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A Coffee Ring Aptasensor for Rapid Protein Detection

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Abstract

We introduce a new biosensing platform for rapid protein detection that combines one of the simplest methods for biomolecular concentration, coffee ring formation, with a sensitive aptamer-based optical detection scheme. In this approach, aptamer beacons are utilized for signal transduction where a fluorescence signal is emitted in the presence of the target molecule. Signal amplification is achieved by concentrating aptamer-target complexes within liquid droplets, resulting in the formation of coffee ring “spots”. Surfaces with various chemical coatings were utilized to investigate the correlation between surface hydrophobicity, concentration efficiency and signal amplification. Based on our results, we found that the increase in coffee ring diameter with larger droplet volumes is independent of surface hydrophobicity. Furthermore, we show that highly hydrophobic surfaces produce enhanced particle concentration, via coffee ring formation, resulting in signal intensities 6-fold greater than those on hydrophilic surfaces. To validate this biosensing platform for the detection of clinical samples, we detected α -thrombin in human serum and 4x diluted whole blood. Based on our results, coffee ring spots produced detection signals 40x larger than samples in liquid droplets. Additionally, this biosensor exhibits a lower limit of detection of 2 ng/mL (54 pM) in serum, and 4 ng/mL (105 pM) in blood. Based on its simplicity and high performance, this platform demonstrates immense potential as an inexpensive diagnostic tool for the detection of disease biomarkers, particularly for use in developing countries that lack the resources and facilities required for conventional biodetection practices.

Keywords

coffee ring; aptasensor; protein detection; α -thrombin

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

Supporting Information

Characterization of coffee ring growth of serum and blood. Bright field and fluorescent images of coffee rings from serum and blood droplets. This material is available free of charge via the Internet at <http://pubs.acs.org>.

INTRODUCTION

The detection of biomolecules (proteins, DNA, RNA) provides valuable information for a broad range of applications including clinical diagnosis, water and environmental analysis, food safety, and biodefense.¹ Current gold standard technologies for the detection of proteins heavily rely on antibody-based immunoassays such as enzyme-linked immunosorbent assay (ELISA), Western blot and lateral flow. However, these methods generally involve timely sample processing, expensive laboratory equipment and highly trained technicians.² In recent years, the global health and biomedical communities have identified an urgent need for low cost and simple diagnostic tests for use in developing countries, where the burden of infectious disease is the highest.³ Recently, aptamers have been widely utilized in biosensors due to their high binding affinities, versatile design strategies and adaptability for multiple sensing platforms and schemes.⁴ Various approaches have been explored in developing aptamer-based sensors for thrombin detection including those based on electrochemistry,^{5–9} electrochemiluminescence,^{10–12} piezoelectric transduction,¹³ surface enhanced resonance Raman scattering,^{14,15} and optical detection.^{16–19} In particular, aptamer-based optical sensors offer several attractive characteristics including high sensitivity and the ability to be used for label-free, in vitro detection.^{20,21} In this approach, aptamers generally adopt two or more conformations where one allows for binding to a target molecule. When combined with a reporter-quencher pair, changes in aptamer conformation are detected by the emission of a fluorescence signal.²² Similar to molecular beacons, aptamer beacons can employ a stem-loop structure where the reporter and quencher molecules are positioned at each end of the stem. In this configuration, the fluorescent signal from the reporter is absorbed by the quencher due to their adjacent proximity. The loop sequence contains a binding site for a target molecule and in its presence, the aptamer undergoes a conformational change causing the loop structure to break apart, separating the reporter and quencher. As a result, the reporter is no longer quenched and a fluorescent signal is emitted. In this work, we adopt another generalized design scheme for aptamer beacons which utilizes an aptamer labeled with a reporter and a complementary oligonucleotide containing a quencher.^{23,24} In the presence of the target, the binding equilibrium is shifted causing the oligonucleotide to dissociate from the aptamer, allowing the reporter to fluoresce. We combine this sensing scheme with an extremely simple method for biomolecular concentration based on the coffee ring effect. This everyday phenomenon occurs when a droplet of colloid solution evaporates on a solid surface, resulting in the formation of a coffee ring spot.²⁵ The underlying mechanisms that explain this effect are contact line pinning and capillary-induced flow, which drives suspended particles edge-ward, concentrating them at the droplet perimeter.^{26–29} Recent studies have employed this principle for concentrating nanoparticles to create three-dimensional microstructures^{30,31} and for biomolecular separation^{32,33}. In this work, we employ the coffee ring effect to concentrate aptamer-thrombin complexes within liquid droplets for subsequent signal amplification. Using this approach, we are able to achieve significant signal enhancement of our optical-based biosensor without the use of additional equipment or labor-intensive sample amplification, thus offering a simple and inexpensive diagnostic platform requiring minimal economic and technological resources.

EXPERIMENTAL METHODS

Materials and Reagents

Colloid solutions of fluorescently dyed (excitation/emission: 580/605 nm) polystyrene microspheres with a stock concentration of 2% solid and particle diameter of 0.02 μm were from Invitrogen (Carlsbad, CA). Solutions were diluted in purified deionized (DI) water (Barnstead EASYpure) with a resistivity of $\sim 18.3 \text{ M}\Omega/\text{cm}$. Pre-cleaned microscope glass slides were from Fischer Scientific (Pittsburg, PA). HDMS (hexamethyldisilazane) was from the Nanoelectronics Research Facility at UCLA and RainX was from a local vendor. Anti-thrombin aptamer beacon sequences used in this study were AlexaFluor®647-5'-CACTGTGGTTGGTGTGGTTGG-3', labeled as FLUORapt, and 5'-CAACCACAGTG-3'-3BHQ2, labeled as BHQ2apt. AlexaFluor®647 has excitation/emission wavelengths of 650/670 nm and exhibits exceptional photostability. All oligomers were from Integrated DNA Technologies (Coralville, IA) and human α -thrombin was from Haematologic Technologies, Inc. (Essex Junction, VT). Human serum and whole blood were from Bioreclamation, LLC, (Westbury, NY). Proteins and aptamers were stored at -20°C prior to experimentation.

Preparation of surface coatings and surface characterization

Three different surfaces were utilized in this study: glass, HDMS-treated glass and RainX-treated glass. HDMS surface treatment: glass slides were cleaned in isopropanol, rinsed in DI water and dried at 150°C for 5 min. The slides were exposed to saturated HDMS vapor for 30 min. RainX surface treatment: RainX is a synthetic, hydrophobic surface treatment that is commonly used on automobile windshields. Surface treatment was carried out by dispensing RainX solution onto glass slides and repetitively polishing the surface until all residual liquid was removed. Samples were immediately treated prior to contact angle (CA) and coffee ring characterization measurements to minimize variations in surface properties that could result from prolonged storage. All three surfaces were characterized by performing static CA measurements using a First Ten Angstroms FTA4000 contact angle measurement system (Portsmouth, VA). DI water droplets of 5 μL were dispensed onto the surfaces and images were captured and analyzed using FTA software.

Coffee ring formation and characterization

Coffee ring formations of colloidal solutions were created as previously demonstrated by Wong.³³ Briefly, colloid solutions were diluted from stock concentration to a volume fraction of $\sim 0.0001\%$. Droplets of 0.5, 1, 1.5, 3 and 5 μL were dispensed onto the three different surfaces using a pipette and allowed to dry under normal atmospheric conditions (temperature of $\sim 25^\circ\text{C}$ and relative humidity of $\sim 46\%$). Coffee ring formations were imaged under a green fluorescent filter (555 nm) using a Nikon Eclipse TE2000 inverted microscope. Coffee ring formations on glass and HDMS surfaces were imaged using a 2x objective and RainX surfaces were imaged using a 10x objective. Images were captured using a Photometric (Tucson, AZ) CoolSNAP CCD camera and fluorescent signals were analyzed using RS Image software.

Aptamer preparation and thrombin detection

Assays were performed to detect α -thrombin at serial concentrations from 1 to 500 ng/mL. Anti-thrombin aptamer beacons were generated by mixing FLUORapt and BHQ2apt together in Tris-EDTA (TE) buffer (10mM Tris-HCl, 1mM EDTA), heating the mixture to 90°C for 3 min and cooling to 25°C at 0.2°C/s. Aptamer preparation was carried out using a MJ Research thermo cycler. Thrombin was diluted in either signaling buffer (20mM Tris pH 8.3, 5 mM KCl, 1mM MgCl₂), serum or 4x diluted whole blood (in DI water), mixed with the aptamer beacons (diluted to 1 μ g/mL) and incubated for 30 min at 25°C, 31°C, 37°C, and 43°C. Following incubation, 1 μ L droplets of the aptamer-thrombin mixture were dispensed onto RainX-treated glass slides, dried, and analyzed using fluorescent microscopy. Droplets of unconcentrated aptamer-thrombin mixtures were imaged immediately after being dispensed onto the substrate. Each measurement was taken within 5 sec and negligible photobleaching was observed. All fluorescence intensity measurements were performed at ~25°C using a Nikon Eclipse TE2000 inverted microscope. Measurements in signaling buffer were imaged using a 10x objective, and measurements in serum and whole blood were imaged using a 4x objective. All detection assays were imaged under a red fluorescent filter (647 nm), which exhibits negligible autofluorescence of blood and serum.

RESULTS AND DISCUSSION

Surface hydrophobicity influences coffee ring formation

Coffee ring spots were formed using colloid solutions of fluorescent microbeads (at a concentration of 200 μ g/mL) on three different surfaces: glass, HDMS-treated glass, and RainX-treated glass. Each of these surfaces exhibits varying degrees of surface hydrophobicity: glass is hydrophilic (CA $\sim 26^\circ \pm 3^\circ$), HDMS-treated glass is mildly hydrophobic (CA $\sim 65^\circ \pm 4^\circ$) and RainX-treated glass is hydrophobic (CA $\sim 96^\circ \pm 3^\circ$). Droplets having volumes of 0.5 to 5 μ L were used to investigate the correlation between surface hydrophobicity and coffee ring diameter. As shown in Fig. 1, more hydrophobic surfaces produce smaller ring diameters for all droplet volumes. The primary underlying mechanism that describes this response is the liquid-to-surface contact area and the corresponding pinning perimeter.³⁴ For example, a droplet on a hydrophobic surface (RainX) has a smaller contact area than the same droplet on a hydrophilic surface (glass). Consequently, the contact line perimeter, i.e. where the droplet is pinned to the surface, is condensed and produces a smaller coffee ring. One of the characteristics of coffee ring formation that is particularly well-suited for biosensing applications is its ability to form compact ring structures from droplets of various types of liquids such as buffer, serum and blood. This is an important feature, especially when utilizing this sensor for the detection of clinical samples.

Quantitative analysis to compare the growth behavior of coffee rings was performed by normalizing the data to the ring diameter at 0.5 μ L (Fig. 2A). From this plot, we show that the ring diameter growth (from a smaller to larger droplet) is comparable on all surfaces and thus independent of surface hydrophobicity. Measurements were also performed using serum and blood (Fig. S1 in Supplementary Information), which exhibited similar drying

behaviors as in buffer. The equivalence in diameter growth can be explained by the hydrodynamics of coffee ring formation, i.e. contact line pinning. The ring diameter is determined by the droplet volume and contact line pinning perimeter, which is dictated by the CA. By fixing the CA, the ring diameter becomes a function of the droplet volume. We also show that coffee rings on hydrophobic surfaces become wider as the perimeter is decreased, which results enhanced particle concentration. Quantitative analysis of coffee ring concentration on different surfaces was performed by measuring the fluorescence signals, as peak intensity per unit area, of coffee ring spots (Fig. 2B). RainX surfaces produced the largest signal intensities, up to 6-fold greater, compared with those on hydrophilic surfaces at all droplet volumes. In addition to providing optimal signal amplification, the RainX surface produces coffee ring spots that are confined within a narrow field of view, even for varying droplet volumes. This characteristic is particularly favorable for optical-based biosensing systems, which would only require a fixed magnification objective, enabling for facile integration with inexpensive, portable detectors.^{35,36} It should also be noted that the generation of coffee ring spots results in relatively uniform structures with minimal geometric variability, allowing for enhanced repeatability, which is a highly desirable attribute for analytical measurements.

Temperature characterization for optimal aptamer binding activity

Studies were performed to determine the optimal temperature for aptamer binding to obtain the highest detection signal. Coffee ring spots containing aptamer-protein complexes were generated in signaling buffer at various temperatures and the resulting fluorescence signal was measured. We utilize an aptamer beacon consisting of a fluorophore tagged anti-thrombin aptamer and a secondary oligonucleotide tagged with a quencher. In its initial conformation, the aptamer and oligonucleotide are hybridized such that the fluorophore and quencher are adjacent to one another, inhibiting the beacon from fluorescing (Sch. 1A). The aptamer is designed to preferentially bind to thrombin; therefore, when thrombin is present, a shift in binding equilibrium causes the protein to bind to the aptamer and the oligonucleotide to dissociate (Sch. 1B). As a result, the fluorophore is no longer quenched resulting in fluorescence emission. Using this design strategy, the concentration of thrombin was determined by quantifying the peak fluorescence intensity of coffee ring spots.

The optimal conditions for aptamer binding is dependent on several factors, including the internal chemical structure of the aptamer, and external factors such as the composition of the sample media and the aptamer binding temperature.^{20,37} Because this sensor was utilized for the detection of clinical samples where the composition cannot be controlled, we focused our efforts on optimizing the aptamer binding temperature. RainX surfaces were exclusively used for the remaining studies in this work, owing to its ability to produce the greatest level of signal amplification and highest detection intensities. Fig. 3 presents quantitative data of signal intensity, as signal-to-background ratios (SBRs), of coffee ring spots at various temperatures ranging from 25°C to 43°C and various protein concentrations ranging from 1 to 500 ng/mL. From this plot, it can be observed that highest detection signal is obtained at 37°C throughout the entire range of protein concentrations. These results match closely with those previously described by Hall et al.²³ At temperatures < 37°C, the detection signals are substantially lower than those at 37°C, particularly from 1 to 30 ng/mL. While this data

demonstrates that lower temperatures minimize aptamer binding activity, it shows that the detection signals do not increase correspondingly with temperatures above 37°C. Specifically, temperatures beyond this threshold result in diminished aptamer binding activity and reduced aptamer kinetics (e.g. protein recognition, folding and conformational changes).

Performance of the coffee ring biosensor

The detection of α -thrombin in human serum and 4x diluted whole blood was performed to demonstrate the applicability of this platform for the detection of clinical samples. Unconcentrated proteins in droplets of buffer solution were also measured to compare the improvement in amplification realized using coffee ring concentration. Fig. 4 shows quantitative data of peak signal intensities, as SBRs, in serum (Fig. 4A) and blood (Fig. 4B). From these plots, we can see that the detection signals of coffee ring spots are substantially higher (~10–40x) than those suspended in buffer. In particular, the fluorescent signals of unconcentrated droplet samples were undetectable even at the highest tested protein concentration, 500 ng/mL, in both serum and blood. In contrast, the formation of coffee ring spots enabled for the aptamer-protein complexes to be substantially concentrated, resulting in enhanced signal amplification (Fig. S2 in Supplementary Information). Based on serial dilution assays, this biosensor exhibits a limit of detection of 2 ng/mL (54 pM) in serum and 4 ng/mL (105 pM) in blood, which is comparable to previously reported thrombin aptasensors.³⁸ These values are in the range of many clinically relevant biomarkers, making this biosensor suitable for various diagnostic applications.³⁹ Additionally, by comparing this data with that of detection in buffer (Fig. 3), we can see that slightly enhanced performance is achieved in serum and whole blood. Since the aptamer activity and binding performance is affected by the sample composition, we speculate that the enhanced aptamer activity in these clinical samples is a result of the additional components (biomolecules, electrolytes, etc.) that are not present in buffer. Previous studies have shown that nucleic acids are influenced by interactions with other molecules (i.e. intracellular proteins) and ions, affecting their folding structure and binding activity.^{40–43} Based on these reports, we deduce that the stability of our aptamer-thrombin complexes is improved in blood and serum where the separation of the aptamer from its quenching oligonucleotide is enhanced, resulting in amplified fluorescent signals even at lower thrombin concentrations.

Detection in whole blood resulted in fluorescent intensities that were slightly lower than those in serum. Because whole blood contains other components (blood cells, fibrinogen) that are not present in serum, they can obstruct the aptamer binding activity, thereby minimizing the fluorescent signal. Additionally, measurements in whole blood required longer imaging (0.55 sec) than those performed in serum (0.02 sec), which we attribute to the presence of blood cells. Specifically, the fluorescence emission is absorbed and obstructed by the blood cells, which results in lower detection signals relative to those in serum. We also observed that the formation of coffee rings in serum and whole blood required slightly shorter incubation times compared to those generated in buffer. This can be attributed to the varying compositions of serum and blood, which contain higher concentrations of solutes compared to the buffer solution. Although measurements in serum and blood required several slight modifications to the experimental protocol, the coffee ring

effect was still effective in concentrating aptamer-thrombin complexes for enhanced signal amplification, resulting in higher detection signals compared with samples in liquid droplets.

Further data analysis was performed by plotting the detection intensities in a log-log format to study the biosensor performance over a broad concentration range. As shown in Fig. 5, measurements in blood exhibit a linear correlation over the entire range of tested concentrations from 1 to 500 ng/mL. In particular, the data exhibits a linear relationship in a log-log format which can be fitted to the power law $y = ax^n$ where the intensity, y , is proportional to the concentration, x , by an exponential factor n . We determined n by fitting linear regression lines to the data, which is shown in the plot. Within the tested concentration range, we obtained a value of 0.37 for n with a correlation coefficient of 0.97. Taking into account the previous results presented in this work, we demonstrate that this coffee ring biosensor is a highly effective in concentrating proteins for signal amplification, which offers substantially enhanced signal intensity. When combined with a sensitive aptamer-sensing scheme, this platform provides a simple and low cost^{35,36,44} method for biomolecular detection, which offers several main advantages over conventional laboratory-based assays that often require costly equipment and labor-intensive processes for sample amplification and high-sensitivity measurements.

CONCLUSIONS

We report a new coffee ring aptasensor for biomolecular detection based on an inexpensive, highly sensitive biosensing platform. This technology utilizes an extremely simple signal amplification strategy based on coffee ring concentration. Substrates with varying surface properties were investigated, which revealed that highly hydrophobic surfaces resulted in concentration levels up to 6-fold greater compared with hydrophilic surfaces. Additionally, hydrophobic surfaces confine coffee ring diameters within a narrow field of view, facilitating integration with portable, low cost detection units. Coupled with a sensitive aptamer beacon sensing scheme, we utilized this platform for the detection of clinically relevant proteins. In particular, α -thrombin was used as a model system which could be detected at concentrations down to 2 ng/mL (54 pM) in serum and 4 ng/mL (105 pM) in 4x diluted whole blood. Moreover, coffee ring-concentrated aptamer-thrombin complexes produced fluorescence signal 40x larger compared with those in liquid droplets. In summary, this biosensing platform offers a simple and cost-effective detection technology, foregoing the need for labor-intensive sample amplification and expensive, high-sensitivity sensors. Furthermore, we envision that this platform can be employed for diagnostic testing in resource limited settings and developing countries to minimize the burden of infectious diseases, such as human immunodeficiency virus (HIV), malaria and tuberculosis, thereby improving global healthcare.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Fluorescent images of coffee ring formations on glass, HDMS-, and RainX-treated surfaces. Hydrophobic surfaces concentrate particles more effectively than hydrophilic surfaces, resulting in smaller ring diameters. Glass and HDMS surfaces were imaged at 2x magnification and RainX-treated surfaces were imaged at 10x magnification.

Figure 2.

(A) Growth of coffee ring diameter with increasing droplet volumes. Data sets are normalized to the ring diameter at 0.5 μL . (B) Fluorescence signals of coffee rings from increasing droplet volumes on various surfaces. Each bar represents the mean $\pm$ standard deviation (SD) of three individual measurements.

Figure 3.

The influence of temperature on aptamer binding activity and subsequent protein detection. α -thrombin was detected in buffer at 25°C, 31°C, 37°C, and 43°C using coffee ring formations on RainX surfaces. These temperatures are those at which the aptamer solutions were prepared and the droplets were dried. The data is plotted in relative fluorescent units (RFU) and represents the signal-to-background ratio (SBR) of each measurement. Each data point represents the mean $\pm$ SD of three individual measurements.

Figure 4.

Detection of α -thrombin in (A) human serum, and (B) 4x diluted whole blood from coffee ring spots and liquid droplets. Aptamer binding was performed at 37°C and coffee rings were generated on RainX surfaces. The data is plotted in relative fluorescent units (RFU) and represents the SBR of each measurement. Each bar represents the mean $\pm$ SD of three individual measurements.

Figure 5.

Log-log plot of signal intensity vs. α -thrombin concentration in 4x diluted whole blood. The data exhibits a linear correlation between 1 to 500 ng/mL which can be fitted to a power law relationship. Fitting linear regression lines to the data resulted in exponential factor, n , of 0.37 with a correlation coefficient of 0.97. Each data point represents the mean $\pm$ SD of three individual measurements.

Scheme 1.

Illustration of the aptamer beacon sensing scheme. (A) In its initial state, the anti-thrombin aptamer is hybridized with a secondary oligonucleotide. Due to the proximity of the reporter and quencher molecules, no fluorescence is emitted. (B) In the presence of thrombin, the oligonucleotide is dissociated from the aptamer, allowing the reporter to fluoresce.