PEG-DNAzymes

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Structure Formation and Catalytic Activity of DNA Dissolved in Organic Solvents**

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The concept that structured oligonucleotides should remain folded and potentially active in organic solvents is interesting because their structure and function evolved in an aqueous environment. Nucleic acids that have catalytic activity, without protein assistance, are known as ribozymes or deoxyribozymes (DNAzymes).[1] Their catalytic activity offers great advantages for biotechnology, therapy, or synthetic chemistry.[2] Nucleic acids are also exciting materials for the fabrication of structured molecular devices in the field of nanotechnology.[3] However, they are usually only soluble in water, which restricts their use for some applications. We believe that being able to place them in an extreme environment, such as dissolving them in an organic solvent, could be one way to expand their properties and versatility.

Herein, we report a method to make oligonucleotides soluble in most organic solvents by simply covalently attaching a polyethylene glycol unit (PEG) to the 5′ DNA terminus.[4] The goal was to create a novel structured oligonucleotide that is capable of functioning as a DNA catalyst (i.e. a DNAzyme) in organic solvents. PEG-modified proteins are soluble in most organic solvents, as well as in water, and can maintain their enzymatic activity in organic solvents.[5] This property led us to design and synthesize PEG-conjugated DNA (PEG-DNA) molecules with a biologically important structure and catalytic activity in organic solvents (Figure 1).

Several studies have reported methods to dissolve DNA in organic solvents, though native DNA is intrinsically insoluble in pure organic solvents.[4a,6] Quaternary alkyl ammonium salts, which associate with DNA phosphates, formed DNA complexes that are soluble in many organic solvents.[6a,b] A mixture of DNA and PEG could be dissolved in selected organic solvents.[6c] Nanoparticles formed by DNA and a synthetic cationic copolymer were also soluble and stable in organic solvents.[6d] When the DNA complex is pre-annealed in an aqueous solvent, DNA-templated organic synthesis has been performed in organic solvents.[6e]

To date, various DNAzymes have been discovered.[1b–d,7] However, to our knowledge, no DNAzyme that can work in organic solvents has been reported. In this study, we used a peroxidase DNAzyme, because structured DNA molecules (i.e. G-quadruplexes) are an important component of its activity.[2b,7b,8] Quadruplex DNA, formed from G-rich sequences, plays an important role in biological processes, one

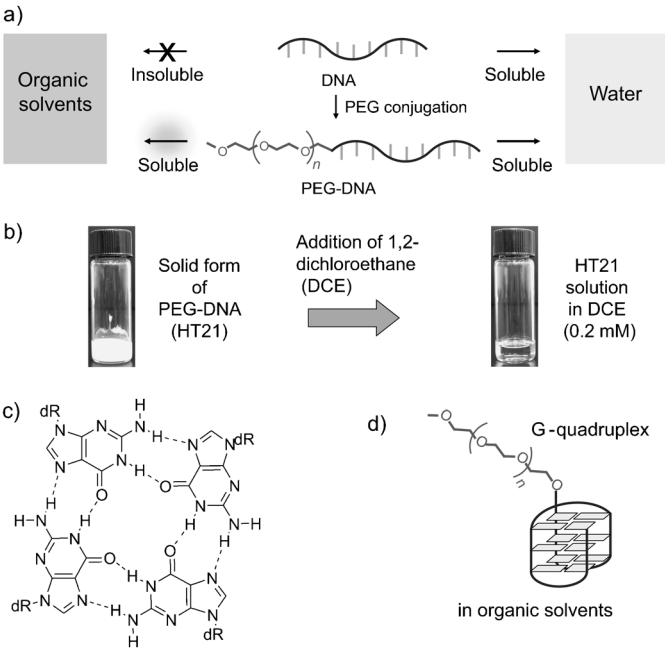

Figure 1. a) PEG conjugation makes DNA soluble in both organic solvents and water. b) PEG-DNA (HT21) is soluble in DCE. c) Chemical formula of a G quartet. d) Schematic model of a G-quadruplex formed by PEG-DNA with a human telomere motif (HT21) in DCE.[13a]

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example is the telomere sequence which forms G-quadruplexes (Figure 1 c,d).[9] Hence, we examined whether a PEG-modified DNAzyme had the required structure and gave catalytic activity in organic solvents.

Herein, we first studied the solubility of PEG-DNA in various organic solvents. Next, the structure of PEG-modified G-rich DNA in organic solvents was analyzed using circular dichroism (CD) measurements.[10] We found that G-rich PEG-DNAs dissolved in organic solvents gave G-quadruplex structures. We also demonstrated that a DNAzyme consisting of PEG-DNA and iron protoporphyrin IX (hemin) had peroxidase-like catalytic activity in an organic solvent.

PEG-DNAs (Table 1) were synthesized by coupling a PEG-*N*-hydroxysuccinimide ester (molecular weight (MW) 10000) with 5′-amino-modified DNAs (Supporting Information, Figure S1). After conjugation with PEG, the DNA was purified using reverse-phase HPLC.[11] In the first step, we performed a solubility test for PEG-DNA using N15, which has a DNA sequence of 15 nucleotides (nt; Table 1). N15 was found to dissolve in various organic solvents, including 1,4-dioxane, 1,2-dichloroethane (DCE), acetonitrile, and methanol, at 0.1 mM, although unmodified DNA was not soluble in these solvents. These solutions were diluted 20-fold with more of the same solvent and the absorption spectra were measured (Figure S2 a). All spectra showed similar intensities, indicating that PEG-DNA N15 dissolved completely in each solvent at that concentration. We also compared the solubility of DNA conjugated to PEG of different molecular weights (MW 2000, 5000, or 10000) in an organic solvent, DCE (Figure S3). We found that longer PEG moieties made DNA more soluble in DCE. Based on this result, we used PEG with a MW of 10000 throughout this study. Next, the CD spectra of two complementary 15 nt PEG-DNAs (N15 and N15-2, Table 1) were measured in DCE (Figure S2 b). They exhibited a smaller Cotton effect compared with that detected in aqueous buffer. This result suggests that single-stranded PEG-DNA forms a less-ordered duplex structure in DCE, probably because of weakened stacking interactions between nucleobases.

By measuring the CD spectra of the conjugate, we tested whether PEG-DNA dissolved in organic solvent gave a G-quadruplex.[10,12] We designed two G-rich PEG-DNAs (termed HT6 and HT21, Table 1) that included one or three repeats of the human telomeric motif (TTAGGG), respectively. Control sequences (CON21 and CON6) were also designed that contained the same number of each of the bases but included randomly rearranged sequences of HT21 or HT6, respectively.

Table 1: PEG-modified DNA sequences[a] used in this study.

Name	Sequence
N15	5′ PEG-d(GTA GCA AGT CAT AGT) 3′
N15-2	5′ PEG-d(ACT ATG ACT TGC TAC) 3′
HT21	5′ PEG-d(GGG TTA GGG TTA GGG TTA GGG) 3′
CON21	5′ PEG-d(GGA GTG TGT GTG AGG TGA GTG) 3′
HT6	5′ PEG-d(TTA GGG) 3′
CON6	5′ PEG-d(GTG TAG) 3′

[a] Molecular weight of PEG = 10000.

First, we tested whether HT21 gave a G-tract-specific structure when dissolved in DCE, since it has been shown to form various G-quadruplex structures in aqueous solution.[12,13] The structure varies according to which species of monovalent cations are included in the solution.[12] Initially, PEG-DNA was annealed in aqueous buffer containing Na^+ or K^+ ions. To measure the CD spectrum of the molecule in DCE, the solution was lyophilized and redissolved in DCE. The resultant CD spectra are shown in Figure 2. In the presence of Na^+ ions, a nearly identical spectrum was obtained for HT21 in DCE as that observed in buffer (Figure 2 a,b); both exhibited a strong positive band at 295 nm and a strong negative band at 265 nm, which are normally interpreted as being characteristic of antiparallel G-quadruplexes.[10,12] In the presence of K^+ ions, a strong positive band was seen at 295 nm in the spectra of HT21 in buffer (Figure 2 d) and in DCE (Figure 2 c), although their shape differed somewhat between 250 and 280 nm.[10,12] In

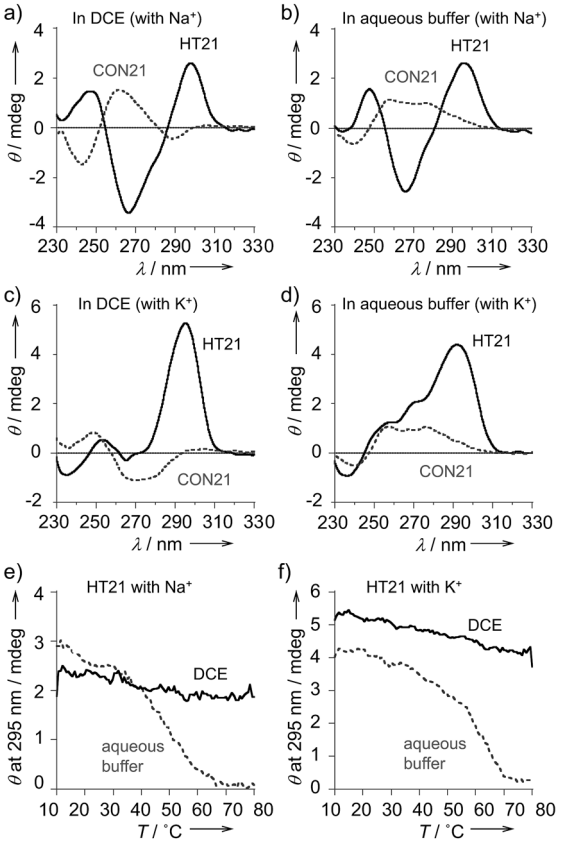

Figure 2. CD spectra of PEG-DNA in DCE. All spectra were recorded at a DNA concentration of 1.4 μM and at 20 °C. a) HT21 (solid line) and CON21 (dashed line) dissolved in DCE, after annealing in buffer-Na (10 mM Na phosphate (pH 7.0) and 100 mM NaCl) and lyophilization. b) HT21 and CON21 in buffer-Na. c) HT21 and CON21 dissolved in DCE after annealing in buffer-K (10 mM K phosphate (pH 7.0), 100 mM KCl) and lyophilization. d) HT21 and CON21 in buffer-K. Thermal denaturation profiles of HT21 in e) DCE (Na^+) (solid line) and buffer-Na (dashed line), and f) DCE (K^+) (solid line) and buffer-K (dashed line).

contrast, the spectra of CON21 did not exhibit these characteristics in solvents with Na^+ or K^+ (Figure 2a–d). These measurements led us to conclude that HT21 forms G-tract-specific structures that seem to be G-quadruplexes based on their CD spectra, although their exact structures have yet to be determined.

Thermodynamically, G-quadruplex structures are more stable in the presence of K^+ ions than in the presence of Na^+ ions.[12,13d] To determine the thermal stability of the G-quadruplex in DCE, the ellipticity at 295 nm was monitored as a function of temperature.[12] As shown in Figure 2e and f, the ellipticity did not decrease greatly in the temperature range of 10–80 °C in the presence of either metal ion in DCE, whereas the Na^+- or K^+-induced structures in aqueous buffer melted at a temperature (T_m) of 50 °C and 59 °C, respectively. These data suggest that the structures in DCE are significantly more stable than those in aqueous buffer.

We also tested whether HT21 was present as G-tract-specific structures in organic solvents other than DCE (in methanol, acetonitrile, and 1,4-dioxane; Figure S4). In all solvents, the spectra of HT21 exhibited positive bands at 295 nm, which could be interpreted as the formation of G-quadruplexes.[10] We also measured the ^{1}H NMR spectrum of HT21 in methanol in the presence of K^+ ions.[13a] We detected proton signals between $\delta = 11.5$ and 12.5 ppm, which could be assigned to the guanine imino protons that are involved in G-tetrad formation (Figure S5).[13a]

We concluded that G-quadruplexes exsisted for the PEG-modified 21 nt sequence (HT21) when dissolved in the organic solvents tested. The shorter PEG-DNA HT6 was then examined for structure formation in organic solvents (Table 1, Figure 3).[14] In aqueous solution, the 6 nt telomeric sequence d(TTAGGG) forms an all-parallel G-quadruplex in the presence of K^+ ions (Figure 3a and Figure S6).[14] In principle, a G-quadruplex could be formed by alignment of four separate strands, by alignment of two hairpins, or unimolecularly.[13c] The question arises as to whether the PEG-DNA HT6 can exsist as an intermolecular G-quadruplex in organic solvents, as the formation of the structure is expected to be entropically more unfavorable than intramolecular structure formation. As shown in Figure 3b, HT6 exhibited different CD spectra in organic solvents compared with those recorded in aqueous buffer, with a relatively wide positive band between 260 and 295 nm.[10,14b] Based on the spectra, the structure found in the organic solvents seems to be a mixture of parallel and antiparallel quadruplexes. In contrast, the CD spectrum of CON6, which contained the control sequence d(GTGTAG), showed no pronounced CD band, either in aqueous buffer or in organic solvents (Figure 3c). This result suggests that HT6 was present as G-quadruplexes in organic solvents as well as in aqueous buffer, although the exact structure has not yet been determined.

Finally, the catalytic activity of PEG-modified DNAs was tested after binding to iron(III) protoporphyrin IX (hemin) in organic solvents. In aqueous solution, it has been shown that the interaction of hemin with a G-quadruplex results in the formation of a DNAzyme that is capable of accelerating an oxidative reaction with peroxidase-like activity (Figure 4a).[2b,7b,8] It can use 3-aminophthalic hydrazide (luminol)

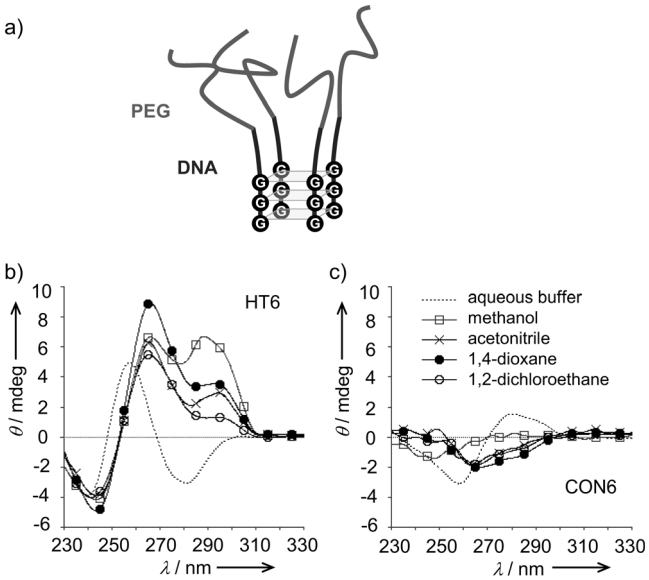

Figure 3. G-quadruplex formation by a hexameric DNA in organic solvents. a) Schematic model of the PEG-DNA G-quadruplex.[14] CD spectra of b) HT6 or c) CON6 in various organic solvents (10 μM DNA, 20 °C). Samples were prepared by annealing the DNA at a concentration of 10 μM in aqueous buffer (50 mM K phosphate (pH 7.0), 300 mM KCl), lyophilizing the sample, and redissolving it in organic solvents (methanol, acetonitrile, 1,4-dioxane, or DCE). The CD spectrum in aqueous buffer is also shown.

as a substrate to generate chemiluminescence in the presence of hydrogen peroxide (Figure 4a).[2b]

In aqueous solution, the G-quadruplex formed by the hexameric sequence d(TTAGGG) forms a complex with hemin, with 1:1 stoichiometry.[15] As a control experiment, we checked the peroxidase activity of the hexameric sequence d(TTAGGG) in aqueous solution (Figure S7). The hexamer was annealed to form a G-quadruplex $[d(TTAGGG)]_4$ in an aqueous buffer containing K^+ ions; subsequently, hemin was added to the solution. The peroxidase activity of the complex was assessed by the addition of luminol and hydrogen peroxide into the mixture of DNA and hemin and by measuring the resulting chemiluminescence. The G-quadruplex-forming sequence d(TTAGGG) exhibited strong peroxidase activity in aqueous buffer with hemin, whereas both the control sequence d(GTGTAG) and the PEG-modified sequence HT6 had no detectable activity (Figure S7). We assume that the binding of hemin to a G-quadruplex formed by HT6 was inhibited by the presence of the PEG moiety. Next, a complex of the PEG-DNA HT6 with hemin was prepared in aqueous buffer, lyophilized, and redissolved in methanol. Methanol was used as the organic solvent because of the limited solubility of luminol in the other organic solvents. The comparison of the peroxidase activity of various DNAs (HT6, CON6, d(TTAGGG), or d(GTGTAG)) in methanol revealed that HT6 exhibited the strongest luminescence (Figure 4b,c). Only weak luminescence was detected in reactions using DNAs other than HT6, probably because of the low solubility of the unmodified DNA d(TTAGGG) or the absence of structure formation for CON6. Integrated photon counting revealed that the complex of HT6 with

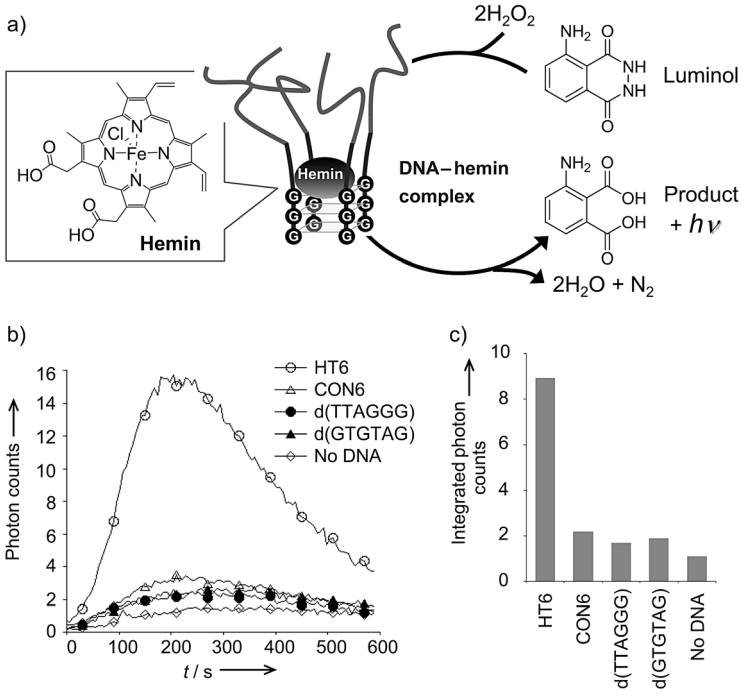

Figure 4. Peroxidase activity of the PEG-DNA–hemin complex in methanol. a) Schematic representation of the peroxidase reaction catalyzed by the PEG-DNA–hemin complex.[15] b) Time course of chemiluminescence, to measure the peroxidase activity of hexameric DNAs with hemin in methanol. The DNAs used were: HT6, CON6, d(TTAGGG), and d(GTGTAG). The composition of the reaction mixture was: 1 μM DNA, 0.25 μM hemin, 1 mM luminol, and 12 mM H_2O_2. c) Integrated photon counts of the chemiluminescence reaction.

hemin was the most efficient catalyst, as it exhibited 4.1- and 8.3-times higher luminescence compared with that of CON6 and no DNA, respectively (Figure 4c). This result demonstrated that the DNAzyme formed by the PEG-DNA–hemin complex exhibits oxidative activity in an organic solvent. It should be noted that the efficiency of the catalyst in methanol and in aqueous solution cannot simply be compared based on chemiluminescent intensity, because the luminescent quantum efficiency is dependent on the solvent (Figure S7b). The chemiluminescent intensity was much higher in aqueous solution than it was in methanol when the same amount of luminol was oxidized in either solvent. The peroxidase activity of HT21 was also measured in methanol. Although HT21 exhibited activity in methanol, the sequence specificity was lower than that seen with the shorter hexamers (Figure S8). Regarding the 21 nt sequences, the peroxidase activity was not G-quadruplex specific in aqueous solution (Figure S8). The broad substrate specificity of porphyrins for the interaction with DNA might explain the results seen for these 21 nt DNAs.[16] G-quadruplex-specific binding was also not seen for these 21 nt DNAs when another porphyrin derivative, 5,10,15,20-tetrakis(*N*-methylpyridinium-4-yl)-21*H*,23*H*-porphyrin (TmPyP4; which is cationic and contains no metal ion) was tested in the interaction (Figure S9, S10).[16a,b,17]

In conclusion, we demonstrated that PEG-DNA is soluble in many types of organic solvents. The force that promotes the molecular interactions that occur in nonpolar organic solvents differs dramatically from that of water. The electrostatic interaction or hydrogen-bonding interaction increases, whereas the hydrophobic interaction or the π–π stacking interaction decreases. These changes in molecular interactions should affect the formation of DNA structures in organic solvents. However, HT21 maintained a similar G-quadruplex structure in DCE as that formed in aqueous buffer, which was assessed using CD measurements (Figure 2). Moreover, the structure was much more stable in DCE than in water under the thermal conditions tested (Figure 2).[18] We infer that the strong electrostatic interaction or hydrogen-bonding interaction of the G-quartet and monovalent metal ions compensated for the weakened hydrophobic interaction and π–π stacking interaction in organic solvents.[13d]

The PEG-modified hexameric DNA (HT6) gave an intermolecular G-quadruplex when dissolved in various organic solvents (Figure 3). Furthermore, this G-quadruplex structure associated with hemin in methanol and formed a DNAzyme that was capable of performing catalytic oxidative reactions in methanol (Figure 4). To our knowledge, this is the first report showing that a nucleic acid enzyme can function in organic solvents. Further investigations should be carried out to confirm the applicability of PEG-modified DNAs as catalysts in organic solvents, by testing systems other than the peroxidase reaction. As PEG-modified oligonucleotides are soluble in both water and organic solvents, a PEG-modified oligonucleotide library for SELEX could be prepared. Although the DNAzyme tested herein was inspired by a known structure that was evolved in an aqueous environment, the SELEX method could allow us to evolve new structured oligonucleotides that will function in organic solvents.

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