Conjugated polyelectrolyte–DNA complexes for multi-color and one-tube SNP genotyping assays†

Xinrui Duan,[a] Shu Wang*[a] and Zhengping Li*[b]

Received (in Maryland, MD, USA) 8th November 2007, Accepted 26th December 2007
First published as an Advance Article on the web 23rd January 2008
DOI: 10.1039/b717300h

The complexes of a cationic conjugated polymer with DNA are designed as new platforms for homogeneous, sensitive and facile fluorescence assays for SNP genotyping, which interface with single-base extension, multi-step FRET and optical amplification properties of conjugated polymers.

In recent years, it has attracted much attention to use conjugated polymers (CPs) as optical transducers in highly sensitive biosensors. In comparison to typical fluorescent dyes, CPs contain a large number of repeated absorbing units, and the transfer of excitation energy along the whole backbone of the CPs to an acceptor results in the amplification of fluorescence signals by a collective optical response.[1–7] Water-soluble cationic conjugated polymers (CCPs) can form rather stable polyelectrolyte complexes with negatively charged DNA by electrostatic interactions, thus they are of particular interest for designing highly sensitive DNA biosensors.[8–16] Within the CCP–DNA complex, the fluorescence resonance energy transfer (FRET) from the CCP to the energy acceptor (fluorescein) labeled at the terminus of the DNA is used to transduce the events of DNA hybridization,[9–13] cleavage, extension and conformation transitions.[17–20] Here we present a new design of complexes of CCP with two separate DNAs, respectively labeled at the 5′-terminus with fluorescein and Cy3 to generate an energy transfer cascade. FRET from the CCP simultaneously to the labelled fluorescein and Cy3 can be observed and the multi-step FRET, from the CCP to the fluorescein, followed from the fluorescein to the Cy3 (see Fig. S1†), can also occur. Furthermore, the emission intensities of fluorescein and Cy3 using 380 nm excitation in the presence of poly[(9,9-bis(6′-*N*,*N*,*N*-trimethylammonium)hexyl)fluorenylene phenylene] (PFP) are approximately eight and five times larger than that obtained by direct excitation at the absorption maximum of fluorescein (480 nm) and Cy3 (543 nm) in the absence of PFP, respectively, due to the optical amplification by the conjugated polymers (Fig. 1). The increased fluorescein and Cy3 emissions by the FRET impart this assay platform high sensitivity. The energy transfer process regulates the fluorescence intensity of the CCP, fluorescein and Cy3 components, which provide a chance for multi-color bioassays. As demonstrated here, the multi-step FRET can be used for genotyping of single nucleotide polymorphisms (SNPs).

SNPs are the most frequent form of sequence variation in the human genome.[21,22] Genotyping of SNPs will take a deep insight into understanding complex diseases and identifying disease-causing genes. As genomic markers, SNPs can also be used to study genome structure and function, and to clinically diagnose inherited diseases.[23,24] Various technologies for SNP genotyping have been reported,[25–33] however, most of these methods are based on electrophoresis separation or heterogeneous assay formats, which are time- and labor-intensive because of the multiple separation or wash steps. The homogeneous pyrosequencing assay for SNPs contains an enzyme cascade in which more chemical reactions and several additional enzymes are involved during the detection. This makes the assay complex and expensive. Although fluorescence-based homogeneous assays have also been developed,[30–33] such as molecular beacon, TaqMan, and fluorescence polarization assays, they either require expensive dual-labeled special probes or complex detection procedures. Moreover, the homogeneous assays generally have low sensitivities because of their low fluorescence intensities. Therefore, a convenient, sensitive and cost-effective SNP genotyping assay is desirable. To attain this goal, here, we use CCP–DNA complexes combining with a single-base extension reaction for SNP genotyping, thus offering a new assay strategy. Based on the strongly electrostatic interaction of CCP–DNA and the amplification of fluorescence signals resulted from the FRET between CCP

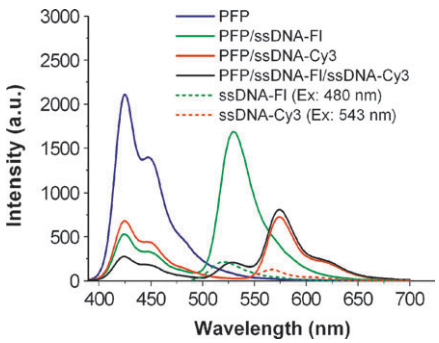


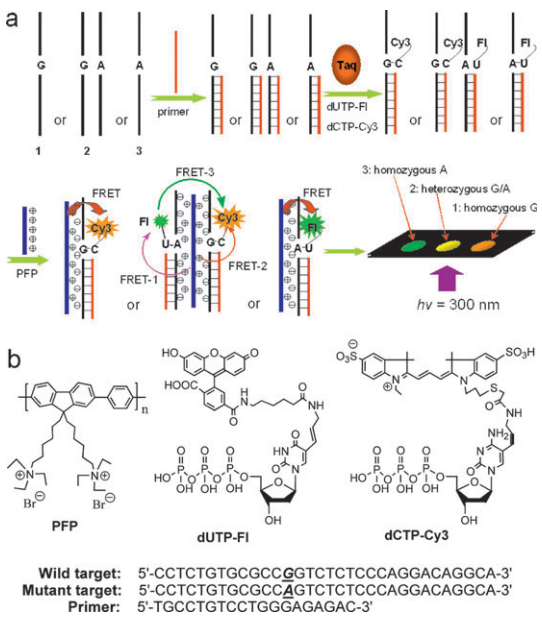
Fig. 1 Emission spectra from solutions containing PFP and dye-labeled DNAs in HEPES buffer solution (25 mM, pH = 8.0). The excitation wavelength is 380 nm. [PFP] = 1.5×10^{-6} M in RUs, [ssDNA-Fl] = [ssDNA-Cy3] = 1.0×10^{-8} M.

[a] *Beijing National Laboratory for Molecular Science, Key Laboratory of Organic Solids, Institute of Chemistry, Chinese Academy of Sciences, Beijing 100080, P. R. China. E-mail: wangshu@iccas.ac.cn; Fax: +86 10 6263 6680; Tel: +86 10 6263 6680*
[b] *College of Chemistry and Environmental Science, Hebei University, Baoding 071002Hebei Province, P. R. China. E-mail: lzpbd@mail.hbu.edu.cn*

† Electronic supplementary information (ESI) available: Fig. S1 and experimental details for SNP genotyping assays. See DOI: 10.1039/b717300h

and fluorescent dyes, the new assay for SNP genotyping can be simply achieved in homogeneous solution with high sensitivity without any requirement for separation steps. More importantly, to perform the single-base extension reactions with multi-color-labeled dNTPs, the multi-step FRET between CCP and the different fluorescence dyes can identify the homozygous and heterozygous genotypes of SNPs within one-tube.

Our new SNP genotyping assay is illustrated in Scheme 1. Water-soluble PFP (see its chemical structure in Scheme 1b)[34] is used as the cationic conjugated polymer in FRET experiments. The fluorescein labeled in dUTP and Cy3 labeled in dCTP are chosen as an acceptor pair. PFP acts as the donor for fluorescein and Cy3, fluorescein acts as the acceptor for PFP and the donor for Cy3 to satisfy the overlap integral requirement for FRET (Fig. S1b†).[35] A sequence on exon 8 of human p53 containing a polymorphic site is used as a DNA target in which the nucleotide G in the wild type is replaced by A in the mutant target. This G → A point mutant alters the coding of an amino acid (Arg → Trp).[36] Thus three kinds of SNP genotypes are possible: homozygous G, heterozygous G/A and homozygous A. The primer is complementary to the wild target and the mutant target immediately upstream of the polymorphic site. Taq DNA polymerase, dUTP-Fl and dCTP-Cy3 (see their chemical structures in Scheme 1b) are used for primer extension reactions. For the homozygous G, only dCTP-Cy3 is incorporated into the primer, and upon adding PFP, the strong electrostatic interactions between negatively charged DNA and cationic PFP bring them close and efficient FRET from PFP to Cy3 occurs. For the homozygous A, only dUTP-Fl is incorporated into the primer, and efficient FRET from PFP to Fl occurs. For the heterozygous G/A, both dUTP-Fl and dCTP-Cy3 are, respectively incorporated into the primers for the mutant target and the wild target. In this



Scheme 1 (a) Schematic representation of the SNP genotyping assays. (b) Chemical structures of dUTP-Fl, dCTP-Cy3 and PFP and DNA sequences used in the assay. Underlined italics in the wild and mutant targets are allele sequences of the SNP site.

case, upon exciting PFP at 380 nm, multi-step FRET (FRET-1, FRET-2 and FRET-3) occurs. By triggering the shift in emission color or the change of emission intensity of fluorescein and Cy3, it is possible to assay the three kinds of SNP genotypes in one extension reaction. Because the ddATP, ddUTP, ddCTP and ddGTP labeled with fluorescein or Cy3 are all available commercially, our new platform could be applied universally to detect any SNP combination (A/T/C/G) by choosing suitable fluorescent ddNTPs. In addition, the application of the current platform in real sample analysis can be easily achieved by using the PCR product of p53 fragment as a template.

Fig. 2 shows the emission spectra and integrated emission intensities of extension products with additions of PFP ([PFP] = 2.5×10^{-7} M in monomer repeat units (RUs)) with the excitation wavelength of 380 nm. The extension products were diluted by 120 times with HEPES buffer solution (25 mM, pH = 8.0) before fluorescence measurements. A no-template control (NTC) was used as the blank. The emission maximum of PFP itself in buffer solution appeared at around 425 nm and no emissions of fluorescein at 530 nm and Cy3 at 574 nm were observed. For homozygous A, the efficient FRET from PFP to fluorescein led to a significant quenching of PFP emission at 425 nm and the appearance of the fluorescein emission at 530 nm. The solution exhibited a green emission color. For homozygous G, the emission of Cy3 was observed and the solution emitted an orange color. For heterozygous G/A, both emissions of fluorescein and Cy3 were observed and the solution demonstrated a yellow-green color emission. The inset in Fig. 2a shows images of extension products with PFP dropped onto a glass slide under 300 nm UV light with transmission mode. A band-pass optical filter was used to filter the emission of the excitation source and PFP. As shown in the inset image, three kinds of genotypes could be clearly distinguished with different colors.

To investigate the dynamic range of the target concentration in our experiments, the emission spectra of a series of extension products containing various concentrations of heterozygous G/A were measured upon adding PFP with the excitation wavelength of 380 nm (Fig. 3a). Fig. 3b shows the fluorescent

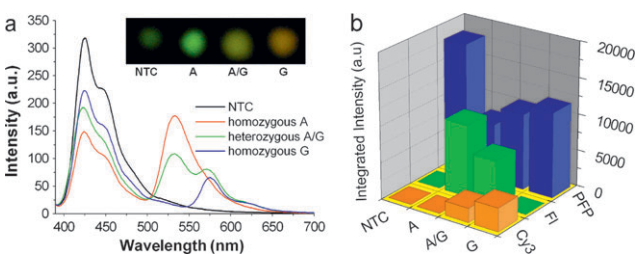


Fig. 2 Emission spectra (a) and integrated emission intensities (b) from solutions containing PFP and extension products of homozygous A, heterozygous G/A and homozygous G. A no-template control (NTC) was used as the blank. The extension solutions were diluted by 120 times with HEPES buffer solution (25 mM, pH = 8.0) before fluorescence measurements. Amounts: 0.42 pmol target DNA, 4.2 pmol primer, 8.4 pmol dUTP-Fl, 8.4 pmol dCTP-Cy3, [PFP] = 2.5×10^{-7} M in RUs. The excitation wavelength is 380 nm. Inset in Fig. 2a shows the images of extension products under a 300 nm UV light with a 550–650 nm band-pass optical filter.

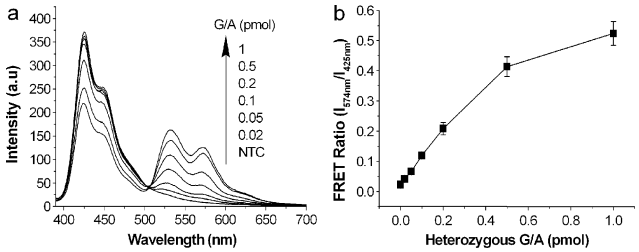


Fig. 3 Emission spectra (a) and FRET ratios (b) from solutions containing PFP and extension products in the presence of varying concentrations of heterozygous G/A. A no-template control (NTC) was used as the blank. The extension reactions were performed in Takara Taq buffer solution (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, pH 8.3). Amounts: heterozygous G/A = 0, 0.02, 0.05, 0.1, 0.2, 0.5 or 1 pmol, primer = 10 pmol, dUTP-Fl = dCTP-Cy3 = 20 pmol. For the fluorescence measurements, 5 μL of extension solution was diluted by 120 times with HEPES buffer solution (25 mM, pH = 8.0) containing PFP ([PFP] = 2.5×10^{-7} M in RUs). The excitation wavelength is 380 nm. Error bars represent the standard deviation of three experiments.

intensity ratio (I_{574nm}/I_{425nm}) as a function of heterozygous G/A concentration. The dynamic range of the target DNA is from 0.02 to 1 pmol. The limit of detection (LOD) of this method is obtained from eqn (1) by seven independent measurements:[37]

$$\mathrm{LOD} = 3 \times \frac{S_0}{S} \qquad (1)$$

where S_0 is the standard deviation of the background and S is the sensitivity. The relative standard deviation for seven measurements of the blank is 5.7%. Accordingly, a limit of detection (LOD) as low as 5.3 fmol was achieved with the proposed method. Thus the method affords high sensitivity for an SNP genotyping assay.

In summary, the complexes of conjugated polymer–DNA can be utilized as probes for homogeneous, sensitive and facile fluorescence assays for SNP genotyping, which interface with the multi-step FRET and optical amplification properties of conjugated polymers. In contrast to previous reports, this method does not require the design of dye-labeled probes, which should significantly reduce the cost (the cost is about 0.7 USD for a SNP site). This method can identify all three kinds of SNP genotypes in one extension reaction, and isolation steps were avoided (the entire protocol takes 1.0 h from start to finish). The detection can be carried out under UV light and no expensive detection instrumentation is needed. Such fluorescence assay systems could be expanded to high-throughput mode. These advantages will make the CCP–DNA system ideal for SNP genotyping and provides a complementary method to commonly used electrophoresis assays.

The authors are grateful for financial support from the National High-Tech R&D Program (No. 2006AA02Z130), the National Natural Science Foundation of China (20725308, 20675023) and the program for New Century Excellent Talents in University (NCET-05-0258).

Notes and references

1 D. T. McQuade, A. E. Pullen and T. M. Swager, *Chem. Rev.*, 2000, **100**, 2537.
2 B. Liu and G. C. Bazan, *Chem. Mater.*, 2004, **16**, 4467.
3 H. A. Ho, A. M. Béra and M. Leclerc, *Chem.–Eur. J.*, 2005, **11**, 1718.
4 S. W. Thomas III, G. D. Joly and T. M. Swager, *Chem. Rev.*, 2007, **107**, 1339.
5 K. E. Achyuthan, T. S. Bergstedt, L. Chen, R. M. Jones, S. Kumaraswamy, S. A. Kushon, K. D. Ley, L. Lu, D. McBranch, H. Mukundan, F. Rininsland, X. Shi, W. Xia and D. G. Whitten, *J. Mater. Chem.*, 2005, **15**, 2648.
6 U. H. F. Bunz, *Chem. Rev.*, 2000, **100**, 1605.
7 M. R. Pinto and K. S. Schanze, *Proc. Natl. Acad. Sci. U. S. A.*, 2004, **101**, 7505.
8 K. P. R. Nilsson and O. Inganäs, *Nat. Mater.*, 2003, **2**, 419.
9 B. S. Gaylord, A. J. Heeger and G. C. Bazan, *Proc. Natl. Acad. Sci. U. S. A.*, 2002, **99**, 10954.
10 H. Xu, H. Wu, F. Huang, S. Song, W. Li, Y. Cao and C. Fan, *Nucleic Acids Res.*, 2005, **33**, e83.
11 S. Wang, B. S. Gaylord and G. C. Bazan, *J. Am. Chem. Soc.*, 2004, **126**, 5446.
12 B. S. Gaylord, A. J. Heeger and G. C. Bazan, *J. Am. Chem. Soc.*, 2003, **125**, 896.
13 B. Liu and G. C. Bazan, *J. Am. Chem. Soc.*, 2004, **126**, 1942.
14 K. Lee, J.-M. Rouillard, T. Pham, E. Gulari and J. Kim, *Angew. Chem., Int. Ed.*, 2007, **46**, 4667.
15 C. J. Yang, M. Pinto, K. Schanze and W. Tan, *Angew. Chem., Int. Ed.*, 2005, **44**, 2572.
16 K. Lee, L. K. Povlich and J. Kim, *Adv. Funct. Mater.*, 2007, **17**, 2580.
17 F. Feng, Y. Tang, F. He, M. Yu, X. Duan, S. Wang, Y. Li and D. Zhu, *Adv. Mater.*, 2007, **19**, 3490.
18 X. Duan, Z. Li, F. He and S. Wang, *J. Am. Chem. Soc.*, 2007, **129**, 4154.
19 F. He, Y. Tang, S. Wang, Y. Li and D. Zhu, *J. Am. Chem. Soc.*, 2005, **127**, 12343.
20 F. He, Y. Tang, M. Yu, F. Feng, L. An, H. Sun, S. Wang, Y. Li, D. Zhu and G. C. Bazan, *J. Am. Chem. Soc.*, 2006, **128**, 6764.
21 International Human Genome Sequencing Consortium, *Nature*, 2001, **409**, 860.
22 J. C. Venter, *et al.*, *Science*, 2001, **291**, 1304.
23 M. K. Halushka, J. B. Fan, K. Bentley, L. Hsie, N. Shen, A. Weder, R. Cooper, R. Lipshutz and A. Chakravarti, *Nat. Genet.*, 1999, **22**, 239.
24 C. Ding, *Trends Biotechnol.*, 2007, **25**, 279.
25 M. M. Shi, *Clin. Chem. (Washington, D. C.)*, 2001, **47**, 164.
26 R. J. Meagher, J. A. Coyne, C. N. Hestekin, T. N. Chiesl, R. D. Haynes, J. Won and A. E. Barron, *Anal. Chem.*, 2007, **79**, 1848.
27 I. K. Litos, P. C. Ioannou, T. K. Christopoulos, J. Traeger-Synodinos and E. Kanavakis, *Anal. Chem.*, 2007, **79**, 395.
28 C. H. Liu, Z. P. Li, A. A. Du, X. R. Duan and Y. C. Wang, *Anal. Chem.*, 2006, **78**, 3738.
29 A. C. Syvänen, *Nat. Rev. Genet.*, 2001, **2**, 930.
30 S. Tyagi, D. P. Bratu and F. R. Kramer, *Nat. Biotechnol.*, 1998, **16**, 49.
31 D. Whitcombe, J. Brownie, H. L. Gillard, D. McKechnie, J. Theaker, C. R. Newton and S. Little, *Clin. Chem. (Washington, D. C.)*, 1998, **44**, 918.
32 X. N. Chen, L. Levine and P. Y. Kwok, *Genome Res.*, 1999, **9**, 492.
33 S. Latif, I. Bauer-Sarsdina, K. Ranade, K. J. Livak and P. Y. Kwok, *Genome Res.*, 2001, **11**, 436.
34 M. Stork, B. S. Gaylord, A. J. Heeger and G. C. Bazan, *Adv. Mater.*, 2002, **14**, 361.
35 J. R. Lakowicz, *Principles of Fluorescence Spectroscopy*, Kluwer Academic/Plenum Publishers, New York, 1999.
36 Z. P. Li, H. Tsunoda, K. Okano, K. Nagai and H. Kambara, *Anal. Chem.*, 2003, **75**, 3345.
37 B. R. Eggins, *Chemical Sensors and Biosensors*, John Wiley & Sons Ltd, West Sussex, England, 2002.

