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Detection of rolling circle amplified DNA molecules using probe-tagged magnetic nanobeads in a portable AC susceptometer

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ABSTRACT

Here, the volume-amplified magnetic nanobead detection assay (VAM-NDA) is for the first time applied for detection of rolling circle amplified (RCA) DNA molecules in a portable, commercial AC susceptometer that operates at ambient temperatures and with an analysis time of about 20 min. The performance of the assay is investigated using three different magnetic nanobead sizes: 50, 130 and 250 nm. The performance of the assay using the AC susceptometer is compared to the performance achieved using a superconducting quantum interference device (SQUID).

It is found that the performance of the assay is comparable in the two setups with a quantitative detection limit of ~4 pM for all bead sizes under study.

The findings show that the VAM-NDA holds promise for future wide-spread implementation in commercial AC susceptometer setups thus opening up for the possibility to perform magnetic bead-based DNA detection in point-of-care and outpatient settings.

1. Introduction

Rapid detection and read-out of specific biomolecules like DNA, proteins and enzymes, are on today's wish list in many areas of clinical diagnostics (Justino et al., 2010; Sun et al., 2005; Teles and Fonseca, 2008; Teles, 2011). One of the most commonly used methods for DNA detection is the polymerase chain reaction (PCR) (Duggan et al., 1999; Fenn et al., 1994; Haynes and Westerneng, 1996). Although PCR is a very sensitive method (Saiki et al., 1985), it requires access to expensive equipment and is rather sensitive to contamination (Lo and Chan, 2006). When it comes to cost-effectiveness, magnetic nanoparticle-based bioassays offer an attractive route for detection of biomolecules, because of the high physical and chemical stability and the potentially low-cost of production of such nanoparticles (henceforth referred to as beads). Furthermore, magnetic bead-based bioassays require small amount of sample and simple preparation procedures compared to traditional detection methods (Fornara et al., 2008; Öisjöen et al., 2010; Teles and Fonseca, 2008). Several magnetic biosensors have been developed in the past years. These include giant magnetoresistance (GMR) (Baselt et al., 1998; Miller et al., 2001; Schotter et al., 2004), giant magnetoimpedance (GMI) (Chen et al., 2011; Kurlyandskaya and Levit, 2005), inductive (Lany et al., 2005), spin valves (Graham et al., 2002) and micro-Hall devices (Besse et al., 2002; Boero et al., 2003). Magnetic biosensors measure the superposition of stray fields due to magnetic nanoparticles. The nanoparticle stray fields are determined by the applied magnetic field amplitude (both DC and AC magnetic fields may be used) and the superparamagnetic response of the nanoparticles. The magnetic field sensor surface (or surfaces in case of sensor arrays) can, e.g., be functionalized with probe molecules or one may use a disposable cartridge containing a surface with probe molecules that can bind to the target molecules attached to the bead surface (Baselt et al., 1998; Graham et al., 2004; Lany et al., 2005; Miller et al., 2001; Schotter et al., 2004). In these cases one measures the equilibrium superparamagnetic response of the immobilized magnetic nanoparticles. The GMI sensor, as described in Chen et al. (2011) and Kurlyandskaya and Levit (2005), is not a close proximity sensor, since it is not needed to immobilize the magnetic nanoparticles at short distance from the sensor surface.

The principle of the magnetic Brownian relaxation readout was theoretically described by Connolly and St Pierre (2001) and was experimentally demonstrated for protein detection by Astalan et al. (2004) where binding of prostate specific antigen (PSA) proteins onto antibody-tagged magnetic beads increased the hydrodynamic volume of the bead, and thereby shifted the Brownian rotational

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frequency. The main difference between this readout strategy and the strategies mentioned above is that the Brownian relaxation readout measures the non-equilibrium magnetic response of the nanoparticles in the carrier liquid in response to the AC magnetic field by varying the frequency of the field.

We have previously demonstrated a new substrate-free detection method which combines padlock probe target recognition and rolling circle amplification (RCA) with the Brownian relaxation method; the volume-amplified magnetic nanobead detection assay (VAM-NDA) (Strömberg et al., 2008a). The assay begins with padlock probe target recognition where the padlock probes are synthetic single-stranded DNA molecules that become circularized through enzymatic ligation upon recognition of specific single stranded DNA sequences (Landegren et al., 2003; Nilsson et al., 1997, 1994). The circular DNA molecules are then copied using RCA (Fire and Xu, 1995; Liu et al., 1996; Lizardi et al., 1998; Nilsson et al., 2006) generating long concatemers consisting of tandem repeated sequences complementary to the circularized probe. Each RCA molecule spontaneously collapses into micrometer-sized random-coil DNA molecules (Blab et al., 2004) and are then detected by oligonucleotide-tagged magnetic beads binding to the RCA molecules by base-pair hybridization. Since the oligonucleotide-tagged magnetic beads only immobilize if the oligonucleotide is complementary to the sequence of the DNA-coil, the bead-coil interaction is highly sequence specific (Strömberg et al., 2008a). This binding of beads causes a dramatic decrease of the Brownian relaxation frequency, since it is inversely proportional to the hydrodynamic volume of the beads. The concentration of target molecules can then be monitored by measuring the decrease of the amplitude of the Brownian relaxation peak of free beads. The VAM-NDA has up to now been investigated using a superconducting quantum interference device (SQUID) for measuring the magnetic response of the beads (Akhtar et al., 2010; Göransson et al., 2010; Strömberg et al., 2008b, 2009; Zardán Gómez de la Torre et al., 2010). The SQUID is a very sensitive instrument, but it is expensive, bulky and requires liquid He cooling of the sensing element. Furthermore, the analysis time is long (approximately 2 h) and is therefore not optimal for point-of-care testing.

The overall aim of the current study is to evaluate the possibility of implementing the VAM-NDA assay on a more simple and easy-to-handle high sensitivity AC susceptometer platform that allows for shorter analysis times and thus for a more wide-spread use of the assay. Here, the assay is for the first time applied for detection of RCA DNA molecules in a portable, commercial AC susceptometer that operates without cryogenic cooling and with an analysis time of about 20 min. The performance of the assay is investigated in terms of sensitivity using three different bead sizes: 50, 130 and 250 nm.

2. Materials and methods

2.1. Coupling of oligonucleotides to magnetic nanobeads

Synthetic single-stranded oligonucleotides were conjugated to spherical amine-functionalized magnetic beads with nominal bead diameters of 50, 130 and 250 nm (Micromod Partikeltechnologie GmbH, Germany), by a heterobifunctional crosslinker sulfo-SMCC chemistry. The magnetic beads are of core-shell type consisting of a core made up of a magnetite nanoparticle aggregate, which is coated with dextran (130 and 250 nm beads) or starch (50 nm beads). The iron-oxide content of the beads, which is determined using a spectrophotometric method, is typically in the range of 75–85% (w/w). The iron-oxide nanoparticle size is 10–15 nm, which would indicate a superparamagnetic behavior at room temperature. However, because of magnetic interparticle interactions within the aggregate, each magnetic bead exhibits a blocked magnetic moment and the low-field non-equilibrium magnetic response of beads in solution can be described by the Brownian relaxation model (see Supplementary material, section A, for further information relating to the non-equilibrium magnetic response of magnetic nanoparticles). Still, at thermodynamic equilibrium the behavior of the beads, which using an AC susceptometer can be studied at low enough frequency of the AC magnetic field, is that of a superparamagnetic system. Magnetization versus field curves for non-functionalized 50, 130 and 250 nm beads appear in Supplementary material, Fig. S5.

The synthesis procedure for one batch of 200 μl oligonucleotide-tagged 130 and 250 nm beads was performed as follows: 0.5 mg of sulfo-SMCC (Thermo Scientific, USA) was dissolved in 20 μl of DMSO (Sigma–Aldrich, Sweden). The bead solution was washed with 1 × PBS (pH 7.5) three times (once for 50 nm beads) and resuspended in 50 μl 1 × PBS (pH 7.5) before adding the sulfo-SMCC solution. The mixture was incubated at room temperature for 2 h and thereafter washed four times (three times for 50 nm beads) with 1 × PBS (pH 7.5) in order to remove non-reacted sulfo-SMCC. The washing procedure of the beads was performed as follows: the beads were collected to the bottom of the Eppendorf tube using a magnetic separator and the liquid phase was removed with a micropipette. Thirty microliters of 83 μM fluorescently labeled and tris (2-carboxyethyl) phosphine (TCEP)-reduced oligonucleotide solution was added to the mixture. See Table 1 for details about the oligonucleotide sequence used. The TCEP-reduction of the thiolated oligonucleotides was performed as follows: a mixture consisting of 50 mM TCEP (pH 7.5), 10 mM EDTA and 135 μM thiolated oligonucleotides was prepared and incubated in a water bath at 37 °C for 1 h. The mixture was spun (1 min at 3000 rpm) one time through a MicroSpin G50-column (GE Healthcare, Sweden) in order to remove TCEP excess. The column was prior to this equilibrated with 1 × PBS (pH 7.5). The mixture was vortexed and incubated over night at room temperature. The suspension of beads was washed three times (once for 50 nm beads) with 1 × PBS (pH 7.5) and resuspended in the original volume. The synthesis procedure for a 100 μl batch of oligonucleotide-tagged 50 nm beads was similar to the synthesis procedure for a batch of 130 and 250 nm beads except for the number of washing steps (see above). Moreover, the washing steps for the smallest beads employed a centrifuge (10,000 rpm for 10 min) instead of a magnetic separator.

The average number of oligonucleotides per bead was estimated by comparing the fluorescence of the oligonucleotide-coupled magnetic nanobeads to a dilution series containing free oligonucleotides and non-functionalized beads (Strömberg et al., 2008a). The emission scans were performed using a fluorometer (Infinite® 200, Tecan, Sweden).

2.2. Padlock probe target recognition, ligation and amplification

The procedure for making a 5 nM RCA DNA solution was performed as follows: 20 nM padlock probe (Biomers, Germany) was hybridized and ligated, templated by 60 nM target DNA (DNA Technology, Denmark) in a solution consisting of 50 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 1 mM ATP (Fermentas, Lithuania) and 0.02 U/μl T4 DNA ligase (Fermentas) at 37 °C for 15 min.

Circularized padlock probes were replicated in 50 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 167 μM dNTP, 0.2 μg/μl BSA (NEB, USA) and 0.067 U/μl phi29 DNA polymerase (Fermentas) for 1 h at 37 °C. Enzymes were inactivated at 65 °C for 5 min. A hybridization mix consisting of 100 mM Tris–HCl (pH 8.0), 100 mM EDTA, 0.5% Tween-20 and 2.5 M NaCl was added to the RCA DNA molecules. Target and padlock probe sequences are listed in Table 1.