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Bead Assembly Magnetorotation as a Signal Transduction Method for Protein Detection

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Abstract

This paper demonstrates a proof-of-principle for a new signal transduction method for protein detection called Bead Assembly Magnetorotation (BAM). In this paper, we chose to focus on the protein thrombin, a popular choice for proof-of-principle work in this field. BAM is based on using the protein target to mediate the formation of aptamer-coated 1 μm magnetic beads into a bead assembly, formed at the bottom of a 1 μL hanging droplet. The size, shape and fractal dimension of this bead assembly all depend on the protein concentration. The protein concentration can be measured in two ways: by magnetorotation, in which the rotational period of the assembly correlates with the protein concentration, or by fractal analysis. Additionally, a microscope-free magnetorotation detection method is introduced, based on a simple laser apparatus built from standard laboratory components.

Keywords

Magnetorotation; bead; assembly; thrombin; aptamers; fractals

1. Introduction

Magnetic beads have several advantages that make them attractive candidates for use as biosensor signal transducers, including their biological inertness, physical stability, and the absence of competing magnetic signals in biological materials (Gijs et al. 2010; Pamme 2006). A number of magnetism-based protein detection techniques have been developed, including giant magnetoresistance (GMR) (Osterfeld et al. 2008; Tamanaha et al. 2008; Wang and Guanxiong 2008), magnetic relaxation (Chung et al. 2004; Lee et al. 2008), Hall Probes (Besse et al. 2002; Sandhu et al. 2010) and amplification of an optical signal by magnetic rotation (Patolsky et al. 2003; Willner and Katz 2003). Additionally, there have

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been reports of using proteins to mediate the formation of one-dimensional magnetic bead chains (Petkus et al. 2006; Ranzoni et al. 2011). However, to the best of our knowledge, there have been no previous reports studying the shape or drag of two-dimensional magnetic microbead assemblies for their use as biomarker signal transducers.

This work builds on previous reports on the use of asynchronous magnetorotation for biological applications. Asynchronous magnetorotation was employed in a sandwich-type biosensor, where the magnetic beads acted as labels attached to nonmagnetic spheres, whose rotational frequency depended on the number of attached magnetic bead labels (Hecht et al. 2011a; Hecht et al. 2011b). Asynchronous magnetorotation was also used to measure the growth rate of attached bacteria (Kinnunen et al. 2011; McNaughton et al. 2007; Sinn et al. 2012), and morphological changes of cancer cells (Elbez et al. 2011).

The beads used in this study are 1 μm Invitrogen™ Dynabeads. These beads, which exhibit superparamagnetic behavior, are composed of maghemite nanoparticles ($\gamma\text{-Fe}_2\text{O}_3$), with a mean diameter of 8 nm, dispersed in a polystyrene matrix (Fonnum et al. 2005). In the absence of a magnetic field, these beads exhibit no net magnetization, but within a magnetic field, the magnetic moments of the bead align with the field, making the beads magnetic.

It has previously been shown that within a two-dimensional rotating magnetic field, the dominant physical mechanism driving the rotation of the beads is the Neel relaxation of the $\gamma\text{-Fe}_2\text{O}_3$ nanoparticles embedded inside the bead (Connolly and St Pierre 2001; Fannin et al. 2006; Hecht et al. 2011a; Hecht et al. 2011b; Janssen et al. 2009). At low driving field frequencies, the beads rotate in-phase with the driving field. At high driving field frequencies, the beads rotate out-of-phase, asynchronously, with the driving field. It is this asynchronous rotation, which depends on the physical properties of the beads, that allows for their rotation to be used to make biological measurements.

The use of surface-attached DNA molecules to control the assembly of micro- and nano-scale materials into highly-ordered crystalline structures has been shown before (Biancaniello et al. 2005; Nykypanchuk et al. 2008; Park et al. 2008). However, while those projects focused on the creation of single crystalline structures, the present project advances the concept one step further by focusing on the creation of structurally tunable assemblies, i.e. assemblies that can assume a variety of structures, depending on the protein concentration

The protein target used in this project is the blood coagulation factor thrombin, and the affinity molecules used are the well-studied thrombin aptamers (Bock et al. 1992; Tasset et al. 1997). Thrombin was chosen because of its popularity as an analyte in proof-of-principle studies, which facilitates evaluation of our method in the context of other methods. There are many papers demonstrating proof-of-principle work on signal transduction methods, all using the same thrombin aptamers in buffer, with reported limits of detection (LOD) ranging from 20 nM down to 6 fM. (An et al. 2010; Bai et al. 2012; Cai et al. 2006; Centi et al. 2007; Cho et al. 2008; Hansen et al. 2006; Hianik et al. 2005; Ho and Leclerc 2004; Hu et al. 2012; Huang et al. 2004; Kim et al. 2009; Li et al. 2007; Liu et al. 2012; Pavlov et al. 2004; Radi et al. 2005; Rahman et al. 2009; Song et al. 2009; Tennico et al. 2010; Zhang et al. 2009; Zhou et al. 2012). To demonstrate the utility of our magnetorotation method, we studied the same biomarker, thrombin, using the same aptamers, and under similar conditions. Thus, while we intend to pursue biomarker detection in serum at a later time, here we limit our focus primarily to work on thrombin in buffer.

2. Materials and Methods

2.1 Materials

The magnetic beads used were 1 μm streptavidin-coated Dynabeads T1 MyOne beads (Invitrogen, Carlsbad, CA). The 1 μm beads used were selected after examining a wide range of beads, from 100 nm to 5 μm , optimizing parameters such as sedimentation rate, available binding sites, and magnetic volume. Human α -thrombin was purchased from Haematologic Technologies (Essex Junction, VT). Two anti-thrombin aptamers were used, the 15-mer (Bock et al. 1992) (5'-GGT TGG TGT GGT TGG) and 29-mer (Tasset et al. 1997) (5'-AGT CCG TGG TAG GGG CAG GTT GGG TGA CT), with 20-base polyT tails (Centi et al. 2007), and were synthesized with a 5'-biotinylation by Integrated DNA Technologies (Coralville, IA). Standard salts and Tween-20 were purchased from Sigma Aldrich (St. Louis, MO). Bovine serum albumin (BSA) 10% blocker solution was purchased from Thermo Scientific (Waltham, MA). Teflon-coated glass slides with 1 mm exposed glass spots were purchased from Tekdon, Inc. (Myakka City, FL). AS568-016 BN70 nitrile O-rings were purchased from Orings, Inc. (Los Angeles, CA). G661 general purpose lubricant was purchased from Novagard (Cleveland, OH).

A two-dimensional rotating magnetic field was constructed from orthogonal Helmholtz coils. The coils were built by 3D-printing a plastic mold consisting of four orthogonal rings, and wrapping coils of 22 gauge copper wire around the rings. Two sinusoidal waves, 90 degrees out-of-phase, were used to power the field and were generated within a LabVIEW program (National Instruments, Austin, TX), passed through a LabVIEW DAQ Board (NI USB-6211 or NI SCB-68 connected to NI PCIe-6321), amplified by a TX-PA40D two-channel amplifier (Radio Design Labs, Prescott, AZ), and then passed to the coils. The field had a frequency of 50 Hz and an amplitude of 4 mT. The field rotated in a two-dimensional plane, which drove the rotation of the bead assemblies. The magnetic beads have a susceptibility (χ) of $8.1 \times 10^{-4} \text{ m}^3/\text{kg}$ (Fonnum et al. 2005).

Videos for magnetorotation analysis were captured on an Olympus IX71 inverted microscope (Olympus, Melville, NY) through a Basler piA640-210gm camera (Basler, Highland, IL). Videos were analyzed using the StAT Tracker (Milne 2007) program written in LabVIEW. Higher resolution images for fractal analysis were captured on an Olympus BX50WI upright microscope through a Mightex BCE-B050-U camera (Mightex Systems, Toronto, Ontario, Canada). Images were analyzed using the FracLac plugin (Karperien 1999-2012) for ImageJ (National Institutes of Health, Bethesda, MD).

For the laser-photodiode detection setup, a 650 nm, 20 mW laser module was purchased from Information Unlimited (Amherst, NH). FDS100 photodiodes were purchased from ThorLabs (Newton, NJ). The components were secured using standard optic table equipment. Conditioning circuitry was built using standard electronics components. The conditioned signal was passed through the NI SCB-68 DAQ board and processed and analyzed in LabVIEW.

2.2 Preparation of Bead Assemblies

Wash buffer (1 M NaCl, 20 mM Tris, 1 mM EDTA, pH = 7.5), aptamer binding buffer (1 M NaCl, 5 mM Tris, 1 mM EDTA, pH = 7.5) and thrombin binding buffer (140 mM NaCl, 50 mM Tris, 1 mM MgCl_2 , 10 mM KCl, 0.1% Tween-20, 0.1% BSA, pH = 7.4) were prepared (Centi et al. 2007; Huang et al. 2004). A 10 μL aliquot of the magnetic beads (10 mg/mL) was washed three times with the wash buffer, and then resuspended in 100 μL of aptamer binding buffer, and split into two portions. To each of the portions was added 3 μL of 50 μM aptamer solution (either the 15-mer or the 29-mer), which represented a ten-fold molar excess, ensuring that the beads would be well functionalized with the aptamer, and they

were incubated with gentle mixing for one hour. The biotinylated aptamers were attached to the streptavidin-coated beads via the biotin-streptavidin bond. The beads were washed three times with thrombin binding buffer, and resuspended at various concentrations (7 $\mu\text{g/mL}$, 22 $\mu\text{g/mL}$, 70 $\mu\text{g/mL}$, 220 $\mu\text{g/mL}$). Solutions of thrombin protein were diluted to various concentrations (ranging from 10 fM to 1 nM) with thrombin binding buffer. 100 μL of thrombin solution was mixed with 5 μL of a solution of 29-mer beads, incubated with gentle mixing for 10 minutes, followed by the addition of 5 μL of a solution of 15-mer beads at the same bead concentration. For control solutions, 100 μL of thrombin binding buffer, with no thrombin added, was used.

Hanging droplets were prepared on a Teflon-coated slide, with an O-ring greased with lubricating grease in the middle (for sealing). Sixteen 1 μL droplets of thrombin-bead solution were placed on the 1-mm exposed glass spots on the slide in a 4-by-4 array. The other side of the O-ring was greased, and a clear glass slide was placed on top, creating a seal around the droplets (this was to prevent evaporation as well as to minimize air currents disrupting the droplets). The slides were then inverted, so that the droplets hung down from the Teflon-coated slide. The slides were allowed to sit for 30 minutes, during which the beads fell through the solution, under the influence of gravity (bead density = 1.9 g/cm^3). As the beads fell towards the tip of the droplet, if they encountered a thrombin molecule, the beads could bind to each other. In the absence of thrombin molecules, the beads collected in a tight cluster in the center of the droplet. The slides were then placed in the rotating magnetic field for magnetorotation and further analysis.

3. Results and Discussion

A schematic showing the formation of the bead assemblies is shown in Figure 1. A schematic showing the setup used for performing magnetorotation is shown in Supporting Information Figure S1. After the beads are mixed in a centrifuge tube, and transferred to the hanging droplet, the beads fall to the bottom of the droplet, and form an assembly. In the absence of thrombin, there is nothing to bind the beads together, and they form a tight hexagonally-packed assembly, as shown in Figure 1d and in the image in Figure 1f. As the concentration of thrombin increases, the likelihood increases that an aptamer on the surface of one of the beads will bind to a thrombin molecule, and that it will encounter a second bead that has a free complementary aptamer on its surface, and that the thrombin molecule will bind to the second aptamer, linking the beads together. As the concentration of thrombin increases, the number of beads that bind to each other increases. This results in a less-densely packed assembly of beads, one that has increased lacunarity (“gapiness”), and a decreased fractal dimension. At high thrombin concentrations, where each bead has many thrombin molecules bound to its surface, the conditions approach those of diffusion-limited aggregation: the bead density is relatively low, the primary means of transport is diffusion, and the beads bind instantly and irreversibly upon contact (Witten and Sander 1981). This results in the highly-branched bead assembly shown in Figure 1e, and in the image in Figure 1g.

The theory underlying the magnetorotation of superparamagnetic beads has been discussed previously (Connolly and St Pierre 2001; Fannin et al. 2006; Hecht et al. 2011a; Hecht et al. 2011b; Janssen et al. 2009), and is summarized in the Supporting Information. Briefly, the rotational period of a superparamagnetic material in the asynchronous regime depends on a number of factors, including the volume of the magnetic material, the total hydrodynamic volume of the assembly, the magnetic susceptibility of the material, the strength of the magnetic field, the viscosity of the solution, and the shape factor of the material. In these experiments, we effectively hold constant all parameters except the total hydrodynamic volume of the assembly. Therefore, the rotational period of the assembly is directly

proportional to the volume of the assembly, which is determined by the thrombin-induced binding of the beads.

Bead assemblies were prepared using four different bead concentrations: 7 $\mu\text{g/mL}$, 22 $\mu\text{g/mL}$, 70 $\mu\text{g/mL}$ and 220 $\mu\text{g/mL}$, which enabled an increased overall dynamic range. Figure 2 shows a series of images of bead assemblies produced with a bead concentration of 220 $\mu\text{g/mL}$, illustrating the effects of thrombin concentration on the size, shape, fractal dimension and lacunarity of the bead assemblies. Additional images, with bead concentrations of 22 and 70 $\mu\text{g/mL}$, are shown in the Supporting Information Figure S2.

Figure 3 shows a series of dose-response curves for the magnetorotation of the bead assemblies at the four different bead concentrations. The data is normalized over the range of 1-10 seconds to facilitate comparison between the different bead concentrations (the non-normalized data, shown in Supporting Information Figure S3, ranges from 1-15 seconds). The data was normalized through the logistic Hill equation that fit the data. After the original data was fit with the Hill equation, for the parameters of the Hill equation, the data was normalized by setting the rotational period at zero concentration to 1 s/s, and the rotational period at infinite concentration to 10 s/s, and creating a linear relationship to scale the original logistic fit to the normalized logistic fit. This relationship was then applied to the data. The dynamic range shifts to the right and limit of detection (LOD) increases as the concentration of beads increases. This is because the dynamic range and LOD depend on the ratio of thrombin molecules per bead (this is discussed in further detail in the Supporting Information and Table S1). Based on the average number of thrombin molecules and beads in solution at the LOD, and the energy of the thrombin-aptamer bond (see Supporting Information for a detailed discussion), it seems possible that only a single thrombin molecule is needed to bind any two beads together, as a step in the formation of a bead assembly, in the hanging droplet. Each data point on the graph represents the average of ten bead assemblies ($\pm$ SD). The coefficient of variation decreases with increasing bead concentration, because a larger number of beads increases the uniformity of the assemblies.

The LOD for each bead concentration, as shown in Figure 3b, is calculated based on the average rotational period of the control plus three standard deviations, and then determining the concentration that corresponds with that period along the logistic curve that fits the data (Long and Winefordner 1983). The data is fit with the logistic Hill equation (Baud 1993). The LOD of the lowest bead concentration, 7 $\mu\text{g/mL}$, is 80 fM, which is among the lowest LODs reported for a thrombin-based diagnostic system in buffer (An et al. 2010; Bai et al. 2012; Cai et al. 2006; Centi et al. 2007; Cho et al. 2008; Hansen et al. 2006; Hianik et al. 2005; Ho and Leclerc 2004; Hu et al. 2012; Huang et al. 2004; Kim et al. 2009; Li et al. 2008; Li et al. 2007; Liu et al. 2012; Pavlov et al. 2004; Radi et al. 2005; Rahman et al. 2009; Song et al. 2009; Tennico et al. 2010; Zhang et al. 2009; Zhou et al. 2012). Of these papers, only three (Centi et al. 2007; Cho et al. 2008; Song et al. 2009) report quantitative results measured in whole or diluted serum, all of which show much higher limits of detection than in buffer. We believe that these observations reflect the challenges associated with using the specific thrombin aptamers in a complex matrix, such as serum. This problem may require better tuned aptamers or antibodies, irrespective of the detection method. Data showing the performance of BAM in serum can be found in Supporting Information Figure S4.

Figure 4 shows the fractal dimension and lacunarity (the measure of gappiness of a structure) of the bead assemblies for three different bead concentrations, 22 $\mu\text{g/mL}$, 70 $\mu\text{g/mL}$ and 220 $\mu\text{g/mL}$, (7 $\mu\text{g/mL}$ assemblies were too small to accurately quantify using this method) as measured by the ImageJ plugin FracLac (Karperien 1999-2012). The fractal dimension of a solid two-dimensional object is 2, and the fractal dimension of a diffusion-

limited aggregate is approximately 1.71 (Witten and Sander 1981). Figure 4a suggests that at high thrombin concentrations, the bead assemblies might have a structure similar to that of diffusion limited aggregates. Figure 4c shows the relationship between fractal dimension, lacunarity, and the normalized rotational period, for the 22 $\mu\text{g/mL}$ bead assemblies.

There are two primary advantages that the rotational period analysis has over image analysis (fractal dimension and lacunarity) as a method for measuring the protein concentration. The first is that assemblies from the lowest bead concentration (7 $\mu\text{g/mL}$) could not be accurately measured by image analysis, because the relatively small number of beads in the assembly results in an unacceptably high level of fluctuations/noise in the measured values, which is not the case with the rotational period measurements. Therefore, analysis by the rotational period method allows for greater sensitivity and dynamic range. The second advantage is that the rotational period can be measured using just a portable laser-and-photodiode setup (discussed below), in contrast to the microscope required for image analysis, which would allow for a portable, rugged and inexpensive instrument for decentralized (“in the field”) measurement locations. Additionally, it should be noted that magnetorotation is still required for performing image analysis. The rotating magnetic field is needed to pack the beads into the dense hexagonal configuration, without which the fractal dimension and lacunarity cannot be accurately measured at low protein concentration (without the field, the beads will diffuse away from the center of the assembly via Brownian motion, creating the appearance of higher lacunarity and lower fractal dimension).

The data in Figure 4b suggest that among image analysis methods, for the data presented here, the fractal dimension appears to provide more useful information than the lacunarity. We included the lacunarity to show that it could also be used as a method for measuring protein concentration through bead assembly. We examined additional image analysis methods; including measuring the physical size of the assembly via the radius of gyration of the assembly and measuring the angles between adjacent nearest-neighbor beads, but those methods did not provide as much information as fractal dimension and lacunarity.

In order to facilitate the performance of BAM without the use of a microscope, it would be necessary to develop a simple, inexpensive and portable detection apparatus. To this effect, we designed a laser-and-photodiode detection apparatus from inexpensive, readily available laboratory components, as shown in Figure 5a. When a low power (20 mW, 650 nm) laser diode is shined on a droplet containing a bead assembly, the droplet acts as a lens, focusing the beam through its center, where the assembly lies. This creates a projection of the bead assembly that is magnified by beam spreading caused by the droplet focusing (in practice, a bead assembly that is approximately 100 μm in diameter is magnified to approximately 4-5 cm over a distance of approximately 20 cm). This rotation of the projection of the bead assembly is captured by a 3-by-3 array of photodiodes located underneath the droplet. The signals from each of the photodiodes are recorded in LabVIEW, and analyzed with a Fourier transform to determine the rotational period of the assembly (a screenshot of this program is shown in Supporting Information Figure S5). Having multiple redundant signals (each of the nine diodes calculates the rotational period independently, creating a set of redundant signals) helps to increase confidence in the output.

The performance of the laser-and-photodiode apparatus was validated by measuring the magnetorotation of a series of 220 $\mu\text{g/mL}$ bead assemblies on the microscope and then on the laser-and-photodiode apparatus, as shown in Figure 5b. The difference in the relative values of the rotational periods between the two systems is due to differences in the strengths of the magnetic field (4 mT on the microscope, 4.7 mT on the laser-photodiode setup) on the two setups. However, the shapes of the two curves closely follow each other, suggesting that a simple, inexpensive laser-and-photodiode system is capable of measuring

the magnetorotation, and therefore the thrombin concentration, of magnetic bead assemblies without the use of a microscope.

Conclusion

This paper introduces Bead Assembly Magnetorotation as a viable signal transduction method for measuring the concentration of a protein in solution through two different methods, magnetorotation and fractal analysis. The 80 fM LOD of this system for the protein thrombin is very competitive with existing detection methods. The simple laser-and-photodiode portable detection setup represents a potential for the development of BAM into a point-of-care detection system. In future work, we intend to demonstrate the performance of BAM in serum with more clinically relevant protein targets.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Bead Assembly Magnetorotation is demonstrated as a viable signal transduction method

- Limit of detection for thrombin in buffer is 80 fM
- Portable laser-and-photodiode system demonstrated for microscope-free detection
- Fractal analysis demonstrated as a secondary signal transduction method

Figure 1.

Schematic illustration of the bead assembly magnetorotation. (a), Beads coated with 29-mer thrombin aptamer are mixed with thrombin in a microcentrifuge tube, followed by addition of the 15-mer aptamer coated beads. (b) A 1 μ L droplet of the bead solution is transferred to an inverted droplet. (c) The beads fall to the bottom of the droplet. A rotating magnetic field is then applied, to pull together beads which are not bound to thrombin. (d) In the case of no or low protein concentration, the beads assume a tightly-packed hexagonal arrangement. (e) In the case of high protein levels, the beads assume a highly branched structure. (f) A brightfield microscope image of hexagonally packed beads in the absence of protein. Imaged through a 40 $\times$ water-immersion objective. Scale bar = 5 μ m. (g) A brightfield microscope image of a loosely-packed bead assembly in the presence of high protein concentration. Imaged through a 40 $\times$ water-immersion objective. Scale bar = 5 μ m.

Figure 2.

A series of brightfield microscope images illustrating the effects of protein concentration on the shape, lacunarity and fractal dimension of the bead assemblies. Initial bead concentration was $220 \mu\text{g/mL}$. The fractal dimension for each bead assembly is provided. Left column was taken using a $20\times$ water-immersion objective, right column was taken using a $40\times$ water-immersion objective. Scale bar = $20 \mu\text{m}$.

Figure 3.

(a) Dose-response curves for the normalized rotational period of bead assemblies from 4 bead concentrations, 7 µg/mL, 22 µg/mL, 70 µg/mL and 220 µg/mL. The curves are fit with the logistic Hill equation. The rotational periods of the bead assemblies were normalized, based on the Hill equation fit through each point, such that the curve had a minimum value of 1 s/s and a maximum value of 10 s/s. This was done to make it easier to compare the behaviors of the different curves. Each point represents the average of the rotational period of ten bead assemblies, and the error bars are $\pm$ SD. (b) A table showing the limit of detection (LOD) for each bead concentration, as well as the normalized rotational periods of the control bead assemblies (no thrombin) for each bead concentration. The limit of detection was calculated as the mean control value $\pm$ 3 SD (Long and Winefordner 1983).

Figure 4.

(a) Fractal dimension, and (b) Lacunarity of bead assemblies. Three bead concentrations are shown, 22 $\mu\text{g/mL}$, 70 $\mu\text{g/mL}$ and 220 $\mu\text{g/mL}$. For both plots, each point represents an average over ten bead assemblies; error bars are $\pm\text{SD}$. Curve is the logistic Hill equation (Hecht et al. 2010) fit to the data. (c) The relationship between rotational period, fractal dimension and lacunarity. The data shown are for the 22 $\mu\text{g/mL}$ bead concentration.

Figure 5.

(a) A schematic of the laser-and-photodiode setup. A low-power red laser is aimed at the center of the droplet holding the self-assembled aggregate. The droplet optically focuses the beam through the aggregate, creating a projection of the rotating aggregate onto an array of photodiodes. The periodic signal captured by the photodiodes is analyzed by a computer program, from which it calculates the rotational period using a Fourier transform. (b) Rotational period of bead assemblies as captured by the microscope (blue, left axis) and the laser-photodiode setup (red, right axis). Each point represents the mean $\pm$ SD of 10 assemblies. Both data sets were fit with the logistic Hill equation.