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Development and application of DNA-aptamer-coupled magnetic beads and aptasensors for the detection of *Cryptosporidium parvum* oocysts in drinking and recreational water resources

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Abstract

Environmentally stable and disinfectant-resistant oocysts of *Cryptosporidium* spp. shed in the feces of infected humans and animals frequently contaminate water resources with subsequent spread via potable and recreational waters. The current monoclonal antibody-based methods for detecting them in water are slow, labor-intensive, and skill demanding while requiring interpretation. We have developed DNA-aptamer-based aptasensors, coupled with magnetic beads, to detect and identify the oocysts of *C. parvum* for monitoring sources of recreational and drinking waters. A sensitive and specific electrochemical aptasensor (3′-biotinylated R4-6 aptamer) was used as a secondary ligand to bind the streptavidin-coated magnetic beads. This was incorporated into a probe using gold nanoparticle-modified screen-printed carbon electrodes (GNP-SPCE). Square wave voltammetry (SWV) allowed for specific recognition of *C. parvum* oocysts. The aptamer-coated probes had an oocyst detection limit of 50. It did not bind to the cysts of *Giardia duodenalis,* another common waterborne pathogen, thus indicating its high specificity for the target pathogen. The system could successfully detect *C. parvum* oocysts in spiked samples of the raw lake and river waters. Therefore, the combined use of the aptasensor and magnetic beads has the potential to monitor water quality for *C. parvum* oocysts in field samples without relying on monoclonal antibodies and skill-demanding microscopy.

Keywords: *Cryptosporidium*; oocysts; water; DNA aptamers; aptasensors; magnetic beads

Introduction

Enteric protozoa of the genus *Cryptosporidium* (Fayer et al. 2000) are the cause of cryptosporidiosis, an acute and potentially fatal form of diarrhea in humans and animals throughout the world (Bouzid et al. 2013; Chalmers and Davies, 2010). The fecally discharged oocysts can survive in the environment for months (Armon et al. 2016). Source water contamination with the oocysts occurs regularly from the feces of humans and livestock, as well as from feral and wild animals (Ramsay et al. 2014). As a result, drinking and recreational waters are both common vehicles for these organisms (Fayer et al. 2000), with small (Karanis et al. 2007) and large (Baldursson and Karanis, 2011) waterborne outbreaks.

Better protection and treatment of source waters can reduce oocyst levels with a corresponding reduction in health risks (Goh et al. 2005). Due to their high resistance to water-disinfecting chemicals, such as chlorine, reliable physical removal of the oocysts via flocculation and filtration is crucial (Quilez et al. 2005; Erickson and Ortega 2006). Malfunctioning of physical removal is known to cause waterborne outbreaks (Pollock et al. 2008).

Alternative water disinfection strategies (e.g., ultraviolet light and ozone treatment) may be effective in oocyst inactivation (Erickson and Ortega 2006), but do not obviate the need for physical removal of the oocysts.

As the levels of *Cryptosporidium* oocysts are generally present at much lower numbers in surface waters compared to bacterial indicators of fecal pollution, multi-liter volumes of source waters require concentration prior to testing (Kitajima et al. 2014). This is often accomplished by using cartridge filters to reduce the sample volumes to more manageable levels, followed by a second concentration step using immuno-magnetic separation. The oocysts are detected in concentrated

samples by fluorescence microscopy using oocysts-specific monoclonal antibodies (USEPA method 1623). This method is time-consuming and labor-intensive, as well as being dependent upon subjective judgment by the analyst. Considerable expertise is required to accurately and consistently identify oocysts among the debris on microscope slides.

Aptamers are single-stranded, synthetic oligonucleotides (DNA or RNA) which can fold into 3-dimensional shapes capable of binding non-covalently and with high affinity to target molecules (Ruscito and DeRosa, 2016). Such targets may range from simple organic (Ellington and Szostak, 1990) and inorganic (Hofmann et al. 1997) molecules to large and complex structures such as carbohydrates (Su et al. 2010), Proteins (Zhang et al. 2019), nucleic acids (Ku et al. 2015) and even whole organisms (Zhang et al. 2019). Aptamers are generated via an *in vitro* process known as the Systematic Evolution of Ligands by EXponential enrichment (SELEX) (Tuerk, and Gold, 1990).

SELEX comprises of three steps: selection, partitioning, and amplification (Ruscito and DeRosa, 2016). Since aptamers have high specificities and binding affinities to their targets, they are suited to the development of a variety of electrochemical biosensors for the detection of small molecules, metal ions, and proteins (Citartan et al. 2016, Zhang and Tao, 2016, Wu et al. 2016). In diagnostics, aptamers act as sensor agents to capture their respective targets (Pinto et al. 2016), and the capture event detected via a labelled- (Wang et al. 2015) or label-free approach (Yang et al. 2015).

The strong target-binding affinities of aptamers, combined with their higher stability under heat or pH variations (Hamaguchi et al. 2001, Ng et al. 2006), make them desirable alternatives to antibodies. Furthermore, aptamers can be readily produced, isolated, and modified *in vitro.* Their automated detection and measurement are now possible using electrochemical aptasensors based

on amperometry, impedimetry, and potentiometry (Escamilla-Gómez et al. 2008, Labib et al. 2012, Hernández et al. 2014).

We recently developed a label-free electrochemical aptasensing method to detect *Cryptosporidium parvum (C. parvum)* oocysts in spiked fruit juices (Iqbal et al. 2015) as an alternative to conventional methods such as microscopy and PCR. Herein, we present a novel electrochemical sensing strategy for the detection of *C. parvum* oocysts based on a specific aptamer by using streptavidin-modified magnetic beads for signal amplification. The main objective here was to select and apply DNA-aptamer-based aptasensors and magnetic beads to detect the oocysts of *Cryptosporidium* in raw waters used for recreation and as a source for drinking water. The proposed technology is designed to overcome the limitations of the current and widely used methodology based on monoclonal antibodies.

Materials and Methods

Aptamer-coupled magnetic beads

We have previously described the single-stranded DNA aptamer ((R4-6) used in this study (Iqbal et al. 2015). Magnetic beads coupled to R4-6 were generated by the following procedure; 100 µL of 20 µM of the R4-6 aptmer-3′-TEG-biotin and ssDNA library-3′-TEG-biotin (Integrated DNA Technologies, San Diego, CA, USA) in Dulbecco's phosphate buffered saline with $CaCl_2$ and $MgCl_2$ (DPBS) (Cat. # 114-059-101, VWR, Mississauga, ON, Canada), were denatured for 5 min at 95°C then renatured on ice for 10 min. R4-6 aptamer-3′-TEG-biotin was incubated with 1 µL of 10 mg/mL of streptavidin-coated magnetic nanoparticles (Cat. # 9-19-132, micromod Partikeltechnologie GmbH, Rostock, Germany) for 2 h, at room temp. Similarly, ssDNA library-3′-TEG-biotin was used for control experiments. The capture aptamer-magnetic beads (MB) were

collected for 2 min using a Dyna Mag™-2 Magnet (Cat. # 12321D, Invitrogen, Burlington, ON, Canada) collection rack. Fluid was carefully siphoned off with a pipet tip, and the R4-6 aptmer-3′-TEG-biotin –magnetic bead complex was washed with 200 uL of DPBS, and finally resuspended in 100 uL DPBS.

Aptamer-magnetic beads and C. parvum oocyst complex

Prior to experimentation, 1 x 10^5 *C. parvum* oocysts (Waterborne, Inc., New Orleans, LA, USA) were washed twice in DPBS at 14,000 x g for 5 min and resuspended in DPBS. Low protein-binding microcentrifuge 1.7 mL tubes (VWR, Cat. # 3207, Corning Life Sciences, New York, NY, USA) were used in all experiments. A 100 uL volume of washed *C. parvum* oocysts was incubated with 100 uL of R4-6 aptamer-3′-TEG-biotin – magnetic beads complex for 2 h at room temperature**.**

Aptasensor preparation

A gold nanoparticle-modified screen-printed carbon electrode (GNP-SPCE) (DRP-110, L33xW10xH0.5, Metrohm, Mississauga, ON, Canada, Inc.) was washed thoroughly with deionized nuclease-free water (Cat. # 10977-015, Invitrogen) and then dried with N_2 gas. Following this, the R4-6 aptamer without biotin label (Integrated DNA Technologies) was denatured by heating for 5 min at 95°C and snap-cooled on ice for 10 min. The thiol-modified primer (Integrated DNA Technologies,) was heated at 60°C for 5 min before use to prevent aggregation. This was followed by reduction of the thiol-modified primer, 5'-/5ThioMC6-D/GGC TTC TGG ACT ACC TAT GC-3', modified at the 5' position with a 6-hydroxyhexyl disulfide group. Briefly, 4 µL of 100 µM thiol-modified primer were mixed with 8

μL of 10 mM tris-(2-carboxyethyl) phosphine (TCEP) (Cat. # 646547, Sigma-Aldrich, Mississauga, ON, Canada) and incubated for 1 h at room temp. Subsequently, 2 μM of the reduced primer were incubated with 2 μM of the denatured R4-6 aptamer for 1 h at room temp. to form a DNA hybrid. Finally, the electrode was incubated overnight in a humidity chamber, with 50 μL of the hybrid**.** The electrode was rinsed with DPBS and incubated with 0.1 mM 2-mercaptoethanol for 30 min to reduce the background oxygen contributions and nonspecific interactions between the probe and the gold surface, and allow the probe to adopt an upright position and measure the baseline (Herne and Tarlov, 1997, Liu and Liu, 2017). Next, the electrodes were dried with N_2 gas, and incubated with 100 uL of 3′-Aptamer-magnetic beads and *C. parvum* oocysts complex for 2 h at room temperature, electrochemical measurements performed and results recorded. It should be noted that electrochemical analysis of the oocysts required a vigorous de-aeration of the measurement buffer. This was carried out using pure N_2 gas for 30 min prior to analysis.

Electrochemical measurements

Square wave voltammetry (SWV) was performed using an electrochemical analyzer (CH Instruments, Inc., Austin, Texas, USA) connected to a computer. All measurements were taken at room temp. in an enclosed and grounded Faraday Cage (CH Instruments). A conventional three-electrode configuration printed on a ceramic substrate, including an aptamer-modified GNP-SPCE electrode as the working electrode, a carbon counter electrode, and a silver pseudo-reference electrode, were used. A three-electric contact edge connector (Metrohm, Canada) was used to connect the screen-printed electrode with the potentiostat. The open-circuit or rest-potential of the system was measured prior to all electrochemical experiments to prevent any

sudden potential-related changes in the self-assembled monolayer. SWV measurements were carried out in the range of –300 to 500 mV with a step potential of 4 mV, amplitude of 5 mV and frequency of 10 Hz. Electrochemical measurements were performed in phosphate buffered saline (PBS, pH 7.4), containing 2.5 mM of K4[Fe $(CN)_6$] and 2.5 mM of K3[Fe$(CN)_6$]. All measurements were repeated a minimum of three times with separate electrodes to obtain statistically significant results.

Filtration of water samples

One sample of three to five liters was collected from three separate sites in southern Ontario (Laurel creek, Waterloo; Lake Ontario and Credit River near Toronto). The river water was collected from the surface of the river with care to avoid any floating debris. A 500 mL volume of water from each sampling point was separately spiked with 5,000 oocysts and left overnight. Spiked water samples were filtered using a flatbed membrane filtration unit system through Supor® white gridded membrane filters with a 0.2 µm pore size and 47 mm diameter (PALL Corporation, Cat. # 66234, -VWR, Toronto, ON, Canada) by applying a vacuum. After filtration, the oocysts were collected from the top of the filter using a cell scraper and centrifuged at 14,000 x g for 15 min at 4°C. Finally, the sediment was resuspended in 100 uL of PBS by vortexing.

Results

Aptamer immobilization for use in AptaCapture assays

A critical step in an AptaCapture assay is the immobilization of the aptamer on the surface platform without compromising its affinity for the target. Among several conjugation methods

used for this purpose is non-covalent binding to biotin-streptavidin (Citartan et al. 2014, Shim et al. 2014).

A 3′-biotinylated R4-6 aptamer was used as a secondary ligand to bind the streptavidin magnetic bead (MB) complex and its concentration was optimized. The sequence of this aptamer was from a previous publication (Iqbal et al. 2015). For this purpose, testing was carried out to find the optimum concentration of streptavidin-MB for use in the sandwich assay. The assay was performed by incubating the magnetic beads with 3′-biotin-R4-6 aptamer. An easy and low-cost electrochemical aptasensor was used for evaluation of 3′- biotinylated R4-6 aptamer-magnetic bead binding with *C. parvum* oocysts (5,000 oocysts) complex. This was carried out by incubating a 5′-thiol-modified R4-6 self-assembled monolayer on gold nanoparticle-modified screen-printed carbon electrode (GNP-SPCE), which served as the aptasensor platform. Square wave voltammetry (SWV) evaluated the selectivity of the aptasensor. As shown in **Figure 1,** the current response for aptamer-MB-oocysts complex was considerably higher than the aptamer without *C. parvum* oocysts. These results suggested that the aptasensor had good selectivity for the oocysts.

The advantages of this approach were the ease of preparation and potential portability of the aptasensor **(Figure 2).** To ensure accuracy, square wave voltammetry measurements were performed before and after capturing the oocysts to guard against any possible variations in the baseline current intensity that might be caused by the different conformations adopted by aptamers on the electrode surface.

Limit of detection of aptamer-MB and *C. parvum* oocysts complex

SWV is a suitable tool to demonstrate the successful immobilization of molecules on to the electrode surface. Aliquots of varying concentrations of *C. parvum* oocysts (ranging from 50 to 900) in 50 µL of DPBS were incubated with 3′-biotinylated R4-6 aptamer-magnetic bead complex for 2 h at 25°C. Next, the mixture was incubated with the probe-modified GNPs-SPCE at 25 °C for 1 h in a dark humidity chamber. SWV was performed at each concentration, and it was observed that the binding between 3'-biotinylated R4-6 aptamer-magnetic bead with *C. parvum* oocysts and the immobilized aptamer caused an increase in the current intensity and a cathodic shift, as shown in **Figure 3A.** The measurement time was less than 10 s. A rational explanation for this behavior may be as follows; in the present aptasensor system, the complex formed through hybridizing the electrode-bound R4-6 aptamer to 3′-biotinylated R4-6 aptamer-magnetic beads with *C. parvum* oocysts allows for quick electron transfer due to the presence of magnetic nanoparticles that act as tiny conduction centers (Citartan et al. 2016). Hence, the modulation of the electrochemical signal was recorded as a function of the current intensity *(I)* and peak potential *(E)*. As shown in **Figure 3A,** *I* value increased linearly with an increase in the number of oocysts, in the range from 50 to 900 oocysts.

A regression equation of $y = 0.0412x + 2.3459$ ($R^2 = 0.9756$) was obtained, where y is the value in µA and x is the logarithmic number of *C. parvum* oocysts, as shown in **Figure 3B.** Selectivity is an important index for evaluating the performance of the aptasensor. Beyond 900 oocysts, the response became non-linear, indicating the saturation of the surface with the target molecules, and the limit of detection (LOD) was 50 oocysts. The median infectious dose for *C. parvum* ranges from less than 10 to over 1,000 oocysts based on human volunteer studies (Ryan et al. 2014).

Water sample analysis

The electrochemical aptasensor was used to detect the presence of *C. parvum* oocysts in spiked water samples collected from Laurel Creek, Lake Ontario and Credit River. The coated aptasensor detected the presence of *C. parvum* by SWV in the three spiked water samples. As shown in **Figure 4**, , the three raw water samples showed different levels of debris, with the one from Laurel Creek having the highest level and that from Lake Ontario the lowest.

In order to confirm the electrochemical findings, a parallel control experiment was run using DPBS only. As evidenced by the relative increase in the current intensity (**Figure 5),** the 3′-biotinylated R4-6 aptasensor was capable of detecting the oocysts in the spiked water samples. Testing was performed using DPBS alone, and different concentrations of spiked *C. parvum* oocysts in the water samples (0, 100, 200, 300, 400, 500, 600 and 700 oocysts). SWV was performed at each concentration and it was observed that the binding between oocysts in the water samples and the immobilized aptamer caused an increase in the current intensity. The, ΔI value increased linearly with an increase in the number of oocysts, in the range from 100 to 700 oocysts. A regression equation of $y = 0.0795x - 3.38$ ($R^2 = 0.9375$) was obtained.

Discussion

Cryptosporidium spp. continues to be a globally significant cause of waterborne and potentially fatal diarrhea in humans. The risk of spread of the parasite continues to rise with increasing fecal pollution of water sources along with unprecedented demands on them. The high environmental stability and disinfectant resistance of *Cryptosporidium* spp. oocysts exacerbate the problem and indicate the need for routine monitoring of source waters in addition to the use of effective water

filtration systems and/or alternative disinfection technologies (e.g., ultraviolet light, ozone) to reduce the risk of waterborne transmission.

The present study describes a highly sensitive and specific electrochemical aptasensor for the detection of *C. parvum* oocysts in water samples based on the use of aptamer-coated magnetic beads. Through the use of streptavidin-conjugated magnetic beads, the 3'-biotinylated R4-6 aptamer could be firmly immobilized onto the GNP-SPCE. *C. parvum* oocysts could then be immobilized onto the electrode through the specific recognition of streptavidin and biotin. Based on the results of this study SWV analysis of MB-coated aptasensor exhibited a LOD of 50 oocysts. In addition to the high sensitivity of this technology, the aptasensor also displayed specificity towards *C. parvum* oocysts over *G. duodenalis,* another common waterborne parasite, providing a promising method for oocyst detection in various water samples.

Immobilization of aptamers on to a surface is commonly achieved by modification at the 5′- or 3′-end with biotin, thiol, or amino functional groups, which will conjugate with the appropriate complement. Immobilization can also be achieved by the Watson–Crick base-pairing interaction using an additional sequence appended to the aptamer, and a complementary sequence immobilized to the surface (Citartan et al. 2016). This complementary sequence is conjugated with the functional groups at the 5′-or 3′-end. It is important that the conjugation strategy chosen should avoid any steric interference or possible debilitation of the binding affinity during the incorporation of the functional groups to the aptamer (Citartan et al. 2016). Wang et al. (2015) recently reported that the immobilization orientation, immobilization method, and spacers used affect the accessibility of the aptamer to target cells. Hence, the optimization of aptamer (3'-biotinylated R4-6) orientation or immobilization can maximize its recognition of the oocysts.

The aptamer-based system reported in the present study addresses the limitations of the current monoclonal antibody-based methodologies used in testing water samples for the presence of *Cryptosporidium* spp. The combined use of magnetic beads and probes enables rapid and specific detection of the target pathogen through changes in the electrical signal. This removes the need for microscopy thus enabling simple and rapid detection of *C. parvum* oocysts, which could be performed onsite with minimal technical expertise. This approach is also feasible for field applications and automation in water sample analyses, yielding objective and quantitative data.

In conclusion, the aptasensor developed in this study is highly effective in detecting *C. parvum* oocysts in both raw water samples and drinking water, and may have important applications for the water supply industry. As guidelines and policies related to drinking water rely on accurate assessments of risk, it is important to determine not only the levels of *Cryptosporidium* spp. oocysts in drinking and source waters but also their potential for transmission. *C. parvum* is a zoonotic pathogen and is common in humans and animals alike. However, numerous other *Cryptosporidium* species and genotypes are also infectious to humans (Xiao, 2010) and may be found in various water sources. Further study is required, therefore, to determine whether the R4-6 aptamer, and the aptasensor itself, is specific to *C. parvum*, or if it cross-reacts with other species and genotypes, particularly the human species, *C. hominis*.

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Figure 1. A**nalysis of aptamer-MB complex.** Square wave voltammogram of developed aptasensor obtained after incubation with R4-6 (dash lines) and coupled with 3′-TEG-biotin-R4-6 aptamer-magnetic bead complex with 5,000 *C. parvum* oocysts (solid line).

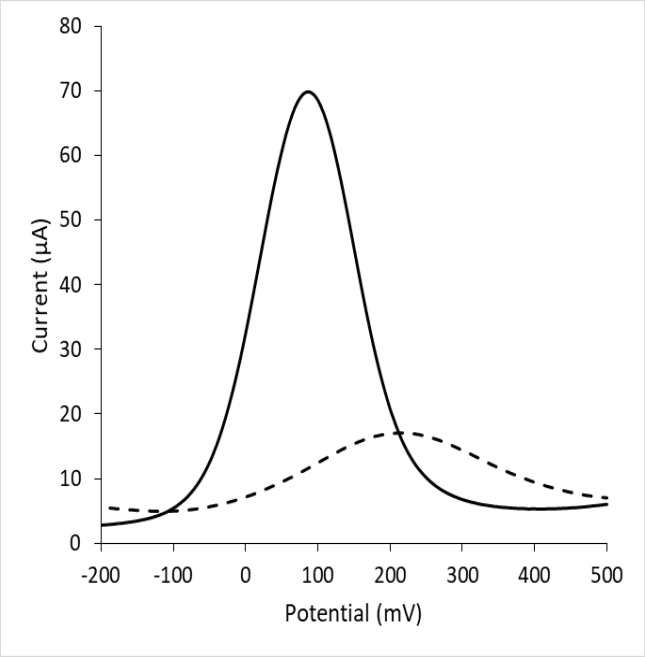
Figure 2. Selectivity of the aptasensor. Square wave voltammogram of the selective experiment performed by incubating aptasensor with buffer alone (dotted line), 3′-TEG-biotin R4-6 aptamer-magnetic bead complex with 900 *C. parvum* oocysts (solid line) and 1,000 *Giardia duodenalis* cysts (dashed line).

Figure 3. **Limit of detection of the aptamer-magnetic bead sensor.** (A) Square wave voltammogram obtained after incubating the 3′-TEG-biotin-R4-6 aptamer-MB complex with 0, 50, 100, 200, 300, 400, 500, 600, 700, 800 and 900 *C. parvum* oocysts. (B) Calibration plot of the change in current intensity (ΔI) vs. number of *C. parvum* oocysts. A regression equation of $y = 0.0412x + 2.3459$ ($R^2 = 0.9756$) was obtained, where y is the value in μA and x is the logarithmic number of *C. parvum* oocysts.

Figure 4. Membrane filtration of raw waters: Membrane filters showing the different levels of debris present in *C. parvum* oocyst-spiked water samples collected from Laurel Creek, Lake Ontario and Credit River in Southern Ontario.

421 **Figure 5. Detection of *C. parvum* oocysts in experimentally spiked water samples.** Square
422 wave voltammogram of developed aptasensor obtained after incubation with buffer alone
423 (Blank), 3′-TEG-biotin-R4-6 aptamer-magnetic bead complex with 5,000 *C. parvum* oocysts, in
424 spiked water samples from Lake Ontario (LO), Credit River (CR) and Laurel Creek (LC).

Figure 1. Analysis of aptamer-MB complex. Square wave voltammogram of developed aptasensor obtained after incubation with R4-6 (dash lines) and coupled with 3′-TEG-biotin-R4-6 aptamer-magnetic bead complex with 5,000 *C. parvum* oocysts (solid line).



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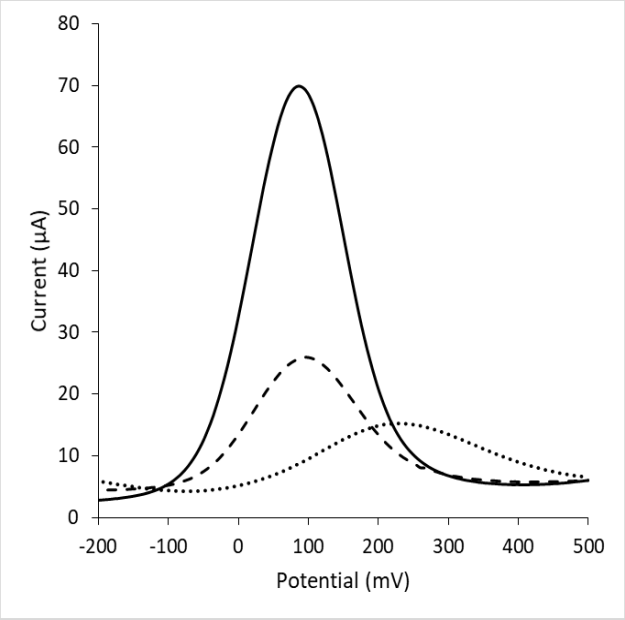

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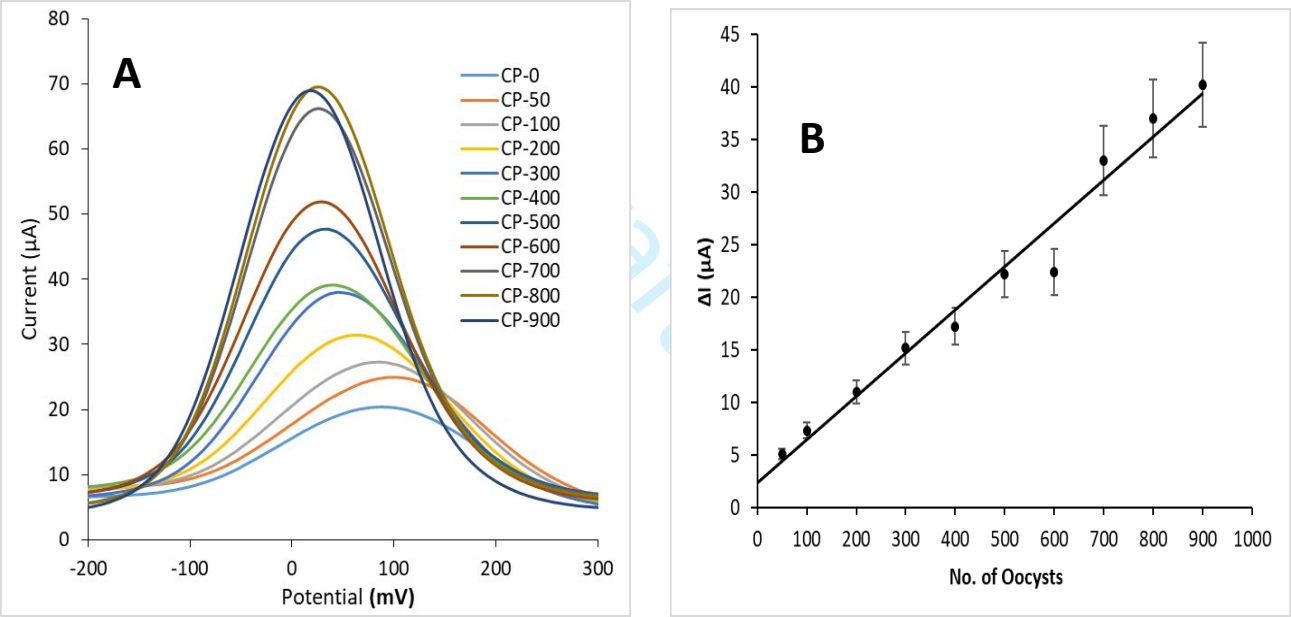

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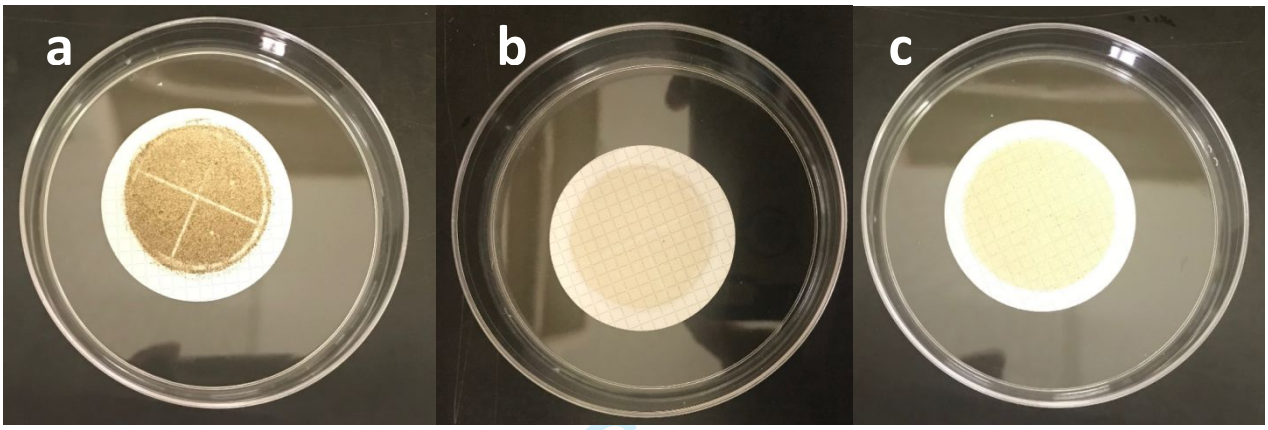

a) Laurel Creek (LC) **(b) Lake Ontario (LO)** **(c) Credit River (CR)**

Figure 5. Detection of *C. parvum* oocysts in experimentally-spiked water samples. Square wave voltammogram of developed aptasensor obtained after incubation with buffer alone (Blank), 3′-TEG-biotin-R4-6 aptamer-magnetic bead complex with 5,000 *C. parvum* oocysts, in spiked water samples from Lake Ontario (LO), Credit River (CR) and Laurel Creek (LC).