Carbohydrate Microarrays

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DNA-Based Carbohydrate Biochips: A Platform for Surface Glyco-Engineering**

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Carbohydrates and glycoconjugates play a major role in key biological events such as cell–cell recognition, pathogenesis, and inflammation.[1,2] As a consequence, there is a need to understand the structural parameters governing the recognition of carbohydrates by their receptors. This knowledge will be of use for both fundamental research and potential applications in diagnostics or therapeutics. However, research in this field is slowed by the wide diversity of carbohydrate structures and by the minute amounts of materials available for experimentation. The design of sensitive and high-throughput technologies for the characterization of oligosaccharide/protein interactions[3] is therefore emerging as an attractive tool for chemists and biochemists. Available techniques such as isothermal calorimetry, enzyme-linked lectin assay, and even crystallographic studies provide data on carbohydrate/protein interactions, but they are often limited by the amount of available material.

Carbohydrate microarray technology[4–16] is a promising approach for probing carbohydrate/protein interactions, and it permits the simultaneous screening of a number of biological interactions with only minute amounts of material. A large family of carbohydrate derivatives has been designed for immobilization on surfaces by various means.[5–14] However, this technology has various limitations. Relative surface densities of bound ligands are often not assessed. A careful optimization of the orientation and the distance separating the carbohydrate probe from the surface is often required. The interactions of oligosaccharides with lectins are usually weak (mM range) and can be enhanced using the "cluster effect" with multivalent ligands.[17–20] In the latter case, the distance between the residues should be optimized for binding.[21–23] Finally, the syntheses of functionalized oligosaccharide ligands are labor intensive.

We report herein an original approach for the surface immobilization of oligosaccharides using glycoconjugate molecules that present a DNA sequence for anchoring onto DNA chips through hybridization. This approach has been used in the field of protein microarrays,[24,25] but to our knowledge this is the first time that such a strategy has been reported in the field of glycoarrays.

Several syntheses of glycoconjugated oligonucleotides have been reported, but none are suitable for introducing different carbohydrate moieties.[26–28] We designed a conjugate that incorporates carbohydrate residue(s) for interacting with a lectin, an oligonucleotide sequence for anchoring on the surface, and a fluorescent tag at the 5′-end for the determination of relative surface densities (Figure 1). These

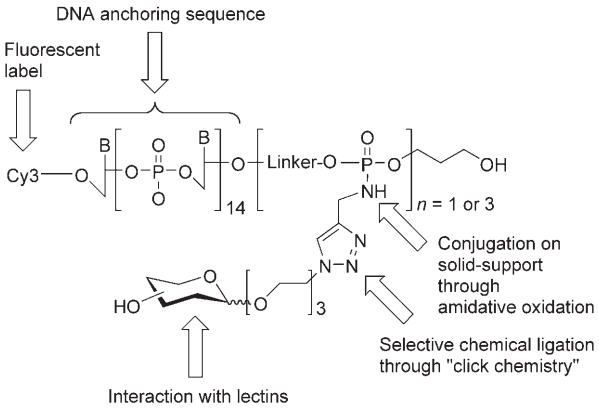

Figure 1. Schematic structure of the glycoconjugates.

moieties were assembled through a combination of automated oligonucleotide synthesis, and amidative oxidation and 1,3-dipolar cycloaddition ("click" chemistry) performed on a solid support (Scheme 1).[29] We introduced either one or three saccharide residues to take advantage of the "cluster effect". The distance between the carbohydrate moieties was also varied by using cyclohexanedimethanol (L1) and tetra-

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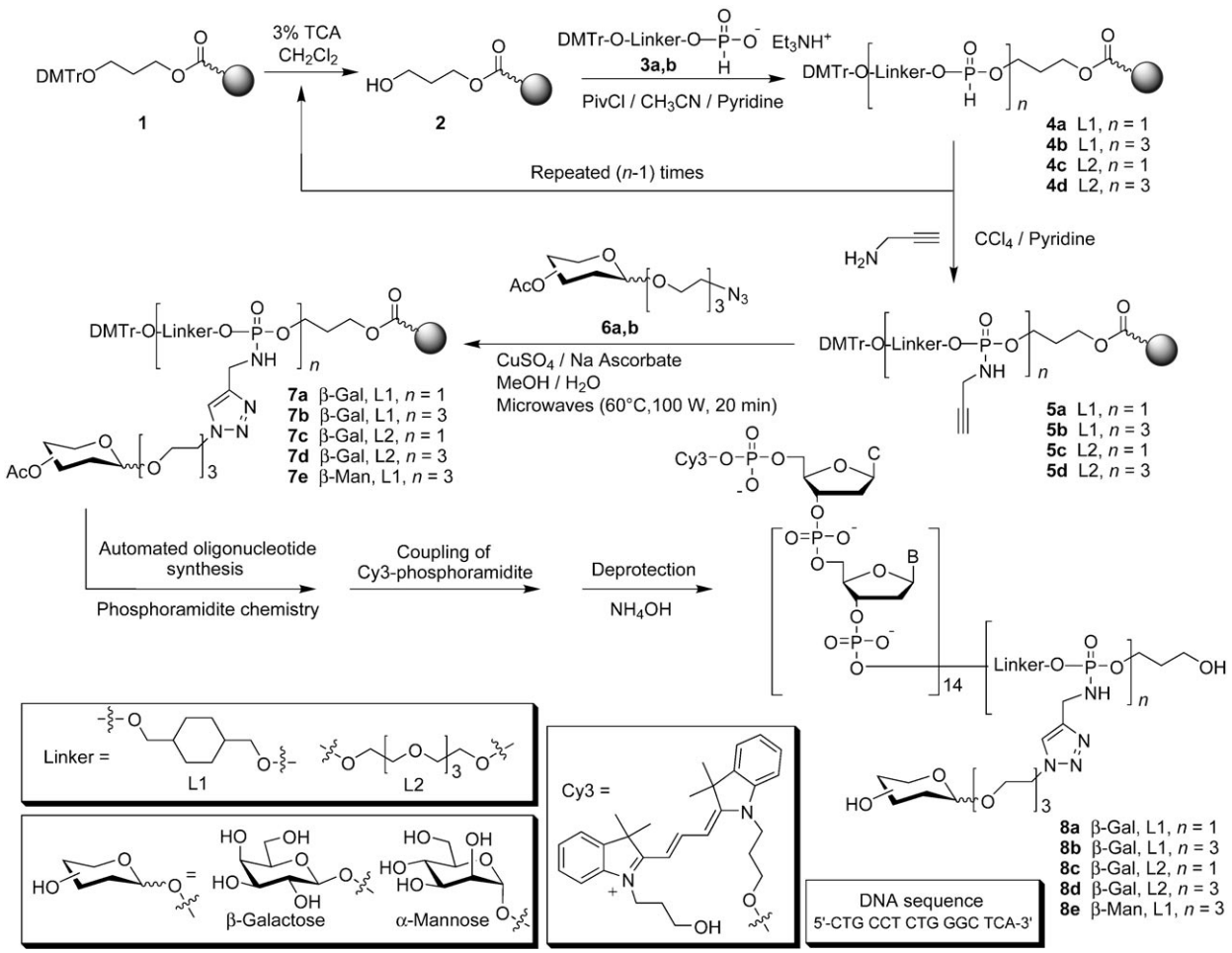
Supporting information for this article (experimental procedures for the preparation of all new compounds and for the design of the carbohydrate microarray) is available on the WWW under http://www.angewandte.org or from the author.



Scheme 1. General synthetic scheme for the preparation of glycoconjugates **8a–e**. TCA = trichloroacetic acid, Piv = pivaloyl; for structures **8a–8e** C denotes cytosine and B represents nucleobase.

ethyleneglycol (L2) as linkers. The L1 linker[29] was selected as a semirigid and hydrophobic scaffold and the L2 linker as a more flexible and hydrophilic one.

Starting from the solid support **1**,[30] removal of the dimethoxytrityl (DMTr) group afforded alcohol **2**.[29] We then introduced either one or three *H*-phosphonate monoester building blocks **3a**,**b**, which contain the linkers L1 and L2, respectively, to afford diesters **4a–d** (Scheme 1). Amidative oxidation of the *H*-phosphonate by carbon tetrachloride in the presence of propargylamine led to mono- and triynes **5a–d**, which were then conjugated with the galactose azide derivative **6a** by means of microwave-assisted 1,3-dipolar cycloaddition, affording solid-supported conjugates **7a–d**. The corresponding trimannoside derivative **7e** was synthesized from **6b** as a negative control for the lectin studied herein. The oligonucleotide was then synthesized and labeled with the fluorescent residue Cy3. Chimeric 5′-Cy3–3′-oligosaccharide oligonucleotides **8a–e** were obtained after deprotection by treatment with aqueous ammonia. Each conjugate was purified by preparative HPLC and characterized by MALDI-TOF mass spectrometry.[31]

The DNA microarray was designed to probe the interactions of these conjugates with lectins using two different approaches. In the first strategy ("on-chip" approach) a DNA chip would be fabricated, the conjugate bearing the complementary DNA sequence would be addressed selectively on the desired spot of the surface by hybridization, and finally cyanine 5 fluorescent dye (Cy5)-labeled lectin would be added to bind to the conjugate before detection (Figure 2a). In a second method ("in-solution" approach) the order of the last two steps of this process would be changed such that the steps would be: 1) fabrication of a DNA chip, 2) biological recognition of the conjugate by the lectin in solution, and 3) selective hybridization of the Cy5-labeled lectin/conjugate complex onto the surface before detection (Figure 2b). In theory, the in-solution strategy should allow for further analysis of effect of the surface on carbohydrate/protein binding.

3′-Amine oligonucleotides were covalently immobilized onto functionalized 52-well glass slides, leading to a surface bearing 4×10^{11} oligonucleotides per cm^2.[32] The DNA anchoring platforms consisted of wells carrying either a complementary sequence of the conjugates for immobilization (S1) or a noncomplementary one (S2) as a control for nonspecific adsorptions. Fluorescence scanning of the Cy3 signal displayed a homogeneous hybridization of conjugates

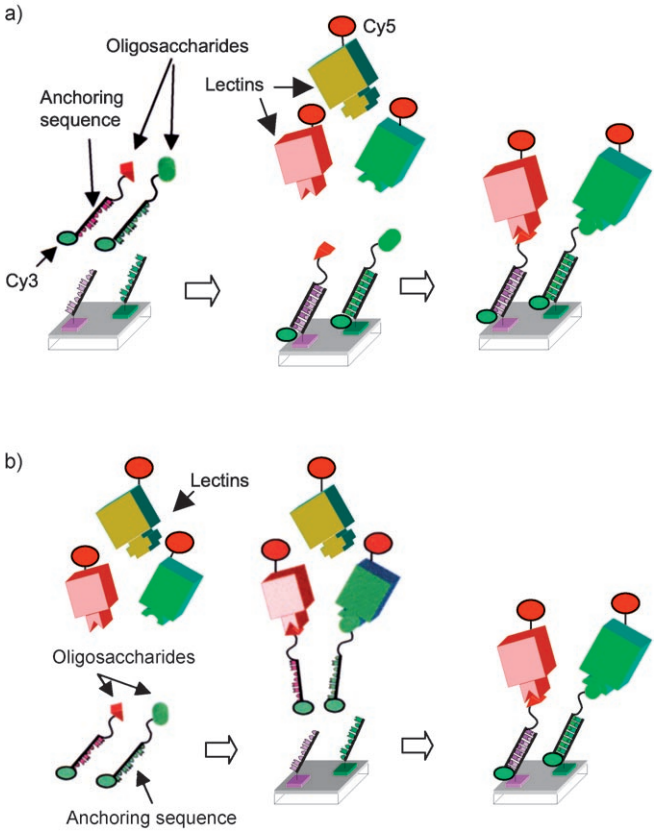


Figure 2. Schematic representation of the design of carbohydrate microarrays using DNA-anchoring platform. a) In the "on-chip" approach lectin/carbohydrate binding is performed on solid support after hybridization of the glycoconjugates. b) The "in-solution" method can be used to address on the chip the lectin/conjugate complex resulting from the interaction of the lectin with the conjugate in solution.

8a–e (maximum mean fluorescence ratio of 1.9:1 between the different spots) with S1, while the fluorescence signal was at background level with S2. The reported concentrations of oligosaccharide solutions used for immobilization are usually in the mM range.[5,7–9] Here, a 1 μM solution was used, which is one of the lowest concentrations reported.

Next, we studied the interaction of **8a–e** with the galactose-binding lectin RCA120 by both the on-chip and the in-solution approaches. The selectivity of RCA120 for its ligand over a non-ligand residue (mannose) was addressed,

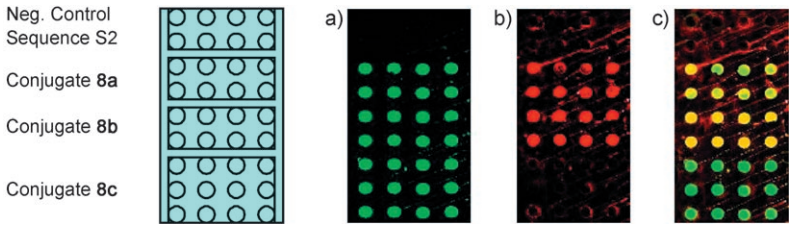


Figure 3. Fluorescence images recorded after hybridization of conjugates **8a**, **8b**, and **8e** with the DNA platform and incubation with Cy5-labeled RCA120 using the on-chip approach. Fluorescence was measured a) for Cy3 and b) for Cy5; c) ratio of fluorescence signals emitted by Cy5 and Cy3.

and also the possible "cluster effect" for binding with an increasing number of carbohydrate residues and the influence of the linkers was examined. In the case of the on-chip approach, Cy5-labeled RCA120 was deposited in each well at 2 μM concentration. After incubation and washing of the glass slide, fluorescent images of the carbohydrate microarray at 532 and 635 nm were obtained. The bright fluorescence of Cy5 was observed as a result of the binding of RCA120 with the galactosyl residues (Figure 3). The Cy5 signal was at background level when S2 or the mannosyl residues were

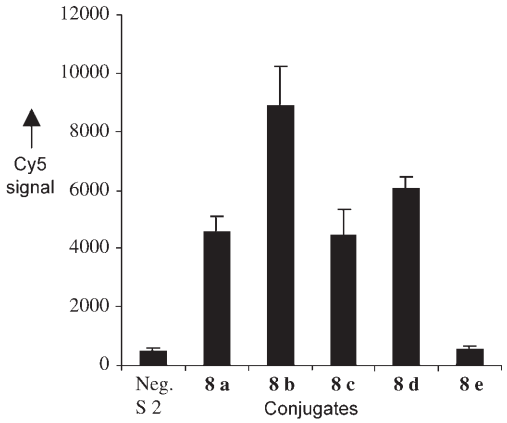


Figure 4. Selectivity of RCA120 (2 μM) for the galactose-coated wells (conjugates **8a–d**) versus mannose-coated wells (conjugate **8e**) using the on-chip approach. The fluorescence signal of each conjugate was determined as the average of the mean fluorescence signal of eight spots. Negative control consisted of covalent immobilization of sequence S2, which is not complementary to the DNA tag of the conjugates.

immobilized (Figure 4). The results obtained clearly demonstrate the selectivity of RCA120 for the mono- and trigalactosyl-coated wells. The mean Cy5 signal of **8b** bearing three galactose residues is 20 times higher than that of **8e** bearing three mannose residues, although the Cy3 signal ratio of **8b** and **8e** is similar (4:3), indicating that the surface density of the carbohydrate residues is similar. Furthermore, the Cy5 signal observed for **8b** is twice as strong as that of **8d** indicating that the linker plays a role in the biorecognition process (Figure 4). The higher affinity of the lectin for conjugate **8b** may be related to the higher hydrophobicity, greater rigidity, or shorter distance of linker L1 in comparison to L2. No cluster effect could be evidenced. The ratio of the fluorescence signals between the monogalactosyl conjugates **8a** or **8c** and the trigalactosyl conjugates **8b** or **8d** is only 1:4, close to the 1:3 ratio expected for a statistical effect.

The lowest lectin concentration required for a signal-to-noise ratio above 3:1 represents the lower detection limit of such devices. Glass slides bearing conjugates **8a**, **8b**, and **8e** were incubated with lectin solutions at 2, 0.2, 0.02, 0.002, and 0.0002 μM (Figure 5). The ratio of the

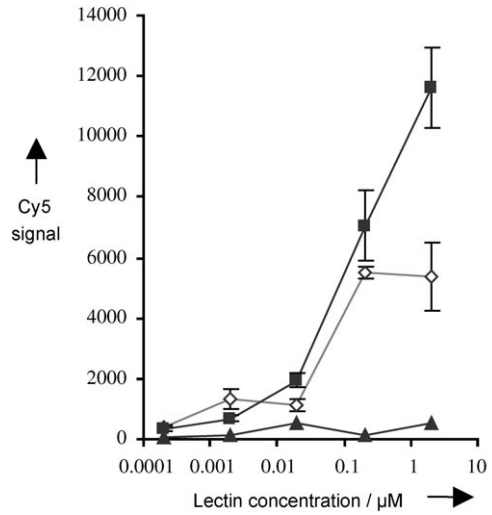


Figure 5. Determination of the lower detection limit of the carbohydrate microarray. Recognition was tested using the on-chip approach with five solutions of Cy5-labeled RCA120 (2, 0.2, 0.02, 0.002, and 0.0002 μM). Conjugates immobilization solution was at a concentration of 1 μM. ◇ conjugate **8a**, ■ conjugate **8b**, ▲ conjugate **8e**. The number of galactose residues does not influence significantly the affinity of the lectin, although the selectivity of the lectin is conserved between 20 and 2 nM as relative to mannose (conjugate **8e**).

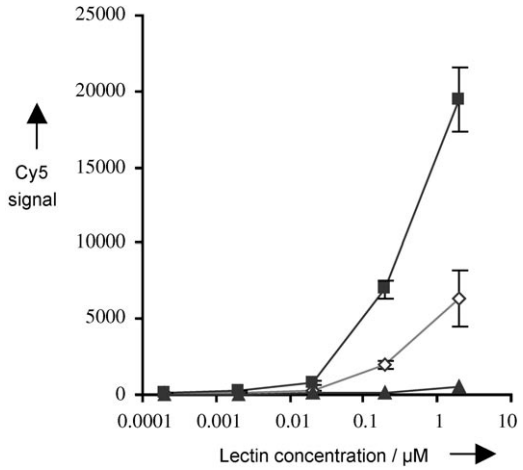


Figure 6. Recognition was performed using the in-solution approach with five solutions of Cy5-labeled RCA120 (2, 0.2, 0.02, 0.002, and 0.0002 μM). ◇ conjugate **8a**, ■ conjugate **8b**, ▲ conjugate **8e**. The effect of the number of galactose residues (comparing **8a** to **8b**) is clearly observed down to at least 20 or even 2 nM.

Cy5 signals of galactosyl-bearing spots to mannosyl-bearing spots is 3:1 when the lectin concentration is between 0.02 and 0.002 μM. These results compare favorably with those reported previously.[33] Our results are corroborated by those of Wacker et al.,[25,34] who used DNA-modified proteins for anchoring. They observed a similar improvement which they attributed to the reversibility of DNA hybridization enabling a denser packing and to the narrow and rigid structure of the DNA duplex.

Finally, we investigated the in-solution methodology (Figure 2b). The lectin was used at concentrations of 2, 0.2, 0.02, 0.002, and 0.0002 μM and incubated with conjugates **8a**, **8b**, and **8e** (1 μM). After incubation, the resulting solution was deposited in the appropriate wells (Figure 6). The Cy5 signal was at the background level when S2 was displayed. For lectin concentrations of 2 μM and 0.2 μM, the mean Cy5 signal for an assay solution containing conjugate **8b** bearing trigalactosyl residues is 30 times higher than that observed for the assay solution containing conjugate **8e** bearing trimannosyl residues. This ratio is still 9:1 at a lectin concentration of 20 nM and drops to 3:1 at 2 nM. Therefore, we can determine that the detection limit in this case is once again between 20 and 2 nM.

In conclusion, the synthesis of five chimeric carbohydrate/oligonucleotide molecules was achieved successfully on a solid support and using microwave-assisted "click chemistry". A DNA-chip-based glycoarray was obtained with an efficient immobilization by hybridization of the oligosaccharide (1 μM). The in-solution approach was performed by allowing first saccharide/lectin recognition before immobilization by hybridization on the surfaces and subsequent detection. We also demonstrated that the carbohydrate microarray was reusable (see the Supporting Information). Future work will focus on kinetic studies for the in-solution approach in order to better understand this process and to obtain clear evidence for the hybridization of the ligand/receptor complex on the support. We also hope to improve the lower detection limit by using higher number of galactose residues or by monitoring the length of the covalently immobilized oligonucleotide.

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[1] A. Varki, *Glycobiology* **1993**, *3*, 97.
[2] N. Sharon, H. Lis, *Glycobiology* **2004**, *14*, 53R.
[3] T. Feizi, F. Fazio, W. C. Chai, C. H. Wong, *Curr. Opin. Struct. Biol.* **2003**, *13*, 637.
[4] D. Wang, *Proteomics* **2003**, *3*, 2167.
[5] O. Blixt, S. Head, T. Mondala, C. Scanlan, M. E. Huflejt, R. Alvarez, M. C. Bryan, F. Fazio, D. Calarese, J. Stevens, N. Razi, D. J. Stevens, J. J. Skehel, I. van Die, D. R. Burton, I. A. Wilson, R. Cummings, N. Bovin, C. H. Wong, J. C. Paulson, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 17033.
[6] Y. Chevolot, J. Martins, N. Milosevic, D. Leonard, S. Zeng, M. Malissard, E. G. Berger, P. Maier, H. J. Mathieu, D. H. G. Crout, H. Sigrist, *Bioorg. Med. Chem.* **2001**, *9*, 2943.
[7] S. Park, M. R. Lee, S. J. Pyo, I. Shin, *J. Am. Chem. Soc.* **2004**, *126*, 4812.
[8] M. A. Brun, M. D. Disney, P. H. Seeberger, *ChemBioChem* **2006**, *7*, 421.
[9] B. T. Houseman, M. Mrksich, *Chem. Biol.* **2002**, *9*, 443.
[10] F. Fazio, M. C. Bryan, O. Blixt, J. C. Paulson, C. H. Wong, *J. Am. Chem. Soc.* **2002**, *124*, 14397.
[11] M. R. Lee, I. Shin, *Angew. Chem.* **2005**, *117*, 2941; *Angew. Chem. Int. Ed.* **2005**, *44*, 2881.
[12] M. Schwarz, L. Spector, A. Gargir, A. Shtevi, M. Gortler, R. T. Altstock, A. A. Dukler, N. Dotan, *Glycobiology* **2003**, *13*, 749.
[13] X. C. Zhou, J. H. Zhou, *Biosens. Bioelectron.* **2006**, *21*, 1451.
[14] J. C. Manimala, T. A. Roach, Z. T. Li, J. C. Gildersleeve, *Angew. Chem.* **2006**, *118*, 3689; *Angew. Chem. Int. Ed.* **2006**, *45*, 3607.

[15] C. Ortiz Mellet, J. M. Garcia Fernandez, *ChemBioChem* **2002**, *3*, 819.
[16] D. Wang, S. Liu, B. J. Trummer, C. Deng, A. Wang, *Nat. Biotechnol.* **2002**, *20*, 275.
[17] J. Hirabayashi, *Trends Biotechnol.* **2003**, *21*, 141.
[18] D. M. Ratner, E. W. Adams, M. D. Disney, P. H. Seeberger, *ChemBioChem* **2004**, *5*, 1375.
[19] Y. C. Lee, R. T. Lee, *Acc. Chem. Res.* **1995**, *28*, 321.
[20] J. J. Lundquist, E. J. Toone, *Chem. Rev.* **2002**, *102*, 555.
[21] K. Matsuura, M. Hibino, Y. Yamada, K. Kobayashi, *J. Am. Chem. Soc.* **2001**, *123*, 357.
[22] E. A. L. Biessen, D. M. Beuting, H. C. P. F. Roelen, G. A. Van de Marel, J. H. Van Boom, T. J. C. Van Berkel, *J. Med. Chem.* **1995**, *38*, 1538.
[23] M. Kohn, J. M. Benito, C. Ortiz Mellet, T. K. Lindhorst, J. M. Garcia Fernandez, *ChemBioChem* **2004**, *5*, 771.
[24] C. Boozer, J. Ladd, S. F. Chen, S. T. Jiang, *Anal. Chem.* **2006**, *78*, 1515.
[25] R. Wacker, H. Schroder, C. M. Niemeyer, *Anal. Biochem.* **2004**, *330*, 281.
[26] T. S. Zatsepin, T. S. Oretskaya, *Chem. Rev.* **2004**, *104*, 1401.
[27] J. D'Onofrio, M. de Champdore, L. De Napoli, D. Montesarchio, G. Di Fabio, *Bioconjugate Chem.* **2005**, *16*, 1299.
[28] Y. Singh, O. Renaudet, E. Defrancq, P. Dumy, *Org. Lett.* **2005**, *7*, 1359.
[29] C. Bouillon, A. Meyer, S. Vidal, A. Jochum, Y. Chevolot, J. P. Cloarec, J. P. Praly, J. J. Vasseur, F. Morvan, *J. Org. Chem.* **2006**, *71*, 4700.
[30] F. Seela, K. Kaiser, *Nucleic Acids Res.* **1987**, *15*, 3113.
[31] Mass spectrometric data obtained using MALDI-TOF (*m*/*z* calcd/found): **8a** 5746.02/5745.23, **8b** 6905.08/6906.40, **8c** 5796.92/5795.26, **8d** 7054.81/7056.45, **8e** 6909.97/6906.40.
[32] V. Dugas, G. Depret, B. Chevalier, X. Nesme, E. Souteyrand, *Sens. Actuators B* **2004**, *101*, 112.
[33] Except for reference [14] and V. I. Dyukova, E. I. Dementieva, D. A. Zubtsov, O. E. Galanina, N. V. Bovin, A. Y. Rubina, *Anal. Biochem.* **2005**, *347*, 94.
[34] R. Wacker, C. M. Niemeyer in *Current Protocols in Nucleic Acid Chemistry* (Eds.: S. L. Beaucage, D. E. Bergstrom, G. D. Glick, R. A. Jones), Wiley, New York, **2005**, p. 12.7.1.

