Mismatch Formation in Solution and on DNA Microarrays:
How Modified Nucleosides Can Overcome Short-Comings of Imperfect Hybridization
Caused by Oligonucleotide Composition and Base Pairing

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1. Synthesis and characterization of oligonucleotides

A series of oligonucleotides (10-14, 20-24, 27-36) were synthesized by solid phase synthesis in a 1 µmol scale employing the regular phosphoramidites and the phosphoramidite building blocks 25-26. For the 5’-amino modification of 30 and 32-35 the phosphoramidite of a terminal aminohexyl linker group (26) (Glen Research, US) was used which makes these oligonucleotides suitable for the immobilization on aldehyde-modified microarray surfaces (Figure S1). The oligonucleotides 31 and 36 were functionalized with the phosphoramidite of a fluorescent cyanine dye (25) (Cy-5; Glen Research, US) at their 5’-end (Figure S1). The syntheses of the regular and the modified oligonucleotides were carried out according to the standard procedure of solid phase synthesis of oligonucleotides. Deprotection was performed with 25% aqueous ammonia solution (60°C, 16 h) and the oligonucleotides were purified by reversed-phase HPLC, RP-18 (see the experimental part). All oligonucleotides were characterized by MALDI-TOF mass spectra. The calculated masses were in good agreement with the measured values (Table S1).

![Figure S1 Phosphoramidite building blocks.](image-url)
Table S1 Molecular masses ([M + H]⁺) of oligonucleotides measured by MALDI-TOF mass spectrometry.

<table>
<thead>
<tr>
<th>Oligonucleotides</th>
<th>[M + H⁺] [Da]</th>
</tr>
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<tbody>
<tr>
<td>5′-d(H₂N-(CH₂)₆-TAG GTC AAT ACT TAG GTC AAT ACT) (30)</td>
<td>7530 7530</td>
</tr>
<tr>
<td>5′-d(Cy5-AGT ATT GAC CTA AGT ATT GAC CTA) (31)</td>
<td>7884 7885</td>
</tr>
<tr>
<td>5′-d(H₂N-(CH₂)₆-TAG GGC AAT ACT TAG GGC AAT ACT) (32)</td>
<td>7580 7581</td>
</tr>
<tr>
<td>5′-d(H₂N-(CH₂)₆-TAG GCC AAT ACT TAG GCC AAT ACT) (33)</td>
<td>7500 7500</td>
</tr>
<tr>
<td>5′-d(H₂N-(CH₂)₆-TAG GAC AAT ACT TAG GAC AAT ACT) (34)</td>
<td>7548 7548</td>
</tr>
<tr>
<td>5′-d(H₂N-(CH₂)₆-TAG GTC AAT ACT) (35)</td>
<td>3825 3825</td>
</tr>
<tr>
<td>5′-d(Cy5-AGT ATT GAC CTA) (36)</td>
<td>4179 4179</td>
</tr>
</tbody>
</table>

Cy5 = indodicarbocyanine dye

2. Preparation of the oligonucleotide microarrays

The DNA microarrays were manufactured with a GMS 417 Arrayer (Genetic MicroSystems, US) employing a four pin print head for spotting. Therefore, the 5′-amino modified target oligonucleotides were dissolved in a Micro Spotting Solution Plus (TeleChem, US) in various concentrations (0.1 – 1.5 A₂₆₀ units/ml) and spotted on aldehyde-functionalized microarray substrates (Super Aldehyde Slides, TeleChem, US). The aldehyde groups on the surface of the microarrays and the primary amino groups of the oligonucleotides form a Schiff base (Scheme S1). After a reaction time of 12 h in a desiccator at 30°C under reduced pressure the unbound DNA was washed off with an aqueous 0.1% sodium SDS solution followed by washing in bidistilled water. The microarray substrate was treated with a NaBH₄ solution to reduce the Schiff base to a secondary amine which is stable towards hydrolysis. Also, the unreacted aldehyde groups were transferred to alcohol groups.
The reduction step was followed by further washing steps to remove the reagents from the surface. For more details see the experimental section.

Scheme S1 Covalent binding of oligonucleotides carrying a 5’-amino linker to an aldehyde functionalized microarray surface.

For the hybridization experiments the target oligonucleotides were dissolved in a hybridization buffer containing 0.1 M NaCl, 1mM EDTA, 10 mM Na-cacodylate buffer, 0.1% SDS and 0.2 mg/ml Top Block, pH = 7. A few drops of the hybridization buffer containing a definite amount of the probe oligonucleotide were applied to the microarray surface carrying the immobilized probe molecules. The hybridization solution was allowed to incubate in a sealed humid chamber to avoid drying-out. After incubation, the remaining non-hybridized target oligonucleotides were washed-off by stringent washes in sodium citrate buffer solutions (SSC buffer) with decreasing ionic strength and dried (for details see the experimental part).

For the detection of the immobilized duplexes the microarray substrates were applied to a GMS 418 Microarray Scanner (Genetic MicroSystems, US). The emission signals of the fluorescent Cy-5 dye were collected and converted into false colour data.
3. Experimental Part

3.1 General

All chemicals were purchased from *Fluka*. The phosphoramidites of the 5'-amino linker (6-(4-monomethoxytritylamino)hexyl-(2-cyanoethyl)-(N,N-diisopropyl)-phosphoramidite) and the Cy-5 dye (1-[3-(4-monomethoxytrityloxy)propyl]-1'-(3-[[(2-cyanoethyl)-(N,N-diisopropyl phosphoramidityl)propyl]-3,3',3'-tetramethylindodicarbocyanine chloride) were obtained from *Glen Research* (Virginia, US). Melting curves were measured with a *Cary-1/3* UV/VIS spectrophotometer (*Varian*, Australia) equipped with a *Cary* thermoelectrical controller. The temperature was measured continuously in the reference cell with a Pt-100 resistor, and the thermodynamic data of duplex formation were calculated by the Meltwin 3.0 program.²

3.2 Synthesis, purification and characterization of the oligonucleotides

The oligonucleotide syntheses were performed in an automated ABI 392 DNA synthesizer (*Applied Biosystems*, Weiterstadt, Germany) at 1 µmol scale according to the standard protocol for 3’-(2-cyanoethyl phosphoramidites) employing the regular phosphoramidites and the phosphoramidite building blocks 25-26.¹ For the 5’-amino modification of 30 and 32-35 the phosphoramidite of a 5’-amino C6-linker was used (*Glen Research*, US) and for the 5’-Cy-5 modification of 31 and 36 the phosphoramidite of the indocarbocyanine Cy-5 dye (*Glen Research*, US) was employed. After cleavage from the solid support, the oligonucleotides were deprotected in 25% aq. ammonia solution for 14-16 h at 60°C. Purification of the 5’-dimethoxytrityl and 5’-monomethoxytrityl oligomers was performed by reversed-phase HPLC (RP-18) with the following solvent gradient system [A: 0.1 M (Et₃NH)OAc (pH 7.8)/MeCN 95:5; B: MeCN]: 5 min, 10-15% B in A, 15 min, 15-50% B in A, 20 min, 50% B in A and 25 min, 10% B in A with a flow rate of 1.0 ml/min. The
oligonucleotide solutions were dried and treated with 2.5% CHCl₂COOH/CH₂Cl₂ for 5 min at 0°C to remove the 4,4'-dimethoxytrityl or 4-monomethoxytrityl residues. The detritylated oligomers were purified by reversed-phase HPLC with the gradient: 0-20 min, 0-20% B in A, flow rate 1.0 ml/min. For the deprotected Cy-5 functionalized oligonucleotides 31 and 36 the gradient of the trityl-on purification was used. The oligomers were desalted (RP-18, silica gel) and lyophilized to yield solids which were frozen at -24°C.

The molecular masses were determined by MALDI-TOF with a Biflex-III instrument (Bruker Saxonia, Leipzig, Germany) and 3-hydroxypicolinic acid (3-HPA) as a matrix (Table S1).

3.3 Preparation of DNA microarrays

Glass slides (Super Aldehyde Slides, 25 x 76 mm) with aldehyde-functionalized surfaces were obtained from TeleChem (TeleChem International, US). Oligonucleotides 30, 32-34 employing an amino-linker group at their 5'-end were each dissolved in diluted Micro Spotting Solution Plus (Micro Spotting Solution Plus: bidistilled water, 1 : 1; 600 µl) (TeleChem International, US) at various concentrations (0.1 – 1.5 A₂₆₀ units/ml). The oligonucleotide solutions were spotted on the surface of the microarray substrates utilizing a GMS 417 Arrayer (Genetic Microsystems, US) equipped with four pin print head employing the Pin-and-Ring Technology. To obtain a higher accurarcy and repeatability, from each oligonucleotide solution six identical spots were produced.

After spotting the microarray substrates were kept in a desiccator at 30°C (reduced pressure, 12 h). The microarrays were twice washed with an aqueous 0.1% sodium dodecyl sulphate solution (SDS) (2 min, r.t.) followed by bidistilled water (2 min, r.t.). Next, the microarray substrates were soaked in an aqueous 11.5 mM NaBH₄ solution (215 ml; phosphate buffered saline solution : bidest. water, 1 : 9; containing 43% ethanol) while stirring (5 min). The microarrays were again washed with bidistilled water and aqueous 0.1% SDS solution (each
2 min, r.t.). Then, the microarrays were incubated in boiling bidestilled water for 3 min and in ice-cold ethanol for 30 sec. The microarrays were dried with compressed air and stored.

3.4 Hybridization experiments performed on microarrays

The oligonucleotide 31 carrying a fluorescent Cy-5 dye at its 5’-end was dissolved in a 100 mM NaCl, 1 mM EDTA, 10 mM Na-cacodylate buffer solution containing 0.1% SDS and 0.2 mg/ml Top Block at various concentrations (0.05 – 1.5 A_{260} units/ml). A few drops of the oligonucleotide solution of 31 were put on the surface of the microarray substrates. The solution was covered with microscope cover slips and the microarray was allowed to stand in a sealed humid reaction chamber. After hybridization, the microarray substrates were entirely washed in a 30 mM citrate, 300 mM NaCl, pH = 7 aqueous solution (2x SSC buffer) containing 0.1% sodium dodecyl sulphate followed by washing in a 15 mM citrate, 150 mM NaCl, pH = 7 aqueous solution (1x SSC buffer) and a 7.5 mM citrate, 75 mM NaCl, pH = 7 aqueous solution (0.5x SSC buffer) (for each washing step 5 min, r.t.). The microarrays were dried with compressed air.

3.5 Detection of duplex formation on microarrays

Detection of the immobilized duplexes was performed with a GMS 418 Array Scanner (Genetic MicroSystems, US) equipped with a laser beam. The microarrays were irradiated with a laser beam at 635 nm. The emission signals of the fluorescent Cy-5 dye were collected by a photo-sensitive device and converted into graphical images which are 16-bit numerical representations of microarray surfaces with intensity values ranging from 0 to 65536 (2^{16}). The intensity values were correlated with a colour wherein blue refers to a low intensity value and a red to white refers to a high intensity value. The relative fluorescence intensity of the
fluorescence signal for a spot representing surface bound duplexes was calculated as an average of the absolute number of the fluorescence intensity of six identical spots.

References
