

Enzymatic Manipulations of DNA Oligonucleotides on Microgel: Towards Development of DNA-Microgel Bioassays

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General:

All DNA oligonucleotides were synthesized using automated DNA/RNA synthesis (Integrated DNA Technologies and MOBIX lab at McMaster University) following the standard phosphoramidite chemistry, and purified by 10% denaturing PAGE before use. Microgel was synthesized following the previously reported protocol for VAA-PNIPAM 3.3 (please see reference 8b in the main text). The fluorescently labeled DNA oligonucleotide was obtained from Invitrogen and purified by HPLC. EDTA and EDC were purchased from Sigma-Aldrich. Sulfo-NHS was purchased from Pierce. The enzymes used in this study (M-MuLV RT, T4 DNA ligase, Phi29 DNA polymerase and Taq1) were purchased from MBI Fermentas (supplied with relevant reaction buffers). [α -³²P]dGTP and [γ -³²P]ATP were purchased from GE Healthcare. Agarose was obtained from Bioshop (Burlington, Canada). The buffer composition for coupling reaction was 50 mM MES, pH 6.6. Water used in this work is double-distilled. The autoradiogram and fluorescent images of the PAGE gels were obtained using Typhoon and analyzed using ImageQuant software (Molecular Dynamics). Confocal fluorescent images were taken using Axiovert 100M–Zeiss microscope and analyzed by the LSM 510 program.

3'- Radio-labeling of DNA1:

The 5'-amine modified DNA (DNA1) was radio-labeled at the 3' end by primer extension reaction with M-MuLV reverse transcriptase. Typically the reaction was set in 50- μ L volume. 26 μ L of a template DNA oligomer (5'-CCGACTAAGCACCTGT-3') (510 pmol) was mixed with 9 μ L of DNA1 (500 pmol) in water (9 μ L), heated at 90 °C for 1 min and then cooled to room temperature for 10 min. 10 μ L of the 5 \times M-MuLV buffer (supplied by the manufacturer) was added to the reaction mixture followed by 2 μ L of [α -³²P]dGTP and 1 μ L of M-MuLV RT (200 U). The reaction mixture was incubated at 37 °C for 1 h. Then, the reaction was quenched by adding 30 mM EDTA (final concentration) followed by heating at 70 °C for 10 min. After ethanol precipitation, the radio-labeled DNA was purified by 10% denaturing PAGE.

Covalent coupling of DNA1 with Microgel (MG):

0.2 mg of MG was dissolved in 100 μ L of coupling buffer in pre-weighed microcentrifuge tube and kept at room temperature with occasional vortexing for at least 1 h to disperse the colloidal particles. To this suspension of MG, EDC/NHSS (200 mM/50 mM, final concentration, dissolved in the coupling buffer that was freshly prepared just before use) was added, followed by addition of 50 pmol (2 μ L) of 3' radio-labeled DNA1. Total reaction volume was approximately 125 μ L. The reaction mixture was covered with aluminium foil to protect from light and left at room temperature in a shaker over night. An aliquot of this reaction mixture (10 μ L) was analyzed by 10% denaturing PAGE to check the amount of coupled DNA. The quantification of the coupled and uncoupled bands by ImageQuant software indicated that 30% of the radioactive DNA was coupled to the microgel (Fig. 1 A shown below). To the

rest of reaction mixture, 300 μ L of 6 M Urea was added with brief vortexing. After short heating (10 to 20 sec at 90 °C), the MG-DNA1 conjugate was precipitated down by centrifugation at 13000 rpm for 30 min at room temperature. The supernatant was removed carefully with a pipette and the precipitant was re-suspended in 300 μ L of 6 M urea by agitating with a pipette tip and brief vortexing. This washing step was repeated three more times. The reaction mixture was further washed with a washing buffer (20 mM sodium phosphate, 150 mM NaCl, 0.1% Tween 20, pH 7.5) followed by water. In order to know how much microgel can be recovered, we dried the microcentrifuge tube after all the washing steps with the MG-DNA conjugate by heating at 90 °C for few minutes followed by speed vac. The pellete of microgel was calculated by subtracting the weight of the microcentrifuge tube and it was found to be 0.16 mg which is 80% of the initial amount of MG. Finally, this precipitate was re-suspended in 20 μ L of H₂O. 10 μ L of this reaction mixture was mixed with 10 μ L of 2 \times gel loading buffer (80 mM EDTA, pH 8.0 at 25 °C, 16 M urea, 180 mM Tris, 180 mM boric acid, 20% sucrose (w/v), 0.05% xylene cyanol and 0.05% bromophenol blue) and subjected to analysis by 10% denaturing PAGE.

Ligation of RCA-P with MG-DNA by T4-DNA ligase:

MG-DNA1 conjugate was prepared in similar way as described above but with non-radiolabeled DNA1. Then, 20 pmol of DNA2 (Fig. 2A) was 5'-phosphorylated with [γ -³²P]ATP using T4 polynucleotide kinase (PNK) following our previously published protocol.^[1, 2] This radio-labeled primer was dissolved in 20 μ L of H₂O and divided into two portions in two individual microcentrifuge tubes. The first portion was set as a control experiment and the second one as the test. Each portion of radioactive primer was annealed with 20 μ L of MG-DNA1 conjugate and 1 μ L (10 pmol) of the template DNA-T (Fig. 2A) by heating at 90 °C for 1 min followed by cooling at room temperature for 10 min. 5 μ L of 10 \times ligase buffer (400 mM Tris-HCl, 100 mM MgCl₂, 100 mM DTT, 5 mM ATP (pH 7.8 at 25 °C) was added to each of the reaction mixture. To the test sample, 1 μ L of T4 DNA ligase (5 U) was added whereas to the control one 1 μ L of H₂O was added. The volume of both the reactions was adjusted to 50 μ L with H₂O. Each reaction proceeded at room temperature overnight. Similarly as above, an aliquot of the reaction mixture (5 μ L) was analyzed by 10% denaturing PAGE to check efficiency of ligation. The bands of the ligated and unligated were quantified by ImageQuant software similarly as above. Ligation efficiency was found to be more than 60% (Fig. 1B shown below). The rest of the reaction mixture was then washed the same way as described above for covalent coupling. The reaction mixture was dissolved in 20 μ L of H₂O. 10 μ L of each sample was mixed with 10 μ L 2 \times gel loading buffer and analyzed by 10% denaturing PAGE.

Rolling circle amplification (RCA): 50 μ L reaction volume

Non-radioactive MG-DNA1-DNA2 conjugate was prepared as described above. 2 μ L (10 pmol) of circularized RCA-CT (circular template for RCA, its sequence shown in Fig. 2A) was added to 20 μ L MG-DNA1-DNA2 conjugate followed by addition of 5 μ L of 10 \times Phi29 DNA polymerase buffer (330 mM Tris-acetate (pH 7.9 at 37 °C), 100 mM Mg-acetate, 660 mM K-acetate, 1% (v/v) Tween 20, 10 mM DTT) and 10 μ L of H₂O. The mixture was heated at 90 °C for 1 min and cooled to room temperature for 10 min. Then, 10 μ L of dNTPs (2 mM each, the final concentration of each nucleotide was 0.4 mM). 1 μ L of radioactive [α -³²P]dGTP was added to this reaction mixture. Finally, 2 μ L of Phi29 DNA polymerase (10 U) was added and the reaction mixture was incubated at 30 °C for 3 h.

Two control experiments were also conducted similarly as described above. In the first control, DNA2 and MG were used instead of MG-DNA1-DNA2 conjugate. In the second control, DNA2 (but no MG) was used. The RCA products of the test and the first control experiment were washed similarly as described above, whereas the RCA product of the second control experiment was isolated by ethanol

precipitation. All the samples were dissolved in 20 μL of H_2O . 2 μL of each sample was mixed with 20 μL of the 1 \times gel loading dye and analyzed by 10% denaturing PAGE.

Digestion of RCA products by Taq1 polymerase: 20 μL reaction volume

10 μL of each sample from above experiment was taken in three individual microcentrifuge tubes. 2 μL of the 10 \times Taq1 buffer (10 mM Tris-HCl, pH 8.0 at 37 $^\circ\text{C}$, 5 mM MgCl_2 , 100 mM NaCl, 0.1 mg/ml BSA) and 7 μL of H_2O were added to each tube followed by 1 μL of Taq1 (10 U). The reaction mixtures were incubated at 65 $^\circ\text{C}$ for 1 h, stopped by heating at 90 $^\circ\text{C}$ for 5 min, mixed with 20 μL of the 2 \times gel loading buffer, and analyzed by 10% denaturing PAGE.

Analysis of fluorescent signal by probe hybridization

Three MG samples were prepared as follows:

- 1) MG alone (control 1): 2 pmol of fluorescent probe was mixed with MG.
- 2) MG-DNA1-RCA-M (control 2): 20 pmol RCA-M was allowed to ligate to approximately 20 μL of MG-DNA1 conjugate; the amount of DNA1 in MG-DNA1 conjugate was assumed to be 10 pmol according to the coupling efficiency; after ligation and subsequent washing it was redispersed in 50 μL H_2O .
- 3) MG-DNA1-RCA-P (the test sample): 20 pmol of RCA-P was allowed to ligate to the same amount of MG-DNA1 conjugate as the control 2 and after ligation the sample was washed similarly. Because of the use of the same procedure, we assume that the same amount of RCA-M and DNA2 were ligated onto the MG. Subsequently, the test sample was subjected to RCA reaction in 50- μL volume similarly as described earlier.

5- μL aliquots of the control 2 sample and the test sample were taken into individual microcentrifuge tubes (4 aliquots from each sample, Fig. 3B), diluted to 10 μL with PBS (20 mM sodium phosphate, 100 mM NaCl, pH 7.0). Then, 2, 4, 6 and 8 pmol of fluorescent probes were added to the subsequent aliquots respectively, heated to 90 $^\circ\text{C}$ for 1 min and cooled to room temperature for 10 min to allow DNA hybridization. These aliquots were analyzed by 0.6% agarose gel.

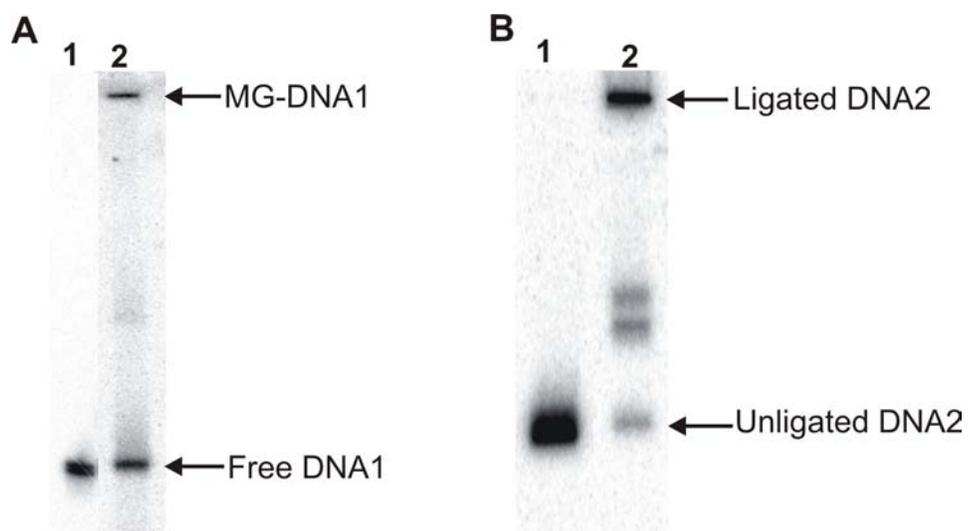
Fluorescent Images analyzed by Confocal Microscopy

Samples from lane 2, 6 and 10 of Fig. 3B were washed with PBS including 0.01% Tween 20 to remove the nonspecifically bound fluorescent probe. Then the fluorescent images of these samples were taken by confocal microscope.

Supporting Table 1.

Physical properties of the microgel (VAA-PNIPAM 3.3 in reference 8b in the main text).

Size at 25 $^\circ\text{C}$	Volume phase transition temperature (VPTT)	-COOH
280 nm	52 $^\circ\text{C}$	0.24 mmol/g



Supporting Figure 1. 10% denaturing PAGE for analysis of the crude reaction mixtures of coupling and ligation, A) Lane 1, DNA1 as marker; lane 2, crude reaction mixture of coupling before wash, B) lane 1, DNA2 as marker; lane 2, crude reaction mixture of ligation before wash.

References:

1. Z. Liu, S. H. Mei, J. D. Brennan and Y. Li, *J Am Chem Soc* 2003, **125**, 7539-7545.
2. a) S. A. Kandadai and Y. Li, *Nucleic Acids Res* 2005, **33**, 7164-7175.