Formation and stabilization of G-quadruplex in nanosized water pools†

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We demonstrate here that G-quadruplex structure can form and exhibits strong stability in nanosized water pools, providing new insight into investigating G-quadruplexes in the cellular environment.

Guanine-rich oligonucleotides are able to form four-stranded G-quadruplex structures by associating stacks of G-quartets which are stabilized by Hoogsteen hydrogen bonding.[1,2] Recently, G-quadruplex structures have drawn lots of attention in areas such as biology, medicinal chemistry, supramolecular chemistry and nanotechnology.[1–5] G-quadruplex structures display great conformational diversity due to strand direction difference, loop polymorphism and glycosidic torsion angle variation.[5,6] For human telomeric G-quadruplex (ht-quadruplex), various structures have been reported.[5–8] To know the exact structures of G-quadruplexes under physiological conditions, many researchers have used molecular crowding agent poly(ethylene glycol) (PEG) to mimic the conditions *in vivo* because a cell is crowded with various biomolecules such as proteins, nucleic acids and polysaccharides.[9–16] However, the genomic DNA strands are confined in cell nuclei, and little is known about the structure of G-quadruplexes in cell-sized water droplets until now. We report here a strategy that employs reverse micelles (RM) to mimic the confined environment *in vivo*, and find that the guanine-rich clusters, human telomeric sequence (HTS), can form G-quadruplex structures in the water pools confined in the nanocages formed by RM. Moreover, the G-quadruplex structures also exhibit strong stability in such nanosized water droplets. Cell nuclei and RM can both provide compartmentalization and a confined environment for DNA, although they are of different sizes. Furthermore, the water pools in the RM are considered to be similar to those found at polar/apolar interfaces *in vivo*, whose properties are often quite different from those of normal bulk water.[17–19] The RM forming surfactant we chose is a particular anionic surfactant, Aerosol OT (AOT). The reasons for using AOT are listed below. Firstly, it is a widely-used surfactant for forming RM and the properties of such RM are well characterized in the literature. Secondly, the most important reason is that the size of its water pools are a little larger than the sizes of G-quadruplexes. Therefore, the AOT RM is one of the best candidates for encapsulation of G-quadruplexes and is suitable for simulation of the confinement effect.

Firstly, evidence for HTS encapsulation in the RM was checked by UV absorption spectroscopy (Fig. S1, ESI†). As shown in Fig. S1, the spectrum of HTS within RM was the same as the sum of the individual spectra of HTS in bulk water and that of RM in the absence of HTS. Therefore, the UV measurements clearly imply the presence of HTS in RM. Next, we used circular dichroism (CD) spectroscopy to investigate the conformation of HTS in RM. From Fig. 1A, we observed that the CD spectrum of HTS in bulk water has weak positive and negative peaks around 295 and 265 nm, respectively, indicating that a small quantity of antiparallel ht-quadruplex is formed. The spectrum of HTS in pure water is consistent with the result in buffer without cations reported previously.[15,20] However, when HTS was in water pools confined in RM, both the positive and negative peaks of its CD spectrum were stronger than those in bulk water. Furthermore, the CD spectrum of HTS in RM is similar to that in sodium solution, not like that in potassium solution, suggesting that HTS forms an antiparallel G-quadruplex structure, not a hybrid one in RM.[20]

The formation of G-quadruplex structure in water pools confined in RM was further demonstrated by fluorescence resonance energy transfer (FRET) and thermal difference spectra (TDS). As shown in Fig. 1B, the fluorescence spectrum of fluorophore-labeled HTS in bulk water shows a strong fluorescence peak at 515 nm and a shoulder peak at 580 nm, indicating that a small fraction of HTS forms G-quadruplex structure, but the structure of most HTS is unstructured single strand, which is in line with the above CD results. However, HTS exhibits a different fluorescence spectrum from that in water pools confined in nanocages. Like the results in sodium and potassium solution,[21] we also observed the fluorescence peak at 580 nm in water pools confined in nanocages, which is the characteristic spectrum of FRET. These results further demonstrate that the G-quadruplex structure is formed in water pools confined in nanocages.

Furthermore, we obtained the TDS of HTS by subtracting the low temperature (15 °C) absorption spectrum from the high temperature one (95 °C) (Fig. 1C); it shows a major positive peak at 243 nm, a shoulder peak at 273 nm and a negative peak at 294 nm. This TDS shows the features of antiparallel G-quadruplex and is consistent with the previous results.[22] The result observed is in agreement with the conclusion obtained from CD and FRET spectroscopies. It

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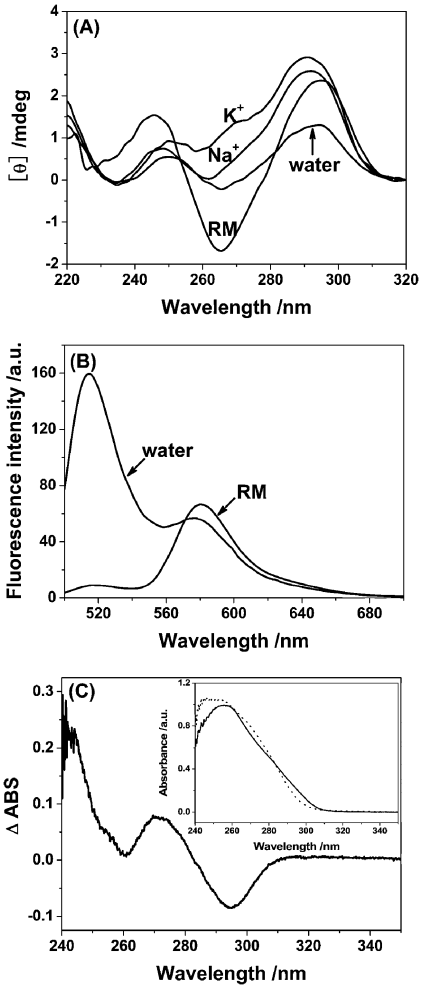

Fig. 1 (A) CD spectra of 4 μM HTS in bulk water, in water pools formed by AOT reverse micelles (w_o = 20) and in 10 mM Tris-HCl buffer (pH 7.4) containing 150 mM K^+ or Na^+; (B) fluorescence spectra of 100 nM HTS in bulk water and in water pools formed by AOT reverse micelles (w_o = 20); (C) thermal difference spectra of HTS in water pools formed by AOT reverse micelles (w_o = 20) resulting from the subtraction of the 15 °C spectrum from the 95 °C one. The inset shows the absorption spectra of HTS at 15 °C (—) and 95 °C (···).

should be noted that the AOT we used here is the sodium salt, although most of the sodium ions are distributed in the micellar periphery due to charge attraction,[19] a small amount of Na^+ will be distributed in the water pools. To eliminate the effect of Na^+, we investigated the properties of HTS in the water pools of RM formed by CTAB (cetyltrimethylammonium bromide). The CD and fluorescence results indicated that HTS can also form G-quadruplex structures in the water pools of RM (Fig. S2, ESI†).

To investigate the stability of G-quadruplexes in the water pools confined in the nanocages of RM, a UV melting method was used. We found that G-quadruplex has a high thermal stability, and its T_m is 81 °C in the water pools confined in nanocages (Fig. 2), whereas its T_m is 35 °C in bulk water (Fig. S3, ESI†). When the temperature is higher than 90 °C, the enhanced absorption may result from the phase transition

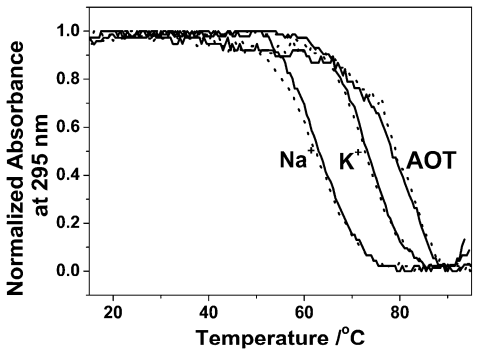

Fig. 2 The melting (—) and annealing (···) curves of HTS at 295 nm in water pools formed by AOT reverse micelles (w_o = 20) and in 10 mM Tris-HCl buffer (pH 7.4) containing 150 mM K^+ or Na^+.

of RM (Fig. S4, ESI†). It is well known that G-quadruplex structures are very stable in the presence of cations, like sodium and potassium. Here the T_m values of HTS in buffer containing 150 mM Na^+ and K^+ are 63 and 72 °C, respectively, which is consistent with a previous report.[23] From these results, we found that the stability of G-quadruplex is much stronger in the nanosized water pools than in bulk buffer containing 150 mM Na^+ and K^+.

To explore why G-quadruplex can form and has such strong stability in water pools confined in the nanocages of RM, we used fluorescein as a molecular probe to investigate the microenvironment of RM. When we placed this probe in RM, compared to its spectrum in bulk water, the absorption maximum is red-shifted and the intensity is increased (Fig. 3). This result indicates that the environment sampled by the dye within RM differs from that of bulk water. Previous studies have demonstrated that this phenomenon results from the lower polarity of the RM environments than that of bulk water.[24,25] It is known that nucleotides have a large number of hydration sites and form a hydration shell by interaction with water molecules.[26] Sugimoto *et al.* have shown that hydration can alter the structure and thermodynamics of diverse DNAs,[11,26] and that dehydration would stabilize G-quadruplexes.[26,27] Furthermore, water molecules forming a hydration shell play fundamental roles in maintaining the structures of nucleic acids.[26] Therefore, it seems that we can attribute the formation and enhanced stability of G-quadruplexes to the effective modification of water properties in micellar interiors. The modification reflects on the absorption difference between fluorescein in bulk water and in water pools confined inside RM. Therefore, we observed the formation and strong stability of ht-quadruplex structure in water pools confined in RM as molecular crowding can induce G-quadruplex formation under cation-deficient conditions[11,15,28,29] and stabilize G-quadruplexes,[14–16,26–28] respectively.

Another important factor to consider is that the RM is a nanocage, so the confinement effect, which is an intuitive description of effects resulting from encapsulation within a limited space, cannot be neglected. We examined the stability of ht-quadruplex in RM with w_o (w_o is the molar ratio of water to AOT) varying from 20 to 30. From the empirical equation of the radius (r_m) of the AOT micelle inner core, r_m (nm) = $0.15 \times w_o + 0.4$, the corresponding radii of the AOT micelle

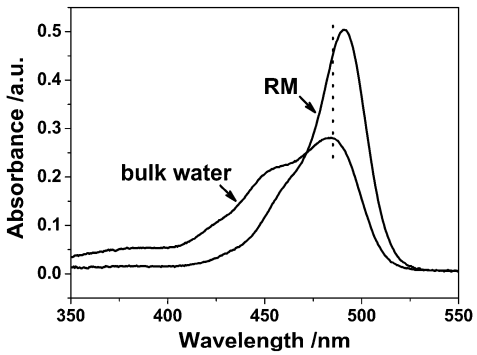

Fig. 3 Absorption spectra of fluorescein in bulk water and in water pools formed by AOT reverse micelles (w_o = 20).

Table 1 The radii of the AOT micelle inner core (r)[a] and melting temperatures (T_m) of G-quadruplexes[b] under different water–surfactant molar ratio (w_o) conditions

w_o	r_m/nm	T_m/°C
20	3.4	81
25	4.1	76
30	4.9	73

[a] The radii were obtained from the empirical equation. [b] The results were drawn from Fig. S5.

inner core are 3.4 to 4.9 nm (Table 1), which are suitable for encapsulation of ht-quadruplex because they are of similar size.[1] We found that the T_m values of ht-quadruplex are 81, 76, and 73 °C in RM with w_o 20, 25 and 30, respectively (Table 1 and Fig. S5, ESI†). Recently, Flynn *et al*. showed that RM are highly effective for probing the influence of confinement, and that RM encapsulation influences the probe molecule in a very similar way to the excluded volume effect induced by molecular crowding.[30] The definition of excluded volume is the space which is excluded from occupation by the target macromolecule due to that space being occupied by other macromolecules. We previously indicated that excluded volume can promote the formation and enhance the stability of ht-quadruplex,[15] thus the confinement we demonstrated here is like the excluded volume effect because both reduce the available volume of target molecules.[30] Therefore, besides the change of water properties, the confinement effect also plays an important role in influencing the formation and stability of ht-quadruplex.

In summary, we show here that guanine-rich oligonucleotides can form G-quadruplex structure in nanosized water pools, and its stability is much higher than that of cation-complexed G-quadruplex in bulk water. To study the features of G-quadruplex structures *in vivo*, molecular crowding co-solutes like PEG are generally used to mimic biomolecules such as proteins, nucleic acids and polysaccharides under physiological conditions.[9–16] However, we should not ignore the fact that genomic DNA strands are confined in cell nuclei. Therefore, in this work, HTS encapsulation in nanosized water pools formed by RM provides new insight into investigating G-quadruplexes in the cellular environment. Inside the cell nuclei, the properties of water molecules are different from those in bulk water. From our results, hydration and confinement, which reflect the conditions *in vivo*, are crucial to the formation and stability of ht-quadruplex in nanosized water pools.

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