

Probing metal-dependent G-quadruplexes using the intrinsic fluorescence of DNA

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K⁺ enhanced the intrinsic fluorescence of a series of G-quadruplex DNA, while Pb²⁺ quenched the fluorescence. The metals showed interesting quadruplex binding kinetics with various DNA sequences.

Apart from carrying the genetic information of living organisms, deoxyribonucleic acid (DNA) has been found to selectively bind to specific molecules (aptamers),^{1,2} catalyze chemical reactions (DNAzymes),³⁻⁶ and even form sophisticated nanostructures based on hybridization.^{7,8} DNAs are often implemented in biosensors, where fluorophores are covalently labelled at either the ends or internally.⁹⁻¹¹ Alternatively, DNA staining dyes such as SYBR Green I, thiazole orange (TO) and thioflavin T (ThT) are added to probe target analyte induced conformational change of DNA.¹²⁻¹⁴

Recently, Gustavsson and Markovitsi shed light on the weak intrinsic fluorescence of DNA, detailing many studies characterizing its emission as short-lived and sequence dependent.¹⁵ For example, poly-adenine DNAs emit at ca. 360 nm,¹⁶ while poly-guanine sequences emit in the 350-420 nm region depending on secondary structures (e.g. the formation of a G-quadruplex).¹⁷ Indeed, even among duplex DNA, emission maxima have been shown to vary widely depending on its base constituents.¹⁸ In some reports, DNA was excited at wavelength near its absorption maximum (260 nm), and emission was observed between 300-350 nm. In other reports, a longer wavelength emission was probed with excitation at 365 nm and emission at ca. 420 nm.^{16,19} On the other hand, fluorescence in the red visible region has been observed in 20-mer strands of all compositions, which was applied for biological imaging.²⁰ Due to the weak nature of this fluorescence, studies have mainly focused on characterizing its origins rather than exploring biochemical or analytical applications.^{21,22}

G-quadruplex (G4) DNA are an important type of DNA secondary structure that has been shown to be related to the development of many diseases such as cancer,²³ amyotrophic lateral sclerosis (ALS),²⁴ as well as bacterial and viral pathogenesis.²⁵ In addition, G4 is also a commonly used motif in nanotechnology and biosensors.²⁶⁻²⁸ Metal ions, such as K⁺, are often required to stabilize G4 structures. The study of G4 structures has mainly relied on circular dichroism (CD) spectroscopy or using dyes such as thioflavin T (ThT).¹² CD is not

available in many labs, while ThT can stabilize certain G4 structures by itself. The intrinsic fluorescence of G4 DNA is a label-free method of monitoring G4 formation.^{29,30} In this work, we communicate the application of the intrinsic fluorescence of G4 DNA for studying the kinetics and affinity of metal binding.

We first measured the steady-state spectra of various G4 DNA (Figure 1A). All exhibited weak fluorescence emission with peaks between 380-400 nm with excitation ranged from 285 nm to 300 nm (Figure 1B).^{17,21} As a comparison, we also measured the fluorescence of A₃₀, which had a stronger emission at 380 nm (excitation at 300 nm). To confirm this DNA emission was not due to impurities, we purified A₃₀ using a Sep-Pak C₁₈ column and observed similar fluorescence post-purification (Figure S1A), as well as UV/Vis spectra (Figure S1B). The sharp peaks between 300 nm and 340 nm represent the water Raman scattering peak, which is quite prominent in samples of weak fluorescence (as seen here). By adding 15 mM K⁺ to the PW17 DNA, a 9-fold increase in fluorescence was observed (Figure 1C). However, when we added Pb²⁺ (another metal that can stabilize a G4 structure),^{31,32} ~60% quenching was observed (Figure 1C).

The large fluorescence enhancement allowed us to perform biochemical assays. We then studied the kinetics of the fluorescence change after adding 15 mM K⁺ or 5 μ M Pb²⁺ to 5 μ M of various G4 DNA (as well as an A₃₀ control, Figure 2A-E). The fluorescence of the K⁺ aptamer and PS2.M increased by less than 1-fold, while that of T30695 and PW17 increased by about 9-fold. This likely reflected the difference in folding of these sequences by K⁺. The addition of higher concentrations of K⁺ to the K⁺ aptamer and PS2.M increased their fluorescence further (Figure S2). Indeed, other previous reports showed that the sequence composition can play a major role in the intensity of fluorescence.^{17,33,34} The kinetics of the fluorescence increase also varied among sequences; the K⁺ aptamer (first order rate constant, $k = 2.0 \times 10^{-2} \text{ s}^{-1}$) and T30695 ($k = 6.7 \times 10^{-3} \text{ s}^{-1}$) were faster while PW17 ($k = 4.5 \times 10^{-3} \text{ s}^{-1}$) and PS2.M ($k = 1.6 \times 10^{-3} \text{ s}^{-1}$) were slower. To understand the slow kinetics of K⁺-dependent G4 DNAs, we then varied the concentration of DNA (Figure S3). For both PW17 and T30695, their kinetics of fluorescence change was independent of DNA concentration, likely reflecting intramolecular folding instead of intermolecular complex formation (e.g., dimers).

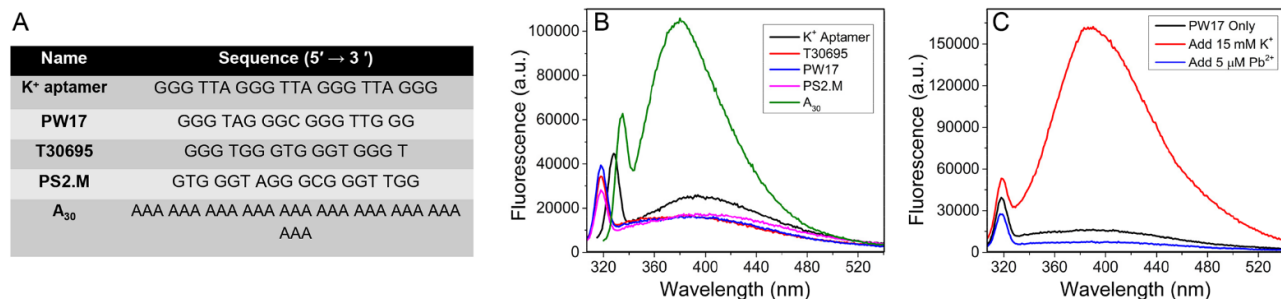


Figure 1. (A) The sequences of the G4 DNAs studied in this work. (B) Fluorescence spectra of various G4 DNA, as well as A₃₀ in water. For PW17, PS2.M and T30695, λ_{exc} = 290 nm. For the K⁺ aptamer and A₃₀, λ_{exc} was 295 and 300 nm, respectively. (C) Fluorescence spectra of PW17 DNA without and with K⁺ or Pb²⁺ in water.

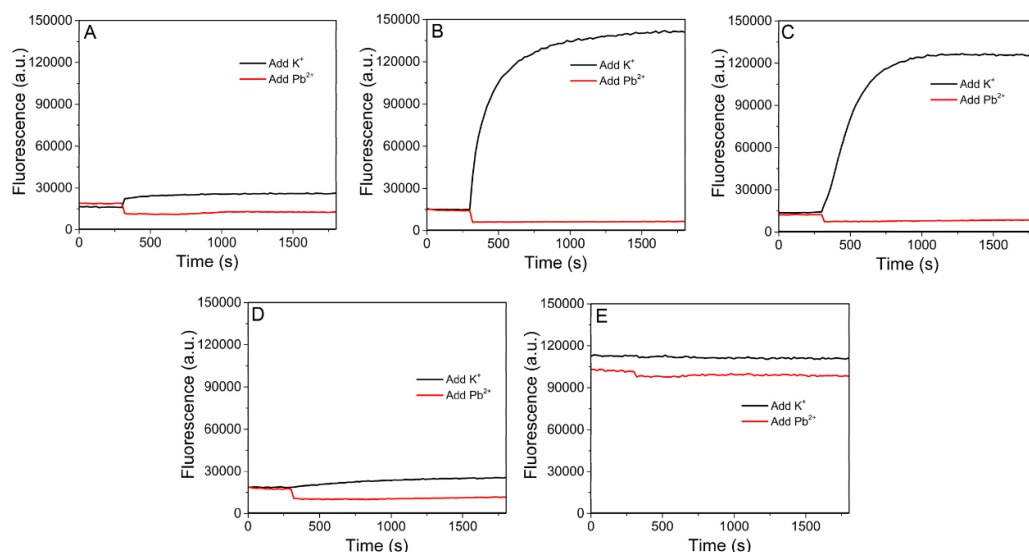


Figure 2. Effect of 15 mM K⁺ and 5 μ M Pb²⁺ on the kinetics of the intrinsic fluorescence of 5 μ M (A) K⁺ aptamer, (B) T30695, (C) PW17, (D) PS2.M and (E) A₃₀. All measurements performed in water without buffer. Excitation and emission wavelengths varied slightly among sequences but generally were between 285–300 nm (excitation) and 380–400 nm (emission).

When we added Pb²⁺, however, the fluorescence was quenched for all the G4 DNAs, which could be related to the heavy metal nature of Pb²⁺. The magnitude of this decrease was also sequence dependent: T30695 was quenched more than PW17, PS2.M and the K⁺ aptamer. Interestingly, the kinetics of quenching was very fast (almost instantaneous) compared to the slow enhancement provided by K⁺ and this was attributed to the higher affinity of Pb²⁺ to DNA. We also performed a similar experiment with a control sequence (A₃₀). In this case, the addition of K⁺ had no effect on the emission, whereas Pb²⁺ slightly quenched its fluorescence (less than quenching of the G4 DNAs). The quenching of A₃₀ was attributed to Pb²⁺ being a relatively soft Lewis acid that can have favourable interactions with all DNA bases (not just G).³⁵

For comparison, we also performed the above experiments with ThT, acquiring both spectra (Figure S4) and kinetic data (Figure S5) in the presence and absence of K⁺ or Pb²⁺. The ThT/G4 fluorescence was quenched by 20–50% with either K⁺ or Pb²⁺. In addition, the kinetic responses were more complicated

in the ThT systems.³⁶ Therefore, the intrinsic fluorescence of DNA can better distinguish between K⁺ and Pb²⁺.

To further explore the contrast between K⁺ and Pb²⁺, we varied the concentration of these metal ions and measured the fluorescence of T30695. We chose this DNA as it had the quickest and largest K⁺/Pb²⁺ responses of all the tested sequences. Stoichiometrically, it has been observed that 2 K⁺ ions or one Pb²⁺ ion is required to stabilize T30695 (Figure 3A).³⁷ With increasing K⁺ concentrations, it was seen that fluorescence increased linearly until ~15 mM (Figure 3B). This confirmed that 15 mM K⁺ was enough to fold 5 μ M T30695. The data was fitted to the Hill equation with two cooperative binding sites, yielding a K_D of 6.8 mM K⁺. We also titrated Li⁺, Na⁺ and Cs⁺. Only with Na⁺ was any enhancement observed; Li⁺ and Cs⁺ caused slight (~20%) quenching. We also fit this data to the Hill equation and acquired a K_D of 10.3 mM, roughly 1.5 $\times$ that of K⁺. Na⁺ is known stabilizer of a G-quadruplex but is less effectively than K⁺ in most cases,³⁸ which is reflected in our data. We also measured the kinetics of fluorescence increase at varying incremental

concentrations of monovalent cations (Figure 3C), showing general saturation of the fluorescence within 5 min at each stage. Overall, this DNA has showed selectivity for K^+ .

Alkaline earth metals can also stabilize G4 structures.³⁹ To explore this with DNA fluorescence, we performed kinetic experiments with T30695 DNA (Figure 3D). Sr^{2+} had the quickest response, and it saturated at the same fluorescence as Ba^{2+} (around a 5-fold enhancement). This is consistent with reports of Sr^{2+} having the best G4 stabilization of the alkaline earth metals.⁴⁰ Ca^{2+} binding was slower and saturated at half the fluorescence of Sr^{2+}/Ba^{2+} , while Mg^{2+} had only a very weak fluorescence increase. In addition, structures stabilized by Sr^{2+}/Ba^{2+} only required a low metal concentration (100 μM), which was added at 300 s. When we added 2 mM of the metals at 1100 s, very little change was observed for Sr^{2+} , with only a small response seen for Ba^{2+} . Therefore, 100 μM was enough to stabilize the G4 structure for these larger metal ions.

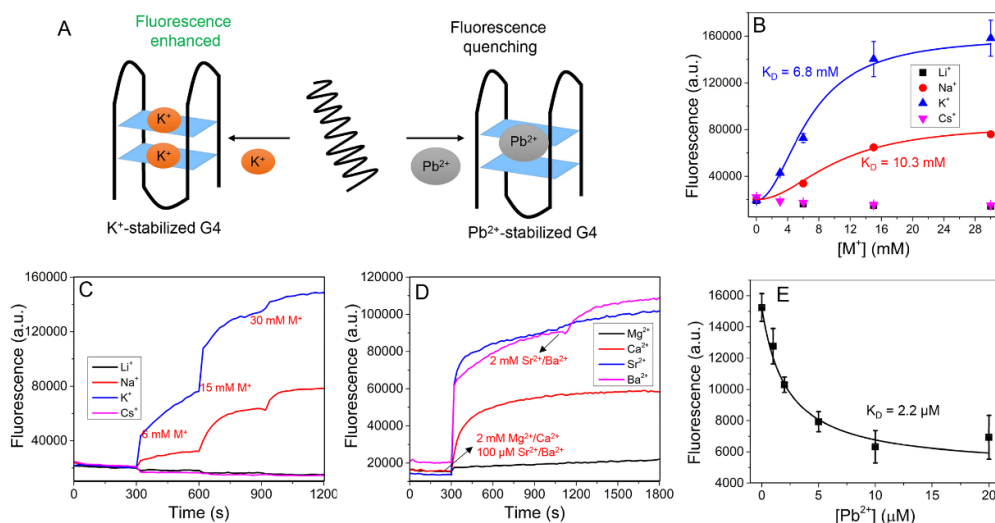


Figure 3. (A) Scheme showing the stoichiometric difference in metal binding for K^+ or Pb^{2+} stabilized G4 DNA. (B) Effect of varying concentrations of monovalent cations on the fluorescence of T30695 DNA in water. Na^+ and K^+ data are fitted using the Hill equation with 2 binding sites. (C) Addition kinetics of various concentrations of monovalent cations to 5 μM T30695 DNA in water. Ions were added every 300 s. (D) Addition kinetics of various alkaline earth metals to 5 μM T30695 DNA in water. For Mg^{2+} and Ca^{2+} , 2 mM was added at 300 s. For Sr^{2+} and Ba^{2+} , 100 μM was added at 300 s, followed by a 2 mM addition at 1100 s. (E) Effect of varying concentration of Pb^{2+} on the fluorescence of T30695 DNA in water.

The different fluorescence responses to K^+ and Pb^{2+} can be used to study their displacement. We first pre-incubated T30695 with different concentrations of Pb^{2+} . Then, the fluorescence was measured for 5 min to confirm a stable background, followed by the addition of 15 mM K^+ (Figure 4A). A significant fluorescence increase was noted in all samples, indicating that K^+ could displace Pb^{2+} from the G4 structure. However, the magnitude of the increase was smaller at higher Pb^{2+} concentrations. When we performed the opposite experiment (i.e., pre-incubation with varying K^+ concentrations, followed by the addition of Pb^{2+}), we observed quenching in all samples (Figure 4B). Interestingly, this quenching was not straightforward; at higher K^+ concentrations, variations in fluorescence was observed in the short time after mixing with Pb^{2+} , which eventually stabilized within ~20 min. This likely reflected complicated internal dynamics within the G-quadruplex before an equilibrium was reached. Therefore, monitoring the kinetics of fluorescence increase or decrease can provide information about the relative affinities of these metal ions to the stabilization of a G4 DNA.

For Pb^{2+} , quenching plateaued at 10 μM (Figure 3E). This data was fitted with the equation for one Pb^{2+} binding site, giving a K_D of 2.2 μM . To confirm that this decrease was specific to Pb^{2+} , other metal ions (Fe^{3+} , Zn^{2+} , Mn^{2+} , and Cu^{2+}) were also tested at similar concentrations (Figure S6). Only Cu^{2+} induced some fluorescence quenching, though the magnitude was less than that of Pb^{2+} . This is reasonable as, of the metals tested, Cu^{2+} is a paramagnetic and the “softest” metal, which would most likely interact favourably with the bases of G4 DNA.⁴¹ While Pb^{2+} showed the highest fluorescence quenching, this DNA is unlikely to be useful for the detection of Pb^{2+} due to the limited sensitivity (in drinking water, Pb^{2+} needs to be detected down to ~70 nM).⁴² We used 5 μM DNA, which was low enough to probe K^+ at mM concentrations but too high to measure Pb^{2+} at nM concentrations.

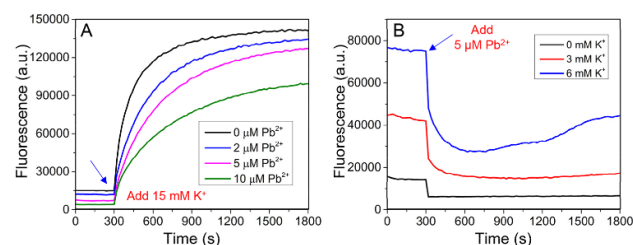


Figure 4. Exploring the competitive nature of K^+ and Pb^{2+} in T30695. (A) Fluorescence of 5 μM T30695 with varying concentrations of Pb^{2+} , followed by the addition of 15 mM K^+ at 300 s. (B) Fluorescence 5 μM T30695 with varying K^+ concentrations, followed by the addition of 5 μM Pb^{2+} at 300 s. All experiments were performed in Milli-Q water.

In this work, we demonstrated that the intrinsic fluorescence of DNA can be used to monitor metal-dependent folding of G4 DNA and acquire useful binding information. There has only been one report utilizing the intrinsic fluorescence of G4 DNA in analytical or biochemical applications.⁴³ We demonstrated here the first work to detail both the kinetics and sensitivity/specificity of metal ion binding to G4 DNA. Compared

to CD, the needed DNA concentration is comparable (1–10 μM).^{44–46} Both are label-free techniques, but the fluorescence technique is more readily available. In addition, the large amount of signal change can be used to study the kinetics of DNA folding and metal ion competition, which is not often seen in CD spectroscopy. CD can provide more detailed structural information such as parallel and anti-parallel G4 structures. We have characterized the binding of K^+ , Na^+ and Pb^{2+} and a few other metal ions using T30695 as a model G4 DNA. Finally, we found that fluorescence could be used to monitor the competition between K^+ and Pb^{2+} for G4 binding sites.

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Conflicts of interest

There are no conflicts of interest to declare

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