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Organic additives stabilize RNA aptamer binding of malachite green

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Abstract

Aptamer-ligand binding has been utilized for biological applications due to its specific binding and synthetic nature. However, the applications will be limited if the binding or the ligand is unstable. Malachite green aptamer (MGA) and its labile ligand malachite green (MG) were found to have increasing apparent dissociation constants \((K_d)\) as determined through the first order rate loss of emission intensity of the MGA-MG fluorescent complex. The fluorescent intensity loss was hypothesized to be from the hydrolysis of MG into malachite green carbinol base (MGOH). Random screening organic additives were found to reduce or retain the fluorescence emission and the calculated apparent \(K_d\) of MGA-MG binding. The protective effect became more apparent as the percentage of organic additives increased up to 10% v/v. The mechanism behind the organic additive protective effects was primarily from a \(~5X\) increase in first order rate kinetics of MGOH\(\rightarrow\)MG \((k_{\text{MGOH}\rightarrow\text{MG}})\), which significantly changed the equilibrium constant \((K_{\text{eq}})\), favoring the generation of MG, versus MGOH without organic additives. A simple way has been developed to stabilize the apparent \(K_d\) of MGA-MG binding over 24 hours, which may be beneficial in stabilizing other triphenylmethane or carbocation ligand-aptamer interactions that are susceptible to SN1 hydrolysis.

Keywords

* Abbreviations

MG, malachite green; MGOH, malachite green carbinol base; MGA, malachite green aptamer; ACN, acetonitrile; DMSO, Dimethyl sulfoxide; EtOH, ethanol.
Malachite green aptamer, malachite green, ligand hydrolysis, adaptive binding, equilibrium

1. Introduction

Oligonucleotide aptamers have been widely studied in biological applications such as biosensing [1-4], cell imaging [5, 6], drug/siRNA delivery [7, 8] and in vivo therapeutics [9], due to their specific recognition, adaptive binding and synthetic nature [10, 11]. Compared to antibodies, aptamers possess advantages such as low molecular weights, facile modification and low immunogenicity [12, 13]. However, lability of ligands affects the binding stability with aptamers, limiting their applications [14]. In this study, malachite green aptamer (MGA) with its labile ligand malachite green (MG) was chosen as a model system to study the aptamer-ligand binding stability over time based on MG’s enhancement of fluorescence upon aptamer adaptive binding. The MGA-MG complex is fluorescent due to an extended π-system of the MG that is forced to become planar by the dynamic binding of the MGA [15, 16].

MGA has been utilized for sensing the banned fungicide MG [17] with a limit of detection down to 5 nM [15]. Detection of such low concentrations is due to the unique enhanced quantum yield (2000X) upon MGA adaptive folding around MG as seen in Fig. 1B [16]. MG is currently employed in as a dye in paper, leather, inks, waxes, and textiles and is known for its use in algal inhibition in fish farming, although this practice has been banned in most developing countries due to acute toxicities in fish and humans [18]. MG belongs to a large family of triphenylmethane compounds that have considerable economic importance, as they are used in the printing industry (to brighten carbon black), sensor applications and as chrome dyes for wool and color formers in self-replicating/thermoresponsive papers. The latest known commercial statistics shows over 25 kilotons of product per year of various triphenylmethanes with unknown amounts ending in industrial waste waters and treatment plants [19]. Thus there exists a need for development of stable, fast, and accurate sensor development towards triphenylmethane derivatives, all of which are more or less susceptible to hydrolysis attack. Detection assays must then incorporate aspects of portability, specificity, and sensitive quantitation of MG in a range of aqueous environments. Additionally, it has been reported that MG can convert to its derivatives such as malachite green carbinol base (MGOH) [20, 21] and
leucomalachite green [22], which affects the sensitivity and specificity of the MGA-MG binding complex (Fig. 1A).

Fig. 1

However, assessments of temporal $K_d$ of MG to MGA and prevention of MG hydrolysis (and related triphenylmethane derivatives) have not been investigated to our knowledge, despite the economic importance of detecting triphenylmethane derivatives in dye industrial wastewaters and within the food supply.

In this work, the adaptive binding behavior of MG to its RNA aptamer was assessed by for temporal fluorescence with various organic additives added to prevent the decreasing fluorescence intensities. The apparent dissociation constant, $K_d$ was determined to be comparable to the reported value of 200 nM [23] at time 0. However, the fluorescence intensity and the apparent $K_d$ decreased as a function of time, but stabilized after 5 h, which suggested a change in equilibrium in the RNA aptamer or MG. We noted that when organic additives were initially employed to dissolve various triphenylmethane dyes, the $K_d$ was less sensitive to time. Investigation of the organic additives allowed more sensitive and reproducible determination of MG, which is a simple strategy with little to no impact on $K_d$. The increase in stability minimizes deviations in triphenylmethane detection and facilitates the further development of RNA aptamer-based applications. More importantly, the discovery of kinetic stabilization of the MGA-MG model may provide a simple method of stabilization for triphenylmethane, triarylmethane, or other carbocation-susceptible ligands that are known to undergo hydrolysis.

2. Materials and methods

2.1. Materials

Desalted RNA aptamer (MGA) (5'-GGAUCCCGACUGGCAGGAGCCAGGUACGAAUGGAUCC-3') was purchased from AITbiotech Pte Ltd Singapore. Malachite green oxalate (MG) and malachite green carbinol base (MGOH) were purchased from Sigma Aldrich, Singapore. Dimethyl sulfoxide (DMSO) was purchased from Alfa Aesar, USA. Ethanol (EtOH) was purchased from Fisher Scientific, Singapore. HPLC grade acetonitrile (ACN) was purchased from Tedia, USA. Polystyrene microplates (96-well) were purchased from Corning, USA. All chemicals and materials were used as received.
2.2. \( K_d \) determination of MG to MGA

Binding buffer (pH = 6.7 unless otherwise specified) which consisted of 5.65 mM NaH\(_2\)PO\(_4\), 4.35 mM Na\(_2\)HPO\(_4\), 10 mM MgCl\(_2\) and 1 mM NaCl was prepared according to the literature [23, 24]. The effect of pH on binding was studied and the results (Fig. S1) indicated that the suitable range for binding was pH 6-8.5. Thus, pH at 6.7 was chosen for comparison to literature [23]. Malachite Green Aptamer (MGA) was denatured at 95 °C for 1 min prior to the binding experiment. The binding of MG to MGA was determined by fluorescence enhancement [16]. \( K_d \) was determined varying concentrations of MGA with MG concentration (150 nM) held constant in binding buffer with or without organic additive present at 25 °C. MG fluorescence was immediately assayed or time delayed from 0.5-24 h in 96-well polystyrene plates with a Tecan Microplate spectrofluorometer (Infinite M200, Tecan Asia PTE LTD, Singapore) at ex/em setting of 620 nm/656 nm. Based on the MG fluorescence enhancement upon aptamer binding described above [16], fluorescence intensity of MG (in fixed concentration) rises with increasing MGA concentration, and reaches a plateau when 100% MG are bound. Thus, the fluorescence signal of MG against added MGA concentration can be utilized for monitoring the binding. By combining the change in fluorescence intensity with the known concentrations of MGA and MG in the binding system, dissociation constant \( K_d \) value can be determined by the non-linear fitting in section 2.3 based on the literature by Wang et al [25, 26].

Additionally, to test the recovery effect of organic additive on fluorescence (of MGA-MG complex) after reduction, DMSO was added into the MGA-MG binding complex 5 h after the binding was performed.

2.3. Fitting of dissociation constant \( K_d \)

The equation (Equation 1) used for fitting of \( K_d \) is displayed below, which is based on previous investigations by Wang et al [25, 26].:

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

\( F \) and \( F_0 \) are fluorescence units (FLU) in the presence and absence of aptamer, respectively. \( F_{\text{max}} \) is the FLU value upon 100% binding to aptamer. \([MG]_0\) and \([MGA]\) are the initial concentrations within the binding system, and the \( K_d \) is the dissociation constant. Non-linear fitting and statistical analysis were performed by OriginPro 9.1.

2.4. HPLC-DAD study on stability of MG
An Agilent 1200 series high-performance liquid chromatography system with diode array detector (DAD) was used to monitor the stability of MG. A Ultisil™ C18 column (Welch Materials Inc.) of 5 µm and 4.6 × 250 mm was used for chromatographic separation. The system was operated with a mobile phase flow rate of 1.0 mL•min⁻¹, and the diode array detector was set to continuously wavelength scans of 200 to 800 nm with λ_max of 265 and 620 nm for MGOH and MG, respectively. Fresh MG (30 µM) in binding buffer was injected (20 µL) at 0 h and 24 h and MGOH (30 µM) as a standard in acetonitrile was injected (20 µL) at 0h. Elution profile was 50 % mobile phase A (25 mM ammonium acetate, pH 6.8) / 50 % mobile phase B (acetonitrile) 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.

2.5. Kinetic study on conversion between MG and MGOH

MG and MGOH were dissolved in pH 6.7 binding buffer with or without 10% (V/V, the same to all description on percentage of organic additive in this article) organic additive respectively. Then the sample was transferred to a quartz cuvette and placed in a Varian Cary 50 UV-Vis spectrophotometer. The UV-Vis spectra were recorded every 5 min for 12 hours. Absorbance maximum of MG at 618 nm was used to calculate the change of MG. The decrease in MG at MG → MGOH and the increase in MG at MGOH → MG were fitted by OriginPro 9.1 with first order equation.

2.7 Statistics

All statistics were performed with OriginPro 9.1 (OriginLab Corporation, Northampton, MA 01060 USA). Linear regressions R² were determined by ordinary least squares. Statistical differences between two groups were performed by Student’s t-test with significance level P < 0.05 (two-tailed test).

3. Results

3.1. Fluorescence intensity and apparent affinity of MGA-MG diminish over time

Based on the fluorescence enhancement of MG, the initial dissociation constant (K_d) is determined to be 200 nM as seen in Fig. 2A and 2B, which compares well to the analysis by Costa et al [23]. However, fluorescence intensity and apparent K_d of MG decrease with time, but reach a plateau at 5 h as shown in Fig. 2A. At 24 h, 40.8% of the fluorescence intensity at maximum [MGA]/ratio (6000 nM, MGA:MG = 40:1) remains while the apparent K_d increases > 10000 nM. The fluorescence intensity at maximum [MGA]/ratio undergoes a first order decay with k = 2.5×10⁻⁴ s⁻¹ (Fig. 2C).
This decrease is attributed to the hydrolysis of MG in aqueous environment. Minute
changes in pH are also ruled out, as only minor shifts in $K_d$ are seen across pH 6-8.5
(Fig. S1). Thus, the shifts in $K_d$ are attributed to the flattened asymptote to linear-
profiles after FLU decay.

3.2. Organic additives prevent fluorescence decay while retaining $K_d$

Binding buffer with 3%-10% v/v acetonitrile (ACN), dimethyl sulfoxide (DMSO)
or ethanol (EtOH), additives typically employed to increase solubility of organic
ligands, is mixed into the MGA-MG binding experiments (Fig. 3) towards assessment
of temporal fluorescent intensity. The MGA-MG fluorescence displays observable
increases across the largest concentrations/ratios of MGA:MG. With organic
additives, > 9000 FLU are observed at [MGA] above 3000 nM as seen in Fig. 3A-C,
about 40% higher than 5500 FLU at time 0 h in Fig. 2A. This suggests MG$\rightarrow$MGOH
conversion is present even before the time 0 h measurements in Fig. 2A were taken.
The addition of organic additives changes the equilibrium constant, decay kinetics, or
both. In other words, known stocks of [MG] can never truly be prepared in aqueous
solutions, because of the fast hydrolysis equilibrium. To further quantitate the effects
of organic additives, the apparent $K_d$ values are calculated and presented in Fig. 3D.
The apparent $K_d$ shows a strong correlation with percentage of ACN, but weak to
moderate correlation in either DMSO or EtOH. Thus, only ACN weakens the overall
$K_d$, while DMSO and EtOH show little to no changes in $K_d$.

3.3. Organic additives retain over >80% of fluorescence intensity after 24 h

The stability of MGA-MG binding after 24 h in the organic additive-buffers is
investigated towards preventing fluorescence decay. Fig. 4A-C illustrates the MGA-
MG $K_d$ fittings at 0 h and 24 h in the presence of ACN from 3 - 10% v/v. Similar
trends are found for DMSO and EtOH (data not shown). Fig. 4B,C summarize the
apparent $K_d$ and FLUs at the highest [MGA]/ratio at 0 h and 24 h vs. conc. of organic
additive. FLU and apparent $K_d$ are less prone to decay or increase, respectively, as the
organic additive increases, suggesting a change in $K_{eq}$, decay kinetics, or both. For
example, with no additives, the FLU at maximum [MGA]/ratio decreases to 40.8% of
the original value and the apparent $K_d$ increases ~ 65-fold after 24 h, **Fig. 2A**. The addition of the 8-10% v/v DMSO additive shows no statistical difference in FLU units after 24 h (at the 40:1 ratio), but a slight shift in the overall binding profile (which incorporates all FLU ratios), doubles the $K_d$. EtOH has the inverse effect. Overall, organic additives cause considerable shifts on the FLU decay kinetics as shown in **Table 1**. The first order decay kinetics, as measured by FLU, decreased as the v/v% increased from 3 to 10%, which indicates organic additives shifted the $K_{eq}$ (MG$\leftrightarrow$MGOH) towards MG, and increasing the overall conc. of the fluorescent MGA-MG complex.

**3.4. Loss of MG fluorescence is attributed to hydrolysis, formation of MGOH**

HPLC in-line with a diode array absorbance detector allows the quantification and identification of MG derivatives through their absorbance spectra. MG (λ$_{max}$ = 620 nm) and MGOH (λ$_{max}$ = 265 nm) absorbance spectra are displayed in the **Fig. 5A,B** insets. HPLC chromatogram overlays of MG 0 h, MG 24 h, MGOH standard is shown in **Fig. 5A**. Under the HPLC separation conditions (see Methods), MG 0 h displays a major peak at 11.1 min with a minor observed peak at 18.2 min attributed to the MGOH (malachite green carbinol base).

The MG peak decreases 36.3 % after 24 h (**Fig. 5A**) while the MGOH peak increases 38.6%, nearly the same amount as seen in **Fig. 5B**. The MGOH peak in the MG 0h sample has 19% AUC compared to the 30 µM standard MGOH; in other words 5.7 µM MGOH is detected in the (as prepared) 30 µM MG 0h sample. In the MG 24h sample, MGOH conc. nearly triples to 17.1 µM, and there is no observed formation of other minor peaks during 0-25 min elution. By plotting 100% MG at 10,000 FLU (**Fig. 4C**), 81% MG at 5,500 FLU (**Fig. 2A and 5B**), and 43% MG at 2,000 FLU (**Fig. 2A and 5A,B**), a reasonable linear fit of $R^2 = 0.86$ is noted.

**3.5. Organic additives shift the $K_{eq}$ towards MG formation**
The kinetics of conversion (without aptamer) of MG→MGOH and MGOH→MG with and without 10% v/v organic additive (ACN, DMSO, EtOH) is conducted by following the lambda max of MG, as seen in Fig. 6. The recorded wavelength scans from 400 nm to 800 nm are seen in Fig. S2, which show similar results as noted in the HPLC characterization above, that MG cannot be prepared pure in aqueous solutions due to a t½ of ~19 min (ln 2/6.2×10⁻⁴ s⁻¹), while MGOH can (t½ = 2.8 h). In buffer without any organic additive, the conversion of MG→MGOH occurs nine times faster (Fig. 6A, k_MG→MGOH = 6.2×10⁻⁴ s⁻¹) than the conversion of MGOH→MG (Fig. 6B, k_MGOH→MG = 0.69×10⁻⁴ s⁻¹). Additionally, the conversion of MG→MGOH reaches equilibrium after 2 h, while the conversion of MGOH to MG does not reach equilibrium even after 12 h, as seen in Fig. 6B. When MG→MGOH in the presence of 10% organic additives (Fig. 6A), the k_MG→MGOH is retarded by 23%, 9%, and 5% for ACN, DMSO, and EtOH, respectively. From the FLU decay kinetics, the aptamer retards the k_MG→MGOH by 60% with no additives present (Fig. 2C and 6A).

Surprisingly, the conversion of MGOH→MG in the presence of 10% organic additives (Fig. 6B) displays a comparably large shift in k_MGOH→MG. Organic additives increased k_MGOH→MG over buffer by 360%, 450%, and 460% for ACN, DMSO, and EtOH, respectively. This acceleration on MGOH→MG conversion by organic additives results in a large shift in K_eq compared to the MG in buffer or buffer + MGA. This shift in K_eq is summarized in Table 2. To investigate any effects the aptamer has on k_MGOH→MG, various ratios of MGA were mixed with freshly prepared MGOH and the MGA-MG fluorescent complex was monitored over time under various MGOH:MGA ratios. At high ratios of 20 and 40:1, there was no statistical difference in the FLU rate of MGA-MG fluorescent complex formation versus the ABS rate of MG formation, as seen in Fig. S3. Thus, high aptamer ratios have no effect on k_MGOH→MG kinetics.

Table 2 summarizes k_MG→MGOH in different additives and in the presence and absence of aptamer (MGA). The molecular properties of water and organic additives are also listed for any evident correlations. Without aptamer, organic additives had only a slight effect on MG→MGOH conversion. However, in the presence of aptamer, the conversion was 40% slower, indicating the protective environment the aptamer provides the MG in terms of hydrolysis. More importantly, this protection effect combined with the organic additives provides a synergistic outcome towards the stable formation of the MGA-MG fluorescent complex. The adjusted k_MG→MGOH as
measured by FLU was 500% slower in 10% ACN and 2950% slower with 10% EtOH. No significant decay in fluorescence was observed with the addition of 10% DMSO, preventing any measurement of $k_{MG\rightarrow MGOH}$ under these conditions. Additionally, Pearson’s $R$ values indicate strong correlations between kinetic constant and intrinsic properties of the organic additives. We speculate that this may allow prediction of even more effective additives. Without aptamer, no distinct correlation between kinetic constant and intrinsic properties is observed ($0.5 > R > -0.5$). However, in the presence of aptamer, kinetic constant has a strong correlation with molecular polar surface area (MPSA) ($R > 0.99$) and a strong inverse correlation to additive volume ($R < -0.99$). The 10% DMSO is excluded in calculation due to its unavailable kinetic constant. The lack of decay follows the strong correlation of the intrinsic molecular properties of DMSO (small MPSA and relatively large volume).

![Fig. 6](image)

### Table 2

#### 3.6 Organic additives reverse decay of fluorescence through MGOH dehydration

The shift in $K_{eq}$ values as seen in Table 2 suggests that organic additives can immediately change the reaction kinetics to favor MG as the major equilibrium product. This hypothesis is challenged by the addition of 3-10% v/v DMSO to MGA:MG aqueous buffer solutions that have reached a stable FLU after 5 h of incubation. Fig. 7 displays FLU recovery of 3 and 5% DMSO after 1 and 12 h incubation. The results support an immediate shift in $K_{eq}$ by organic additives, with a minimum of 5% v/v DMSO to recover the 0 h fluorescence intensity after 12 h. Higher conc. of DMSO yielded FLU values that approached 10,000 FLU units (at MGA:MG ratios > 20:1), similar to the values seen in Fig. 4C (data not shown).

![Fig. 7](image)

### 4. Discussion

MG and its aptamer have been utilized for various applications such as cell imaging and probing for RNA nanoparticles based on enhanced fluorescence of MG after induced binding [14, 27, 28]. However, due to the known instability of MG in
aqueous buffers, MG and MGA-MG binding will decrease over time. However the
temporal $K_d$ or fluorescent intensity of the MGA-MG complex is seldom reported.
Wang et al. has experimentally and theoretically argued that MGA prevents MG
hydrolysis by measuring absorbance maxima in bound vs. unbound MG—no data on
changes in fluorescence was described [29]. Decreased hydrolysis kinetics was also
observed by FLU measurements where MGA is in buffer and buffer + organic
additives, but our work differs in conclusions drawn. Additionally, we also monitored
the apparent $K_d$ of MGA-MG over time, and found that fluorescence intensity
decreased while apparent $K_d$ increased over time. Moreover, we report for the first
time how the presence of common organic additives prevented temporal shifts in
fluorescence intensity and $K_d$ within a 24 h timeframe. This stabilization is beneficial
for MGA-based applications towards accurate and repeatable detection assays based
on MG’s fluorescence enhancement by MGA [30, 31]. Finally, a synergistic
stabilization mechanism is proposed based on both fluorescence and absorbance
kinetics combined with previous reports on MG kinetics in aqueous environments [32,
33].

4.1. Hydrolysis of MG to MGOH prevents aptamer binding

The $K_d$ was determined by MG’s unique fluorescence enhancement upon induced
aptamer binding, but when the FLU signal decays, the dissociation constant will be
directly affected. The fluorescent response of MG upon binding to MGA is illustrated
in Fig. 2. In Fig. 2A, the increase in apparent $K_d$ and subsequent decrease in
fluorescence intensity through 24 h (at 40:1 ratio) indicate an unstable association
between MGA and MG. In two preliminary experiments, MG and MGA were
separately added into the MGA-MG complex respectively after 24h to isolate the
cause of fluorescence decay. The addition of fresh MG recovered the fluorescence
intensity instantly, but continued to decay. The addition of fresh MGA displayed no
fluorescence recovery (Fig. S4). Such behavior indicated that the decrease in FLU
was due to events centered on MG but not the aptamer, ruling out RNA misfolding or
degradation. To investigate integrity of MGA in binding buffer (containing 10 mM
Mg$^{2+}$) over 48 h, gel electrophoresis on MGA over time was performed. As seen in
Fig. S5, no obvious change in the MGA band was observed from 0 h to 48 h. The
binding interactions of RNA aptamers to ligands are dynamic and do not follow a
typical ‘lock and key’ static enzyme mechanism, but follows an ‘induced fit’
mechanism that more closely resembles RNA folding around the ligand, where both
the RNA and ligand have induced changes in structure [34]. Thus, a clear explanation of how RNA folding changes after a ligand hydrolysis event hasn’t been explored, but creates another level complexity best removed if possible, which is yet another reason for the addition of the organic additives shown herein. HPLC investigations display only MG and MGOH as the major reactants and products. The 1:1 conversion of MG peak to MGOH peak combined with the UV profile spectra confirms the conversion of MG to MGOH with no other peaks of interests detected [21, 35, 36]. No other MG variants (i.e. leucomalachite green, dimers) were detected, nullifying any hypothesized side reactions. Another hypothesis, that aptamer prevented MGOH$\rightarrow$MG dehydration, was proven false, as pure MGOH was incubated with varying ratios of aptamer, with increasing fluorescence noted as MG was formed at the predicted kinetics. The observation that FLU decay is completely reversible by organic additives (see Fig. 7) speculates that MG undergoes a time dependent change in electronic resonance or charge redistribution within the aptamer binding pocket. This would lower the quantum yield or shift its fluorescent planar ‘induced fit’ configuration. As seen in Fig. 8, we speculate that the hydrolysis intermediate, MG-OH$_2$, is responsible for the FLU decay through electronic and charge redistribution within the aptamer binding pocket, as the C$^+$-OH$_2$ carbocation is further stabilized and becomes the preferred resonance structure. Shifts in MG $^{13}$C NMR peaks have confirmed this shift in charge distribution between unbound and bound MG [34]. However, due to the long timescale requirement of $^{13}$C measurements, MG intermediates are unlikely to be distinguished from MG. Alternatively, the RNA base stacking forces may be weakened through the more hydrophilic C$^+$-OH$_2$ carbocation, relaxing the induced fluorescent planar geometry [37]. The addition of organic additives prevents or reverses it.

**Fig. 8**

The hydrolysis of MG resulted in a significant impact in the apparent $K_d$ (Fig. 2A), due to the less available MG, reduced affinity MG-OH$_2$ transition state, or both. The binding of one ligand to MGA will reduce the competitiveness, even with ligands of higher affinity, due to the induced fit model [23]. Thus, competitive binding experiment (between MG and MGOH to MGA) was conducted by simultaneously introducing various ratios of MGOH:MG with a fixed amount of MGA. As shown in
Fig. S6, up to 7-fold (limited by aqueous solubility) molar excess of MGOH was added into a fixed MGA:MG ratio, but no significant changes were observed in FLU (no competitive binding) of the MGA-MG complex. Even when MGOH and MGA were the initial conditions (Fig. S3), the formation of MG and the subsequent MGA-MG fluorescent complex still matched absorbance kinetics (buffer-only $k_{\text{MGOH}}\rightarrow\text{MG}$), suggesting there is no competitive or non-specific interactions from buffer containing primarily MGOH. The failed affinity of MGOH to MGA is speculated to be from MGOH’s neutrally charged structure, as long range electrostatic forces provided by cationic amines need to be present [15, 24].

4.2. Organic additives shift $K_{eq}$ with limited impact on $K_d$

Organic additives are known to increase the $K_d$ of molecular recognition elements [15]. Towards MGA-MG binding at time 0 h, the organic additives increased $K_d$ in the following additive order: ACN > EtOH > DMSO. The lowered affinity was proportional to the additive concentration, but increased the fluorescence intensity of MGA-MG complex (Fig. 3A-C, compared to Fig. 2A). This paradox can be explained by the shift in $K_{eq}$ and by noting that freshly prepared MG solutions always contained various concentrations of MG and MGOH, as seen in Fig. 5. Fig. 6 demonstrates how organic additives slow MG$\rightarrow$MGOH while accelerating the MGOH$\rightarrow$MG conversion. Hence, the presence of organic additives results in synergistic shift towards MG, thus enhances the formation of MGA-MG based fluorescence. Although the aptamer binding is normally performed in aqueous only environments, the presence of organic additives simplifies the $K_{eq}$ towards MG and allows a much longer window of measurement towards assay development and may extend the application of aptamers and more exotic ligands, i.e. modifications of hydrophobic prodrugs for delivery [38]. Indeed, 40% of ‘new chemical entities’ developed by the pharmaceutical industry are practically insoluble and organic additives are often added to mimic in vivo solubility parameters, widening the window of application and selection of ligands with poor aqueous solubility [39-42]. Additional benefits include the facilitated competitive binding of organic ligands and antibacterial properties.

The loss of fluorescence intensity and increase in $K_d$ were prevented upon addition of organic additives. This discovery provides a simple way to stabilize aptamer binding, which is beneficial for applications that rely on MGA for accurate detection of MG. This inhibition strategy may also be applied to other triphenylmethane compounds that are susceptible to hydrolysis, for instance, to stabilize aptasensors of
crystal violet [43-46]. To date, more than $10^5$ triphenylmethane compounds ((Ph)$_3$-C) and 9,000 substructures of MG have been reported to date (SciFinder). Triphenylmethane compounds have been commercially and analytically used as bacteriological stains, pH indicators, therapeutic agent and food dyes [45]. However, the use of some triphenylmethane compounds has been banned for farming due to their potential toxicity, thus the detection and monitoring of triphenylmethane compounds is of consumer importance [17]. Additionally, it is well known that the triphenylmethane and other triarylmethane structures can form carbocations, which is susceptible to nucleophilic attack (i.e. hydrolysis). The discovery in this work paves a way to stabilize aptamer binding to ligands with triphenylmethane or similar carbocation structures.

Moreover, the decreasing trend of rate constants with increasing additive concentration (Table 1) shows a strong correlation. In other words, the rate constant may be tuned by the type of organic additive, which may benefit alternative designs for in-line or flow aptasensors, i.e. sensing within specific time frames.

4.3. Synergistic stabilization of ligand by aptamer and organic additives

To further understand the MG hydrolysis and MGOH dehydration, kinetics in both directions was evaluated independently without aptamer by absorbance (Fig. 6). The results indicate a relatively fast hydrolysis reaction from MG to MGOH in pH 6.7 buffer (Fig. 6A-B). The larger rate constant (compared to the one in aptamer binding in Fig. 2C) indicates the aptamer can interfere with MG hydrolysis, but not prevent it completely. Wang et al. has shown that the MGA could prevent MG well from hydrolysis over time due to the steric hindrance effect [29]. However, our results showed the protective effect is weaker, which is likely due to the difference in experimental method of kinetics and affinity evaluation. Wang et al. evaluated MG hydrolysis through changes in MG’s absorbance maxima, while we based ours on fluorescence. Absorbance changes may not be apparent between complexed MG and the intermediate MG-OH$_2$, thus accounting for their perceived observation of complete quenching of hydrolysis. If the bound intermediate MG-OH$_2$ is responsible for the FLU decay, Wang’s results provide further evidence that MG-OH$_2$ intermediate can still provide enough affinity interactions to keep the aptamer folded, while MGOH cannot. Without aptamer, the organic additives had a slight inhibition on kinetics of MG to MGOH (Fig. 6A), but catalyze the dehydration of MGOH to MG (Fig. 6B). Addition of organic additive not only accelerated the conversion of
MGOH to MG, but the conversion appeared to shift the equilibrium in favor of MG (Table 2). The impact on kinetics and equilibrium defines how the organic additives stabilized the fluorescence intensity of the MGA-MG complex over 24 h.

Synergistic stabilization on MG was observed by the combination of rates changes through the organic additives and aptamer (Table 2). Pearson’s R correlation shows a strong correlation between rate constants and the molecular properties of the organic additives in the presence of aptamer. Less polar surface area, larger solvent volume, or a combination of both, correlates with decreasing rate constants of MG hydrolysis. These results are supported by Wang et al, showing minimal space between the MGA-MG interface and the prevention of hydroxyl ion attack, which is an unlikely mechanism of hydrolysis in a pH 6.7 buffer [29, 32]. Nguyen et al. argued that MGA can change the conformation and charge distribution of MG upon binding [34], which may result in a shift of hydrolysis kinetics and thermodynamics. However, it has not yet been reported what the impact of organic additives may have on the stability of MG inside the aptamer binding pocket. In this study, the results show that organic additives; 1) Shift equilibrium to MG in the presence of aptamer, 2) had minor effect on MG hydrolysis without aptamer, 3) catalyze MGOH dehydration and 4) had only minor shifts on affinity and $K_d$. Based on these primary 4 observations, a mechanism is proposed in Fig. 8. At pH 6.7, MG undergoes slow hydrolysis to intermediate MG-$\text{OH}_2$, which transforms to MGOH immediately. 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 (Fig. 8A). Since $[\text{OH}^-]$ equals to $5.0 \times 10^{-8}$ M at this pH, thus $k_{\text{OH}^-}[\text{OH}^-] < k_{\text{H}_2\text{O}}$ and results in $k_{\text{obs}} \approx k_{\text{MG} \rightarrow \text{MGOH}}$ [47] (Fig. 8B). As proposed in Fig. 8C, MGA retards MG hydrolysis (where aptamer is in excess of MG) by retarding the conversion of MG-$\text{OH}_2$ to MGOH, which results in a decreased $k_{\text{MG} \rightarrow \text{MGOH}}$, as summarized in Table 2. However, this inhibition effect by MGA is insufficient to make $k_{\text{MG} \rightarrow \text{MGOH}} \leq k_{\text{MGOH} \rightarrow \text{MG}}$, and thus the conversion still favors to MGOH generation, suggested by fluorescence decrease of MGA-MG complex over time as displayed in Fig. 2A. With the presence of organic additives, $k_{\text{MGOH} \rightarrow \text{MG}}$ increases by few hundred percent (as shown in Fig. 6B), which results in $k_{\text{MG} \rightarrow \text{MGOH}} \geq k_{\text{MGOH} \rightarrow \text{MG}}$ combining with the effect from MGA. The synergistic effect of aptamer and organic additives (decrease of $k_{\text{MG} \rightarrow \text{MGOH}}$ and increase of $k_{\text{MG} \rightarrow \text{MGOH}}$) results in a $K_{\text{eq}}$ favoring prevention of MG hydrolysis (Fig. 4) and MGOH dehydration. MGOH dehydration and observed $K_{\text{eq}}$ was challenged by repeating the FLU decayed experimental
conditions in Fig. 2A, with the addition of DMSO (3-10% v/v) post decay. The results in Fig. 7 show that a minimum of 5% v/v DMSO was enough to recover the 0 h fluorescence after an incubation time of 12 h. Higher conc. of DMSO yielded FLU values that approached 10,000 FLU units (data not shown).

5. Conclusions

Aptamers as new materials for pharmaceutics and diagnostics have unique advantages as molecular recognition elements. However, the aptamer-ligand affinity is seldom investigated long term. In this work, we investigated the fluorescence intensity of aptamer-malachite green complexes over 24 h and quantified the kinetics of malachite green hydrolysis under various environmental conditions. The loss of fluorescence intensity and increase in apparent $K_d$ was due to the hydrolysis of malachite green into speculated intermediates and malachite green carbinol base, as indicated by spectrofluorometry, HPLC-diode array detector, and absorbance spectra. Organic additives prevented the decay of the fluorescence intensities and were found to minimize shifts in apparent $K_d$. A mechanism was proposed and challenged that suggests shifts in $K_{eq}$ prevent the formation of malachite green carbinol base, or if formed, cause dehydration back into the malachite green. This work demonstrated a synergistic effect of both aptamer and organic additive on the prevention of carbocation hydrolysis, allowing larger time frames for aptamer biosensor assays.

Acknowledgments

This work was supported by the Ministry of Education Tier 1 Grant RG54/13: “Photochrome Aptamer Switch Assay: A Universal Bioassay Device”; and the Campus for Research Excellence and Technological Enterprise (CREATE) programme (13-04-00364 A), which is supported by the National Research Foundation, Prime Minister’s Office, Singapore.

Appendix A. Supplementary materials

Please refer to the individual file for supplementary data.

References


Tables

Table 1. Decay rate constant ($k_{\text{MG} \rightarrow \text{MGOH}}$) of fluorescence intensity at maximum [MGA]/ratio with various percentages of organic additives.

<table>
<thead>
<tr>
<th>Media</th>
<th>$k_{\text{MG} \rightarrow \text{MGOH}} \times 10^{-4}$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3% Acetonitrile</td>
<td>3.1±0.4</td>
</tr>
<tr>
<td>5% Acetonitrile</td>
<td>2.6±0.1</td>
</tr>
<tr>
<td>8% Acetonitrile</td>
<td>1.3±0.2</td>
</tr>
<tr>
<td>10% Acetonitrile</td>
<td>1.0±0.1</td>
</tr>
<tr>
<td>3% Dimethyl sulfoxide</td>
<td>2.7±0.6</td>
</tr>
<tr>
<td>5% Dimethyl sulfoxide</td>
<td>0.2±0.3*</td>
</tr>
<tr>
<td>8% Dimethyl sulfoxide</td>
<td>&lt; 0.01, NSD</td>
</tr>
<tr>
<td>10% Dimethyl sulfoxide</td>
<td>&lt; 0.01, NSD</td>
</tr>
<tr>
<td>3% Ethanol</td>
<td>4.0±2.1</td>
</tr>
<tr>
<td>5% Ethanol</td>
<td>1.5±0.1</td>
</tr>
<tr>
<td>8% Ethanol</td>
<td>0.4±0.1</td>
</tr>
<tr>
<td>10% Ethanol</td>
<td>0.2±0.1</td>
</tr>
</tbody>
</table>

* Fitting coefficient of determination $R^2 < 0.6$. NSD: No statistical difference was seen between 0 and 24 h FLU.
Table 2. Rate constant ($k_{\text{MG\rightarrow MGOH}}$) of MG hydrolysis with 10% percentages of organic additives in the presence and absence of aptamer (maximum [MGA]/ratio), and the theoretical properties of water and additives.

<table>
<thead>
<tr>
<th>Media</th>
<th>With aptamer</th>
<th>Without aptamer</th>
<th>Solvent</th>
<th>Molecular properties*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_{\text{MG\rightarrow MGOH}}$ ($\times 10^{-4}$) (s$^{-1}$)</td>
<td>$K_{eq}$ (s$^{-1}$)</td>
<td>$k_{\text{MG\rightarrow MGOH}}$ ($\times 10^{-4}$) (s$^{-1}$)</td>
<td>$K_{eq}$</td>
</tr>
<tr>
<td>buffer</td>
<td>2.5±0.2</td>
<td>3.62</td>
<td>6.2±0.1</td>
<td>8.99</td>
</tr>
<tr>
<td>10% Acetonitrile</td>
<td>1.0±0.1</td>
<td>0.40</td>
<td>4.8±0.1</td>
<td>1.92</td>
</tr>
<tr>
<td>10% Ethanol</td>
<td>0.2±0.1</td>
<td>0.06</td>
<td>5.9±0.1</td>
<td>1.84</td>
</tr>
<tr>
<td>10% Dimethyl sulfoxide</td>
<td>NSD*</td>
<td>&lt; 0.05*</td>
<td>5.7±0.0</td>
<td>1.84</td>
</tr>
<tr>
<td>Water</td>
<td>29.3</td>
<td>19.3</td>
<td>Water</td>
<td>23.8</td>
</tr>
<tr>
<td>Acetonitrile</td>
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<td>46.1</td>
<td>Acetonitrile</td>
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</tr>
<tr>
<td>Ethanol</td>
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<td>54.0</td>
<td>Ethanol</td>
<td>17.1</td>
</tr>
<tr>
<td>Dimethyl sulfoxide</td>
<td>17.1</td>
<td>71.4</td>
<td>Dimethyl sulfoxide</td>
<td></td>
</tr>
</tbody>
</table>

Pearson’s $R$ correlation, $k_{\text{MG\rightarrow MGOH}}$ vs. MPSA: +0.999** +0.189
Pearson’s $R$ correlation, $k_{\text{MG\rightarrow MGOH}}$ vs. Solvent Volume: -0.991** -0.293

* No statistical difference in 0 and 24 h FLU values under 10% DMSO additive.
**: 10% Dimethyl sulfoxide is excluded in the correlation due to its unavailable kinetic constant.
**Figures**

**A**

Malachite green aptamer (MGA)
38 nucleotides PubMed ID: IQBN
(Not fluorescent)

Malachite green (MG) (Fluorescent)
Malachite green carbinol base (MGOH) (Not fluorescent)

\[
K_{eq} = \frac{k_{MG\rightarrow MGOH}}{k_{MGOH\rightarrow MG}}
\]

**B**

\[
K_d = \frac{[\text{MGA}][\text{MG}]}{[\text{MGA-MG}]}
\]

**Fig. 1.** A: Structure of malachite green aptamer (MGA), malachite green (MG) and malachite green carbinol base (MGOH). B: Binding of MG to MGA, with fluorescence obtained.
Fig. 2. A: $K_d$ determination of MGA to MG in aqueous buffer from 0 to 24 h. B: An illustration of the fitting of $K_d$ ($R^2 > 0.99$). $F_0$ and $F_{\text{max}}$: the fluorescence intensity at zero and infinite aptamer [MGA]; [MG]$_0$ and [MGA]$_0$: initial concentrations of MG and MGA, respectively; $K_d$: dissociation constant. D: Normalized fluorescence decay at the highest MGA concentration/ratio for MG. The fluorescent decrease has a high correlation to first order decay.
Fig. 3. Apparent $K_d$ in organic additives at time 0 h. A: In 3% and 10% ACN. B: In 3% and 10% DMSO. C: In 3% and 10% EtOH. D: Comparison of the apparent $K_d$ values * = $p < 0.05$: significant difference vs. apparent $K_d$ with 0% organic additive. Generally, the apparent $K_d$ increases with the percentage of organic additives, while acetonitrile displayed the largest shifts.
**Fig. 4.** A: $K_d$ determination of MG to MGA with different percentage of ACN at 0 h and 24 h. B: Plots of calculated $K_d$ values vs. %v/v additive at 0 h and 24 h. C: Fluorescence intensity at maximum [MGA]/ratio at 0 h and 24 h with various percentages of organic additives. The decrease of fluorescence intensity and increase of apparent $K_d$ after 24 h becomes smaller and smaller as the percentage of organic additive increases from 3% to 10%. NSD = no statistical difference, $p < 0.05$. 

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<table>
<thead>
<tr>
<th>MGA:MGA</th>
<th>MGA:MGA</th>
</tr>
</thead>
<tbody>
<tr>
<td>![Graph A]</td>
<td>![Graph B]</td>
</tr>
<tr>
<td>![Graph C]</td>
<td>![Graph D]</td>
</tr>
</tbody>
</table>

**Legend:**
- **Graph A**: Determination of $K_d$ for MG to MGA with different percentages of ACN at 0 h and 24 h.
- **Graph B**: Plots of calculated $K_d$ values vs. %v/v additive at 0 h and 24 h.
- **Graph C**: Fluorescence intensity at maximum [MGA]/ratio at 0 h and 24 h with various percentages of organic additives.

*NSD = no statistical difference, $p < 0.05$.***
Fig. 5. HPLC of MG, MGOH and the monitoring of the conversion of MG. A: Detection wavelength = 620 nm. B: Detection wavelength = 265 nm. As time goes on, the MG peak decreases while the MGOH peak increases, which indicates the conversion of MG to MGOH.
Fig. 6. Kinetic evolution between MG and MGOH. A: MG converts to MGOH in binding buffer and with 10% organic additives. B: MGOH converts to MG in binding buffer and with 10% organic additives. The kinetics of MG to MGOH is much larger than MGOH to MG in binding buffer, while organic additives induces a slightly smaller kinetics of the conversion of MG to MGOH, but a much larger kinetics of the conversion of MGOH to MG.
Fig. 7. Addition of DMSO into MGA-MG complex at 5h. Fluorescence of MGA-MG complex decreases significantly at 5h, while the addition of DMSO at 5h recovers the fluorescence. Stronger effect of recovery occurs while higher percentage of DMSO is added.
Fig. 8. A: Equilibrium and intermediates of MG, MG-OH$_2$ and MGOH. B: Rate of MG elimination. At pH 6.7, [OH$^-$] is $5.0 \times 10^{-8}$ M and thus, $k_{OH^-} [OH^-] << k_{H2O}$ [47]. C: Proposed mechanism of the conversions between MG$^+$, MG-OH$_2^+$ and MGOH in the presence of MGA. In the absence of MGA, the rate constants $k_{MG\rightarrow MGOH}$ (hydrolysis) and $k_{MGOH\rightarrow MG}$ (dehydration) favor MGOH as the major equilibrium product (A). While excess MGA is present, it retards MG hydrolysis, which results in a decreased $k_{MG\rightarrow MGOH}$. With the presence of organic additives, $k_{MGOH\rightarrow MG}$ increases by few hundred percent (C), resulting in a “stabilizing” effect.
Electronic Supplementary Information (ESI)

for

Organic additives stabilize RNA aptamer binding of malachite green

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Method for electrophoresis of MGA

MGA in binding buffer (containing 10 mM Mg$^{2+}$) over 48 hours were analyzed by electrophoresis. Samples were stained with AMRESCO EZ-Vision® One (with amaranth indicator) and loaded on 2% agarose gel, and then electrophoresed at 100 V for 40 minutes in pH = 8.0 1X tris-acetate-EDTA (TAE) buffer. BioLabs 100 bp DNA ladder (100-1517 bp) was used as marker for reference. The gel was visualized under 365 nm UV exposure.
Fig. S1. Binding curves (A) and apparent $K_d$ values (B) in different pH at time 0. The result indicates that the suitable pH for the binding experiment is 6-8.5.
**Fig. S2.** UV-vis spectra of conversion between MG and MGOH as a function of time. A: MG converted to MGOH. B: MGOH converted to MG.
Fig. S3. Rate constant of MGOH to MG ($k_{\text{MGOH}\rightarrow\text{MG}}$) in binding buffer in the presence of MGA.
Fig. S4. Fluorescence intensity of MGA-MG complex (32:1) at 24 h binding and with the subsequent addition of original amount of MG (blue square) and MGA (red circle).
**Fig. S5.** Agarose gel electrophoresis of MGA in different time points in binding buffer (with Mg$^{2+}$ presented).
Fig. S6. Competitive binding of MGOH with MG to MGA. No significant change in fluorescence intensity of MGA-MG was observed in the presence of 7-fold excess of MGOH (poor solubility of MGOH in water limits the maximum ratio to 8X in our experimental setup).