DNA-nanoparticle micelles as supramolecular fluorogenic substrates enabling catalytic signal amplification and detection by DNAzyme probes

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General methods

All reagents were purchased from Sigma-Aldrich and used without further purification except DNA synthesis reagents and modifiers purchased from Glen Research and ACZO. Anhydrous toluene and dichloromethane were purified using a Dow-Grubbs two-column purification system (Glasscontour System, Irvine, CA) - Pangborn, A. B.; Giardello, M. A.; Grubbs, R. H.; Rosen, R. K.; Timmers, F. J. Organometallics 1996, 15, 1518-1520. (N-glycine)-5-norborene-exo-2,3-dicarboximide was prepared as described by Biagini et al. (Tetrahedron, 1995, 51, 7247). (IMesh2)(C2H3N2)(C1)2Ru=CHPh was prepared as described by Biagini et al. (Organometallics, 2001, 20, 5314). Polymerizations were performed under dry dinitrogen atmospheres with anhydrous solvents. All DNA except DNAzyme-2 were synthesized on an ABI-391 via standard solid phase synthesis on controlled pore glass supports. Fluorescence measurements were performed with a microplate reader (Perkin Elmer, HTS7000 Plus, λex = 485 nm, λem = 535 nm) using black 96 well plates. Reaction volumes of 100 µL were used in all fluorescence experiments of this type. HPLC purifications of DNA strands were performed on a Clarity 5u Oligo-RP phenosex column (150 x 4.60 mm) with a binary gradient using a Hitachi-Elite LaChrom L-2130 pump equipped with UV-Vis detector (Hitachi- Elite LaChrom L-2420). Gradient: (Solvent A: 50 mM triethylammonium acetate, pH 7.5; Solvent B: 100% methanol; gradient: 10-45% B from 0-28 minutes, 45-60% B from 28-34 minutes, and 60-70% B from 34-40 minutes, Flow rate: 1 ml/min). To confirm DNA molecular weight, MALDI-TOF mass spectrometry was performed on an ABI MALDI Voyager (equipped with Thermolaser Science, VSL-337ND) using THAP matrix (2,4,6-trihydroxyacetonaphone monohydrate) (18 mg), ammonium citrate (7 mg), acetonitrile:water (1 mL, 1:1). Polymer polydispersity and molecular weight were determined by size-exclusion chromatography (Phenomenex Phenogel 5u 10, 1K-75K, 300 x 7.80 mm in series with a Phenomenex Phenogel 5u 10, 10K-1000K, 300 x 7.80 mm (DMF)) or (Jordi Gel DVB 1000A, 500 x 10 mm, (CHCl3)) using a Hitachi-Elite LaChrom L-2130 pump equipped with a multi-angle light scattering detector (DAWN-HELIOS: Wyatt Technology) and a refractive index detector (Hitachi L-2490) normalized to a 30,000 MW polystyrene standard. Particle and DNA concentrations were determined via UV-Vis on a Hitachi U-2810 spectrophotometer. TEM images were acquired on a carbon grid (Ted Pella, INC) with 1% uranyl acetate stain on a FEI Tecnai G2 at 200 KV. 1H (400 MHz) and 13C (100 MHz) NMR spectra were recorded on a Varian Mercury Plus spectrometer. Chemical shifts (δH) are reported in δ (ppm) relative to the CDCl3 residual proton peak (7.27 ppm). Chemical shifts (δ13C) are reported in δ (ppm) relative to the CDCl3 carbon peak (77.00 ppm). Mass spectra were obtained at the UCSD Chemistry and Biochemistry Molecular Mass Spectrometry Facility.

DNA synthesis

Preparation of DNAzyme-1

5'-GGAGAGAGATCCGAGCCGGTCAAGGTTGCGA-3'

A 1 µmol dA-CPG was utilized as the support. The oligonucleotide was synthesized in the standard manner leaving the final base protected with a DMT group. Following cleavage and deprotection with ammonium hydroxide overnight, the oligonucleotide was purified by HPLC (retention time = 26 min), treated with acetic acid, followed by solvent removal and characterization by MALDI-MS. Mass calcd: 10029.6; Mass obs: 10040.7.

Preparation of DNAzyme-2

5'-AACACACACTCCGAGCCGGTCAAGCTTTCTGAT-3'

DNAzyme-2 was purchased from Sigma-Aldrich, and the Mass is 10685.

Preparation of fluorogenic ssDNA substrate (F-ssDNA)

Fluorescein-5'-TCGCACCAGTCTCTCTCC-3'-Dabcyl (rA is the RNA base)

A 1 µmol 3'-Dabcyl -CPG from Glen Research was utilized as the support, with a 5'- Fluorescein phosphoramidite as the terminus. An RNA base (rA) was incorporated as a TOM-protected base. The oligonucleotide was synthesized in the standard manner. Following cleavage and deprotection by ammonium hydroxide/ethanol in 3:1 ratio for 4 hrs, the oligonucleotide was purified by HPLC (retention time = 33 min), and characterized by MALDI-MS. Mass calcd: 6670.7; Mass obs: 6674.5.

Preparation of DNA-1 for particl-1 shown in Fig 1.

NH2-5'-TCGCACCCAGTCTCTCTCC-3'-PEG-Fluorescein

A 1 µmol Fluorescein-CPG was used as the support and rA was incorporated as a TOM-protected base. The oligonucleotide was synthesized in the standard manner, with a 5’-amino group (5’-amino modifier 5, Glen Research) at the terminus. Following cleavage and deprotection by ammonium hydroxide/ethanol in a 3:1 ratio for 4 hrs, the oligonucleotide was purified by HPLC (retention time = 30 min), and characterized by MALDI-MS. Mass calcd: 7086.7, Mass obs: 7100.4.

Preparation of DNA-2 for particle-2 shown in Fig 2C.

NH2-5'-ATCAGAAAGGTrAGGTGTTGTT-PEG-3'-Fluorescein

A 1 µmol 3'- Fluorescein phosphoramidite was utilized as the support and rA was incorporated as a TOM-protected base. The oligonucleotide was synthesized in the standard manner, with a 5’-amino group at the terminus. Following cleavage and deprotection by ammonium hydroxide/ethanol in a 3:1 ratio for 4 hrs, the oligonucleotide was purified by HPLC (retention time = 29 min), and characterized by MALDI-MS. Mass calcd: 8277.5, Mass obs: 8290.1.
Supplementary Information

Preparation of Target DNA (DNA-T)

$5^\prime$-GGAGAGAGATGGGTGCGAGAGCGTCAGTAT$-3^\prime$

A 1 $\mu$mol $3^\prime$-Fluorescein phosphoramidite was utilized as the support. The oligonucleotide was synthesized in the standard manner leaving the final base protected with a DMT group. Following cleavage and deprotection by ammonium hydroxide overnight, the oligonucleotide was purified by HPLC (retention time = 29 min), treated with acetic acid followed by solvent removal and characterization by MALDI-MS. Mass calc: 8973.8; Mass obs: 8988.4.

Preparation of Inhibitor (DNA-Inh)

$5^\prime$-ATACTGACGCTCTCGCACCCATCTCTCTCC$-3^\prime$

To a stirred solution of N-benzylamine (2.85 g, 26.6 mmol) in dry toluene (125 mL) was added 5-norbornene-2,3-dicarboxylic anhydride (4.10 g, 25.0 mmol) and triethylamine (3.83 mL, 27.5 mmol). The reaction was stirred at room temperature and washed with 10% HCl (3 x 50 mL) and brine (2 x 50 mL). The aqueous layers were combined and extracted with EtOAc. The organic layer was precipitated by addition to cold MeOH to give the homopolymer (Block A) as an off white solid. 1H NMR of the polymer confirms the absence of monomer (no olefin peak at 6.30 ppm) and the presence of broad trans and cis olefin peaks of the polymer backbone at 5.73 and 5.50 ppm respectively. SEC-MALS: homopolymer of Block A: $M_w$ = 9814, $M_w/M_n = 1.019$, $t_{n}$ = 18. Copolymer of 1-b-2: $M_w = 15380$, $M_w/M_n = 1.023$, 2 = 18.

Polymer synthesis

Backbone Copolymer (1_{38b-2_{19}}) – Proceeds as shown in Figure 1 below:

To a stirred solution of 1 (0.400 g, 1.58 mmol) in dry CH$_2$Cl$_2$ (2 mL) cooled to -78°C was added a solution of the catalyst ((IMesH$_2$)(C$_5$H$_5$N)$_2$(Cl)$_2$Ru=CHR) (41.0 mg, 0.00564 mmol) in dry CH$_2$Cl$_2$ (1 mL) also cooled to -78°C. After 5 min the cold bath was removed and the reaction was left to stir under nitrogen while warming to room temperature. After 40 min a 0.25 mL aliquot was removed and quenched with ethyl vinyl ether as shown in Fig. 1 below. After 25 min the polymer was precipitated by addition to cold MeOH to give the homopolymer (Block A) as an off white solid. To the remaining reaction mixture a solution of 2 (0.164 g, 0.517 mmol), in dry CH$_2$Cl$_2$ (1 mL) was added. The mixture was left to stir under N$_2$ for 40 min followed by quenching with ethyl vinyl ether (100 mL). After 25 min the solution was concentrated to approx. 1/3 the original volume then precipitated by addition to cold MeOH to give the copolymer as an off white solid. 1H NMR of the copolymer confirms the absence of monomer (no olefin peak at 6.30 ppm) and the presence of broad trans and cis olefin peaks of the polymer backbone at 5.73 and 5.50 ppm respectively. SEC-MALS: homopolymer of 1: $M_w$ = 9814, $M_w/M_n = 1.019$, $t_{n}$ = 18. Copolymer of 1-b-2: $M_w = 15380$, $M_w/M_n = 1.023$, 2 = 18.

Figure 1S. General method utilized in polymerization reactions. For analysis purposes a sample of the first block in the copolymer is quenched prior to addition of the second monomer. This is used to confirm block size and is compared with weight fraction analysis of the copolymer by SEC-MALS.

Supplementary Material (ESI) for Chemical Communications
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DNA conjugation

0.05 µmol of copolymer was dissolved in 0.25 mL of chloroform, followed by addition of 1 equiv. of N,N-Diisopropylethylamine (DIPEA) and 0.2 µmol of HBTU to which 0.1 mL of acetonitrile was added. Freshly synthesized CPG-support-bound-DNA was removed from the capsule and placed in a 1.5 mL eppendorf tube to which was added copolymer mixture described above. This reaction was shaken at room temperature overnight, deprotected in ammonium hydroxide/ethanol (3:1 ratio) for 4 hrs, and reduced in vacuo to approx. 50 µL.

Spherical micelle formation

To the DNA-brush copolymer in 50 µL of water was added 1 mL of Tris buffered water (20 mM, pH 7.4). This solution was then transferred to 10,000 MWCO dialysis tubing. Buffer was changed three times, once per day.

DNAzyme catalysis experiments

Fluorescence was directly measured at time points of 2 min, 5 min, 10 min, and 15 min after adding DNAzyme to F-ssDNA solutions in a 96-well plate. Maximum fluorescence for the DNAzyme (5 nM) added to micellar particles (0.14 g/L) at 15 minutes was set to a value of 1 for normalization of data. Complete consumption of particle bound substrate was confirmed by a calibration curve plotted for fluorescence vs concentration of a fluorescein-modified oligonucleotide (DNA-1 – see above for synthesis and sequence). i.e. plateau in this figure is due to consumption of substrate not product inhibition. Each assay was repeated three times with error bars representing standard deviation for each time point. Fluorescent product from shell DNA cleavage was measured from supernatant that passed through a 10,000 MWCO spin tube filter. i.e. particles are left behind, and fluorescent product comes through for analysis at each time point.

Characterization of cleaved DNA from micellar particles described in Fig. 1B. Main Text.

The flow-through from 10,000 MWCO centrifugation of cleaved DNA fragments post treatment with DNAzyme was collected and concentrated in vacuo to 50 µL. The product was desalted and characterized by MALDI-MS. Mass calcd: 4187.9, Mass obs: 4194.4 (Figure 4S).

DNA conjugation

![Figure 2S. SEC-MALS traces of homopolymer 1$_{38}$ (blue) (MW = 9814) and copolymer 1$_{38-b}$-2$_{18}$ (red) (MW = 15380) (0.5 mL/min CHCl$_3$).](image)

![Figure 3S. SEC-MALS traces of copolymer 1$_{38-b}$-2$_{18}$ (red) (MW = 15380) and DNA-copolymer 1$_{38-b}$-DNA-1 (black) (1.0 mL/min DMF).](image)

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![Figure 4S MALDI-TOF of cleaved DNA fragment from sequence shown in Fig. 1 Main text, treated with DNAzyme.](image)

![Fig. 5S Plot of product concentrations versus time points (A) and standard plot of concentration versus fluorescent intensity (B). Conditions: P-1 and F-ssDNA (1 µM). DNAzyme (5 nM). Buffer: Tris (20 mM, pH 7.4), MgCl$_2$ (50 mM), room temp.](image)
Supplementary Information