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A colorimetric aptasensor for the diagnosis of malaria based on cationic polymers and gold nanoparticles

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ABSTRACT

Malaria, a major burden of disease caused by parasites of the genus *Plasmodium*, is widely spread in tropical and subtropical regions. Here, we have successfully developed a diagnostic technique for malaria. The proposed method is based on the interaction among the *Plasmodium* lactate dehydrogenase (pLDH), which is a biomarker for malaria, and pL1 aptamer against *Plasmodium vivax* lactate dehydrogenase (*Pv*LDH) and *Plasmodium falciparum* lactate dehydrogenase (*Pf*LDH). In addition, the cationic polymers, poly(diallyldimethylammonium chloride) (PDDA) and poly(allylamine hydrochloride) (PAH), aggregate gold nanoparticles (AuNPs) that should be possible to observe the change in color from red to blue, which depends on the concentration of pLDH. Using this aptasensor, pLDH proteins were successfully detected with low detection limits. Moreover, the specificity test proved that the aptasenor is very specific in targeting proteins over other interfering proteins. In addition, the pLDH from infected blood samples of the two main species of malaria were also detected. The limits of detection for *P. vivax* were determined as 80 parasites/μl for PDDA and 74 parasites/μl for PAH. The aptasenor has great advantages that can simply and rapidly diagnose malaria. Thus, the developed aptasensor for detection of pLDH can offer an effective and sensitive diagnosis of malaria.

Malaria is a major burden of disease caused by parasites of the genus *Plasmodium*. According to the 2011 World Malaria Report by the World Health Organization, an estimated 3.3 billion people were at risk for malaria and 216 million cases of malaria resulted in an estimated 655,000 deaths in 2010 [1]. Thus, malaria diagnosis and treatment are very important to save human lives. In the clinical field, malaria is commonly diagnosed using parasitological confirmation obtained by microscopy or malaria rapid diagnostic tests [2]. However, both of these methods have several limitations. Microscopy needs a highly trained, experienced expert, and there is difficulty in preparing blood samples. Rapid diagnostic tests are commercially available and easy to use, but they exhibit inadequate sensitivity and specificity at a concentration of less than 1000 parasites/μl [3]. To address these limitations, a rapid and accurate diagnostic method for malaria is essential. Hence, we developed a rapid, accurate, sensitive, and specific single-stranded DNA (ssDNA)[1] aptamer for the diagnosis of malaria. In addition, we used the developed aptamer for the detection of malaria in real samples.

Aptamers are ssDNA or RNA molecules that are considered to rival antibodies in both therapeutic and diagnostic applications [4]. Aptamers have many advantages such as their ease of discovery and analysis, specificity to targets, thermal stability, and convenience of chemical synthesis [5–8]. Thus, research is ongoing on the development of an aptamer. In previous work, our group reported the development of a pL1 DNA aptamer (5′-GTTCGATTGGATTGTGCCGGAAGTGCTGGCTCGAAC-3′) against *Plasmodium vivax* lactate dehydrogenase (*Pv*LDH) and *Plasmodium falciparum* lactate dehydrogenase (*Pf*LDH), which is a biomarker for malaria, using SELEX (systematic evolution of ligands by exponential enrichment) [9].

Gold nanoparticles (AuNPs) have been used in biosensors because of unique properties such as good biocompatibility, easy conjugation of biomolecules, a large surface area, and a high absorption coefficient [10–12]. In particular, AuNP-based colorimetric biosensors, using AuNP aggregation-based methods, are very attractive because they are visible to the naked eye. Thus, these biosensors have been studied for use in the detection of nucleic acids and small molecules [13,14]. In general, unmodified AuNP-based biosensors exploit the aggregation of nanoparticles

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[1] *Abbreviations used:* ssDNA, single-stranded DNA; *Pv*LDH, *Plasmodium vivax* lactate dehydrogenase; *Pf*LDH, *Plasmodium falciparum* lactate dehydrogenase; AuNP, gold nanoparticle; PDDA, poly(diallyldimethylammonium chloride); PAH, poly(allylamine hydrochloride); pLDH, *Plasmodium* lactate dehydrogenase; UV–Vis, ultraviolet–visible; RBC, red blood cell; TEM, transmission electron microscope; BSA, bovine serum albumin.

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that occurs at a high concentration of salts. However, this approach suffers from relatively low detection limits [15]. On the other hand, water-soluble cationic polymers such as poly(diallyldimethylammonium chloride) (PDDA) and poly(allylamine hydrochloride) (PAH) exhibit not only a significant advantage in relation to the aggregation of AuNPs but also electrostatic interactions with DNA [16–18]. Using these properties, Wu and coworkers proposed a novel colorimetric biosensor for metal ions [19]. However, whether this can be applied to biomolecules such as proteins has not yet been confirmed. Here, we developed a biosensor for malaria diagnosis based on cationic polymers and *Plasmodium* lactate dehydrogenase (pLDH) aptamer-mediated aggregation of AuNPs. The proposed biosensor is rapid and accurate and can be applied to actual patient blood samples.

Materials and methods

Preparation of pLDH

The *Pv*LDH gene was cloned into pET-28a and transformed into BL21(DE3). The cells containing the plasmid were induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and grown at 37 °C for 4 h. Harvested cells were resuspended in a lysis buffer (20 mM Tris–HCl [pH 8.0], 500 mM NaCl, 0.5 mM β-mercaptoethanol, 10 mM imidazole, and 5% [w/v] glycerol) and lysed by sonication at 4 °C. The supernatant was loaded onto the Hi-Trap Ni-NTA affinity column and purified using a 10- to 300-mM imidazole gradient. For further purification, the eluted solution was loaded onto the MonoQ anion exchange column and then applied to the Superdex 200 HL gel filtration column, equilibrated with a storage buffer (50 mM Hepes [pH 7.5], 5 mM β-mercaptoethanol, and 5% [w/v] glycerol). The *Pf*LDH was expressed and purified using the same procedures.

Preparation of AuNPs

The 13-nm diameter AuNPs were prepared by citrate reduction of $HAuCl_4$ following the procedure outlined in the literature [20]. Aqua regia was prepared by mixing 3:1 concentrated HCl and HNO_3. It was used for cleaning all glassware, and the glassware was rinsed with a copious amount of deionized water. An aqueous $HAuCl_4$ solution (final 0.40 mM, 100 ml) was brought to a reflux while stirring, and trisodium citrate (final 1.52 mM, 1 ml) was then added quickly. The color changed from pale yellow to deep red in 1 min. After the color change, the solution was refluxed for an additional 1 min and cooled to room temperature (23–25 °C) with stirring. Finally, 4.9 nM AuNPs solution was obtained (100 ml). The concentration of these AuNPs was determined by ultraviolet–visible (UV–Vis) spectroscopy using an extinction coefficient of $2.7 \times 10^8\ M^{-1}\ cm^{-1}$ at $\lambda = 520$ nm for 13 nm AuNPs [21].

Optimization of sensing conditions

To optimize the sensing conditions, varying concentrations of PDDA were added to a buffer solution (400 μl, 20 mM Hepes, pH 7.4), followed by 100 μl of a solution of AuNPs. The absorption spectra and the absorbance values of the AuNP solutions were obtained by using a UV–Vis spectrophotometer. The values showed that 3.5 nM PDDA was suitable to aggregate all of the AuNPs. The concentration of the pL1 aptamer was determined at a fixed concentration of 3.5 nM PDDA. First, varying concentrations of pL1 (0, 0.1, 1, 2.5, 5, 10, 25, and 50 nM) were incubated in a buffer solution containing 3.5 nM PDDA for 20 min, and 100 μl of constant AuNP solution was added. The results showed that 10 nM of the aptamer is suitable to detect target proteins. The optimization of the sensing conditions for PAH was obtained using the same procedures.

To optimize the sensing conditions for the detection of malaria in the infected blood samples, a suitable concentration of the cationic polymer was measured to avoid interference by charged biomolecules. The sensing was determined not by the concentration of the aptamer, cationic polymer, or AuNPs but rather by the ratio of all these. Hence, the concentrations of the aptamer and AuNPs were fixed as above. The 10 μl of pretreated noninfected blood samples was added to buffer solution. Varying concentrations of PDDA or PAH were then added with the AuNPs. The solutions were mixed thoroughly for 20 min, and the absorbance was measured with a UV–Vis spectrophotometer.

Preparation of RBC-lysed whole blood samples

The blood samples were provided by J. W. Park (Gachon University of Medicine and Science). The red blood cell (RBC)-lysed whole blood samples were prepared according to previous reports [9]. Before the experiments, varying concentrations of infected blood samples were prepared by serial dilution of blood samples containing a high concentration (23,334 parasites/μl for *P. vivax* and 20,000 parasites/μl for *P. falciparum*) with noninfected human whole blood samples. The infected and noninfected blood samples (50 μl) were added to an RBC lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, and 10 μM ethylenediaminetetraacetic acid [EDTA], 100 μl), incubated with shaking at room temperature for 10 min, and disrupted by sonication for 1 min. Next, the mixtures were centrifuged at 13,000 rpm for 5 min to remove the sediments. Red and clear supernatant liquid was carefully taken and applied to the biosensor. Blood collection performed for this study was conducted following informed consent of the patients and adhering to the institutional ethical guidelines reviewed and approved by the ethics committee of Gachon University of Medicine.

In vitro detection of pLDH

For the detection of pLDH, varying concentrations of *Pv*LDH and *Pf*LDH were incubated with 10 nM pL1 in a buffer solution for 20 min at room temperature. The cationic polymer was then added and incubated for 20 min. Subsequently, 100 μl of AuNPs was added and mixed thoroughly. The final volume of the solution was maintained at 500 μl, and the absorbance was measured at 650 and 520 nm. The absorbance spectra and the value of the absorbance (A_{650}/A_{520}) were obtained. This experiment was performed in triplicate.

Detection of pLDH in blood samples of patients

To diagnose the malaria in the blood samples from malaria patients, 10 μl of the pretreated samples was added in a buffer solution. Concentrations of 40 nM for PDDA and 100 nM for PAH were used to detect the pLDH in the blood samples. The other procedures were followed, as mentioned in the previous section.

Results and discussion

Colorimetric aptasensor for diagnosis of malaria

Fig. 1 shows a schematic representation of the biosensor for pLDH detection. Free ssDNA aptamer interacts with cationic polymers, such as PDDA and PAH, through electrostatic interactions. The cationic polymer and the aptamer form a duplex structure [19]. In the absence of pLDH, additional AuNPs cannot aggregate because PDDA or PAH is not sufficient for aggregation. On the other

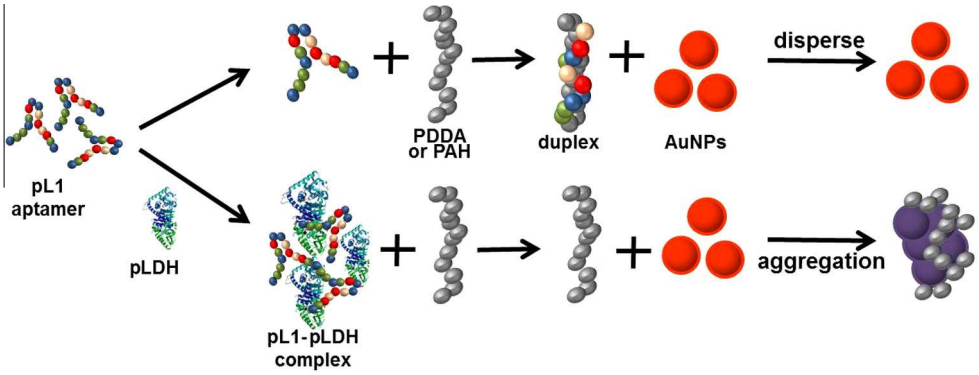

Fig.1. Schematic illustration of the aptasensor for pLDH detection.

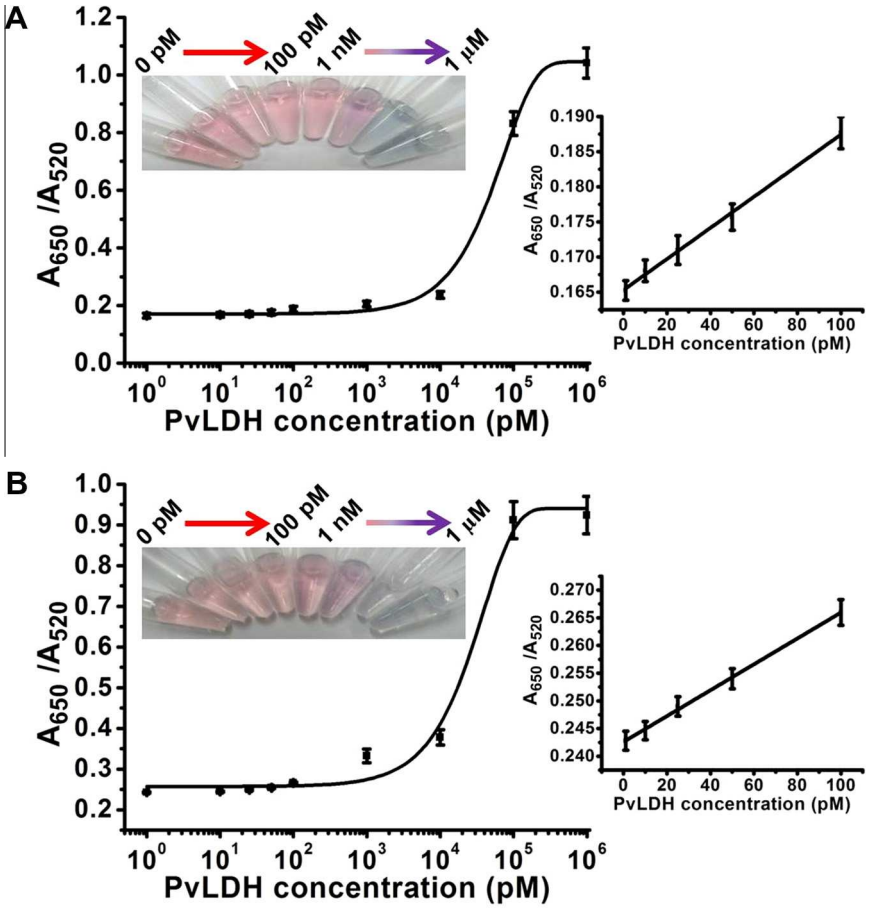

Fig.2. Absorption calibration curve of sensing solutions treated with varying concentrations of *Pv*LDH for PDDA (A) and PAH (B). The inset figures represent the visual color changes of the AuNP solutions, and the plots are the values of A_{650}/A_{520} at a low concentration range with a linear fitted plot.

hand, in the presence of pLDH, the aptamer interacts with pLDH and the remaining PDDA or PAH can aggregate the AuNPs. The aggregation of AuNPs leads to a change in color from red to blue. Therefore, this scheme provides a colorimetric assay for the detection of pLDH.

Optimization of biosensor conditions for in vitro detection

For pLDH detection, the concentration of the cationic polymer and the aptamer is a critical factor because the relative proportions of these determine the aggregation of AuNPs. Thus, first we optimized the sensing conditions by varying the concentrations of PDDA or PAH added in a buffer solution (20 mM Hepes, pH 7.4). A constant AuNP solution was then added to a buffer solution containing the cationic polymer. We found that the absorption spectra and the absorbance ratios of the AuNP-containing solutions increased significantly and became saturated at concentrations of more than 3.5 nM for PDDA and 25 nM for PAH (see Fig. S1 in supplementary material). Thus, these concentrations of cationic polymers were enough to aggregate AuNPs and were considered to be suitable concentrations for the biosensor. Similarly, the concentration of the pL1 aptamer was determined against the cationic

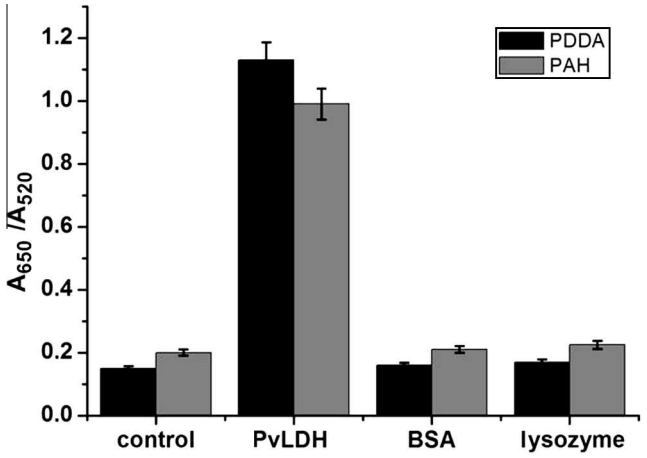

Fig.3. Specificity of aptasensor for pLDH. The values of A_{650}/A_{520} for each competing protein were measured at the same concentration of 1 μM.

polymer and the AuNPs. The results confirmed that 10 nM of the pLDH aptamer completely formed a duplex structure without aggregation of the AuNPs with both PDDA and PAH (Fig. S2).

In vitro detection of pLDH

The optimized sensor was applied to the detection of purified *Pv*LDH, which was monitored visually with the naked eye and with a UV–Vis spectrophotometer and transmission electron microscope (TEM) imaging. In the absence of *Pv*LDH, the AuNP solution containing pL1 and the cationic polymer was a red color because the AuNPs remained in their dispersed state (Fig. 2). Varying concentrations of *Pv*LDH (1 pM, 10 pM, 100 pM, 1 nM, 10 nM, 100 nM, and 1 μM) were then applied to the biosensor. The experimental results revealed that the color of the AuNP solution underwent a significant change from red to purple at the maximum concentration of 1 nM *Pv*LDH, The color change was further confirmed by absorption spectra, which showed that the absorbance values of A_{650}/A_{520} were increased (Fig. 2; see also Fig. S3 in supplementary material). These results confirm that the color change was correlated with the degree of aggregation of the AuNPs caused by the interaction of the AuNPs with PDDA, depending on the concentration of *Pv*LDH. The state of the AuNP solutions was further confirmed by TEM imaging. The images showed that the AuNPs were efficiently aggregated in the presence of *Pv*LDH (1 μM, for PDDA) (Fig. S4). Therefore, these results demonstrate that the proposed biosensor successfully detected *Pv*LDH.

The limit of detection for the naked eye was 1 nM *Pv*LDH for the PDDA and PAH biosensor. The values of absorption (A_{650}/A_{520}) were plotted and fitted to a logistic plot. The constantly increased plots were fitted to linear to determine the limit of detection based on previous reports [19,20,22]. The limits of detection for *Pv*LDH were determined as 8.7 pM for PDDA and 8.3 pM for PAH. In addition, the *Pf*LDH was also detected, and the limits of detection were 10.3 pM for PDDA and 12.5 pM for PAH (Fig. S5).

Distinctive specificity of aptasensor

Diagnostic sensors must have specificity for target molecules. For this reason, the specificity of the biosensor was tested for non-target proteins such as lysozyme and bovine serum albumin (BSA). For the specificity test, the concentrations of the aptamer and the cationic polymer were fixed as above. The competitive proteins

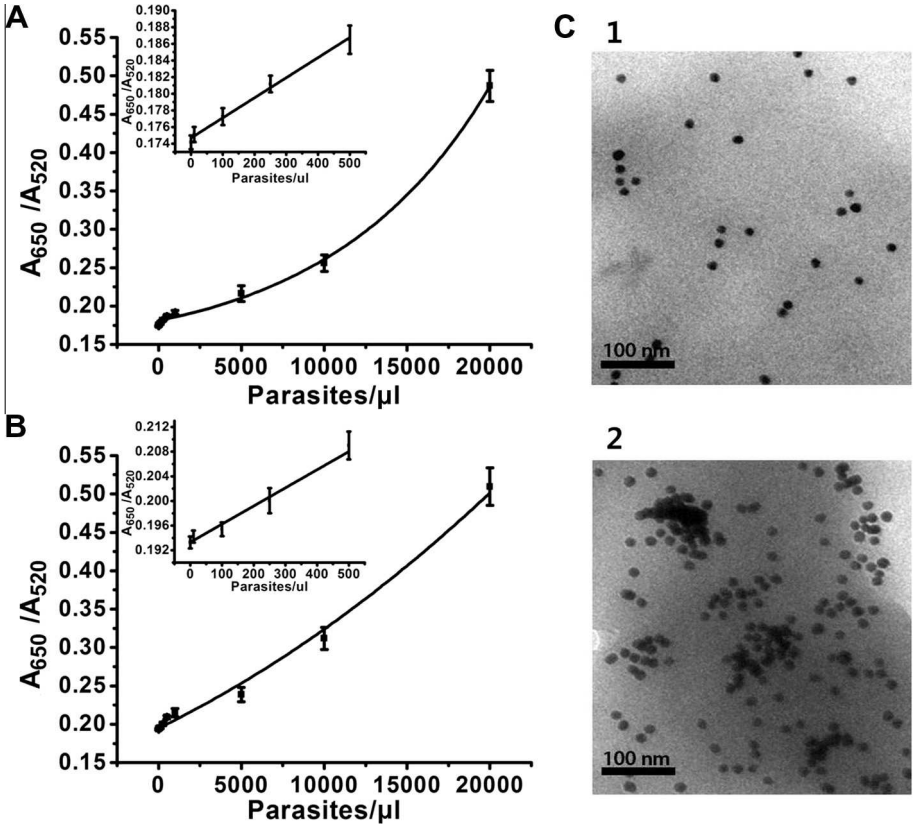

Fig.4. Calibration curves of sensing solutions treated with varying concentrations of *P. vivax* in blood samples for PDDA (A) and PAH (B). The inset plots are the values of A_{650}/A_{520} at a low concentration range with a linear fitted plot. (C) TEM images of AuNPs treated with a noninfected blood sample (image 1) and an infected blood sample (20,000 parasites/μl, *P. vivax*) for PDDA (image 2).

were incubated individually with the pL1 aptamer in a buffer solution, followed by the addition of PDDA or PAH and AuNPs to the sensor solution. The value of A_{650}/A_{520} was then measured. Fig. 3 shows the values of absorbance of the AuNP solutions containing 1 μM of each protein. In comparison with the control solution, the values of *Pv*LDH increased. As expected, the A_{650}/A_{520} values of the competitive proteins, BSA and lysozyme, showed no significant difference when compared with the control solution. These results demonstrate that this aptasensor has specificity for *Pv*LDH.

Optimization of biosensor conditions for detection of pLDH in blood samples of patients

To ensure the effectiveness of the biosensor, this sensing method was applied to detect pLDH in malaria-positive blood samples. Blood contains many negatively and positively charged biomolecules such as nucleic acids, lipids, and proteins that can disrupt the electrostatic interaction between pL1 and the cationic polymer. Hence, the biosensor conditions were freshly optimized to detect pLDH in malaria-positive blood samples. Before the experiments, malaria-negative RBCs were lysed because pLDH proteins exist in serum and in the cytosol of erythrocytes [23].

A suitable concentration of the cationic polymer was then determined by similar procedures, followed by the optimization of in vitro detection, by which the aggregation of AuNPs was induced. The optimal concentrations of PDDA and PAH required for the aggregation of the AuNPs were 40 and 100 nM, respectively (Fig. S6). The difference in the concentrations of the cationic polymer for in vitro detection may have arisen as a result of nonspecific binding of negatively charged biomolecules in the blood. Despite interruption by the charged biomolecules, the value of A_{650}/A_{520} increased significantly and the optimal concentration of the cationic polymer was determined for the blood sample.

Clinical evaluation of blood samples from infected patients

Before the detection of pLDH in blood samples from patients, the concentration of parasites in each malaria-positive blood sample was measured by traditional microscopic analysis. Serial diluted blood samples were then applied to the biosensor. The ratio of A_{650}/A_{520} increased for both PDDA and PAH (Fig. 4A and 4B), depending on the concentration of the infected blood samples. However, the color change associated with the aggregation of the AuNPs could not be detected visually owing to the red color of the blood samples. To verify whether the increase of A_{650}/A_{520} values was due to the aggregation of AuNPs, the sensor solutions containing infected and noninfected blood samples were monitored by TEM imaging. Although the background of TEM image came out blurry, because of ground substance of blood, the state of AuNPs was clearly shown. The AuNP solution containing the noninfected blood samples remained in their dispersed state, whereas the AuNP solution containing the infected blood samples (20,000 parasites/μl for *P. vivax*) was partially aggregated due to the interaction with the cationic polymer, as expected (Fig. 4C).

The limit of detection of pLDH in the infected blood samples was calculated using the linear fit of the values of absorbance. The limits of detection for *P. vivax* were determined as 80 parasites/μl for PDDA and 74 parasites/μl for PAH. In addition, *P. falciparum* was also detected, with the limits of detection being 92 parasites/μl for PDDA and 97 parasites/μl for PAH (Fig. S7).

Conclusion

We have successfully developed a diagnostic technique for malaria that is simple, rapid, and highly sensitive. The proposed method is based on the interaction between pLDH, a biomarker for malaria, and a pL1 aptamer. In addition, the cationic polymers, PDDA and PAH, aggregate the AuNPs. It should be possible to observe the change in color from red to blue, which is dependent on the concentration of pLDH. Using this aptasensor, pLDH proteins were successfully detected at low detection limits. The limit of detection for the naked eye was 1 nM *Pv*LDH for the PDDA and PAH biosensor. The calculated limits of detection for *Pv*LDH were determined as 8.7 pM for PDDA and 8.3 pM for PAH. In addition, the *Pf*LDH was also detected, and the limits of detection were 10.3 pM for PDDA and 12.5 pM for PAH.

Moreover, the specificity test proved that the aptasenor was very specific to target proteins compared with protein competitors. In addition, pLDH from infected blood samples containing the two main species of malaria was detected at low detection limits. The limits of detection for *P. vivax* were determined as 80 parasites/μl for PDDA and 74 parasites/μl for PAH. In addition, *P. falciparum* was also detected, with the limits of detection being 92 parasites/μl for PDDA and 97 parasites/μl for PAH.

Furthermore, the aptasenor has a major advantage in that it can simply and rapidly diagnose malaria. Thus, the developed aptasensor for the detection of pLDH can offer effective and sensitive diagnosis of malaria.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ab.2013.03.032.

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