# Twin Probes as a Novel Tool for the Detection of Single-Nucleotide Polymorphisms

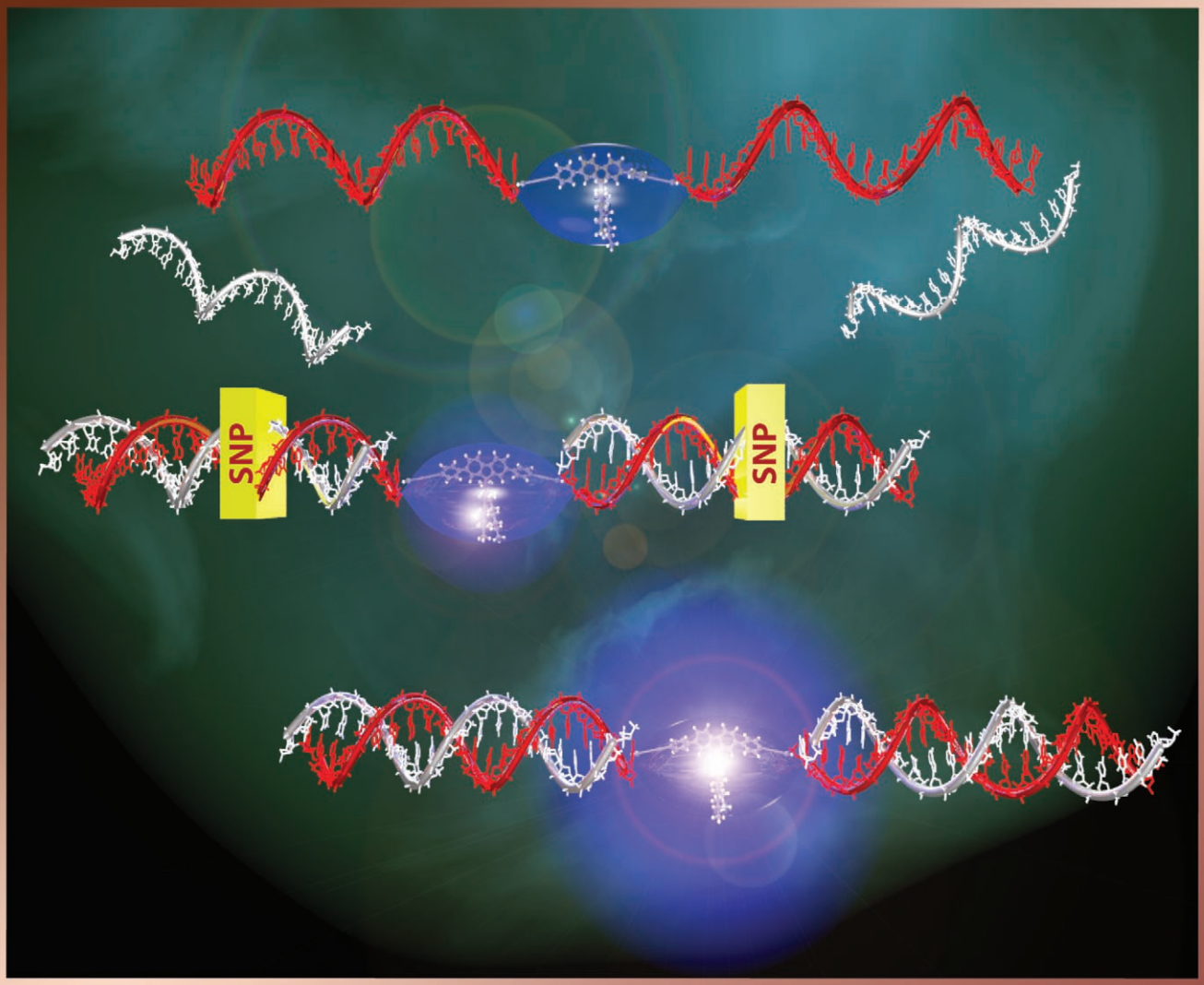
Ergen, Erhan; Weber, Markus; Jacob, Josemon; Herrmann, Andreas; Müllen, Klaus

*Published in:*
Chemistry : a European Journal



*Publication date:*
2006

# Twin Probes:
## A Novel Tool for SNP Detection




For more information see the following pages.

CHEMISTRY
A EUROPEAN JOURNAL

DOI: 10.1002/chem.200501526

VIP

# Twin Probes as a Novel Tool for the Detection of Single-Nucleotide Polymorphisms

**Erhan Ergen, Markus Weber, Josemon Jacob, Andreas Herrmann,* and Klaus Müllen*[a]**

**Abstract:** Single-nucleotide polymorphisms (SNPs) are the most common form of DNA sequence variation. There is a strong interest from both academy and industry to develop rapid, sensitive and cost effective methods for SNP detection. Here we report a novel structural concept for DNA detection based on fluorescence dequenching upon hybridization. The so-called "twin probe" consists of a central fluorene derivative as fluorophore to which two identical oligonucleotides are covalently attached. This probe architecture is applied in homogeneous hybridization assays with subsequent fluorescence spectroscopic analysis. The bioorganic hybrid structure is well suited for sequence specific DNA detection and even SNPs are identified with high efficiency. Additionally, the photophysical properties of the twin probe were investigated. The covalent attachment of two single stranded oligonucleotides leads to strong quenching of the central fluorescence dye induced by the nucleobases. The twin probe is characterized by supramolecular aggregate formation accompanied by red-shifted emission and broad fluorescence spectra.

**Keywords:** DNA detection · DNA · fluorescent probes · hybridization

## Introduction

With the completion of the human genome project, maps of human sequence variations have been reported and deposited to public databases.[1–3] Among these variations, single-nucleotide polymorphisms (SNPs) are the most frequent and according to the International SNP Map Working Group, the occurrence of SNPs in human genes is approximately 1.42 million, 60000 of them being located in exons.[4] Since SNPs are assumed to play an important role in determining the genetic predisposition towards inherited diseases, disease diagnosis, and personalized medicine,[5] there is a strong interest in developing rapid, sensitive, and cost effective detection methods. Various detection schemes are presently available for SNPs.[6,7] When utilizing hybridization in homogeneous solution, a few formats have been established which make use of fluorescence resonance energy transfer (FRET).[8] The spatially close arrangement of a donor and an acceptor is realized in TaqMan probes[9,10] and molecular beacons.[11,12] Instead of using interactions between two extrinsic probes, interactions of one fluorophore with DNA bases can be used for specific detection of nucleic acid sequences.[13,14] Besides the use of fluorescein,[15,16] very recently fluorene has also been incorporated into the loop structure of a hairpin architecture.[17] A single base mismatch was detected by de-quenching upon hybridization with the fully matched target and quenching upon hybridization with a target containing a single base mutation.

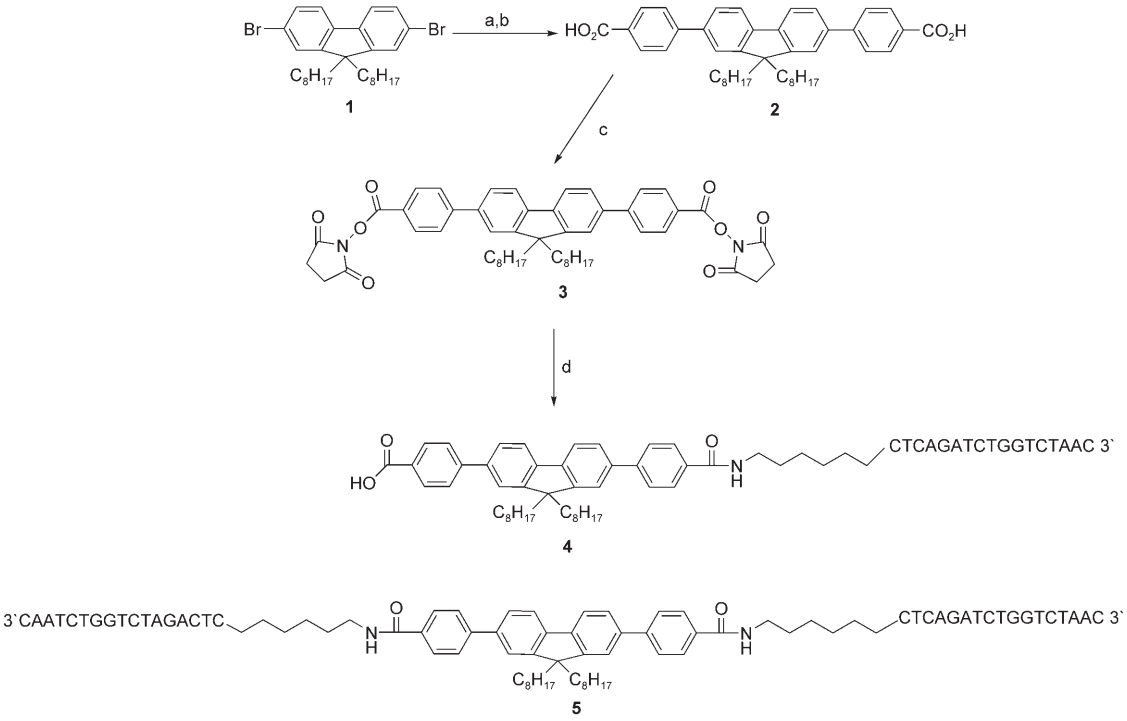
In this paper, we report a novel structural concept for DNA detection. The so-called "twin probe" consists of a single central fluorophore to which two identical oligonucleotides have been covalently attached. The twin probe is applied in a homogeneous DNA detection assay. SNPs could be detected upon hybridization of the twin probe and a target sequence with high efficiency.

## Results and Discussion

As a central emitter, the fluorene derivative **2** was chosen for two reasons. First, it is an efficient blue emitter with a high fluorescence quantum efficiency in organic solvents[18,19] as well as water.[20] Second, at a later stage it will be advantageous to extend this concept from monomeric to polymeric emitters, which have already found applications as biosensors.[21–23]

[a] E. Ergen, M. Weber, Dr. J. Jacob, Dr. A. Herrmann, Prof. Dr. K. Müllen
Max Planck Institute for Polymer Research
Ackermannweg 10, 55128 Mainz (Germany)
Fax: (+49) 6131-379-350
E-mail: andherrm@mpip-mainz.mpg.de
muellen@mpip-mainz.mpg.de




Scheme 1. Synthesis of fluorescent core and oligonucleotide conjugates: a) 4-Carboxymethylphenylboronic acid, $K_2CO_3$, THF/$H_2O$, [$Pd(PPh_3)_4$], 80 °C, 16 h, 73 %; b) KOH, EtOH/$H_2O$, reflux, 3 h, 88 %; c) *N*-hydroxysuccinimide (NHS), dicyclohexylcarbodiimide (DCC), DMF, RT, overnight; d) 5′-amino modified oligonucleotide, DMF/sodium tetraborate decahydrate buffer (pH 8.5), RT, overnight.

The synthesis of the core fluorophore started from the well-known fluorene derivative **1** (Scheme 1).

2,7-Dibromo-9,9-di-*n*-octylfluorene (**1**) was treated in a palladium-catalyzed Suzuki coupling[24] with 4-carboxymethylphenylboronic acid. Alkaline hydrolysis of the resulting diester generated the biscarboxylic acid **2**. Subsequent activation of the carboxylic acids was carried out with *N*-hydroxysuccinimide. The corresponding active ester **3** was treated with a 17-mer oligonucleotide sequence (5′-CTCAGATCTGGTCTAAC-3′) carrying a 5′-amino linker to generate the desired twin probe **5** along with the monoconjugate **4** as a by-product. Purification of the target product **5** and monoconjugate **4** was performed by polyacrylamide gel electrophoresis (PAGE) using a 20 % denaturing gel. The purity of **4** and **5** was proven by PAGE analysis (Figure 1A).

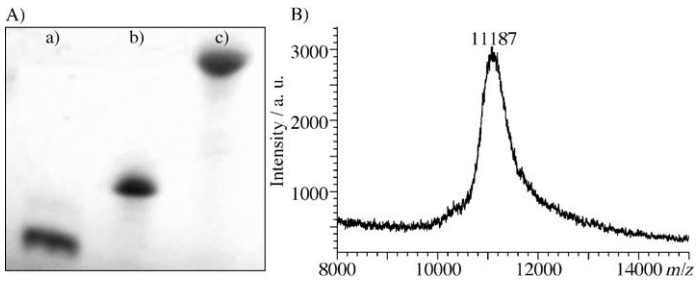



Figure 1. A) Polyacrylamide gel electrophoresis analysis of **4** and **5**. a) Oligonucleotide (5′-$NH_2$-$(CH_2)_6$-CTCAGATCTGGTCTAAC-3′); b) monoconjugate **4**; c) twin probe **5**. B) MALDI-TOF mass spectrum of **5** (calcd: 11241 g mol$^{-1}$; found: 11187 g mol$^{-1}$).

Compounds **4** and **5** exhibited a significant lower electrophoretic mobility than the amino modified oligonucleotide, which was used as a starting material. With MALDI-TOF mass spectrometry the identity of **5** was confirmed (Figure 1B).

Two identical 5′-amino modified oligonucleotide sequences were used for coupling to the fluorophore core as these modifications can be more easily accessed by automated synthesis in contrast to 3′-modifications. Furthermore, 5′-modified oligonucleotides are commercially available so that the twin probe **5** can be synthesized in a standardized fashion without the need for an oligonucleotide synthesizer.

To study the influence of the fluorene moiety and the attached C6-alkyl spacers on duplex formation, the melting temperature ($T_m$) of the twin probe **5** and its complementary sequence with that of the free oligonucleotide with the same sequence were compared. The melting temperature $T_m$ of **5** was 58 °C; whereas, the free oligonucleotides exhibited a $T_m$ of 57 °C. This small difference in $T_m$, which lies within the experimental error of the measurement, indicates that there are only weak interactions between the chromophore and the attached oligonucleotide sequence.

To test the suitability of twin probe **5** for SNP detection, hybridization experiments were carried out (Table 1, Figure 2). The nonhybridized twin probe **5** showed low fluorescence intensity in buffer solution upon excitation with 350 nm. Hybridization of the twin probe **5** with the target sequence ODN1, which fully matched the probe sequence, led to a ten-fold increase in fluorescence intensity. Simulta-

neously, a hypsochromic shift of the emission band occurred from 432 to 417 nm. When **5** was incubated with a noncomplementary target ODN2 only a 1.6-fold enhancement of the fluorescence intensity was observed, which is accompanied by a hypochromic shift of the emission maxima from 432 to 439 nm. On the basis of the hybridization results of the twin probe **5** with the fully matching and a noncomplementary sequence, the possibility of detecting a single-nucleotide mismatch was investigated. Accordingly, two target sequences ODN3 and ODN4 containing an A to C and an A to G mismatch, respectively, were examined. Upon hybridization, the mismatch is located within the helix of the probe and target sequences (Table 1). When **5** was hybridized with the mismatched sequences ODN3 and ODN4, the emission intensities dropped to 49 and 25%, respectively, compared with the signal obtained with the fully matched sequence ODN1. Upon duplex formation of the mismatched sequences ODN3 and ODN4 with probe **5**, the emission maxima shifted hypsochromically from 432 to 420 nm (Figure 1, Table 1). Very similar emission behavior of **5** upon hybridization was found for probe concentrations ranging from $10^{-5}$ to $10^{-8}$ M. Especially, the lower limit is of importance since a typical polymerase chain reaction results in equal amounts of DNA.[25] The fluorescence measurements (Table 1), clearly demonstrated that the novel twin probe architecture is well suited for sequence specific DNA assays and even a single base mismatch could be detected with a discrimination factor of up to 4.

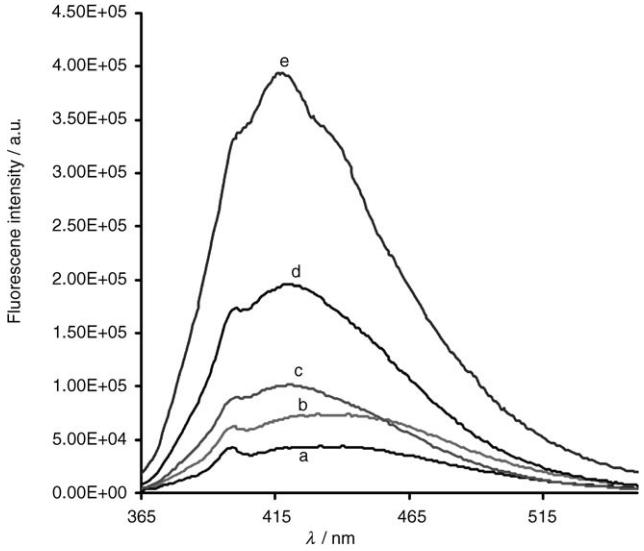



Figure 2. Emission spectra of the twin probe **5** ($1.7\times10^{-7}$ M) and corresponding duplexes of **5** with ODN1 to ODN4. All oligonucleotides have been used in two-fold excess with respect to **5**: a) nonhybridized twin probe **5**, b) noncomplementary ODN2 + **5**, c) A to G mismatch ODN4 + **5**, d) A to C mismatch ODN3 + **5**, and e) complementary ODN1 + **5**. Fluorescence spectra were obtained using an excitation wavelength of 350 nm and were recorded at room temperature. Hybridization buffer solutions contained 100 mM Tris base, 100 mM NaCl, and 50 mM $MgCl_2$.

Table 1. Fluorescence spectroscopic analysis of the twin probe **5** and the corresponding hybridization experiments with different target sequences. Mismatches are in italic.

| | Sequence | Rel. fluorescence int. [%] | Em. max. [nm] |
|---|---|---|---|
| **5** | probe: 5′$NH_2$-CTCAGATCTGGTCTAAC-3′ | 11 | 432 |
| ODN1 | comp: 3′-GAGTCTAGACCAGATTG-5′ | 100 | 417 |
| ODN2 | noncomp: 3′-*ACTCTTATCACATACGC*-5′ | 18 | 439 |
| ODN3 | A to C: 3′-GAGTC*C*AGACCAGATTG-5′ | 49 | 420 |
| ODN4 | A to G: 3′-GAGTC*G*AGACCAGATTG-5′ | 25 | 420 |

Hybridization experiments were also carried out with the fluorene derivative **4** connected only to a single-oligonucleotide sequence. This compound exhibited high fluorescence intensity in buffer solution. Upon hybridization with its complementary sequence, a decrease in fluorescence intensity was observed (data not shown). Since the aim was to generate a system, which produces an increase in the fluorescence signal upon hybridization, monoconjugate **4** was not suited for such detection purposes. However, to gain further insight into the factors that determine the fluorescence behavior of the central fluorophore within twin probe **5**, monoconjugate **4** is well suited for comparative studies. To investigate the effect of the four different nucleotides occurring in DNA on the optical properties of the fluorene moiety, additional quenching experiments were performed with **4** and $A_{16}$, $G_{16}$, $C_{16}$, $T_{16}$, respectively. Monoconjugate **4** has been utilized in the quenching experiments because contrary to **2**, it is readily soluble in buffer solutions. The fluorescence intensities of the **4** ($1.6\times10^{-7}$ M) in the presence of various 16-mer concentrations ranging from $7.0\times10^{-5}$ to $1.5\times10^{-3}$ M were measured and expressed in Stern–Volmer plots.[26] With increasing concentrations of $A_{16}$, $G_{16}$, $C_{16}$, and $T_{16}$, a decrease in the emission intensity of **4** was observed. An exponential relationship in the Stern–Volmer plot was obtained for quencher concentrations higher than $5.0\times10^{-4}$ M (data not shown), which can be explained by sphere-of-action quenching also present in other polyelectrolyte systems.[27] For oligonucleotide concentrations below $5.0\times10^{-4}$ M, a linear dependence in the Stern–Volmer plot was obtained (Figure 3) from which $K_{SV}$ values were calculated. The most efficient quencher was $G_{16}$ with a $K_{SV}$ of $5.1\times10^{-4}\,M^{-1}$. The $K_{SV}$ values measured for $C_{16}$ and $T_{16}$ were $3.8\times10^{-4}\,M^{-1}$ and $3.5\times10^{-4}\,M^{-1}$, respectively, the difference in magnitude being within the uncertainty of the measurement. The oligonucleotide with the lowest effect on the emission intensity of the fluorene moiety was $A_{16}$ with a $K_{SV}$ of $1.3\times10^{-4}\,M^{-1}$. The data from the quenching experiments were also in agreement with the results obtained from hybridization. An A to G mismatch in ODN4 led to a higher extent of quenching than the A to C mismatch in ODN3 underpinning the fact that G was the most efficient quencher for fluorene connected to oligonucleotides in conjugates **4** and **5**.

Fluorescence quenching by nucleobases also referred to as base quenching has been mentioned previously. Photoin-

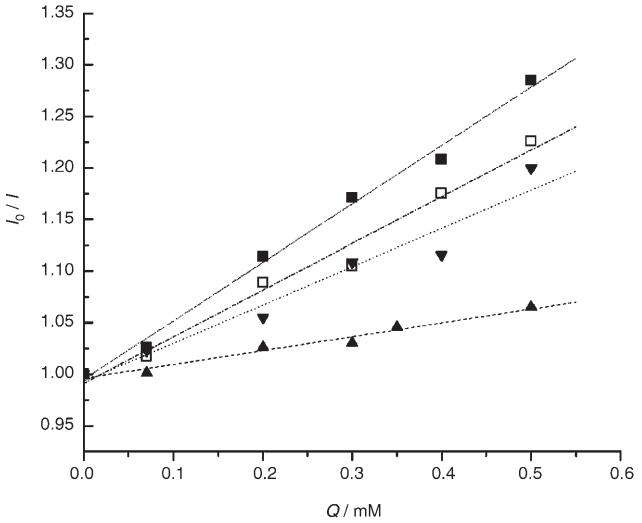



Figure 3. Stern–Volmer plots measured in hybridization buffer for $G_{16}$ (■), $C_{16}$ (□), $T_{16}$ (▼), and $A_{16}$ (▲) with the monoconjugate **4** (conc. $1.6\times10^{-7}$ M). The emission spectra were measured upon excitation at 350 nm.

duced electron transfer plays an important role for the mechanism of quenching of the excited dyes. However, other effects like coupled proton transfer and hydrophobic interactions need to be considered as well in the context of fluorescence quenching.[28] Due to the good electron donating properties of guanosine residues, this base has been reported to quench the emission of other fluorescent dyes efficiently.[15,29] The fluorescence measurements performed with the monoconjugate **4** suggest that through the covalent attachment of the second oligonucleotide chain, as realized in the bisconjugate **5**, the quencher concentration is becoming high enough to induce a low fluorescence emission from the fluorene emitter in the nonhybridized probe **5**.

The broad fluorescence spectra of **5**, and its hybridization products, are a hint that besides base quenching, also aggregate formation of the hydrophobic fluorene units influences the emission behavior of twin probe **5**. The aggregation of fluorene units, leading to long wavelength emission and broadened spectra, is well known and has been investigated intensively in the context of polymeric materials.[30] To gain further insight into aggregate formation of the central emitter unit, avidin–biotin binding was applied. A biotin moiety was introduced into the target strands, which are either fully complementary or noncomplementary to the probe sequence of **5**. Hybridization of twin probe **5** with the biotinylated oligonucleotides led in the case of the fully matching sequence to a 5.4-fold increase of the fluorescence intensity, whereas with the noncomplementary sequence only a 1.6-fold increase was obtained (Figure 4, Table 2). The addition of avidin to both hybridization solutions resulted in a further increase of the fluorescence intensity for the matching ODN5 and no changes for ODN6. Equal amounts of avidin with respect to biotin groups were used. More important, the biotin–avidin interaction induced a significant hypsochromic shift of the emission wavelengths from 445 to 402 nm for the hybridization product of **5** and ODN5 (Table 2, Figure 4). For the noncomplementary oligonuleotide ODN6 no changes of the fluorescence spectrum was observed.

This shift of the emission wavelengths suggests that the binding of biotin and avidin molecules in the hybridization buffer interferes with the π-stacking and/or hydrophobic interactions of the emitter units, supporting the hypothesis that supramolecular aggregate formation plays an important role for the twin probe nucleic acid detection system.

After elucidating the factors that determine the photophysical properties of the twin probe system, the generality of the new detection system regarding variations in sequence composition needs to be demonstrated. It should be

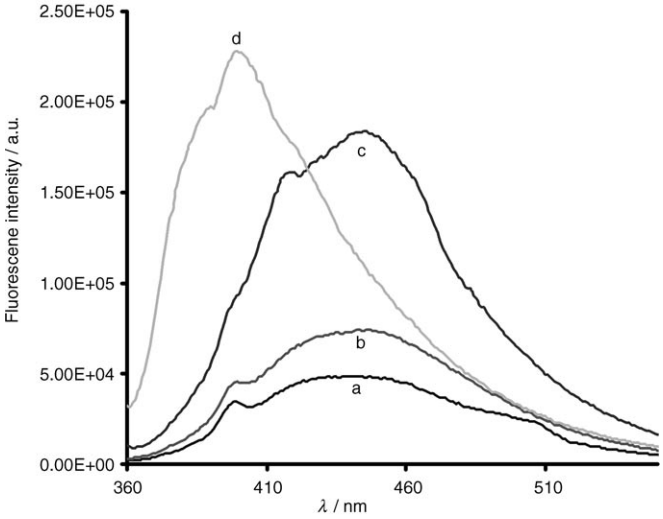



Figure 4. Emission spectra of the twin probe **5** ($2.8\times10^{-7}$ M) and the corresponding hybridization products with biotinylated target sequences without and in the presence of avidin. The target sequences were added in twofold excess with respect to **5**. a) Nonhybridized twin probe **5**, b) noncomplementary biotin modified ODN5 + **5**, c) biotin-modified complementary ODN6 + **5**, d) biotin-modified complementary ODN6 + **5** in the presence of equimolar amounts of avidin with respect to biotin groups. Fluorescence spectra were obtained using an excitation wavelength of 350 nm and were recorded at RT. Hybridization buffer solutions contained 100 mM Tris base, 100 mM NaCl, and 50 mM $MgCl_2$.

Table 2. Fluorescence spectroscopic analysis of the hybridization experiments of the twin probe **5** and biotinylated target sequences in the presence of and without avidin. The biotin-modified bases are indicated by $^{B}$.

| | Sequence | Rel. fluorescence int. [%] | Em. max. [nm] |
|---|---|---|---|
| **5** | probe: 5′$NH_2$-CTCAGATCTGGTCTAAC-3′ | 26 | 446 |
| ODN6 | comp-biotin: 3′-GAGTCT$^{B}$AGACCAGATTG-5′ | 100 | 442 |
| ODN6 | comp-biotin + avidin: 3′-GAGTCT$^{B}$AGACCAGATTG-5′ | 114 | 403 |
| ODN5 | noncomp biotin: 3′-AATACT$^{B}$CACTTTACACT-5′ | 42 | 447 |
| ODN5 | noncomp biotin + avidin: 3′-AATACT$^{B}$CACTTTACACT-5′ | 45 | 448 |

noted that the 17-mer oligonucleotide sequence (5′-CTC*A-GA*TCTGG*TCT*AAC-3′) of **5** can adopt a loop structure by base pairing of the italicized oligonucleotides which might influence the results of fluorescence measurements. Therefore, twin probes **6** (5′-CGCTTCATTTGTTCTCCC-3′) and **7** (5-CTGACTGGGTTGAAGGCTCT-3′) were synthesized by the same procedure as described above. The sequences of **6** and **7** were designed to avoid any secondary structure formation. The results of the hybridization experiments with subsequent fluorescence spectroscopic analysis are summarized in Table 3.

Table 3. Fluorescence spectroscopic analysis of the twin probes **6** and **7** as well as the corresponding hybridization experiments with different target sequences. Mismatches are in italic.

| | Sequence | Rel. fluorescence int. [%] | Em. max. [nm] |
|---|---|---|---|
| **6** | probe:5′$NH_2$-CGCTTCATTTGTTCTCCC-3′ | 48 | 436 |
| ODN7 | comp: 3′-GCGAGAACAAATGAAGCG-5′ | 100 | 438 |
| ODN8 | C to A: 3′-GCGAA*A*TAAACAAGAGGG-5′ | 66 | 442 |
| ODN9 | noncomp: 3′-*TGTACTGCCTCCAACA*-5′ | 50 | 444 |
| **7** | probe: 5′$NH_2$-CTGACTGGGTTGAAGGCTCT3′ | 20 | 421 |
| ODN10 | comp: 3′-GACTGACCCAACTTCCGAGA-5′ | 100 | 430 |
| ODN11 | T to G: 3′-GACTG*G*CCCCAACTTCCGAGA-5′ | 62 | 439 |
| ODN12 | noncomp: 3′-*AAGTTAGGTGGGCCTAATTT*-5′ | 38 | 421 |

For twin probes **6** and **7**, a similar behavior as for **5** was detected. When not hybridized, **6** and **7** exhibit a low fluorescence intensity. Upon base pairing with the fully complementary sequences, the emission increased two- and fivefold, respectively. Addition of the noncomplementary sequences only slightly increased the fluorescence intensity. As described for **5**, SNPs were also detected with **6** and **7**.

These measurements demonstrated that secondary structure formation of the attached ODNs to the fluorene emitter only slightly influenced the performance of the detection assay. More important, the general applicability of the novel twin probe system with respect to sequence composition was proved.

In TaqMan probes[9,10] and molecular beacons,[11,12] donor and acceptor dyes must be covalently attached to the termini of the probe sequences. Even though both methods are applied commercially, each method suffers from dual-labeling of DNA. This ultimately results in low yields, while the presence of impurities can adversely affect the sensitivity of the assay.[31] Another drawback is that although design algorithms for the sequences are well elaborated, both probe sequences require significant optimization and redesign. The twin probe system offers several advantages, most noticeably, the ability to introduce the fluorene moiety **3** (Scheme 1) between virtually any sequence of choice. Also, the need for dual labeling of an oligonucleotide at both the 5′- and 3′-ends can be eliminated, which makes the twin probe assay relatively inexpensive. In addition, one of the 3′-ends in the twin probe, or one of the alkyl chains of the fluorene moiety,[32] could be used for immobilization to transfer the twin probe assay into a chip-based format.

## Conclusion

In summary, a novel architecture for DNA detection was presented, the so-called "twin probe", where two identical oligonucleotide sequences were attached to a central fluorene emitter. The synthesis of the twin probe was carried out in four straightforward synthetic steps. The twin probe system appears to be well suited for sequence specific DNA detection by means of fluorescence in a homogeneous hybridization assay. The twin probe shows a remarkable selectivity that even allows identification of different SNPs. The general applicability of this nucleic acid detection scheme was demonstrated regarding sequence composition. Two factors seem to influence the photophysical properties of the twin probe. First, the attachment of two oligonucleotides to the central emitter leads to efficient quenching of the fluorene by the nucleobases. Second, supramolecular aggregate formation induced by the fluorene leads to broad red-shifted emission. And finally, the aggregates can be destroyed by the strong biotin–avidin interaction.

To broaden the field of application of twin probes, such as real-time PCR, the sites of oligonucleotide attachment will be varied. Future work will also be dedicated to transfer our novel detection concept to oligo- and polyfluorenes.

## Experimental Section

Unless otherwise specified, materials were obtained from commercial suppliers and used without further purification. 2,7-Dibromofluorene was purchased from Aldrich and used as such. *N*-Hydroxysuccinimide (NHS) was purchased from Acros, *N*,*N*′-dimethylformamide (DMF) from Fluka, and 1,3-dicyclohexylcarbodiimide (DCC) from Merck. Dimethoxytrityl (DMTr) protected phosphoramidites, trityl protected C6-amino linker and polystyrene/divinylbenzene support were purchased from Perbio, Glen Research and Amersham Biosciences, respectively. $^1$H and $^{13}$C NMR spectra were recorded on a Bruker AMX 250 (250 and 62.5 MHz, respectively) spectrometer. Molecular weights were determined using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS), using a Bruker MALDI-TOF (Reflex-TOF) mass spectrometer. Field desorption (FD) mass spectra were recorded on a VG Instruments ZAB2-SE-FPD spectrometer.

**2,7-Dibromo-9,9-di-*n*-octylfluorene (1)**: Synthesized according to the literature.[33]

**2,7-Bis(4-phenylcarboxylic acid)-9,9-di-*n*-octylfluorene (2)**: Diacid functionalized fluorene **2** was synthesized in two steps starting from **1** (Scheme 1).

**a) 2,7-Bis(4-phenylcarboxylic acid methyl ester)-9,9-di-*n*-octylfluorene**: 2,7-Dibromo-9,9-di-*n*-octylfluorene (**1**; 1.06 g, 1.94 mmol), 4-carboxymethylphenylboronic acid (0.91 g, 5.03 mmol) and $K_2CO_3$ (0.54 g, 3.88 mmol) were dissolved in THF (12 mL) and water (6 mL) in a Schlenk flask. The solution was purged with argon for 15 min, tetrakis(triphenylphosphine)palladium(0) (67 mg, 58 μmol) was added, and the reaction mixture was heated at 80 °C for 16 h. The product was extracted

into dichloromethane, washed with brine, and dried over $MgSO_4$. The crude product was recrystallized from ethanol to generate the pure diester as light yellow shiny crystals (0.93 g, 73%). $^1$H NMR (250 MHz, $CD_2Cl_2$, 25°C): $\delta$ = 8.12 (d, $J$=8.5 Hz, 4H), 7.80 (m, 6H), 7.67 (m, 4H), 3.93 (s, 6H), 2.10 (t, $J$=8.9 Hz, 4H), 1.20–1.09 (m, 20H), 0.83–0.68 (m, 10H); $^{13}$C NMR (62.5 MHz, $CD_2Cl_2$, 25°C): $\delta$ = 167.53, 152.79, 146.63, 141.54, 139.80, 130.74, 129.70, 127.78, 127.05, 122.49, 121.09, 56.27, 52.74, 41.05, 32.51, 30.68, 29.94, 29.92, 24.59, 23.34, 14.56; FDMS: $m/z$: calcd: 658.93; found: 659.40; elemental analysis calcd (%): C 82.03, H 8.26; found C 81.87, H 8.26.

**b) 2,7-Bis(4-phenylcarboxylic acid)-9,9-di-*n*-octylfluorene (2)**: A mixture of above diester (0.70 g, 1.06 mmol), KOH (0.80 g, 14 mmol), THF (10 mL) and $H_2O$ (5 mL) was heated under reflux for 16 h. The reaction was cooled to room temperature and then acidified with concentrated HCl. The precipitated white solid was washed several times with water and then ethanol, and dried under vacuum (0.59 g, 88%). $^1$H NMR (250 MHz, [D$_6$]DMSO, 25°C): $\delta$ = 13.03 (br s, 2H), 8.07 (d, $J$=8.5 Hz, 4H), 7.91 (m, 8H), 7.74 (d, $J$=7.9 Hz, 2H), 2.11 (m, 4H), 1.20–0.56 (m, 30H); $^{13}$C NMR (62.5 MHz, [D$_6$]DMSO, 25°C): $\delta$ = 167.10, 151.49, 144.49, 140.24, 138.06, 129.88, 129.36, 126.72, 125.99, 121.22, 120.64, 55.06, 40.45, 31.03, 28.99, 28.35, 28.33, 23.21, 21.91, 13.74; FDMS: $m/z$: calcd: 630.88; found: 630.70.

**Disuccimidyl ester of 2,7-bis(4-phenylcarboxylic acid)-9,9-di-*n*-octylfluorene (3)**: Diacid **2** (20 mg, 0.03 mmol) and NHS (18.25 mg, 0.15 mmol) were dissolved in dry *N*,*N*′-dimethylformamide (DMF) (2 mL) under argon. To this solution, DCC (18.55 mg, 0.09 mmol) dissolved in dry DMF (1.5 mL) was added dropwise and stirred overnight at room temperature. The completion of reaction was determined by TLC (methylene chloride) and FDMS. FDMS: $m/z$: calcd: 825.0; found: 825.24.

**5′-Amino-modified oligodeoxyribonucleotide (ODN)**: The amino modified oligonucleotide, 5′-$NH_2$-$C_6$-CTCAGATCTGGTCTAAC-3′ was synthesized by standard solid-phase DNA chemistry using the phosphoramidite method[34] on a polystyrene/divinylbenzene support. After the synthesis, deprotection of the oligonucleotides was carried out in a suspension of 37% ammonia at 50°C, overnight. The trityl group was deprotected under standard conditions by stirring in acetic acid/$H_2O$ (4:1) for 2 h at room temperature. The resulting oligonucleotide was dried under vacuum overnight and purified by ion-exchange HPLC.

**ODN–fluorene conjugates**: Active ester **3** (0.5 mg, 0.6 μmol) was dissolved in a small amount of dry DMF (25 μL) and added to the 5′-amino modified oligonucleotide (ODN)[14] (19 mg, 3.6 μmol) in a total volume of 250 μL of 0.10 M sodium tetraborate buffer (pH 8.5) and shaken overnight at room temperature. Purification of the products was performed using a 20% denaturing polyacrylamide gel (100 V, 2 h) with trisborate/EDTA buffer (90 mM Tris, 90 mM boric acid, 2 mM EDTA) as the running buffer. Identification of conjugates **4** and **5** was achieved by UV shadowing. The respective bands were excised from the gel and incubated in Tris buffer (pH 7) overnight at 37°C. Products **4** and **5** were obtained after filtration through a filter with 22μm pores.

## Acknowledgements

This work, as part of the European Science Foundation EUROCORES Programme BIONICS, was supported by funds from the DFG and the EC Sixth Framework Programme.

[1] http://genome.ucsc.edu/
[2] http://www.ensembl.org/
[3] http://www.ncbi.nlm.nih.gov/
[4] R. Sachidanandam, D. Weissman, S. C. Schmidt, J. M. Kakol, L. D. Stein, G. Marth, S. Sherry, J. C. Mullikin, B. J. Mortimore, D. L. Willey, S. E. Hunt, C. G. Cole, P. C. Coggill, C. M. Rice, Z. M. Ning, J. Rogers, D. R. Bentley, P. Y. Kwok, E. R. Mardis, R. T. Yeh, B. Schultz, L. Cook, R. Davenport, M. Dante, L. Fulton, L. Hillier, R. H. Waterston, J. D. McPherson, B. Gilman, S. Schaffner, W. J. Van Etten, D. Reich, J. Higgins, M. J. Daly, B. Blumenstiel, J. Baldwin, N. S. Stange-Thomann, M. C. Zody, L. Linton, E. S. Lander, D. Altshuler, *Nature* **2001**, *409*, 928.
[5] http://www.ornl.gov/sci/techresources/Human_Genome/project/journals/insights.html/
[6] M. M. Shi, *Clin. Chem.* **2001**, *47*, 164.
[7] P.-Y. Kwok, *Annu. Rev. Genomics Hum. Genet.* **2001**, *2*, 235.
[8] R. M. Clegg, *Methods Enzymol.* **1992**, *211*, 353.
[9] K. J. Livak, S. J. A. Flood, J. Marmaro, W. Giusti, K. Deetz, *PCR Methods Appl.* **1995**, *4*, 357.
[10] M. M. Shi, S. P. Myrand, M. R. Bleavins, F. A. Iglesia, *J. Clin. Pathol.* **1999**, *52*, 295.
[11] G. Bonnet, S. Tyagi, A. Libchaber, F. R. Kramer, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 6171.
[12] S. A. E. Marras, F. R. Kramer, S. Tyagi, *Genet. Anal.* **1999**, *14*, 151.
[13] O. Kohler, D. Venkatrao, D. V. Jarikote, O. Seitz, *ChemBioChem* **2005**, *6*, 69.
[14] T. Ishiguro, J. Saitoh, H. Yawata, M. Otsuka, T. Inoue, Y. Sugiura, *Nucleic Acids Res.* **1996**, *24*, 4992.
[15] A. O. Crockett, C. T. Wittwer, *Anal. Biochem.* **2001**, *290*, 89.
[16] C. P. Vaughn, K. S. J. E. Johnson, *Am. J. Pathol.* **2003**, *1*, 29.
[17] G. T. Hwang, Y. J. Seo, B. H. Kim, *J. Am. Chem. Soc.* **2004**, *126*, 6528.
[18] A. W. Grice, D. C. C. Bradley, M. T. Bernius, M. Inbasekaran, W. W. Wu, E. P. Woo, *Appl. Phys. Lett.* **1998**, *73*, 629.
[19] C. Ego, D. Marsitzky, S. Becker, J. Y. Zhang, A. C. Grimsdale, K. Müllen, J. D. MacKenzie, C. Silva, R. H. Friend, *J. Am. Chem. Soc.* **2003**, *125*, 437.
[20] M. Stork, B. S. Gaylord, A. J. Heeger, G. C. Bazan, *Adv. Mater.* **2002**, *14*, 361.
[21] L. H. Chen, D. W. McBranch, H. L. Wang, R. Helgeson, F. Wudl, D. G. Whitten, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 12287.
[22] D. L. Wang, X. Gong, P. S. Heeger, F. Rininsland, G. C. Bazan, A. J. Heeger, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 49.
[23] H. A. Ho, M. Boissinot, M. G. Bergeron, G. Corbeil, K. Dore, D. Boudreau, M. Leclerc, *Angew. Chem.* **2002**, *114*, 1618; *Angew. Chem. Int. Ed.* **2002**, *41*, 1548.
[24] N. Miyaura, A. Suzuki, *Chem. Rev.* **1995**, *95*, 2457.
[25] J. Sambrook, D. W. Russel, *Molecular Cloning*, Cold Spring Harbour Laboratory Press, New York, **2001**.
[26] B. Valeur, *Molecular Fluorescence: Principles and Applications*, Wiley-VCH, Weinheim, **2001**.
[27] J. Wang, D. Wang, E. K. Miller, D. Moses, G. C. Bazan, A. J. Heeger, *Macromolecules* **2000**, *33*, 5153.
[28] C. A. M. Seidel, A. Schulz, H. M. Sauer, *J. Phys. Chem.* **1996**, *100*, 5541.
[29] T. Heinlein, J. P. Knemeyer, O. Piestert, M. Sauer, *J. Phys. Chem. B* **2003**, *107*, 7957.
[30] S. Setayesh, A. C. Grimsdale, T. Weil, V. Enkelmann, K. Müllen, F. Meghdadi, E. J. W. List, G. Leising, *J. Am. Chem. Soc.* **2001**, *123*, 946.
[31] J. P. Knemeyer, N. Marme, M. Sauer, *Anal. Chem.* **2000**, *72*, 3717.
[32] X. Chen, J.-L. Liao, Y. Liang, M. O. Ahmed, H.-E. Tseng, S.-A. Chen, *J. Am. Chem. Soc.* **2003**, *125*, 636.
[33] M. Fukuda, K. Sawada, K. Yoshino, *J. Polym. Sci. Part A: Polym. Chem.* **1993**, *31*, 2465.
[34] M. H. Caruthers, *Acc. Chem. Res.* **1991**, *24*, 278.

Received: December 6, 2005
Published online: March 7, 2006