Label-Acquired Magnetorotation As a Signal Transduction Method for Protein Detection: Aptamer-Based Detection of Thrombin

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ABSTRACT: This paper presents a new signal transduction method, called label-acquired magnetorotation (LAM), for the measurement of the concentration of proteins in solution. We demonstrate the use of LAM to detect the protein thrombin using aptamers, with a limit of detection of 300 pM. LAM is modeled after a sandwich assay, with a 10 μm nonmagnetic “mother” sphere as the capture component and with 1 μm magnetic “daughter” beads as the labels. The protein-mediated attachment of daughter beads to the mother sphere forms a rotating sandwich complex. In a rotating magnetic field, the rotational frequency of a sandwich complex scales with the number of attached magnetic beads, which scales with the concentration of the protein present in solution. This paper represents the first instance of the detection of a protein using LAM.

One of the primary goals in a point-of-care diagnostic system is measuring the concentration of a protein in order to assess the overall health of a patient. Effective screening methods have been shown to improve patient health, such as a recent large study on a population at high risk for lung cancer, that found a 20% decrease in mortality due to better early screening.1,2 There are three primary components in a protein measurement system: the target biomarker to be measured, the affinity molecules used to capture the target, and the method of transducing a successful binding event into a quantifiable signal. There are several popular signal transduction methods, including optical, electrochemical, and magnetic schemes. This paper presents the development of a new, optomagnetic signal transduction method, called label-acquired magnetorotation (LAM), which has the potential for eventual incorporation into a point-of-care diagnostic system. Previously, we published proof-of-principle work demonstrating the concept of LAM using a biotin and streptavidin system as protein and aptamer mimics.3 Here, we demonstrate the next step by showing LAM used to detect a protein in solution using aptamers.

The most common setup for measuring the concentration of a protein in solution is the sandwich assay, where the target is first captured by an affinity molecule bound to a surface and is then sandwiched by a signal transducer attached to another affinity molecule.4 Optical signal transduction methods include sandwich-based ELISA,5–9 fluorescence signaling10–12 or quantum dots,13,14 and the nonsandwich based surface plasmon resonance methods,13–15. Electrochemical signal transduction methods include sandwich-based amperometric enzymatic methods16,17 and nonsandwich-based impedimetric sensing.18,19

Magnetic beads are advantageous for use as signal transducers because they are biologically inert, they are physically stable under most biological environments, and biological materials have no native magnetism that could interfere with a signal from the beads.20,21 Because of these advantages, magnetic beads have been used as signal transducers in a variety of applications, including giant magnetoresistance (GMR),22–24 Hall probes,25,26 and magnetic relaxation.27,28 Additionally, magnetic beads have been used as carriers for magnetophoresis and to facilitate detection by other signal transduction methods.29–31 In contrast, the method described here uses optical detection of the magnetic behavior. The beads used in this study are 1 μm commercial beads that exhibit superparamagnetic behavior (DynaBeads). These beads are composed of maghemite (γ-Fe₂O₃) nanoparticles, with a mean diameter of 8 nm dispersed within a polymer matrix. The beads are 25.5% Fe by mass.32 In the absence of a magnetic field, these beads have no net magnetization, but within a magnetic field, the magnetic moments of the beads align with the field and they become strongly magnetic.32

The work presented here uses these beads in a rotating magnetic field. Previous studies have examined and characterized the behavior of these beads in alternating magnetic fields. It was first shown that in a one-dimensional alternating magnetic field, the dominant relaxation mechanism of such superparamagnetic beads is the Neel relaxation of the nanoparticles embedded within the bead.33 It was later shown that in a two-dimensional rotating magnetic field, at high driving frequencies, the dominant mechanism driving the rotation of these same beads is also Neel relaxation.34 Brownian rotational effects are not significant for these beads because the time constant for the Brownian relaxation of a sphere with a diameter on the order of a micrometer is on the order of seconds, while the time constant for the Neel relaxation of the inner magnetic nanoparticles is on the order of nanoseconds.

In a two-dimensional rotating magnetic field, at low driving frequencies, magnetic beads are able to rotate synchronously with the field. At higher driving frequencies (above the critical frequency35), these beads are not able to stay in phase with the field and rotate asynchronously. In the asynchronous regime, the
rotational frequency of the bead depends on a number of factors, including the magnetic moment of the bead, the amplitude and frequency of the driving field, the hydrodynamic volume of the bead, and the viscosity of the solution. This asynchronous rotation has already been demonstrated to be a useful tool for making biological measurements, specifically for monitoring the growth and antibiotic susceptibility of bacteria.\textsuperscript{36–39}

Thrombin is a coagulation factor that participates in the first step in the coagulation cascade that leads to the formation of a blood clot, so as to stem blood loss. Aptamers are single- or double-stranded nucleic acid sequences that bind to proteins through favorable electrostatic interactions, with affinities similar to those of antibodies.\textsuperscript{40,41} One of the earliest aptamers to be identified binds to the fibrin exosite on thrombin and has the following 15-base pair sequence: $5'$-GGTTGGTTGGTTGG-3'.\textsuperscript{42} Later, a second, 29-base pair sequence against thrombin was identified, which binds to the heparin exosite: $5'$-AGTCCGGTTAGGGCAGGTTGGGTGACT-3'.\textsuperscript{43} Since these aptamers bind to opposite sides of the thrombin molecule, they represent an ideal system for the development of an aptamer-based sandwich assay and have been used in the development of many such assays.\textsuperscript{44–46}

\section*{EXPERIMENTAL SECTION}

A schematic of LAM is shown in Figure 1. The mother spheres used were 10 $\mu$m nonmagnetic streptavidin-coated ProActive microspheres (Bangs Laboratories, Fishers, IN). The daughter beads used were Dynal MyOne 1 $\mu$m streptavidin-coated Dynabeads that exhibit superparamagnetic behavior (Invitrogen, Carlsbad, CA). Human $\alpha$-thrombin was purchased from Haematologic Technologies (Essex Junction, VT). Biotinylated aptamers (with a 5$'$-polyT\textsubscript{10} tail for improved binding)\textsuperscript{47} were purchased from Integrated DNA Technologies (Coralville, IA). Salts (NaCl, KCl, MgCl\textsubscript{2}, EDTA, and Tris-HCl) and Tween-20 were purchased from Sigma Aldrich (St. Louis, MO). Bovine serum albumin (BSA) blocker solution was purchased from Thermo Scientific (Waltham, MA). Zero-thickness glass coverslips were obtained from Electron Microscopy Sciences (Hatfield, PA). OPI Top Coat clear nail protector was purchased from OPI Products Inc. (North Hollywood, CA). Formulations for wash buffer, aptamer binding buffer, and thrombin binding buffer (containing 0.1% BSA, and with the addition of 10 mM KCl\textsuperscript{48}) were based on previously published work.\textsuperscript{47}

An aliquot of 50 $\mu$L of the magnetic beads was washed three times by centrifugation in 200 $\mu$L of wash buffer, then resuspended in 500 $\mu$L of aptamer binding buffer, at a concentration of 1 mg/mL beads in a microcentrifuge tube. An aliquot of 50 $\mu$L of the mother spheres was washed three times by centrifugation in 200 $\mu$L of wash buffer, then resuspended in 1 mL of aptamer binding buffer, at a concentration of 0.5 mg/mL spheres. A 10 $\mu$L aliquot of biotinylated-15-mer aptamer was added to the superparamagnetic beads, and a 10 $\mu$L aliquot of biotinylated-29-mer aptamer was added to the mother spheres. The two solutions were briefly vortexed then incubated on an end-over-end rotator for 1 h. They were then washed (by magnetic separation and centrifugation, respectively) three times and resuspended in thrombin binding buffer. An aliquot of human $\alpha$-thrombin was serially diluted over a concentration range of 50 nM to 100 pM in thrombin binding buffer. In a separate tube, 100 $\mu$L of thrombin solution was mixed with 40 $\mu$L of mother sphere solution and then incubated on an end-over-end rotator for 90 min. Finally, 10 $\mu$L of magnetic bead solution was added to the mother spheres and thrombin and incubated on an end-over-end rotator for 90 min.

Microfluidic flow cells were prepared from two zero-thickness glass coverslips (the bottom coverslip was coated with a thin layer of clear nail protector, to reduce particle sticking) separated by a single piece of double-sided Scotch tape (3M, St. Paul, MN). The solution containing the mother spheres and the magnetic beads was diluted with 140 $\mu$L of 0.2% Tween-20, and 20 $\mu$L of this solution was pipetted into the coverslip flow cell. The coverslip flow cell was then placed in a rotating magnetic field (amplitude 1.25 mT, frequency 200 Hz) built from two pairs of orthogonally oriented Helmholtz coils driven by a pair of sinusoidal waves 90° out of phase with each other. The magnetic field was located on top of an IX71 inverted microscope (Olympus, Melville, NY). The rotation of the sandwich complexes was observed through a 100\times oil-immersion objective, imaged through a Basler piA640-210gm camera (Basler, Highland, IL) and recorded by an in-house program written in LabVIEW (National Instruments, Austin, TX). Videos were analyzed using the St. Andrews particle tracker\textsuperscript{49} and an in-house program written in MATLAB.

\section*{THEORY}

The theory governing the behavior of superparamagnetic particles and beads in rotating magnetic fields has been discussed in detail elsewhere.\textsuperscript{33,34,50} Briefly, starting from the equation for the magnetic torque, $\tau = m \times B$, where $m$ is the magnetic moment of the bead and $B$ is the external magnetic field, assuming steady-state rotation (allowing for the equating of rotational driving forces with drag forces, $\tau = \kappa \eta V(t) (d\theta/dt)$, where $\kappa$ is the shape factor (equal to 6 for a sphere), $\eta$ is the viscosity of the surrounding fluid, and $V(t)$ is the hydrodynamic volume), and, making some simple substitutions, $B = \mu_0 H$, $m = MV_m$, $M = \chi H$ and $\chi = \chi' - i \chi''$, (where $H$ is the magnetizing field, $\mu_0$ is the

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Schematic of LAM with thrombin as the analyte. (1) 10 $\mu$m nonmagnetic mother spheres coated with the 29-mer antithrombin aptamer are mixed with thrombin, which binds to the mother spheres. (2) 1 $\mu$m magnetic beads coated with the 15-mer antithrombin aptamer are mixed with the thrombin-coated mother spheres. The magnetic beads bind to the thrombin attached to the mother sphere, forming a sandwich complex. (3) The sandwich complex is transferred to a rotating magnetic field, where the rotational frequency of the sandwich complex depends on the number of attached magnetic beads.}
\end{figure}
permeability of free space, $M$ is the volume magnetization, $V_m$ is the volume of the bead’s magnetic material, $\chi$ is the bead susceptibility, $\chi'$ is the real component of the bead susceptibility, and $\chi''$ is the imaginary component of the bead susceptibility, we can obtain an expression for the rotational frequency, $d\theta/dt$:

$$
\frac{d\theta}{dt} = \frac{1}{k_B \mu_0 V_m} \mu_0 V_m \chi'' H^2
$$

The definition of imaginary susceptibility, $\chi''$, is $\chi'' = \chi(\Omega \tau_N)/(1 + \Omega^2 \tau_N^2)$, where $\chi_0$ is the dc susceptibility and $\Omega$ is the frequency of the driving field. The definition of Neel relaxation time, $\tau_N$, is $\tau_N = \tau_0 \exp((KV_m)/(k_B T))$, where $\tau_0$ is the attempt frequency, $K$ is the anisotropy constant (equal to $5 \times 10^4$ J/m$^3$ for maghemite nanoparticles$^{[5]}$), $V_p$ is the volume of the maghemite nanoparticles, $k_B$ is Boltzmann’s constant, and $T$ is the ambient temperature. The magnetic nanoparticles are not perfectly uniform; for a size distribution with $n$ intervals, with average nanoparticle volume $V_p$, the total volume of nanoparticles in the distribution is $V_n$. The expression for Neel relaxation time, $\tau_N$, can be substituted into the expression for imaginary susceptibility, $\chi''$, which along with considering the effects of the nanoparticle size distribution, can then be substituted into eq 1 to create a single expression describing the rotation of a superparamagnetic object in a magnetic field:$^{[34]}

$$
\frac{d\theta}{dt} = \frac{1}{k_B \mu_0 V_m} \mu_0 V_m H^2 \sum_n V_n^2 \sum_{1}^{n} \frac{\Omega \tau_0 \exp\left(\frac{KV_p}{k_B T}\right)}{1 + \Omega^2 \exp\left(\frac{2KV_p}{k_B T}\right)} V_n^2
$$

In the low driving frequency ($\Omega \ll 1$ kHz) regime used in this paper, $\Omega^2 \exp\left(\frac{2KV_p}{k_B T}\right) \ll 1$, so eq 2 can be simplified:

$$
\frac{d\theta}{dt} = \frac{1}{k_B \mu_0 V_m} \mu_0 V_m H^2 \chi_0 \sum_n V_n^2 \sum_{1}^{n} \frac{\Omega \tau_0 \exp\left(\frac{KV_p}{k_B T}\right)}{1 + \Omega^2 \exp\left(\frac{2KV_p}{k_B T}\right)} V_n^2
$$

\[eq 3\]

### RESULTS AND DISCUSSION

To test whether the sandwich complexes follow the model of eq 3, we observed the response of the sandwich complexes to changes in amplitude and frequency. Holding constant all variables except for field amplitude, eq 3 reduces to $(d\theta/dt) \approx H^2$. Figure 2a shows indeed that the rotational frequency of a sandwich complex is directly proportional to the square of the amplitude of the driving field. Holding constant all variables except for field driving frequency, eq 3 reduces to $(d\theta/dt) \approx \Omega$. Figure 2b shows that the rotational frequency of a sandwich complex does increase with the frequency of the driving field, but it does not exactly demonstrate the linear relationship that eq 3 suggests.

We examined the stability of the rotation of sandwich complexes over 60 min of observation. The rotational frequency of four sandwich complexes was measured every 5 min for 60 min, as shown in Figure 3a. The coefficient of variation (standard deviation divided by the mean, multiplied by 100%) of the complexes (A–D) was 3.3%, 2.5%, 1.5%, and 1.6%, respectively, demonstrating that the rotation of a sandwich complex is fairly stable over a 60 min observation period. All other measurements reported here were made within an hour of the sandwich complexes being injected into the coverslip fluidic cell.

A dose–response curve of LAM used for measuring the concentration of thrombin in solution is shown in Figure 3b. At each thrombin concentration, the rotation of 15 sandwich complexes was measured, and each point in the figure represents the average of those 15 measurements ($\pm$ standard deviation). The data was fit using the four-parameter logistic Hill equation.$^{[22,55]}$ The dynamic range of the curve extends from about 1 nM to about 20 nM. Above 20 nM, the curve plateaus. Below 1 nM, there is still a detectable signal down to 300 pM. In the 300 pM to 1 nM range, there was still binding of beads to the mother sphere, but there was no significant difference between the different concentrations. Below 300 pM, no binding of beads to the mother sphere was observed. Similarly, in a control sample (no thrombin), there was also no binding detected. In the absence of the aptamers, thrombin does not bind to the spheres and beads. Figure 3b demonstrates the viability of LAM as a tool for measuring the concentration of a protein in solution, with a limit of detection of 300 pM.

Screenshots of the rotation of five of the sandwich complexes from Figure 3b are shown in Figure 4. These images show that the number of beads attached to each complex increases with the
concentration of thrombin and that the rotational frequency of the complexes increases with the number of attached beads. These images also show that a qualitative estimate of the protein concentration can be made merely by looking at the complexes under a microscope, without using rotation.

One of the advantages of using the thrombin aptamers are their popularity; many groups have used these aptamers for demonstration of signal transduction techniques. When examining other methods that are sandwich-based and use single-step (nonamplified) methods, reported LODs typically are in the 0.1–1 nM range, including electrochemical detection, quantum dots, Si-nanowire FETs, and fluorescent molecular beacons. There are many clinically relevant biomarkers found in plasma at concentrations around 1 nM. Within this context, we believe that LAM is certainly competitive with other detection technologies. Moreover, LAM has the advantage of simplicity, robustness, and low cost, without requiring sensitive optical readers or other expensive and stationary sensing equipment.

We generated a model in MATLAB to simulate the optimal performance of LAM, assuming perfect mixing and no nonspecific interactions, based on a previously reported two-site immunoassay model. Considering only specific interactions, there are two primary reactions that take place in our system:

\[
Q_1 + \frac{k_1}{k_{-1}} P \rightarrow Q_1P
\]

\[
Q_1P + \frac{k_2}{k_{-2}} Q_2 \rightarrow Q_1Q_2
\]

where \( P \) is the protein of interest, \( Q_1 \) is the capture aptamer, and \( Q_2 \) is the detection aptamer. There are two possible side reactions:

\[
Q_2 + \frac{k_3}{k_{-3}} Q_2P
\]

\[
Q_2P + \frac{k_4}{k_{-4}} Q_1Q_2
\]

The model is carried out in two parts, capture and detection. In the capture phase, only eq 4 is considered. After the capture reaction has reached equilibrium, the detection phase commences, in which eqs 4–7 are all considered. The rate constants for the thrombin aptamers were obtained from previously published work. The model is generated by simultaneously solving the six differential equations below:

\[
\frac{d[Q_1]}{dt} = -k_1[Q_1][P] + k_{-1}[Q_1P] - k_4[Q_1][Q_2P] + k_{-4}[Q_1Q_2]
\]

\[
\frac{d[Q_2]}{dt} = -k_3[Q_2][P] + k_{-3}[Q_2P] - k_2[Q_1P][Q_2] + k_{-2}[Q_1Q_2]
\]

\[
\frac{d[P]}{dt} = -k_1[Q_1][P] + k_{-1}[Q_1P] - k_3[Q_2][P] + k_{-3}[Q_2P]
\]
simple, compact-disc-like, laser-and-photodiode setup, 59 to-  

top of the predicted dose  

Figure 3b, and a logistic curve  

Also included in the plot are experimental data (  

a model based on the binding kinetics of the aptamers with thrombin.  

Figure 5.  

The rather abrupt plateau at the top of the  

imperfect mixing, suboptimal aptamer-bead attachment, or ex-  

interactions between the aptamers and other proteins in solution,  

steps must be taken. We plan to translate LAM o  

a million thrombin molecules could bind to the mother sphere.  

only a few hundred beads can bind to the mother sphere, but over  

Response curve could be due to nonspeci  

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CONCLUSIONS  

In summary, we have demonstrated that label-acquired mag-  

netorotation is a viable signal transduction method for measuring  

the concentration of a protein in solution, with a limit of  

detection of 300 pM of thrombin when using the classic  

thrombin aptamers. We have shown that the amplitude and  

frequency response of a sandwich complex generally follow the  

behavior predicted by the equations that describe superparamag-  

netic bead behavior. It is our hope for the future that, with further  

work, LAM will be developed into a viable signal transduction  

method for point-of-care testing.  

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Figure 5. Simulated dose—response curve (dotted line) for LAM from  

a model based on the binding kinetics of the aptamers with thrombin.  

Also included in the plot are experimental data (filled circles), from  

Figure 3b, and a logistic curve fit (dashed line). The abrupt plateau at  

the top of the predicted dose—response curve represents the saturation  

of the sensor.  

\[
\begin{align*}
\frac{d[Q_1P]}{dt} &= k_1[Q_1][P] - k_{-1}[Q_1P] - k_2[Q_1P][Q_2] \\
&+ k_{-2}[Q_1PQ_2] \\
\frac{d[Q_2P]}{dt} &= k_3[Q_2][P] - k_{-3}[Q_2P] - k_4[Q_1][Q_2P] \\
&+ k_{-4}[Q_1PQ_2] \\
\frac{d[Q_1PQ_2]}{dt} &= k_2[Q_1P][Q_2] + k_4[Q_1][Q_2P] \\
&- (k_{-2} + k_{-4})[Q_1PQ_2]
\end{align*}
\]