Published in final edited form as:

Anal Chem. 2018 September 04; 90(17): 10487–10493. doi:10.1021/acs.analchem.8b02484.

Comprehensive Regression Model for Dissociation Equilibria of Cell-Specific Aptamers

Yifan Lyu^{†,‡,§}, I-Ting Teng[‡], Liqin Zhang^{‡,§}, Yian Guo[‡], Ren Cai^{‡,§}, Xiaobing Zhang[§], Liping Qiu^{*,§}, and Weihong Tan^{*,†,‡,§}

[†] Institute of Molecular Medicine, Renji Hospital, School of Medicine and College of Chemistry and Chemical Engineering, Shanghai Jiao Tong University, Shanghai 200240, People's Republic of China

[§] Molecular Science and Biomedicine Laboratory, State Key Laboratory of Chemo/Biosensing and Chemometrics, College of Chemistry and Chemical Engineering, College of Life Sciences, Aptamer Engineering Center of Hunan Province, Hunan University, Changsha, Hunan 410082, People's Republic of China

[‡] Department of Chemistry and Department of Physiology and Functional Genomics, Center for Research at the Bio/Nano Inter-face, Health Cancer Center, UF Genetics Institute, McKnight Brain Institute, University of Florida, Gainesville, Florida 32611-7200, United States

Abstract

A comprehensive nonlinear regression model for dissociation equilibria of cell-specific aptamers is proposed by considering the effect of receptor expression level. Benefiting from the global regression of simultaneous equations, the fitted parameters reach a very significant level, indicating the statistical validity of this updated model. According to the fitting results, we found that dissociation constants fitted using the previous model are obviously larger than the updated values, which can be explained by the effect of receptor number on curve fitting. In addition, equivalent receptor density can be estimated using the updated model, which may lead to some new judgments about reported results of cell-SELEX.

Aptamers have been used widely as “chemical antibodies” since they were first reported in the 1990s.^{1,2} Benefiting from the basic principle of in vitro selection of aptamers, known as Systematic Evolution of Ligands by Exponential enrichment (SELEX; Figure S1), as well as the diversity of tertiary structures of nucleic acids, the aptamer selection process can be performed against a wide range of targets, including ions, small molecules, peptides, purified proteins, and even whole live cells.^{3–6} For the past few decades, the use of live cells

*Corresponding Authors: tan@chem.ufl.edu. qiuliping@hnu.edu.cn.

Author Contributions

The manuscript was written through the contribution of all authors.

The authors declare no competing financial interest.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.anal-chem.8b02484.

Additional information about the updated regression model for K_d determination, including experimental schemes and methods, some supplementary global regression results, and a table containing sequences used in this experiment (PDF).

as targets for aptamer selection, termed cell-SELEX, which can successfully isolate aptamers without prior knowledge of a cell's molecular signature, such as the number and type of proteins on the cell membrane surface, has gained popularity and made such aptamers promising for applications in molecular medicine.⁷

An aptamer's primary parameter is its dissociation constant (K_d), which is inversely related to the binding affinity between the aptamer and its target. For a cell-specific aptamer, K_d is an equilibrium constant that assesses the tendency of an aptamer—target complex to dissociate into separate components (aptamer and target molecule on the cell surface). The determination of K_d is usually performed with a fixed concentration of either the aptamer or target molecule and an elevated amount of the other component to achieve binding equilibrium data. Several ways have been devised to evaluate this equilibrium experimentally, for example, chromatography,^{8,9} capillary electrophoresis,¹⁰ equilibrium dialysis,¹¹ ultrafiltration,¹² gel electrophoresis,¹³ surface plasmon resonance (SPR),¹⁴ isothermal titration calorimetry (ITC),¹⁵ fluorescence polarization,¹⁶ UV—vis absorption,¹⁷ and circular dichroism (CD).¹⁴ These methods, however, often suffer from experimental drawbacks, such as time and sample consumption, obvious nonspecific adsorption, low sensitivity, inevitable effects of the molecular label or probe structure, and large solution volume. More importantly, most of these methods are not suitable for cell-specific aptamers owing to the unknown target molecule and complex cellular morphology.

Existing methods for K_d determination of cell-specific aptamers, like fluorescence correlation spectroscopy and flow cytometry,⁵ are usually based on nonlinear regression of a previously reported monovalent binding model as

$$Y = B_{\max} \frac{X}{X + K_d} \quad (1)$$

where Y , $B_{\max}$, and X are the amount of bound target (e.g., bound number of moles or fluorescent intensity of cells caused by aptamer binding in flow cytometry, which is linearly related to the bound number of moles), saturated binding of target, and total aptamer concentration, respectively.

Importantly, however, this equation presupposes that the number of aptamers used for incubation with cells exceeds the number of receptors on the cell surface so that the concentration of free aptamer can be approximated by the total aptamer concentration. However, as knowledge about cell phenotypes becomes more available, especially information about the density of surface receptors, it is generally understood that some receptors on cell surfaces are highly expressed. For example, the density of acetylcholine receptors on some cells of the electric ray is in the range of 10^{11} receptors μm^{-2} , which is much higher than the average value of receptor density ($10^3 \sim 10^4$ receptors μm^{-2}).¹⁹ Furthermore, cancer cells, which are typically used as the target cells of cell-specific SELEX, are also reported to have an overexpression of some specific receptors.²⁰ All of these highly expressed, or overexpressed, receptors will generally become potential targets when performing cell-SELEX, and the number of potential targets may be the same, or even

greater than, the number of aptamers in regular experimental conditions. This indicates that the previous model may not be accurate for K_d determination in some cases and that the concentration of bound aptamers should be considered.

In this paper, we propose an updated monovalent binding model (eq 2) of nonlinear curve regression to refine the dissociation constant of cell-specific aptamers by considering the effect of receptor number as

$$Y = \frac{n \cdot B_{\max} + K_d + X - \sqrt{(n \cdot B_{\max} + K_d + X)^2 - 4 \cdot n \cdot B_{\max} \cdot X}}{2 \cdot n} \quad (2)$$

where X is total aptamer concentration, Y is the amount of bound target, $B_{\max}$ is the saturated binding of target, and n is the intensity coefficient (defined in this model), which indicates the relationship between the amount of bound target and the concentration of bound target, as we will discuss in the following sections. Compared with the previously reported model, eq 2 is more reasonable when describing the chemical equilibrium and shows a large nonlinear correlation coefficient according to some statistical tests. K_d values fitted using the new model are smaller than previous values, indicating the effects of receptor number. Although the binding between aptamer and target cell is multivalent, we chose to improve the monovalent binding model to avoid overfitting caused by the power coefficient used for describing multivalent binding.²¹ Different from established models for aptamer-protein interactions, the most conspicuous challenge for describing aptamer-cell interaction is the uncertainty of target concentration. In order to solve this problem, we define the intensity coefficient n and use global regression to fit simultaneous equations. The physicochemical significance of the two constants, $B_{\max}$ and n , is also discussed and further used to give an evaluation of receptor density on the cell surface, as a result of deeper understanding of the dissociation equation. The importance of this work lies in the significant role our updated K_d equation may play in the future development of cell-SELEX and aptamer characterization, since it offers a more reasonable and accurate nonlinear curve regression model for K_d determination, as well as a comprehensive clarification of the dissociation equilibrium. We believe that the strategy we use to build the updated model is also universal for other models and will find broad usage.

EXPERIMENTAL SECTION

DNA Synthesis (Including Normal Oligonucleotides and dZ/dP-Containing Oligonucleotides)

DNA sequences were synthesized using standard phosphoramidite chemistry on glass supports (CPG) on an ABI 394 DNA synthesizer. The synthesis protocol was set up according to the requirements specified by the reagents' manufacturers. Protected dZ and dP phosphoramidites were purchased from Firebird Biomolecular Sciences LLC (Alachua FL, www.firebirdbio.com). Other DNA synthesis reagents were purchased from Glen Research (Sterling, VA).

For oligonucleotides containing dZ and dP, the CPG-bound DMT-off DNA molecules were incubated with acetonitrile/triethylamine (1:1 v/v, 1.5 mL) for 1 h at 25 °C, followed by removal of supernatant. The CPG-bound oligonucleotides were then treated with another 1.5 mL of acetonitrile/triethylamine (1:1 v/v) overnight at 25 °C. After removal of supernatant, the CPG-bound oligonucleotides were incubated with 1.0 mL of DBU in anhydrous CH₃CN (1 M) at room temperature for ~18 h to remove the protecting groups on dZ. After removal of CH₃CN, dZ- and dP-containing oligonucleotides were retreated with NH₄OH (55 °C, overnight). The product mixture was resolved by denaturing PAGE (7 M urea) and extracted with TEAA buffer (0.2 M, pH = 7.0). The product was then desalted by SepPac Plus C18 cartridges (Waters).

For normal oligonucleotides, the CPG-bound DMT on DNA products was deprotected and cleaved from CPG by incubating with 2 mL of AMA (ammonium hydroxide and 40% methylamine, 1:1) for 30 min at 65 °C in a water bath. The cleaved DNA product was transferred to a 15 mL centrifuge tube and mixed with 200 μ L of 3.0 M NaCl and 5.0 mL of ethanol, after which the sample was placed in a freezer at -20 °C for ethanol precipitation. Afterward, the DNA product was spun at 4000 rpm at 4 °C for 30 min. The supernatant was removed, and the precipitated DNA product was dissolved in 400 μ L of 0.1 M triethylamine acetate (TEAA) for HPLC purification. HPLC purification was performed with a cleaned C18 column on an Agilent 1260 HPLC instrument. A solution of 0.1 M TEAA was used as HPLC eluent A, and HPLC-grade acetonitrile from Oceanpak (Sweden) was used as HPLC eluent B. The collected DNA product was dried and processed for detritylation by dissolving and incubating in 200 μ L of 80% acetic acid for 20 min. The detritylated DNA product was mixed with 20 μ L of 3.0 M NaCl and 500 μ L of ethanol and placed in a freezer at -20 °C for 30 min. Afterward, the DNA product was spun at 14000 rpm at 4 °C for 5 min. The DNA product was dried by a vacuum dryer and redissolved in ultrapure water, followed by desalting with desalting columns. The DNA products were quantified using a Nano Drop 2000 spectrophotometer (Thermo Scientific) and stored in ultrapure water for subsequent experiments. The detailed sequences are given in Table S1.

Cell Culture

All cells were obtained from ATCC (American Type Culture Collection, Manassas, VA, U.S.A.). Cell lines HepG2 (liver cancer cell line), CCRF-CEM (Human T-cell ALL), Ramos (Human B-cell Burkitt's lymphoma), and HeLa (cervical adenocarcinoma) were cultured in RPMI 1640 or DMEM medium supplemented with 10% fetal bovine serum (FBS, heat inactivated) and penicillin (100 U/mL) — streptomycin (100 μ g/mL) in a cell culture incubator at 37 °C with 5% CO₂. Cell density was determined by using a TC 10 automated cell counter (Bio-Rad). For adherent cell lines, short-term (30 s⁻¹ min) trypsin treatment was adopted to dissociate cells from the culture flask or dish.

Binding Affinity of Aptamer

Aptamer binding was studied using flow cytometry (BD FACSVerse) and fluorescence correlation spectroscopy. The binding affinity of aptamer was measured by incubating target cells (5×10^5) with a series of fluorophore-labeled aptamers dissolved in a 200 μ L volume of binding buffer (4.5 g/L glucose, 5 mM MgCl₂, 0.1 mg/mL tRNA, and 1 mg/mL BSA, all

in Dulbecco's PBS) containing 10% FBS on ice for 30 min. Cells were then washed twice with 0.6 mL of binding buffer containing 0.1% sodium azide, after which the cells were suspended in 0.4 mL of binding buffer with 0.1% sodium azide and subjected to flow cytometric analysis. The fluorophore-labeled ssDNA library was used as the control sample to determine the nonspecific binding. After subtracting the mean fluorescence value of the control sample, the mean fluorescence intensity of target cells bound with aptamers was used to calculate the equilibrium dissociation constants (K_d) of the aptamer—cell interaction. The apparent K_d of the aptamer—cell interaction was determined by the global regression using SigmaPlot 12.5 (Jandel Scientific, San Rafael, CA). For fluorescence correlation spectroscopy, cells were grown in 8-well Nunc chambers (Nalge Nunc Inc., Naperville, IL) to a density of approximately 1000 cells per well. The cells were washed before and after aptamer incubation with Dulbecco's phosphate buffer (Sigma) containing 5 mM $MgCl_2$. The fluorescence intensity and autocorrelation of bound aptamers on a single cell membrane were monitored for 90 min by using FCS. The apparent K_d of the aptamer—cell interaction was determined by the global regression using SigmaPlot 12.5 (Jandel Scientific, San Rafael, CA).

RESULTS AND DISCUSSION

In order to appreciate the refinement of the updated model for K_d determination, a full understanding of the derivation of the previous model is needed.

For a simple 1:1 binding equilibrium between aptamer and target, we present the following dissociation equation (eq 3):

Here we use [total aptamer], [free aptamer], [bound aptamer], [total target], [free target], and [bound target] to represent the concentrations of total aptamer, unbound aptamer, bound aptamer, total target, unbound target, and bound target, respectively. The dissociation constant K_d can be described as

$$K_d = \frac{[\text{free aptamer}][\text{free target}]}{[\text{bound target}]} \quad (4)$$

where

$$[\text{bound target}] = [\text{bound aptamer}] \quad (5)$$

Eq 4 indicates that the dissociation constant of an aptamer has units of molarity and that the value of K_d is equivalent to the concentration of free aptamer when half the number of targets are bound with aptamers ($[\text{free target}] = [\text{bound target}]$). A lower K_d indicates less dissociation and, hence, higher binding affinity between aptamer and target, just like an antibody/antigen interaction.

Since

$$[\text{total target}] = [\text{free target}] + [\text{bound target}] \quad (6)$$

the ratio of total target concentration to bound target concentration can be shown as

$$\frac{[\text{total target}]}{[\text{bound target}]} = \frac{[\text{free target}] + [\text{bound target}]}{[\text{bound target}]} = \frac{[\text{free target}]}{[\text{bound target}]} + 1 \quad (7)$$

According to eq 4, we know that

$$\frac{[\text{free target}]}{[\text{bound target}]} = \frac{K_d}{[\text{free aptamer}]} \quad (8)$$

Then eq 7 can be transformed into

$$\frac{[\text{total target}]}{[\text{bound target}]} = \frac{K_d}{[\text{free aptamer}]} + 1 = \frac{K_d + [\text{free aptamer}]}{[\text{free aptamer}]} \quad (9)$$

Considering the linear relationship between the amount of bound target (B ; for saturated binding of target, we use $B_{\max}$, as defined above) and concentration of bound target, eq 9 can be subsequently derived as

$$\frac{[\text{total target}]}{[\text{bound target}]} = \frac{B_{\max}}{B} = \frac{K_d + [\text{free aptamer}]}{[\text{free aptamer}]} \quad (10)$$

After further transformation, we obtain eq 11 as [free aptamer]

$$B = B_{\max} \frac{[\text{free aptamer}]}{[\text{free aptamer}] + K_d} \quad (11)$$

According to eq 11, the amount of bound target is described as a function of the concentration of free aptamer. However, free aptamer concentration is difficult to determine owing to the inhomogeneity of cellular samples. For the previous model in eq 1, a key hypothesis holds that the concentration of aptamer is always in large excess compared to that of target for each binding sample, which means that bound aptamer concentration is assumed to be negligible when compared with total aptamer concentration as

$$[\text{total aptamer}] = [\text{bound aptamer}] + [\text{free aptamer}] \approx [\text{free aptamer}] \quad (12)$$

Based on this assumption, we obtain eq 13:

$$B = B_{\max} \frac{[\text{total aptamer}]}{[\text{total aptamer}] + K_d} \quad (13)$$

Hence, eq 1 can be obtained by plotting aptamer concentration as the X axis and bound amount as the Y axis and further used with nonlinear curve regression to determine K_d , as well as $B_{\max}$. In spite of the existence of several alternative methods, flow cytometry (Figure S2) is still the most widely used method for determining the binding affinity of a cell-specific aptamer.⁵ This is because flow cytometry is one of the primary techniques used during cell-SELEX, and the fluorescence signal given by flow cytometry indicates the direct binding performance between aptamer and cell (receptor).

When using flow cytometry for K_d determination, a widely accepted method involves the binding of cells with a gradient of aptamer and library DNA concentrations from low to high. In particular, the mean fluorescence intensity of target cells bound to labeled aptamers is used to calculate the specific binding amount by subtracting the mean fluorescence intensity of nonspecific binding from unselected library DNAs. Since each data point given by flow cytometry describes the overall fluorescence of a single cell, a theoretical linear dependence exists between the amount of bound target and fluorescence intensity. Hence, fluorescence intensity of cells after binding with aptamers can be regarded as B for nonlinear curve regression, and in this case, when the concentration of aptamer is infinitely high, the binding of target by aptamer will reach a saturation point, giving saturated $B_{\max}$. The equilibrium K_d of the aptamer—cell interaction is obtained by fitting the dependence of fluorescence intensity of specific binding on the total aptamer concentration to eq 1.

This model is reasonable when the concentration of total aptamer is always in large excess compared to that of total targets on the cell membrane during the concentration gradient, and it has been used for about 10 years in a considerable number of studies for cell-specific aptamer screening, giving hundreds of K_d values from picomolar to nanomolar range. By ignoring the concentration of bound target, eq 1 attributes all the binding difference to the effect of K_d . However, with our increasing knowledge of cellular morphology and the development of high-resolution imaging, it is clear that the density of aptamer target on the cell surface is so high that the concentration of bound aptamer cannot be ignored, especially for some aptamers with high binding affinity. For example, the density of PTK 7, which is a confirmed target of aptamer *sgc8*, was determined to be 1300 ± 190 receptors/ μm^2 on human leukemia CCRF-CEM cells.¹⁸ Thus, for a sample containing 5×10^5 CEM cells in $200 \mu\text{L}$ of solution, the concentration of PTK 7 can be estimated to be in the nanomolar range, which is sufficient to have an obvious effect on K_d determination and further influence an accurate understanding of binding affinity, as well as a reasonable comparison of aptamers and other binding ligands, such as antibodies, if the previous model is used. This calls for building an updated model by considering the concentration of bound aptamer as

$$[\text{total aptamer}] = [\text{bound aptamer}] + [\text{free aptamer}] \quad (14)$$

Thus, our new derivation starts from a transformation of eq 10 as

$$\frac{[\text{total target}]}{[\text{bound target}]} = \frac{B_{\max}}{B} = \frac{K_d + [\text{total aptamer}] - [\text{bound aptamer}]}{[\text{total aptamer}] - [\text{bound aptamer}]} \quad (15)$$

Here we set

$$[\text{total target}] = n \cdot B_{\max} \quad (16)$$

by introducing the intensity coefficient n , the reciprocal of which describes the average contribution of each unit of bound concentration to the bound amount; therefore,

$$[\text{bound target}] = n \cdot B \quad (17)$$

After substitution of $[\text{bound target}]$ and $[\text{total target}]$, we obtain

$$\frac{B_{\max}}{B} = \frac{K_d + [\text{total aptamer}] - n \cdot B}{[\text{total aptamer}] - n \cdot B} \quad (18)$$

Then we describe B as a function of $[\text{total aptamer}]$ as

$$B = \frac{n \cdot B_{\max} + K_d + [\text{total aptamer}]}{2 \cdot n} - \frac{\sqrt{(n \cdot B_{\max} + K_d + [\text{total aptamer}])^2 - 4 \cdot n \cdot B_{\max} \cdot [\text{total aptamer}]}}{2 \cdot n} \quad (19)$$

By plotting fluorescence intensity (B) on the Y axis and total aptamer concentration as X axis, we obtain the updated model eq 2, as previously introduced:

$$Y = \frac{n \cdot B_{\max} + K_d + X - \sqrt{(n \cdot B_{\max} + K_d + X)^2 - 4 \cdot n \cdot B_{\max} \cdot X}}{2 \cdot n}$$

Here, $B_{\max}$, K_d , and n can be fitted with a data set containing X and Y . Simulated curve shapes of eqs 2 and 1 are shown in Figure 1.

In order to give a convenient and visual comparison, K_d , determined by the previous model eq 13, is denoted as K_{d1} , which can be derived as

$$K_{d1} = \frac{B_{\max} \cdot [\text{total aptamer}]}{B} - [\text{total aptamer}] \quad (20)$$

while K_d , determined by the updated model eq 19, is denoted as K_{d2} , which can be derived as

$$K_{d2} = \frac{B_{\max} \cdot [\text{total aptamer}]}{B} - [\text{total aptamer}] - n \cdot B_{\max} + n \cdot B \quad (21)$$

For the same set of data, we have the same $B_{\max}$ and the same data points, including total concentration of aptamer and amount of bound target. The difference in K_d (K_d) can be calculated by a simple subtraction, as follows:

$$\Delta K_d = K_{d1} - K_{d2} = n \cdot B_{\max} - n \cdot B = [\text{total target}] - [\text{bound target}] \quad (22)$$

indicating that the K_d determined by the previous model is always larger than the true value (Figure 1). For the updated model (eq 2), considering the lower degree of freedom (df), a more reasonable approach involves using global regression to fit simultaneous curves as

$$\begin{aligned} Y_1 &= \frac{n \cdot B_{\max1} + K_{d1} + X_1 - \sqrt{(n \cdot B_{\max1} + K_{d1} + X_1)^2 - 4 \cdot n \cdot B_{\max1} \cdot X_1}}{2 \cdot n} \\ Y_2 &= \frac{n \cdot B_{\max2} + K_{d2} + X_2 - \sqrt{(n \cdot B_{\max2} + K_{d2} + X_2)^2 - 4 \cdot n \cdot B_{\max2} \cdot X_2}}{2 \cdot n} \\ Y_3 &= \frac{n \cdot B_{\max3} + K_{d3} + X_3 - \sqrt{(n \cdot B_{\max3} + K_{d3} + X_3)^2 - 4 \cdot n \cdot B_{\max3} \cdot X_3}}{2 \cdot n} \\ &\vdots \\ Y_m &= \frac{n \cdot B_{\maxm} + K_{dm} + X_m - \sqrt{(n \cdot B_{\maxm} + K_{dm} + X_m)^2 - 4 \cdot n \cdot B_{\maxm} \cdot X_m}}{2 \cdot n} \end{aligned} \quad (23)$$

Here, n is a shared parameter for all equations in eq 23 because n is defined as an intensity coefficient describing the relationship between bound concentration and bound amount. When using other parameters like fluorescence intensity as Y , n is determined by the experimental conditions, for example, the properties of the probe or instrumental parameters, and should be a constant for similar experiments. The global regression of simultaneous curves is quite efficient for elimination of overfitting, which is the major source of errors in fitted parameters. With a sufficient number of samples, the P values in a hypothesis test for n , as well as K_{dm} and $B_{\max}$, given by the global regression of m simultaneous equations gradually reaches a significant level (<0.05 or <0.01).

We use a recently selected aptamer family JHIT for human hepatoma cell HepG2 as the first example²³ to validate the reliability of the updated model. Figure 2 shows the global regression result, as well as the decreasing p -value with increasing numbers of simultaneous equations. With enough curves, it is quite clear that the fitted values of n (Figure 2a), K_d (Figure 2b), and $B_{\max}$ (Figure S3) become extremely significant ($P < 0.01$), indicating the statistical validity of global regression. Because the effect of target concentration was

involved in our model, the fitted K_d was obviously smaller than the previously reported value (dotted line in Figure 2b), which stands in agreement with our theoretical prediction.

Another aptamer family for HepG2 cells reported by our group, the LZH family, is taken as still another example.²⁴ The LZH family was a landmark result obtained using the artificially expanded genetic information system (AEGIS). In spite of the addition of artificial bases Z and P (Z: 6-amino-5-nitro-3-(1'- β -D-2'-deoxyribo-furanosyl)-2(1H)-pyridone; P: 2-amino-8-(1'- β -D-2'-deoxyribofuranosyl)-imidazo[1,2-*a*]-1,3,5-triazin-4(8*H*)-one), containing nitro groups that do not occur in natural DNA (Figure S4),^{25,26} oligonucleotides containing Z and P have been demonstrated to be compatible with PCR amplification²⁷ and deep sequencing technology.²⁸ The generated aptamers using libraries containing Z and P bases will still follow the typical binding equilibrium. As such, they can also be fitted using our updated model. The fitted K_d values are listed in Table 1. Apart from adherent cells like HepG2, data for other aptamer families for suspension cells like Ramos cells were also refitted by the updated model (Figures S5 and S6). Compared with the overestimated values fitted by previous models, the updated K_d values were generally lower. This difference will become much more obvious when the aptamer has a stronger binding affinity and a highly expressed receptor because such binding will give a huge difference between [total aptamer] and [free aptamer]. Here we chose aptamer TE13 with low binding affinity and a receptor with low expression, and we used both the previous and the updated models to fit the binding curve. Both models give a perfect coincidence in the fitted curve and K_d , indicating the small effect of receptor concentration for such a weak aptamer (Figure S7).

Since the coefficient n and $B_{\max}$ can be acquired by global regression, the physicochemical meaning of the chemical equilibrium equation can be further investigated because the concentration of total target in our model can be calculated as an equivalent value. Here, we denote the equivalent target concentration [equivalent target], which is given as

$$[\text{equivalent target}] = n \cdot B_{\max} \quad (24)$$

For a given sample, an average receptor density on a single cell can be determined as an equivalent receptor density. Table 1 gives a summary of the parameters for different aptamers with equivalent receptor density acquired using the updated model.

The calculated equivalent receptor densities are in the range of 10^6 /cell, which is higher than the receptor density in most normal cells (10^3 – 10^4), but in the same range as that for previously reported receptor densities in cancer cells (10^6), which, as previously noted, can be attributed to the typical overexpression of cancer-related receptors in cancer cells. The equivalent receptor density is not the accurate density of aptamer receptors on the cell membrane, but it should be positively correlated with the true value. Theoretically, the equivalent values should be larger than true values because in real samples, the receptor has an extremely high local concentration on the cell surface, which will have a positive effect on binding affinity. Not all research groups employing cell SELEX may be equipped with instruments for receptor density determination; therefore, this equivalent value will help to

give a first-time comparison of receptor densities. In addition, the equivalent receptor density should be of great value when, for example, judging the feasibility of further receptor identification by considering both binding affinity and receptor density, or it can even be used as part of the evidence to study whether some screened aptamers bind with the same receptors when they are calculated to have similar receptor densities (Figure 3).

Another investigation inspired by the updated equilibrium equation is the relationship between expression level of receptors and shift of flow cytometry peaks. By using the intensity coefficient n , the saturated shift of the flow peak is directly related to the expression level of receptors. From this viewpoint, when screening cell-specific aptamers, flow cytometry results with different shifts should be given equivalent attention because all potential receptors should have significant biological and theranostic meaning, even though their expression levels may vary by 1 or 2 orders of magnitude.

Our updated model was then tested by directly fitting bound aptamer numbers acquired by fluorescence correlation spectroscopy (FCS) because of the high sensitivity of FCS as a biophysical tool for studying molecular interactions on live cells.¹⁸ The femtoliter-sized observation volume created by FCS allows fluorescence-aptamer detection down to two molecules so that the binding number can be accurately determined (Figure S8). The regression results are shown in Figure 4. The updated model gives a K_d of 263 ± 86 pM ($P < 0.001$), while the previous model gives a much larger K_d of 936 ± 290 pM. This difference can be explained by the effect of target concentration, as we have mentioned before.

In summary, the updated model proposed in this paper offers a comprehensive approach to understand the binding equilibrium between a cell-specific aptamer and target cell by considering the concentration of total receptors on the cell membrane. When used for K_d determination, the effect of target concentration can be involved, especially for aptamers with high binding affinities and highly expressed receptors. Since this is a more detailed model, a more accurate value of K_d can be fitted, and additional information about receptor density can be acquired. Benefiting from the power of global regression, a significant degree of relevance for each parameter was observed by analyzing several sets of data, indicating the reasonability and credibility of this updated model. With the help of fitted intensity coefficient n , the calculated equivalent receptor density could become a universal and comparable parameter between different batches. Apart from the global regression used in the current paper to acquire the n value, there should be many other experimental approaches to determine this coefficient, because this coefficient, as mentioned above, is determined in part by experimental conditions and signal intensity. This updated model for equilibria of aptamer binding could also change the way we judge the reported results of cell-SELEX.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

This work is supported by Renji Hospital, and the National Institutes of Health Maximizing Investigators' Research Award R35 127130i, NSF1645215, and NSFC Grants (21521063). We thank Qiuqi Li for her valuable suggestions on this paper.

REFERENCES

- (1). Tuerk C; Gold L Science 1990, 249, 505–510. [PubMed: 2200121]
- (2). Ellington AD; Szostak JW Nature 1990, 346, 818. [PubMed: 1697402]
- (3). HuiZenga DE; Szostak JW Biochemistry 1995, 34, 656–665. [PubMed: 7819261]
- (4). Bock LC; Griffin LC; et al. Nature 1992, 355, 564. [PubMed: 1741036]
- (5). Shangguan D; Li Y; Tang Z; Cao ZC; Chen HW; Mallikaratchy P; Sefah K; Yang CJ; Tan W Proc. Natl. Acad. Sci. U. S. A 2006, 103, 11838–11843. [PubMed: 16873550]
- (6). Li S; Xu H; Ding H; Huang Y; Cao X; Yang G; Li J; Xie Z; Meng Y; Li XJ Pathol. 2009, 218, 327–336.
- (7). Xiong X; Lv Y; Chen T; Zhang X; Wang K; Tan W Annu. Rev. Anal. Chem 2014, 7, 405–426.
- (8). Deng Q; German I; Buchanan D; Kennedy RT Anal. Chem 2001, 73, 5415–5421. [PubMed: 11816567]
- (9). Hage DS; Tweed SAJ Chromatogr., Biomed. Appl 1997, 699, 499–525.
- (10). Drabovich AP; Berezovski M; Okhonin V; Krylov SN Anal. Chem 2006, 78, 3171–3178. [PubMed: 16643010]
- (11). Cruz-Aguado JA; Penner GJ Agric. Food Chem 2008, 56, 10456–10461.
- (12). Hall B; Arshad S; Seo K; Bowman C; Corley M; Jhaveri SD; Ellington AD Current protocols in nucleic acid chemistry 2010, 9.3. 1–9.3. 27.
- (13). Jaouen S; De Koning L; Gaillard C; Muselřková-Polanská E; Štros M; Strauss FJ Mol. Biol 2005, 353, 822–837.
- (14). Lin P-H; Chen R-H; Lee C-H; Chang Y; Chen C-S; Chen W-Y Colloids Surf., B 2011, 88, 552–558.
- (15). Potty AS; Kourentzi K; Fang H; Jackson GW; Zhang X; Legge GB; Willson RC Biopolymers 2009, 91, 145–156. [PubMed: 19025993]
- (16). Cruz-Aguado JA; Penner G Anal. Chem 2008, 80, 8853–8855. [PubMed: 18947191]
- (17). Guédin A; Lacroix L; Mergny J-L Methods Mol. Biol 2010, 613, 25–35. [PubMed: 19997875]
- (18). Chen Y; Munteanu AC; Huang YF; Phillips J; Zhu Z; Mavros M; Tan W Chem. - Eur. J 2009, 15, 5327–5336. [PubMed: 19360825]
- (19). Miyazawa A; Fujiyoshi Y; Unwin N Nature 2003, 423, 949–955. [PubMed: 12827192]
- (20). Chen YN; Mickley LA; Schwartz AM; Acton EM; Hwang J; Fojo AT J. Biol. Chem 1990, 265, 10073–10080. [PubMed: 1972154]
- (21). Gokulrangan G; Unruh JR; Holub DF; Ingram B; Johnson CK; Wilson GS Anal. Chem 2005, 77, 1963–1970. [PubMed: 15801725]
- (22). Wilmes S; Staufienbiel M; Liße D; Richter CP; Beutel O; Busch KB; Hess ST; Piehler J Angew. Chem., Int. Ed 2012, 51, 4868–4871.
- (23). Xu J; Teng IT; Zhang L; Delgado S; Champanhac C; Cansiz S; Wu C; Shan H; Tan W PLoS One 2015, 10, e0125863. [PubMed: 25938802]
- (24). Zhang L; Yang Z; Sefah K; Bradley KM; Hoshika S; Kim M-J; Kim H-J; Zhu G; Jimenez E; Cansiz S; Teng IT; Champanhac C; McLendon C; Liu C; Zhang W; Gerloff DL; Huang Z; Tan W; Benner SA J. Am. Chem. Soc 2015, 137, 6734–6737. [PubMed: 25966323]
- (25). Georgiadis MM; Singh I; Kellett WF; Hoshika S; Benner SA; Richards NG J. J. Am. Chem. Soc 2015, 137, 6947–6955.
- (26). Yang Z; Hutter D; Sheng P; Sismour AM; Benner SA Nucleic Acids Res. 2006, 34, 6095–6101. [PubMed: 17074747]
- (27). Yang Z; Chen F; Chamberlin SG; Benner SA Angew. Chem., Int. Ed 2010, 49, 177–180.

- (28). Yang Z; Chen F; Alvarado JB; Benner SA J. Am. Chem. Soc 2011, 133, 15105–15112. [PubMed: 21842904]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 1. Curves of eqs 1 (a) and 2 (b). Curve domains in green regions are used for nonlinear fitting. K_d indicates the real dissociation constant, while K_d' indicates the dissociation constant fitted by each curve. For eq 1, the fitted K_d' is always larger than the real K_d .

Figure 2.

Global regression to determine K_d using the updated model (eq 2) and data for aptamer family JHIT for human hepatoma HepG2. (a) With an increasing number of simultaneous equations, the fitted n value gradually reaches a very significant level. (b) With an increasing number of simultaneous equations, the fitted K_d gradually reaches a very significant level. The dotted level indicates the K_d value fitted using the previously reported model (eq 1), which is obviously larger (* $P < 0.1$; ** $P < 0.05$, *** $P < 0.01$).

Figure 3.

Calculated equivalent receptor densities of JHIT family and LZH family. Values are listed from low to high so that aptamers with similar equivalent receptor densities can be easily distinguished.

Figure 4. Comparison of regression results between the updated model and previous model. (a) Regression results using eq 2. (b) Regression results using eq 1.

Some Aptamers with Updated K_d and Equivalent Receptor Density

Table 1.

name of aptamer	target cell	previous K_d (nM)	updated K_d (nM)	equiv receptor density ($\times 10^6$ per cell)
JHIT-1	HepG2	206.6 $\pm$ 35.5	191.2 $\pm$ 26.0	3.80–9.69
JHIT-2	HepG2	64.4 $\pm$ 11.0	31.7 $\pm$ 7.2	6.22–15.85
JHIT-3	HepG2	272.1 $\pm$ 22.7	218.8 $\pm$ 67.0	10.37–26.40
JHIT-4	HepG2	349.0 $\pm$ 69.9	336.5 $\pm$ 47.0	2.57–6.54
JHIT-5	HepG2	334.0 $\pm$ 47.6	305.5 $\pm$ 37.7	5.10–12.98
JHIT-6	HepG2	249.4 $\pm$ 83.6	228.1 $\pm$ 46.2	4.25–10.83
JHIT-7	HepG2	289.0 $\pm$ 56.5	261.9 $\pm$ 37.1	4.91–12.50
LZH-1	HepG2	14.0 $\pm$ 3.1	3.9 $\pm$ 2.9	3.54–9.01
LZH-2	HepG2	24.2 $\pm$ 3.7	18.5 $\pm$ 3.4	1.32–3.36
LZH-3	HepG2	24.3 $\pm$ 4.8	10.3 $\pm$ 3.3	2.68–6.83
LZH-4	HepG2	36.0 $\pm$ 4.9	22.3 $\pm$ 4.8	3.40–8.66
LZH-5	HepG2	41.4 $\pm$ 7.1	19.9 $\pm$ 5.2	5.99–15.27
LZH-6	HepG2	47.2 $\pm$ 5.1	24.9 $\pm$ 4.5	5.97–15.22
LZH-7	HepG2	55.1 $\pm$ 7.6	37.5 $\pm$ 6.1	4.12–10.50
LZH-8	HepG2	95.5 $\pm$ 10.5	83.8 $\pm$ 9.0	2.16–5.51
LZH-9	HepG2	214.2 $\pm$ 26.2	204.7 $\pm$ 41.1	1.64–4.17
LZH-10	HepG2	233.6 $\pm$ 53.3	216.9 $\pm$ 49.6	3.38–8.61
LZH-11	HepG2	246.7 $\pm$ 24.9	221.4 $\pm$ 26.1	4.50–11.46
LZH-12	HepG2	298.3 $\pm$ 37.4	284.9 $\pm$ 32.5	2.23–5.67
LZH-13	HepG2	326.9 $\pm$ 53.4	318.6 $\pm$ 76.6	1.34–3.42
LZH-14	HepG2	346.3 $\pm$ 50.5	325.6 $\pm$ 54.8	3.21–8.17
LZH-15	HepG2	727.1 $\pm$ 83.6	674.3 $\pm$ 87.9	6.60–16.81
LZH-16	HepG2	964.8 $\pm$ 294.0	942.9 $\pm$ 287.5	2.34–5.97
LZH-17	HepG2	2549.2 $\pm$ 1118.3	2516.3 $\pm$ 1192.7	2.61–6.64