was dissolved in ethyl acetate (35 mL). Extraction with ethyl acetate and subsequent column chromatography delivered 1.15 g of product **4** (88%). ^{1}H NMR (300 MHz, $CDCl_3$, TMS): $\delta = 2.92-2.99$ (1H; CH_2), 3.07–3.23 (6H; CH_2), 4.10 (1H; CH_2), 6.51 (1H; CH), 6.54 (1H; CH), 6.60 (1H; CH), 6.65 (1H; CH), 6.68 (1H; CH), 6.81 (1H; CH), 7.39 (1H; CH); ^{13}C NMR (75 MHz, $CDCl_3$, TMS): $\delta = 34.80$, 35.32, 35.45, 36.38, 125.79, 127.50, 131.74, 132.51, 133.28, 133.53, 136.59, 136.81, 138.01, 138.52, 139.71, 139.94, 140.10, 140.12, 140.80, 141.24, 143.50, 145.10, 159.51; IR (KBr): $\tilde{\nu} = 513$, 630, 693, 790, 852, 902, 994, 1040, 1122, 1163, 1255, 1516, 1757, 2853, 2898, 2926, 2955, 3015 cm^{-1}; MS (70 eV): m/z: 418 [M^+], 314 [$C_8H_7CO_2C_6F_5^+$], 251 [$C_{16}H_{15}CO_2^+$], 235 [$C_{16}H_{15}CO^+$], 131 [$C_8H_7CO^+$], 104 (100) [$C_8H_8^+$], 77 [$C_6H_5^+$].

5: Compound **4** was polymerized using a self-designed CVD installation consisting of a sublimation zone, a pyrolysis zone, and a deposition chamber.[21] Compound **4** (30 mg, 0.07 mmol) was placed in the sublimation zone and a sample, such as a gold-coated silicon substrate, was fixed in the deposition chamber at 45 °C. The pressure was adjusted to 0.2 mbar and the pyrolysis zone was heated to 600 °C. Subsequently, **4** was sublimated slowly by increasing the temperature of the sublimation zone from 120 to 130 °C. Under these conditions, the deposition rate was 0.4 Å s^{-1}.

The spectroscopic ellipsometry was done on a variable-angle spectroscopic ellipsometer (J. A. Woollam Inc., USA) using a Cauchy model for curve fitting. AFM studies were conducted in tapping mode on a NanoScope III (Digital Instruments Inc., USA).

XPS (atomic ratios): F_{1s}/C_{1s}: 31.7% (calcd: 21.7%), O_{1s}/C_{1s}: 7.4% (calcd: 8.7%), C–F/C–C: 28.3% (calcd: 31.3%), C–O/C–C: 5.7% (calcd: 6.3%), C=O/C–C: 5.4% (calcd: 6.3%); IR (grazing angle 85°): $\tilde{\nu} = 658$, 829, 997, 1036, 1176, 1246, 1450, 1471, 1497, 1523, 1762, 2862, 2931, 3025, 3054 cm^{-1}.

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Hapten-Functionalized DNA-Streptavidin Nanocircles as Supramolecular Reagents in a Competitive Immuno-PCR Assay**

Christof M. Niemeyer,* Ron Wacker, and Michael Adler

The self-assembly of small building blocks to form structural and functional elements is an important goal of molecular nanotechnology.[1] In this respect, DNA is a promising construction material which has already been employed in the fabrication of nanometer-scale scaffolds and surface architectures,[2] to selectively position proteins[3] and nanoclusters,[4] and to generate mechanical molecular devices.[2] We recently reported the nanostructured oligomeric conjugates **3**, formed by self-assembly of bisbiotinylated DNA **1** and the biotin-binding protein streptavidin (STV) **2** (Scheme 1).[5] Despite its tetravalent binding capacity for biotin, the STV molecules are predominantly present as bi- or trivalent linkers between double-stranded DNA (dsDNA) fragments within **3**. Because of this remaining biotin-binding capacity, the DNA–STV oligomers **3** are powerful reagents for immuno-PCR (IPCR; PCR = polymerase chain reac-

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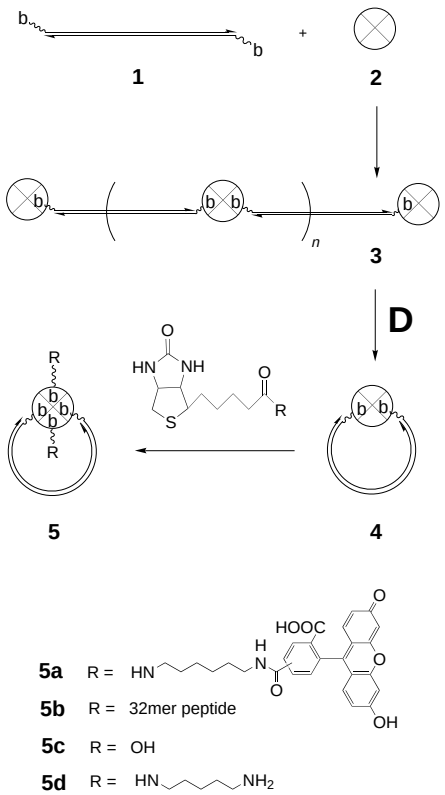
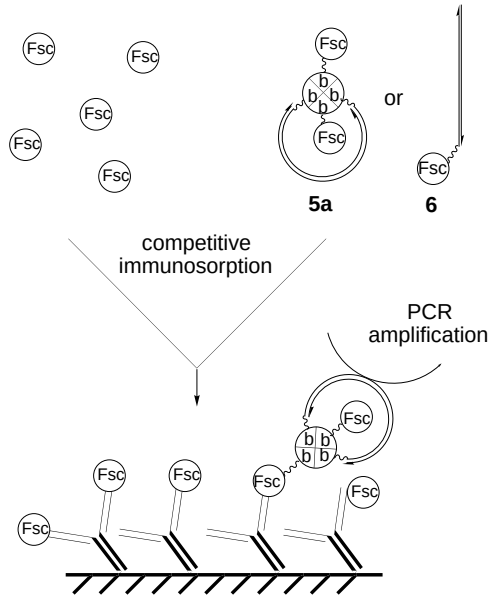


Scheme 1. Synthesis of hapten-functionalized DNA – STV nanocircles **5** from 5′,5′-bisbiotinylated 169 bp dsDNA fragments (b = biotinyl) and STV **2**. The oligomeric conjugates **3** are transformed into the nanocircles **4** by thermal treatment, and the crude products are coupled with an excess of biotinylated fluorescein (Calbiochem; **5a**), biotinylated 32mer peptide β-endorphine (EGKKYANKIIANKFLWVLPWQSKESWMFGGYK-biotin, Sigma; **5b**), D-Biotin (**5c**), or aminobiotin[15] (**5d**), and purified by electrophoresis. The complementary DNA strands are drawn as parallel lines. The 3′-ends are indicated by the arrow heads.

tion),[5] a highly sensitive method for the trace analysis of proteins and other antigens.[6] Moreover, the oligomers **3** can be transformed by thermal treatment into well-defined supramolecular nanocircles **4** which have two unoccupied biotin-binding sites.[7] Herein, we report the functionalization of **4** with biotinylated hapten groups, as well as the use of the conjugates **5** as reagents in a novel competitive immuno-PCR (cIPCR) assay for the ultrasensitive detection of low molecular weight substances.

The specific detection of hormones, toxins, pesticides, and other low molecular weight analytes is commonly achieved by methods based on chromatography, mass spectrometry, and biosensors. In addition, antibody-based techniques, such as radioimmunoassay (RIA) or competitive enzyme-linked immunosorbent assays (cELISA) are often applied.[8] The competitive assay format is necessary since for steric reasons the low molecular weight analyte can only bind to a single antibody. Typically, in cELISA, a conjugate of the traceable hapten and a signal-generating enzyme is employed. The signal-generating conjugate competes with the free analyte for binding sites on a surface.[8d] As a result of our experience with the ultrasensitive detection of proteins by means of IPCR,[5] we attempted to investigate whether the PCR amplification of a DNA marker might be applicable to the competitive detection of low molecular weight analytes (Scheme 2). In such a cIPCR, the hapten-functionalized dsDNA fragment **6** might be used as the signal-generating conjugate. Conjugates such as **6** can be obtained by chemical oligonucleotide synthesis and preparative PCR. However, despite the power of today's solid-phase DNA synthesis, this



Scheme 2. Detection of low molecular weight analytes by cIPCR, illustrated with the model analyte fluorescein (Fsc). The free analyte Fsc competes with the immunosorptive binding of the signal-generating hapten – DNA conjugate to an antibody-modified surface. The covalent Fsc functionalized dsDNA **6** or the hapten functionalized nanocircle **5a** are employed as the hapten – DNA conjugate. After competitive binding and washing, the surface-immobilized hapten – DNA conjugate can be detected by means of PCR amplification.

approach would be laborious since, for instance, appropriate hapten – phosphoramidite precursors would need to be prepared for each particular application. In comparison, the coupling of a hapten with a biotin group is often easier to achieve.[9] Thus, the modular generation of hapten – DNA conjugates by means of the highly specific biotin – STV interaction appeared very attractive to us. Because of their simple and efficient preparation, as well as their well-defined stoichiometry and structure, the DNA – STV nanocircles **4** were chosen as basic building blocks of a supramolecular construction kit for generating hapten – DNA conjugates.

To experimentally verify our concept, the nanocircle **5a** was prepared containing the model hapten fluorescein. According to the established procedure,[7] oligomers **3** were generated from 5′,5′-bisbiotinylated 169 base pair (bp) dsDNA fragments and STV, the oligomers were thermally denatured, and the crude products were coupled with an excess of biotinylated fluorescein. Subsequently, **5a** was purified by native electrophoresis. The intact circular structure of hapten-functionalized **5** was verified by its characteristic electrophoretic mobility, which is identical to that of **4**. The presence of the fluorescein moiety in **5a** was verified by antibody-induced gel-shift. Since the hapten-containing nanocircles

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were supposed to be applied as storable stock solutions for the cIPCR, we investigated the thermal stability of **5a** by incubating aliquots of **5a** for 90 seconds at various temperatures and subsequent analysis by gel electrophoresis (Figure 1). As determined by densitometry, the purity of **5a** was about 93% (position c in Figure 1). The brief heating of the nanocircle led to the temperature-dependent formation of degradation products which were assigned by their electrophoretic mobility as free DNA (position a), a STV conjugate containing single-stranded DNA (position b), the linearized, opened dsDNA–STV nanocircle (position d), as well as the $(dsDNA)_2$–STV conjugate (position e). To differentiate between the closed and linearized (opened) nanocircles, which show an almost identical electrophoretic mobility (position c and d, respectively, in Figure 1), an excess of STV was added to an aliquot of the heat-denatured products. This procedure retarded the movement of the opened circle and allowed the quantification of the band intensity of the remaining intact nanocircles. An increase in temperature led to an increase in the amount of degradation products, and a distinctive change in degradation-product distribution was observed between 75 and 80 °C. At temperatures greater than 80 °C an approximately constant amount of a single-stranded DNA–STV conjugate was formed.[10] The further increase of the temperature over 90 °C led to almost complete degradation of nanocircle **5a**.

Similar experiments were carried out with the nanocircles **5b**–**d** (Scheme 1). Based on product distributions determined by densitometry, temperature dependent degradation curves were prepared in which the amount of intact nanocircle **5** remaining was plotted against the temperature (Figure 2). To compare the relative thermal stability of **5a**–**d**, the temperature was extrapolated to give the temperature at which 50% of nanocircles remained intact (TD_{50} value). As indicated from the TD_{50} values listed in the legend to Figure 2, the thermal stability of **5a** through **5d** continuously decreases. It can be estimated that the stability of fluorescein-modified nanocircle **5a** is reduced by about 100 J mol^{-1}, while the stability of aminobiotin-modified circle **5d** is enhanced by

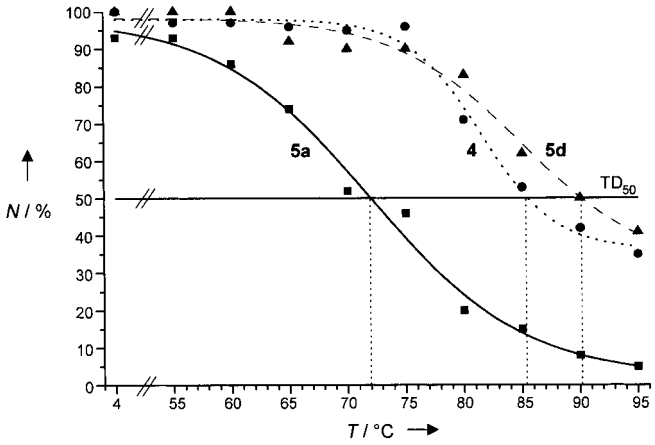
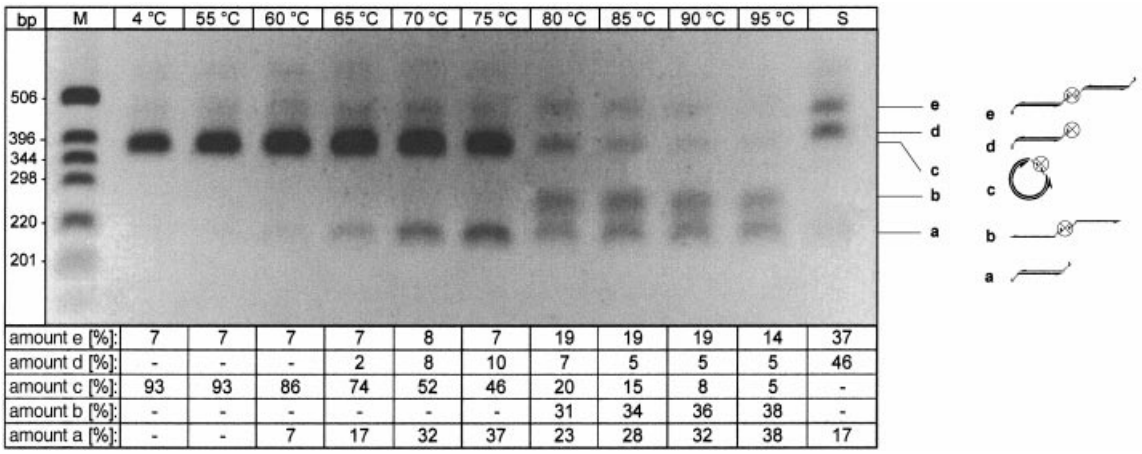


Figure 2. Thermal stability of nanocircles **4** and **5**. The relative product distributions from the experiments in Figure 1 were determined by densitometric analysis. The percentage amounts (N) of the remaining intact nanocircles **4** or **5** are plotted against the temperature. The temperature point at which 50% of the nanocircles remained intact (TD_{50} value) was extrapolated to be 85 ± 3 °C (**4**), 73 ± 3 °C (**5a**), 77 ± 3 °C (**5b**), 80 ± 3 °C (**5c**), and 90 ± 3 °C (**5d**).

about 40 J mol^{-1} compared to that of unmodified circle **4**. Functionalization with the biotinylated 32mer neuropeptide β-endorphine[11] in **5b** and the coupling of D-Biotin in **5c** induces a small destabilization of about 40–50 J mol^{-1} compared to unmodified **4**. The destabilization of **5a** by the coupling of biotinylated fluorescein, and the slight stabilization of **5d** with aminobiotin might be a result of Coulomb interactions between the negatively charged phosphate backbone of the dsDNA and the negatively charged fluorescein groups in **5a**, or the positively charged amino groups in **5d**.[12]

To demonstrate the functionality of the hapten-nanocircles **5** in a model cIPCR assay, samples containing a constant amount of **5a** were mixed with increasing amounts of free fluorescein. For competitive immunosorption, these mixtures were added to wells of a microplate previously coated with an antibody directed against fluorescein (Scheme 2). After incubation and washing of the microplate, the DNA immunosorbed was amplified by PCR, and the PCR products were



amount e [%]:	7	7	7	7	8	7	19	19	19	14	37
amount d [%]:	-	-	-	2	8	10	7	5	5	5	46
amount c [%]:	93	93	86	74	52	46	20	15	8	5	-
amount b [%]:	-	-	-	-	-	-	31	34	36	38	-
amount a [%]:	-	-	7	17	32	37	23	28	32	38	17

Figure 1. Thermal stability of fluorescein-modified nanocircle **5a**. A nondenaturing 1.5% agarose gel stained by ethidiumbromide is shown. Samples of purified **5a** were briefly heated to the temperatures listed at the top of the lanes. The percentage yields of the various products formed, listed at the bottom of the lanes, were determined by densitometric analysis. The products a–e were assigned by comparison of the mobility of standards, such as, conjugates of STV and monobiotinylated dsDNA applied to lane S. Lane M contains a DNA molecular-weight marker; the lengths are given in base pairs.

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quantified.[13] For comparison, similar experiments were carried out using the DNA conjugate **6**, which contains a covalently bound fluorescein moiety. As shown in Figure 3, the signals obtained in the cIPCR assays clearly indicate that this novel immunoassay allows trace amounts of the model analyte to be detected. Although direct comparison of **5a** and **6** reveals greater signal intensities for **6**, the use of **5a** led to significantly smaller errors as well as an improved limit of detection of about 10 pM of the analyte. Further experiments indicated that, as expected, the intensity and the detection limit of cIPCR depends on the amount of both the hapten–DNA conjugate and the antibody immobilized. No reference data on the ELISA detection of fluorescein were available for comparison. However, typical limits of detection of low molecular weight haptens are about ≥ 10 nM by using mass spectrometry, or about ≥ 100 pM by using ELISA and RIA.[14] Thus, the detection limit of 10 pM for cIPCR observed here with the nonoptimized model-system fluorescein, is similar to the 10- to 1000-fold enhancement of the detection limit regularly obtained by the transition from ELISA to the analogous IPCR assay.[5]

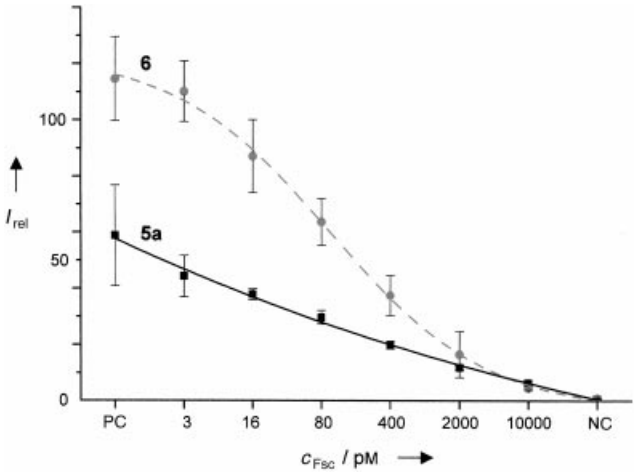

Figure 3. Functionality of the fluorescein–DNA conjugates **5a** and **6** in the cIPCR assay (see Scheme 2). A constant amount of **5a** or **6** was mixed with increasing amounts of free fluorescein (c_{Fsc}) and the mixtures were incubated in microplate wells, previously coated with an antibody directed against fluorescein. After competitive immunosorption, the DNA conjugate immobilized was amplified by PCR. Quantification of the PCR products led to signals which were normalized to relative intensities (I_{rel}) using a PCR negative control (NC) in which no DNA-conjugate was applied to the wells. PC = positive control.

We reported here the simple and efficient formation of well-defined functional DNA–protein conjugates by noncovalent assembly of biotinylated dsDNA, biotinylated hapten groups, and streptavidin. The supramolecular complexes can be used as reagents in a novel PCR-based immunoassay, which we term competitive immuno-PCR (cIPCR). Our model studies suggest that cIPCR allows for around a 10- to 1000-fold improvement over the detection limit of conventional antibody-based assays. Further optimization of the cIPCR and applications regarding the detection of biomedical-relevant analytes are in progress. For instance, these studies indicate that the model system **5a** can be applied to the β-endorphine–peptide nanocircle **5b**, as well as to similar conjugates prepared from biotinylated testosterone.[9]

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