Magnetic Nanosensors for the Detection of Oligonucleotide Sequences

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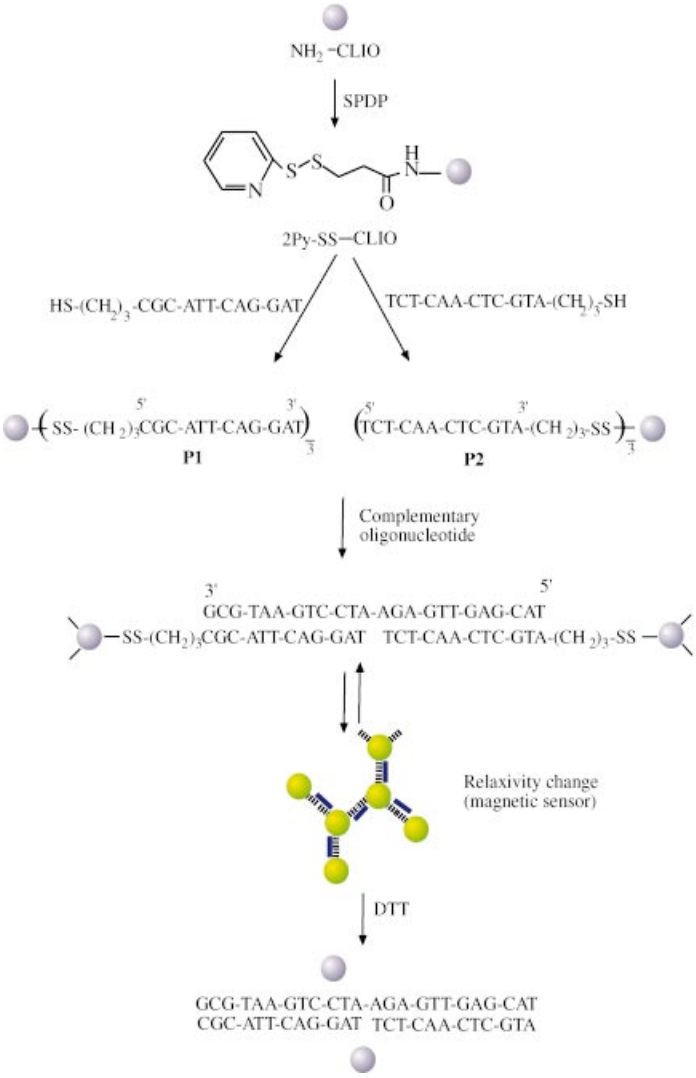
The rapid detection and read-out of specific nucleic acid sequences has become a fundamental necessity in medicine and biotechnology. Photosensitive reporters such as fluorophores,[1, 2] gold particles,[3] and chemiluminescent probes[4] bound to complementary oligonucleotides have now largely replaced older detection systems based on radioisotopes. A number of biological applications, however, dictate fast sample throughput, simplified sample preparation, and measurements in turbid/obscure tissue-type media. As one method, bioelectronic DNA chip technology has recently been developed which relies on the detection of electrical current generated by oxidation and reduction of labeled nucleic acid targets.[5] We report here on a novel magnetic nanosensor technology as an alternative method for the detection of specific oligonucleotides.

The starting material consisted of monodisperse, fluid-phase nanoparticles containing an icosahedral core of superparamagnetic, crystalline Fe_2O_3/Fe_3O_4 (3 nm), caged by epichlorohydrin cross-linked dextran and functionalized with amine groups (CLIO-NH_2).[6, 7] We then coupled thiolated oligonucleotides to CLIO-NH_2 using *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) as a linker (Scheme 1). The physical properties of the conjugates **P1** (CLIO-(SS-$(CH_2)_6$-CGC-ATT-CAG-GAT)$_{\bar{3}}$) and **P2** ((TCT-CAA-CTC-GTA-$(CH_2)_3$-SS)$_{\bar{3}}$-CLIO) are summarized in Table 1. **P1** and **P2** each had an average of three oligonucleotides per particle based on a single crystal per particle, and 2064 iron atoms per crystal.[7] They could be stored at room temperature or 4 °C for several weeks without precipitation.

Mixtures of **P1** and **P2** were incubated with a complementary oligonucleotide to examine the ability of **P1** and **P2** to hybridize. The sample became slightly turbid within 3 – 4 hours at room temperature, with a brown precipitate forming after 16 hours (Figure 1 B). The precipitate moved to the side of the tube on application of a hand-held magnet. Tubes containing the **P1**/**P2** mixture alone (Figure 1 A), the **P1**/**P2** mixture with noncomplementary olgonucleotide (Figure 1 C), and the **P1**/**P2** mixture with half-complementary oligonucleotide (Figure 1 D) did not show turbidity or precipitate formation even after several weeks at room temperature.

Gel electrophoresis was performed to further elucidate the interaction of **P1**/**P2** with a complementary oligonucleotide. Under nondenaturing conditions and without 1,4-dithiothreitol (DTT) the precipitate (Figure 1 B) remained at the top of the gel (Figure 2 A, lane 1). Treatment with DTT (Figure 2 A, lane 2) resulted in a single band of hydridized oligonucleotide. Under denaturing conditions and with DTT added (Figure 2 B) two bands were observed, the slower one consisting of complementary oligonucleotide and the faster one of a mixture of 3′ and 5′ oligonucleotides.



Scheme 1. Alkanethio-substituted oligonucleotides were treated with SPDP-activated nanoparticles to form the **P1** and **P2** nanosensors. **P1** and **P2** hybridize with complementary oligonucleotide and result in oligomerization and changes in the magnetic relaxivity. Treatment with DTT breaks the bond between the nanoparticle and alkanethio-substituted oligonucleotide.

Table 1. Size and relaxivities before and after hybridization.

Compound	Hybridization[a]	Size [nm]	$R1$[b] [s^{-1} mM^{-1}]	$R2$[b] [s^{-1} mM^{-1}]	$R2/R1$
P1	no	53 ± 11	27.7 ± 0.3	75 ± 2	2.7 ± 0.1
P2	no	53 ± 11	26.7 ± 0.3	71 ± 10	2.6 ± 0.4
P1 + **P2** + oligo noncomplement.	no	65 ± 25	25.8 ± 0.4	67 ± 1	2.6 ± 0.1
P1 + **P2** + oligo complement.	yes	215 ± 19	23.0 ± 1.0	128 ± 3	5.6 ± 0.2

[a] Determined by gel electrophoresis. [b] Relaxivities are the slopes of plots of relaxation rate ($1/T$, s^{-1}) against nanosensor concentration (Fe, mM); values are plotted as means ± standard deviation from three determinations. Size refers to the unimodal size distribution as determined by light scattering, mean ± standard deviation over six measurements.

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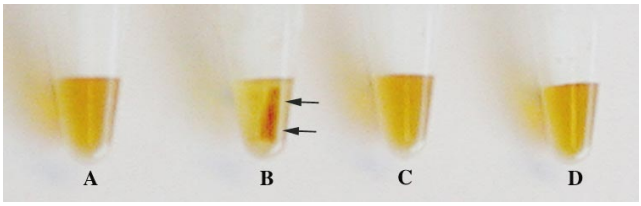

Figure 1. Effect of incubating nanoparticle probes with oligonucleotides: A) **P1** and **P2;** B) **P1** and **P2** plus complementary oligonucleotide; C) **P1** and **P2** plus noncomplementary oligonucleotide; D) **P1** and **P2** and half-complementary oligonucleotide.

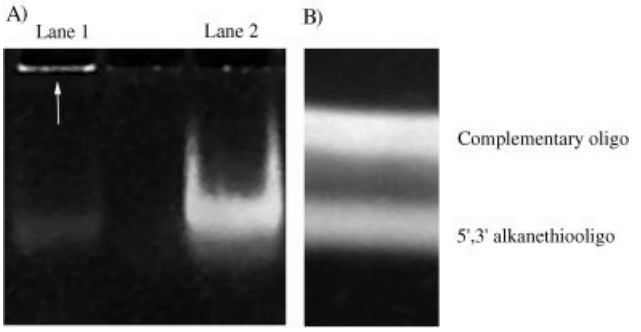

Figure 2. Gel electrophoresis of **P1**/**P2**/oligonucleotide precipitate: A) Nondenaturing conditions: lane 1) no DTT, lane 2) with DTT. B) Denaturing conditions with DTT.

The nanoparticles **P1** and **P2** are potent enhancers of the spin–spin and spin–lattice relaxation processes (Table 1). Interestingly, the spin–spin relaxation was significantly enhanced by oligonucleotide hybridization, and rendered the particles potential "magnetic nanosensors". Figure 3 shows the effect of adding oligonucleotide to an aqueous

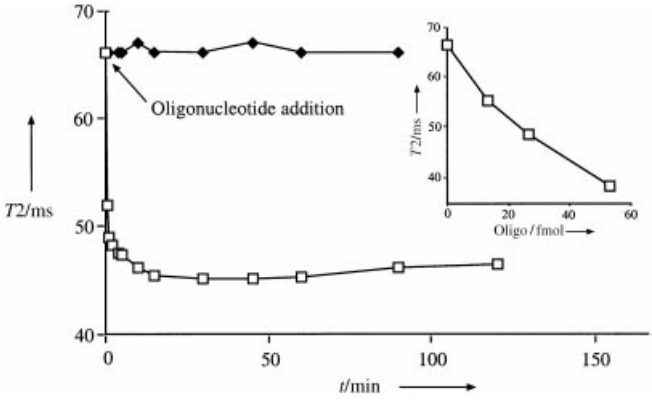

Figure 3. Temporal change of water $T2$ relaxation times with (□) and without complementary oligonucleotide (◆). The insert shows the effect of increasing concentrations of complementary oligonucleotide on the $T2$ value. All data points shown represent the average of three measurements with standard deviations ranging between 0.4–0.6 ms for the $T2$ values (too small to graph).

solution of **P1**/**P2** on the relaxation times $T2$. The value of $T2$ decreased from 63 ms to 45 ms within several minutes, and this effect persisted for the period of observation (2 h). Table 1 summarizes the concentration-independent relaxivities $R1$ and $R2$ before and after hybridization. Hybridization primarily affected $R2$ with a doubling of the $R2/R1$ ratio being observed. Concomitant laser light scattering indicated a significant size increase of the hybridized conjugates, which presumably affected the spin–spin relaxation. Similar changes in relaxivity were also observed in turbid and tissuelike samples (data not shown).

We next investigated the effect of temperature cycling on the hybridization of the nanosensors by measuring changes in the $T2$ values (Figure 4). Hybridization was minimal at 80 °C and changes in the $T2$ values were small. Representative changes in the $T2$ values were observed during multiple cycles of heating and cooling. Furthermore, the oligonucleotides were cleaved from the nanosensors upon addition of DTT, and the $T2$ value did not change during further temperature cycling.

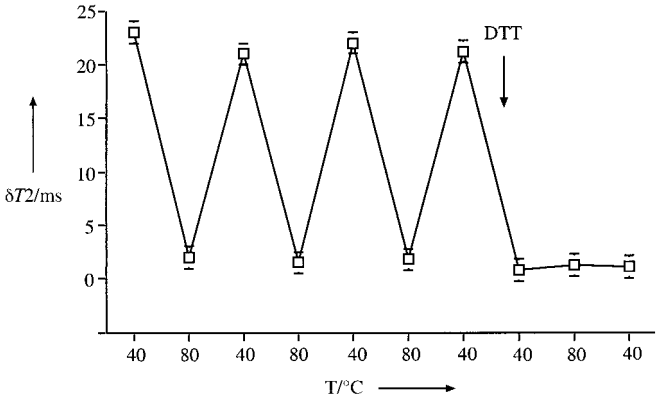

Figure 4. Changes in the $T2$ values of an aqueous solution of **P1**/**P2**/complementary oligonucleotide as a function of temperature cycling.

The above results indicate that oligonucleotide hybridization efficiently changes the spin–spin relaxation time of adjacent water protons, that these effects occur within minutes, that the magnetic effects are fully reversible, and that the oligonucleotides are recoverable from the sensors. We believe that the described nanosensors may have considerable applications in biotechnology and biomedicine. Reduced sample sizes required by modern magnetic bench-top readers should enable the attomole detection of oligonucleotides at high throughputs, while detection systems with higher sensitivity, such as magnetic force microscopy or sensitive oscillating magnetic field readers, should allow detection of single strands of hybridized oligonucleotides.[8] Finally, since the developed nanosensors are essentially nontoxic to mammalian cells,[9] nondegradable oligonucleotide analogues (for example, peptide nucleic acid (PNA)) may be coupled to nanoparticles and used to image sequences of nucleic acids in vivo.[10]

Experimental Section

Synthesis of magnetic nanoparticles: Biocompatible, fluid-phase magnetic nanoparticles (CLIO-NH_2) were synthesized as described in more detail elsewhere[6] and treated with *N*-succinimidyl-3-(2-pyridyldithio)-propionate (SPDP) to yield 2Py-SS-CLIO (Scheme 1).[6] The 5′-alkanethiol oligonucleotide (HS-$(CH_2)_6$-CGC-ATT-CAG-GAT) and 3-alkanethiol oligonucleotide (TCT-CAA-CTC-GTA-$(CH_2)_3$-SH) were synthesized at a 1 μmol scale using standard phosphoramidite chemistry. The sulfhydryl

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groups were protected with a thioalkyl linker. The oligonucleotides were deprotected with DTT immediately before reaction with 2Py-SS-CLIO.[11]

Synthesis of magnetic oligonucleotide nanoparticles: Complementary (5′TAC-GAG-TTG-AGA-ATC-CTG-AAT-GCG3′), half-complementary (5′TAC-GAG-TTG-AGA-GAG-TGC-CCA-CAT3′), and noncomplementary (5′ATG-CTA-AAT-GAC-GAC-TGC-CCA-CAT3′) oligonucleotides were synthesized using standard phosphoramidite chemistry (underlined bases will hybridize). Either 5′- or 3′-alkanethio-oligonucleotide (550 μg) was added to of 2Py-SS-CLIO (1.1 mL; 3 mg of Fe in 0.1 M phosphate buffer, pH 8.0), and the mixture incubated overnight at room temperature. The mixture was purified using an LS + high-gradient magnetic separation column (Miltenyi Biotec, Auburn, CA) equilibrated with 0.1 M phosphate buffer, pH 7.5. The number of oligonucleotides attached per particle was determined by treatment with DTT, followed by separation of the iron and oligonucleotide using a microconcentrator as described.[6] The oligonucleotide concentration was then determined from absorbance spectroscopy at 260 nm using an extinction coefficient of $1.2 \times 10^5\,M^{-1}\,cm^{-1}$. The probes are denoted **P1** (CLIO-SS-$(CH_2)_6$-CGC-ATT-CAG-GAT) and **P2** (TCT-CAA-CTC-GTA-$(CH_2)_3$-SS-CLIO).

Hybridization: For Figure 1 equal volumes (25 μL) of **P1** and **P2** (both at 550 μg Fe mL^{-1}) were mixed with a solution of 1 M NaCl and 0.1 M phosphate at pH 7.5 (14 μL). Various oligonucleotides (2 μL, 400 ng) were then added. The mixture was heated to 50 °C for 5 min and allowed to react at room temperature overnight. The precipitate shown in Figure 2B was obtained after overnight incubation of **P1**/**P2** with complementary oligonucleotide; the precipitate was washed with 0.1 M NaCl and 0.1 M phosphate buffer, and re-suspended in the same buffer (300 μL). The sample was split into two portions (15 μL) and examined by electrophoresis without DTT (lane 1) or with 4 mM DTT (lane 2) under nondenaturing conditions (Figure 2A) or denaturing conditions (Figure 2B).

Gel electrophoresis: Nondenaturing gels (10% polyacrylamide) and denaturing gels (20% polyacrylamide) were used. Gels were stained with SYBR Gold dye (Molecular Probes,Eugene OR).

Determination of proton relaxation times: Relaxation time measurements were performed at 0.47 T and 40 °C or 80 °C (Bruker NMR Minispec, Billerica, MA). To determine the effect of hybridization on the $T2$ values of water, equal amounts of **P1** and **P2** (5 μL) were diluted in a solution of 1 M NaCl and 0.1 M phosphate buffer at pH 7.5 (1 mL) to give a total iron content of 10 μg mL^{-1}. $T2$ values were obtained before and after addition of 1 μL (390 ng) of complementary, half-complementary, or noncomplementary oligonucleotides and plotted as a function of time. The relaxivity was determined by plotting $1/T2$ and $1/T1$ values of water as a function of the iron concentration. The size of the conjugates was determined by light scattering (Coulter N4, Hialeah, FL).

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Do More Electrophilic Aldehydes/Ketones Exhibit Higher Reactivity toward Nucleophiles in the Presence of Lewis Acids?

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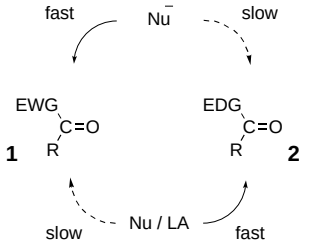
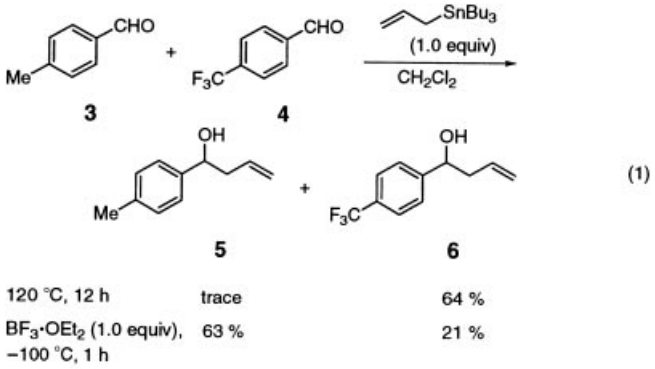
Lewis acid mediated electrophilic reactions of carbonyl compounds are among the most fundamental and important reactions in modern organic synthesis.[1] It is well known that the coordination of carbonyl groups to Lewis acids exerts a dramatic effect on the rates and selectivities of reactions at the carbonyl centers. While much research into the Lewis acid mediated stereoselective or regioselective reactions has been carried out, less attention has been paid to the chemoselective reactions in the presence of Lewis acids.[2–4] It is evident for organic chemists that more electrophilic aldehydes and ketones **1** react with nucleophiles (Nu^-) much faster than less electrophilic analogues **2**. We found that the reverse is the case in the Lewis acid mediated reactions: more electrophilic aldehydes and ketones **1** react much slower than the less electrophilic analogues **2** in the presence of Lewis acids (Scheme 1), with a chemoselectivity that is not attainable under ordinary conditions.



Scheme 1. Chemoselective reactions of equimolar mixtures of **1** and **2**. EWG = electron-withdrawing group, EDG = electron-donating group.

For many years, we have researched the Lewis acid mediated reaction of allyl stannanes and related organometallic compounds.[5] An interesting observation was made in the reaction of allyltributylstannane with aldehydes. The reaction of a 1:1 mixture of *p*-tolualdehyde (**3**) and α,α,α-trifluoro-*p*-tolualdehyde (**4**) with one equivalent of allylstannane at 120 °C gave the homoallylic alcohol **6** derived from **4**, in 64% yield, along with trace amounts of the homoallylic alcohol **5** derived from **3** [Eq. (1)]. This is an expected result,



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