



Preliminary Development of a Magnetically Assisted Test Strip (MATS) Cartridge and Fluorescent DNA Aptamer-Magnetic Bead Quantum Dot Sandwich Assays for Multiplexed Food Safety Applications

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Abstract

Preliminary development of a simple mesofluidic multi-channel plastic cartridge with underlying external magnet to drag DNA aptamer-coated paramagnetic beads through fluids in the channels while developing a sandwich assay with quantum dot-conjugated reporter aptamers is described. This approach is superior to traditional lateral flow test strips in several ways including: 1) the ability to control the speed of lateral flow in the channel versus conventional nitrocellulose analytical membranes with fixed wicking times. 2) The use of aptamers for potentially greater affinity and consistency from batch-to-batch versus comparable antibodies. 3) Superior fluorescence efficiency and intensity provided by quantum dots versus conventional fluorescent dyes and 4) the ability to multiplex based on the various colored emissions of different sized quantum dots when excited with a single ultraviolet source. Development of the system from concept to prototype is described along with illustration of sensitive system performance for several food safety-related targets. The system is also clearly adaptable to rapid multiplex detection and sensitive quantitation of clinical biomarkers, drugs, environmental, veterinary or other target analytes.

Keywords: Aptamer; Fluorescence; Magnetic bead; Mesofluidic; Multiplex; Quantum dot; SELEX.

Introduction

Lateral flow (LF) or immunochromatographic (IC) test strips have long dominated the rapid detection markets in the areas of food safety, clinical (especially pregnancy), drug and other testing for their convenience, speed, portability, minimal training requirements, and low cost which is especially important in resource-limited areas or underdeveloped countries [1]. However, LF or IC assays have several drawbacks including a general lack of sensitivity compared to fluorescence or chemiluminescence methods which stems in part from the use of antibodies and in part from the use of visual assessment of colloidal gold or colored latex particle test lines [1]. While the use of fluorophores or even quantum dots (QDs) with LF or IC test strips has proven to increase sensitivity at least ten-fold [2-6], the use of antibodies still contributes some lack of consistency in performance due to the little acknowledged, but quite real, variability in antibody performance from batch-to-batch [7,8]. DNA aptamers developed by the Systematic Evolution of Ligands by EXponential enrichment (SELEX) process may solve the potential antibody problems because they are proven to have higher affinity than comparable antibodies in some cases [9,10] even leading to low zeptomole level detection [11]. And once an aptamer's DNA sequence is known it can be reproduced with extremely high fidelity by chemical synthesis from batch-to-batch [9], thus leading to very high assay consistency.

Therefore, our group and many others have been investigating application of aptamers for food safety assays [4,12-29], because food safety testing is an area of "zero tolerance," i.e., no detectable foodborne pathogens are allowed in tested foods or enrichment broth

cultures derived from tested foods. Thus, ultrasensitivity is desired in food safety assays to reduce culture enrichment times enabling faster clearance and sale of refrigerated or frozen food products which saves money for food producers and increases profitability. Our group has demonstrated that aptamers it has developed against *Campylobacter jejuni* and *Listeria monocytogenes* can be used in QD or enzyme-linked fluorescence aptamer-paramagnetic bead (MB) sandwich assays to achieve detection limits below 10 colony forming units (cfu) or cells per ml [13,15]. Our aptamers and ultralow detection limits even in various foods or culture enrichment broths have been validated by other researchers [11, 16-18] and independent laboratories [13] and led to a recent second place finish among 49 competitors in the U.S. FDA's inaugural Food Safety Testing Technology Challenge [30-32]. While, our aptamers have proven useful for improving detection limits in traditional LF test strip formats with QDs [4] or tube-based MB fluorescent sandwich assays [13,15], they have thus far only been applied to single target tests. In the present work, we describe our first attempts to develop a multiplexed cartridge in which various aptamer-MB plus aptamer-QD sandwich assays are added to multiple linear mesofluidic channels in a plastic cartridge or tray for simultaneous multiplex testing. These assays are assisted by an external magnet to traverse the channels while the sandwich assays develop and are separated from and washed free of excess aptamer-QD conjugates and any potential food particles or other debris to generate a purified fluorescent assay on MBs for visual or fluorometric detection with a single UV excitation source [33].

More complex external and internal magnetic pumping systems [34-36] including the rotating magnetic wheel pump developed at the Naval Research Laboratories [37] are ingenious and highly useful, but we sought the simplest possible and least expensive system which would still enable the concentrating and purifying advantages of MBs

in a small portable package for users in resource-limited environments. Thus, we focused on development of a Magnetically Assisted Test Strip (MATS) cartridge with a simple manually operated or automated external magnet to pull aptamer-coated MBs through fluid or membrane-filled mesofluidic or microfluidic channels as described herein and elsewhere [38]. Only linear movement of the external magnet is required to move the capture aptamer-MB conjugates through the channels to areas of dried and rehydrated reporter aptamer-QD or aptamer-enzyme conjugates and the operator has complete control over the speed of MB movement, thus enabling a major advantage over traditional LF analytical membranes with fixed capillary migration times. Greater reaction times can lead to greater sensitivity by ensuring that binding reactions come to completion or equilibrium. The fluorescence assays can also be washed in the channels with clean buffers following completion of the sandwich assay and prior to analysis in the detection windows shown in the figures.

Materials and Methods

DNA Aptamers, Magnetic Beads, Quantum Dots and Other Reagents or Materials

DNA aptamer development (SELEX) for each of the targets cited in the figures has been described elsewhere in the literature [13, 15, 39]. The actual DNA sequences are not divulged here due to their potential commercial value, but the aptamers used herein are among a subset of the aptamer DNA sequences reported in patents and patent applications [40, 41]. Aptamers biotinylated on their 5' ends were synthesized and purchased from Integrated DNA Technologies (Coralville, IA). Streptavidin-coated M280 (2.8 μm diameter) Dynal paramagnetic MBs and a sampler kit (catalogue no. Q10151MP) of variously colored streptavidin-coated-QDs were purchased from Life Technologies, Inc. (Carlsbad, CA) and Evident Technologies, Inc. (Troy, NY). Sephadex G25 (PD-10) size-exclusion columns were purchased from GE Healthcare, Inc. Silanizing reagent (~ 5% dimethyldichlorosilane in heptane) was purchased from Sigma-Aldrich, Inc. For some experiments, translucent polypropylene 8-channel low-profile troughs or reservoirs were purchased from Corning Axygen Scientific, Inc. to act as MATS cartridge surrogates for developmental purposes. *Campylobacter jejuni* (strain 29428) was purchased from American Type Culture Collection (ATCC; Manassas, VA) and staphylococcal enterotoxin B (SEB) was purchased from Sigma-Aldrich, Inc.

Aptamer Conjugation to Magnetic Beads and Quantum Dots

One mg of various 5' biotinylated capture aptamers specific for each assay target were dissolved in 500 μl of sterile phosphate buffered saline (PBS, pH ~ 7.2) and added to 500 μl of streptavidin-coated M280 MBs (~ 3 x 10⁷ MBs) and gently mixed for 1 h at room temperature (~ 25°C). Aptamer-biotin-streptavidin-MBs were washed three times in 1 ml of sterile PBS after collection of the MBs on a Dynal MPC-S magnetic rack. Five hundred μg of various 5' biotinylated reporter aptamers specific for each assay target were added to 50 μl of each type of colored streptavidin-QD in 1 ml of sterile PBS and gently mixed at room temperature for 1 h. Aptamer-5'-biotin-streptavidin-QD conjugates were then purified in the void volume (pooled fourth and fifth 1 ml eluted fractions) of a sterile PBS-equilibrated PD-10 column.

Magnetically-Assisted Test Strip (MATS) Cartridge, Automated Assay Processor and Assays

A credit card-sized MATS prototype cartridge (Figures 1 and 2) was milled out of polycarbonate plastic slabs by Precision Mold and Tool Group (PMTG), Inc. (San Antonio, TX). The automated magnetic MATS assay processor was designed and built around the use of 8-channel plastic troughs or reservoirs by Taboada Research Instruments, Inc. (TRI; San Antonio, TX). TRI's unique external magnet design involved putting the North and South poles of a very strong rare Earth magnet in close proximity to one another instead of simply using the edge of a rectangular permanent magnet. The resulting geometry looks something like a vise that is closing when viewed from the side. The magnetic field lines are concentrated at both edges and create a powerful (> 4 Tesla) trap for the magnetic beads, which align themselves in a tight rectangular area that is ideal for fluorescence detection in the final detection window. A screw gear mechanism and servo motor were used to slide the external magnet in a linear fashion underneath the 8-channel trough which emulated the MATS cartridge for initial experiments.

In general, 20 μl of aptamer-QD conjugates were added about 3 cm from the entrance of each channel of the 8-channel troughs or MATS cartridge and allowed to dry overnight. Then 50 μl of aptamer-MB conjugates were added to the entrance of various channels in the 8-channel troughs which were pre-treated with silanizing reagent for 30 min and washed in deionized water prior to experiments to help avoid trailing of the MBs. Varied amounts of the target analytes (*Campylobacter jejuni* bacteria and staphylococcal enterotoxin B (SEB)) in 50 μl of PBS were added to the channels as shown in the figures and allowed to bind the capture aptamer-MB complexes for 5 min at room temperature. Blanks without the targets were also run to assess background fluorescence levels.

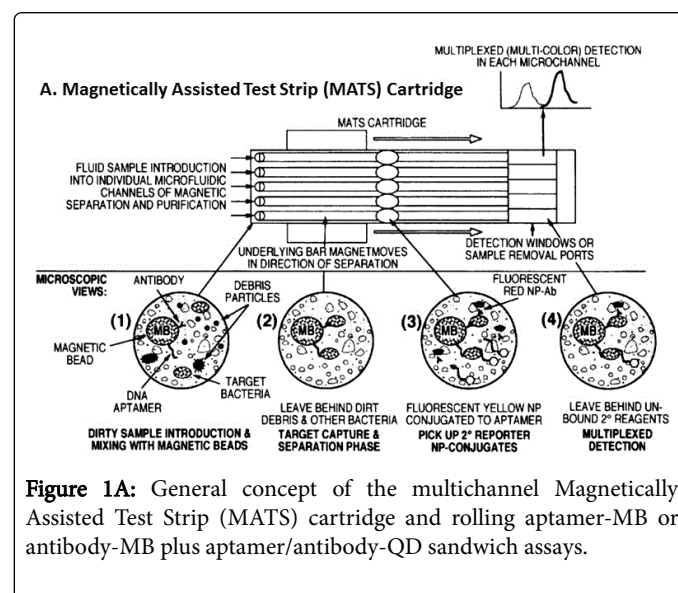


Figure 1A: General concept of the multichannel Magnetically Assisted Test Strip (MATS) cartridge and rolling aptamer-MB or antibody-MB plus aptamer/antibody-QD sandwich assays.

Thereafter, 500 μl of PBS was added to each channel and the underlying external magnet was slowly pulled along the length of the channel either manually or using the automated servo motor mechanism. Reaction times were varied, but 5 min of total time to traverse the channels with the external magnet gave the best results. Once the sandwich assays had formed and been processed at the end of each channel, they were assessed visually, digitally photographed

with and without an external UV light source, and siphoned out of the channels (~ 100 µl total volume). Sandwich assays on the MBs were then resuspended in 2 ml of PBS in clear plastic cuvettes and assessed

using the UV channel of a handheld PicoFluor™ fluorometer (Turner BioSystems, Inc., currently the QuantiFluor™ by Promega Corp., Sunnyvale, CA).

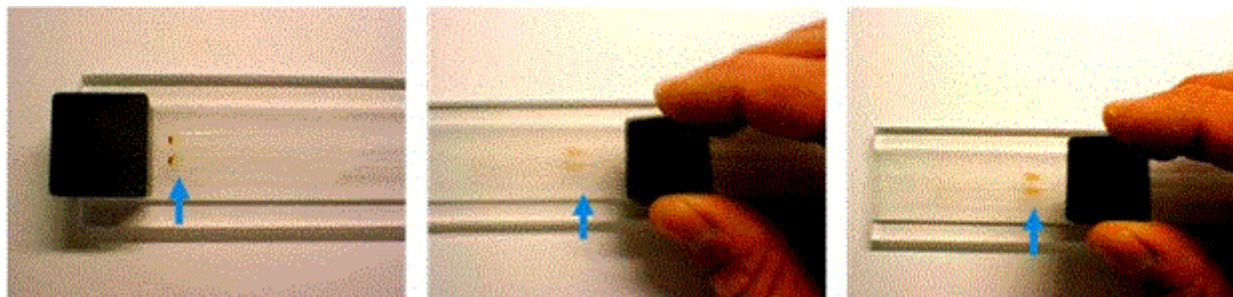


Figure 1B: Simple proof-of-concept experimental results showing that MBs can be pulled along the length of a narrow mesofluidic (2 mm wide) channel cut into polycarbonate by means of the edge of a permanent magnet.

Results

Figure 1A illustrates the basic concept of the MATS cartridge in which a “rolling” fluorescent sandwich assay is developed on the surface of capture aptamer- or antibody-coated MBs added to the entrance of the cartridge, followed by the sample containing the target analyte. After binding to the target analytes, the MBs are pulled to the end of the channel where the sandwich assay on the MBs has left behind much of the matrix and debris or interfering materials. At this point, the stationary MBs which are held in place by the external magnet can be further purified by suctioning out the matrix and washing the MBs several times with clean buffer followed by fluorescence detection resulting from QDs and captured analytes on the MB surfaces. It should be noted here that multiplex detection is possible both by detection of different colored QDs in different channels or different colored QDs in the same channel [33]. Figure 1B shows the results of a very simple proof-of-principle experiment to prove that MBs could be pulled along a buffer-filled mesofluidic (2 mm wide) channel by the edge of a strong permanent magnet.

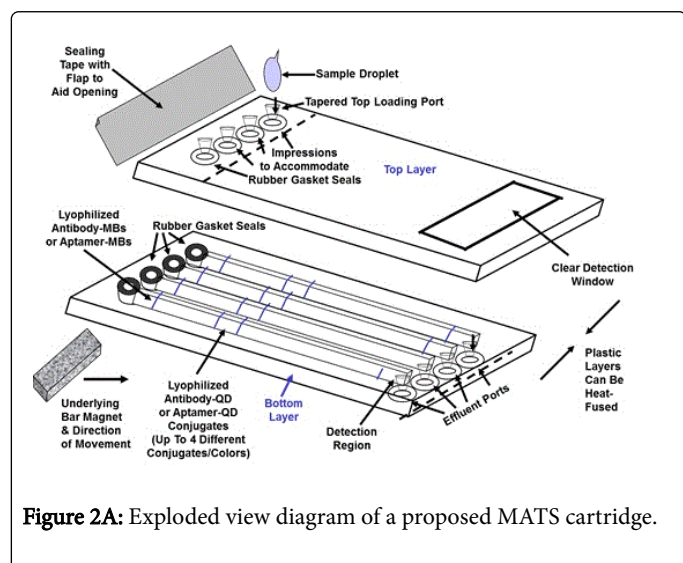


Figure 2A: Exploded view diagram of a proposed MATS cartridge.

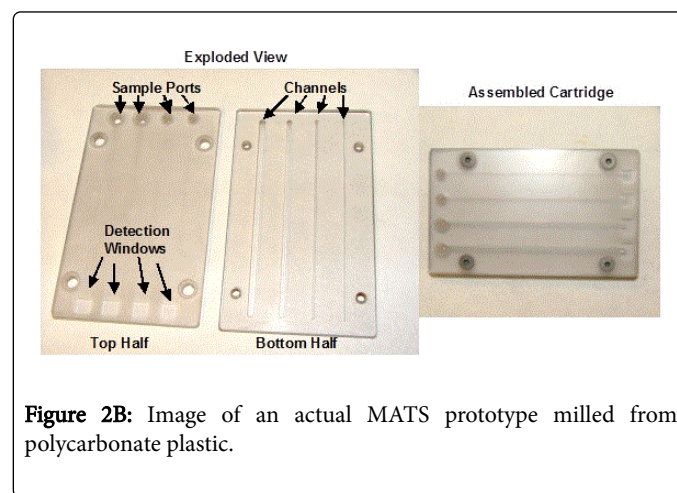


Figure 2B: Image of an actual MATS prototype milled from polycarbonate plastic.

Figure 2A illustrates an exploded view of a credit card-sized prototype MATS cartridge. This design was milled from polycarbonate to produce the prototype cartridge shown in Figure 2B with channels having various diameters from 0.5 to 2 mm. The 1 and 2 mm diameter channels proved to be preferable in experiments because they clogged less frequently at the levels of MBs and target analytes used.

Figure 3A illustrates tracking of reporter aptamer-QDs in a PD-10 (Sephadex G25) column during the purification process to better enable capture of the fluorescent aptamer-QDs in the column's void volume. Figure 3B illustrates the ease of multicolored or multiplex detection of various QDs in the prototype MATS cartridge with a single UV excitation source (simple mineral light).

Figure 4A is a photo of the special underlying magnet with N and S poles held about 5 mm apart from one another to create a rectangular trap area for the MBs. This special underlying magnet was used in the automated servo motor-driven MATS (8-channel trough) processor shown in Figure 4B. The brown aptamer-MBs are shown at the beginning of each channel in Figure 4C and after stopping to react with the dried reporter aptamer-QDs several cm down the channels in Figure 4D.

A potential future epifluorescence optical quantitation system for the MATS cartridges in central laboratories is sketched in Figure 5.

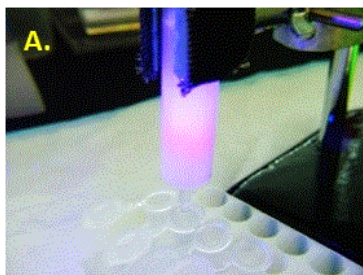


Figure 3A: Purification of aptamer-biotin-streptavidin-QD conjugate in a Sephadex G25/PD-10 column visualized by UV light excitation.

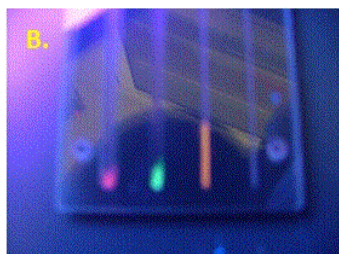


Figure 3B: Image of multi-colored types (various sizes) of QDs in the MATS cartridge prototype showing that only one UV light source is required to induce all three fluorescent color emissions [33]. The far right channel is empty.

The sketch illustrates the possible use of objective lenses to magnify fluorescence from the aptamer-QD plus aptamer-MB sandwich assay complexes following excitation through the objective lenses from a UV light source that is reflected down onto the MATS channels by dichroic mirrors. Fluorescent emission light from QDs in the various channels would then be sent through the objective lenses through fluorescence emission filters and to photodiode detectors for sensitive detection and fluorescence quantitation. While this detector design is more sensitive and accurate, it is more complicated and expensive and not required for simple visual assessment in low-resource settings. Indeed, simple manual movement of the underlying magnet and visual assessment of

fluorescence from a UV mineral light are the minimal detection requirements.

An example of simple visual assessment of red QD 655 fluorescence following an aptamer-MB *Campylobacter jejuni* experiment is shown in Figure 6. In the figure, duplicate positive channels containing 100,000 live *C. jejuni* cells (designated plus (+) in Figure 6) are shown aggregated around the MBs at the end of the channels with strongly visible orange-red fluorescence while the two blank (designated minus (-) in Figure 6) channels demonstrated little or no orange-red fluorescence.

Figure 7A illustrates the concept of the aptamer-based MB and aptamer-QD sandwich assay, while Figures 7B and 7C illustrate validation of *Campylobacter* cell capture from the assay shown in Figure 6. In Figure 7B, the *C. jejuni* bacteria were stained with Coomassie blue dye and washed in PBS to make the aptamer-MB-captured cells visible under brightfield microscopy at 1,000X total magnification.

When this same sample was subjected to UV fluorescence microscopy, red patches or clumps corresponding to the locations of the *C. jejuni* cells under brightfield microscopy emerged as shown in Figure 7C. Figure 7D illustrates that this assay was sensitive to a level of at least 10 *C. jejuni* cells per ml as previously published for similar tube-based aptamer-MB plus aptamer-QD assays following assessment of siphoned MB samples resuspended in 2 ml of PBS using a Picofluor™ handheld fluorometer [14,15].

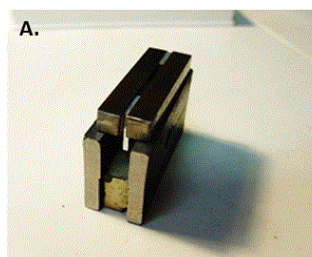


Figure 4A: Custom designed underlying magnet for movement of MBs along the MATS channels having N and S poles adjacent to one another across a small (5 mm) gap to create a rectangular MB trap.

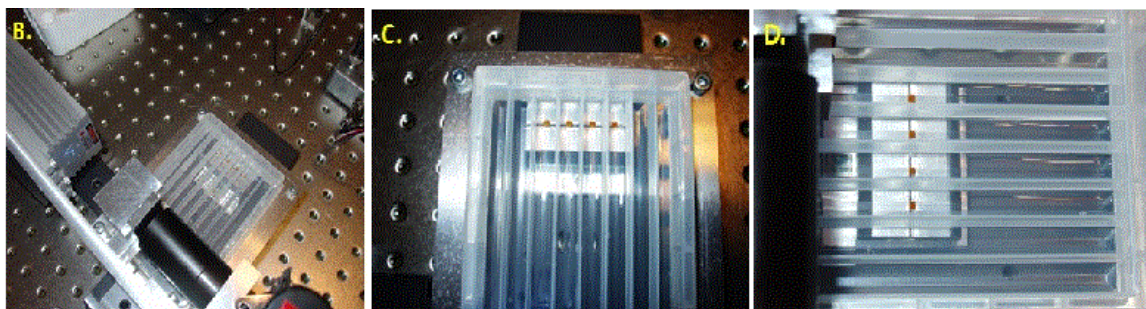


Figure 4B-D: Various views of the automated MATS processor using an 8-channel trough and servo motor screw gear mechanism to move the underlying magnet slowly along the MATS channels to develop the fluorescence sandwich assays.

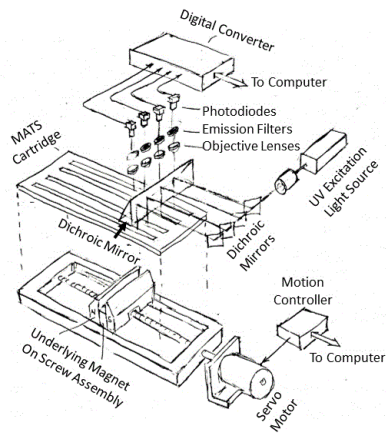


Figure 5: Proposed epifluorescence optical design for more sensitive and accurate quantitation of target analytes in the MATS cartridge versus simple visual assessment using a handheld UV light and image analysis.

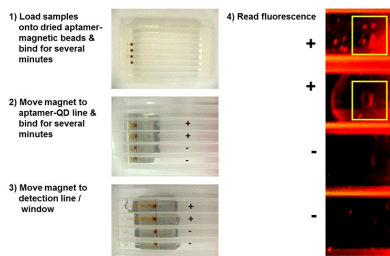


Figure 6: Visual results of a *Campylobacter jejuni* aptamer-MB/QD sandwich assay in an 8-channel trough which emulates a MATS cartridge. The two (+) channels received ~ 100,000 live *C. jejuni* cells per channel while the two (-) channels represented blanks without any bacteria. Problematic trailing MBs is shown in part 3, but this was later solved by first silanizing the channels to maintain a tight band of MBs throughout the assay development process.

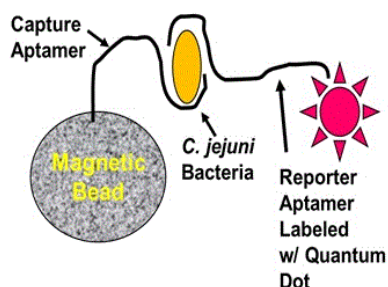


Figure 7A: Diagram of the general aptamer-MB plus aptamer-QD sandwich assay.

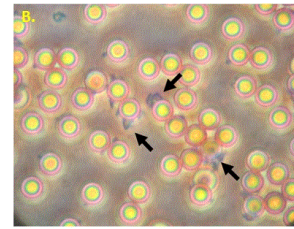


Figure 7B: 1,000X brightfield microscopy image showing Coomassie blue-stained *Campylobacter jejuni* cells captured by aptamers on the surface of MBs (arrows).

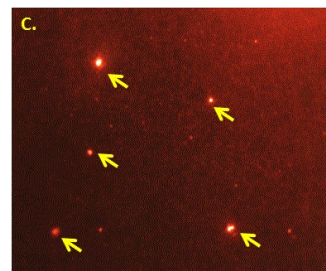


Figure 7C: A fluorescence microscopy image (1,000X) of the same sample as in (B) showing red QD 655 *C. jejuni* cell detection (arrows).

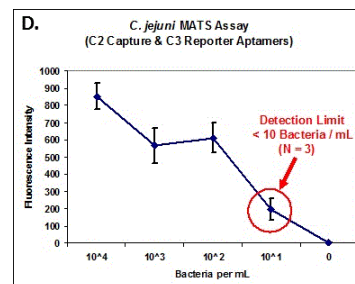


Figure 7D: Titration curve of the *C. jejuni* fluorescence aptamer-MB/QD sandwich assay performed in a prototype MATS cartridge demonstrating ultrasensitive detection to a level of 10 or fewer cells per ml as previously reported [15]. Errors bars represent standard deviations of the mean fluorescence values for three independent measurements (N=3). Results were quantified after MB resuspension in 2 ml of PBS using a Picofluor™ handheld fluorometer.

Finally, Figure 8 illustrates that the MATS aptamer-MB/QD assays can be extended to biotoxins such as SEB in PBS buffer with a likely limit of detection (LOD) of 100 ng or less per sample using aptamers previously cited by Bruno and Kiel [39]. Again, SEB aptamer-MB/QD assay samples were siphoned from the end of the MATS channels and fluorescence was quantified using a Picofluor™ handheld fluorometer after resuspension of samples in clear plastic cuvettes with 2 ml of PBS.

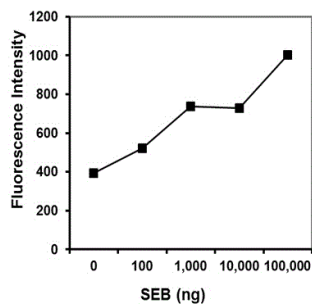


Figure 8: Titration curve for staphylococcal enterotoxin B (SEB) using aptamers developed by Bruno and Kiel [39] used in the fluorescent aptamer-MB/red QD 655 sandwich assay in a prototype MATS cartridge with a 100 ng limit of detection (LOD) in PBS. Results were quantified after resuspension of MBs in 2 ml of PBS using a Picofluor™ handheld fluorometer.

Discussion

The present report documents preliminary development of a simple mesofluidic system designed to be facile and work at low cost in resource-limited environments without sacrificing detection sensitivity, multiplexing ability, or speed. To achieve these goals, our group has built upon FDA award winning [30-32] aptamer-MB plus aptamer-QD sandwich assay technology and transferred that technology first to lateral flow test strips without MBs [4] and then to the mesofluidic channels of the prototype MATS cartridge as reported herein.

While the system has been shown to function well and produced sensitive detection of food safety-related analytes (Figures 6 and 8) the MATS design can potentially be improved in several ways. Firstly, the MATS channels might benefit from addition of a paper or other porous solid matrix to better hold back or filter out food or other debris and interferents while still enabling the relatively free movement of the aptamer-MBs and aptamer-QDs in the channels as the sandwich assay rolls along and fully develops at the end. The addition of a paper matrix in the channels could decrease or eliminate washing or back flushing with buffer to purify the MB assay prior to fluorescence detection. One such paper material is Fusion 5 membrane manufactured by Whatman Inc. [42]. Fusion 5 is used a single membrane replacement for traditional multilayered lateral flow test strips in a method known as “boulders in the stream” lateral flow immunoassay [42] and may be suitable for use in MATS channels. Our group is currently investigating this Fusion 5 matrix possibility in MATS channels. In the authors’ long experience with MB-based and lateral flow assays, we have only encountered one other patent application which claimed to move antibody-coated MBs or magnetic particles into and through the analytical membrane of a lateral flow test strip [43], but this patent application has not yet resulted in a commercial product to date.

More sophisticated and sensitive (vs. the human eye) QD fluorescence optical detection and quantitation systems for multiplex (multi-colored) detection in single or multiple channels of a MATS cartridge such as the proposed design shown in Figure 5 are also being investigated. The Figure 5 system will be compared to simple UV light exposure of assays conducted in MATS cartridges followed by color image analysis of digital photos to determine which engineering

approach yields the most sensitive and discriminatory fluorescence color quantitation at the best overall manufacturing and operating costs.

In the end, we hope to deliver an ultrasensitive, multiplexed system to the food testing market which combines the concentrating and purifying power of aptamer-MB conjugates with the high affinity and high signal-to-noise ratios of aptamer-QD conjugates in a single easy to use multiplexed cartridge. Of course aptamers are not necessary to the system and aptamers could be replaced by antibodies, if desired, but most aptamers will probably confer greater affinity [9-11], specificity [44,45], and reproducibility [9] to the end user. Finally, it should be clear that if MATS is a success in complex diluted food or enrichment broth samples [13,15], it could also function with body fluids including whole blood, serum and urine, making MATS technology amenable to the much broader clinical biomarker and related diagnostics markets.

Acknowledgments

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