

Single Nucleotide Polymorphism Detection Using a Biocompatible, Fluorogenic and DNA-Templated Reaction of Cyanine Dye Formation

Abstract

Water-soluble Peptide Nucleic Acids (PNAs) functionalised with fluorogenic cyanine dye precursors (i.e. a Fisher's base aldehyde and a 2-methylene indolenine) are employed for detecting single-nucleotide polymorphism in vitro under near physiological conditions of salt and pH. This quick and versatile fluorogenic system has been designed so that fluorescence is formed only upon simultaneous hybridisation of both PNAs to a fully complementary DNA strand. The reaction yield increased up to around 10% upon addition of the DNA template, even in the presence of a large excess of Calf-Thymus competitor DNA.

Introduction

Since the final sequencing of the human genome was completed, in 2003, considerable research effort has been devoted to the identification and analysis of variations among individual genomes [1-4]. Although more than 99.9% of human DNA sequences are the same across the population, variations can have a major impact on how humans respond to diseases or drug therapies [5,6]. Amongst the most common forms of genomic variation, single-nucleotide polymorphism (SNP) accounts for over 80% of sequence variants [7-9]. Current estimates suggest that SNPs occur as frequently as every 100 to 300 bases and that they are stably inherited, making them a unique tool/marker for identifying numerous genetic and inherited complex diseases such as cancers and diabetes [7-9]. Therefore, there is an increasing need for high throughput technologies suitable for rapid and sensitive SNP genotyping [10-12]. So far, methods using short oligonucleotides or oligonucleotide mimics as probes to reveal the presence of complementary sequences have been successfully developed [13,14]. Of particular interest are oligonucleotide-templated reactions that can be monitored with high sensitivity by the appearance/disappearance of a fluorescent signal upon binding to the oligonucleotide target [15-17]. Representative examples of such technologies include the use of fluorogenic probes (e.g. molecular beacons [18,19]), or rely on fluorogenic reactions of chemical ligation [20] or primer extension [21]. Most recent reports are based on the Staudinger reaction [22-27] diamine-catalysed aldol condensation [28] organomercury-activated [29] or S_NAr reactions [30]. Among these examples, a small proportion only was successfully applied for the detection of SNPs in vitro. Briefly, two modified oligonucleotides (or oligonucleotide analogues) are designed so that they can hybridize in a sequence-specific manner to a unique nucleic acid template through Watson-Crick base pairing. Upon hybridization of the synthetic probes to the complementary DNA strand, both fluorogenic moieties are brought in close enough proximity to react



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with each other, thus generating the fluorescent product. In case of a partial or uncomplete hybridization of one of the modified oligonucleotides (e.g. as a consequence of a SNP), both probes are no longer positioned favorably to react with each other, thus leading to a significant decrease of reaction efficiency (i.e. a weaker fluorescence). Within such systems, genetic information can be directly linked to the appearance of a characteristic fluorescence signal.

Herein, we report a biocompatible and fluorogenic system that uses two water-soluble PNAs functionalised at their N- or C- terminus with non-fluorescent cyanine dye precursors for SNPs detection. Briefly, two 5-mer PNAs were synthesized on solid support that also contained two ϵ -N,N-dimethyl-Lysines to ensure water-solubility and were functionalised either at their N- or C-terminus with a Fischer's base aldehyde and 2-methylene-indolenine, respectively [31,32]. Upon simultaneous hybridization of both PNAs to a complementary DNA template only, both fluorogenic probes will be orientated optimally to react with each other, thus producing irreversibly a highly fluorescent symmetrical trimethine cyanine dye (Figure 1).

Materials and Methods

General

DNase- and RNase-free water, potassium phosphate buffer and Calf-Thymus DNA were purchased from Sigma-Aldrich. PNA monomers were purchased from ASM Research Chemicals (Germany) and Rink amide resin for solid phase synthesis from Merck Biosciences (UK). DNA oligonucleotides were purchased from Sigma and were all HPLC purified (Table 1). Synthesis and Characterisation (HR-MALDI) of both purified fluorogenic PNAs (**Pna1** and **Pna2**) was recently reported by us [31,32].

Sensing experiments

Stock solutions (50 μ M) of **Pna1**, **Pna2** and DNA were prepared in water. In a typical experiment, 20 μ L of a potassium phosphate buffer solution (100 mM, pH = 7.4) and 10 μ L of each stock solution were transferred into one well to make the final concentrations of

Table 1: Oligonucleotide sequences (given from the 5' to 3' end)^a.

Name	Sequence	Name	Sequence
WT0	<u>GCATCTCGGC</u>	Mut5	<u>GCATCCTTCGGT</u>
WT1	<u>GCATCTTCGGC</u>	Mut6	<u>GCATCCTTCGGC</u>
WT2	<u>GCATCCTTCGGC</u>	Mut7	<u>GCATCCTT<u>X</u>CGGC^b</u>
WT3	<u>GCATCCTTTCGGC</u>	Mut8	<u>GCAT<u>X</u>CCTTTCGGC^b</u>
WT4	<u>GCATCCTTTTCGGC</u>	Mut9	<u>GCATCCTT<u>A</u>AGGC</u>
Mut1	<u>GCATCCT<u>X</u>CGGC^b</u>	Mut10	<u>GCATCCTT<u>AA</u>AGC</u>
Mut2	<u>GCATCCTT<u>T</u>GGC</u>	Mut11	<u>GCAT<u>A</u>CTT<u>AC</u>GGC</u>
Mut3	<u>GCATCCTT<u>C</u>TGC</u>	Mut12	<u>GCA<u>AA</u>CTT<u>A</u>AGGC</u>
Mut4	<u>GCATCCTTC<u>G</u>TC</u>		

^aUnderlined sequences are that complementary to Pna1 and Pna2;
^bX represents A, T, C or G.

10 μM Pna1, 10 μM Pna2, 10 μM DNA and 40 mM buffer. Reaction time-courses were determined in a 384-well plate using a SpectraMax M5 fluorescence plate reader (Molecular devices, UK). Reactions were carried out at 37°C with λ_{exc} = 540 nm and λ_{em} = 573 nm.

Results and Discussion

The ability of Pna1 and Pna2 to react with each other in the absence and in the presence of various DNA sequences used as templates was investigated by fluorescence spectroscopy by monitoring the formation of a symmetrical trimethine cyanine dye (λ_{exc} = 540 nm, λ_{em} = 573 nm). Reactions were carried out in potassium phosphate buffer (40 mM, pH 7.4) at 37°C using a stoichiometric mixture of Pna1, Pna2 and DNA (10 μM each, total volume 50 μL) and the efficiency of the fluorogenic reaction was monitored over three hours in a fluorescence microplate reader. Importantly, no fluorescence was ever detected when incubating Pna1 and Pna2 under such conditions of concentration and pH and in the absence of any DNA template (Figure 3, grey curve).

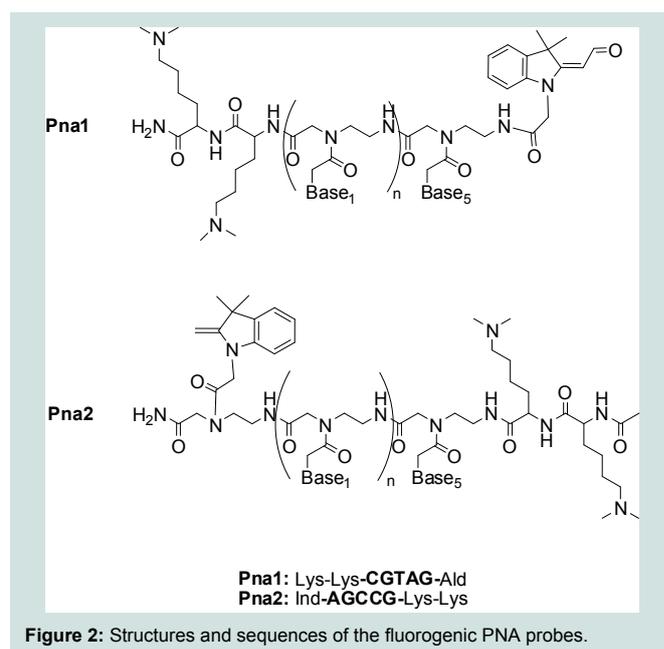
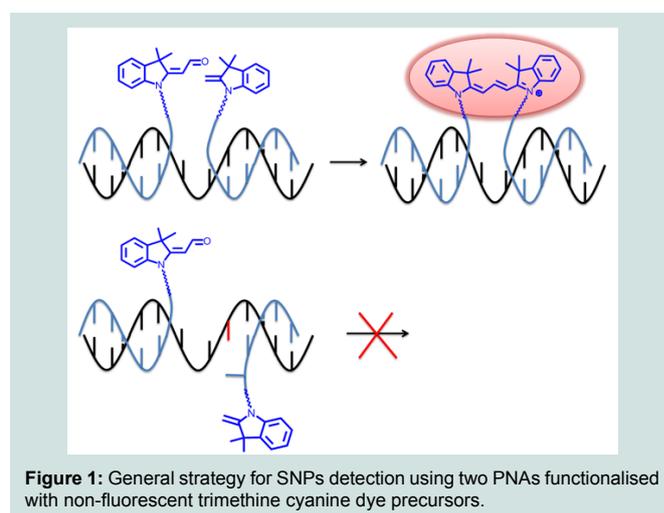
We first investigated the influence of the distance (i.e. number of nucleotides) between both PNAs on the efficiency of the fluorogenic reaction. Five DNA strands (WT0-4, Table 1), all containing both sequences complementary to Pna1 and Pna2 but separated by a variable number of nucleobases (from 0 to 4), were evaluated as potential candidates capable of templating the reaction of cyanine dye formation. While short distances between both PNAs (WT0-2) proved equally good at templating the fluorogenic reaction, slightly more flexible systems (e.g. WT3 where PNA/DNA duplexes formed upon hybridisation of Pna1 and Pna2 with the DNA template are separated by three nucleotides) appeared more favorable (Figure 3). This tendency was confirmed when increasing the distance even further (up to 7 nucleotides, data not shown), although never exceeding the maximum fluorescence observed with a 3 nucleotide gap (WT3).

Next, both WT2 and WT3 were selected for a more detailed study. The effect of systematic point mutations within the DNA template on the reaction efficiency was investigated. In the case of WT3, none of the single mutations tested led to a significant change in reaction efficiency, regardless of the nature and position of the mutation (Figure 4A). Significant inhibition of the fluorogenic reaction was observed only when mutating at least two consecutive residues in WT3 while near-complete inhibition was obtained when mutating at

minimum of three residues, hence preventing hybridization of at least one of the fluorogenic PNAs (Figure 4B).

Although WT3 seemed optimal for templating the reaction of cyanine dye formation, it appeared that a 3-nucleotide gap between both PNA complementary sequences was probably making the PNA-DNA complex too flexible to be responsive to minimal changes such as single mutations.

Therefore, and despite the fact that WT2, as a template, was not quite as efficient as WT3, we reasoned that a 2-nucleotide gap could provide a more rigid environment for the fluorogenic reaction so that the correct alignment of both non-fluorescent cyanine dye precursors would be more responsive to SNPs. DNA sequences derived from WT2 but carrying single nucleotide mutations at various positions (Mut1-6, Table 1) were tested for their ability to template the fluorogenic reaction. The reaction time-courses were determined by fluorescence spectroscopy on a microtiter plate



format and compared to that obtained with the corresponding native sequence **WT2** (Figure 5).

A significant decrease (up to 5-fold) in fluorescence intensity was observed when mutating one nucleotide within the sequence of **WT2** complementary to **Pna2** (**Mut1-5**, Table 1). As previously observed by others, the strongest “inhibitory” effects were obtained with single mutations located near the indole fluorogenic probe (e.g. **Mut1** vs **Mut5**). However, the nature of the mutation had no influence on the reaction time-course. For instance, similar time courses were obtained for **Mut1**, regardless of the nature of X (A, C or G). Surprisingly, DNA strands carrying mutations within the sequence of **WT2** complementary to **Pna1** (**Mut6**, Table 1) were found to be almost equally efficient templates when compared to the native sequence **WT2**. This is likely due to a greater conformational freedom of the Fischer’s base aldehyde allowing its reaction with the neighbouring indole even if **Pna1** is not fully hybridized to the DNA template. Altogether, these results suggest that a constrained system in which two 5-mer fluorogenic PNAs can hybridize, simultaneously and two nucleotides apart, to a unique DNA strand serving as a reaction template, can be used for SNPs detection. Most sensitive detection is achieved when the mutation is located opposite the indole’s neighbouring nucleobase (**Mut1**) although the system remains sensitive enough for detecting mutations located further away in the DNA template (**Mut2-4**).

Finally, in order to estimate the efficiency of the fluorogenic reaction upon addition of complementary DNA, a water-soluble analogue of the symmetrical trimethine cyanine dye reaction product was synthesized and used as a standard for quantifying the amount of dye formed in the absence or presence of various DNA templates. Using this external standard, the yield of the reaction templated by **WT2** was found to be approximately $10 \pm 5\%$ while no fluorescent product was ever detectable under similar conditions but in the absence of template (Figure 6). Interestingly, addition of a large excess (20 $\mu\text{g/mL}$) of competitor Calf-Thymus DNA in the reaction mixture resulted in no significant loss of reaction efficiency (data not shown), thus confirming the high specificity of our fluorogenic system.

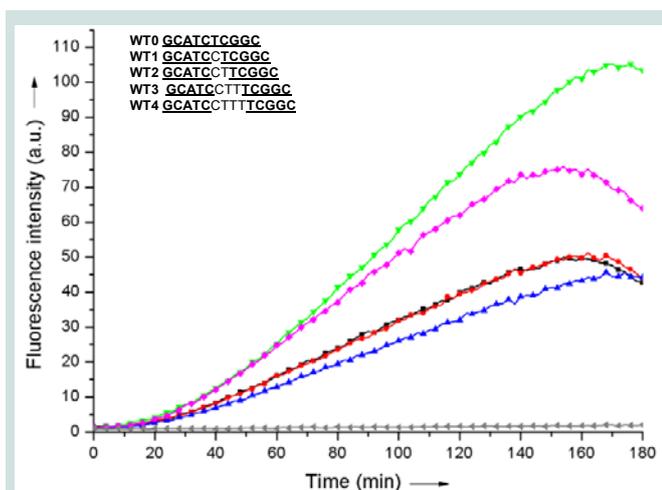


Figure 3: Reaction time-course of a stoichiometric mixture of **Pna1** and **Pna2** (10 μM each) in the absence (grey) and in the presence of a stoichiometric amount of **WT0** (blue), **WT1** (red), **WT2** (black), **WT3** (green) or **WT4** (pink). Reaction was monitored by fluorescence spectroscopy ($\lambda_{\text{exc}} = 540 \text{ nm}$, $\lambda_{\text{em}} = 573 \text{ nm}$).

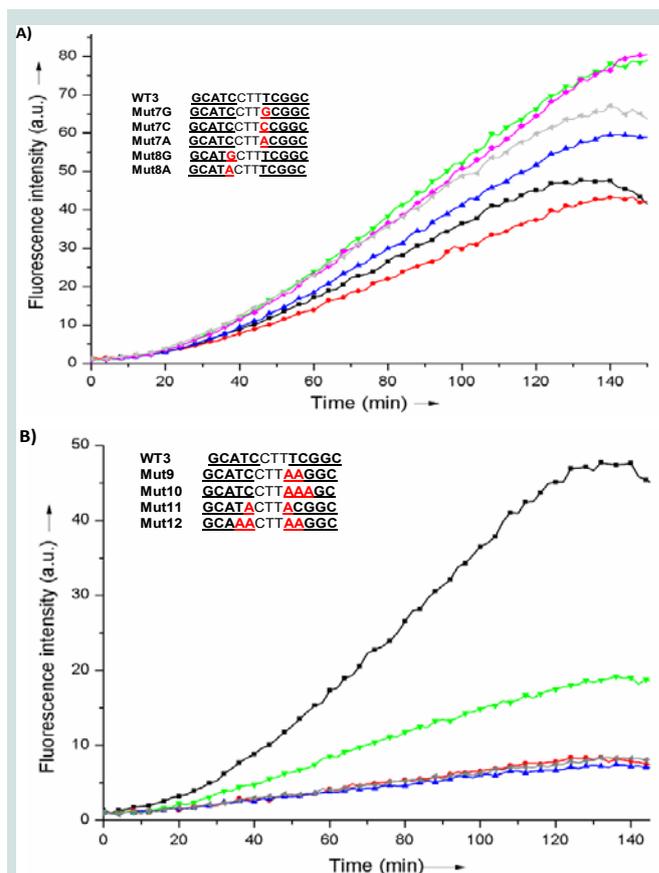


Figure 4: Reaction time-course of a stoichiometric mixture of **Pna1** and **Pna2** (10 μM each) in the presence of a stoichiometric amount of **WT3** (black) or one of the mutated sequences **Mut7G** (red), **Mut7C** (blue), **Mut7A** (green), **Mut8G** (pink) or **Mut8A** (grey) (Fig. 4A), or **Mut9** (red), **Mut10** (blue), **Mut11** (green) or **Mut12** (grey) (Fig. 4B). Reaction was monitored by fluorescence spectroscopy ($\lambda_{\text{exc}} = 540 \text{ nm}$, $\lambda_{\text{em}} = 573 \text{ nm}$).

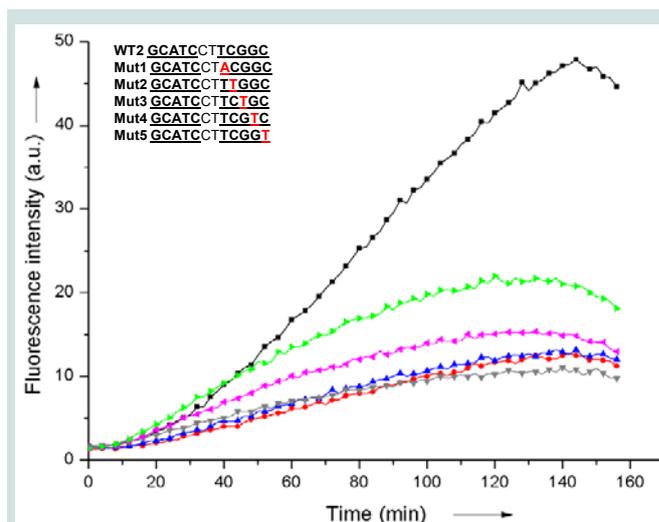


Figure 5: Reaction time-course of a stoichiometric mixture of **Pna1** and **Pna2** (10 μM each) in the presence of a stoichiometric amount of **WT2** (black), **Mut1** (red), **Mut2** (blue), **Mut3** (grey), **Mut4** (pink), **Mut5** (green). Reaction was monitored by fluorescence spectroscopy ($\lambda_{\text{exc}} = 540 \text{ nm}$, $\lambda_{\text{em}} = 573 \text{ nm}$).

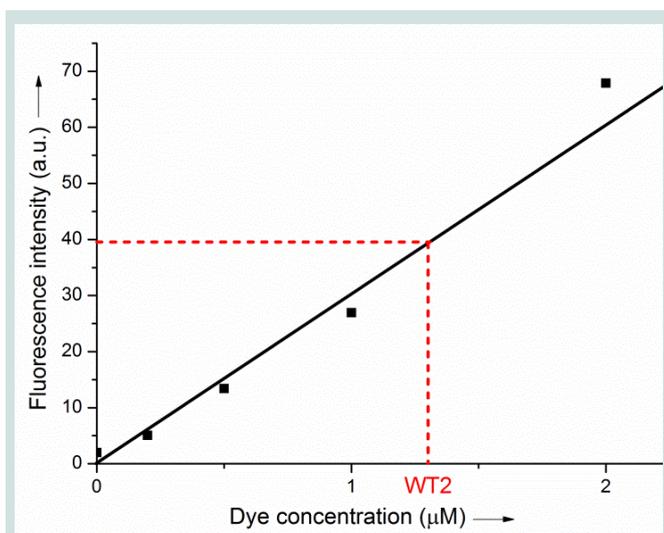


Figure 6: Fluorescence calibration curve (fluorescence intensity versus dye concentration, $\lambda_{exc} = 540$ nm, $\lambda_{em} = 573$ nm) obtained from an analytically pure sample of symmetrical trimethine cyanine dye similar to the product of the fluorogenic reaction (inset). The fluorescence intensity obtained at equilibrium (after 3 hours) for a stoichiometric 30 mixture of **Pna1** and **Pna2** (10 μ M each) in the presence of a stoichiometric amount of **WT2** is highlighted in red.

Conclusion

In conclusion, we have reported the use of a fast, versatile, biocompatible and fluorogenic DNA-templated reaction of cyanine dye formation for the detection of SNPs in vitro. The system offers the advantage of a reasonably high sensitivity due to the high absorptivity and moderately high fluorescence quantum yield of the cyanine dye formed [33] and requires minimal amounts (500 pmol) of both DNA and PNA probes when the reaction is carried out in a 384-well microtiter plate. The design of optimized (e.g. more rigid, multi-coloured) systems to further improve the sensitivity of the detection is currently underway in our laboratory.

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