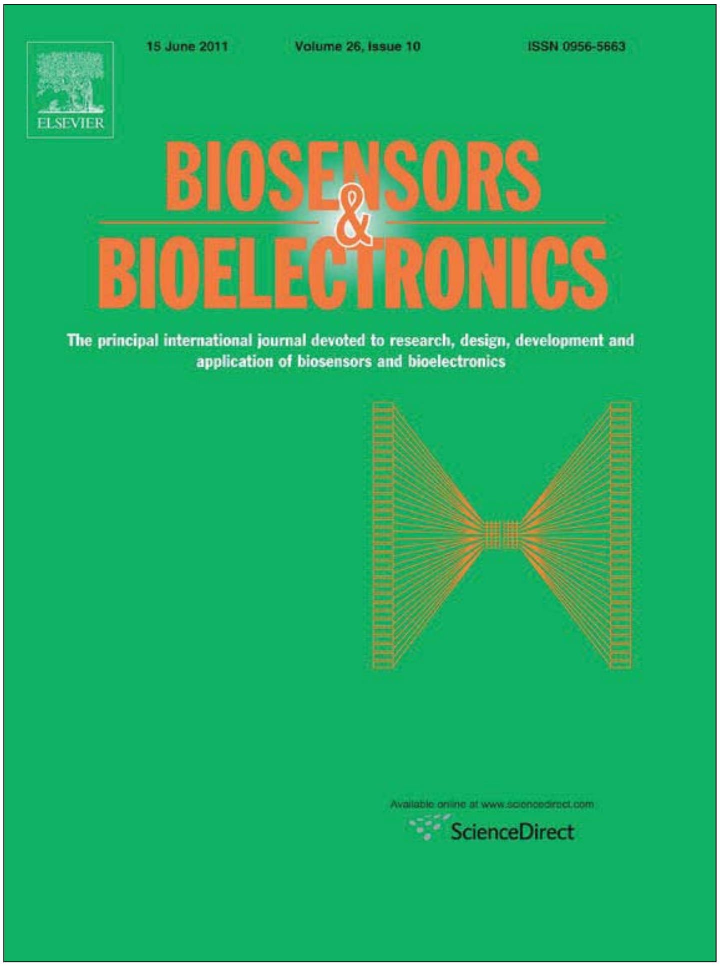
15 June 2011
Volume 26, Issue 10
ISSN 0956-5663
ELSEVIER
BIOSENSORS & BIOELECTRONICS
The principal international journal devoted to research, design, development and application of biosensors and bioelectronics
Available online at www.sciencedirect.com
ScienceDirect

Biosensors and Bioelectronics 26 (2011) 4099–4103

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Real-time aptamer quantum dot fluorescent flow sensor

Anastasia Bogomolova*, Matt Aldissi

Smart Polymers Research Corporation, 108 4th Street, Belleair Beach, FL 33786, USA

ARTICLE INFO

Article history:
Received 24 January 2011
Received in revised form 21 March 2011
Accepted 1 April 2011
Available online 9 April 2011

Keywords:
Aptamer
Quantum dot
Sensor
Real-time
Flow
Environmental

ABSTRACT

The goal of this work was to develop and test a novel real-time biosensing approach which can be adapted to either environmental or clinical monitoring of biological pathogens. We have developed a working prototype of a real-time aptamer-based fluorescent flow sensor. The sensor utilizes a competitive displacement approach to measure the binding of the analyte, which keeps the nonspecific binding below detectable levels. The complex of surface-immobilized DNA aptamer with fluorescent complementary oligonucleotide releases the oligonucleotide upon binding with a specific target, which is translated by a decrease in fluorescence. Bright and stable fluorescence of quantum dots is utilized for prolonged detection of the analyte in flow conditions. The real-time sensor prototype is developed with previously characterized ATP-specific aptamer and is capable of specifically detecting 0.1 mM of ATP in biological buffer, with a quantitative response up to 5 mM. The developed prototype is portable and easy to use and its design allows further miniaturization and multiplexing. The developed real-time sensing approach can be adapted to a variety of targets of environmental and clinical significance.

1. Introduction

A critical need exists for a real-time biosensing approach which can be adapted to either environmental or clinical monitoring of biological pathogens. It should be capable of direct (reagent-free) detection and quantification of pathogens of various nature, such as bacteria, bacterial spores, bacterial toxins and viruses. The sensor design should be adaptable to multispecific simultaneous detection, and allow further miniaturization and automation. Making such a sensor economical, robust and user-friendly will widen its applicability. Given the various nature of biological pathogens, the recognition moiety should be adaptable to various analytes and ensure highly selective detection. Utilizing competitive binding with antibody-like recognition moieties would result in higher specificity compared to measurement of direct analyte binding.

DNA aptamers (30–70 base oligonucleotides, selected as highly specific binders for desired targets) offer unique advantages for biosensing compared to antibodies, such as their small size, resulting in higher density of immobilized binding sites; better stability and heat-resistance, lower cost, and, most importantly, endless possibilities for design. Aptamers have been selected since 1992 (Bock et al., 1992). A number of biopathogen-specific DNA aptamers, which can be potentially used for environmental monitoring, has been selected (Bruno and Kiel, 1999, 2002; Vivekananda and Kiel, 2006; Ngundi et al., 2006; Nitsche et al., 2007; Bruno et al., 2008, 2009; Joshi et al., 2009; Dwivedi et al., 2010). Extensive aptamer research, as well as biosensing, diagnostic and clinical applications of aptamers have been recently reviewed (Tombelli and Mascini, 2009; Cho et al., 2009; Sefah et al., 2009). Aptamers are suitable for multiplex analyte detection (Liu et al., 2007; Xie and Walton, 2010) as well as creation of aptamer arrays (Collett et al., 2005; Rowe et al., 2009). The most useful property for biosensing is their ability to change conformation upon binding of the target, which is used in design of the signaling aptamers and molecular beacons (Hall et al., 2009; Li et al., 2008, 2010). Such conformational changes, along with the ability to switch between binding a complementary oligonucleotide and a target analyte can be used for real-time electrochemical and fluorescent detection. Thus, cocaine was detected in real-time in micromolar concentrations electrochemically (Swensen et al., 2009), and γ-interferon was detected by FRET (fluorescence resonance energy transfer) in nanomolar range (Tuleuova and Revzin, 2010).

To design a real-time flow sensor prototype, we have chosen to combine a well characterized ATP-specific aptamer (Zheng et al., 2009) with stable quantum dot (QD) fluorescence. QDs are nanometer-size atom clusters of a semiconductor material (cadmium mixed with selenium or tellurium), which exhibit bright and photostable fluorescence, which is very important for prolonged fluorescence detection. The physical size of the nanocrystal defines the wavelength of emitted fluorescence, thus a single excitation source can be used for multiplex analysis. Wide use of QD bioconjugates in immunoassays, fluorescent biosensing and imaging applications has been recently reviewed (Xing et al., 2009; Algar et al., 2010; Frasco and Chaniotakis, 2010).

* Corresponding author.
E-mail address: abogomol@hotmail.com (A. Bogomolova).

0956-5663/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.bios.2011.04.001

Combining QD fluorescence and aptamer recognition for biosensing has been performed since 2005 (Levy et al., 2005), and was used for rapid detection of proteins (thrombin (Choi et al., 2006; Huang and Zhu, 2009), Mucin 1 (Cheng et al., 2009) and platelet derived growth factor (Kim et al., 2009)); small biomolecules (cocaine (Golub et al., 2009; Freeman et al., 2009) and ATP (Chen et al., 2008; Huang et al., 2010)), as well as the food-born pathogenic bacteria *Campylobacter jejuni* (Bruno et al., 2009). The QD-aptamer-based detection times are in the minute(s) range, however, to our knowledge, real-time detection has not been reported yet. Below we present a design of a real-time aptamer-QD fluorescent flow sensor prototype, whose specificity can be later multiplexed and adapted to a variety of targets.

2. Materials and methods

All chemicals, buffers and deionized sterile DNase, RNase-free water were purchased from Sigma.

2.1. Oligonucleotide probes

ATP aptamer probe and detector oligonucleotides design was based on the work of Mirkin and co-workers (Zheng et al., 2009), although the detector probes in our case were shorter. Oligonucleotide synthesis, purification and modification were performed by Operon (Huntswille, Alabama). All probes were modified at the 5′ end. The detector oligonucleotides were complementary to the 3′ end of the aptamer probe. The sequences of the probes were the following:

(1) ATP-specific aptamer probe "ATPA": 5′ biotin-A_{12} CCTGGGGGAGTATTGCGGAGGAAGGTGTCACA
(2) "long" complementary detector "detL": 5′ biotin-A_{12} TGACACCTTCCT
(3) "short" complementary detector "detS": 5′ biotin-A_{12} GACACCTTCC

All oligonucleotides were designed to have A_{12} leg to distance the quantum dot label (in case of the detector) or the attachment site (in case of the aptamer) from the functional molecular switch. Possible secondary structures of the aptamer were predicted with Quikfold program (http://dinamelt.bioinfo.rpi.edu/results/quikfold) for 20–25 °C, 150 mM NaCl, 5 mM $MgCl_2$.

2.2. Fluorescence detection system and flow cell design

The portable fluorescent detection flow system consisted of a miniature light source (LED 400 nm, Ocean Optics, Dunedin FL); two fiber optic cables; a flow cell built in-house connected to a mini peristaltic pump (Cole-Parmer) and supplied with an optional sample injection valve, miniature fiber optic spectrometer (USB 2000, Ocean Optics) connected through a USB cable to a laptop with Spectra Suite spectral acquisition software (Ocean Optics). The total weight of the prototype setup, together with the computer, was under 2 kg.

Our flow cell consisted of a standard UV-transparent (340–800 nm) 10 mm × 10 mm disposable polystyrene cuvette with a tight cap (Perfector Scientific, Atascadero, CA), containing 10 mm × 22 mm glass coverslip (C&L Instruments, Hershey, PA), secured diagonally, inlet and an outlet. The solution entered the flow cell at the bottom, flowed along both sides of a modified coverslip and exited at the top (Fig. A1). The flow cell was positioned in the 4-way cuvette holder (Ocean Optics), with excitation and acquired fluorescence beams 90° relative to each other (Fig. A2). In our experience, positioning the coverslip at an angle of 50–55° relative the excitation beam instead of the customary 45° angle allows better signal acquisition due to the elimination of the excitation beam reflection from the inner surfaces of the glass coverslip.

2.3. Sensing surface preparation

The glass coverslips were modified on both sides using the procedure outlined below, which was optimized after multiple rounds of testing. All incubations were carried out in TN buffer (TE+ 150 mM NaCL, pH 8), in 20–30 μl drops between sterile parafilm sheets, in humidified chambers, with 3× gentle rinsing of each coverslip with 2 ml of TN buffer between incubations. The oligonucleotide probes were denatured by placing them at 65 °C for 2 min, followed by placing them on ice prior to use. The coverslip modification procedure included six steps:

(1) 11 mm × 22 mm × 0.2 mm glass coverslips were silanized by dipping in concentrated 3-glycidoxypropyl-trimethoxysilane, air-dried and hand-polished.
(2) Streptavidin was attached from 5 μM solution in TN buffer, for 48–72 h at 4 °C.
(3) Biotinylated aptamer was freshly denatured at 65 °C for 2 min, followed by placing it on ice. 10 μM aptamer solution in TN buffer was used to attach to streptavidin layer for 18–48 h at 4 °C.
(4) Free streptavidin binding sites were blocked with 5 mM biotin in TN buffer for 1–2 h at RT.
(5) Surface-immobilized aptamer was annealed with the freshly denatured biotinylated detector oligonucleotide (20 μM solution in TN buffer) for 5–7 h @ RT, followed by 16 h at 4 °C.
(6) QD–streptavidin conjugate (523 nm, Invitrogen) was attached to the detector oligonucleotide from 0.1 μM solution in TN buffer for 48–96 h at 4 °C. Prolonged incubation up to one week was possible but did not result in enhanced fluorescence. The surface modification procedure is summarized in Fig. 1A.

2.4. Fluorescence measurements

Fluorescence of the modified coverslip, excited by 400 nm LED source (Ocean Optics) was acquired by USB 2000 spectrometer. The flow cell with a coverslip, modified with the QD sensing complex was washed with TNM (TN + 5 mM $MgCl_2$, pH 8) or PBSM buffer (PBS + 5 mM, $MgCl_2$, pH 7.4) for 30 min at 0.5 ml/min prior to measurements. The spectra 370–800 nm were recorded and processed with Spectra Suite acquisition software. Spectra were acquired in stop-low conditions, with the flow cell filled with a freshly prepared solution of ATP (0–5 mM) in PBSM buffer.

Real-time fluorescence changes of the QD-labeled sensing surface (523 nm) were recorded either at a single wavelength of 523 nm, or as averaged fluorescence 510–537 nm versus time at a rate of 1 measurement per second (recording averaged fluorescence resulted in a smoother baseline, compared to single wavelength acquisition of 523 nm). The real-time fluorescence measurements took place during the flow of 0.1–2 ml/min of PBSM buffer or ATP/PBSM solution through the flow cell for up to 2 h. Prior to real-time fluorescence measurements of the ATP binding, the baseline was recorded until stabilized for an average of 30 min. Although it was possible to measure ATP binding in TNM buffer, sensitivity was higher in PBSM buffer.

3. Results and discussion

The mechanism of aptamer-based QD detection is schematically presented in Fig. 1B. The sensing complex consisted of

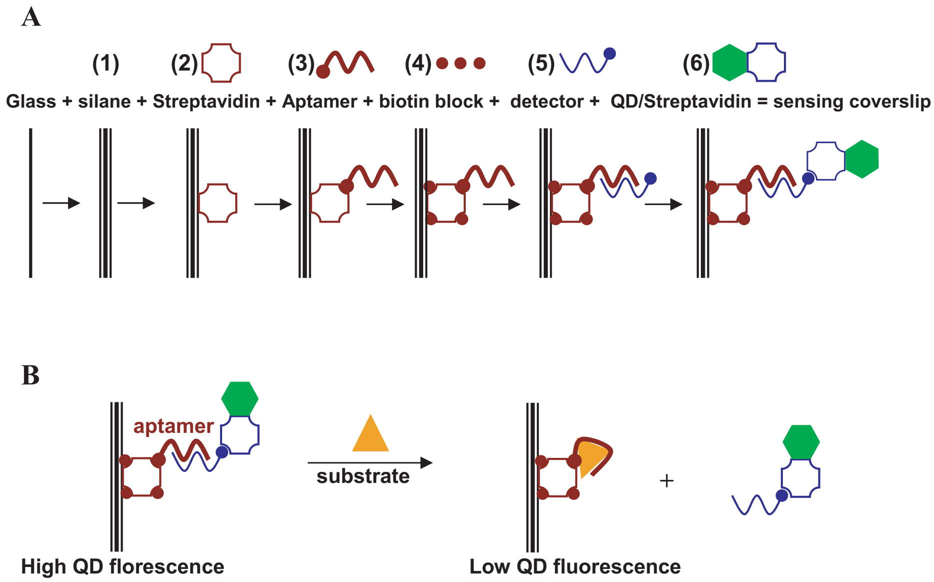

Fig. 1. Scheme of aptamer-QD sensing coverslip preparation and substrate detection. (A) Six-step sensing coverslip preparation (both sides of a glass coverslip are modified); (B) ATP detection with modified sensing coverslip.

glass-immobilized aptamer (ATPA), annealed with the detector oligonucleotide/QD complex. Two detector oligonucleotides, complementary to the 3′ end of the ATP-specific aptamer by 10 and 12 nucleobases (detS and detL) were tested. Both detector oligonucleotides produced stable complexes with the immobilized aptamer. The complexes retained their florescence under flow of up to 2 ml/min for at least 8 h and remained active towards ATP detection even after up to 1 week storage at 4 °C.

Binding of the substrate causes structural changes in the aptamer and releases the detector oligonucleotide/QD complex, resulting in decreased fluorescence of a sensing surface. The fluorescent detector oligonucleotide/QD complex is released in solution (becoming undetectable) and/or is carried away by the flow. Although complexes prepared with both detectors were active towards ATP, the sensing complex prepared with a shorter detector (ATPA/detS-QD) responded more readily towards addition of ATP, compared to the similar complex with longer detector ATPA/detL-QD.

The modified glass coverslips were designed to be disposable and were not regenerated, however they could be repeatedly used as long as they were fluorescent. Substrate detection experiments were taking place in the presence of 5 mM $MgCl_2$ to allow aptamer folding and binding to the substrate. Two buffers were tested for ATP detection: TNM (TN + 5 mM $MgCl_2$, pH 8) and PBSM (PBS + 5 mM $MgCl_2$, pH 7.4). We have found that using PBSM buffer allows higher sensitivity of detection, compared to TNM buffer.

Fig. 2A presents sensor response to ATP obtained under stop-flow conditions. The corresponding changes in QD fluorescence (523 nm) versus time are plotted in Fig. 2B. The strongest response was observed during the first 30 min, and the reaction was 90% complete after 3 h.

The examples of real-time ATP detection are presented in Fig. 3. Fig. 3A demonstrates prolonged continuous response to 5 mM ATP recorded for ∼1.5 h, performed with ATPA/detS-QD complex in PBSM buffer at 0.33 ml/min. Similarly to the behavior observed in Fig. 2A, the strongest ATP-related fluorescence decrease is observed during the first half hour, with a characteristic sharp fluorescence drop immediately after the addition of ATP. It is interesting to note that this sharp drop can be seen only at concentrations above 0.5 mM, while addition of ATP at lower concentrations causes a clear but more linear response (Fig. 3B). Thus we have used the

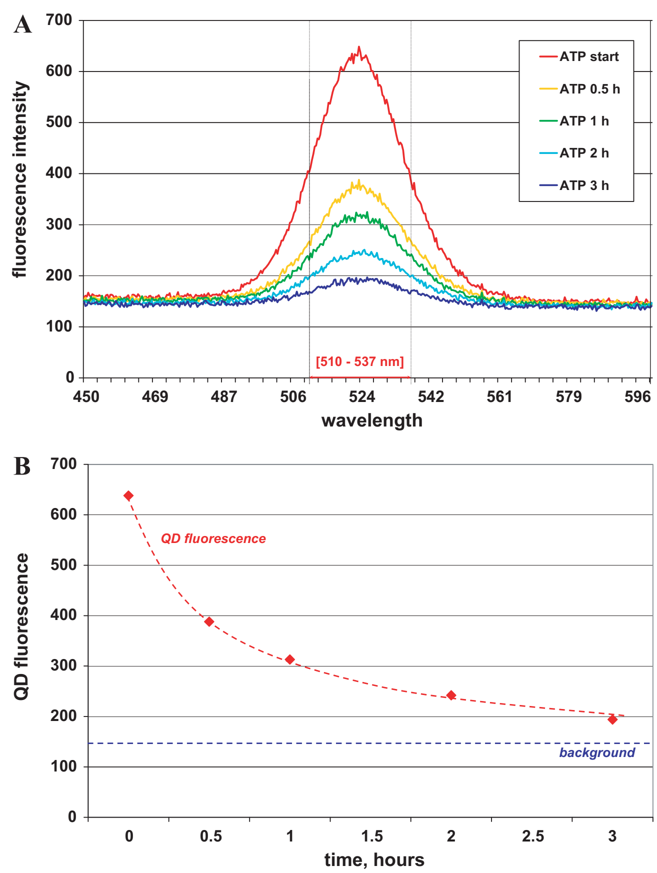

Fig. 2. Detection of ATP on a QD fluorescent sensing coverslip in stop-flow conditions. (A) Overlay of fluorescence spectra [400–650 nm], recorded with time intervals; (B) changes in QD peak fluorescence (524 nm), plotted versus time (same experiment). Reaction with 2 mM ATP performed in TNM buffer (10 mM Tris–HCl, pH 8, 1 mM EDTA, 150 mM NaCl, 5 mM $MgCl_2$) for 3 h using coverslip modified with ATPA/detectorS–QD complex.

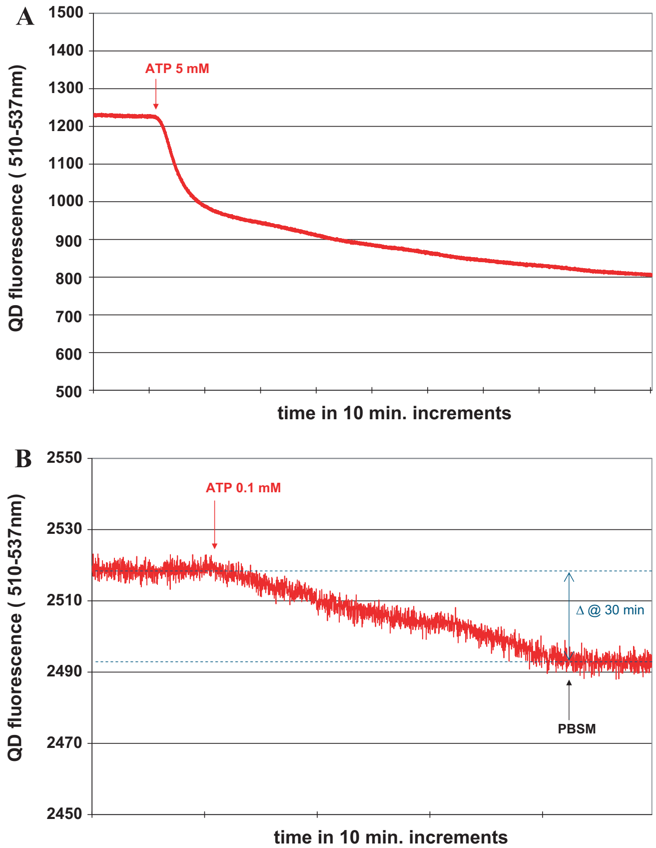

Fig. 3. Real-time detection of ATP in the flow. Coverslips modified with ATPA/detectorS–QD complex were exposed to the flow (0.33 ml/min) of (A) 5 mM ATP in PBSM buffer for ~1.5 h and (B) 0.1 mM ATP in PBSM for 30 min, followed by PBSM buffer for 10 min. Estimated baselines are shown in dotted lines. Note the difference in scale between A and B. Binding of the aptamer with ATP releases detector–QD oligonucleotide, visible as fluorescence decrease. Changes in averaged (510–37 nm) fluorescence after 30 min (Δ @ 30 min) were used to build calibration plot.

parameter of fluorescence decrease after 30 min "Δ at 30 min" to compare and quantify the ATP-related fluorescence response. Replacing the flow of ATP solution by the buffer stops the release of the fluorescent complex from the sensing surface and is registered as a plateau on the curve in Fig. 3B.

It would be logical to suppose that the "saturation" behavior of the real-time ATP response in Fig. 3A is related to the depletion of the aptamer sensing complexes on the surface of the modified coverslip. In such case, repeated injections of ATP solution of the same concentration, alternated with the flow of the buffer would produce decreased responses for subsequent ATP injections. However, the situation appears to be more complex:

Fig. 4 demonstrates repetitive real-time detection of 1 mM ATP, performed on the same coverslip, with alternating flows of ATP, buffer, ATP, buffer, AMP (as a negative control), ATP, buffer, for 30 min each step. Three ATP fragments resulted in comparable fluorescence decrease, all with a noticeable "drop" behavior, most pronounced in the first ATP fragment. That leads us to suggest the presence of the two populations of the sensing complex, existing in equilibrium, with the first population being more responsive to ATP and providing rapid fluorescence release during the first ~10 min of ATP flow, while the second population being responsible for slower but more continuous fluorescence release (Figs. 3 and 4). When the flow of ATP is replaced by the flow of the buffer (Fig. 4), the concentration of the first "rapid" population, previously depleted, is being (partially) restored, which results in repeated "drop" behavior during subsequent ATP injections. The longer the coverslip is allowed to "rest" between the repetitive detection steps, the more reproducible is the "drop" behavior.

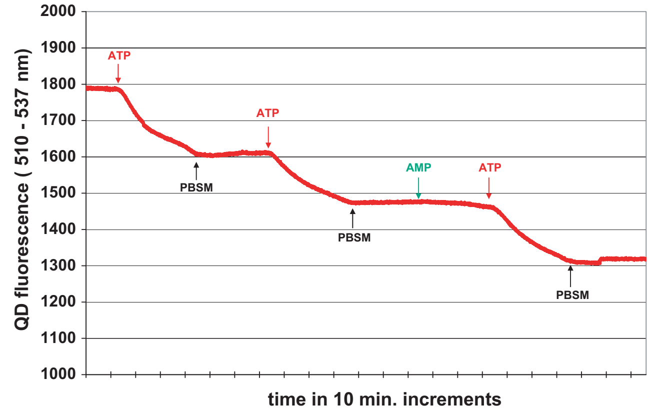

Fig. 4. Real-time repetitive detection of ATP. Real-time fluorescence measurements on coverslip modified with ATPA/detectorS–QD exposed to the flow (0.33 ml/min) of 1 mM ATP/PBSM, 30 min, alternating with PBSM buffer for 30 min 3 times. The flow of 1 mM AMP for 30 min was used as specificity control. Repetitive exposure to ATP results in reproducible fluorescence decrease. Exposure to AMP did not cause any changes.

The "rapid response" sensing complex population can be explained through the presence of aptamer-detector duplexes with partially melted ends, existing in equilibrium with fully annealed duplexes, which represent the "slow response" population. Whether such kinetic phenomena is universal for our sensing approach remains to be discovered in the course of ongoing experimental work with the other aptamers and substrates.

The specificity of the sensing approach is defined by the specificity of the used aptamer. Nonspecific closely related substrate, AMP, did not result in fluorescence changes (Fig. 4). The real-time response to ATP was quantitative, i.e. the higher ATP concentration resulted in stronger response (Fig. 5). The fluorescence decrease after 30 min of ATP flow was used to quantify the results. The reproducibility of detection was slightly better at lower concentrations of ATP. Both reproducibility and sensitivity depend on the uniformity and density of sensing surface modification, and such optimization, along with miniaturization of the flow cell, has been planned. Here, we report the mere possibility of real-time sensor creation, which has been proved with quite simple means.

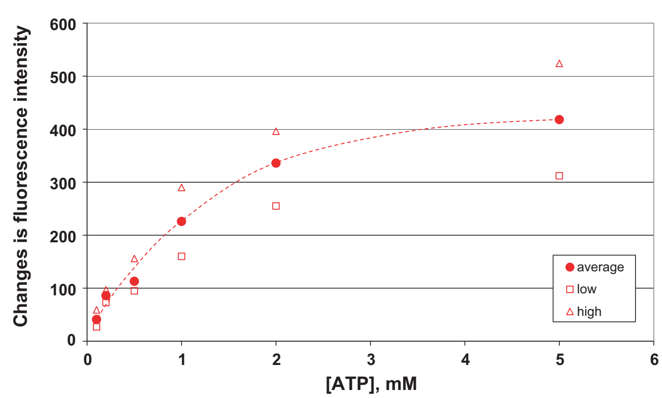

Fig. 5. Calibration plot for real-time ATP detection in 2 ml flow cell. 3–7 repeated measurements of fluorescence changes after 30 min were used for each concentration. Detection range 0.1–5 mM ATP in PBSM buffer, LOD 0.1 mM.

4. Conclusions

We have developed a working prototype of a real-time aptamer-based fluorescent flow sensor. The sensor utilizes a competitive displacement approach to measure the binding of the analyte, which keeps the nonspecific binding below detectable levels. Bright and stable fluorescence of quantum dots is utilized for prolonged detection of analyte in the flow. The real-time sensor prototype is developed with previously characterized ATP-specific aptamer and is capable of specifically detecting 0.1 mM of ATP in biological buffer, with quantitative response up to 5 mM. The developed prototype is portable and easy to use. We plan to further optimize the sensitivity and perform significant sensor miniaturization and proceed to design of multispecific real-time sensor. Our approach is novel and can be adapted to a variety of environmental and clinical targets.

Acknowledgement

This work has been supported by EPA SBIR Phase I EP-D-10-039.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2011.04.001.

References

Algar, W.R., Tavares, A.J., Krull, U.J., 2010. Anal. Chim. Acta 673, 1–25.
Bock, L.C., Griffin, L.C., Latham, J.A., Vermaas, E.H., Toole, J.J., 1992. Nature 355, 564–566.
Bruno, J.G., Carrillo, M.P., Phillips, T., 2008. J. Biomol. Technol. 19, 109–115.
Bruno, J.G., Kiel, J.L., 1999. Biosens. Bioelectron. 14, 457–464.
Bruno, J.G., Kiel, J.L., 2002. Biotechniques 32, 178–180, 182–183.
Bruno, J.G., Phillips, T., Carrillo, M.P., Crowell, R., 2009. J. Fluoresc. 19, 427–435.
Chen, Z., Li, G., Zhang, L., Jiang, J., Li, Z., Peng, Z., Deng, L., 2008. Anal. Bioanal. Chem. 392, 1185–1188.
Cheng, A.K., Su, H., Wang, Y.A., Yu, H.Z., 2009. Anal. Chem. 81, 6130–6139.
Cho, E.J., Lee, J.W., Ellington, A.D., 2009. Annu. Rev. Anal. Chem. 2, 241–264.
Choi, J.H., Chen, K.H., Strano, M.S., 2006. J. Am. Chem. Soc. 128, 15584–15585.
Collett, J.R., Cho, E.J., Ellington, A.D., 2005. Methods 37, 4–15.
Dwivedi, H.P., Smiley, R.D., Jaykus, L.A., 2010. Appl. Microbiol. Biotechnol. 87, 2323–2334.
Frasco, M.F., Chaniotakis, N., 2010. Anal. Bioanal. Chem. 396, 229–240.
Freeman, R., Li, Y., Tel-Vered, R., Sharon, E., Elbaz, J., Willner, I., 2009. Analyst 134, 653–656.
Golub, E., Pelossof, G., Freeman, R., Zhang, H., Willner, I., 2009. Anal. Chem. 81, 9291–9298.
Hall, B., Cater, S., Levy, M., Ellington, A.D., 2009. Biotechnol. Bioeng. 103, 1049–1059.
Huang, H., Tan, Y., Shi, J., Liang, G., Zhu, J.J., 2010. Nanoscale 2, 606–612.
Huang, H., Zhu, J.J., 2009. Biosens. Bioelectron. 25, 927–930.
Joshi, R., Janagama, H., Dwivedi, H.P., Senthil Kumar, T.M., Jaykus, L.A., Schefers, J., Sreevatsan, S., 2009. Mol. Cell. Probes 23, 20–28.
Kim, G.I., Kim, K.W., Oh, M.K., Sung, Y.M., 2009. Nanotechnology 20, 175503.
Levy, M., Cater,.S.F., Ellington, A.D., 2005. Chembiochem 6, 2163–2166.
Li, D., Song, S., Fan, C., 2010. Acc. Chem. Res. 43, 631–641.
Li, J., Cao, Z.C., Tang, Z., Wang, K., Tan, W., 2008. Methods Mol. Biol. 429, 209–224.
Liu, J., Lee, J.H., Lu, Y., 2007. Anal. Chem. 79, 4120–4125.
Ngundi, M.M., Kulagina, N.V., Anderson, G.P., Taitt, C.R., 2006. Expert Rev. Proteomics 3, 511–524.
Nitsche, A., Kurth, A., Dunkhorst, A., Pänke, O., Sielaff, H., Junge, W., Muth, D., Scheller, F., Stöcklein, W., Dahmen, C., Pauli, G., Kage, A., 2007. BMC Biotechnol. 7, 48.
Rowe, W., Platt, M., Day, P.J., 2009. Integr. Biol. (Camb.) 1, 53–58.
Sefah, K., Phillips, J.A., Xiong, X., Meng, L., Van Simaeys, D., Chen, H., Martin, J., Tan, W., 2009. Analyst 134, 1765–1775.
Swensen, J.S., Xiao, Y., Ferguson, B.S., Lubin, A.A., Lai, R.Y., Heeger, A.J., Plaxco, K.W., Soh, H.T., 2009. J. Am. Chem. Soc. 131, 4262–4266.
Tombelli, S., Mascini, M., 2009. Curr. Opin. Mol. Ther. 11, 179–188.
Tuleuova, N., Revzin, A., 2010. Cell. Mol. Bioeng. 3, 337–344.
Vivekananda, J., Kiel, J.L., 2006. Lab. Invest. 86, 610–618.
Xie, S., Walton, S.P., 2010. Biosens. Bioelectron. 25, 2663–2668.
Xing, Y., Xia, Z., Rao, J., 2009. IEEE Trans Nanobiosci. 8, 4–12.
Zheng, D., Seferos, D.S., Giljohann, D.A., Patel, P.C., Mirkin, C.A., 2009. Nano Lett. 9, 3258–3261.