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Single nucleotide polymorphism analysis using different colored dye dimer probes

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Abstract

Fluorescence quenching by dye dimer formation has been utilized to develop hairpin-structured DNA probes for the detection of a single nucleotide polymorphism (SNP) in the penicillin target gene *pbp2x*, which is implicated in the penicillin resistance of *Streptococcus pneumoniae*. We designed two specific DNA probes for the identification of the *pbp2x* genes from a penicillin susceptible strain R6 and a resistant strain *Streptococcus mitis* 661 using green-fluorescent tetramethylrhodamine (TMR) and red-fluorescent DY-636, respectively. Hybridization of each of the probes to its respective target DNA sequence opened the DNA hairpin probes, consequently breaking the nonfluorescent dye dimers into fluorescent species. This hybridization of the target with the hairpin probe achieved single nucleotide specific detection at nanomolar concentrations via increased fluorescence.

1. Introduction

Since the first description of molecular beacons in 1996 [1], many self-quenching hairpin probes containing quenchers, such as Dabcyl, gold nanoparticles [2] or guanine residues [3], have been developed. Some of these probes are already being applied to a wide range of DNA-based diagnostic systems. It has been known for many years that at high concentrations many fluorescent dyes form nonfluorescent dye dimers as predicted by the excitation theory [4,5]. We have exploited dimerization induced fluorescence quenching for the construction of a new type of self-quenching hairpin-structured DNA probes. Upon hybridization of the dimer probes to a complementary target DNA the hairpin-structure is opened and thus preventing dye dimerization that results in an up to 10-fold increase of the fluorescence intensity with respect to the closed probe.

As a model system, we utilized sequences of genes implicated in penicillin resistance of *Streptococcus pneumoniae* that differ by only one nucleotide. *S. pneumoniae*, one of the major human pathogens, has become a paradigm for the evolution of antibiotic resistance, and strains with high level penicillin resistance are isolated with increasing frequency worldwide since the late 1970s [6]. Penicillin resistance in *S. pneumoniae* is due to alterations of penicillin-target enzymes, the penicillin-binding proteins (PBPs). Three PBPs are implicated in high level penicillin, with mutations in PBP2x being the basis and the prerequisite for high resistance levels [7]. Whereas penicillin susceptible *S. pneumoniae* isolates contain highly conserved *pbp* genes, altered PBPs of resistant clinical isolates are encoded by highly variable *pbp* genes that contain regions differing up to 20% on the DNA sequence level. These altered *pbp* regions are the result of gene transfer involving related commensal species such as *S. mitis* [7]. Since many different

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pbp2x variants have been described covering different regions of the *pbp2x* gene, identification of the highly conserved sensitive gene sequences is the method of choice to discriminate between sensitive and resistant strains; however, one nucleotide discrimination has to be ensured to obtain reliable test results. We have chosen now a region of the *pbp2x* gene known to be highly variable in resistant isolates where one to four nucleotide changes at seven different sites are altered in a resistant isolate: 5′-CAAAAAGAA **A** TTGTGGGAAATCCTGT-3′ from the susceptible laboratory strain *S. pneumoniae* R6, and the second sequence 5′-CAAAAAGAA **G** TTGTGGGAAATCCTGT-3′ occurring in resistant *S. mitis* strains such as *S. mitis* 661 (Hakenbeck, unpublished results). Oligonucleotides complementary to these sequences, also containing complementary nucleotides at both ends (underlined) to ensure a hairpin-structure of the probe, were designed: probe A = 5′-CAGGATTTCCCACAA **T** TTCTTTCCTG-3′ and probe B 5′-CAGGATTTCCCACAA **C** TTCTTTCCTG-3′.

2. Dimerization of the fluorescent dyes

With increasing concentration many fluorescent dyes form dimers that are classified as so-called J-type ('face-to-edge' conformation) and H-type ('face-to-face' conformation). It is supposed that dimer formation in solution is mainly due to hydrophobic interactions [8]. Usually only H-type dimers are observed in aqueous solution due to their higher stability [9], whereas J-type dimers can be formed in weakly polar solvent or in the adsorbed state [8,10]. Dimerization has been described for a wide variety of fluorescent dyes, e.g., the xanthene [11,12], phenothiazine [13], oxazine [14], squarine [15], BODIPY [16,17] and cyanine [5] dyes. As predicted by excitation theory [4,5], H-type dimers show a blue-shifted absorption band and the fluorescence intensity is strongly decreased compared to the monomers. The fluorescence quenching induced by dye dimerization has been exploited in several analytical methods. In enzyme assays for proteases, for example, a peptide substrate is labeled at both sides of the cleavage site with an identical fluorescent dye, whereby aggregation of the dyes decreases the fluorescence intensity in the intact (uncleaved) substrate peptide. Upon cleavage by the enzyme the spatial contact of the dyes is no longer maintained and the fluorescence intensity increases [18–20]. For these tests, tetramethylrhodamine (TMR) has been used frequently. It shows a dimerization constant of 7300 M^{-1} and 18 000 M^{-1} for the 5′-isomer and the 6′-isomer, respectively [21].

In the present publication we have constructed two different DNA hairpin probes labeled at both ends with the same dye, so that in the hairpin-structure the dyes can form dimers that result in strong fluorescent quenching. In the presence of a complementary DNA strand the hairpin structure is opened, thus the dimers are broken and fluorescence is restored. Two different dyes were used: the well known green-fluorescent tetramethylrhodamine (TMR, Molecular Probes), and the red-fluorescent DY-636 (Dyomics GmbH, Jena, Germany) for which we investigated the dimerization behavior for the first time. We recorded the absorption spectra of DY-636 ($\lambda_{abs} = 636$ nm) and TMR ($\lambda_{abs} = 560$ nm) solutions at different concentrations (Fig. 1). With increasing concentration the characteristic absorption band for the dimer is formed at 590 nm and 515 nm, respectively. The measurements were carried out in PEG-coated cuvettes to avoid adsorption effects of the highly hydrophobic DY-636.

For this dye, a very strong aggregation tendency was found and the dimerization constant estimated to be approx. 54 000 M^{-1}. For the calculation of the dimerization constant the extinction coefficient of the dye in water has to be known. Two measurements of the same dye concentration (5×10^{-7} M), one in ethanol ($\varepsilon = 200\,000$ L mol^{-1} cm^{-1})

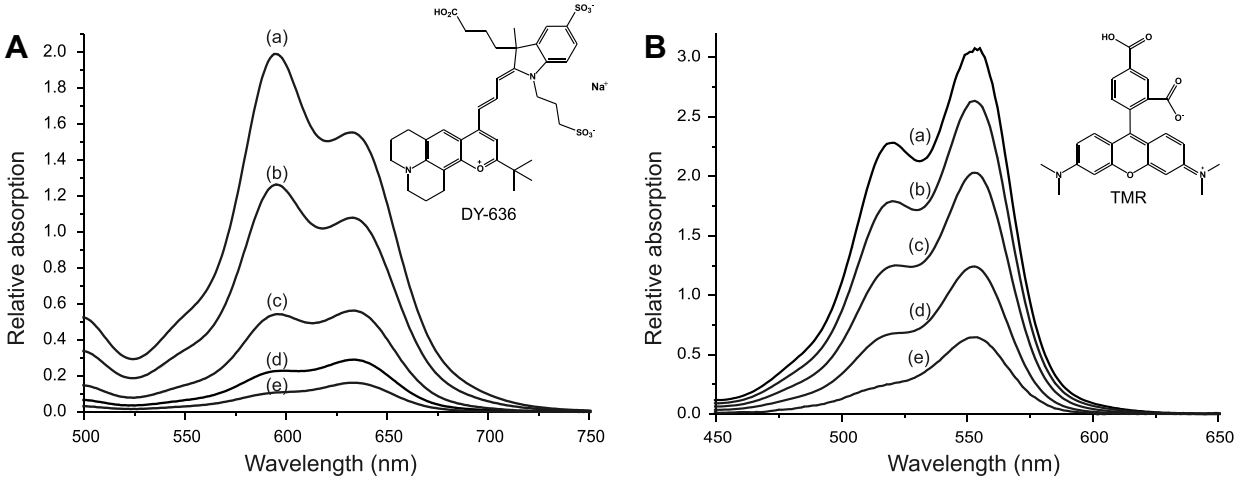

Fig. 1. (A) Absorption spectra of DY-636 at different concentrations: (a) 6×10^{-5} M, (b) 4×10^{-5} M, (c) 2×10^{-5} M, (d) 1×10^{-5} M, (e) 5×10^{-6} M. (B) Absorption spectra of TMR at different concentrations: (a) 1×10^{-4} M, (b) 8×10^{-5} M, (c) 6×10^{-5} M, (d) 3.6×10^{-5} M, (e) 2×10^{-5} M. Measurements were carried out in PEG-coated cuvettes with an optical pathway of 3 mm in PBS.

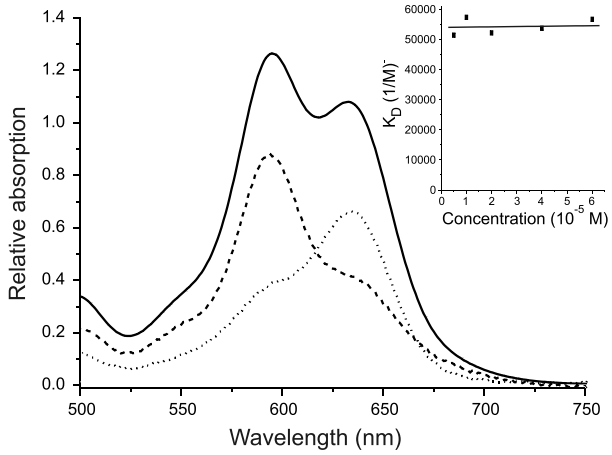

Fig. 2. Linear combination of the absorption spectrum (solid line) from a 4×10^{-5} M solution of DY-636 (4×10^{-5} M) in PBS and the resulting monomer spectrum (dotted) and dimer spectrum (dashed). Inset: calculated dimerization constants from the five measurements from Fig. 1 using this dimer spectrum (dashed line).

and one in aqueous solution, gives an extinction coefficient of 138 000 L mol^{-1} cm^{-1} for the DY-636 in water. We have determined the dimerization constant from a series of measurements using different dye concentrations. The monitored absorption spectra (Fig. 1a) can be separated by linear combination into a monomer and a dimer spectrum as shown in Fig. 2. Using the extinction coefficient of 138 000 L mol^{-1} cm^{-1} for DY-636 in water, the concentration of the monomeric dye can be calculated and thus also the dimer concentration and the dimerization constant. Because the spectrum of the pure dimer is unknown the calculation were done with different shapes of the dimer spectrum obtained by different linear combinations. Fig. 2 shows the linear combination which gives similar results for all measurements (Fig. 2, inset).

3. Properties of the DNA probes

The absorption spectra of the doubly TMR and DY-636 labeled hairpin probes before and after addition of the complementary target DNA are shown in Fig. 3. In agreement with the higher dimerization constant of DY-636 compared to TMR, the DY-636 labeled hairpin shows a stronger formation of H-type dye dimers (Fig. 3a,b). The DY-636 labeled hairpin probe shows a three times higher fluorescence increase than the TMR labeled hairpin (Fig. 3c) after hybridization to a complementary target sequence. In this context it is important to remember that the efficiency of dye dimerization with double-labeled hairpin probes is also dependent on the structure as well as the linker between probe and dye. Often the geometry of the probe prevents the efficient formation of dye dimers.

With respect to the free dye, the closed TMR probe shows just 25% of the fluorescence intensity. Even upon hybridization, i.e., in the presence of complementary DNA, the fluorescence intensity (75%) does not reach the

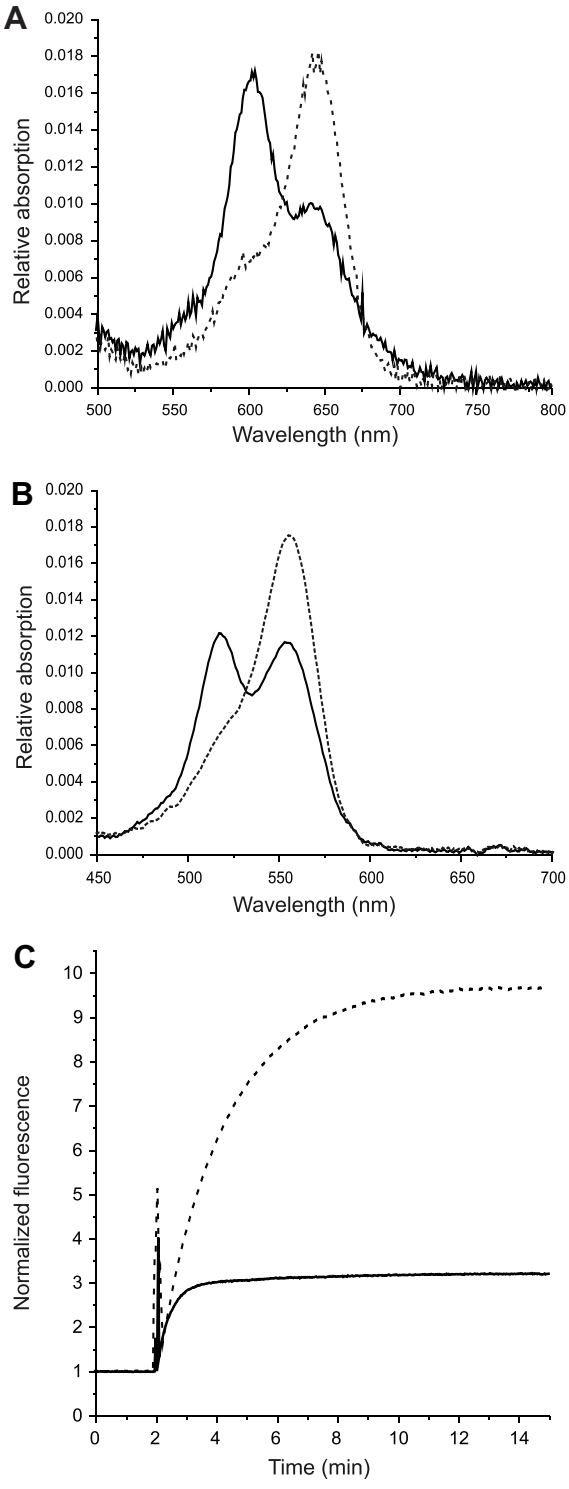

Fig. 3. Absorption spectra of the hairpin-structured probes (6×10^{-7} M) labeled with (A) two DY-636 dyes and (B) two TMR dyes in the absence (solid line) and the presence of a 10-fold excess of the matching target DNA (dashed line). Measurements were carried out in cuvettes with an optical pathway of 3 mm in PBS. (C) Increase of fluorescence intensity of the TMR-probe (solid) and the DY-636 probe (dashed) upon addition of a 10-fold excess of the target DNA at 2 min. The narrow peak at 2 min is due to scattering light while mixing the solution. In order to slow down the fluorescence increase the measurement was carried out at 10 °C.

fluorescence of the free dye. A possible explanation for this reduction in fluorescence intensity of the probe is unspecific quenching of the dyes by guanine residues via a photoinduced electron transfer reaction [22,23]. The fluorescence intensity of DY-636 depends much more on the molecular environment than that of TMR. For example, in ethanol, DY-636's fluorescence intensity is approx. ten times higher than in water. The DY-636 probe shows 42% of the fluorescence intensity of the free dye in water and 400% after hybridization to its target sequence. Indeed, upon addition of an excess of the complementary target DNA, we observed a 10-fold fluorescence increase for the DY-636 probe and a 3-fold increase for the TMR probe. At room temperature both probes hybridize very fast to the target DNA and the maximum fluorescence increase is achieved immediately. Therefore, the measurements were carried out at 10 °C, where the opening kinetics of the probes are significantly slower. As can be seen in Fig. 3c, under these conditions the TMR probe opens faster (50% of the maximum fluorescence increase is achieved after approx. 20 sec.) compared to the DY-636 probe (50% of the increase after approx. 90 sec.) which is in agreement with their dimerization constants. Due to stronger interactions between the two DY-636 dyes compared to the two TMR dyes, the DY-636 hairpin probe is more stable.

4. Discrimination between single nucleotide mutations

We have now used two probes that differed by one nucleotide, one labeled with TMR (probe A), corresponding to the *S. pneumoniae* R6 *pbp2x*, and the other with DY-636 corresponding to the *S. mitis* 661 (probe B). The probes were mixed at equimolar amounts and the DNA sample of interest was added. The mixture was heated to 65 °C and allowed to cool to room temperature at a rate of 1 °C/2.5 min. The assay system is schematically shown in Fig. 4. Due to the very slow temperature decrease, the probes have sufficient time to reach the thermodynamic equilibrium. Thus, the exact complementary probe hybridizes at a higher temperature to the target DNA compared to the mismatch probe. For this experiment, it is important that the concentration of the fluorescent probes is in excess of the target DNA, since in the reverse situation, i.e., if the target DNA is in excess, hybridization can also occur with the mismatch probe.

Fig. 5 shows the results of the experiments. The fluorescence spectra of a mixture of both hairpin probes (10^{-7} M) in absence and presence of different DNA target sequences can be seen in Fig. 5a. After cooling of the reaction mixture as described, the sample was measured twice, with an excitation wavelength of 550 nm and 640 nm, in order to record the fluorescence of both, the TMR probe and the DY-636 probe as well. The emission maximum of the TMR probe is 585 nm and that of the DY-636 probe 670 nm. In the absence of any target DNA, both probes are closed and the resulting fluorescence spectra are depicted in Fig. 5a, solid line. In agreement with the more efficient quenching of the DY-636 probe, the fluorescence intensity at 670 nm is lower than that at 585 nm. The narrow peaks at the excitation wavelengths (550 nm and 640 nm) are due to light scattering in the sample. These peaks show neglectable values at 585 nm and 670 nm, respectively (data not shown). In the presence of 7.5×10^{-8} M of the R6 target DNA (Fig. 5a, dashed line) the fluorescence intensity at 585 nm i.e., the TMR probe increases from 4.8 to approx. 11.0 corresponding to a 2.3-fold increase. However, the fluorescence at 670 nm, i.e., the DY-636 probe, increases only by a factor of 1.3, which corresponds only to approx. 3% of the maximum increase (10-fold). In the presence of the same amount of

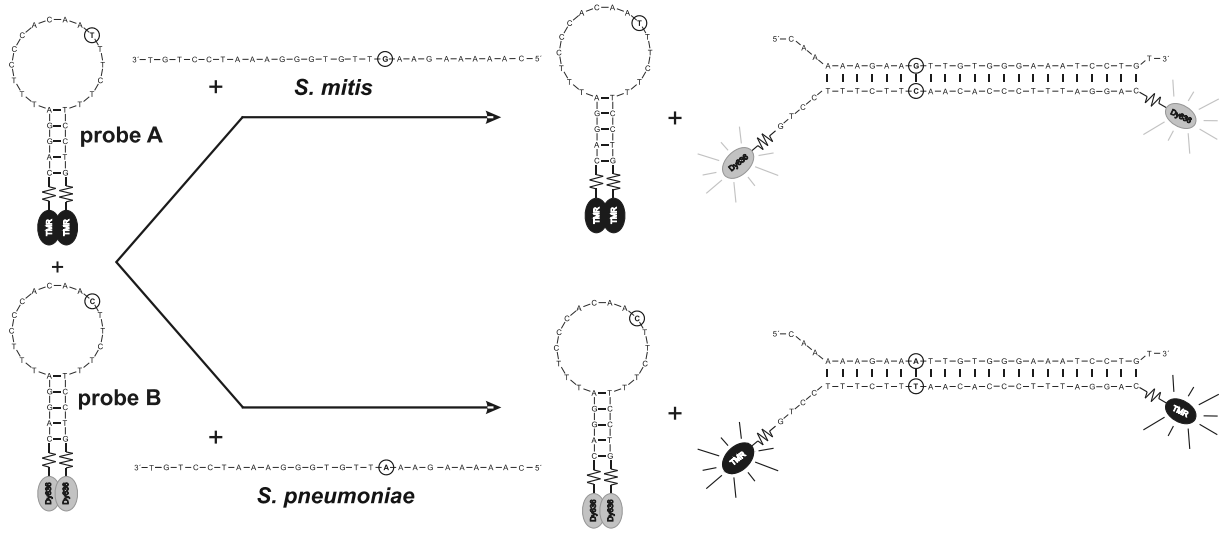

Fig. 4. Schematic diagram of the 'two-color' identification of a single mismatch between two DNA sequences. In the presence of the *S. pneumoniae* R6 target sequence the TMR-dimer probe forms a DNA double helix, causing a fluorescence increase at 585 nm, while the DY-636-dimer probe remains closed. If the *S. mitis* 661 sequence is present, the fluorescence of the DY-636 at 670 nm increases and the TMR-dimer probe remains in the hairpin-structure.

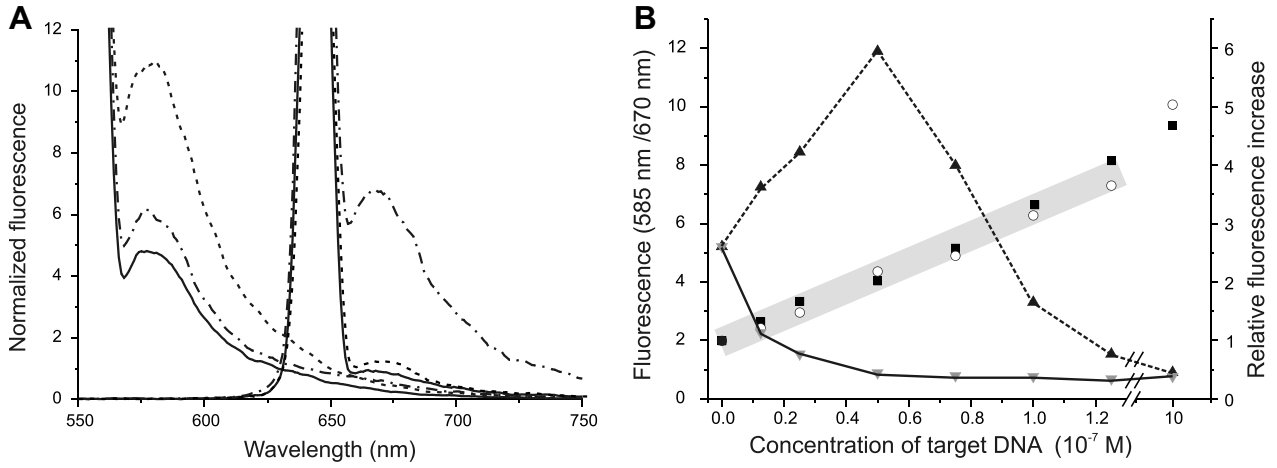

Fig. 5. (A) Fluorescence spectra of an equimolar mixture of both DNA probes (10^{-7} M) in PBS recorded with an excitation wavelength of 550 nm and 640 nm. The spectra were measured in the absence of target DNA (black line) and after addition of 7.5×10^{-8} M of the *S. pneumoniae* R6 target sequence (dashed line) and the *S. mitis* 661 target sequence (dotted-dashed line), respectively. (B) Ratio of the fluorescence maxima at 585 nm and 670 nm obtained from measurements with an equimolar mixture of both DNA probes (10^{-7} M in PBS) with various concentrations of the *S. pneumoniae* R6 target sequence (dashed line) and the *S. mitis* 661 target sequence (solid line). The squares (for R6 target) and the circles (for 661 target) show the relative overall fluorescence intensity, which is the sum of the max. intensities at 585 nm and 670 nm.

the 661 target DNA, a 7-fold fluorescence increase at 670 nm was observed, whereas only a 1.2-fold increase of fluorescence intensity, which is 10% of the maximum increase (3-fold), was obtained at 585 nm (Fig. 5a, dotted-dashed line).

In order to further demonstrate the sensitivity and specificity of this assay system, the experiment described before was carried out using different concentrations of the respective target DNA sequences. The results were analyzed by calculating the ratio of the fluorescence at 585 nm and the fluorescence at 670 nm (Fig. 5b). This has a major advantage over the calculation of the absolute fluorescence increase compared to a reference sample for several reasons. First, the fluorescence intensity of the reference and the sample of interest can vary significantly due to different adsorption to the plastic reaction tubes during the relatively long temperature shift of the experiment. Second, the power of the excitation source or the detection efficiency may fluctuate between different measurements. We found that the fluorescence intensity between different reference samples varied up to 30%, but the ratio between the fluorescence at 585 nm and 670 nm stayed constant with a value around 5.1.

In the presence of the *S. pneumoniae* R6 target sequence the complementary TMR probe opens better compared to the DY-636 probe; accordingly the fluorescence intensity at 585 nm increases more than at 670 nm and thus this ratio increases (Fig. 5b, dashed line) whereas in the presence of the *S. mitis* 661 sequence the ratio decreases (Fig. 5b, solid line). At a concentration of 12 nM (0.12×10^{-7} M) target DNA a significant change in the fluorescence ratio occurs and it can easily be discriminated between the two sequences. Up to a concentration of 50 nM (0.5×10^{-7} M) of the target DNA, the ratio between the two fluorescence intensities increases and a good discrimination is achieved. If higher concentrations of the target DNA are used, the ratios converge and the discrimination gets worse, since an excess of the target DNA leads to an opening of both hairpin probes. That means that each value of this ratio can be caused by two different concentrations of the respective target DNA. Therefore, it is important to measure the overall fluorescence intensity (which is the sum of the intensity at 585 nm and at 670 nm) which increases with the total amount of target DNA. With this information the proper DNA concentration can be assigned to the measured ratio.

In conclusion, we have shown that the formation of nonfluorescent dye dimers can be used for developing efficient hairpin-structured DNA probes. The DY-636 shows a very strong aggregation tendency and its dimerization constant of approx. 54 000 M^{-1} is much higher than that of TMR, which has been usually applied for dimerization experiments. By using two probes labeled with different colored dyes, an assay system has been developed that is able to discriminate between two DNA sequences that differ in only one nucleotide in one cuvette at nanomolar sensitivity through standard fluorescence spectroscopy.

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