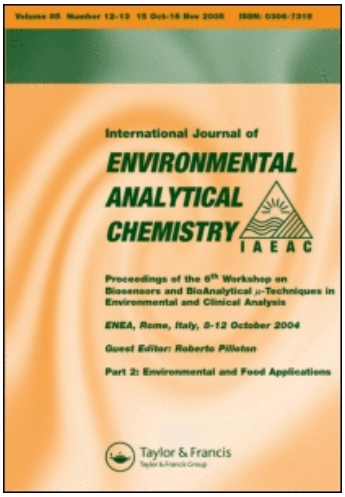

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A new assay for single nucleotide polymorphism analysis based on displacement reactions in PNA–DNA double helices

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In this article, we describe a new method for single nucleotide polymorphism analysis using the displacement reaction of the DNA in a PNA–DNA double helix by the target DNA. Thereby, the probe consists of a TMR-labeled PNA and a Cy5-labeled DNA forming a FRET system. Due to the displacement of the labeled DNA strand by the target DNA, the FRET is invalidated and the fluorescence of the donor dye (TMR) increases. Investigations of the exchange reaction show that increasing salt concentration and temperature accelerate the exchange rate.

Keywords: PNA–DNA systems; Fluorescence-resonance-energy-transfer; Single nucleotide polymorphism analysis; DNA assay

1. Introduction

Highly-specific DNA detection methods are becoming increasingly important in many fields such as criminology, environmental studies, biochemical research and especially health care. Beside cardiovascular diseases, cancer and infection diseases are the most frequently cause of death. Single nucleotide polymorphism (SNP) analysis plays a major role in modern early-stage diagnostics of tumor diseases [1–3] and infections like HIV [4, 5] as well as tuberculosis [6, 7]. Furthermore, tuberculosis bacteria and their antibiotic resistant forms in particular are becoming ever more important in human medicine as they are spreading at a rapid pace even in Europe. Their identification frequently takes too long due to time-consuming assays and as such often leading to the death of the respective patient. Most resistant agents are due to an exchange of a single nucleic base in the respective gene.

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Detection of specific DNA sequences can be classified into homogeneous and heterogeneous assay formats. Commonly, in heterogeneous assays, fluorescently labeled probes hybridize to the target-DNA, which has to be immobilized on a solid surface, e.g. via another hybridzation reaction [8]. After removal of the unhybridized probes using several cleaning steps, the remaining fluorescence intensity is measured. Unfortunately, the requirement of removing unhybridized probe molecules precludes the use of this technique for online monitoring of hybridization. In addition, unspecific adsorption of dye-labeled probes on the surface decreases the sensitivity of regular heterogeneous assays. Homogeneous assays allow time-efficient detection of DNA without cleaning or separation steps. In essence, the probe consists of a short fluorescently-labeled oligonucleotide, which is complementary to the target sequence. Due to a higher mass after hybridization to the target, the diffusion time increases and can be measured by FCS (fluorescence correlation spectroscopy) [9, 10]. Other assays are based on double-labeled probes in which the dyes form a FRET-pair (fluorescence-resonance-energy-transfer) [11]. More recent methods, e.g. the molecular beacons developed by Tyagi and Kramer [12], use probes that report the presence of a specific DNA sequence via a significant increase of their fluorescence intensity. These probes are based on oligonucleotides labeled with a fluorescent dye and a quencher molecule like DABCYL. The last 4–6 nucleic bases of both ends of the oligonucleotide are complementary to each other, and thus the probe forms a hairpin-structure with the fluorescence dye and the quencher molecule in close proximity, effecting an efficient quenching of the fluorescence intensity.

The sequence of the loop of the hairpin-structured probe is complementary to the target-DNA. By hybridizing to the target, a rigid double helix is formed and the hairpin is opened. Thus, the dye and quencher molecule lose contact and the fluorescence intensity increases. By optimizing the probes and measuring at a certain temperature, these probes can be used for single nucleotide polymorphism (SNP) analysis [13]. Instead of organic quencher molecules like DABCYL or the more effective *black hole quencher* (BHQ), gold nano-particles have been successfully applied [14]. Hence, the fluorescence of several oxazine and rhodamine derivatives is quenched by the nucleic base guanosine [15], the oligonucleotide itself can effect a quenched probe, so called 'Smart Probes'. Thereby, the dye-labeled end, e.g. the 5-prime, contains several cytosines and the opposite end accordingly contains several guanosines. In the closed hairpin form the guanosine quenches the fluorescence of the dye, whereas opening the hairpin leads to an increase of fluorescence intensity [16]. The sensitivity of homogeneous assays can be elevated to the pico-molar (10^{-12} M) range by using single-molecule detection methods [17, 18].

By dint of these self-quenching probes it is possible to construct heterogeneous assays that do not require any cleaning or separation steps [19, 20]. For example, the smart probes were linked by a biotin-streptavidine system to a BSA coated glass surface and the fluorescence of the individual probes was detected by *fluorescence lifetime imaging microscopy* (FLIM) [21]. With this method, a DNA-target concentration of 10^{-13} M can be detected.

Several assays for SNP exploit enzymatic reactions. The commercially available *template directed dye terminator incorporation* (TDI) [22] or the TaqMan assay [23] are based on polymerases chain reaction (PCR). Furthermore, ligase that connects two oligonucleotides, which are hybridized directly neighboured to a specific target, is applied in the LDR (ligase detection reaction) assay [24].

In recent years, some methods based on *peptide nucleic acid* (PNA) probes have been developed. In PNAs, the negatively charged sugar-phosphate backbone of the DNA is replaced with a neutral charged pseudopeptide chain. Originally, they were designed as a mimic of a DNA oligonucleotide for major-groove-binding and triplex-formation [25–27]. However, PNA also forms stable double helices by hybridizing to single-stranded PNA, RNA and DNA obeying the Watson–Crick hydrogen-bonding rules [28]. Furthermore, they can form PNA_2–DNA or PNA–DNA_2 triple helices [29] and even PNA–DNA quadruple helices [30, 31]. In contrast to DNA, PNA is stable against nucleases and proteases, thus making it a promising agent with regards to gene therapy [32–35].

Using PNA instead of DNA probes may be advantageous because they hybridize to single stranded DNA and RNA more specifically and the resulting helices are more stable than the respective DNA analogs [36]. The special properties of the PNA–DNA double helix can be used for SNP analysis. For example, a PNA–DNA double helix consisting of the PNA probe and the DNA of the target sample, can be marked by the 3,3′-diethylthiadicarbocyanine dye ($DiSC_2(5)$) that changes its absorption maximum from 534 nm to 652 nm due to intercalation into the PNA–DNA helix. In the presence of a mismatch, the PNA–DNA double helix is destroyed by an enzymatic reaction [37]. Furthermore, PNA–DNA double helices can be used for constructing primers suitable for PCR online monitoring [38]. All these assays require higher temperatures for the enzymatic reaction or for denaturation steps.

In this article we present an assay for detection of SNP at room temperature based on displacement reactions in a PNA–DNA double helix. The exchange of short PNA strands in PNA–DNA double helices has been reported by developing DNA-templated cleavage reactions of carboxylic ester catalysed by copper complexes [39]. Thereby, a copper complex and an ester respectively are linked to short PNAs. Due to hybridization to the DNA template, the ester and the copper complex are in close proximity and the reaction takes place. Afterwards, the PNA carrying the cleaved ester can be replaced by another PNA linked to an uncleaved ester. Based on the same principle, several drug releasing systems have been developed, in which DNA or RNA act as the template [40]. This strategy aims for efficient therapies of cancer diseases [41], while side effects will be minimized by the specific release of the drugs in the cancer cells containing cancer specific RNA or DNA sequences.

For the novel highly specific DNA assay, a displacement of the DNA-strand of the PNA–DNA helix is required. Thus, we investigated the DNA exchange reaction under varying reaction conditions first. Thereby, salt concentration, solvent and temperature were of major interest. Secondly, we evaluated the possibility of applying this reaction in a DNA assay.

2. Experimental

The PNA-synthesis (TMR-TCACAACTAKKK) as well as the labeling of the tetramethylrhodamine to the N-terminus of the PNA was kindly carried out by Prof. Dr R. Krämer (Institute of Inorganic Chemistry, University of Heidelberg, Germany). The tetramethylrhodamine (TMR) as well as DABCYL were purchased from Molecular Probes (Göttingen, Germany) and Cy5 from Amersham Pharmacia Biotec (Freiburg, Germany) as NHS-ester.

The oligonucleotides (5′-C_6-amino-AGTGTTGAT-3′; 5′-AGTGT(C_6-amino)TGAT-3′; 5′-AGTGTTGAT-3′; 5′-AGTTTTGAT-3′; 5′-AGTGTGGAT-3′) were custom-synthesized by IBA (Göttingen, Germany). For the coupling reaction 5 nmol of the amino modified DNA is dissolved in 100 μL $NaHCO_3$ buffer (0.1 M; pH 8.5). A measure of 10 nmol of the NHS-ester of the quenching molecule DABCYL was dissolved in 30 μL acetonitrile and mixed with the solved DNA. The solution was incubated for 3 h at room temperature and afterwards purified by reversed-phase (Hypersil-ODS column) HPLC (Agilent Technologies, Waldbronn, Germany) using a linear gradient of 0–75% acetonitrile in 0.1 M aqueous triethylammonium acetate. Molecular weights were determined by mass spectrometry. Reaction yields of 20–50% were achieved.

The determination of the concentration via absorption measurements were carried out with a Cary 500 UV-Vis-NIR spectrometer (Varian, Darmstadt, Germany) at room temperature. The extinction coefficient of the TMR is 96.000 M^{-1}cm and approximately 250.000 M^{-1}cm for Cy5 and Cy3. Steady-state emission spectra were measured with a temperature controlled Cary Eclipse fluorescence spectrometer. In order to avoid an overlay of scattering and fluorescence light, the excitation wavelength was 10 nm lower than the absorption maximum of the measured dye. For monitoring the DNA exchange reaction the excitation wavelength (550 nm) and the wavelengths for fluorescence detection (590 nm and 670 nm) were kept constant for half an hour. Every 2 s a data point was taken. Because of the hydrophobic PNA-probes all measurements were taken in PEG-coated standard quartz cuvettes. In all measurements the concentration was kept strictly below 1 μM to avoid re-absorption and re-emission effects. Relative fluorescence quantum yields, $\Phi_{f,rel}$, were measured with respect to the fluorescence intensity of the free dye.

3. Results and discussion

For the investigation of the displacement of the DNA-strand of a PNA–DNA double helix, we synthesized self-quenching PNA–DNA helices that report the exchange of the DNA by an increase of the fluorescence intensity (figure 1). Therefore, a PNA 9mer containing three lysine residues in order to raise solubility is labeled at the N-terminus with tetramethylrhodamine (TMR). At the complementary DNA-strand a quenching molecule DABCYL or rather an acceptor dye (Cy5) is attached either at the 5prime or at an internal amino modified thymidine. In the double helix, the dye and the quencher are in close proximity and a fluorescence quenching or a *fluorescence resonance energy transfer* (FRET) occurs.

However, the fluorescence of the TMR attached to the PNA is reduced. Due to displacement of the DNA-strand by an identical but unlabeled DNA-strand, the quencher or acceptor dye is removed and the fluorescence intensity increases.

In general, the fluorescence of the TMR-labeled PNA is 2–3 times worse quenched by using DABCYL as quencher compared to the FRET system using Cy5 as an acceptor. Thereby, the quenching is better if the quencher is labeled to the 5prime. More effective is utilization of a FRET system with Cy5 acting as an acceptor dye. We did not observe significant differences in the decrease of the TMR fluorescence between the DNA-strand labeled at the 5prime or internally at the thymidine. However, we found the TMR–Cy5 FRET system, in which the Cy5 is labeled internally, to be a suitable

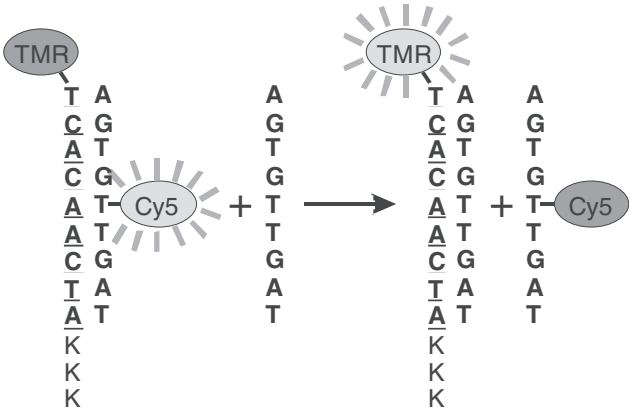

Figure 1. Working mechanism of the new probes. The fluorescence intensity of the TMR-labeled PNA (PNA: underlined) is decreased by forming a FRET system with a Cy5-labeled DNA. Due to an exchange of the DNA-strand by the target DNA, fluorescence intensity of TMR increases.

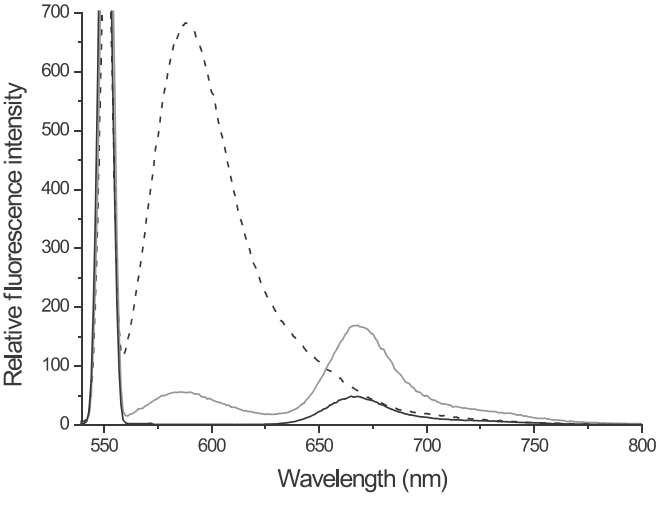

Figure 2. Fluorescence spectra of 10^{-7} M tetramethylrhodamine labeled PNA (TMR-TCA CAA CTA KKK; K = lysine) (dashed line), of 10^{-7} M TMR–PNA mixed with an equimolar amount of Cy5 labeled DNA (Cy5-DNA; 5′-TAG T(Cy5)TG TGA-3) (gray line) and 10^{-7} M Cy5-DNA (black line).

probe for monitoring the exchange reaction. The distance between both dyes is five base pairs corresponding to approximately 15 Å. Figure 2 shows the fluorescence properties of the FRET system. The TMR–PNA has an absorption maximum at 560 nm, the emission maximum is 590 nm. After adding a three-fold excess of complementary Cy5 labeled DNA a PNA–DNA double helix is formed spontaneously and an efficient FRET is observed. The fluorescence of TMR decreases at 590 nm more than 10-fold and a new band at 670 nm appears in the emission spectrum. In order to estimate the cross-talk, a reference sample of Cy5-DNA without TMR–PNA (black line) was excited at 550 nm. It shows nearly no fluorescence at 590 nm and only little fluorescence at 670 nm. All shown fluorescence measurements are already corrected

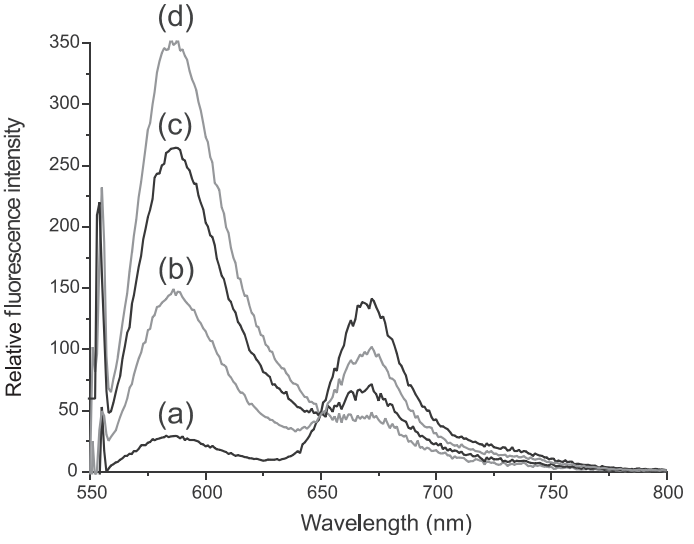

Figure 3. Fluorescence spectra of 10^{-7} M PNA–TMR after adding 3×10^{-7} M DNA–Cy5 (a). Unlabeled DNA with the same sequence is added subsequently with a concentration of (b) 1.5×10^{-7} M, (c) 5×10^{-7} M and (d) 1×10^{-6} M. All spectra are corrected by the fluorescence occurred be Cy5–DNA itself. Measurements were carried out in aqueous solution (pH 7.0) containing 30 nM NaCl and 5 mM $MgCl_2$ at 20°C.

by this cross-talk. To demonstrate the exchange of the DNA-strand of the PNA–DNA double helix, we added different concentrations of unlabeled DNA with the same sequence as the Cy5-labeled DNA (figure 3).

The higher the concentration of the DNA, the more the TMR signal at 590 nm increases and accordingly the Cy5 signal at 620 nm decreases. In order to study the kinetics of the DNA displacement, we monitored the fluorescence signal at 590 nm and 690 nm during the exchange reaction. Thereby, the excitation wavelength was kept constant at 550 nm. Figure 4 shows an example of a DNA exchange at 20°C in aqueous solution (pH 7.0), containing 5 mM $MgCl_2$. After waiting 12 min until the fluorescence intensity has reached a constant level, a two-fold excess of Cy5-labeled DNA was added to a 5×10^{-8} M TMR–PNA solution. The fluorescence decrease at the beginning of the measurement is likely due to adsorption effects of the hydrophobic PNA to the glass walls of the cuvette. Due to formation of the PNA–DNA double helix, the fluorescence at 590 nm drops from 310 to 15 within seconds, whereas the fluorescence at 690 nm increases from 0 to 90. The signal at 690 nm, representing the Cy5 fluorescence is already corrected by the signal caused by TMR (approximately 8% of the signal at 590 nm) and by direct excitation of Cy5, respectively. At 18.3 min, a two fold excess of unlabeled DNA referring to Cy5–DNA was added. The narrow peak in the spectrum is due to scattering light caused by the pipette.

The TMR fluorescence increases nearly linearly during the first 2 min, then becomes slower and ends at 200. That is consistent with the 2 : 1 ratio of unlabeled DNA to DNA–Cy5. Correspondingly, the Cy5 fluorescence decreases from 100 to less than 50.

We found the exchange rate to be extremely dependent on the reaction conditions. First, we investigated the influence of the salt concentration. To that end, an univalent salt (NaCl) as well as a bivalent salt ($MgCl_2$) was tested. For each investigated salt concentration, a kinetic study was executed as previously described (figure 4).

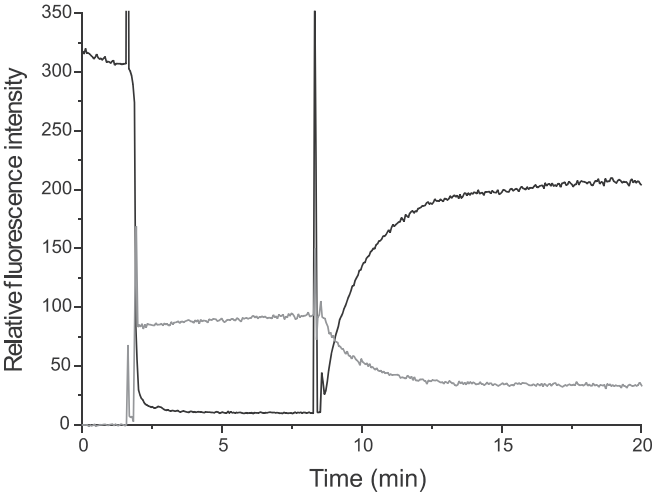

Figure 4. Fluorescence signal of TMR–PNA (5×10^{-8} M) at 590 nm (black line) and 690 nm (gray line) with an excitation wavelength of 550 nm monitored over a time period of 20 min. After 2 min Cy5–DNA (10^{-7} M) and at 8 min unlabelled DNA with the same sequence (2×10^{-7} M) was added. Measurement was carried out in aqueous solution containing 5 mM $MgCl_2$ at 20°C.

There are several aspects that ought to be considered in the analysis of the experiment. Under some conditions, the displacement reaction is very slow, and thus the interpretation of the kinetic analysis often causes difficulties. Furthermore, the adsorption tendency of the hydrophobic PNA increases with the amount of salt and the FRET efficiency depends on the salt concentration. Generally, the FRET of the PNA–DNA system is reduced by adding salts. That might be due to a strengthening of the interaction between the PNA and the tetramethylrhodamine dye that is relatively hydrophobic, as well. Thus, the rotation of the dipole is hindered, effecting a smaller FRET efficiency.

However, to compare all measurements, we took the fluorescence signal of the TMR at 590 nm 60 s after adding the unlabeled DNA and calculated the fraction of the fluorescence increase with respect to the expected increase at the equilibrium (figure 5). Due to the two fold excess of unlabeled DNA, we expected a maximum fluorescence intensity of two thirds of the value of pure TMR–PNA. Under most conditions, the expected fluorescence was reached within 15 min. In pure water no significant change in fluorescence was observed, thus the value of the fraction is zero. If the full increase would be reached within a minute, the fraction would be one. Increasing salt concentration, especially $MgCl_2$ accelerates the exchange reaction of the DNA-strand of the PNA–DNA double helix significantly. Already, 5 mM $MgCl_2$ effects a rapid DNA displacement, thus after 60 s 45% of the maximum fluorescence increase is accomplished. By adding up to 10 mM $MgCl_2$, a strong increase of the exchange rate is observed, whereas further increase of the $MgCl_2$ concentration shows much smaller effects. The univalent NaCl also accelerates the DNA displacement but a much higher concentration of salt is needed. For example, 45 mM NaCl is required to achieve a similar exchange rate as observed by adding just 5 mM $MgCl_2$ and 100 mM NaCl has an equal effect as approximately 10 mM $MgCl_2$. Concentrations higher than 100 mM effect only a small increase of the exchange rate. These results indicate that

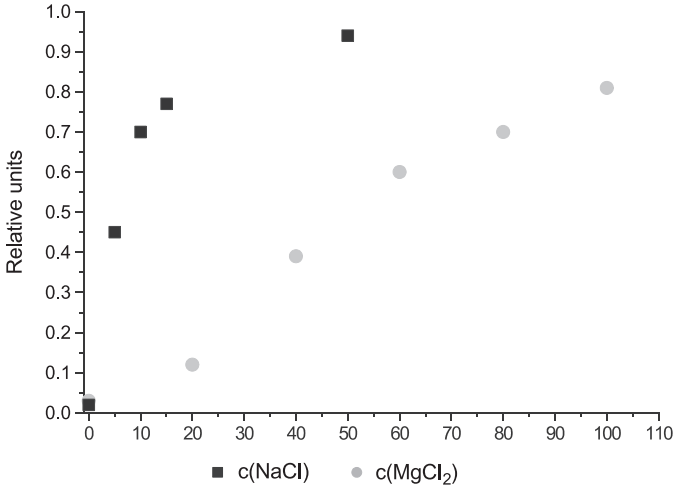

Figure 5. Measurements (described in figure 3) were performed under different conditions. The fluorescence increase at 590 nm was measured 60 s after adding unlabelled DNA. This value was divided by the expected maximum increase, which is approximately two thirds of the value before adding the dye-labeled DNA. Measurements and analysis were done for a aqueous solution (pH 7.0) containing different amounts of $MgCl_2$ (■) and NaCl (●).

the approach of the negatively charged DNA-strand to the also negatively charged PNA–DNA double helix is the rate limiting step of the exchange reaction. In the absence of positively charged ions the electric repulsion prevents a displacement reaction. The more cations are present, the better the negatively charge is protected and the approach is less hindered. Thus, salts and in particular bivalent salts advance an effective displacement of the DNA-strand.

Like most chemical reactions, the velocity of the investigated DNA displacement depends on temperature, as well (figure 6). The reaction conditions, aqueous solution containing 20 mM NaCl, for this experiment were chosen in such a way that a slow but significant exchange reaction could be observed at 10°C. At this temperature, the slope at the beginning of the exchange reaction is about $400\,s^{-1}$, whereas the velocity approximately doubles ($894\,s^{-1}$) by increasing the temperature by 10 to 20°C according to the rule-of-thumb by van't Hoff.

Further increase of the temperature to 30°C effects a dramatic increase of the exchange rate ($3800\,s^{-1}$) that cannot be explained by van't Hoff or the Arrhenius equation. This behavior may be due to the approach to the melting point of the PNA–DNA double helix that is estimated to about 40°C for the used concentrations (data not shown).

A major aim of the article is to apply the described reaction to DNA analytics. Therefore we investigated the specificity of the exchange reaction with respect to DNA-strands containing mismatches. Firstly, suitable reaction conditions have to be selected. They have to enable a fast exchange reaction and the dye labeled PNA–DNA double helix has to show an efficient FRET, and thus the increase of the TMR fluorescence caused by the displacement of the DNA-strand is as high as possible. Thus, on the one hand, salts accelerate the exchange reaction and on the other, they hinder an efficient FRET, and hence a compromise has to be found. We used aqueous solutions containing 50 mM NaCl and 5 mM $MgCl_2$. Under these conditions,

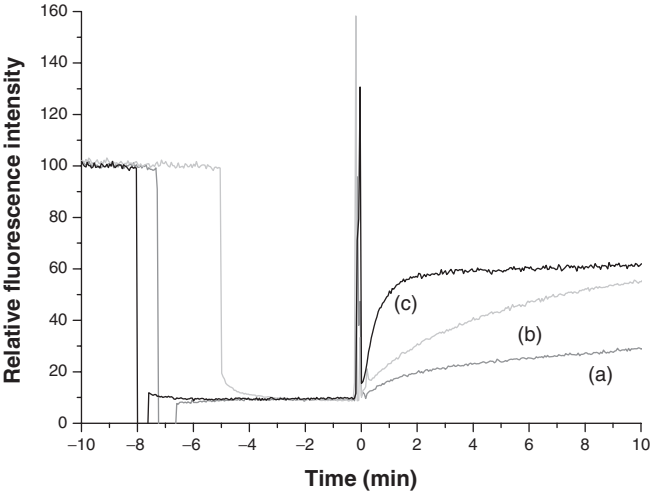

Figure 6. Fluorescence signal at 590 nm (excitation 550 nm) of PNA–TMR (5×10^{-8} M) mixed with DNA–Cy5 (10^{-7} M) in aqueous solution (pH 7.0; 20 mM NaCl). At 0 min unlabelled DNA (2×10^{-7} M) was added. Measurements were carried out at 10°C (a), 20°C (b) and 30° (c). Because of temperature dependence of the fluorescence of the dye the curves were normalized at a starting fluorescence of PNA–TMR.

the fluorescence intensity at 590 nm of a 1×10^{-8} M TMR–PNA solution drops to a tenth by the addition of a two fold excess of Cy5-labeled DNA. By adding 6×10^{-8} M of target DNA that has the identical sequence as the Cy5-labeled DNA the fluorescence intensity increases by a factor of seven within 2 min, whereas the addition of the same amount of a DNA containing one mismatch effects no significant fluorescence increase (figure 7). Only with a 10 to 20-fold excess of the mismatched DNA, an increase of the fluorescence intensity can be observed. DNA-strands containing two or more mismatches do not lead to measurable increases, even on adding high excesses.

To achieve a significant increase of the fluorescence intensity, the concentration of the target-DNA should be at least in the same region as the Cy5-labeled DNA, and thus the detection limit in this experimental setup was about 10^{-8} M. In order to detect lower DNA-concentrations, the concentration of the PNA–DNA probe has to be decreased. Using probe concentrations below 10^{-8} M leads to several problems. First, a higher excess of dye-labeled DNA is required to obtain a well quenched system. This might be due to concentration dependence of the melting temperature of the PNA–DNA double helix. The concentration of the dye-labeled DNA cannot be raised arbitrarily because the excessive DNA competes with the target DNA. Furthermore, low target concentrations do not lead to an efficient displacement reaction. However, below a target concentration of 10^{-8} M, no 'suitable' assay could be achieved with the used PNA–DNA system.

One way to avoid the excess of the dye labeled DNA would be to connect the PNA and DNA strand via a linking molecule, e.g. an oligopeptide. By this method the ratio of dye-labeled PNA and DNA is always 1 : 1, nevertheless each PNA should be hybridized to a DNA carrying an acceptor dye and a quencher molecule, respectively. The linkage should effect stabilization of the double helix similar to DNA-hairpins. However, to still enable an efficient exchange reaction of the DNA strand, one or more

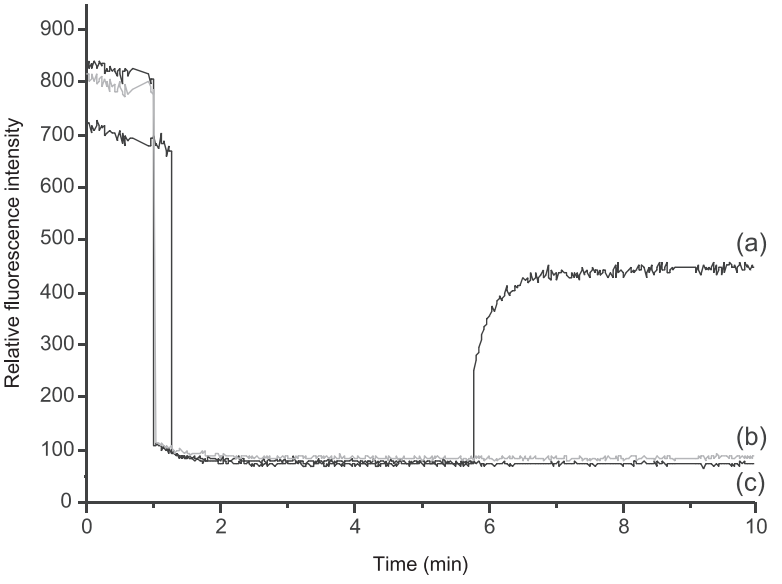

Figure 7. Fluorescence signal at 590 nm (excitation 550 nm) of PNA–TMR (5×10^{-8} M) in aqueous solution (pH 7.0; 25°C; 50 mM NaCl, 5 mM $MgCl_2$) mixed after 1 min with DNA–Cy5 (10^{-7} M). After about 6 min, unlabeled DNA (2×10^{-7} M) with the same sequence or rather a mismatch were added (a) 5′-AGTGTTGAT-3′; (b) 5′-AGT**T**TTGAT-3′; (c) 5′-AGTGT**G**GAT-3′.

mismatches need to be incorporated. In principle, the presented assay system should be suitable for chip technology. Therefore, the dye-labeled PNA-strand has to be covalently linked to the surface. Applying immobilized probes might be a useful method to increase the sensitivity.

4. Conclusion

The results shown demonstrate the feasibility of detecting single nucleotide polymorphisms at room temperature with self-quenching PNA–DNA probes. The fluorescence quenching can be achieved either by applying quencher molecules or better yet by using a FRET system, whereby the fluorescence of the donor chromophore is quenched. The probes are based on the displacement of the quencher labeled DNA of the PNA–DNA double helix by the target DNA. We found that the FRET system consists of the TMR-labeled PNA and the Cy5-labeled DNA to be a good candidate to monitor the exchange reaction and can be used to detect specific DNA sequences over a broad temperature range. Especially by their high specificity, these PNA–DNA systems may become ideal probes for identification of single nucleotide polymorphisms.

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