NANYANG
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**PHOTOCHROME APTAMER SWITCH ASSAY (PHASA) FOR
SMALL MOLECULE DETECTION AND QUANTIFICATION**

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SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

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Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research and has not been submitted for a higher degree to any other University or Institution.

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Abstract

In order to detect small analytes with high specificity and efficiency that cannot otherwise be achieved by antibody-based immunoassays, the development of a new Photochrome Aptamer Switch Assay (PHASA) was explored. In this assay, aptamer which can be synthesized chemically and can bind small molecules specifically is utilized as the molecular recognition element. Stilbene compounds having a unique reporting power via an instant conformational change upon irradiation, and whose decay rate change depends on medium viscosity are used as the photochrome probe. Two scopes—competitive binding and adaptive binding were proposed and were applied on malachite green (MG)-MG aptamer model system.

The competitive binding scope involves the competition for the aptamer binding pocket between MG analyte and stilbene-MG conjugate. The conjugate needs to possess both strong binding affinity and fluorescence decay. Four stilbene-MG conjugates: “fused” stilbene-MG, MG-CH₂-NH-Stilb-OMe, MG-CH₂-NAC-Stilb-OMe and MG-COO-Stilb-NO₂ were synthesized. Their binding experiments with MG aptamer and decay kinetics measurements in MG aptamer binding buffer based aqueous mixtures were conducted. Results of the measured dissociation constant demonstrate that $K_{d(MG)} (200 \text{ nM}) < K_{d(\text{“fused” stilbene MG})} (500 \text{ nM}) < K_{d(-CH_2-NAC-)} (1.7 \text{ }\mu\text{M}) < K_{d(-CH_2-NH-)} (2.4 \text{ }\mu\text{M}) < K_{d(-COO-)} (6.6 \text{ }\mu\text{M})$. That is, “fused” stilbene-MG conjugate has the strongest binding affinity, and there is literally no binding of MG-COO-Stilb-NO₂ to MG aptamer among the studied stilbene-MG conjugates. For the decay kinetics, the decay of both “fused” stilbene-MG and MG-COO-Stilb-NO₂ conjugates are quenched while the decay of MG-CH₂-NH-Stilb-OMe and MG-CH₂-NAC-Stilb-OMe are preserved. Combining the binding affinity and decay kinetics, the MG-CH₂-NAC-Stilb-OMe conjugate is the most promising candidate for PHASA competitive binding scope.

The adaptive binding scope requires the conjugation between stilbene compounds and MG aptamer, and thiol-maleimide click chemistry was chosen as the cross-linker. The dissociation constant K_d value of four SH-modified MG aptamers are measured in the range of 700~900 nM, preserving their binding ability towards MG analyte. Thiol conjugation of

stilbene maleimides proves to restore their quenched fluorescence decay in DMSO ($v\% \leq 10\%$) and MG aptamer binding buffer solution mixtures, allowing subsequent detection based on decay rate change in stilbene-MG complex without further separation. Three stilbene maleamic acid compounds were also synthesized for better water solubility, their decay rates' dependences on medium viscosity are confirmed and decay kinetics in DMSO and buffer solution mixtures are preserved instead of being quenched.

Besides medium viscosity probing, further applications of studied stilbene compounds were also explored. The fluorescence decay rate of dimethylamino-stilbene-maleamic acid (DMASMA) is linearly quenched by EtOH in the concentration range of $0.004 \sim 0.1 \text{ mol L}^{-1}$ where no effect from methanol and isopropanol (IPA) is observed. The photophysical properties of all three stilbene maleimic acids are sensitive to the solution pH. That is, the studied stilbene compounds can have promising applications in pH or EtOH sensing apart from medium viscosity probing.

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Abbreviations

ACN	Acetonitrile
DMSO	Dimethyl sulfoxide
DCM	Dichloromethane
EtOH	Ethanol
MeOH	Methanol
IPA	Isopropyl alcohol
PC	Propylene carbonate
PBS	Phosphate buffered saline
QS	Quinine sulfate
FP	Fluorescence polarization
QY	Quantum yield
HPLC	High performance liquid chromatography
TLC	Thin layer chromatography
NMR	Nuclear magnetic resonance
UV-Vis	Ultraviolet-visible
BSA	Bovine Serum Albumin
MG	Malachite green
BPA	Bisphenol A
BVA	4,4-Bis(4-hydroxyphenyl)valeric acid
DMAAS	4-Dimethylamino-4'-aminostilbene
MAS	4-Methoxy-4'-aminostilbene
DMASMI	Dimethylamino-stilbene-maleimide
MSMI	Methoxy-stilbene-maleimide
DMASMA	Dimethylamino-stilbene-maleamic acid
MSMA	Methoxy-stilbene-maleamic acid
HSMA	Hydroxy-stilbene-maleamic acid
PHASA	Photochrome aptamer switch assay

Chapter 1

Introduction

Nowadays, a majority of analyte detection is based on enzyme-linked immunosorbent assay (ELISA) attributed to its specificity. However, ELISA requires several hours or even days of incubation and laborious purification and washing protocols, making the detection time-consuming. Based on stilbenes and aptamers, a new photochrome aptamer switch assay (PHASA) is proposed whose detection depends on the instant conformational change of stilbene compounds. Hence, the assay allows detection and quantification of analytes within minutes, making real-time or on-site detection possible. This chapter presents the background information of stilbene compounds and aptamers, the proposed hypotheses and the detailed approach for analyte detection of PHASA. Also, it gives a brief introduction to each chapter in this thesis and points out the major findings obtained in the results chapters.

1.1 Background information

Increased preoccupation in diagnostic testing paved the way for the elaboration of alternative, state-of-the-art analytical devices, known as bioassay or biosensing devices or biosensors which comprise two major elements: “molecular recognition entity” and “sensing or reporter entity”. Currently, the most widely studied bioassays are ELISAs which are antibody-based. But their detection processes are both labor and time consuming. Besides, the recognition element–antibody suffers from batch to batch variation and some toxins’ corresponding antibody is not available. This poses limitations in their further development. On the other hand, aptamers which are single strand DNA or RNA oligonucleotides have been attracting attention due to their ease of manufacturing, little batch variation, and adaptive binding to their targets with strong affinity and excellent specificity even for small molecular weight targets. As a promising substituent for antibody, numerous detection sensors based on aptamer have been developed.

So far, stilbene compounds have never been used as molecular sensing entity in bioassays, although they possess all the desired features of ideal reporter molecules, such as chemical and biological stability, low toxicity, synthetic availability, high photochemical sensitivity, rapid response and easy regeneration. Among molecules reported for such applications, stilbenes have the unique ability to rapidly shift fluorescence upon excitation.

Figure 1.1. Conformational structure of *trans* and *cis* isomer of stilbene and the conversion under UV light excitation.

As shown in **Figure 1.1**, there are two possible conformational isomers for stilbene compounds: *trans* and *cis*. The *trans* isomer is planar in both the ground and excited state while the geometry of the *cis*-stilbene isomer in both the ground and excited state is non-planar because of the steric hindrance between the two phenyl rings and the consequent phenyl torsion. Attributed to the structural difference, the *trans* form of the stilbene is

strongly fluorescent while the *cis* form is essentially non-fluorescent. Upon excitation, the *trans-cis* photoisomerisation can occur and acts as a quenching funnel on fluorescence emission, thereby depriving the fluorescence probe of its essential photochemical stability. However, the actual reporting power of the stilbenes is not in their fluorescence, but in a rapid loss of their fluorescence via an instant conformational change upon irradiation with the excitation light. This makes the stilbene, among molecules reported for such applications, switches unique in the sense that most fluorescent reporters (labels or probes) either do not possess this intramolecular switchable nature or require the separation of adjacent fluorophores. Previous research in stilbenes [1-3] has confirmed the *trans-cis* isomerization process, where the fluorescence decay rate was found to be strongly dependent on steric hindrance and medium viscosity. These attributes allow applications in new photochrome methods based on local medium effects. For example, stilbene phase transitions can be used as the basis for photochrome-based bioassays in such specific environments as biological membranes.

1.2 Hypotheses

Combining the specificity and sensitivity of aptamers' kinetic adaptive binding [4-8] with phase transitions of stilbenes' photo-isomerization process, it is hypothesized that hapten binding to the aptamer will change stilbene's surrounding environment. Thus, the stilbene's photo-isomerization rate will be influenced and this decay rate difference can in turn be used to indicate the presence of and even quantify the specific target within seconds to minutes. Specifically, for this Photochrome Aptamer Switch Assay (PHASA), two scopes-competitive binding and adaptive binding are proposed and their details are presented below.

Hypothesis 1: *Stilbene-analyte conjugate encapsulated in the aptamer binding pocket will have slow fluorescence decay. With analyte addition, the conjugate will be released, resulting in faster decay and higher decay rate constant. When different analyte concentrations are added, a calibration curve of fluorescence decay rate constant vs. analyte concentration can be generated. (Covered in chapter 4)*

As the name implies, competitive binding shown in **Figure 1.2** involves the competition between the stilbene-analyte conjugate and the analyte of interest for the aptamer binding

pocket. Initially, upon addition of the corresponding *trans*-stilbene-analyte molecule to its aptamer, they form a complex. Being squeezed in the aptamer binding site, the *trans*-stilbene-analyte molecule exhibits slow fluorescence decay upon irradiation at the excitation maximum of the *trans*-isomer. When a sample solution containing analyte is introduced, the free analytes will competitively bind to analyte aptamer, resulting in stilbene-analyte conjugate which is previously located inside the binding pocket of the aptamer being released into free solutions. Thus, under excitation, the fluorescence decay process will be much faster with higher decay rate constant.

Figure 1.2. Competitive binding—when the *trans*-stilbene-analyte molecule is encapsulated inside the aptamer binding pocket, its fluorescence decay is slow. In the presence of analyte, the molecule is replaced and released to free solutions, resulting in fast fluorescence decay.

The quantification is achieved by measuring the steady-state fluorescence decay rate constant at different concentrations of the analyte, as shown in **Figure 1.3**. The obtained calibration curve can be used to determine the unknown concentration of the analyte of interest by recording its steady-state fluorescence decay curves in the competitive binding system.

Hypothesis 2: When stilbene molecule is attached to aptamer, it is not constrained and its fluorescence decay is fast. In the presence of analyte, aptamer folding towards analyte is induced, which sterically hinders the stilbene's decay and produces slower fluorescence

decay. By varying the analyte concentration, a calibration curve of decay rate constant vs. analyte concentration is obtained. (Covered in chapter 5)

In adaptive binding, aptamers are covalently modified with stilbene compounds. Based on the folding and unfolding behavior of aptamer around the attached stilbene molecule, there are two cases (**Figure 1.4 A and B**) for the detection mechanism.

Figure 1.3. With the addition of different amount of analyte, fluorescence decay curves having different decay rate constants are obtained. Plotting the rate constant against the corresponding analyte concentration, a calibration curve is generated.

The first case is shown in **Figure 1.4 A**. After stilbene molecule is attached onto the aptamer, no folding is induced. So there is no steric hindrance experienced by the stilbene and its fluorescence decay rate is fast. With the addition of analyte, aptamer adaptively binds to the analyte and results in stilbene being squeezed inside the aptamer together with the analyte. And this squeezing considerably retards and slows down the fluorescence decay. When different analyte concentrations are added into the solution, a series of fluorescence decay curves are obtained as well as a calibration curve as can be seen in **Figure 1.3**. But the fluorescence decay rate constant is inversely proportional to the analyte concentration.

Hypothesis 3: After being attached to aptamer, stilbene is encapsulated by the aptamer, giving rise to slow fluorescence decay. The encapsulation is removed when analyte is added and the stilbene decays faster. Based on this decay rate difference, analyte detection and quantification can be achieved. (Covered in chapter 5)

Figure 1.4 B shows the second case of adaptive binding. Right after the covalent bonding of stilbene onto an aptamer, the aptamer folds around the stilbene, sterically hindering the

twisting motion of the stilbene in the excited state and resulting in slow fluorescence decay. When the analyte is added, the aptamer folds around the analyte, exposing the stilbene to solution and thus, the fluorescence decay becomes fast. Similarly, changing the analyte concentration can generate fluorescence decay curves with different rate constants, from which a calibration curve can be obtained (**Figure 1.3**).

Figure 1.4 Adaptive binding–**A**. The fluorescence decay of the stilbene component is fast initially. After the analyte addition, the stilbene compound is constrained inside the binding pocket, giving rise to a slow decay. **B**. The fluorescence decay of the stilbene fragment is slow in the beginning as it is trapped inside the binding pocket. In the presence of analyte, the stilbene fragment is replaced out of the pocket, resulting in a fast fluorescence decay.

A third case for adaptive binding is possible, where stilbene kinetics is independent of aptamer folding. This system is not applicable for analyte detection and quantitation based on aptamer binding, but the stilbene molecules may be used in other applications such as pH or micro-viscosity assessment etc.

Hypothesis 4: The fluorescence decay kinetics of stilbene can be influenced by the surrounding proton concentration. Different pH solutions thus produce different stilbene fluorescence decay curves, which can be used for pH sensing. (Covered in chapter 6)

Hypothesis 5: Solvent molecules surrounding stilbene compound can change its solvation shell, which changes its electron distribution and its fluorescence decay kinetics, and this can be used to indicate the presence of a specific solvent molecule. (Covered in chapter 6)

Upon excitation, *trans*-stilbene is converted to *cis*-stilbene and results in fluorescence decrease. The competition between *trans-cis* isomerization and fluorescence emission of stilbene is influenced by medium viscosity as it mediates the torsion of the isomerization process [1-3]. It is also affected by stilbene substituents and solvent polarity [9, 10], as they change the electron distribution of the stilbene excited states and make one state more stabilized and preferred. By interacting with stilbene molecules through hydrogen binding and ionic interaction etc., other medium parameters such as the solvent itself, the acidity of the solvent can also change the electron distribution. Hence, they can affect the fluorescence decay behavior of stilbene molecule, which can be used for sensing.

1.3 Objectives and scope

From the above discussions, the ultimate goal of the project is to design a universal detection assay (PHASA) based on aptamer and stilbene compounds, which would allow the state-of-the-art analyte detection and quantification of small molecules such as toxins, explosives, drugs and pollutants which are difficult to be detected using antibody-based assays with high sensitivity and specificity, in minutes.

To achieve the objective, the entire scope is presented below. And for the author's part, synthesis, characterization and promising applications of stilbene compounds are the focus.

In this thesis, the analyte malachite green (MG) and malachite green aptamer were focused on, as the MG aptamer makes an ideal test system because of the following advantages; it has various fluorescent ligands with their binding affinities determined, its X-ray crystal and NMR structure is available, and single point mutation investigations have identified nucleic acids that can be modified. The approach is as follows:

- Synthesis of various stilbene-malachite green conjugates for competitive aptamer binding (**Figure 1.2**).
- Synthesis of stilbene-maleimide/maleamic crosslinking compounds towards click chemistry conjugation to thiol-modified MG aptamer in adaptive binding (**Figure 1.4 A & B**);
- Photophysical characterization including absorption and excitation, emission spectrum, fluorescence quantum yield and fluorescence decay rate measurement for the parent stilbenes, stilbene-MG conjugates and stilbene maleimide and maleamic compounds;
- Medium viscosity study of these prepared stilbene compounds, conjugates, maleimide compounds, based on fluorescence decay and fluorescence polarization experiments in solutions having different viscosities;
- Effects of organic and aqueous environment on the fluorescence intensity and fluorescence decay behavior.
- Dissociation constants between aptamer and stilbene-analyte conjugate or aptamer-stilbene conjugate with analyte.
- As applicability of the photochrome sensors towards alternative aqueous environments, including pH sensing and ethanol detection.

1.4 Dissertation overview

The thesis addresses the competitive and adaptive binding format design of Photochrome Aptamer Switch Assay (PHASA) for small molecules detection based on aptamers and stilbene molecules as well as alternative applications.

Chapter 1 provides the background information for the research and elaborates the proposed hypothesis. It also outlines the goal and scope and offers a brief summary for each chapter of the thesis.

Chapter 2 reviews several common molecular recognition elements and emphasizes the numerous advantages of aptamers against other recognition elements. Currently available aptamer based sensors; mainly optical sensors are elaborated as well as their associated disadvantages. Literature work regarding photochrome molecules is also included. The *trans-cis* photoisomerization process of stilbene molecules, the effect from different substituents or solvents, the dependence of stilbenes' decay rate on their surrounding medium viscosity together with the application of stilbenes in antibody based bioassays are reviewed.

Chapter 3 discusses the rational for the synthesis of different stilbene-analyte conjugates, stilbene maleimides and maleamic acids etc. although their detailed synthesis procedures are presented in the respective results chapter 4 and 5. Moreover, the principles underlying various techniques and instruments employed such as UV-Vis spectrometer, spectrofluorometer, and fluorescence decay and fluorescence polarization are briefly introduced. Detailed protocols for all the photophysical characterization (absorption, emission spectrum and quantum yield determination etc.) and medium viscosity study of stilbene compounds are also included.

Chapter 4 elaborates the photophysical properties of two parent stilbenes as well as their fluorescence decay rate and fluorescence polarization dependence on medium viscosity. Following that, results of photophysical characterization, stability study, and decay rate measurement in buffer based aqueous environment and binding interaction with malachite green aptamer are presented and analyzed for the four stilbene-MG conjugates (competitive binding in **Figure 1.2**). For comparison, similar studies are also performed for the negative control (Rhodamine B-DMAAS).

Chapter 5 elaborates the photophysical study of the synthesized stilbene maleimide and maleamic acid molecules together with the response of their fluorescence decay rate and fluorescence polarization to their surrounding medium environment. Their decay rate study in aqueous environment shows that stilbene maleamic acids have better aqueous adaptability. To mimic the conjugation with thiol modified MG aptamer, the conjugate MSMI-6MCH is synthesized and its photophysical characterization and decay rate dependence on medium viscosity are also presented.

Chapter 6 demonstrates the promising applications of the synthesized stilbene maleimide

and maleamic acid compounds in probing the presence of thiol containing molecules, in low concentration ethanol detection and in pH sensing.

Chapter 7 summarizes all the results obtained in this thesis and their significance towards the proposed hypothesis is also pointed out. Based on the results, recommendations for future work to further optimize the detection system are provided. Besides, some preliminary results for the trial system–Bisphenol A (BPA)/BPA aptamer are also presented.

1.5 Findings and outcomes

This research leads to several novel outcomes by:

1. Combining aptamers and stilbene compounds together for the first time for new sensor design. It utilizes the adaptive binding property of aptamer and the decay rate dependence on medium viscosity of stilbenes so that the problems associated with proteins and in intensity based sensors are avoided.
2. Successfully synthesizing different stilbene-malachite green conjugates. Their photophysical study, medium viscosity dependence study and their binding experiment with MG aptamer provide very useful information in finding the optimal conjugate suitable for the subsequent competitive binding experiments.
3. Synthesizing different stilbene maleimide and maleamic acid compounds. They are able to be conjugated to thiol containing macromolecules, making their labeling possible for further detection applications.
4. Exploring the potential applications of stilbene compounds in areas such as fast and easy thiol probing, low concentration ethanol detection and pH indicator etc.

References

- [1] G.I. Likhtenshtein, R. Bishara, V. Papper, B. Uzan, I. Fishov, D. Gill, A. Parola, *Journal of Biochemical and Biophysical Methods*, 33 (1996) 117-133.
- [2] P.P.-B. Arye, N. Strashnikova, G.I. Likhtenshtein, *Journal of Biochemical and Biophysical Methods*, 51 (2002) 1-15.
- [3] N. Strashnikova, V. Papper, P. Parkhomyuk, G.I. Likhtenshtein, V. Ratner, R. Marks, *Journal of Photochemistry and Photobiology A: Chemistry*, 122 (1999) 133-142.
- [4] T. Dieckmann, E. Suzuki, G. Nakamura, J. Feigon, *RNA*, 2 (1996) 628-640.
- [5] T. Dieckmann, S.E. Butcher, M. Sassanfar, J.W. Szostak, J. Feigon, *Journal of Molecular Biology*, 273 (1997) 467-478.
- [6] T. Hermann, D.J. Patel, *Science*, 287 (2000) 820-825.
- [7] S.D. Gilbert, F.E. Reyes, A.L. Edwards, R.T. Batey, *Structure*, 17 (2009) 857-868.
- [8] D.J. Patel, A.K. Suri, F. Jiang, L. Jiang, P. Fan, R.A. Kumar, S. Nonin, *Journal of Molecular Biology*, 272 (1997) 645-664.
- [9] V. Papper, D. Pines, G.I. Likhtenshtein, E. Pines, *Journal of Photochemistry and Photobiology A: Chemistry*, 111 (1997) 87-96.
- [10] V. Papper, G.I. Likhtenshtein, *Journal of Photochemistry and Photobiology A: Chemistry*, 140 (2001) 39-52.

Chapter 2

Literature Review

This chapter starts with a brief description of biosensors and introduces its two main components: a molecular recognition element and a transducer. In terms of Photochrome Aptamer Switch Assay (PHASA) design, the choice of aptamer as the molecular recognition element was defended against others such as enzyme, antibody and molecular imprint. Detection of common aptamer based optical biosensors depends on the change of fluorescence intensity, which suffers from fluorescence intensities of detection backgrounds. Photochromic molecules respond to their surrounding environment by a fluorescence decay which is independent of the fluorescence intensity and avoids the effect from backgrounds. Stilbene was chosen as the transducer for PHASA against azobenzene especially. The trans-cis isomerization and decay rate dependence on medium viscosity of stilbenes were reviewed as well as their applications in detection. The PHASA based on aptamer and stilbene promises fast (within minutes) and fluorescence intensity independent detection.

2.1 Biosensor

Biosensor, by definition of IUPAC, is an integrated receptor–transducer device which can provide selective quantitative or semi-quantitative analytical information of the analyte. Compared with those traditional detection methods such as high-performance liquid chromatography (HPLC) and gas chromatographic-mass spectrometry (GC-MS) whose detection, identification and quantification process can be complex, expensive and time consuming, biosensors aim to be rapid, simple to use, portable, and sensitive. In a typical biosensor, there are two main components: a molecular recognition element and a transducer. The recognition element is responsible for recognizing, binding to and interacting with the target of interest while the transducer converts the molecular recognition event into a quantifiable event through optical, electrical, magnetic, or other means.

2.2 Molecular recognition elements

Due to their requirements in bio-sensing, molecular recognition elements come in variety of materials. Nature predominantly relies on protein based enzymes, receptors and antibodies for molecular recognition. But other materials offer easier laboratory synthesis, such as aptamers and polymer molecular imprinting.

2.2.1 Enzyme

Among those molecular recognition elements found in nature, enzyme and antibody are predominant. Glucose sensors [1] utilize the enzyme glucose oxidase to catalyze glucose oxidation to gluconolactone as seen in eq. 2.1:

The glucose concentration is then determined indirectly through electrochemically detection of hydrogen peroxidase, H_2O_2 . Enzyme-based biosensors have found applications in many aspects of our life such as health care, food industry and environmental monitoring due to their catalytic activity, specific binding capability and a variety of measurable events, including pH, redox reactions, heat exchange, and gas detection [2]. However, enzyme recognition elements are limited by their instability and cofactor requirements. Due to the synthetic nature of aptamers, they can be heat

regenerated and can be stored more than one year in powder form, and there is no cofactor needed.

2.2.2 Antibody

Attributed to their specific binding affinity towards their corresponding antigen, antibodies have been predominantly used as a molecular recognition element for small molecules and bio-macromolecules. One assay that based on the specific antibody-antigen interaction is the enzyme-linked immunosorbent assay (ELISA) (**Figure 2.1**).

Figure 2.1. A schematic illustration of the sandwich ELISA format.

The detection of this assay relies on the reaction between the linked-enzyme with its corresponding substrate, which gives rise to colorimetric, fluorescent or electrochemical signal. Compared to radioimmunoassay, ELISA is safer and simpler as there is no radioactive labels involved and the analyte quantity is associated with the amount of signal obtained. Based on ELISA, literature has reported the detection of various targets from proteins such as human serum antibody biomarkers and serum immunoglobulin G (IgG) etc. [3, 4] to virus such as hepatitis E virus (HEV), plant virus and chikungunya virus in mosquitoes etc. [5-12]. Also, high detection sensitivity can be achieved in ELISA. For example, in Wang and coworkers' work [3], the detection limit of 1.1 pg/mL was obtained for the biomarker of *Candida albicans* infection. However, to make sure the only presence of the enzyme-linked antibody that binds to the analyte of interest, tedious washing steps are required to eliminate other unbound and non-specifically bound antibodies. These washing steps increase assays times on the order of hours and even days. In the above biomarker detection, the average detection time for each serum sample is 6 hours [3]. This significantly limits the high-throughput applications of ELISA. Moreover, antibodies are primarily generated through immunogen injection into animals such as mice, rabbits and

goats etc. to provoke an adaptive immune response. This method of synthesis introduces batch to batch variations and can kill the animals if the antigens are toxic. Since aptamers are chemically synthesized *in vitro*, there is little or no batch to batch variation and aptamers for toxic antigens can be generated without taking the risk of killing the animals. Besides, no separation is needed in PHASA and the detection can be completed within minutes.

2.2.3 Molecular imprinted polymer

Unlike the above molecular recognition elements that are primarily isolated from biological system, molecular imprinted polymers are generated by polymer synthesis in the presence of the target of interest. A generalized scheme describing the synthesis process is shown in **Figure 2.2** [2].

Figure 2.2. A schematic representation of the preparation of molecular imprinted polymer.

Target molecules are used as templates and are mixed with functional monomers which assemble around the templates accordingly. After polymerization, an imprinted matrix with templates is formed. Removing the templates, a molecular imprinted polymer with cavities complementary in size and shape to the templates is obtained. The cavities are then used for selective recognition of the target molecules. “Lock and key” model is applied in its recognition and ensures its high specificity. Together with other advantages such as cheap fabrication, versatility and stability etc, it has been promisingly used for detection of various proteins, cancer biomarkers and small molecules like TNT explosives, virus and

even chemical vapors (NH_3 , CO_2 and SO_2) [13-21]. However, there are also limitations involved such as the complex template removal process and matrix fabrication. More importantly, as aforementioned, the recognition of molecular imprinted polymer relies on fitting, unlike the “adaptive binding” [22-26] of aptamer in the presence of the corresponding analyte. Since PHASA involves binding of the recognition element to the analyte as well as the stilbene-analyte conjugates, the use of an aptamer is more suitable.

2.2.4 Aptamer

Aptamers, since their discovery by two groups independently in 1990 [27, 28], have been considered as a promising molecular recognition element. One unique feature of aptamers is their “adaptive binding” [22-26] to the corresponding targets. In the absence of ligands, aptamers are unstructured in solution. But in the presence of ligands, aptamers fold round the ligands, which become part of the aptamer architecture, and a three-dimensional aptamer complex structure forms. This folding provides numerous discriminatory intermolecular contacts, including base stacking, shape complementarity, electrostatic interactions, and hydrogen bonding [29-32]. That is, aptamers interact with ligands to form the stable complex structure, involving the properties of both aptamer and ligand. This is different from the ligand recognition process in proteins by a preformed binding pocket where the binding does not depend on the presence of the ligand, except for local changes due to “induced fit”. This allosteric property of aptamers changes their conformation and their surrounding micro-environment in the presence of ligands, which can be used for ligand detection with no washing steps needed.

Unlike the way from which antibodies are generated, aptamers are obtained based on an iterative process of in vitro selection and amplification namely Selective Evolution of Ligands by Exponential Enrichment (SELEX) [33], in which a large library containing randomly generated oligonucleotide sequences are incubated with the target molecule and the sequences that bind the strongest to the target is used as the binding receptors. Based on SELEX, aptamers for a wide range of targets such as AMP, peptides and proteins have been developed with the dissociation constant K_d in the range of nM ~ μM [24]. As SELEX is done in vitro, theoretically all antigens can have a corresponding binding aptamer for their recognition. This process enables the fabrication of aptamers for non-immunogenic

and toxic targets that are otherwise difficult through immunological means. Advantages of aptamers versus antibodies are summarized in **Table 2.1**, explaining why aptamers are an attractive molecular recognition element for biosensors that operate in ambient conditions. In spite of the numerous advantages of aptamers, sensitivity (pg/mL to ng/mL range) [3, 34, 35] and selectivity achieved in antibody based biosensors is difficult to be realized in aptamer based sensors. Since aptamer folding around arbitrary ligand cannot display perfect shape complementary, aptamer can bind to more than one ligand [36]. In the context of PHASA which aims fast and portable detection and involves aptamer binding to its ligand as well as stilbene-ligand conjugates, aptamer's less-than-perfect folding, possible modification and detection with no separation make it a better molecular recognition element than antibody.

Table 2.1. Comparison of aptamer and antibody as recognition element [37].

Antibody	Aptamer
Limitations against targets representing constituents of the body and toxins	Toxins and molecules that do not elicit good immune response can be used to generate high affinity aptamer
Kinetic parameters of antibody-antigen interactions cannot be changed on demand	Kinetic parameters such as on/off rates can be changed on demand
Antibodies are more sensitive to storage time, temperature, and pH in terms of tertiary denaturation	Denatured aptamer can be heat regenerated and tends to have longer dry storage
Antibody recognition work best under physiological conditions at which they were produced	Selection conditions can be manipulated to obtain aptamer with properties desirable target environments
Production suffers from batch to batch variation	Synthesized by chemical process resulting in little or no batch to batch variation
Production is dependent on animal or dedicated cell lines, chemical synthesis is too costly.	Aptamer is selected via SELEX related methods, requiring no living organisms.
Antibody labelling is more problematic in terms of off affinity and amino acid specificity	Reporter molecules can be attached to aptamer at precise locations during chemical synthesis

2.2.4.1 Aptamer based biosensors

Since aptamers can bind to targets with high affinity and specificity like antibodies, aptamer based biosensors that analogous to immunosensors have also been developed. For example, Jo and coworkers [38] reported the development of a pair of bisphenol A (BPA) aptamers, and a detection limit of 1 $\mu\text{M}/\text{mL}$ has been achieved for BPA detection based on the sandwich format. While due to the unique “adaptive binding” behavior of aptamers, new detection formats that based on its conformational change and thus its surrounding environment change in the presence of targets have also been developed. According to the way the signals are generated, they can be divided into optical, electrochemical and mass-sensitive detection methods.

Electrochemical sensors convert the binding event to the change of electrochemical signal. Since aptamer is immobilized on electrode and labelled or surrounded with electro-active groups, upon target binding, the conformational change of aptamer changes their interaction or the surface properties of electrodes changes. Based on this, electrochemical biosensors for the detection of thrombin and platelet derived growth factor and even small molecules like cocaine and ATP have been developed with detection limits as low as those of ELISA. [39-46]. However, electrochemical sensors cannot be applied in complex or “dirty” environments as signals from background interactions interfere with the detection results significantly. In PHASA, aptamers are dissolved in solutions instead of being immobilized on electrodes, avoiding the limitation of aptamer loading issue in electrochemical sensors. The detection in PHASA based on the fluorescence decay of stilbene compounds, which significantly reduces the effect from backgrounds.

Mass sensitive sensors detect the bound target mass, ultimately changing surface properties on which the aptamers are bound. This allows it to be a label free detection method. SPR (surface plasma resonance) sensors recognise the mass change through differential refractive index; QCM (quartz crystal microbalance) sensors detect analytes through resonance frequency of a vibrating crystal and micromechanical cantilever-based aptasensors rely on the bending of the cantilever after target binding. Mass sensitive sensors for the detection of HIV-1 Tat Protein, hepatitis C virus helicase and thrombin etc. have been reported in literature [47-52]. But in these designs, targets are bio-macromolecules that can induce substantial mass variations. Hence, they are more applicable to targets on

the order of the aptamer recognition element [53]. Moreover, mass sensitive sensors are also surface-based like electrochemical sensors, and washing is involved. So the detection timescale of several minutes in PHASA cannot be achieved in mass sensitive biosensors.

2.2.4.2 Aptamer based optical biosensors

Unlike aptamer based electrochemical and mass sensitive biosensors, optical biosensors are solution based and independent of surface. Due to the wide variety of fluorophores and the simple measurement instruments, a large number of detection strategies have been developed. One common format is molecular beacon, in which a fluorophore is covalently labelled at one end of the aptamer and a quencher at the other end, shown in **Figure 2.3 A**. This format has been used in the detection of such as metal ions and proteins [54-57]. As single strand oligonucleotides, aptamers can also bind to a complementary sequence to form a duplex structure, apart from binding to their ligands. Based on this feature, another format shown in **Figure 2.3 B** has been proposed and applied for ATP sensing [58, 59]. Both formats involve labelling of aptamers or nucleic acids, making the detection complex and costly.

Figure 2.3 A. The molecular beacon format: in the absence of target, the fluorescence intensity of the fluorophore is quenched. But in the presence of target, the stem structure is destroyed, resulting in separation of the fluorophore from the quencher and giving rise to high fluorescence intensity. **B.** The competition format between target and a complementary sequence of the aptamer. With target addition, aptamer folds around target and restores fluorescence intensity.

For the development of “label free” aptamer based optical sensors, fluorophores whose fluorescence properties change greatly when aptamer conformational change occurs, are introduced. A common example is malachite green (MG), which itself has low fluorescence but its fluorescence intensity increases up to 2000 fold when bound to MG

aptamer [60]. Based on this feature, an aptasensor for the detection of adenosine has been fabricated by Xu and coworkers [61] whose design is presented in **Figure 2.4 A**. Similar to MG, Oil Green (OG) exhibits a fluorescence enhancement upon binding to ssDNA, based on which, the detection of potassium ions using an ATP-binding aptamer has been reported by Huang and coworkers [62]. Another kind of fluorophores namely intercalators such as Thiazole Orange (TO), SYBR Green I (SG) and $[\text{Ru}(\text{phen})_2(\text{dppz})]^{2+}$, which when inserted into the double helix structure can lead to strong fluorescence enhancement, are also widely used (**Figure 2.4 B**). Based on them, the detection of ATP, thrombin and other proteins like IgE and PDGF-BB has been presented in literature [63-65].

Figure 2.4 A. With no target, the bridging strand forms duplex structure with MG aptamer and adenosine aptamer. After addition of adenosine, the bridging strand is released and MG aptamer binds to MG in the free solution, resulting in fluorescence enhancement. **B.** Detection based on intercalators, analyte-TO (Thiazole Orange) conjugate is pre-bound to the aptamer and fluorescence is observed due to the intercalation of TO to the double helix structure. The target addition replaces the conjugate and fluorescence decrease observed.

For the aforementioned aptamer based optical biosensor formats, either “labelled” or “label free”, the detection and quantification is predominantly based on the change of fluorescence intensity. This poses limitations to their further development since fluorescence intensity is sensitive to measurement conditions, instrumental conditions and backgrounds especially in biological environments. Hence, besides fluorescence intensity, fluorophores’ other photophysical parameters such as peak wavelength and absorbance have been in used for ATP, cocaine and PDGF detection [66-68]. However, in these formats, the quantification is difficult and the fluorophores cannot be used for wide

applications. Photochromic molecules, under excitation by light or heat, convert from one isomer to other and produces a fluorescence decay curve. This curve is independent of fluorescence intensity and thus eliminates the interference from backgrounds. Besides, they can be chemically tailored to adapt to wide analyte detection applications. Therefore, photochromic molecules are selected as the transducer to construct PHASA.

2.2.5 Peptide

Peptides have been used as a recognition element for the fabrication of novel biosensors for a number of reasons, including their diversified structure, high affinity to targets, matured synthesis protocol and simple attachment to reporter molecules etc. [69, 70]. Similar to SELEX for aptamers, high affinity peptide sequences can also be obtained through various screening methods.

The five combinatorial peptide library methods generally used for the screening process include: 1) biological libraries such as phage display [71], 2) spatially addressable libraries like multipin technology [72] and SPOT synthesis method [73], 3) mixture libraries that require deconvolution such as the positional scanning method [74], 4) the “one-bead one-compound (OBOC)” combinatorial library method [75,76] and 5) solution peptide libraries with affinity chromatography [77]. Based on phage display, peptide ligands for various targets such as $\alpha_5\beta_1$ integrin [78], monoclonal antibody (3-E7) [79], virus [80, 81], surface receptors [82, 83] etc. have been isolated and identified. One major limitation of phage display peptide library is, however, that only peptides with L-amino acids can be displayed. With OBOC library, the incorporation of non-natural components, such as D-amino acids or cyclic or branched ligands can be achieved, which facilitates the identification of ligands that are resistant to proteolytic degradation and suitable for *in vivo* applications [84]. Peptide ligands for $\alpha_4\beta_1$ integrin [85], inhibitors of protein kinases [86], surface receptors of human cancer cells [87, 88] and HIV anti-p24 antibodies [89] etc. have been identified using the OBOC library screening method.

Combining with the various transduction methods, electrochemical [89-93] and optical [94-106] peptide biosensors have been largely fabricated for various targets from proteins, metallic ions, nucleic acids and bacterial etc. Similar to optical aptamer sensors, the detection of optical peptide based sensors depends on environmental sensitivity [94-97],

fluorescence quenching/fluorescence resonance energy transfer (FRET) [98-104] and the wavelength shifts in monomer-excimer [105, 106]. Based on the binding capability of aptamers to their complementary nucleotides as well as their targets, aptamer sensors for ATP sensing [58, 59] have been constructed. Similarly, based on the enzymatic catalysis of peptide substrates, peptide sensors have been used in monitoring the enzyme activity of proteases and kinases [70, 107].

As molecular recognition element, both aptamers and peptides are comparable in terms of selectivity and sensitivity. However, the majority of peptide ligands' targets are proteins, antibodies, bacterial and receptors. That is, they are very promising in drug development and biomedical research. Although specific peptide ligands have been identified for small dye molecules such as indigo carmine, crystal violet and rhodamine etc. in Lam et al [108] and Wennemers et al' [109] work, they stressed caution in attaching fluorophores to peptides to avoid label-induced artifacts, instead of their direction application in detection. Therefore, under the thesis project scope of small molecule detection (<1000 Da), aptamers are potentially favored. Together with photochromic molecules whose detection is based on fluorescence decay rather than fluorescence intensity, photochromic aptamer switch assay (PHASA) for small molecule detection has been explored in the project.

2.3 Photochromic molecular switch

Photochromism can be defined as a reversible change of a single species between its two states having distinguishable absorption spectra, such change being induced in at least one direction after exposure to electromagnetic irradiation which are normally in the region of ultraviolet or visible. For organic compounds that exhibit photochromism, the conversion from one state to another is achieved through different processes, including *trans-cis* isomerization, heterolytic cleavage, photo-induced ring closing reactions and photo-tautomerism [110, 111].

Figure 2.5. Examples of photochromic compounds and their conversion processes.

Figure 2.5 gives some examples of common organic photochromism compounds and their associated conversion processes. Among them, ring closing reaction and heterolytic cleavage lead to more electron delocalized structure, and the isomers obtained are generally colored. Compared with the *trans*-*cis* isomerization, in which the *cis* isomer is non-fluorescent due to the steric hindrance and torsion experienced, the fluorescence decay kinetics of compounds undergoing ring closing reaction or heterolytic cleavage are more complicated. Thus, they are not chosen for PHASA. For the *trans*-*cis* isomerization process, it is observed for both stilbene and azobenzene compounds. However, their isomerization mechanism, photophysical parameters and decay kinetics behavior are different due to different compound structure, substituents and environment parameters, making them suitable for different applications.

2.3.1 Azobenzene and its derivatives

Azobenzene is composed of two phenyl rings linked by an N=N bond. As mentioned above, the photochromism of azobenzene compounds are realized by *trans*-*cis* isomerization. This photoreaction only causes the rearrangement of the electronic and nuclear structure of the molecule and no bond breaking or formation is involved. **Figure 2.6** shows schematically the *trans*-*cis* isomerization process. After exposure to a light irradiation $h\nu_1$, the *trans* isomer is promoted to its excited state, from which relaxation occurs and *cis*

isomer can be obtained. Likewise, *trans* isomer can also be generated from the *cis* isomer by irradiation but with a shorter wavelength. Besides, because of *trans* isomer's thermodynamical stability, it can also be generated thermally as the *cis*–*trans* reaction can take place in dark.

Figure 2.6. A schematic illustration of *trans*–*cis* isomerization of azobenzene [110].

The photophysics of azobenzene compounds' isomerization process has been extensively studied [112-118]. But there is still controversy with regards to their detailed isomerization mechanism. Generally, it has been envisaged that the isomerization process takes place along two different pathways, one that involves the torsional motion around the N=N bond passing through a conical intersection and another relates to the in-plane inversion about one of the nitrogen centers. Either way, the photo-isomerization of azobenzene is reported to be extremely rapid, occurring in picosecond and even femtoseconds timescales [110]. Based on the rapid and reversible conformational change, they have been extensively developed as photo-switches for biomolecules, allowing spatial and temporal control of a variety of biological processes [119]. This isomerization timescale is several magnitudes faster than that of stilbene compounds under the same conditions, meaning the conversion barrier is much lower. Hence, the conformational change of aptamer may not be able to exert a significant influence on the measured steady-state fluorescence decay curve of azobenzene compounds, and detection based on decay rate change will be impossible. Besides faster photo-isomerization process, the fluorescence quantum yields of azobenzene compounds are much lower than that of stilbenes. In Han and other researchers' work, their

quantum yields have been reported to be in the range of 10^{-7} to 10^{-5} [120-122], which is more than 100 times lower, as the quantum yields of stilbene compounds are generally greater than 10^{-3} [123]. As PHASA relies on the change of fluorescence decay rate, low fluorescence quantum yield is not appropriate as it results in low signal to noise ratio and can even make the decay behavior not observable. Therefore, stilbene compounds are preferred for PHASA.

2.3.2 Stilbene and its derivatives

By stilbene compounds, they refer to systems that are made up of stilbene units. In all compounds of this type, 1,2-ethenediyl groups link benzene rings. That means, the N=N bond in azobenzene compounds is replaced by the CH=CH bond. Similarly, there are two isomers for stilbene compounds: *trans* and *cis*. And their *trans* isomer is fluorescent while *cis* isomer is non-fluorescent due to the steric hindrance and torsion between the two phenyl rings.

2.3.2.1 *Trans-cis* photo-isomerization process

Like that observed in azobenzene compounds, the *trans* and *cis* isomer can be inter-converted by light irradiation. In the photolysis of *trans* stilbene, the major products are *trans* and *cis* stilbene. In the case of *cis* stilbene, the major products are the same. But dihydrophenanthrene which is the photo-cyclization product from the *cis* isomer can be formed. The reaction is believed to be minor but is commonly used in the synthesis of phenanthrenes. For the photo-isomerization processes, the currently accepted potential energy scheme is represented by the sketch in **Figure 2.7** proposed by Saltiel [124, 125]. In the ground electronic state a large barrier exists between the *trans* isomer and the *cis*. In the first excited state of *trans* stilbene, there is a potential minimum and a much smaller barrier for the twisting motion about the ethylene bond. After crossing the twisting barrier, the energy of the excited configuration gradually decreases as the angle of twist evolves from the *trans* value to the 90° form (twisted form), where the excited state has the minimum and correlates with the ground state. Then rapid decay to the ground state surface occurs. Once on the ground state surface, the product molecule branches to either the *trans* or the *cis* form. The isomerization from the *cis* to the *trans* form is more or less the same

except that the initial irradiation wavelength is shorter and there is little or no barrier to the torsional motion. The *cis*–*trans* isomerization rate is very fast and has been reported to be less than 2 ps [126-128]. This is likely attributed to little or no twisting barrier present. And this also explains the picosecond timescale photo-isomerization rate in azobenzene compounds. But the *trans*–*cis* isomerization process of stilbene compounds is more than 10 times slower.

A simplified view of the stilbene *trans*–*cis* isomerization process can be described in the following manner; upon electronic excitation the bond order of the ethylene moiety is reduced and twisting about this bond is possible during the excited-state lifetime (ns range). Based on Saltiel and coworkers' effort, three mechanisms have been proposed [124, 125]. The commonly accepted mechanism involves twisting about the ethylenic bond in the first excited singlet state to a twisted singlet intermediate state ("phantom state"), where there is an avoided crossing with the ground state (**Figure 2.7**). Saltiel and Sumitani and coworkers [129-132] have supported that this singlet mechanism is more self-consistent and applicable to the majority of stilbenes through investigations of *cis/trans*-quantum yield measurements, triplet sensitization and quenching of isomerization.

Figure 2.7. A schematic illustration of *trans*–*cis* isomerization of stilbenes.

The *trans* isomer of stilbene compounds is strongly fluorescent while the *cis* isomer has an insignificant quantum yield. When the *trans* isomer is irradiated, both radiative fluorescence emission and *trans*–*cis* twisting around the double bond are possible during the excited–state lifetime. A competition between the two processes is induced and the photo-isomerization from *trans* to *cis* acts like a quenching funnel for the fluorescence of the *trans* isomer. Hence, constant irradiation of the fluorescent *trans* stilbene compounds at the excitation maximum can produce a substantial change of the fluorescence emission which can then be monitored as a time trace (kinetics decay) signal or emission gradient. A typical fluorescence decay curve may be fitted into two first–order exponential decay curves generally. The first represents the reversible *trans*–*cis* photo-isomerization process and the second is probably a result of some photo-destruction processes and usually appears after approximately 30% of photo-conversion. The first exponential decay is characterized by the apparent decay rate constant k_{app} , which is the sum of the forward (*trans* to *cis*) and back (*cis* to *trans*) reaction rate constants. A number of studies have demonstrated that the apparent decay rate k_{app} can be calculated from the slope of the fluorescence decay rate $-dI/dt$ against the fluorescence intensity I plot [133–137].

2.3.2.2 Substituent & solvent effect

The *trans*–*cis* isomerization rate or the fluorescence decay rate depends strongly on the substituted groups of stilbene compounds as well as their medium solvents. Activation energy investigation of the isomerization process for non-polar stilbenes in homologous series of solvents has been performed and the activation energy tends to decrease in more polar solvents. This indicates that the twisted transition state (phantom state) is polarizable and involves zwitterionic structure which can be stabilized in polar solvents, lowering the barrier to the torsional motion and facilitating the isomerization reaction [138]. But for stilbenes that have donor or acceptor groups or even both, reaction barrier excitation is much more complicated, especially in polar solvents.

Detailed studies regarding the effect from both substituents and solvents on the isomerization rate have been extensively conducted by Papper and coworkers [137, 139]. In their publications, based on the substituents, stilbene compounds were divided into three groups. Group one consists of non-polar or slightly polar stilbenes without strong donor–

acceptor groups. Charges are separated and localized around the twisted zwitterion state. So both polar substituents and solvents stabilize the more polar p^* state (phantom state), giving rise to a much easier torsional motion and a faster decay rate. In group two, stilbene compounds have a strong donor group $-N(CH_3)_2$ in their para position. This strong donor stabilizes more the singlet excited state than the “phantom” state. Therefore, a high activation energy and a slow isomerization rate is resulted. For group three, strong donor–acceptor pairs are substituted in stilbene compounds. This leads to large charge delocalization between the donor and acceptor aromatic moieties and hence large dipole moment. In other words, the twisted state has low polarity and the excited singlet state is stabilized preferentially. This results in very high twisting barrier and *trans*–*cis* isomerization cannot even be observed just like the case in *trans*-4-dimethylamino-4'-carbomethoxy stilbene (DACMS).

Besides the high twisting barrier resulted from the highly polarized excited state, for stilbene compounds with strong donor–acceptor pair substituents, a second state–twisted intramolecular charge transfer (TICT) state is proposed to have formed which is more stable and thus make the *trans*–*cis* isomerization process even less preferred. The formation of TICT state is supported by the observation of two emissive bands in several stilbene compounds such as *trans*-4-dimethylamino-4'-cyanostilbene (DACS) and DACMS but a non-emissive TICT state has been assumed for nitro-substituted stilbenes due to a specific interaction of the nitro group which quenches the charge transfer state emission. Extensive work [140-145] including both theoretical and experimental has been performed to confirm the existence of the TICT state and explore the associated mechanism.

2.3.2.3 Medium viscosity dependence and areas of application

One unique feature of stilbene photochemistry is their strong dependence on the medium parameters such as temperature, pressure and especially medium viscosity. This is because the *trans*–*cis* isomerization process of stilbenes involves the torsional rotation of the ethylene double bond, so steric hindrance or viscous medium can hinder the rotation and thus decrease the fluorescence decay rate, as schematically shown in **Figure 2.8**.

Detailed investigations of the medium viscosity effect on the decay kinetics have been performed. In Strashnikova and coworkers' work [133], several stilbene derivatives were

immobilized on quartz surfaces which were coated with proteins. Compared with those stilbenes in free solution, their apparent fluorescence decay rates were 3-4 times lower. This indicates that the quartz surface and protein sterically hinder the rotation of the stilbene fragment around the olefinic double bond in the excited state. To further study the micro-viscosity effect on the photo-isomerization decay of stilbenes, a series glycerin–water mixtures were prepared by changing the relative concentration (% v/v) of glycerin, in which the decay rates were measured. A logarithmic dependence of the *trans*–*cis* isomerization rate on the reciprocal of the medium viscosity was observed. This strong dependence has made stilbene compounds extremely attractive in probing the micro-environments of various organized media such as biological membranes and surface systems [133-137].

Figure 2.8 A. In free solution (~ 1.0 cP), *trans* stilbene gradually converts to the *cis* isomer, resulting in a fast fluorescence decay. B. When the solution is viscous (several hundreds of cP) or *trans* stilbene is sterically hindered, the conversion to *cis* isomer is prohibited, and a slower fluorescence decay curve is resulted.

Besides the biological applications, stilbene compounds have also been applied in immunoassays for detection. In Chen and coworkers' study [146], a rapid and sensitive immunoassay based on stilbene compounds and antibodies has been constructed for TNP (2,4,6-trinitrophenol) detection. The design format is shown in **Figure 2.9**.

The stilbene–antigen analogue probe was first synthesized and then bound within the binding site of antiTNP antibody on one side and with a second globular protein (lysozyme) on the other side. The *trans*–*cis* photo-isomerization rate of the probe in the constricted environment is markedly inhibited when compared with that in free solution. In other words, when there is no TNP in the solution, a slow fluorescence decay curve for the probe is obtained. But when the analyte TNP is added, the probe is replaced out of the binding site, leading to a faster fluorescence decay. This change in apparent decay rate can certainly be used to indicate the presence and even determine the concentration of the target. This study not only confirms again the strong dependence of stilbene compounds' decay rate on their surrounding medium viscosity, it also paves the way for their extensive applications in bio-detection.

Figure 2.9 A. The immunoassay design for TNP detection based on stilbene and antibody. B. Different fluorescence decay kinetics with or without the addition of the TNP analyte.

In Chen's study, antibody has been used as the molecular recognition element. However, as have been discussed in section 2.2.2 and section 2.2.4, antibody suffers from batch to batch variation, instability and animal based production compared to aptamer, and it does not have the "adaptive binding" feature of aptamer. Hence, it is proposed to replace antibody with aptamer and take full advantage of stilbene's fluorescence decay rate dependence on surrounding medium viscosity to construct a biosensor for detection, as presented in detail in the hypothesis section.

References

- [1] J. Wang, Chemical Reviews, 108 (2008) 814-825.
- [2] J.P. Chambers, B.P. Arulanandam, L.L. Matta, A. Weis, J.J. Valdes, DTIC Document 2008.
- [3] Y. Wang, Z. Ju, B. Cao, X. Gao, Y. Zhu, P. Qiu, H. Xu, P. Pan, H. Bao, L. Wang, ACS Nano, 9 (2015) 4475-4483.
- [4] D.D. Erdman, L.J. Anderson, D.R. Adams, J.A. Stewart, L.E. Markowitz, W.J. Bellini, Journal of Clinical Microbiology, 29 (1991) 1466-1471.
- [5] H. Xu, B. Di, Y. Pan, L. Qiu, W. Hao, L. He, K. Yuen, X. Che, Journal of Clinical Microbiology, 44 (2006) 2872-2878.
- [6] S.A. Tsarev, T.S. Tsareva, S.U. Emerson, A.Z. Kapikian, J. Ticehurst, W. London, R.H. Purcell, Journal of Infectious Diseases, 168 (1993) 369-378.
- [7] R. Singh, B. Sreenivasa, P. Dhar, L. Shah, S. Bandyopadhyay, Veterinary Microbiology, 98 (2004) 3-15.
- [8] S. Hundekar, J. Thakare, M. Gokhale, S. Barde, Indian Journal of Medical Research, 115 (2002) 144.
- [9] K. Chand, S.K. Biswas, A. De, B. Sing, B. Mondal, Journal of Virological Methods, 160 (2009) 189-192.
- [10] M.F. Clark, A. Adams, Journal of General Virology, 34 (1977) 475-483.
- [11] P.D. Skottrup, M. Nicolaisen, A.F. Justesen, Biosensors and Bioelectronics, 24 (2008) 339-348.
- [12] D. Haines, E. Clark, E. Dubovi, Veterinary Pathology Online, 29 (1992) 27-32.
- [13] F. Berti, S. Todros, D. Lakshmi, M.J. Whitcombe, I. Chianella, M. Ferroni, S.A. Piletsky, A.P.F. Turner, G. Marrazza, Biosensors and Bioelectronics, 26 (2010) 497-503.
- [14] H. Bao, T. Wei, X. Li, Z. Zhao, H. Cui, P. Zhang, Chinese Science Bulletin, 57 (2012) 2102-2105.
- [15] Y. Wang, Z. Zhang, V. Jain, J. Yi, S. Mueller, J. Sokolov, Z. Liu, K. Levon, B. Rigas, M.H. Rafailovich, Sensors and Actuators B: Chemical, 146 (2010) 381-387.
- [16] G. Guan, B. Liu, Z. Wang, Z. Zhang, Sensors, 8 (2008) 8291-8320.
- [17] Y. Uludağ, S.A. Piletsky, A.P. Turner, M.A. Cooper, Febs Journal, 274 (2007) 5471-5480.

- [18] R. Liang, L. Chen, W. Qin, *Scientific Reports*, 5 (2015) 12462.
- [19] D. Cai, L. Ren, H. Zhao, C. Xu, L. Zhang, Y. Yu, H. Wang, Y. Lan, M.F. Roberts, J.H. Chuang, *Nature Nanotechnology*, 5 (2010) 597-601.
- [20] Q. Deng, J. Wu, X. Zhai, G. Fang, S. Wang, *Sensors*, 13 (2013) 12994-13004.
- [21] M. Pan, G. Fang, Z. Duan, L. Kong, S. Wang, *Biosensors and Bioelectronics*, 31 (2012) 11-16.
- [22] D.J. Patel, A.K. Suri, F. Jiang, L. Jiang, P. Fan, R.A. Kumar, S. Nonin, *Journal of Molecular Biology*, 272 (1997) 645-664.
- [23] S.D. Gilbert, F.E. Reyes, A.L. Edwards, R.T. Batey, *Structure*, 17 857-868.
- [24] T. Hermann, D.J. Patel, *Science*, 287 (2000) 820-825.
- [25] T. Dieckmann, S.E. Butcher, M. Sassanfar, J.W. Szostak, J. Feigon, *Journal of Molecular Biology*, 273 (1997) 467-478.
- [26] T. Dieckmann, E. Suzuki, G.K. Nakamura, J. Feigon, *RNA*, 2 (1996) 628-640.
- [27] A.D. Ellington, J.W. Szostak, *Nature*, 346 (1990) 818-822.
- [28] C. Tuerk, L. Gold, *Science*, 249 (1990) 505-510.
- [29] X. Ye, A. Gorin, R. Frederick, W. Hu, A. Majumdar, W. Xu, G. McLendon, A. Ellington, D.J. Patel, *Chemistry & Biology*, 6 (1999) 657-669.
- [30] S. Rowsell, N.J. Stonehouse, M.A. Convery, C.J. Adams, A.D. Ellington, I. Hirao, D.S. Peabody, P.G. Stockley, S.E.V. Phillips, *Nature Structure Molecular Biology*, 5 (1998) 970-975.
- [31] M.A. Convery, S. Rowsell, N.J. Storehouse, A.D. Ellington, I. Hirao, J.B. Murray, D.S. Peabody, S.E.V. Phillips, P.G. Stockley, *Nature Structure Molecular Biology*, 5 (1998) 133-139.
- [32] X. Ye, A. Gorin, A.D. Ellington, D.J. Patel, *Nature Structure Molecular Biology*, 3 (1996) 1026-1033.
- [33] W. Mok, Y. Li, *Sensors*, 8 (2008) 7050-7084.
- [34] R. Kuruto-Niwa, Y. Tateoka, Y. Usuki, R. Nozawa, *Chemosphere*, 66 (2007) 1160-1164.
- [35] J. Zhou, S. Zhao, J. Zhang, L. Zhang, Y. Cai, L. Zhou, *Analytical Methods*, 5 (2013) 1570-1576.
- [36] C. Baugh, D. Grate, C. Wilson, *Journal of Molecular Biology*, 301 (2000) 117-128.

- [37] C.K. O'Sullivan, *Analytical and Bioanalytical Chemistry*, 372 (2002) 44-48.
- [38] M. Jo, J.Y. Ahn, J. Lee, S. Lee, S.W. Hong, J.W. Yoo, J. Kang, P. Dua, D. Lee, S. Hong, S. Kim, *Oligonucleotides*, 21 (2011) 85-91.
- [39] L. Zhou, L.J. Ou, X. Chu, G.L. Shen, R.Q. Yu, *Analytical Chemistry*, 79 (2007) 7492-7500.
- [40] P. He, L. Shen, Y. Cao, D. Li, *Analytical Chemistry*, 79 (2007) 8024-8029.
- [41] R. Polsky, R. Gill, L. Kaganovsky, I. Willner, *Analytical Chemistry*, 78 (2006) 2268-2271.
- [42] G.S. Bang, S. Cho, B.-G. Kim, *Biosensors and Bioelectronics*, 21 (2005) 863-870.
- [43] X. Zuo, S. Song, J. Zhang, D. Pan, L. Wang, C. Fan, *Journal of the American Chemical Society*, 129 (2007) 1042-1043.
- [44] M. Mir, I. Katakis, *Mol. Biosyst.*, 3 (2007) 620-622.
- [45] A.E. Radi, J.L. Acero Sánchez, E. Baldrich, C.K. O'Sullivan, *Journal of the American Chemical Society*, 128 (2006) 117-124.
- [46] Y. Xiao, A.A. Lubin, A.J. Heeger, K.W. Plaxco, *Angewandte Chemie International Edition* 44 (2005) 5456-5459.
- [47] K.S. Hwang, S.-M. Lee, K. Eom, J.H. Lee, Y.-S. Lee, J.H. Park, D.S. Yoon, T.S. Kim, *Biosensors and Bioelectronics*, 23 (2007) 459-465.
- [48] C.A. Savran, S.M. Knudsen, A.D. Ellington, S.R. Manalis, *Analytical Chemistry*, 76 (2004) 3194-3198.
- [49] A. Bini, M. Minunni, S. Tombelli, S. Centi, M. Mascini, *Analytical Chemistry*, 79 (2007) 3016-3019.
- [50] T. Hianik, V. Ostatná, Z. Zajacová, E. Stoikova, G. Evtugyn, *Bioorganic & Medicinal Chemistry Letters*, 15 (2005) 291-295.
- [51] S. Tombelli, M. Minunni, E. Luzi, M. Mascini, *Bioelectrochemistry*, 67 (2005) 135-141.
- [52] E. Luzi, M. Minunni, S. Tombelli, M. Mascini, *TrAC Trends in Analytical Chemistry*, 22 (2003) 810-818.
- [53] E.J. Cho, J.W. Lee, A.D. Ellington, *Annual Review of Analytical Chemistry*, 2 (2009) 241-264.
- [54] X. Fang, A. Sen, M. Vicens, W. Tan, *ChemBioChem*, 4 (2003) 829-834.

- [55] T.M. Lerga, C.K. O'Sullivan, *Analytica Chimica Acta*, 610 (2008) 105-111.
- [56] R. Yamamoto, P.K. Kumar, *Genes to Cells*, 5 (2000) 389-396.
- [57] Z. Tang, P. Mallikaratchy, R. Yang, Y. Kim, Z. Zhu, H. Wang, W. Tan, *Journal of the American Chemical Society*, 130 (2008) 11268-11269.
- [58] R. Nutiu, Y. Li, *Journal of the American Chemical Society*, 125 (2003) 4771-4778.
- [59] R. Nutiu, Y. Li, *Chemistry—A European Journal*, 10 (2004) 1868-1876.
- [60] J.R. Babendure, S.R. Adams, R.Y. Tsien, *Journal of the American Chemical Society*, 125 (2003) 14716-14717.
- [61] W. Xu, Y. Lu, *Analytical Chemistry*, 82 (2009) 574-578.
- [62] C.C. Huang, H.T. Chang, *Chemical Communications*, (2008) 1461-1463.
- [63] Y. Jiang, X. Fang, C. Bai, *Analytical Chemistry*, 76 (2004) 5230-5235.
- [64] Y. Tan, X. Zhang, Y. Xie, R. Zhao, C. Tan, Y. Jiang, *Analyst*, 137 (2012) 2309-2312.
- [65] R. Pei, J. Rothman, Y. Xie, M.N. Stojanovic, *Nucleic Acids Research*, 37 (2009) e59-e59.
- [66] M.N. Stojanovic, D.W. Landry, *Journal of the American Chemical Society*, 124 (2002) 9678-9679.
- [67] K. Yamana, T. Iwai, Y. Ohtani, S. Sato, M. Nakamura, H. Nakano, *Bioconjugate Chemistry*, 13 (2002) 1266-1273.
- [68] C.J. Yang, S. Jockusch, M. Vicens, N.J. Turro, W. Tan, *Proceedings of the National Academy of Sciences of the United States of America*, 102 (2005) 17278-17283.
- [69] E. Pazos, O. Vazquez, J.L. Mascarenas, M. Eugenio Vazquez, *Chemical Society Reviews*, 38 (2009) 3348-3359.
- [70] Q. Liu, J. Wang, B.J. Boyd, *Talanta*, 136 (2015) 114-127.
- [71] J.K. Scott, G.P. Smith, *Science*, 249 (1990) 386.
- [72] H.M. Geysen, R.H. Meloen, S.J. Barteling, *Proceedings of the National Academy of Sciences of the United States of America*, 81 (1984) 3998-4002.
- [73] R. Frank, *Tetrahedron*, 48 (1992) 9217-9232.
- [74] C. Pinilla, J.R. Appel, P. Blanc, R.A. Houghten, *Biotechniques*, 13 (1992) 901-905.
- [75] K.S. Lam, S.E. Salmon, E.M. Hersh, V.J. Hruby, W.M. Kazmierski, R.J. Knapp, *Nature*, 354 (1991) 82-84.
- [76] K.S. Lam, M. Lebl, V. Krchňák, *Chemical Reviews*, 97 (1997) 411-448.

- [77] Z. Songyang, K.L. Carraway, M.J. Eck, S.C. Harrison, R.A. Feldman, M. Mohammadi, J. Schlessinger, S.R. Hubbard, D.P. Smith, C. Eng, M.J. Lorenzo, B.A.J. Ponder, B.J. Mayer, L.C. Cantley, *Nature*, 373 (1995) 536-539.
- [78] E. Koivunen, D.A. Gay, E. Ruoslahti, *Journal of Biological Chemistry*, 268 (1993) 20205-20210.
- [79] S.E. Cwirlla, E.A. Peters, R.W. Barrett, W.J. Dower, *Proceedings of the National Academy of Sciences of the United States of America*, 87 (1990) 6378-6382.
- [80] T. Heiskanen, A. Lundkvist, A. Vaheri, H. Lankinen, *Journal of Virology*, 71 (1997) 3879-3885.
- [81] T. Pulli, E. Koivunen, T. Hyypiä, *Journal of Biological Chemistry*, 272 (1997) 21176-21180.
- [82] J. Doorbar, G. Winter, *Journal of Molecular Biology*, 244 (1994) 361-369.
- [83] R.J. Goodson, M.V. Doyle, S.E. Kaufman, S. Rosenberg, *Proceedings of the National Academy of Sciences*, 91 (1994) 7129-7133.
- [84] C.F. Cho, B. Behnam Azad, L.G. Luyt, J.D. Lewis, *ACS Combinatorial Science*, 15 (2013) 393-400.
- [85] X. Chen, S.S. Gambhir, *Nature Chemical Biology*, 2 (2006) 351-352.
- [86] K.S. Lam, R. Liu, S. Miyamoto, A.L. Lehman, J.M. Tuscano, *Accounts of Chemical Research*, 36 (2003) 370-377.
- [87] O.H. Aina, J. Marik, R. Liu, D.H. Lau, K.S. Lam, *Molecular Cancer Therapeutics*, 4 (2005) 806.
- [88] N. Yao, W. Xiao, X. Wang, J. Marik, S.H. Park, Y. Takada, K.S. Lam, *Journal of Medicinal Chemistry*, 52 (2009) 126-133.
- [89] J.Y. Gerasimov, R.Y. Lai, *Chemical Communications*, 46 (2010) 395-397.
- [90] J. Adjémian, A. Anne, G. Cauet, C. Demaille, *Langmuir*, 26 (2010) 10347-10356.
- [91] R. Li, H. Huang, L. Huang, Z. Lin, L. Guo, B. Qiu, G. Chen, *Electrochimica Acta*, 109 (2013) 233-237.
- [92] H. Li, Y. Cao, X. Wu, Z. Ye, G. Li, *Talanta*, 93 (2012) 358-363.
- [93] N. Zhao, Y. He, X. Mao, Y. Sun, X. Zhang, C. Li, Y. Lin, G. Liu, *Electrochemistry Communications*, 12 (2010) 471-474.
- [94] G.K. Walkup, B. Imperiali, *The Journal of Organic Chemistry*, 63 (1998) 6727-6731.

- [95] B.P. Joshi, J.-Y. Park, K.-H. Lee, *Sensors and Actuators B: Chemical*, 191 (2014) 122-129.
- [96] J. Kim, C.R. Lohani, L.N. Neupane, Y. Choi, K. Lee, *Chemical Communications*, 48 (2012) 3012-3014.
- [97] Y. Huang, F. Hu, R. Zhao, G. Zhang, H. Yang, D. Zhang, *Chemistry – A European Journal*, 20 (2014) 158-164.
- [98] J. Hirata, L.P. Chung, F. Ariese, H. Irth, C. Gooijer, *Journal of Chromatography A*, 1081 (2005) 140-144.
- [99] B.P. Holskin, M. Bukhtiyarova, B.M. Dunn, P. Baur, J. Dechastonay, M.W. Pennington, *Analytical Biochemistry*, 227 (1995) 148-155.
- [100] Y. Li, L. Li, X. Pu, G. Ma, E. Wang, J. Kong, Z. Liu, Y. Liu, *Bioorganic & Medicinal Chemistry Letters*, 22 (2012) 4014-4017.
- [101] B.R. White, H.M. Liljestrand, J.A. Holcombe, *Analyst*, 133 (2008) 65-70.
- [102] F. Bianying, G. Linjie, W. Lihua, L. Fan, L. Jianxin, G. Jimin, F. Chunhai, H. Qing, *Analytical Chemistry*, 85 (2013) 7732-7737.
- [103] A. Torrado, G.K. Walkup, B. Imperiali, *Journal of the American Chemical Society*, 120 (1998) 609-610.
- [104] K.J. Oh, K.J. Cash, V. Hugenberg, K.W. Plaxco, *Bioconjugate Chemistry*, 18 (2007) 607-609.
- [105] K.J. Oh, K.J. Cash, K.W. Plaxco, *Journal of the American Chemical Society*, 128 (2006) 14018-14019.
- [106] J. Wu, Y. Zou, C. Li, W. Sicking, I. Piantanida, T. Yi, C. Schmuck, *Journal of the American Chemical Society*, 134 (2012) 1958-1961.
- [107] E.L. Volk, J.J. Pankuch, K.J. Chave, J.K. Coward, E. Schneider, *Biotechniques*, 35 (2003) 926-928, 930, 932.
- [108] K.S. Lam, Z.-G. Zhao, S. Wade, V. Krcňák, M. Lebl, *Drug Development Research*, 33 (1994) 157-160.
- [109] H. Wennemers, W.C. Still, *Tetrahedron Letters*, 35 (1994) 6413-6416.
- [110] J. García-Amorós, D. Velasco, *Beilstein Journal of Organic Chemistry*, 8 (2012) 1003-1017.
- [111] F. Hamon, F. Djedaini-Pilard, F. Barbot, C. Len, *Tetrahedron*, 65 (2009) 10105-10123.

- [112] T. Cusati, G. Granucci, M. Persico, *Journal of the American Chemical Society*, 133 (2011) 5109-5123.
- [113] Y. Ootani, K. Satoh, A. Nakayama, T. Noro, T. Taketsugu, *The Journal of Chemical Physics*, 131 (2009) 194306.
- [114] G. Tiberio, L. Muccioli, R. Berardi, C. Zannoni, *ChemPhysChem*, 11 (2010) 1018-1028.
- [115] M. Han, D. Ishikawa, T. Honda, E. Ito, M. Hara, *Chemical Communications*, 46 (2010) 3598-3600.
- [116] H.D. Bandara, T.R. Friss, M.M. Enriquez, W. Isley, C. Incarvito, H.A. Frank, J. Gascon, S.C. Burdette, *The Journal of Organic Chemistry*, 75 (2010) 4817-4827.
- [117] H.D. Bandara, S.C. Burdette, *Chemical Society Reviews*, 41 (2012) 1809-1825.
- [118] H. Rau, D. Rötger, *Molecular Crystals and Liquid Crystals*, 246 (1994) 143-146.
- [119] A.A. Beharry, G.A. Woolley, *Chemical Society Reviews*, 40 (2011) 4422-4437.
- [120] M.R. Han, Y. Hirayama, M. Hara, *Chemistry of Materials*, 18 (2006) 2784-2786.
- [121] H. Satzger, S. Spörlein, C. Root, J. Wachtveitl, W. Zinth, P. Gilch, *Chemical Physics Letters*, 372 (2003) 216-223.
- [122] M. Han, D. Ishikawa, E. Muto, M. Hara, *Journal of Luminescence*, 129 (2009) 1163-1168.
- [123] J.L. Charlton, J. Saltiel, *The Journal of Physical Chemistry*, 81 (1977) 1940-1944.
- [124] J. Saltiel, *Journal of the American Chemical Society*, 90 (1968) 6394-6400.
- [125] J. Saltiel, *Journal of the American Chemical Society*, 89 (1967) 1036-1037.
- [126] F. Doany, R. Hochstrasser, B. Greene, R. Millard, *Chemical Physics Letters*, 118 (1985) 1-5.
- [127] D.C. Todd, J.M. Jean, S.J. Rosenthal, A.J. Ruggiero, D. Yang, G.R. Fleming, *The Journal of Chemical Physics*, 93 (1990) 8658-8668.
- [128] S. Abrash, S. Repinec, R. Hochstrasser, *The Journal of Chemical Physics*, 93 (1990) 1041-1053.
- [129] M. Sumitani, K. Yoshihara, *Bulletin of the Chemical Society of Japan*, 55 (1982) 85-89.
- [130] L. Brey, G.B. Schuster, H. Drickamer, *Journal of the American Chemical Society*, 101 (1979) 129-134.

- [131] S. Malkin, E. Fischer, *The Journal of Physical Chemistry*, 66 (1962) 2482-2486.
- [132] J. Saltiel, J.T. D'Agostino, *Journal of the American Chemical Society*, 94 (1972) 6445-6456.
- [133] N. Strashnikova, V. Papper, P. Parkhomyuk, G.I. Likhtenshtein, V. Ratner, R. Marks, *Journal of Photochemistry and Photobiology A: Chemistry*, 122 (1999) 133-142.
- [134] V. Papper, G.I. Likhtenshtein, N. Medvedeva, D. Khoudyakov, *Journal of Photochemistry and Photobiology A: Chemistry*, 122 (1999) 79-85.
- [135] G.I. Likhtenshtein, R. Bishara, V. Papper, B. Uzan, I. Fishov, D. Gill, A. Parola, *Journal of Biochemical and Biophysical Methods*, 33 (1996) 117-133.
- [136] G.I. Likhtenshtein, D. Khudjakov, V. Vogel, *Journal of Biochemical and Biophysical Methods*, 25 (1992) 219-229.
- [137] V. Papper, G.I. Likhtenshtein, *Journal of Photochemistry and Photobiology A: Chemistry*, 140 (2001) 39-52.
- [138] D.H. Waldeck, *Chemical Reviews*, 91 (1991) 415-436.
- [139] V. Papper, D. Pines, G.I. Likhtenshtein, E. Pines, *Journal of Photochemistry and Photobiology A: Chemistry*, 111 (1997) 87-96.
- [140] C.K. Lin, Y.F. Wang, Y.C. Cheng, J.S. Yang, *The Journal of Physical Chemistry A*, 117 (2013) 3158-3164.
- [141] Y. Amatatsu, *Chemical Physics*, 274 (2001) 87-98.
- [142] Z.R. Grabowski, J. Dobkowski, *Pure and Applied Chemistry*, 55 (1983) 245-252.
- [143] J.F. Letard, R. Lapouyade, W. Rettig, *Journal of the American Chemical Society*, 115 (1993) 2441-2447.
- [144] E. Gilabert, R. Lapouyade, C. Rullière, *Chemical Physics Letters*, 145 (1988) 262-268.
- [145] E. Abraham, J. Oberlé, G. Jonusauskas, R. Lapouyade, C. Rulliere, *Chemical Physics*, 214 (1997) 409-423.
- [146] O. Chen, R. Glaser, G.I. Likhtenshtein, *Journal of Biochemical and Biophysical Methods*, 70 (2008) 1073-1079.

Chapter 3

Experimental Methodology

This chapter begins with an overview of the properties required for the stilbene compounds based on the hypothesis. It also introduces the model system—malachite green (MG) and MG aptamer used to test the hypothesis. The rationalities for different stilbene compounds' synthesis and characterization are presented, together with the principles and theories involved in the characterization instruments and techniques. Detailed procedures in obtaining photophysical parameters such as molar extinction coefficient, quantum yield and apparent decay rate constant etc. and in conducting the medium viscosity effect studies are also fully explained and illustrated.

3.1 Overview

As introduced in chapter 1, PHASA depends on the fluorescence decay rate change of stilbene components for analyte detection. Since the synthesis of stilbene-analyte conjugates and stilbene-aptamer complexes are the focus for competitive binding (**Figure 1.2**) and adaptive binding scope (**Figure 1.4**), respectively, the following features of them are required for PHASA analyte detection.

Competitive binding scope (stilbene-analyte conjugates)

- Suitable fluorescence quantum yield (greater than 0.001) to allow detection within the nmol range or less;
- Steady state fluorescence, first order decay rates of stilbene compounds within 0.1 to 10 s⁻¹;
- Good water solubility greater than 1 mg mL⁻¹ (to be able to mix with aptamers in aqueous buffer solutions);
- Preserved fluorescence intensity and observable fluorescence decay in aqueous buffer environments with less than 20% organic solvent;
- Binding affinity (K_d) to analyte aptamer remains competitive.

Adaptive binding scope (stilbene-aptamer complex)

- Preserved binding affinity of aptamer after being modified;
- Suitable fluorescence quantum yield (greater than 0.001) for nmol range detection;
- Preserved fluorescence intensity and observable fluorescence decay of the stilbene fragment in the complex in aqueous buffer environments with less than 20% organic solvent.

3.2 Model system for PHASA: malachite green aptamer & malachite green

Malachite green (**Figure 3.1**) has been extensively used in aquaculture as a parasiticide since 1936 due to its biocidal nature in controlling fungal attacks, protozoan infections etc. It has also been used in food, health and textile industries as food colouring agent, food additive and dye. However, recently malachite green has been reported to be highly toxic and it causes carcinogenesis, mutagenesis and respiratory toxicity [1]. In spite of the high risks and threats that malachite green poses to animals and mankind, it is still being used due to its low cost, ready availability and efficacy. So portable and one-step method for the

detection of malachite green is required.

An RNA aptamer has been developed to bind malachite green in 1999 by Grate and coworkers [2]. It was later found to bind more tightly to rigidly planar analogues like tetramethylrosamine (TMR) and pyronin Y compared with the non-planar, rotationally mobile MG but not to rhodamine B and crystal violet (**Figure 3.1**) [3]. Due to the tighter binding, the crystal structure of MG aptamer was determined with TMR instead of MG. These studies have consistently concluded that the malachite green aptamer binding pocket consists of a base quadruple and a Watson–Crick base pair with two base pairs and a U turn motif providing the supporting framework [3, 4]. The respective contribution of these different structural components to the overall stability of the aptamer–target complex has also been investigated [4]. Moreover, it was also shown that unlike the complex formed by ATP & FMN aptamer with their targets in which hydrogen bonding plays a significant role for stabilization, steric hindrance and electrostatic interaction are the major stabilizing factors. Therefore, besides the overall structural configuration, electronic distribution of the target is also important for the aptamer binding [4, 5].

Figure 3.1. Structure of malachite green and its analogues and their respective binding dissociation constant K_d value.

The key feature of malachite green (MG) is its fluorescence enhancement upon binding to MG aptamer [6]. Based on this feature, the binding affinities (binding dissociation constant

K_d) measurement of the stilbene-MG conjugates or the modified malachite green aptamer can be performed by fluorescence measurement which is simple, easy and fast. Together with the aforementioned well-defined crystal structure of MG aptamer which makes aptamer mutation with minimal effect on its binding capacity possible, and its various binding targets, malachite green aptamer–malachite green is considered as a perfect model system for the project.

3.3 Strategies for stilbene compounds synthesis

3.3.1 Stilbene–malachite green (MG) conjugates

Competitive binding scope (**Figure 1.2**) involves the competition between malachite green (MG) analyte and stilbene-MG conjugate for the MG aptamer binding pocket. Hence, conjugation between a MG derivative and a stilbene derivative needs to be conducted first. A *para*-substituent on one ring of the stilbene derivative must be sufficiently reactive to conjugate with an analyte (MG) derivative. An example of R_2 is an amino or hydroxyl substituent. In order to ensure a high fluorescence quantum yield, a *para*-substituent R_1 on the opposite ring of the stilbene derivative should be a strong donor group because strong donor substituents stabilize more the singlet excited state than the “phantom” state, leading to less isomerization and more irradiative emission as introduced in literature review section (**Section 2.3.2.2**). Examples are dimethylamino, methoxy and hydroxyl. Taking into account the aforementioned requirements for the stilbene molecule, *trans*-4-dimethylamino-4'-aminostilbene (DMAAS) and *trans*-4-methoxy-4'-aminostilbene (MAS) (**Figure 3.2**) were chosen as parent stilbenes for the conjugation and assay.

Figure 3.2. Requirements for the two stilbene substituents and the structures of two parent stilbenes.

Previous publications have shown that besides target configuration, electron distribution plays an important role in MG aptamer binding [4, 5]. In addition, Papper et al. [7, 8] have shown that the steady state fluorescence decay of stilbene compounds upon irradiation with the excitation light depends strongly on the substituents nature. Thus, the electron donating/accepting properties of the stilbene-MG conjugate have a significant impact on its binding affinity towards the MG aptamer as well as the fluorescence decay of its stilbene fragment. Therefore, several different cross-linkers were designed based on available chemical reagents and reaction pathways to find the most suitable stilbene-MG conjugates for the proposed format. In addition, a rhodamine B–stilbene conjugate was also synthesized to provide a negative control, since it has been confirmed that there is no specific binding interaction between rhodamine B and MG aptamer.

3.3.2 Stilbene maleimides and stilbene maleamic acids

In adaptive binding scope (**Figure 1.4**), the conjugation between an aptamer and a stilbene compound is to be done in the preparation step of the assay prior to actual detection of the analyte molecules.

In general, maleimides are known to react with sulfhydryl groups. This is because of the strong nucleophilicity of a sulfhydryl group, which can readily react with maleimides, haloacetyls and disulfide bonds. Such high reactivity of maleimides towards sulfhydryl groups is widely used in click chemistry. Therefore, the “home-made” new stilbene maleimides were bound to the thiolated MG aptamer and used in the PHASA adaptive binding scope.

MG aptamer binding buffer is essential for binding pocket formation in the final binding experiment of adaptive binding scope. To reduce the fluorescence intensity or fluorescence decay quenching effect from the aqueous environment, stilbene compounds with good water solubility are required. The intermediates during the synthesis of stilbene maleimides are stilbene maleamic acids which have carboxyl groups, making them more water compatible than the stilbene maleimides. Moreover, they also have the double bonds to react with sulfhydryl groups and thus thiolated MG aptamer. Therefore, stilbene maleamic acids were also synthesized and applied under adaptive binding scope.

Before the conjugation of the obtained stilbene maleimides or stilbene maleamic acids with

thiolated MG aptamer was conducted, the reaction of one stilbene maleimide and a small thiol molecule—6-mercapto-1-hexanol (6MCH) was first tried to acquire the optimal reaction conditions.

3.4 Photophysical characterization of stilbene compounds

When some of the light energy matches the energy needed for a possible electronic transition within a molecule, the energy will be absorbed to promote the electrons to a higher energy orbital. After that, the excited electrons reach the lowest excited state, from which they relax to the ground state either non-radiatively or radiatively (fluorescence or phosphorescence). For stilbene compounds, isomerization between their *trans* and *cis* isomers by twisting offers another relaxation pathway. Since stilbene substituents, solvents affect the relative role they play, after the stilbene compounds are successfully synthesized with their structures confirmed, their photophysical properties such as absorption, emission spectrum, quantum yield and fluorescence decay kinetics were measured. This is ensure they have the required fluorescence intensity and fluorescence decay for competitive binding or adaptive binding scope.

3.4.1 Absorption spectrum and molar extinction coefficient ϵ

Absorption spectrum is measured by a spectrometer which typically has three components: light source, monochromator and detector. It is generated by recording the absorbance value at each wavelength within a certain wavelength range depending on the optical property of the molecule. The absorbance A is obtained by

$$A = \log\left(\frac{I_0}{I}\right) \quad (3.1)$$

Where I_0 is the fluorescence intensity passing through the reference and I is the intensity through the sample. Considering the capacity of the detectors in distinguishing the light intensities, the observed absorbance range is generally 0~1, that is, from no absorption by the molecule to 90% absorption.

For all the stilbene compounds, their absorption spectra were measured using UV-Vis Spectrometer UV-2700 from Shimadzu, Japan. The light sources of this spectrometer are halogen lamp (50 W) and deuterium lamp. The detection wavelength range is from 185 nm

to 900 nm. Besides, to minimize the interruption from sample containers, quartz cuvettes with the two sides perpendicular to the light transmitting direction being transparent only were used throughout all the absorption spectrum measurements.

With regards to the detailed experimental procedures, the absorption spectrum measurement for the parent stilbene–DMAAS in DMSO was presented as an example. Firstly, the wavelength range for absorbance scanning was set from 250 nm to 550 nm. Then two cuvettes containing only the solvent DMSO were placed in the reference holder and sample holder respectively, from which baseline was measured. After that, the cuvette in the sample holder was taken out and DMSO was discarded. After proper cleaning and drying, the cuvette was filled with a DMAAS in DMSO solution having concentration of 10^{-7} mol L⁻¹ (this concentration range gives rise to absorbance from 0 to 1) and put back to the sample holder. Then the absorption spectrum with absorption peak at 368 nm was obtained for DMAAS in DMSO. Taking into account experimental errors as well as for the extinction coefficient calculation, the measurements for another four samples (absorbance in the range of 0~1) were also conducted. For other stilbene compounds' absorption spectrum measurement, the scanning wavelength range and solution concentration need to be changed accordingly.

From absorption spectrum measurement, besides the absorption peak ($\lambda_{\max}$), the molar absorptivity or molar extinction coefficient (ϵ) at absorption peak can also be calculated and used to compare the relative strength of light absorbing function among various stilbene compounds. It is defined as

$$\epsilon = \frac{A}{c * l} \quad (3.2)$$

Where A is the measured absorbance, c is the sample concentration in mol/L and *l* is the length of light path through the cuvette in cm, which is usually 1 cm. To be more precise, ϵ is commonly generated as the slope of the A vs. (*c***l*) plot. Thus, the absorbance values of several concentrations need to be measured as mentioned above. Also, they need to be within the range from 0.1 to 1 to ensure the linearity of the A vs. (*c***l*) plot based on Beer–Lambert law. For the above solution of DMAAS in DMSO, the absorbance values from five solutions were measured and plotted against their respective concentration * 1 cm. By linear fitting the data using OriginPro 9.1 software, the molar extinction coefficient is

obtained as 24.02 ± 0.85 (L*mmol⁻¹*cm⁻¹) at 368 nm.

3.4.2 Emission and excitation spectrum

One of the excited electrons' relaxation pathways is fluorescence emission which is accompanied by a photon emission during their relaxation process to the ground state. Emission spectrum is the wavelength distribution of an emission measured under a single constant excitation wavelength. While excitation spectrum is the dependence of fluorescence intensity over a range of excitation wavelengths when the emission wavelength is fixed. Both emission and excitation spectrum can be recorded with a spectrofluorometer, which has three main components: light source, monochromator and detector, just like those in a UV-Vis spectrometer. But the setup is slightly different. The detector is placed not along but perpendicular to the direction of the excitation light so that the influence from the strong excitation light can be avoided.

In an emission spectrum measurement, the excitation wavelength and scanning emission wavelength range are inputted first. The emission range needs to include the emission peak of the compound being measured. The absorption peak from the measured absorption spectrum is used as the excitation wavelength. Due to the non-irradiative losses in the excited states, the obtained emission peak is always in the red side of the absorption peak and is also independent of the excitation wavelength since fluorescence is always emitted from the lowest excited state. While for an excitation spectrum measurement, it works similarly by setting the emission wavelength and scanning excitation wavelength range. Usually the peak generated from the emission spectrum measurement is used as the emission wavelength. As excitation spectrum only presents wavelengths that are able to produce emission or fluorescence, it also termed as "fluorescence detected absorption". For those wavelengths that dissipate their energy as heat instead of photons on their way back to the ground states, they do not appear in the excitation spectrum.

All the fluorescence emission and excitation spectra presented in this thesis were recorded by Horiba Jobin Yvon FluoroLog®-3 modular spectrofluorometer using a xenon lamp. They were corrected for instrumental sensitivity at different excitation and emission slits using the instrument internal excitation-emission matrix (EEM) correction [9]. Due to the different setup, 1 cm. sq. quartz cuvette (four sides transparent) was employed for all the

excitation and emission measurements. To minimize the inner filter effect, the solution concentrations of less than 0.1 absorbance value were chosen.

Hence, for the emission spectrum measurement of DMAAS in DMSO, its absorption peak (λ_{max}) 368 nm was used as the excitation wavelength and the scanning emission range was from 370 nm to 600 nm. Slit widths of 5 nm for both excitation and emission slits were kept constant across all investigations unless otherwise noted. Before the emission spectrum measurement of DMAAS in DMSO, the baseline solvent DMSO's emission spectrum was recorded. After baseline subtraction, the sample emission spectrum was acquired. The obtained emission peak λ_{max} for DMAAS in DMSO was 420 nm. For other stilbene compounds or in other solvents, similar procedures were followed unless noted. For the excitation spectrum measurement of DMAAS in DMSO, the emission peak λ_{max} 420 nm was utilized as the emission wavelength and the scanning excitation wavelength range was set from 250 nm to 400 nm. Similarly, the spectrum of DMSO baseline was recorded before that of DMAAS in DMSO solutions. The λ_{max} excitation peak was measured to be 366 nm which is similar to the λ_{max} absorption wavelength of 368 nm.

3.4.3 Quantum yield ϕ_f

Since fluorescence emission intensity is strongly dependent on the concentration of the emitting species, spectrofluorometer hardware, light source and other experimental conditions, it is not accepted in general to use fluorescence emission intensity as a parameter in the photophysical experiments. Instead, quantum yields, which are usually independent of the aforementioned parameters, are reported. By definition, fluorescence quantum yield is the ratio between the number of photons absorbed to the number of photons radiatively emitted (for example, in a fluorescence emission process). In other words, it is the probability of the electrons in the excited states to be deactivated via the radiative pathway, rather than non-radiative pathways.

There are two methods in quantum yield determination: the absolute measurement and the relative measurement by comparing to a reference fluorophore with a well-known quantum yield. The relative one is easier and more frequently used and there are both single-point method and comparative method [10]. The single-point method uses the integrated emission intensities from a single sample and reference pair at identical concentration to

determine the quantum yield. In the comparative method, quantum yield is generated based on calculating the slope of the integrated fluorescence intensity vs. absorbance plot from multiple concentrations.

Experimentally, under the same measurement conditions, the relative quantum yield of a molecule by the single-point method is calculated as

$$\phi = \phi_r \frac{\eta^2}{\eta_r^2} \frac{I}{A} \frac{A_r}{I_r} \quad (3.3)$$

Where ϕ and ϕ_r are the quantum yield of the sample and reference respectively; η is the refractive index of the solvent; I is the integrated fluorescence intensity and A is the absorbance at the excitation wavelength. For the comparative method, $\frac{I}{A} \frac{A_r}{I_r}$ is replaced by the ratio of I vs. A plot slope from sample to that from the reference [10]. In this thesis, all the absorbance values and emission intensities required for quantum yield calculation were measured from the Shimadzu UV-Vis Spectrometer and Horiba spectrofluorometer respectively.

3.4.3.1 Quantum yield calibration

Quantum yield values of some common reference fluorophores under detailed measurement conditions are available in literature. To confirm the reliability of these values as well as check the performance of the instruments, cross calibration between the reference fluorophores was first conducted. Taken into account that the same excitation wavelength is to be used to get the emission spectrum, reference fluorophores rhodamine 6G and rhodamine B in EtOH were chosen. The comparative method was applied to guarantee the accuracy of the measurement results.

Stock solutions of rhodamine 6G and rhodamine B in EtOH were prepared and their absorption spectra from 0.2 μM to 8.0 μM were measured and shown in **Figure 3.3 A** and **B**, respectively. The molar extinction coefficient for rhodamine 6G in EtOH at the absorption peak 530 nm is $99.4 \pm 0.9 \text{ L} \cdot \text{cm}^{-1} \cdot \text{mmol}^{-1}$ and for rhodamine B at 559 nm is $111.3 \pm 0.3 \text{ L} \cdot \text{cm}^{-1} \cdot \text{mmol}^{-1}$. Based on their overlapped absorption spectra, 540 nm was chosen as the common excitation wavelength for the subsequent emission measurements. The recorded emission spectra are presented in **Figure 3.3 C**. The integrated fluorescence

emission intensities from 500 nm to 700 nm of the three concentrations (0.2, 0.3 and 0.4 $\mu\text{mol L}^{-1}$) for both rhodamine 6G and rhodamine B were calculated. They were then plotted against their corresponding absorbance at λ_{abs} of 540 nm as shown in **Figure 3.4**, from which the slopes were obtained.

Figure 3.3. The absorption spectra of rhodamine 6G (A) and rhodamine B (B) in ethanol (EtOH) and their emission comparison under the excitation of 540 nm (C).

Figure 3.4. The relationship between the integrated fluorescence intensity area and the corresponding absorbance for rhodamine 6G (A) and rhodamine B (B).

Since the common solvent EtOH was used, η equals to η_r . Taken rhodamine B as the “sample” and rhodamine 6G as the “reference” whose quantum yield is reported to be 0.95 [11, 12], the quantum yield of rhodamine B can be calculated as

$$\phi = \phi_r \frac{\eta^2}{\eta_r^2} \frac{I}{A} \frac{A_r}{I_r} = \phi_r \frac{k}{k_r} = 0.95 * \frac{3.9E10}{6.4E10} = 0.579. \quad (3.4)$$

This value is within the reported quantum yield range 0.41 to 0.97 for rhodamine B in the literature [13, 14], so the reliability of the calculation methods and the instrument performance are confirmed.

3.4.3.2 Quantum yield determination

For the quantum yield (QY) determination of the stilbene compounds in this thesis, the single point method instead of the comparative method was used since it is fast, and the instruments have sufficient accuracy for this method to be used. Since the stilbene compounds and their conjugates absorb more in the UV range of 320-380 nm, quinine sulfate (QS) whose absorption peak is 347 nm was chosen as the reference fluorophore. Its quantum yield is reported to be 0.58 in 0.1N H₂SO₄ [10]. The stock solution of QS was prepared and the absorption spectrum of five concentrations of QS were measured, from which the molar extinction coefficient ϵ at each required wavelength can be obtained.

The previously measured absorption peak 368 nm of DMAAS in DMSO was employed as the common excitation wavelength for the quantum yield determination. The maximum absorption wavelength of the studied stilbene compound is used as the common excitation wavelength because the quantum yield of quinine sulfate is high. This rationale was applied to all the quantum yield determination measurements. Solutions of 0.1 $\mu\text{mol L}^{-1}$ DMAAS in DMSO and 0.1 $\mu\text{mol L}^{-1}$ QS in 0.1N H₂SO₄ were prepared and their respective emission spectrum under λ_{Ex} of 368 nm were obtained. Their integrated fluorescence emission intensities were calculated. After obtaining the molar extinction coefficients for the two solutions at 368 nm, their corresponding absorbance at 0.1 $\mu\text{mol L}^{-1}$ was generated. Since η_r (refractive index of water) is 1.33 and that of the DMSO solvent (η) is 1.479, according

to $\phi = \phi_r \frac{\eta^2}{\eta_r^2} \frac{I}{A} \frac{A_r}{I_r}$ (equation (3.3)) where ϕ_r is 0.52, the quantum yield of DMAAS in

DMSO was calculated to be 0.81.

3.4.4 Fluorescence decay kinetics–apparent decay rate constant

As discussed in the literature review section (**Figure 2.8**), under excitation light irradiation, the fluorescent *trans*-stilbene isomer is converted to the non-fluorescent *cis*-isomer, resulting in decrease in fluorescence with time or a fluorescence decay. The dependence of its fluorescence decay rate on the medium viscosity makes them promising probes. Since dynamic equilibrium always exists between the *trans*- and *cis*- isomers, continuous illumination is essential in recording the steady-state decay curve measured by a regular spectrofluorometer. Instruments equipped with pulse-based light sources are not suitable for the steady-state decay measurements of the stilbene compounds.

Shimadzu RF-5301PC spectrofluorophotometer which employs a 150 W Xenon lamp, was used for recording the steady-state fluorescence decay curves of the stilbene compounds. Its wavelength scales from 220 nm to 900 nm, and its sensitivity is very high with the signal to noise (S/N) ratio being 150 or higher for the Raman line of distilled water (350 nm excitation wavelength and 5 nm bandwidth). Excitation and emission slit widths were set to 5 nm for all steady-state fluorescence decay measurements. The overall time scan was always 180 sec, and the absorption and emission peaks from the above measurements of the studied compound were used as the excitation and emission wavelength respectively. For DMAAS in DMSO, its measured maximum absorption wavelength is 368 nm and emission wavelength is 420 nm. Under this excitation and emission wavelength, the decay curve of the baseline solvent DMSO was recorded first which was expected to be a flat line with no fluorescence intensity change. Then, the decay curve of 0.1 $\mu\text{mol L}^{-1}$ DMAAS in DMSO was measured under the same conditions. After subtraction the baseline, the fluorescence decay curve of DMAAS in DMSO was obtained. After normalization, the first five seconds' decay (the time varies for different stilbene compounds depending on their specific decay behavior) was fitted by an exponential equation

$$I = a + c * \exp(bt) \quad (3.5)$$

Where a is 0.550 ± 0.002 , b is -0.547 ± 0.007 and c equals to 0.460 ± 0.002 .

The decay behavior of stilbene compounds has been extensively studied by G. I. Likhtenshtein and coworkers [7, 15, 16]. In their work, they have concluded that the decay behavior follows pseudo first order kinetics and can be described by

$$-\frac{dI}{dt} = k_{app} * I + C \quad (3.6)$$

Where k_{app} is the apparent decay rate constant and I is the fluorescence intensity. Hence, the rate constant k_{app} can be calculated from the slope of the $-\frac{dI}{dt}$ vs. I plot. That is,

$$k_{app} = \frac{d(-dI/dt)}{dI} \quad (3.7)$$

Based on the fitted equation $I = a + c * \exp(bt)$ (equation (3.5)), it was concluded that $k_{app} = -b$ as supported by the calculation as follows.

$$k_{app} = \frac{d(-dI/dt)}{dI} = \frac{d(-d(a + c * \exp(bt))/dt)}{dI} = \frac{d(-bc * \exp(bt))}{dI} \quad (3.8)$$

Since $I = a + c * \exp(bt)$, $c * \exp(bt) = I - a$. This leads to

$$k_{app} = \frac{d(-b(I - a))}{dI} = -b \frac{d(I - a)}{dI} = -b \quad (3.9)$$

Then, the fluorescence decay rate of DMAAS in DMSO is 0.547 ± 0.007 . Therefore, for the decay rate calculations, after fitting the decay curve by the exponential equation, $-b$ is directly taken as k_{app} and used for *trans-cis* isomerization rate comparison among different stilbene compounds in different solvents.

3.5 Medium viscosity dependence study

The hypothesis in this thesis is based on the fact that the surrounding environment of stilbene compounds such as steric hindrance or medium viscosity changes their fluorescence decay rate constants. To check the studied stilbene compounds' sensing ability towards their surrounding medium viscosity change, their fluorescence decay rates' dependence on medium viscosity was studied before the binding experiment.

To get solutions having different viscosity, glycerol was introduced. Seven solution mixtures consisting of different DMSO to glycerol volume ratios (28 to 72, 48 to 52, 60 to 40, 68 to 32, 80 to 20, 88 to 12 and 96 to 4) were prepared. Since glycerol is quite viscous, in the preparation of these mixtures, measurement of glycerol was performed based on mass instead of volume. Taking the volume ratio of 60 to 40 as an example, the required glycerol mass

$$m = \rho * v_t * v\% \quad (3.10)$$

Where ρ is the density of glycerol (1.26 g/cm³), v_t is the total volume of the solution mixture and $v\%$ is the volume percentage of glycerol 40%. To these glycerol solutions, the corresponding DMSO volumes were added. The obtained mixtures were well mixed by a Vortex Mixer. After that, they were left undisturbed until the air bubbles generated during vortexing disappear. The viscosities of the seven prepared DMSO and glycerol mixtures were measured then by Brookfield viscometer DV-II+ Pro LV series which is simple, easy to use and needs only 500 μ L testing samples. The proper spindle for the mixtures was found to be CPE-40. The measured viscosities are summarized in **Table 3.1**.

Table 3.1. The viscosity values summary of the seven DMSO/glycerol mixtures.

DMSO to glycerol ratio (v : v)	Viscosity (cP)	Temperature (°C)
28 : 72	215.8	25.6
48 : 52	38.6	25.6
60 : 40	18.3	25.6
68 : 32	11.4	25.6
80 : 20	6.23	25.6
88 : 12	3.99	25.6
96 : 4	2.68	25.6

3.5.1 Fluorescence decay rate vs. medium viscosity

After obtaining the viscosities of the DMSO and glycerol mixtures, the same solution mixtures containing the studied stilbene compound were prepared and characterized. Taking DMAAS as an example, using its stock solution in DMSO, 1 μ mol L⁻¹ and 0.1 μ mol L⁻¹ DMAAS in the seven different DMSO and glycerol mixtures were prepared for the absorption and emission/decay measurements, respectively. In addition, the same seven mixtures without DMAAS were prepared as baseline in the measurements. The absorption and emission peaks of DMAAS in each sample were obtained from their respective spectra. Under these excitation and emission wavelengths, the fluorescence decay curves of DMAAS in these seven DMSO/glycerol mixtures were measured. After subtracting the baseline, the obtained fluorescence decay curves were normalized, and their apparent decay rate constants k_{app} were calculated based on the aforementioned method. The calculated decay rate constants were plotted against the reciprocal of the DMSO/glycerol mixtures'

viscosity values, for which the corresponding steady-state fluorescence decay curves of the stilbene compound were recorded. The linear relationship between the rate constant and the reciprocal of the viscosity in a log scale was obtained.

3.5.2 Fluorescence polarization vs. medium viscosity

Fluorescence polarization results reflect a molecule's relative rotation rate in different mediums, and can be used to indicate the medium viscosity property of the environment. To further confirm the dependence of stilbene's fluorescence decay rate constant on medium viscosity rather than other interactions, the fluorescence polarization measurement of stilbene compounds in solutions having different viscosities should also be conducted.

Figure 3.5. A simple illustration of the working principles of fluorescence polarization.

Figure 3.5 shows its basic working principles. Upon exposure to polarized excitation light, fluorophores having their absorption transition moments oriented along the electric vector of the incident light are preferentially excited. If the fluorophore itself is small in size and its surrounding medium is non-viscous, the rotation and diffusion of these excited fluorophores are very fast, randomizing their directions and giving rise to depolarized emission. The fluorescence polarization is low and close to zero. On the other hand, if the fluorophore is bulky or has been attached to a binding target and the solutions are very viscous, its rotation and diffusion are expected to be very slow. So the excited fluorophores keep their directions, resulting in polarized emission and high fluorescence polarization. There are two quantities describing the degree of the fluorescence polarization process: polarization P and anisotropy r . The polarization P is defined as:

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}} \quad (3.11)$$

Anisotropy r is defined as

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} \quad (3.12)$$

Where $I_{\parallel}$ is the observed emission intensity when the emission polarizer is oriented parallel to the excitation plane and $I_{\perp}$ is the intensity when the emission polarizer is placed perpendicular to the excitation plane. Considering the fact that monochromator usually has different transmission efficiencies for vertically and horizontally polarized light, G factor describing the instrumental preference of the emission optics for the horizontal orientation to the vertical orientation is introduced and defined as

$$G = \frac{I_{HV}}{I_{HH}} \quad (3.13)$$

Where I_{HV} corresponds to horizontally polarized excitation and vertically polarized emission and I_{HH} refers to horizontally polarized excitation and horizontally polarized emission. Then, taking into account the G factor, anisotropy is re-defined as

$$r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}} \quad (3.14)$$

All the fluorescence polarization experiments in this thesis were conducted using Cary Eclipse fluorescence spectrophotometer which is equipped with automated polarization accessory from Agilent Technologies. It uses unique xenon flash lamp technology and thus is less destructive. Similar to the fluorescence decay measurements, 0.1 $\mu\text{mol L}^{-1}$ DMAAS in the seven DMSO to glycerol mixtures were prepared and well mixed. The excitation and emission slit widths and the photomultiplier tube (PMT) voltage were first optimized so that the I_{HV} , I_{HH} , I_{VV} and I_{VH} values are high enough to produce reasonable G factor and fluorescence polarization value. For DMAAS, 5 nm slit widths for both excitation and emission and medium PMT voltage were set, and these parameters were applied in its entire fluorescence polarization measurement. After that, based on the obtained excitation and emission wavelength for each sample, the corresponding G factor and polarization value P were measured. For each DMSO to glycerol ratio, five measurements were conducted with G factor being measured in each irradiation and then applied in polarization P calculation. The average value of the five measurements was used as the P value for that

DMSO/glycerol ratio. The obtained P values in the seven DMSO/glycerol mixtures were plotted against their corresponding medium viscosities and a linear dependence of P on the viscosity in log scale was observed.

3.6 Photophysical characterization in aqueous environment

In the final binding experiments, PBS binding buffer is essential for the MG aptamer binding pocket formation (solubility). However, previous work [17-20] has suggested that through exciplex formation, hydrogen binding interaction, and proton or electron charge transfer during the fluorophores' excited states and etc., water can quench the fluorescence intensity and influence the photophysical properties of the fluorophores significantly. Therefore, prior to the binding experiment, samples of stilbene compounds in PBS buffer with low volume percentage (3, 5, 8 or 10%) of organic solvent which dissolves the stilbene compounds were prepared, and their photophysical studies in these solvent mixtures were performed. This will ensure that the stilbene compounds are fluorescent in these solvent mixtures, and their steady state fluorescence decay can be observed.

The stilbene compounds were initially dissolved in organic solvents, and then water or PBS buffer was added. For example, DMSO (3%) and water (97%) solution mixture was used for the measurements of DMAAS. During its absorption spectrum measurement, after baseline correction, 10 μL 1.0 mmol L^{-1} stock solution of DMAAS in DMSO was prepared, to which 970 μL DI water and 20 μL DMSO were added in (total volume used for absorption measurement is 1.0 mL). After sufficient mixing, the DMAAS's absorption spectrum in this DMSO and water mixture was recorded. Similarly, 1 $\mu\text{mol L}^{-1}$ DMAAS in 3% DMSO and 97% water was prepared, and its emission spectrum was recorded under the excitation of the obtained absorption peak. Based on the excitation and emission wavelength, its steady state fluorescence decay curve was measured. The above procedures were also applied for the photophysical property studies of other stilbene compounds in other organic solvent/aqueous solvent mixtures.

3.7 Aptamer binding experiment with stilbene-analyte conjugate

After confirming that the stilbene-MG compounds are fluorescent and their fluorescence decay are observed in PBS based solvent mixtures (volume percentage of organic solvent

is not more than 20%), their binding experiments with aptamer have to be conducted. This is because besides the observable fluorescence decay, high binding affinity is also required for the MG-stilbene conjugates to competitively bind to the aptamer binding pocket with MG analyte, and generate a decay rate constant vs. analyte concentration calibration curve. The procedures for the binding experiment between MG aptamer and rhodamine B–DMAAS conjugate which was used as a negative control were explained in detail as an example.

One aliquot of MG aptamer dissolved in PBS binding buffer was denatured after heating at 90 °C for 1 min. It was then stored in ice and ready to be used. Stock solution of rhodamine B–DMAAS conjugate in DMSO was prepared as it is not soluble in PBS buffer. Solutions with a series of MG aptamer to the conjugate ratio (including 0; 1:10; 1:4; 1:1; 10:1; 40:1 etc.) were prepared, in which the amount of MG aptamer was varied while the conjugate concentration ($0.1 \mu\text{mol L}^{-1}$) was kept constant. In all solutions, the final DMSO volume percentage was kept at 5%. The prepared solutions were incubated at room temperature for 30 mins. In order to ensure that the same incubation period was applied to all the MG aptamer and the rhodamine B-DMAAS mixtures, 5 mins interval was allocated for the preparation between two solutions to compensate the time needed for the subsequent fluorescence decay measurements. After that, their steady state fluorescence decay curves under λ_{Ex} of 350 nm and λ_{Em} of 445 nm (these excitation and emission wavelengths come from the absorption and emission spectrum measurement of the rhodamine B–DMAAS conjugate in 5% DMSO and PBS buffer mixture) were measured and the apparent decay rate constants were calculated. The decay rates were plotted against the corresponding MG aptamer/conjugate ratio to get the influence of increasing MG aptamer on the fluorescence decay of the conjugate's stilbene fragment. For comparison, MG aptamer in the above experiments was replaced by BSA and exactly the same measurements were conducted. For the negative control, besides the fluorescence decay rate measurements, fluorescence polarization was also performed to check the binding affinity of MG aptamer towards the conjugate's rhodamine B component. Similarly, different samples with various MG aptamer to rhodamine B-DMAAS ratios where the conjugate concentration ($0.1 \mu\text{mol L}^{-1}$) was kept constant were prepared. After 30 mins incubation, their fluorescence polarization values were obtained. The same fluorescence polarization experiments were also

performed between MG aptamer and rhodamine B. The obtained fluorescence polarization results were used to plot the FP vs. MG aptamer to conjugate or rhodamine B ratio.

For MG-stilbene conjugates, the same experimental procedures apply except that instead of fluorescence decay, fluorescence emission spectrum from 500 to 800 nm (the emission range characterizing the MG component in the MG-stilbene conjugates) was measured. This is because binding to MG aptamer enhances the fluorescence intensity of MG significantly. As the emission spectrum measurement is faster than the fluorescence decay, 2 mins interval rather than 5 mins was allocated for the preparation between two samples to guarantee the same incubation duration. Then, fluorescence intensity vs. MG aptamer to MG-stilbene conjugate ratio was plotted, from which the binding affinity was estimated.

References

- [1] S. Srivastava, R. Sinha, D. Roy, *Aquatic Toxicology*, 66 (2004) 319-329.
- [2] D. Grate, C. Wilson, *Proceedings of the National Academy of Sciences*, 96 (1999) 6131-6136.
- [3] C. Baugh, D. Grate, C. Wilson, *Journal of Molecular Biology*, 301 (2000) 117-128.
- [4] J. Flinders, S.C. DeFina, D.M. Brackett, C. Baugh, C. Wilson, T. Dieckmann, *ChemBioChem*, 5 (2004) 62-72.
- [5] D.H. Nguyen, S.C. DeFina, W.H. Fink, T. Dieckmann, *Journal of the American Chemical Society*, 124 (2002) 15081-15084.
- [6] J.R. Babendure, S.R. Adams, R.Y. Tsien, *Journal of the American Chemical Society*, 125 (2003) 14716-14717.
- [7] V. Papper, G.I. Likhtenshtein, *Journal of Photochemistry and Photobiology A: Chemistry*, 140 (2001) 39-52.
- [8] V. Papper, D. Pines, G.I. Likhtenshtein, E. Pines, *Journal of Photochemistry and Photobiology A: Chemistry*, 111 (1997) 87-96.
- [9] Horiba Scientific 2014.
- [10] J.R. Lakowicz, *Principles of Fluorescence Spectroscopy*, 2nd Edition ed., Kluwer Academic, New York, 1999.
- [11] M. Fischer, J. Georges, *Chemical Physics Letters*, 260 (1996) 115-118.
- [12] D. Magde, R. Wong, P.G. Seybold, *Photochemistry and Photobiology*, 75 (2002) 327-334.
- [13] T. Karstens, K. Kobs, *The Journal of Physical Chemistry*, 84 (1980) 1871-1872.
- [14] R.F. Kubin, A.N. Fletcher, *Journal of Luminescence*, 27 (1983) 455-462.
- [15] O. Chen, R. Glaser, G.I. Likhtenshtein, *Journal of Biochemical and Biophysical Methods*, 70 (2008) 1073-1079.
- [16] N. Strashnikova, V. Papper, P. Parkhomyuk, G.I. Likhtenshtein, V. Ratner, R. Marks, *Journal of Photochemistry and Photobiology A: Chemistry*, 122 (1999) 133-142.
- [17] G. Dobretsov, T. Syrejschikova, N. Smolina, *Biophysics*, 59 (2014) 183-188.
- [18] S. Tobita, K. Ida, S. Shiobara, *Research on Chemical Intermediates*, 27 (2001) 205-218.
- [19] J. Oshima, T. Yoshihara, S. Tobita, *Chemical Physics Letters*, 423 (2006) 306-311.

[20] C. A. T. Laia, S. M. B. Costa, *Journal of the Chemical Society, Faraday Transactions*, 94 (1998) 2367-2373.

Chapter 4

Fluorescence and binding studies of parent stilbenes and their malachite green conjugates

In this chapter, the photophysical characterization and medium viscosity study for two reactant stilbenes (DMAAS and MAS) were conducted first. Their fluorescence decay and decay rate dependence on medium viscosity were confirmed. For PHASA competitive binding scope, besides a negative control conjugate, a “fused” stilbene-MG (malachite green) conjugate and three other conjugates with different linkers such as $-\text{COO}-$, $-\text{CH}_2\text{-NH}-$ and $-\text{CH}_2\text{-NAC}-$ were synthesized. To check the binding of stilbene-MG conjugates to MG aptamer, their binding experiments with the MG aptamer were conducted. The dissociation constant K_d values were found as follows: $K_{d(\text{MG})} < K_{d(\text{“fused” stilbene MG})} < K_{d(-\text{CH}_2\text{-NAC}-)} < K_{d(-\text{CH}_2\text{-NH}-)} < K_{d(-\text{COO}-)}$. The fluorescence decay properties of these conjugates were also studied. The “fused” stilbene-MG conjugate had no observable fluorescence decay in several organic solvents, such as DMSO, acetonitrile (ACN) and ethanol (EtOH). This also applied to the conjugate with the $-\text{COO}-$ linker. Conjugates with the $-\text{CH}_2\text{-NH}-$ and $-\text{CH}_2\text{-NAC}-$ linker both exhibited fluorescence decay in organic solvent, as well as in aqueous buffer. As the conjugate with the $-\text{CH}_2\text{-NAC}-$ linker has lower K_d value and thus stronger binding affinity to MG aptamer, which makes it more competitive with MG analyte for the MG aptamer, it was considered to be the best candidate for PHASA competitive binding scope.

4.1 Introduction

Competitive binding scope (**Figure 1.2**) relies on the competition between malachite green (MG) and the stilbene–MG conjugate for the MG aptamer binding pocket. The stilbene–MG conjugates having the $-\text{COO}-$, $-\text{CH}_2\text{-NH}-$ and $-\text{CH}_2\text{-NAc}-$ linkers have been synthesized from two parent stilbenes and *leuco*-MG-COOH (**3**), as shown in **Figure 4.1**. Since these three linkers have different electron-donating and accepting abilities, the resulting stilbene-MG conjugates have different electron density distribution along the molecule. This affects the binding affinity of the conjugates towards the MG aptamer and their fluorescence decay behavior. Further, in order to increase the binding affinity of the conjugate, a “fused” stilbene-MG conjugate (**5**; **Figure 4.1**) was also synthesized. Since rhodamine B cannot bind to the MG aptamer, the rhodamine B–DMAAS conjugate (**4**; **Figure 4.1**) has been prepared and studied as the negative control. The photo-physical properties, fluorescence decay kinetics, medium viscosity dependence and binding affinities for these stilbene-MG conjugates were studied and discussed.

4.2 Experimental section

4.2.1 Materials

Two parent stilbenes: 4-dimethylamino-4'-aminostilbene (**1**) and 4-methoxy-4'-aminostilbene (**2**) were purchased from TCI, Japan. For the synthesis work, reactants such as *N,N*-dimethylaniline, 4-carboxybenzaldehyde, (E)-4-styrylbenzaldehyde and rhodamine B, and solvents including triethylamine, dry dichloromethane (DCM), ethyl acetate and etc. were purchased from Sigma Aldrich, Singapore. TLC (thin layer chromatography) alumina plates used for reaction monitoring were ordered from Sigma Aldrich. All the organic solvents used for spectroscopy measurements such as acetonitrile (ACN), ethanol (EtOH), dimethyl sulfoxide (DMSO) and propylene carbonate (PC) were purchased in HPLC grade from Sigma Aldrich, Singapore. Glycerol in the medium viscosity study was purchased from Fisher Scientific, Singapore. MG aptamer was purchased in its powder form from AITBiotech, Singapore. The PBS buffer was prepared from 5.65 mmol L⁻¹ NaH₂PO₄ (Sigma), 4.35 mmol L⁻¹ Na₂HPO₄ (Sigma), and 10 mmol L⁻¹ MgCl₂ (Sigma) and 1 mmol L⁻¹ NaCl unless otherwise stated. Deionised (DI) water was supplied by Milli-Q® water system.

Figure 4.1. Structure of various stilbene–MG conjugates and rhodamine B–DMAAS conjugate studied in competitive binding scope.

4.2.2 Synthesis of rhodamine B-DMAAS conjugate **4** (Figure 4.1)

Conjugate **4** was synthesized as a negative control for the stilbene–MG conjugates in the binding experiments. **Figure 4.2** shows its synthesis pathway and the detailed reaction procedures and conditions were described below.

Figure 4.2. Reaction route for the synthesis of conjugate **4**.

Equal molar amounts of rhodamine B (0.2 g; 0.42 mmol L⁻¹) and triethylamine (0.043 g) were dissolved in dry CH₂Cl₂ (8 mL) at 0 °C. Then ethyl chloroformate was added over 1 to 2 minutes under N₂. After stirring the mixture at 0 °C for 30 minutes, the cooling bath was removed and the reaction was brought to room temperature for 30 minutes. The reaction mixture was filtered, washed with H₂O (1×50 mL), saturated NaCl solution (1×50

mL) and dried over anhydrous Na_2SO_4 . After solvent evaporation, the rhodamine B anhydride was obtained and used directly in the second step.

To a solution of DMAAS (0.23 mmol L^{-1}) and triethylamine (0.45 mmol L^{-1}) in dry CH_2Cl_2 (5 mL) was added a solution of rhodamine B anhydride (0.13 g) obtained above in dry CH_2Cl_2 (2 mL) under nitrogen. The resulting mixture was stirred for 3 hours at room temperature. Then the mixture was diluted with chloroform (10 mL), washed with H_2O ($2 \times 25 \text{ mL}$), saturated NaCl ($1 \times 25 \text{ mL}$) and dried over anhydrous Na_2SO_4 . After solvent removal, the obtained residue was purified by column chromatography using ethyl acetate as the eluting phase. The fractions obtained were evaporated and ^1H NMR was conducted. ^1H NMR, 400 MHz, CDCl_3 : 1.15 (6H; d; $-\text{N}(\text{CH}_2\text{CH}_3)_2$); 1.41 (6H; d; $-\text{N}^+(\text{CH}_2\text{CH}_3)_2$); 3.06 (6H; s; $-\text{N}(\text{CH}_3)_2$); 3.41 (4H; d; $-\text{N}(\text{CH}_2\text{CH}_3)_2$); 4.07 (4H; d; $-\text{N}^+(\text{CH}_2\text{CH}_3)_2$); 5.56 (s, H; s); 6.07 (1H; d); 5.89 (1H; d); 6.22 (1H; d); 6.27 (1H; d); 7.03 (1H; d); 6.76 (2H; d); 7.66 (2H; d); 6.95 (s; 2H; vinyl H); 7.56 (1H; d); 7.65 (1H; d); 7.51 (1H; d); 7.62 (1H; d); 7.84 (2H; d); 8.00 (2H; d); 9.15 (s; CONH).

4.2.3 Synthesis of “fused” stilbene-MG conjugate **5** (Figure 4.1)

The synthesis pathway for the conjugate **5** is shown in **Figure 4.3**, in which its *leuco* form **9** was obtained first.

Figure 4.3. Reaction pathway for the synthesis of conjugate **5**.

Dimethylaniline (286.1 mg; 2.36 mmol L^{-1}), *trans*-4-stilbenecarboxaldehyde (95.7 mg; 0.43 mmol L^{-1}) and 37% HCl (129 mg) were mixed in 0.6 mL isopropyl alcohol. The reaction mixture was heated with weak reflux in argon for 24 hours. Then KOH (20%) was added and white precipitate was collected on filter. After washing with water and methanol, 80 mg white solid **9** (43% yield) was obtained. ^1H NMR, 400 MHz, CDCl_3 : 1.55 (s; H_2O); 2.908 (12H; s; $\text{N}(\text{CH}_3)_2$); 5.372 (1H; s; CH); 6.668 (4H; d; 8.6 Hz); 6.992 (4H; d; 8.6 Hz); 7.020-7.061 (1H; d; 16.4 Hz; CH vinyl); 7.070-7.111 (1H; d; 16.4 Hz; CH vinyl); 7.122 (2H; d; 8.1 Hz); 7.214-7.251 (1H + CHCl_3 -distorted triplet); 7.337 (2H; t; 7.6 Hz); 7.406

(2H; d; 8.0 Hz); 7.490 (2H; d; 7.8 Hz). ^{13}C NMR, CDCl_3 : 40.734 ($\text{N}(\text{CH}_3)_2$); 54.792 (CH); 77.019 (t; 32 Hz; CDCl_3); 77.222 (CHCl_3); 112.562; 126.303; 126.419; 127.388; 127.952; 128.630; 128.667; 129.679; 129.935; 132.684; 134.932; 137.564; 145.214; 149.014. MALDI TOF (*neat*, reflectron mode, positive) m/z 434.41 $[\text{M}+\text{H}]^+$ (calculated 433.26).

The above obtained *leuco* “fused” stilbene–MG conjugate **9** (80 mg) and chloranil (45 mg) were heated in 10 mL ethyl acetate at 55 °C in dark. Blue-green color was developed quickly and dark precipitate accumulated. After 4 hours heating, the mixture was cooled down and diluted with 10 mL of ether for precipitation. NMR spectrum for the obtained conjugate **5** was recorded. ^1H NMR, 400 MHz, CDCl_3 : 1.81 (broad s; H_2O); 3.397 (12H; s; $\text{N}(\text{CH}_3)_2$); 6.995 (4H; d; 7 Hz); 7.187–7.228 (1H; d; 16.4 Hz; CH vinyl); 7.268 (s; CHCl_3); 7.328–7.432 (10H; overlapping doublets and triplets); 7.587 (2H; d; 7.5 Hz); 7.681 (2H; d; 8.2 Hz). ^{13}C NMR, CDCl_3 : 41.108 ($\text{N}(\text{CH}_3)_2$); 113.927; 126.519; 127.013; 127.074; 128.674; 128.851; 132.911; 135.584; 140.766. MALDI TOF (*neat*, reflectron mode, positive) m/z 433.48 $[\text{M}]^+$ (calculated 431.25).

4.2.4 Synthesis of stilbene-MG conjugates with linkers of –COO– (**6**), –CH₂–NH– (**7**) and –CH₂–NAc– (**8**) (Figure 4.1)

Figure 4.4. Detailed synthesis procedures for the stilbene–MG conjugates **6**, **7** and **8**.

The three stilbene-MG conjugates were synthesized from parent stilbenes and *leuco* MG-COOH **3**. Since they were obtained similarly, they were introduced together. **Figure 4.4** schematically shows their synthesis pathways. Parent stilbene **1** (DMAAS) is not involved because of its strong donor group—dimethylamino, which makes the final oxidation and purification process unstable and complex.

Synthesis of leuco MG-COOH 3

4-carboxybenzaldehyde (1.5g; 10 mmol L⁻¹) and dimethylaniline (4 mL; 31.5 mmol L⁻¹) were mixed with ethanol (3 mL) and 37% HCl (0.9 mL). The reaction mixture was heated at 80 °C overnight. White precipitate was collected on a filter and thoroughly washed with ethanol. After drying, 1.45 g of white solid (**3**) was obtained. ¹H NMR, 400 MHz, CDCl₃: 7.977 (2H; d; 8.0 Hz); 7.261 (s; CDCl₃); 7.237 (2H; d; 8.0 Hz); 6.960 (4H; d; 8.5 Hz); 6.668 (4H; d; 8.5 Hz); 5.423 (1H; s; CH); 2.915 (12H; s; N(CH₃)₂). ¹³C NMR, DMSO-d₆: 39.52 (DMSO-d₆); 40.20 (N(CH₃)₂); 54.10 (CH); 112.40; 128.32; 129.00; 129.21; 129.39; 131.45; 148.85; 150.67; 167.22 (-COOH). MALDI TOF (*neat*, reflectron mode, positive) m/z 375.15 [M+H]⁺ (calculated 375.20).

Synthesis of MG-COO-Stilb-NO₂ 6

Compound **3** (30.9 mg; 0.082 mmol L⁻¹) and **10** (17.4 mg; 0.072 mmol L⁻¹) were suspended in 1 mL anhydrous CH₂Cl₂. To the mixture, 47.2 mg of N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) was added and a clear yellow solution formed. The solution was stirred for 3 days, then 2 mL NaHCO₃ was added. After extra 30 mins stirring, 5 mL CH₂Cl₂ was added for extraction. The obtained product was purified by chromatography on silica. The fractions were evaporated, dried and 16 mg (37%) of conjugate **14** was obtained. ¹H NMR, 400 MHz, DMSO-d₆: 2.502 (q; DMSO-d₅H); 2.860 (12H; s; N(CH₃)₂); 3.323 (H₂O); 5.477 (1H; s; CH); 6.680 (4H; d; 8.7 Hz); 6.925 (4H; d; 8.7 Hz); 7.305-7.326 (2H; d; 8.3 Hz); 7.321-7.342 (2H; d; 8.6 Hz); 7.420-7.461 (1H; d; 16.5 Hz); 7.566-7.607 (1H; d; 16.5 Hz); 7.775 (2H; d; 8.7 Hz); 7.882 (2H; d; 8.9 Hz); 8.061 (2H; d; 8.4 Hz); 8.252 (2H; d; 8.9 Hz). ¹³C NMR, DMSO-d₆: 39.520 (septet; 21 Hz; DMSO-d₆); 40.202 (N(CH₃)₂); 54.121 (CH); 112.439; 122.401; 124.031; 126.359; 126.582; 127.303; 128.213; 129.413; 129.800; 132.304; 134.146; 143.953; 146.215; 148.879;

150.813; 152.157; 164.397. MALDI TOF (*neat*, reflectron mode, positive) m/z 599.17 $[M+H]^+$ (calculated 598.26).

The above obtained **14** (14.5 mg) was dissolved in a solution mixture of 1.5 mL ethyl acetate and 0.1 mL chloroform, to which 9 mg chloranil was added. The reaction mixture was heated at 60 °C for 90 mins. A green precipitate (**6**) formed and was collected by centrifugation. ^1H NMR, 400 MHz, DMSO- d_6 : 2.503 (q; 1.85 Hz; DMSO- $d_5\text{H}$); 3.331 (12H; s; $\text{N}(\text{CH}_3)_2$); 3.343 (s; H_2O in DMSO); 7.124 (2H; d; 9.5 Hz); 7.367 (4H; d; 9.5 Hz); 7.418 (2H; d; 8.7 Hz); 7.446-7.487 (1H; d; 16.4 Hz; CH-vinyl); 7.570 (2H; d; 8.4 Hz); 7.593-7.634 (1H; d; 16.4 Hz; CH-vinyl); 7.822 (2H; d; 8.7 Hz); 7.898 (2H; d; 8.9 Hz); 8.266 (2H; d; 8.9 Hz); 8.345 (2H; d; 8.4 Hz); 10.237 (1H; broad s; OH). ^{13}C NMR, DMSO- d_6 : 39.443 (septet; 21 Hz; DMSO- d_6); 40.550 ($\text{N}(\text{CH}_3)_2$); 114.267; 120.602; 122.225; 123.980; 123.980; 126.416; 126.701; 127.271; 128.235; 128.428; 129.695; 131.627; 132.158; 134.177; 134.390; 139.855; 143.824; 143.964; 144.342; 146.194; 150.563; 156.451; 163.854. MALDI TOF (*neat*, reflectron mode, positive) m/z 597.30 $[M]^+$ (calculated 596.25).

Synthesis of leuco MG-CONH-Stilb-OMe 11

Parent stilbene **2** (65 mg; 288.5 $\mu\text{mol L}^{-1}$) and **3** (114 mg; 304.3 $\mu\text{mol L}^{-1}$) were added to 3.75 mL CH_2Cl_2 containing 137.5 mg of ethylcarbodiimide hydrochloride (716.7 $\mu\text{mol L}^{-1}$) in ice bath. To this mixture, 4-dimethylaminopyridine (20 mg; 164 $\mu\text{mol L}^{-1}$) was also added. The obtained mixture was left stirred for two days at room temperature. After diluting with CH_2Cl_2 , aqueous NaHCO_3 was added. The mixture was extracted and layers were separated. The organic layer was collected, washed, and then recrystallized from CH_2Cl_2 /i-PrOH (25 mL each). Yield of 77% for conjugate **11** was produced. ^1H NMR, 400 MHz, CDCl_3 : 1.2553; 1.58 (H_2O); 2.919 (s; 12H); 3.829 (s; 3H); 5.294 (s; CH_2Cl_2); 5.428 (s; 1H); 6.660-6.682 (4H; d; 8.7 Hz); 6.885-6.907 (2H; d; 8.6 Hz); 6.924-6.963 (1H; d; 15.9 Hz); 6.958-6.9793 (4H; d; 8.6 Hz); 7.001-7.042 (1H; d; 16.2 Hz); 7.248-7.268 (2H; d; 8.2 Hz); 7.256 (CHCl_3); 7.433-7.455 (2H; d; 8.6 Hz); 7.471-7.492 (2H; d; 8.6 Hz); 7.605-7.626 (2H; d; 8.4 Hz); 7.745-7.766 (2H; d; 8.3 Hz); 7.796 (broad s; 1H). ^{13}C NMR, CDCl_3 : 40.675; 53.419 (CH_2Cl_2); 54.923; 55.334; 77.020 (t; 32 Hz; CDCl_3); 77.227

(CHCl₃); 112.555; 114.153; 120.167; 125.963; 126.860; 126.897; 127.543; 127.629; 129.797; 129.914; 130.220; 131.848; 132.487; 133.954; 137.127; 149.145; 150.013; 159.243; 165.593. MALDI TOF (*neat*, reflectron mode, positive) *m/z* 582.88 [M+H]⁺ (calculated 582.30).

Synthesis of MG-CH₂-NH-Stilb-OMe 7

Compound **11** (215 mg; 0.37 mmol L⁻¹) was suspended in 20 mL anhydrous THF (tetrahydrofuran) under argon. 1 mL of 1 mol L⁻¹ lithium aluminum hydride (LAH) solution in diethyl ether was added. The reaction mixture was stirred until the completion of gas evolution. Then it was heated and stirred for 24 hours. The excess of LAH was quenched by isopropanol and the reaction mixture slurry was filtered. After washing with THF, light yellow solid (**12**) was obtained. ¹H NMR, 400 MHz, CDCl₃: 1.589 (broad s; H₂O); 2.903 (12H; s; N(CH₃)₂); 3.812 (3H; s; -OCH₃); 3.97-4.15 (1H; broad hump; NH); 4.289 (2H; s; Ar-CH₂NH-); 5.359 (1H; s; Ar₃CH); 6.61 (2H; d; 8.6 Hz); 6.66 (4H; d; 8.8 Hz); 6.818-6.859 (1H; d; 16.2 Hz; CH=CH vinyl); 6.858-6.879 (2H; d; 8.3 Hz); 6.873-6.912 (1H; d; 16.2 Hz; CH=CH vinyl); 6.98 (4H; d; 8.7 Hz); 7.11 (2H; d; 8.1 Hz); 7.242-7.262 (2H; d; 8.1 Hz); 7.250 (s; CHCl₃); 7.32 (2H; d; 8.6 Hz); 7.40 (2H; d; 8.8 Hz). ¹³C NMR, CDCl₃: 40.734; 48.082; 54.737; 55.318; 112.560; 112.916; 114.066; 124.113; 126.883; 127.153; 127.274; 127.439; 129.618; 129.899; 130.998; 132.755; 136.546; 144.717; 147.656; 148.997; 158.673. MALDI TOF (*neat*, reflectron mode, positive) *m/z* 567.33 [M+H]⁺ (calculated 568.32).

The above conjugate **12** (7 mg) was dissolved in 0.7 mL ethyl acetate, to which 3.7 mg chloranil was added. The mixture was heated at 53 °C for 1 hour and green precipitate formed. After washing with ethyl acetate, the obtained solid was dissolved in 1 mL CHCl₃, which was then evaporated and dried. ¹H NMR shows the decrease of the -CH peak, meaning the *leuco* form **12** has been oxidized and **7** has formed. But the obtained conjugate **7** is not pure.

Synthesis of MG-CH₂-NAc-Stilb-OMe 8

Leuco MG-CONH-Stilb-OMe—compound **12** (16 mg) was dissolved in 0.5 mL CH₂Cl₂. To the solution was added 0.5 mL aqueous NaHCO₃ followed by 10 μL acetic anhydride. The

mixture was vortexed well and left overnight. The organic layer was then separated in a separating funnel and the solvent was evaporated using Buchi rotary evaporator. *Leuco* MG-CH₂-NAc-Stilb-OMe (**13**) with yield of 30% was obtained. ¹H NMR, 400 MHz, CDCl₃: 1.901 (s); 2.085 (s; 3H; CO-CH₃); 2.895 (s; 12H; N(CH₃)₂); 3.833 (s; 3H; O-CH₃); 4.850 s + 4.862 s (2H; Ar-CH₂-N(Ac)) ; 5.330 s + 5.425 s (1H; Ar-CH-(Ph-N(CH₃)₂)₂); 6.643-6.664 (4H; d; 8.7 Hz C aryl-H, orto to N(CH₃)₂); 6.88-7.15 (16 H; overlapping doublets C aryl-H and CH vinyl); 7.258 (s; CHCl₃); 7.405-7.425 (2H; d; 8.4 Hz C aryl-H); 7.431-7.452 (2H; d; 8.8 Hz C aryl-H).

Compound **13** (14 mg) was mixed with 9 mg of chloranil in 5.0 mL ethyl acetate. The solution was heated for 1 hour at 60 °C and green crystals were precipitated. The greenish-colored organic layer was decanted and removed. The obtained crude crystals were thus mixed with 1 mL H₂O/5 μL 37% HCl and 0.5 mL ethyl acetate/0.5 mL hexane. After thorough mixing, the organic layer was separated and discarded. Then, the aqueous layer was washed with ethyl acetate/hexane (1:1) twice and hexane once. Finally water was evaporated, leaving green solid (**8**). ¹H NMR, 400 MHz, DMSO-d₆: 1.910 s + 1.926 (board s; 3H; CH₃C=O); 2.507 (q 1.8 Hz; dmsoD5H); 3.278 (s; 12H; N(CH₃)₂); 3.770 s + 3.776 (shoulder on s.) (3H; OCH₃); 4.864 s + 5.041 s (CH₂N); 6.934-6.955 (2H; d; 8.4 Hz); 7.059-7.082 (4H; d; 9.2 Hz); 7.087-7.127 (1H; d; 16.1 Hz C-H vinyl); 7.208-7.248 (1H; d; 16.1 Hz C-H vinyl); 7.267-7.303 (8H; overlapping doublets); 7.451-7.470 (2H; d; 7.7 Hz); 7.525-7.546 (2H; d; 8.4 Hz); 7.586-7.607 (2H; d; 8.4 Hz). ¹³C NMR, DMSO-d₆: 22.904; 39.975; 40.961; 52.130; 55.664; 114.422; 114.676; 125.499; 126.885; 127.503; 128.368; 128.439; 128.661; 129.402; 129.487; 129.942; 134.891; 138.438; 140.539; 141.762; 143.713; 156.893; 159.618; 170.093; 175.551. MALDI TOF (*neat*, reflectron mode, positive) m/z 608.90 [M]⁺ (calculated 608.33).

4.3 Results & discussions

4.3.1 Obvious fluorescence decay & high quantum yield of parent stilbenes (**1&2**) are observed in organic solvents

The photophysical properties of two parent stilbenes **1** and **2** (Figure 4.1) were characterized in ACN and DMSO since they dissolve a wide range of compounds, both polar and non-polar. Figure 4.5 summarizes the measured absorption spectrum, emission

spectrum and excitation spectrum. Their absorption, emission and excitation peaks and the molar extinction coefficients are tabulated in **Table 4.1**. When measured in the same solvent, the absorption and excitation peaks of **2** are blue shifted compared with that of **1**. This is because the dimethylamino substituent in **1** has stronger electron-donating ability than the methoxy group in **2**, which facilitates the charge delocalization in the ground state 1t of **1** and results in a lower energy and longer wavelength transition to the Frank-Condon state [1, 2]. While for the same stilbene molecule, when the solvent is changed from ACN to DMSO, the absorption, excitation and emission peaks are all red shifted. This is attributed to the higher polarity of DMSO which stabilizes more both the ground and excited states.

Figure 4.5. Absorption spectrum (solid line), emission spectrum (dash line) and excitation spectrum (dot line) summary for **1** (Black) and **2** (Grey) in ACN (A) and DMSO (B).

Based on their absorption and emission peaks, the fluorescence decay curves of two parent stilbene **1** and **2** in both ACN and DMSO were measured and summarized in **Figure 4.6**. Their respective apparent decay rate constants (k_{app} values) were calculated and presented in **Table 4.1**. Compared with the solvation effect from ACN to DMSO, the substituent plays a more significant role in decay rate constant. The faster *trans-cis* isomerization (higher k_{app} value) of stilbene **2** than **1** indicates that **2** experiences a smaller energy barrier during its rotation from excited state $^1t^*$ to the phantom state P^* .

Figure 4.6. Fluorescence decay curves of **1** (Black) and **2** (Grey) in ACN (solid line) and DMSO (dot line).

Table 4.1. Photophysical data summary two parent stilbenes in ACN and DMSO.

Stilbene	Solvent	$\lambda_{\text{abs}} / \lambda_{\text{Ex}}$ (nm)	λ_{Em} (nm)	ϵ ((mM*cm) ⁻¹)	QY	k_{app} (s ⁻¹)
1	ACN	357 / 349	420	23.83±0.94	0.60	0.565±0.004
	DMSO	368 / 366	420	24.02±0.85	0.81	0.547±0.007
2	ACN	337 / 317	410	20.36±0.58	0.038	0.721±0.006
	DMSO	350 / 343	426	21.14±0.66	0.072	0.83±0.02

Table 4.1 also displays the quantum yield (QY) results. Opposite to that observed in fluorescence decay rate constant, **1** has higher quantum yields than **2** in both solvents. Based on the photophysics of stilbenes [1, 2], the fluorescence *trans* isomer can be converted to the non-fluorescence *cis* isomer through torsional rotation around the double bond. So the *trans-cis* isomerization process acts as a quenching funnel or the irradiative emission process. When the isomerization process dominates the excited states deactivation process, the chance of fluorescence emission is low, resulting in low quantum yield and vice versa.

4.3.2 Decay rate of stilbenes **1** and **2** depends on medium viscosity

Shown in **Table 4.1**, the quantum yields of the two parent stilbenes **1** and **2** are more than 0.02 and their fluorescence decay is obvious (k_{app} values greater than 0.5 s⁻¹), satisfying the requirements of competitive binding scope. In literature, the decay rate dependence of stilbene compounds on their surrounding medium viscosity has been extensively studied [3-5]. So seven solution mixtures consisting of different DMSO to glycerol ratios were

prepared. After measuring their viscosities (results shown in **Table 3.1**), the fluorescence decay curves of **1** and **2** in these solution mixtures were recorded.

Figure 4.7. Fluorescence decay curves of stilbene **1** (A) and **2** (B) in solution mixtures having different volume ratios of DMSO to glycerol. The relationship of their decay rate constant (C) and fluorescence polarization (D) with viscosity.

Figure 4.7 A and **B** shows the normalized fluorescence decay curves of **1** and **2** in the first 20 secs. Their decay varies in different solution mixtures. The apparent decay rate constants were calculated and then plotted against the reciprocal of the corresponding viscosities, shown in **Figure 4.7 C**. Linear relationships between the decay rate constant and the reciprocal of viscosity in a log scale are observed for both parent stilbenes in the viscosity range studied (2.7 cP~215.8 cP), and they can be expressed in equation 4.1 and 4.2 for parent stilbene **1** and **2** respectively.

$$k_{app} = (0.599 \pm 0.003) + (0.065 \pm 0.003) * \log(1/\eta) \quad (4.1)$$

$$k_{app} = (0.916 \pm 0.007) + (0.160 \pm 0.004) * \log(1/\eta) \quad (4.2)$$

Where η is the medium viscosity value. Stilbene **2** is more “sensitive” to the surrounding viscous environment than **1** in the way that greater fluorescence decay rate change is resulted with the same amount of viscosity change.

Besides fluorescence decay kinetics, fluorescence polarization (FP) measurements for the two stilbene compounds in the seven solution mixtures were also conducted and the results are summarized in **Figure 4.7 D**. Similarly, linear dependence of FP on the medium viscosity in log scale is observed for both stilbene molecules, and the relationships are expressed in equation 4.3 and 4.4 for **1** and **2** respectively.

$$FP = (-0.01 \pm 0.01) + (0.24 \pm 0.01) * \log \eta \quad (4.3)$$

$$FP = (0.093 \pm 0.001) + (0.208 \pm 0.001) * \log \eta \quad (4.4)$$

Therefore, the strong dependence of both parent stilbenes’ fluorescence decay rate and fluorescence polarization on their surrounding medium viscosity is confirmed. Based on this dependence, the sensing ability of stilbene compounds can be applied in PHASA which is associated with environmental change caused by aptamer kinetics binding.

4.3.3 Fluorescence decay of **1** and **2** retains in aqueous environment

To preserve the binding interactions between MG aptamer and MG analyte in forming the binding pocket, MG aptamer binding buffer (PBS buffer: 5.65 mmol L⁻¹ NaH₂PO₄, 4.35 mmol L⁻¹ Na₂HPO₄, 10 mmol L⁻¹ MgCl₂ and 1 mmol L⁻¹ NaCl) is essential in the final competitive binding experiment. Hence, fluorescence decay kinetics measurement of the parent stilbenes in water and the above binding buffer is necessary.

Figure 4.8. The fluorescence decay curves of stilbene **1** (A) and **2** (B) in 3% organic solvent (ACN/DMSO) and aqueous (water/buffer) mixtures.

Figure 4.8 presents the normalized fluorescence decay curves of the two stilbenes in 3% ACN/DMSO and 97% water/buffer aqueous mixtures. The addition of low volume percentage of organic solvents is to enhance the low solubility of the two stilbenes in aqueous solvents. Organic solvent concentrations less than 10% does not significantly influence the binding interactions between MG analyte and MG aptamer and it can enhance the MG stability, preventing it from hydrolysis [6]. The absorption maxima, emission peaks, and apparent decay rate kinetics of stilbene **1** and **2** in the above four aqueous solvents are summarized in **Table 4.2**.

Table 4.2. Photophysical data summary of stilbene **1**, **2** and conjugate **4** in aqueous mixtures.

Compound	Solvent	λ_{abs} (nm)	λ_{Em} (nm)	k_{app} (s ⁻¹)
1	ACN (3%) and water	337	441	0.641±0.004
	DMSO (3%) and water	337	442	0.621±0.004
	ACN (3%) and buffer	338	442	0.658±0.006
	DMSO (3%) and buffer	339	440	0.637±0.006
2	ACN (3%) and water	331	432	0.529±0.003
	DMSO (3%) and water	326	432	0.506±0.005
	ACN (3%) and buffer	329	432	0.535±0.003
	DMSO (3%) and buffer	329	431	0.536±0.005
Conjugate 4	DMSO (10%) and buffer	350	445	0.456±0.006

In the aqueous mixtures, both parent stilbenes' absorption peaks shift to the blue side while their emission peaks shift to the red side compared to their respective organic solvents. The shift (10~20 nm) is not great as ~10 nm shift is observed when ACN is changed to DMSO, considering that water is a polar protic solvent and hydrogen bonding interactions can occur. So both parent stilbenes are likely surrounded by organic solvent shell although the bulk environment is aqueous. The fluorescence decay of both parent stilbenes **1** and **2** are preserved with the apparent decay rate constants of **1** being faster than **2**. Compared with their fluorescence decay in organic solvents (**Table 4.1**), the decay rate constants of **2** decrease while that of **1** have no great change. That is, the *trans-cis* isomerization process of **2** is more sensitive to electronic distribution variations. The preservation of both stilbenes' fluorescence decay in aqueous solution indicates that stilbene-MG conjugates' decay can also survive, and analyte detection based on their decay rate change is possible.

4.3.4 Rhodamine B–DMAAS conjugate **4** (Figure 4.1) has no binding affinity to MG aptamer

In order to test whether there will be additional non-specific binding of stilbene-MG conjugates to MG aptamer caused by the stilbene conjugation, a negative control for the stilbene–MG conjugates in competitive binding scope is necessary. Since rhodamine B does not binding to MG aptamer [7], rhodamine B-DMAAS conjugate (**4**) was synthesized. Before the binding experiments of conjugate **4** with MG aptamer were performed, the photophysical characterization of **4** and its fluorescence decay rate dependence on medium viscosity was conducted first.

Photophysical characterization and medium viscosity study

The absorption, emission and excitation spectrum of **4** in DMSO (10%) and PBS buffer system as well as its fluorescence decay curves are presented in **Figure 4.9**. The absorption, emission and excitation maxima together with the apparent decay rate constant are summarized in **Table 4.2**. The absorption and emission spectra of **4** have no insignificant shifts from those of parent stilbene **1**. More importantly, the conjugate has clearly observed fluorescence decay kinetics. These observations suggest that the rhodamine B and stilbene fragments are electronically decoupled as the amide linkage breaks the electronic conjugation between the two parts. Hence, the photophysical properties, particularly the fluorescence decay kinetics, of the stilbene fragment is not affected by conjugation with rhodamine B via the amide linkage.

Figure 4.9. Normalized absorption spectrum (solid line), emission spectrum (dash line) and excitation spectrum (dot line) summary (**A**) and fluorescence decay (**B**) of conjugate **4** in DMSO (10%) and buffer.

To further explore the response of the conjugate to its surrounding medium, its fluorescence decay curves and the fluorescence polarization were measured in the seven DMSO to glycerol solution mixtures having different viscosities (**Table 3.1**). The normalized decay curves are shown in **Figure 4.10 A**. The plot of the calculated apparent rate constant vs. the reciprocal of the viscosity value in a log scale and fluorescence polarization vs. viscosity in a log scale are shown in **Figure 4.10 B** and **4.10 C**, respectively.

Figure 4.10. Decay curves of conjugate 4 in different DMSO to glycerol mixtures (**A**), the relationship between its decay rate constant and viscosity (**B**) and fluorescence polarization and viscosity (**C**).

Linear relationship between fluorescence polarization (FP) and viscosity is observed and can be expressed as

$$FP = (0.05 \pm 0.02) + (0.18 \pm 0.02) * \log \eta \quad (4.5)$$

For the decay rate constant, linear dependence is not applied in the entire studied viscosity range, but in the range from 2.68 cP to 38.6 cP, where the relationship is expressed as

$$k_{app} = (0.103 \pm 0.002) + (0.016 \pm 0.001) * \log(1/\eta) \quad (4.6)$$

For the higher viscosity value 215.8 cP, the measured rate constant is 14% higher than that obtained from the relationship equation. Comparing the conjugate's decay rate dependence on medium viscosity with that of the parent stilbene **1**, the slope for the conjugate **4** is four times lower, meaning the response of the conjugate's decay rate to its surrounding viscosity change is less sensitive. This observation also applies to the conjugate's fluorescence polarization dependence on medium viscosity compared to the parent stilbene **1**. This is probably because of the conjugate's three times greater rotational volume than the parent stilbene **1** after rhodamine B addition.

Binding reaction between 4 and MG aptamer

In the binding experiment between conjugate **4** and MG aptamer, solutions having different MG aptamer to the conjugate ratios were prepared, in which the conjugate's concentration was kept constant. In the same way, the conjugate was also mixed with BSA (Bovine Serum Albumin) for comparison. Fluorescence polarization (FP) measurements in which rhodamine B and the conjugate were mixed separately with MG aptamer were also conducted, and the concentration of rhodamine B and **4** was kept constant.

Figure 4.11 A. The decay rate constant for the binding of conjugate **4** with MG aptamer/BSA. B. The fluorescence polarization results for the MG aptamer binding with the conjugate and rhodamine B.

Figure 4.11 presents the obtained decay rate constants with the change of aptamer/BSA to the conjugate ratio as well as the FP values with respect to different MG aptamer to the conjugate/rhodamine B ratio. **Figure 4.11 A** shows that there is no difference observed when the amount of MG aptamer is 100 times more than the conjugate. This constant

fluorescence decay rate with increasing MG aptamer to conjugate ratio indicates that there is no binding of MG aptamer towards conjugate **4** (negative control) and stilbene conjugation to rhodamine B does not cause binding interaction.

The non-responsiveness of MG aptamer towards **4** is also supported by the FP results shown in **Figure 4.11 B**. With the ratio of MG aptamer to conjugate up to 100, there is no significant difference observed, just like that observed for rhodamine B which has been confirmed to have no binding with MG aptamer. The measured higher polarization of the conjugate than rhodamine B is because of its 1.5 times higher molecular weight, making its rotation slower. Therefore, both the fluorescence decay and fluorescence polarization results support the conclusion that stilbene conjugation to MG derivatives does not cause binding and the binding of stilbene-MG conjugates to MG aptamer only results from the MG fragment. Thus, the final MG analyte detection in competitive binding scope of PHASA is not influenced by stilbene conjugation.

4.3.5 “Fused” stilbene-MG conjugate **5** (Figure 4.1)–strong binding to MG aptamer but no fluorescence decay

To preserve the electrostatic interactions while minimizing the geometry changes within the adaptive binding MG aptamer binding pocket, the “fused” stilbene-MG conjugate (**5**) was synthesized.

Figure 4.12. Direct binding experimental results of MG (A) and **5** (B) to MG aptamer under various MG aptamer to MG/5 ratios where MG and conjugate **5** concentration is kept constant.

Figure 4.12 presents the direct binding experimental results of MG to MG aptamer and conjugate **5** to MG aptamer, as assessed by project team member Y. Zhou. Their calculated

binding dissociation constants K_d are also shown in the figure. The binding assay relies on the following mechanism; the fluorescence quantum yield of MG is enhanced more than 2000 times when MG's aryl rings are sterically limited to a planar structure [8], which is the case when trapped within the MG aptamer binding pocket. Hence, in the direct binding assay, the concentration of MG and **5** are held constant and the fluorescence intensity was monitored for a series of MG aptamer to MG/conjugate **5** ratios.

To the binding of MG aptamer, the binding dissociation constant K_d of MG analyte is 200 nM while that of **5** is 500 nM. This means that with the 1.3 times higher molecule volume (the “additional” double bond and benzene ring) of conjugate **5**, its binding affinity to the MG aptamer is not strongly interrupted and its binding competitiveness remains. From **Figure 4.12**, the plateau intensity of **5** is about 10 times lower than that of the MG analyte. Since the fluorescence enhancement of MG upon binding is achieved by reducing the vibrational deactivation of excited states through a more constrained and planar structure, the ~10% of the fluorescence intensity increase in conjugate **5** compared to MG analyte indicates that there might be changes in the electronic distribution of **5**, resulting in less light absorption and fluorescence intensity.

Since the binding affinity of **5** to MG aptamer is adequate, its fluorescence decay was checked. During the photophysical characterization, colour change from green to white was observed, indicating changes in electronic structure. Hence the long term stability of **5** was monitored from its absorption spectrum.

Figure 4.13 A. Absorption spectrum of conjugate **5** in DMSO with time. **B.** The comparison of **5**'s absorption spectrum with that of **9** (*leuco* form).

Figure 4.13 A displays the decay of **5**'s absorption spectra in DMSO within 70 mins. After only 10 mins, there is more than 30 % decrease in absorbance value. When the absorbance value of absorption wavelengths above 350 nm decreases with increasing time, increase in wavelengths below 350 nm observed. Comparing the absorption spectrum of **5** with its *leuco* form **9** in **Figure 4.13 B**, one possible explanation is that the conjugate **5** is reduced to its *leuco* form. To avoid this reduction, all subsequent measurements were conducted using fresh prepared solutions.

Based on the absorption maxima in **Figure 4.13**, the emission spectra of **5** were measured. Following that, its fluorescence decay curve in DMSO was obtained, presented in **Figure 4.14**. The fluorescence decay is not obvious and the emission intensity is low compared to parent stilbene **2** with 1000 times higher fluorescence intensity under similar experimental conditions. The decay kinetics of the conjugate **5** in other water soluble, polar organic solvents such as ACN and EtOH (**Figure 4.14**), methanol, IPA, acetone and PC (propylene carbonate) were also examined in order to find optimal conditions of high quantum yield (> 100 fluorescence intensity units) and decay times less than 60 s. However, none of these solvents gave minimal parameters that would support conjugate **5**'s further development towards PHASA.

Figure 4.14. The fluorescence decay curves of **5** in ACN, EtOH and DMSO.

After conversion to its *leuco* form **9**, fluorescence decay in acetone is observed. This decay is preserved even with the addition of water or PBS buffer. The only difference between **5** and its reduced form is the charge conjugation. Combining the observation of plateau fluorescence intensity difference between **5** and MG analyte in **Figure 4.12**, it is proposed that within conjugate **5**, electronic conjugation occurs in the entire molecule, not only the

MG component. Resonance structure shown in **Figure 4.15** forms, which prevent the *trans-cis* isomerization process, and no fluorescence decay can be observed. Therefore, in order to preserve the fluorescence decay and fluorescence intensity of the stilbene fragment within stilbene-MG conjugates, a linker should be introduced to isolate the stilbene electronics from malachite green.

Figure 4.15. Proposed electronic conjugation within conjugate **5**.

4.3.6 MG-COO-Stilb-NO₂ conjugate **6** (Figure 4.1)–no binding to MG aptamer and non-observable fluorescence decay

The fluorescence decay of conjugate **5** is quenched because of the electronic conjugation of the stilbene fragment with the MG fragment. Hence, a linker needs to be introduced to electronically decouple the two components. Based on the reactivity between the carboxyl group of *leuco* MG-COOH (**3**) and the hydroxyl group of **10**, *leuco* MG-COO-Stilb-NO₂ (**14**) with an ester linker was synthesized. **Figure 4.16 A** displays the normalized absorption and emission spectrum of *leuco* MG-COO-Stilb-NO₂ (**14**) in DMSO. Absorption peaks are observed only in the UV range and no peak around 640 nm is seen as expected. Its decay curve in **Figure 4.16 B** shows that there is measureable fluorescence decay but the intensity is weak (less than 0.1% of that stilbene **2**).

Upon oxidation of the *leuco* form **14**, MG-COO-Stilb-NO₂ (**6**) was obtained. Its stability was studied by recording its absorption spectrum in DMSO with time, shown in **Figure 4.17 A**. Similar to that observed in conjugate **5**, conjugate **6** is unstable. The absorbance value at peak 650 nm decreases 35% after 10 mins, and two days later, the two peaks of 440 nm and 650 nm disappear. The remaining absorption spectrum is similar to that of its *leuco* form **14** (**Figure 4.16 A**). So conjugate **6** is prone to be reduced to its *leuco* form, just like what has been observed in conjugate **5** (“fused” stilbene conjugate). Regardless of

the instability of conjugate **6**, electronic decoupling between stilbene and MG fragments via the ester linkage is confirmed. Comparing the absorption spectrum of **6** (**Figure 4.17 A**) with that of **5** (**Figure 4.13 A**), there is an “extra” peak ~360 nm in **6**, corresponding to the characteristic absorption of stilbene fragment. The rest three peaks in **6** corresponds to the absorption spectrum of its MG fragment. This conclusion is also supported by the emission spectrum and decay kinetics measurements.

Under the excitation of the four absorption peaks (321, 362, 440 and 650 nm), conjugate **6**'s emission spectra were measured and shown in **Figure 4.17 B**. Its fluorescence decay curves in DMSO were also recorded and summarized in **Figure 4.17 C**. The fluorescence intensities of the emission spectra under the excitation of 440 and 650 nm are lower than 0.01% of parent stilbene **2** under the same experimental conditions. This is consistent with the fact that malachite green is non-fluorescent under non-planar conditions. In **Figure 4.17 C**, fluorescence decay is observed only under Ex 362 nm (the peak representing the stilbene fragment) and Em 520 nm. Moreover, the decay parameters and kinetics are close to these of the *leuco* form **14** (**Figure 4.16**). This means oxidation of the MG fragment does not influence the decay kinetics of the stilbene fragment, supporting the conclusion that MG and stilbene fragments are electronically decoupled in conjugate **6**.

Figure 4.16 A. Normalized absorption spectrum (solid line) and emission spectrum (dash line) of *leuco* MG-COO-Stilb-NO₂ (**14**). **B.** Fluorescence decay of **14** in DMSO.

Figure 4.17 **A.** The absorption spectrum of conjugate **6** with time in DMSO. **B.** The emission spectra of **6** under the excitation of the four absorption peaks. The fluorescence decay curves of **6** in DMSO (**C**) and 90% water and DMSO (**D**).

Low intensities (less than 0.01% of parent stilbene **2**) are obtained under the excitation of shorter wavelengths (321 and 362 nm), it is likely due to the formation of acceptor-acceptor structure by the ester linkage and nitro stilbene substituent as it has been reported that ester linkage tends to reduce the intramolecular charge transfer or the electron density delocalization in nitro-stilbene derivatives compounds and thus quench the fluorescence together with its electron-withdrawing effect [9]. This explains the “flat” fluorescence decay curve obtained under Ex 321 nm/Ex 520 nm in which the fluorescence intensity is too weak to make the decay observable. This fluorescence intensity quenching may also be the reason for the fluorescence decay curve obtained under Ex 362 nm/Em 520 nm in 90% water and DMSO (**Figure 4.17 D**). Thus, the ester linker in conjugate **6** electronically decouples the stilbene and MG fragments, but the nitro group quenches the fluorescence

intensity, resulting in non-observable decay. Hence nitro group shall be modified to a donor group to restore the intensity and decay.

The binding affinity of conjugate **6** to MG aptamer was estimated by project team member Y. Zhou and the results are shown in **Figure 4.18**. Unlike the previous binding curves obtained from direct binding, competitive binding involving the competition between MG and conjugate **6** towards MG aptamer was conducted. The concentrations of MG and MG aptamer were kept constant and that of conjugate **6** increases. With the increasing amount of conjugate **6**, MG aptamer adaptively binds to **6** and releases MG analyte, resulting in fluorescence intensity decrease. The binding dissociation constant K_d is $6.6\ \mu\text{M}$, 30 times greater than that of MG analyte ($200\ \text{nM}$). So the binding affinity of conjugate **6** to MG aptamer is very weak and conjugate **6** can't be used in PHASA. In MG binding to MG aptamer, both steric configuration and electronic interactions contribute to the binding pocket formation [7, 10, 11]. Compared to MG analyte, conjugate **6** with the addition of stilbene fragment to MG is expected to sterically interrupt the binding affinity, but its 30 times greater K_d value than MG should also be caused by the disruption in electronic interactions. Since there is no electronic coupling with the stilbene fragment, the ester linker should have played a role in interfering the electronic distribution of MG fragment in conjugate **6**. In the binding experiment of MG aptamer with MG NHS ester (**Figure 4.19**) having the ester linkage as well, no binding was observed. So the conclusion that ester linkage interferes the electronic distribution of MG fragment in **6** and results in weak binding is further supported.

Figure 4.18. The competitive binding results of MG-COO-Stilb-NO₂ (**6**) and the binding dissociation constant K_d .

Figure 4.19 proposes the possible electronic distributions in conjugate **6** due to the ester linkage. There is partial charge separation between the third phenyl ring of MG fragment and the ester linkage. This electronic distribution profile is significantly different from that reported in Nguyen's work [10]. The charge distribution difference might result in weakened electronic interactions and weak binding to MG aptamer. Hence, a link that does not interact with MG fragment should be introduced. Combining with the conclusion from conjugate **6**'s decay kinetics study that donor stilbene substituents are preferred, MG-CH₂-NH-Stilb-OMe conjugate **7** was studied.

Figure 4.19. Structures of conjugate **6** with different electronic distributions due to the ester link.

4.3.7 MG-CH₂-NH-Stilb-OMe conjugate **7** (Figure 4.1) – obvious fluorescence decay but binding is not strong

Based on the reactivity between the carboxyl group of *leuco* MG-COOH (**3**) and the amino group of MAS (**2**), *leuco* MG-CONH-Stilb-OMe (**11**) was obtained. After reduction of the amide linker, conjugate **11** was converted to *leuco* MG-CH₂-NH-Stilb-OMe (**12**), from which MG-CH₂-NH-Stilb-OMe (**7**) was generated after oxidation. However, NMR results show that complete oxidation of **12** is difficult. Although conjugate **7** is synthesized in the oxidation mixture but its *leuco* form (**12**) still remains as a minor component. Through the introduction of -CH₂-NH- linker, it is expected that the stilbene and MG components are electronically decoupled, the interruptions to the electronic distributions of the MG fragment are reduced, and the obtained conjugate **7** will have both fluorescence decay and

binding affinity to MG aptamer. The oxidation mixture containing both conjugate **7** and its *leuco* form **12** was used for characterization.

Figure 4.20 **A.** The absorption spectrum of the oxidation mixture in DMSO (**12** and **7**) with time. **B.** The emission spectra under the excitation of four absorption peaks. The fluorescence decay curves under Ex of 324/356 nm and Em of 432 nm (**C**) and other Ex and Em combinations (**D**).

Before the photophysical characterization of the oxidation mixture, its stability was checked by recording its absorption spectrum with time, shown in **Figure 4.20 A**. Within 80 mins, there is no significant change of absorbance values. Comparing with conjugate **6** where there is more than 30 % absorbance decrease after 10 mins (**Figure 4.17 A**), this oxidation mixture is more stable. This might be due to the presence of the *leuco* form (**12**) in the fresh mixture solution so that the conversion between **12** and **7** has reached the equilibrium. Under the excitation of the four absorption peaks (324, 356, 440 and 634 nm) from the absorption spectrum, the emission spectra (**Figure 4.20 B**) were measured. Two categories of emission behaviour are observed. Under the excitation of the shorter absorption peaks (324 and 356 nm), ~100 fluorescence emission intensity units (under the

same measurement conditions) are observed. However, the emission intensities under the excitation of the two longer absorption peaks (440 and 634 nm) are approximately 100 times (~ 1 fluorescence intensity unit) lower. This is because the 440 and 634 nm peaks characterize the MG fragment which is non-fluorescence in free solution, and they cannot excite the stilbene fragment. Based on the four absorption peaks and their respective emission peaks, the fluorescence decay curves of the oxidation mixture under each Ex/Em were measured and summarized in **Figure 4.20 C and D**.

When the fluorescence decay was measured under the excitation of the two longer peaks (440 and 634 nm), there is no obvious decay and the fluorescence intensity is less than 5 units. It is expected as the two longer peaks come from the MG component of the conjugate **7** in the oxidation mixture. When the two shorter absorption peaks (324 and 356 nm) were utilized for excitation, fluorescence decay curves with obvious decay and high intensity (more than 200 fluorescence emission intensity units) were generated. Hence, for PHASA whose detection depends on fluorescence decay rate change, the decay curve of stilbene-MG conjugates should be recorded based on its stilbene component's property.

As to the two short absorption peaks (324 and 356 nm) of the oxidation mixture, 324 nm is suspected to be attributed to the conjugate **7** and 356 nm to its *leuco* form **12** from the following aspect. In **Figure 4.20 C and D**, the fluorescence decay rate constants under excitation of 324 nm ($k_{app} = 0.512 \pm 0.003 \text{ s}^{-1}$; $0.478 \pm 0.03 \text{ s}^{-1}$) are always lower than that under 356 nm ($k_{app} = 0.709 \pm 0.003 \text{ s}^{-1}$; $0.659 \pm 0.03 \text{ s}^{-1}$ correspondingly). Based on the conclusion from section 4.3.5 that stilbene component is better electronically decoupled and suffers less fluorescence decay quenching in the conjugate's *leuco* form than the conjugate itself, the decay rate constants under the excitation of 356 nm decreases $\sim 20\%$ and $\sim 42\%$ under 324 nm compared to the decay rate constant of parent stilbene **2** in DMSO ($k_{app} = 0.829 \pm 0.021 \text{ s}^{-1}$; **Table 4.1**). Moreover, the absorption peak 356 nm is closer to 350 nm which is the absorption peak of **2** in DMSO (**Table 4.1**). To be 100% sure, the fluorescence decay kinetics of the oxidation mixture in aqueous solution mixtures under both conditions, that is under the excitation wavelength of 324 nm and emission wavelength of 432 nm (Ex 324 nm/Em 432 nm) and the excitation and emission of 356 nm and 432 nm (Ex 356 nm/Em 432 nm), were measured to check the quenching effect.

Figure 4.21. The fluorescence decay curves (A, B), normalized decay (C, D) of the oxidation mixture in four water and DMSO mixtures and the relationship between the apparent decay rate and the water percentage (E, F) under Ex 324 nm/Em 432 nm and Ex 356 nm/Em 432 nm respectively.

Figure 4.21 summarizes the recorded decay curves in four water (80, 90, 95 and 98%) and DMSO solution mixtures under Ex 324 nm/Em 432 nm and Ex 356 nm/Em 432 nm. The normalized decay curves and the plots of decay rate constant vs. water percentage are also

presented. Under both decay measurement conditions, the fluorescence decay of the oxidation mixture is preserved, and the fluorescence intensity is quenched to be less than 30 emission intensity units. Under Ex 324 nm/Em 432 nm, the decay rate constant shows no dependence on water percentage in the four studied water and DMSO mixtures, but under Ex 356 nm/Em 432 nm, the decay rate constant decreases with increasing water percentage.

Also, the same decay experiments of the oxidation mixture were conducted in four PBS buffer (80, 90, 95 and 98%) and DMSO mixtures. The normalized fluorescence decay curves and the decay rate constant vs. buffer percentage plots under both Ex 324 nm/Em 432 nm and Ex 356 nm/Em 432 nm are shown in **Figure 4.22**. The results of decay rate constants as well as the relationships between decay rate and buffer percentage are similar to those obtained in the water and DMSO mixtures.

Figure 4.22. The normalized fluorescence decay curves (**A**, **B**) of the oxidation mixture in four PBS buffer and DMSO mixtures. The relationship between its decay rate constant and the buffer percentage (**C**, **D**) under Ex 324 nm/Em 432 nm and Ex 356 nm/Em 432 nm respectively.

In both water/DMSO and buffer/DMSO aqueous mixtures (v% of DMSO is not greater than 20%), the fluorescence decay of the oxidation mixture (containing **12** and **7**) is not quenched under both Ex 324 nm/Em 432 nm and Ex 356 nm/Em 432 nm conditions. Hence, it is proven that conjugate **7** has obvious fluorescence decay and the decay is preserved in aqueous mixtures. This makes the detection based on **7**'s decay rate change in MG aptamer binding buffer environment possible.

Since the fluorescence decay of conjugate **7** in buffer/DMSO mixtures is confirmed, its binding affinity to MG aptamer is studied by project team member Y. Zhou based on its competitive binding with MG towards MG aptamer. The results are summarized in **Figure 4.23**. The obtained binding dissociation constant K_d for conjugate **7** is 2.4 μM which is 12 times greater than MG analyte (200 nM). However, compared to conjugate **6** ($K_d = 6.6 \mu\text{M}$), the binding affinity of conjugate **7** has been increased ~ 3 times. This indicates that the $-\text{CH}_2\text{-NH}-$ linker in conjugate **7** does not initiate partial charge separation and reduces interruption to electronic distribution of MG fragment. Since the binding affinity of conjugate **7** ($K_d = 2.4 \mu\text{M}$) is still more than 10 times greater than the MG analyte, further enhancement in its binding to MG aptamer may be possible.

Figure 4.23. The competitive binding experimental results and binding dissociation constant K_d of conjugate **7**.

Compared to malachite green, crystal violet (**Figure 3.1**) has an additional dimethylamino group on the third phenyl ring. Because of this dimethylamino group, crystal violet has no binding affinity to MG aptamer. In Baugh et al's work [7], they explained that some of the positive charge localized to the two amine groups in MG is distributed to the third amine group in crystal violet. This reduces the electronic interactions between the amino and the

backbone phosphate groups. Since the $-\text{CH}_2\text{-NH}-$ linker in conjugate **7** also has the ability to share positive charges, it is proposed that decreasing the linker's electron-donating property by acetylation can produce a stilbene-MG conjugate with stronger binding affinity.

4.3.8 MG- $\text{CH}_2\text{-Nac-Stilb-OMe}$ conjugate **8** (Figure 4.1) – obvious decay and strong binding affinity (the most promising candidate)

To decrease the electron-donating property of the $-\text{CH}_2\text{-NH}-$ linker in conjugate **7** to reduce the impact on charge distributions on MG fragment, the linker was acetylated and stilbene-MG conjugate MG- $\text{CH}_2\text{-Nac-Stilb-OMe}$ **8** was synthesized.

Figure 4.24 **A.** The absorption spectra of conjugate **8** with time. **B.** The emission spectra under the excitation of absorption peaks. The fluorescence decay curves under Ex 327 nm/ Em 389 nm (**C**) and other Ex/Em combinations (**D**) in DMSO.

Similarly, the stability of conjugate **8** was firstly checked by measuring its absorption spectrum in DMSO with time. From the results shown in **Figure 4.24 A**, within 80 mins, there is no significant decrease of the absorbance values within 80 mins, and 20% decrease

was observed after 5 hours. Hence, conjugate **8** is stable compared to the stilbene-MG conjugate **5** and **6**.

Three absorption peaks (327, 441 and 634 nm) displayed in **Figure 4.24 A** were used as excitation wavelengths for the emission spectrum measurement. The obtained emission spectra are presented in **Figure 4.24 B**. Like that observed in conjugate **7** (**Figure 4.20 B**), there are two categories: under the excitation of the two longer absorption peaks (441 and 634 nm), the fluorescence emission intensities are ~1.0 unit, less than 0.1% of the parent stilbene **2** under the same measurement conditions, preventing their use in bio-sensing. Under the excitation of 327 nm, the emission peak intensity is enhanced more than 30 times (~60 fluorescence intensity unit) compared to that under 441 nm. The obtained fluorescence intensities under the excitation of the two longer absorption peaks (441 and 634 nm) is due to the MG fragment, which has low quantum yield under non-planar conditions. The major emission peak from excitation at 327 nm matches the parent stilbene **2**, indicating the -CH₂-NAC- linker has achieved electronic decoupling between MG and stilbene fragments.

The fluorescence decay curves of conjugate **8** are listed in **Figure 4.24 C** and **D**. When the conjugate **8** is excited under 441 nm or 634 nm, no decay was observed and fluorescent intensity was less than 0.1% of parent stilbene **2**. Decay observed under excitation at 327 nm demonstrated additional evidence of stilbene decay isolated away from MG. Excitation at 327 nm and emission at 389 nm were carried forth for aqueous decay validation.

Figure 4.25 A and **B** summarize the fluorescence decay curves and the normalized decay of **8** in five water (80, 85, 90, 95 and 98%) and DMSO solution mixtures respectively. Compared with the fluorescence intensity in DMSO (**Figure 4.24 C**), the conjugate has 10 times lower fluorescence intensities in all the five studied solution mixtures due to the quenching effect from the aqueous environment. With higher water percentage, the intensity is lower. However, all the measured fluorescence decay curves have measurable decay rates. Presented in **Figure 4.25 C**, the plot of the apparent decay rate constant k_{app} vs. the water percentage shows that there is no clear dependence of decay rate constant on water percentage.

Figure 4.25 A. Fluorescence decay curves of conjugate 8. B. The normalized decay of conjugate 8 under Ex 327 nm/Em 389 nm in five water/DMSO mixtures. C. The relationship between 8's decay rate constant and water percentage.

Figure 4.26 A. The normalized fluorescence decay curves of 8 in four PBS buffer and DMSO solution mixtures under Ex 327 nm/Em 389 nm. B. The relationship between conjugate 8's decay rate constant and the buffer percentage.

Besides water and DMSO mixtures, the fluorescence decay kinetics of conjugate **8** were also studied in four PBS buffer (80, 90, 95 and 98%) and DMSO solution mixtures. The obtained decay curves as well as the decay rate constants are shown in **Figure 4.26**. Similar to the results obtained in water/DMSO mixtures, there is no significant difference among the decay rate constants in the four studied buffer and DMSO aqueous mixtures. The preservation of conjugate **8**'s fluorescence decay in both studied aqueous mixtures (water/DMSO and buffer/DMSO) confirms its applicability in MG aptamer binding buffer environment. With no clear dependence on organic solvent percentage (as measure from 3~20%), its flexibility is extended towards broader assay development.

As the fluorescence decay of conjugate **8** in buffer/DMSO aqueous mixtures has been confirmed, its binding was assessed by project team member Y. Zhou. The results are presented in **Figure 4.27**. With increasing ratios of MG-CH₂-NAc-Stilb-OMe conjugate **8** to MG aptamer, the MG's fluorescence intensity was non-linearly enhanced. The obtained binding dissociation constant K_d of conjugate **8** (MG-CH₂-NAc-Stilb-OMe) is 1.7 μ M. It is 30% lower than that of conjugate **7** (2.4 μ M; MG-CH₂-NH-Stilb-OMe), and three times higher than that of conjugate **5** ("fused" stilbene-MG; 500 nM). Therefore, after acetylation of the -CH₂-NH- linker in conjugate **7**, the obtained conjugate **8** with the -CH₂-NAc- linker has stronger binding affinity to MG aptamer. This makes **8** more competitive than **7** in final binding experiment. Together with the preserved fluorescence decay kinetics of **8** in aqueous environment, conjugate **8** is a better candidate than **7** for competitive binding scope of PHASA.

Figure 4.27. Direct binding results of conjugate **8** to MG aptamer and its binding dissociation constant K_d .

In PHASA competitive binding scope (**Figure 1.2**), the synthesis of stilbene-MG conjugates with both fluorescence decay kinetics and binding affinity to MG aptamer is the focus, as mentioned in the introduction. Among the four stilbene-MG conjugates studied in this chapter, conjugate **8** is the most promising candidate. In order to preserve the decay kinetics of stilbene fragment, a linker between the MG and stilbene fragment is needed. This makes minimal steric interruption of conjugate (conjugate **5**) to its binding to MG aptamer not applicable. Moreover, to minimize the disruption to the electronic distribution of conjugate's MG fragment, the linker should neither cause partial charge separation with MG fragment's third phenyl ring nor have strong electron-donating ability to share the positive charges localized to the other rings. Therefore, unreactive linkers that have very weak electron-donating property or have little or no electronic interactions with MG fragment are preferred for further synthesis of stilbene-MG conjugates for PHASA competitive binding.

4.3.9 Rationale for the analyte detection range of PHASA

Based on the competitive binding format in PHASA, the binding process is influenced by various parameters such as the initial concentration of MG-stilbene conjugate, the ratio of MG aptamer to MG-stilbene conjugate, the binding affinity of MG-stilbene conjugate to the aptamer and the decay property of the MG-stilbene conjugate. To have a clear understanding of their correlations in determining the detection sensitivity of the analyte, their relationships are thus mathematically analysed.

Before the addition of analyte MG, the MG aptamer-MG stilbene conjugate complex which is denoted as $MGA \bullet MGS$ is formed based on the reaction,

Where MGA and MGS refer to the MG aptamer and MG stilbene conjugate compound respectively.

$$\text{Thus, } K_{d(MGS)} = \frac{[MGA]_F * [MGS]_F}{[MGA \bullet MGS]} \quad (4.2)$$

Where $[MGA]_F$ and $[MGS]_F$ represent the free or unbound state of MG aptamer and MG stilbene conjugate compound in the solution respectively.

Based on the law of mass conservation,

$$[MGA]_T = [MGA]_F + [MGA \bullet MGS] \quad (4.3)$$

$$[MGS]_T = [MGS]_F + [MGA \bullet MGS] \quad (4.4)$$

Where $[MGA]_T$ and $[MGS]_T$ represent the total amount of MG aptamer and MG stilbene conjugate compound in the solution respectively. Set the binding ratio of MG-stilbene

$$\text{conjugate to MG aptamer } \frac{[MGA \bullet MGS]}{[MGS]_T} = B \quad (4.5)$$

Set the ratio of total amount of MG-aptamer to MG stilbene conjugate

$$\frac{[MGA]_T}{[MGS]_T} = R \quad (4.6)$$

Then, combining equations 4.3, 4.4 and 4.2,

$$K_{d(MGS)} = \frac{([MGA]_T - [MGA \bullet MGS]) * ([MGS]_T - [MGA \bullet MGS])}{[MGA \bullet MGS]} \quad (4.7)$$

Substitute $[MGA]_T$ and $[MGA \bullet MGS]$ in equation 4.7 with $[MGS]_T$ based on equation 4.5 and 4.6, respectively,

$$K_{d(MGS)} = \frac{(R * [MGS]_T - B * [MGS]_T) * ([MGS]_T - B * [MGS]_T)}{B * [MGS]_T} = \frac{(R - B) * (1 - B) * [MGS]_T}{B}$$

$$\text{So } B^2 - (R + 1 + \frac{K_{d(MGS)}}{[MGS]_T}) * B + R = 0 \quad (4.8)$$

Based on equation 4.8 and the fact that the range of the binding ratio value B is 0~1, so

$$B = 0.5 * (\frac{K_{d(MGS)}}{[MGS]_T} + R + 1 - \sqrt{(\frac{K_{d(MGS)}}{[MGS]_T} + R + 1)^2 - 4R}) \quad (4.9)$$

By fixing the binding affinity $K_{d(MGS)}$ or the total amount of MG-stilbene conjugate, the relationship of B vs. R can be generated under different conditions as shown in **Figure 4.28**. Both graphs show that when the aptamer to MG-stilbene conjugate ratio R increases, the binding ratio value B increases. Higher binding ratio B is also achieved under conditions of higher binding affinity $K_{d(MGS)}$ and higher MG-stilbene conjugate $[MGS]_T$, as expected.

Figure 4.28 **A.** The binding ratio of MG-stilbene conjugate verse $[MGA]_T/[MGS]_T$ ratio at different $[MGS]_T$ amount when the binding affinity $K_{d(MGS)}$ is fixed at 1.7 μM. **B.** The relationship between the binding ratio of MG-stilbene conjugate and $[MGA]_T/[MGS]_T$ ratio at different binding affinity $K_{d(MGS)}$ when the $[MGS]_T$ amount is fixed at 5 μM.

After the MG aptamer MG-stilbene conjugate complex is formed, MG analytes are added and lead to change in fluorescence decay of the MG-stilbene conjugate, based on which the MG analyte detection is achieved. The process involves the competition of MG analyte and MG-stilbene conjugate for the MG aptamer binding pocket and can be described as

$$\text{For reaction 4.10, } K_{d(MGS)} = \frac{[MGA]_F * [MGS]_F}{[MGA \bullet MGS]} \quad (4.12)$$

$$\text{For reaction 4.11, } K_{d(MG)} = \frac{[MGA]_F * [MG]_F}{[MGA \bullet MG]} \quad (4.13)$$

$$\text{Based on the mass conservation law, } [MG]_T = [MG]_F + [MGA \bullet MG] \quad (4.14)$$

$$[MGA]_T = [MGA]_F + [MGA \bullet MG] + [MGA \bullet MGS] \quad (4.15)$$

$$[MGS]_T = [MGS]_F + [MGA \bullet MGS] \quad (4.16)$$

Combining the above five equations from 4.12 to 4.16, the relation of the total amount of MG analyte added ($[MG]_T$) as a function of the unbound MG-stilbene conjugate ($[MGS]_F$) is expressed in equation 4.17. The corresponding curves can also be plotted under specific experimental parameters ($[MGS]_T$; $[MGA]_T$; $K_{d(MG)}$ and $K_{d(MGS)}$), taking into account that $[MG]_T \geq 0$ and $[MGS]_F < [MGS]_T$.

$$[MG]_T = \frac{1}{K_{d(MGS)}} * \left(\frac{[MGA]_T}{[MGS]_T - [MGS]_F} - \frac{K_{d(MGS)}}{[MGS]_F} - 1 \right) * [K_{d(MGS)} * ([MGS]_T - [MGS]_F) + [MGS]_F * K_{d(MG)}] \quad (4.17)$$

From **Figure 4.29 A**, it can be seen that when $[MGA]_T$ and $[MGS]_T$ are fixed, the amount of unbound MG-stilbene conjugate $[MGS]_F$ (the interception with the x axis) is lower with high binding affinity $K_{d(MGS)}$ which promotes the binding event. The same observation can be generated as $[MGA]_T$ is increased as shown in **Figure 4.29 B**. These correspond to the curves of MG-stilbene conjugate binding ratio verse $[MGA]_T/[MGS]_T$ ratio in **Figure 4.28**.

Figure 4.29 A. The relationship between the total amount of MG added $[MG]_T$ and the amount of MGS unbound $[MGS]_F$ at different $K_{d(MGS)}$ values when the $[MGA]_T = 10 \mu M$ and $[MGS]_T = 5 \mu M$. **B.** The correlation between the total amount of MG added $[MG]_T$ and the amount of MGS unbound $[MGS]_F$ at different concentrations of $[MGA]_T$ and $[MGS]_T$ while the binding affinity is fixed at $1.7 \mu M$.

At lower initial MG-stilbene binding ratios (high $[MGS]_F$), the sensitivity is higher especially at the starting point, as seen in **Figure 4.29**. In other words, for the same amount of MG analyte added, greater amounts of $[MGS]_F$ can be released, resulting in larger extent of fluorescence decay rate change. This is reasonable since stronger pre-binding of MG-stilbene conjugate to the MG aptamer makes the subsequent release more difficult, thus higher amount of MG is required. This is at the cost of a decreased detection range since the amount of MG-stilbene conjugate can be released is restricted. This situation may be improved by enhance $[MGS]_T$ (the magenta line in **Figure 4.29 B**), but the amount of MGA needs to be increased also, which make the assay more expensive. Hence, all the parameters ($[MGS]_T$, $[MGA]_T$ and $K_{d(MGS)}$) need to be properly selected, which in turn makes it

possible of designing the right MG-stilbene conjugate and choosing respective $[MGS]_T$ and $[MGA]_T$ for the desired applications.

As mentioned above, the fluorescence decay behaviour of the unbound MG-stilbene conjugates $[MGS]_F$ is a major factor in the determination of the MG analyte detection range and limit. To simplify the deduction process, it is assumed that the decay curve is “flat” under complete bound conditions (binding percentage $B \geq 0.95$) as shown in **Figure 4.30** and fluorescence intensity can be utilized to roughly represent the fluorescence decay rate and thus the binding environment. A signal to noise ratio of four ($S/N = 4$) is assumed as the lowest detectable change in decay rate. Then, the minimum percentage (%) of unbound MG-stilbene conjugate ($[MGS]_F$) required can be estimated as,

$$\%([MGS]_F) = \frac{4 * N}{1 - FLU_1} * 100\% = \frac{4 * N}{\Delta FLU} * 100\% \quad (4.18)$$

Where FLU_1 is the normalized fluorescence intensity of MG-stilbene conjugate at t (sec) when measured in free solutions (aqueous buffer environment with no presence of MG aptamer) and N is the noise ratio. When the noise ratio N is small and the FLU_1 value close to 0, the minimum percentage of $[MGS]_F$ is lower, as displayed in **Figure 4.30**. This means when other parameters are the same, a lower $[MG]_T$ amount is needed to generate a detectable decay signal, indicating a lower MG limit of detection.

Figure 4.30 A. The illustration of MG-stilbene conjugate's fluorescence decay curves under different conditions. B. Minimum percentage of $[MGS]_F$ for a detectable decay signal verse noise ratio N under conditions of different ΔFLU .

To exemplify the above deduction, the MG analyte detection based on the binding and decay behaviour of MG-stilbene conjugate **8** is shown. From the normalized fluorescence

decay curve of conjugate **8** in 90% PBS buffer and DMSO (the smoothed decay curve is shown in **Figure 4.26 A**), its FLU_1 value at $t = 5$ sec is 0.40 and the noise ratio N is estimated to be 0.08. According to equation 4.18, the minimum percentage (%) of $[MGS]_F$

is calculated as $\%([MGS]_F) = \frac{4 * N}{1 - FLU_1} * 100\% = \frac{0.32}{0.6} * 100\% = 53.3\%$ That is the

binding percentage of MG-stilbene conjugate needs to be less than 46.7 % in order for the decay behaviour to be distinguished from the background. The concentration used for the decay measurement of conjugate **8** is $5 \mu\text{mol L}^{-1}$. Since its binding affinity has been determined to be $1.7 \mu\text{M}$, based on equation 4.9 and **Figure 4.28**, the MGS binding percentage is 56.3% if the $[MGA]_T$ to $[MGS]_T$ ratio is 1. That is, according to equation 4.17, the minimum amount of MG analyte can be detected will be $1.33 \mu\text{mol L}^{-1}$. When the aqueous environment is changed to 95% PBS buffer and DMSO, ΔFLU is smaller, leading to even higher $[MGS]_F$ required and thus higher limit of detection of MG analyte. Therefore, for the best candidate conjugate **8**, its fluorescence decay property in aqueous environment needs to be further improved to achieve lower noise N and higher ΔFLU and thus lower percentage of $[MGS]_F$ for decay measurement.

4.4 Conclusions

Under competitive binding scope, two reactant stilbenes (DMAAS and MAS) with strong donor groups were selected. Their photophysical characterization results confirm their high quantum yields and fast fluorescence decay, and the medium viscosity studies show a linear dependence of their fluorescence decay rate constant on the reciprocal of their surrounding medium viscosity (2.7~215.8 cP) in log scale. Therefore, they are good reactant stilbenes for PHASA.

For PHASA competitive binding scope, four stilbene-MG conjugates and a negative control–rhodamine B-DMAAS conjugate were synthesized. Their fluorescence decay kinetics and binding affinity towards MG aptamer were studied in context as a model biosensor for MG detection, as summarized in **Table 4.3**.

Table 4.3. Fluorescence decay and binding results summary for four stilbene-MG conjugates and a negative control.

Compound	Structure	Fluorescence decay in aqueous environment	Dissociation constant K_d	Remarks
4		Yes	$K_d > 1 \text{ mM}$ No binding	Negative control
5		No	$K_d = 500 \text{ nM}$	Highest binding affinity
6		No	$K_d = 6.6 \text{ }\mu\text{M}$	Lowest binding affinity
7		Yes	$K_d = 2.4 \text{ }\mu\text{M}$	Not pure
8		Yes	$K_d = 1.7 \text{ }\mu\text{M}$	The best candidate

There is no binding of the negative control **4** towards MG aptamer, so stilbene conjugation to MG analyte does not cause any binding and MG aptamer binding pocket formation is solely due to MG fragment. The K_d value of conjugate **5** is 500 nM, 2.5 times higher than that of MG analyte (200 nM). Conjugate **5** has the highest binding affinity to MG aptamer among the four studied stilbene-MG conjugates. However, no measurable fluorescence decay in several organic solvents are observed, due to the electronic conjugation which prevent the decay process. So **5** can't be used in PHASA whose detection is based on decay rate change. So a linker was introduced to electrically decouple the two components. With the -COO- linker, highest dissociation constant (K_d is 6.6 μM) was obtained in conjugate **6**, as the ester can cause partial charge separation with the third phenyl ring of MG fragment.

After reduction, the obtained conjugate **7** with the -CH₂-NH- linker has preserved decay kinetics and its K_d (2.4 μM) decreases ~3 times than conjugate **6**. After further acetylation to reduce the linker's electron-donating property, the conjugate **8** with -CH₂-NAc- linker has a smaller K_d of 1.7 μM. Together with its retained decay kinetics in binding buffer aqueous environment, conjugate **8** is the most promising candidate for PHASA competitive binding scope. For further synthesis of more promising stilbene-MG conjugates, a linker is essential to preserve the decay kinetics of the stilbene fragment. Moreover, the linker should be unreactive and does not interact with conjugate's MG fragment to get a strong binding affinity with MG aptamer.

References

- [1] V. Papper, G.I. Likhtenshtein, *Journal of Photochemistry and Photobiology A: Chemistry*, 140 (2001) 39-52.
- [2] V. Papper, D. Pines, G.I. Likhtenshtein, E. Pines, *Journal of Photochemistry and Photobiology A: Chemistry*, 111 (1997) 87-96.
- [3] P.P.-B. Arye, N. Strashnikova, G.I. Likhtenshtein, *Journal of Biochemical and Biophysical Methods*, 51 (2002) 1-15.
- [4] G.I. Likhtenshtein, R. Bishara, V. Papper, B. Uzan, I. Fishov, D. Gill, A. Parola, *Journal of Biochemical and Biophysical Methods*, 33 (1996) 117-133.
- [5] N. Strashnikova, V. Papper, P. Parkhomyuk, G.I. Likhtenshtein, V. Ratner, R. Marks, *Journal of Photochemistry and Photobiology A: Chemistry*, 122 (1999) 133-142.
- [6] Y. Zhou, H. Chi, Y. Wu, R.S. Marks, T.W.J. Steele, *Talanta*, 160 (2016) 172-182.
- [7] C. Baugh, D. Grate, C. Wilson, *Journal of Molecular Biology*, 301 (2000) 117-128.
- [8] J.R. Babendure, S.R. Adams, R.Y. Tsien, *Journal of the American Chemical Society*, 125 (2003) 14716-14717.
- [9] F. Gao, L. Yang, L. Yang, H. Li, S. Zhang, *Journal of Fluorescence*, 20 (2010) 353-364.
- [10] D.H. Nguyen, S.C. DeFina, W.H. Fink, T. Dieckmann, *Journal of the American Chemical Society*, 124 (2002) 15081-15084.
- [11] J. Flinders, S.C. DeFina, D.M. Brackett, C. Baugh, C. Wilson, T. Dieckmann, *ChemBioChem*, 5 (2004) 62-72.

Chapter 5*

Fluorescence decay study of stilbene maleimide and stilbene maleamic acid compounds

This chapter focus on adaptive binding scope, as described in chapter 1. Based on the click chemistry reaction between thiol-labelled aptamers and maleimides, two stilbene maleimide conjugates (DMASMI and MSMI) were designed. Their fluorescence decay rates show strong dependence on their surrounding medium viscosities and linear relationships between the decay rate constant and the reciprocal of viscosity were observed. Before the conjugation with thiol modified MG aptamer, a model reaction between MSMI and a small thiol molecule 6MCH was conducted. The obtained MSMI-6MCH conjugate confirms the feasibility of stilbene maleimide conjugation to thiol modified MG aptamer. Also, this conjugate restores the fluorescence decay of the stilbene maleimide (MSMI) whose decay is quenched. To improve stilbene compounds' water solubility and reduce maleimide-mediated quenching, three stilbene maleamic acids (DMASMA, MSMA and HSMA) were synthesized. Their fluorescence decay in ACN, DMSO and EtOH and their aqueous solution mixtures are confirmed. Reaction monitoring between stilbene maleamic acids and the small thiol molecule 6MCH indicates the conjugation.

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5.1 Introduction

Under adaptive binding scope (**Figure 1.4**), the fluorescence decay rate of stilbene compounds is dependent on the aptamer folding. In this regard, attachment of stilbene compounds to MG aptamer is required within the design of the stilbene conjugate. Since the click reaction between maleimide and thiol molecules (**Figure 5.1**) has been extensively studied [1-3] and aptamers can be readily modified with thiols, maleimide groups are selected as the cross-linker. Thus, MG aptamer was thiol modified and stilbene maleimide compounds were synthesized from the two parent stilbenes **1** and **2** (**Figure 4.1**). **Figure 5.2** shows the structure of the two designs of stilbene maleimides conjugates; DMASMI (**15**) and MSMI (**16**). To improve stilbene compounds' water solubility, three stilbene maleamic acids were also synthesized and their structures are summarized in **Figure 5.2**. Besides DMASMA (**17**) and MSMA (**18**), HSMA (**19**) having hydroxyl stilbene substituent is also included. Before the reaction of the stilbene maleimide or maleamic acid compounds to thiol modified MG aptamer, their conjugation to a small thiol molecule 6-Mercapto-1-hexanol (6MCH) was first tested to explore the optimum reaction conditions.

Figure 5.1. Click reaction chemistry between thiol molecules and maleimides.

Figure 5.2. Structures of the studied stilbene maleimides and maleamic acids.

5.2 Experimental section

5.2.1 Materials

Chemical reagents for the synthesis of stilbene maleimide and maleamic acid compounds such as maleic anhydride, acetic anhydride and anhydrous sodium acetate were purchased from Sigma, Singapore. Two parent stilbenes: 4-dimethylamino-4'-aminostilbene (**1**) and 4-methoxy-4'-aminostilbene (**2**) and 4-hydroxy-4'-aminostilbene were ordered from TCI, Japan. Organic solvents like DCM (dichloromethane), DMF (dimethylformamide) and isopropanol were purchased from Sigma. 6-Mercapto-1-hexanol (6MCH), triethylamine and methanol for the synthesis of MSMI-6MCH conjugate were also purchased from Sigma. Four MG RNA aptamers modified with C₆SH label (5' end of C38, A9 and C20 and 3' end of U4) were purchased from AITbiotech, Singapore. TCEP (tris(2-carboxyethyl)phosphine) used to activate the thiol molecules was ordered from Sigma. All the organic solvents used for spectroscopy measurements such as acetonitrile, ethanol, dimethyl sulfoxide and propylene carbonate were purchased in HPLC grade from Sigma Aldrich, Singapore. Glycerol in the medium viscosity study was purchased from Fisher Scientific, Singapore. The PBS buffer for MG aptamer binding was prepared from 5.65 mmol L⁻¹ NaH₂PO₄ (Sigma), 4.35 mmol L⁻¹ Na₂HPO₄ (Sigma), and 10 mmol L⁻¹ MgCl₂ (Sigma) and 1 mmol L⁻¹ NaCl unless otherwise stated. Deionised (DI) water was supplied by Milli-Q® water system.

5.2.2 Synthesis of stilbene maleimides (**15**, **16**) and maleamic acids (**17**, **18** and **19**)

Maleamic acid is the intermediate during the synthesis of maleimide. That is, both stilbene maleimide and its corresponding stilbene maleamic acid can be obtained in the synthesis process of the stilbene maleimide.

Figure 5.3. Reaction pathway for the synthesis of stilbene maleimide and stilbene maleamic acid compounds.

As shown in the reaction pathway in **Figure 5.3**, the stilbene maleamic acid was obtained

first by reacting maleic anhydride with the amine group of the parent stilbene (**1** and **2**). Then stilbene maleimide was generated through cyclisation of the obtained stilbene maleamic acid by dehydration with acetic anhydride and sodium acetate as described in the literature [4, 5].

*Synthesis of stilbene maleimide DMASMI **15** & stilbene maleamic acid DMASMA **17***

Maleic anhydride (4.34 g; 44.3 mmol) and 50 mL of DCM were introduced into a round-bottom flask equipped with a condenser and a heated magnetic stirrer. After dissolution, **1** (7.95 g; 33.3 mmol) was added and the mixture was left stirred at room temperature for 4 hours. Crude dark red precipitate was collected by vacuum filtration, washed with DCM and recrystallized from boiling DMF. Upon recrystallization, it was collected, washed with DI water and DCM and then vacuum dried. 9.61 g (28.6 mmol) of compound **17** was obtained with 85.7% yield. ¹H NMR, 400 MHz, DMSO-d₆: 2.93 (6H; s); 6.31-6.34 (1H; d; 12.13 Hz); 6.46-6.49 (1H; d; 12.06 Hz); 6.71-6.73 (2H; d; 8.65 Hz); 6.91-6.95 (1H; d; 16.48 Hz); 7.05-7.09 (1H; 16.36 Hz); 7.40-7.42 (2H; d; 8.65 Hz); 7.49-7.51 (2H; d; 8.46 Hz); 7.60-7.62 (2H; d; 8.46 Hz); 10.46 (1H; s); 13.17 (1H; broad s). ¹³C NMR, DMSO-d₆: 167.30; 163.49; 150.30; 137.55; 134.14; 131.97; 131.13; 128.28; 127.84; 126.72; 125.64; 123.62; 120.16; 112.80; 40.48. MALDI-TOF (*neat*, reflectron mode, positive) m/z 337.65 [M+H]⁺ (calculated 337.15).

The above obtained maleamic acid **17** (7 mmol) was mixed with 0.35 g of anhydrous sodium acetate and 10 mL of acetic anhydride. Upon stirring and heating at 100 °C, the suspension was dissolved and the resulting solution gradually changed color from dark red to dark yellow. 45 mins later, the reaction mixture was allowed to cool for 1 h and poured on ice. Stirring the mixture for 30 mins resulted in a separation of a dark red precipitate, which was collected by vacuum filtration, washed with DI water and vacuum dried. The product was purified with the flash chromatography on the silica gel with DCM eluent. Yield of the purified compound **15** is 71%. ¹H NMR, 400 MHz, DMSO-d₆: 2.95 (6H; s); 6.72-6.75 (2H; d; 8.91 Hz); 6.99-7.03 (1H; d; 16.42 Hz); 7.16-7.20 (1H; d; 16.29 Hz); 7.19 (2H; s); 7.28-7.30 (2H; d; 8.53 Hz); 7.44-7.47 (2H; d; 8.84 Hz); 7.62-7.64 (2H; d; 8.53 Hz). ¹³C NMR, DMSO-d₆: 112.67; 123.03; 125.17; 126.50; 127.32; 128.14; 130.12; 130.17; 135.16; 137.85; 150.58; 170.44. MALDI-TOF (*neat*, reflectron mode, positive) m/z 318.15

$[M+H]^+$ (calculated 319.14).

Synthesis of stilbene maleimide MSMI 16 & stilbene maleamic acid MSMA 18

Stilbene compound **2** (5 g; 22.2 mmol) was suspended in 100 mL DCM in a 250 mL beaker. It was gently heated to enhance dissolution. To the warm stirred dark yellow solution, maleic anhydride (3 g; 30.6 mmol) was added in portions. Dissolution of the maleic anhydride was observed followed by the solution color change to red. The mixture was left stirred to cool down to room temperature. Then red precipitates were collected by vacuum filtration and washed thoroughly with DCM. For further purification, the precipitates were dissolved in 100 mL hot DMF and the hot solution was filtered. 25 mL water was added to the clear filtrate while keeping the mixture hot and precipitate formation was noticed. After cooling, the precipitates were collected and washed with methanol (2X20 mL). Upon drying, 6.12 g (18.9 mmol; 85.3%) of red solid (**18**) was obtained. ^1H NMR, 400 MHz, DMSO- d_6 : 13.07 (1H; broad s); 10.48 (1H; s); 7.65-7.63 (2H; d; 8.65 Hz); 7.55-7.53 (2H; d; 8.61 Hz); 7.54-7.51 (2H; d; 8.72 Hz); 7.16-7.12 (1H; d; 16.44 Hz); 7.07-7.03 (1H; d; 16.39 Hz); 6.96-6.93 (2H; d; 8.75 Hz); 6.50-6.47 (1H; d; 12.08 Hz); 6.35-6.32 (1H; d; 12.03 Hz); 3.77 (3H; s). ^{13}C NMR, DMSO- d_6 : 167.34; 163.55; 159.33; 138.07; 133.60; 131.97; 131.09; 130.28; 128.10; 127.57; 127.10; 126.10; 120.12; 114.65; 55.61. MALDI-TOF (*neat*, reflectron mode, positive) m/z 324.54 $[M+H]^+$ (calculated 324.12).

Similar to the way **15** was obtained from **17** as described above, stilbene maleimide **16** was also generated through cyclizing the maleamic acid **18** by dehydration with acetic anhydride and sodium acetate. After purification with the flash chromatography, light lemon-yellow crystals were obtained. ^1H NMR, 400 MHz, DMSO- d_6 : 7.60-7.58 (2H; d; 8.53 Hz); 7.50-7.48 (2H; d; 8.72 Hz); 7.36-7.34 (2H; d; 8.46 Hz); 7.12-7.08 (1H; d; 16.23 Hz); 7.02-6.98 (1H; d; 16.29 Hz); 6.94-6.92 (2H; d; 8.65 Hz); 6.88 (2H; s); 3.86 (3H; s). ^{13}C NMR, DMSO- d_6 : 170.40; 159.60; 137.32; 135.19; 130.72; 129.96; 129.43; 128.40; 127.33; 126.88; 125.65; 114.68; 55.65. MALDI-TOF (*neat*, reflectron mode, positive) m/z 306.13 $[M+H]^+$ (calculated 306.11).

Synthesis of stilbene maleamic acid HSMA 19

4-hydroxy-4'-aminostilbene (0.31 g; 1.46 mmol) was dissolved in 5 mL DMSO, to which

maleic anhydride (0.28 g; 2.86 mmol) was added. After stirring for 2 hours, the mixture was diluted with 60 mL water and yellow precipitate was formed. It was collected by vacuum filtration and washed with water, generating 0.27 g of yellow solid (0.86 mmol; 58.7%). Additional purification was conducted by flash chromatography on C18 RP (12 g Sepacore cartridge; 0.1% aq NaHCO₃/EtOH gradient; 12% EtOH elution) and yielded 0.21 g of bright yellow solid. ¹H NMR, 400 MHz, DMSO-d₆: 6.31-6.34 (1H; d; 12 Hz); 6.46-6.49 (1H; d; 12.13 Hz); 6.76-6.78 (2H; d; 8.59 Hz); 6.95-6.99 (1H; d; 16.42 Hz); 7.06-7.10 (1H; 16.42 Hz); 7.40-7.42 (2H; d; 8.65 Hz); 7.50-7.52 (2H; d; 8.72 Hz); 7.61-7.64 (2H; d; 8.72 Hz); 9.57 (1H; s); 10.47 (1H; s); 13.07 (1H; broad s). ¹³C NMR, DMSO-d₆: 116.02; 120.14; 125.09; 126.95; 127.99; 128.19; 128.69; 131.11; 131.97; 133.80; 137.87; 157.65; 163.53; 167.32. MALDI-TOF (*neat*, reflectron mode, positive) *m/z* 310.48 [M+H]⁺ (calculated 310.10).

5.2.3 Synthesis of MSMI-6MCH conjugate **20** and DMASMI-SH-MG aptamer

Before the conjugation of stilbene maleimide or maleamic acid with thiol modified MG aptamer, the reaction with 6MCH was conducted first. **Figure 5.4** shows the reaction scheme for MSMI-6MCH conjugate **20**. For the reaction with thiol modified MG aptamer, PBS buffer was utilized and TCEP was added to activate the thiol groups before the addition of stilbene compound. The thiol conjugation reactions are relatively simple and the detailed experimental protocols for conjugate **20** and DMASMI-SH-MG aptamer were described below.

Figure 5.4. Schematic pathway for the synthesis of conjugate **20**.

*Synthesis of MSMI-6MCH conjugate **20***

Stilbene maleimide **16** (0.272 mmol) was dissolved in 4 mL of DCM. Isopropanol and triethylamine (0.4 mL each) were added, followed by 6-mercaptohexanol (70 μ L; 0.51 mmol). The mixture was stirred for 4 h to complete the reaction, the progress of which was monitored with silica-TLC. Then it was evaporated and the remaining white residue was

washed with isopropanol, methanol and vacuum dried. A reaction yield of 75% was obtained. ^1H NMR, 400 MHz, DMSO- D_6 : 7.69-7.67 (2H; d; 8.46 Hz); 7.59-7.56 (2H; d; 8.78 Hz); 7.29-7.25 (1H; d; 16.67 Hz); 7.26-7.24 (2H; d; 8.46 Hz); 7.17-7.13 (1H; d; 16.48 Hz); 6.98-6.96 (2H; d; 8.78 Hz); 4.37-4.34 (1H; t; 5.15 Hz; -OH); 4.13-4.09 (1H; dd; 9.12 Hz; 4.14 Hz); 3.79 (3H; s); 3.41-3.38 (2H; t; 6.25 Hz); 2.86-2.74 (2H; m); 2.72-2.67 (1H; dd; 18.31 Hz; 4.10 Hz); 1.65-1.53 (2H; m); 1.46-1.28 (6H; m). ^{13}C NMR, DMSO- d_6 : 176.42; 174.78; 159.65; 138.03; 131.36; 129.91; 129.72; 128.45; 127.56; 126.92; 125.57; 114.69; 61.09; 55.65; 36.67; 32.86; 31.02; 29.18; 28.56; 25.53. MALDI-TOF (*neat*, reflectron mode, positive) m/z 440.46 $[\text{M}+\text{H}]^+$ (calculated 440.19).

Synthesis of DMASMI-SH-MG aptamer

MG RNA aptamer A9 modified with C_6SH label (5 nmol) as solution in 40 μL of PBS buffer was treated with TCEP (1 μL ; 40 mmol L^{-1} in PBS buffer) for 30 mins at room temperature to activate the thiol group. Then 340 μL DMSO and 24 μL of **15** stock solution in DMSO (8.67 mmol L^{-1}) were added. Vortexing resulted in clear solution, which was left at room temperature overnight. RNA was precipitated with 5 mol L^{-1} aqueous ammonium acetate (90 μL) and isopropanol (1.7 mL). Upon keeping at $-20\text{ }^\circ\text{C}$ and centrifugation (13,000 rpm; 30 mins), a pellet was formed. After removing the supernatant, the pellet was washed with isopropanol (3X1 mL) and then re-dissolved in 600 μL PBS buffer. Based on the OD (260 nm) of 0.579, the yield of the DMASMI-SH-MG aptamer was calculated to be 14 %. Its purity was accessed to be more than 99% based on the HPLC results [4]. Theoretical m/z , 12514, $[\text{M}+\text{H}]^+$; Supplier m/z , A9 modified with C_6SH label, 12515, $[\text{M}+\text{H}]^+$; MALDI-TOF (THAP matrix, reflection mode, positive), A9 modified with C_6SH label, 12615, $[\text{M}+\text{H}+\text{DMSO}+\text{H}_2\text{O}]^+$; MALDI-TOF (THAP matrix, reflectron mode, positive), DMASMI-SH-MG aptamer, 12833, $[\text{M}+\text{H}]^+$.

5.2.4 Reaction monitoring between stilbene maleamic acid (Figure 5.2) and 6MCH

The reaction between stilbene maleamic acid and 6MCH was monitored by absorption, emission and fluorescence decay measurement. The reaction was conducted in three different environments: DMSO, DMSO : PBS buffer (v% ratio is 1 : 1) and DMSO : buffer (v% ratio is 1 : 9). To promote the reaction, the 6MCH concentration was twice higher

than MSMA (**18**). Based on the stock solution of 2 mM **18** in DMSO and 16 mM 6MCH in DMSO, the highest concentration of **18** can be achieved in the environment of DMSO : buffer (1 : 9) is $\frac{4}{5} * \frac{1}{10} * 2 \text{ mmol} * L^{-1} = 0.16 \text{ mmol} * L^{-1}$, which is also used in the other two conditions. According to the volume required for absorption, emission and fluorescence decay measurement, total volume of 2.0 mL reaction mixture was prepared in which the required corresponding volume of **18**, 6MCH, DMSO and PBS buffer for the three different conditions are shown in **Table 5.1**. To guarantee sufficient reaction time, the three prepared reaction mixtures were left under dark for one day before measurement.

Table 5.1. Respective amounts of **18**, 6MCH, DMSO and buffer for the monitoring reaction of **18** and 6MCH in three different environments.

DMSO : buffer (v : v)	Volume of 18 (μL)	Volume of 6MCH (μL)	Volume of DMSO (μL)	Volume of buffer (μL)
1 : 0	160	40	1800	0
1 : 1	160	40	800	1000
1 : 9	160	40	0	1800

Based on the absorption spectrum of **18** in DMSO, 0.02 mM was chosen as the measurement concentration. For comparison, the absorption spectrum of 0.02 mM 6MCH under the three conditions were also measured. After obtaining the absorption peaks, the emission spectra were recorded for the mixtures in which the MSMA concentration was further diluted to 4 μM . Under the absorption and emission peaks, the fluorescence decay curves were measured for the reaction mixtures as well as **18**. The monitoring reaction experiment between DMASMA (**17**) and 6MCH was also conducted similarly.

5.3 Results & discussions

5.3.1 Thiol-modified MG aptamers retain ability to bind MG ligand

Based on the known crystal structure of MG aptamer binding pocket in the presence of MG analyte, four locations—5' end of C38, A9 and C20 and 3' end of U4 of the aptamer were selected to be modified with –SH group because of their non-essential nature, distance from the binding pocket and surface exposure to the solvent [6-8].

From the direct binding experiment between the four thiol modified MG aptamers and MG analyte, their binding dissociation constant K_d values (obtained by project team member Y.

Zhou) are in the range of 700 nM to 1 μ M, with the thiol-C38 having the highest K_d of 1 μ M. C38 thiol modified MG aptamer to MG ratio was demonstrated in **Figure 5.5** as a representative of the four thiol nucleotide modifications. Compared to the wild type MG aptamer (MG K_d is 200 nM), the binding affinities of these modified aptamers are 4 to 5 times weaker to MG analyte.

Figure 5.5. Direct binding experiment of C38 thiol modified MG aptamer to MG analyte and its binding dissociation constant value K_d .

5.3.2 Obvious fluorescence decay of stilbene maleimide **15** and **16** (Figure 5.2) in ACN and DMSO

Figure 5.6 summarizes the normalized absorption, emission and excitation spectrum of the two stilbene maleimide compounds (**15** and **16**) in both ACN and DMSO. Their spectral parameters including the absorption and emission peak, molar extinction coefficient and the quantum yield are tabulated in **Table 5.2**.

Figure 5.6. Absorption (solid line), emission (dash line) and excitation (dot line) spectrum summary of **15** (Black) and **16** (Grey) in ACN (**A**) and DMSO (**B**).

Comparing the parameters between **15** and **16**, there is a strong red shift of the absorption and emission peak in **15**. This is because its dimethylamino group is a stronger donor than methoxy in **16**. This strong donor facilitates the charge delocalization in the ground states and results in a lower energy (longer wavelength) transition to the excited states. It also further stabilizes the excited states of **15** giving rise to larger Stock shift. For both **15** and **16**, their absorption and emission maxima in DMSO shift to the red side compared with these in ACN. This is explained by the stronger polarity of DMSO which enhances the electron delocalization. But this influence is weaker than that from a strong donor group. Compared with their parent stilbenes **1** and **2** (Table 4.1), the quantum yield of both stilbene maleimide **15** and **16** becomes lower. The decrease is more than four times in **15** and two times in **16**. The attached maleimide fragment can be considered as an electron-acceptor and it intrinsically shows negligible fluorescence. Since the dimethylamino group has strong donor ability, more electronic energy transfer occurs in **15**, resulting in more non-irradiative loss and thus more decrease in quantum yield.

Figure 5.7. Normalized fluorescence decay curves of **15** (black) and **16** (grey) in ACN (solid line) and DMSO (dash line).

Table 5.2. Spectral parameters summary for **15** and **16** in ACN and DMSO.

	Solvent	$\lambda_{\text{abs}} / \lambda_{\text{Ex}}$ (nm)	λ_{Em} (nm)	ϵ ((mM*cm) ⁻¹)	QY	k_{app} (s ⁻¹)
15	ACN	359 / 349	445	28.84±0.90	0.09	0.914±0.004
	DMSO	367 / 361	452	30.93±2.80	0.18	0.834±0.003
16	ACN	324 / 326	392	29.55±0.90	0.018	0.567±0.006
	DMSO	330 / 328	391	29.30±2.04	0.037	0.557±0.007

The fluorescence decay curves of the two maleimide compounds were recorded and presented in **Figure 5.7**. The calculated apparent decay rate constants are included in **Table**

5.2. Although the attached maleimide fragment reduces the quantum yield of both stilbene maleimides, their fluorescence decay behaviour remains obvious.

5.3.3 Decay rate constant of **15** and **16** depends on medium viscosity

After the photophysical characterization, the fluorescence decay kinetics of both maleimide compounds in solutions having different DMSO to glycerol ratios were performed in order to study the effect of medium viscosity on their *trans-cis* isomerization process. **Figure 5.8** **A** and **B** show the normalized fluorescence decay curves in these solution mixtures for **15** and **16** respectively. With the increase of glycerol volume percentage in the DMSO/glycerol mixtures, the fluorescence decay gradually becomes slower.

Figure 5.8. Fluorescence decay curves of **15** (**A**) and **16** (**B**) in solution mixtures having different DMSO to glycerol ratios. **C.** The plot of decay rate constant vs. the reciprocal of viscosity for **15** and **16**. **D.** Fluorescence polarization vs. viscosity for **15** and **16**.

With the viscosity values of the seven DMSO to glycerol mixtures (**Table 3.1**), the calculated decay rate constant in these solution mixtures were plotted against the reciprocal of the corresponding medium viscosity in log scale for both stilbene maleimide compounds, shown in **Figure 5.8 C**. Besides the fluorescence decay measurement, the fluorescence polarization was also measured. The obtained fluorescence polarization (FP) values vs. viscosity in log scale curve is also displayed in **Figure 5.8 D**. For both **15** and **16**, their decay rate constants are linearly related to the reciprocal of their medium viscosity in logarithmic scale and their FP values have a linear dependence on the logarithmic viscosity. The detailed relationships are expressed as follows. For **15**:

$$k_{app} = (1.07 \pm 0.01) + (0.31 \pm 0.01) * \log (1/\eta) \quad (5.1)$$

$$FP = (0.233 \pm 0.008) + (0.15 \pm 0.01) * \log \eta \quad (5.2)$$

For **16**:

$$k_{app} = (0.73 \pm 0.01) + (0.083 \pm 0.005) * \log (1/\eta) \quad (5.3)$$

$$FP = (0.276 \pm 0.006) + (0.117 \pm 0.007) * \log \eta \quad (5.4)$$

Where η denotes the medium viscosity. But these equations are not applied in the entire viscosity range studied (2.68~215.8 cP). In terms of fluorescence decay rate, the linear relationship works in a lower viscosity region (2.68~18.3 cP) for **15** and higher viscosity region (6.23~215.8 cP) for **16**. While for fluorescence polarization, the obtained equations are only valid in the viscosity range from 2.68 cP to 18.3 cP for both stilbene maleimide compounds. The dependence of stilbene maleimide compounds' fluorescence decay rate on their surrounding medium viscosity confirms their response of decay rate change to changing environment, such as aptamer folding.

5.3.4 Fluorescence decay of **15** and **16** is not observed in aqueous solutions

To preserve the binding affinity of the thiol modified MG aptamer, its reaction to stilbene maleimide needs to be conducted in aqueous environment. Therefore, the fluorescence decay of stilbene maleimides in buffer aqueous environment needs to be confirmed as the detection relies on the decay rate change of the attached stilbene fragment.

The fluorescence decay kinetics of both **15** and **16** were studied in DMSO (2%, 4%, 5% and 8%) and PBS buffer solution mixtures. **Figure 5.9** summarizes the obtained fluorescence decay curves. For both stilbene maleimides in the studied DMSO and buffer

solution mixtures, their fluorescence decay is not observed. Their fluorescence intensities are less than 0.1% of the parent stilbene **2** (under similar conditions). The “flat” fluorescence decay curves may be attributed to the low fluorescence intensities of the two stilbene maleimides in buffer and DMSO mixtures which make the decay non-observable, as has been observed in stilbene-MG conjugate **6** in Section 4.3.6. While the low fluorescence intensities are likely caused by the aqueous environment quenching and their attached maleimide group which is electron accepting and non-fluorescent.

Figure 5.9. Fluorescence decay curves of **15** (A) and **16** (B) in different DMSO and buffer solution mixtures.

Since thiol conjugation can decrease the electron accepting power of the maleimide group, the obtained conjugate is expected to have enhanced fluorescence intensity and observable fluorescence decay, as demonstrated with MSMI-6MCH conjugate **20**.

5.3.5 Click chemistry product MSMI-6MCH conjugate **20** (Figure 5.4) restores stilbene fluorescence

The photophysical properties including the absorption, excitation and emission spectrum of the MSMI-6MCH conjugate **20** in ACN and DMSO were studied together with its fluorescence decay kinetics. The normalized spectra as well as its fluorescence decay curves are presented in **Figure 5.10**. **Table 5.3** summarizes its absorption and emission peaks, molar extinction coefficients, quantum yields and apparent decay rate constants.

Comparing the photophysical parameters of **20** summarized in **Table 5.3** with these of MSMI **16** in **Table 5.2**, no significant change (less than 10%) is observed other than the

quantum yield. In ACN, the quantum yield of **20** is two times lower than **16**, but 25% higher in DMSO. So in DMSO, thiol conjugation decreases the electron-accepting power of the maleimide group, reduces non-irradiative loss and enhances the quantum yield. The absence of the enhancement in ACN may be attributed to its lower polarity.

Figure 5.10 **A.** Normalized absorption (solid line), emission (dash line) and excitation spectrum (dot line) of conjugate **20** in ACN (black) and DMSO (grey). **B.** The fluorescence decay curves of **20**.

Table 5.3. Summary of conjugate **20**'s photophysical parameters.

	Solvent	$\lambda_{\text{abs}} / \lambda_{\text{Ex}}$ (nm)	λ_{Em} (nm)	ϵ ((mM*cm) ⁻¹)	QY	k_{app} (s ⁻¹)
20	ACN	324 / 314	388	33.30±1.90	0.009	0.578±0.003
	DMSO	330 / 322	391	33.74±2.17	0.047	0.561±0.003

Figure 5.11. Fluorescence decay curves of conjugate **20** in DMSO (2, 4 and 8%) and PBS buffer solution mixtures.

To check the adaptability of **20** in MG aptamer binding buffer, the fluorescence decay curves of the conjugate in DMSO (2%, 4% and 8%) and PBS buffer solution mixtures were measured and the results are presented in **Figure 5.11**.

Comparing to **Figure 5.9**, the fluorescence decay of **20** in buffer based aqueous environment is preserved and the fluorescence intensity is enhanced more than 20 times than stilbene maleimide **16** under similar conditions. The fluorescence decay of stilbene maleimide compounds are quenched in DMSO/buffer aqueous mixtures but restored after conjugation with the thiol modified MG aptamer. This has significance importance to PHASA whose detection is based on decay rate change. The decay restoration confirms the thiol conjugation and the subsequent detection can be conducted without further separation and purification, since the decay of stilbene maleimides are quenched and do not interfere with the analyte detection process.

5.3.6 Fluorescence decay of conjugate **20** depends on its surrounding medium viscosity

To investigate the decay rate response of conjugate **20** to viscosity environment changes, the fluorescence decay curves of **20** in solutions having different viscosities were measured. **Figure 5.12 A** summarizes the normalized decay curves of the conjugate in seven solution mixtures having different DMSO to glycerol ratios. The apparent decay rate constants are calculated and plotted against the reciprocal of medium viscosity in log scale as shown in **Figure 5.12 B**. Fluorescence polarization (FP), a measure of molecular tumbling, also displays dependence on viscosity, displayed in **Figure 5.12 C**.

From **Figure 5.12**, it can be concluded that there is strong dependence of both the conjugate's fluorescence decay rate constant and fluorescence polarization on its surrounding medium viscosity. The equations for the linear relationships can be expressed as:

$$k_{app} = (0.824 \pm 0.009) + (0.098 \pm 0.005) * \log (1/\eta) \quad (5.5)$$

$$FP = (0.377 \pm 0.003) + (0.037 \pm 0.002) * \log \eta \quad (5.6)$$

Like that already seen for **16**, the equations cannot be applied to the entire studied viscosity range and the linear dependence is observed in high viscosity range (6.23~215.8 cP) for fluorescence decay and low viscosity range (2.68~38.6 cP) for FP. Comparing the decay

rate vs. medium viscosity plot of **20** (equation 5.5) with that of **16** (equation 5.3), the difference between their slopes is less than 15%. However, for their FP vs. viscosity plots (equation 5.6 and 5.4), the slope of **20** is three times lower than **16**. This means after the conjugation of 6MCH, the sensitivity of the fluorescence decay rate to medium viscosity is not significantly influenced, but the sensitivity of fluorescence polarization to medium viscosity is lower more than three times. The addition of 6MCH increases 40% molecular weight of **20** than **16**. Thus, the difference in their sensitivity change is suspected to be the reason that stilbene fluorescence decay involves the rotation of the double bond, but fluorescence polarization measurement involves the rotation of the entire molecule. The 40% molecular weight is added to one side of conjugate **16** and forms a long tail, retarding the rotation of the entire molecule **20** and resulting in less effective response to the medium viscosity change.

Figure 5.12 **A.** Normalized fluorescence decay curves of **20** in different DMSO/glycerol ratio solution mixtures. **B.** The relationship between its decay rate constant and medium viscosity. **C.** The plot of fluorescence polarization vs. viscosity.

Combining **20**'s fluorescence decay rate dependence on its surrounding medium viscosity and the observation that thiol conjugation restores the fluorescence decay of stilbene maleimides in buffer/DMSO solution mixtures, it can be concluded that after conjugating the stilbene maleimides to the thiol modified MG aptamer, the stilbene component of the obtained complex can still respond to the changes of its surrounding environment and be employed for bio-sensing.

As already seen for section 5.2.3, the synthesis of stilbene maleimide-SH-MG aptamer complex has been accomplished. Fluorescence enhancement was observed when the obtained DMASMI-C₆SH-MG aptamer complex was mixed with MG analyte. This means the binding affinity of the obtained complex to MG analyte is retained. However, fluorescence decay results were unable to be obtained from the DMASMI-C₆SH-MG aptamer complex. As described in the experimental section 5.2.3, the amount of MG A9 modified aptamer is 5 nmol and the amount of stilbene maleimide **15** is 208 nmol ($n = c * v = 8.67 \text{ mM} * 24 \mu\text{L} = 208 \text{ nmol}$). Hence, based on the reaction yield of 14 %, total obtained DMASMI-C₆SH-MG aptamer complex amount is 0.7 nmol. The highest concentration, since the smallest cuvette is 50 μL , thus can be used for decay measurement is 14 nmol L⁻¹ which is extremely low (The concentrations used for the decay measurement of parent stilbenes **1** and **2** are hundreds of nmol, not speak of their 5 to 6 times higher quantum yield than stilbene maleimide **15** and **16**). That is, the low synthesis yield and the low quantum yield of DMASMI-C₆SH-MG aptamer complex make its decay results difficult to be obtained. Considering the cost of aptamers, stilbene maleamic acids having better water solubility than **15** and **16** are proposed to enhance the production yield. In addition, the accepting ability of maleimide group in **15** and **16** is reduced and can lead to high quantum yield.

5.3.7 Obvious fluorescence decay of three stilbene maleamic acids (17, 18 and 19) in organic solvents

Based on the observation that aqueous solution mixtures quench the fluorescence intensities of stilbene maleimide compounds and results in non-observable fluorescence decay, stilbene compounds with more water solubility are preferred to reduce the quenching effect from aqueous environment. Hence, three stilbene maleamic acid

compounds—DMASMA (**17**), MSMA (**18**) and HSMA (**19**) having carboxylic group were synthesized.

The photophysical properties of the three stilbene maleamic acids were characterized. Since the carboxylic group in three maleamic acids is reactive, it may interact with surrounding solvent to shift electronic distribution and thus change photophysical properties. Therefore, besides ACN and DMSO, the characterization was also conducted in a strong polar aprotic solvent—propylene carbonate (PC) and a polar protic solvent—ethanol (EtOH). **Figure 5.13** summarizes their normalized absorption, excitation and emission spectra in the above four organic solvents. Their normalized fluorescence decay curves are presented in **Figure 5.14**. The calculated fluorescence decay rate constants together with other spectral parameters such as absorption, excitation and emission peak, molar extinction coefficient and quantum yield of the three stilbene maleamic acid compounds are tabulated in **Table 5.4**.

Figure 5.13. Normalized absorption (solid line), emission (dash line) and excitation (dot line) spectrum of **17** (Black), **18** (Grey) and **19** (Light grey) in ACN (**A**), DMSO (**B**), EtOH (**C**) and PC (**D**).

Figure 5.14. The fluorescence decay curves of **17** (black), **18** (red) and **19** (blue) in ACN (solid line) and DMSO (dash line) (**A**), and in EtOH (solid line) and PC (dash line) (**B**).

The absorption and emission spectra of **18** and **19** are almost identical. This is explained by the similar donor properties of their respective methoxy and hydroxy group. On the other hand, as explained above, dimethylamino group is a very powerful donor compared to hydroxyl and methoxy, which leads to much larger electron delocalization in **17** than in **18** or **19**, and the smaller energy gap between the ground and excited states of the molecule. There is almost no observable spectrum shifts due to solvent effect in ACN and DMSO. This manifests for much stronger intramolecular stabilization due to *para*-substitution effect than intermolecular stabilisation due to solvent effect. The emission spectra in PC is considerably red-shifted and correlates with the polarity effect of the solvent, when more polar solvent is able to efficiently stabilize the excited state of a molecule, thereby decreasing its energy. The exception is EtOH, in which both absorption and emission peaks of the three stilbene maleamic acids are blue shifted. The blue shift in EtOH is probably caused by its protic nature which may lead to specific interactions with the stilbene maleamic acid compounds' carboxyl group at both ground states and excited states. The interactions with **17**, especially its ground states, may be the strongest since it has the largest Stoke shift and blue shift of its absorption peak. Besides, its molar extinction coefficient is more than two times lower than the rest two (**18** and **19**).

Table 5.4. Spectral parameters summary of three stilbene maleamic acid compounds.

	Solvent	$\lambda_{\text{abs}} / \lambda_{\text{Ex}} \text{ (nm)}$	$\lambda_{\text{Em}} \text{ (nm)}$	$\epsilon \text{ ((mM*cm)}^{-1})$	QY	$k_{\text{app}} \text{ (s}^{-1})$
17	ACN	379 / 350	418	32.29±0.30	0.0011	0.337±0.001
	DMSO	377 / 365	417	38.18±0.24	0.0058	0.495±0.004
	EtOH	343 / 342	402	9.07±0.11	0.0032	N.A
	PC	375 / 360	430	16.64±1.22	0.0803	0.420±0.006
18	ACN	348 / 320	412	29.64±1.53	5.39E-4	0.697±0.005
	DMSO	345 / 335	415	35.48±0.15	0.00277	0.789±0.007
	EtOH	325 / 310	391	24.03±0.96	0.00262	0.550±0.003
	PC	348 / 373	430	19.32±0.90	0.0204	0.67±0.01
19	ACN	350 / 315	408	29.85±1.09	0.0016	0.692±0.003
	DMSO	348 / 338	416	33.12±0.05	0.0023	0.755±0.005
	EtOH	333 / 312	393	23.25±1.79	0.0022	0.462±0.003
	PC	348 / 373	407	17.59±1.07	0.0203	0.65±0.02

The above conclusions regarding the solvent and substituent effect are also supported by the fluorescence decay results. As shown in **Figure 5.14** and **Table 5.4**, in ACN, DMSO and PC, **18** and **19** have similar decay profile and fluorescence decay rate constants, just as was observed in their absorption and emission spectra. In EtOH, their decay rate is the lowest among the four studied solvents due to the proposed interaction between EtOH and the carboxyl group. For compound **17**, due to the presence of the stronger dimethyl donor group, it has the lowest decay rate constant compared to **18** or **19** under the same conditions. Moreover, its decay in EtOH is completely quenched. The *trans-cis* isomerization dynamics of *trans*-stilbene in *n*-alcohols has been studied by Sundstrom *et al* [9] and they concluded that the *trans-cis* isomerization yield is 0.54±0.05. From Schulte-Frohlinde and coworkers' investigation, a more comprehensive conclusion that the isomerization process strongly depends on the solvent and stilbene substituent can be generated. When stilbenes are not substituted with strong donor-acceptor groups, their decay is observed. However, when strong donor-acceptor groups are present such as in 4-nitro-4'-aminostilbene and 4-nitro-4'-dimethylaminostilbene, their decay in polar solvents such as ethanol and dimethylformamide are completely quenched [10]. This is consistent with the experimental results that the decay quenching is observed only in compound **17**. In their study, they attributed the quenching to solvation and orientation polarization. But the protic nature of the solvent which can promote the special interactions such as hydrogen binding is also

believed to have played a great role in stabilizing and quenching the fluorescence decay of **17** in ethanol rather than in ACN, DMSO and PC which are also polar solvents. As to the quantum yield (QY) results in Table 5.4, all the stilbene maleamic acid molecules have the lowest quantum yield value in ACN and that of **18** is 100 times lower than the rest. This indicates that ACN may not be a good organic solvent for the application of the three stilbene maleamic acids.

5.3.8 Fluorescence decay of three stilbene maleamic acids response to environment having different viscosity

To study the fluorescence decay rate response of the three stilbene maleamic acids to their surrounding medium having different viscosities, their fluorescence decay kinetics were studied in seven solution mixtures consisting of different volume ratios of DMSO to glycerol. The obtained fluorescence decay curves for **17**, **18** and **19** are normalized and summarized in **Figure 5.15 A, B and C**, respectively. Their fluorescence decay rate constants are calculated and plotted against the reciprocal of medium viscosity in log scale as shown in **Figure 5.15 D**.

The fluorescence decay rate constants of all three stilbene maleamic acids show strong dependence on their surrounding medium viscosity and linear relationships with the reciprocal of the viscosity in log scale are observed. The equations of these linear relationships are expressed in the following equation 5.7, 5.8 and 5.9 for three stilbene maleamic acid **17**, **18** and **19** respectively.

$$k_{app} = (0.521 \pm 0.005) + (0.100 \pm 0.005) * \log (1/\eta) \quad (5.7)$$

$$k_{app} = (0.979 \pm 0.012) + (0.223 \pm 0.009) * \log (1/\eta) \quad (5.8)$$

$$k_{app} = (0.878 \pm 0.011) + (0.177 \pm 0.006) * \log (1/\eta) \quad (5.9)$$

These equations cannot be applied to the whole studied viscosity range (2.68~215.8 cP). For **18** and **19**, their fluorescence decay rate responses to their surrounding medium viscosity are similar, and there is 20% difference in the slopes of obtained plots. Both the slopes are ~2 times higher than that of **17**. This means they are more sensitive to the medium viscosity change than **17**. Regarding the viscosity range of detection, both **18** and **19** are suitable for higher viscosities values (6.23~215.8 cP).

Figure 5.15. The fluorescence decay curves of **17** (A), **18** (B) and **19** (C) in solution mixtures having different volume percentages of DMSO and glycerol. **D.** The relationship between their apparent decay rate constant and the medium viscosity.

5.3.9 Fluorescence decay kinetics of three stilbene maleamic acids (**17**, **18** and **19**; Figure 5.2) retains in aqueous environment

To confirm the aqueous quenching effect on the three stilbene maleamic acids, their fluorescence decay kinetics were studied in aqueous systems, which consist of low volume percentage (3, 5, and 8%) of organic solvents and water or PBS binding buffer. **Figure 5.16** summarizes the normalized fluorescence decay curves of the three stilbene maleamic acids in 3% organic solvent (ACN, DMSO and EtOH) and water/PBS buffer. The obtained fluorescence decay rate constants are presented in **Table 5.5** together with other spectral parameters. Propylene carbonate (PC) is not included in this study because the above basic photophysical characterization results of three stilbene maleamic acids in PC do not offer extra advantage in terms of fluorescence decay rate compared to ACN or DMSO. The decay curves in solution mixtures having 5% and 8% organic solvent are not shown, but their associated parameters such as maximum absorption and emission wavelengths, molar

extinction coefficient and apparent decay rate constant are tabulated in **Table 5.6**, **5.7** and **5.8** for **17**, **18** and **19** respectively.

Figure 5.16. Normalized fluorescence decay curves of **17** (A), **18** (B) and **19** (C) in 3% ACN (red), DMSO (green) and EtOH (blue) and water (solid line)/PBS buffer (dash line) solution mixtures.

For all three stilbene maleamic acids, when the organic solvent percentage is varied from 3% to 8% in the same organic solvent–aqueous system, there is no obvious change in their maximum absorption and emission wavelengths. Comparing with the absorption peaks summarized in **Table 5.4**, the differences among the three organic solvent–aqueous systems are smaller. This indicates that the blue shift in ACN/DMSO–aqueous systems is larger than in EtOH–aqueous system. In other words, both EtOH and water have similar impact on blue shifting the stilbene maleamic acids’ absorption peak. Thus the suspected specific interaction in the ground states is likely to be hydrogen bonding. As to the emission peaks, the presence of water results in more non-irradiative loss and more red shift as observed in ACN/DMSO–aqueous systems. However, weaker red shift is seen in EtOH–aqueous environment. This suggests that the presence of EtOH prevents further excited

states stabilization against water. Also, it needs to be noticed that replacing water with binding buffer do influence the effect from EtOH, as the maximum absorption and emission wavelengths in EtOH–buffer mixtures are longer than in EtOH–water mixtures. This impact is weak and might be caused by the involvement of hydrogen phosphate and dihydrogen phosphate ions from the PBS buffer in the interactions between EtOH and the ground/excited states of stilbene maleamic acids.

Table 5.5. Spectral parameters summary of **17**, **18** and **19** in 3% organic solvent (ACN, DMSO, EtOH) and water/buffer mixtures.

	Solvent	λ_{abs} (nm)	λ_{Em} (nm)	ε ((mM*cm) ⁻¹)	k_{app} (s ⁻¹)
17	ACN (3%) and water	346	441	23.2±0.9	0.772±0.004
	DMSO (3%) and water	341	440	8.62±0.16	0.837±0.005
	EtOH (3%) and water	348	415	12.7±0.3	N.A.
	ACN (3%) and buffer	350	440	27.6±1.23	0.732±0.005
	DMSO (3%) and buffer	351	441	19.37±0.77	0.783±0.003
	EtOH (3%) and buffer	350	426	14.4±0.42	0.58±0.06
18	ACN (3%) and water	334	426	29.1±0.7	0.69±0.01
	DMSO (3%) and water	333	428	6.90±0.34	0.690±0.007
	EtOH (3%) and water	321	389	12.4±0.6	0.575±0.009
	ACN (3%) and buffer	334	430	32.3±0.64	0.643±0.008
	DMSO (3%) and buffer	334	431	20.68±1.10	0.620±0.006
	EtOH (3%) and buffer	326	386	9.3±0.46	0.53±0.03
19	ACN (3%) and water	333	428	24.02±1.3	0.646±0.005
	DMSO (3%) and water	335	426	21.30±0.73	0.624±0.005
	EtOH (3%) and water	321	407	7.97±0.14	0.527±0.008
	ACN (3%) and buffer	334	427	25.23±1.12	0.590±0.004
	DMSO (3%) and buffer	334	425	22.21±0.59	0.599±0.004
	EtOH (3%) and buffer	333	428	23.64±0.99	0.587±0.002

The fluorescence decay kinetics results support the above effect from the PBS binding buffer. In EtOH only, the fluorescence decay of **17** is completely quenched. This quenching is also observed in three EtOH–water solution mixtures. While in EtOH–buffer solution mixtures, the quenching occurs only in 8% EtOH and buffer mixture. In the other two mixtures having lower EtOH volume percentage (3% and 5%), the fluorescence decay restores. Hence, for **17** in EtOH–buffer mixtures, as long as EtOH is less than 8% in volume, its fluorescence decay is observable and measurable. In ACN/DMSO–water/buffer systems, all three stilbene maleamic acid compounds have obvious decay in all the studied organic

solvent volume percentages (3, 5 and 8%). Also, in the same organic solvent–water/buffer system, the change in volume percentage from 3% to 8% does not significantly affect their apparent decay rate. Hence, compared to the two stilbene maleimides (**15** and **16**), the carboxyl group in three maleamic acids reduces the aqueous quenching on fluorescence decay, making them promising in PHASA which is binding buffer based.

Table 5.6. Summary of **17**'s photophysical parameters in 5% and 8% organic solvent and water/buffer mixtures.

Solvent	λ_{abs} (nm)	λ_{Em} (nm)	ϵ ((mM*cm) ⁻¹)	k_{app} (s ⁻¹)
ACN (8%) and water	346	438	27.24±1.84	0.787±0.005
ACN (5%) and water	346	442	21.21±0.80	0.771±0.005
ACN (8%) and buffer	350	440	26.65±1.37	0.752±0.005
ACN (5%) and buffer	350	440	27.52±1.07	0.748±0.005
DMSO (8%) and water	344	440	8.86±0.11	0.851±0.005
DMSO (5%) and water	340	444	8.92±0.41	0.801±0.004
DMSO (8%) and buffer	352	439	21.25±0.70	0.762±0.003
DMSO (5%) and buffer	353	440	20.24±0.67	0.776±0.003
EtOH (8%) and water	347	415	10.75±0.28	N.A.
EtOH (5%) and water	347	414	11.94±0.10	N.A.
EtOH (8%) and buffer	350	416	13.03±0.17	N.A.
EtOH (5%) and buffer	349	427	14.14±0.20	0.5±0.1

Table 5.7. Summary of **18**'s photophysical parameters in 5% and 8% organic solvent and water/buffer mixtures.

Solvent	λ_{abs} (nm)	λ_{Em} (nm)	ϵ ((mM*cm) ⁻¹)	k_{app} (s ⁻¹)
ACN (8%) and water	335	424	24.63±0.1.32	0.713±0.011
ACN (5%) and water	336	426	28.82±0.49	0.676±0.010
ACN (8%) and buffer	335	431	27.18±1.16	0.616±0.007
ACN (5%) and buffer	335	430	28.63±0.47	0.628±0.008
DMSO (8%) and water	335	428	9.97±0.38	0.688±0.006
DMSO (5%) and water	334	425	8.69±0.50	0.680±0.004
DMSO (8%) and buffer	335	430	22.22±0.76	0.632±0.004
DMSO (5%) and buffer	336	430	21.31±0.74	0.622±0.005
EtOH (8%) and water	320	391	11.25±0.30	0.597±0.006
EtOH (5%) and water	321	392	12.22±0.48	0.588±0.007
EtOH (8%) and buffer	326	385	9.23±0.35	0.527±0.033
EtOH (5%) and buffer	325	384	8.29±0.41	0.459±0.032

Table 5.8. Summary of **19**'s photophysical parameters in 5% and 8% organic solvent and water/buffer mixtures.

Solvent	λ_{abs} (nm)	λ_{Em} (nm)	ϵ ((mM*cm) ⁻¹)	k_{app} (s ⁻¹)
ACN (8%) and water	335	425	24.56±0.78	0.644±0.004
ACN (5%) and water	334	426	25.12±0.77	0.637±0.004
ACN (8%) and buffer	335	426	25.62±1.16	0.599±0.004
ACN (5%) and buffer	334	429	25.00±0.13	0.588±0.004
DMSO (8%) and water	335	425	21.72±0.65	0.648±0.005
DMSO (5%) and water	335	424	22.59±1.02	0.641±0.005
DMSO (8%) and buffer	335	426	22.15±0.65	0.592±0.004
DMSO (5%) and buffer	336	426	21.97±0.67	0.588±0.004
EtOH (8%) and water	320	403	10.19±0.52	0.619±0.009
EtOH (5%) and water	322	408	9.38±0.30	0.544±0.007
EtOH (8%) and buffer	328	418	15.36±0.73	0.466±0.005
EtOH (5%) and buffer	332	428	22.97±0.80	0.583±0.002

5.3.10 Reaction between stilbene maleamic acid and 6MCH indicates conjugation

Based on the reaction between stilbene maleimide (**15**) and thiol modified MG aptamer (Section 5.3.6), it can be deduced that the conjugation between the modified MG aptamer and stilbene maleamic acid is also applicable, as the stilbene maleamic acid compounds also have the double bond to be reduced.

Figure 5.17 presents the reaction results of MSMA (**18**) + 6MCH (1 : 2) mixtures in three different environments: DMSO, DMSO : buffer (1 : 1) and DMSO : buffer (1 : 9). The absorption spectrum summary in **Figure 5.17 A** shows that there is no significant difference between MSMA (**18**) and MSMA (**18**) + 6MCH mixture in DMSO and DMSO : buffer (1 : 1). However, when the DMSO volume percentage is decreased to 10%, the absorption peaks of both MSMA (**18**) and the mixture shift to the blue side and the absorbance values of the mixture is only ~60% of that MSMA (**18**). If MSMA-6MCH conjugate is formed in the mixture, it has smaller extinction coefficient than **18**.

Figure 5.17. The absorption spectrum (A), fluorescence decay kinetics (B) and the corresponding apparent decay rate (C) summary of MSMA (18) (black) and MSMA (18) + 6MCH (molar ratio is 1 : 2) mixtures (red) in DMSO (solid line), DMSO (50%) and buffer (dash line) and DMSO (10%) and buffer (dot line).

For the fluorescence decay results, as expected, the fluorescence intensity becomes lower when the buffer percentage increases, as shown in **Figure 5.17 B**. Under the three conditions, the decay curves of both **18** and the mixture are obvious and measurable. The apparent decay rate constants in **Figure 5.17 C** show that with decreasing of DMSO, the decay rate decreases. Moreover, in three studied solution mixtures, the decay rate of **18** is higher than that of the MSMA (18) + 6MCH mixture, and the difference is more than five times greater in DMSO : buffer (1 : 1) and DMSO : buffer (1 : 9) than in DMSO solvent only. That is, the effect of aqueous environment quenching on decay rate constant is more severe for the MSMA-6MCH conjugate than **18**. Therefore, based on the decrease in the absorbance and apparent decay rate constant of the reaction mixture observed from the reaction monitoring results, the formation of the MSMA-6MCH conjugate from MSMA

(**18**) and 6MCH is suspected to be achievable. Similar results were obtained for the experiments between HSMA (**19**) and 6MCH. Hence, like stilbene maleimide compounds, stilbene maleamic acids (**17**, **18** and **19**) can also be attached to thiol modified MG aptamer. However, the same problem is encountered, that is, the fluorescence decay behaviour of the stilbene maleamic acid SH MG aptamer complex cannot be obtained. Comparing **Table 5.2** and **5.4**, when stilbene maleimide compounds are converted to their corresponding stilbene maleamic acids, their quantum yields are 10 times lowered. To compensate the reduced quantum yields, the production yields for the synthesis of stilbene maleamic acids and SH modified MG aptamer have to be increased more than 10 times which is impractical since the production yield for DMASMI-SH-MG aptamer is 14%. Besides, the above monitoring reaction (section 5.3.10) between **18** and MCH supports that the aqueous quenching effect on the fluorescence decay of the formed conjugate is more severe. Therefore, instead of being used for MG analyte detection, other promising applications for the three stilbene maleamic acids such as ethanol or pH sensing were further explored and presented in chapter 6 in detail. As to the MG analyte detection, since the major barrier is the fluorescence decay of stilbene compound probes in aqueous environment, water soluble stilbene compound (4-Acetamido-4'-isothiocyanato-2,2'-stilbenedisulfonic acid disodium salt) was introduced. The detection based on the water soluble stilbene and NH₂ modified MG aptamer for MG and TMR (tetramethylrosamine) has been conducted by my project team member Y. Zhou and detection limits of 1.85 µg and 0.101 µg have been achieved for MG and TMR, respectively.

5.4 Conclusions

For adaptive binding scope, thiol modification retains the binding affinity of MG aptamer to MG analyte. Based on the click reaction between maleimide and thiol molecules, two stilbene maleimide compounds (DMASMI **15** and MSMI **16**) were synthesized. Their fluorescence decay are non-observable in DMSO (v% is less than 10%) and PBS buffer solution mixtures. However, after being conjugated to a small thiol molecule 6MCH, the fluorescence decay of the obtained MSMI-6MCH conjugate **20** restores. Hence, after stilbene maleimide conjugation to thiol modified MG aptamer, analyte detection based on the decay rate change of the attached stilbene fragment is applicable, and this detection can

be conducted without separation or purification from the stilbene maleimide compounds. Moreover, this restoration of fluorescence decay of stilbene maleimide after thiol conjugation makes stilbene maleimide compounds promising thiol probes and can be used for fast thiol detection.

By introducing the carboxylic group to stilbene maleimide compounds, the obtained stilbene maleamic acids show obvious fluorescence decay in aqueous solution mixtures. This is, their decay is more robust than stilbene maleimides in aqueous detection environment. For DMASMA **17**, its fluorescence decay is quenched in EtOH and EtOH/water mixtures, and in PBS buffer based aqueous mixtures, its decay restores when v% of EtOH is less than 8%. Hence, besides medium viscosity, EtOH can also change stilbene compound **17**'s fluorescence decay through interacting with carboxylic group, and EtOH detection based on **17** may be possible. The interaction of stilbene maleimide acids with other solvent parameters such as pH and thus their detection application may also be explored.

References

- [1] S.S. Ghosh, P.M. Kao, A.W. McCue, H.L. Chappelle, *Bioconjugate Chemistry*, 1 (1990) 71-76.
- [2] R.J. Pounder, M.J. Stanford, P. Brooks, S.P. Richards, A.P. Dove, *Chemical Communications*, (2008) 5158-5160.
- [3] C.W. Scales, A.J. Convertine, C.L. McCormick, *Biomacromolecules*, 7 (2006) 1389-1392.
- [4] M.P. Cava, A.A. Deana, K. Muth, M.J. Mitchell, *Organic Syntheses*, 41 (1961) 93.
- [5] T.W.J. Steele, W.T. Shier, *Pharmaceutical Research*, 27 (2010) 683-698.
- [6] D. Grate, C. Wilson, *Proceedings of the National Academy of Sciences*, 96 (1999) 6131-6136.
- [7] J. Flinders, S.C. DeFina, D.M. Brackett, C. Baugh, C. Wilson, T. Dieckmann, *ChemBioChem*, 5 (2004) 62-72.
- [8] C. Baugh, D. Grate, C. Wilson, *Journal of Molecular Biology*, 301 (2000) 117-128.
- [9] V. Sundström, T. Gillbro, *Chemical Physics Letters*, 109 (1984) 538-543.
- [10] D. Schulte-Frohlinde, H. Blume, H. Güsten, *The Journal of Physical Chemistry*, 66 (1962) 2486-2491.

Chapter 6*

Other promising applications of stilbene compounds

This chapter explores promising applications of stilbene compounds beyond those designed for the PHASA technology. Besides medium viscosity, other solvent parameters such as pH and solvation influence stilbene's fluorescence decay by shifts in electronic distribution. pH dependence on UV-Vis absorbance, emission and fluorescence decay kinetics of three stilbene maleamic acids were explored. Decay rates correlate with decreasing pH values below 4.5. Fluorescence decay of DMASMA is quenched in the presence of ethanol, as noted in Chapter 5. So the change of DMASMA's fluorescence decay rate constant in solutions having different ethanol concentration was studied for possible ethanol detection strategies. A linear relationship was obtained in ethanol concentration range from 4–100 mM, in which alcohol analogues of isopropanol (IPA) and methanol show no decay dependence.

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6.1 Introduction

The promising applications of stilbene compounds based on their fluorescence decay rate constants' strong dependence on their surrounding medium viscosity have been studied [1-5]. This dependence behavior has been confirmed in chapter 4 and 5 for studied stilbene compounds. New observations have been obtained, based on which other applications of studied stilbene compounds were explored.

The previous experimental results (**Section 5.3.7**) show that the fluorescence decay of DMASMA **17** in ACN and DMSO is obvious but completely quenched in EtOH. The same observation was seen in their water mixtures and there is no decay observed even in 3% EtOH and water mixture. It has been proposed that the polar and protic nature of ethanol makes it strongly interacts with **17** having a strong dimethylamino donor group, changes its electronic distribution and results in quenched fluorescence decay. By monitoring the relationship between the decay rate change of DMASMA **17** and the amount of EtOH present, a calibration curve might be obtained which can be used for EtOH detection and quantification.

The decay kinetics study of DMASMA **17** (**Section 5.3.9**) shows that when v% of ethanol in EtOH/buffer mixtures is less than 8%, fluorescence decay restores. This is suspected to be resulted from the involvement of the hydrogen phosphate or dihydrogen phosphate ions in interrupting the interactions between ethanol and **17**. Based on the sensitivity of **17**'s decay behaviour to charge distribution and the surrounding interactions, and the fact the dimethylamino and carboxylic groups of **17** can be protonated and deprotonated respectively, it is reasonable to propose that protons and hydroxyl ions can also influence the decay behaviour of **17**, and the resulted decay rate constant change can be used to indicate the pH of the studied solutions.

6.2 Experimental section

6.2.1 Materials

The stilbene maleimide and stilbene maleamic acid compounds were synthesized as introduced in chapter 5 and literature [6]. 6-Mercapto-1-hexanol (6MCH) and TCEP (tris(2-carboxyethyl)phosphine) were ordered from Sigma, Singapore. Organic solvents like ethanol, methanol, isopropanol (IPA) and DMSO were purchased from Sigma. Sulfuric

acid (95~97%) used in pH study was purchased from Honeywell. Hydrochloric acid fuming (37%) was purchased from Merck and sodium hydroxide pellets (99.9%) was received from Schedelco. PBS buffer was prepared from 5.65 mmol L⁻¹ NaH₂PO₄ (Sigma), 4.35 mmol L⁻¹ Na₂HPO₄ (Sigma), 10 mmol L⁻¹ MgCl₂ (Sigma) and 1 mmol L⁻¹ NaCl unless otherwise stated. Deionised (DI) water was supplied by Milli-Q® water system.

6.2.2 Ethanol effect study on DMASMA 17 (Figure 5.2)

Since the fluorescence decay of **17** in 5% ACN and water mixture is obvious, the fluorescence decay kinetics of 6 μM **17** in 5% ACN and water was set as the reference. All the fluorescence decay curves were measured under the decay conditions of **17** in 5% ACN and water—λ_{Ex} 346 nm/λ_{Em} 442 nm (Table 5.6).

To get solutions having low EtOH concentrations, two different solutions—solution **a** which is 6 μmol L⁻¹ **17** in 5% ACN and water (300 μL) with 2 μL EtOH added and solution **b** which is 6 μmol L⁻¹ **17** in 5% ACN and H₂O with no EtOH addition were firstly prepared. Then, after obtaining the decay curve of the reference, 2 μL solution **a** and 298 μL solution **b** (total volume was 300 μL) were mixed and its fluorescence decay curve was measured. Following that, the volume of solution **a** was gradually increased, and a range of mixtures with different EtOH volume were obtained. For high ethanol concentration (> 0.05 mol L⁻¹), EtOH was directly mixed with solution **b**.

The fluorescence decay curves of **17** in the presence of a series amount of EtOH were measured and their apparent decay rate constants were calculated. The EtOH concentration in the solution mixture can be calculated based on

$$c = \frac{n}{v_t} = \frac{1}{v_t} * \frac{m}{M} = \frac{1}{v_t} * \frac{\rho * v}{M} \quad (6.1)$$

Where v is the added EtOH volume, ρ (0.789 g/cm³) and M (46.07 g/mol) are the density and molecular mass of EtOH respectively. v_t is the total volume of the solution mixture which is 300 μL. Then the decay rate constant vs. EtOH concentration was plotted.

6.2.3 pH effect study for the three stilbene maleamic acids (Figure 5.2)

Firstly, solutions with different pH values were prepared by adding different volume of HCl or NaOH into the PBS buffer whose pH is 6.7. HCl or NaOH were chosen because

their addition does not introduce any foreign ions to the PBS buffer. Stock solution (1.0 mol L^{-1}) of HCl and NaOH were prepared from the fuming hydrochloric acid (37%) and sodium hydroxide pellets respectively.

pH values were measured by Sartorius Docu-pH meter. It has a pH measuring range from -2.000 to 20.000 with readability of 0.001 and accuracy of ± 0.005 . Besides, a temperature sensor has been integrated inside to correct the pH measurements due to temperature change. Before measurement, calibration of the Docu-pH meter using three standard buffer solutions ($\text{pH} = 4$, $\text{pH} = 7$ and $\text{pH} = 10$) was conducted. Then, PBS buffer in a beaker was used as the base, in which the electrode was fully immersed. To accelerate the homogenization process after the addition of HCl or NaOH, a magnet stirrer was also put inside the beaker for continuous stirring. Small amount of 1.0 M HCl stock solution was gradually added into the PBS buffer until a stable value of 6.5 was obtained. Then, 4 mL was taken out and labelled. HCl was further added until the next stable value 6.0 was achieved. Similarly, 4 mL of the solution was taken out. The process continued until a series solutions having pH values from 6.5 to 2.0 with 0.5 interval were obtained. For the preparation of solutions having pH values from 7.0 to 8.0 , the same procedures were applied and NaOH instead of HCl was added. Solutions with pH values higher than 8.0 were not prepared since they turned to suspension and became translucent due to the presence of HPO_4^{2-} and H_2PO_4^- ions in the PBS buffer.

Taking into account the low solubility of stilbene compounds in aqueous environment, solution mixtures of stilbene compounds with 10% DMSO and 90% above obtained buffer solutions having different pH (from 2.0 to 8.0 with 0.5 interval) were prepared for measurement. Then, their absorption spectrum, emission spectrum and fluorescence decay kinetics were measured and the apparent decay rate constants were calculated.

6.3 Results & discussions

6.3.1 Milli-molar concentrations of ethanol in aqueous solvents shift the fluorescence decay of DMASMA 17 (Figure 5.2)

ACN (5%) and water was used as the baseline solvent in these measurements. A series solutions having $6 \mu\text{M}$ DMASMA 17 but different amount of EtOH (from 0 to 450 mM) were prepared and characterized. Their normalized fluorescence decay curves were

summarized in **Figure 6.1**. With increasing concentrations of ethanol, the decay of **17** is retarded. **Figure 6.2** shows the plot of the calculated fluorescence decay rate constants against the corresponding EtOH concentration. The relationship obtained can be divided into three regions. From 0 to 5 mmol L⁻¹ EtOH, there is no significant difference among the decay rate constants. From 5 to 100 mmol L⁻¹, linear dependence ($r^2 = 0.92$) of **17**'s fluorescence decay rate constant on EtOH concentration is observed in **Figure 6.2**'s inset. When the EtOH concentration is 100 to 450 mmol L⁻¹, no linear correlation is seen.

Figure 6.1. The fluorescence decay curves of **17** in 5% ACN and water with increasing EtOH addition.

Figure 6.2. The relationship between the decay rate constant of **17** and the corresponding EtOH concentration, and the inserted graph showing the linear dependence in the EtOH concentration range from 5 mmol L⁻¹ to 100 mmol L⁻¹.

Hence, linear relationship is obtained only in the second region from 5 to 100 mmol L⁻¹.

To make the effective detection zone more precise and get a more reliable calibration equation, the same experiment was performed again with more focus on low EtOH concentrations. **Figure 6.3** displays the plot of fluorescence decay rate constant vs. the corresponding EtOH concentration for the range from 0 to 4 mmol L⁻¹ and 4 mmol L⁻¹ to 100 mmol L⁻¹ in **A** and **B** respectively. Similar to the previous results, no correlation was found for the lower concentration range (**Figure 6.3 A**). For the higher concentration range (**Figure 6.3 B**), linear relationship exists and can be described by:

$$k_{app} = 0.71 \pm 0.02 - (4.4 \pm 0.4) * [C_{EtOH}] \quad (6.2)$$

Figure 6.3. The relationship between the decay rate constant of **17** and the EtOH concentration for the EtOH concentration range from 0 to 4 mmol L⁻¹ (**A**) and from 4 mmol L⁻¹ to 100 mmol L⁻¹ (**B**) respectively.

Figure 6.4. The relationship between the decay rate constant of **17** and the IPA concentration for the studied IPA concentration range from 0 to 2.5 mol L⁻¹ (**A**) and from 0 to 60 mmol L⁻¹ (**B**) respectively.

Similar experiments were conducted for ethanol's analogues such as methanol and

isopropanol (IPA) in a concentration range including 4~100 mmol L⁻¹. So different amount of methanol and IPA were added into the solutions of DMASMA **17** in ACN (5%) and water, and the fluorescence decay curves of the solution mixtures were measured under the same decay conditions. **Figure 6.4** describes the relationship between the decay rate constant of **17** and the present IPA concentration. In **Figure 6.4 A**, when the IPA concentration is more than 500 mmol L⁻¹, the decay rate constant slowly increases. But in the lower IPA concentration range from 0 to 500 mmol L⁻¹ in which the effective EtOH detection zone is included, there is no significant difference observed for the decay rates as shown clearly in **Figure 6.4 B**. This means that under the conditions of low concentration detection, IPA does not interfere with the EtOH detection process.

Figure 6.5. The change of **17**'s apparent decay rate constant in ACN (5%) and water with methanol addition in the concentration range from 0 to 4.0 mol L⁻¹ (**A**) and from 0 to 80 mmol L⁻¹ (**B**).

For methanol addition, similar results were obtained. In **Figure 6.5 A**, when methanol concentration is higher than 1.0 mol L⁻¹, the decay rate constant gradually increases with methanol concentration. However, in the lower concentration range from 0 to 80 mmol L⁻¹ as shown in **Figure 6.5 B**, there is no significant difference observed. That is, in the effective detection region of EtOH (4~100 mmol L⁻¹), methanol will not play a great role in changing the fluorescence decay kinetics of **17** under low concentration conditions. This conclusion has also been observed in the experiment with IPA. Thus, based on the calibration curve obtained between ethanol concentration and decay rate constant of **17** in **Figure 6.3**, real time ethanol detection can be possible. In fermentation, accumulation of ethanol concentration slows down the reaction and the fermentation process. So the decay rate of DMASMA **17** may be used to reflect the ethanol concentration in real time, and

promote the fermentation process by in time ethanol removal.

As has been fully explained in section 5.3.7, the observed quenching of compound **17**'s fluorescence decay in ethanol is attributed to two factors: the strong donor substituent (dimethylamino group) of **17** and the polar and protic nature of ethanol. As analogues, the polarity and protic nature of methanol and IPA are similar to that of ethanol, and methanol is even more polar than ethanol. So, the observed fluorescence quenching in ethanol alone suggests that there are special molecular interactions or electronic interactions between **17** and ethanol which stabilize the excited state of **17** and prevent its conversion to the *cis* state. Further investigation of **17**'s decay behaviour in other alcohol solvents, solvent mixtures and complex environment as well as the detailed quenching mechanism of ethanol are recommended for future work.

6.3.2 pH influence on the photophysical properties of three stilbene maleamic acids

After preparation of solutions having different pH values based on the binding PBS buffer, the absorption, emission spectra and the fluorescence decay curves of the three stilbene maleamic acids **17**, **18** and **19** (**Figure 5.2**) were measured.

6.3.2.1 Photophysical properties of DMASMA **17** (**Figure 5.2**) depends on pH

Among the three stilbene maleamic acids, the dimethylamino substituent of **17** has the strongest donor ability. Its interactions with different pH solutions are expected to be the strongest. pH effect on **17**'s photophysical properties and fluorescence decay kinetics was studied first. **Figure 6.6 A** summarizes the absorption spectra of **17** in eight freshly prepared buffer solutions having pH value ranging from 2.5 to 6.0. Their normalized spectra are presented in **Figure 6.6 B**. The measured emission spectra and their corresponding normalized spectra in these eight solutions are shown in **Figure 6.6 C** and **D**.

Figure 6.6. The absorbance spectra (A), normalized absorption spectra (B), fluorescence emission spectra (C) and the normalized emission spectra (D) of **17** in solutions having different pH from 2.5 to 6.0.

In **Figure 6.6**, when solution mixture pH is decreased, the maximum absorption wavelength of **17** gradually shifts to the blue side and the absorbance value decreases. In the emission spectra, when pH is decreased, the fluorescence intensity of **17** decreases. Also, within the pH range of 6.0 to 4.5, there is no significant difference observed in terms of emission spectrum shape and the emission peak. However, when pH is lower than 4.5, red shift of the emission peak occurs with decreasing pH. Simultaneously, a new fluorescence peak on their blue side gradually develops and its emission intensity increases with the decreasing pH. The formation of a new emissive state might be explained by the intramolecular charge transfer in the excited states but other reasons such as solvation, hydrogen bonding or even dimers formation are also possible.

Figure 6.7. The fluorescence decay curves of **17** in solutions having different pH (A) and the relationship between the decay rate constant and pH (B).

The fluorescence decay curves of **17** in the eight solutions were measured. Their normalized decay curves and the plot of the apparent decay rate constant versus the corresponding pH are shown in **Figure 6.7**. From both figures, there are two different trends and pH 4.5 is the critical point, just as that observed in the normalized emission spectra. When the solution pH is higher than 4.5, there is no observable difference seen from the decay rate constants. But when pH is lower than 4.5, the decay rate becomes slower with decreasing pH.

Hence, among the absorption, emission spectra and fluorescence decay kinetics, DMASMA **17**'s absorption spectrum responds to the entire studied pH range by its peak shift and change of absorbance. Its emission intensity decreases with decreasing pH, but the emission spectrum shift is effective for pH lower than 4.5, as observed also for its fluorescence decay rate constant.

6.3.2.2 pH changes the photophysical properties of MSMA **18** (Figure 5.2)

The photophysical characterization of MSMA **18** (Figure 5.2) was conducted in solutions having pH from 2.0 to 8.0. The obtained absorption and emission spectra are illustrated in **Figure 6.8 A** and **B** respectively. In absorption spectra, there are two distinct zones: when pH is lower than 4.0, the absorption spectrum is broad and the peak is not obvious. When solution pH is higher than 4.0, the peaks of the obtained spectra are distinguishable and no shift is observed among the peaks. The absorbance value increases with the increasing pH except from pH 7.0 to pH 8.0. For the emission spectra, in the pH range from 2.0 to 3.0

and 4.5 to 8.0, no obvious change in fluorescence intensity and emission peak is observed. Only in a small pH region from 3.0 to 4.5, fluorescence intensity gradually increases with increasing pH. The same trend is seen in its fluorescence decay kinetics results.

Figure 6.8. The absorption spectra (A) and the emission spectra (B) of **18** in solutions having pH from 2.0 to 8.0.

Figure 6.9 A shows the normalized decay curves, and the plot of the calculated apparent decay rate constant versus solution pH is displayed in **Figure 6.9 B**. The decay rate constants have no significant difference in solutions having pH greater than 4.0. Increase in decay rate is only observed when pH is increased from 3.0 to 4.0. That is, by either its emission spectrum or fluorescence decay kinetics, **18** can only roughly indicate whether the solution pH is higher or lower than 4.0. The response of its absorption spectrum to pH is effective in the pH range from 4.0 to 7.0. The less effectiveness of **18** in pH probing than **17** is suspected to be attributed to its methoxy stilbene substituent having weaker donor ability.

Figure 6.9. The normalized fluorescence decay curves of **18** in different pH solutions (A) and the plot of its apparent decay rate constant vs. pH (B).

6.3.2.3 Photophysical properties of HSMA **19** (Figure 5.2) depends on pH

The same photophysical characterization and fluorescence decay kinetics measurements were conducted for HSMA **19** in solutions having different pH values. **Figure 6.10 A** demonstrates the change of **19**'s absorption spectrum with increasing pH from 2.0 to 8.0. The emission spectrum obtained under the same measurement conditions are summarized in **Figure 6.10 B**.

Figure 6.10. The absorption spectra (A) and the emission spectra (B) of **19** in solutions having different pH.

Figure 6.11. The normalized fluorescence decay kinetics of **19** in solutions having different pH (A) and the relationship between its apparent decay rate constant with pH (B).

Though methoxy and hydroxyl substituents have close electron-donating abilities, the trend observed in their absorption spectrum are different. With pH increasing from 2.0 to 4.5, the absorbance values of **19** increases and its maximum absorption wavelength shifts to the red side. When pH is higher than 4.5, no significant difference can be seen among the spectra. While for the emission spectrum, the results are similar to that obtained in **18**. In

solutions having pH higher 4.5, **19**'s emission spectra are nearly the same. The fluorescence intensity increase with increasing pH is only valid when pH ranges from 3.0 to 4.5.

Figure 6.11 summarizes the normalized fluorescence decay curves and presents the relationship between the decay rate constant and the solution pH value. There is no dependence of decay rate constant on the solution pH when pH is higher than 5.0. When pH is lower than 5.0, the decay rate increases with the increasing pH. Hence for **19**, its reactive region for pH probing is 2.0~4.5 by either its absorption spectrum or the fluorescence decay kinetics.

Table 6.1. The effective pH sensing region for three maleamic acids (**17**, **18** and **19**) based on their absorption, emission and fluorescence decay kinetics.

Compound	Structure	Absorption	Emission	Decay kinetics
17		2.5~6.0 (peak shift & absorbance)	2.5~6.0 (fluorescence intensity)	2.5~4.5 (decay rate constant)
18		4.0~7.0 (absorbance)	3.0~4.5 (fluorescence intensity)	3.0~4.0 (decay rate constant)
19		2.0~4.5 (peak shift & absorbance)	3.0~4.5 (fluorescence intensity)	2.0~4.5 (decay rate change)

As a summary for the three stilbene maleamic acid compounds (**17**, **18** and **19**), their respective pH sensing region based on their absorption, emission and decay kinetics are presented in **Table 6.1**. The responses of their absorption and emission spectrum to different pH solutions are different, but their decay rate constants' pH dependence profiles are similar. They share the common critical point of pH 4.5. In solutions having pH higher than 4.5, there is no dependence of the decay rate constant on pH, and when pH is lower than 4.5, decay rate constant increases with increasing pH. Theoretical calculations for **17** have shown that when **17** is protonated, the positively charged dimethylamino group twists to the orthogonal configuration resulting in a more planar molecule, thereby stabilising the Franck-Condon state, increasing the activation barrier and decreasing the decay rate [7]. So, when the surrounding pH of **17** is lower than 4.5, conformational change occurs and decrease of its fluorescence decay rate constant is resulted. Since the three stilbene

maleamic acids have similar decay rate constant vs. pH plots, the conclusion also applies to **18** and **19**. As the decay rate constant of three stilbene maleamic acids which is independent of background fluorescence intensity, has common response to pH in the range of 2.0~4.5, the application of stilbene maleamic acids in strong acidic and dirty environment may be possible.

6.4 Conclusions

Based on the fluorescence quenching of DMASMA **17** in the presence of EtOH. A linear relationship between EtOH concentration and **17**'s fluorescence decay rate constant is obtained for the EtOH concentration range of 4 mmol L⁻¹ to 100 mmol L⁻¹. The equation is expressed as: $k_{app} = 0.71 \pm 0.02 - (4.4 \pm 0.4) * [C_{EtOH}]$. Same experiments were conducted for methanol and IPA, and in the effective detection range of EtOH, there is no dependence of **17**'s decay rate on the methanol or IPA concentration. So low ethanol concentration detection based on **17** is possible and this might be applied in fermentation process monitoring. For the results of pH effect on the absorption, emission spectra and fluorescence decay kinetics of three stilbene maleamic acids, the trends of their decay rate constant's dependence on pH are similar. When pH is lower than 4.5, their decay rate constant decreases with pH decreasing, but when pH is above 4.5, their decay rate constants have no dependence on pH. This conclusion makes the maleamic acids more applicable in strong acidic discharge monitoring.

References

- [1] G.I. Likhtenshtein, R. Bishara, V. Papper, B. Uzan, I. Fishov, D. Gill, A. Parola, *Journal of Biochemical and Biophysical Methods*, 33 (1996) 117-133.
- [2] G.I. Likhtenshtein, D. Khudjakov, V. Vogel, *Journal of Biochemical and Biophysical Methods*, 25 (1992) 219-229.
- [3] V. Papper, G.I. Likhtenshtein, N. Medvedeva, D. Khoudyakov, *Journal of Photochemistry and Photobiology A: Chemistry*, 122 (1999) 79-85.
- [4] V. Papper, G.I. Likhtenshtein, *Journal of Photochemistry and Photobiology A: Chemistry*, 140 (2001) 39-52.
- [5] N. Strashnikova, V. Papper, P. Parkhomyuk, G.I. Likhtenshtein, V. Ratner, R. Marks, *Journal of Photochemistry and Photobiology A: Chemistry*, 122 (1999) 133-142.
- [6] V. Papper, O. Pokholenko, Y. Wu, Y. Zhou, P. Jianfeng, T.W.J. Steele, R.S. Marks, *Journal of Fluorescence*, 24 (2014) 1581-1591.
- [7] Y. Wu, V. Papper, O. Pokholenko, V. Kharlanov, Y. Zhou, T.W.J. Steele, R.S. Marks, *Journal of Fluorescence*, 25 (2015) 961-972.

Chapter 7

Summary and future work

This chapter summarizes the entire project as a whole. Based on the obtained results, the hypotheses are deeply addressed and future work is thus pointed out. Some preliminary results for the Bisphenol A (BPA)-BPA aptamer trial system are presented, demonstrating the medium viscosity dependence of the decay rate of two synthesized stilbene-Bisphenol A conjugates. Lastly, according to the challenges encountered and the solutions figured out, some recommendations are given.

7.1 Summary

Based on the dependence of stilbene compounds' fluorescence decay rate on their surrounding medium viscosity and the adaptive binding kinetics of aptamer, a photo-chrome aptamer switch assay (PHASA) has been developed and two scopes: competitive binding and adaptive binding are proposed. In competitive binding, the focus is the competition between analyte and stilbene-analyte conjugate towards aptamer binding. The adaptive binding, on the other hand, relies on decay rate change of stilbene compounds conjugated to aptamer. When bound to malachite green aptamer, the fluorescence quantum yield of malachite green (MG) is enhanced more than 2000 times. Together with other advantages such as well-defined crystal structure, various binding targets and specific site modification, MG–MG aptamer was chosen as the model system for PHASA study. To ensure measurable fluorescence decay and reactivity, two parent stilbenes 4-dimethylamino-4'-aminostilbene (**1**, **Figure 4.1**) and 4-methoxy-4'-aminostilbene (**2**, **Figure 4.1**) were selected. Their fluorescence decay in aqueous mixtures (volume of organic solvent is 3%) shows their adaptability in MG aptamer binding buffer. Their fluorescence decay rate dependence on surrounding medium viscosity makes the detection based on environmental change such as aptamer kinetics possible.

For competitive binding scope, based on the two parent stilbenes, four stilbene-MG conjugates were synthesized to find the optimum conjugate that have both binding affinity to MG aptamer and fluorescence decay in the PBS binding buffer based environment. Besides, a negative control was also synthesized based on rhodamine B and 4-dimethylamino-4'-aminostilbene since it is known that rhodamine B has no binding to MG aptamer. Binding experimental results based on both fluorescence decay and fluorescence polarization show that no binding occurs between MG aptamer and the negative control. This confirms that stilbene conjugation to MG does not cause any additional binding and binding is only attributed to MG fragment of stilbene-MG conjugates.

Among the four stilbene-MG conjugates, a “fused” stilbene MG conjugate (**5**, **Figure 4.1**) was obtained to minimize configurational interruption to MG binding pocket. Its direct binding with MG aptamer gives rise to a dissociation constant K_d value of 500 nM, which is 2.5 times higher than that of MG analyte (200 nM), and is the lowest among the four studied stilbene-MG conjugates. That is, this “fused” stilbene-MG conjugate indeed

preserves the majority of the interactions in the MG aptamer binding pocket formation. However, no obvious fluorescence decay was observed for this conjugate in various organic solvents such as ACN, DMSO, EtOH etc. Thus the conjugate cannot be applied in competitive binding scope. The quenched decay is explained by the possible resonance structure formed from electron conjugation of the stilbene and MG fragment, which prevents the *trans-cis* isomerization of the stilbene fragment and results in non-observable fluorescence decay. To electrically decouple the stilbene and MG fragments and restore the decay behavior of stilbene component, a linker was thus introduced.

Therefore, MG-COO-Stil-NO₂ conjugate (**6**, **Figure 4.1**) with an ester linker and nitro stilbene substituent was synthesized. However, quenched fluorescence decay is observed for this conjugate, and the obtained dissociation constant K_d value from binding experiments is 6.6 μ M which is the highest among the four stilbene-MG conjugates. The weakest binding affinity is suspected to be caused by the ester linker which causes partial charge separation with the third phenyl ring of MG, interrupting the electronic interactions for binding pocket formation. Hence, the reactivity of the linker needs to be reduced. The quenched decay is attributed to the quenched fluorescence intensity, making the decay non-observable. Nitro group is believed to be responsible for the low intensity (<0.1% of parent stilbene **2**), and other donor stilbene substituent needs to be used, such as methoxy group. With the introduction of a -CH₂-NH- linker between the stilbene and MG fragment and a methoxy stilbene substituent, obvious fluorescence decay kinetics in organic solvent as well as PBS buffer based aqueous solution mixtures are observed for the obtained stilbene-MG conjugate MG-CH₂-NH-Stil-OMe (**7**, **Figure 4.1**). This confirms the electron decoupling achieved by the linker, and makes the PHASA detection based on decay rate change possible. The dissociation constant K_d obtained for this conjugate is 2.4 μ M, which is 2.75 times lower than the MG-COO-Stil-NO₂ conjugate (**6**, **Figure 4.1**). This indicates no electronic conjugation between the linker and MG fragment. However, this binding affinity is still 12 times higher than that of MG analyte (200 nM). This makes the final binding experiment in competitive binding scope less effective and thus limit the application. The relative weak binding of **7** is suspected to be caused by its linker's electron donating property that attracts some of the positive charges localized in the other two rings

of the MG fragment. So acetylation of the linker was proposed for binding affinity improvement.

MG-CH₂-NAc-Stil-OMe (**8**, **Figure 4.1**) was synthesized by acetylation of the conjugate **7**. Similarly, electron decoupling was achieved by the -CH₂-NAc- linker and measureable fluorescence decay in buffer based aqueous solution mixtures are observed. The dissociation constant obtained from binding experiments is 1.7 μ M, higher than that of conjugate **7** (2.4 μ M), meaning its binding affinity to MG aptamer has been improved.

Based on the four studied stilbene-MG conjugates for PHASA competitive binding scope, it can be concluded that a linker having weak electron donating/accepting property is essential between the MG and stilbene fragment of stilbene-MG conjugate to prevent their electronic conjugation, preserve the decay kinetics of the stilbene fragment, and maintain the electronic distribution of MG component. Moreover, considering both the fluorescence decay kinetics and the binding affinity to MG aptamer, MG-CH₂-NAc-Stil-OMe conjugate (**8**, **Figure 4.1**) is the most promising candidate for PHASA competitive binding scope.

Under adaptive binding scope, MG aptamer was thiol modified at four different locations (C38, A9, U4 and C20). Their binding experiments with MG analyte produce similar K_d values, in the range of 700 nM ~1 μ M. This means that they still preserve the binding affinity to MG analyte although the bindings are weaker than the wild type MG aptamer (K_d =200 nM). Based on the thiol–maleimide cross-linking reaction, two stilbene maleimide compounds were synthesized. Their decay kinetics in organic solvents (ACN and DMSO) presents obvious fluorescence decay. In the medium viscosity study, the decay rate constants of both stilbene maleimide compounds show strong dependence on their medium viscosity and a linear relationship between their decay rate constant and the reciprocal of viscosity value in log scale has been obtained. When their decay kinetics was measured in DMSO (v% is less than 10%) and PBS buffer solution mixtures, however, both stilbene maleimide compounds' decay are quenched. Under the same measurement conditions, the obtained 6MCH-MSMI conjugate (**20**, **Figure 5.4**) after thiol (6MCH) conjugation of MSMI (**16**, **Figure 5.2**) shows obvious fluorescence decay. So the decay of stilbene maleimide compounds in aqueous environment is restored after thiol conjugation. Based on the decay rate change of stilbene component in the thiol conjugated compounds, subsequent detection can be conducted without further separation of stilbene maleimide

compounds. The linear dependence of the 6MCH-MSMI conjugate's fluorescence decay rate on surrounding medium viscosity is observed. Further supported by the generation of DMASMI-SH MG aptamer complex, it can be concluded that the attached stilbene fragment still preserves its fluorescence decay kinetics in the PBS binding buffer environment, and the change of its fluorescence decay rate can be used for the subsequent MG analyte detection without separation from the stilbene maleimide compounds.

To enhance stilbene compounds' solubility in aqueous solutions and thus minimize the aqueous quenching effect, three stilbene maleamic acid compounds with carboxylic group—DMASMA, MSMA and HSMA (**17**, **18** and **19**; **Figure 5.2**) were synthesized. Their decay kinetics study results in organic solvents (ACN, DMSO and EtOH) and the corresponding water/buffer based aqueous mixtures (v% of organic solvents is less than 10%) show that their fluorescence decay were very obvious except that the decay of DMASMA (**17**, **Figure 5.2**) was quenched in the presence of EtOH. Also, for all the three stilbene maleamic acids, a linear relationship between their decay rate constant and the reciprocal of viscosity in log scale is observed. The results obtained from the real time reaction monitoring between MSMA (**18**)/HSMA (**19**) and 6MCH also indicate that the reaction between stilbene maleamic acids and thiol molecule is successful and the conjugation of stilbene maleamic acids to SH-MG aptamer is achievable. Therefore, for PHASA adaptive binding scope, both stilbene maleimide and stilbene maleamic acid compounds are promising, since after thiol conjugation and being attached to the thiol modified MG aptamer, the fluorescence decay of the attached stilbene component is preserved in MG aptamer binding buffer environment. Based on the decay rate change, MG analyte detection is possible.

Lastly, other applications of the studied stilbene compounds were explored. For the EtOH effect study based on DMASMA (**17**, **Figure 5.2**), an effective EtOH detection range from 0.004 mol L^{-1} to 0.1 mol L^{-1} is obtained, in which a linear relationship between the ethanol concentration and the decay rate of DMASMA is observed. In this detection region, both IPA and methanol show no effect. As to the pH effect study for three stilbene maleamic acids, their absorption, emission spectrum and fluorescence decay kinetics show dependence on their surrounding solution pH, which can be used for pH indication. That is, besides being used in biological systems as viscosity probes, there are wide applications for stilbene compounds in analyte detection and sensing.

7.2 Preliminary results of bisphenol A (BPA)-BPA aptamer trial system

As mentioned in chapter 1, MG-MG aptamer is used as a model system for the investigation of PHASA detection. Therefore, trial experiments were conducted for the bisphenol A (BPA)-BPA aptamer system and some preliminary results under PHASA competitive binding scope were obtained. Based on MAS (**2**, **Figure 4.1**) and BPA's analogue—4,4-Bis(4-hydroxyphenyl)valeric acid (BVA) having an reactive carboxylic group, two stilbene-BVA conjugates **22** and **23** (**Figure 7.1**) were obtained. Their photophysical properties, decay kinetics and decay rate dependence on medium viscosity were studied.

Figure 7.1. Synthesis procedures for two stilbene-BVA conjugates **22** and **23**.

Stilbene-BVA conjugate 22—decay rate depends on surrounding medium viscosity

The photophysical properties such as absorption, emission spectrum and decay kinetics of stilbene-BVA conjugate **22** were studied. The results are summarized in **Figure 7.2 A** and **B**.

There is no electronic conjugation between the BVA and stilbene fragments, and the absorption and emission peaks in **Figure 7.2 A** characterize the stilbene component. The conjugate's absorption and emission peaks are red shifted in DMSO than in ACN. Its decay rate in ACN is higher than in DMSO (**Figure 7.2 B**). The spectrum red shift is attributed to the higher polarity of DMSO which stabilizes the electron distribution of ground states. For the decay kinetics behavior, this is because the amide group is electron-accepting,

making the twisted P* state less stable in more polar solvent and thus giving rise to lower fluorescence decay rate.

Figure 7.2 **A.** Normalized absorption (solid line) and emission (dash line) spectrum of stilbene-BVA conjugate **22**. **B.** The decay kinetics of **22** in ACN (black) and DMSO (grey). **C.** Fluorescence decay curves of **22** in solutions having different DMSO to glycerol ratio. **D.** The plot of **22**'s decay rate vs. 1/viscosity in log scale.

To study the fluorescence decay rate dependence of conjugate **22** on its surrounding medium viscosity. Its decay kinetics in solution mixtures having different DMSO to glycerol volume ratios were measured. The normalized decay curves are shown in **Figure 7.2 C**. Their apparent decay rate constants were also calculated and then plotted against the reciprocal of the corresponding viscosity value in log scale, shown in **Figure 7.2 D**. Strong decay rate dependence on medium viscosity is observed. A linear relationship for the viscosity range (6.23~215.8 cP) is obtained, and is expressed as: $k_{app} = (0.632 \pm 0.009) + (0.098 \pm 0.006) \cdot \log(1/\eta)$. This dependence paves its way for subsequent experiments in PHASA competitive binding.

As aforementioned, the amide linker is electron-accepting, and fluorescence decay of the stilbene component is less preferred than emission in polar solvents. To enhance the decay kinetics, the accepting power of the amide linker is to be reduced. Thus, by reducing the amide linker to $-\text{CH}_2\text{-NH}-$, stilbene-BVA conjugate **23** was obtained.

Stilbene-BVA conjugate 23—fluorescence decay depends on medium viscosity and is preserved in aqueous environment

Figure 7.3 **A.** Absorption spectrum change of conjugate **23** in DMSO with time. **B.** The emission spectra of **23** in DMSO under the excitation of 291 and 359 nm. **C.** The fluorescence decay of **23** at Ex 359 nm/Em 436 nm in DMSO.

Before the photophysical characterization of conjugate **23**, its stability was checked by monitoring its absorption spectrum change in DMSO with time. The results in **Figure 7.3 A** shows that within 70 mins, there is no change in absorption spectrum observed. Since there is no electron coupling between the stilbene and BVA components, the observed absorption peak 359 nm characterizes the stilbene fragment and the shoulder peak 291 nm

is suspected to represent the BVA fragment. Under the excitation of both peaks, the emission spectra were measured and a common emission peak 436 nm was obtained, as shown in **Figure 7.3 B**. The single emissive state originates from the stilbene fragment since BVA is literally non-fluorescent. Therefore, decay conditions of Ex 359 nm/Em 436 nm are used in the subsequent decay experiments. **Figure 7.3 C** presents the fluorescence decay of **23** in DMSO and the calculated decay rate constant. Comparing to the decay rate of stilbene-BVA conjugate **22** under the same conditions, the decay of **23** is enhanced ~40% than **22**. That is, the reduction of the amide linker in **22** indeed enhance the obtained conjugate **23**'s fluorescence decay. This provides conjugate **23** greater flexibility as an optical transducer since decay rate change is used for sensing in PHASA.

Figure 7.4 A. Normalized fluorescence decay curves of **23** in solution mixtures having different viscosities. The relationship of k_{app} vs. $1/\eta$ (**B**) and fluorescence polarization vs. η (**C**) for conjugate **23**.

To confirm the medium viscosity effect on the decay rate response from conjugate **23**, its decay kinetics in solution mixtures having different amount of DMSO and glycerol was

measured. The obtained decay curves were normalized and displayed in **Figure 7.4 A**. Increasing fluorescence decay with decreasing amount of glycerol can be observed among the seven DMSO and glycerol mixtures. The obtained decay rate constants were calculated and plotted against viscosity, shown in **Figure 7.4 B**. In the entire viscosity range studied (2.68~215.8 cP), the conjugate **23**'s decay rate is linearly dependent on the reciprocal of the medium viscosity. This dependence is supported by the fluorescence polarization results in **Figure 7.4 D**. The obtained relationships are expressed as

$$k_{app} = (0.249 \pm 0.003) + (0.041 \pm 0.003) * \log(1/\eta) \quad (7.1)$$

$$FP = (0.289 \pm 0.008) + (0.060 \pm 0.006) * \log(\eta) \quad (7.2)$$

That is, the decay rate of the conjugate **23** responds promptly to the surrounding environment change, which can be caused by aptamer binding kinetics.

Figure 7.5. The fluorescence decay (A), normalized decay kinetics (B) and apparent decay rate constant (C) of conjugate **23** in three water (80, 90 and 95 in v%) and DMSO mixtures.

Similar to the MG-MG aptamer model system, the final binding experimental environment is BPA aptamer binding buffer based. So aqueous quenching effect needs to be studied for

conjugate **23**. Three solution mixtures consisting DMSO and water (80, 90 and 95 in v%) were prepared, in which the decay kinetics of conjugate **23** were measured. The normalized decay curves and apparent decay rate constants are summarized in **Figure 7.5**. Aqueous quenching effect is significant. When water percentage is increased from 80% to 90%, the fluorescence intensity decreases about three times (**Figure 7.5 B**) as well as the decay rate constant (**Figure 7.5 C**). In the solution mixture of 95% water and DMSO, the decay rate is $\sim 0.1\text{ s}^{-1}$, significantly constrains the flexibility. Hence, for the application of **23** in binding buffer based environment, 20% organic solvent (DMSO) will be a better choice.

7.3 Future work

Based on the results obtained in chapter 5, stilbene compounds are found to be promising in other applications besides medium viscosity probes. So some future work are proposed.

- The proof of concept for PHASA has been explored based on MG and MG aptamer which is a RNA aptamer. Since DNA aptamer is more stable than RNA aptamer, the PHASA detection based on BPA/BPA aptamer (DNA aptamer) should be continued based on the results obtained above.
- As previously presented in chapter 6, ethanol can quench the fluorescence decay of DMASMA (**17**, **Figure 5.2**), and a linear detection range has been found. Moreover, there is no interference on **17**'s decay from ethanol's analogues—methanol and isopropanol. The decay kinetics of **17** should be further investigated in other alcohol solvents, solvent mixtures and complex environment and the mechanism of **17**'s quenching in ethanol needs to be addressed. Then, based on the quenching, fast and real-time ethanol detection based on the fluorescence decay rate change of **17** may be developed. This may be used to promote industrial fermentation process by real time controlling of ethanol concentration.
- In chapter 6, the pH effect on the three stilbene maleamic acids (**17**, **18** and **19**; **Figure 5.2**) has been demonstrated. Their photophysical properties, especially the decay rate constant responds to pH values less than 4.5. So their further applications in highly acidic discharge monitoring in real time and on site can be significant.
- In chapter 5, the fluorescence decay of two stilbene maleimide compounds (**15**, **16**; **Figure 5.2**) restores after thiol conjugation in aqueous environment. This

observation may be applied for thiol detection in biological environment, since the detection is based on decay rate change rather than fluorescence intensity. Thus, the noise intensity from background won't affect the sensitivity of the detection process.

7.4 Recommendations

Based on the problems encountered and the useful conclusions obtained in the project, here are some recommendations for future related work:

- To preserve the fluorescence decay kinetics of stilbene fragment in the synthesized stilbene-analyte conjugates under competitive binding scope, a linker that can electronically decouple the two fragments is essential. Otherwise, it is likely that the decay cannot be observed.
- Stilbene substituents that quench stilbene fluorescence should be avoided as the fluorescence quenching results in decay being non-observable.
- Water soluble stilbene compounds should be preferred, since the final binding environment is aptamer binding buffer (aqueous) based and the quenching effect from aqueous environment on stilbene compounds is significant.
- Organic solvents or stilbene substituents are also important in determining the final conjugate's property and thus should be wisely selected.

Appendix

Synthesis of stilbene-BVA conjugate **22** (Figure 7.1)

4,4-Bis(4-hydroxyphenyl)valeric acid (BVA) (760 mg; 2.654 mmol L⁻¹) was dissolved in a solution mixture of 1 mL methanol, 2 mL pyridine and 10 mL diethyl ether. To the solution, 1.0 mL SOCl₂ was added. The mixture was left to stir at room temperature for 3 h. Then, 1 mL methanol, 20 mL ethyl acetate and HCl was added to acidify the aqueous reaction medium. The organic layer was collected, and after solvent evaporation, 980 mg brown oil (**21**) was obtained.

The brown oil (**21**, 2.63 g) was heated with 700 mg MAS (**2**) under nitrogen for 24 h at 200 °C. Then the mixture was dissolved in 20 mL CH₂Cl₂ and filtered via silica pad. After eluting with 80 mL CH₂Cl₂/i-PrOH (1:1) and subsequent solvent evaporation, dark oil was obtained. It was dissolved in 20 mL DMSO, which was poured into 100 mL of 7% NaOH. The aqueous mixture was extracted by toluene/i-PrOH (3:1) mixture (2x130 mL). After discarding the organic layer, the pH of the remaining solution was measured to be 13.7. It was gradually acidified until sudden separation of white emulsion. Then, the mixture was extracted by ethyl acetate/i-PrOH (3:1) (2x100 mL), and pH of the obtained aqueous layer was 9.0. So the aqueous layer was acidified again and extracted with 100 mL ethyl acetate. The extracted organic layers were unified and washed with aqueous NaHCO₃ (100 mL) and water. After solvent evaporation, 1.5 g dark oil left. The oil was dissolved in CH₂Cl₂ and flash chromatography was performed with CH₂Cl₂/i-PrOH gradient. The obtained fractions were evaporated, leaving a brown solid which was suspended in CH₂Cl₂ and collected on a filter. This yielded 210 mg (13%) of a creamy solid. ¹H NMR, 400 Hz, CDCl₃: 1.578 (3H; s; Ar₂C-CH₃); 2.157 (2H; m; -CH₂-); 2.466 (2H; m; -CH₂-); 3.189 (3H; s; Ar-O-CH₃); 6.739 (4H; d; 8.5 Hz); 6.869-6.891 (2H; d; 8.7 Hz); 6.877-6.918 (1H; d; 16.4 Hz); 6.945-6.985 (1H; d; 16.4 Hz); 7.045 (4H; d; 8.5 Hz); 7.386 (2H; d; 8.6 Hz); 7.403 (s; CHCl₃); 7.420 (2H; d; 8.8 Hz); 7.557 (2H; d; 8.6 Hz); 8.517 (2H; s; -Ar-OH); 8.859 (1H; broad s; NH). ¹³C NMR, CDCl₃: 28.053 (-CH₃); 33.217 (-CH₂-); 37.049 (-CH₂-); 44.387; 55.286 (Ar-OCH₃); 114.093; 114.929; 119.730; 126.150; 126.523; 126.754; 127.465; 128.212; 130.222; 132.711; 138.169; 140.136; 154.940; 159.045; 172.115 (HN-C=O). MALDI TOF (*neat*, reflectron mode, positive) m/z 494.09 [M+H]⁺ (calculated

494.23).

Synthesis of stilbene-BVA conjugate 23 (Figure 7.1)

Stilbene-BVA conjugate **22** was dissolved in 20 mL anhydrous dioxane, to which was added 20 mL of 1 mol L⁻¹ lithium aluminium hydride solution in ether. The reaction mixture was heated under nitrogen for 2 days. Then the reaction was quenched by careful addition of 10 mL ethyl acetate. After that, water and acetic acid was added until pH reached 4. The formed precipitate was filtered and the organic layer was separated from the diphasic mixture. After solvent evaporation of a fraction of the obtained organic layer, 200 mg yellow solid was obtained. The solid was dissolved in a solution mixture of 0.6 mL DMSO, 0.3 mL water and 50 µL acetic acid, and flash chromatography on reverse phase (Sepacore 12 g C18 cartridge) was conducted by eluting 0.2% acetic acid/i-PrOH gradient. The respective fractions were collected, and after evaporation, 26 mg of a yellow solid was obtained. ¹H NMR, 400 Hz, CDCl₃: 1.417 (2H; m; -CH₂-); 1.573 (3H; s; C-CH₃); 2.116 (2H; m; CH₂); 3.072 (2H; t); 3.817 (3H; s; -OCH₃); 6.505 (2H; d); 6.718 (4H); 6.814-6.855 (1H; d; 16.4 Hz); 6.867-6.908 (1H; d; 16.4 Hz); 6.872 (2H; d); 7.040 (2H; d); 7.296 (2H; d); 7.396 (2H; d). MALDI TOF (*neat*, reflectron mode, positive) m/z 479.14 [M+H]⁺ (calculated 480.25).

Rational for PHASA sensitivity estimation

The limit of detection (LOD) for PHASA detection is based on the results obtained so far and several assumptions. In the final binding experiment of PHASA competitive scope, stilbene-analyte conjugates are allowed to bind to analyte aptamer first before the addition of the analyte.

Assumption 1: stilbene-analyte conjugates are fully bound inside the aptamer binding pockets and a “flat” fluorescence decay curve will be obtained.

Subsequently, when analytes are added, analyte aptamer prefers to bind to analyte and releases the stilbene-analyte conjugates, resulting in fluorescence decay rate decrease. The minimum amount of analyte can be detected thus depends on the minimum decay rate signal for measurement. Based on the definition of limit of detection (3σ), assumption 1

and the method for decay rate constant calculation, the minimum rate $k_{\min(\text{app})}$ ($\sim 0.08 \text{ s}^{-1}$ under the instrumental conditions in this thesis) can be estimated.

Assumption 2: the linear relationships between decay rate constant and the reciprocal of medium viscosity in log scale can be used directly in the aptamer system.

For demonstration and simplification, the sensitivity of parent stilbene's decay rate to medium viscosity is used for the LOD estimation for its corresponding stilbene-analyte conjugate. To achieve the minimum decay rate detection signal $k_{\min(\text{app})}$, the overall viscosity η_1 for stilbene-analyte conjugate can be calculated as:

$$\log(1/\eta_1) - \log(1/\eta_0) = \frac{k_{\min(\text{app})}}{k_{\text{slope}}} \quad (\text{A.1})$$

Where η_0 represents the viscosity environment of stilbene-analyte conjugates when they are fully bound inside the aptamer binding pocket and there is no analyte addition, and k_{slope} refers to the slope of the k_{app} vs. $\log(1/\eta)$ plot for the conjugates' corresponding parent stilbene obtained from medium viscosity study.

Assumption 3: the viscosity environment for stilbene-analyte conjugate is proportional to the amount bound inside the aptamer binding pocket and in free solutions.

Based on assumption 3, to get η_1 for minimum detection, the ratio r_{free} of stilbene-analyte conjugate should be released can be estimated by:

$$r_{\text{free}} * \eta_{\text{free}} + \eta_0 * (1 - r_{\text{bound}}) = \eta_1 \quad (\text{A.2})$$

Where r_{bound} is the ratio of the conjugates still bound inside the aptamer binding pocket, and η_{free} refers to the viscosity value of free solution.

Finally, based on the binding affinity (K_d) of stilbene-analyte conjugate and analyte towards the analyte aptamer, the minimum amount of analyte added can be estimated, which is considered as the limit of detection for the analyte based on PHASA.

From chapter 4, the most promising stilbene-MG conjugate is conjugate **8** (**Figure 4.1**). Assuming the aptamer viscosity η_0 for conjugate **8** is 400 cP and the viscosity in free solution is 1.0 cP, and based on the results for k_{slope} (0.16 s^{-1} ; equation 4.2), the limit of detection for MG analyte is roughly 3 μM (μM range detection limit).