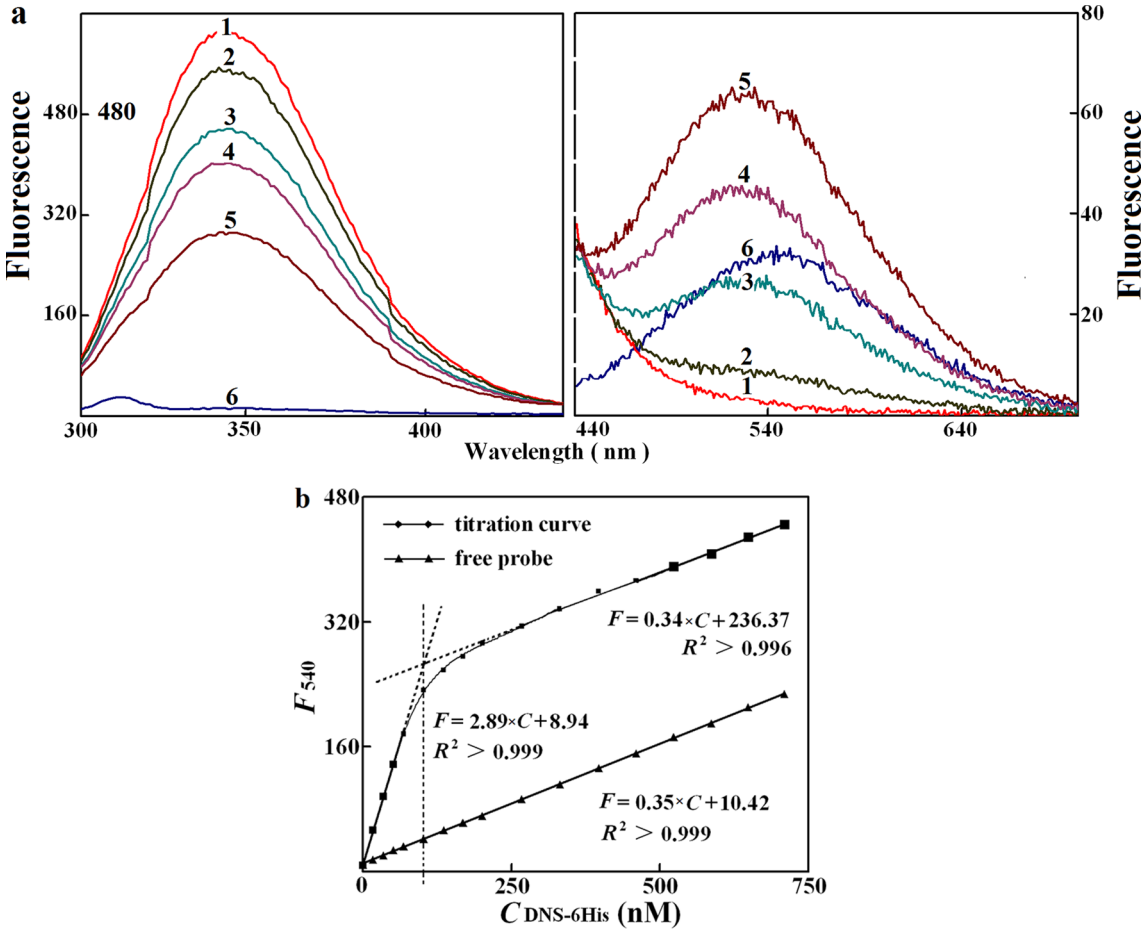

Figure 1. Titration of mcAb-6His by DNS-6His. (a) Emission spectra of complexes of mcAb-6His and DNS-6His. 1 mcAb-6His alone at 225 nM; 2 mcAb-6His +17 nM DNS-6His; 3 mcAb-6His +68 nM DNS-6His; 4 mcAb-6His +168 nM DNS-6His; 5 mcAb-6His +709 nM DNS-6His; 6 DNS-6His 709 nM alone. (b) Titration curve of mcAb-6His recorded as fluorescence at 540 nm under the excitation at 280 nm.

each binding site of a mcAb, regardless of the recording of a titration curve as fluorescence of the mcAb at 340 nm or fluorescence specific for a FRET probe.

$$C_x = \frac{(C_0 + x + K_d) - \sqrt{(C_0 + x + K_d)^2 - 4 \times C_0 \times x}}{2} \quad (1)$$

$$F = \frac{S_1 \times (C_0 - C_x)}{1 + Ksv \times (x - C_x)} + \frac{S_2 \times C_x}{1 + Ksv \times (x - C_x)} + S_3 \times (x - C_x) + \frac{F_b}{1 + Ksv \times (x - C_x)} \quad (2)$$

To simplify fitting of eq 2 to the titration curve, S_3 for either type of probes is determined experimentally. As for titration curves recorded as fluorescence specific for FRET probes, free mcAbs usually have negligible fluorescence at the detection wavelengths and thus S_1 is negligible to simplify fitting to titration curves. With titration curves recorded as fluorescence at 340 nm, however, the initial signals are originated from signals of the mcAb and other components; i.e., they are equal to $S_1 \times C_0 + F_b$. This fact indicates covariance among C_0, F_b, and S_1 and challenges fitting of eq 2 to titration curves recorded as fluorescence at 340 nm. Hence, the fitting of eq 2 to titration curves recorded as fluorescence specific for FRET probes may be easy, whereas fitting of eq 2 to titration curves recorded as fluorescence at 340 nm may encounter the challenge to eliminate covariance among C_0, F_b, and S_1.

Titration Curve of mcAb-6His Recorded as Fluorescence at 540 nm for DNS-6His. The mcAb-6His under analysis was rich in tryptophan residues and had strong affinity for DNS-6His (DataS1, Supporting Information). Under the excitation at 280 nm of reaction mixtures of mcAb-6His and DNS-6His, fluorescence at 340 nm decreased while fluorescence at 540 nm increased, versus increasing quantities of DNS-6His (Figure 1a,b). More importantly, more than 70% of fluorescence at 340 nm was quenched by DNS-6His in great excess, supporting high abundance of tryptophan residues in the vicinity of the binding site of mcAb-6His for 6His. When mcAb-6His was about 5-fold of DNS-6His, free probe should be negligible; quantum yield of the probe in the titration mixture was about 1.6-fold of that of free probe, consistent with those observed before.[8,11,12] However, fluorescence of the titration mixture (complexes) at 540 nm was nearly 8-fold of that of free DNS-6His at the same levels under the excitation at 280 nm, supporting strong FRET in the complexes.[6−9] Moreover, titration curves as fluorescence at 540 nm indicated saturation binding of mcAb-6His by DNS-6His at tested levels (Figures 1b and 2) and the rationality of the approximation approach.

By the excitation at 280 nm to record fluorescence at 540 nm, mcAb-6His had negligible signals and thus S_1 was zero. With S_1 of zero for fitting of eq 2 to titration curves of mcAb-

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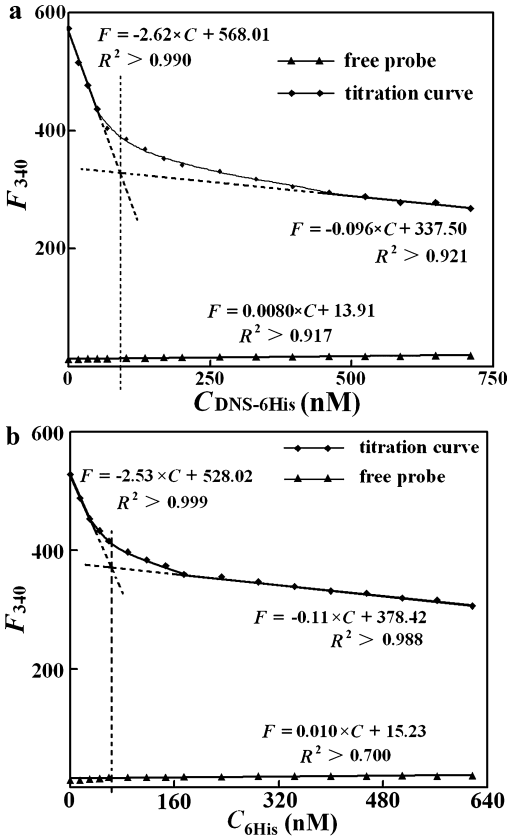

Figure 2. Fluorometric titration of mcAb-6His by fluorescence at 340 nm under the excitation at 280 nm. (a) mcAb-6His at 225 nM by DNS-6His; (b) mcAb-6His at 225 nM by 6His.

6His by DNS-6His recorded as fluorescence at 540 nm, C_0 and K_d were easily estimated with determination coefficient (R^2) over 0.96. By the fluorometric titration approach, C_0 showed CVs below 10% but accounted for about 50% of that calculated from protein quantities determined by absorbance at 280 nm (Table 1; DataS1, Supporting Information). Interestingly, protein quantity in stock solution of mcAb-6His by the Bradford method was just half of that by absorbance at 280 nm (DataS2, Supporting Information). In fact, C_0 of mcAb-6His by the titration approach over a wide range showed good consistency with that derived from protein quantity determined by the Bradford method (DataS2, Supporting Information).

On the other hand, the asymptote for x approaching zero showed much larger slope than that of the asymptote for x approaching saturation binding or that of the free FRET probe (Figure 1b), supporting it was practical to estimate C_0 by the approximation approach. Importantly, C_0 of mcAb-6His by the titration approach displayed smaller CVs and good consistency with those by the approximation approach (Table 1). Albumin is a common auxiliary protein in preparations of antibodies but possesses strong binding of common hydrophobic substances. DNS-6His showed binding to human or bovine serum albumin while negligible binding to antipenicillin mcAb (mcAb-PNG), supporting DNS-6His had strong specificity for mcAb-6His over other antibodies. K_d of DNS-6His to mcAb-6His at tested levels was at nanomolar levels and showed moderate CV (DataS1, Supporting Information); this strong affinity should account for consistency between the slope of the asymptote for x approaching saturation binding and that for the free DNS-6His (Figure 1b). Hence, the proposed titration approach by fitting of eq 2 to titration curves of mcAbs recorded as fluorescence specific for the RFET probes was practical and may even work to screen mcAbs in a library.

Titration Curves Recorded as Fluorescence of mcAb at 340 nm. As detected by sodium-dodecylsulfate-polyacrylamide gel electrophoresis, the tested mcAb-6His and mcAb-PNG had negligible non-mcAb proteins (Note S3, Supporting Information), but F_b at 340 nm for other components still had to be considered. In this case, however, fitting of eq 2 to all titration curves at tested levels of mcAb-6His recorded as fluorescence at 340 nm with 6His or DNS-6His did not converge even if R^2 was over 0.9992 (DataS1 and DataS3, Supporting Information). Specially, the change of lower boundaries of C_0 for fitting greatly altered outputs. As expected, there was close covariance among C_0, S_1 and F_b (Figure 3) and

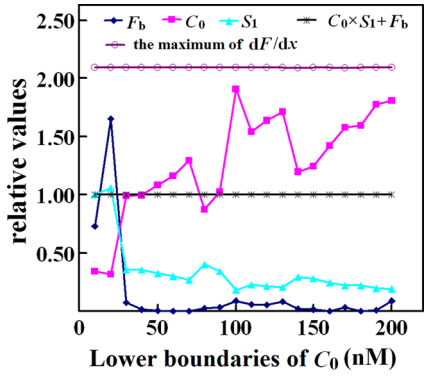

Figure 3. Relative variations of parameters versus lower boundaries of C_0 during fitting of eq 2 to the titration curve in Figure 2b with a fixed upper boundary of C_0 at 400 nM. All parameters were relative values to their means except the maxima of dF/dx were reduced by 50%.

Table 1. Fluorometric Titration of Two mcAbs with Different Probes and Fluorescence Signals[a]

mcAb	probe/ fluorescence	S_1	titrated[b] for 75 nM	approximated[c] for 75 nM	S_1	titrated[b] for 150 nM	approximated[c] for 150 nM	S_1	titrated[b] for 225 nM	approximated[c] for 225 nM
mcAb-6His (n = 3)	6His/F_{340}	5.8	29.7 ± 1.8	28.6 ± 3.1	4.7	77.4 ± 2.0	54.5 ± 3.9[d]	4.2	119.3 ± 0.8	72.0 ± 9.2[d]
	DNS-6His/ F_{340}	7.0	27.7 ± 2.7	28.8 ± 0.8	7.0	54.4 ± 3.6	54.8 ± 6.4	5.2	107.7 ± 4.7	92.0 ± 5.8[d]
	DNS-6His/ F_{540}	ND	27.9 ± 2.2	32.4 ± 5.7	NA	56.3 ± 5.3	58.6 ± 5.0	ND	93.9 ± 4.1	105.3 ± 24.0
mcAb-PNG (n = 2)	penicillin/ F_{340}	2.1	78.0 ± 2.2	17.9 ± 2.0[d]	2.3	144.4 ± 5.3	33.4 ± 4.5	ND	ND	ND

[a] F_{340} and F_{340} were fluorescence at 340 and 540 nm, respectively. S_1 was the mean with CV below 5%. ND: no determination. [b] By the fluorometric titration approach. [c] By approximation of the intersection point of two asymptotes. [d] Significant difference at $P < 0.01$.

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close covariance between F_b and K_{sv} (DataS1 and DataS3, Supporting Information). In detail, to all titration curves of mcAb-6His recorded as fluorescence at 340 nm with DNS-6His or 6His, fitting of eq 2 always gave $(C_0 \times S_1 + F_b)$ bearing CV below 0.02% for increasing lower boundaries of C_0 and good consistency with the initial signals, even estimated F_b showed variations as large as 10-fold. Hence, S_1 had to be estimated separately for fitting of eq 2 to titration curves recorded as fluorescence at 340 nm, while F_b was needed if there were fluorescent components other than the mcAb of interest.

To estimate S_1 experimentally with mcAbs, there should be negligible F_b and thus this fluorometric titration approach is limited to purified mcAbs that are free of other components emitting at 340 nm under the excitation at 280 nm. In practice, when a mcAb has sufficient homogenicity, unwanted antibodies can be negligible; nonantibody proteins can be removed by affinity-purification with protein A. Namely, there can be a F_b of zero for fitting of eq 2 to titration curve of a purified mcAb. In this case, S_1 can be estimated by experiments, regardless of cost and time. In fact, molar quantity of mcAb in any preparation is unknown and experimental estimation of S_1 is still impractical. Fortunately, S_1 can be aprroximated via a special strategy at no additional cost as follows.

The maximum of absolute values of dF/dx loalized at x approaching zero with any group of parameters for best-fitting of eq 2 to a titration curve recorded as fluorescence at 340 nm (Note S2, Supporting Information). At x approaching zero, a dynamic quench should be negligible and C_x can be approximated as x in eq 2 to give eq 3 as long as

$$\lim_{x\to 0} F \approx S_1 \times (C_0 - x) + S_2 \times x \tag{3}$$

the probe has sufficient affinity for the mcAb. Moreover, for the quench of fluorescence of a mcAb at 340 nm, S_1 is larger than S_2. Hence, there are eqs 4 and 5 to support

$$\lim_{x\to 0} F \approx S_1 \times (C_0 - x) \tag{4}$$

$$\lim_{x\to 0} (dF/dx) \approx -S_1 \tag{5}$$

the approximation of S_1 as the maximum of absolute values of first-order derivatives for the best-fitting of eq 2 to a titration curve. Different from $(C_0 \times S_1 + F_b)$, the maxima of absolute values of the first-order derivatives for the best-fitting of eq 2 to any titration curve recorded as fluorescence at 340 nm with 6His or DNS-6His as the probe showed no dependence on lower boundaries of C_0, regardless of the convergence of the fitting (Figure 3). These facts supported the validity of eq 5. However, no estimation of F_b is accessible. Hence, the proposed titration approach with titration curve recorded as fluorescence at 340 nm was limited to purified mcAbs of interest.

There was convergence for the consecutive fitting of eq 2 to any of the same titration curves of mcAb-6His recorded as fluorescence at 340 nm with the approximated S_1 and experimental S_3 plus F_b of zero. The estimation of C_0 from titration curves recorded as fluorescence at 340 nm showed strong resistance to ranges of probe levels for analysis (DataS1, Supporting Information). In general, if data under analysis covered more than 50% of the quenchable fluorescence at 340 nm of mcAb-6His, the estimated C_0 showed good consistency (DataS1, Supporting Information). More importantly, with DNS-6His as the FRET probe, analyses of titration curves recorded as fluorescence at 340 nm and those concomitantly recorded as fluorescence at 540 nm gave consistent C_0; such values of C_0 for the same quantities of mcAb-6His were consistent with those estimated with 6His to record titration curves as fluorescence at 340 nm (Table 1). These consistent results cross-validated the proposed titration approach based on curve-fitting. The overall CVs of all C_0 estimated thereby for mcAb-6His with two types of probes were below 10%, supporting good precision of the proposed titration approach. With the same titration curves recorded as fluorescence at 340 nm, however, the approximation approach gave much smaller C_0 or much larger CVs of C_0 for mcAb-6His (Table 1). This difference supported the advantage of fitting of eq 2 to titration curves for the estimation of C_0. On the other hand, the titration curve of mcAb-PNG by penicillin G itself recorded as fluorescence at 340 nm gave C_0 consistent with that by either the Bradford method or absorbance at 280 nm (DataS4, Supporting Information), supporting universal applicability of the titration approach with haptens nonfluorescent at 340 nm as titration probes. Unfortunately, fitting of eq 2 to titration curves as fluorescence at 340 nm gave unreliable K_d (DataS1, Supporting Information) and thus was unsuitable for screening of mcAbs in a library.

In theory, larger C_0 and stronger affinity of a probe gave higher validity of eq 3 while stronger quenching efficiency of titration probes enhanced validity of eq 4. In general, dansylated compounds of higher hydrophobicity have stronger affinities to proteins; the combination of the formation of complexes and FRET enhances quenching efficiency.[6−9] These properties can account for approximated S_1 with DNS-6His larger than those with 6His for the same levels of C_0 (Table 1). By recording titration curves of mcAb-6His as fluorescence at 340 and 540 nm with DNS-6His and 6His as probes, however, C_0 of mcAb-6His was consistent and accounted for just about 50%, while that of mcAb-PNG titrated with penicillin G accounted for nearly 95% (DataS1, Supporting Information) of those derived from protein quantities determined by absorbance at 280 nm. Unusually, C_0 of mcAb-6His via fitting of eq 2 to such titration curves showed consistency with that derived from protein contents determined by the Bradford method. Moreover, the average of S_1 for fluorescence at 340 nm of mcAb-6His with DNS-6His and 6His as probes was more than twice of that of mcAb-PNG under titration by penicillin G (Table 1), supporting higher abundance of the tryptophan residues nearby binding site of mcAb-6His. Hence, the proposed titration approach was resistant to variations of abundance of tryptophan residues in mcAbs and probe affinities and suited for calibration of quantities of purified mcAbs as long as their titration probes were accessible.

■ CONCLUSION

Quantities of binding site of purified mcAbs were estimated via nonlinear fitting of a universal model to fluorometric titration curves under the excitation at 280 nm. The titration probes included nonfluorescent haptens/epitopes, their conjugates with FRET acceptors of tryptophan residues bearing excitation valleys at 280 nm, or fluorescent haptens with excitation peaks nearby 340 nm and excitation valleys nearby 280 nm. The fluorometric titration approach was resistant to variations in abundance of tryptophan residues in mcAbs and affinities of probes. It was promising for *de novo* calibration of quantities of purified mcAbs and estimation of their absorptivity at 280 nm,

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preparation of reference materials of IgGs/IgMs, and the trace of quantities of mcAbs in bioanalyses.

■ ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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