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Sensitive Molecular Diagnostics Using Volume-Amplified Magnetic Nanobeads

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ABSTRACT

In this letter, we demonstrate a new principle for diagnostics based on DNA sequence detection using single-stranded oligonucleotide tagged magnetic nanobeads. The target DNA is recognized and volume-amplified to large coils by circularization of linear padlock probes through probe hybridization and ligation, followed by rolling circle amplification (RCA). Upon hybridization of the nanobeads in the RCA coils, the complex magnetization spectrum of the beads changes dramatically, induced by the attached volume-amplified target molecules. We show that the magnetization spectrum of the nanobeads can be used for concentration determination of RCA coils down to the pM range, thus creating the opportunity for nonfluorescence-based cost-efficient high-sensitivity diagnostics tool. We also show that the bead incorporation in the coils is diffusion-controlled and consequently may be accelerated by incubating the sample at higher temperatures.

There is a need for novel simple assay formats for sensitive DNA sequence analysis, suitable for the clinical setting. Information about the presence, abundance, and/or nature of DNA sequences are of importance in many areas of clinical diagnostics to guide therapy and make accurate diagnosis of disease. Recently, immunoassay techniques have emerged that use DNA as reporter molecules to indicate detection of specific protein analytes, making DNA analysis techniques useful for all biomolecules of interest for diagnosis (IMMUNO-PCR,[1] IMMUNO-RCA,[2] Biobarcodes,[3] PLA[4]). DNA sequence detection schemes often rely on a hybridization reaction between a target DNA molecule and a probe molecule designed to match the target. It is convenient if hybridizations can be monitored in homogeneous readout formats that do not require separation of an unbound labeled probe from the matching probe−target complexes. One commonly used technique to achieve this is based on detection of the change in fluorescence depolarization when a probe binds its target.[5] Other readout formats for hybridization reactions, suitable for diagnostics, have been explored involving, e.g., electrochemical detection schemes,[6] where monitoring of the hybridization reaction is based on the electrochemical response when probes, labeled with, e.g., organic dyes, metal complexes, and metal nanoparticles, bind their targets. There also exist gravimetric detection schemes such as microcantilever resonance based DNA detection with nanoparticle probes.[7] To achieve high detection sensitivity, in the extreme case single-molecule detection, it is typically necessary to amplify the probe−target complex. One of the most commonly used methods for this is the polymerase chain reaction (PCR).[8] Although the PCR scheme in principle provides unlimited sensitivity and quantitative dynamic range, the technique is considered too complicated for the diagnostic setting, requiring expensive bulky equipment, special laboratories to avoid contamination, and operators trained to set up the reactions and to interpret the data.

An alternative approach for enzymatic detection and amplification for detecting sets of gene sequences with high specificity and selectivity involves the use of circularizing oligonucleotide probes (padlock probes)[9,10] for recognition of the target DNA in combination with enzymatic signal amplification by the rolling circle amplification (RCA) mechanism.[11,12] The 5′ and 3′ ends of the linear padlock probe are designed to base pair next to each

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other on the target strand. Thereafter, if properly hybridized, the ends can be enzymatically joined by a DNA ligase, thereby creating a circularly closed probe−target complex (reacted probe) for each recognized target. Circularized probes can then be amplified by a DNA polymerase using the RCA mechanism, which generates a DNA strand consisting of a large number of tandem copies of the complement to the circularized probe, collapsing into a random-coil DNA macromolecule in solution.[13] Jarvius et al.[14] demonstrated this scheme for DNA single-molecule detection. The RCA products were detected by using fluorescence molecule-tagged probes, designed to hybridize to the repeated sequence of the RCA product, resulting in a confined cluster of fluorophores. These clusters were in turn detected and quantified by pumping the sample through a microfluidic device mounted in a standard confocal fluorescence microscope operating in line-scan mode, thereby allowing for digital quantification. Furthermore, various circularized probe−target complexes, each corresponding to a unique target sequence, could be formed and amplified simultaneously. Hybridization of fluorescence probes with different colors provided the opportunity to perform multiplexed target analysis. Although fluorescence detection of the RCA products, the current state-of-the-art method for DNA sequence detection, has several advantages such as high selectivity and sensitivity, the equipment needed for this is expensive and rather difficult to miniaturize.

Biomolecule detection by measuring changes in the Brownian relaxation frequency of magnetic beads in aqueous solution was originally proposed by Connolly and St Pierre.[15] In their detection scheme, the surfaces of magnetic beads are biofunctionalized with probe molecules, e.g., single-stranded oligonucleotide molecules. When single-stranded DNA molecules, e.g., a PCR product having a sequence that matches the probe oligonucleotides, are added, hybridization reactions occur on the surface of the beads, giving rise to a slightly increased hydrodynamic diameter and, consequently, a decreased Brownian relaxation frequency. This detection principle has been demonstrated in the case of the antigen–antibody reaction by Astalan et al.,[16] albeit with a somewhat modest sensitivity. The complex magnetization spectra were recorded using induction coils and a lock-in amplifier technique. Other examples of magnetic bead based schemes, without fluorescence readout, are high-sensitivity micro-Hall[17–19] and giant magnetoresistive biosensors.[20–22] These techniques are based on the detection of the magnetic fringe field of magnetically labeled biomolecules interacting with complementary biomolecules bound to the surface of the sensor. Common for these magnetic field biosensors is that the size of the sensor can be miniaturized to match the size of a single magnetic bead, thereby increasing the sensitivity of detection. Both types of biosensors have been shown to possess high enough sensitivity to detect the magnetic field from single micrometer-sized magnetic beads. Moreover, single magnetic bead detection for bead sizes as small as 100 nm is anticipated in the near future.[21] It should be noted that these ultrasensitive magnetic field sensors can also be used for dynamic magnetic measurements where changes of the Brownian relaxation frequency are detected.

In this letter, we give a proof-of-concept of a novel nonfluorescence-based DNA sequence detection scheme: the volume-amplified magnetic nanobead detection assay (VAMNDA). Submicrometer-sized magnetic particles, so-called nanobeads, are biofunctionalized with single-stranded oligonucleotide detection probe molecules that contain a sequence complementary to a specific part of the repeating motif in the RCA coils. If the RCA coils are present in the test sample, i.e., if the target DNA was present originally, the probe-tagged magnetic nanobeads will be incorporated into the RCA coils by base pair hybridization. Thus, the dynamic magnetic response of the beads changes dramatically due to the extreme hydrodynamic volume increase of the nanobeads as they attach to the RCA coil. The current DNA sequence detection scheme relying on changes in magnetic properties in combination with the powerful padlock probe and RCA concepts for volume amplification of target molecules opens up potential possibilities to design high-sensitivity genetic point-of-care diagnostic tests at a much lower cost compared to already existing ones.

Figure 1 shows our DNA sequence detection principle step by step. In this figure, a collection of single-stranded target DNA molecules, indicated in blue, is shown. Linear padlock probe molecules designed to exactly match the target DNA are added, which upon hybridization with the target DNA molecules form circularized padlock probes. The ends of the padlock probe molecules are joined together by ligation. The addition of DNA polymerases initiates RCA, and the circularized padlock probes are amplified for a certain time (the RCA time). After the RCA is completed, the solution consists of $\sim$1 μm sized (for RCA time $\sim$1 h) random-coil single-stranded molecules. The RCA coils are detected by addition of single-stranded oligonucleotide biofunctionalized magnetic nanobeads (total hydrodynamic diameter $\sim$150 nm), where the coupled oligonucleotides are complementary to a region of the repeating motif in the RCA coils. The detection probe-tagged beads and the RCA coils will, during their diffusive motion, approach each other, and the probe-tagged beads are then incorporated into the coils by base pair hybridization. The hydrodynamic size of the incorporated beads is strongly increased, now essentially corresponding to the diameter of a RCA coil. Beads that are not incorporated exhibit an unaltered hydrodynamic diameter. When the target DNA is not present, no circularized padlock probes and hence no RCA products are formed. Thus, in this case, all probe-tagged beads remain free in solution. It can be argued, though, that this kind of negative control sample, i.e., completely without RCA coils, is theoretically not the most convenient one to use, see further discussion below in connection to Figure 2. The lower right part of Figure 1 shows the spectrum for the imaginary part of the frequency-dependent complex magnetization for one positive sample (blue curve; in this case, there are three times as many coils as probe-tagged beads) and for a negative control sample (green curve). The negative control sample exhibits a well-defined Brownian relaxation frequency, f_B, defined by the

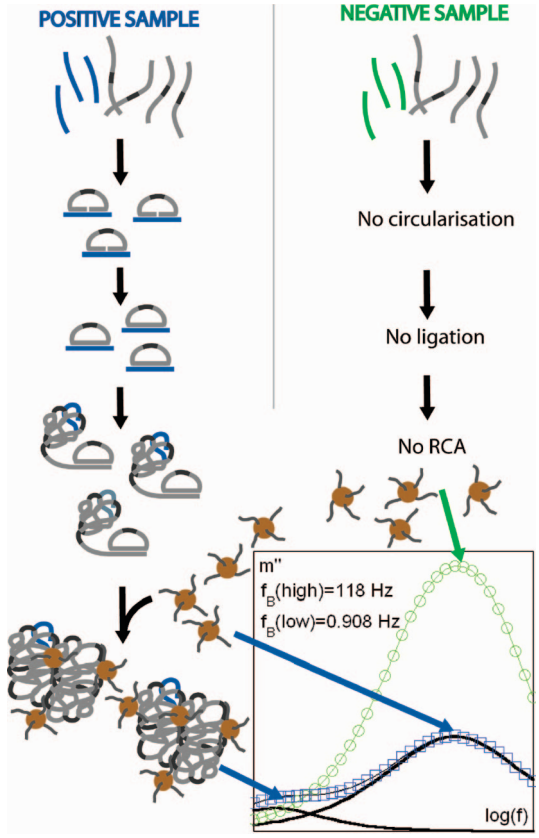

Figure 1. Schematic illustration of the volume-amplified magnetic nanobead detection assay. A collection of single-stranded target DNA molecules are indicated in blue. After addition of matching padlock probes (grey), circularized padlock probes form upon hybridization and the ends of the padlock probe molecules are joined together by ligation. The circularized padlock probes are then amplified by the RCA mechanism, creating random-coiled DNA macromolecules. To detect the presence of RCA products, single-stranded oligonucleotide-functionalized magnetic beads (brown) are added, which are incorporated by base pair hybridization to the RCA coils. The bead incorporation results in a spectrum of the complex magnetization (positive sample, blue magnetization curve) substantially different from the response of a sample containing free beads only, i.e., the absence of RCA products (negative sample, green magnetization curve). The two relaxation events in the blue curve have been resolved (bold black curves) using a Cole–Cole fitting procedure, and the peak frequency values are indicated.

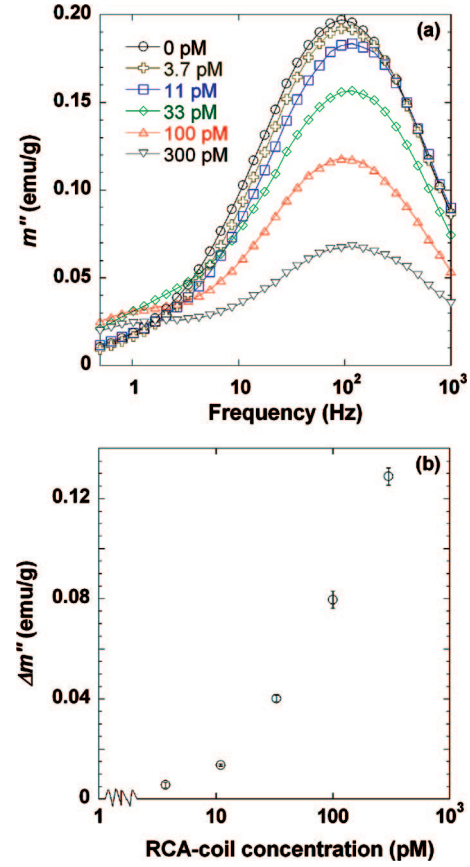

Figure 2. Complex magnetization spectra of nanobeads for different RCA coil concentrations. (a) Imaginary part of the complex magnetization vs frequency measured at 310 K for a series of samples having RCA coil concentrations ranging from 0 to 300 pM, i.e., matching RCA coils. The "0 pM" (negative control) curve corresponds to a sample consisting of RCA coils having a sequence that is noncomplementary (padlock probe B, padlock target B) to the oligonucleotide probes on the beads, see Supporting Information, SI 3, VII in Figure S-2 for details. (b) $\Delta m'' = m''(0) - m''(c)$ at the high frequency peak vs RCA coil concentration c with error bars showing the absolute variation over measurements of three different samples.

frequency at which the imaginary part of the magnetization, $m''(f)$, exhibits a maximum (indicated by green arrow), and the peak height gives a measure of the number of free probe-tagged beads. The positive sample has essentially two relaxation frequencies (indicated by blue arrows), where the low-frequency peak mainly corresponds to single RCA coils with incorporated beads and the high-frequency peak arises from probe-tagged beads that are still free. The two relaxation events have been resolved using the Cole–Cole fitting procedure, and the bold black curves show the two contributions to the blue curve. The extracted peak frequency values are also indicated. By measuring the m'' high frequency peak (HFP) level, discrimination can be made between positive and negative samples and, as described below, a target DNA concentration determination can be performed. For details on Brownian relaxation dynamics and the Cole–Cole model, see Supporting Information SI 1.

Figure 2a shows the imaginary part of the complex magnetization vs frequency at 310 K for positive samples (padlock probe A, padlock target A, Supporting Information SI 3) with various RCA coil concentrations ranging from 0 to 300 pM, i.e., RCA coils that match the bead detection probes. The probe-tagged bead (detection probe A1) concentration used was 1 nM, the RCA time 1 h, and the most optimal sample preparation procedure was applied, see Supporting Information SI 3 and SI 5 for details. The magnetic properties of the synthesized probe-tagged beads were found to be highly reproducible (see Supporting Information SI 5). To obtain a similar chemical environment for the beads in all samples, the "0 pM" (negative control) curve corresponds to a sample consisting of RCA coils having a sequence that is noncomplementary (padlock probe B, padlock target B) to the oligonucleotide probes on the beads, see Supporting Information SI 3, VII in Figure S-2 for details. This kind of negative control sample is from a

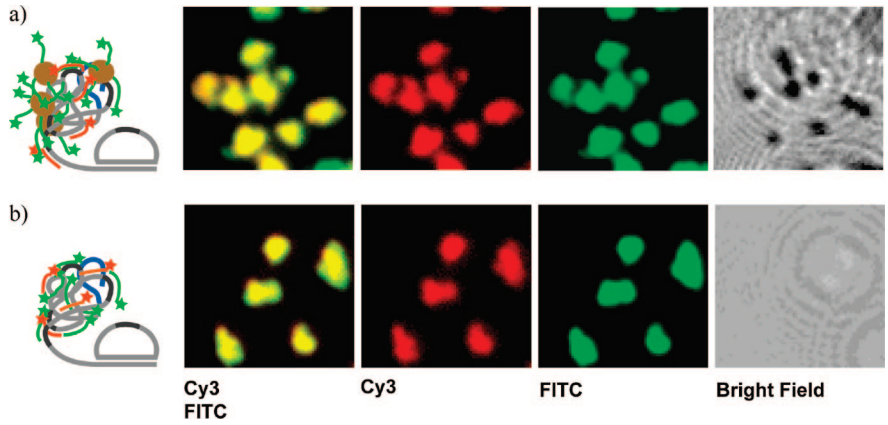

Figure 3. Microscope analysis of immobilized RCA products in the presence or absence of functionalized nanobeads. RCA products immobilized to glass slides were hybridized with two detection probes, labeled with Cy3 and FITC, respectively, and targeting two different regions in the concatemer RCA products. (a) Hybridization with Cy3-labeled probes and magnetic nanobeads functionalized with FITC-probes. (b) Hybridization with Cy3 probes and unconjugated FITC probes.

theoretical point of view the most convenient one to use, although technically one may consider the negative control in Figure 1, i.e., a negative control without RCA coils, to more closely mimic a real case scenario where there is no match between padlock and target probes. Also, it is reasonable to assume that the presence of RCA coils affects the sedimentation speed of the beads, which in turn affects the recorded magnetization spectrum, in particular the m'' HFP level and the low frequency magnitude of m'. In fact, the presence of RCA coils decreases the bead sedimentation rate, which gives a slightly higher m'' HFP level for the negative control having nonmatching RCA coils compared to the negative control having no RCA coils. This is also a reason for using a negative control containing nonmatching RCA coils. Figure 2b shows $\Delta m''$, defined by $\Delta m'' = m''(0) - m''(c)$, at the HFP vs RCA coil concentration, c. Note the very clear correspondence between the HFP m'' level and the RCA product concentration. As the RCA product concentration increases, more probe-tagged beads are incorporated into coils and, therefore, the HFP m'' level decreases. The high- and low-frequency Brownian relaxation peaks correspond to the expected hydrodynamic sizes of free beads and beads incorporated into RCA coils, respectively, as shown in Supporting Information SI 5. This conclusion is also supported by the fact that the low-frequency peak is shifted to higher frequencies when decreasing the RCA time, i.e., coils with smaller diameter are obtained (see Supporting Information SI 5, Figure S-3). Furthermore, the results obtained using a negative control sample containing RCA coils having a sequence that is noncomplementary to the oligonucleotide probes on the beads, see Supporting Information SI 3, VII in Figure S-2 for details, prove that the changes in the complex magnetization spectrum are solely due to bead incorporation by hybridization. We have also shown that the bead incorporation kinetics is significantly improved by using a high incubation temperature (see Figure S-2 in Supporting Information SI 5 where 10 different sample preparation procedures were evaluated). It can be concluded from Figure 2 that a positive sample can be discriminated from a negative control sample at least at the 3 pM level. This limit of detection level compares very favorably with and is even better than fluorescence polarization (FP) measurements of the same RCA products, where a limit of detection of around 10 pM was achieved (see Supporting Information SI 5, Figure S-6). Last, our detection method works with the same sensitivity when a large excess of genomic background material is present in the samples, see Supporting Information SI 5, Figure S-5 and the discussion in connection with this figure.

A general remark should be made here on our DNA sequence detection principle: The RCA coil and probe-tagged bead concentrations investigated in this proof-of-concept study are chosen in order to make magnetic read-out possible using a commercial SQUID magnetometer with comparably low pick-up coil filling factor (the volume fraction of magnetic beads inside the SQUID pick-up coil is estimated to $\sim 10^{-4}$). By constructing a miniaturized magnetic biosensor with size matching the size of the ensemble of beads to be investigated, including microfluidics for sample injection and by using smaller magnetic nanobeads to increase the Brownian relaxation frequency for free beads and the bead diffusion rate, it will be possible to increase the measurement sensitivity even further, thereby allowing for lower concentrations of RCA coils and probe-tagged beads. In particular, by decreasing the amount of probe-tagged beads relative to the number of RCA coils, detection sensitivity can be increased. Experimental evidence for this is given and discussed in Supporting Information SI 5 in connection with Figure S-4. The RCA time and bead surface coverage of oligonucleotides are other factors that may be optimized to further improve the level of detection. In fact, by combining the present concept for molecular diagnostics with a miniaturized high-sensitivity magnetoresistive sensor[21] capable of detecting the fringing magnetic field of single beads, it may even be possible to reach the single-molecule detection limit. Comparing the present concept for molecular diagnostics with the more traditional magnetic bead based techniques, we see some advantages; the present concept is inherently a dynamic measurement technique, implying

narrow bandwidth detection and, if small enough beads are used, it is possible to detect the magnetic signal at high enough frequencies where the intrinsic voltage noise of the sensor is minimal (corresponding to the white noise limit); the present concept implies comparably simple surface chemistry and it implies, through the introduction of the Brownian rotation frequency, more possibilities for multiplex detection.

To visualize the presence of magnetic probes in the RCA products, we immobilized the RCA products on a glass surface and hybridized functionalized magnetic nanobeads to them (detection probe A1, Supporting Information SI 3), Figure 3. The oligonucleotides were labeled with a fluorophore (FITC) at the 3′ end, visualizing the RCA products as bright micrometer-sized objects. A second fluorescent probe (detection probed A2), hybridizing to another region in the concatemer RCA product, was added to prove the presence of RCA products. Local aggregations of beads colocalizing with the fluorescent RCA products were visible as dark objects in the bright-field image. Single magnetic nanobeads, on the other hand, were not visible in the microscope because they are too small. As a control, we hybridized RCA products with unconjugated fluorescent probes. No aggregates were detected in these RCA products. Interestingly, the morphology of the RCA products did not seem to be affected by the incorporated magnetic nanobeads.

Summarizing, we have demonstrated a proof-of-concept of a new principle of specific DNA sequence detection using single-stranded oligonucleotide probe-tagged magnetic beads in combination with padlock probes and RCA, all together having the potential to form a sensitive and cost-effective method for diagnosis. Upon incorporation of the nanobeads in the volume-amplified target DNA RCA coils, the complex magnetization spectrum of the beads changes significantly. A simple and reproducible way to detect this change in magnetic dynamics is to measure the level of m'' at the Brownian relaxation frequency for the free probe-tagged beads. We have shown that this level has a clear correspondence with the RCA product concentration. In the absence of target DNA (negative sample), no RCA coils are formed and, consequently, all added probe-tagged beads remain free. Furthermore, we have shown that the incorporation is diffusion-controlled. Thus, it may be accelerated by incubating the sample at a higher temperature or by reducing the nanobead size. Optimizations of the sample preparation procedure, RCA time, bead surface coverage of oligonucleotides, bead size, etc., may greatly improve the level of detection, which in combination with fabrication of a miniaturized magnetic circuit involving microfluidics, for sample, injection, promise to give a powerful and low-cost diagnostic device. Although we have shown single target detection, this method can easily be generalized to multitarget detection by having several nanobead sizes, one for each target. Furthermore, other kinds of biomolecules than DNA may be detected by using the so-called proximity ligation technique that produce RCA products as a consequence of coincident binding of two or more specific antibodies equipped with DNA strands.[23,24]

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Supporting Information Available: (SI 1) Theoretical considerations, (SI 2) details of the synthesis procedure of oligonucleotide detection probe-tagged beads and analysis of oligonucleotide coupling yield, (SI 3) padlock probe, padlock target, and detection probe sequences, (SI 4) details of sample characterization and sample preparations applied in the optimization procedure, (SI 5) optimization of sample preparation procedure, diffusion, negative control samples, effects of RCA time, effects of using a lower concentration of probe-tagged beads, measurements on samples containing a large excess of genomic background material (evaluation of specificity), and fluorescence polarization, (SI 6) synthesis of oligonucleotide detection probe-tagged beads and analysis of oligonucleotide coupling yield, (SI 7) synthesis of RCA-coil samples, (SI 8) sample preparation and characterization, and (SI 9) fluorescence study. This material is available free of charge via the Internet at http://pubs.acs.org.

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