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1 Aptamer adaptive binding assessed by stilbene
2 photoisomerization towards regenerating aptasensors

3
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* **Abbreviations**

MG, malachite green; MGA, malachite green aptamer; ITC-Stilbene, 4-Acetamido-4'-
isothiocyanato-2,2'-stilbenedisulfonic acid disodium salt; RhB, rhodamine B; TMR,
Tetramethylrosamine; PHASA, photochrome aptamer switch assay.

ABSTRACT

Fluorescent aptasensors are reliant on static fluorescence intensity measurements, which suffer drawbacks such as background interference and laborious separation procedures. A unique aptasensor based on the photochrome aptamer switch assay (PHASA) has been developed that is independent of background fluorescence, requires no analyte separation, and allows rapid quantification within seconds. Malachite green aptamer (MGA) conjugated with a water-soluble stilbene on the MGA 3' C38 terminus was chosen for building the proof-of-concept aptasensor. In the presence of malachite green and tetramethylrosamine ligands, the rate of the stilbene fluorescence decay was found to be linearly dependent on the ligand concentration. Molecular dynamic simulation suggests hydrogen bonding between stilbene sulfonates and neighboring nucleotides is the primary mechanism responsible for rate changes in stilbene photoisomerization. Analysis of the apparent fluorescence decay rate (k_{app}) versus analyte concentration gives a limit of detection (LOD) of 2 μ M for MG and 0.6 μ M for TMR. This aptasensor design opens up a new sensing mode, which is promising for rapid development of SELEX generated molecular recognition elements.

Keywords:

Aptamer

Stilbene

Fluorescence decay

Photoisomerization

Aptasensor

Malachite green

1. Introduction

Aptamers are synthetic single-stranded oligonucleotides obtained by systematic evolution of exponential enrichment (SELEX) technology *in vitro*, which can bind to various targets with extremely high specificity, for instance, drugs, growth factors, peptides, enzymes, spores, toxins, proteins, whole cells and ions [1-6]. As candidates for replacing antibodies, aptamers possess advantages of relatively simple synthetic preparation, low batch-to-batch variability, facile modification, low immunogenicity, and structural robustness [7-10]. In addition, it has been widely reported that aptamers have unique properties of conformational change/adaptive binding, making them more versatile as molecular recognition elements [11-15]. Thus, they have been applied in diagnosis, biosensing, bio-imaging, drug delivery, and drug discovery [16-18]. Electrochemical, fluorescent, chemiluminescent, and colorimetric sensors have employed aptamers for their molecular recognition properties. [16, 19, 20]. Among these sensors, fluorescent aptasensors are under rapid development, but largely rely upon static fluorescence intensity and fluorescence quantum yield measurements. [21]. While simple and straightforward, static fluorescence measurements are susceptible to fluorescence background interference and may require additional washing protocols to increase sensitivity.

Stilbene (1,2-Diphenylethene) fluorophores have been employed as fluorescence probes in our previous work but never in aptasensors [22-28]. This is mainly because of their strong tendency to lose fluorescence rapidly upon excitation via the non-radiative deactivation process connected to the twisted transition in the excited state [25, 29]. The latter is responsible for the *trans-cis* photoisomerization of the molecule and acts as a quenching funnel on fluorescence emission, thereby depriving the fluorescence probe of its photochemical stability. Under constant illumination at excitation maximum, the sterically unhindered *trans*-stilbene molecules in solution rapidly change their molecular configuration with observed fluorescence emission decay. The fluorescence decay kinetics is strongly dependent upon local steric and electronic environments. [23, 30-32]. Such photochemical instability is detrimental if the stilbene compound is considered a classical fluorescence probe, and this is probably the reason why stilbene compounds have never been effectively used as fluorescent reporters (labels or probes) in biosensors.

72 However, the actual reporting power of the stilbenes is not in their fluorescence, but in a
73 rapid loss of their fluorescence via an instant conformational change upon excitation.
74 This makes the stilbene switches unique in the sense that most fluorescent reporters either
75 do not possess this intramolecular switchable nature or require the separation of adjacent
76 fluorophores. One of the most striking features of stilbene photochemistry is its essentially
77 strong dependence on local environments, which can effectively alter or even hinder the
78 photoisomerization of the molecule in the excited state. [27, 33, 34]. This includes
79 grafting of stilbene compounds on oligonucleotides [35].

80 In this proof-of-concept work, we report a new molecular sensor based on stilbene
81 fluorophores conjugated to an RNA aptamer molecular recognition element.
82 Photochrome aptamer switch assay (PHASA) concept, design and preparation of the
83 elements, and the long-term stability of the RNA aptamer have previously been reported
84 [26, 36, 37]. Our previous investigation focused on the synthesis of stilbene maleimide
85 derivatives and prepared an aptamer-stilbene based on maleimide-thiol conjugation.
86 However, the poor synthetic yields and low emission intensities of the synthesized
87 conjugates prevented a quantitative ligand binding analysis. In our current study, we
88 successfully constructed MGA-Stilbene conjugate based on amine-isothiocyanate
89 grafting that attained an 80% synthetic yield with adequate S/N emission intensities. For
90 the first time, the PHASA concept is tested and successfully demonstrated, which
91 presents a novel fluorescence decay-based sensing mode. The PHASA-based ‘aptasensor’
92 detects ligand induced adaptive binding based on changes in the apparent rate of the
93 stilbene fluorescence decay under constant-illumination conditions, which forms our
94 central hypothesis. This proof-of-concept work (**Fig. 1A&B**) incorporates four unique
95 features towards the synthesis and exposition of the PHASA elements: 1) MGA’s
96 (malachite green aptamer) ability to undergo adaptive binding (structural folding around
97 ligand), 2) chemical synthesis of MGA that allows incorporation of reactive functional
98 groups, 3) sensitivity of stilbene’s photoisomerization kinetics to sterical hindrance in
99 different rigid environments, and 4) commercial availability of water soluble stilbenes
100 with reactive functional groups. The combination of MGA with its triphenylmethane
101 ligands, as seen in **Fig. 2**, was chosen as a model study for a number of reasons: 1) the
102 combination allows rapid screening of binding affinities in high throughput microplate

assays [36], 2) MGA has a host of known diaryl- and triarylmethane ligands (see **Fig. 2**) that bind at varying affinities [38-40], 3) MGA's 3D crystal structure and NMR aqueous adaptive binding conformation have already been established [39, 41] and 4) the triarylmethane, an MG ligand, has commercial significance as a pigment, illegal fungicide, and blood detection compound [42-44]. To our knowledge, this is the first successful demonstration of a stilbene 'aptasensor', which provides a path for development of other similar aptasensors based on fluorescence decay kinetics of the stilbene reporters. The design provides a new approach for rapid detection and quantification of ligands within seconds and requires no washing protocols or labelled ligands.

Fig. 1

2. Materials and methods

2.1 Materials

HPLC purified RNA malachite green aptamer 5'-GGAUC CCGAC UGGCG AGAGC CAGGU AACGA AUGGA UCC-3' with -NH₂ and -SH modified nucleotides (see **Table S1**) or wild type (MGA) was purchased from AITbiotech Pte Ltd Singapore. 4-Acetamido-4'-isothiocyanato-2,2'-stilbenedisulfonic acid disodium salt (ITC-Stilbene), malachite green oxalate (MG), rhodamine B (RhB), sodium bicarbonate (NaHCO₃) and sodium carbonate (Na₂CO₃) were purchased from Sigma Aldrich, Singapore. Tetramethylrosamine (TMR) was purchased from Life Technologies, Singapore. Structures of MGA, ITC-Stilbene, MG, TMR and RhB are displayed in **Fig. 2**. All materials were used as received.

Fig. 2

2.2 Synthesis of MGA-Stilbene conjugate

Bioconjugation of MGA-NH₂ (C38) and ITC-Stilbene follows the amine-isothiocyanate reaction (**Fig. 1B**) [45]. Briefly, MGA-NH₂ was mixed with ITC-Stilbene (molar ratio = 1:100) in 0.1 M sodium bicarbonate reaction buffer (pH = 9), with stirring for 12 h at room temperature under dark conditions. Excess ITC-Stilbene was removed by a minimum of eight cycles of ultracentrifugation with 10 kDa molecular cutoff weight Amicon Centrifugal Filter, which also removes other small molecule side reactions with ITC-Stilbene.

2.3 Binding equilibrium dissociation constant, K_d , of MGA-Stilbene with MG

MGA-stilbene/MG binding in 5% DMSO-95% binding buffer was measured based on fluorescence enhancement of MG upon aptamer binding [38, 40]. The addition of DMSO prevents MG hydrolysis, as previously reported [36]. Emission spectrum of MG upon addition of MGA-Stilbene (1 μ M of MGA-Stilbene with 1 μ M of MG) was recorded with SHIMAZU RF-5301PC Spectrofluorophotometer at excitation wavelength of 620 nm. Binding equilibrium dissociation constant (K_d), was performed in a Tecan Microplate spectrofluorometer (Infinite M200, Tecan Asia PTE LTD, Singapore) in triplicate at room temperature as described in [36]. Briefly, increasing concentrations of MGA-Stilbene were added into constant MG (150 nM), and the fluorescence of MG was assayed by the spectrofluorometer with ex/em of 620/656 nm. Finally, K_d was calculated based on the enhancement in fluorescence as reported in [46-48].

2.4 Fluorescence decay measurement of MGA-Stilbene and its aptasensing

Fluorescence decay kinetics of MGA-Stilbene was measured at room temperature with SHIMADZU RF-5301PC Spectrofluorophotometer using the “time” mode, which is a continuous excitation with an excitation/emission slit width of 5 nm, and a simultaneous emission recording. MGA-Stilbene samples (1 μ M) in 5% DMSO-95% binding buffer in 1000 μ L quartz cuvettes were excited at 340 nm, and their fluorescence decay kinetics was recorded at emission wavelength 428 nm. Finally, the apparent fluorescence decay rate constant (k_{app}) for the first 10 second’s fluorescence decay was determined by first-order fitting routine described in [29, 49] using OriginPro 9.1 and based on the fact that upon constant illumination there is always a steady-state equilibrium between *trans* and *cis* Stilbene isomer in solution at any discrete time (any specific moment) of measurements. Sensing of analytes (ligands) was based on the change in the apparent

fluorescence decay rate (k_{app}) of MGA-Stilbene in the presence of ligands. Different concentrations of ligands were mixed with MGA-Stilbene (1 μ M in the final solution) with the fluorescence decay of MGA-Stilbene recorded. As a negative control, non-binding RhB (see **Fig. 2**) was tested for non-specific changes in the photoisomerization kinetics.

2.5 Fluorescence decay measurement of ITC-Stilbene with MG and MGA/MG complex

To investigate any non-specific photoisomerization effects on non-conjugated ITC-Stilbene, ITC-Stilbene fluorescence decay was monitored in the presence of MG and MGA at similar ratios as described above, which followed the same procedure as that of MGA-Stilbene (**Section 2.3**). The concentration of ITC-Stilbene used was 0.6 μ M that matches the concentration of MGA-Stilbene (HPLC indicates the Stilbene conjugation to aptamer at $60 \pm 1\%$).

2.6 Modeling and simulation of TMR/MG bound C38 MGA-Stilbene complex

All modeling was carried out using the Schrödinger modeling suite package (Schrödinger LLC, New York, NY). The modeling approach was adopted from our earlier studies [50]. The starting model was based on the X-ray crystallographic structure of the RNA aptamer in complex with TMR (PDB: 1F1T) [41]. The stilbene conjugate was appended to the terminal C38 at the ribose 3' hydroxyl group, followed by substructure energy minimization using the OPLS3 forcefield [51] with implicit solvent model. Similarly, the MG bound complex was modeled by the replacement of TMR with an overlay MG, followed by substructure energy minimization within binding site. Each of the model complex was solvated by a 15 Å buffer layer of explicit TIP3 water and 0.15 M of NaCl counterions within a rectangular box. Molecular Dynamics simulation was first initialized with the default multistage relaxation protocol, followed by 10 ns of unrestraint production simulation at 300 K and 1atm isothermal isobaric (NPT) condition. The long range electrostatic conditions were evaluated by particle mesh Ewald method under periodic bound condition. The structural changes of MGA were assessed by the RMSD and RMSF based on the phosphorus atom of the RNA backbone phosphates.

2.7 Statistics

Hypothesis testing was performed by Student's t-Test ($n = 3$) with significance level $p < 0.05$ (Two sample t-Test by OriginPro 9.1, OriginLab Corporation, Northampton, MA 01060 USA). Linear regressions R^2 were determined by ordinary least squares.

3. Results

3.1 Synthesis via C38-NH₂ water soluble ITC-Stilbene yields MGA-Stilbene conjugate

The synthesis strategy for the MGA-Stilbene conjugate aptasensor relied on a one-step reaction approach with commercially available reagents. The chemically synthetic nature of aptamer molecular recognition elements allows them to be commercially procured with a choice of functional groups on a specific nucleotide of the user's design. **Table S1** displays the specific nucleotide adaptations with amine or thiol functional groups. Flinders et al has elegantly probed nucleotide positions and their effects on MGA-MG K_d [39]. This information was combined with a visual determination of exposed surface nucleotides in the NMR structure to yield the first targets with U4 (located away from the quadrupole binding site), A9 (sterically flexible nucleotide position), C20 and C38 (both non-critically base-paired and solvent exposed). Maleimide-thiol conjugation was our first priority, but low aqueous solubility of maleimide-stilbenes created certain difficulties in aptasensor operation with this conjugate [26]. According to our experimental results (**Table S1**), C38 MGA-NH₂ retains the tightest binding (compared to wild type) so it is conjugated with commercially available, water-soluble, ITC-Stilbene (see **Fig. 1B**). The synthesized MGA-Stilbene conjugate is characterized by HPLC-DAD for chemical composition and MALDI-TOF mass spectrometry for molar mass. Under the conditions of HPLC-DAD separation, MGA-NH₂ displays a peak retention time (RT) of 1.7 min ($\lambda_{\max} = 260$ nm, **Fig. S2A**), while ITC-Stilbene peak has a peak RT of 8.5 min ($\lambda_{\max} = 340$ nm, **Fig. S2A**), and there is no considerable absorbance at 340 nm of the MGA-NH₂ peak. Similar to MGA-NH₂, MGA-Stilbene conjugate has a RT of 1.7 min. Furthermore, at the MGA-Stilbene peak position, there is a significant absorbance at both 260 nm and 340 nm, as seen in **Fig. S2B**. The DAD wavelength scan (**Fig. S2A&B** inset) also indicates the conjugation, with ITC-Stilbene absorbance spectra appearing at the 1.7 min peak. There is no ITC-Stilbene peak at 8.5 min, indicating excess removal and purification by ultracentrifugation. Peak integration analysis suggests that 60% of the

aptamer is labelled with stilbene with >80% ($84 \pm 4\%$) retention of original aptamer. No further purification is performed, as further chromatography or precipitation methods have a slim chance of improving the labelling percentage, but have a high risk of low yielding (<50%) aptamer retention. For example, isopropanol precipitation of RNA aptamer only yields 14% [26].

MALDI-TOF mass spectrometry was applied to determine the change in molar mass from the MGA-NH₂ upon stilbene conjugation. As seen in **Fig. S2C**, the RNA aptamer MGA-NH₂ has an initial mass to charge ratio (m/z) of 12621 [M+H]⁺ (blue). The conjugation product MGA-Stilbene has a m/z of 13072 [M+H]⁺ with detection of m/z peak of 12615 [M+H]⁺, which is assumed to be starting material. The increase of 457 Da after the conjugation indicates the successful click chemistry crosslinking of the isothiocyanate on ITC-Stilbene to the amine on MGA-NH₂. The theoretical increase in molar mass is predicted to be 454.5 Da (the molar mass of ITC-Stilbene after desalting). The small variation in m/z value is considered to be within instrumental error. Based on the two peak integrations at 12615 and 13072 (assuming similar rates of ionization for both species), the MALDI-TOF analysis suggests the majority of the aptamer molecules are labelled with stilbene, which matches with the HPLC data in **Fig. S2A&B**.

3.2 MGA-Stilbene retains its photoisomerisation and binding affinity to MG

Fluorescence decay and binding affinity of the MGA-Stilbene were investigated to confirm latent activity. **Fig. 3A** displays the fluorescence decay of MGA-Stilbene, demonstrating that the conjugate still shows the fluorescence decay similar to the parent ITC-Stilbene (see **Fig. S3** for comparison of absorbance and emission properties). Slight decrease of the apparent fluorescence decay rate constant k_{app} from 0.379 s⁻¹ (for ITC-Stilbene) to 0.360 s⁻¹ (for MGA-Stilbene) is observed. It was reported that fluorescence quantum yield of MG increases from 8×10^{-5} (free MG) to 0.187 while bound to MGA. The quantum yield and subsequent fluorescence enhancement is used for K_d measurements [38, 40]. As seen in **Fig. 3B**, the enhanced fluorescence properties at ~650 nm of MG bound to MGA-Stilbene is still present. K_d of MGA-Stilbene binding to MG is determined to be 240 ± 10 nM, which does not significantly differ from K_d of MGA-NH₂ binding to MG (200 ± 30 nM).

Fig. 3

3.3 ITC-Stilbene photoisomerisation is unaffected by MG or MGA, implying any changes in fluorescence decay of MGA-Stilbene is only by steric effects.

It was initially speculated that electronic interactions between the stilbene compound and triaryl amines may alter the photoisomerisation of the stilbene molecule. As shown in **Fig. 3C**, a negative control experiment was conducted by monitoring k_{app} of ITC-Stilbene in the presence of 20 and 40 μ M MG. No significant differences between the kinetic curves are observed, thereby confirming that free MG does not affect the stilbene *trans-cis* photoisomerisation. MGA is also added to challenge the null hypothesis that induced aptamer folding by MG (for different MGA/MG ratios) may hinder the *trans-cis* photoisomerisation of the stilbene. As shown in **Fig. 3C&D**, this null hypothesis seems to be false, since no significant difference (at $p < 0.05$) in k_{app} is observed in the presence or in the absence of MG or MGA/MG complex. Furthermore, k_{app} of ITC-Stilbene is also MG concentration-independent (addition of 20 μ M or 40 μ M results in the similar k_{app} , regardless of MGA addition). Overall, this data provides strong evidence that any change in the photoisomerisation of MGA-Stilbene is only through steric effects, for example the aforementioned adaptive binding. This is because the stilbene photoisomerisation is unaffected by the presence of free MG or complexed MGA/MG.

3.4 MGA-Stilbene fluorescence photodecay is only affected by adaptive binding ligands.

The MGA-Stilbene conjugate was exposed to ligands which are capable of MGA adaptive binding. Rhodamine B (RhB) has no binding affinity to MGA, therefore no ligand induced adaptive binding was expected to be observed. Tetramethylrhodamine (TMR) has the highest binding affinity (40 nM) known for MGA, which translates into a rigid, well defined MGA/TMR structure that has already been resolved by x-ray crystallography [41]. MG binds with less affinity resulting in a less rigid structure; it was possible to elucidate only the binding pocket structure using NMR spectroscopy [39]. As shown in **Fig. 4A**, the MGA-Stilbene conjugate in the presence of RhB exhibits no significant change in its fluorescence decay compared to the same experiment but without RhB. The fluorescence decay of MGA-Stilbene is however dramatically inhibited in the presence of TMR. **Fig. 4B** shows the comparison of k_{app} for all three ligands against the

MGA-Stilbene control. The k_{app} for the MGA-Stilbene significantly drops in the presence of TMR, slightly decreases in the presence of MG, and does not undergo any changes in the presence of RhB. This experimental observation enables the versatile use of the aptasensor in detection of the MG and TMR ligands.

Fig. 4

3.5 Proof of concept: Detection of MG, TMR, and MG in ecological conditions

The MGA-Stilbene was used for quantification of the MG ligand. Fluorescence decay of MGA-Stilbene for the first ten seconds was recorded in the presence of MG. As seen in **Fig. 4C&E**, k_{app} decreases with MG concentration increases. **Fig. 4E** shows a linear plot of k_{app} against MG concentration. A good linear range from 0.5 to 50 μM with slope = $-9.4 \times 10^{-4} \text{ s}^{-1} \cdot \mu\text{M}^{-1}$ ($R^2 = 0.98$) is obtained, and the limit of detection (LOD) for MG is calculated to be 10.5 μM , defined as 3σ criterion. As seen in **Fig. 4D**, similar to MG, the photoisomerisation reaction of MGA-Stilbene is inhibited as the conjugate is adaptively binding TMR, but to much higher degree. Linear calibration curve of k_{app} versus TMR concentration gives a slope of $-1.6 \times 10^{-2} \text{ s}^{-1} \cdot \mu\text{M}^{-1}$ ($R^2 = 0.99$) ranging from 0.2 to 10 μM , which is ~ 17 times higher than that for MG, with LOD of 0.6 μM (**Fig. 4F**). To challenge the aptasensor's ability to detect MG under ecological conditions, local procured seawater off the coast of Singapore was spiked with a final concentration of 20-40 μM MG (with no buffer) and assessed. Before spiking with MG, the local seawater samples were filtered through 0.22 μm filters to remove natural particulates. As seen in **Table. S2**, recoveries between 94% and 104% were obtained.

3.6 Reversing photodecay creates a more sensitive regenerating aptasensor

After *trans* to *cis*-Stilbene photoisomerization at 340 nm, an equilibrium of *trans* and *cis*-Stilbene is reached. The observed photodecay of this mixture can be partially reversed to regenerate *trans*-Stilbene when exposed to 280 nm light. The apparent photo-regeneration kinetics will also be dependent on the surrounding microenvironment, expanding the methods of quantification. The regenerating nature of stilbene was used to explore if sensitivity and LOD of the MGA-Stilbene sensor could be improved for MG detection. **Fig. 5A** shows the cycles of MGA-Stilbene with excitation at 340 nm followed by excitation at 280 nm, each for 60 seconds. Additional cycles are monitored with a 2:1

molar ratio of MG:MGA-Stilbene containing 2 μ M of MG. **Fig. 5B** displays the apparent kinetics for both *trans* to *cis* and *cis* to *trans* averaged over 10 cycles, with and without MG. A significant difference between 0 and 2 μ M MG at *cis* to *trans* photoisomerization is noted, which was not possible in the method employed in **Fig. 4E**. This strategy lowers the MG limit of detection to 2 μ M. To demonstrate its durability, the MGA-Stilbene aptasensor is cycled through 100 photoisomerizations (**Fig. S4**). Comparison of the first and last 10 cycles observed no loss of stilbene function.

Fig. 5

3.7 Modeling and simulation of TMR/MG bound C38 MGA-Stilbene complex

Ligand binding to folded DNA can involve either intercalation between planar nucleobases or binding to its the major or minor groove. The X-ray crystallographic structure of TMR- bound MGA consist of an asymmetric internal loop for ligand binding, a U-turn and a 3' and 5' terminal region with traditional nucleobases stacking that forms a distinctively major and minor groove. To examine the interactions of the ITC-Stilbene conjugate at the C38 position to MGA and its effect on photoisomerization, molecular modeling and molecular dynamics simulation were carried out (**Fig. 6**). Replacement of TMR by overlay MG in the modeling of the MG bound complex incurred minimum structural changes to the ligand binding site. The average RMSD for the TMR and MG bound C38 MGA-Stilbene complex was 2.3Å and 2.8Å, respectively (**Fig. 6A**). Both complexes shared comparable dynamic profile (**Fig. 6B**) with the maximum conformation flexibility found at the residue 15-18 hairpin turn region where the maximum RMSF of 5.0Å and 2.9Å for the TMR and MG bound complexes, respectively. The most conformational rigid region for the MGA was at the ligand binding site (residues 7-8, 23, 27-29) where the lowest RMSF of 0.6Å and 1.0Å was observed. The placement of the stilbene at the terminal end of the MGA provides significant conformational freedom. Neither interaction nor binding to the major or minor groove of the terminal MGA region was observed in the MD simulation. Formation of hydrogen bonds with G1 significantly limit the conformational flexibility of stilbene and its ability to be fully extended into the solvent medium, as seen in **Video S1** (MG, 10 ns simulation)

and **Video S2** (TMR, 10 ns simulation). Once formed, the constant exchange of hydrogen bonds between the its charged sulfonates with the amine and terminal 5' hydroxyl group of G1 detain stilbene at close proximity to the terminal base pair (**Fig. 6C&D**).

Fig. 6

4. Discussion

4.1 Biosensors based on aptamer adaptive binding

The observed aptasensing is based on the conjugation of a switchable *trans*-stilbene molecule to aptamer of interest and utilizes the numerous advantages of aptamers, such as their adaptive binding and easy chemical synthesis. The present manuscript is aimed at discussion of the instantly developed adaptive binding format. As mentioned above, in this format, the stilbene molecule is covalently attached to the modified aptamer far from the binding site of the ligand. Monitoring the changes in the fluorescence decay of the stilbene molecule as a result of the aptamer folding around its ligand can lead to generation of a calibration curve for practical applications. In the design we could use the commercially available stilbene compound for direct covalent conjugation to the aptamer using a simple "click-chemistry" type reaction.

Previous investigations have linked stilbenes to oligonucleotides, but not towards aptasensing purposes. For example, stilbene ethers have been successfully conjugated to DNA as linkers in forming stable DNA hairpins [35, 52, 53]. The majority of the stilbene linkers were weakly fluorescent after DNA conjugation, but had an observed retarded photoisomerization compared to non-conjugated, but this wasn't investigated in context to sensing of ligands. In the present studies, we used a C6-NH₂ linker to conjugate with the isothiocyanate (ITC)-stilbene, a simple click-chemistry reaction which ensured high efficiency. The C6-NH₂ linker imparts a 'propeller effect', which in most FRET/polarization assays is detrimental [54], but serves a purpose here in increasing the stilbene's degrees of freedom—the aptamer conjugation only incrementally shifts the photoisomerization k_{app} from $0.379 \pm 0.009 \text{ s}^{-1}$ to $0.360 \pm 0.003 \text{ s}^{-1}$. The conjugation rate of ITC-Stilbene (excess in the reaction mixture) was 60%, which is likely limited to the solid phase synthetic method of C38 functionalization. This is in contrast to our previous

synthetic protocol that relied on isopropanol precipitation and yielded only 14% conjugated aptamer [26]. The C38 MGA-NH₂ was chosen from other modified nucleotides as it presented the strongest binding affinity to MG, which was comparable to the non-modified MGA (**Table S1**).

As seen in **Fig. S2A-C**, MALDI-TOF spectra and HPLC confirm the successful conjugation of the MGA-NH₂ and ITC-Stilbene in terms of chemical purity and structural identity of the obtained conjugate. The obtained conjugate was thoroughly purified from excess of ITC-Stilbene to avoid the undesirable fluorescence background of ITC-Stilbene during the measurements and to increase the signal-to-noise ratio of the aptasensor. Further purification was not worth the risk of obtaining lower yields, which are typical for affinity chromatography or ion exchange chromatography [55, 56]. The synthetic solid phase chemistry synthesis of the RNA aptamer remains rather expensive (~560 USD/mg) and is limited to small oligonucleotide lengths (< 50 nts), which was one of the primary considerations in choosing the malachite green aptamer. Commercial scale up of RNA aptamers can be accomplished by dedicated PCR and continuous rapid thermal cycle systems, but specific nucleotide functionalization remains difficult [57].

The aptasensor relies on the *trans-cis* photoisomerisation of a stilbene compound. The *trans-cis* photoisomerisation rate of the stilbene compound is dependent on the surrounding media, which can be used to indicate the presence of the analyte and its further quantification. Quantification of the biosensor is essentially based on the constant-illumination fluorescence decay of the *trans*-isomer toward the photostationary equilibrium that removes cumbersome and error-prone light intensity calibrations.

Further, as demonstrated above, the MGA adaptive folding around the ligand (or analyte of interest) may significantly slow down the apparent photoisomerisation rate of the stilbene molecule attached to MGA, resulting in inhibition of the fluorescence photodecay of the stilbene molecule (**Fig. 4C**). Based on the obtained experimental results shown in **Fig. 4C** and **4D**, it seems that our hypothesis is supported under these limited conditions, and the adaptive aptamer binding indeed induced steric effects in the local environment of the stilbene molecule. Reviewing the structure of ITC-Stilbene and the RNA aptamer [39, 41, 58], we hypothesized that the steric effects might be attributed to π - π stacking or hydrogen bond formation between stilbene and folded MGA

nucleotides, or both. Molecular modelling seen in **Fig. 6** and **Video S1 & S2** supports this hypothesis and suggests that hydrogen bonding of the stilbene's sulfonates may be the primary mechanism of steric confinement. Inability to bind to the major or minor groove is likely due to the unfavorable electrostatic repulsion between the negatively charged sulfonates group on the stilbene and the phosphodiester backbone of the RNA. This would suggest the placement of our conjugated stilbene is restricted to either the 3' or 5' terminal end of the MGA.

The k_{app} of the conjugate decreased only when the stilbene molecules had been grafted on the C38-aptamer, as indicated by a negative control experiment on free stilbene molecule. When ITC-Stilbene was not grafted on MGA, the MGA/MG binding had no measureable effect on the stilbene fluorescence decay, regardless of the MG concentration (see **Fig. 3C&D**). This strongly supports our central hypothesis that changes in stilbene photoisomerisation kinetics were attributed to the ligand induced aptameric folding (illustrated in **Fig. 1A**).

4.2 Specificity and sensitivity of the MGA-Stilbene aptasensor

The MGA-Stilbene aptasensor responded to the MGA ligands MG and TMR but did not show any reactivity towards RhB (see **Fig. 4A&B**), displaying the wild-type selectivity of the aptamer. This result was actually expected, because Rhodamine B (RhB) has no binding affinity to MGA, therefore no ligand induced adaptive binding was observed. Fluorescence decay kinetic analysis for the TMR ligand displayed a 17-fold higher sensitivity compared to the MG ligand, with LOD in a sub-micromolar level (see **Fig. 4E**). From the reported data, TMR had 20 times smaller K_d value compared to MG [41], which may be a reason for the significant response of the TMR sensing, where strong binding of MGA-Stilbene may result in a more efficient confinement of the *trans*-stilbene molecule, inhibiting its *trans-cis* photoisomerisation. The overall sensitivity of stilbene photoisomerization vs. that of MG fluorescence enhancement combined with the observed dose-response curve suggests our central hypothesis needs further refinement. If photoisomerization kinetics was directly linked to aptamer folding, then ligand sensitivity should be within an order of magnitude of the K_d and have a hyperbolic dose-response curve as observed by malachite green fluorescence enhancement. The disagreement in theory versus empirical results may stem from the photoisomerization kinetics that are

dependent on the intermittent hydrogen bonding and resulting aptamer structural fluctuation, as observed in the molecular dynamic simulations in **Fig. 6**. This is further supported by the unique structural features of the MGA-TMR complex. A comparison of the crystal structure of MGA-TMR with the 2D NMR spectra of MGA-MG complex highlights these differences [39, 41]. The NMR-determined 3D structure of MGA-MG complex revealed flexible nucleotides in the binding site of the complex (i.e., A9 and U25), where they were settled in MGA-TMR binding. Interactions of the TMR planar aryl rings display almost perfect stacking with regards to C28 and G8. This is in contrast to MG's two rotated rings of 57° within the aptamer, which makes it impossible to match the interactions of TMR. Molecular dynamic simulations in **Fig. 6B** displays locations of A9 and U25 as comparatively rigid compared to the high fluctuations observed at the RNA hairpin (residues 15-21) and at the G1/C38 base-pair. We revise our central hypothesis that the differences in fluctuations at the G1/C38 base-pair are responsible for the empirical changes in the C38-stilbene photoisomerization kinetics, which is only partially dependent on aptamer folding. Regardless, the simulations combined with the binding studies confirmed the more rigid crystal structure of MGA-TMR results in a faster and stronger inhibition of the *trans-cis* photoisomerisation of the stilbene molecules, while the flexible MGA-MG complex was speculated to lead to a weaker effect due to intermittent hydrogen bond formation or less steric confinement on stilbene. Future work will attempt to graft stilbene in residues with less fluctuation, where the inhibition of the *trans-cis* photoisomerisation by MG and TMR may be more significant. This difference in sensitivity of the MGA-Stilbene aptasensor towards MG and TMR gives indication on the future design of the sensor, especially for the improvement of its sensitivity. That is, aptamers with stronger binding affinity with rigid conformations in ligand binding would be predicted to have a higher sensitivity. Alternatively, results in **Fig. 5** suggest regeneration and dynamic wavelength excitation may further optimize sensitivity. A potential criticism of the *cis* to *trans* regeneration is the 280 nm excitation wavelength maybe be detrimental absorbed by biomolecules, especially those containing tryptophan. However, *cis* to *trans* regeneration can be alternatively induced at 546 nm with erythrosin sensitizer [25].

In the illegal prophylaxis/treatment of fungal infections within fish farming, MG (as a drug) ranges from 0.5 to 300 μM [59]. The MGA-Stilbene aptasensor should be able to detect MG within 2 - 50 μM , with detection outside this range requiring sample modification. In analysis of fish tissue, concentrated sample extractions would be required. For example, to meet the EU minimum required performance limit (MRPL) (2 $\mu\text{g}\cdot\text{kg}^{-1}$) [40], extraction of MG would require ~ 0.5 kg of fish tissue, concentrated to a 1 mL sample. In comparison, MGA has been utilized for the more sensitive sensing of MG in concentration as low as 5 nM, based on the unique enhanced quantum yield (2000X) of MG upon aptamer binding [40]. The enhanced quantum yield method (ex/em = 620/656 nm) is suitable for visible wavelength excitation. The MG/MGA complex happens to be one of the most sensitive pairs available in the context of ‘light-up aptamers’ [60]. The aptasensor (ex/em = 340/428 nm) while not as sensitive for MGA, would be more beneficial for most aptamer ligands (analytes) without unique fluorescence enhancement, which accounts for the majority of aptamers developed.

Moreover, this sensing strategy would provide a rapid and robust way to determine aptamer adaptive binding (with the structural fluctuation caveats mentioned above) based on the stilbene fluorescence decay. Traditionally, adaptive binding were confirmed by XRD or NMR, requiring time consuming and laborious protocols [61], while determination by stilbene allows a simplified method of conformational change assessment. Other aptasensors have relied on adaptive binding to develop aptasensors with fluorescent “signal on/off” modes based on fluorescence resonance energy transfer (FRET) [20], which is sensitive, rapid, low cost, and flexible in design, but may be affected by background interference and cannot be regenerated. The FRET sensors normally require correct orientation of fluorophores and the free fluorophores may mask the energy transfer. Moreover, FRET-based aptameric molecular beacons usually require multiple grafting of fluorophores, or employing energy acceptors like graphene, quantum dots or carbon nanotubes, which complicates the biosensor fabrication and reduces the yield [21]. Finally, FRET aptasensors are not widely reported to be reusable, probably owing to the difficulty in recovery of the fluorophores to be the original designed orientation prior to sensing. By contrast, stilbene photoisomerization is known to be reversible through a variety of methods [23, 25]. The MGA-Stilbene conjugate

demonstrated allowed 100 cycles of decay and regeneration, (see **Fig. S4**), promising the design to be a regenerable aptasensor, where dynamic photoisomerization can be exploited for refinement of statistical significance and increased sensitivity as seen in **Fig. 5**.

5. Conclusions

The recent developments of SELEX technology and solid phase oligonucleotide synthesis allow rapid identification and design of unique oligonucleotide molecular recognition elements. Our technology aims to exploits these developments for the rapid development of aptasensors once the molecular recognition elements are sequenced and identified. We present a proof-of-concept aptasensor (MGA-Stilbene) based on the malachite green RNA aptamer (MGA). Ligand quantification relies on changes in stilbene fluorescence decay kinetics, which is promising to overcome aptasensors based on traditional “signal on/off” fluorescence modes. This design takes advantage of aptamer adaptive binding and photoisomerization of stilbene, which removes laborious and time-consuming washing procedures and detects ligands in seconds. Malachite green (MG) and tetramethylrosamine (TMR) served as model small molecule ligands whose binding affinity induced the adaptive binding of MGA and MGA-Stilbene. These ligands demonstrated that binding affinity and its effect on MGA’s structural fluctuation had a direct effect on ligand sensitivity. MGA-Stilbene regeneration was possible by dynamic wavelength excitation, which may further optimize ligand sensitivity. Future work will focus on other adaptive binding aptamers where no fluorescence quantitation is reported.

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526

527 **Appendix A. Supplementary material**

528 Supplementary data associated with this article can be found in the online version.

529

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Figures

Fig. 1. (A) Schematic representation of the PHASA and related sensing application based on inhibition of the stilbene fluorescence decay due to aptameric conformational change. In the absence of ligand (analyte), *trans*-stilbene grafted on aptamer undergoes free photoisomerisation (fluorescent *trans*-isomer is converted to non-fluorescent *cis*-isomer under irradiation with excitation light), resulting in fast fluorescence decay. Upon binding to ligand (analyte), conformational change of aptamer induces the inhibition of *trans*-stilbene photoisomerisation, resulting in slowing of the fluorescence decay. (B) Synthesis of malachite green aptamer-stilbene (MGA-Stilbene) conjugate (see **Fig. S1** for details of C38-C6-NH₂ modification).

Fig. 2. Structures of malachite green aptamer (MGA), 4-Acetamido-4'-isothiocyanto-2,2'-stilbenedisulfonic acid disodium salt (ITC-Stilbene), malachite green (MG), Tetramethylrhodamine (TMR) and Rhodamine B (RhB). 3D Structure of MGA (1Q8N) is visualized by Discovery studio 3.5 Visualizer, Accelrys.

Fig. 3. (A) Fluorescence decay of ITC-Stilbene and MGA-Stilbene, $\lambda_{ex} = 340 \text{ nm}$ and $\lambda_{em} = 428 \text{ nm}$. (B) Fluorescence emission of free MG and MG bound to the MGA-Stilbene complex (1:1), $\lambda_{ex} = 620 \text{ nm}$, resulting in λ_{em} of around 650 nm , showing an enhancement induced by aptamer binding. (C) Fluorescence decay curves of ITC-Stilbene in the presence of MG and wild type MGA/MG complex, $\lambda_{ex} = 340 \text{ nm}$ and $\lambda_{em} = 428 \text{ nm}$. (D) k_{app} of ITC-Stilbene upon addition of MG and wild type MGA/MG complex. NSD: No statistical difference at $p < 0.05$ against ITC-Stilbene control (without MG and MGA).

Fig. 4. (A) Fluorescence decay curves of MGA-Stilbene in the presence of 20 μ M MG, TMR and RhB. (B) k_{app} of MGA-Stilbene upon addition of the analytes (error bars refer to the standard deviation). Fluorescence decay curves of MGA-Stilbene in the presence of MG (C) and TMR (D). Calibration plot for k_{app} of MGA-Stilbene as a function of MG

concentration (0.5-50 μM) (E) and TMR concentration (0.2-10 μM) (F). *: $p < 0.05$: significant difference against MGA-Stilbene ($n = 3$).

Fig. 5. Regenerating of the MGA-Stilbene aptasensor. (A) MGA-Stilbene with and without MG excited at ex/em 340 nm/428 nm (*trans* to *cis* photoisomerization) for 60 seconds followed by ex/em 280 nm/428 nm (*cis* to *trans*) for 60 seconds for 5 cycles as illustration. (B) Averaged values of the apparent kinetics over 10 cycles. * $p < 0.05$. Fluorescence measurements on *trans-cis* photoisomerization were conducted at ex/em = 340 nm/428 nm for fluorescence decay and ex/em = 280 nm/428 nm for regeneration. In *cis* to *trans* isomerization, excitation slit of 20 nm was applied to maximize intensity.

Fig. 6. Molecular Dynamics simulation of TMR (black) and MG (grey) bound C38 MGA-Stilbene conjugate complex. A) RMSD plot relative to the starting X-ray structure showed a stable complex throughout the simulation with an average RMSD of 2.3Å and 2.8Å for TMR and MG respectively. B) RMSF of the MGA residues showed lowest structural fluctuation near the ligand binding site (in dotted red box) (residues 7-8, 24-31) and the highest at the RNA hairpin turn (residues 15-21). C) Interatomic distance showed the formation of stable hydrogens bonds between the sulfonate group of stilbene and with G1. D) Final structural snapshot of the TMR (red) and MG (cyan) bound MGA-Stilbene complex showing the stilbene-G1 interaction.

Supplementary material
for
Aptamer adaptive binding assessed by stilbene
photoisomerization towards regenerating aptasensors

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Methods for HPLC and MALDI-TOF:

High-performance liquid chromatography (HPLC)-diode array detector (DAD)

characterization of MGA-Stilbene

An Agilent 1200 series HPLC-DAD system paired with a $5\ \mu\text{m} \times 4.6\ \text{mm} \times 250\ \text{mm}$ Ultisil™ C18 column (Welch Materials Inc.) was used for chromatographic separation and characterization of MGA-Stilbene. Isocratic elution is performed with 1:4 v/v ratio of 25 mM ammonium acetate (pH 6.8) and acetonitrile at $1.0\ \text{mL}\cdot\text{min}^{-1}$ with $10\ \mu\text{L}$ sample injections. The diode array detector continuously scanned wavelength of 200 to 800 nm with 260 nm and 340 nm peaks used for quantification of MGA-NH₂ and ITC-Stilbene, respectively.

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrum characterization of MGA-Stilbene

MALDI-TOF measurement were performed as previously described [1]. The theoretical molar mass of MGA-NH₂ is 12509.7 Da. Before measurement, the samples were desalted with 100 mM ammonium citrate exchange buffer (pH = 6.0) (10 k Amicon Centrifugal Filter) to suppress the impact from metal cations. Then $1\ \mu\text{L}$ of sample (10 μM) was mixed with $1\ \mu\text{L}$ of THAP matrix (20 mg/ml in 4:1 mixture of ethanol/aqueous ammonium citrate (pH = 6; 100 mM)) on a MALDI-TOF sample plate and the mixture was dried at room temperature [2]. The theoretical molar mass of ITC-Stilbene should be 454.5 Da post desalting (replacement of sodium with hydrogen). The dried matrix was assayed in a Shimadzu Axima MALDI-TOF mass spectrometer. All MALDI-TOF spectra were processed with the following parameters: peak width: 20 channels; smoothing-average, 50 channels filter; baseline subtraction 100 channels filter.

Fig. S1. C6-NH₂ modification of the 3' C38 phosphate allows functionalization with the ITC-Stilbene to form the MGA-Stilbene.

Fig. S2. HPLC-DAD and MALDI-TOF characterization of MGA-Stilbene. (A) HPLC chromatography of MGA-NH₂ and ITC-Stilbene. (B) HPLC chromatography of MGA-Stilbene conjugate. The MGA-Stilbene conjugate (RT = 1.7 min) shows UV absorbance at both 260 nm and 340 nm, while there is no free ITC-Stilbene left in the conjugate (RT = 8.5 min). Insets are the UV-vis spectra at corresponding RT. (C) MALDI-TOF spectra of MGA-NH₂ and MGA-Stilbene conjugate. Top (blue): MGA-NH₂ before conjugation. Bottom (red): ITC-Stilbene grafted MGA-NH₂ (MGA-Stilbene conjugate).

Fig. S3. UV absorbance (A) and fluorescence emission (B) of ITC-Stilbene and MGA-Stilbene. Absorbance λ_{max} : 339 nm for ITC-Stilbene, 258 nm & 333 nm for MGA-Stilbene; Emission λ_{max} : 427 nm for ITC-Stilbene, 423 nm for MGA-Stilbene.

Fig. S4. MGA-Stilbene aptasensor from 1 to 10 (A) and 91 to 100 (B) cycles. In an individual cycle, MGA-Stilbene was excited at ex/em = 340 nm/428 nm (*trans* to *cis* photoisomerization) for 60 seconds followed by ex/em = 280 nm/428 mm (*cis* to *trans*) for 60 seconds. Note: Optical excitation intensities of spectrofluorometers vary by instrument and throughout the lifetime of the deuterium/mercury lamps, which may affect absolute values of FL.

Video S1: Animation of the MGA aptamer (ribbons) with docked MG (green) displaying the random placement of C38-stilbene. At 00:53, the formation of hydrogen bonds with G1's amine and 5' hydroxyl group and stilbene's charged sulfonates limit the conformational flexibility of stilbene and its ability to be fully extended into the solvent medium (Modelled time period = 10 ns, MPEG video, 38 MB). Video can be accessed by the following hyperlink: http://labsteele.com/Videos/MGA_MG.mpeg

Video S2: Animation of the MGA aptamer (ribbons) with docked TMR (green) displaying the random placement of C38-stilbene. At 00:30, the formation of hydrogen bonds with G1's amine and 5' hydroxyl group and stilbene's charged sulfonates limit the conformational flexibility of stilbene and its ability to be fully extended into the solvent medium (Modelled time period = 10 ns, MPEG video, 38 MB). Video can be accessed by the following hyperlink: http://labsteele.com/Videos/MGA_TMR.mpeg

Table S1. K_d of modified MGA with Malachite green (MG) ligand in pH 6.7 binding buffer.

MGA Modified Position		Modification Group	K_d (nM)	R^2
Wild type		NA	200±10	0.99
U4	-SH	820±90	0.99
	A9	-SH	690±90	0.98
	C20	-SH	670±100	0.99
	C38	-SH	980±140	0.99
	C38	-NH ₂	200±30 ^a	0.99
	C38	-NH-Stilbene	240±10 ^a	0.99

^a no statistical difference.

Table S2. Determination of MG in seawater samples.

Seawater sample ^a	Added (μM)	Found (μM)	Recovery (%)
1	20	20.1	100.5
2	30	31.2	104.0
3	40	37.7	94.3

^a: Seawater sample was filtered through 0.22 μm filters to remove natural particulates prior to MG spiking.

References

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