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Homogeneous competitive assay of ligand affinities based on quenching fluorescence of tyrosine/tryptophan residues in a protein *via* Fŏrster-resonance-energy-transfer

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ABSTRACT

A new homogeneous competitive assay of ligand affinities was proposed based on quenching the fluorescence of tryptophan/tyrosine residues in a protein *via* Fŏrster-resonance-energy-transfer using a fluorescent reference ligand as the acceptor. Under excitation around 280 nm, the fluorescence of a protein or a bound acceptor was monitored upon competitive binding against a nonfluorescent candidate ligand. Chemometrics for deriving the binding ratio of the acceptor with either fluorescence signal was discussed; the dissociation constant (K_d) of a nonfluorescent candidate ligand was calculated from its concentration to displace 50% binding of the acceptor. N-biotinyl-N′-(1-naphthyl)-ethylenediamine (BNEDA) and N-biotinyl-N′-dansyl-ethylenediamine (BDEDA) were used as the reference ligands and acceptors to streptavidin to test this new homogeneous competitive assay. Upon binding of an acceptor to streptavidin, there were the quench of streptavidin fluorescence at 340 nm and the characteristic fluorescence at 430 nm for BNEDA or at 525 nm for BDEDA. K_d of BNEDA and BDEDA was obtained *via* competitive binding against biotin. By quantifying BNEDA fluorescence, K_d of each tested nonfluorescent biotin derivative was consistent with that by quantifying streptavidin fluorescence using BNEDA or BDEDA as the acceptor. The overall coefficients of variation were about 10%. Therefore, this homogeneous competitive assay was effective and promising to high-throughput-screening.

1. Introduction

Ligands of proteins play important roles in biomedicine and their affinities to proteins, *i.e.*, dissociation constants of the complexes between proteins and ligands, primarily determine their biomedical significance. To determine the affinity of a ligand to a protein, common methods employ competitive binding of the ligand against a reference ligand labeled with radio-active isotopes or fluorophores to facilitate selective quantification of the complexes in reaction mixtures. In general, competitive assays of ligand affinities include the heterogeneous method that separates the complexes from reaction solutions before quantification, and the homogenous method that quantifies the complexes directly in reaction solutions without any separation process. In practice, homogenous competitive assays of ligand affinities are favored for the efficiency, cost and suitability for high-throughput-screening (HTS) of ligands in a library, and they are realized principally with fluorescent reference ligands in either of the following two ways [1–7]. The first way measures changes of fluorescence polarization [1,7–13], the second way measures changes of fluorescence due to Fŏrster-resonance-energy-transfer (FRET) [2–6,14–18], upon the binding of a fluorescent reference ligand to a protein. Usually, the FRET-based homogenous competitive assay is preferable for its sensitivity, cost and no dependence on specialized instrumental items.

Nowadays, in classical FRET-based homogenous competitive assay of ligand affinities, amino acid residue(s) on a protein is/are

Abbreviations: SAV, streptavidin; BBZA, N-biotinyl-benzylamine; BME, methyl biotinyl ester; BDEDA, N-biotinyl-N′-dansyl-ethylenediamine; BETA, N-biotinyl-ethanolamine; BDETA, N-biotinyl-diethanolamine; NHS, N-hydroxylsuccinamide (NHS); NHS-Biotin, N-hydroxylsuccinamide biotinyl ester; BCHA, N-biotinyl-cyclohexylamine; BNEDA, N-biotinyl-N-(1-naphthyl)-ethylenediamine; BMPL, N-biotinyl-morpholine; *BR*, the binding ratio of the reference ligand; EC_{50}, the concentration of a candidate ligand for 50% binding of a reference ligand; HTS, high-throughput-screening.

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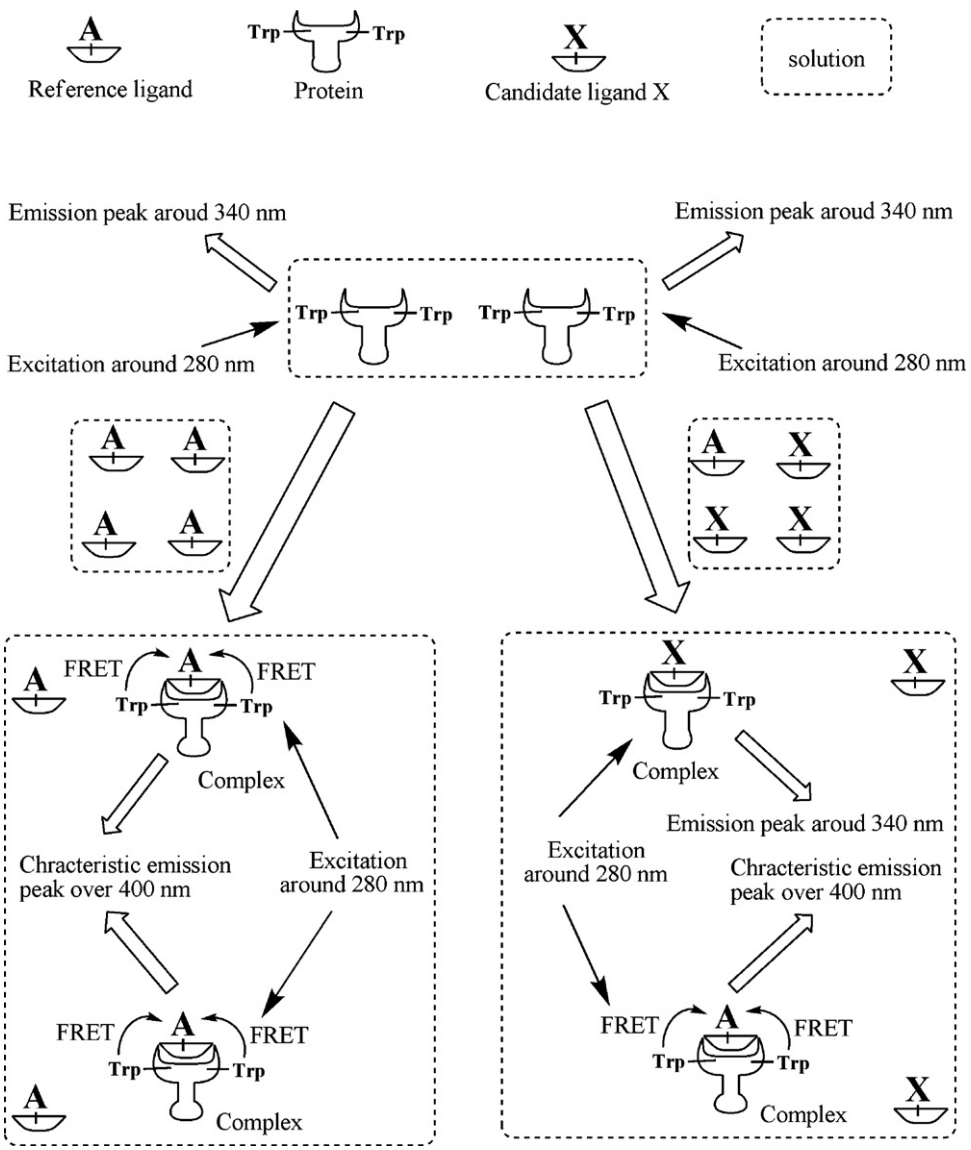


Scheme 1. Principle of this new homogeneous competitive assay of ligand affinity.

labeled with a suitable fluorophore to act as acceptor(s) (donor(s)) while a suitable fluorescent reference ligand acts as the donor (acceptor); in the complexes, the distance between the acceptor(s) and the donor(s) is so short that FRET is greatly enhanced to quench fluorescence of the donor(s) and to produce the characteristic fluorescence of the acceptor(s) [2–6,14–18]. In practice, however, this classical homogenous competitive assay of ligand affinities for HTS tolerates the cost and time for labeling proteins, potential alteration of protein functions by labeling and too many false positive results.

Surely, a FRET-based homogenous competitive assay of ligand affinities is more desirable if the labeling of a protein with a fluorophore is not required so that there will be neither cost/time for the labeling nor the potential alteration on protein functions. In fact, proteins of reasonable sizes contain tyrosine/tryptophan residues that account for the intrinsic fluorescence of cofactorless proteins [6]. In theory, tyrosine/tryptophan residues in a protein can act as intrinsic donors and FRET is expected as long as a suitable fluorescent reference ligand as the FRET acceptor is bound by the protein. Thus, a new FRET-based homogeneous competitive assay of ligand affinities can be developed by measuring the characteristic fluorescence emission of the bound FRET acceptor or the quenched fluorescence of the protein (Scheme 1). In this new homogeneous competitive assay, there is/are neither cost/time for labeling proteins nor problems associated with labeling, and conventional fluorospectrometers can be used to measure steady-state fluorescence with ease.

The quench of fluorescence of tryptophan residues in proteins *via* FRET is in common use to study protein conformation [19–26], but is only occasionally used to quantify ligands [27] and estimate affinities of fluorescent ligands *via* titration rather than by competitive binding [28]. As far as we knew, homogeneous competitive assay of affinities of nonfluorescent ligands to proteins using suitable fluorescent reference ligands as the FRET acceptors and tyrosine/tryptophan residues as intrinsic donors has not been reported. During competitive binding, the concentration of a candidate ligand to displace 50% binding of a reference ligand (EC_{50}) is usually estimated to derive its dissociation constant [29,30]. The binding of biotin derivatives to streptavidin (SAV) is widely used in bioaffinity recognition, and convenient methods to estimate affinities of biotin derivatives are needed [31–33]. The guideline to design fluorescent ligands as FRET acceptors to tyrosine/tryptophan residues in proteins as the intrinsic donors is proposed [34,35], and it is easier to design fluorescent biotin deriva-

tives as the FRET acceptor to SAV [35–38]. In this report, therefore, we discussed chemometrics to derive EC_{50} of nonfluorescent candidate ligands with this homogeneous competitive assay, and two fluorescent biotin derivatives were used as reference ligands and acceptors to test this homogeneous competitive assay by cross-validation.

2. Materials and methods

2.1. Chemicals

D-Biotin was from Biosci Basic Inc. Streptavidin (SAV) was from Promega (Z7041). N-Hydroxylsuccinamide (NHS), dansyl chloride, dicyclohexylcarbodiimide (DCC), and N-(1-naphthyl)-ethylenediamine (NEDA) were from Alfa Aesar. Morpholine, cyclohexylamine, benzylamine, ethanolamine, diethanolamine and other chemicals were domestic analytical products. Water was restilled before use.

2.2. Instruments

A Shimadzu RF5301PC fluorospectrometer was used to record the spectra with the excitation slit at 5 nm and the emission slit at 10 nm. To estimate ligand affinities, a Shimadzu RF540 fluorospectrometer was routinely used to measure the fluorescence with both the excitation slit and the emission slit at 10 nm, unless otherwise stated. API QSTAR LC-Q-TOF was used for electro-spray-ionization high-resolution-mass-spectrometry (ESI-HRMS) to determine formula composition, and DRX 500 M Hz NMR spectrometer was used to record NMR data (Kunming Institute of Botany, Chinese Academy of Sciences, Yunnan 650224).

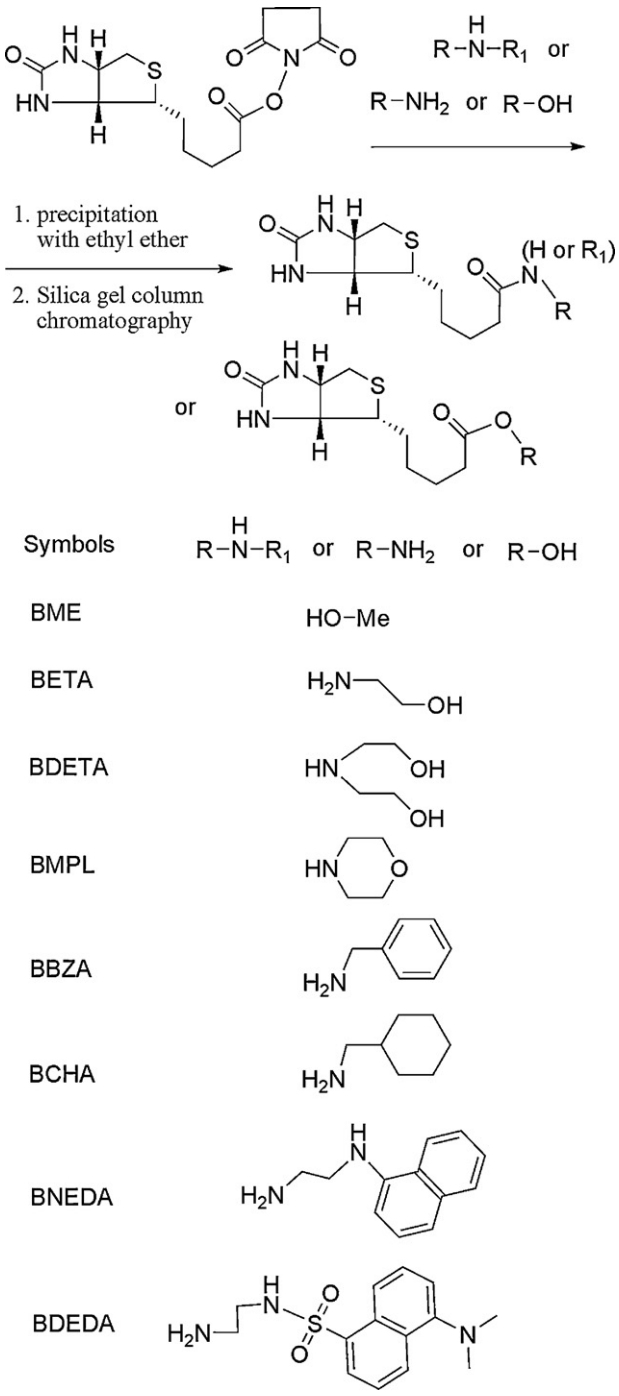
2.3. Experimental procedures

2.3.1. Syntheses of compounds and structural analyses

N-Hydroxylsuccinamide biotinyl ester (NHS-Biotin) was prepared and purified as before [35]. Mono-dansylated ethylenediamine (DEDA) was prepared *via* the reaction of ethylenediamine in great excess with dansyl chloride (30:1) in dichloromethane, washed repeatedly with large volumes of water and 0.3% HCl, followed by purification *via* silica gel chromatography. Reaction of NHS-biotin with each indicated alkyl amine in dichloromethane at room temperature for 24 h gave the desired biotinyl amide (Scheme 2); each intended biotinyl amide, after the removal of most solvents under reduced pressure at 40 °C, was precipitated and washed with ethyl ether, purified by silica gel chromatography *via* the elution with 7% methanol in chloroform. Methyl biotin ester (BME) was prepared with NHS-biotin in methanol at 40 °C for 72 h, followed by removal of methanol, wash with ethyl ether and column chromatography on silica gel. From each column, eluted fractions without contaminants detectable by TLC were pooled, and solvents were removed to give each biotin derivative. Formula compositions were analyzed by ESI-HRMS, and NMR data in DMSO-*d*6 were collected at 25 °C (Note S1, Supplementary Material).

2.3.2. Binding reaction and measurement of fluorescence

BNEDA was calibrated with its absorptivity of 5.3 mM^{-1} cm^{-1} at 325 nm, and BDEDA was determined with its absorptivity of 4.4 mM^{-1} cm^{-1} at 338 nm (Supplementary Material). A stock solution of biotin at 20.0 mM was made in dimethylsulfone and stock solutions of other biotin derivatives at 4.0 mM were prepared in methanol. The amount of binding sites on SAV was titrated with BNEDA and the buffer was 0.10 M sodium phosphate at pH 7.0 as before [35]. Reaction mixtures in total of 3.0 mL at 25 °C contained 100 μL BNEDA (or BDEDA) diluted by the buffer, 100 μL SAV solution and 200 μL solution of biotin (derivative) diluted by the buffer.



Scheme 2. Syntheses and structures of the used biotin derivatives.

As for BNEDA, the excitation was realized at 280 nm to measure the fluorescence of the bound BNEDA at 430 nm or SAV at 340 nm. As for BDEDA, the excitation was made at 280 nm to measure SAV fluorescence at 340 nm, but at 285 nm to measure the fluorescence of the bound BDEDA at 525 nm (the half-frequency scattering of the excitation light was reduced). With BNEDA varying from 0.18 μM to 0.54 μM as indicated, the final levels of SAV ranged from 0.10 μM to 0.30 μM to ensure competitive binding, correspondingly. With BDEDA at 0.50 μM as the reference ligand, the final level of SAV was 0.30 μM, unless otherwise stated. The final level of biotin was below 60.0 μM and that of any biotin derivative was below 12.0 μM to give final contents of organic solvents below 0.4% for negligible interference with fluorescence of components in reaction mixtures. The

binding reaction was initiated by the addition of SAV. Three minutes after the addition of SAV, the fluorescence of reaction mixtures was measured within 5 min. Dissociation constants of BDEDA was estimated *via* competitive binding against biotin [31,35].

2.4. Data processing

Details to approximate binding ratio (*BR*) of any fluorescent reference ligand as the FRET acceptor and to estimate the dissociation constant (K_{dX}) of any nonfluorescent candidate ligand were given in Section 3. Experimental results were in mean ± standard deviation (SD). Comparison was made by Student's *t*-test with $P < 0.05$ for significant difference.

3. Chemometrics for calculating ligand affinities

To simplify the approximation of the binding ratio of a reference ligand, the background fluorescence is corrected for a negligible intercept of linear response with each interested component in the following reaction mixtures. For reaction mixtures containing a protein at a constant level indexed as its binding site(s) (C_{TProt}), a fluorescent reference ligand at a constant level (C_{TRef}) and a candidate ligand at varying levels for competitive binding, the fluorescence (F_X) is the sum of those from interested components including the complex of the protein with the reference ligand (F_{BRef}), the complex of the protein with the candidate ligand (F_{BCan}), the free reference ligand (F_{FRef}), the free protein (F_{FProt}) and the free candidate ligand (F_{FCan}). Obviously, to ensure competitive binding, C_{TRef} should be reasonably larger than C_{TProt}, and Eq. (1) applies as long as F_X is within its linear range and there are no interactions among aforementioned interested components to quench their fluorescence each other.

$$F_X = F_{BCan} + F_{BRef} + F_{FRef} + F_{FProt} + F_{FCan} \quad (1)$$

Assume that the fluorescence of each interested component responds linearly to its concentrations with a negligible intercept. Namely, F_{BRef}, F_{BCan}, F_{FRef}, F_{FProt} and F_{FCan} are directly proportional to their concentrations (C_{BRef}, C_{BCan}, C_{FRef}, C_{FProt} and C_{FCan}, respectively) with slopes of S_{BRef}, S_{BCan}, S_{FRef}, S_{FProt} and S_{FCan}, correspondingly. Therefore, Eq. (2) applies.

$$FX = S_{BCan}\ C_{BCan} + S_{BRef}\, C_{BRef} + S_{FRef}\, C_{FRef} + S_{FProt}\, C_{FProt} + S_{FCan}\, C_{FCan} \quad (2)$$

Upon the excitation around 280 nm, both protein fluorescence around 340 nm and the characteristic fluorescence of the bound reference ligand will be altered *via* FRET during competitive binding, and *BR* is approximated with different fluorescence signals as follows.

3.1. Approximation of BR from protein fluorescence

With protein fluorescence around 340 nm to approximate *BR*, nonfluorescent candidate ligands of asymmetric structures and any reference ligand within limited concentration ranges should have negligible fluorescence at 340 nm. If the concentration of the free protein, C_{FProt}, is negligible with respect to C_{TProt} during competitive binding, the contribution of F_{FProt} to F_X could be neglected. Assigning the dissociation constant of the reference ligand to K_{dR}, there is Eq. (3).

$$C_{FProt} = \frac{C_{BRef}}{C_{FRef}} \times K_{dR} \le \frac{C_{TProt}}{C_{TRef} - C_{TProt}} \times K_{dR} \quad (3)$$

If C_{TRef} minus C_{TProt} is 50 times higher than K_{dR}, C_{FProt} is negligible with respect to C_{TProt} so that the contribution of F_{FProt} to F_X in Eq. (1) is negligible. Namely, Eq. (4) should apply.

$$\frac{C_{FProt}}{C_{TProt}} \le \frac{K_{dR}}{C_{TRef} - C_{TProt}} \le 0.02 \quad (4)$$

C_{TRef} is usually restricted within 5 μM for the linear response of F_X. Consequently, Eq. (4) can be easily validated with K_{dR} of a fluorescent reference ligand below 0.1 μM and C_{TProt} at about 0.1 μM. With K_{dR} of a fluorescent reference ligand over 0.1 μM, the contribution of F_{FProt} should be included to approximate *BR* with inevitable complexity. With negligible F_{FProt}, the fluorescence of reaction mixture at 340 nm under the excitation at 280 nm is approximated as Eq. (5).

$$F_X \approx S_{BCan}\ C_{BCan} + S_{BRef}\, C_{BRef} \quad (5)$$

The sum of C_{BRef} and C_{BCan} is equal to C_{TProt} if C_{FProt} is negligible, and thus Eq. (6) applies.

$$S_{BCan}\ C_{TProt} - F_X \approx (S_{BCan} - S_{BRef}) C_{BRef} \quad (6)$$

Assigning $S_{BRef}\, C_{TProt}$ to F_{min}, which accounts for the fluorescence of all the protein that is bound by the reference ligand, and $S_{BCan}\, C_{TProt}$ to F_{max}, which accounts for the fluorescence of all the protein that is bound by the candidate ligand, there is Eq. (7).

$$C_{BRef} \approx \frac{F_{max} - F_X}{S_{BCan} - S_{BRef}} \quad (7)$$

For the maximal binding of the reference ligand to the protein, C_{BRef} can be taken as C_{TProt}. With *BR* defined as the percentage of C_{BRef} to C_{TProt}, Eq. (8) applies.

$$BR = \frac{C_{BRef}}{C_{TProt}} \approx \frac{F_{max} - F_X}{C_{TProt}(S_{BCan} - S_{BRef})} = \frac{F_{max} - F_X}{F_{max} - F_{min}} \quad (8)$$

F_{min} and F_{max} can be determined *via* titration, or approximated as follows as long as F_{FProt} is negligible. (a) F_{max} can be approximated as the fluorescence of a reaction mixture containing the protein at C_{TProt} and a candidate ligand at a suitable level to bind all the protein as long as the candidate ligand in excess does not affect F_{max}. (b) F_{min} can be approximated as the fluorescence of a reaction mixture containing the protein at C_{TProt} and the reference ligand at a suitable level to bind all the protein as long as the reference ligand in excess doesn't affect F_{min}.

3.2. Approximation of BR from fluorescence of a reference ligand

With any suitable reference ligand that has large enough Stokes shift as the FRET acceptor when tyrosine/tryptophan residues act as the donors, the protein by the excitation around 280 nm should have negligible fluorescence at the wavelength to quantify the characteristic fluorescence of the bound reference ligand. At such a detection wavelength under excitation around 280 nm, free or bound nonfluorescent candidate ligands should also have negligible signals. Therefore, by quantifying the fluorescence of the bound reference ligand upon the excitation around 280 nm, F_X principally originates from the bound and the free reference ligand as described in Eq. (9).

$$F_X \approx S_{BRef}\ C_{BRef} + S_{FRef}\, C_{FRef} \quad (9)$$

Eq. (9) is independent on K_{dR}. Assigning $S_{FRef}\, C_{TRef}$ to F_{min} and $S_{BRef}\, C_{TProt}$ to F_{max}, Eq. (10) applies because C_{TRef} is a constant [35]. Consequently, Eq. (11) applies.

$$F_X - F_{min} \approx (S_{BRef} - S_{FRef}) C_{BRef} \quad (10)$$

$$C_{BRef} \approx \frac{F_X - F_{min}}{S_{BRef} - S_{FRef}} \quad (11)$$

Similar to the derivation of *BR* from protein fluorescence at 340 nm, there is Eq. (12) from the fluorescence of the bound reference ligand under the excitation around 280 nm.

$$BR = \frac{C_{\mathrm{BRef}}}{C_{\mathrm{TProt}}} \approx \frac{F_{\mathrm{X}} - F_{\mathrm{min}}}{(S_{\mathrm{BRef}} - S_{\mathrm{FRef}})C_{\mathrm{TProt}}} = \frac{F_{\mathrm{X}} - F_{\mathrm{min}}}{F_{\mathrm{max}} - S_{\mathrm{FRef}}\ C_{\mathrm{TProt}}} \quad (12)$$

F_{min} for the reference ligand at C_{TRef} is directly determined by experimentation. F_{max} with the protein at C_{TProt} is preferred to be determined *via* titration with the reference ligand. With a reference ligand of K_{dR} below nanomolar, F_{max} can be approximated as the fluorescence of a reaction mixture containing the protein at C_{TProt} and the reference ligand in slight excess after the effect of the free reference ligand in excess is corrected. $S_{\mathrm{FRef}}\,C_{\mathrm{TProt}}$ is calculated from S_{FRef} and C_{TProt} or be approximated as F_{min} if C_{TRef} is just 10% in excess to C_{TProt}. So, Eq. (13) applies.

$$BR = \frac{C_{\mathrm{BRef}}}{C_{\mathrm{TProt}}} \approx \frac{F_{\mathrm{X}} - F_{\mathrm{min}}}{F_{\mathrm{max}} - F_{\mathrm{min}}} \quad (13)$$

3.3. Estimation of dissociation constant of a candidate ligand

K_{dR} can be estimated *via* competitive binding of a reference ligand against a nonfluorescent ligand with a known affinity, or be determined *via* titration when applicable. EC_{50} of any candidate ligand was determined from regression analysis of the linear part of the plot of *BR* to logarithmic concentrations of a nonfluorescent candidate ligand during competitive binding. Assigning the dissociation constant of a candidate ligand to K_{dX}, the depletion of the reference ligand during competitive binding should be considered to derive K_{dX} and there was Eq. (14) [29,30].

$$\frac{K_{\mathrm{dX}}}{K_{\mathrm{dR}}} = \frac{2\mathrm{EC}_{50}}{2C_{\mathrm{TRef}} - C_{\mathrm{TProt}} - 2K_{\mathrm{dR}}} - \frac{C_{\mathrm{TProt}}}{2C_{\mathrm{TRef}} - C_{\mathrm{TProt}}} \quad (14)$$

In competitive binding systems, $(2C_{\mathrm{TRef}} - C_{\mathrm{TProt}})$ was much over nanomolar while K_{dR} for either BNEDA or BDEDA was below picomolar [31,35–38]. Thus, Eq. (15) applied.

$$\frac{K_{\mathrm{dX}}}{K_{\mathrm{dR}}} \approx \frac{2\mathrm{EC}_{50} - C_{\mathrm{TProt}}}{2C_{\mathrm{TRef}} - C_{\mathrm{TProt}}} \quad (15)$$

3.4. Optimization and expected properties

For this new homogenous assay of ligand affinities, the design of suitable fluorescent reference ligands as the FRET acceptors is already discussed [35]. To enhance this homogeneous competitive assay, the following points should be considered. (a) The binding of the reference ligand should quench protein fluorescence as effectively as possible but the binding of any nonfluorescent candidate ligand should quench fluorescence of the protein as negligibly as possible. (b) Due to the potential quench of fluorescence of complexes by a reference ligand or candidate ligand in great excess, F_{min} and F_{max} in both Eq. (8) and Eq. (12) should be checked for their validity. (c) The validity of Eq. (8) requires the affinity of a suitable reference ligand as high as possible. The validity of Eq. (12) requires the quantum yield of fluorescence of a suitable reference ligand as high as possible. When a fluorescent reference ligand as the FRET acceptor has an affinity strong enough to validate Eq. (8) and simultaneously a high enough quantum yield of fluorescence in complexes with the protein, both Eq. (8) and Eq. (12) can be used.

The following properties are expected and can be used for cross-validating this new homogeneous competitive assay. (a) If both Eq. (8) and Eq. (12) are validated with a reference ligand, consistent K_{dX} of a nonfluorescent candidate ligand could be obtained by quantifying the fluorescence of the protein and the fluorescence of the bound reference ligand. (b) If both Eq. (8) and Eq. (12) are validated with two reference ligands, K_{dX} of a nonfluorescent candidate ligand should be consistent, regardless of the use of either reference ligand and the quantification of either fluorescence signal. (c) With a protein of multiple binding sites, deviations are expected if candidate ligands quench excited states of tyrosine/tryptophan resides in adjacent binding sites or the fluorescence of the acceptor bound in adjacent sites. (d) Upon the excitation around 280 nm, if any candidate ligand has obvious signals at the wavelength to quantify the fluorescence of the bound reference ligand, it is prone to cause the deviation in K_{dX} if the quantum yield of fluorescence of the bound reference ligand is relatively small.

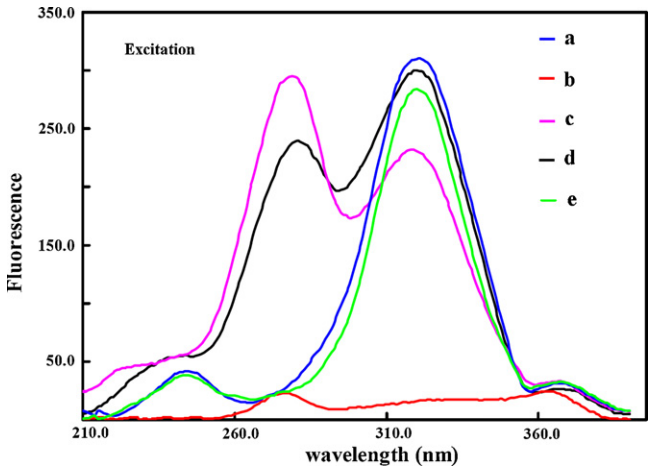


Fig. 1. Excitation spectra of BNEDA, SAV and their complexes detected at 450 nm. (a) BNEDA alone; (b) SAV alone; (c) 0.1 μM SAV + 0.12 μM BNEDA; (d) 0.1 μM SAV + 0.12 μM BNEDA + 0.4 μM biotin; (e) 0.1 μM SAV + 0.12 μM BNEDA + 2.4 μM biotin.

4. Experimental results

Fluorescent biotin derivatives with K_{dR} below 10 nM could be easily designed [35–38]. With tryptophan residues as intrinsic donors, both dansylamide and naphthylamine are effective acceptors [35]. But dansylamide has a quantum yield of fluorescence in aqueous solutions lower than that of naphthylamine. Therefore, to SAV, BNEDA and BDEDA were used as the reference ligands of different quantum yields to cross-validate this homogenous competitive assay.

4.1. Characterization of FRET in complexes

BNEDA and BDEDA had their absorption valleys around 280 nm and 285 nm, and absorption peaks around 325 nm and 340 nm, respectively (Figure S1, Supplementary Material). Upon direct excitation of free BNEDA and free BDEDA at 325 nm and 340 nm, their characteristic emission peaks appeared around 450 nm and 515 nm, respectively, but quantum yield of fluorescence of BDEDA was less than 10% of that of BNEDA. Meanwhile, they showed excitation valleys around 280 nm. After the binding to SAV, excitation of bound BNEDA at 325 nm and bound BDEDA at 340 nm produced their characteristic emission peaks around 430 nm and 525 nm, respectively, but the bound BDEDA still showed much lower quantum yield of fluorescence at 525 nm than that of the bound BNEDA at 430 nm. By excitation of the complexes at 280 nm, there was about 70% reduction of SAV fluorescence at 340 nm by the bound BNEDA or BDEDA and meanwhile there was the characteristic emission of the bound reference ligand. Upon competitive binding against biotin, the excitation peak at 280 nm of SAV to produce fluorescence at 340 nm was rescued but the fluorescence peak at 430 nm with BNEDA or that at 525 nm with BDEDA was reduced, in a manner dependent on biotin concentrations (Figs. 1 and 2, Figure S2, Supplementary Material).

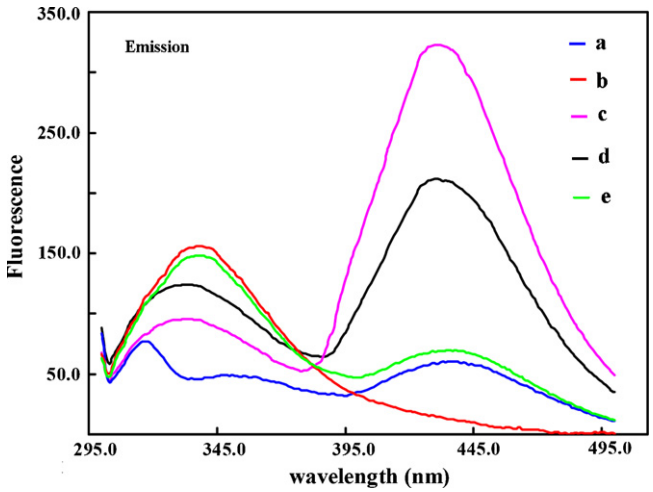


Fig. 2. . Emission spectra of BDEDA, SAV and their complexes excited at 280 nm. Solutions were completely the same as those in Fig. 1.

In solutions with free tryptophan and free BDEDA at levels comparable to those in solutions used above, there were no detectable fluorescence peaks around 525 nm by excitation at 285 nm. So was it with free tryptophan and free BNEDA in solutions by excitation at 280 nm. And NEDA or DEDA at the same level had no effects on SAV fluorescence. In solutions of free tryptophan and free BDEDA (or free BNEDA), averaged distances between the FRET pair were over 15 nm [35]. But such distances in the complex of SAV and BDEDA or BNEDA were within 5.0 nm (Scheme 1). Sensitivity to distances between a donor and acceptor was the nature of FRET [6]. Therefore, FRET in complexes of SAV with BDEDA or BNEDA should principally account for the quench of SAV fluorescence and the characteristic fluorescence of the bound BNEDA or BDEDA.

4.2. Dissociation constants of reference ligands

By quantifying the fluorescence of a bound reference ligand, its K_{dR} could be estimated based on its competitive binding against biotin. (a) For BNEDA, F_{max} at 430 nm upon the excitation at 280 nm was estimated with BNEDA in 10% excess to SAV. When the effect of free BNEDA on F_{max} was corrected, paired *t*-test gave statistically lower K_{dR} of BNEDA by Eq. (12) but the difference was within 3% (Table 1). Averaged K_{dR} of BNEDA was (14 ± 2) fM (n = 12) (Fig. 3a). When concentrations of SAV and BNEDA were varied over wide ranges, BNEDA had consistent K_{dR} by Eq. (12). (b) Free BDEDA had negligible effects on F_{max}. K_{dR} of BDEDA by Eq. (12) was (26 ± 2) fM

Table 1
Dissociation constants of BNEDA and BDEDA based on their competitive binding against biotin. Number in parenthesis was that for independent assays. Other details were described in Section 2.3.

Compound	Conditions (μM)		K_{dR} (fM)	
	C_{prot}	C_{TRef}	Emission via FRET	Quench via FRET
BNEDA	0.30	0.50	15 ± 3 (5)	33 ± 4 (4)[a]
	0.30	0.35	12 ± 2 (2)	14 ± 2 (2)[b]
	0.10	0.18	13 ± 2 (3)	–[c]
	0.025[d]	0.10	14 ± 2 (2)	18 ± 2 (2)
BDEDA	0.30	0.50	26 ± 2 (4)	28 ± 3 (4)

[a] Significantly higher than that of BNEDA under any other condition, but all other results for BNEDA had no differences by Student's two-way *t*-test.
[b] F_{min} was from titration, which was significant higher than that at 0.50 μM BNEDA.
[c] Not determined due to low sensitivity on Shimadzu RF540.
[d] Fluorescence was monitored on Shimadzu RF5301PC.

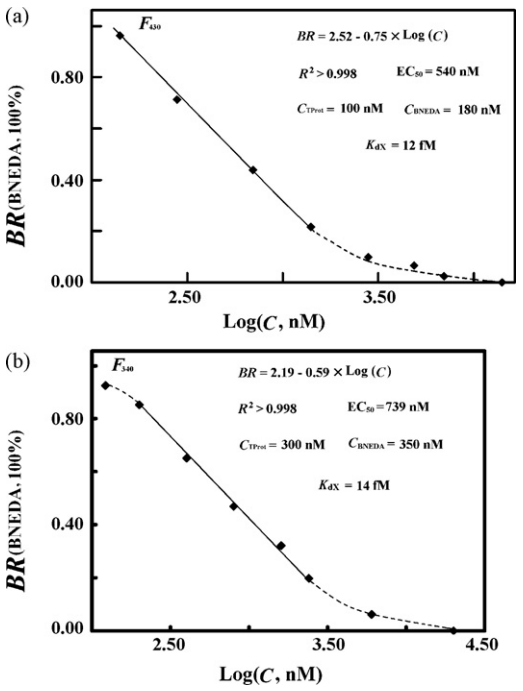


Fig. 3. Estimation of the affinity of BNEDA *via* competitive binding against biotin. (a) With fluorescence of bound BNEDA at 430 nm under the excitation at 280 nm. (b) With fluorescence of SAV at 340 nm under the excitation at 280 nm.

(n = 4) with fluorescence at 525 nm under excitation at 285 nm (Fig. 4a).

Biotin at 20 μM quenched SAV fluorescence by only about 10%, *via* mechanisms other than FRET. By measuring SAV fluorescence at

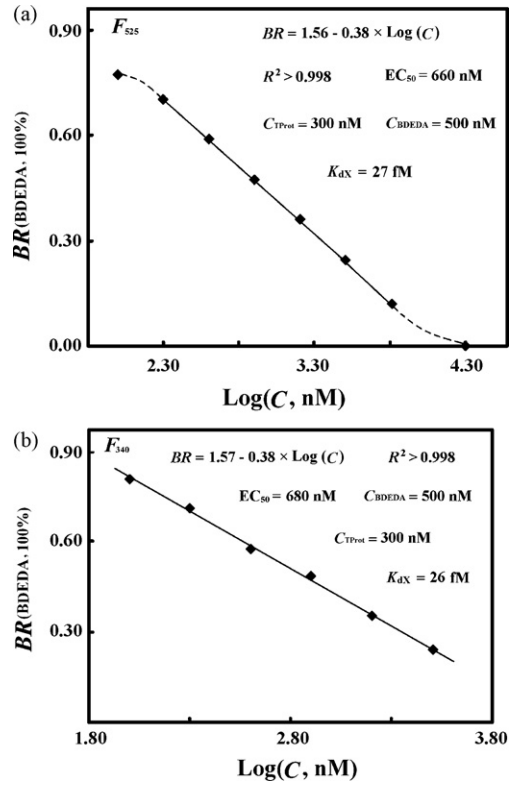


Fig. 4. Estimation of the affinity of BDEDA *via* competitive binding against biotin. (a) With fluorescence of bound BDEDA at 525 nm under the excitation at 285 nm. (b) With fluorescence of SAV at 340 nm under the excitation at 280 nm.

Table 2
Dissociation constants of some nonfluorescent biotin derivatives with either BNEDA or BDEDA as the reference ligand. Each result was the average in duplicate plus standard deviation. All the fluorescence was monitored on Shimadzu RF540.

Compound	K_{dX} (fM, BNEDA)[a]	K_{dx} (fM, BNEDA)[b]	K_{dx} (fM, BDEDA)[c]
BCHA	28 ± 3	29 ± 2	30 ± 3
BMPL	82 ± 3	84 ± 1	83 ± 3
BDETA	188 ± 6	193 ± 6	163 ± 8
BME	43 ± 2	45 ± 2	41 ± 3
BETA	94 ± 5	92 ± 3	87 ± 6
BBZA	42 ± 2	41 ± 2	44 ± 2

[a] Fluorescence was monitored at 430 nm by the excitation at 280 nm with BNEDA as the reference ligand, by presetting C_{TRef} = 0.18 μM, C_{Prot} = 0.10 μM, and K_{dR} = 14 fM.
[b] Fluorescence was monitored at 340 nm by the excitation at 280 nm with BNEDA as the reference ligand, by presetting C_{TRef} = 0.50 μM, C_{Prot} = 0.30 μM, and K_{dR} = 33 fM.
[c] Fluorescence was monitored at 340 nm by the excitation at 280 nm with BDEDA as the reference ligand, by presetting C_{TRef} = 0.50 μM, C_{Prot} = 0.30 μM, and K_{dR} = 27 fM.

340 nm under excitation at 280 nm, affinities of BNEDA and BDEDA were also estimated *via* competitive binding against biotin. (a) BNEDA over 0.35 μM reduced the approximated F_{min} at 340 nm in a concentration-dependent manner. When F_{min} at 340 nm with BNEDA below 0.35 μM were used in Eq. (8), K_{dR} of BNEDA was consistent with that by using fluorescence of the bound BNEDA at 430 nm (Fig. 3b). When F_{min} at 340 nm approximated with BNEDA at 0.50 μM was used in Eq. (8), K_{dR} of BNEDA showed significant positive deviation from that by using its fluorescence at 430 nm (Table 1). (b) BDEDA had no effects on F_{min} at 340 nm. With SAV fluorescence at 340 nm, K_{dR} of BDEDA was consistent with that using its fluorescence at 525 nm (Fig. 4b).

The overall coefficients of variations were about 10%, supporting reasonable reproducibility of this new homogenous competitive assay of ligand affinities based on estimation of EC_{50}.

4.3. Dissociation constants of some nonfluorescent biotin derivatives

Low quantum yield of fluorescence of BDEDA at 525 nm made BDEDA only suitable to approximate *BR via* Eq. (8). By using BNEDA to quantify its fluorescence at 430 nm, K_{dX} of each tested nonfluorescent biotin derivative *via* Eq. (12) was consistent with that *via* Eq. (8) using BDEDA as the reference ligand to quantify SAV fluorescence at 340 nm (Table 2).

By using BNEDA as the reference ligand at 0.50 μM without correction of the effect of BNEDA in excess on F_{min} at 340 nm, K_{dX} of each tested nonfluorescent biotin derivative by Eq. (8) was still consistent with that by using Eq. (13) with the characteristic fluorescence of the bound BNEDA at 430 nm, when K_{dR} of BNEDA under the corresponding conditions was used (Table 2). Affinity of each tested nonfluorescent biotin derivative relative to that of biotin, by using Eq. (8) and parameters with BNEDA at 0.50 μM to calculate K_{dR} and K_{dX}, was consistent with that by using Eq. (8) and BDEDA as the reference ligand (Table S1, Supplementary Material).

4.4. Deviations under special situations

Deviations were observed as follows. (a) By using BNEDA as the reference ligand to monitor its characteristic fluorescence at 430 nm, EC_{50} of BDEDA as a candidate ligand was so small that a negative K_{dX} of no physical meaning was observed (Figure S3, Supplementary Material). The quench of excited tryptophan residues in adjacent site, where BNEDA was bound, may account for negative K_{dX} of BDEDA with BNEDA as the reference ligand [35]. (b) When BDEDA was used as the reference ligand, the fluorescence at 340 nm showed negligible responses to increasing concentrations of BNEDA as a candidate ligand. The comparable quenching effects of BNEDA and BDEDA on SAV fluorescence at 340 nm could account for such deviations. (c) BCHA had some scattering signals over 460 nm. By quantifying the fluorescence at 525 nm of the bound BDEDA that had low quantum yield of fluorescence, the scattering signal of BCHA caused positive deviation to its K_{dX} by Eq. (12) (Figure S4, Supplementary Material). By quantifying SAV fluorescence at 340 nm with BDEDA as a reference ligand, K_{dX} of BCHA by Eq. (8) was consistent with that *via* Eq. (12) by using BNEDA as the reference ligand (Table 2).

5. Discussion and conclusion

The experimental results described above clearly supported our theoretical considerations on this new homogeneous competitive assay of ligand affinities. Other mechanisms may also quench the fluorescence of proteins upon the binding of fluorescent reference ligands. In fact, Eq. (8) only requires the quench of protein fluorescence, and Eq. (12) only requires the transfer of energy from excited tyrosine/tryptophan residues, upon the binding of a fluorescent reference ligand. Hence, this homogeneous competitive assay of ligand affinities should be effective even if other mechanisms also account partly for the quench of protein fluorescence.

This new homogenous competitive assay requires a suitable fluorescent reference ligand as the FRET acceptor to tyrosine/tryptophan residues as intrinsic donors. The general guideline to design such FRET acceptors to common proteins was proposed previously [35]. Typically, this new homogenous competitive assay of ligand affinities should be applicable to the following three types of proteins. (1) Proteins that bind macromolecular ligands, including protein kinases, signaling proteins, receptors of cytokines and DNA polymerases, all of which are important drug targets. (b) Proteins that possess binding sites large enough to accommodate nucleotides including ATP, GTP, cAMP, or cGMP. (c) Proteins such as SAV and albumin whose putative ligands have some groups exposed to solvents, on which suitable fluorophores can be attached to act as acceptors.

Taken together, based on the cost, efficiency, sensitivity, alteration on protein functions and the suitability for routine practice of HTS, this homogeneous competitive assay of ligand affinities can be an advantageous alternative of the classical homogenous competitive assay of ligand affinities when fluorescent reference ligands are available as the suitable FRET acceptors while tyrosine/tryptophan residues in a protein as the intrinsic donors.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.saa.2010.08.021.

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