Equipment-Free Quantitative Aptamer-Based Colorimetric Assay Based on Target-Mediated Viscosity Change

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ABSTRACT: In this paper, we describe an aptamer-based colorimetric assay (ABCA), which integrates enzyme-loaded microparticles for signal amplification with distance measurements for equipment-free quantitative readout. The distance measurement readout is based on target-induced selective reduction in viscosity of reaction solution. Its utility is well demonstrated with inexpensive, sensitive, and selective detection of adenosine (model analyte) in buffer samples and real samples of human serum and urine with the naked eye. This ABCA method just requires operators to simply count the number of colored distance-relevant marked bars on the calibrated glass microsyringes (testing containers) to provide quantitative results. It thus holds great promise for wide applications particularly in limited-resource settings.

1. INTRODUCTION

Aptamers are specific oligonucleotides that are selected from pools of random-sequence DNAs or RNAs.1,2 They are widely considered as the ideal molecular probes for analytical and biomedical applications due to their simplicity of synthesis, ease of labeling, high stability, wide applicability, and excellent specificity.3–6 During the last decade, various aptamer-based assay methods have been developed for detection of metal ions,7–9 small molecules10–14 DNAs,15,16 RNAs17–19 proteins,20–23 cancer cells,24,25 viruses,26,27 and bacteria28 in wide fields, such as medical diagnosis, environmental analysis, and food safety testing. Measurements of signals for these assays are implemented using several techniques, with fluorescence7–9,15,16,19,20,24,25,28,29 chemiluminescence12,13,22 and Raman scattering23 being the two most widely used types. Recent methods include electrochemistry10,11,16,21,26 and aptamer-based equipment-free quantitative analysis would be more beneficial and desirable for use in the resource-limited environments. In our work, we cope with this challenge by designing a novel ABCA with such a merit. This approach is on the basis of the selective change in the viscosity of a soluble starch-contained reaction solution (Figure 1). It integrates SiO2 microparticles labeled with glucoamylase molecules for efficient yet robust signal amplification with naked-eye measurement of distance of a colored reporting reagent (i.e., red ink) in the reaction solution for simple equipment-free quantitative readout. In our initial proof-of-concept study, adenosine, a key cofactor in numerous biochemical processes,37 is chosen as a model analyte. The working principle and analytical procedures of this adenosine assay are illustrated in Figure 1. In brief, superparamagnetic microparticles (SPMs) are immobilized with biotinylated DNA strands that have anchored adenosine’s aptamers via hybridization reaction.38 In the presence of adenosine, each aptamer captures one target to form an aptamer–adenosine complex. The biotin is thus exposed to capture a streptavidin-labeled SiO2 microparticle to transform recognition chemistry into changes in color kind and strength of reaction solutions. Although the existing ABCAs are facile to utilize to achieve rapid, naked-eye qualitative analytical results, most of them still require desktop equipment (typically ultraviolet/visible spectrometers39,40) to perform quantitative detection. In fact, few of the current ABCAs could achieve the extreme economic aim of quantifying target levels in samples without the aid of external electronic readers. In this regard, a universal ABCA that enables equipment-free quantitative analysis would be more beneficial and desirable for use in the resource-limited environments.

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loaded with several glucoamylase tags via the biotin–streptavidin interaction. The glucoamylase is further used to catalyze hydrolysis of soluble starch-producing glucose. As a result, the viscosity of the resultant starch-contained reaction solution is dramatically reduced, which allows the red ink to diffuse a long distance (Figure 1, top). In the absence of the target, on the contrary, the reaction solution containing unhydrolyzed soluble starch could maintain its original, high viscosity and in turn limit the ink’s diffusion (Figure 1, bottom). The diffusion distance of the red ink is positively proportional to the target concentration in sample. The results demonstrate that our method just requires operators to simply count the number of colored distance-relevant marked bars on the calibrated glass microsyringes (testing containers chosen herein) to measure the concentration of adenosine target in buffer as well as complex human body fluids (i.e., serum and urine). To our knowledge, this may be the first study of applying viscosity, one of the basic properties of liquids, to design affordable, instrument-free quantitative ABCAs.

2. RESULTS AND DISCUSSION
The pivotal conception of our ABCA strategy focalizes transition of the adenosine determination into measurement of diffusion distance of red ink in the starch-contained reaction solution. Thus, the starch viscosity-dependent ink’s diffusion was first investigated. Figure 2A displays the images of three soluble starch solutions with different levels (i.e., 10, 1.3, and 0.6 mg/mL) in three test glass microsyringes. Obviously, it is impossible to visually distinguish the concentrations or viscosities of these colorless, transparent solutions. As expected, on the other hand, differentiable diffusions of red ink with specific distances were interestingly observed in the three starch solutions after introduction of 3 μL of the colored reporting reagent into each microsyringe (Figure 2B). The ink’s diffusion distances are inversely associated with the starch’s viscosities that positively rely on its levels. This phenomenon might be explained in view of the effect of intermolecular steric hindrance. That is, it is harder for the ink to freely diffuse in a starch solution with higher viscosity because of the greater intermolecular steric hindrance. Too high starch concentration (viscosity) could even totally stop the diffusion of red ink (Figure S1, Supporting Information). In other words, the number of microsyringe’s marked bars (\(N_{\text{bar}}\)) related to the colored distance could be adopted to indirectly measure the starch viscosity in a certain level range. It was experimentally found that the appropriate applied volume (i.e., 3 μL) and time (i.e., 1 min) for the ink’s diffusion benefited the formation of a visually clear red diffusion end (Figures S2 and S3, Supporting Information). Moreover, because each glass microsyringe had a relatively thick outer wall, such test container enabled stable 1 min diffusions of red ink at wide temperatures ranging from 4 to 60 °C (Figure S4, Supporting Information).

Enzymatic activity of the glucoamylase-coated SiO\(_2\) microparticle probes, which plays a key part in the detection performance of the developed assay, was then characterized. As shown in Figure 3A, although an \(N_{\text{bar}}\) of ~11.5 was counted for a 2.5 mg/mL original starch solution, a much larger \(N_{\text{bar}}\) of ~17 was obtained after its hydrolysis catalyzed by free glucoamylase molecules. The use of functionalized SiO\(_2\) microparticles also led to the production of a similar ink’s diffusion distance (~16.8 in \(N_{\text{bar}}\)), suggesting that the glucoamylase covalently immobi-
lized on the particles still retained the high catalytic activity toward starch. Furthermore, the well-known iodine−starch complexation reaction was used to monitor the starch's hydrolysis.41 That is, the mixing of the original starch solution with a 0.3 mg/mL I₂ solution (in the presence of KI) resulted in a black mixture solution because of the formation of several iodine−starch complexes (Figure 3A, inset, left). On the other hand, after the hydrolysis of the starch by the free glucoamylase molecules or the enzyme-tagged microparticles, only blue solutions containing relatively lower levels of iodine−starch complexes were formed (Figure 3A, inset, middle and right). It should be pointed out that a viscosimeter is routinely utilized to measure a solution’s viscosity. However, the viscosimeter commonly needs tens to even hundreds of milliliters of solution for each measurement and is thus unsuitable for our approach, in which only ~40 μL of a soluble starch solution was consumed for one assay run.

Next, the feasibility of the proposed ABCA with microparticle amplification bioprobes was studied. Assays of a blank sample (i.e., buffer without the analyte) and a 7.5 μM adenosine sample were performed according to the analytical procedures schematically shown in Figure 1. After the starch hydrolysis, the Nbar associated with the ink’s diffusion distance in the reaction mixture for each sample was counted and the iodine−starch complexation for each corresponding reaction mixture was also conducted using an iodine solution (0.3 mg/mL; containing 0.2 mg/mL KI), compared to a background starch solution (2.5 mg/mL). Figure 3B displays that no

Figure 3. (A) Comparison of the number of microsyringe’s marked bars (Nbar) related to the diffusion distance of red ink (3 μL each) in 40 μL of a 2.5 mg/mL soluble starch solution, a mixture formed after 30 min incubation of 20 μL of a 5 mg/mL soluble starch solution and 20 μL of a 5 ng/mL glucoamylase solution containing free enzyme molecules (starch + F-enzyme), and a mixture formed after 30 min incubation of 20 μL of a 5 mg/mL soluble starch solution and 20 μL of a suspension of glucoamylase-immobilized SiO₂ microparticles (50 ng/mL, starch + I-enzyme). (B) Nbar values gained from the analysis of a blank phosphate-buffered saline (PBS) sample (without target) and 7.5 μM adenosine samples using (S−Y) or not using (S−N) the biofunctionalized SiO₂ amplification probes. The insets display the photographs of the above reaction mixtures and the original starch solution, each of which was mixed with a 0.3 mg/mL I₂ solution (containing 0.2 mg/mL KI). Each error bar indicates a standard deviation from three tests of every sample.

Figure 4. (A) Colorimetric results gained in analysis of various samples: (a) 7.5 μM adenosine and 1 mM, (b) cytidine, (c) uridine, and (d) guanosine. (B) The working curve describing a linear relationship between signals of Nbar changes (ΔNbar) and adenosine concentrations (Cadenosine). Its regression equation is: \( y = 0.9779x + 0.2878 \) \((R^2 = 0.9954)\). Each error bar stands for a standard deviation of three parallel tests.

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significant differences are observed in either the \( N_{\text{bar}} \) values or the black mixture solutions of iodine–starch complexes (Figure 3A, inset, left; Figure 3B, inset, left) obtained from PBS and original starch. The \( N_{\text{bar}} \) measured for 7.5 \( \mu \)M adenosine is \( \sim 19 \), which is far higher than the starch’s background \( N_{\text{bar}} \) value (\( \sim 11.5 \)); a light blue iodine–starch complexation mixture was formed in this case (Figure 3B, inset, right). Both the dramatically increased red ink’s diffusion distance and decreased level of iodine–starch complexes gained in adenosine analysis show that after analytes were bound by specific aptamers these recognition events could be further traced by SiO\(_2\) particles loaded with several glucoamylase molecules that subsequently catalyzed the hydrolysis of soluble starch efficiently. Moreover, a low \( N_{\text{bar}} \) of \( \sim 13.5 \) and a dark blue solution of iodine–starch complexes (Figure 3B, inset, middle) were achieved using glucoamylase–streptavidin conjugates for assaying the same adenosine sample. These results confirm that as every functionalized microparticle carried with it more glucoamylase labels per adenosine recognition event the degrees of the reduced starch viscosity and level were greater than those took place in the absence of such amplification probes. Thus, the improved sensitivity for adenosine detection could be expected.

After demonstrating the principle of designed ABCA and the efficient amplification based on the enzyme-loaded microparticles, its analytical selectivity was tested by conducting assays of 7.5 \( \mu \)M adenosine and 1 mM cytidine, uridine, and guanosine. The four types of small molecules belong to the nucleosides family. The corresponding colorimetric results are shown in Figure 4A. As shown in Figure 4A, when the \( N_{\text{bar}} \) value of up to \( \sim 19 \) is observed for adenosine assay, \( N_{\text{bar}} \) values for cytidine, uridine, and guanosine samples are estimated to be only about 11 (close to the value recorded from the blank buffer shown in Figure 3B), although the concentrations of the three aspecific small molecules were \( \sim 133 \) times higher than the analyte level. The data imply that only the target adenosine could be bound selectively by its aptamer strand for triggering the glucoamylase-catalyzed hydrolysis of soluble starch.

The major analytical parameters, namely, level of soluble starch and temperature and time for incubation of aptamer-coated SPM conjugates, adenosine samples, and functionalized SiO\(_2\) microparticle bioprobes have been optimized (Figures S5–S7, Supporting Information). To evaluate the detection performance of the proposed assay, a set of buffer samples having various adenosine concentrations were analyzed under the optimal conditions. The corresponding signal of \( N_{\text{bar}} \) change (\( \Delta N_{\text{bar}} \)) is defined as \( \Delta N_{\text{bar}} = N_{\text{bar,a}} - N_{\text{bar,b}} \) in which \( N_{\text{bar,a}} \) and \( N_{\text{bar,b}} \) are \( N_{\text{bar}} \) values severally measured for each adenosine sample and the blank buffer sample. The relationship among the resultant \( \Delta N_{\text{bar}} \) results and the adenosine level tested is shown in Figure 4B. One can find that the \( \Delta N_{\text{bar}} \) increases as the analyte level increases, clearly displaying adenosine-dependent \( \Delta N_{\text{bar}} \) responses. The proposed method can linearly detect the adenosine target in concentrations ranging from 0.4 to 7.5 \( \mu \)M. The limit of adenosine detection was calculated to be \( \sim 0.18 \) \( \mu \)M (3\( \sigma \)). Furthermore, relative standard deviations (RSDs) obtained in three tests of 0.4, 0.9, 1.8, 3.7, and 7.5 \( \mu \)M adenosine samples were 2.1, 4.3, 5.7, 5.2, and 6.7%, respectively, implying acceptable detection reproducibility. As additionally shown in Table 1, in comparison to several other aptamer-based adenosine assays with fluorescence,\(^{45–48}\) electrochemical,\(^{11,42,49,50}\) or absorbance\(^{35,36}\) measurements, this new technique does not need any extra electronic reader to realize comparable or even better detection performance.

With these good preliminary results in hand, recovery experiments of adenosine in undiluted human serum and urine were further carried out to assess detection reliability and practicability of the designed ABCA system. Adenosine with given levels were mixed with the real samples. Then, these samples were analyzed in light of analytical processes schematically shown in Figure 1. The ratio of calculated adenosine level to the mixed (or total) concentration in the human serum or urine was defined as the recovery. For each sample was performed six times, with recovery results summarized in Table S1 (Supporting Information). As shown in Table S1, recovery ranges gained from the serum and urine samples are 92.4–102 and 96.8–104.5%, respectively, and the calculated RSDs are in the range of 2.7–8.3% (\( n = 6 \)). Significantly, the data imply that the aptamer still presented good recognition ability toward adenosine analyte even in real samples of serum and urine. Moreover, superparamagnetic microparticle-based segregation and washing operations in the analytical procedures could be conducive to minimize undesirable influences of uncaptured reagents and interferences in the above two complex matrices.\(^{32}\)

### 3. CONCLUSIONS

We develop a new ABCA approach on the basis of the analyte-mediated selective change in the liquid viscosity, which enables the quantitative determination of analytes of interest with the naked eye. This should be especially useful for application in resource-limited environments lacking access to public laboratory construction. Our ongoing studies include the (1) development of equipment-free ABCAs with sample-in-answer-out quantitative ability applicable for point-of-need testing uses.
and (2) enhancement of analytical performance by seeking novel chemical detection motifs for more efficient signal amplification.

4. EXPERIMENTAL SECTION

4.1. Reagents and Apparatus. The DNA strands, whose thermodynamic factors were enumerated with the aid of previously reported bioinformatics software, were prepared commercially from Takara BiotecnoLOGY Co., Ltd. (Dalian, China). The capture DNA’s sequence (5′-3′) is CAC TGA CCT GGG GGA GTA TTG CGG AGG AAG GT (adenosine’s binding sequence is underlined). Streptavidin (from Streptomyces avidinii, >17 U/mg) and glutaraldehyde were purchased from Sigma-Aldrich. Glucoamylase (>20 U/mg), adenosine, cytidine, uridine, guanosine, lysine, and bovine serum albumin were provided by Sangon Biotechnology Co., Ltd. (Shanghai, China). Amine-coated SiO2 microparticles (∼0.2 μm in diameter) and amine-modified superparamagnetic microparticles are the products of Tianjin BaseLine Chrom Tech Research Centre (Tianjin, China). Soluble starch was provided by Xilong Chemical Co., Ltd. (Shanghai, China). All other chemicals of analytical grade were used as received. Human serum and urine samples were collected from healthy volunteers. Unless specially stated, ultrapure water (with a resistivity of 18.2 Ω·cm) was used to prepare stock solutions and buffer. The deionized water instrument was gained from Chengdu Yuechun Technology Group Co., Ltd. (Shanghai, China). The used buffer solution is 10 mM phosphate-buffered saline (PBS, pH 6 or 7.4) solution containing 0.3 M NaCl. Red ink is from Shanghai Hero Group Co., Ltd. (Shanghai, China); 50 μL glass microsyringes are the products of Gaoge Industrial Trade Co., Ltd. (Shanghai, China).

4.2. Preparation of Aptamer–SPM Conjugates. In brief, aptamer and biotinylated capture DNA strands in PBS (pH 7.4, 0.5 μM each) were mixed, heated to 90 °C, incubated for 10 min, and finally allowed to cool slowly to room temperature (∼2 h). Duplex DNA strands could be formed through hybridization reactions. Meanwhile, 1 mL of a 1 mg/mL SPM suspension was incubated with 5 mL of a glutaraldehyde solution (5%, w/v) for 3 h at room temperature. The resultant aldehyde-modified SPMs were isolated magnetically and washed with buffer solution (three times) and then dispersed in 1 mL of the 0.5 μM duplex DNA solution mentioned above for 3 h incubation. Moreover, 1 mL of a 1 mM lysine solution in water was utilized to block residual aldehyde on SPMs, followed by magnetic segregation and thorough washings. Such as-prepared aptamer–SPM conjugates were dispersed in 1 mL of buffer solution (pH 7.4) that contains 1.5% poly(ethylene glycol) and finally stored at 4 °C in a refrigerator.

4.3. Preparation of Glucoamylase–SiO2–Streptavidin Conjugates. Briefly, 1 mL of a 1 mg/mL SiO2 microparticle suspension was mixed and reacted with 5 mL of a glutaraldehyde solution (5%, w/v). After 3 h, excess glutaraldehyde was removed from the mixture by centrifuging and washing these SiO2 particles three times with water, followed by redispersion in 5 mL of PBS buffer (pH 7.4). Then, 1 mL of a streptavidin solution (1 mg/mL) and 1 mL of a 10 mg/mL glucoamylase solution were added into the suspension of aldehyde-activated particles and incubated for 3 h at room temperature. After centrifugal separation and washing, the resultant glucoamylase–SiO2–streptavidin conjugates were resuspended in 6 mL of a solution of bovine serum albumin (10 mg/mL, containing 1.5% poly(ethylene glycol)) in PBS buffer (pH 6) and finally stored at 4 °C in a refrigerator.

4.4. Preparation of Glucoamylase–Streptavidin Conjugates. Streptavidin solution (1 mL, 1 mg/mL), glucoamylase solution (1 mL, 2 mg/mL), and glutaraldehyde solution (5 mL, 2.5% w/v, in water) were incubated at 4 °C overnight. Excess cross-linker molecules in the resulting mixture were then removed using a dialysis bag that could retain a molecular weight of ∼3.5 kDa. Finally, PBS (pH 6) was used to dilute the dialyzed solution to its initial volume. The resultant glucoamylase–streptavidin conjugates were stored at 4 °C until used.

4.5. Analytical Procedures for Detection of Adenosine in Buffer Samples. In a typical assay, 10 μL of an adenosine sample in buffer, 10 μL of aptamer-modified SPM bioconjugates, and 10 μL of glucoamylase–SiO2–streptavidin bioconjugates were mixed together. Incubation was carried out for 40 min at 37 °C to allow aptamer–adenosine binding, which exposed the biotin moieties on the capture DNAs to further bind the streptavidin-coated SiO2 microparticles (loaded with a large number of glucoamylase tags) onto the SPM surfaces via the biotin–streptavidin interaction. After the uncaptured functionalized SiO2 particles were magnetically removed, the sediments were resuspended in 40 μL of a 2.5 mg/mL soluble starch solution in water in a 50 μL glass microsyringe. During incubation for 60 min at 37 °C, the glucoamylase on the SiO2 particles catalyzed the hydrolysis of soluble starch. Then, 3 μL of red ink was introduced into the resulting reaction solution. After 1 min, counting the number of marked bars (Nbar) on the microsyringe related to the ink’s diffusing distance with the naked eye permitted the quantitative detection of adenosine. The Nbar is positively proportional to the level of adenosine target in the sample. Moreover, selectivity tests were carried out for analysis of PBS buffer, cytidine, guanosine, or uridine but not adenosine. Comparison tests were also conducted according to the same steps but using glucoamylase–streptavidin conjugates instead of the SiO2 microparticle bioprobes. The corresponding signal of Nbar change (∆Nbar) was defined as ∆Nbar = Nbar−s − Nbar−b, in which Nbar−s and Nbar−b were the Nbar values measured for adenosine sample and a blank PBS sample, respectively.

In addition, the hydrolysis of soluble starch could be monitored using a complexation reaction between iodine and starch by mixing the corresponding reaction solution with 0.3 mg/mL I2 solution (containing 0.2 mg/mL KI). If the iodine–starch complexation was complete, the mixture is blue. In a typical assay, the mixture was incubated at 37 °C for 60 min, and the blue color was monitored. After incubation, 1 mL of a 1 mg/mL SiO2 microparticle suspension was added to the solution, and the suspension was incubated for another 3 h at room temperature. After centrifugation, the blue color was monitored again.

4.6. Analytical Procedures for Assay of Adenosine in Human Serum and Urine. To assess the practicability of the proposed method, recovery experiments of adenosine in human serum and urine were carried out. Different levels of adenosine were mixed with undiluted human serum or urine. The analyte-spiked serum or urine samples were then analyzed according to the above-described analytical processes. The “found” level of adenosine in serum or urine samples was estimated from their signals of Nbar and the regression equation obtained.

ASSOCIATED CONTENT

4. Supporting Information. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.7b01814.
Additional diffusion results of red ink in three soluble starch solutions at relatively high levels (Figure S1); time optimization for the diffusion of red ink in soluble starch solution (Figure S2); volume optimization for the diffusion of red ink in soluble starch solution (Figure S3); effect of environmental temperature on the diffusion of red ink in soluble starch solution (Figure S4); optimization of the starch concentration for the adenosine assay (Figure S5); temperature optimization for the incubation of aptamer–SPM bioconjugates, adenosine sample, and glucoamylase–SiO₂–streptavidin bioconjugates (Figure S6); time optimization for the incubation of the aptamer–SPM bioconjugates, adenosine sample, and glucoamylase–SiO₂–streptavidin bioconjugates (Figure S7); recovery of adenosine in undiluted human serum and urine samples (Table S1) (PDF)

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Notes
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