Analytical Methods

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Site-Specific DNA Cleavage on a Solid Support: A Method for Mismatch Detection**

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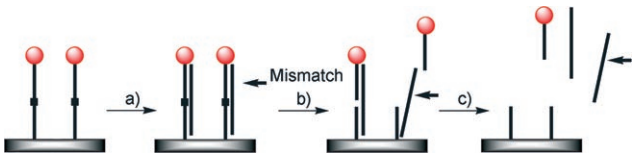
The growing insight into genetically caused diseases induced by single nucleotide polymorphisms (SNPs) calls for the development of analytical techniques to detect these SNPs. The techniques should be quick, reliable, and inexpensive. In recent years several strategies for genetic analyses were introduced, which are based on polymerase chain reactions (PCR),[1] enzymatic digestion,[2] electrochemistry,[3] or methods where target binding directly changes the fluorescence.[4] We now present a new concept for mismatch detection using chemical DNA cleavage on a solid support (Scheme 1): A



Scheme 1. Concept: An oligonucleotide selectively modified with a photolabile cleavage site (black square) in the middle of the strand and a marker tag (red circle) at the end of the strand is immobilized on a solid support. After hybridization of target sequences (a), and subsequent strand cleavage by irradiation (b), the tag remains on the support in the matched case, and is released into the solution in a mismatched case. Subsequent heating quantitatively releases the remaining tag (c).

single DNA strand, which carries a cleavage site at the central position and a detection tag at the 5′-end, is immobilized on the solid support through the 3′-end and hybridized with the target strand. Upon irradiation the immobilized single strand is cleaved and releases the tag-containing fragment into the solution if a mismatch is present. In the matched case, the higher melting point prevents dehybridization.

As the cleavage site we used the 5′-*o*-nitrophenyl thymidine (**1**, **T***; Scheme 2), which selectively recognizes adenosine and quantitatively cleaves the DNA strand upon irradiation (360 nm) in solution, whereby the whole DNA strand is cleaved.[5] This modified nucleotide **1** was incorporated through standard phosphoramidite chemistry into the modified 21mer **2**, which was immobilized on agarose (formation of **3**; Scheme 3).[6] We used dispers red 1 (DR1)

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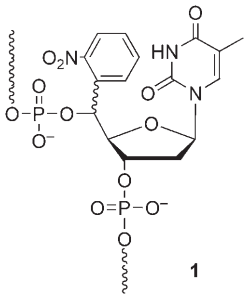

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Scheme 2. The photocleavable site, modified nucleotide **1**.

as a tag,[7] and directly observed the color changes of the beads after irradiation (10 °C, 10 min, $\lambda = 360$ nm). The results were quantified by HPLC analysis of the tagged fragment **4**, which was released into the solutions by irradiation. Subsequent heating of the solid-phase system quantitatively released the remaining tag-containing fragment **4** into solution (Scheme 3, Experimental Section).[8]

Irradiation of the immobilized single-strand **3** released 98 % of the cleavage product **4** into the solvent, and the beads became colorless. Thus, a quantitative cleavage of the modified strand occurs not only in solution[5] but also if the strand is immobilized on agarose. This result is in accord with other photolysis experiments with agarose as a solid support.[9]

Double-strand formation was quantitative when the immobilized strands **3** were hybridized with a slight excess of target strands, even if one or two mismatches were introduced (Experimental Section). But in contrast to the single-strand experiment, irradiation of a matched double strand at 10 °C released only a small amount of 10mer **4** into the solution (15 %), and the beads remained red. Subsequent heating quantitatively released the cleaved strand **4** (the beads became colorless).[10] This behavior changed if mismatches were incorporated. Irradiation of double strands with two mismatches released 70–98 % of the 10mer **4** into solution at 10 °C (Table 1).

The melting points of the photocleaved systems are decreased by two mismatches to such an extent that the tag-containing fragment **4** is released into the solvent. Experiments in homogeneous solution showed the same trend. The melting point of a double-stranded oligonucleotide containing a 21mer of the sequence **2** (T instead of **T***) with a complementary 21mer is 62.6 °C (10 mM KH_2PO_4, 0.5 M NaCl, pH 7.0). Hybridization of the 21mer with two matching 10mers that should mimic the photoinduced cleavage products reduced the melting point to 32.2 °C. But if in this system

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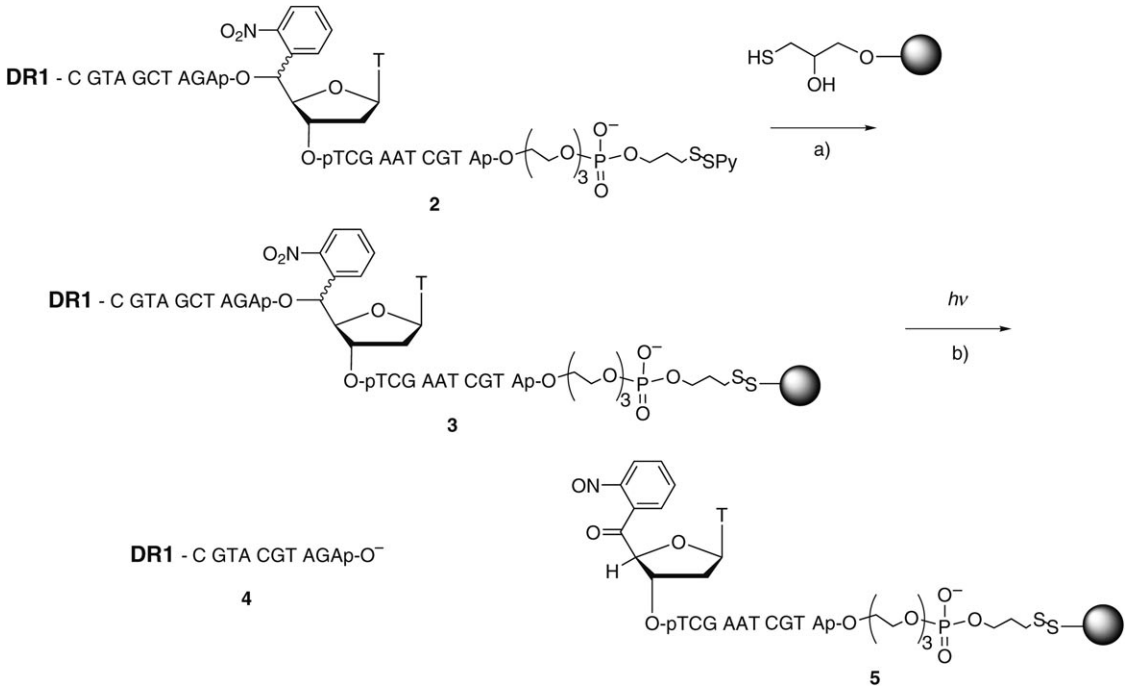

Scheme 3. a) Immobilization of the modified oligonucleotide **2** on an agarose support (1 h, pH 4.4, 35 °C); b) irradiation of the single-stranded system **3** (10 min, pH 7.0, $\lambda = 360$ nm, 10 °C). Py = 2-pyridyl.

Table 1: Results for irradiation of double strands with two mismatches between the immobilized oligonucleotide **3** and counter strands **6**–**12**.

	DR1-CGTAGCTAGAT*TCGAATCGTA[a]	**4** [%][b]
6	3′-GCATCGA**G**CTA AG**A**TTAGCAT	78
7	3′-GCATCGAT**G**TA AG**A**TTAGCAT	85
8	3′-GCATCGA**AG**TA AGCTTAGCAT	94
9	3′-GCATCGAT**GC**A AGCTTAGCAT	85
10	3′-**A**CATCGAT**G**TA AGCTTAGCAT	89
11	3′-GCATCGAT**G**TA AGCT**C**AGCAT	70
12	3′-**T**CATCGATCTA AGCTTAGCA**G**	98

[a] Immobilized single strands **3** hybridized with the counter strands **6**–**12**, mutations in bold. [b] Yields of the photolysis product **4** released into the solution at 10 °C.

Table 2: Results for irradiation of double strands with one mismatch between the immobilized oligonucleotide **3** and counter strands **13**–**25**.

	DR1-CGTAGCTAGAT*TCGAATCGTA[a]	**4** [%][b]
13	3′-GCATCGATCTA AGCT**C**AGCAT	45
14	3′-GCATCGA**C**CTA AGCTTAGCAT	49
15	3′-GCATCGA**A**CTA AGCTTAGCAT	50
16	3′-GCATCGATCTA AG**T**TTAGCAT	51
17	3′-GCAT**C**TATCTA AGCTTAGCAT	68
18	3′-**A**CATCGATCTA AGCTTAGCAT	70
19	3′-GCATCGATCTA AG**G**TTAGCAT	74
20	3′-GCATCGATCTA AG**A**TTAGCAT	79
21	3′-GCAT**C**TATCTA AGCTTAGCAT	82
22	3′-GCATCGATCTA AGCTTA**C**CAT	86
23	3′-GCATCGATC**T**A AGCTTAGCAT	17
24	3′-GCATCGATCTA **T**GCTTAGCAT	30
25	3′-GCATCGATCTA AGCTTAGCA**G**	31

[a] Immobilized single strands **3** hybridized with the counter strands **13**–**25**, mutations in bold. [b] Yields of photolysis product **4** released into the solution at 10 °C.

one or two mismatches are introduced, no melting points above 15 °C are observed.

As the data of Table 2 show, single mismatches (SNPs) could also be detected (strands **13**–**22**, 45–86 % yield of **4** at 10 °C). Only when the SNP was adjacent to the adenine residue opposite T*, did the yield of **4** drop to 17–30 % (strands **23**, **24**), although the efficiency of the photolytic cleavage of the immobilized 21mer was not influenced by the presence of the SNP. Presumably, SNPs at the ends of DNA strands do not influence the melting points very much. This assumption is in accord with the results of recent liquid-phase studies by Richert et al.[11] The yield of the released fragment **4** was also low when the mismatch was at the lower end (5′-end) of the target strand (strand **25**). In contrast, when the mismatch was adjacent to the DR1 tag at the upper end (3′-end) of the target strand, the yield of the released photofragment **4** was high (70 %, strand **18**). This situation is caused by the π–π interaction between the capping aromatic tag and a DNA base at the end of the strand.[11]

To use this method for the detection of a medically relevant SNP, which indicates the disposition to develop diseases, immobilized strands should be used with the respective mutation site being at least two base pairs away from the cleavage site and the spacer. A color change of the beads demonstrates the existence of an SNP, and the solution should be analyzed. In the best case the specific SNP causes a typical yield of the released tag-containing fragment. Thus, the benefit of this simple and quick method is that the

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qualitative response of the beads (the color changes, which indicate mutations in the target strands) can be checked and quantified by analysis of the solutions in a reliable way, because subsequent heating quantitatively releases the remaining tag. Not only the sequence, but also the length of the double strand plays a role. The optimal length of the double strand when using immobilized strand **3** was a 21mer. When the double strand was reduced to a 19mer (one nucleotide shorter at each end of the target strand), even in the matched case 65% of the fragment **4** was released after irradiation at 10°C. However, overhanging ends at the target strand did not influence the efficiency of our analytical method.

In conclusion, A proof of concept for a new method for the detection of single nucleotide polymorphisms is described, where the tag is attached to beads, so that the label-free target strand does not have to be chemically manipulated. This new assay allows a fast detection of SNPs because the hybridization takes only 2 h and is very efficient, the photolysis needs 10 min, and a simple filtration shows directly the results (color changes of the beads).

Experimental Section

DNA synthesis and purification: DNA syntheses were performed on a DNA synthesizer (PerSeptive Biosystems, Expedite). Commercially available modifiers (spacer9 phosphoramidite, modified solid support; Eurogentec) were incorporated in the DNA as indicated by the supplier. The modified nucleotide **1** was incorporated as described in ref. [5]. DR1, transferred into the corresponding phosphoramidite, was coupled to the DNA (15 min). After deprotection and purification by denaturing polyacrylamide gel electrophoresis (PAGE; 20%, 130 V, 15 h) and desalting over NAP columns the disulfide of the oligonucleotide was reduced (30 min, 30°C) with the reduction buffer (100 mM dithiothreitol (DTT), 40 mM tris(hydroxymethyl)aminomethane (Tris), 0.5 M NaCl, 1 mM ethylendiamine tetraacetic acid (EDTA), pH 8.3), extracted with ethylacetate, incubated with 2,2′-dithiodipyridine (30 min, 30°C), and extracted two times with ethylacetate. Final purification of **2** was performed by RP-HPLC. Unmodified oligonucleotides (Microsynth AG) were used after purification by RP-HPLC. All oligonucleotides were characterized by MALDI-TOF mass spectrometry.

Immobilization: Commercially available 2-pyridyldisulfide activated agarose (Pharmacia Biotech AB, Sepharose 6B) was first reduced with the reduction buffer (30 min, 30°C), the modified oligonucleotide **2**, in immobilization buffer (370 mM, NaOAc, 0.5 mM NaCl, 1 mM EDTA, pH 4.4), was added (1 h, 35°C), and excess thiols were capped using cap-solution I (60 mM *S*-(2-thiopyridyl)-2-mercapto-ethanol, 40 mM MES, 40 mM $MgCl_2$, 0.5 M NaCl, 1 mM EDTA) and cap-soution II (200 mM *S*-(2-thiopyridyl)-2-mercaptoethanol in EtOH). Loading density was determined by RP-HPLC using an internal standard oligonucleotide.

Hybridization: Counter strands (1.2 to 2.0 equiv) were added to the immobilized strands in a phosphate buffer (10 mM KH_2PO_4, 100 mM NaCl, pH 7.0). The mixture was heated with stirring to 85°C and cooled down to 35°C in 1.5 h In all cases the yields were quantitative (or nearly quantitative) as determined by RP-HPLC, using an oligonucleotide as internal standard.

SNP detection by irradiation: Buffered solutions of the double strands were irradiated under stirring (10°C, 10 min, pH 7.0,) by a high-pressure mercury arc lamp ($\lambda = 360$ nm, 500 W, Osram). The solutions were filtered, and the color changes of the beads could be directly observed. To quantify the effects, the solutions were analyzed by RP-HPLC with an external standard. The solutions could also be analyzed by UV spectroscopy. Addition of a new portion of the buffer to the beads, heating to 85°C, and filtration after 3 mins quantitatively released the remaining tagged nucleotide **4** into solution.

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