

# Collection of Trace Amounts of DNA/mRNA Molecules Using Genomagnetic Nanocaptors

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**The collection and then the separation of rare DNA/mRNA targets with single-base mismatches in a complex matrix is critically important in human disease diagnostics, gene expression studies, and gene profiling. The major result of this work is the development and application of a novel genomagnetic nanocaptor (GMNC) for the collection, separation, and detection of trace amounts of DNA/RNA molecules with one single-base difference. The GMNC is constructed by bioconjugating molecular beacon DNA probes onto magnetic nanoparticle surfaces. We have successfully applied the GMNC in artificial buffer solution samples and in cancer cell samples, both containing different proteins and random DNA sequences. Our method has three distinctly useful features: highly efficient collection of trace amount of DNA/mRNA samples down to femtomolar ( $10^{-15}$  M) concentrations; excellent ability to differentiate single-base-mismatched DNA/mRNA samples by combining the exceptional specificity of molecular beacons and the separation power of magnetic nanoparticles; and real-time monitoring and confirmation of the collected gene products. The newly developed genomagnetic nanocaptors will be highly useful for the collection of trace amounts of DNA/mRNA targets in a variety of sample sources in forensic, medical, and biotechnological fields.**

The separation and collection of trace amounts of gene products are of great interest in disease diagnostics, biomedical studies, and biotechnology development. This is especially true for single-base-mismatched DNA/mRNA strands in complex mixtures such as cell lysates and tissue samples. It has been reported that most cancers originate from genetic mutations starting with a single-base change in DNA sequences.<sup>1</sup> The detection and collection of DNA/mRNA sequences with single-base differences should thus play a major role in mutation detection and in gene expression studies. Currently, however, there is a lack of efficient biotechnologies to collect trace amounts of target DNA/mRNA from a complex matrix with single-base accuracy. Reversed-phase HPLC and ion-exchange HPLC are

routinely used in DNA synthesis and purification because of their high separation capacity,<sup>2</sup> but these techniques are based on hydrophobicity or DNA lengths instead of DNA sequences.<sup>3</sup> Gel electrophoresis is a common method for DNA separation and collection.<sup>4–6</sup> However, it does not have sequence recognition capability. The problem becomes even more severe when low-abundance DNA strands are targeted in situations where living cells are involved. Therefore, the collection of rare gene products from a complex mixture has been a significant challenge.

DNA hybridization is one of the most selective molecular recognition mechanisms capable of detecting target DNA sequences from a mixture. However, discriminating between two DNA sequences that differ by only one single base is difficult for linear DNA probes due to a small difference in thermodynamic stability between the two DNA duplexes. Usually, the melting temperatures between two DNA duplexes formed by linear DNA targets with a one-base difference are less than 10 °C.<sup>7</sup> Better DNA probes are thus needed for more effective discrimination between two DNA strands with a single-base difference. Recently, molecular beacons (MBs) have been developed for real-time DNA/RNA detection with high specificity and sensitivity.<sup>7–8</sup> As a result of the stem–loop structure, MBs have a much better capability to discriminate perfect complementary DNA from single-base-mismatched DNA.<sup>7</sup> MBs also have an excellent built-in signal transduction mechanism that enables them to be used as gene probes for “detection without separation”.<sup>8</sup> Since the introduction of MBs, they have been used in real-time monitoring of DNA/RNA in solution and living specimens, protein–DNA interaction studies, and biosensor development.<sup>9–14</sup> To take advantage of MBs’ high sequence selectivity and signaling capability in gene detection

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and separation, a suitable molecular carrier between liquid and solid phases is required.

Nanomaterials are excellent molecular carriers due to their high surface-to-volume ratio and ultrasmall size.<sup>15</sup> Among several types of nanomaterials, nanoparticles have demonstrated unique advantages when they are combined with biomolecules for bioanalysis.<sup>16,17</sup> We have recently developed magnetic nanoparticles<sup>18</sup> that are ideal molecular carriers for gene separation due to their high separation efficiency in a magnetic field.<sup>19–21</sup> The separation of biotinylated DNA and nonbiotinylated DNA has been carried out using avidin-coated magnetic nanoclusters.<sup>22</sup> However, the collection and separation of single-base-mismatched DNA strands has not yet been demonstrated.

In this work, we report the development of a genomagnetic nanocapturer (GMNC) using magnetic nanoparticles functionalized with MBs. The high sequence selectivity and excellent detection sensitivity of MBs are combined with the superior separation capability of the magnetic nanoparticles<sup>18</sup> for the separation of trace amounts of DNA/mRNA strands with a single-base difference. Single-base-mismatched DNA has been easily separated, collected, and determined from perfectly matched complementary DNA by applying a magnetic field and controlling the temperature. The separation and collection processes can be monitored with the changes of the intrinsic fluorescence signal of MBs. To demonstrate the applicability of the GMNC, we have also collected trace amounts of mRNA targets from both an artificial complex matrix and cultured cells using the GMNC. We expect that the GMNC described in this work will be useful in treating various samples such as those obtained from laser capture microdissection.<sup>23</sup>

## EXPERIMENTAL SECTION

**Chemicals and Apparatus.** All biochemicals were purchased and used as received. Lysozyme (Lyz), hemoglobin (Hb), and beef serum albumin (BSA) were purchased from Sigma. Ferric chloride, ferrous chloride, Triton X-100 (polyoxyethylene (10) isooctyl phenyl ether, 4-(C<sub>8</sub>H<sub>17</sub>)C<sub>6</sub>H<sub>4</sub>(OCH<sub>2</sub>CH<sub>3</sub>)<sub>n</sub>OH,  $n \sim 10$ ), and tetraethyl orthosilicate (TEOS) were purchased from Aldrich Chemical Co. Inc. (Milwaukee, WI). Cyclohexane, *n*-hexanol, and

sodium hydroxide were obtained from Fisher Scientific Co. (Pittsburgh, PA). Distilled deionized water (Easy Pure LF) was used for the preparation of all aqueous solutions in the synthesis of magnetic nanoparticles.

An FS20 ultrasonicator (Fisher Scientific Co.), a 5810 R centrifuge (Eppendorf), a Hitachi H-7000 transmission electron microscope, and a MPMS-5S superconducting quantum interference device (SQUID) magnetometer were used for the synthesis and characterization of magnetic nanoparticles.<sup>18</sup> A Fluorolog TAU-3 spectrofluorometer (Jobin Yvon-Spex, Instruments S.A., Inc.) was used to detect fluorescence intensity at different temperatures.

**Design of the Molecular Beacon.** One molecular beacon (MB1, TriLink BioTechnologies Inc.) was designed with a 15-nucleotide loop and 5-nucleotide arms. The loop sequence, 5'-ATC AAT ATT TAA CAA-3', is complementary to a disease-related DNA sequence known as the anthrax lethal factor. Fluorescein was chosen as the fluorophore and DABCYL [4-(4'-dimethylaminophenylazo)benzoic acid] as the quencher. Another molecular beacon (MB2, TriLink BioTechnologies Inc.) was designed with an 18-base loop and 5-base arms. This loop sequence is complementary to a section of a rat 204nt  $\gamma$ -actin mRNA. To compare linear DNA probes with molecular beacons, a 15-base linear DNA probe, which has the same sequence as the loop sequence of MB1, was designed with fluorescein as the fluorophore. Targets to the linear DNA probe were labeled with DABCYL as the quencher. Several different target DNA sequences (synthesized by Integrated DNA Technologies Inc.) have been designed for DNA hybridization studies with the designed MBs and linear DNA probes. All DNA sequences are shown in Table 1.

**Synthesis of Magnetic Nanoparticles.** The silica-coated magnetic nanoparticles were synthesized using a reverse microemulsion method previously reported.<sup>18</sup> The microemulsion was prepared using Triton X-100 surfactant. FeCl<sub>2</sub> and FeCl<sub>3</sub> were used to form iron oxide nanoparticles. The silica layer was formed by adding TEOS to the microemulsion. The resulting nanoparticles were characterized using transmission electron microscopy (TEM) and SQUID magnetometry.

**Fabrication of the Genomagnetic Nanocapturer.** Through avidin–biotin linkage,<sup>24</sup> the MB was immobilized onto the magnetic nanoparticle surface. The avidin was first immobilized onto the magnetic nanoparticle surface by incubating silica-coated magnetic nanoparticles in an avidin solution of 2 mg/mL in 10 mM phosphate buffer (pH = 7.3) for 14 h in a refrigerator. The positively charged avidin was adsorbed onto the negatively charged magnetic nanoparticle surface, where the negative charges were from the products of the polymerization of TEOS during the nanoparticle synthesis process. The nanoparticles were subsequently washed with 0.5 mL of buffer three times. The avidin layer was stabilized by cross-linking the coated nanoparticles with 1% glutaraldehyde in 100 mM potassium phosphate buffer for 1 h at room temperature. The avidin-coated magnetic nanoparticles were then incubated in Tris-HCl buffer for 3 h in a refrigerator after being washed three times with 0.5 mL of 1 M Tris-HCl buffer (pH = 7). Then, biotinylated molecular beacon (1.0 × 10<sup>-6</sup> M) was incubated with the nanoparticles for 12 h at 4 °C. To make

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Table 1. Sequence of DNA Probes and Targets

MB1	5'-(6FAM)-CGA GCA TCA ATA TTT AAC AAG C (biotin-dT)C G (DABCYL)-3' 5'-TTG TTA AAT ATT GAT-3'
target DNA1 (perfect complementary to MB1 loop sequence)	5'-TTA TTA AAT ATT GAT-3'
target DNA2 (single-base mismatch to MB1 loop sequence)	5'-TAG TTA TAA ATT GTT-3'
random DNA3	5'-TAG TTA TAA ATT ATT-3'
random DNA4	5'-TMR(-C6Am)GCA CGT CCA TGC CCA GGA AGG AAC G (biotin-dT)GC(DABCYL)-3' 5'-TTC CTT CCT GGG CAT GGA-3'
MB2	TTC CTT CCT GGG CAT GGA
target DNA5 (perfect complementary to MB2 loop sequence)	5'-fluorescein/C ATC AAT ATT TAA CAA-3'
bases 815-832 of 204 nt $\gamma$ -actin mRNA (complementary to MB2 loop sequence)	5'-TTG TTA AAT ATT GAT G/DABCYL-3'
linear DNA probe	5'-TTA TTA AAT ATT GAT G/DABCYL-3'
target DNA 6 (perfect complementary to linear DNA probe)	
target DNA7 (single-base mismatch to linear DNA probe)	

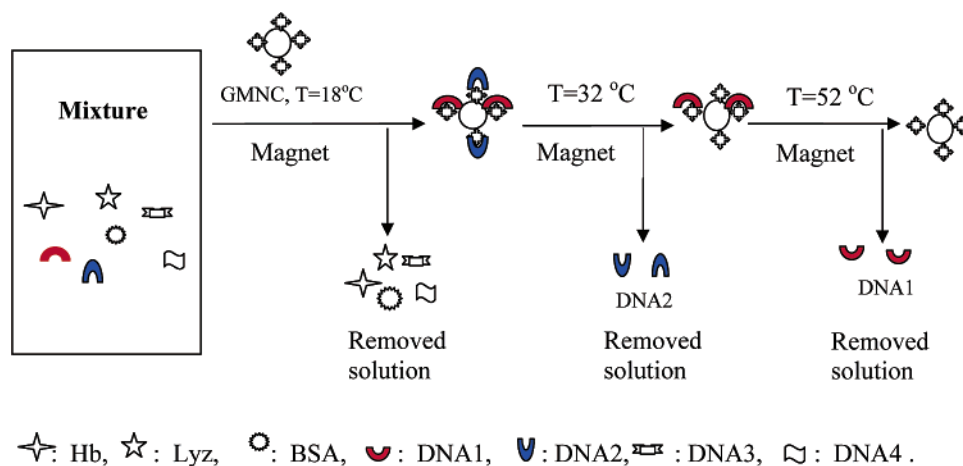


Figure 1. Schematic diagram of the working principle of the genomagnetocapturer for separation and collection of gene products with a one-base difference in sequence.

sure each exposed biotin group on the MB was connected to the avidin molecules on the magnetic nanoparticles (each avidin molecule has four biotin binding sites), the amounts of avidin were in excess. The resulting GMNC was then washed three times with 20 mM Tris-HCl/5 mM MgCl<sub>2</sub> buffer (pH = 8) and stored at 4 °C for future use.

**Preparation of  $\gamma$ -Actin mRNA.** The mRNA was isolated from rat lung tissues and then reverse transcribed to cDNA with oligo(dT) primer using a cDNA cycle kit (Introgen BV). The primer pair, 5'-GCG CTT CCG GTG TCC AGA-3' and 5'-GCC AGG GCT GTG ATC TCC-3', were used for PCR with 25 cycles. The PCR product of a 204-bp DNA fragment was cloned using the PCR2.1 vector (TA Cloning Kit, Invitrogen). Then, the recombinant plasmid was transformed to *Escherichia coli* INV $\alpha$  cells. Minipreps of the DNA was carried out and linearized with *Bam*HI. To produce  $\gamma$ -actin mRNA, 1.0  $\mu$ g of linearized plasmid DNA template was used in Ambion Megascript for the T7 transcription reaction. After purification, 204-nt rat  $\gamma$ -actin mRNA was obtained.

**Separation and Collection of DNA Sequences from the Mixture.** The artificial matrix was a 9.90-mL solution containing trace amounts of target DNA1 and DNA2, large amounts of random DNA 3 and DNA4 (100 times more concentrated), and BSA, Hb, and Lyz proteins (1000 times more concentrated). DNA1 was a perfectly complementary target to the MB while DNA2 was

a single-base-mismatched sequence. Three separation steps were performed in the experiments as illustrated schematically in Figure 1.

First, separation and collection of target sequences, including perfectly matched and single-base-mismatched sequences, from the mixture: A 100- $\mu$ L (25 nM MB) solution of GMNC was added to the mixture for 30 min at 18 °C. DNA1 and DNA2 were specifically hybridized with the MB on the GMNC surface while the random DNA sequences and proteins would not hybridize. When the mixture was exposed to a magnet, the GMNCs carrying trace amounts of DNA1 and DNA2 were collected and separated from the mixture.

Second, separation and collection of DNA2 from the mixture of DNA1 and DNA 2: The separation of DNA1 and DNA2 is based on the melting temperature difference between the two DNA duplexes. A 20- $\mu$ L solution of 20 mM Tris-HCl/5 mM MgCl<sub>2</sub> buffer was added to the GMNCs. The DNA1 and DNA2 were separated by bathing the GMNC solution at temperature of 32 °C for 15 min. At this temperature, DNA2 completely dissociated from the GMNC, while DNA1 remained bound on the GMNC surface. The solution was then exposed again to a magnetic field, and DNA1 bound GMNCs was removed. The supernatant contained DNA2, realizing the individual collection of DNA1 and DNA2 from a mixture.

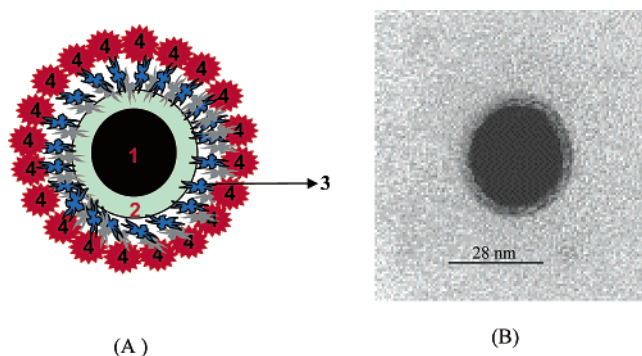


Figure 2. Structure of the genomagnetic nanocaptor. (A) Schematic representation of a genomagnetic nanocaptor: 1, magnetic nanoparticle; 2, silica layer; 3, biotin-avidin linkage; 4, molecular beacon DNA probe. (B) TEM image of a silica-coated magnetic nanoparticle. The diameter is in the range of 28 nm.

Third, collection of DNA1: A 20- $\mu$ L solution of 20 mM Tris-HCl 5 mM MgCl<sub>2</sub> buffer was added to the DNA1-bound GMNC. The temperature of the solution was raised and fixed at 52 °C for 15 min, resulting in the complete dissociation of DNA1 from the GMNCs. The genomagnetic nanocaptor was then removed from the solution using a commercial small piece of magnet. (The exact strength of the magnet is unknown.)

**Separation of mRNA from the Cultured Cells.** The breast cancer cells, MDA-MB-231 adenocarcinoma, breast pleural, were cultured in 90-mm cell flasks according to the instructions from American Type Cell Culture. Cell lysis was then completed by adding BME solution (guanidine thiocyanate extraction buffer) to the cells with continuous vortexing for 1 min. To clear the homogenate of cell debris and precipitated proteins, the lysate was centrifuged at 12000*g* for 10 min at room temperature. The mRNA-containing supernatant was decanted and denatured at 70 °C for 5 min. GMNCs were then mixed with the lysate in the dilution buffer with 1%  $\beta$ -mercaptoethanol. By subjecting the mixture to a magnet, the GMNCs were separated from the solution and the fluorescence intensity was detected using a spectrofluorometer directly.

## RESULTS AND DISCUSSION

**Genomagnetic Nanocaptors for Gene Recognition, Separation, and Collection.** To capture target DNA sequences from a mixture, an effective GMNC has been developed where the magnetic nanoparticles serve as magnetic carriers and MB probes act as recognition elements and indicators for specific gene sequences. Figure 1 schematically shows the mechanism of how the GMNC discriminates, separates, and collects DNA sequences with single-base differences. The structure of the GMNC is shown in Figure 2A. The GMNC's ability to capture trace amounts of DNA targets from a complex matrix depends strongly on the amounts of the MB immobilized on the nanoparticle surfaces. After interacting with a target, the GMNC's ability to separate trace amounts of targets from the matrix relies mainly on the nanoparticle's magnetic properties. Therefore, the efficiency of the MB immobilization on nanoparticle surfaces and the magnetic properties of the nanoparticles are the two most important factors for the preparation and application of the GMNC.

**Effect of Silica Coating on the Nanoparticle's Magnetic Property.** We first synthesized magnetic nanoparticles using the

water-in-oil microemulsion method.<sup>18</sup> The characterization of nanoparticle size and distribution as well as the measurements of the nanoparticles' magnetic properties were conducted using methods similar to those previously described.<sup>18</sup> The size of the magnetic nanoparticles can be controlled by changing the water-to-surfactant molar ratio ( $W_0$  value; here,  $W_0 = 10$ ). Using a Triton X-100/cyclohexane/1-hexanol system, we were able to obtain silica-coated magnetic nanoparticles with uniform size and small diameter ( $28 \pm 3$  nm). The TEM image of a silica-coated magnetic nanoparticle is shown in Figure 2B. The magnetic nanoparticles can be made smaller if needed, as demonstrated previously.<sup>18</sup>

A thin silica-coating layer encapsulating the bare magnetic nanoparticle was formed by a base-catalyzed hydrolysis and polymerization reaction of TEOS in a microemulsion, which can be observed in the TEM image (outside layer shown in Figure 2B). The silica layer serves as a biocompatible and versatile group for further biomolecular functionalization. The thickness of the silica layer can be controlled by both TEOS amount and TEOS polymerization time. As the amount of TEOS decreases or the polymerization time reduces, the silica layer will be thinner. The amount of TEOS in the microemulsion significantly affects the nanoparticle's magnetic property. For instance, as the TEOS concentration is increased, the thickness of the silica layer is also increased, resulting in a decrease of the nanoparticles' magnetivity. This effect would reduce the separation efficiency at low magnetic fields. On the other hand, as the TEOS concentration is lowered down to  $9.32 \times 10^{-4}$  M in the microemulsion, the magnetic nanoparticles could not be completely coated with a silica layer. By using SQUID magnetometric measurements,<sup>18</sup> room-temperature magnetization curves of magnetic nanoparticles synthesized with various concentrations of TEOS were obtained (data not shown). The results showed that  $2.33 \times 10^{-3}$  M TEOS was the optimal concentration for the preparation of silica-coated nanoparticles with the strongest magnetivity.

**Efficiency of the MB Immobilization on the Magnetic Nanoparticle Surface.** To effectively capture trace amounts of DNA targets from a matrix, MBs need to be immobilized reproducibly on the magnetic nanoparticle surfaces, in suitable excess and active for target hybridization. The MB's immobilization efficiency was investigated by comparing fluorescence intensities of the MB incubation supernatant solution, washing solutions, and the GMNC. Concentrations of the MB were determined in all three samples after the preparation of the GMNC. The GMNCs were washed three times with a buffer. For the fluorescence intensity measurements, buffer solution was added to the GMNC after it was separated from the supernatant. Three samples, GMNC solution, supernatant solution, and the washing solutions, were obtained, with the MBs dispersed in all three samples. Since the MB fluorescence signal is weak at low concentration, an excess amount (6-fold) of complementary DNA1 was added to all three samples in order to obtain a detectable fluorescence signal for the determination of MB distribution in the three samples. As shown in Figure 3, curves 1 and 2, it was clear that the majority of the MBs were immobilized onto the nanoparticle surfaces while a small amount of the MBs was left in the supernatant solution and the washing solutions (curve 3). The MB in the washing solutions could be ignored because of its insignificant amount (Figure 3, curve 3). The concentration of the MB in the

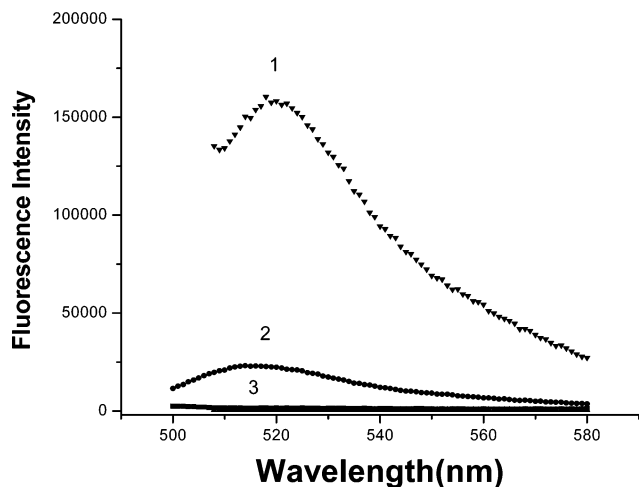


Figure 3. Comparison of amounts of the MBs immobilized onto the magnetic nanoparticles vs those left in the solutions. A 60- $\mu\text{L}$  solution of 1  $\mu\text{M}$  DNA1 and 30  $\mu\text{L}$  of buffer were mixed with 10  $\mu\text{L}$  of each of the three solutions (genomagnetic nanocapture solution, supernatant solution, washing solutions). The fluorescence intensities of MB–DNA1 hybrids were detected after 30 min of hybridization of the MB with DNA1: 1. GMNC solution; 2. supernatant solution; 3. washing solutions.

supernatant was determined using a calibration curve for the MB. The curve was obtained based on the hybridization of 6-fold excess of standard DNA1 solutions with the MBs. The results indicated a 7.8 nM MB concentration in the supernatant solution, as compared to the original MB concentration of 100 nM. It is thus believed that 92.2% of MB probes were immobilized onto the nanoparticles for the preparation of GMNCs for gene recognition, separation, and collection. The immobilization efficiency was high and could be controlled by immobilization time and MB concentration.

**Selection of Melting Temperature for DNA Separation and Collection.** To separate perfectly matched DNA sequences from one-base-mismatched target, a common procedure is to use a melting temperature at which hybridization to a complementary target is stable, while the duplex formed with the mismatched is unstable.<sup>25</sup> For linear DNA probes, the difference in melting temperatures between perfectly complementary duplexes and single-base-mismatched duplexes is rather small.<sup>26</sup> In contrast, molecular beacons can provide a much larger melting temperature difference between the two DNA duplexes. To compare a linear DNA probe with a molecular beacon, we used a linear DNA probe with the same sequence as the loop of the MB1. The melting temperature difference between the two duplexes (DNA 6 is the perfect complement and DNA 7 is a complement with a one-base mismatch) was only 7.5  $^{\circ}\text{C}$ . This result is similar to those found in other studies.<sup>25</sup> As shown in Figure 4, when the MB-coated GMNC was used as the DNA probe, the melting temperature difference between the two duplexes was significantly higher (21  $^{\circ}\text{C}$ ). This rather large difference in melting temperatures is due to the stem–loop structure of MBs.<sup>25</sup> It allows the single-base-mismatched DNA targets to be separated from the complementary ones by simply controlling the temperature. To effectively separate

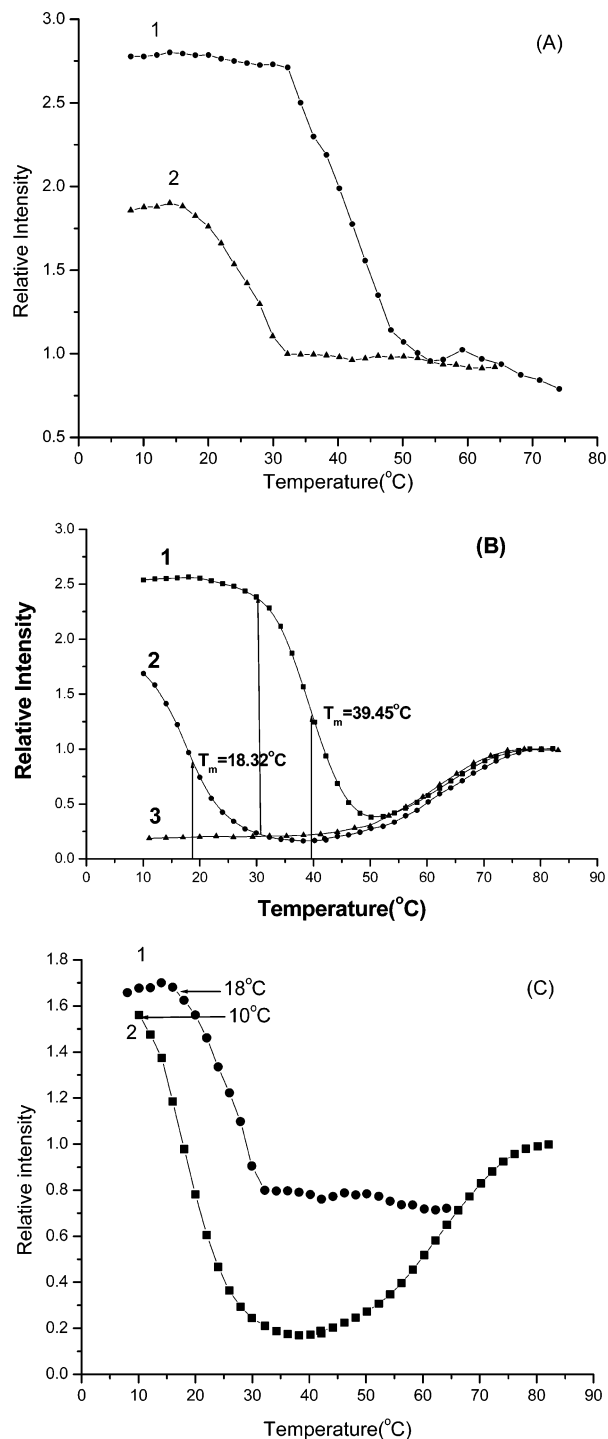


Figure 4. Melting temperature profiles of molecular beacons on different substrates: (A) the genomagnetic nanocaptors with MBs immobilized. A 6-fold excess of DNA1 (0.6  $\mu\text{M}$ , curve 1) or DNA2 (0.6  $\mu\text{M}$ , curve 2) was added to the GMNC in the buffer. Fluorescence intensities of the solutions were monitored as a function of temperature. The temperature was slowly increased in steps of 2  $^{\circ}\text{C}$  from 8 to 75  $^{\circ}\text{C}$ , holding at each temperature for 5 min to equilibrate. (B) The MB (not immobilized on a GMNC surface) and its target duplexes in buffer solutions. Conditions and experimental procedures were the same as those in (A). The solution contained 0.6  $\mu\text{M}$  DNA1 and 0.1  $\mu\text{M}$  MB for curve 1 and 0.6  $\mu\text{M}$  DNA2 and 0.1  $\mu\text{M}$  MB for curve 2. Curve 3 is for molecular beacon only in solution. (C) Direct comparison of the melting profiles of the duplexes formed by MB and DNA2: curve 1 is for MB immobilized on the GMNC surface, while curve 2 is for MB in the buffer solution. (In (B), there are three vertical lines. The middle one is not labeled.)

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DNA1 and DNA2, the DNA1 duplex should be stable over a broad temperature range. As shown in Figure 4A, the melting temperature curves for the duplexes formed with MBs on the GMNCs were different for the two different DNA targets. At low temperatures, both duplexes on the GMNC surfaces fluoresced well as MBs were opened due to hybridization. As the temperature rose, the duplex dissociated into a much less fluorescent MB and a DNA1 or DNA2 strand, resulting in a markedly diminished fluorescence. The one-base-mismatched duplex had considerably lower fluorescence intensities compared to the complementary DNA duplex. As the temperatures rose over 18 °C, the mismatched duplex became unstable. When the temperature was raised to 32 °C, DNA2 duplex was completely dissociated while DNA1 duplex was still stable with only 3% dissociation. Therefore, it is possible to separate DNA1 from DNA2 by raising the temperature to 32 °C. At this temperature, 100% of DNA2 can be separated with only 3% DNA1 dissociated into the solution, which can be further separated by repeating the separation process if extra separation efficiency is needed.

There are two major factors for the enhanced capability of the GMNC to discriminate two similar DNA targets with one-base difference and for the effective separation from a mixture. The first one is the above-mentioned MB's stem-loop structure for single-base-mismatch discrimination, and the second one is the use of nanoparticles. To demonstrate the nanoparticle's unique role in separations with GMNCs, melting temperature profiles of the MB duplexes were investigated both on GMNC surfaces and in solution without the nanoparticles. As shown in Figure 4, there is a sharp difference between the melting profiles of the duplexes formed by MB on the GMNC surface (A) and in solution (B). The melting temperature profiles for MB duplexes with both DNA1 and DNA2 were shifted to higher temperature when the nanoparticles were used. As shown in Figure 4C, the DNA2 duplex dissociation begins at 18 °C on the GMNC, which is 8 °C higher than that without using the nanoparticles. This means that a convenient 18 °C could be used for collection of DNA1 and DNA2 from the matrix instead of a much lower temperature (10 °C). Therefore, the nanoparticles not only serve as a magnetic carrier for the separation of DNA sequences but also contribute to the efficient and easy separation and collection of the DNA targets based on their melting temperature profiles. The fact that nanoparticles are able to affect DNA melting temperature profiles was reported in studies of gold nanoparticle probes in a DNA array.<sup>27</sup> It is worth noting that the MB's melting curves obtained in solution are smoother than those from the nanoparticle probes. However, this does not deter the application of the magnetic nanoparticles for efficient DNA separation and collection.

**Selectivity of the GMNC for the Collection of Target DNA Sequences in a Mixture.** Usually, in real life samples, a target DNA sequence is in a complicated surrounding. Various proteins, DNA, and other biomolecules coexist in any biological samples containing the target DNA. To determine how selective the GMNCs are in binding target DNAs, the influence of the matrix on the GMNC's collection ability was investigated. Three different general proteins, Hb, BSA, and Lyz, and two 15-base random oligonucleotides are used in an artificial mixture. The GMNC's binding with each component were first tested individually (data

not shown). The results demonstrated that the GMNC has a significant capacity to bind target DNA1 and DNA2 with a marked difference between them. The other components, even at higher concentrations (Hb, Lyz, and BSA: 1000× more concentrated than DNA1/DNA2. DNA3 and DNA4: 100× more concentrated than DNA1/DNA2) did not show appreciable binding with the GMNCs. Next, the mixture of Hb, BSA, Lyz, DNA3, and DNA4 in the presence of 3 fmol (0.313 pM) of DNA1 and 3 fmol (0.313 pM) of DNA2, was tested using the GMNC. The separation was carried out according to the procedures described in the Experimental Section (shown in Figure 1). After separation, the fluorescence signals of DNA1 and DNA2 were measured. The results indicated that the GMNC had a high capacity of capturing trace amounts of target DNA sequences. Based on the detection of the recovered target DNA from the matrix, we were able to collect complementary DNA1 from concentrations down to  $3 \times 10^{-15}$  M and  $\sim 9 \times 10^{-15}$  M for one-base-mismatched DNA2. The absolute amounts of DNA targets that could be collected by the GMNCs under our current experimental conditions are 30 and 90 amol for DNA1 and DNA2, respectively. This clearly demonstrates the potential to use GMNCs to capture rare gene products from a complex mixture. The absolute amount of target probes can be further reduced by more sensitive detection of the recovered targets. With better detection methodologies adapted from our single-molecule studies,<sup>28</sup> we would be able to detect captured target with much lower concentrations than shown in this study.

The process of the GMNC collection of target DNA sequences is also an enrichment method for gene products. After separation and collection, the DNA targets were enriched by 1000× based on the reduced volume. This easy enrichment by GMNCs will be highly useful for collection of low-abundance mRNA from a variety of sample sources such as cancer cells and tissues.

**Separation Efficiency of DNA Targets with One-Base Difference.** The separation efficiency of the GMNC with DNA1 and single-base-mismatched DNA2 was further investigated. To compare the results, the fluorescence of each DNA1 or DNA2 solution was determined before and after the collection. For example, the fluorescence spectra of two solutions, 25 nM DNA1 and 25 nM DNA2 hybridized with an excess of molecular beacons on GMNC, were obtained. At the same time, a mixture of 25 nM DNA1 and 25 nM DNA2 was prepared and then separated according to the procedures described in Figure 1. After separation and collection from the mixture, the same amount of the GMNC probes was added to the separated DNA1 and DNA2 solutions, respectively. There was no significant difference in the amounts of DNA1 or DNA2 before and after separation and collection. The efficiency for separation and collection is above 97% at 25 nM concentration of the targets. To determine the collection efficiency of the method at other target concentrations, a few complex samples with lower concentrations of DNA1 and DNA2 were tested with the GMNCs. The results, which represent the average of five repeated experiments under the same conditions, are summarized in Table 2. In each mixture, the concentrations of potential interferences were the same. Even at low target concentrations, the collection efficiency has always been close to or higher than 90% except for one sample (No. 4 in Table 2).

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Table 2. Efficiency of the Separation of DNA1, DNA2, and mRNA from the Mixture<sup>a</sup>

DNA sample	DNA 1			DNA2		
	std value (pM)	mead value (pM, <i>n</i> = 5)	recovery (%)	std value (pM)	measd value (pM, <i>n</i> = 5)	recovery (%)
1	8.25	8.56 ± 0.28	103.4 <sup>b</sup>	100	98.6 ± 1.01	98.6
2	12.5	11.8 ± 0.74	94.4	50	48.5 ± 0.72	96.9
3	25	24.4 ± 0.45	97.6	25	24.3 ± 0.63	97.2
4	50	46.9 ± 0.71	93.8	12.5	11.1 ± 0.57	88.8
5	8.25	7.57 ± 0.52	91.8	8.25	8.15 ± 0.44	98.8
mRNA sample	std value (pM)		mead value (pM)	recovery (%)		
6	10.0		9.30 ± 0.73	93.0		
7	15.0		14.7 ± 0.79	98.7		
8	20.0		18.9 ± 0.47	94.5		

<sup>a</sup> In each sample, the concentrations of Hb, BSA, and Lyz are  $1 \times 10^{-7}$  M; the concentrations of DNA3 and DNA3 are  $1 \times 10^{-8}$  M. <sup>b</sup> May be due to errors in small volume measurements.

**Collection of Trace Amounts of  $\gamma$ -Actin mRNA from a Mixture.** To determine the ability of GMNCs to collect mRNA and PCR products from a mixture, artificial mixtures containing 204-nt rat  $\gamma$ -actin mRNA fragments (bases 782–985, bases 815–832 are complementary to the MB2 loop sequence) were prepared. These mixtures also included the proteins and random DNA sequences that were used in the previously described DNA separations. To confirm the mRNA hybridization ability with the MBs, two hybridization experiments were carried out. One used 18 bases of perfect complementary target DNA5 for MB2. The other used 204-nt rat  $\gamma$ -actin mRNA for the MBs in the buffer solution. After hybridization, the fluorescence intensities of both solutions were seen to increase similarly. This result demonstrated that the MB was able to hybridize with the target mRNA even though the sequence of target was over 10 times longer than the MB. When the concentration of mRNA is low, the hybridization time for the mRNA and its target is longer. The mixtures containing different concentrations of mRNA were added to the GMNC solutions. Considering lower mRNA concentration samples, a hybridization time of 60 min was used to ensure all mRNA molecules were collected. The GMNCs were separated from the mixture using an applied magnetic field and following the same separation procedures described in the DNA separation section. The collected mRNA amounts were investigated by detecting fluorescence intensities of the solutions after hybridizing the MB to the mRNA. The collection efficiencies of the GMNC for mRNA are showed in Table 2.

**Capture of mRNA from Cultured Cells.** To test the GMNC's applicability in real biological samples, we further collected an mRNA sequence with 156 bases from cultured MDA-MB-231 amdenocarcinoma cells using the GMNCs. Among the 156 bases in the target mRNA, an 18-base section is perfectly complementary to the MB2 loop sequence. Meanwhile, there are no other mRNA molecules possessing the same section of sequence inside the cell. Following the procedure of extracting mRNA from the cells described in the Experimental Section, the mRNA molecules were specifically captured by the GMNC, as indicated by the obvious fluorescence signal from the separated GMNC (Figure 5, curve a). To further confirm that the fluorescence signal was indeed from the hybridization of MB with target mRNA, two control experiments were performed. First, before lysing the cells,

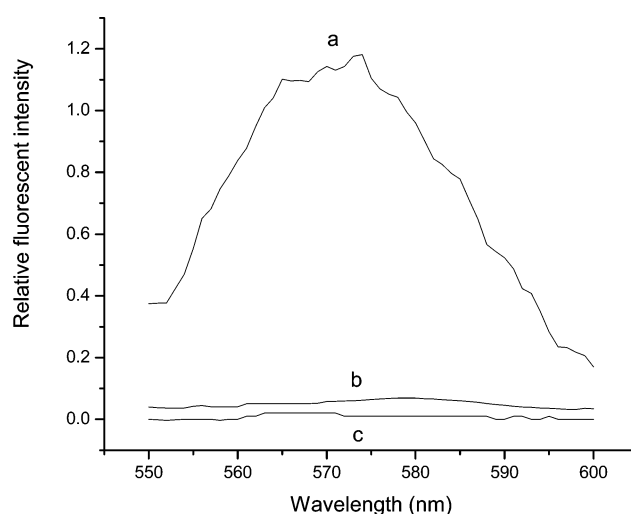


Figure 5. Collection of mRNA from the MDA-MB-231 cells. (a) cell lysates from six pieces of 90-mm flask of cells incubated with 100  $\mu$ L of 1.0  $\mu$ M MB2. Fluorescence signal was detected in Tris-HCl/5 mM MgCl<sub>2</sub> buffer (pH = 8). (b) The cells were not lysed. Other conditions are same as the curve a. (c) MB2 was replaced by a different sequence MB. Other conditions are same as for curve a. All the curves were subtracted by the control of pure MB in buffer solution.

GMNCs were added to the cultured cells and then followed by magnetic separation and washes. As shown in curve b, there was no detectable fluorescence signal when the GMNC solution was detected in the same condition as shown in Figure 5, curve a. Second, a different sequence of MB immobilized on GMNC was used instead of MB2 to incubate with the cell lysates under the same conditions. Still, there was no detectable fluorescence in the GMNC solution (Figure 5, curve c).

## CONCLUSIONS

A genomagnetic nanocapturer has been developed for the separation and collection of trace amounts of DNA/mRNA from mixtures and cancer cells. The GMNC was fabricated using a magnetic nanoparticle as a magnetic carrier, which was then functionalized with a molecular beacon as a DNA probe for gene recognition and collection. The use of MBs and the magnetic nanoparticles enabled a significant melting temperature difference

for complementary DNA and one-base-mismatched DNA targets both in solution and on the nanoparticle surface. We have successfully applied the GMNC in artificial buffer solution samples and in cancer cell samples, both containing different proteins and random DNA sequences. The GMNC has three distinctly useful features over existing technologies: highly efficient collection of trace amount of DNA/mRNA samples down to femtomolar ( $10^{-15}$  M) concentrations; excellent ability to differentiate single-base-mismatched DNA/mRNA samples by combining the exceptional specificity of molecular beacons and the separation power of magnetic nanoparticles; and real-time monitoring and confirmation of the collected gene products. The complementary DNA target sequence can be captured down to a  $3 \times 10^{-15}$  M concentration, and the one-base-mismatched sequence can be captured down to  $\sim 9 \times 10^{-15}$  M. The GMNC served three functions in gene separation, collection, and detection. It acted as both a magnetic carrier for the separation and collection of DNA/mRNA sequences and also as a fluorescence marker when different targets were hybridized to the molecular beacons. Therefore, each separation process was monitored in real time and confirmed by detecting the GMNC fluorescence signal. The GMNCs' DNA collection

efficiency is  $\sim 90\%$  or higher. Over 200-nt mRNA fragments can be easily collected from a mixture or cell lysates using the GMNC. The application of GMNCs in disease diagnosis, drug development, rare mRNA capturing in disease mechanism studies, and bioterrorist situations should be significant and will be further investigated.

#### ACKNOWLEDGMENT

We thank Dr. William Farmerie and Mr. Holland Kevin for providing mRNA samples, Ms. Marie C. Vicens-Contreras for her assistance in culture of the MDA-MB-231 cells, and Dr. Arthur Hebard and Ms. Nikoleta Theodoropoulou for their help in the magnetic nanoparticle characterization with SQUID. This work is supported in part by NIH R01 NS39891, by NIH NCI CA92581, and by a Packard Foundation Interdisciplinary Science Program Award.

Received for review March 31, 2003. Accepted April 24, 2003.

AC0343300