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Design and Optimisation of Photochrome Aptamer Switch Assay (PHASA)

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ABSTRACT

Photochrome Aptamer Switch Assay (PHASA) is our unique and recently developed fluorescent biosensing platform that exploits the combination of aptamer adaptive binding and photoisomerisation kinetics of stilbenes. The PHASA is based on converting any suitable stilbene-ligand-aptamer complex into a reversible biosensor. Binding affinity, aptamer concentration, and stilbene molecular structure determine the PHASA performance criteria. Consequently, understanding of the structure-activity relationship of the stilbene-ligand conjugate is extremely important when predicting and optimising the biosensor sensitivity. In the present manuscript, new analytical methods relating to calculation of the limit of detection (LOD) of the PHASA are proposed and discussed. Using these methods, it is possible to optimise the independent or dependent physicochemical parameters of the stilbene-ligand conjugates and evaluate their general sensing performance. The proposed analytical methods are based on the inter-dependent relationships between the sensitivity and the range of analyte detection and provide starting conditions for known aptamer binding constants in various PHASA applications.

Keywords:

Aptamer, Stilbene, Photochrome Aptamer Switch Assay (PHASA), Limit of detection, Fluorescence assay, Photoisomerisation

1. Introduction

Biosensors are commonly used today in medical diagnostics, drug discovery, environmental monitoring, and food safety testing. Fluorescence spectroscopy is considered to be one of the most sensitive biosensing techniques [1-4]. A new biosensing technique, known as Photochrome Aptamer Switch Assay (PHASA), recently developed by our team, offers a simple and rapid analytical method for fluorescence sensing of analytes in the process of adaptive binding in stilbene-ligand-aptamer complexes [5, 6]. The PHASA allows state-of-the-art one-step analyte detection in several minutes and may overcome many common problems of the existing fluorescence methods, such as prolonged time and complexity of the fluorescence bioassays, signal dependence on fluorescence intensity, and consequently, the relatively low signal-to-noise ratio (S/N) at low concentration of analytes, and strong ambient interference.

The PHASA outperforms also regular immunoassays in many ways. The advantages of using aptamers in the PHASA are inherent to their initial design. Too many disadvantages of antibodies used in an immunoassay are easily foreseen. Antibodies denature quite easily, their decomposition makes limited shelf-lives and most antibodies have strong background fluorescence. Moreover, the PHASA is essentially a "no-separation" assay, whereas for comparison, the regular ELISA test for bisphenol detection in water, as an example, may take up to 6 hours due to many steps of separation and washing. Aptamers are relatively small in size, can be stable in organic/aqueous solvents, and achieve low lot-to-lot variability [7-9]. Therefore, they are a more logical choice for bioassays than antibodies, including the fact that higher surface densities can be placed in a microtiter plate well - the

higher the density, the greater the sensitivity, especially when combined with metal enhanced fluorescence.

The PHASA combines the advantages of the aptamer adaptive binding assay [5, 6, 10-12] and fluorescence-photochrome assay based on stilbene photoisomerisation [13-18]. The PHASA relies on the photoisomerisation of a fluorescent *trans*-stilbene to a non-fluorescent *cis*-stilbene - an intramolecular process taking less than several nanoseconds. The switchable reporter entity of the PHASA is thus comprised of the stilbene molecule conjugated to analyte (ligand) of interest [5, 6]. The stilbene reporter molecule is unique in the way that it can reversibly adapt in solution to any of its stereochemical configurations upon UV irradiation at the excitation maximum of the corresponding isomer. In case of an unsubstituted stilbene molecule in a model liquid solvent of relatively low polarity and viscosity, the stereochemical *trans*-isomer is strongly fluorescent, while the *cis*-isomer is non-fluorescent. The light-induced process of *trans-cis* photoisomerisation acts like a quenching funnel on fluorescence of the stilbene molecule, which is typically equilibrated in solution toward a more stable *trans*-isomer. Therefore, irradiation of the fluorescent *trans*-form of the reporter stilbene molecule at its excitation maximum produces a substantial change of the fluorescence emission as the excited mixture of the isomers reaches its new steady-state equilibrium, the process that can be monitored as a time trace signal (fluorescence kinetic decay) or emission gradient under constant-illumination conditions.

It has been previously demonstrated [13-15, 17] that the reaction dynamics of the *trans-cis* photoisomerisation reaction is strongly dependent on the properties of surrounding micro-environment. The reported results clearly indicate that the stilbene molecule has serious steric constraints for the light induced *trans-cis* isomerisation within various micro-

environments [13-15, 17]. If the highly fluorescent *trans*-isomer of the stilbene molecule cannot undergo any geometrical changes, it cannot be transformed into its non-fluorescent *cis*-form, and no change in fluorescence decay (photoisomerisation) occurs.

As shown in **Fig. 1A**, the PHASA for analyte detection and concentration measurement in a sample is based on the competitive binding of the free analyte molecules present in the sample to their binding site in the stilbene-ligand-aptamer complex. The ligand is actually an analyte molecule conjugated to stilbene. Initially, the *trans*-stilbene-ligand conjugate forms a complex with the aptamer of interest. Being squeezed in the aptamer binding site, the fluorescent *trans*-isomer exhibits very slow fluorescence decay upon irradiation at its excitation maximum. In other words, the aptamer inhibits the fluorescence decay of the *trans*-stilbene-ligand conjugate. Introduction of a sample solution containing the analyte molecules results in their competitive binding to the aptamer. This event subsequently releases the fluorescent *trans*-stilbene-ligand conjugate molecules from the aptamer active site into solution. What follows is a significant acceleration of the fluorescence decay of the *trans*-isomer upon irradiation with the excitation light, because of the rapid conversion of the *trans*-isomer to its non-fluorescent *cis*-isomer. The latter connects to the apparent *trans-cis* photoisomerisation rate constant k_{app} of the stilbene molecule that is experimentally measured by the steady-state fluorescence technique [13, 14]. The exact value of the analyte concentration in the sample is obtained from the calibration of the *trans-cis* photoisomerisation rate constant, since it is dependent on the concentration of the analyte. It actually indicates an equilibrium between the free and bound stilbene-ligand conjugate. This requires the constant k_{app} be independent of fluorescence intensity.

The PHASA is dependent on various aptamer parameters, such as total aptamer concentration and dissociation constant, that significantly affect assay sensitivity and detection ranges (see **Fig. 1A** and **1B**). Understanding correlations between these parameters allows choosing the proper transducer design for a specific PHASA-based biosensor, for example fibre optics or lateral flow membrane [19, 20]. In the present manuscript, new analytical methods relating to the PHASA are discussed. These methods assess the experimental values of the dissociation constant K_d within the known reagent concentration ranges. This allows choosing the proper aptamer/conjugate ratios required to perform the PHASA and predict the limit of detection (LOD) of the PHASA-based biosensor. The developed analytical models may provide a theoretical framework towards future applications of the PHASA-based biosensors.

Fig. 1. A: Schematic illustration of the PHASA competitive binding format, which is based on the competitive binding of the free analyte molecules present in the sample to their binding site in the stilbene-ligand-aptamer complex. Initially, the *trans*-stilbene-ligand conjugate forms a complex with the aptamer of interest and exhibits very slow fluorescence decay upon irradiation at its excitation maximum. Introduction of a sample containing the analyte molecules results in their competitive binding to the aptamer, subsequent release of the fluorescent *trans*-stilbene-ligand conjugate molecules from the aptamer active site into solution and significant acceleration of the fluorescence decay. B: The exemplary fluorescence decay curves of a stilbene-ligand conjugate in the PHASA competitive binding format. The pink curve shows the fluorescence decay of the stilbene-ligand conjugate complexed to an aptamer. In this case, the ideal concentration of the free

stilbene-ligand conjugate in solution is zero ($[\text{CON}] = 0$). The dark yellow curve shows the fluorescence decay of the stilbene-ligand conjugate completely released into solution ($[\text{CON}] = [\text{CON}]_{\text{T}}$). The limit of detection (LOD) is indicated by the light blue band, while the sensing capacity is indicated by the orange band. To measure a reliable signal, the background noise should be taken into account, and this will narrow down the sensing window to be between the LOD and sensing capacity. An acceptable sensing signal-to-noise ratio (S/N) should be no less than 3 (or the 3σ criterion), which defines the LOD and sensing capacity in PHASA.

2. Methods and Results

2.1. Calculation of the amount of the stilbene-ligand conjugate complexed to the aptamer

To establish the binding relationship between an aptamer and stilbene-ligand conjugate, the following method of data analysis is proposed. The equilibrium reaction between the aptamer and stilbene-ligand conjugate can be written as follows, where *APT*, *CON* and *APT·CON* represent aptamer, conjugate and stilbene-ligand-aptamer complex, respectively:

The total concentration of the aptamer in the assay $[APT]_T$ is actually a sum of the concentrations of the free aptamer $[APT]$ (without the complexed stilbene-ligand conjugate) and the stilbene-ligand-aptamer complex $[APT \cdot CON]$:

$$[APT]_T = [APT] + [APT \cdot CON] \quad [2]$$

Similarly, the total concentration of the stilbene-ligand conjugate $[CON]_T$ is the sum of the concentrations of the free stilbene-ligand conjugate in solution $[CON]$ and the stilbene-ligand-aptamer complex $[APT \cdot CON]$ in the assay:

$$[CON]_T = [CON] + [APT \cdot CON] \quad [3]$$

According to definition, the dissociation constant K_d of the stilbene-ligand-aptamer complex can be calculated as:

$$K_d = \frac{[APT] \times [CON]}{[APT \cdot CON]} \quad [4]$$

The fraction α of the stilbene-ligand conjugate bound to the aptamer is:

$$\alpha = \frac{[APT \cdot CON]}{[CON]_T} \quad [5]$$

Inserting the above values of $[APT]_T$, $[CON]_T$ and α from the Equations [2], [3] and [5] into the Equation [4] will yield the following equation for the dissociation constant:

$$K_d = \frac{\left(\frac{[APT]_T}{[CON]_T} - \alpha\right) \times (1 - \alpha) \times [CON]_T}{\alpha} \quad [6]$$

Thus, it is possible to calculate the fraction α (being in the range between 0 and 1 inclusively) by solving the Equation [6] as follows:

$$\alpha = 0.5 \times (A + B + 1) - 0.5 \times \sqrt{(A + B + 1)^2 - 4 \times B} \quad [7],$$

where A and B are defined as:

$$A = \frac{K_d}{[CON]_T} \quad [7'],$$

$$B = \frac{[APT]_T}{[CON]_T} \quad [7'']$$

2.2. Calculation of the analyte concentration

To calculate the analyte (ligand) concentration $[LIG]_T$ in a sample, it is necessary to solve the system of the two following chemical kinetics equations, where *LIG* and *APT·LIG* stand for ligand and ligand-aptamer complex, respectively:

The system of these two equations represents the competitive reaction between the analyte molecules and the stilbene-ligand-aptamer complex for the aptamer binding site. The initial total concentration of the analyte (ligand) in the sample $[LIG]_T$ can be expressed as a sum

of concentrations of the free (unbound) analyte [LIG] remaining in solution after its binding to the aptamer and the formed ligand-aptamer complex [APT · LIG]:

$$[\text{LIG}]_{\text{T}} = [\text{LIG}] + [\text{APT} \cdot \text{LIG}] \quad [9]$$

Then, the total concentration of the aptamer in the assay [APT]_T after the competitive reaction occurs will be as follows:

$$[\text{APT}]_{\text{T}} = [\text{APT}] + [\text{APT} \cdot \text{LIG}] + [\text{APT} \cdot \text{CON}] \quad [10]$$

The dissociation constant K_d^L of the ligand-aptamer complex can be calculated as:

$$K_d^L = \frac{[\text{APT}][\text{LIG}]}{[\text{APT} \cdot \text{LIG}]} \quad [11]$$

Thus, by combining the Equation [4], the dissociation constants' ratio is obtained as follows:

$$\frac{K_d}{K_d^L} = \frac{[\text{CON}][\text{APT} \cdot \text{LIG}]}{[\text{LIG}][\text{APT} \cdot \text{CON}]} \quad [12]$$

By rearranging Equation [12], the concentration of the formed ligand-aptamer complex can then be expressed as:

$$[\text{APT} \cdot \text{LIG}] = \frac{K_d[\text{LIG}][\text{APT} \cdot \text{CON}]}{K_d^L[\text{CON}]} \quad [13]$$

Now, inserting the Equation [13] into the Equation [9], the initial total concentration of the ligand in the sample [LIG]_T will be:

$$[\text{LIG}]_{\text{T}} = [\text{LIG}] \left(1 + \frac{K_d[\text{APT} \cdot \text{CON}]}{K_d^L[\text{CON}]} \right) \quad [14]$$

Again, taking into account the Equation [3] representing the relationship between the total concentration of the stilbene-ligand conjugate [CON]_T and the stilbene-ligand-aptamer complex [APT · CON], the Equation [14] can be re-written as:

$$[\text{LIG}]_{\text{T}} = [\text{LIG}] \left(1 + \frac{K_d([\text{CON}]_{\text{T}} - [\text{CON}])}{K_d^L[\text{CON}]} \right) \quad [15]$$

Solving the Equation [15] for the concentration [LIG] of the free (unbound) analyte remaining in solution after its binding to the aptamer, will result in:

$$[\text{LIG}] = \frac{K_d^L [\text{LIG}]_T [\text{CON}]}{K_d^L [\text{CON}] + K_d ([\text{CON}]_T - [\text{CON}])} \quad [16]$$

Now, on the other hand, taking the [APT] concentration from the Equation [4] and combining it with the ligand-aptamer complex concentration [APT · LIG] from the Equation [13], the total aptamer concentration [APT]_T represented by the Equation [10] can be rearranged as follows:

$$[\text{APT}]_T = [\text{APT} \cdot \text{CON}] \left(\frac{K_d}{[\text{CON}]} + \frac{K_d [\text{LIG}]}{K_d^L [\text{CON}]} + 1 \right) \quad [17]$$

Since according to the Equation [3], the total concentration of the stilbene-ligand conjugate, complexed to the aptamer and free in solution, [CON]_T is the sum of the concentrations of the free stilbene-ligand conjugate in solution [CON] and the stilbene-ligand-aptamer complex [APT · CON], the total concentration of the aptamer in solution [APT]_T can then be expressed as:

$$[\text{APT}]_T = ([\text{CON}]_T - [\text{CON}]) \left(\frac{K_d}{[\text{CON}]} + \frac{K_d [\text{LIG}]}{[\text{CON}] K_d^L} + 1 \right) \quad [18]$$

Combining the Equations [16] and [18], we can finally calculate the total concentration of the analyte in the sample [LIG]_T using only the known and measurable values:

$$[\text{LIG}]_T = \frac{1}{K_d} \left(\frac{[\text{APT}]_T}{[\text{CON}]_T - [\text{CON}]} - \frac{K_d}{[\text{CON}]} - 1 \right) (K_d^L [\text{CON}] + K_d ([\text{CON}]_T - [\text{CON}])) \quad [19]$$

The values K_d , K_d^L , [APT]_T and [CON]_T are normally known or predetermined, while the concentration of the free stilbene-ligand conjugate in solution [CON] can be calculated from the apparent *trans-cis* photoisomerisation rate constant k_{app} of the stilbene which is

experimentally measured by the steady-state fluorescence technique [13, 14]. Thus, the concentration of the analyte in the sample $[LIG]_T$, and consequently, the LOD of the sensor, can be calculated according to the Equation [19] based on the measurable k_{app} of the stilbene-ligand conjugate.

2.3. Experimentally assessing the α and B values

The present format of the PHASA, based on the combined adaptive and competitive binding of an analyte to an aptamer, requires the initial formation of the stilbene-ligand-aptamer complex. Therefore, the simplified chemical kinetics model described above is initially applied to formation of this complex, where the variation in a pre-bound stilbene-ligand conjugate will tune the balance between sensitivity and detection range of the assay. For example, the almost completely pre-bound conjugate provides a wide detection range but on account of decreasing sensitivity, and vice versa. To ensure practical sensing, some assumptions are empirically made.

For instance, 50-90% of the bound conjugate is required for the initial conditions of the competitive assay. That gives some flexibility based on the application, where 50% of the bound conjugate allows more sensitivity and 90% ensures a wide range of the analyte detection. Additionally, K_d should not be more than 2 μM for successful binding and for keeping concentration of the stilbene fluorophores in the sub-micromolar range, and $[APT]_T$ should not be more than 10 μM because of solubility and cost. Furthermore, $[CON]_T$ should be maximised, as the increase of $[CON]_T$ results in a strong enhancement of the fluorescence intensity, and consequently high signal-to-noise ratio (S/N). Taking into account the relatively low quantum yields of most of the stilbene derivatives in aqueous

environments, the limit of detection of a conventional spectrofluorometer requires sub-micromolar $[\text{CON}]_{\text{T}}$ concentration. To illustrate, $[\text{CON}]_{\text{T}}$ of 500 nM results in the stilbene concentration range of 50-250 nM at 50-90% of the bound conjugate. This is our defined range of initial conditions. This concentration range is compared to the empirical data for two aqueous stilbene derivatives (see **Fig. 2**):

4'-*N,N*-dimethylamino-4-aminostilbene (**DMAAS**) [13-15], and

4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid disodium salt (**SITS**) [15].

The method of the fluorescence decay measurements used in the present experiments has already been described in detail in our previous work [4, 13 and 14]. **DMAAS** at its concentration of 100 nM exhibits the S/N ratio of 200 ($\Delta\text{FLU}/\text{STD-DEV}$). **SITS** at its concentration of 1 μM (ten times the concentration of **DMAAS**) shows a much longer and much noisier decay with S/N ratio of 125 ($\Delta\text{FLU}/\text{STD-DEV}$). The initial fluorescence intensity of **SITS** is however only about twice the initial fluorescence of **DMAAS** because of the **SITS**'s much lower fluorescence quantum yield (see **Fig. 2**). These results imply that the PHASA may require the application of a certain concentration of stilbene fluorophore in the assay in order to maintain the fluorescence intensity and fluorescence decay, as well as to reduce the noise.

Fig. 2. Fluorescence decay curves of 100 nM **DMAAS** (1) and 1 μM **SITS** (2). The fluorescence decay curves were recorded in dH_2O at room temperature and at the constant illumination conditions with SHIMADZU RF-5301PC spectrofluorophotometer. The stilbenes were dissolved in 5% DMSO/ dH_2O . **DMAAS** (1) at 100 nM concentration gives $\text{S/N} = 200$. **SITS** (2) at 1 μM concentration exhibits much slower fluorescence

decay with S/N = 125.

According to the Equations [7], [7'] and [7''], with a given $[\text{CON}]_T$, α can be calculated as a function of the two parameters: K_d and B (the ratio of the starting reagents), which is illustrated in **Fig. 3**. In practical experiments, the value of K_d is known, and the concentration ratio B is defined by a practitioner. The relationship between the reagent concentration and B ratio can then be optimised to determine the specific ratio of the bound stilbene-ligand conjugate for applicable sensitivity and detection range. For example, in order to yield a 90% pre-bound stilbene-ligand conjugate, the following parameters should be chosen: $K_d = 500$ nM, $[\text{APT}]_T = 9$ μM , and B is calculated to be 1.8 (see the dotted blue lines in **Fig. 3B**). On the other hand, **Fig. 3** also suggests that the correlation between the parameters B and α is dependent on both $[\text{CON}]_T$ (as seen in **Fig. 3A** and **3B**) and K_d (as seen in **Fig. 3C** and **3D**). Thus, the optimal value of B can be found by calibrating the total conjugate concentration or optimising the K_d value, which helps extending the application range of the assay.

Fig. 3. Illustration of the relationship between the fraction α of the stilbene-ligand conjugate bound to the aptamer and the B ratio of the aptamer to stilbene-ligand conjugate concentrations, with the known K_d and conjugate concentration values.

A: $[\text{CON}]_T = 1$ μM , while K_d varies from 50 nM to 2 μM .

B: $[\text{CON}]_T = 5$ μM , while K_d varies from 50 nM to 2 μM .

C: $K_d = 1$ μM , while $[\text{CON}]_T$ varies from 50 nM to 5 μM .

D: $K_d = 2 \mu\text{M}$, while $[\text{CON}]_T$ varies from 50 nM to 5 μM .

As noted above, the higher is $[\text{CON}]_T$, the stronger is a fluorescence signal, and as a result, the higher is a S/N ratio. As shown in **Fig. 1**, the apparent rate constant k_{app} is calculated by fitting the first-order fluorescence decay, where the S/N ratio determines the standard deviation σ of the k_{app} value, and consequently, the limit of detection (LOD) and sensing window (see **Fig. 1B**). In general, the S/N ratio ≥ 3 is required to define the LOD. This is so called the "3 σ criterion". On the other hand, if there is a stilbene-ligand conjugate having a high fluorescence quantum yield, the higher $[\text{CON}]_T$ produces high fluorescence intensity signals which result in less noise (σ), ultimately lowering the LOD. Thus, a strategy for obtaining smaller LOD is to maximize $[\text{CON}]_T$ or fluorescent intensity.

2.4. Prediction and optimisation of the LOD and detection range

In the present format of the PHASA based on the combination of adaptive and competitive binding of an analyte to an aptamer, the k_{app} constant correlates to $[\text{CON}]$, which is the concentration of the free (unbound) stilbene-ligand conjugate released from the aptamer complex into solution by the analyte molecules competing for the binding of the aptamer (see **Fig. 1A**). Therefore, the relationship between $[\text{LIG}]_T$ and $[\text{CON}]$ presented in Equation [19] will allow predicting the LOD and sensing window/detection range. The $[\text{CON}]$ value is measured empirically by determining the k_{app} constant using the constant-illumination steady-state fluorescence technique developed by the present authors [4, 13-15, 17, 21].

Consequently, the empirically measured $[\text{CON}]$ is used to calculate $[\text{LIG}]_T$ and further predict the LOD of the particular analyte. In the present empirical calculations, the values

of K_d , K_d^L , $[APT]_T$ and $[CON]_T$ are static and user-defined. **Fig. 4** displays representative curves with empirically relevant values of $K_d^L = 200$ nM, and $[CON]_T = 5$ μ M. When the values K_d or $[APT]_T$ are varied (see **Fig. 4A-4F**), suitable amount of the added analyte $[LIG]_T$ (plotted as a Y axis) is required to release the same or corresponding amount of the stilbene-ligand conjugate $[CON]$ (plotted as an X axis). Such release is observed as an acceleration of the steady-state fluorescence decay under constant illumination conditions and can be used to predict the LOD of the assay.

In order to predict the LOD, we assume that $k_{app} > k_{app}^o + 3\sigma$ ($S/N > 3$), where k_{app}^o is the *trans-cis* photoisomerisation rate constant of the stilbene-ligand conjugate bound to the aptamer. In other words, we assume that the fluorescence signal corresponding to $[CON]$ is significantly higher than the background signal (or noise) and therefore, the fluorescence measurements in the assay can be reliable. In that case, $[LIG]_T$ required to release the proposed amount of $[CON]$ for achieving $k_{app} > k_{app}^o + 3\sigma$ is the LOD of the assay.

Similarly, assuming that at a certain value of $[CON]$ for the stilbene-ligand conjugate released into solution, $k_{app} > k_{app}^f - 3\sigma$, where k_{app}^f is the photoisomerisation rate constant of the free stilbene-ligand conjugate in solution (when the infinite amount of the analyte is added), the value $[LIG]_T$ releasing this $[CON]$ is the upper limit of detection defining the entire detection range (see **Fig. 1B**).

As shown in **Fig. 4C**, for example, when $K_d^L = 200$ nM, $K_d = 500$ nM, $[CON]_T = 5$ μ M, $[APT]_T = 10$ μ M, where minimum 500 nM of the free stilbene-ligand conjugate in solution (10% of $[CON]_T$) is required to distinguish its fluorescence decay from that of the stilbene-ligand conjugate bound to the aptamer ($k_{app} > k_{app}^o + 3\sigma$ ($S/N > 3$)), the LOD is then

predicted to be 1044 nM (see the blue point in **Fig. 4C**). As explained above, this value is calculated using Equation [19]. Similarly, with the same parameters as above, assuming that 4.5 μM of the free stilbene-ligand conjugate in solution (90% of $[\text{CON}]_{\text{T}}$) is the maximum amount producing fluorescence decay distinguishable from that of the total amount of the free stilbene-ligand conjugate in solution ($[\text{CON}]_{\text{T}} = 5 \mu\text{M}$), and taking into account the same pre-determined parameters as above and that $k_{app} > k_{app}^f - 3\sigma$, the detection range of the analyte is predicted to be from 1 μM to 43 μM .

Furthermore, Equation [19] also reflects the impact on the LOD from the structure-activity relationship of the stilbene-ligand conjugate. The conjugate structure actually determines its activity in the aptamer binding and its fluorescence property. When a ligand is tagged with stilbene, its binding affinity may vary, but will likely have less affinity than the native ligand. **Fig. 3** and Equation [7] suggest that smaller K_d (or stronger binding) requires less $[\text{APT}]_{\text{T}}$ to reach a pre-set α (i.e., 90%), reducing the amount of the aptamer and making the assay less expensive. However, the small K_d requires considerably more $[\text{LIG}]_{\text{T}}$ to release the same $[\text{CON}]$, thereby lowering the assay sensitivity (when $[\text{APT}]_{\text{T}}$ is the same) (see **Fig. 4A-4C**). On the other hand, as mentioned above, strong fluorescence intensity of the conjugate lowers the LOD of the analyte. Thus, the structure of the stilbene molecule (its substituents pattern) has an essential effect on the sensitivity and should be carefully considered during the design step of the assay [14, 15, 17].

Finally, according to Equation [19], in order to obtain the LOD of 100 nM at the conditions of $K_d^L = 200 \text{ nM}$, $[\text{CON}]_{\text{T}} = 5 \mu\text{M}$, $K_d = 1 \mu\text{M}$, and $[\text{CON}] = 742 \text{ nM}$ and to distinguish the fluorescence decay of the free stilbene-ligand conjugate in solution from that of the

conjugate bound to the aptamer ($S/N > 3$), then 10 μM of $[\text{APT}]_{\text{T}}$ should be applied (see the orange curve in **Fig. 4E**).

Fig. 4. Illustration of the relationship between the analyte concentration $[\text{LIG}]_{\text{T}}$ and free stilbene-ligand conjugate $[\text{CON}]$ ratios with the known values of $K_d^L = 200 \text{ nM}$, and $[\text{CON}]_{\text{T}} = 5 \mu\text{M}$.

A-C: K_d varies between 50 nM and 2 μM , with $[\text{APT}]_{\text{T}} = 1 \mu\text{M}$, 5 μM and 10 μM , respectively.

D-F: $[\text{APT}]_{\text{T}}$ varies between 500 nM and 20 μM , with $K_d = 500 \text{ nM}$, 1 μM and 2 μM , respectively.

3. Discussion and Conclusions

The present format of the PHASA based on the combined adaptive and competitive binding of an analyte to a stilbene-ligand-aptamer complex provides a novel fluorescence technique for biosensing, which exploits the unique properties of both aptamers and stilbenes. This method may overcome the drawbacks of the existing bioassays as described in the present manuscript. In the proposed assay format, the competitive binding and the resulted fluorescence decay are characterised by the kinetic parameters K_d^L and K_d and concentration of reagents (stilbene-ligand conjugate, aptamer and analyte). The constant K_d and total amount of the stilbene-ligand conjugate $[CON]_T$ are the parameters determining the concentration of aptamer $[APT]_T$ to be taken for the particular fraction α of the stilbene-ligand conjugate bound to the aptamer. This work demonstrates the relationship between these parameters and allows predicting and optimising the aptamer-to-conjugate ratio B and the LOD of the assay. Thus, the analytical models based on these parameters and their relationships are established in the present work and provide a theoretical basis to accurately predict the LOD and detection range of the assay.

The K_d value for a particular stilbene-conjugate and its minimal concentration $[CON]_T$, at which fluorescence emission can be detected, are determined by intrinsic biochemical and physicochemical properties of the ligand and conjugate. These properties are inherent to the structure of the ligand and conjugate and unlikely to be easily user-modified.

The K_d value of a stilbene-ligand conjugate less than 2 μM is acceptable in the majority of practical applications. For example, if a stilbene-ligand conjugate has K_d of 2 μM and undetectable fluorescence decay at $[CON]_T$ of 20 μM , the $[APT]_T$ concentration required for 90% bound conjugate is calculated according to Equation [7] to be more than 36 μM .

This calculation can be performed with an assumption that fluorescence decay of this conjugate can be observed at $[\text{CON}]_{\text{T}} > 20 \mu\text{M}$. Since such amount ($36 \mu\text{M}$) of aptamers is expensive and non-practical, that prediction would immediately remove the unsuitable conjugate candidates from consideration at the preliminary development stage of the assay and suggest using a different stilbene compound in designing the assay, thereby allowing a range of parameters to be known and targeted.

The proposed model also suggests that a smaller K_d value allows reducing the amount of the aptamer in the assay. However, the small K_d also limits the release of the conjugate under the same conditions, resulting in much lower $[\text{CON}]$ which leads to the decreased sensitivity of the assay (see **Fig. 4**, showing that when other parameters are constant, the smaller K_d value requires more $[\text{LIG}]_{\text{T}}$ to release the same amount of the conjugate). This information helps to design and synthesise a structurally optimal stilbene-ligand conjugate having an ideal K_d for a particular assay.

Thus, the analytical model proposed in this manuscript presents a viable approach to design and prepare a stilbene-ligand conjugate that would be structurally the most suitable for a particular PHASA format, and predict an optimal concentration ratio B between an aptamer and this conjugate.

Regarding the LOD and detection range of the assay, the directly proportional correlations (linear ranges) of the empirical curves, which are shown in **Fig. 4**, correspond to these values. Only the k_{app} constant is experimentally measured, while the parameters K_d^L , K_d , $[\text{CON}]_{\text{T}}$ and $[\text{APT}]_{\text{T}}$ are empirically determined and user-defined as discussed above, thereby allowing predictable and tunable sensitivity and detection ranges of the assay, provided that the free conjugate can be measured with k_{app} . For example, if $K_d^L = 0.2 \mu\text{M}$,

$K_d = 0.8 \mu\text{M}$ and the free conjugate concentration range in solution is $0.5\text{--}4.5 \mu\text{M}$ out of the total concentration $[\text{CON}]_{\text{T}} = 5 \mu\text{M}$, varying the aptamer concentration would allow to easily tune the LOD and detection range of the assay. This is according to Equation [19], when the chosen $[\text{CON}]$ range of $0.5\text{--}4.5 \mu\text{M}$ allows distinguishing the fluorescence decay of the stilbene-ligand conjugate bound to the aptamer from that of the free stilbene-ligand conjugate in solution (the k_{app} change is observable, $S/N > 3$). Thus, for instance, in order to arrive to the LOD of $1 \mu\text{M}$, the amount of aptamer required is $12.7 \mu\text{M}$ under the aforementioned conditions. Moreover, when it is required to directly detect the analyte ranges up to $30 \mu\text{M}$, the amount of the aptamer added to the assay should be $9.8 \mu\text{M}$. Thus, the analytical model proposed in the present manuscript also provides a simple way to predict and tune the LOD and detection range of the assay.

Working aptamer concentrations for biosensors can be up to $50 \mu\text{M}$, but generally they are less than $10 \mu\text{M}$ [20, 22-25], in order to maintain the low cost of the assay. For example, a colorimetric aptasensor applies 200 nM aptamer for immobilisation on gold nanoparticles surface [25]. Thus, when optimising the conditions for enhancing the PHASA sensitivity, the low aptamer concentration should always be preferable.

Moreover, in the assay of real analytes detection, this model could provide a preliminary theoretical support for the initial design. For instance, according to Equation [7] and Equation [19], some ligand-stilbene conjugate candidates with weak binding affinity, low fluorescence quantum yield, or slow decay can be eliminated. Since the PHASA assay is kinetics-based, background fluorescence from sample complex may affect the fluorescence intensity but not fluorescence decay, making the assay more robust in turbid applications. However, it will be more sensitive to microenvironment changes, such as apparent

viscosity and temperature changes. Thus, to avoid the inaccurate results, internal, non-conjugate stilbene standards would likely be required to account for microenvironment variations.

Finally, as discussed above, optimising the K_d value of the conjugate is another approach to improve the LOD and detection range (see **Fig. 4A-4C** and **Fig. 1B**). However, this approach requires a more dedicated molecular biology optimisation than directly varying the aptamer concentration.

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References

1. Ping, J., et al., *Recent advances in aptasensors based on graphene and graphene-like nanomaterials*. Biosensors and Bioelectronics, 2015. **64**: p. 373-385.
2. Zhou, Y., et al., *Organic additives stabilize RNA aptamer binding of malachite green*. Talanta, 2016. **160**(Supplement C): p. 172-182.
3. Zhou, Y., et al., *Real-time colorimetric hydration sensor for sport activities*. Materials & Design, 2016. **90**(Supplement C): p. 1181-1185.
4. Wu, Y., et al., *New Photochrome Probe Allows Simultaneous pH and Microviscosity Sensing*. Journal of Fluorescence, 2015. **25**(4): p. 961-972.
5. Papper, V., et al., *Novel Photochrome Aptamer Switch Assay (PHASA) for Adaptive Binding to Aptamers*. Journal of Fluorescence, 2014. **24**(6): p. 1581-1591.
6. Zhou, Y., et al., *Aptamer adaptive binding assessed by stilbene photoisomerization towards regenerating aptasensors*. Sensors and Actuators B: Chemical, 2018. **257**(Supplement C): p. 245-255.
7. Li, F., et al., *Electrochemical aptamer-based sensors for food and water analysis: A review*. Analytica Chimica Acta, 2018.
8. Qian, J., et al., *Magnetically controlled fluorescence aptasensor for simultaneous determination of ochratoxin A and aflatoxin B1*. Analytica Chimica Acta, 2018. **1019**: p. 119-127.
9. Rossetti, M. and A. Porchetta, *Allosterically regulated DNA-based switches: From design to bioanalytical applications*. Analytica Chimica Acta, 2018. **1012**: p. 30-41.
10. Hermann, T. and D.J. Patel, *Adaptive Recognition by Nucleic Acid Aptamers*. Science, 2000. **287**(5454): p. 820-825.
11. Lu, C.-H., et al., *Amplified Aptamer-Based Assay through Catalytic Recycling of the Analyte*. Angewandte Chemie International Edition, 2010. **49**(45): p. 8454-8457.
12. Neves, M.A.D., C. Blaszykowski, and M. Thompson, *Utilizing a Key Aptamer Structure-Switching Mechanism for the Ultrahigh Frequency Detection of Cocaine*. Analytical Chemistry, 2016. **88**(6): p. 3098-3106.

13. Likhtenshtein, G.I., et al., *Novel fluorescence-photochrome labeling method in the study of biomembrane dynamics*. Journal of Biochemical and Biophysical Methods, 1996. **33**(2): p. 117-133.
14. Papper, V., et al., *Photophysical characterization of trans-4,4'-disubstituted stilbenes*. Journal of Photochemistry and Photobiology A: Chemistry, 1997. **111**(1): p. 87-96.
15. Papper, V. and G.I. Likhtenshtein, *Substituted stilbenes: a new view on well-known systems: New applications in chemistry and biophysics*. Journal of Photochemistry and Photobiology A: Chemistry, 2001. **140**(1): p. 39-52.
16. Papper, V., et al., *Theoretical and Experimental Studies of N,N-Dimethyl-N'-Picryl-4,4'-Stilbenediamine*. Journal of Fluorescence, 2014: p. 1-7.
17. Pines, D., et al., *Dual Fluorescence Phenomenon in 'Push-Pull' Stilbenes*, in *Reviews in Fluorescence 2015*, D.C. Geddes, Editor. 2016, Springer International Publishing: Cham. p. 337-352.
18. Zhou, Y., et al., *Stilbene Switch Activated by Click Chemistry*. Procedia Technology, 2017. **27**(Supplement C): p. 10-11.
19. Jauset-Rubio, M., et al., *Aptamer Lateral Flow Assays for Ultrasensitive Detection of β -Conglutin Combining Recombinase Polymerase Amplification and Tailed Primers*. Analytical Chemistry, 2016. **88**(21): p. 10701-10709.
20. Luo, Z., et al., *An aptamer based method for small molecules detection through monitoring salt-induced AuNPs aggregation and surface plasmon resonance (SPR) detection*. Sensors and Actuators B: Chemical, 2016. **236**(Supplement C): p. 474-479.
21. Chen, J.-W., et al., *Detection of adenosine using surface-enhanced Raman scattering based on structure-switching signaling aptamer*. Biosensors and Bioelectronics, 2008. **24**(1): p. 66-71.
22. Kim, Y.S., et al., *A novel colorimetric aptasensor using gold nanoparticle for a highly sensitive and specific detection of oxytetracycline*. Biosensors and Bioelectronics, 2010. **26**(4): p. 1644-1649.
23. Dong, Z.M. and G.C. Zhao, *Quartz crystal microbalance aptasensor for sensitive detection of mercury(II) based on signal amplification with gold nanoparticles*. Sensors (Basel), 2012. **12**(6): p. 7080-94.

24. Emrani, A.S., et al., *Colorimetric and fluorescence quenching aptasensors for detection of streptomycin in blood serum and milk based on double-stranded DNA and gold nanoparticles*. Food Chemistry, 2016. **190**: p. 115-121.
25. Kim, U.-J. and B.C. Kim, *A colorimetric assay for detection of 6-OH-BDE-47 using 6-OH-BDE-47-specific aptamers and gold nanoparticles*. Sensors and Actuators B: Chemical, 2017. **248**: p. 298-304.

Figures

Figure 1. A: Schematic illustration of the PHASA competitive binding format, which is based on the competitive binding of the free analyte molecules present in the sample to their binding site in the stilbene-ligand-aptamer complex. Initially, the *trans*-stilbene-ligand conjugate forms a complex with the aptamer of interest and exhibits very slow fluorescence

decay upon irradiation at its excitation maximum. Introduction of a sample containing the analyte molecules results in their competitive binding to the aptamer, subsequent release of the fluorescent *trans*-stilbene-ligand conjugate molecules from the aptamer active site into solution and significant acceleration of the fluorescence decay. B: The exemplary fluorescence decay curves of a stilbene-ligand conjugate in the PHASA competitive binding format. The pink curve shows the fluorescence decay of the stilbene-ligand conjugate complexed to an aptamer. In this case, the ideal concentration of the free stilbene-ligand conjugate in solution is zero ($[CON] = 0$). The dark yellow curve shows the fluorescence decay of the stilbene-ligand conjugate completely released into solution ($[CON] = [CON]_T$). The limit of detection (LOD) is indicated by the light blue band, while the sensing capacity is indicated by the orange band. To measure a reliable signal, the background noise should be taken into account, and this will narrow down the sensing window to be between the LOD and sensing capacity. An acceptable sensing signal-to-noise ratio (S/N) should be no less than 3 (or the 3σ criterion), which defines the LOD and sensing capacity in PHASA.

Figure 2. Fluorescence decay curves of 100 nM **DMAAS** (1) and 1 μ M **SITS** (2). The fluorescence decay curves were recorded in dH₂O at room temperature and at the constant illumination conditions with SHIMADZU RF-5301PC spectrofluorophotometer. The stilbenes were dissolved in 5% DMSO/dH₂O. DMAAS (1) at 100 nM concentration gives the S/N ratio = 200. SITS (2) at 1 μ M concentration exhibits much slower fluorescence decay with the S/N ratio = 125.

Figure 3. Illustration of the relationship between the fraction α of the stilbene-ligand conjugate bound to the aptamer and the B ratio of the aptamer to stilbene-ligand conjugate concentrations, with the known K_d and conjugate concentration values. A: $[CON]_T = 1 \text{ }\mu\text{M}$, while K_d varies from 50 nM to 2 μM . B: $[CON]_T = 5 \text{ }\mu\text{M}$, while K_d varies from 50 nM to 2 μM . C: $K_d = 1 \text{ }\mu\text{M}$, while $[CON]_T$ varies from 50 nM to 5 μM . D: $K_d = 2 \text{ }\mu\text{M}$, while $[CON]_T$ varies from 50 nM to 5 μM .

Figure 4. Illustration of the relationship between the analyte concentration $[LIG]_T$ and free stilbene-ligand conjugate $[CON]$ ratios with the known values of $K_d^L = 200 \text{ nM}$, and $[CON]_T = 5 \text{ } \mu\text{M}$. A-C: K_d varies between 50 nM and $2 \text{ } \mu\text{M}$, with $[APT]_T = 1 \text{ } \mu\text{M}$, $5 \text{ } \mu\text{M}$ and $10 \text{ } \mu\text{M}$, respectively. D-F: $[APT]_T$ varies between 500 nM and $20 \text{ } \mu\text{M}$, with $K_d = 500 \text{ nM}$, $1 \text{ } \mu\text{M}$ and $2 \text{ } \mu\text{M}$, respectively.

A

B

Highlights

- Photochrome Aptamer Switch Assay (PHASA), a new biosensing platform based on combination of aptamer adaptive binding and stilbene photoisomerisation, utilises the unique structural, biochemical and photophysical properties of aptamers and stilbenes and overcomes the drawbacks of the existing bioassays.
- A new analytical model presented in this work, based on the relationship between the kinetic parameters of the PHASA, allows optimising the aptamer-to-conjugate ratio, accurately predicting the limit of detection and the detection range of the assay and designing a structurally optimal stilbene-ligand conjugate having an ideal dissociation constant K_d for a particular assay.
- Optimising the dissociation constant K_d of the stilbene-ligand-aptamer complex is another approach to improve the limit of detection and detection range of the assay.

- Photochrome Aptamer Switch Assay (PHASA) is a biosensing platform based on combination of aptamer adaptive binding and stilbene photoisomerization. PHASA utilizes the unique structural, biochemical and photophysical properties of aptamer-stilbene constructs to overcome drawbacks of existing bioassays.
- The analytical model presented is based on the relationships between the kinetic parameters of the PHASA to optimize the aptamer-to-conjugate ratio, limit of detection, and detection range to yield a structurally optimal stilbene-ligand conjugate having an ideal dissociation constant for a particular assay.
- Optimizing the dissociation constant of the stilbene-ligand-aptamer complex is another approach to improve the limit of detection and detection range of the assay.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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