# Enzymatic manipulations of DNA oligonucleotides on microgel: towards development of DNA–microgel bioassays†

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**We demonstrate that DNA oligonucleotides covalently coupled to colloidal microgel can be manipulated by T4 DNA ligase for DNA ligation and by Phi29 DNA polymerase for rolling circle amplification (RCA). We also show that the long single-stranded RCA product can generate intensive fluorescence upon hybridization with complementary fluorescent DNA probe. We believe DNA–microgel conjugates can be explored for the development of DNA based bioassays and biosensors.**

DNA not only serves as a carrier of genetic information in living organisms but has also found important applications in many areas such as disease diagnosis,[1] gene therapy,[2] biosensor,[3] and nanotechnology.[4] In many cases, DNA is combined with suitable polymers or solid supports to achieve its full potential. DNA microarray (or gene chip) is one good example where DNA is immobilized onto a solid support to facilitate simultaneous analysis of all RNA transcripts in a given organism.[5] Therefore, surface immobilization of nucleic acids is one of the most important criteria to consider for developing DNA based bioassays or detection technology.

In recent years, latex colloidal particles with submicron size have been shown to be suitable materials for the surface immobilization of biomolecules such as proteins and DNA, through both physical adsorption and covalent coupling.[6] The large surface area, low dispersity, and versatility of functional groups on the surface make colloidal particles particularly desirable for this purpose. It has been shown that cationic latex particles conjugated to DNA oligonucleotides can be used in the ELOSA (Enzyme Link Oligonucleotide Sorbent Assay) technique to detect nucleic acids with increased sensitivity.[6,7] Among the colloidal particles, poly(*N*-isopropylacrylamide) (poly(NIPAM)) microgels (MGs) are a class of cross-linked colloidal particles possessing interesting physical properties of swelling and shrinking under external stimuli such as temperature, pH, salt concentration and solvents.[8] Because of these attractive properties, MGs have been extensively investigated for use in many biomedical and industrial applications such as ink jet printing, molecular separation, drug delivery and environmental clean-up.[6c,8,9] As an on-going effort of diversifying the application of MGs and developing DNA-MG based bioassays, we set out to investigate whether DNA-MG conjugates were compatible with enzymatic reactions that are commonly used for manipulations of DNA in the design of DNA based bioassays or biosensors.

We chose to examine two such enzymes: T4 DNA ligase and Phi29 DNA polymerase. T4 DNA ligase is one of the most widely used enzymes in molecular biology and biotechnology due to its ability to join, in the presence of ATP, any 5′-phosphorylated DNA strand (called donor DNA) to the 3′-hydroxyl group of a second DNA strand (acceptor DNA) in the presence of a third DNA strand (template DNA) that can align the acceptor DNA next to the donor DNA *via* Watson–Crick duplex structure formation (Fig. 1, step II).

Rolling circle amplification (RCA) is a simple and elegant technique for the generation of long single stranded DNA molecules with tandem repeats under isothermal conditions.[10] In RCA, Phi29 DNA polymerase can synthesize a large number (hundreds or more) of tandem DNA repeats in the presence of a short DNA primer, deoxyribonucleoside 5′-triphosphates (dNTPs) and circular DNA template (Fig. 1, step III). If the 5′ end of the RCA primer is attached to a solid support, the long RCA product will remain with the solid support which can be detected either directly with the use of a fluorescently labeled or radiolabeled dNTP during the RCA reaction or indirectly through the hybridization of a fluorescently labeled complementary oligonucleotide probe. RCA has been explored as a reliable strategy for probe/signal amplification in DNA-based diagnosis.[11] It has been demonstrated that RCA can be successfully accomplished on solid supports to perform multiplex DNA detection.[12] Streptavidin coated magnetic beads and gold nanoparticles have also been

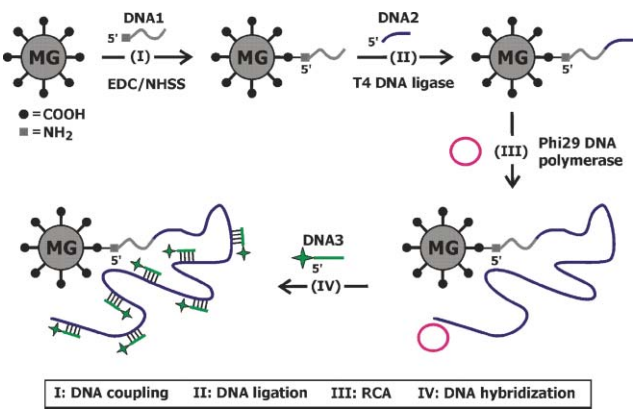


**Fig. 1** Schematic illustration of DNA manipulations on MG examined in this study. (I) Covalent coupling of DNA with MG by EDC/NHSS; (II) DNA ligation; (III) RCA; (IV) Signal generation by hybridization with a fluorescent DNA probe.

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explored for DNA amplification by RCA.[12,13] However, DNA manipulation on bare gold is difficult due to the instability of thiol modified DNA on gold surface in presence of dithiothretol (DTT).[13] Without DTT the efficiency of the polymerase is significantly lower.[14] Furthermore, streptavidin modified nanoparticles are costly. Therefore, development of convenient method for generating and processing signal enhancement by DNA amplification on a suitable support is still highly demanded. To the best of our knowledge, however, RCA reactions (as well as other enzymatic DNA manipulations such as DNA ligation) on water-soluble and stimuli-responsive MGs have never been reported.

Our investigative plan in this study is illustrated in Fig. 1, which includes: (I) covalent coupling of a 5′-amine modified DNA oligonucleotide (denoted DNA1) to carboxylic groups of MG by EDC (EDC: *N*-Ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide) and NHSS (NHSS: *N*-hydroxysulfosuccinimide), (II) ligating the coupled DNA1 with a second DNA oligonucleotide (DNA2) in the presence of T4 DNA ligase and a template oligonucleotide, (III) RCA by Phi29 DNA polymerase, and (IV) hybridization with a fluorescent DNA probe (DNA3).

We synthesized the carboxyl-containing MG following a previously reported protocol for VAA-PNIPAM 3.3[8b] to conjugate a 5′-amine modified DNA1 (its sequence is given in Fig. 2A) using EDC/NHSS. Some important physical properties of the microgel have been depicted in the Electronic Supporting Information (ESI, Table 1†). In order to confirm the covalent conjugation, DNA1 was radiolabeled at the 3′ end by primer extension reaction (see ESI†). Two reactions were conducted to confirm covalent conjugation and removal of the non-specifically bound DNA from MG. In the control sample EDC/NHSS was omitted, and in the test sample EDC/NHSS was added (the detailed description of the coupling reaction is available in the ESI†). After washing, both samples were subjected to 10% denaturing PAGE. The gel image (Fig. 2B) shows that the control sample did not produce any radioactive band in the gel (Fig. 2B, lane 2) indicating complete removal of the non-specifically bound DNA with MG. However, the test sample produced a radioactive band on the top of the gel (Fig. 2B, lane 3). The gel image also reveals that the MG–DNA conjugate did not migrate into the gel (remained in the well) due to large particle sizes. Interestingly, the swelling and shrinking properties of MG gave us a convenient way for washing because slight heating caused the MG–DNA conjugate to precipitate easily by simple centrifugation at room temperature to isolate the conjugate. Importantly, the MG–DNA conjugate can be re-dispersed easily after adding water and agitating with a pipette tip followed by short vortexing. This result proves that DNA does not retain inside the MG during shrinking, indicating that the swelling and shrinking properties of MG did not affect DNA.

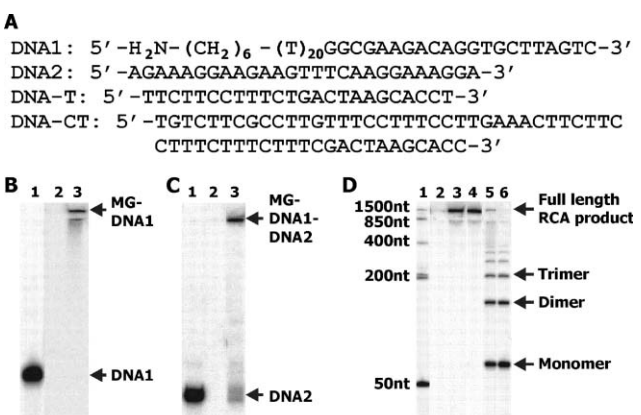


**Fig. 2** (**A**) The sequences of DNA oligonucletides used for DNA ligation and RCA. (**B**) PAGE analysis of the coupling product. Lane **1**, radiolabeled DNA1 only; **2**, DNA1 and MG without EDC/NHSS; **3**, DNA1 and MG with EDC/NHSS. (**C**) PAGE analysis of the ligation mixture. Lane **1**, radiolabeled DNA2 only; **2**, DNA2 and MG–DNA1 (nonradioactive) without T4 DNA ligase; **3**, DNA2 and MG–DNA1 with T4 DNA ligase. (**D**) PAGE analysis of the RCA product. Lane **1**, DNA markers; **2**, RCA with MG and uncoupled DNA2; **3**, RCA in solution; **4**, RCA with MG–DNA1–DNA2; **5**, the RCA product of lane **4** digested by Taq1; **6**, the RCA product of lane **3** digested by Taq1.

The DNA ligation reaction with DNA–MG conjugate was examined next. For this purpose, we coupled the non-radioactive DNA1 to MG as described above. The second oligonucleotide DNA2 (Fig. 2A) was first phosphorylated with $\gamma$-$^{32}$P-ATP and PNK (T4 polynucleotide kinase) and ligated to MG–DNA1 in the presence of DNA–T, (Fig. 2A) and T4 DNA ligase (see ESI† for experimental details). A control experiment was also conducted in which T4 DNA ligase was absent. After washing, the two reaction mixtures were analyzed by 10% denaturing PAGE. The control sample (Fig. 2C, lane 2) did not produce any radioactive band whereas the test sample generated a DNA band on the top of the gel (Fig. 2C, lane 3). These results clearly revealed that T4 DNA ligase was able to perform the ligation reaction with DNA on MG.

We next performed an RCA reaction on MG. We first synthesized the same MG–DNA1 conjugate and ligated the non-radioactive DNA2 to the conjugate following the same procedure as described above. The circular template was then mixed with MG–DNA1–DNA2 (see details in the ESI†). The RCA reaction was initiated by the addition of dNTPs, followed by Phi29 DNA polymerase. In order to analyze the RCA product, we added trace amount of radioactive dGTP ($\alpha$-$^{32}$P-dGTP) in the reaction mixture. Two control reactions were also carried out. The first control was performed with DNA2 in solution mixed with MG (designed to confirm that the non-specifically bound RCA product on MG can be washed away), and the second control was done with DNA2 but without MG (employed to compare the efficiency of the free RCA reaction to that on MG). Upon analysis by 10% denaturing PAGE, we found that the first control did not produce any radioactive band (Fig. 2D, lane 2) indicating that non-covalently bound DNA could indeed be removed from MG by washing. The RCA product either in the solution or on the MG produced similar DNA bands on the top of the gel (Fig. 2D, lanes 3 and 4). For further confirmation, a portion of the RCA product from each sample of lane 3 and lane 4 was subjected to digestion with Taq1 restriction enzyme that cleaves RCA products at the TCGA site. PAGE analysis of these samples produced DNA bands corresponding to monomer, dimer, trimer and so on (Fig. 3, lanes 5 and 6) as previously reported.[13] These results show that Phi29 DNA polymerase was able to perform RCA smoothly on MG.

Since DNA detection by fluorescence is a widely used method, we investigated visualization of the RCA product by hybridization with a fluorescently labelled oligonucleotide (DNA3, Fig. 3A). For this purpose, three MG samples were prepared: (1) MG alone (CS1 or control sample 1); (2) MG–DNA1–RCA-M (CS2) in

 

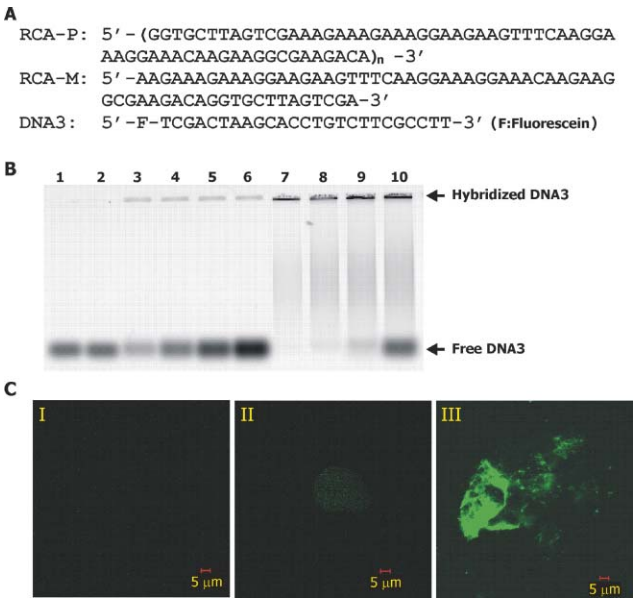


**Fig. 3** (**A**) The sequences of concerned DNA molecules. RCA-P, the RCA product, where *n* is the number of tandem repeating units; RCA-M, RCA monomer; DNA3, the fluorescent probe for hybridization with the RCA product. (**B**) A 0.6% agarose gel image of the RCA product. Lane 1, 2 pmol of DNA3 only; lane 2, 2 pmol of DNA3 mixed with MG; lane 3 to 6, MG-monomer with increasing amount of DNA3 (from 2 pmol to 8 pmol with 2 pmol interval); lane 7 to 10, MG-RCA-P with increasing amount of DNA3 (from 2 pmol to 8 pmol with 2 pmol interval). (**C**) Fluorescent images. I, II, III show the confocal microscopic images of the MG/DNA samples used for lanes 2, 6 and 10, respectively (after washing).

which an oligonucleotide denoted "RCA-M" representing the monomeric RCA unit (see Fig. 3A for its sequence) was ligated to MG–DNA1; (3) MG–DNA1–DNA2–RCA-P (TS or test sample) in which DNA2 was ligated to MG–DNA1, which was subsequently subjected to the RCA reaction as described earlier. It should be noted that equal amounts of MG–DNA1 were ligated with equivalent RCA-M and DNA2 individually, and therefore, it is reasonable to assume that the same amount of RCA-M and DNA2 was loaded on MG.

Fig. 3B shows the results from agarose gel electrophoresis of CS1, CS2 and TS upon hybridization with DNA3. As expected, CS1 did not have the ability to retain DNA (lane 2). When CS2 (which contained $\sim$0.5 pmol of RCA-M) was incubated with 2, 4, 6 and 8 pmol of DNA3, a weakly fluorescent band was observed on the top of the gel (Fig. 3C, lanes 3 to 6, respectively); however, the band intensity did not increase with the increase of DNA3 concentration (comparing the intensities of the top DNA band in lanes 3–6). In contrast, when TS was incubated with increasing amounts of DNA3 (Lanes 7–9, 2–8 pmol), more intensive top DNA bands were observed. Comparing the intensity distribution of the fluorescent bands in lane 6 and lane 10, we estimated that *ca*. 15-fold fluorescence enhancement was produced by TS over CS2. We further analyzed the MG samples of lanes 2, 6 and 10 by confocal fluorescent microscopy after washing away unhybridized DNA3, and the images obtained are depicted in Fig. 3C as I, II and III. The imaging results are consistent with the findings from the agarose gel analysis, confirming the high DNA loading capability of the MG containing the RCA product.

In conclusion we have demonstrated that DNA oligonucleotides can be covalently coupled to microgels and that DNA molecules on microgels can still be manipulated by DNA-processing enzymes such as T4 DNA ligase and phi29 DNA polymerase. We believe that these findings will lay a solid foundation for the development of DNA–microgel based bioassays with high chemical stability, reduced cost, and unique signal amplification capability.

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## Notes and references

1 (*a*) J. Bai, K. Yokoyama, S. Kinuya, K. Shiba, R. Matsushita, M. Nomura, T. Michigishi and N. Tonami, *Eur. J. Nucl. Med. Mol. Imaging*, 2004, **31**, 1523–1529; (*b*) B. Tavitian, *Gut*, 2003, **52**, 40–47.
2 (*a*) Y. Fichou and C. Ferec, *Trends Biotechnol.*, 2006, **24**, 563–570; (*b*) B. Bordier, M. Perala-Heape, G. Degols, B. Lebleu, S. Litvak, L. Sarih-Clottin and C. Helene, *Proc. Natl. Acad. Sci. U. S. A.*, 1995, **92**, 9383–9387; (*c*) L. J. Maher, 3rd, B. Wold and P. B. Dervan, *Antisense Res. Dev.*, 1991, **1**, 277–281; (*d*) J. F. Lee, G. M. Stoval and A. D. Ellington, *Curr. Opin. Chem. Biol.*, 2006, **10**, 282–289; (*e*) G. M. Emilsson and R. R. Breaker, *Cell. Mol. Life Sci.*, 2002, **59**, 596–607.
3 (*a*) E. D. Brody and L. Gold, *Rev. Mol. Biotechnol.*, 2000, **74**, 5–13; (*b*) J. Liu, D. Mazumdar and Y. Lu, *Angew. Chem., Int. Ed.*, 2006, **45**, 7955–7959; (*c*) R. Y. Lai, K. W. Plaxco and A. J. Heeger, *Anal. Chem.*, 2007, **79**, 229–233; (*d*) N. K. Navani and Y. Li, *Curr. Opin. Chem. Biol.*, 2006, **10**, 272–281.
4 (*a*) S. H. Um, J. B. Lee, N. Park, S. Y. Kwon, C. C. Umbach and D. Luo, *Nat. Mater.*, 2006, **5**, 797–801; (*b*) C. Lin, Y. Liu and H. Yan, *Nano Lett.*, 2007, **7**, 507–512; (*c*) Y. He, Y. Chen, H. Liu, A. E. Ribbe and C. Mao, *J. Am. Chem. Soc.*, 2005, **127**, 12202–12203; (*d*) N. C. Seeman, *Annu. Rev. Biophys. Biomol. Struct.*, 1998, **27**, 225–248; (*e*) Y. He, Y. Tian, A. E. Ribbe and C. Mao, *J. Am. Chem. Soc.*, 2006, **128**, 15978–15979.
5 (*a*) C. Sears and S. A. Armstrong, *Adv. Cancer Res.*, 2007, **96**, 51–74; (*b*) A. Dutt and R. Beroukhim, *Curr. Opin. Oncol.*, 2007, **19**, 43–49; (*c*) L. Westin, X. Xu, C. Miller, L. Wang, C. F. Edman and M. Nerenberg, *Nat. Biotechnol.*, 2000, **18**, 199–204.
6 (*a*) T. Basinska, *Macromol. Biosci.*, 2005, **5**, 1145–1168; (*b*) A. Ellaissari, F. Ganachaud and C. Pichot, *Top. Curr. Chem.*, 2003, **227**, 169–193; (*c*) C. Pichot, *Curr. Opin. Colloid Interface Sci.*, 2004, **9**, 213–221.
7 T. Delair, F. Meunier, A. Elaissari, M.-H. Charles and C. Pichot, *Collloids Surf., A*, 1999, **153**, 341–353.
8 (*a*) R. Pelton, *Adv. Colloids Interface Sci.*, 2000, **85**, 1–33; (*b*) T. Hoare and R. Pelton, *Macromolecules*, 2004, **37**, 2544–2550; (*c*) T. Hoare and R. Pelton, *Langmuir*, 2004, **20**, 2123–2133; (*d*) T. Hoare and R. Pelton, *J. Colloid Interface Sci.*, 2006, **303**, 109–116; (*e*) T. Hoare and R. Pelton, *Langmuir*, 2006, **22**, 7342–7350.
9 V. C. Lopez, J. Hadgraft and M. J. Snowden, *Int. J. Pharm.*, 2005, **292**, 137–147.
10 P. M. Lizardi, X. Huang, Z. Zhu, P. Bray-Ward, D. C. Thomas and D. C. Ward, *Nat. Biotechnol.*, 1998, **19**, 225–232.
11 (*a*) M. Nilsson, F. Dahl, C. Larsson, M. Gullberg and J. Stengerg, *Trends Biotechnol.*, 2006, **24**, 83–88; (*b*) V. V. Demidov, *Expert Rev. Mol. Diagn.*, 2002, **2**, 89–95; (*c*) Z. Cheglakov, Y. Weizmann, B. Basnar and I. Willner, *Org. Biomol. Chem.*, 2007, **5**, 223–225; (*d*) E. J. Cho, L. Yang, M. Levy and A. D. Ellington, *J. Am. Chem. Soc.*, 2005, **127**, 2022–2023; (*e*) Y. Tian, Y. He and C. Mao, *ChemBioChem*, 2006, **7**, 1862–1864.
12 A. Hatch, T. Sano, J. Misasi and C. L. Smith, *Gen. Anal. Biomol. Eng.*, 1999, **15**, 35–40.
13 (*a*) W. Zhao, Y. Gao, S. A. Kandadai, M. A. Brook and Y. Li, *Angew. Chem., Int. Ed.*, 2006, **45**, 2409–2413; (*b*) W. Zhao, Y. Gao, M. A. Brook and Y. Li, *Chem. Commun.*, 2006, 3582–3584.
14 A. G. Kanaras, Z. Wang, A. D. Bates, R. Cosstick and M. Brust, *Angew. Chem., Int. Ed.*, 2003, **42**, 191–194.