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To achieve linear response for competitive bioaffinity assay of ligand: criteria of optimized interaction system†

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For linear response by competitive bioaffinity assay of a ligand, an optimized system required C_{RT} over 3-fold of C_{PT}, C_{PT} over 50-fold of K_{dR} while K_{dR} over 260-fold of K_{dX}, based on chemometrics for bioaffinity interaction (C_{RT} and C_{PT} are the concentrations of the probe and the biomacromolecule, K_{dX} and K_{dR} are the dissociation constants of complexes with the ligand and the probe, respectively). These criteria were tested for competitive bioaffinity assay of biotin. The probe was the conjugate of monomethyl-poly-(ethylene glycol)-5000, 1-naphthyl-ethylenediamine and biotin. The complex of the probe with streptavidin was quantified by fluorescence at 430 nm based on FÖrster-resonance-energy-transfer with tryptophan residues as the intrinsic donors. By fluorometric titration, K_{dR} of the probe was 5.4 ± 1.4 nM (n = 4). At 1.5 μM probe plus 0.50 μM streptavidin, there was linear decrease of fluorescence at 430 nm to biotin concentrations ranging from ~36 to ~500 nM; the linear response slope was consistent with that for fluorescence at 430 nm to concentrations of the complex of streptavidin and the probe. Biotin at 81 and 414 nM was estimated with variation coefficients below 7%. These proposed criteria may be universally applicable for linear responses by competitive assays of ligands.

Introduction

The specific reversible binding of a ligand (the guest) to a biomacromolecule (the host) is denoted bioaffinity interaction, making each the counterpart of the other. There are many small and macromolecular ligands of biological importance. Putatively, a small ligand exerts bioaffinity interaction with just one biomacromolecule, but a macromolecular ligand can concomitantly exert bioaffinity interactions with multiple counterparts. Common ligands in mixtures have no distinctive signals suitable for direct quantification; the quantitative analyses of such ligands in mixtures based on bioaffinity interactions are highly desired for their excellent selectivity, which are denoted bioaffinity assay and have pivotal applications in laboratory medicine, food and drug monitor, environment monitor and forensic medicine. For bioaffinity assay of a ligand, the signals of a component involved in bioaffinity interaction(s) with the ligand should be quantified; a calibration curve of the quantified signals to ligand concentrations in bioaffinity interaction system is thus developed to derive ligand quantities in samples and reflects analytical performances of bioaffinity assay. Clearly, analytical performances are determined by the quantification performances reflected by the response of the instrument signals to concentrations of the quantified component bearing an easy-to-detect property, and the response performances represented by the relationship of concentrations of the quantified component to concentrations of the ligand in bioaffinity interaction system. The quantification performances are reflected primarily by the quantification range, precision, sensitivity and selectivity of the component bearing the easy-to-detect signal. The quantification precision is determined by the instrument while quantification range, sensitivity and selectivity are greatly affected by both the signal of the quantified component and instrument. Indeed, there are a huge number of reports on the use of nanomaterials bearing signals of higher quantification sensitivity and selectivity as labels for bioaffinity assay.[1] The response performances of bioaffinity assay are determined by parameters of bioaffinity interaction system, whose effects have hardly been discussed to date. In this report, the effects of parameters of bioaffinity interaction system are discussed for better analytical performances of bioaffinity assay.

To discuss the effects of parameters of bioaffinity interaction system, the response performances for bioaffinity assay of a ligand are reflected mainly by (a) response sensitivity as the first-order derivative of the concentration of the quantified component to the concentration of the ligand in the interaction system, (b) response precision as the relative standard deviation (RSD) of the concentration of the quantified component at a given quantity of the ligand in bioaffinity interaction system, (c) response range from the lower limit of quantification (LOQ) to the upper limit of quantification (UOQ) of the ligand in the interaction system, (d) response selectivity as the ratios of the affinity of the counterpart biomacromolecule for the ligand to those for others in the

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interaction system (higher response selectivity for a ligand needs molecular engineering of its counterpart, and will not be discussed in this report). For bioaffinity assays of any ligands, the concurrent enhancement of response sensitivity, precision and ranges is always highly desired, but inherently infeasible with small ligands based on current conventional approaches for bioaffinity assay.

To develop a calibration curve for bioaffinity assay of a ligand, the signal of the free ligand, the free biomacromolecule, or their complex should be quantified. Usually, however, none of those components in the bioaffinity interaction system have satisfactory quantification selectivity and sensitivity. An analytical probe bearing both a bioaffinity interaction moiety and an easy-to-detect signal is thus needed, to give a competitive and noncompetitive bioaffinity assay. Noncompetitive bioaffinity assay of a ligand usually has a limited response range, but a linear response under optimized conditions for better response precision due to weaker propagation of the error from the concentration of the quantified component to the derived ligand concentration.[2] Competitive bioaffinity assay of a ligand usually has a wide response range, but always a nonlinear response associated with lower response precision due to greater propagation of error. Moreover, for bioaffinity assay, response sensitivity of a nonlinear response is lower than that of a linear response. In biomedicine, the qualitative judgment of the quantities of a ligand in mixture samples relative to its established cutoff has crucial roles,[3] making response sensitivity and precision more important than response range and thus noncompetitive bioaffinity assay more favorable. For noncompetitive bioaffinity assay of a macromolecular ligand, a small counterpart,[4] or multiple macromolecule counterparts,[5] are suitable probes and accessible. For noncompetitive bioaffinity assay of a small ligand, however, only its counterpart biomacromolecule is the suitable probe, but none of the resulted components have satisfactory quantification sensitivity and selectivity. With common small ligands, thus, only competitive bioaffinity assays are applicable because probes of the same bioaffinity moiety as the ligands are always accessible.[6] However, there are usually low response sensitivity and precision.

Based on chemometrics for bioaffinity interaction, it is expected that the optimization of parameters of competitive bioaffinity interaction system may provide an approximated linear response, giving higher response sensitivity and better response precision over a reasonable response range. Herein, for an approximated linear response by competitive bioaffinity assay of a ligand, the optimization criteria of those parameters were proposed based on chemometrics for bioaffinity interaction, and proved effective with competitive assay of biotin as the model.

Results and discussion

Optimization of competitive bioaffinity interaction system for a reasonable linear response range

The following parameters are defined with concentrations in the bioaffinity interaction system.

K_{dX}: the dissociation constant of the ligand of interest;
K_{dR}: the dissociation constant of the probe;
m: the ratio of K_{dR} to K_{dX};
C_{RT}: the total concentration of the probe;
C_{RF}: the concentration of the free probe;
n: the ratio of C_{RT} to C_{PT};
C_{RB}: the concentration of the bound probe;
C_{RB0}: C_{RB} in the absence of the ligand of interest;
C_{RBL}: C_{RB} for the ligand of interest at the LOQ;
C_{RBU}: C_{RB} for the ligand of interest at the UOQ;
C_{XT}: the total concentration of the ligand of interest;
C_{XF}: the concentration of the free ligand of interest;
C_{XB}: the concentration of the bound ligand of interest;
C_{XBL}: C_{XB} for C_{XT} at the LOQ;
C_{XBU}: C_{XB} for C_{XT} at the UOQ;
C_{PT}: the total concentration of the counterpart macromolecule;
C_{PF}: the concentration of the free counterpart macromolecule;
C_{PF0}: the concentration of the free counterpart for C_{XT} at zero;

According to mass conservation for 1:1 complex of the probe or the ligand with the same counterpart, Eq.(1) through Eq.(5) are defined for competitive bioaffinity interaction. The combination of Eq.(4) and Eq.(5) thus gives Eq.(6). With $Abs(C_{PF}-C_{XF})$ negligible to C_{XT}, there is an approximated linear response of the concentrations of the quantified component to the concentrations of the ligand, which is reflected by Eq.(7) when the signal of the bound probe or the complex is quantified, or by Eq.(8) when the signal of the free probe is quantified. The optimization of parameters for Equ.(7) and Equ.(8) is discussed to get an approximated linear response.

$$K_{dR}=(C_{RF}\times C_{PF})/C_{RB} \quad (1)$$
$$K_{dX}=(C_{XF}\times C_{PF})/C_{XB} \quad (2)$$
$$C_{RB}=C_{RT}-C_{RF} \quad (3)$$
$$C_{XB}=C_{XT}-C_{XF} \quad (4)$$
$$C_{PT}=C_{RB}+C_{XB}+C_{PF} \quad (5)$$
$$C_{PT}-C_{RB}=C_{XT}+(C_{PF}-C_{XF}) \quad (6)$$
$$C_{PT}-C_{RB}\approx C_{XT} \quad (7)$$
$$C_{PT}-C_{RT}+C_{RF}\approx C_{XT} \quad (8)$$

According to the principle of error propagation,[2] RSD of C_{XT} is equal to that of $(C_{PT}-C_{RB})$ when Eq.(7) applies, and equal to that of $(C_{PT}-C_{RT}+C_{RF})$ when Eq.(8) applies. The error in the quantified signal of either the bound probe as Eq.(7) or the free probe as Eq.(8) will not be amplified into C_{XT}, but RSDs of the concentrations of the ligand at levels slightly over LOQ with Eq.(7) will inevitably be larger than those with Eq.(8). Meanwhile, from either Eq.(7) or Eq.(8), the first-order derivative as the slope of the response curve is approximately 1.0 over the response range, supporting that response sensitivity reaches the maximum when there is linear response (Data S1,ESI†). The parameters of competitive bioaffinity interaction system should thus be optimized systematically to validate Eq.(7) or Eq.(8) over the desired response range of the concentrations of the ligand.

A desired response range is reflected by a pair of LOQ and UOQ. If $Abs(C_{PF}-C_{XF})/C_{XT}$ is below 10%, $(C_{PF}-C_{XF})$ is negligible to C_{XT}.[7] C_{PF}/C_{XT} and C_{XF}/C_{XT} are positive values bearing monotonic continuous associations with C_{XT}. When $C_{PF}/C_{XT}<10\%$ and $C_{XF}/C_{XT}<10\%$ are validated from LOQ to UOQ, $Abs(C_{PF}-C_{XF})/C_{XT}<10\%$ is validated over the response range. Classical noncompetitive bioaffinity assays have response ranges with UOQ about ten-fold of the paired LOQ.[8] Therefore, the related parameters for a competitive bioaffinity assay system are optimized

for $C_{PF}/C_{XT} < 10\%$ and $C_{XF}/C_{XT} < 10\%$, over a pair of UOQ and LOQ bearing the ratio of about ten-fold.

When $C_{PF}/C_{XT} < 10\%$ is validated at a smaller C_{XT}, it is validated at any higher C_{XT} due to the use of a larger denominator while a smaller C_{PF} by competitive binding. C_{PF0} is surely larger than any C_{PF} for C_{XT} over zero. The validity of $C_{PF0}/\mathrm{LOQ} < 10\%$ ensures $C_{PF}/C_{XT} < 10\%$ at any C_{XT} within the linear range. With C_{RT} equal to $n \times C_{PT}$ (n > 1.0) for competitive binding, C_{PF} at C_{XT} equal to LOQ is determined by K_{dR}, C_{PT}, C_{RT}, C_{XT} and K_{dX} as described by Eq.(1) and Eq.(2). Consequently, Eq.(9) reflects the upper bound of K_{dR} for the desired LOQ. However, Eq.(9) involves too many parameters for computation. Fortunately, it is easy to calculate C_{PF0} and thus C_{RB0} as the difference in C_{PT} and C_{PF0}. Clearly, C_{RB0} is larger than C_{RBL} due to competitive binding and Eq.(10) refines the upper bound of K_{dR}. Hence, the association of those parameters for a desired LOQ is approximated with Eq.(10); UOQ for linear response is restricted by C_{PT}, and thus LOQ should be smaller than $0.10 \times C_{PT}$ for a ten-fold range while below $0.25 \times C_{PT}$ for a four-fold range.

$$K_{dR} = (C_{RT}\text{-}C_{RBL}) / C_{RBL} \times C_{PF} < (n \times C_{PT}/C_{RBL} - 1) \times 0.1 \times \mathrm{LOQ} \quad (9)$$

$$K_{dR} < (n \times C_{PT}/C_{RB0} - 1) \times 0.1 \times \mathrm{LOQ} \quad (10)$$

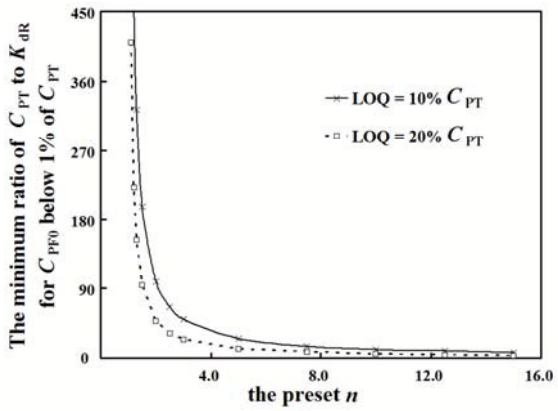


Fig. 1. Response of the minimum ratios of C_{PT} to K_{dR} to the preset n for C_{PF0} below 10% of the required LOQ

By computation, a smaller K_{dR}, a higher C_{PT}, a larger n facilitate achieving C_{PF0} below $0.10 \times \mathrm{LOQ}$ and thus the desired LOQ (Data S2, ESI†). With the same n, there are consistent minimal ratios of C_{PT} to K_{dR} for C_{PF0} below $0.10 \times \mathrm{LOQ}$ when C_{PT} is varied from those comparable to K_{dR} to those nearly 100-fold of K_{dR} (Data S2, ESI†). For LOQ < $0.25 \times C_{PT}$, there can be much smaller ratios of C_{PT} to K_{dR} to have C_{PF0} below $0.10 \times \mathrm{LOQ}$ (Fig. 1). Moreover, for C_{PF0} below $0.10 \times \mathrm{LOQ}$, the minimal ratios of C_{PT} to K_{dR} have negligible dependence on preset levels of C_{PT}, but an exponential association with n (Fig. 1). These associations predict the optimal n for C_{PF0} below $0.10 \times \mathrm{LOQ}$ with a known K_{dR}. For instance, the use of n of 3.0 needs C_{PT} over 50-fold of K_{dR} to yield C_{PF0} below $0.10 \times \mathrm{LOQ}$; the use of a probe of K_{dR} below 20 nM and C_{PT} below 1.0 μM thus can give the LOQ smaller than $0.10 \times C_{PT}$.

On the other hand, Eq.(11) always applies for the same C_{PF} under competitive binding in the same interaction system; Eq.(12) thus applies when $C_{XF}/C_{XT} < 10\%$ is validated. With C_{PF} negligible to C_{XT} for the desired LOQ, the difference between C_{PT} and C_{RBU} is an approximate of C_{XBU} so that Eq.(13) applies. Meanwhile, the difference between C_{PT} and C_{RBL} is an approximate of C_{XBL} and thus Eq.(14) applies. Clearly, when K_{dR} already validates Eq.(13), it also validates Eq.(14) because C_{XBU} is surely larger than C_{XBL}. However, Eq.(13) still involves too many parameters. Fortunately, UOQ is larger than C_{XBU} since the small ligand can not be bound completely by the counterpart biomacromolecule for competitive bioaffinity assay. The validity of Eq.(15) thus ensures the validity of Eq.(13); Eq.(15) thus refines the lower bound of m for the desired UOQ and suits for calculating the association of C_{PT}, C_{RT} and m for a desired UOQ by competitive bioaffinity assay.

$$m \times C_{RB}/C_{RF} = C_{XB}/C_{XF} \quad (11)$$

$$m \times C_{RB}/(C_{RT} - C_{RB}) = C_{XT}/C_{XF} - 1 > 9 \quad (12)$$

$$m > 9 \times (n \times C_{PT}/(C_{PT} - C_{XBU}) - 1) \quad (13)$$

$$m > 9 \times (n \times C_{PT}/(C_{PT} - C_{XBL}) - 1) \quad (14)$$

$$m > 9 \times (n \times C_{PT}/(C_{PT} - \mathrm{UOQ}) - 1) \quad (15)$$

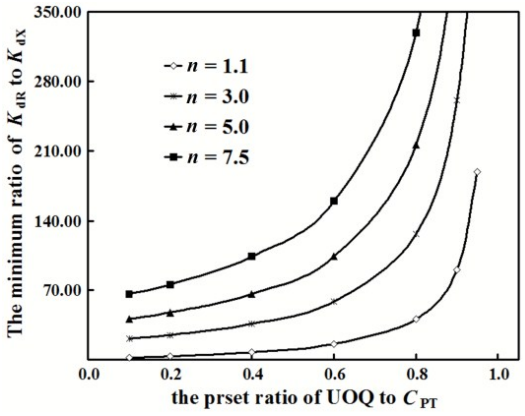


Fig. 2 The minimum ratio of K_{dR} to K_{dX} for a required UOQ

By computation, a higher UOQ with a known K_{dR} needs a larger m (Data S2, ESI†). The minimal values of m moderately and linearly increase for UOQ below $0.80 \times C_{PT}$, but exponentially for UOQ over $0.80 \times C_{PT}$; the use of a larger n leads to a rapider increase of the minimal values of m for the same ratios of UOQ to C_{PT}; for the same m, a larger n reduces the achievable UOQ (Fig. 2). For instance, with n of 3.0, the minimal ratio of m should be about 60 to have UOQ of about $0.60 \times C_{PT}$, and about 260 for UOQ of about $0.90 \times C_{PT}$ (Data S2, ESI†). Moreover, a larger m enhances the feasibility for linear response and 1:1 displacement of the probe from the complex by the ligand to have the maximal response sensitivity of 1.0; any m smaller than 1.0 leads to a smaller ratio of the displacement, lower response sensitivity and in fact no practical linear response range. Hence, for linear response over a reasonable range, m should be much larger than 1.0.

Clearly, to achieve a desired linear range at favourable cost, there are sophisticated associations of the optimized values of C_{PT}, K_{dR}, n and m. In general, a larger m facilitates the optimization of a bioaffinity interaction system for a desired linear range. In practice, it is easy to design a probe for K_{dR} larger than K_{dX}, but is challenging to engineer the counterpart biomacromolecule for a smaller K_{dX}. Moreover, the minimal ratios of C_{PT} to K_{dX} for a desired LOQ show weaker and negative dependence on the preset n for a linear range within 6-fold, but stronger and negative dependence on the preset n for a linear range over 6-fold (Fig. 3). C_{PT} below 1.0 μM plus n from 1.5 to 3.0 is practical considering operation errors; K_{dR} should

thus be no more than 20 nM for LOQ below $0.10\times C_{PT}$ while over 260-fold of K_{dX} for UOQ over $0.90\times C_{PT}$ (Data S2, ESI†). Consequently, for a desired linear range, K_{dX} should be smaller than a threshold since K_{dR} has an upper bound; a desired linear range is thus applicable only to a ligand of strong affinity to its counterpart. On the other hand, in practice, C_{PT} and C_{RT} can be easily adjusted, and n slightly over 1.5 is practical considering operation errors. It is easy to reduce LOQ with an optimized pair of C_{PT} and C_{RT}, but it is difficult to increase UOQ with known K_{dR} and K_{dX} (Data S2, ESI†). With known K_{dR} and K_{dX}, the use of a larger n and/or larger C_{PT} reduces ratios of both LOQ and UOQ to C_{PT}, supporting the use of a larger C_{PT} plus a smaller n for a desired linear range.

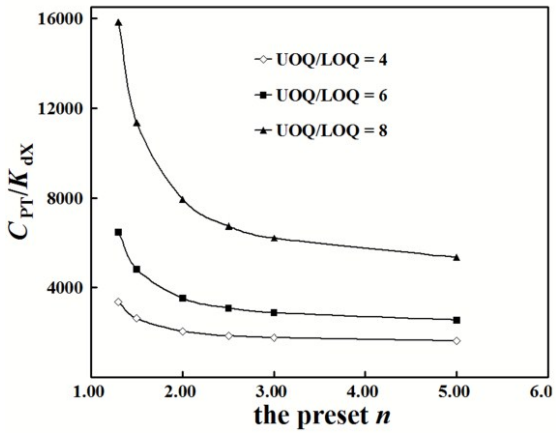


Fig. 3 Response of the minimal ratios of C_{PT} to K_{dX} to the preset n for a desired linear range

Taken together, with known K_{dR} and K_{dX}, steps to optimize C_{PT} and C_{RT} are proposed below. First, m as the ratio is calculated from the known pair of K_{dR} to K_{dX}. Second, based on the requirement of the minimal value of m for a preset ratio of UOQ to C_{PT}, the maximal ratio of UOQ to C_{PT} together with the upper bound of n is estimated with the calculated m (Fig. 2; Data S3, ESI†). Third, for a desired linear range, the largest LOQ is determined with the known UOQ for a practical C_{PT}. Fourth, for the desired LOQ, the combination of the largest n and the smallest C_{PT} is sought (Data S3, ESI†). Fifth, the combination of n and C_{PT} is sought for applicability to K_{dR} at 99% confidence due to its experimental errors (Data S3, ESI†).

Design, preparation and characterization of a fluorescent probe

Biotin is a vitamin bearing no physicochemical signals suitable for direct quantification. Bioaffinity assays of biotin have long been reported based on competitive binding of probes to streptavidin (SAV) but usually tolerate nonlinear responses. K_{dX} of biotin to SAV is wonderfully as low as 40 fM.[8a] Hence, the criteria and strategies for the optimization of bioaffinity interaction system were tested with competitive bioaffinity assay of biotin as the model.

For bioaffinity assay, the signal of a component can be quantified after its separation from the interaction system to make a heterogeneous assay, or be quantified directly in the interaction system without separation to make a homogeneous assay. A homogenous assay always has better analytical precision and higher analytical efficiency. Förster-resonance-energy-transfer (FRET) is susceptible to the distance between a donor and its respective acceptor. Bioaffinity interaction easily drives a ligand and its counterpart closer to form a complex and exert FRET for homogeneous assay. For homogenous competitive assay of biotin, the use of tryptophan residues in SAV as intrinsic donors is advantageous; many fluorophores can serve as the acceptors of tryptophan residues to prepare the probes.[4a, 4c, 4e, 9] Hence, homogenous competitive bioaffinity assay of biotin is tested with tryptophan as donors, but the quantification of the complex inevitably leads to larger RSDs at low biotin levels (Data S4, ESI†).

To achieve LOQ below $0.10\times C_{PT}$ and UOQ over $0.90\times C_{PT}$ with C_{PT} below 1.0 μM and C_{RT} at $3.0\times C_{PT}$, a fluorescent biotin derivative should have K_{dR} from 10 pM to 20 nM (Data S2, ESI†). The conjugates of biotin to aliphatic amino groups of some fluorophores give the probes,[4c, 9b, 10] whose affinities are sometimes even higher than biotin. For instance, 1-naphthylamine is an acceptor of tryptophan; the amide of the aliphatic amino group of 1-naphthylethylenediamine (NEDA) and biotin, BNEDA, has a quantum yield over 0.5 but an affinity stronger than biotin.[4c] However, a conjugate of monomethyl poly-(ethylene glycol) (mPEG) and biotin has nanomolar K_{dR};[10a] the conjugate of NEDA, biotin and mPEG of 5000 Dalton (mPEG5k-BNEDA) may be a suitable probe. Hence, mPEG5k-BNEDA was prepared (Scheme 1), and tested as the probe for competitive bioaffinity assay of biotin.

There was FRET in the complex of the probe with SAV to give strong fluorescence at 430 nm under the excitation at 280 nm; the competitive binding against biotin caused significant decrease of the FRET fluorescence (Data S5, ESI†). The quantum yield of the free probe was about 0.26 ± 0.02 (n = 2), accounting for about 40% of that of BNEDA.[4c,9b] The quantum yield of the probe bound to SAV was 0.23 ± 0.02 (n = 2), indicating a smaller decrease in the quantum yield of the bound probe than the bound BNEDA.[4c]

The fluorescence at 430 nm under the excitation at 280 nm of interaction system (F_X) is approximated as Eq.(16).[4c, 9b, 11] In Eq.(16), S_1 is the slope for the response of fluorescence at 430 nm of the complex excited at 280 nm to its concentrations minus the slope for linear response of fluorescence at 430 nm of the free probe excited at 280 nm to its concentrations; F_0 is the F_X when all the probe is free. Based on Eq.(6), Eq.(16) is converted into Eq.(17) and Eq.(18) by assuming that F_1 equal to F_0 plus the fluorescence at 430 nm calculated for the complex at the concentration of C_{PT} with the stated S_1. Clearly, at C_{XT} of zero, F_X is approximated by F_1, which is the largest value and gives a larger SD together with a larger LOQ. When the approximated linear response is achieved, S_1 should be consistent with that estimated by other approaches. The titration curve as fluorescence at 430 nm under the excitation at 280 nm of interaction system (F_X) to varying concentrations of SAV for a given concentration of the probe, or varying concentrations of the probe for a given concentration of SAV, is analyzed by MATLAB 7.11 as described previously, to estimate K_{dR}.[4d, 4e]

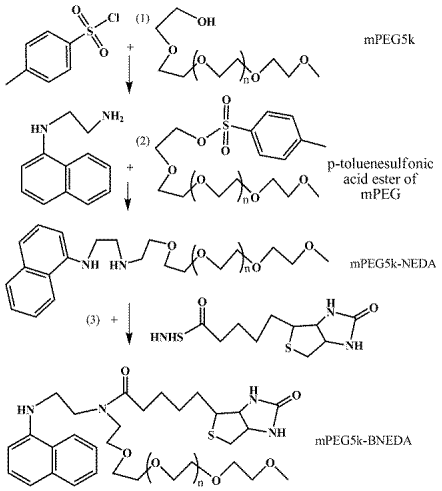
$$F_X \approx F_0 + S_1 \times C_{RB} \quad (16)$$

$$F_X \approx F_0 + S_1 \times C_{PT} - S_1 \times C_{XT} \approx F_1 - S_1 \times C_{XT} \quad (17)$$

$$\Delta F_X \approx F_1 - F_X = S_1 \times C_{XT} \quad (18)$$

There was a challenge to estimate the molar quantity of mPEG5k-BNEDA since there were multiple derivatives bearing laddered molecular weights, besides leftover mPEG5k, NEDA and BNEDA as the contaminants (Data S6, ESI†). As an alternative, the molar quantity of mPEG5k-BNEDA was estimated as its binding

equivalency to SAV. In the probe, only NEDA and its derivatives have significant absorbance at 325 nm. The molar content of NEDA plus its derivatives in the probe was thus estimated by the absorbance at 325 nm to facilitate calibration of the quantity of the probe; the binding equivalency of the probe was titrated with SAV and data were analyzed by an approximation approach.[4c,4d,4e] The binding equivalency of mPEG5k-BNEDA was about (71 ± 2)% (n = 2) of the molar content of NEDA (Data S7, ESI†).



Scheme 1 Preparation of the probe mPEG5k-BNEDA

There was BNEDA contaminated in the probe. Biotin at just $0.10\times C_{PT}$ still reduced F_X of the bound mPEG5k-BNEDA with C_{RT} at $3.0\times C_{PT}$, indicating few unoccupied sites of SAV homotetramer and strong affinity of the probe to SAV. There was a nonlinear decrease in F_X to biotin levels over C_{PT} (Data S4, ESI†), supporting the contamination of other probes bearing affinities comparable to that of biotin. BNEDA has stronger affinity than biotin,[4c, 9b] and may be the contaminant (Scheme S1, ESI†). F_X at biotin of about 102% C_{PT} was taken as the contribution of the bound BNEDA, indicating about 12% of BNEDA in mPEG5k-BNEDA considering different quantum yields of their complexes with SAV.[4c,9b]

K_{dR} was estimated by a fluorometric titration assay.[4d, 4e, 11] To titrate the probe with SAV, there will be a constant background signal from the bound BNEDA after SAV levels were high enough to sequester all BNEDA. The use of a titration model considering the signal of the bound BNEDA as a constant background for fitting the titration curve in the presence of SAV levels high enough gave K_{dR} of (5.7 ± 0.2) nM (n = 2) (Data S8, ESI†). BNEDA had a stronger affinity and formed complex with SAV before the probe did. In this case, the slope for the response of F_X to the concentrations of the complex reflected the signal of the bound BNEDA, which was 1.3 ± 0.1 (n = 2) (Data S8, ESI†). To titrate SAV with the probe, there were varying levels of BNEDA. The percentage of BNEDA in mPEG5k-BNEDA was fixed at 12%; the slope for linear response of F_X to concentrations of the complex with BNEDA was fixed at 2.2-fold of that to concentrations of the complex with the probe for correcting the interference of BNEDA. The fitting without the correction of such interference gave K_{dR} of tiny difference from that after the correction of such interference (Data S8, ESI†). Moreover, the correction of such interference with the contaminated BNEDA below 6% of the molar quantity of NEDA derivatives usually caused non-convergence of the fitting with the same model. For the contaminated BNEDA from 9% to 15% in the probe, there was the convergence of the fitting and less than 10% differences in the estimated K_{dR} (Data S8, ESI†). Interestingly, the slope for the response of F_X to the concentrations of the complex was about 0.65 after the correction of the role of BNEDA (Data S8, ESI†), which was consistent with the following titration of the complex of the probe and SAV with biotin (Fig. 4), but was just half of that of the complex of BNEDA. Such differences were consistent with the ratio of the quantum yields of their complexes with SAV. Taking together, K_{dR} was (5.4 ± 1.4) nM (n = 4), comparable to that of an analogue.[10]

Characterization of competitive bioaffinity assay of biotin

K_{dR} of mPEG5k-BNEDA required a C_{PT} over 0.30 μM with C_{RT} at $3.0\times C_{PT}$ for a LOQ below 30 nM while the small K_{dX} of biotin easily gave a UOQ over $0.95\times C_{PT}$ (Data S3, ESI†). Due to potential errors of K_{dR}, 0.50 μM SAV plus 1.50 μM mPEG5k-BNEDA was used (Data S3, ESI†). During competitive binding of biotin against the probe, F_X indeed linearly decreased to biotin concentrations up to nearly 100% of C_{PT} (Fig. 4). Due to FRET, the probe quenched SAV fluorescence at 340 nm more strongly than biotin, and thus there was a concomitant but much smaller linear increase of the fluorescence of SAV at 340 nm to biotin concentrations during competitive binding (Data S4, ESI†). The LOQ of biotin was about 36 nM (Data S4, ESI†). If just the deviation from linear response was considered, the LOQ reached zero that was impractical (Data S4, ESI†). The UOQ was nearly 100% of C_{PT}. For competitive assay of biotin, therefore, these results supported that there was indeed a linear response over the predicted range.

Interestingly, the slope for the linear response of $\triangle F$ to concentrations of biotin was about 0.63, while that for the titration of SAV by the probe was about 0.65 after the correction of the contribution of BNEDA (Data S8, ESI†); this consistency supported the validity of Eq.(7) and Eq.(17) and the maximal response sensitivity over the linear range. For biotin at 81 nM and 414 nM, RSDs for their F_X as the quantification precision of the signal by the instrument were just about 0.4% and 1.7% (n = 5), but 6.4% and 4.6% for their $\triangle F$ (Data S4, ESI†), respectively. After calculation from $\triangle F$ according to Eq.(18), RSDs of the two biotin concentrations were comparable to those of $\triangle F$, supporting that there was no significant amplification of random errors into ligand concentrations from the quantified signals, as predicted.

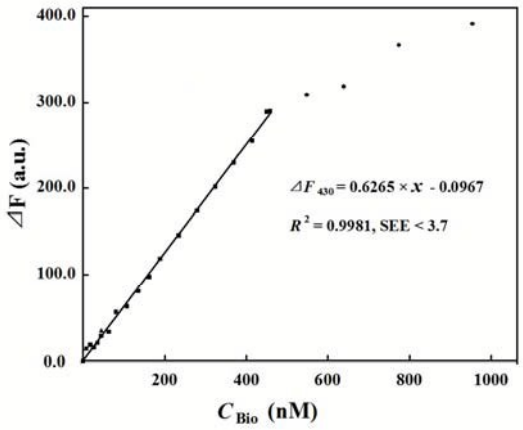


Fig. 4. Calibration curves for the titration of the complex by biotin
The probe was 1.5 μM and SAV was 0.50 μM.

Further discussion on competitive bioaffinity assay of a ligand

For bioaffinity assay of a ligand, the overall analytical performances are characterized mainly by (a) analytical sensitivity, which is the response sensitivity times the quantification sensitivity; (b) analytical selectivity, which is the response selectivity times the quantification selectivity; (c) the analytical range, which is the cross-section of the response range and the quantification range, (d) analytical precision, which is RSD of ligand quantity in a sample.[2]

Notably, for bioaffinity assay, the quantification precision is always much better than the response precision, making the response precision a reasonable approximate of analytical precision; the quantification range is always much wider than the response range and thus the analytical range is primarily determined by the response range. To enhance analytical sensitivity for competitive bioaffinity assay of a ligand, the straightforward strategy is the use of a probe of higher quantification sensitivity. The use of nanomaterials as labels for bioaffinity assay can greatly enhance analytical sensitivity.[1] With such probes, however, classical approaches for competitive bioaffinity assays of ligands still give nonlinear responses. After logarithmic conversion of concentrations of ligands, those classical approaches usually give linearized responses over several orders of magnitude of ligand concentrations.[1,12] Notably, for competitive bioaffinity assay of a ligand, the linear response achieved by the proposed optimization criteria is much different from a linearized response. Clearly, the analytical range for linear response by the proposed optimized criteria is much narrower than that for a linearized response. However, with such a linearized response, the use of logarithmic ligand concentration as the independent variable causes much worse analytical precision at ligand levels approaching the LOQ.[12] And analytical sensitivity for a linear response with the same probe by the proposed optimization criteria is much larger than that with a linearized response.[6g, 12] Therefore, the parameters of bioaffinity interaction system still need optimizations to enhance the analytical performances for competitive bioaffinity assays of ligands.

In theory, the proposed criteria and steps for optimization to achieve linear responses by competitive bioaffinity assays are applicable to common small and macromolecular ligands. The improvement of the analytical sensitivity and ranges by the enhancement of *m* or the affinity ratios of the probes to the ligands has already been observed.[12a, 12b, 12c] However, in those reports, there were no systematic optimizations of parameters for bioaffinity interactions, and thus nonlinear responses and unsatisfactory analytical precision at low levels of the ligands. Moreover, by competitive bioaffinity assays with nonlinear responses, there are usually much larger RSDs in the low ligand levels when the signals of complexes are quantified.[12b] Hence, the quantification of the free probe is usually preferable for better analytical precision by competitive bioaffinity assay.

For a desired linear range by competitive bioaffinity assay, a small K_{dX} is the prerequisite. Most tight-binding ligands have K_{dX} < 1.0 nM.[9b, 11] The multi-valent interactions with biomacromolecules reduce K_{dX};[13] aptamers can have K_{dX} < 1.0 nM for common small ligands and biomacromolecules.[14] Namely, the desired linear ranges can be achieved with some ligands since the required counterpart biomacromolecules are accessible, but the requirement of proper pairs of K_{dX} and K_{dR} makes the optimization process a topic of molecular engineering rather than analytical sciences alone. From this aspect, bioaffinity assay is not a work of analytical sciences only, but should be integrated with molecular engineering. Clearly, such integration makes bioaffinity assay a challenging topic of chemical biology that is a new concept to biomedical researches. However, some biomarkers in mixture samples are repetitively measured in laboratory medicine, forensic medicine and the monitor of food, drug, and environment; molecular engineering of their required counterparts and probes for better analytical performances has the practical significance. On the other hand, the design of suitable probes is a prerequisite for the practice of the optimization criteria. Fortunately, when a counterpart biomacromolecule is available, the design of a probe of weaker affinity is facile. For example, the structure of the complex of the counterpart biomacromolecule with the ligand of interest can provide valuable information on the design of the required probe; all approaches for structure-based design of ligands can be applicable for the design of the probe to have a required *m*. Therefore, the technical challenge on the practice of the proposed optimization criteria should be the access to the counterpart biomacromolecule, whose engineering is a focus of both modern biotechnology and chemical biology.

Conclusion

Some criteria and associated steps for optimization of competitive bioaffinity assay of ligands are proposed to achieve approximated linear responses and thus better analytical precision and sensitivity. For a reasonable linear range by competitive bioaffinity assay, a suitable pair of the probe and counterpart is required for K_{dR} below 20.0 nM and K_{dX} below 1.0 nM. The use of larger C_{PT} and larger ratio of C_{RT} to C_{PT} reduces LOQ at affordable cost. The criteria and the associated steps of optimization may be universally applicable for desired linear ranges by competitive bioaffinity assays of ligands, which can significantly enhance analytical performances of competitive bioaffinity assays and respective biosensors.

Materials and Methods

Chemicals and materials

Streptavidin (SAV) was bought from Promega (Shanghai, China); 1-naphthylamine and biotin were purchased from Alfa-Aesar (Tianjing, China); monomethyl poly-(ethylene glycol) of 5,000 Dalton (mPEG5k) was provided by Sigma-Aldrich (St Louis, MO, USA).

Preparation of the probe

Three stages of reactions were utilized. (a) Sodium naphthalene was prepared with equal moles of naphthalene and solid sodium in anhydrous tetrahydrofurane under room temperature for 12 h to yield a dark green solution, and reacted with mPEG5k in toluene under room temperature to generate the anion of mPEG5k for the subsequent reaction with paratoluensulfonyl chloride in 10% excess. The resulted mixture of the paratoluenesulfonate of mPEG5k was filtered, and the solution was precipitated with at least three-fold volume of ethyl ether; the precipitates were further washed with ethyl ether twice. (b) The aqueous solution of 1-naphthylethylenediamine (NEDA) (di-hydrochloride) was alkalinized

with concentrated sodium hydroxide, and extracted with chloroform; the evaporation of chloroform yielded the white powder of NEDA. The paratoluenesulfonate of mPEG5k was reacted with NEDA in chloroform under reflux to yield *N*'-mPEG5k-*N*-(1-naphthyl)-ethylenediamine (mPEG5k-NEDA), which was purified by chromatography through silica column eluted with ethyl acetate: methanol at 1:3. (c) The molar quantity of mPEG5k-NEDA was determined by absorbance at 325 nm with the millimolar absorption coefficient of 5.3 $(mM)^{-1}\cdot cm^{-1}$ and MAPADA UV 1600 PC spectrophotometer.[4c,9b] *N*-hydroxysuccinamide (NHS) ester of biotin was prepared as before,[4c, 9b] and reacted with mPEG5k-NEDA in 5% excess in dimethylforamide to yield the probe, which was purified via precipitation with ethyl ether, and chromatography through silica twice. The quantity of the probe in the final preparation was determined by absorbance at 325 nm as well. The molecular weights of the probe and mPEG5k-NEDA were determined by matrix-assisted-laser-desorption-ionization time-of-flight mass-spectrophotometer (MALDI-TOF-MS) and compared to verify the linkage of biotin to mPEG5k-NEDA (Data S6, ESI†).

Determination fluorescence

Agilent Carry Eclipse fluorospectrophotometer was used, with excitation at 280 or 325 nm while emission at 430 nm, unless otherwise stated.[4d, 4e] The reaction buffer was 20 mM sodium phosphate at pH 7.0, after filtration through 0.22 μm membrane. The probe was mixed with a sample before the addition of SAV. Fluorescence was recorded after reaction for 5.0 min since the addition of SAV to the mixture of the probe and a sample.

Data processing

Of a ligand by competitive bioaffinity assay, LOQ and UOQ are estimated as follows. A linear equation for the response of the quantified signals to the concentrations of the ligand over a narrow central range is developed to estimate the standard error of estimate (SEE) and predict the nearest data exceeding the analyzed range with preset independent variables. The range for estimating SEE and predicting the nearest data is widened step-by-step until the following LOQ and/or UOQ are/is reached (Data S9, ESI†). The lowest concentration of the ligand that causes the change of the quantified signal bearing a deviation from the predicted value within twice the SEE and the RSD below 35% serves as the LOQ,[15] while the largest concentration of the ligand that causes the change of the quantified signal with a deviation from the predicted value below twice the SEE and RSD below 10% serves as the UOQ.[2]

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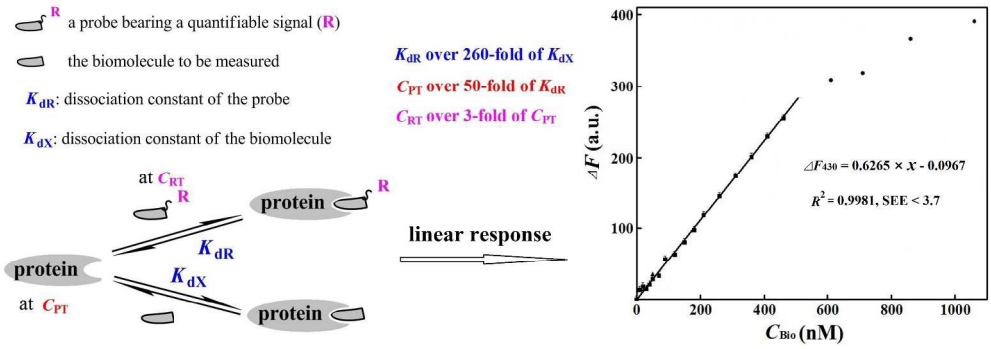

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