Microfluidic Platform for Aptamer based Fluorimetric Analysis of Analytes

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Abstract: In this work, we are reporting fabrication of a simple and low cost setup for fluorescence detection based on aptamer probes. For this reason, we fabricated a PMMA-PMMA microfluidic chip using easily available laboratory techniques and combined the chip with a simple fluorescence detection setup using optical fiber, filter, detector and a commercial spectroscopy software. In this new approach, we used two different strategies to use aptamers as probe. In first strategy, detection of any nucleic acid could be targeted using simple DNA hybridization with aptamer probe. Such strategy can be used in analysis of samples with specific nucleic acid sequence, such as pathogen. We proved this using known sequence of ssDNA aptamer probe immobilized on detection zone on microchip and its FAM labeled complementary strand was passed over it using microfluidic condition. In other strategy, we attempted detection of any protein or biomarker using sandwich fluorimetric technique with primary and labeled secondary aptamer immobilized on sensing region. For this, we used thrombin as model target to validate our setup. Both the strategies proved satisfactory on our setup. Even more, LOD was also impressive. In future, this setup could further be miniaturized by using a small on-chip CCD array detector, microcontroller based electronics and LabVIEW software based control.

1 INTRODUCTION

Aptamers are single stranded nucleic acids, DNA or RNA, which selectively and specifically bind to a target molecule such as nucleic acids, proteins, cells, microorganisms, etc. interacting via weak molecular forces. They often complement antibodies in terms of binding efficiency and specificity, yet are more stable and cheaper to produce. Aptamers are picked for a particular target from a process called SELEX (Systematic evolution of ligands by exponential enrichment) which is comparatively simple process compared to typical antibody production using hybridoma technique. In addition, host animal is also not required for the production of aptamers. Extra resistance against denaturation and ease of chemical modifications make aptamers friendlier to use in biosensing (Tennico, 2010; Song, 2012).

Meanwhile, microfluidics has replaced many analytical and biomedical techniques due to its reduced size, cost and reagents utilization. Aptamers and their application in microfluidics are inspiring researchers to create a bridge between two drastically different fields of biological and analytical techniques. Aptamers with microfluidics have already been used for analysis of biological targets/analytes like thrombin, VEG-165, peptides, cancer cells, C-reactive protein, viruses, microbes and various other proteins or biomarkers (Xu, 2010). Out of which, viruses, pathogens or microbes are identified by these aptamers due to their specific microbial proteins, lipopolysaccharides or nucleic acids. On the other hand, biomarkers (proteins) originating in the case of cancer allow their detection using aptamers (Viscidi, 1987; Su, 2015).

Detection of various diseases associated with microbes was made possible by simple hybridization of aptamer probe labelled with dye to the complementary nucleic acid strand from the microbe (Tennico, 2010). Besides, aptamers and hybridization principle has been used for DNA microarrays, single nucleotide polymorphism detection, gene expression studies and nucleic acid diagnostic applications (Wang, 2011; Abu-Salah, 2015). Furthermore, various proteins and biomarkers have been identified using primary and dye labeled secondary aptamers sandwich assay like ELISA technique. The use of fluorescent dyes in such

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sandwich protocol required sophisticated instrumentation including a microscope (Tennico, 2010; Fenzyl, 2016). Hence, in our work, we developed a simple setup for fluorescence detection using PMMA-PMMA microfluidic chip and aptamers as probe.

The choice of polymethyl methacrylate (PMMA) slides as a substrate was due to its sturdiness and possibility for immobilization of aptamer as a probe. PMMA is also a choice in fabrication of microfluidics chips because of its low cost and optical clarity (Tennico, 2010). Various techniques are available for fabrication of microfluidic chips like lithography, micromachining, laser ablation, thermal embossing etc. While, in this work, we used simple techniques like laser ablation for cutting of and engraving of channels on PMMA, and thermal and UV bonding for sticking the two layers.

Apart from developing a PMMA microchip, we also developed a simple setup for fluorescence detection. Majorly, the strategy employed for detection of various analytes using aptamers involves simple hybridization with dye labeled complementary nucleic acid or sandwich assay. Here, we have used both the strategies to check our fluorescence detection setup. Aptamers were immobilized using hexamethylene diamine (HMDA) and glutaraldehyde for both the strategies. For hybridization, we used an aminated sequence of aptamer whose complementary 6-carboxyfluorescein (FAM) labeled strand was made to interact. While in other strategy, thrombin was used as a model analyte to validate sandwich assay using our setup. The setup for fluorescence detection is shown in Figure 1. This setup enabled low cost fluorescence detection without use of bulky microscopes. In addition, this can be miniaturized further via using a different detector and LabVIEW software.

2 MATERIALS AND METHODS

2.1 Materials

All chemicals were of analytical grade and purchased from Sigma or Merck. Aptamer sequences were purchased from Integrated DNA Technologies (IDT). Sequences for first and second strategy were : 5'-/5AmMC6/GCC AAA TTG TTT GAC GAG A-3', 5'-/56-FAM/ TCT CGT CAA ACA ATT TGG C-3' and 5'-/5AmMC6/ TTT TTG GTT GGT GTG GTT GG-3', 5'-/56-FAM/TTT TTT TTT TTT TTT AGT CCG TGG TAG GGC AGG TGG GGG TGA CT-3'. Cyan LED 490nm, PMMA sheets and optical narrow bandpass interference filter of 530 nm were purchased from eBay India Online Store and Optics & Allied Engg. Pvt. Ltd. respectively.

2.2 PMMA-PMMA Chip Fabrication

PMMA sheets of thickness 1.5 and 5 mm were cut into substrates of 60 x 20 mm using CO_2 laser cutting machine. Same technique was used to engrave channel of depth 130µm, puncture for inlet tubing, sensing area and outlet tubing, and detection area. The detection area was made in such a way that it will be at 90º angle with LED source for fluorescence detection. Further, substrates were ultrasonicated in Isopropyl alcohol (IPA) for 20 minutes and dried using nitrogen purging.

For bonding of both layer of substrates, UV and

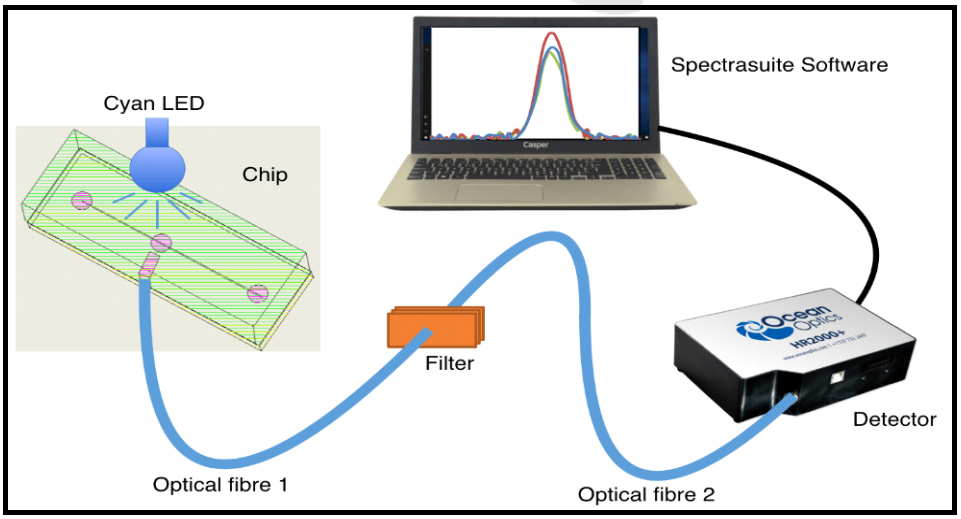

Figure 1: Fluorescence detection setup used with microfluidic chip.

solvent assisted thermal bonding were used. First, ethanol was poured on both substrates, aligned over each other avoiding any air bubble, clipped properly and kept for UV bonding for 20 minutes. Then, after removing clips, chips were kept on hot plate for 2 hours under 1 Kg weight at 120ºC. Further, weights were removed when substrate cooled to room temperature. Next, they were soaked in IPA for 10 minutes and rinsed with double distilled water. Design of PMMA-PMMA microfluidic chip is shown in Figure 2. Here, detection area was an aperture in PMMA substrate at 90º to sensing area where optical fiber was inserted for fluorescence detection. Sensing area is the area where aptamers were immobilized for interaction with analyte. Inlet and outlet in the microchip were made for insertion of inlet and waste tubing.

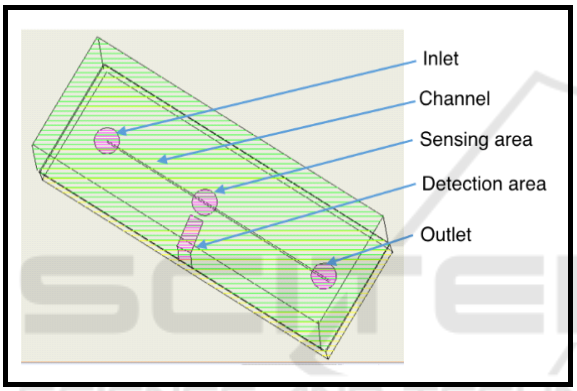

Figure 2: Design of PMMA-PMMA microfluidic chip.

2.3 Sensing Area Modification and Aptamer Immobilization

Sensing area on PMMA was treated with a solution of 10% HMDA in 100 mM borate buffer of pH 11.5 for 2 hours. Following this, the surface of sensing area was washed 3-4 times with distilled water.

Afterwards, surface was activated with 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7 for 2 hours at room temperature followed by washing with 0.1 M phosphate buffer. Then, aminated aptamer sequences were drop casted on sensing area for immobilization and kept for 1 hour at room temperature. Next, channels were washed with 1× Tris EDTA (TE) buffer using syringe pump with flow rate of 5 µl/min. In our two different strategies, different sequences were immobilized using same protocol. Immobilization step was confirmed by immobilizing an aptamer 5'-/5AmMC6/GCC AAA TTG TTT GAC GAG A-3' and passing FAM labeled complementary strand 5'-/56-FAM/ TCT CGT CAA ACA ATT TGG C-3'. Fluorescence intensity from sensing zone was checked before and after passing complementary strand into the microchannel.

2.4 Instrumentation

The instrumentation involved in our setup had a Cyan LED of 490nm as excitation source. LED was placed just above sensing area which was at 90º to detection area. Optical fiber was inserted into detection area of PMMA-PMMA chip followed by an optical narrow bandpass interference filter of 530 nm. This optical fiber passed fluorescence signal picked from the sensing zone of the microchip. Next, the signal from filter was further collected at the photodetector (Ocean Optics HR2000+) and changes were read via commercial Spectrasuite software from Ocean optics.

2.5 Sensing Procedure

In our first strategy, immobilized aptamer 5'-/5AmMC6/GCC AAA TTG TTT GAC GAG A-3' was made to interact with different concentrations of complementary FAM labeled DNA 5'-/56-FAM/ TCT CGT CAA ACA ATT TGG C-3'. Concentra-

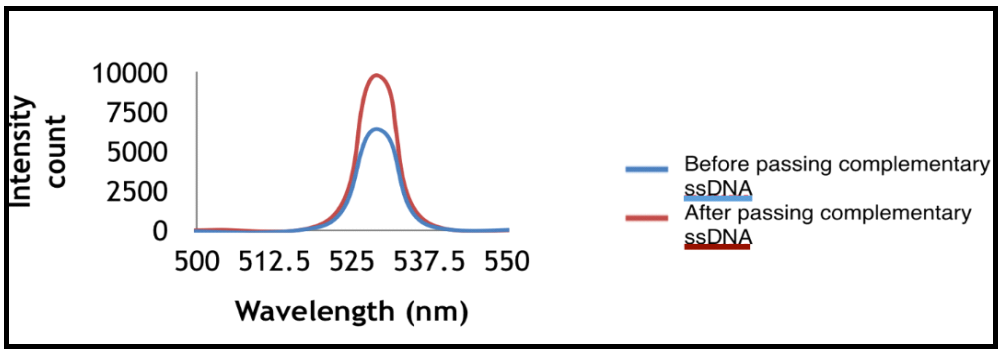

Figure 3: Increase in fluorescence after passing FAM labeled complementary ssDNA.

tion from 0.6-1000ng of complementary DNA was passed through channel for 45 minutes and then channels were washed with 1×TE buffer for 15 minutes. Both steps were performed at 5µl/min. Fluorescence was noticed before and after passing complementary DNA.

For confirmation of sandwich assay protocol, immobilized primary aptamer 5'-/5AmMC6/ TTT TTG GTT GGT GTG GTT GG-3' was made to interact with model analyte thrombin. Different concentrations of thrombin, from 6-600 ng were passed through channel for 45 minutes and then channels were washed using 1×TE buffer for 15 minutes. Both steps were performed at 5 µl/min. Afterwards, FAM labeled secondary aptamers 5'-/56-FAM/TTT TTT TTT TTT TTT AGT CCG TGG TAG GGC AGG TGG GGG TGA CT-3' were passed over the detection zone. Fluorescence intensities from detection zone were recorded before and after passing secondary aptamers.

3 RESULTS AND DISCUSSION

3.1 Confirmation of Sensing Protocol

We checked immobilization of aptamer by passing its FAM labeled complementary strand in the microchannel post immobilization of aptamer in the sensing area. We found an increase in fluorescence intensity post hybridization as shown in Figure 3. This increase shows that hybridization occurred on binding of FAM labeled complementary DNA strand which increased number of FAM molecules in sensing area. Hence, it proved successful immobilization of aptamer.

Further, we tested our first strategy of fluorescence detection using simple hybridization of FAM labeled complementary strand. Increase in fluorescence was seen with increase in concentration of complementary ssDNA as shown in Figure 4. A linear calibration curve was also obtained for the same, as shown in Figure 5. Both figures show proportionate increase in fluorescence with increase in concentration of complementary DNA strand. When complementary DNA strand was passed through channel of microfluidic chip, it hybridized with immobilized aptamer. According to the arrangement in our setup, when FAM labeled complementary DNA was targeted with light of cyan LED source, dye molecules got excited and showed fluorescence which was detected at 90° angle to detection area using attached fiber optic probe and coupled CCD array spectrometer. Therefore, it proved direct relationship between concentration of analyte and fluorescence. According to figure 5, the linear range of detection was between 6 to 1000 ng of ssDNA with 6 ng as the practical LOD for measurement. Hence, this setup could be used for detection of nucleic acid sequences using simple hybridization.

3.2 Sandwich Assay Protocol

As our second detection strategy, we tested fluorescence detection using thrombin protein to validate sandwich assay using primary and FAM labeled secondary aptamers. Again, increase in fluorescence was observed proportionate to the concentration of analyte thrombin as seen in Figure 6. When thrombin was passed through microchannel in microfluidic chip, it interacted with its immobilized primary aptamer. Then, FAM labeled secondary aptamer for thrombin was passed through channel. This secondary aptamer attached wherever thrombin analyte was bound to primary aptamer and hence, any increase in fluorescence was directly related to thrombin concentration, as secondary aptamer would not bind in absence of thrombin in sensing area. As per figure 6, the linear range of detection was found as 125-600 ng with 125ng as the practical LOD for measurement. Therefore, the

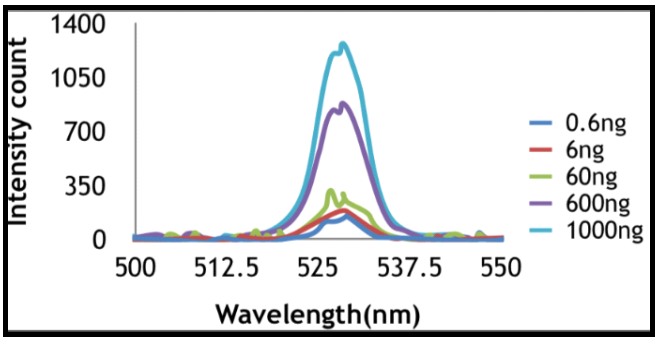

Figure 4: Increase in fluorescence proportionate with concentration of complementary ssDNA.

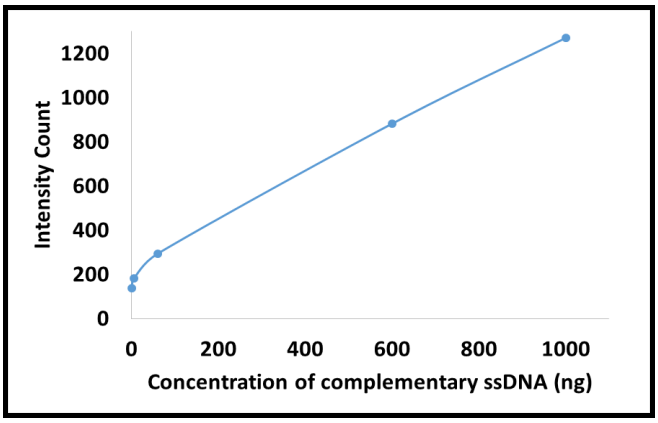

Figure 5: Calibration curve for complementary ssDNA.

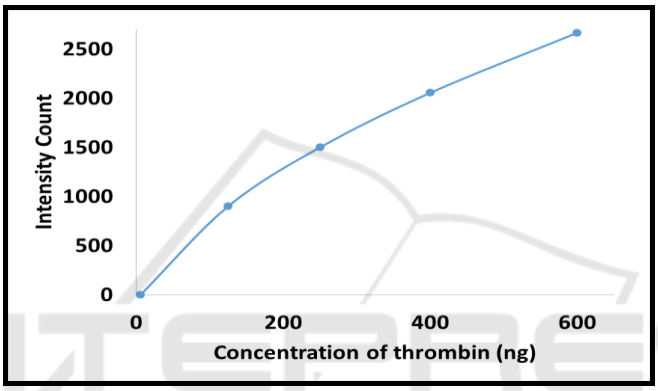

Figure 6: Increase in fluorescence proportionate to increasing concentration of thrombin.

protocol was suitable for detection of diseases such as some types of cancer, microbial diseases, myocardial infarction etc. that gives rise to altered concentration of biomarkers such as protein in blood (Viscidi, 1987).

4 CONCLUSION

We have reported a simple and low cost setup for fluorescence detection using aptamers as probe without using any bulky devices like microscope. We fabricated a PMMA-PMMA microfluidic chip using simple and easily available laboratory techniques and combined the chip with a simple fluorescence detection setup. In this new approach, we used two different strategies to use aptamers as probe. In first strategy, diseases which can be identified by nucleic acid hybridization could be analyzed. We proved this using known sequence of aptamer and its FAM complementary strand. Here, we found that whole setup showed reliable linear range of detection. In other strategy, diseases that can be identified using analysis of any proteinic biomarker could be detected using our sandwich technique. In this, we used thrombin as model target to validate our setup. We found that the minimum concentration of thrombin which could be detected out using our setup was impressive. Both the strategies proved successful with our setup. In future, this setup size could further miniaturized by using a small CCD array, microcontroller and LabVIEW software.

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