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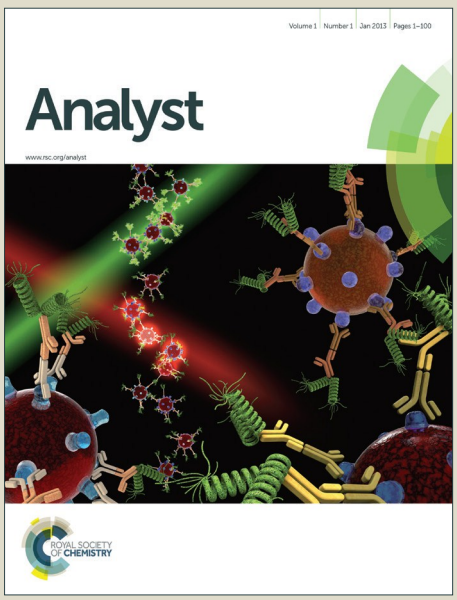

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ARTICLE

Small molecule detection by lateral flow strips via aptamer-gated silica nanoprobes

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A fast, sensitive and ratiometric biosensor strategy for small molecule detection was developed through nanopore actuation. The new platform engineers together a highly selective molecular recognition element, aptamers, and a novel signal amplification mechanism, gated nanopores. As a proof of concept, aptamer gated silica nanoparticles have been successfully used as a sensing platform for the detection of ATP concentrations at a wide linear range from 100 µM up to 2 mM.

Introduction

Lateral flow assays are chromatography based simplified sensor formats for rapid on-site detection of ligands. They are cost effective platforms for point-of-care applications in medical, diagnostics, food and environmental applications.[1] Quantitative or qualitative detection is based on the application of fluid samples on prefabricated strips of nitrocellulose membrane containing dry reagents that activates a signalling mechanism, usually colorimetric or fluorimetric.[2, 3] The lateral flow platform has specifically been developed for single use at point-of-care locations. Successful and popular applications include home pregnancy tests for on/off signal and glucose meters for quantitative assessments with portable instrumental reading [4]. However, if a small molecule is the target of the assay, signalling mechanism becomes a challenge. Competitive assay format is the most suitable strategy for small molecule detection with lateral flow. Since small molecules cannot bind more than one capture molecules (antibodies or aptamers), labelled target molecules are usually used for designing competitive assays.

Adenosine-5´-triphosphate (ATP) is a purine nucleotide present in all living cell with a molecular weight of 507.18 g mol^{-1}. Extracellular ATP is found in many biological processes including neurotransmission, muscle contraction, cardiac function, platelet function, vasodilatation, signal transduction and secretion.[5] Also, ATP has been commonly used as the biomarker for assessing cell viability because the amount of ATP in cells is directly proportional to the health of cells.[6] Therefore, there is an interest in quantitative monitoring of ATP. For example, ATP bioluminescence has been used for hygiene monitoring in food industries, and hospitals for rapid screening of surfaces. Research efforts have continued for improving hygiene monitoring by investigating fast techniques for detecting ATP. Aptamers have many advantages, such as stability under thermal and ionic extremes, in vitro selection procedure and easy modification for chemical conjugation [7, 8]. Many sensor formats combined with aptamers were exploited to develop sensors for small molecules in the past such as acoustic,[9] chemiluminescent,[10] electrochemical,[11-13] or nanomaterial associated.[14] However, lateral flow biosensors are attractive because of their speed, simplicity and easy handling. Lateral flow assays have been developed widely for detecting proteins and whole cells. Although lateral flow assays for small molecules included pesticides, toxins and drugs, the main obstacle is the difficulty to design a simple signalling mechanism for small molecules lateral flow assay.

We previously demonstrated that a homogenous solution of aptamer-gated silica nanoparticles loaded with fluorescein could be used as a sensing method for small molecules.[15] Aptamer gate molecules block the nanopores on the surface of particles by entrapping fluorescein molecules inside. When the target molecules are added to assay solution, a conformational change of the gate molecule results in opening of the nanopores and allowing the release of trapped fluorescent molecules. Aptamer gates can be designed from any aptamer sequence in hairpin form by adding a short sequence that is complementary to its other end. Duplex-to-complex behaviour of an aptamer sequence upon target binding results in denaturation of duplex region and intramolecular conformational change. We showed that an aptamer hairpin

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undergo strikingly different structural rearrangement upon binding to ATP than the aptamer sequence itself.[16] Therefore, incorporation of such an aptamer gate near the mouth of a nanopore creates an on-off state of valve function, blocking the release of guest molecules in duplex form and allowing their release upon ligand-complex formation. A similar application of gated silica has been demonstrated in recent homogenous sensors for thrombin[17] and genomic DNA. Many forms of this strategy have been designed as reviewed comprehensively in a recent article.[18] Here, we demonstrated that aptamer-gated silica nanoparticles can be used to construct lateral flow assays, allowing to develop sensitive detection for small molecules on paper strips. This design is advantageous because the detection is achieved without requiring any modification on the target molecules, thus it is a direct method, depending on the interaction of the target molecule and aptamers. The advantages of aptamer gate strategy for small molecule detection is twofold; i) it overcomes requirement for labelling of target molecules, extending applicability of lateral flow tests on difficult (small size) targets, and ii) it amplifies the signal because of multiple reporter molecules (e.g. fluorophores) trapped inside the pores.

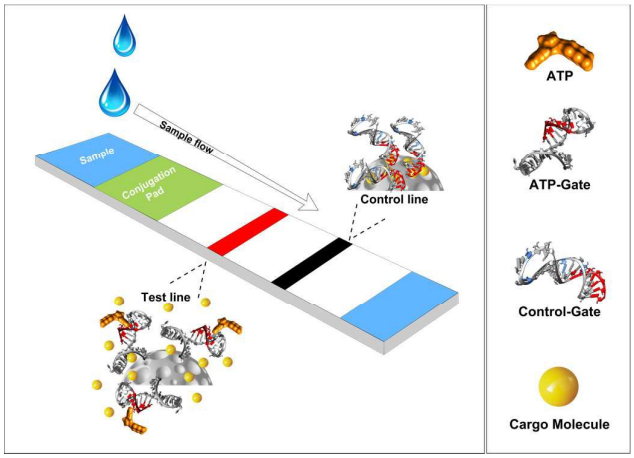

Fig. 1 Assay principle. ATP-binding aptamer-gated silica nanoparticles were fixed on test line and a mutated form of the aptamer (not responding to ATP) on control line of a lateral flow strip.

Fig. 1 is a schematic drawing to explain the assay principle developed in this report. ATP aptamer-gated nanoparticles were immobilized on the test line while a mutated aptamer sequence (non-responsive to ATP) were immobilized on the control line of a lateral flow strip. When samples containing ATP were applied on the sample pad, ATP molecules flow through test and control lines. Since ATP causes opening of aptamer gates, Rhodamine B molecules inside the nanopores are released and washed by flowing buffer. The release of fluorophore molecules can be monitored by quantifying the decrease of fluorescent signal in the test line.

The main objective of this study is to use nanopore-controlled inorganic nanoparticles for developing lateral flow sensors for small molecules. As a proof of study, ATP responsive aptamer sequence was first converted to gating structure and immobilized on the surface of silica nanoparticles to encapsulate fluorophore molecules inside the pores. The aptamer-gated fluorophore-loaded nanoparticles were then fixed on test region of a lateral flow strip. This novel strategy could provide development of paper-based detection by direct interaction of small molecules with affinity agents.

Results and discussion

Synthesis of aptamer-gated silica nanoparticles

Aptamer-gated nanoparticles were prepared according to Ozalp & Schafer [15]. About 1 mg of mesostructured silica nanoparticles were loaded with Rhodamine B molecules. After grafting with amino silane, the surface of silica nanoparticles was functionalized with thiol labelled ATP-responding gate sequences via sulfo-GMBS linkers. To obtain ATP specific molecular gate, the aptamer sequences (Table I) were converted to a hairpin structure as reported previously for three different aptamers.[19-21] The aptamer gate consists of the aptamer sequence and a short-stem DNA sequence complementary to the other end of the aptamer sequence. The interaction of ATP molecules with the aptamer sequence disrupts the hairpin structure and leads to rearrangement of the structure, resulting in nanoscale changes in molecular conformation. This leads to opening of pores and release of cargo fluorescent molecules. We demonstrated previously that aptamer gates respond to ATP by opening and releasing the cargo molecules at a rate proportional to the concentration of ATP molecules in the solution [22]. The nanoparticles had an average diameter of 185 nm with a uniform size dispersion and electron microscopy images confirmed the mesoporous structure (Fig. 2). We first characterized the ability of aptamer gates to block and to allow the release upon ATP interaction (Data not shown) [15]. The amount of entrapped Rhodamine B molecules was at similar values to previous study.

Table I. Aptamer gate oligonucleotides used in this study.

ATP-Gate	**5'-CACCTGGGGGAGTATTGCGGAGGAAGGTTCCAGGTG-SH-3'**
Control-Gate	**5'-CACCTAGGAGAGTAATGCCGAGGAAGGTTCCAGGTG-SH-3'**

SH: Thiol-C6-modification, **Black:** Huizenga ATP-Binding aptamer sequence1, **Red:** Added nucleotides for creating hairpin, **Blue:** Mutated nucleotides in the control sequence

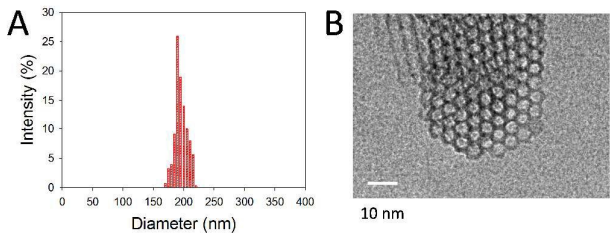

Fig. 2 Characterization of silica particles. A) Size distribution was determined by dynamic light scattering analysis and average hydrodynamic diameter of the particles was 185 ± 3.1 nm. B) A typical Transmission Electron Microscopy (TEM) image showing nanometer size pores on the silica particle surface.

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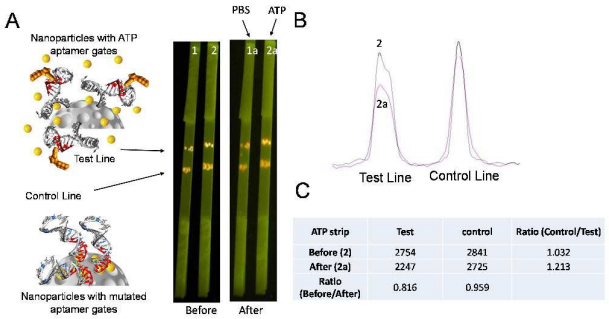
Preparation of the strip assay

The strip sensors were prepared by immobilizing aptamer-gated-Rhodamin B loaded silica nanoparticles on the test region of nitrocellulose membrane strips. A mutated form of the ATP aptamer sequence was used as gating molecule on Rhodamine loaded silica particles and immobilized in control line of the same strip (Fig. 3A). When ATP molecules in the sample were applied on the strip pad, they moved along the liquid phase, reached silica nanoparticles on the test and control zones and interacted with the aptamer gates. The intensity of fluorescence on the test line depends on the concentration of analyte-ATP diffusing through the test line zone. The ATP molecules have to reach the aptamer gate molecules on the surface of the silica particles in order to trigger conformational change that will release the Rhodamine B dye, which will be washed away and thus decrease the fluorescence intensity on the test line. Fig. 3 shows the results of ATP assay with LFA strips prepared with aptamer-gated silica nanoparticles. In a typical experiment, ATP was applied on sample pad region of the test strips in PBS buffer at 1.0 mM concentration.

Fig. 3A shows the strips before and after the application of ATP solution. Subsequently, the results in Fig. 3A were converted into fluorescent intensities. As Fig. 3B shows, the fluorescence intensities of the test line decreased with ATP sample application (Strips 2, 2a) while the control strip with PBS application (Strips 1, 1a) maintained unchanged fluorescence reading. The intensity change was calculated by dividing the control line value by that of the test line. In this example, 1.0 mM ATP application resulted in about 18.4 % decrease of fluorescence intensity on the test line and only 4.1 % in the control line. Thus, the leakage of fluorophore molecules from aptamer gates were limited to less than 5 %. A ratiometric value (Φ) of fluorescence decrease was calculated by dividing the control line value by that of the test line. This normalized value represented changes in test line signal independent of the strip and allowed comparison between experiments. This value was found to be 1.021 for control strip (1, 1a) and 1.231 for ATP sample strip (2, 2a).



ATP strip	Test	control	Ratio (Control/Test)
Before (2)	2754	2841	1.032
After (2a)	2247	2725	1.213
Ratio (Before/After)	0.816	0.959	

Fig. 3 Lateral flow strips were prepared by depositing ATP binding aptamer-gated silica nanoparticles loaded with Rhodamine B fluorophore on the test line. A control line was obtained by depositing silica nanoparticles loaded with Rhodamine B and capped with a mutated aptamer gate. (A) Application of ATP or PBS were presented before and after the application. (B) The change in the fluorescence intensity of control and test line region were quantified by estimating the intensities of the bands. C) The fluorescence changes were normalized by calculating ratios.

Strip assay for ATP determination

The aptamer-gated LFA strips for ATP were tested with a series of ATP concentrations (Fig. 4A). The strips were contacted with ATP at a series of concentrations (10 µM to 10 mM) in binding buffer (i.e. PBS + 5mM $MgCl_2$). A fluorescence decrease (increase in Φ value) in frequency was detected with each ATP application. The affinity coefficient (kD) was determined as 956 µM by fitting to a two ligand parameter binding curve. The literature cites the affinity constants of the ATP-binding DNA aptamer in homogeneous solution to be in the range of 6 µM to 100 µM depending on the method used in the measurement.[23] A higher affinity constant for gate structure of ATP aptamer in solution was reported at 273 µM.[24] Changes in the affinity of aptamer after modification is commonly observed for optical biosensors based on structural transduction mechanisms. With the strips developed in this study, a linear dynamic range was obtained between 100 µM and 2 mM ATP (Fig. 4B). The limit of detection was determined to be 69 µM with 3σ method. In fact, the ATP or any adenine containing molecules will interact with the aptamer sequence immobilized on silica surface because the ATP aptamer sequence[25] was selected for binding affinity from adenine moiety of the molecule.[23, 24] The specificity of the aptamer gates was also tested with GTP application and no significant response was observed (Fig. 3B).

Two dominant types of tests have been employed in lateral flow assay development; direct or competitive. Sandwich assay is one of the most common assay type in which one biorecognition molecule (e.g. antibody) can be used to capture the target molecules in the sample and then a labeled biorecognition molecule can produce the signal. Since two molecules cannot bind to one small molecule due to its small molecular structure, competitive assays were frequently employed. LFA for small molecules detection were reported mostly in competitive format with various labels. The target molecule is immobilized on test region and a labelled antibody on the conjugation pad interacts with immobilized target depending on the presence of target molecules in the samples [26]. However, the antigen (targets) should commonly be conjugated to a carrier protein for immobilization on the test line and allowing the access of labeled biorecognition molecules for interaction. For example, Yang *et. al.* designed a lateral flow competitive assay for estradiol in milk samples by conjugating gold nanoparticles on the antibody molecules.[27] In the strategy reported here, small molecules can interact with aptamer gates and results in fluorescent signal changes in a direct assay. Finally, the lateral flow strips were tested in whole blood samples spiked with 1 mM ATP and similar results to PBS experiments were obtained (1.214 ± 0.0309; n=3).

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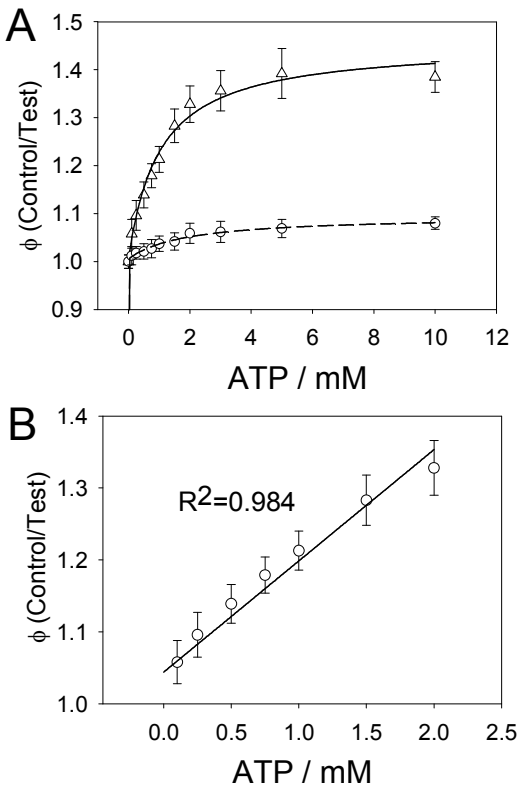

Fig. 4 A) The normalized fluorescent signal change (Ø) of strips was plotted against ATP (line) or GTP (dotted line) concentrations, and fitted (black line) to a ligand binding curve. B) Each experimental point represents the average of three independent experiments and the error bars are the corresponding standard deviations. The linear line follows the equation (Y=0.01411X+1.062).

Experimental

Materials and reagents

MCM-41 type hexagonal-ordered mesoporous silica particles were obtained from Sigma-Aldrich (Sigma-Aldrich, Germany). The characteristic properties of silica particles were supplied by the company as follows; the unit cell size: 4.6-4.8 nm; pore volume: 0.98 cm^3/g; pore size: 2.3-2.7 nm; specific surface area: ~1000 m^2/g (BET); bp: 2230 °C; mp: >1600 °C and bulk density: 0.34 g/mL. (3-Mercaptopropyl) triethoxysilane (CAS number: 14814-09-6), Rhodamine B and all other chemicals were purchased from Sigma-Aldrich. Sulfo-GMBS (Sulfo-N-succinimidyl 4-maleimidobutyrate sodium salt, CAS number: 185332-92-7) was from Fisher Scientific. The DNA oligonucleotides in Table I were synthesized by IDTDNA.

Preparation of Rhodamine loaded silica particles

The particles with aptamer gates were prepared by following a previous report.[19, 28] Briefly, 10mM Rhodamine B was loaded into silica nanoparticles suspended in PBS buffer (0.1 M phosphate buffer, 137 mM NaCl, pH=8.0) by incubating the mixture overnight at room temperature with vigorous stirring. After centrifugation, supernatant was discarded and the surface of silica particles was functionalized with thiol groups by silanization with 5% (3-Mercaptopropyl) triethoxysilane in 95% ethyl alcohol. 5´-Amine modified ATP aptamer of 100µM was added and the mixture was incubated for 30 min with shaking at room temperature, then it was dried and stored at 4°C.

Basic lateral flow assay principle

Lateral flow strips were prepared as described previously.[29] Briefly, 50 µg of nanoparticle solution of sensor particles or control particles was mixed in 5% acrylamide solution and manually deposited on the corresponding zones of nitrocellulose membrane (HF240MC100, Millipore) of lateral flow strips. Sample pad (CFSPOO 1700, Millipore) were assembled on an overlapping sequence on the adhesive tape at both ends of nitrocellulose membrane. The strips were stored in dry conditions at 25 °C in dark for at most two weeks before use. The stability of the strips at these conditions were checked by measuring a sample of 1 mM ATP sample for 14 days. There was no significant differences for ϕ values during this time (Data not shown). During assays, 200 µl of sample was directly applied on sample pad, which migrates the whole strip within 3-5 min. The samples were prepared in PBS buffer with 5 mM $MgCl_2$. The ATP or any adenine containing molecules will interact with the aptamer sequence immobilized on the silica surface. The nanoprobe-LFA strips were scanned on a slide with a GenePix 4100A (Molecular Devices, California, USA). Rhodamine B was excited at 535 nm and emission was recorded at 600 nm. The intensities of fluorescent bands on the strips were determined by ImageJ 1.46r.[30]

Conclusions

In this study, a simple and sensitive lateral flow strip assay was developed for measurement of ATP, based on the release of fluorophore molecules through aptamer gates. The specific interaction of ATP and the aptamer gates causes conformational changes in aptamer structure and unblocks the pores of silica nanoparticles. This assay was demonstrated to be a powerful tool in detection of small molecule in lateral flow format. The proposed assay system provides a new and effective strategy to monitor small molecules since specific aptamers can be obtained for any desired target.

Acknowledgements

The acknowledgements come at the end of an article after the conclusions and before the notes and references.

Notes and references

‡ Footnotes relating to the main text should appear here. These might include comments relevant to but not central to the matter under discussion, limited experimental and spectral data, and crystallographic data.

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