NANYANG
TECHNOLOGICAL
UNIVERSITY

PHOTOCHROME APTAMER SWITCH ASSAY

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SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

2017

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A thesis submitted to the Nanyang Technological University
in partial fulfilment of the requirement for the degree of
Doctor of Philosophy

2017

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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20 July 2016

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Abstract

Aptamers are known to undergo adaptive conformational change upon ligand binding, causing micro-environment rearrangements that may be linked with stilbene transducers. Stilbene displays fluorescence decay upon continuous excitation, which is sensitive to micro-environment sterics. This thesis hypothesizes that fluorescence decay based biosensing can be achieved by incorporating the molecular recognition properties of aptamer with time-dependent stilbene *trans-cis* photodecay. Two designs were proposed and tested according to the hypothesis: 1) “competitive binding of stilbene-analyte conjugate with analyte” and 2) “stilbene-aptamer conjugate fluorescence decay correlates to analyte binding”. Malachite green aptamer (MGA) and its ligand malachite green (MG) were chosen as a model study to test the hypothesis. Prior to hypothesis investigation, experimental conditions were established and optimized. The MGA/MG binding was found to have hydrolysis-dependent photodecay, but organic additives were found to reverse the hydrolysis and ultimately stabilize the binding. For the design 1), MG-stilbene conjugates obtained for the hypothesis testing had micromolar binding affinities with appreciable photodecay. Based on the MG-stilbene conjugates analyzed, electron donating needs to be optimized towards retaining the stilbene fluorescence decay property in MG-stilbene conjugate. In the design 2), stilbene was grafted on aptamer to fabricate the stilbene aptasensor through amine-isothiocyanate click chemistry. This conjugate maintained properties of aptamer binding and stilbene fluorescence decay, where presence of MG inhibited the fluorescence decay of MGA-SITS. The fluorescence decay of this aptasensor was also sensitive to ligand tetramethylrosamine (TMR) but presented no response to another non-binding triphenyl molecule rhodamine B (RhB), displaying good selectivity. The sensitivity of the MGA-SITS aptasensor was low for MG, when compared to TMR. Future work aims to improve the performance of the fluorescence decay based aptasensor.

Acknowledgements

Finally, here is a space allowing writing without rationality and logic. Yes, this page is all about emotional appreciations, which seems common but really essential. Let's go.

Definitely, I would like to present my deepest appreciation to my supervisor, Professor Terry W. J. Steele, for his excellent guidance, help, support and suggestions. His critical thinking always lights up my ideas on research work, which will play an important role throughout my research life. Without his help and support, nothing is still noting---this dissertation and the published papers would have not been possible.

I am very thankful for my beloved family for their always love and support on my study and research, even though they do not really understand what I am doing. I would really appreciate all the encouragement from my family.

I wish to express my gratitude towards my thesis committee members Professor Hu Xiao and Professor Ali Gilles Tchenguise Miserez for their time and valuable suggestions on my project, presentation and the thesis.

I would also like to thank my project partner Ms. Wu Yuanyuan. Her contribution greatly helps improve the project and the thesis. Thank Dr. Vladislav Papper for his professional help on photochemistry and stilbene characterization. Particularly, thank Dr. Oleksandr Pokhonenko for his help in my research and for the materials he provides. Specially, thank Dr. Ping Jianfeng for his kindly help, mentoring and suggestions on my experimental work on biosensors.

Thank Professor Robert S. Marks for his suggestions and supporting on my topic.

Thank Professor Souhir Boujday for her kindly guidance on teaching me new knowledge on interface.

I am also very thankful to all the members of our group, both the current and the former group members. It is my pleasure to be in the same group with Raghavendra (Dr.), Alok (Dr.), Sing Shy (Dr.), Jianlin (Dr.), Hong (Dr.), Kumar (Dr.), Richard (Dr.), Ivan (Dr.), Lu (Dr.), Himansu (Dr.), Vishal (Dr.), Su Yin, Divya, Feng, Ting, Yuanyuan, Raphael, Ankur (Shah), Shardul, Ankur (Ruhela), Harshini, Tobias, Teilo and Eda.

I would like to give sincere appreciation to all my teachers on my course work. I also appreciate the help from all the MSE technical and administrative staff on my research.

Thank MSE and NTU for providing me the research scholarship and the chance to study in such an amazing place. Thank MOE Tier 1 and CREATE programme for funding the project. Thank MSE laboratories, especially the B1 Research Focus Laboratory, for providing the modern experimental equipment for my project.

I would also like to thank my friends for their support in my research, and my basketball-mate for helping me release from the hard lab work.

Finally, thanks to my youth.

Table of Contents

Abstract.....	i
Acknowledgements	iii
Table of Contents	v
Table Captions.....	xi
Figure Captions	xiii
Abbreviations	xxv
 Chapter 1 Introduction.....	 1
1.1 Background	2
1.2 Research Motivation	3
1.3 Hypothesis.....	4
1.4 Objectives and Scope	4
1.4.1 Competitive Stilbene-Analyte Conjugate with Analyte (Figure 1.3)	4
1.4.2 Stilbene-Aptamer Conjugate Fluorescence Decay Correlates to Analyte Binding (Figure 1.5).....	7
1.5 Dissertation Overview.....	8
1.6 Findings and Outcomes/Originality	9
References.....	10
 Chapter 2 Literature Review	 11
2.1 Biosensors	12

2.1.1 Definition of Biosensors	12
2.1.2 Two Pivotal Elements of Biosensors	12
2.2 Aptamers-Molecular Recognition Elements	14
2.2.1 Definition and Present Situation of Aptamers	14
2.2.2 Features of Aptamers	15
2.2.3 Methods for Measurement of Aptamer Binding Affinity	17
2.2.4 Aptamers in Biosensing Application	18
2.2.5 Aptamers Applied in Fluorescent Biosensors	19
2.2.6 Malachite Green Aptamer (MGA)-A Model Study	20
2.2.7 Summary	22
2.3 Stilbene Compounds-Reporters	22
2.3.1 Current Reporters and Definition of Stilbene	22
2.3.2 Fluorescence Properties of Stilbene	22
2.3.3 Variation in Photoisomerization of Stilbene Compounds.....	23
2.3.4 Regeneration of Decayed Stilbene Compounds.....	25
2.3.5 Azobenzenes	26
2.3.6 Summary	26
References	27
Chapter 3 Experimental Methodology	33
3.1 Rationale for selection	34
3.2 Selection of Model Aptamer – Malachite Green Aptamer (MGA) and Malachite Green (MG).....	34
3.3 Determination of MGA Binding Affinity by High-Throughput Microplate Assay ...	36
3.3.1 High-Throughput Binding by Fluorescence Enhancement of MG	36
3.3.2 Fitting Equation for K_d Determination in Direct Binding	40

3.3.3 Determination of K_d in Competitive Binding	43
3.4 Fluorescence Emission and Fluorescence Decay Measurements of Stilbene Compounds by Spectrofluorophotometer	44
3.5 Preparation of MGA-SITS Conjugate (5) (Figure 3.1).....	47
3.5.1 Synthesis and Purification of MGA-SITS Conjugate	47
3.5.2 Characterization of MGA-SITS Conjugate.....	49
3.6 Kinetic Study on MG Conversion.....	53
3.7 Statistical Analysis	55
References	55
 Chapter 4 Conditions for Malachite Green Aptamer Binding	59
4.1 Introduction.....	60
4.2 Materials and Methods.....	61
4.2.1 Materials.....	61
4.2.2 MGA/MG Binding Assay	62
4.2.3 High Performance Liquid Chromatography (HPLC)-Diode Array Detector (DAD) Study on MG Hydrolysis	62
4.2.4 UV-Vis Spectral Study on Kinetic Conversion between MG and MGOH.....	63
4.2.5 Electrophoresis of MGA	63
4.3 Results and Discussion.....	63
4.3.1 Effect of pH on Aptamer Binding Affinity	64
4.3.2 Fluorescence Intensity and Apparent Affinity of MGA/MG Complex Diminish Over Time	65
4.3.3 Addition of MG Reverses Decreased Fluorescence and MGA Keeps Stable Over Time	66
4.3.4 Loss of Fluorescence Is Attributed to Hydrolysis of MG to MGOH.....	68

4.3.5 Organic Additives Retain Fluorescence Intensity and Binding Affinity of MG with Limited Impact on K_d	69
4.3.6 Organic Additives Dehydrate MGOH and Shift K_{eq}	75
4.3.7 Organic Additives Reverse Decreased Fluorescence through MG Dehydration	77
4.3.8 Synergistic Stabilization of Ligand by Aptamer and Organic Additives.....	79
4.4 Conclusions.....	83
References.....	83
 Chapter 5 Binding and Fluorescence Properties of Malachite Green-Stilbene Conjugates	85
5.1 Introduction.....	86
5.2 Materials and Methods.....	87
5.2.1 Materials.....	87
5.2.2 MG/RhB-Stilbene Conjugates	88
5.2.3 Binding Tests of MGA to MG, RhB and MG/RhB-Stilbene Conjugates.....	88
5.2.4 Fluorescence Decay of Stilbene Compounds.....	89
5.3 Results and Discussion.....	89
5.3.1 Binding of MGA with MG (1) and Fluorescence Decay of (CH ₃) ₂ -N-stilbene-NH ₂ (9).....	89
5.3.2 MGA Binding and Fluorescence Decay of RhB-stilbene-N-(CH ₃) ₂ (11).....	91
5.3.3 MG-COO-stilbene-NO ₂ (12).....	94
5.3.4 MG-CH ₂ -NH-stilbene-OMe (2).....	96
5.3.5 MG-CH ₂ -NAc-stilbene-OMe (13)	98
5.3.6 MG-fused-stilbene (14).....	100
5.4 Conclusions.....	102

References.....	104
Chapter 6 Malachite Green Aptamer-Stilbene Conjugate.....	107
6.1 Introduction.....	108
6.2 Materials and Methods.....	109
6.2.1 Materials.....	109
6.2.2 Synthesis of MGA-SITS Conjugate (5).....	110
6.2.3 Characterization of MGA-SITS by High Performance Liquid Chromatography (HPLC)-Diode Array Detector (DAD)	111
6.2.4 Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) Mass Spectrum Characterization of MGA-SITS	112
6.2.5 K_d Determination of MGA-SITS in Binding with MG.....	112
6.2.6 Measurement of MGA-SITS Fluorescence Decay and Its Aptasensing.....	112
6.2.7 Measurement of SITS Fluorescence Decay in the Presence of MG and MGA/MG Complex	113
6.3 Results and Discussion.....	113
6.3.1 Successful Synthesis of MGA-SITS Conjugate with Complete Removal of Free SITS	114
6.3.2 Optimizations on Bioconjugation Reaction	117
6.3.3 MGA-SITS Keeps Properties of Aptamer Binding and Stilbene Fluorescence Decay	119
6.3.4 Fluorescence Decay of MGA-SITS Is Retarded upon MG Binding	121
6.3.5 Delayed Fluorescence Decay Isolated To MGA-SITS	122
6.3.6 The MGA-SITS Conjugate Shows Selectivity of Ligands	124
6.4 Conclusions.....	125
References.....	126

Chapter 7 Summary, Implications and Future Suggestions	129
7.1 Summary	130
7.2 Implications and Future Suggestions	133
References	141
List of Publications.....	142

Table Captions

Table 2.1 Summary of advantages of aptamers over antibodies.

Table 4.1 Decay rate constant ($k_{\text{MG} \rightarrow \text{MGOH}}$) of fluorescence intensity at MGA:MG = 40:1 (maximum ratio) with various organic additive percentages.

Table 4.2 MG hydrolysis rate constant ($k_{\text{MG} \rightarrow \text{MGOH}}$) with 10% of organic additives in the presence and absence of MGA (MGA:MG = 40:1), and the theoretical calculated properties of water and additives.

Table 5.1 Summary of the binding and fluorescence results of conjugates and controls.

Table 6.1 MG binding K_d of modified MGA.

Figure Captions

Figure 1.1 Schematic illustration of the fluorescence decay of *trans*-stilbene upon irradiation. (A) Stilbene exists in *trans* form and *cis* form. (B) The *trans*-stilbene exhibits fluorescence when excited, followed by a conversion to the non-fluorescent *cis*-stilbene, where a steady-state equilibrium is reached.

Figure 1.2 Overview of the aptasensor project with project team members of PhD candidate Wu Yuanyuan and Dr. Oleksandr Pokholenko.

Figure 1.3 Schematic illustration of fluorescence decay of stilbene in competitive binding.

Figure 1.4 Fluorescence decay correlates to analyte concentrations.

Figure 1.5 Schematic illustration of the hypothesized modes of stilbene fluorescence decay upon aptameric allostery. (A) When a stilbene is grafted on aptamer, its fluorescence decay behavior is kept, and the decay is inhibited due to the adaptive aptamer binding in presence of analyte. (B) When a stilbene is grafted on aptamer, its fluorescence decay behavior is inhibited, and the decay is recovered due to the adaptive aptamer binding in presence of analyte. (C) There is no variation in the fluorescence decay of aptamer-conjugated stilbene in the presence of analyte.

Figure 2.1 Schematic illustration of working principle of biosensors.

Figure 2.2 Illustration of applications of aptamers.

Figure 2.3 Schematic illustration of conformational change of aptamer upon binding.

Figure 2.4 A label-free colorimetric aptasensor based on the color change of gold

nanoparticles (AuNPs) upon aggregation for biosensing.

Figure 2.5 Scheme of a typical graphene oxide-based fluorescent aptameric biosensor using fluorescence resonance energy transfer (FRET).

Figure 2.6 Structure of malachite green aptamer (MGA) with MG bound. 3D structure of MGA is presented by Discovery studio 3.5 Visualizer, Accelrys. Nucleic Acid Sequence: 38mer
GGAUCCCGACUGGCGAGAGCCAGGUAACGAAUGGAUCC. 3D structure of MGA (1Q8N) is presented by Discovery studio 3.5 Visualizer, Accelrys. Preferred positions for modification are marked according to the 3D conformation and the structure activity relationship.

Figure 2.7 *Trans*-isomer and *cis*-isomer of stilbene and schematic illustration of the fluorescence decay property of *trans*-stilbene upon UV irradiation.

Figure 2.8 Inhibition of stilbene photoisomerization by being placed in an octa acid capsule. Conformations were evidenced by ^1H NMR.

Figure 2.9 Schematic illustration of fluorescence change of stilbene upon photoisomerization. *Trans-cis*: excitation 350 nm, emission 438 nm, and *cis-trans*: excitation 300 nm, emission 438 nm. Graph is redrawn from reference.

Figure 2.10 Chemical structures of stilbene-like compounds. (A) *Trans*-isomer and *cis*-isomer of azobenzene. (B) *N*-benzylideneaniline.

Figure 3.1 Structure of malachite green aptamer (MGA), malachite green (MG) (**1**), MG-CH₂-NH-stilbene-OMe (**2**), 4,4'-Diisothiocyanatostilbene-2,2'-disulfonic acid disodium salt (DIDS) (**3**), 4-Acetamido-4'-isothiocyanato-2,2'-stilbenedisulfonic acid disodium salt (SITS) (**4**), MGA-SITS (**5**) and

malachite green carbinol base (MGOH) (**6**). 3D structure of MGA (1Q8N) is presented by Discovery studio 3.5 Visualizer, Accelrys.

Figure 3.2 Jablonski diagram illustration of fluorescence emission.

Figure 3.3 Fluorescence enhancement of MG upon binding to MGA at excitation wavelength of 620 nm.

Figure 3.4 An illustration of K_d determination by fitting equation EQ (1) based on the fluorescence enhancement of MG (150 nM) at ex/em = 620 nm/656 nm upon binding to MGA.

Figure 3.5 An illustration of K_d determination by fitting equation EQ (13) based on the fluorescence enhancement of MG (150 nM) at ex/em = 620 nm/656 nm upon binding to MGA.

Figure 3.6 An illustration of K_d determination by competitive binding of a MG-stilbene conjugate (**2**) to MGA/MG complex, ex/em = 620 nm/656 nm.

Figure 3.7 Fluorescence emission spectrum of stilbene compound DIDS (**3**) with λ_{ex} = 340 nm.

Figure 3.8 Fluorescence decay spectrum at wavelength of emission maximum (λ_{max}) and the corresponding apparent fluorescence decay rate constant k_{app} (calculated by EQ (15)) of stilbene compound DIDS (ex/em = 340 nm/414 nm).

Figure 3.9 Synthesis of MGA-SITS conjugate (**5**).

Figure 3.10 Schematic simplified working flow/principle of HPLC and DAD.

Figure 3.11 C₁₈ reversed-phase HPLC-DAD chromatography of MGA-NH₂.

Figure 3.12 Schematic principle of MALDI-TOF mass spectrometry.

Figure 3.13 MALDI-TOF spectra of MGA-NH₂ and MGA-SITS conjugate (**5**). Top (blue): MGA-NH₂ before conjugation. Bottom (red): SITS grafted MGA-NH₂ (MGA-SITS conjugate (**5**)). The *m/z* increases for 457 units after the conjugation, indicating the molecular weight of the aptamer molecule increases 457 Da upon attachment of the SITS fragment. The MALDI-TOF results indicate the SITS is successfully conjugated on MGA.

Figure 3.14 Schematic working principle of UV-Vis spectrophotometer.

Figure 3.15 An example of kinetic study on MG conversion by UV-Vis spectrophotometer.

Figure 4.1 (A) Structure of malachite green aptamer (MGA) (**6**), malachite green (MG) (**1**) and malachite green carbinol base (MGOH). (B) Binding of MGA to MG, with fluorescence enhancement obtained.

Figure 4.2 MGA/MG binding curves at *ex/em* = 620 nm/656 nm (A) and corresponding apparent *K_d* values (B) in various pH.

Figure 4.3 (A) Binding *K_d* of MGA/MG in binding buffer from 0 to 24 h. (B) Normalized fluorescence decay at MGA:MG of 40:1 (the highest ratio).

Figure 4.4 (A) Fluorescence intensity of MGA/MG complex after 24 h binding and with the subsequent addition of original amount of MG (blue square) and MGA (black circle). (B) Agarose gel electrophoresis of MGA over time in binding buffer.

Figure 4.5 HPLC of MG 0h, MG 24 h and MGOH. (A) Detection $\lambda = 620$ nm. (B) Detection $\lambda = 265$ nm.

Figure 4.6 Apparent K_d values with organic additives at time 0 h. (A) With 3% and 10% ACN. (B) With 3% and 10% DMSO. (C) With 3% and 10% EtOH. (D) Variation of the apparent K_d values in different percentages of organic additives. * = $p < 0.05$: significant difference against apparent K_d without organic additive.

Figure 4.7 (A) Binding curves of MGA to MG with different percentage of ACN at 0 h and 24 h. (B) Plots of calculated K_d values with different percentages of organic additives at 0 h and 24 h. (C) Plots of fluorescence intensity at MGA:MG = 40:1 at 0 h and 24 h with different percentages of organic additives. NSD = no statistical difference, $p < 0.05$.

Figure 4.8 Kinetic evolution between MG and MGOH (without aptamer). (A) MG converts to MGOH in binding buffer and in the presence of 10% organic additives. (B) MGOH converts to MG in binding buffer and in the presence of 10% organic additives.

Figure 4.9 Rate constant of MGOH to MG ($k_{\text{MGOH} \rightarrow \text{MG}}$) in binding buffer in the presence of MGA.

Figure 4.10 Addition of DMSO into MGA/MG complex at 5 h. (A) 3% v/v DMSO for 1 h. (B) 3% v/v DMSO for 12 h. (C) 5% v/v DMSO for 1 h. (D) 5% v/v DMSO for 12 h.

Figure 4.11 (A) Equilibrium of MG (**1**), MG-OH₂ (**7**) (intermediates) and MGOH (**6**). (B) Rate of MG elimination. At pH 6.7, $[\text{OH}^-]$ is 5.0×10^{-8} M and thus, $k_{\text{OH}^-} [\text{OH}^-] \ll k_{\text{H}_2\text{O}} [\text{H}_2\text{O}]$. (C) Proposed mechanism of the conversions between

MG, MG-OH₂ and MGOH with MGA presented. Without MGA, the rate constants $k_{\text{MG} \rightarrow \text{MGOH}}$ (hydrolysis) and $k_{\text{MGOH} \rightarrow \text{MG}}$ (dehydration) favor MGOH as the major equilibrium product (A). When excess MGA is present, it retards MG hydrolysis, resulting in a decreased $k_{\text{MG} \rightarrow \text{MGOH}}$. With the addition of organic additives, $k_{\text{MGOH} \rightarrow \text{MG}}$ increases by few hundred percent (C), which results in a “stabilizing” effect.

Figure 5.1 Chemical structures of malachite green (MG) (**1**), 4-Amino-4'-(N,N-dimethylamino)stilbene ((CH₃)₂-N-stilbene-NH₂) (**9**), rhodamine B (RhB) (**10**) and MG/RhB-stilbene conjugates: RhB-stilbene-N-(CH₃)₂ (**11**), MG-COO-stilbene-NO₂ (**12**), MG-CH₂-NH-stilbene-OMe (**2**), MG-CH₂-NAC-stilbene-OMe (**13**) and MG-fused stilbene (**14**).

Figure 5.2 MGA/MG binding curve and its calculated K_d based on the fluorescence enhancement of MG (**1**) at ex/em = 620 nm/656 nm upon aptamer binding.

Figure 5.3 Fluorescence decay of (CH₃)₂-N-stilbene-NH₂ (**9**), ex/em = 344 nm/439 nm.

Figure 5.4 Influence of medium viscosity on fluorescence decay rate constant (k_{app}) of (CH₃)₂-N-stilbene-NH₂ (**9**). Results are from my project partner Ms. Wu Yuanyuan.

Figure 5.5 Binding of MGA to with RhB (**10**). No significant change in anisotropy is observed.

Figure 5.6 Fluorescence decay of RhB-stilbene-N-(CH₃)₂ (**11**), ex/em = 350 nm/445 nm.

Figure 5.7 Binding of MGA to with RhB-stilbene-N-(CH₃)₂ (**11**). No significant change in anisotropy is observed.

Figure 5.8 (A) Fluorescence decay of RhB-stilbene-N-(CH₃)₂ (**11**) in the presence of different concentration of MGA. (B) Normalized decay curves of A.

Figure 5.9 k_{app} of RhB-stilbene-N-(CH₃)₂ (**11**) in the presence of different concentration of MGA.

Figure 5.10 Competitive binding of MG-COO-stilbene-NO₂ (**12**) to MGA/MG complex and its calculated K_d .

Figure 5.11 Fluorescence decay of MG-COO-stilbene-NO₂ (**12**) at ex/em = 362 nm/520 nm. Results are from my project partner Ms. Wu Yuanyuan.

Figure 5.12 Competitive binding of MG-CH₂-NH-stilbene-OMe (**2**) to MGA/MG complex and its calculated K_d .

Figure 5.13 (A) Fluorescence decay of MG-CH₂-NH-stilbene-OMe (**2**) in different percentage of DMSO/binding buffer at ex/em = 324 nm/432 nm. (B) Normalized fluorescence decay of A. C: Apparent fluorescence decay rate constant (k_{app}) of the decay curves. Results are from my project partner Ms. Wu Yuanyuan.

Figure 5.14 Binding of MG-CH₂-NAc-stilbene-OMe (**13**) with MGA and its calculated K_d based on fluorescence enhancement.

Figure 5.15 (A) Fluorescence decay of MG-CH₂-NAc-stilbene-OMe (**13**) in different percentage of DMSO at ex/em = 327 nm/389 nm. (B) Normalized fluorescence decay of A. C: Apparent fluorescence decay rate constant (k_{app}) of the decay curves. Results are from my project partner Ms. Wu Yuanyuan.

Figure 5.16 Binding of MG-fused-stilbene (**14**) with MGA and its calculated K_d based on fluorescence enhancement.

Figure 5.17 Fluorescence decay of MG-fused-stilbene (**14**) in different organic solvents. Ex/em = 318 nm/381 nm for DMSO, 312 nm/398 nm for ACN and 314 nm/398 nm for EtOH, respectively. Results are from my project partner Ms. Wu Yuanyuan.

Figure 6.1 Schematic illustration of the aptasensor based on photochrome switch of stilbene and adaptive binding of aptamer. Without analyte (ligand), *trans*-stilbene grafted on aptamer undergoes free photoisomerization (fluorescent *trans* form to non-fluorescent *cis* form), resulting in rapid fluorescence decay. Upon analyte binding, conformational change of aptamer induces an inhibition of the stilbene photoisomerization, resulting in decrease in fluorescence decay kinetics, which can be utilized for biosensing.

Figure 6.2 Structures of malachite green aptamer (MGA), malachite green (MG) (**1**), 4-Acetamido-4'-isothiocyanato-2,2'-stilbenedisulfonic acid disodium salt (SITS) (**4**), rhodamine B (RhB) (**10**), tetramethylrosamine (TMR) (**15**), aminocaproic acid (EACA) (**16**) and lysine (**18**). MGA 3D structure (PDB ID: 1Q8N) is presented by Discovery studio 3.5 Visualizer, Accelrys.

Figure 6.3 Synthesis of MGA-SITS conjugate (**5**).

Figure 6.4 HPLC-DAD characterization of (A) MGA-NH₂, (B) SITS (**4**) and (C) MGA-SITS conjugate (**5**). Insets are the UV-Vis spectra (from DAD) at corresponding RT displaying the variation in absorbance.

Figure 6.5 MALDI-TOF spectra of MGA-NH₂ (top blue) and MGA-SITS conjugate (**5**) (bottom red).

Figure 6.6 (A) Reaction of EACA (**16**) with SITS (**4**). (B) Reaction progress at 4 deg C. (C) Reaction progress at room temperature.

Figure 6.7 (A) Reaction of lysine (**17**) with SITS (**4**). (B) Reaction progress at 4 deg C. (C) Reaction progress at room temperature.

Figure 6.8 (A) HPLC monitoring of absorbance increase in UV340 (grafting of SITS (**4**)) over time at RT 1.7 min. (B) The calculated degree of conjugation as a function of time.

Figure 6.9 (A) Fluorescence decay of MGA-SITS (**5**) at ex/em = 340 nm/428 nm. (B) Fluorescence emission of MG in the absence and presence of MGA-SITS at excitation wavelength of 620 nm.

Figure 6.10 Binding of MGA-NH₂ and MGA-SITS (**5**) with MG, ex/em = 620 nm/656 nm.

Figure 6.11 (A) Fluorescence decay curves of MGA-SITS (**5**) in the presence of increasing MG concentration. (B) Calibration plot of k_{app} of MGA-SITS as a function of MG concentration (0.5-50 μ M).

Figure 6.12 (A) Fluorescence decay curves of SITS (**4**) in the presence of MG and MGA/MG complex. (B) k_{app} of SITS upon the presence of MG and MGA/MG complex. *: $p < 0.05$: significant difference against SITS control (without MG and MGA) ($n = 3$).

Figure 6.13 (A) Fluorescence decay curves of MGA-SITS (**5**) in the presence of 20 μ M MG, TMR (**15**) and RhB (**10**). (B) k_{app} of MGA-SITS upon the addition of the analytes. *: $p < 0.05$: significant difference against MGA-SITS ($n = 3$).

Figure 6.14 (A) Fluorescence decay curves of MGA-SITS (**5**) in the presence of increasing concentration of TMR (**15**). (B) Calibration plot of MGA-SITS k_{app} as a function of TMR concentration (0.2-20 μ M).

Figure 7.1 Recycling of computational modelling and synthesis on MG-stilbene conjugates for optimal binding affinity and fluorescence decay.

Figure 7.2 Competitive binding of MG with prebound MGA-SITS/TMR complex for MG sensing.

Figure 7.3 Conjugation of MeO-stilbene-maleimide (**20**) with 6-Mercapoto-1-hexanol (MCH) (**21**).

Figure 7.4 MGA-SH/MG binding curve and its calculated K_d , ex/em = 620 nm/656 nm.

Figure 7.5 Schematic illustration of regeneration of aptamer (A), stilbene (B) and the regenerated aptamer-stilbene conjugate sensor (C).

Abbreviations

ACN	Acetonitrile
DAD	Diode array Detector
DIDS	4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid Disodium Salt
DMSO	Dimethyl Sulfoxide
ELISA	Enzyme-Linked Immunosorbent Assay
EtOH	Ethanol
FI	Fluorescence Intensity
FP	Fluorescence Polarization
HPLC	High-performance Liquid Chromatography
LMG	Leucomalachite Green
LOD	Limit of Detection
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization Time-of-Flight
MG	Malachite Green
MGA	Malachite Green Aptamer
MGA-NH ₂	Amine Modified Malachite Green Aptamer
MGOH	Malachite Green Carbinol Base
MPSA	Molecular Polar Surface area
NMR	Nuclear Magnetic Resonance
SEC	Size Exclusion Chromatography
RT	Retention Time
SELEX	Systematic Evolution of Ligands by Exponential Enrichment
SITS	4-acetamido-4'-isothiocyanato-2,2'-stilbenedisulfonic acid disodium salt
TMR	Tetramethylrosamine
UV-Vis	Ultraviolet-visible
XRD	X-ray Diffraction

Chapter 1

Introduction

This chapter describes the background of biosensors, aptamers and stilbenes (section 1.1), followed by an introduction of the research motivation, hypothesis and scopes (section 1.2-1.4). The advantages of aptamers and stilbenes are illustrated, providing the rationale for the hypothesis. Two designs according to the hypothesis are displayed with corresponding scopes (section 1.4). Finally, the overview of the dissertation (section 1.5) and the outcomes of the research (section 1.6) are also outlined.

1.1 Background

Aptamers are a kind of synthetic oligonucleotides discriminated by Systematic Evolution of Ligands by EXponential enrichment (SELEX) technique and *in vitro* screening, which can bind to a wide range of targets (small molecules, proteins, or whole cells). As molecular recognition elements, aptamers have some advantages over antibodies in fabrication of the biosensors including *in vitro* screening and non-animal synthetic origins [1-3]. Especially, aptamers undergo adaptive conformational change upon ligand recognition, inducing micro-environment rearrangements. Currently, fluorescence biosensors mainly rely on fluorescence intensity or quenching of fluorescence reporters (probes or labels) for analyte quantification. However, this detection mode suffers from several drawbacks, such as the laborious and tedious washing procedures for the separation and background autofluorescence.

Stilbene compounds so far have never been used as the molecule sensing entity in the biosensing devices and bioassays, although they possess many of the desired features of ideal reporter molecules, for instance, chemical and biological stability, biocompatibility, synthetic availability, high photochemical sensitivity, rapid response and easy regeneration. Stilbene molecules are capable of changing their molecular configurations with fluorescence decay upon UV irradiation, which is sensitive to micro-environment sterics. There are two possible stilbene conformational isomers: *trans* and *cis*, as shown in **Figure 1.1A**. In case of a stilbene molecule, the stereochemical *trans*-isomer is strongly fluorescent while the *cis*-isomer is non-fluorescent. Hence, irradiation of the fluorescent *trans* form of the reporter molecule at the excitation maximum produces a substantial change of the fluorescence emission that can be monitored as a time trace (kinetic decay) signal or emission gradient, illustrated in **Figure 1.1B** [4-6].

Figure 1.1 Schematic illustration of the fluorescence decay of *trans*-stilbene upon irradiation. (A) Stilbene exists in *trans* form and *cis* form. (B) The *trans*-stilbene exhibits fluorescence when excited, followed by a conversion to the non-fluorescent *cis*-stilbene, where a steady-state equilibrium is reached.

1.2 Research Motivation

The molecular recognition properties of aptamers and the fluorescence decay feature of stilbene compounds make them promising materials in the development of robust, simple, and rugged fluorescent biosensing assay. To date, research on an aptasensor using stilbene as reporter has not been found yet. Herein, we attempt to utilize aptamers and stilbene compounds to develop a fluorescence decay-based biosensor device, regardless of absolute fluorescence intensity, which can remove laborious washing procedures.

Figure 1.2 displays the overlapping contribution with my project team members, PhD candidate Wu Yuanyuan and Dr. Oleksandr Pokholenko.

Figure 1.2 Overview of the aptasensor project with project team members of PhD candidate Wu Yuanyuan and Dr. Oleksandr Pokholenko.

1.3 Hypothesis

Changes in surrounding steric and viscosity micro-environment can be quantitated by the fluorescence decay of stilbene; aptamers are known to change their micro-environments upon binding ligands or undergo dynamic binding upon ligand docking; therefore by conjugating ligands to stilbene or stilbene directly to an aptamer, changes in aptamer binding or dynamic folding can be directly quantitated through stilbene fluorescence decay.

1.4 Objectives and Scope

Here are two designs and scopes for the hypothesis:

1.4.1 Competitive Stilbene-Analyte Conjugate with Analyte (Figure 1.3)

A stilbene-analyte conjugate has fluorescence decay property similar to stilbene. When this conjugate binds to corresponding aptamer, the whole conjugate is enclosed or immobilized by the aptamer and the fluorescence decay is retarded. When analyte is added in the aptamer/conjugate complex, it will compete and free the aptamer bound, resulting in an analyte-dependent recovery of the fluorescence decay.

Figure 1.3 Schematic illustration of fluorescence decay of stilbene in competitive binding.

Scope for design “competitive stilbene-analyte conjugate with analyte”:

- 1) Determination of the binding dissociation constant (K_d) of the *trans*-stilbene-analyte conjugate. The binding ability of the conjugate will be obtained by determining the K_d .
- 2) Fluorescence decay measurement of the aptamer-bound conjugate. The fluorescence decay feature of the conjugate upon binding to aptamer will be correlated to conjugate fluorescent decay.
- 3) Fluorescence decay measurement of the aptamer-bound conjugate in competition against target analyte. The fluorescence decay of the “aptamer/conjugate” complex upon adding analyte will be correlated against initial fluorescent decay.
- 4) Calculation of the first order decay rate constant and calibration of the system. Calibration curve of first order decay rate constant vs. analyte concentration, establishing a standard curve for quantitation (**Figure 1.4**).

Figure 1.4 Fluorescence decay correlates to analyte concentrations.

1.4.2 Stilbene-Aptamer Conjugate Fluorescence Decay Correlates to Analyte Binding (Figure 1.5)

When a stilbene is modified on aptamer, its fluorescence decay behavior will be different in the absence and presence of analyte, due to the aptamer conformational rearrangement upon analyte binding, which can affect the micro-environment surrounding stilbene.

Figure 1.5 Schematic illustration of the hypothesized modes of stilbene fluorescence decay upon aptameric allostery. (A) When a stilbene is grafted on aptamer, its fluorescence decay behavior is kept, and the decay is inhibited due to the adaptive aptamer binding in presence of analyte. (B) When a stilbene is grafted on aptamer, its fluorescence decay behavior is inhibited, and the decay is recovered due to the adaptive aptamer binding in presence of analyte. (C) There is no variation in the fluorescence decay of aptamer-conjugated stilbene in the presence of analyte.

Scope for design “stilbene-aptamer conjugate fluorescence decay correlates to analyte binding”:

- 1) Determination of the K_d of the *trans*-stilbene-aptamer conjugate to the analyte.
- 2) Fluorescence decay measurement of the *trans*-stilbene-aptamer in the presence and absence of analyte.
- 3) Calculation of the first order decay rate constant as a function of analyte concentration. (**Figure 1.4**).

If any of the designs above can be proved to have fluorescence decay change due to the addition of analyte, the hypothesis will be supported by the scope described above and challenged against the malachite green aptamer (MGA).

1.5 Dissertation Overview

The thesis consists of the following seven chapters:

Chapter 1 briefly describes the research background of the study, followed by an introduction of research motivation, hypothesis and scopes, and finally outlines the overview and outcomes of the thesis.

Chapter 2 reviews the historical and state-of-the-art background of biosensors, aptamers and stilbenes, with research gaps highlighted. The advantages of aptamers and stilbenes are reviewed within a biosensor perspective. A model system, comprising of malachite green aptamer, is proposed to challenge the hypothesis above.

Chapter 3 introduces the main experimental methods or techniques and their corresponding principles, including K_d determination, bioconjugation, and fluorescence decay analysis.

Chapter 4 refines the conditions for MGA/MG binding. pH and organic additives

correlations on MGA/MG binding is demonstrated. The stability of MGA, MG and bound MGA/MG is experimentally assessed and vindicated. Organic additives and their effects on MG hydrolysis and subsequent MGA binding are summarized.

Chapter 5 demonstrates MG-stilbene conjugate binding affinities and inherent fluorescence decay kinetics. The relationship between the design of MG-stilbene conjugates and their binding provides structure activity relationships in aptasensor construction.

Chapter 6 summarizes fluorescent biosensing using the MGA-stilbene aptasensor conjugate. Binding affinities of the conjugate and the aptamer before conjugation are compared, demonstrating little to no loss in affinity after conjugate construction, and conjugate maintains fluorescence decay of stilbene. The aptasensor conjugate supports our hypothesis under the limited experimental conditions described in the scope.

Chapter 7 concludes the results according to the hypothesis, and gives recommendations towards more advanced aptasensors based on stilbene transducers.

1.6 Findings and Outcomes/Originality

This research led to several novel outcomes:

1. Optimized binding conditions for MGA/MG binding were found and a simple way to stabilize MGA binding was developed with common organic additives. Inhibition effect of aptamer and organic additives on ligand lability was presented.
2. MG-stilbene conjugates were synthesized and their binding affinities to MGA and fluorescence properties were measured for the first time, providing structure activity relationships required in ligand-stilbene aptameric biosensors.
3. MGA-stilbene conjugate was synthesized successfully, with a linear correlation of

fluorescence decay kinetics and concentrations of MG and tetramethylrosamine (TMR) as model analytes. Aptasensor based on stilbene-aptamer conjugates is demonstrated for the first time.

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Chapter 2

Literature Review

This chapter introduces the state-of-the-art background of biosensors, aptamers and stilbenes. The basic information of biosensors and their principal elements are reviewed, with research gaps described. Advantages and unique properties of aptamers (like adaptive binding) and stilbenes (like fluorescence decay) are described and compared to other biosensor elements. Finally, a model biosensor malachite green aptamer (MGA) is introduced.

2.1 Biosensors

2.1.1 Definition of Biosensors

Biosensors are devices that use a molecular recognition element coupled to a signal reporter (transducer) to detect an analyte of interest [1,2]. The advantages of biosensors such as high sensitivity, high specificity, portability and rapid analysis for both *in situ* and laboratory detection have made biosensors powerful tools for detection in medical diagnostics, environmental monitoring, security applications and food safety, among many others [3-8]. In 1962, scientist Leyland C. Clark developed an “enzyme electrode”, which started the history of biosensors [8,9].

2.1.2 Two Pivotal Elements of Biosensors

From the definition, a biosensor includes two main elements: molecular recognition element and signal reporter (transducer). An analyte interacts with the biological molecular recognition element with biological response generated, and then the recognition process is transduced to be a signal by the reporter. Finally, the signal from reporter will be usually read and recorded by corresponding equipment [2]. The working principle of biosensors is schematically shown in **Figure 2.1** [2,10,11].

Figure 2.1 Schematic illustration of working principle of biosensors [2,10,11].

Molecular recognition element is the core of a biosensor. To date, there are different kinds of molecular recognition elements that have been discovered and commonly used: antibodies, aptamers, enzymes, phages, cell-surface molecules, oligonucleotide probes, tissues, receptors, hormones, molecularly imprinted polymers and so on [11-15]. Enzymes are widely used in biosensors and their commercial biosensors for glucose measurement in blood have occupied the main part of the market. However, they suffer from lifespan and robustness [14,16]. Antibodies providing high affinity and specificity have been applied in enzyme-linked immunosorbent assay (ELISA) optical sensing, which is a widespread application [8,17]. However, the intrinsic shortcomings of antibodies limit their applications, which will be compared with aptamers in the following **section 2.2.2**. Among these molecular recognition elements, aptamers as recognition molecules have unique merits, such as chemical stability, adaptive binding

and ease of modification, which antibodies do not possess [18].

As for reporters, electrochemical, thermal, optical, mass, piezoelectric, acoustic and mechanical reporters have been utilized in biosensing [11,14,19]. Different reporters can be selected according to the properties of molecular recognition elements and specific purposes. Optical reporters have been extensively involved in biosensors for toxins and foodborne pathogens detection [2], and optical aptameric biosensors have simple operation, remarkable sensitivity and rapid detection [20]. Fluorescence-based (optical) reporters are often used for biosensing due to their high sensitivity and simple operation [14], especially in the combination use of aptasensors [21]. However, the fluorescence-based reporters (probes or labels) are not free of shortcomings. They are now mainly based on the “Signal on/off” mode [22] and they rely on fluorescence intensity quantification or fluorescence quenching, which can be easily affected by various factors, resulting in inaccurate results. Moreover, enzyme-linked immunosorbent assay (ELISA)–type fluorescent biosensors require time-consuming washing steps, and do not allow real-time detection [17,23]. Stilbene molecules are fluorescent reporters that undergo instant *trans-cis* conformational conversion upon irradiation. At the same time, the quantum yield diminishes because of the *trans-cis* conversion [24,25]. This decrease in quantum yield leads to a fluorescence decay that can be employed to correlate shifts in local micro-environments and ultimately develop a separation-free biosensor.

2.2 Aptamers-Molecular Recognition Elements

2.2.1 Definition and Present Situation of Aptamers

The term aptamer is from Latin *aptus*, meaning “to fit” [26]. It was first discovered by Gold’s and Szostak’s groups in 1990 and named by Szostak’s group [27,28]. Aptamers are typically artificial oligonucleic acid molecules (single stranded DNA or RNA ligands) which can bind to a broad range of non-nucleic acid targets with high specificity and affinity, including drugs, small molecules, growth factors, peptides, enzymes, spores, toxins, proteins, whole cells and ions [29-32]. Hydrogen bonds, stacking interactions,

electrostatic interactions and hydrophobic effect are the driving force commonly involved in the binding events, and they are related to the functional groups of targets [33,34]. Aptamers as biomaterials have been studied in a range of fields, such as diagnosis, biosensing, bioimaging, drug delivery, drug discovery, etc [18,35]. Applications of aptamers are shown in **Figure 2.2** [35].

Figure 2.2 Illustration of applications of aptamers [35].

2.2.2 Features of Aptamers

Aptamers can be obtained from random pools of RNA or DNA *in vitro* via Systematic Evolution of Ligands by EXponential enrichment (SELEX) [27,28]. Aptamers are termed “chemical antibodies” because of this artificial process, which have been suggested to be good candidates towards the replacement of antibodies in biosensing [36-38]. From the SELEX process, aptamers can be selected with a wide range of targets [39,40].

A significant advantage of aptamers is their large-scale chemical synthesis, which makes them more cost-effective and avoids any animal origins. This reduces batch-to-batch variability and allows them to work under a range of conditions where antibodies may be hindered.

Aptamers' synthetic nature allows them to be easily modified [41], which is problematic for other types of molecular recognition elements. This allows them to be easily tagged with reporters such as fluorophores, nanoparticles and quantum dots [18,42].

A unique feature of aptamers is their “adaptive binding” [43], which means aptamers will undergo a conformational change upon binding [44]. Aptamers exhibit random structures in the absence of targets while they form a defined structure upon adaptive binding [45]. Various aptameric sensors have been reported widely based on the structural switching of aptamers [46-51]. **Figure 2.3** illustrates the conformational change of aptamer upon binding to ligand [52,53]. Micro-environment rearrangements can induce a difference in signal for sensing [21,52]. The “adaptive binding” results in a rigid and stable structure that can protect them from nuclease digestion [54]. Moreover, aptamers, like other nucleotide constructs, can recover their native structures upon thermal annealing or washing [55], allowing biosensors to be designed for multiple-used analyses with lower cost. **Table 2.1** summarizes the advantages of aptamers over antibodies [56-58].

Figure 2.3 Schematic illustration of conformational change of aptamer upon binding [52,53].

Table 2.1 Summary of advantages of aptamers over antibodies [56-58].

Properties	Aptamers	Antibodies
Design of structural change	Easy	Hard
Construction of conjugate	Easy	Hard
Affinity	Low nM-pM	Low nM-pM
Range of targets	Wide: small molecules, drug, organic molecules, ions, toxin, peptides, proteins, whole cells, etc.	Narrow: only immunogenic compounds
Chemical modification	Easy and straightforward	Limited
Thermal denaturation	Reversible	Irreversible
Production	<i>In vitro</i> chemical process	<i>In vivo</i> biological system
Batch to batch variation	Little or no	Significant
Stability	High	Low

2.2.3 Methods for Measurement of Aptamer Binding Affinity

Various techniques have been developed to measure aptamer binding affinity (or equilibrium dissociation constant, K_d): dialysis, filtration, fluorescence intensity (FI), fluorescence polarization (FP), ultraviolet-visible (UV-Vis) absorption, surface plasmon resonance (SPR), isothermal titration calorimetry (ITC), etc [59]. Among these techniques, FI can give a simple, rapid and sensitive measurement. By incorporating the microplate reader, high-throughput FI method can be developed for K_d determination. This technique, utilizing changes in fluorescence signal, requires modification of aptamer or fluorescent signal change of ligand upon binding. FI strategy is preferred for ligands whose fluorescence can be activated by aptamer binding. FP method which distinguishes light polarization between bound and free status, is a preferred method for fluorescent molecules, without requiring absolute fluorescence intensity change in binding events. This method is suitable for K_d determination of aptamers with fluorescent ligands in relatively small molecule weight.

2.2.4 Aptamers in Biosensing Application

Compared to the therapeutic field, aptamers are more attractive in the biosensing field. Aptamers have opened up exciting fields in the research of sensing interest [34,44,60]. An aptamer-based protein precipitation kits (AMSBIO) has been marketed capable for reuse for several times [61]. The K_d of aptamers binding to targets are commonly in the picomolar to micromolar range [21], which compares well to that of antibodies. Although there are many other commonly used recognition molecules such as antibodies, enzymes, ssDNA, peptides and receptors [14], aptamers have their advantages over other recognition molecules of biosensor: ease in synthesis and generation, a diverse range of possible target molecules, adaptive binding, easy modification, high target affinity, structural and functional robustness, and so on [39,62]. Aptamers have been proven to be suitable for molecular recognition of targets in biosensing [34,44]. For instance, a label-free colorimetric aptasensor has been developed based on the color change of gold nanoparticles (AuNPs) upon aggregation (**Figure 2.4**) [63] and similar strategy can also been applied for other aptamers [64,65]. As a molecular recognition element in biosensing, aptamers have comparable binding specificity and affinity to antibodies, which are well known and extensively used biosensors, and thus aptamers are widely known as a substitute for antibodies [18]. Moreover, as seen in **Table 2.1** aptamers possess various unique advantages and overcome the weaknesses of antibodies [18,66]. Particularly, detection of small molecules (like malachite green (MG)) by aptasensors is a promising direction as it is difficult to generate antibodies for small molecules, although this kind of aptasensing still remains a challenge currently [22,48,67]. Additionally, the adaptive binding of aptamers enables their sensing strategies based on conformational change, such as fluorescence resonance energy transfer (FRET) [44,68], which directly provides foundation for the hypothesis.

Figure 2.4 A label-free colorimetric aptasensor based on the color change of gold nanoparticles (AuNPs) upon aggregation for biosensing [63].

2.2.5 Aptamers Applied in Fluorescent Biosensors

Optical aptasensors have attracted great interest from researchers due to their high sensitivity, simple operation, and rapid detection. Briefly, optical aptasensors utilize signals of colorimetry, fluorescence, chemiluminescence, surface plasmon resonance (SPR), and so on. Among these optical methods, fluorescent signaling is one of the most popular optical approaches for aptamers because of its remarkable sensitivity, high selectivity, high efficiency and easy operation, as well as the unique features of aptamers. As a consequence, various fluorescence-based aptasensors have been developed in biosensing for a wide range of detections: adenosine triphosphate (ATP), ochratoxin A, Cocaine, Adenosine, etc. [20]. **Figure 2.5** illustrates a typical graphene oxide-based fluorescence resonance energy transfer (FRET) aptasensor that realized by fluorescence resonance energy transfer [44,68]. This strategy illustrates an example in sensitive fluorescent sensing based on aptamer's conformational change through adaptive binding.

Figure 2.5 Scheme of a typical graphene oxide-based fluorescent aptameric biosensor using fluorescence resonance energy transfer (FRET) [44,68].

2.2.6 Malachite Green Aptamer (MGA)-A Model Study

Malachite green aptamer (MGA) was first discovered by Wilson Charles and his lab for site-specific inactivation of RNA transcripts [69] (**Figure 2.6**). Wilson Charles continued to find that the MGA can not only bind to malachite green (MG) but also bind to some dyes having similar structures as MG, and a crystal structure of MGA bound to tetramethylrosamine (TMR) was obtained [70-72]. When MG binds to MGA, the charge distribution and conformation of MG results in 2000 fold enhancement of MG quantum yield [72-75]. The conformational change in MG or MGA indicates a rearrangement in micro-environment surrounding, which provides possibility of providing an analyte concentration correlation in fluorescent decay kinetics. Based on the unique fluorescence enhancement of MGA on MG, a simple and label-free approach for the determination of binding affinity (or K_d) can be developed in both direct and competitive binding formats. Fluorescence polarization (FP) may also be employed for K_d determination as MGA can also bind to other fluorescent ligands (small molecules). Immobilized MG was originally used in affinity chromatography to select the MGA, which indicates MGA has ability to bind to MG conjugates, allowing a strategy to conjugate stilbene [70,76]. The successful use of MGA for detection and analysis of MG and leucomalachite green (LMG) residues in aquaculture testifies to its commercial viability [77]. Previous investigations on MGA have detailed the inviolable nucleotides and structure activity relationships required for biosensing [78]. According to the report, the research also compared the 3D crystal (X-Ray Diffraction, XRD) and solution (nuclear magnetic resonance, NMR) structure of

MGA, which gives more information of the MGA and give indication on where should be modified. For instance, U4 is away from the binding site, A9 mutant within binding site has tinny impact on K_d , C20 is close to the tetraloop and C38 is the end modification commonly used. These positions should be considered in our design. On the other hand, MG as an efficient fungicide with commercial prevalence has been banned for fish farming in Canada, the United States and European union owing to its potential toxicity [79]. Detection of MG is in need, and the designed sensor according to our hypothesis (if true) may directly realize a novel commercial assay for the detection of toxin MG.

To summarize, MGA is a model system towards stilbene-based aptasensor development because K_d is easily measured with commercial plate readers, has relative high affinity to MG and TMR analytes, has a known crystal (XRD) and solution structure after binding with MG analyte (NMR), and retains its ability to bind immobilized MG-conjugates.

Figure 2.6 Structure of malachite green aptamer (MGA) with MG bound. 3D structure of MGA is presented by Discovery studio 3.5 Visualizer, Accelrys. Nucleic Acid Sequence: 38mer GGAUCCCGACUGGCGAGAGCCAGGUAACGAAUGGAUCC. 3D structure of MGA (1Q8N) is presented by Discovery studio 3.5 Visualizer, Accelrys. Preferred positions for modification are marked according to the 3D conformation and the structure activity relationship.

2.2.7 Summary

In summary, as discussed above, the significant advantages of aptamers make them become increasingly powerful tools for food safety, environmental monitoring, drug discovery, diagnostics, therapeutics, and so on [37,43,80,81]. MGA with their targets which is well studied should be reasonable for the model study of the stilbene-based aptasensor quantitated by fluorescence decay.

2.3 Stilbene Compounds-Reporters

2.3.1 Current Reporters and Definition of Stilbene

Fluorescence-based optical reporters are commonly used due to their high sensitivity [14]. Fluorescence reporters which are sensitive, rapid, simple, and cost-effective are the most commonly used reporters in aptamer-based biosensors [22]. However, the fluorescence-based reporters (probes or labels) are now mainly based on the “signal on-off” mode which relies on fluorescence intensity quantification or fluorescence quenching. This mode can be easily affected by various factors, resulting in inaccurate results.

Stilbene is a fluorescent molecule existing in two forms: *trans*-isomer and *cis*-isomer. The structures of *trans*-stilbene and *cis*-stilbene can be seen in **Figure 2.7**.

2.3.2 Fluorescence Properties of Stilbene

The *trans*-isomer stilbene is strongly fluorescent, but upon excitation, it undergoes a conformation shift to the *cis*-isomer which is non-fluorescent. Determination of apparent fluorescence decay rate constant (k_{app}) of stilbene molecules has been developed [82] and defined as classical photoisomerization in organic photochemistry [25]. This fluorescence decay may take only seconds to reach a steady state equilibrium, a property we can exploit for rapid sensing. **Figure 2.7** shows the typical structures of *trans*-stilbene and *cis*-stilbene, and the fluorescence decay of *trans*-stilbene upon irradiation [24,25].

Derivatives of stilbene also show similar fluorescence behavior [83-90] as non-modified stilbene, which makes stilbene have the potential towards sensing micro-environment rearrangements, such as those present in aptamer adaptive binding.

Figure 2.7 *Trans*-isomer and *cis*-isomer of stilbene and schematic illustration of the fluorescence decay property of *trans*-stilbene upon UV irradiation [24,25,91].

2.3.3 Variation in Photoisomerization of Stilbene Compounds

The photoisomerization/switching of stilbene kinetics can be affected by factors such as steric hindrance, viscosity, temperature, and solvent. Studies have shown that *trans-cis* switching of stilbenes can be affected or inhibited by various causes [86,89-91]. Steric

hindrance or viscous micro-environment is one of the most significant factors of this effect [89,92]. When a *trans*-stilbene molecule was put into a constrained capsule or was enclosed by a large molecule, photodecay kinetics were correlated to the steric hindrance (**Figure 2.8**) [88,89,93,94]. While stilbene derivatives were fixed on protein coated quartz surface, their apparent fluorescence decay rate were times slower than when they were in solution, which also suggested the influence of immobilizing environments. Moreover, this research also shows the impact on the decay rate from absolute viscosity [92]. In the stilbene-based aptasensors, long chain aptamers may also act like a capsule or large molecule to enclose *trans*-stilbene by “adaptive binding”, resulting in constrained micro-environment of stilbene and inhibition of the photoisomerization. This can be correlated to stilbene photodecay rates due to the allosteric effect of aptamers upon binding. Because the stilbene-based aptasensors are based on the fluorescence decay of *trans*-stilbene, compared to FI-based “signal on/off” sensors it allows separation free detection of bound and unbound targets. These properties of stilbene fluorophores may make stilbene-based aptasensors overcome the shortcomings of the “signal on/off” aptasensors.

Figure 2.8 Inhibition of stilbene photoisomerization by being placed in an octa acid capsule. Conformations were evidenced by ^1H NMR [93].

2.3.4 Regeneration of Decayed Stilbene Compounds

As sensors, the devices capable of multiple-used analytes and continuous monitoring are welcomed. Thus, a reusable device is prior to a single-used one. The reversible *trans-cis* switching of stilbene compounds makes it an ideal reporter element in multiple-used sensing. Even though the conversion of *trans*-stilbene to *cis*-stilbene is relatively efficient, the *cis* form can be turned back to *trans* form under certain condition, e.g. UV-Vis irradiation in another wavelength [95]. Studies show that this “turning back” conformational change makes stilbene molecule recover fluorescent to almost the initial level [86]. This regeneration ability of stilbene combining with the reversible allostery of aptamer makes them an ideal couple of sensing element which may have a lower cost of detection compared to the disposable elements. **Figure 2.9** gives an example of the fluorescence change of stilbene derivative from *cis* form to *trans* form [86].

Figure 2.9 Schematic illustration of fluorescence change of stilbene upon photoisomerization. *Trans-cis*: excitation 350 nm, emission 438 nm, and *cis-trans*: excitation 300 nm, emission 438 nm. Graph is redrawn from reference [86].

2.3.5 Azobenzenes

Besides stilbenes, there are diverse organic photochromic molecules undergoing switching upon activation: azobenzenes, fulgides, diarylethenes, chromenes, spiropyranes and so on [96]. Specifically, azobenzenes are structurally similar to stilbenes (with -N=N- instead of -C=C- compared to stilbene). **Figure 2.10A** displays the structures of *trans*-azobenzene and *cis*-azobenzene and their photoisomerization. The time for *trans*-to-*cis* isomerization of azobenzenes is in picosecond or even femtosecond scale, while the time for stilbenes is mainly in picosecond scale [96-99]. This difference on azobenzenes can be applied while faster signal reporting is needed. However, it should be noted that the fluorescence quantum yield of azobenzenes is 3-5 orders smaller than the fluorescence quantum yield of stilbenes, which limits their fluorescence-based applications [100-103]. Finally, as another type of stilbene-like compounds, *N*-benzylideneaniline (**Figure 2.10B**) compounds (with -C=N- instead of -C=C- compared to stilbene) also possess the property of isomerization upon UV excitation, having merits of convenient synthesis and lower activation energy [104]. However, *N*-benzylideneaniline compounds are rarely used for photoswitch sensing because of the easy hydrolysis of imines [105].

Figure 2.10 Chemical structures of stilbene-like compounds. (A) *Trans*-isomer and *cis*-isomer of azobenzene. (B) *N*-benzylideneaniline.

2.3.6 Summary

In summary, taking the discussion above into account, stilbene and its derivatives have the excellent features which fulfill the requirements of an ideal sensing element for our

design. The high photochemical sensitivity, permission to be modified, fast response and the ability of regeneration make stilbene a promising reporter in sensing. Other stilbene-like compounds are also reviewed. Azobenzenes are possible substitutes of stilbenes for rapid assay if their fluorescence quantum yield can be sufficiently enhanced. *N*-benzylideneaniline compounds are hardly used due to the easy hydrolysis of imines.

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Chapter 3

Experimental Methodology

This chapter introduces the main experimental methods and corresponding principles used in this thesis. Section 3.1 describes briefly the rationale of selection of the methods. Section 3.2 explains the reason for using the model aptamer-malachite green aptamer (MGA). Section 3.3 introduces the high-throughput technique and principle in determination of aptamer binding affinity. Section 3.4 shows the measurement of stilbene fluorescence emission and decay spectra. Section 3.5 is about the techniques used in synthesis and characterization of aptamer-stilbene conjugate. Strategy on monitoring the kinetics of MG conversion is described in section 3.6. Additionally, methods employed for statistical analysis are also introduced in section 3.7. This chapter mainly focuses on the rationale of methods chosen in our experiments, with examples given to show the rationality and appropriateness of such methods. Detailed experimental procedures will be introduced in Result chapters.

3.1 Rationale for selection

To test the hypothesis, an appropriate model aptamer needs to be selected foremost. Our designs require an ideal aptamer to be as small as possible, nM binding affinity (K_d less than 1 μ M), fast K_d determination, amenable in modification and known 3D structure upon binding. Malachite green aptamer (MGA)/ malachite green (MG) as aptamer/ligand pair is chosen as an ideal model aptamer system as it possesses all the properties required and additionally has advantages beneficial for the designs within the scopes in this thesis, such as the fluorescence enhancement of MG upon binding to MGA towards rapid K_d assessment. Binding affinity determination based on fluorescence enhancement of MG allows using minimal amounts of MGA which is the most expensive consumable in our project. Shimadzu RF-5301PC Spectrofluorophotometer providing continuous fluorescent excitation is employed in the fluorescence emission and decay spectra measurement of stilbene compounds while high-throughput TECAN M200 microplate reader can only supply pulse excitation, which is not applicable for stilbene excitation. In the synthesis of aptamer-stilbene conjugate, amine modified MGA and 4-Acetamido-4'-isothiocyanato-2,2'-stilbenedisulfonic acid disodium salt (SITS) are selected which are commercially available. Additionally, SITS which has significant fluorescence decay in aqueous system is a preferred choice for our experimental design. Finally, for characterization of MGA-SITS conjugate, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry is applied to assess the molecular weight of MGA before and after conjugation, while high-performance liquid chromatography-diode array detector (HPLC-DAD) is used for indication of conjugation and purity monitoring. Ultraviolet-visible (UV-Vis) measurement is chosen for studies on kinetics of MG conversion based on the significant UV-Vis absorbance and the known conversion of MG. Methods chosen for statistical analysis in this thesis are the commonly used approaches in data explanation.

3.2 Selection of Model Aptamer – Malachite Green Aptamer (MGA) and Malachite Green (MG)

To test our hypothesis, an appropriate aptamer/ligand model possessing the ideal properties is preferred: as small length of nucleotide as possible, binding to small molecules with high affinity (K_d less than $1\ \mu\text{M}$), ease of binding affinity determination, amendable for modification and well-known 3D NMR or crystal structure. After searching in an online aptamer database (<http://aptamer.icmb.utexas.edu/>) established by Ellington and co-workers [1], MGA/MG that meets the requirements above has been chosen as an ideal model for our study. There are also other aptamers meet most of the requirements above but ultimately ruled out, which can be considered as candidates in the future. For instance, kanamycin B aptamer has a K_d of 180 nM, but its length is more than 100 nucleotides, dramatically increasing the cost on aptamer. A 29-mer sulforhodamine B aptamer binds to sulforhodamine B with a K_d of 190 nM, but no report of its NMR or XRD structure is found. **Figure 3.1** displays the structures of MGA and MG (1). Besides the properties above, MGA can also bind to other targets such as tetramethylrosamine (TMR), and thus TMR can be a candidate or positive control in our hypothesis testing. The XRD (1F1T) and NMR (1Q8N) structures of MGA has been revealed and used in computational modelling [2,3], which explains the position and interaction between MGA and stilbene molecules. Binding affinities of the MGA point mutations have also been reported [3], which give indications on the modification of MGA in our designs. For example, A9 mutant (within binding site) has tinny impact on K_d (< 2 times increase in K_d when A9 was replaced by C9). Commercially available derivatives of MG facilitate the synthesis of MG-stilbene conjugates. On the other hand, MG has commercial prevalence as an efficient fungicide but has been banned for fish farming in the United States, Canada and European union due to its potential toxicity [4]. Successful development of an aptasensor according to the hypothesis will provide a new approach for detection of MG. However, it also needs to be noted that MGA is a RNA aptamer, which has a more costly synthesis than DNA.

Figure 3.1 Structure of malachite green aptamer (MGA), malachite green (MG) (1), MG-CH₂-NH-stilbene-OMe (2), 4,4'-Diisothiocyanatostilbene-2,2'-disulfonic acid disodium salt (DIDS) (3), 4-Acetamido-4'-isothiocyanato-2,2'-stilbenedisulfonic acid disodium salt (SITS) (4), MGA-SITS (5) and malachite green carbinol base (MGOH) (6). 3D structure of MGA (1Q8N) is presented by Discovery studio 3.5 Visualizer, Accelrys.

3.3 Determination of MGA Binding Affinity by High-Throughput Microplate Assay

3.3.1 High-Throughput Binding by Fluorescence Enhancement of MG

There are various techniques used for binding affinity measurements. For instance, fluorescence intensity (FI), fluorescence polarization (FP), ultraviolet-visible (UV-Vis) absorption, surface plasmon resonance (SPR) and isothermal titration calorimetry (ITC)

are commonly applied in K_d determination [5]. FI method usually utilizes the enhancement or quenching in fluorescence of ligand or aptamer (fluorophore-labelled) upon binding, which is a rapid, sensitive and simple method. However, it requires special properties of ligand or modification of aptamer. FP utilizes the difference in anisotropy of emission light for distinguishing the bound or free fluorescent molecules, which displays different rotational relaxation time. FP is available for binding assay of various fluorescent molecules [6]. However, it has disadvantages like requiring fluorescent/fluorophore-labelled molecules and suitable size of ligands. SPR is a powerful technique for determination for binding affinity based on changes in refractive index, which can perform sensitive, label-free and real-time monitoring. However, this method requires relatively complicated immobilization steps and suffers interference from non-specific binding [7]. ITC is a label-free technique requires no immobilization, but at least 1 mL of samples is needed in ITC measurements (at least 10 times more than FI method under our experimental conditions), which is not suitable for aptamer binding affinities. According to the methods described above, the simple, sensitive and rapid FI method based on the fluorescence enhancement of MG is selected for binding affinity determination. This method has been developed by us to be a high-throughput approach with a common microplate reader, which is an ideal option for our K_d determination requiring small sample consumption.

Fluorescent technique has been widely used in various research fields such as biological, chemical and medical studies. Fluorescence process includes excitation and subsequent emission of light in a further wavelength. At ground state, a fluorophore can absorb energy of a specific wavelength after is excited by light, and then stays in excited state for a finite time with partially energy dissipation, and finally returns to ground state with light emission in a certain wavelength. The processes are illustrated by the simple electronic-state diagram (Jablonski diagram) in **Figure 3.2**. Fluorescence possesses advantages such as high sensitivity, high specificity, wide concentration range, relative simplicity and low time consumption, while it also has shortcomings like suffering interferences from impurities, bubbles or environment (pH, temperature, etc). The obvious advantages of fluorescence enable it to be a powerful tool for our MGA/MG

binding assay which will be described below.

Free non-planar MG has fluorescence quantum yield of 0.790×10^{-4} without aptamer binding. However, when MG binds to MGA, it can lead to a unique enhanced quantum yield to 0.187 because of the more planar structure of MG upon binding, which results in significant enhancement in fluorescence ($\sim 2000\times$) [8]. This fluorescence enhancement can be utilized for monitoring the MGA/MG binding event and measuring the K_d in our study. **Figure 3.3** displays the fluorescence enhancement of MG at ~ 650 nm upon binding to MGA.

Figure 3.2 Jablonski diagram illustration of fluorescence emission.

Figure 3.3 Fluorescence enhancement of MG upon binding to MGA at excitation wavelength of 620 nm.

MG derivatives bind to MGA or MGA derivatives bind to MG with small K_d (less than 1 μM) is a necessary parameter for success biosensor evaluation, along with specific fluorescence decay properties of stilbene conjugates. This necessitates a high-throughput and rapid binding assay with minimal amounts of aptamer consumption. Fluorescence measurements in 96-well microplate by TECAN M200 microplate reader meet the requirements above. In brief, a typical assay measurement using 150 nM MG with various ratios of MGA in binding buffer are mixed and transferred into a black 96-well microplate and measured by a Tecan Microplate spectrofluorometer (Infinite M200, Tecan Asia PTE LTD, Singapore), triplicate for each concentration. Excitation/emission wavelengths are set to 620 nm/656 nm [9]. By using this high-throughput fluorescence method, only 5.5 ng of MG is needed for each well. **Figure 3.4** is representative data of MG fluorescence enhancement upon binding to MGA, where a saturation of MGA is obtained after $\sim 10\times$ of MGA is present.

Figure 3.4 An illustration of K_d determination by fitting equation EQ (1) based on the fluorescence enhancement of MG (150 nM) at ex/em = 620 nm/656 nm upon binding to MGA.

3.3.2 Fitting Equation for K_d Determination in Direct Binding

The equation for K_d determination of MGA/MG binding has been deduced fundamentally, which has been reported [10-12] with some modifications:

$$F = F_0 + (F_{\max} - F_0) \times \frac{([MG]_0 + [MGA] + K_d) - \sqrt{([MG]_0 + [MGA] + K_d)^2 - 4[MG]_0 \times [MGA]}}{2 \cdot [MG]_0} \quad \text{EQ (1)}$$

F and F_0 are the fluorescence intensity in the presence and absence of aptamer, respectively. $F_{\max}$ is the fluorescence intensity while $[MGA] \gg [MG]_0$. $[MG]_0$ and $[MGA]$ are the concentrations of MG and MGA added into the binding system, and the K_d is the dissociation constant of the binding.

Process of deduction:

Set the fraction of binding as

$$f = \frac{F - F_0}{F_{\max} - F_0} \quad 0 \leq f \leq 1 \quad \text{EQ (2)}$$

and

$[MGA]'$: concentration of free MGA in solution.

$[MGA \cdot MG]$: concentration of MGA MG complex in solution.

$[MG]$: concentration of free MG in solution.

Thus,

$$MGA \cdot MG \xrightleftharpoons{K_d} MGA + MG \quad \text{EQ (3)}$$

$$[MGA]' = K_d \times [MGA \cdot MG] / [MG] \quad \text{EQ (4)}$$

$$[MGA \cdot MG] = [MG]_0 \times f \quad \text{EQ (5)}$$

$$[MG] = [MG]_0 \times (1 - f) \quad \text{EQ (6)}$$

$$[MGA] = [MGA]' + [MGA \cdot MG] \quad \text{EQ (7)}$$

Based on EQ (7) and EQ (4),

$$[MGA] = K_d \times [MGA \cdot MG] / [MG] + [MGA \cdot MG] \quad \text{EQ (8)}$$

Based on EQ (5), EQ (6) and EQ (8),

$$[MGA] = K_d \times \frac{f}{1-f} + [MG]_0 \times f \quad \text{EQ (9)}$$

From EQ (9),

$$[MG]_0 \times f^2 - ([MG]_0 + [MGA] + K_d) \times f + [MGA] = 0 \quad \text{EQ (10)}$$

Solve the mathematical equation EQ (10), and get

$$f = \frac{([MG]_0 + [MGA] + K_d) \pm \sqrt{([MG]_0 + [MGA] + K_d)^2 - 4[MG]_0[MGA]}}{2 \cdot [MG]} \quad \text{EQ (11)}$$

And, $0 \leq f \leq 1$, so

$$f = \frac{([MG]_0 + [MGA] + K_d) - \sqrt{([MG]_0 + [MGA] + K_d)^2 - 4[MG]_0[MGA]}}{2 \cdot [MG]_0} \quad \text{EQ (12)}$$

Combine EQ (2) and EQ (12), and an equation for fitting of binding curve is obtained:

$$F = F_0 + (F_{\max} - F_0) \times \frac{([MG]_0 + [MGA] + K_d) - \sqrt{([MG]_0 + [MGA] + K_d)^2 - 4[MG]_0[MGA]}}{2 \cdot [MG]_0} \quad \text{EQ (1)}$$

All the fitting calculations are performed by OriginPro 9.1. Fitting of the binding data in **Figure 3.4** by equation EQ (1) results in a high R^2 (> 0.99) and the K_d obtained compares well to the reported value.

Practically, there are two common methods for determination of K_d in direct binding assay. Besides the equation described above, another simple equation (Michaelis–Menten equation) is also applied to fit the binding data:

$$F = \frac{[F_{\max}] \times [MGA]}{K_d + [MGA]} \quad \text{EQ (13)}$$

However, equation is applicable while $[MG]_0$ is well below the K_d in experimental setup (Under this situation, $[MGA] \approx [MGA]'$) [13]. Even through a high R^2 (> 0.99) and related similar K_d can also be obtained using EQ (13) for fitting, as shown in **Figure 3.5**, EQ (1) is preferred for this thesis.

Figure 3.5 An illustration of K_d determination by fitting equation EQ (13) based on the fluorescence enhancement of MG (150 nM) at ex/em = 620 nm/656 nm upon binding to MGA.

3.3.3 Determination of K_d in Competitive Binding

In some cases, MG derivatives or MG-stilbene conjugates may not have fluorescence enhancement upon binding to MGA. Thus, based on the fluorescence enhancement of MG upon binding to MGA, competitive binding assay is employed. In competitive binding mode, binding affinity is determined by varying concentrations of MG derivatives or MG-stilbene with MGA/MG complex concentration held constant. If the MG derivatives or MG-stilbene conjugates binds to MGA with competitively high affinity compared to MGA/MG binding, the fluorescence intensity of MGA/MG complex will decrease. The K_d can be determined by Cheng-Prusoff equation (EQ (14)), which is widely employed in competitive binding assay [14]:

$$K_d = \frac{IC_{50}}{1 + \frac{[MG]}{K_d(MG)}} \quad \text{EQ (14)}$$

K_d : dissociation constant of the conjugate

IC_{50} : concentration of the conjugate at 50% inhibition of MGA/MG complex

$[MG]$: concentration of MG

$K_d(MG)$: dissociation constant of MG

Figure 3.6 shows an example of competitive binding of an MG-stilbene conjugate (MG-CH₂-NH-stilbene-OMe (**2**) (**Figure 3.1**)) to MGA/MG complex.

Figure 3.6 An illustration of K_d determination by competitive binding of a MG-stilbene conjugate (2) to MGA/MG complex, ex/em = 620 nm/656 nm.

3.4 Fluorescence Emission and Fluorescence Decay Measurements of Stilbene Compounds by Spectrofluorophotometer

Hypothesis of this thesis (section 1.3) focuses on the effect of aptamer binding on fluorescence decay of stilbene. Fluorescence decay property of stilbene molecules is another critical element in our designs while detectable fluorescence emission/fluorescence intensity is needed for fluorescence decay measurement. The basic principle of fluorescence emission has been described above (section 3.3.1). Fluorescence decay in emission maximum of stilbene molecules is due to the *trans-cis* isomerization of them upon UV irradiation. Like stilbene compounds, there are also other similar photoswitchable molecules undergo isomerization. Azobenzene compounds (with -N=N-) undergo isomerization with a higher rate compared to stilbene compounds [15], which can be applied while faster signal reporting is needed. The time for *trans-to-cis*

isomerization of azobenzenes is in picosecond or even femtosecond scale, while the time for stilbenes is mainly in picosecond scale [16-19]. Even limited by quantum yield, azobenzenes are still possible candidates for stilbenes in rapid sensing as methods for azobenzene fluorescence quantum yield enhancement have been developed [20,21], though the enhanced quantum yield is still small compared to that of stilbenes. *N*-benzylideneaniline compounds (with -C=N-) with advantages of lower activation energy and convenient synthesis also have property of isomerization upon UV excitation [22]. However, they are rarely used for photoswitch due to the easy hydrolysis of imines [23]. Stilbene compounds are chosen in our design because of their appropriate detectable fluorescence decay time. However, if our hypothesis is supported by experimental data, when faster detection or monitoring is needed, azobenzene compounds should be good candidates, for instance, for detection in a microplate reader with pulse excitation.

In this study, fluorescence emission and fluorescence decay spectra are recorded by Shimadzu RF-5301PC spectrofluorophotometer (Japan). RF-5301PC provides continuous excitation which is suitable for fluorescence decay experiments of stilbene compounds, but M200 microplate reader can only supply pulse excitation. Briefly, for fluorescence emission spectra recording, appropriate concentration of stilbene sample is placed in a quartz cuvette and the fluorescence emission spectrum is recorded by the Spectrofluorophotometer. Wavelength of emission maximum (λ_{max}) will be obtained by fluorescence emission spectrum scan, which will be used as emission wavelength in subsequent fluorescence decay measurement. Excitation/emission wavelengths, testing solvent systems and concentrations vary according to certain stilbene compounds based on their specific fluorescent properties. **Figure 3.7** displays the fluorescence emission spectrum of a stilbene molecule 4,4'-Diisothiocyanatostilbene-2,2'-disulfonic acid disodium salt (DIDS) (**3**) (**Figure 3.1**). For fluorescence decay measurement, appropriate concentration of stilbene sample (varies in different kinds of stilbene compounds) is placed in a quartz cuvette, and the fluorescence intensity at λ_{max} is recorded as a function of time, and the apparent decay rate is analyzed by a first order fitting which will be introduced below.

Figure 3.7 Fluorescence emission spectrum of stilbene compound DIDS (**3**) with $\lambda_{\text{ex}} = 340$ nm.

Analysis of apparent fluorescence decay rate of stilbene molecules is adapted from literature [24]. Calculation of apparent fluorescence decay rate follows the equation $dI_t/dt = -k_{\text{app}} \times I_t + \text{constant}$, which can be rearranged into a first order equation for convenient fitting analysis:

$$I_t = A \times e^{-k_{\text{app}} \times t} + B \quad \text{EQ (15)}$$

t: decay time.

k_{app} : apparent fluorescence decay rate constant (first order).

I_t : fluorescence intensity at time t.

A: total change of fluorescence intensity.

B: final fluorescence intensity.

Figure 3.8 shows the fluorescence decay spectrum and apparent fluorescence decay rate constant of DIDS (**3**). All the fitting calculations are performed by OriginPro 9.1.

Figure 3.8 Fluorescence decay spectrum at wavelength of emission maximum ($\lambda_{\max}$) and the corresponding apparent fluorescence decay rate constant k_{app} (calculated by EQ (15)) of stilbene compound DIDS (**3**) (ex/em = 340 nm/414 nm).

3.5 Preparation of MGA-SITS Conjugate (**5**) (Figure 3.1)

3.5.1 Synthesis and Purification of MGA-SITS Conjugate

There are several usual approaches used for oligonucleotide/protein bioconjugation with ligand: amine-isothiocyanate reaction, azide-alkyne click reaction, thiol-maleimide, amino-N-hydroxysuccinimide ester [25]. Selection of reaction approaches mainly depends on the active groups and difficulty in obtaining of aptamer or stilbene compounds. In this study, commercially available 4-Acetamido-4'-isothiocyanato-2,2'-stilbenedisulfonic acid disodium salt (SITS) (**4**) (**Figure 3.1**) with one active group (isothiocyanate) is paired with amine modified MGA (MGA-NH₂), purchased from AITbiotech Pte Ltd Singapore. The synthesis of MGA-SITS follows a typical amine-isothiocyanate reaction protocol (**Figure 3.9**). In our study, the reaction is conducted

based on previous methods [26]. Briefly, MGA-NH₂ reacts with 100X excess of SITS in pH = 9 sodium bicarbonate reaction buffer for 12 hours at room temperature.

Figure 3.9 Synthesis of MGA-SITS conjugate (5).

Purification of conjugate and removal of excess SITS is done by ultrafiltration with Amicon Centrifugal Filter based 10k Da membrane. The reaction mixture was centrifuged, concentrated, and rediluted with binding buffer to be centrifuged again. The cycle was repeated 9 times to yield a SITS-free MGA-SITS. A product yield of > 80% can be obtained. Nanodrop UV-Vis spectrometer (260 nm) and HPLC-DAD are used to monitor the purification. A simplified schematic description of HPLC-DAD on the typical working flow/principle has been drawn in **Figure 3.10**. C₁₈ reversed-phase chromatography is able to separate the MGA-NH₂ and SITS compounds for analytical purpose. Additionally, DAD detector can record a full spectrum of the peaks in a specific retention time. However, DAD has shortcomings such as relatively low sensitivity and large noise compared to UV-Vis HPLC detector, but this is not an issue in our analysis as our analytes are in relatively high concentration. This HPLC-DAD system can give information on how much free (not conjugated) SITS in the purified product and thus ensure the purity of the final product. **Figure 3.11** gives an example of our C₁₈ reversed-

phase HPLC-DAD chromatography on analysis of MGA-NH₂ with mobile phase of ammonium acetate buffer/acetonitrile, where the spectrum of MGA-NH₂ and the full spectra over the elution period are also displayed.

3.5.2 Characterization of MGA-SITS Conjugate

HPLC-DAD and MALDI-TOF mass spectrometry are used for the characterization of the synthesized conjugate. HPLC-DAD is used for monitoring the change in UV-Vis spectrum of the MGA-NH₂ peak (Absorbance at 260 nm). If the SITS is successfully grafted on MGA, absorbance at 340 nm should increase at the peak retention time of MGA-NH₂, indicating the successfully grafting of SITS on MGA. The procedure of HPLC-DAD measurement is the same as that described in **section 3.5.1**.

Figure 3.10 Schematic simplified working flow/principle of HPLC and DAD.

Figure 3.11 C₁₈ reversed-phase HPLC-DAD chromatography of MGA-NH₂.

MALDI-TOF mass spectrometry is a technique for measurement of molecular weight of targeted compounds based on the time of flight of the charged compound. The working principle of MALDI-TOF mass spectrometry is shown in **Figure 3.12** [27]: Crystals form upon the mixing of analyte and saturated matrix; then ionization takes place after laser irradiation (typical wavelength is 337 nm); the formed sample ions will be accelerated by a high voltage (from 15 kV to 25 kV) within the electrostatic field; then the molecules in sample will be separated in the time of flight tube according to the time of flight, which depends on the mass-to-charge ratio (m/z) of molecules in defined experimental conditions: $(mv^2)/2 = zeEs$ — while experimental conditions e , E and s are fixed, velocity v decrease as mass-to-charge ratio m/z increase, resulting in an increase of time of flight to reach the detector; finally, sample ions will reach the detector and corresponding spectrum will be record. Values of m/z which is indicated by flight of time can be directly reflected in MALDI-TOF mass spectrum.

The relatively small error (less than 0.5%) and high sensitivity of MALDI-TOF mass spectrometry system enables it to tell the difference of MGA-NH₂ before and after conjugation with SITS, which is sufficient for characterization of the conjugate. 2', 4', 6'-Trihydroxyacetophenone monohydrate (THAP) and 3-Hydroxypicolinic acid (3-HPA) are excellent matrices for oligonucleotide in MALDI-TOF mass spectrometry, which are able to promote the ionization of oligonucleotide and reduce fragmentation [28]. THAP

matrix is mixed with matrix: MGA-SITS = 1:1 (v/v). Prior to the test, MGA-SITS undergoes desalting pretreatment by Amicon Centrifugal Filter with 100 mM ammonium citrate (pH = 6), which removes the interference from metal ions (i.e. Na^+ and Mg^{2+}). Results display that the mass spectrum obtained is sufficient to suggest the conjugation of SITS on MGA (**Figure 3.13**).

Size exclusion chromatography (SEC) is also an approach for characterization of the conjugation reaction based on column retention time. However, the resolution of most SEC column is inadequate to separate the molecular weight difference of a SITS molecule (~500 Da) based on the retention time or with static light. Moreover, SEC method requires large sample amount (10-100 μg) for injection. Thus, SEC method is not an ideal technique under our experimental conditions. NMR is another method directly showing the structure of RNA. Compared to the methods above, NMR may even give the information on *trans-cis* isomerization of SITS grafted on MGA. However, NMR experiments require a large amount of costly oligonucleotide, and the signal of SITS molecule may also be interfered by oligonucleotide signals. Thus, MALDI-TOF mass spectrometry should be an appropriate method for the characterization of the MGA-SITS conjugate, while HPLC can also give confirmation on the successfully conjugation of stilbene to aptamer.

Figure 3.12 Schematic principle of MALDI-TOF mass spectrometry.

Figure 3.13 MALDI-TOF spectra of MGA-NH₂ and MGA-SITS conjugate (**5**). Top (blue): MGA-NH₂ before conjugation. Bottom (red): SITS grafted MGA-NH₂ (MGA-SITS conjugate (**5**)). The m/z increases for 457 units after the conjugation, indicating the molecular weight of the aptamer molecule increases 457 Da upon attachment of the SITS fragment. The MALDI-TOF results indicate the SITS is successfully conjugated on MGA.

3.6 Kinetic Study on MG Conversion

Kinetic study on MG or malachite green carbinol base (MGOH) (**6**) (**Figure 3.1**) conversion is accomplished by monitoring UV-Vis absorbance of MG at absorbance maximum (~620 nm). Briefly, MG or MGOH solution is placed into a Varian Cary 50 UV-Vis spectrophotometer and the UV-Vis spectra are recorded every 5 min for 12 hours. According to Beer-Lambert law, concentration of analyte is directly proportional to the specific absorbance. The working principle of UV-Vis spectrophotometer is introduced in

Figure 3.14. MG has a significant absorbance maximum at wavelength around 620 nm while MGOH has no significant absorbance at the region around 620 nm. Studies by HPLC have indicated that MG can hydrolyze to MGOH under certain conditions. Thus, UV-Vis recording at wavelength of MG absorbance maximum is a rapid and sufficient method to monitor the hydrolysis of MG to MGOH, and vice versa. **Figure 3.15** shows an example of kinetic study of MG conversion monitored by UV-Vis spectrophotometer.

Figure 3.14 Schematic working principle of UV-Vis spectrophotometer.

Figure 3.15 An example of kinetic study on MG conversion by UV-Vis spectrophotometer.

3.7 Statistical Analysis

All the statistics were performed by OriginPro 9.1 (OriginLab Corporation, Northampton, MA 01060 USA). Linear regressions R^2 were determined by ordinary least squares with a minimum of $n = 3$ data points. To test the statistical difference between two groups of values, hypothesis testing was performed by Student's t-test with significance level $p < 0.05$ (two-tailed test).

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Chapter 4

Conditions for Malachite Green Aptamer Binding

This chapter studies the binding conditions for malachite green aptamer (MGA) binding with malachite green (MG), and the stability of MGA, MG and their binding, which pave the foundation for the hypothesis testing. Binding affinity and fluorescence intensity of MGA/MG binding were found to decrease over time. It was hypothesized the hydrolysis of MG to malachite green carbinol base (MGOH) resulted in the loss of fluorescence, while our results suggest this conversion and confirm the integrity of MGA over 48 hours. Interestingly, we noted the employment of organic additives retained the fluorescence and binding over time, and synergistic effect from both organic additives and aptamer on stabilizing MG lability were found and discussed. Limited impact from organic additives on binding affinity was found. Regarding to the hydrophobicity of stilbene, organic additives are required in MGA/MG binding. Additionally, effect of pH on MGA/MG binding was also investigated. The results obtained in this chapter establish optimal binding conditions for hypothesis testing and provide useful information on the future aptameric sensing.

*This section published substantially as: Y. Zhou; H. Chi; Y. Wu; R. S. Marks; T. W. J. Steele *Talanta* **2016**, 160, 172-182.

4.1 Introduction

Prior to the test of the hypothesis, basic experimental conditions need to be defined for reliable binding. Malachite green aptamer (MGA) with its ligand malachite green (MG) (**1**) is a model study in the thesis, while triphenylmethane compound MG may undergo hydrolysis and the RNA aptamer MGA may degrade. It was reported that MG can transfer to its derivatives like leucomalachite green and malachite green carbinol base (MGOH) (**6**) in aqueous environment (**Figure 4.1A**) [1,2]. On the other hand, RNA is known to be susceptible to degradation. Thus, it is essential to investigate the stability of MGA, MG and their binding, and establish suitable experimental conditions for the binding, which is the prerequisite for hypothesis testing. As reviewed in literature (**section 2.2.8**), MGA/MG binding results in an enhanced fluorescence quantum yield (more than 2000X) upon recognition, which can be employed for study the binding interaction (**Figure 4.1B**) [3]. The initial binding affinity (or equilibrium dissociation constant, K_d) was determined to be comparable to literature at time 0. However, it was found that MGA/MG binding had increasing apparent K_d as determined through the first order rate loss of their fluorescence intensity over time, indicating variation in MGA, MG, or their binding. Electrophoresis analysis suggests MGA kept its integrity with 48 hours while HPLC results indicated MG converted to MGOH over time, and thus the hydrolysis of MG to MGOH was hypothesized to be the cause of fluorescence intensity loss (**Figure 4.1A**). Various organic additives (acetonitrile (ACN), dimethyl sulfoxide (DMSO) and ethanol (EtOH)) were investigated to retain the binding stability [4]. Both fluorescence intensity and apparent K_d were less sensitive to time in the presence of the organic additives, and the protection became more apparent with increasing organic additive percentage (up to 10%, v/v, the same to all description on organic additive percentage). Additionally, the presence of the organic additives only has limited impact on the 0 time K_d . In fact, hydrophobic stilbene (miLog P = 4.5, calculated by Molinspiration Cheminformatics 2016: www.molinspiration.com) normally requires the involvement of organic additives due to its solubility issue, and the results enable the application of these additives on MGA/MG binding for the test of hypothesis. This simple strategy minimizes deviations in the MGA/MG binding analysis, and also indicates an approach for

stabilizing the aptamer binding of other triphenylmethane, triarylmethane, or carbocation-susceptible ligands bindings susceptible to S_N1 hydrolysis. Additionally, other studies like pH effect investigation are also conducted. Various results and conclusions obtained in this chapter establish optimal conditions for MGA/MG binding as well as hypothesis testing, and provide meaningful information and evidence for the future MGA based biosensing.

Figure 4.1 (A) Structure of malachite green aptamer (MGA) (6), malachite green (MG) (1) and malachite green carbinol base (MGOH). (B) Binding of MGA to MG, with fluorescence enhancement obtained. (Reprinted with permission from reference [4].)

4.2 Materials and Methods

4.2.1 Materials

Malachite green aptamer (MGA) RNA (5'-GGAUCCCGACUGGCGAGAGCCAGGUAACGAAUGGAUCC-3') was purchased from AITbiotech Pte Ltd Singapore. Acetonitrile (ACN) (HPLC grade) was purchased from Tedia, USA. Dimethyl sulfoxide (DMSO) was purchased from Alfa Aesar, USA. Ethanol (EtOH) was purchased from Fisher Scientific, Singapore. Malachite green oxalate (MG) (1) and malachite green carbinol base (MGOH) (6) were purchased from

Sigma Aldrich, Singapore. Polystyrene microplates (96-well) were purchased from Corning, USA. All chemicals and materials were used as received.

Binding buffer (pH = 6.7 unless otherwise specified) consisting of 5.65 mM NaH_2PO_4 , 4.35 mM Na_2HPO_4 , 10 mM MgCl_2 and 1 mM NaCl was prepared according to the literature [5,6].

4.2.2 MGA/MG Binding Assay

The MGA/MG binding was determined by monitoring the fluorescence enhancement of MG upon binding according to literature [3], and the method has been described in **section 3.3.1** and **section 3.3.2**. In details, K_d was determined varying ratios 0:1 to 40:1 MGA:MG with MG concentration held constant (150 nM) in binding buffer or organic additive/binding buffer mixture. Fluorescence of MG was immediately assayed from 0 h to 24 h in 96-well polystyrene plates with a Tecan Microplate spectrofluorometer (Infinite M200, Tecan Asia PTE LTD, Singapore), at ex/em = 620 nm/656 nm.

Fitting of binding equilibrium dissociation constant K_d was achieved by using function EQ (1) in **section 3.3.2**, where the rationale of such fitting is also described. Non-linear fitting and statistical analysis were performed by OriginPro 9.1.

In the investigation of organic additives on fluorescence of MGA/MG complex post decay, DMSO was added into the complex 5 h after the binding experiment started.

4.2.3 High Performance Liquid Chromatography (HPLC)-Diode Array Detector (DAD) Study on MG Hydrolysis

An Agilent 1200 series HPLC system with DAD was used to investigate the stability of MG. Chromatographic separation was conducted using a $5\ \mu\text{m} \times 4.6\ \text{mm} \times 250\ \text{mm}$ UltisilTM C18 column (Welch Materials Inc.). The HPLC-DAD system was operated in $1.0\ \text{mL}\cdot\text{min}^{-1}$ flow rate and 20 μL sample injection. The DAD was set to λ of 265 nm and

620 nm for simultaneously detection of MGOH and MG respectively, with continuously full spectrum scans from 200 to 800 nm. Fresh MG in binding buffer (30 μ M) was injected at 0 h and 24 h, while MGOH in HPLC grade ACN (30 μ M) as a standard was injected at 0 h. Elution profile for liquid chromatography was: 50% mobile phase A (25 mM ammonium acetate, pH 6.8)/50% mobile phase B (ACN, HPLC grade) for 5 min, a gradient to 15% A/85% B from 5 to 15 min, a 15% A/85% B from 15 to 18 min, followed by a ramp back to initial conditions from 18 to 21 min and held for 4 min thereafter.

4.2.4 UV-Vis Spectral Study on Kinetic Conversion between MG and MGOH

MG and MGOH were dissolved in binding buffer (pH 6.7) with or without 10% (v/v) organic additive, respectively. The sample was loaded in a quartz cuvette and measured in a Varian Cary 50 UV-Vis spectrophotometer. The UV-Vis spectra were recorded every 5 min, with a total period of 12 hours. MG $\lambda_{\max}$ at 618 nm was employed to calculate the change of MG. The decrease of MG in conversion MG $\rightarrow$ MGOH and the increase of MG in conversion MGOH $\rightarrow$ MG were fitted using first order equation which were performed by OriginPro 9.1.

4.2.5 Electrophoresis of MGA

Electrophoresis measurement was applied to analyze the integrity of MGA (RNA) in binding buffer over 48 hours. Samples stained with AMRESCO EZ-Vision® One (with amaranth indicator) were loaded on agarose gel (2%), and electrophoresed for 40 minutes at 100 V in 1X tris-acetate-EDTA (TAE) buffer (pH = 8.0). BioLabs 100 bp DNA ladder (from 100 bp to 1517 bp) was used as marker for reference. Finally, 365 nm UV exposure was applied to visualize the gel.

4.3 Results and Discussion

Prior to the test of the hypothesis, it is critical to get optimal conditions for reliable MGA/MG binding. Initially, pH effect was studied to obtain a suitable pH (6.7) for the binding according to our results and literature [6]. Particularly, owing to the known instability of MG and RNA, it is worth noting that the MG, MGA or MGA/MG binding may become unstable within a certain period. However, the fluorescence intensity and apparent K_d of MGA/MG binding is seldom reported. Wang et al. has experimentally and theoretically reported that MGA could prevent hydrolysis of MG in aptamer binding by monitoring the absorbance maxima in bound and free MG, but no fluorescence data was mentioned [7]. According to our study on MGA/MG complex, fluorescence intensity decreased while apparent K_d increased as a function of time. For the first time, we report how the addition of common organic additives retained both fluorescence intensity and binding affinity, and thus stabilized the binding (within a 24 hour timeframe). This stabilization is beneficial for MGA-based applications towards accurate and repeatable detection assays. A synergistic stabilization mechanism based on kinetic analysis is proposed. Additionally, stability of MGA and MG was investigated by electrophoresis and HPLC respectively. All the results obtained in this chapter illustrate optimal experimental conditions for MGA/MG binding as well as the hypothesis testing.

4.3.1 Effect of pH on Aptamer Binding Affinity

Considering the possible impact of pH on aptamer binding and MG, binding assays were tested in binding buffer with pH from 5 to 8.5. The results (**Figure 4.2A** and **Figure 4.2B**) suggest that the suitable range for binding is from pH 6 to pH 8.5, which results in K_d close to 200 nM. Thus, pH at 6.7 was chosen for the MGA/MG binding assay and hypothesis testing, which has been reported previous [6].

Figure 4.2 MGA/MG binding curves at ex/em = 620 nm/656 nm (A) and corresponding apparent K_d values (B) in various pH. (Reprinted with permission from reference [4].)

4.3.2 Fluorescence Intensity and Apparent Affinity of MGA/MG Complex Diminish Over Time

As displayed in **Figure 4.3A**, the initial K_d is determined to be 200 nM based on the MG fluorescence enhancement upon aptamer binding, which compares well to the result reported by Costa et al [6]. However, as presented in **Figure 4.3A**, fluorescence intensity and apparent binding affinity of MGA/MG complex reduce with time, but reach a plateau at 5 h. At 24 h, 40.8% of the fluorescence intensity at MGA:MG of 40:1 (maximum ratio) remains and the apparent K_d increases to be more than 10000 nM. The apparent K_d was determined based on the unique fluorescence enhancement of MG upon induced aptamer binding, but when the fluorescence signal decays, the apparent K_d will be directly affected. The fluorescence intensity at MGA:MG of 40:1 undergoes a first order decay over 24 hours with decay rate constant $k = 2.5 \times 10^{-4} \text{ s}^{-1}$ (**Figure 4.3B**). This decrease suggests an unstable association between MGA and MG, where more results will be presented to find out the cause below. Minute variations in pH are also ruled out since only minor shifts in K_d are observed across pH 6 to 8.5 (**Figure 4.2B**). These results indicate an instable binding, which may be due to the loss of MGA or MG over time. This instability may affect the hypothesis testing and thus it is critical to find a way to prevent this instable binding.

Figure 4.3 (A) Binding K_d of MGA/MG in binding buffer from 0 to 24 h. (B) Normalized fluorescence decay at MGA:MG of 40:1 (the highest ratio). (Reprinted with permission from reference [4].)

4.3.3 Addition of MG Reverses Decreased Fluorescence and MGA Keeps Stable Over Time

In two individual preliminary tests, MG and MGA were separately added into the MGA/MG complex after 24 hours respectively to investigate the cause of fluorescence decay. From the results shown in **Figure 4.4A**, the presence of fresh MG recovers the fluorescence intensity instantly, which continues to decay. However, the presence of fresh MGA displays no recovery on fluorescence. Such observation indicates the decrease in fluorescence intensity was owing to events centered on MG rather than MGA, indirectly ruling out RNA aptamer degradation or misfolding.

To isolate the cause on fluorescence diminishing over time, stability of MGA (RNA aptamer) in binding buffer was investigated. To analyze the integrity of MGA over time, gel electrophoresis on MGA was conducted. As observed in **Figure 4.4B**, no obvious change in the MGA band is observed from 0 h to 48 h. The kept integrity of MGA over 48 h enables its flexible use in aptamer binding for the hypothesis testing.

Figure 4.4 (A) Fluorescence intensity of MGA/MG complex after 24 h binding and with the subsequent addition of original amount of MG (blue square) and MGA (black circle). (B) Agarose gel electrophoresis of MGA over time in binding buffer. (Reprinted with permission from reference [4].)

4.3.4 Loss of Fluorescence Is Attributed to Hydrolysis of MG to MGOH

HPLC-DAD allows the identification and quantification of MG derivatives through their chromatography and absorbance spectra. **Figure 4.5A&B** insets present the UV-Vis spectra of MG and MGOH obtained by the DAD. HPLC chromatogram overlays of MG 0 h, MG 24 h, MGOH standard are shown in **Figure 4.5A** (with $\lambda = 620$ nm for MG) and **Figure 4.5B** (with $\lambda = 265$ nm for MGOH). MG 0 h shows a major peak at 11.1 min with a minor peak at 18.2 min observed, attributed to MGOH. The peak of MGOH in the MG 0 h sample presents 19% AUC compared to the MGOH standard (30 μ M). In other words, 5.7 μ M of MGOH is detected to exist in the 30 μ M of MG 0 h sample (as prepared). After 24 h, the MG peak AUC decreases 36.3% (**Figure 4.5A**) while the MGOH peak AUC increases 38.6% (**Figure 4.5B**), nearly the same variation in AUC. In the MG 24 h sample, MGOH concentration nearly triples to 17.1 μ M, and there is no observed formation of other minor peaks during 0-25 min elution. In summary, according to the HPLC results, only MG and MGOH are the major reactants and products in the binding condition. The 1 to 1 shift of MG peak to MGOH peak associated with the UV-Vis profile spectra confirms the conversion of MG to MGOH, with no other peaks of interests detected [1,2,8], nullifying other side reactions. This discovery confirms the cause of the instable MGA/MG binding, providing clue on how to stabilize the binding, which is beneficial for the hypothesis testing.

Figure 4.5 HPLC of MG 0h, MG 24 h and MGOH. (A) Detection $\lambda = 620$ nm. (B) Detection $\lambda = 265$ nm. (Reprinted with permission from reference [4].)

4.3.5 Organic Additives Retain Fluorescence Intensity and Binding Affinity of MG with Limited Impact on K_d

Binding buffer with different percentages (3%-10%, v/v) of acetonitrile (ACN), dimethyl sulfoxide (DMSO) or ethanol (EtOH) (organic additives usually applied in biological recognition for solubility enhancement) is mixed into the binding experiments of MGA with MG, and the binding at 0h are analyzed (**Figure 4.6**). Interestingly, there is observable increase in MGA/MG fluorescence across the highest MGA:MG ratio in the

presence of organic additives (**Figure 4.6A-C**). In the presence of organic additives, more than 9000 fluorescence intensity unit are observed at MGA concentration above 3000 nM (**Figure 4.6A-C**), where the 5500 fluorescence intensity at time 0 h in **Figure 4.3A** is about 40% lower. This raises the possibility that MG→MGOH conversion (**Figure 4.5**) exists even before the time 0 h binding measurements in **Figure 4.3A** were conducted, and the presence of organic additives shifts the conversion equilibrium constant, decay kinetics, or both. Further analysis will be presented in the following sections. Organic additives are known to increase the K_d of molecular recognition elements [9]. To quantitate the impact from organic additives on binding affinity, the apparent K_d values are calculated and presented in **Figure 4.6D**. Only ACN weakens the overall K_d , while DMSO and EtOH show little to no changes in K_d . Furthermore, the apparent K_d shows a strong correlation with percentage of ACN, but weak to moderate correlation with percentage of DMSO or EtOH, as seen in **Figure 4.6D**. In other words, the involvement of DMSO and EtOH results in little or no impact on K_d , while ACN slightly weakens the overall apparent binding affinity, and the lowered affinity was proportional to the concentration of additives. Although the aptamer binding is typically conducted in aqueous environments, these results indicate the availability of organic additives in the MGA/MG binding assay. In particular, it is beneficial for the employment of stilbene or its conjugates with poor aqueous solubility, facilitating their MGA binding based assay in the hypothesis testing.

Figure 4.6 Apparent K_d values with organic additives at time 0 h. (A) With 3% and 10% ACN. (B) With 3% and 10% DMSO. (C) With 3% and 10% EtOH. (D) Variation of the apparent K_d values in different percentages of organic additives. * = $p < 0.05$: significant difference against apparent K_d without organic additive. (Reprinted with permission from reference [4])

The stability of MGA/MG binding after 24 h in the organic additive-buffer systems is investigated to see the effect of organic additives on the decreasing fluorescence intensity and binding affinity. **Figure 4.7A** illustrates the MGA/MG binding curves at 0 h and 24 h in the presence of different concentrations of ACN. Similar trends are found for DMSO and EtOH (data not shown). **Figure 4.7B&C** summarize the apparent K_d and fluorescence intensity at MGA:MG = 40:1 at 0 h and 24 h obtained against concentrations organic additives. More fluorescence intensity and apparent binding affinity are remained as the concentration of organic additive increases, suggesting that the organic additives prevent hydrolysis and stabilize binding, which may be the result of

a change in MG conversion K_{eq} , decay kinetics or both. For instance, the fluorescence intensity at MGA:MG = 40:1 decreases to 40.8% compared to the initial value and the apparent K_d increases to ca. 65-fold after 24 h binding (**Figure 4.3A&B**). By contrast, as displayed in **Figure 4.7B&C**, a 2.7-fold decrease in fluorescence decay (21.7% vs. 59.2%) occurs with an impact on apparent K_d increasing being significant (~3.2-fold vs. 65-fold compared to time 0) in the presence of 3% DMSO, where this impact is more apparent as the DMSO percentage increases. ACN and EtOH display the similar effect. Furthermore, organic additives lead to considerable shifts on the fluorescence decay kinetics (at MGA:MG = 40:1) as shown in **Table 4.1**. The first order decay kinetics of fluorescence decreased as the organic additive percentage increased from 3% to 10%, which implies that organic additives shifted the K_{eq} ($MG \leftarrow \rightarrow MGOH$) towards MG, and thus increased the overall amount of the fluorescent MGA/MG complex. The binding interactions between RNA aptamers and ligands are dynamic without a typical ‘lock and key’ static mechanism of enzyme, but follows an ‘induced fit’ dynamic mechanism that more closely resembles RNA folding surrounding its ligand, where both the RNA and ligand exhibits structural induced changes [10]. Thus, a clear explanation on how RNA folding varies after ligand hydrolysis hasn’t been explored, but brings a high level complexity best removed if possible, and this is another reason for the employment of organic additives herein.

The fluorescence intensity loss and K_d increase were prevented upon the addition of organic additives. This observation provides a simple approach to stabilize aptamer binding, which is beneficial for applications relying on MGA for accurate MG sensing. The strategy may also be employed to other triphenylmethane compounds susceptible to hydrolysis. [11-14]. According to SciFinder, more than 100000 triphenylmethane compounds ((Ph)₃-C) and 9000 substructures of MG have been reported to date. However, some triphenylmethane compounds have been banned for the use of farming owing to their potential toxicity, thus the monitoring of triphenylmethane compounds is of consumer significance [15]. Additionally, the triphenylmethane and other triarylmethane structures may form carbocations, which is susceptible to nucleophilic attack (i.e. hydrolysis in aqueous media). The discovery by using organic additives paves a way for

stabilizing aptamer binding to ligands which have triphenylmethane or similar carbocation structures. Moreover, the decreasing trend of fluorescence decrease rate constants with increasing additive concentration (**Table 4.1**) displays a strong correlation. From another view, the rate constant may be tuned by the type and concentration of organic additives, which may be beneficial for alternative designs for flow or in-line biosensors, for instance, sensing within specific time frames. These results indicate organic additives result in more MG and stabilize its aptamer binding as well as fluorescence intensity, providing optimal conditions for MGA/MG binding in the hypothesis testing.

Table 4.1 Decay rate constant ($k_{MG \rightarrow MGOH}$) of fluorescence intensity at MGA:MG = 40:1 (maximum ratio) with various organic additive percentages.

Media	$k_{MG \rightarrow MGOH} (\times 10^{-4}) (s^{-1})$
3% Acetonitrile	3.1 ± 0.4
5% Acetonitrile	2.6 ± 0.1
8% Acetonitrile	1.3 ± 0.2
10% Acetonitrile	1.0 ± 0.1
3% Dimethyl sulfoxide	2.7 ± 0.6
5% Dimethyl sulfoxide	$0.2 \pm 0.3^*$
8% Dimethyl sulfoxide	< 0.01, NSD
10% Dimethyl sulfoxide	< 0.01, NSD
3% Ethanol	4.0 ± 2.1
5% Ethanol	1.5 ± 0.1
8% Ethanol	0.4 ± 0.1
10% Ethanol	0.2 ± 0.1

* Fitting coefficient of determination $R^2 < 0.6$.

NSD: No statistical difference was seen between 0 and 24 h FLU.

Figure 4.7 (A) Binding curves of MGA to MG with different percentage of ACN at 0 h and 24 h. (B) Plots of calculated K_d values with different percentages of organic additives at 0 h and 24 h. (C) Plots of fluorescence intensity at MGA:MG = 40:1 at 0 h and 24 h with different percentages of organic additives. NSD = no statistical difference, $p < 0.05$. (Reprinted with permission from reference [4])

4.3.6 Organic Additives Dehydrate MGOH and Shift K_{eq}

To further understand the MG hydrolysis, kinetics in both directions of $MG \rightarrow MGOH$ and $MGOH \rightarrow MG$ was evaluated independently without aptamer. The test on $MG \leftrightarrow MGOH$ conversion kinetics (in the absence of aptamer) with and without organic additives (10% v/v ACN, DMSO or EtOH) is investigated by following the MG lambda max, as shown in **Figure 4.8**. The results is similar as noted in the HPLC characterization in **Figure 4.5**, that MG cannot be prepared pure in aqueous media owing to a $t_{1/2}$ of ~19 min ($\ln 2/6.2 \times 10^{-4} \text{ s}^{-1}$), while MGOH can with $t_{1/2} = 2.8 \text{ h}$. In binding buffer without any organic additive, the $MG \rightarrow MGOH$ conversion occurs 9-fold faster (**Figure 4.8A**, $k_{MG \rightarrow MGOH} = 6.2 \times 10^{-4} \text{ s}^{-1}$) than the $MGOH \rightarrow MG$ conversion (**Figure 4.8B**, $k_{MGOH \rightarrow MG} = 0.69 \times 10^{-4} \text{ s}^{-1}$). Moreover, the $MG \rightarrow MGOH$ conversion reaches equilibrium after 2 h, while the $MGOH \rightarrow MG$ conversion does not reach equilibrium after 12 h, as shown in **Figure 4.8B**. When MG converts to MGOH with 10% organic additives (**Figure 4.8A**), the $k_{MG \rightarrow MGOH}$ is retarded by 23%, 9%, and 5% for ACN, DMSO, and EtOH, respectively. From the fluorescence intensity decay kinetics, the aptamer without additives retards the $k_{MG \rightarrow MGOH}$ by 60% (**Figure 4.3B** and **Figure 4.8A**). These results suggest aptamer retards the $k_{MG \rightarrow MGOH}$ while organic additives have not huge impact on $k_{MG \rightarrow MGOH}$.

Surprisingly, the $MGOH \rightarrow MG$ conversion with 10% organic additives (**Figure 4.8B**) presents a comparably big shift in $k_{MGOH \rightarrow MG}$. Organic additives increase $k_{MGOH \rightarrow MG}$ (over pure buffer) by 360%, 450%, and 460% for ACN, DMSO, and EtOH, respectively. This acceleration on the conversion of $MGOH \rightarrow MG$ by organic additives leads to a large shift in K_{eq} compared to the MG in buffer or (MGA + buffer). To investigate if the MGA impact $k_{MGOH \rightarrow MG}$, MGA were mixed with freshly prepared MGOH and the fluorescence of generated MGA/MG complex was monitored over time using different MGOH:MGA ratios. As displayed in **Figure 4.9**, at high ratios of 20:1 and 40:1, there is no statistical difference in fluorescence rate of MG formation with MGA, compared to the UV-Vis absorbance rate of MG formation without aptamer. In other words, high ratios of aptamer have no effect on $k_{MGOH \rightarrow MG}$. Thus, the assumption that aptamer affected $MGOH \rightarrow MG$

conversion, was proven to be false, as increasing fluorescence (MG generation) was formed with the predicted kinetics. Combining the results of $\text{MG} \rightarrow \text{MGOH}$ conversion (**Figure 4.8A**), the results shows MGA retards $k_{\text{MG} \rightarrow \text{MGOH}}$ while organic additives slow $k_{\text{MGOH} \rightarrow \text{MG}}$. This total effect may be the reason why organic additives retain fluorescence intensity and binding affinity of MG (**Figure 4.7**), where the K_{eq} is shifted by the synergistic effect from both aptamer and organic additives. Towards MGA/MG binding at 0 h in **Figure 4.6A-C**, organic additives lower the affinity, but increase the fluorescence intensity of MGA/MG complex (compared to **Figure 4.3A**). This paradox can be explained by the K_{eq} shift and by noting that MG solutions (freshly prepared) always contain various ratios of MG to MGOH (**Figure 4.5**). **Figure 4.8** illustrates the accelerating $\text{MGOH} \rightarrow \text{MG}$ conversion by organic additives. Hence, the presence of organic additives results in synergistic shift towards MG (with aptamer), thus enhances the formation of MGA/MG based fluorescence.

Figure 4.8 Kinetic evolution between MG and MGOH (without aptamer). (A) MG converts to MGOH in binding buffer and in the presence of 10% organic additives. (B) MGOH converts to MG in binding buffer and in the presence of 10% organic additives. (Reprinted with permission from reference [4])

Figure 4.9 Rate constant of MGOH to MG ($k_{\text{MGOH} \rightarrow \text{MG}}$) in binding buffer in the presence of MGA. (Reprinted with permission from reference [4])

4.3.7 Organic Additives Reverse Decreased Fluorescence through MG Dehydration

The results above demonstrate the stabilizing effect on fluorescence intensity of organic additives by dehydrating MGOH. However, can the organic additives recover the decreased fluorescence intensity of MGA/MG complex? Thus, DMSO was added into MGA:MG buffer solutions which have reached a stable fluorescence intensity after 5 h of binding, and **Figure 4.10** shows the fluorescence recovery of 3% and 5% DMSO after 1h and 12 h incubation with the MGA/MG complex. The data suggests that organic additives can immediately vary the reaction kinetics to favor MG as the major equilibrium product. A minimum of 5% v/v DMSO is needed to recover the fluorescence intensity after 12 h. Higher DMSO concentration can result in fluorescence intensity values of 10000 (at MGA:MG > 20:1), similar to the values displayed in **Figure 4.7** (data not shown). The observation that the decreased fluorescence is completely reversible by the addition of organic additives (**Figure 4.10**) speculates that MG undergoes a time dependent variation in charge redistribution or electronic resonance within the binding site of MGA, which might reduce the quantum yield of MG or shift its fluorescent planar configuration formed by ‘induced fit’ [3,10]. We speculate the intermediate of MG hydrolysis, MG-OH₂, is responsible for the fluorescence decay through electronic or charge redistribution inside the binding pocket of aptamer [16], as the C⁺-OH₂

carbocation is further stabilized and turns to the preferred resonance structure. Shifts in NMR peaks of MG ^{13}C have confirmed the charge distribution change between free and bound MG [10]. However, because ^{13}C measurements require long timescale, it is unlikely to distinguish MG intermediates from MG. Alternatively, the more hydrophilic C^+-OH_2 carbocation may weaken the RNA base stacking forces, relaxing the induced fluorescent planar configuration [17]. The employment of organic additives prevents or reverses this. This observation also extends the application window of MGA/MG binding for hypothesis testing, where organic additives can be employed for stabilizing the binding even after the MGA/MG binding has started.

Figure 4.10 Addition of DMSO into MGA/MG complex at 5 h. (A) 3% v/v DMSO for 1 h. (B) 3% v/v DMSO for 12 h. (C) 5% v/v DMSO for 1 h. (D) 5% v/v DMSO for 12 h. (Reprinted with permission from reference [4])

4.3.8 Synergistic Stabilization of Ligand by Aptamer and Organic Additives

Table 4.2 summarizes the MG kinetic constant ($k_{\text{MG} \rightarrow \text{MGOH}}$) and K_{eq} in various organic additives in the presence and absence of MGA, and lists the molecular properties of water and organic additives for evident correlations. In the absence of aptamer, organic additives only had a slight impact on the conversion of $\text{MG} \rightarrow \text{MGOH}$. However, the conversion was 60% slower with MGA, suggesting the protective effect from aptamer in terms of hydrolysis. More importantly, this protective effect combined with the organic additives exhibits a synergistic result towards a stable formation of fluorescent MGA/MG complex. The adjusted $k_{\text{MG} \rightarrow \text{MGOH}}$ obtained by fluorescence intensity in binding buffer was 5 times faster than in buffer with 10% ACN and 29.5 times fast than in buffer with 10% EtOH. No detectable conversion of MG was observed with 10% DMSO, preventing the measurement of $k_{\text{MG} \rightarrow \text{MGOH}}$ under the experimental conditions. In addition, Pearson's R analysis implies the correlations between kinetic constant and intrinsic molecular properties of the organic additives. We speculate this may lead to the prediction of even more effective organic additives. In the absence of aptamer, no distinct correlation between kinetic constant and intrinsic properties of the organic additives is observed ($0.5 > R > -0.5$). With aptamer, kinetic constant has a strong direct correlation with molecular polar surface area (MPSA) ($R > 0.99$) and a strong inverse correlation with volume ($R < -0.99$). The 10% DMSO is excluded in calculation because of its unavailable kinetic constant. However, the lack of fluorescence decay follows the strong correlation of the DMSO intrinsic molecular properties (small in MPSA and relatively large in volume).

Table 4.2 MG hydrolysis rate constant ($k_{MG \rightarrow MGOH}$) with 10% of organic additives in the presence and absence of MGA (MGA:MG = 40:1), and the theoretical calculated properties of water and additives.

Media	With Aptamer		Without Aptamer		Solvent	Molecular Properties [#]	
	$k_{MG \rightarrow MGOH}$ ($\times 10^{-4}$) (s ⁻¹)	K_{eq}	$k_{MG \rightarrow MGOH}$ ($\times 10^{-4}$) (s ⁻¹)	K_{eq}		Molecular Polar Surface Area (MPSA) (Å ²)	Volume (Å ³)
buffer	2.5±0.2	3.62	6.2±0.1	8.99	Water	29.3	19.33
10% Acetonitrile	1.0±0.1	0.40	4.8±0.1	1.92	Acetonitrile	23.8	46.05
10% Ethanol	0.2±0.1	0.06	5.9±0.1	1.84	Ethanol	20.2	54.02
10% Dimethyl sulfoxide	NSD*	< 0.05*	5.7±0.0	1.84	Dimethyl sulfoxide	17.1	71.43
Pearson's <i>R</i> correlation, $k_{MG \rightarrow MGOH}$ vs. MPSA	+0.999**		+0.189				
Pearson's <i>R</i> correlation, $k_{MG \rightarrow MGOH}$ vs. Solvent Volume	-0.991**		-0.293				

* No statistical difference in 0 and 24 h FLU values under 10% dimethyl sulfoxide additive.

** : 10% Dimethyl sulfoxide is excluded in the correlation due to its unavailable kinetic constant.

[#]: Calculated by Molinspiration Cheminformatics 2015: www.molinspiration.com.

The kinetic study results (**Figure 4.8**) suggest a relatively fast $MG \rightarrow MGOH$ hydrolysis reaction in binding buffer. The larger $k_{MG \rightarrow MGOH}$ (compared to the one with aptamer binding in **Figure 4.3B**) shows the evidence that the aptamer is able to interfere with the hydrolysis of MG, without completely preventing. Wang et al. has argued MGA could prevent MG from hydrolysis well over time, owing to its steric hindrance effect [7]. Nonetheless, our results display that a weaker protective effect, which is likely because of the different experimental methods of affinity and kinetics evaluation. Wang et al. evaluated the hydrolysis of MG by monitoring MG's absorbance maxima changes, where we based ours on fluorescence measurement. Changes in absorbance maxima may not be

apparent between aptamer complexed MG and the intermediate MG-OH₂, thus accounting for their perceived observation of full stopping of hydrolysis. If the bound intermediate MG-OH₂ has similar UV-Vis absorbance but is responsible for the decrease of fluorescence, Results from Wang's report provide further evidence that MG-OH₂ intermediate may still have enough binding affinity to keep the MGA folded, while MGOH has not. In the presence of aptamer, the organic additives exhibited a slight inhibition on $k_{\text{MG} \rightarrow \text{MGOH}}$ (**Figure 4.8A**), but catalyze the MGOH dehydration to MG (**Figure 4.8B**). Employment of organic additive not only accelerated the MGOH $\rightarrow$ MG conversion, but the accelerated conversion appeared to shift the equilibrium in favor of MG (**Table 4.2**). This impact on kinetics and equilibrium defines the approach how the organic additives stabilized the MGA/MG complex fluorescence intensity over 24 h.

By combining the rates changes caused by the organic additives and aptamer, synergistic effect of stabilization on MG was observed (**Table 4.2**). With aptamer, a significant correlation between rate constant and the molecular properties of the organic additives can be obtained by Pearson's *R* analysis. Decreasing MG hydrolysis rate constants correlates with less MPSA, larger volume, or a combination of both. Nguyen et al. reported the conformation and charge distribution of MG can be changed upon MGA binding [10], which may result in a shift in kinetics and thermodynamics of MG hydrolysis. Nonetheless, to our knowledge, no report has been found on what the influence of organic additives may bring on the MG stability inside the binding pocket of aptamer. The results in this chapter suggest that the organic additives: 1) shift equilibrium towards MG with aptamer, 2) had minor effect on MG hydrolysis in the absence of aptamer, 3) catalyze the MGOH dehydration and 4) had only minor shifts on affinity and K_d . Based on these primary four observations, a proposed mechanism is displayed in **Figure 4.11**.

Figure 4.11 (A) Equilibrium of MG (1), MG-OH₂ (7) (intermediates) and MGOH (6). (B) Rate of MG elimination. At pH 6.7, $k_{\text{obs}} \approx k_{\text{MG} \rightarrow \text{MGOH}}$ [16]. (C) Proposed mechanism of the conversions between MG, MG-OH₂ and MGOH with MGA presented. Without MGA, the rate constants $k_{\text{MG} \rightarrow \text{MGOH}}$ (hydrolysis) and $k_{\text{MGOH} \rightarrow \text{MG}}$ (dehydration) favor MGOH as the major equilibrium product (A). When excess MGA is present, it retards MG hydrolysis, resulting in a decreased $k_{\text{MG} \rightarrow \text{MGOH}}$. With the addition of organic additives, $k_{\text{MGOH} \rightarrow \text{MG}}$ increases by few hundred percent (C), which results in a “stabilizing” effect. (Reprinted with permission from reference [4])

In pH 6.7 binding buffer, MG hydrolyzes to MG-OH₂ (intermediate), which transforms to MGOH (**Figure 4.11A**). Thus, in the absence of aptamer, the rate constants $k_{\text{MG} \rightarrow \text{MGOH}}$ (hydrolysis) and $k_{\text{MGOH} \rightarrow \text{MG}}$ (dehydration) favor MGOH as the major equilibrium product.

Additionally, as displayed in **Figure 4.11B**, $k_{\text{obs}} \approx k_{\text{MG} \rightarrow \text{MGOH}}$ in this pH [16]. Thus, as proposed in **Figure 4.11C**, when MGA is in excess of MG, it retards MG hydrolysis by slowing the $\text{MG-OH}_2 \rightarrow \text{MGOH}$ conversion, resulting in a decreased $k_{\text{MG} \rightarrow \text{MGOH}}$ (summarized in **Table 4.2**). However, such inhibition by MGA is still not able to enable $k_{\text{MG} \rightarrow \text{MGOH}} \leq k_{\text{MGOH} \rightarrow \text{MG}}$. Thus, the $\text{MG} \leftrightarrow \text{MGOH}$ conversion still favors to MGOH production, which can be evidenced from the result of fluorescence decrease of MGA/MG complex over time (**Figure 4.3**). With organic additives, $k_{\text{MGOH} \rightarrow \text{MG}}$ rises by hundreds of percentage (**Figure 4.8B**), resulting in $k_{\text{MG} \rightarrow \text{MGOH}} \leq k_{\text{MGOH} \rightarrow \text{MG}}$ combining with the synergistic effect from MGA. This synergistic effect by both aptamer (decrease of $k_{\text{MG} \rightarrow \text{MGOH}}$) and organic additives (increase of $k_{\text{MG} \rightarrow \text{MGOH}}$) results in a global K_{eq} favoring inhibition of MG hydrolysis (**Figure 4.7**).

4.4 Conclusions

It is critical to establish optimal conditions for MGA/MG binding, prior to the hypothesis testing. In this chapter, we investigated the factors that may affect the MGA/MG binding, like pH, stability of MGA, MG and their binding. It was found that there was loss of fluorescence intensity and increase in apparent K_d in MGA/MG binding, which was due to the hydrolysis of MG into speculated intermediates and MGOH. This phenomenon may affect our aptasensing assay in the proposed conjugate sensor according to our hypothesis. Interestingly, organic additives were found to prevent the fluorescence decrease (over time) and minimize shifts of apparent K_d . A synergistic mechanism through both aptamer and organic additive on the prevention of carbocation hydrolysis is proposed. Various results and discoveries obtained in this chapter establish optimal conditions for hypothesis testing and provide useful information and foundation on the future aptameric sensing.

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Chapter 5

Binding and Fluorescence Properties of Malachite Green-Stilbene Conjugates

This chapter investigates the binding and fluorescence properties of synthetic malachite green (MG)-stilbene conjugates. Aptamer binding affinity and stilbene fluorescence decay are required parameters of MG-stilbene conjugates towards hypothesis testing and aptasensor design. Structure activity relationships were focused on conjugate covalent linkers and stilbene side groups. MG-stilbene conjugate with amide covalent linker and -methoxy stilbene electron donating groups provided the most efficient aptasensor design.

5.1 Introduction

To examine the hypothesis according to design “competitive stilbene-analyte conjugate with analyte” (section 1.4.1), several conjugates of malachite green (MG) (**1**) and stilbene were analyzed towards aptamer binding and stilbene photoisomerization. The conjugate design attempts to retain MG’s aptamer binding properties while incorporating and stilbene’s fluorescence decay. Rhodamine B (RhB)-stilbene conjugate (**11**) was designed as a negative control, as RhB has no malachite green aptamer (MGA) binding. This rules out stilbene conjugation as a mechanism of non-specific aptamer binding. Binding of MGA to MG and fluorescence decay of stilbene molecule $(\text{CH}_3)_2\text{-N-stilbene-NH}_2$ (**9**) were employed as positive controls for each parameter. The chemical structures of the MG, RhB (**10**), $(\text{CH}_3)_2\text{-N-stilbene-NH}_2$ and their conjugates are listed in **Figure 5.1**. The results show the MG-stilbene conjugate molecules possess either low binding affinity or weak fluorescence decay, which limits its design as a sensitive aptasensor, but provides proof of concept data of the design. Steric and electronic structure activity relationships are discussed for future designs on MG-stilbene conjugates.

Figure 5.1 Chemical structures of malachite green (MG) (1), 4-Amino-4'-(N,N-dimethylamino)stilbene ((CH₃)₂-N-stilbene-NH₂) (9), rhodamine B (RhB) (10) and MG/RhB-stilbene conjugates: RhB-stilbene-N-(CH₃)₂ (11), MG-COO-stilbene-NO₂ (12), MG-CH₂-NH-stilbene-OMe (2), MG-CH₂-NAc-stilbene-OMe (13) and MG-fused stilbene (14).

5.2 Materials and Methods

5.2.1 Materials

Malachite green aptamer (5'-GGAUCCCGACUGGCGAGAGCCAGGUAACGAAUGGAUCC-3') (MGA) used in this study was purchased from AITbiotech Pte Ltd Singapore. 4-Amino-4'-(N,N-dimethylamino)stilbene ((CH₃)₂-N-stilbene-NH₂, *trans*-isomer), rhodamine B (RhB) and malachite green oxalate (MG) were purchased from Sigma Aldrich, Singapore. Dimethyl

sulfoxide (DMSO) was purchased from Alfa Aesar, USA. HPLC grade acetonitrile was purchased from Tedia, USA. Binding buffer (pH = 6.7) consisted of 5.65 mM NaH₂PO₄, 4.35 mM Na₂HPO₄, 10 mM MgCl₂ and 1 mM NaCl. 96-well polystyrene microplates were purchased from Corning, USA. All materials were used as received.

5.2.2 MG/RhB-Stilbene Conjugates

Stilbenes and their MG-conjugates are in *trans* isomers as default. RhB-stilbene-N-(CH₃)₂ was synthesized by the project partner PhD Candidate Ms. Wu Yuanyuan. MG-COO-stilbene-NO₂ (**12**), MG-CH₂-NH-stilbene-OMe (**2**) (not pure), MG-CH₂-NAC-stilbene-OMe (**13**) and MG-fused stilbene (**14**) were synthesized by our group member Dr. Oleksandr Pokholenko. The synthesized conjugates were characterized by NMR and HPLC [1].

5.2.3 Binding Tests of MGA to MG, RhB and MG/RhB-Stilbene Conjugates

Direct binding experiments of MGA to MG or MG-stilbene were conducted with conjugates that retained MG's characteristic fluorescence enhancement, as described in **section 3.3.1**, **section 3.3.2** and **section 4.2.2**.

MG-stilbene conjugates without fluorescence enhancement were analyzed by competitive binding against MG as described in **section 3.3.3**. Simply summarizing, the concept of competitive binding is to monitor the fluorescence decrease of MGA/MG complex with increasing competitor (MG-stilbene conjugate).

For binding of MGA with RhB (similar to MG in chemical structure) and RhB-stilbene-N-(CH₃)₂ as negative control, fluorescence polarization (FP)/fluorescence anisotropy (FA) was applied as RhB has no fluorescence enhancement. Generally, increasing [MGA] was added to constant (60 nM) of RhB or RhB-stilbene-N-(CH₃)₂, and the anisotropy value was recorded in an Agilent Cary Eclipse fluorescence spectrophotometer (G9800A), with ex/em = 350 nm/445 nm (obtained by UV-Vis absorption and emission spectrum

measurements). RhB with fluorescence emission wavelength of around 580 nm shifts the wavelength to 445 nm after conjugation with another dye stilbene, which is speculated to be the result of energy transfer between RhB and stilbene moiety post conjugation.

5.2.4 Fluorescence Decay of Stilbene Compounds

Methods for fluorescence decay measurements and data analysis are described in **section 3.4**. In this chapter, conjugate photoisomerization was conducted by my project partner PhD candidate Ms. Wu Yuanyuan, while the first order fitting analysis of the 10 s fluorescence decay was performed by OriginPro 9.1 as mentioned in **section 3.4**. Briefly, fluorescence emission intensities of stilbene compounds were monitored and recorded over time in a SHIMADZU RF-5301PC Spectrofluorophotometer under constant excitation in corresponding wavelengths.

5.3 Results and Discussion

5.3.1 Binding of MGA with MG (1) and Fluorescence Decay of $(\text{CH}_3)_2\text{-N-stilbene-NH}_2$ (9)

MGA/MG binding positive control is displayed in **Figure 5.2**, which shows binding affinity of $K_d = 200$ nM. This provides a reference for the binding of MGA to MG-stilbene conjugates. MGA/MG binding affinity is retained in the presence of up to 10% organic additives (ACN, DMSO or EtOH) [2], which extends its application window. Stilbene requires the addition of organic additives due to the poor water solubility. Fluorescence decay of $(\text{CH}_3)_2\text{-N-stilbene-NH}_2$ in 5% DMSO/binding buffer is demonstrated in **Figure 5.3**. Stilbene photoisomerization dependence on viscosity [3-8], was confirmed (**Figure 5.4**), indicating the micro-environment variation caused by aptamer-ligand recognition may be transduced by stilbene signaling. MG binding and stilbene fluorescence decay displayed in **Figure 5.3** and **Figure 5.4** serve as positive controls, and will be used for comparison of MG-stilbene conjugate.

Figure 5.2 MGA/MG binding curve and its calculated K_d based on the fluorescence enhancement of MG (1) at ex/em = 620 nm/656 nm upon aptamer binding.

Figure 5.3 Fluorescence decay of $(\text{CH}_3)_2\text{N}$ -stilbene- NH_2 (9), ex/em = 344 nm/439 nm.

Figure 5.4 Influence of medium viscosity on fluorescence decay rate constant (k_{app}) of $(CH_3)_2$ -N-stilbene- NH_2 (**9**). Results are from my project partner Ms. Wu Yuanyuan.

5.3.2 MGA Binding and Fluorescence Decay of RhB-stilbene-N- $(CH_3)_2$ (**11**)

RhB is chemically similar to MG, but has no binding affinity to MGA. MGA mixed with RhB displays no binding, as seen in **Figure 5.5**. RhB (**10**) displays no fluorescence anisotropy variation as [MGA] increases up to 3000 nM [MGA]. Since MGA does not specific bind to RhB, its stilbene conjugate RhB-stilbene-N- $(CH_3)_2$ can be employed as a negative control to investigate if stilbene conjugates have non-specific binding to MGA. RhB-stilbene-N- $(CH_3)_2$ photoisomerization is retained, as seen in **Figure 5.6**. No significant variation in the fluorescence anisotropy of RhB-stilbene-N- $(CH_3)_2$ is observed with increasing concentration of MGA (**Figure 5.7**), suggesting no binding interaction between MGA and RhB-stilbene-N- $(CH_3)_2$. Additionally, the presence of MGA does not interfere the fluorescence decay curves of RhB-stilbene-N- $(CH_3)_2$ (**Figure 5.8A&B**), and no significant change in k_{app} is observed with increasing MGA concentration (**Figure 5.9**), indicating the fluorescence decay of conjugate will not be affected without specific aptamer binding. Indications are obtained from the results above: 1) fluorescence decay of stilbene in MG-stilbene conjugates is retained, 2) no evidence of MGA non-specific

binding is observed with stilbene conjugates and 3) no significant difference is seen between the fluorescence decay of stilbene conjugate and its non-binding aptamer mixture. The observation from RhB-stilbene-N-(CH₃)₂ conjugate, presenting no binding with MGA while retaining fluorescence decay, allows us to explore structure activity relationships of MG-stilbene.

Figure 5.5 Binding of MGA to with RhB (**10**). No significant change in anisotropy is observed.

Figure 5.6 Fluorescence decay of RhB-stilbene-N-(CH₃)₂ (**11**), ex/em = 350 nm/445 nm.

Figure 5.7 Binding of MGA to with RhB-stilbene-N-(CH₃)₂ (**11**). No significant change in anisotropy is observed.

Figure 5.8 (A) Fluorescence decay of RhB-stilbene-N-(CH₃)₂ (11) in the presence of different concentration of MGA. (B) Normalized decay curves of A.

Figure 5.9 k_{app} of RhB-stilbene-N-(CH₃)₂ (11) in the presence of different concentration of MGA.

5.3.3 MG-COO-stilbene-NO₂ (12)

After the control investigations above, MG-COO-stilbene-NO₂ conjugate was examined to see if it possesses the required aptamer binding properties before the -NO₂ is further

modified. As preliminary results displayed that the MG moiety had no fluorescence enhancement of this conjugate upon the addition of MGA, competitive binding was applied to investigate its binding affinity with MGA. As seen in **Figure 5.10**, a K_d up to 6560 nM is obtained. This K_d (> 6000 nM) is inefficient and thus further development was arrested for higher priority conjugates. In addition, according to the fluorescence decay experiments, there is no detectable fluorescence decay observed in this conjugate in 10% DMSO-water, likely due to the $-\text{NO}_2$ electron withdrawing group [9,10] (**Figure 5.11**). MGA/MG binding is mainly attributed to the electrostatic and basic pair stacking/special interaction [11,12]. The weak binding ($K_d > 6000$ nM) of MG-COO-stilbene- NO_2 to MGA may be due to the large molecular volume of the conjugate ($553.92 \text{ \AA}^3$ vs. $\text{MG} = 332.38 \text{ \AA}^3$, calculated by Molinspiration Cheminformatics 2016, the same to all the calculations on molecular volume in this chapter) bothering the base pair stacking, or the strong electron disturbing effect from $-\text{COO}-$ and $-\text{NO}_2$ groups affecting the electrostatic interaction, or both. Additionally, the loss of fluorescence decay is probably due to the strong electron accepting effect from $-\text{NO}_2$ groups, which has been reported [9,10]. Thus, electron donating/accepting effects from linkers/side groups and the size factor of conjugates is further optimized below.

Figure 5.10 Competitive binding of MG-COO-stilbene- NO_2 (**12**) to MGA/MG complex and its calculated K_d .

Figure 5.11 Fluorescence decay of MG-COO-stilbene-NO₂ (**12**) at ex/em = 362 nm/520 nm. Results are from my project partner Ms. Wu Yuanyuan.

5.3.4 MG-CH₂-NH-stilbene-OMe (**2**)

In order to achieve better binding affinity and fluorescence decay in MG-stilbene conjugate, conjugate MG-CH₂-NH-stilbene-OMe (volume: 557.37 Å³, similar to MG-COO-stilbene-NO₂ = 553.92 Å³) was investigated. Different from MG-COO-stilbene-NO₂, the -CH₂-NH- linker decouples the electronics of MG and stilbene. MG-CH₂-NH-stilbene-OMe displays no fluorescence enhancement in the presence of MGA, but competitive binding assay shows a $K_d = 2380$ nM (**Figure 5.12**). Photoisomerization results in **Figure 5.13A&B** confirm significant fluorescence decay in 5% DMSO/binding buffer ($k_{app} = 0.442$ s⁻¹). Furthermore, the decay presents even in 2%-20% DMSO/binding buffer (**Figure 5.13C**), which allows hypothesis confirmation under aqueous environments [11,13,14]. CH₂-NH-stilbene-OMe keeps the properties of stilbene fluorescence decay and exhibits measureable binding affinity. Despite the ignorable difference in molecular size as previously described (< 10 Å³), compared to -COO- and -NO₂ groups, linkers like -CH₂-NH- and the stilbene functional group of -OCH₃ is beneficial for the MGA binding and stilbene fluorescence decay, respectively. This

confirms our prediction of electronic decoupling by the $-\text{CH}_2-$ linker and electron donating group of $-\text{OCH}_3$ on stilbene. However, although the binding affinity of $\text{CH}_2\text{-NH-stilbene-OMe}$ rises ca. 2.8 times compared to $\text{MG-COO-stilbene-NO}_2$ (2380 nM vs. 6560 nM), it is still low compared to our MG reference (2380 nM vs. 200 nM). A simple variant, $\text{MG-CH}_2\text{-NAC-stilbene-OMe}$, is further tested to improve binding by adding hydrogen acceptors at cost of increasing molecular volume.

Figure 5.12 Competitive binding of $\text{MG-CH}_2\text{-NH-stilbene-OMe}$ (2) to MGA/MG complex and its calculated K_d .

Figure 5.13 (A) Fluorescence decay of MG-CH₂-NH-stilbene-OMe (**2**) in different percentage of DMSO/binding buffer at ex/em = 324 nm/432 nm. (B) Normalized fluorescence decay of A. C: Apparent fluorescence decay rate constant (k_{app}) of the decay curves. Results are from my project partner Ms. Wu Yuanyuan.

5.3.5 MG-CH₂-NAc-stilbene-OMe (**13**)

Regarding to the electrostatic interaction between MGA and MG [11,12], MG-CH₂-NAc-stilbene-OMe conjugate was tested to see if the -N-C=O linker can enhance the MGA binding by strengthening the hydrogen bonding and electrostatic interaction. The modification has the unexpected benefit of retaining MG's fluorescent enhancement within the MG-CH₂-NAc-stilbene-OMe conjugate after MGA binding. Direct binding assessment results in a $K_d = 1700$ nM (**Figure 5.14**) and the conjugate retained photoisomerisation (**Figure 5.15**). Similar to -CH₂NH-, -CH₂-N(COCH₃)- linker had fluorescence decay in 5% DMSO/binding buffer with $k_{app} = 0.352$ s⁻¹, and the decay existed in 2%-20% DMSO/binding buffer (**Figure 5.15A-C**), which makes it possible for

hypothesis testing. This MG-CH₂-NAc-stilbene-OMe conjugate has an improved K_d compared to the similar conjugate MG-CH₂-NH-stilbene-OMe (1700 vs. 2380 nM, respectively). This was attributed to hydrogen bonding, electrostatic effect, or combination thereof within the -N-C=O functional group, even though the MG-CH₂-NAc-stilbene-OMe has a larger volume (593.29 Å³) compared to MG-CH₂-NH-stilbene-OMe (557.37 Å³). This conjugate may be a possible “candidate” for the proof of concept testing but may be limited by sensitivity. Results from this conjugate indicate the introduction of hydrogen bonding and electrostatics with -N-C=O may enhance the MGA binding, but size effect may still be an important factor affecting the aptamer binding. To improve binding, a design to lower molecular volume and retain MG electrostatics was designed into the MG-fused stilbene.

Figure 5.14 Binding of MG-CH₂-NAc-stilbene-OMe (**13**) with MGA and its calculated K_d based on fluorescence enhancement.

Figure 5.15 (A) Fluorescence decay of MG-CH₂-NAc-stilbene-OMe (13) in different percentage of DMSO at ex/em = 327 nm/389 nm. (B) Normalized fluorescence decay of A. C: Apparent fluorescence decay rate constant (k_{app}) of the decay curves. Results are from my project partner Ms. Wu Yuanyuan.

5.3.6 MG-fused-stilbene (14)

Considering the base pair stacking/steric effect on MGA binding [11,12], MG-fused-stilbene with relatively small molecular size may be a good choice for strong aptamer binding. This molecule not only fuses the benzene ring of MG and stilbene, but also removes the side group of stilbene. As a result, this molecule has a size of only 431.21 Å³, which is ca. 100 Å³ larger than MG, but 100+ Å³ smaller than all the three MG-stilbene conjugates above. MG-fused-stilbene retains fluorescence enhancement, and the K_d is measured to be 480 nM (Figure 5.16), which is comparable to MG and ideal for our design to investigate the hypothesis in terms of binding affinity. It should be noted that the absolute enhanced fluorescence intensity is only 11% of MGA/MG binding (Figure

5.2), which may be due to its less planar structure upon binding [15,16]. The lower fluorescence enhancement upon binding suggests the planar interference from the fused stilbene. However, the fluorescence decay of the fused stilbene structure is not retained. **Figure 5.17** displays the results of fluorescence decay characterization. Even in pure organic solvents acetonitrile (ACN), dimethyl sulfoxide (DMSO) or ethanol (EtOH), no applicable fluorescence decay is observed. This loss in fluorescence decay may be due to the significant shift in stilbene electronics from fused MG. Thus, this MG-fused-stilbene conjugate cannot be used in the hypothesis testing. However, the results in this conjugate give a critical clue on conjugate design---“Fused” structure with relative small volume (i.e. $< 430 \text{ \AA}^3$) is good for the retaining of binding affinity, but the loss of fluorescence decay of stilbene needs to be remedied, possibly through the appropriate electron donating or withdrawing group (**Figure 5.17**).

Figure 5.16 Binding of MG-fused-stilbene (**14**) with MGA and its calculated K_d based on fluorescence enhancement.

Figure 5.17 Fluorescence decay of MG-fused-stilbene (**14**) in different organic solvents. at Ex/em = 318 nm/381 nm for DMSO, 312 nm/398 nm for ACN and 314 nm/398 nm for EtOH, respectively. Results are from my project partner Ms. Wu Yuanyuan.

5.4 Conclusions

The structure activity relationships of conjugate binding to aptamer and photoisomerization led to conjugates that allow ongoing proof of concept testing. **Table 5.1** summarizes the binding and fluorescence decay results. RhB-stilbene-N-(CH₃)₂ as a negative control indicates that 1) no evidence of MGA non-specific binding is observed with stilbene conjugates and 2) no significant difference is seen between the fluorescence decay of stilbene conjugate and its non-binding aptamer mixture. MG-COO-stilbene-NO₂ shows negligible binding affinity and fluorescence decay, losing both the properties of MG and stilbene. This observation suggests electron disturbing groups like -COO- should not be linked on MG, and electron accepting groups like -NO₂ should not be chosen as side substituent of stilbene as reported [9,10]. Linkers decoupling MG from stilbene should be considered and electron donating groups may be beneficial for stilbene fluorescence decay. MG-CH₂-NH-stilbene-OMe presents fluorescence decay. The

outcomes suggest the -OCH₃ side group design may be beneficial for retaining the fluorescence decay of stilbene, and the -CH₂- spacer may be helpful for MG binding by decoupling the MG from stilbene, but the low binding affinity (> 2000 nM) remains an issue. To improve the binding affinity, factors like size or electrostatic interaction may be considered based on the reported MGA/MG interactions [11,12]. Compared to MG-CH₂-NH-stilbene-OMe, MG-CH₂-NAC-stilbene-OMe providing stronger hydrogen bonding and electrostatic interaction brings the K_d down to 1700 nM, with similar fluorescence decay kept. The results indicate an approach to enhance the binding affinity by introduction of hydrogen bonding and electrostatics, but size effect may still be an importing factor on binding. MG-fused-stilbene shows a remarkable retaining of binding affinity which is comparable to MG. However, the fluorescence decay of stilbene losses in this conjugate, probably due to the electronic and steric effect from the nearby fused MG, and thus attention is suggested to be focused on retaining the fluorescence decay (i.e. the introduction of the appropriate electron donating group on stilbene) on the design of “fused” conjugates in the future. Some indications on structure activity relationships can be referred from all the results in this chapter. For binding affinity (MG-COO-stilbene-NO₂ < MG-CH₂-NH-stilbene-OMe < MG-CH₂-NAC-stilbene-OMe < MG-fused-stilbene < MG), small molecular size, suitable decoupling linker for MG, and stronger electrostatic interaction of conjugate may be beneficial for MGA binding. For fluorescence decay, electron donating groups should be preferred while the stilbene should be far from the effect of MG.

Table 5.1 Summary of the binding and fluorescence results of conjugates and controls.

Compound	Linker/Side Group	K_d (nM)	Method for K_d Determination	Fluorescence Decay in Aqueous Environment	Molecular Volume ($\text{\AA}^3$)*	Remarks
MG (1)	-	200	FI direct binding	-	332.38	Positive Control
(CH ₃) ₂ -N-stilbene-NH ₂ (9)	-	-	-	Yes	-	Positive control
RhB (10)	-	NA	FP direct binding	-	-	-
RhB-stilbene-N-(CH ₃) ₂ (11)	-CO-NH-/ -N(CH ₃) ₂	NA	FP direct binding	Yes	-	Negative control
MG-COO-stilbene-NO ₂ (12)	-COO-/ -NO ₂	6560	FI competitive binding	No	553.92	Weakest binding
MG-CH ₂ -NH-stilbene-OMe (2)	-CH ₂ -NH-/ -OCH ₃	2380	FI competitive binding	Yes	557.37	Weak binding, not pure
MG-CH ₂ -NAc-stilbene-OMe (13)	-CH ₂ -N(COCH ₃)-/ -OCH ₃	1700	FI direct binding	Yes	593.29	Weak binding, not ideal
MG-fused stilbene (14)	-	480	FI direct binding	No	431.21	Strongest binding

*Calculated by Molinspiration Cheminformatics 2016: www.molinspiration.com.

NA: no quantitative K_d is available.

-: Not applicable.

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Chapter 6

Malachite Green Aptamer-Stilbene Conjugate

This chapter presents the hypothesis according to the design “stilbene-aptamer conjugate fluorescence decay correlates to analyte binding”. Firstly, stilbene was grafted on malachite green aptamer (MGA) by click chemistry to fabricate the conjugate, and the properties of both MGA in binding and stilbene in fluorescence decay were retained in the conjugate. Fluorescence decay of the conjugate was inhibited in the presence of ligands malachite green and tetramethylrosamine. MGA’s adaptive binding may confine the free isomerization of stilbene, supporting our central hypothesis. Analysis of apparent fluorescence decay rate (k_{app}) vs. ligand concentration gives a limit of detection (LOD) of 10.5 μM for malachite green and 0.7 μM for tetramethylrosamine. The novel aptasensor is capable of quantitative sensing within ten seconds of fluorescence decay. This original assay reveals a new fluorescent aptasensing mode employing fluorescence kinetics, which is reported for the first time. The results presented in this chapter support the hypothesis of this thesis within the scope for “stilbene-aptamer conjugate fluorescence decay correlates to analyte binding”, under the limited conditions.

6.1 Introduction

As reviewed in Chapter 2, it has been widely reported that aptamers have unique properties of conformational change or structural switching upon binding, making them versatile molecular recognition elements [1-4]. On the other hand, stilbene fluorophores may be used as kinetic fluorescent probes [5-7], whose photoisomerization can be influenced by the surrounding micro-environment such as steric hindrance or viscosity [8-10]. Thus, “stilbene-aptamer conjugate fluorescence decay correlates to analyte binding” (aptamer-stilbene in short) strategy has been designed according to our hypothesis (section 1.4.2), which takes advantages of both conformational change of aptamer and kinetic fluorescence decay of stilbene.

To test the hypothesis regarding the aptamer-stilbene design, a malachite green aptamer (MGA)-stilbene conjugate has been synthesized as an aptasensor and challenged against known ligands. Results show both the MGA's adaptive binding (with MG) and stilbene's fluorescence decay behavior were retained in the conjugate. In the presence of ligand MG, stilbene's fluorescence decay kinetics decreases, due to the conformational change of MGA upon binding. A ligand with higher affinity, tetramethylrosamine (TMR), was quantitated by the synthesized conjugate and the results confirmed the concept of the kinetic fluorescence decay detection mode according to the hypothesis (**Figure 6.1**). These results support the hypothesis under our limited experimental conditions. The aptasensor shows a never-before detection mode for MG.

Figure 6.1 Schematic illustration of the aptasensor based on photochrome switch of stilbene and adaptive binding of aptamer. Without analyte (ligand), *trans*-stilbene grafted on aptamer undergoes free photoisomerization (fluorescent *trans* form to non-fluorescent *cis* form), resulting in rapid fluorescence decay. Upon analyte binding, conformational change of aptamer induces an inhibition of the stilbene photoisomerization, resulting in decrease in fluorescence decay kinetics, which can be utilized for biosensing.

6.2 Materials and Methods

6.2.1 Materials

Malachite green aptamer (5'-GGAUCCCGACUGGCGAGAGCCAGGUAACGAAUGGAUCC-3') (MGA) and its 3'-amino-modified variant (C38) MGA-NH₂ used in this study were purchased from AITbiotech Pte Ltd Singapore. Aminocaproic acid (EACA) (**16**), lysine (**18**), 4-Acetamido-4'-isothiocyanato-2,2'-stilbenedisulfonic acid disodium salt (SITS) (**4**), rhodamine B (RhB) (**10**), malachite green oxalate (MG) (**1**) were purchased from Sigma Aldrich, Singapore. Tetramethylrosamine (TMR) (**15**) was purchased from Life Technologies, Singapore. **Figure 6.2** displays the structures of MGA, MG, SITS, RhB, TMR, EACA and lysine. Binding buffer (pH = 6.7) consisted of 5.65 mM NaH₂PO₄, 4.35 mM Na₂HPO₄, 10 mM MgCl₂ and 1 mM NaCl unless specified elsewhere. Amicon

Centrifugal Filters with 10 kDa molecular weight cut-off were purchased from Merck, Singapore. 96-well polystyrene microplates were purchased from Corning, USA. All materials were used as received. Stilbenes used in decay studies refer to the *trans*-isomers.

Figure 6.2 Structures of malachite green aptamer (MGA), malachite green (MG) (1), 4-Acetamido-4'-isothiocyanato-2,2'-stilbenedisulfonic acid disodium salt (SITS) (4), rhodamine B (RhB) (10), tetramethylrhodamine (TMR) (15), aminocaproic acid (EACA) (16) and lysine (18). MGA 3D structure (PDB ID: 1Q8N) is presented by Discovery studio 3.5 Visualizer, Accelrys.

6.2.2 Synthesis of MGA-SITS Conjugate (5)

Synthesis of MGA-SITS conjugate follows the classical amine-isothiocyanate reaction commonly used in bioconjugation [11] (**Figure 6.3**). Briefly, 50 μ M of MGA-NH₂ was mixed with 5 mM of SITS in pH = 9 sodium bicarbonate reaction buffer, with magnetic

stirring at room temperature for 12 hours and the reaction was prevented from light. After reaction, the excess SITS was eliminated by ultrafiltration with Amicon Centrifugal Filter (10 kDa).

Figure 6.3 Synthesis of MGA-SITS conjugate (5).

6.2.3 Characterization of MGA-SITS by High Performance Liquid Chromatography (HPLC)-Diode Array Detector (DAD)

An Agilent 1200 series HPLC system with DAD was used for the characterization of MGA-SITS conjugate. Chromatographic separation was conducted using a 5 $\mu\text{m} \times 4.6 \text{ mm} \times 250 \text{ mm}$ UltisilTM C18 column (Welch Materials Inc.), and constant mobile phase that consists of 20% 25 mM ammonium acetate (pH 6.8) and 80% acetonitrile was applied. The HPLC-DAD system was operated in 1.0 $\text{mL} \cdot \text{min}^{-1}$ flow rate and 10 μL sample injection. The DAD was set to 260 nm and 340 nm for simultaneously detection of MGA-NH₂ and SITS respectively, with full spectrum scans from 200 to 800 nm. Herein, HPLC-DAD characterization provided information in both conjugation degree and purity of the synthesized conjugate (removal of free SITS).

6.2.4 Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) Mass Spectrum Characterization of MGA-SITS

Procedures of MALDI-TOF mass spectrum measurement were adapted from literature [12] and the MALDI-TOF experiments were conducted by my group member Dr. Oleksander Pokholenko. Prior to measurement, the samples were desalted with 100 mM ammonium citrate exchange buffer (pH = 6.0) (with 10k Amicon Centrifugal Filters) to suppress the impact from metal cations. After the desalting, 1 μ L of 10 μ M sample was mixed with 1 μ L of THAP matrix (20 mg/ml in 4:1 mixture of ethanol/aqueous ammonium citrate (pH = 6; 100 mM)) on a MALDI-TOF sample plate and the mixture was dried at room temperature [13]. The dried matrix was analyzed in a Shimadzu Axima MALDI-TOF mass spectrometer (in positive linear mode and 1000 profiles).

6.2.5 K_d Determination of MGA-SITS in Binding with MG

Similar to MGA/MG binding, K_d determination of MGA-SITS binding with MG was measured according to the fluorescence enhancement of MG upon aptamer binding [14,15], which has been discussed in **section 3.3**. Fluorescence emission spectrum of MG upon MGA-SITS addition was measured with SHIMADZU RF-5301PC Spectrofluorophotometer in “spectrum” mode. In the spectral measurement, mixed solution containing 1 μ M of MGA-SITS and 1 μ M of MG (1:1) was excited at wavelength of 620 nm, with the emission spectrum recorded.

6.2.6 Measurement of MGA-SITS Fluorescence Decay and Its Aptasensing

Trans-cis photoisomerization of MGA-SITS conjugate was measured with SHIMADZU RF-5301PC Spectrofluorophotometer with the “time” mode at room temperature, which is described in **section 3.4**. MGA-SITS samples (1 μ M) in 5% DMSO-95% binding buffer were excited in quartz cuvette at 340 nm, with their fluorescence decays recorded at $\lambda_{em} = 428$ nm, while the excitation and emission slits were set to 5 nm. The apparent fluorescence decay rate constant (k_{app}) of fluorescence decay for the first 10 second's was

determined by first order fitting, as described in **section 3.4**. Sensing of analytes was conducted based on the variation in k_{app} of MGA-SITS in the presence of various analytes.

6.2.7 Measurement of SITS Fluorescence Decay in the Presence of MG and MGA/MG Complex

To investigate the impact of MG and MGA on fluorescence decay of non-conjugated stilbene molecule SITS, fluorescence decay of SITS was monitored in the presence of free MG and MGA/MG (1:20) complex. Same concentrations of SITS and MGA were employed as that used in the MG sensing experiments. Fluorescence decay measurement of SITS (in the presence/absence of MG and MGA) can be referred to **section 3.4**.

6.3 Results and Discussion

A novel aptasensor based on fluorescence decay has been developed, which supports the hypothesis according to the “aptamer-stilbene” design. This aptasensor quantitation mechanism may overcome the existed drawbacks in the traditional “signal on/off” fluorescence sensing mode [16]. Amine MGA and thiol MGA in different position were tested towards binding affinity with MG. The results in **Table 6.1** show C38 amine modification presents the lowest K_d . Considering the commercially available isothiocyanate stilbene compounds and the ease in large-scale synthesis, C38 amino-modified MGA was chosen for conjugation. MALDI-TOF and HPLC-DAD analyses confirm the successfully synthesis of MGA-SITS conjugate. A quantitative fluorescence decay based sensing of MG is obtained according to the variation of k_{app} . This inhibition effect only appears when the SITS is attached on aptamer, indicated by a negative control experiment with non-conjugated SITS. Additionally, the MGA-SITS sensor has significantly stronger response on TMR (another MGA ligand) compared to MG, while RhB not binding to MGA has no effect on the fluorescence decay of the sensor, showing the selective and multiplex assay of the sensor.

Table 6.1 MG binding K_d of modified MGA.

Modified Position	Modification Group	K_d (nM)	R^2
C38	Amine	200 $\pm$ 30	0.99
C38	Thiol	980 $\pm$ 140	0.99
A9	Thiol	690 $\pm$ 90	0.98
U4	Thiol	820 $\pm$ 90	0.99
C20	Thiol	670 $\pm$ 100	0.99

6.3.1 Successful Synthesis of MGA-SITS Conjugate with Complete Removal of Free SITS

HPLC-DAD and MALDI-TOF mass spectrum were applied for the characterization of the synthesized MG-SITS. Characterization by HPLC-DAD indicates the successful reaction and removal of free SITS (**4**) (**Figure 6.4**) post purification. Under the HPLC conditions in **section 6.2.3**, MGA-NH₂ peak at retention time (RT) 1.7 min has significant absorbance at 260 nm but no significant absorbance at 340 nm (**Figure 6.4A**), while SITS peak at RT 8.5 min has absorbance at 340 nm but no strong absorbance at 260 nm (**Figure 6.4B**). As shown in **Figure 6.4C**, similar to MGA-NH₂, the synthesized MGA-SITS conjugate peak has a RT of 1.7 min with significant absorbance at 260 nm. Additionally, the peak also presents absorbance at 340 nm, which is the characteristic of SITS but not MGA-NH₂ (MGA-NH₂ has ignorable UV absorbance at 340 nm). This feature indicates the formation of MGA-SITS conjugate, which should theoretically have UV absorbance at both 260 nm for MGA-NH₂ and 340 nm for SITS according to **Figure 6.4A** and **Figure 6.4B**. Further information in UV-Vis spectra obtained from the DAD also indicates the achievement of the conjugation, where the UV-Vis spectrum of MGA-SITS possesses both the feature of MGA-NH₂ and SITS (insets of **Figure 6.4A-C**). Finally, there is no detectable SITS peak in the chromatography of MGA-SITS, suggesting the complete removal of free SITS in the conjugate (**Figure 6.4C**). The totally removal of free SITS indicated by **Figure 6.4C** confirms the fluorescence decay behavior of the synthesized product only belongs to the MGA-SITS conjugate, but not excess SITS.

Figure 6.4 HPLC-DAD characterization of (A) MGA-NH₂, (B) SITS (**4**) and (C) MGA-SITS conjugate (**5**). Insets are the UV-Vis spectra (from DAD) at corresponding RT displaying the variation in absorbance.

As seen in **Figure 6.5**, starting MGA-NH₂ has m/z of 12621 $[M+H]^+$ (blue). For the spectrum of the product MGA-SITS (red), it possesses m/z of 13072 $[M+H]^+$, while the value of initial MGA-NH₂ is 12615 $[M+H]^+$ within the same spectrum (red). The 457 unit increasing in m/z post conjugation indicates the aptamer molecule weight increases 457

Da due to the attachment of SITS molecule (after replacement of sodium with hydrogen, SITS has a theoretical molecular weight of 454.5 Da). The small variation in m/z value in the results can be considered within instrumental error. The presence of MGA-NH₂ peak in the spectrum of MGA-SITS suggests the remained starting MGA-NH₂ in the conjugate or SITS was cleaved during laser ablation, or combination thereof. Assuming the worst case scenario of non-conjugated MGA-NH₂, its presence will not affect the fluorescence decay of the MGA-SITS but may reduce overall sensitivity of the aptasensor. In summary, MALDI-TOF spectra results combining with the HPLC-DAD support the synthesis of MGA-SITS without an excess of free SITS.

Figure 6.5 MALDI-TOF spectra of MGA-NH₂ (top blue) and MGA-SITS conjugate (**5**) (bottom red).

6.3.2 Optimizations on Bioconjugation Reaction

Reaction conditions were initially optimized on the reaction of SITS with inexpensive aminocaproic acid (EACA) (**16**) and lysine (**18**) (**Figure 6.2**). HPLC-DAD was employed for monitoring the reaction by tracing the absorbance of SITS. **Figure 6.6A** displays the EACA-SITS reaction while **Figure 6.7A** shows the lysine-SITS reaction, at 4 deg C and room temperature. Less than 50% of SITS was reacted at 4 deg C (**Figure 6.6B**) with EACA. At 12 h at room temperature, reaction was nearly complete (**Figure 6.6C**). In the reaction with lysine, completion was reached at 24 h at 4 deg C (**Figure 6.7B**), or 4 h at room temperature (**Figure 6.7C**). Although lysine reacts faster than EACA with SITS, owing to the single -NH₂ group on MGA, EACA reaction should be a more proper reference for the reaction of MGA-NH₂ with SITS. Thus, room temperature and at least 8 h should be chosen for a relatively rapid and complete reaction with amino-modified aptamer.

Figure 6.6 (A) Reaction of EACA (**16**) with SITS (**4**). (B) Reaction progress at 4 deg C. (C) Reaction progress at room temperature.

Figure 6.7 (A) Reaction of lysine (**17**) with SITS (**4**). (B) Reaction progress at 4 deg C. (C) Reaction progress at room temperature.

The reaction of MGA-NH₂ with SITS at room temperature was monitored by HPLC-DAD, based on the increase of SITS peak ($\lambda = 340$ nm) over time on the RT of MGA-NH₂ peak (**Figure 6.8 A**). As shown in **Figure 6.8 B**, the conjugation is nearly complete at 12 h. Although reaction time up to 48 h is more beneficial for the degree of reaction, considering the stability of RNA aptamer and reaction system, reaction time 12 h was chosen.

Figure 6.8 (A) HPLC monitoring of absorbance increase in UV340 (grafting of SITS (4)) over time at RT 1.7 min. (B) The calculated degree of conjugation as a function of time.

6.3.3 MGA-SITS Keeps Properties of Aptamer Binding and Stilbene Fluorescence Decay

Prior to sensing applications/hypothesis testing, MGA-SITS conjugate needs to be assessed for both the fluorescence decay behavior (property of SITS) and binding to MG

(property of MGA). **Figure 6.9A** exhibits the fluorescence decay of MGA-SITS, presenting its retained fluorescence decay property of SITS (k_{app} : 0.360 s^{-1} for MGA-SITS vs. 0.379 s^{-1} for SITS). Based on the fluorescence enhancement of MG upon aptamer binding, as described in **section 3.3.1**, the direct binding between MGA-SITS and MG was confirmed [14,15]. **Figure 6.9B** displays fluorescence emission (enhancement) of MG at wavelength around 650 nm upon addition of MGA-SITS, suggesting the binding between MGA-SITS and MG. For the determination of binding affinity, according to the method in **section 3.3.2**, the K_d of MGA-SITS binding with MG was determined to be $240 \pm 10\text{ nM}$, which compares well to the K_d of MGA-NH₂ (starting material for the synthesis of MG-SITS) binding to MG ($200 \pm 30\text{ nM}$), showing no significant difference between each other (**Figure 6.10**). These results confirm that MGA-SITS keeps the properties of both aptamer in MG binding and stilbene in fluorescence decay, which allows for the hypothesis to be challenged under limited conditions.

Figure 6.9 (A) Fluorescence decay of MGA-SITS (5) at ex/em = 340 nm/428 nm. (B) Fluorescence emission of MG in the absence and presence of MGA-SITS at excitation wavelength of 620 nm.

Figure 6.10 Binding of MGA-NH₂ and MGA-SITS (5) with MG, ex/em = 620 nm/656 nm.

6.3.4 Fluorescence Decay of MGA-SITS Is Retarded upon MG Binding

The MGA-SITS conjugate was incubated with the ligand MG, which should retard the stilbene's *trans-cis* photoisomerization according to our proposed hypothesis under the “aptamer-stilbene” design. Fluorescence decay of MG-SITS was measured according to the method in **section 6.2.6** in the presence and absence of MG. As seen in **Figure 6.11A**, decay kinetics, k_{app} , of MGA-SITS is delayed with increasing [MG]. The linear correlation of k_{app} vs. [MG] suggests adaptive folding by MGA confines the free photoisomerization of the C38 SITS conjugate, resulting in an inhibited photodecay. NMR characterization by others has shown that MGA forms G1-C38 base pair under adaptive binding with MG [17]. Additionally, isomerization of *trans*-stilbene is subjected to the surrounding environment [9,10]. Thus, change in local environment induced by aptamer binding, or more specifically, increase in local viscosity is suspected to be the reason for the inhibition of SITS fluorescence decay, in the presence of MG. Considering

the structures of SIST and the folded MGA, this impact on fluorescence decay may be attributed to hydrogen bond or stacking interactions between SITS and MGA, or both. **Figure 6.11B** presents the linear relationship between k_{app} and MG concentration. From 0.5 to 50 μM , a good linear range can be obtained ($R^2 = 0.98$), with slope = $-9.4 \times 10^{-4} \text{ s}^{-1} \cdot \mu\text{M}^{-1}$, and the limit of detection (LOD) for the sensing of MG is calculated to be 10.5 μM (defined as 3σ criterion, σ is the standard deviation of response). The results on MG sensing by the MGA-SITS conjugate illustrate a novel aptameric structure-switching strategy quantitating by fluorescence decay kinetics. However, as shown in **Figure 6.11B**, it should also be noted that the impact on MGA-SITS fluorescence decay is not dramatic as the concentration of MG increases (slope = $-9.4 \times 10^{-4} \text{ s}^{-1} \cdot \mu\text{M}^{-1}$). The relatively weak response resulting in relatively low sensitive and high LOD (10.5 μM) may be due to the relatively weak fluorescence intensity. To improve the sensitivity, higher concentration of MGA-SITS may be used in the future, or through another more efficient way, functionalized stilbene with high fluorescence quantum yield should be employed for the conjugation. In summary, the results on MG sensing by the MGA-SITS provide preliminary proof-of-concept support towards our hypothesis.

Figure 6.11 (A) Fluorescence decay curves of MGA-SITS (**5**) in the presence of increasing MG concentration. (B) Calibration plot of k_{app} of MGA-SITS as a function of MG concentration (0.5-50 μM).

6.3.5 Delayed Fluorescence Decay Isolated To MGA-SITS

MG inhibits the fluorescence decay of MGA-SITS as shown in the results of **section**

6.3.4. In order to prove further support for our central hypothesis, we challenged the central hypothesis with two null-hypotheses pertaining to the delayed photoisomerization inhibition: 1) MG interaction with SITS is responsible for the fluorescence decay regardless of aptamer binding and 2) Wild type MGA/MG binding complex can inhibit the fluorescence decay of SITS through non-specific binding interactions. The null-hypotheses were conducted by monitoring the k_{app} of SITS in the presence of MG or MGA/MG complex. **Figure 6.12A** presents the fluorescence decay curves of SITS with and without the addition of MG or MGA/MG binding complex. **Figure 6.12B** compares the corresponding k_{app} values of SITS in **Figure 6.12A** and shows no significant difference in the absence and presence of MG or MGA/MG complex (significance level $p < 0.05$, $n = 3$). Additionally, the k_{app} of SITS is also independent of MG concentration, since the addition of 20 μM or 40 μM MG results in the similar k_{app} , regardless of the presence of MGA (**Figure 6.12B**). By contrast, MG leads to change in K_{app} of MGA-SITS under the same experimental conditions (**Figure 6.11A** and **Figure 6.11B**). The results suggest the fluorescence decay inhibition on MGA-SITS induced by MG is not due to the interaction of SITS molecule with MG or MGA/MG complex when SITS is not grafted on aptamer. These evidences indicate the influence of MG on SITS fluorescence decay (**Figure 6.11**) is only owing to ligand induced aptameric folding of SITS attached MGA (illustrated in **Figure 6.1**), and the two null-hypotheses are reported false under the given conditions.

Figure 6.12 (A) Fluorescence decay curves of SITS (**4**) in the presence of MG and MGA/MG complex. (B) k_{app} of SITS upon the presence of MG and MGA/MG complex. *: $p < 0.05$:

significant difference against SITS control (without MG and MGA) ($n = 3$).

6.3.6 The MGA-SITS Conjugate Shows Selectivity of Ligands

Other triphenyl dyes RhB and TMR (see **Figure 6.2** for structures) similar to MG were tested regarding to the selectivity and multiplex detection of the MGA-SITS sensor. RhB does not specifically bind with MGA (**section 5.3.2**) while TMR binds with MGA with high affinity [18]. There is no significant change in MGA-SITS fluorescence decay in the presence of 20 μM RhB, while the decay is dramatically inhibited in the presence of 20 μM TMR, which displays a more sensitive than all known concentrations of MG (**Figure 6.13A**). As shown in **Figure 6.13B**, analysis on k_{app} of MGA-SITS quantitatively confirms the observations in **Figure 6.13A**. Against MGA-SITS blank control, there are significant differences on k_{app} in the presence of MG and TMR, but no significant difference k_{app} in the presence of RhB (significance level $p < 0.05$), which displays a good selectivity of the MGA-SITS sensor.

Figure 6.13 (A) Fluorescence decay curves of MGA-SITS (**5**) in the presence of 20 μM MG, TMR (**15**) and RhB (**10**). (B) k_{app} of MGA-SITS upon the addition of the analytes. *: $p < 0.05$: significant difference against MGA-SITS ($n = 3$).

TMR displays a greater retardation in fluorescence decay of the MGA-SITS conjugate. Similar to the case of MG sensing, the decay kinetics of MGA-SITS decreases upon its binding to TMR (**Figure 6.14A**). Linear correlation of k_{app} vs. TMR concentration gives a slope of $-1.4 \times 10^{-2} \text{ s}^{-1} \cdot \mu\text{M}^{-1}$ ($R^2 = 0.92$) ranging from 0.2 to 20 μM , which is 15 times

more sensitive than the MG linear fit (-1.4×10^{-2} vs. -9.4×10^{-4}). LOD of TMR is calculated to be $0.7 \mu\text{M}$ (**Figure 6.14B**). In short, TMR sensing by the MGA-SITS sensor presents more than 10-fold higher sensitivity compared to MG sensing, with LOD in sub-micromolar level. From the reported data, TMR has up to 5 times smaller K_d value than MG in the binding with MGA [15,18], which may be a potential reason for the higher sensitivity of TMR linear correlation. TMR's strong binding affinity with MGA-SITS may induce more folded aptamer. In addition, the structural feature of MGA/ligand complex may be another reason. MGA presents different bound structure when it binds to TMR and MG [17,18], which may induce a different impact on stilbene isomerization. This speculation needs to be examined by computational modelling in the future work. This difference between the sensing of MG and TMR gives indication on the future design of the sensor based on the hypothesis, especially for the improvement of the sensitivity. For instance, employ MGA-SITS/TMR complex for the sensing of MG by competitive binding, which might induce a higher sensitivity in MG detection. Additionally, optimizations of binding conditions for higher affinity may also be beneficial for the sensitivity enhancement.

Figure 6.14 (A) Fluorescence decay curves of MGA-SITS (**5**) in the presence of increasing concentration of TMR (**15**). (B) Calibration plot of MGA-SITS k_{app} as a function of TMR concentration (0.2-20 μM).

6.4 Conclusions

In this work, we have developed a MGA-SITS conjugate which can challenged our central hypothesis under the scope design “stilbene-aptamer conjugate fluorescence decay correlates to analyte binding”. This design takes advantages of conformational changes present upon aptamer binding and stilbene’s fluorescence decay sensitivity. The MGA-SITS aptasensor developed is capable of selectivity toward a range of ligand affinities. Difference in response between ligands of TMR and MG sensing suggests high binding affinity and competitive design of MG to MGA-SITS/TMR complex may result in a higher sensitivity, giving indications for future strategies on the sensor optimization. Additionally, influence on SITS fluorescence decay from the interactions with MG or MGA/MG complex is ruled out by two null hypotheses. A novel fluorescence decay based sensing mode has been reported for the first time. Future work should focus on the improvement in sensitivity of the aptasensor like MG competitive binding with MGA-SITS/TMR complex, grafting stilbene on various positions of MGA, analysis of stilbene photoisomerization on the bound aptamer, employing functionalized stilbene with high fluorescence quantum yield, improving the bioconjugation efficiency, and enhancing the binding affinity.

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Chapter 7

Summary, Implications and Future Suggestions

This chapter summarizes the results throughout the thesis, and presents implications from the outcomes. Both the successful and failed data in this thesis give significant hints on future designs of the fluorescence decay based biosensors, incorporating the adaptive binding of aptamer and fluorescence decay of stilbene. Based on the results obtained and literature, some strategies are proposed for future work, such as the employment of computational molecular modelling on optimal malachite green (MG)-stilbene synthesis, involvement of new click chemistry for higher reaction efficiency, regeneration of the sensor and competitive binding of MG to MGA-SITS/TMR complex. These strategies should be able to improve the sensing performance of the fluorescence decay based aptasensor.

7.1 Summary

Combining the adaptive binding behavior of aptamer and the fluorescence decay property of stilbene, an aptasensor quantitating by fluorescence decay has been proposed in this work. Fluorescence decay quantitation may overcome some disadvantages in typical “signal on/off” fluorescent biosensors, such as the laborious and tedious washing procedures for the separation of adjacent fluorophores and being susceptible to interference like autofluorescence. Owing to the reported merits, malachite green aptamer (MGA) and its ligand malachite green (MG) were chosen as a model study to test the hypothesis of this thesis. MG was reported to have significant fluorescence enhancement upon MGA binding [1], which can be utilized for the determination of binding affinity. However, the MGA/MG binding was found to be unstable over time, as evidenced by the variation on apparent equilibrium dissociation constant (K_d) and observed fluorescence intensity, due to the hydrolysis of MG. This phenomenon may introduce difficulties in our hypothesis testing, especially in accurate detection of MG during long-time operations. Organic additives acetonitrile (ACN), dimethyl sulfoxide (DMSO) and ethanol (EtOH) were found to prevent MG hydrolysis, and thus stabilize the MGA/MG binding, with an aptamer associated synergistic stabilizing mechanism proposed [2]. This discovery may also be beneficial for carbocation aptamer ligands susceptible to S_N1 hydrolysis. Additionally, other factors such as pH and the stability of MGA were also investigated to obtain optimal conditions for MGA/MG binding. The total outcomes obtained pave the foundation and provide optimal experimental conditions for the hypothesis testing.

To test our hypothesis, two designs were investigated within the scopes: 1) “competitive stilbene-analyte conjugate with analyte” and 2) “stilbene-aptamer conjugate fluorescence decay correlates to analyte binding”. The design 1) “competitive stilbene-analyte conjugate with analyte” focuses on the performance of MG-stilbene conjugate. It is supposed that MG-stilbene conjugate is able to keep both the binding of MG (with MGA) and fluorescence decay of stilbene, which are the critical properties for the investigation of the hypothesis. Thus, MG-stilbene conjugates have been studied towards their MGA

binding and stilbene fluorescence decay, including a rhodamine B (RhB)-stilbene conjugate as a negative control. Studies on negative control RhB-stilbene conjugate give some indications: 1) fluorescence decay of stilbene in MG-stilbene conjugates is retained, 2) No evidence of MGA non-specific binding is observed with stilbene conjugates and 3) no significant difference is seen between the fluorescence decay of stilbene conjugate and its non-binding aptamer mixture. This RhB-stilbene conjugate showing no binding (with MGA) and significant fluorescence decay provides a good negative control on the MG-stilbene conjugate design. The structure activity relationships of conjugate binding to aptamer and photoisomerization led to conjugates that allow ongoing proof of concept testing. In particular, structure activity relationships were focused on conjugate covalent linkers and stilbene side groups. MG-COO-stilbene-NO₂ conjugate displays extremely low binding affinity with MGA and no detectable fluorescence decay (as expected). Conjugates MG-CH₂-NH-stilbene-OMe and MG-CH₂-NAC-stilbene-OMe exhibit fluorescence decay, but the binding affinity is still relatively low compared to MG, which is not ideal for the hypothesis testing. MG-fused stilbene conjugate with smaller molecular size solves the problem in binding affinity, with K_d comparable to MG, but it loses the fluorescence decay property. Although no ideal MG-stilbene conjugate candidate is obtained for hypothesis testing, some indications on structure activity relationships can be referred from the performance of the conjugates, associated with their molecular properties (**Table 5.1**). By analysis of the conjugates structure, smaller molecular size, introduction of decoupling groups between MG and stilbene, and strengthening of electrostatic interaction with MGA may be beneficial for enhancing the binding affinity of MG-stilbene conjugates. It has been reported that electrostatic and base pair stacking interaction are the two main forces in MGA/MG interactions [3,4]. On the other hand, introduction of electron donating groups as side groups may help keep the fluorescence decay property. The results show the MG-stilbene conjugate molecules possess either low binding affinity or weak fluorescence decay, which limits its design as a sensitive aptasensor, but provides proof of concept data of the design.

According to design 2) “stilbene-aptamer conjugate fluorescence decay correlates to analyte binding”, stilbene molecule needs to be grafted on aptamer to fabricate the

biosensor. Aptamer-stilbene conjugate MGA-SITS was synthesized by classical amine-isothiocyanate click chemistry in aqueous environment, under the reaction conditions optimized. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) and high performance liquid chromatography (HPLC)-diode array detector (DAD) have confirmed the successful conjugation and complete removal of the free starting stilbene molecule. The MGA-SITS conjugate has comparable binding affinity (compared to MGA-NH₂) and exhibits significant fluorescence decay similar to the starting SITS, which is available for the hypothesis testing. Thus, MG was subsequently tested by the MGA-SITS conjugate. Significantly, the presence of MG results in inhibition on the fluorescence decay of the MG-SITS conjugate, with quantitative reduced apparent fluorescence decay rate constant (k_{app}). This novel discovery supports the hypothesis of the thesis under our limited experimental conditions within the scope (**Figure 6.1**). Results from negative control experiments on SITS have ruled out the interference from interactions between MG and SITS, or between MGA/MG complex and SITS, which support that the inhibition on fluorescence decay is only due to SITS grafted MGA binding. Moreover, the fluorescence decay of MGA-SITS is only sensitive to MGA ligands MG and tetramethylrosamine (TMR), but not to the non-binding triphenyl compound RhB, showing selectivity and the capability of multiplex detection of ligands. Linear ranges of 0.5-50 μ M for MG and 0.2-20 μ M for TMR have been obtained by the MGA-SITS conjugate sensor. Compared to the sensing of MG, sensing of TMR by MGA-SITS presents higher sensitivity and lower limit of detection (LOD). This difference in response indicates that higher affinity or different bound configuration of MGA may result in a better sensing performance, which also gives suggestions on the further optimization of aptasensors based on this “aptamer-stilbene” design, for example, the competitive binding of MG to MGA-SITS/TMR complex. A novel sensing mode based on fluorescence decay kinetics has been invented according to the hypothesis of the thesis. This mode takes advantages of conformational change of aptamer binding and fluorescence decay of stilbene by grafting stilbene molecule on aptamer. However, sensitivity and LOD of the aptasensor still needs to be improved by further optimization. The results supporting the hypothesis set up a significant starting point for the development of fluorescence decay based aptasensor.

7.2 Implications and Future Suggestions

Besides the discovery and outcomes achieved in the study, some indications are obtained from the experimental results, which should be valuable for the future design of the aptasensors based on the hypothesis of this thesis. Additionally, some strategies and designs will also be suggested, which may be beneficial for the construction of fluorescence decay based aptasensors according to our hypothesis.

For the design 1) “competitive stilbene-analyte conjugate with analyte”, some implications can be obtained from the results, which may help improve the performance of MG-stilbene conjugates. Electron accepting groups (i.e. $-\text{COO}-$ and NO_2 groups) should be avoided for stilbene side groups, which may decrease the fluorescence decay. In other words, electron donating groups (like $-\text{OCH}_3$) are preferred for stilbene fluorescence decay retaining. Additionally, small molecular size of conjugate is preferentially recommended but the fused MG and stilbene may heavily disturb the fluorescence properties of stilbene. Finally, increase of electrostatic effect and spacers preventing the MG from electron disturbing groups should be considered to enhance the aptamer binding. The evidences on structure activity relationships above will be beneficial for the synthesis of new MG-stilbene conjugates in the future.

Some other strategies are also suggested for the future work. The first idea is to apply computational molecular modelling to guide the synthesis. To date, NMR and XRD structure of MG or TMR bound MGA have been revealed [3,4]. In fact, some computational molecular modelling studies on MGA/MG complex have been reported, which presents the configuration of MGA and the conformation and circumstances of the bound MG [5-8]. Compared with practical lab synthesis, computational modelling dramatically saves time, labor force and materials. Additionally, some preliminary results on MG-stilbene have been obtained in this study as described above, which may be beneficial for improving the modelling parameters. Thus, employment of the data we have to computational modelling may give helpful hints and implications on the future

synthesis of MG-stilbene conjugates in keeping both the MG binding and stilbene fluorescence decay properties. In turn, the experimental results on new synthesis MG-stilbene conjugate can also put some feedbacks on the computational modelling (**Figure 7.1**). Finally, incorporation of cycles of synthesis and modelling will create a database, which presents guideline on the synthesis of MG-stilbene conjugates suitable for hypothesis investigation as well as other aptamer ligand-stilbene conjugates.

Figure 7.1 Recycling of computational modelling and synthesis on MG-stilbene conjugates for optimal binding affinity and fluorescence decay.

Another proposal is to conduct a new round of systematic evolution of ligands by exponential enrichment (SELEX) selection. The purpose is to obtain a new aptamer simultaneous for both MG and the MG-stilbene conjugate with fluorescence decay (i.e. MG-CH₂-NAC-stilbene-OMe), and there requires competitive binding between the two ligands in the aptamer. The example of MGA has shown the possibility that aptamer can bind to multiple ligands. This strategy removes the consideration of keeping the binding affinity of MG-stilbene conjugates, since the aptamer binding both ligands will be selected post the synthesis of MG-stilbene conjugate. On the other hand, selection of aptamer with competitive binding of MG and stilbene is another strategy. If an aptamer

binds to both MG and stilbene, then MG can release the pre-bound stilbene molecular by competitive binding and probably change the fluorescence kinetics of stilbene due to the variation of micro-environment rearrangements. However, it might be challenging to obtain this kind of aptamer simultaneous binding to two targeted ligands in practical operations.

For the design 2) “stilbene-aptamer conjugate fluorescence decay correlates to analyte binding”, hypothesis has been supported by data under the scope and our experimental conditions. However, there are still some issues need to be solved and some further development should be conducted in the future.

The sensitivity of the MGA-stilbene sensor is relatively low, and thus more effort should be made to enhance the sensitivity. According to the sensing results from MG and TMR by the MGA-SITS conjugate sensor, aptamer-ligand with higher binding affinity and or different bound configuration of MGA may be preferred for higher sensitivity. Optimizations for enhancing the binding affinity should be an approach to enhance the conjugate sensitivity. Additionally, some modification on stilbene molecule may be performed to enhance the fluorescence properties of the conjugate and subsequently enhance the sensitivity, as higher quantum yield and k_{app} of the stilbene molecule can reduce the minimum amount of conjugate sensor needed for sensing, which may result in a stronger response to aptamer ligands. Additionally, decoration of stilbene molecules (i.e. introduction of electrostatic strengthening groups) may increase the electrostatic, base stacking or hydrogen bonding interactions between the stilbene molecule and the fold MGA, which results in a strong inhibition of stilbene fluorescence decay and thus enhances the sensitivity. However, the real situation of stilbene in the bound MGA has not been reported yet. NMR or XRD study on the MGA-SITS before and after aptamer binding should be conducted in the future to reveal the configuration of stilbene and the mechanism on how the aptamer folding affects the stilbene isomerization. Furthermore, computational molecular modelling on the dynamic change of stilbene during aptamer binding should also help discover the mechanism and give indications on optimal designs on the aptamer-stilbene conjugate biosensors.

Competitive binding strategy may be helpful in enhancing the sensitivity of the conjugate. According to our results, MGA-SITS is more sensitive to TMR compared to MG. Thus, MG sensing strategy by releasing the TMR from prebound MGA-SITS/TMR complex is proposed for the future work and **Figure 7.2** shows the concept. In this design, MG will replace the TMR in MGA-SITS/TMR complex. The decrease of MGA-SITS/TMR complex will dramatically promote the fluorescence decay, while the increase of MGA-SITS/MG complex will have relatively limited impact on the inhibition of fluorescence decay, due to the difference in MGA-SITS conjugate sensitivity for TMR and MG. This design may results in a higher sensitivity for MG sensing.

Figure 7.2 Competitive binding of MG with prebound MGA-SITS/TMR complex for MG sensing.

The bioconjugation reaction is still inefficient, which increase the cost of the aptasensor. The remained not reacted MGA-NH₂ may bind to ligands and thus reduce the sensitivity. Future work should also be focused on increasing the reaction efficiency, for instance, using other bioconjugation strategies. Maleimide-thiol reaction is a click reaction with high efficiency. My project partner Ms. Wu Yuanyuan (PhD candidate) has conducted the conjugation of MeO-stilbene-maleimide (**20**) with thiol molecule 6-Mercapto-1-hexanol (MCH) (**21**), which mimics the reaction of maleimide stilbene with SH-aptamer (**Figure 7.3**). The MeO-stilbene-maleimide-MCH (**22**) conjugate displays significant fluorescence decay in aqueous environment, which implies the possible conjugation of MeO-stilbene-maleimide (**20**) with thiol modified aptamer, with fluorescence decay behavior on the conjugate. On the other hand, C38 thiol modified MGA (MGA-SH) binds to MG with $K_d = 980$ nM from our preliminary study (**Figure 7.4**), which enables the possible application of the MGA-SH with guaranteed binding affinity after the aptamer is conjugated with MeO-stilbene-maleimide (**20**). According to the results above, conjugation of MGA-SH and maleimide stilbene molecules (like MeO-stilbene-maleimide (**20**)) can be conducted in the future, in order to reach higher reaction efficiency as well as better sensing performance.

Figure 7.3 Conjugation of MeO-stilbene-maleimide (**20**) with 6-Mercapto-1-hexanol (MCH) (**21**).

Figure 7.4 MGA-SH/MG binding curve and its calculated K_d , ex/em = 620 nm/656 nm.

In addition, different positions on aptamer for stilbene grafting should also be considered, which may vary the sensing performance, for instance, increasing the binding affinity or the response to ligands of the MGA-stilbene biosensor. It has been reported by Flinders et al that some base mutants on MGA only results in tiny impact on its binding affinity [4]. Thiol modified MGA on various positions (U4, A9, C20 and C38) have also been investigated by us, showing applicable binding affinities (less than 1000 nM) with MG (**Table 6.1**). The conjugation of stilbene on different positions of MGA may results in different efficiency of stilbene photoisomerization, which may enhance the sensitivity of the conjugate. Thus, by grafting stilbene on suitable location of the MGA, it can be proposed that the binding will not be influenced significantly. However, the fluorescence decay behavior of stilbene molecule may be different due to the different surround chemical and biological environment. Especially, the different fluorescence decay of stilbene molecule in different positions may reveal a law or relationship, which enables flexible tuning on fluorescence decay based on specific requirements. Furthermore,

owing to the well-known NMR and XRD 3D structure of the aptamer, computational molecular modelling should also help on implying the properties of MGA-stilbene when the stilbene molecule is grafted on different base of the MGA.

Computational molecular modelling may also help reveal the different response of the sensor in the sensing of MG and TMR. Sensing of TMR displays higher sensitivity and lower LOD compared to the MG sensing. It is hypothesized in Chapter 6 that may be due to the higher binding affinity and or different bound configuration of MGA in TMR binding. Computational modelling can be used to examine the hypothesis. By computational modelling, the view of the stilbene surrounding environment can be obtained, revealing the interaction between aptamer and stilbene (i.e. steric stacking and hydrogen bonding). Additionally, the freedom of stilbene isomerization can also be monitored upon the MGA-SITS binding to ligands, and the different stilbene surrounding environment induced by MG and TMR should be a key point to explain why the two ligands result in different fluorescence decay responses.

Moreover, although the MGA-stilbene biosensor quantitating by fluorescence decay has been developed, the use of RNA aptamer may cause some problem in the practical applications. RNA is susceptible to ribonuclease degradation, while DNA is known to have much better stability. Thus, to achieve a robust biosensor, DNA aptamer should be preferential under the same conditions. Additionally, the price for DNA is normally much lower than that of RNA, and the yield of DNA is higher in synthesis. Recently, Wang et al has successfully selected DNA aptamer for MG [9]. It is worth applying the same strategies in this work to the DNA MG aptamer for the development of a more practical and robust aptasensor, which also reduces the cost in sensor fabrication.

Furthermore, a reusable device is prior to a single-used one. Thus, regeneration of the used MGA-SITS sensor is also important. In fact, it is possible to reuse the MGA-SITS sensor because both the MGA and trans-stilbene can be regenerated. Aptamers can undergo reversible thermal denaturation (different from antibodies). Normally, denaturation by heat can destroy the bound aptamer/ligand complexed structure and thus

regenerate the aptamer for multiple uses [10]. Aptamer can even be regenerated by washing procedures when it binds to small molecule like cocaine [11,12]. On the other hand, the reversible *trans-cis* switching of stilbene compounds makes it an ideal reporter element in sensing. Even though the conversion of *trans*-stilbene to *cis*-stilbene is a relatively complete process with a high reaction rate, the *cis* form can be turned back to *trans* form under certain condition, e.g. heating or UV-Vis irradiation in another wavelength. Study shows that this “turning back” conformational change makes stilbene molecule recover fluorescent to almost the initial level [13]. **Figure 7.5A** presents the regeneration of aptamer and **Figure 7.5B** displays the *cis* to *trans* conversion of stilbene, which may towards the regeneration of aptamer-stilbene conjugate sensor (**Figure 7.5C**). Since both aptamer and stilbene can be regenerated for reusing, future work is suggested to regenerate the used aptamer-stilbene biosensor for multiple uses, which can reduce the cost of quantitation. If possible, approaches and conditions able to simultaneously regenerate the aptamer and stilbene are preferred, for instance, heating the used sensor with a proper temperature.

Figure 7.5 Schematic illustration of regeneration of aptamer (A), stilbene (B) and the regenerated aptamer-stilbene conjugate sensor (C).

Finally, if possible, apply this strategy to other aptamers to see if this sensing mode can be used in other aptamers. Especially, aptamers with high binding affinities and with small organic molecule ligands should be tried based on the results obtained in this work. Successful employment of this fluorescence decay based strategy will further support the hypothesis of the thesis.

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List of Publications

1. **Y. Zhou**, *et al.* Aptamer adaptive binding assessed by stilbene photoisomerization towards new aptasensors. *In progress*.
2. **Y. Zhou**, H. Chi, Y. Wu, R. S. Marks, T. W. J. Steele. Organic Additives Stabilize RNA Aptamer Binding of Malachite Green. *Talanta*, 2016, 160, 172-182.
3. **Y. Zhou**, H. Han, H. P. P. Naw, A. V. Lammy, C. H. Goh, S. Boujday, T. W. J. Steele. Real-Time Colorimetric Hydration Sensor for Sport Activities. *Materials & Design* 2016, 90, 1181-1185.
4. J. Ping†, **Y. Zhou**†, Y. Wu, V. Papper, S. Boujday, R. S. Marks, T. W. J. Steele. Recent Advances in Aptasensors Based on Graphene and Graphene-Like Nanomaterials. *Biosensors and Bioelectronics* 2015, 64 (0), 373-385. (†equal contribution)
5. Y. Wu, V. Papper, O. Pokhonenko, V. Kharlanov, **Y. Zhou**, T. W. J. Steele, R. S. Marks. New Photochrome Probe Allows Simultaneous pH and Microviscosity Sensing. *Journal of Fluorescence* 2015, 1-12.
6. V. Papper, O. Pokhonenko, Y. Wu, **Y. Zhou**, P. Jianfeng, T. W. J. Steele, R. S. Marks. Novel Photochrome Aptamer Switch Assay (PHASA) for Adaptive Binding to Aptamers. *Journal of Fluorescence* 2014, 24 (6), 1581-1591.