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DNA-probes for the highly sensitive identification of single nucleotide polymorphism using single-molecule spectroscopy

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Abstract **This article presents a new, highly sensitive method for the identification of single nucleotide polymorphisms (SNPs) in homogeneous solutions using fluorescently labeled hairpin-structured oligonucleotides (smart probes) and fluorescence single-molecule spectroscopy. While the hairpin probe is closed, fluorescence intensity is quenched due to close contact between the chromophore and several guanosine residues. Upon hybridization to the respective target SNP sequence, contact is lost and the fluorescence intensity increases significantly. High specificity is achieved by blocking sequences containing mismatch with unlabeled oligonucleotides. Time-resolved single-molecule fluorescence spectroscopy enables the detection of individual smart probes passing a small detection volume. This method leads to a subnanomolar sensitivity for this single nucleotide specific DNA assay technique.**

Keywords: DNA hairpin probes; Fluorescence spectroscopy; Single-molecule spectroscopy; Single nucleotide polymorphism

1. Introduction

We have developed a simple method using self-quenching, hairpin-structured DNA-probes (smart probes) for sensitive and specific single nucleotide polymorphism (SNP) analysis in homogeneous solutions. Highly specific DNA detection methods are becoming increasingly important in many fields such as criminology, environmental studies, biochemical research and especially health care. Single Nucleotide Polymorphism analysis plays a major role in modern early-stage diagnostics of tumor diseases [1–3] and infectious diseases, e.g. AIDS [4,5] and tuberculosis [6,7] which are the most frequent causes of death after cardiovascular diseases. Identification of specific DNA sequences can be performed in homogeneous and heterogeneous assay formats. In contrast to most of the latter, homogeneous assays allow time-efficient detection of DNA without purification steps. Usually, homogeneous assays for identification of SNPs are based on enzymatic reactions. For instance, PCR (Polymerase Chain Reaction) is used in the *template-directed dye terminator incorporation* (TDI) [8] or the TaqMan assay [9]. The most powerful non-enzymatic assays utilize hairpin-structured self-quenching oligonucleotides, e.g. molecular beacons [10]. Hairpin oligos are labeled at opposing ends with a fluorescent dye and quencher, respectively. Due to close proximity of the dye and quencher in the closed hairpin, fluorescence intensity is efficiently suppressed. The hairpin is designed to complement the target DNA sequence. Thereby the target sequences can hybridize to the hairpin forming a double helix. Due to helix formation, the fluorescent dye and quencher are separated, producing an increase in fluorescence intensity. In addition to organic quencher molecules like DABCYL and the more effective *black hole quencher* (BHQ), gold nano-particles have been successfully applied [11]. All of these probes require at least two different labels. It is also possible to use two identical chromophores that quench each other due to the formation of non-fluorescent H-type dye dimers [12]. In recent years, we developed DNA-probes based on mono-labeled hairpin-structured oligonucleotides (smart probes) [13–15]. Instead of a quencher molecule, guanine residues are employed to quench the fluorescence intensity of several oxazine and rhodamine dyes. Applying hairpin-structured probes to single nucleotide polymorphism analysis requires probe sequence and temperature optimization [16].

In this work, we present a new approach for a simple and highly sensitive identification of SNPs using smart probes. Single nucleotide specificity is achieved by including unlabeled oligonucleotides that are complementary to DNA sequences containing the mismatches. Thereby, these mismatched, non-targeted sequences are blocked and only the perfectly complementary sequence does consequently open the fluorescently labeled probe. Subnanomolar sensitivity is realized by using time-resolved fluorescence spectroscopy that enables the detection of individual molecules. For each molecule passing the detection volume parameters, such as fluorescence intensity or fluorescence lifetime can be determined. The combination of several parameters gives us information on whether or not the fluorescently labeled smart probe is hybridized to the matching target sequence.

2. Materials and methods

For preparation of *smart probes* the amino-modified oligonucleotide 5′-CCCTCTGGTCCATGAATTGAGGG-3′ (IBA GmbH,

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Göttingen, Germany) is fluorescently labeled by the *N*-hydroxysuccinimidyl ester of the dye ATTO 655 (ATTO-TEC, Siegen, Germany). The coupling reactions are carried out in hydrogen carbonate buffer (100 mM, pH 9.0) and the products is purified by HPLC (Hewlett-Packard). Due to the sequence complementarity (underlined) of the ends, the oligonucleotide is forced into a hairpin-structure. The bold oligonucleotide indicates the position of the investigated mismatch. The sequences for the unlabeled "blocking oligonucleotides" are 5′-CCCTCTGGACCATGAATTGAGGG-3′ (block A), 5′-CCCTCTGGCCCATGAATTGAGGG-3′ (block C), and 5′-CCCTCTGGGCCATGAATTGAGGG-3′ (block G). The oligonucleotides 5′-CCCTCAATTCATGGACCA-3′ (target A), 5′-CCCTCAATTCATGGTCCA-3′ (target T), 5′-CCCTCAATTCATGGCCCA-3′ (target C), and 5′-CCCTCAATTCATGGGCCA-3′ (target G) are used as target sequences.

For single-molecule experiments, we applied a standard inverted confocal microscope (Zeiss, Jena, Germany) coupled to a 635 nm laser diode excitation source (PDL800, Picoquant, Berlin, Germany) with a highly sensitive photo-avalanche diode (EG&G, Optoelectronics, Canada) for the detection of fluorescence light. The setup for single-molecule detection is described in more detail in the literature [17,18]. Low concentration of the samples and the small detection volume ensure that an average of less than 0.1 molecules are in the laser focus at the same time. If a fluorescent molecule passes the detection volume, the fluorescence increases immediately and a fluorescence burst is detected. The data analysis is described elsewhere [13,19]. Briefly, all fluorescence bursts that have a maximum count rate over certain threshold, in this case 30 kHz, were extracted from the fluorescence trace. For each of these bursts the maximum intensity, the fluorescence lifetime and the burst duration are determined.

Sample preparation. The ATTO655-labeled smart probe (5×10^{-10} M) is mixed in PBS buffer (140 mM NaCl, pH 7.4) with an excess of the "blocking oligonucleotides" (1×10^{-6} M) and various concentrations of different target sequences. Before measuring the fluorescence signal, the samples are heated to 65 °C and cooled down to 25 °C at the rate of 1 °C per 2.5 min. Afterwards, the sample is transferred to a PEG coated microscope slide with a well, covered with a 170-μm thin cover slide and placed face down above the objective of the confocal microscope. For each sample the fluorescence signal is recorded three times for 60 s.

3. Results and discussion

To demonstrate the specificity and the sensitivity of this novel DNA-assay system we employ a smart probe with the sequence 5′-ATTO655-CCCTCTGG **T** CCATGAATT GAGGG-3′ and vary in the respective target sequence. Because of complementary nucleotides at both ends (underlined), the smart probe forms a hairpin-structure with a five base pair stem and a 13 nucleotides containing loop (Fig. 2). Due to the close proximity to the guanine residues in the closed DNA-hairpin, the fluorescence intensity of the ATTO655 dye is reduced to a relative quantum yield of 0.15 with respect to the free dye. Upon hybridization to an excess of the matching target DNA, we observe a 6-fold fluorescence increase with a standard fluorescence spectrometer. With confocal fluorescence spectroscopy, it is possible to measure much lower concentrations of the smart probe (5×10^{-10} M) and also of the target DNA. The increase in fluorescence signals (intensity and frequency of the fluorescence bursts) upon hybridization is obvious as can be seen in Fig. 1. For example the number of bursts with a maximum count rate above 30 kHz rises from approximately four to over 120 bursts per second in the presence of the target DNA in a concentration of 1×10^{-9} M. On the other hand, the averaged fluorescence intensity increases only from 6.5 kHz to 18.5 kHz which is approximately half of the maximum fluorescence increase observed at higher concentrations with standard fluorescence spectrometry.

However, this smart probe does not allow discrimination between oligonucleotide target sequences that differ by only one nucleotide. In general, hairpin-structured probes (e.g. Molecular Beacons) are appropriate for SNP discrimination if the sequence of the probe and the temperature of the measurements are optimized. Very recently we have shown that well designed

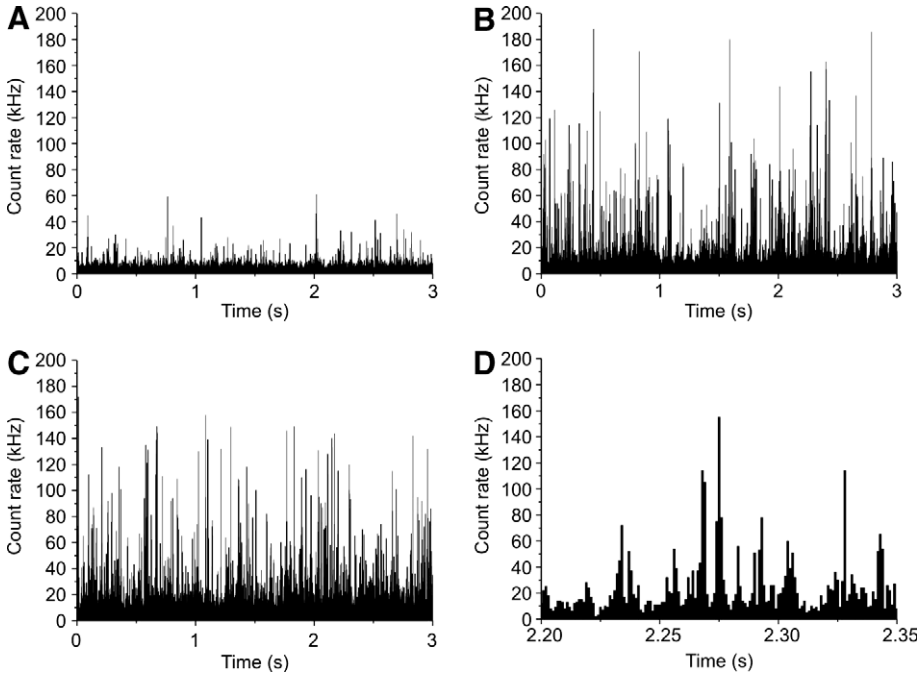

Fig. 1. Fluorescence intensity traces of the smart probe (5×10^{-10} M) (A) in the absence and (B) the presence of an excess of the target sequence (target A; 1×10^{-9} M) and (C) a mismatched sequence (target C; 1×10^{-9} M). (D) Shows a cut-out of the trace (B).

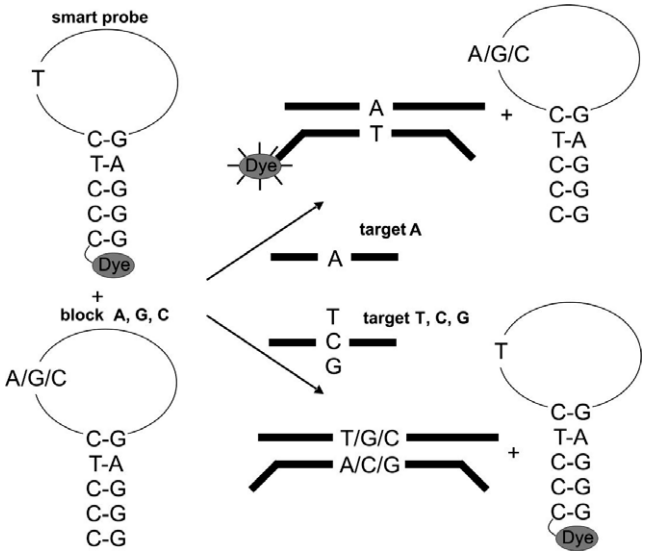

Fig. 2. Proposed mechanism of the DNA probe (smart probe) in combination with the "blocking oligonucleotides" that prevent the probe from hybridizing to DNAs containing a mismatch.

smart probes can also be used for the identification of a certain SNP in PCR products of *Mycobacterium tuberculosis* [19]. For the method presented here, hairpin optimization was not necessary, since we apply unlabeled "blocking oligonucleotides" that hybridize to the DNA-sequences containing the mismatch. Thus, the hybridization of the fluorescent smart probe (Fig. 2) to the DNA sequence containing the mismatch is prevented. The blocking oligonucleotides (*block A*, *G*, and *C*) have the same sequences as the fluorescently labeled smart probe but containing an A, G or C at the position of the investigated SNP.

Fig. 3 shows the fluorescence intensity traces of the smart probe mixed with a 2000-fold excess of the three different blocking oligonucleotides (*block A*, *block C* and *block G*) in the presence of a nanomolar concentration of the matching target DNA and after adding 1×10^{-8} M of each of the three mismatched oligonucleotides. The fully matching target sequence results in a dramatical increase in fluorescence bursts whereas the mixture of the mismatched sequences is efficiently blocked and consequently no significant change is found. In order to detect lower DNA concentrations the fluorescence signal is analyzed in more detail. The fluorescence lifetime, the burst duration and the maximum count rate of a burst can be determined for each detected fluorescence burst. The distributions of these parameters are shown in Fig. 4 for the case of a reference sample (smart probe in the absence of any target DNA) and for a completely opened smart probe (due to the addition of a 200-fold excess of target DNA). It can be seen that all three parameters increase significantly upon hybridization of the smart probe.

In both cases 8800 bursts are analyzed. Above a value of 2 ns (fluorescence lifetime), 50 kHz (max. count rate), and 2 ms (burst duration) more bursts for the hybridized probe are found than for the closed smart probe. Counting all bursts that show values above the thresholds of these parameters leads to picomolar sensitivities. This multi-parameter analysis enables an efficient discrimination between closed and hybridized smart probes and is described in more detail elsewhere [13,19]. The smart probe concentration of the different samples can vary up to 30% due to adsorption to the plastic vials during the hybridization procedure. Therefore, the number of bursts meeting the above mentioned criteria is related to the number of all detected bursts. This ratio is relatively constant, even if the overall concentration of the smart probe varies. This analysis is done for the smart probe without any blocking

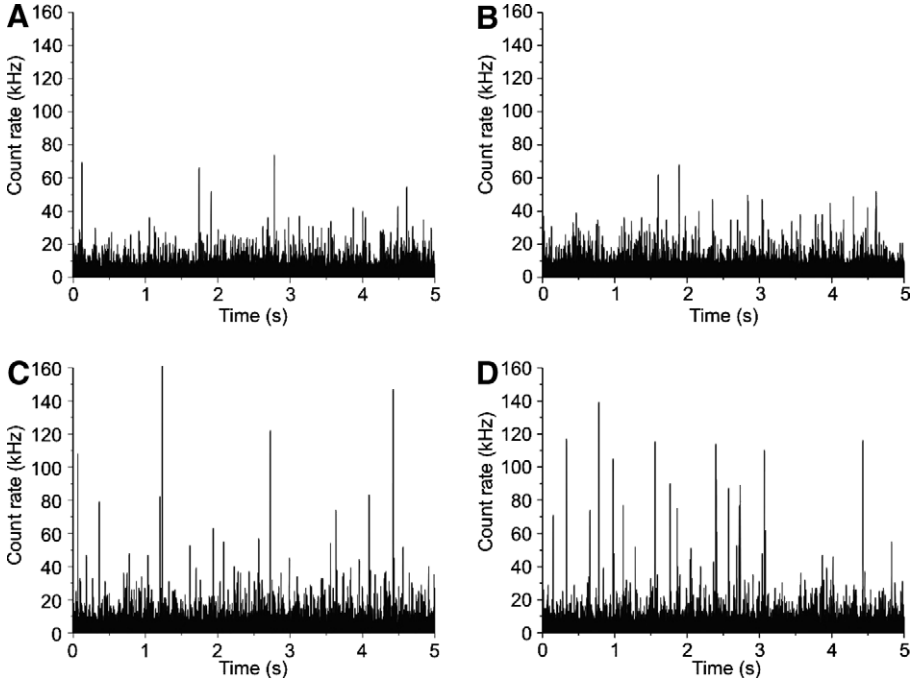

Fig. 3. Fluorescence intensity traces of the smart probe (5×10^{-10} M) mixed with the blocking oligonucleotides block A, block C and block G (each 1×10^{-6} M) in the absence of target DNA (A) and in the presence of 5×10^{-9} M (C) and 1×10^{-8} M (D) of the matching target DNA (target A). For the control experiment (B) a mixture of the mismatched target sequences target C, target G, target T (each 10^{-8} M) was added.

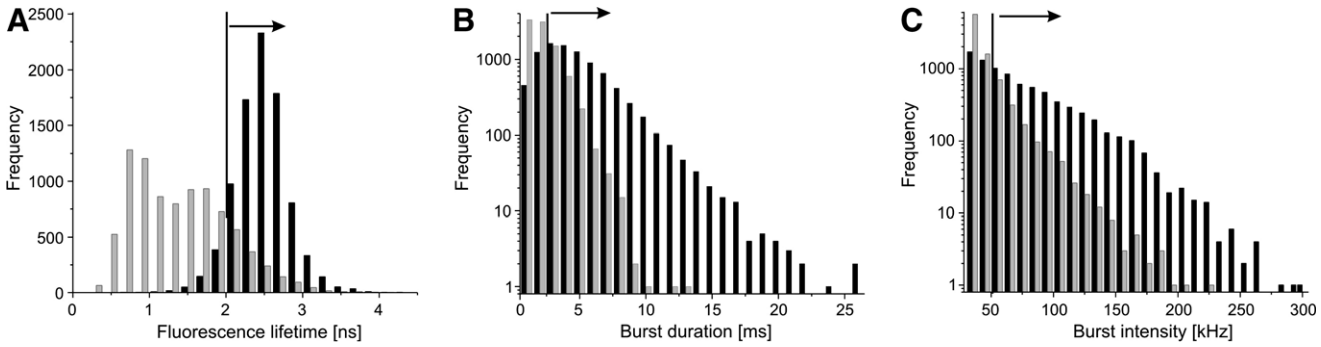

Fig. 4. Distributions of the fluorescence lifetime (A), the burst duration (B) and the burst intensity (C) measured for a non-hybridized (gray) and an hybridized (black) smart probe. For both samples 8800 bursts were analyzed.

oligonucleotides (Fig. 5, black) and mixed with all blocking oligonucleotides as described above (Fig. 5, green) in the presence of different concentrations of the target (filled circles) and mismatched DNA (opened circles). It can be clearly seen that the use of these additional unlabeled blocking oligonucleotides dramatically enhances the specificity, but on the other hand decreases sensitivity. The detection limit degrades by approximately two orders of magnitude to the nanomolar range. Most likely the relatively high concentration of the blocking oligonucleotides (2000-fold excess compared to the smart probe) partly masks the matching target sequence. Investigating all three blocking oligonucleotides separately shows that this effect is dominated by the guanosine containing blocking oligonucleotide (Fig. 5, red), whereas the effect of the other two blocking oligonucleotides is substantially smaller (Fig. 5, blue, pink). This can be explained by formation of a weak A–G base pair, which is significantly less stabile than a C–G or A–T base pair, but it stabilizes a DNA double helix comparable to a T–G, T–C or A–C mismatch [20,21]. In many medical or biological challenges a certain mutation has not be discriminated to all other nucleotides, but often a defined exchange has to be monitored. In such cases only one blocking oligonucleotide has to be added which leads to a better sensitivity. For example, our system is able to monitor an A–T or A–C exchange with high specificity and subnanomolar sensitivity.

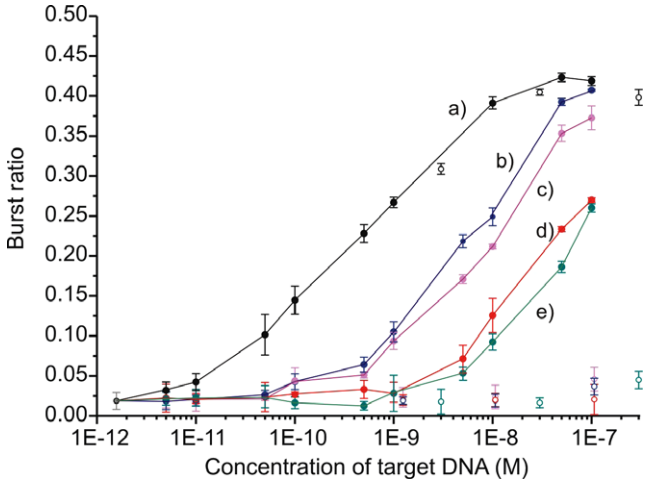

Fig. 5. Ratio of bursts with fluorescence lifetime >2.0 ns, duration >2.0 ms, and intensity (maximum count rate) >50 kHz to overall number of detected bursts for different samples in the presence of different concentrations of target DNA (target A). Black (a): smart probe (5×10^{-10} M) without any blocking oligonucleotides; blue (b): smart probe (5×10^{-10} M) mixed with block C (1×10^{-6} M); pink (c): smart probe (5×10^{-10} M) mixed with block A (1×10^{-6} M); red (d): smart probe (5×10^{-10} M) mixed with block G (1×10^{-6} M), green (e): smart probe (5×10^{-10} M) mixed with all three blocking oligonucleotides (each 1×10^{-6} M). The reference sample without any target DNA is represented by the gray data point. For control experiments (opened circles) the respective target sequences containing the mismatch were added (black: target T, C, G; blue: target G; pink: target T; red: target C, and green: target T, C, G). For each data point, three 60-seconds measurements were analyzed and averaged. The error bars were set in a way that each measurement is within the error bars.

In conclusion, an efficient and highly-sensitive method for SNP analysis using self-quenching probes in combination with blocking oligonucleotides is reported. This method enables the detection of specific DNA sequences among a huge excess (up to 100- to 1000-fold) of target oligonucleotides differing by a single nucleotide mismatch. A high sensitivity between 10^{-9} and 10^{-11} M, depending of the investigated mismatch, can be realized by applying time-resolved single-molecule spectroscopy and a multi-parameter analysis.

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