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Improved single nucleotide polymorphisms detection using conjugated polymer/surfactant system and peptide nucleic acid

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Abstract

An improved assay for the detection of single nucleotide polymorphisms (SNP) of mutant DNA using a combination of peptide nucleic acid (PNA) probes, a cationic conjugated polymer (CCP) and non-ionic surfactant is reported. A comparison between CCP/surfactant and CCP alone shows enhancement in the discrimination between mutant and wild type DNA by a factor of two. A discrimination factor of 70% and 92% was calculated for single and five bases mismatched mutants, respectively when using CCP/surfactant. Furthermore, CCP/surfactant provides a strong emissive donor which increases signal to noise ratio and prevents fluctuation in the output signal caused by the suspension nature of the CCP (due to polymer aggregation) in water. The fluorescence resonance energy transfer (FRET) ratio which defined as the ratio of PL emission of the acceptor to that of the donor, was found to be 20% better when the location of the mutation is five bases away from the duplex terminal compared to that in the centre of the duplex. The enhance discrimination referred to the difference in the FRET and reabsorption rates in different types of duplex. The FRET ratio can be very sensitive to the sample excitation strength, emission collection and spectrometer setting.

Keywords: SNP; Conjugated polymer; Surfactant; PNA; Fluorescence; Quenching

1. Introduction

Recent knowledge in human genome sequencing has revealed that there are great amounts of DNA sequence variation among individuals. Differences in DNA sequence, i.e. polymorphisms, can be single nucleotide changes or changes in the number of copies of a small repeated sequence (mini or micro satellites) (Hartwell et al., 2006). More than 90% of the genetic variability among humans is thought to consist of single nucleotide polymorphisms (SNPs), and efforts are on going to map more than 300,000 SNPs (Collins et al., 1998; Brookes, 1999). Many of the DNA variations seen between individuals are silent, i.e. they have no effect on phenotype. However, specific SNPs have been found to be associated with certain diseases or traits. Example of such SNPs related diseases is the well known point mutation in human β-globin gene that leads to sickle cell anemia (Strittmatter et al., 1993) and Alzheimer's disease (Strittmatter and Roses, 1995). Mutation in the p53 gene has also been implicated in a wide variety of human cancers (Greenblatt et al., 1994). Another important impact of recent identified SNPs is in the drug-resistant mutants of *ABL* portion of the *BCR-ABL* oncogene that reduce drug binding while retaining aberrant kinase activity (Shannon, 2002). To monitor the emergence of drug resistance in patients treated with imatinib (Glivec®) (2-phenylaminopyrimidine derivative), there is a great need to develop an easy, rapid and reliable method for the screening of mutation in the *BCR-ABL* oncogene (Shannon, 2002; Shah et al., 2002; Carter, 2005).

A wide variety of techniques have been proposed for SNP detection, and many of these methods begin with PCR amplification of the gene region to be tested, typically followed by an enzymatic allele discrimination reaction, and then the detection and identification of the reaction products (Landegren et al., 1998; Irving et al., 2004). Irving et al. have successfully detected the mutations in exons 4 and 6 of the *ABL* gene using denaturing HPLC (DHPLC) as a method of screening. Recently, SNP detection schemes based on fluorescence quenching or fluorescence resonance energy transfer (FRET) using conjugated polymers

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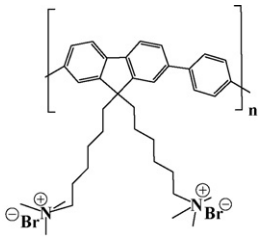
Cationic Conjugated Polymer (CCP)
Poly[6'-bromohexylfluoren-2, 7-diyl)-*alt* – co-(benzene-1, 4-diyl) **1**



Surfactant ($C_{12}E_5$)
$H\text{-}(OCH_2CH_2)_5OCH_2(CH_2)_{10}CH_3$ **2**

PNA reader
Flu-OO-ATG-AAC-TCA-**A**TG-AT -3` **3**

DNA targets
Mut-t, 5`-ATC A**T**T GAG TTC ATG ACC TAC GGG- 3` **4**
Wild-t, 5`-ATC A**C**T GAG TTC ATG ACC TAC GGG- 3` **5**
Non-Compl. 5`-**C**TC A**C**T GA**A** TTC A**CA** ACC TAC GGG- 3` **6**

Mut-m, 5`- TCT ATA TCA TCA **T**TG AGT TCA TGA 3` **7**
Wild-m, 5`- TCT ATA TCA TCA **C**TG AGT TCA TGA 3` **8**

DNA probe
Flu-OO-ATG-AAC-TCA-**A**TG-AT- 3` **9**
Alexa-OO-ATG-AAC-TCA-**A**TG-AT -3` **10**

Fig. 1. Chemical structure of water soluble polyfluorene poly(6′-bromohexylfluoren-2,7-diyl)-*alt*-co-(benzene-1,4-diyl) (CCP) **1**, the non-ionic surfactant *n*-odecylpentaoxyethylene glycol ether ($C_{12}E_5$) **2**, PNA-Flu* **3**, the different targeted ssDNAs **4–8** that corresponds to *BCR-ABL* oncogene (T315I) and ssDNA probes labelled with fluorescien and Alexa Fluor 430, **9** and **10**.

has gain interest because the DNA sequence detection does not require a PCR amplification stage (Chen et al., 1999; Leclere, 1999; Kushon et al., 2002; Gaylord et al., 2002). In our previous work (Al Attar and Monkman, in press), we have improved the initial approach proposed by Gaylord et al. (2002), showing that both sensitivity and selectivity can be enhanced by using non-ionic surfactant which increases the fluorescence quantum yield (FQY) of the conjugated polymer and reduces the hydrophobic interaction between the CCP as a donor and the labelled peptide nucleic acid (PNA) probe as an acceptor. The proposed scheme for DNA detection using conjugated polymer is illustrated by Gaylord et al. (2002). In this scheme the DNA sequence detection is based on optical amplification of a probe signal by light harvesting from a water soluble conjugated polymer via FRET, the FRET efficiency is directly proportional to the FQY of the donor. It is well known that water soluble conjugated polymers are highly aggregated and their FQY is very low in water compared to their counter part non-ionic polymers. This is due to interchain interaction that leads to quenching the fluorescence of the stacked polymer chains. For instant, the FQY of the poly (9,9-bis(2-ethylhexyl)fluorene-2,7-diyl) in chlorobenzene is around 0.8 ± 0.1. However, using similar polymer with a charge group (compound **1** in Fig. 1) the FQY reduces to 0.3 ± 0.1, (Al Attar and Monkman, 2007; Xu et al., 2004). This is mainly due to the strong hydrophobic interaction between the CCP and the strong polar solution (water). We have shown that non-ionic surfactant can break-up this aggregation, promptly leading to an increase in the FQY by a factor of three approaching the quantum yields of normal polyfluorene solution in organic solvent.

In this work we have discussed the effect of non-ionic surfactant on the SNP detection using the conjugated polymer as a donor and a labelled PNA-Flu* as an acceptor in assays containing sequence DNA's that is complementary (SNP), single base mismatched (Wild type) and non-complementary (five-base pair mismatch). The effect of FRET and self-quenching by reabsorption on the SNP detection and identification has been analyzed. The discrimination efficiency (Michelson contrast) for SNP and five bases mismatch has been calculated.

2. Materials and methods

The CCP used was the cationic water-soluble conjugated polymer poly(6′-bromohexylfluoren-2,7-diyl)-*alt*-co-(benzene-1,4-diyl) **1**, molecular weight around $(25.7 \times 10^3$ g/mol), yielding a 35 monomer units (fluorine-phenylene) per polymer chain (Mallavia et al., 2005). The absorption and emission peaks of the CCP are at 380 nm and 420 nm, respectively. The non-ionic *n*-dodecylpentaoxyethylene glycol ether ($C_{12}E_5$) **2** Fig. 1, was purchased from Aldrich and diluted to the required concentration using distilled and deionised water Millipore filtration system, (18 MΩ/□). A 14-base fluorescein labelled PNA probe (reporter) obtained from Applied Biosystem, with a base sequence, Flu-OO-ATG-AAC-TCA-**A**TG-AT (Fig. 1, **3**). The absorption and emission peaks of the fluorescein are at 495 nm and 520 nm, respectively. PNAs are DNA analogs where the four nucleotides (A, T, G and C), are attached to a *N*-(2-aminoethyl)glycine backbone instead of the negatively charged deoxyribose phosphate backbone in DNA (Nielson et al., 1991; Engholm et al., 1992).

Hydrogel Purity electrophoresis (HYPUR®)-purified 24-base mutant DNA sequence (complementary to the PNA), a wild type (single base mismatch to the PNA) and a five-base pair mismatched (partly non-complementary DNA sequences to the PNA-Flu* Fig. 1, **4–8**) were obtained from MWG Biotech. On designing the PNA-Flu* probe the Flu* probe was attached to the 5′ terminal of the PNA leaving the amino acid terminal of the PNA to be equivalent to the 3′ terminal of the DNA. This allows formation of the preferable antiparallel hybridization duplex structure. Two sets of 24-base DNA sequence were used where the mutation location is either four bases away from the 5′ terminal denoted as a terminal (t) or in the middle of the DNA sequence denoted as (m), to evaluate the effect of the mutant position on the FRET strength. The captured PNA probe was designed to be a complementery with the targeted DNA that corresponds to a portion of mutant *ABL*-gene sequence (T315I). Two DNA probes having similar sequence to the PNA probe, one labelled with fluorescein and the other with Alexa Fluor **9** and **10**, were used to study the effect of the absorption and the emission overlap of the acceptor dye on the FRET and the self-quenching by reabsorption. The concentrations of the PNA-Flu* and DNAs were determined by measuring the absorbance at 260 nm in 3 ml quartz cells using a Perkin Elmer Lambda 19 spectrophotometer. Photoluminescence was measured using a Jobin Yvon Fluorolog spectrophotometer. An equi-molar amount (0.1 μM) of PNA-Flu* was mixed with each one of ssDNA **4–8**, and each mixture was annealed at 45 °C for 20 min before being cooled very slowly to allow the hybridization process to occur. Hybridization was accomplished in 10 mM phosphate buffer (pH = 7.4) unless it is stated. CCP untreated with surfactant looks as a suspension in the water due to aggregation. The detection was