On-chip-based electrochemical biosensor for the sensitive and label-free detection of Cryptosporidium

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Cryptosporidium, an intestinal protozoan pathogen, is one of the leading causes of death in children and diarrhea in healthy adults. Detection of Cryptosporidium has become a high priority to prevent potential outbreaks. In this paper, a simple, easy to fabricate, and cost-effective on-chip-based electrochemical biosensor has been developed for the sensitive and label-free detection of Cryptosporidium oocysts in water samples. The sensor was fabricated using standard lithography using a mask with a 3-electrode design and modified by self-assembling a hybrid of a thiolated protein/G and the specific anti-Cryptosporidium monoclonal antibodies (IgG3). The electrochemical impedance spectroscopy (EIS) was employed to quantitate C. parvum in the range of 0 to 300 oocysts, with a detection limit of approximately 20 oocysts/5 µL. The high sensitivity and specificity of the developed label-free electrochemical biosensor suggest that this novel platform is a significant step towards the development of fast, real-time, inexpensive and label-free sensing tool for early warning and immediate on-site detection of C. parvum oocysts in water samples, as compared to the traditional methods (such as PCR and microscopy). Furthermore, under optimized conditions, this label-free biosensor can be extended to detect other analytes and biomarkers for environmental and biomedical analyses.

Cryptosporidium spp. is a common intestinal protozoan parasite occurring in many animal species and humans worldwide. Recently, cryptosporidiosis has become a global public health concern. Water contamination with Cryptosporidium represents a significant challenge in delivering safe drinking water and a significant threat to human health. Cryptosporidium oocysts have high resistance to the most common disinfectants and can remain infective and survive outside the host for up to 16 months. Cryptosporidium can cause mortality in immunocompromised individuals, especially patients with AIDS and children, and can lead to severe gastroenteritis, cryptosporidiosis in healthy adults. In developing countries, an estimation of 30% to 50% of childhood deaths is caused by Cryptosporidium. In developed countries, Cryptosporidium represents a significant risk in the water supply. One of the major outbreaks caused by Cryptosporidium was in Milwaukee in 1993. More than 400,000 people were infected, and over 100 deaths were reported. Furthermore, Cryptosporidium harms the economy in both developing and developed countries. For example, the cost of illness in the 1993 Milwaukee waterborne Cryptosporidium outbreak was 96 million USD.

The major challenge in detecting Cryptosporidium oocysts’ early detection (without using a pre-concentration method) is their low number in a large volume of water. The existing methods for detecting Cryptosporidium include the Environmental Protection Agency (EPA) 1623, immunoassay techniques such as Enzyme-linked immunosorbent assay (ELISA), and molecular techniques such as polymerase chain reaction (PCR). The EPA 1623 is often insufficient, complicated with variable and low recovery efficiencies. The method requires labelling Cryptosporidium with an immuno-fluorescence label. After successful labelling, a fluorescence microscope is used to visualize the labelled oocysts. The steps involved in this detection method make it expensive and time-consuming. Furthermore, the EPA has a limited sensitivity due to the cross-reactivity and background noise generation. Additionally, it needs well-trained personnel, extensive sample preparation and is not suitable for on-site detection.

In the last decade, immunological methods have gained significant market acceptance for detecting pathogens in environmental samples. These techniques include western blotting, enzyme-linked immunosorbent assay...
platforms to detect different pathogens in minute volumes rapidly. This allows many analyses to be performed of samples with excellent specificity and sensitivity, reproducibility, and throughput. Thus, enabling rapid powerful emergent tools for developing miniaturized sensing platforms for on-site detection and diagnostics. remotely outside the laboratory (at the point of need). Therefore, on-chip detection could be used as the bridging gap between detection in advanced laboratories and detection on-site, providing substantial advancements in the field of point-of-care (POC) diagnostic.

Recently, a screen-printed aptamer-based electrochemical biosensor was developed for detecting Cryptosporidium oocysts in spiked fresh fruits. In this study, DNA aptamers were developed and immobilized via a thiolated ssDNA primer onto a screen-printed carbon electrode modified with gold nanoparticles. The immobilized DNA aptamer was used as the molecular recognition element. The square wave voltammetry (SWV) method was used as the detection technique. The reported detection limit of the sensor was reported as 100 oocysts/30 µL. One point to emphasize regarding this study is the cost/labor associated with developing aptamers and their commercial availability. Furthermore, sensor modification with gold nanoparticles was used to enhance the sensitivity.

This paper presents a simple, easy to fabricate, cost-effective, and scalable chip-based electrochemical biosensor for sensitive and label-free Cryptosporidium detection. The biosensor’s sensing interface is based on forming a self-assembled monolayer of thiolated-protein/G/anti-Cryptosporidium antibodies (commercially available) as the capture probe onto a microfabricated gold electrode. The self-assembled monolayer (SAM) created on the chip-based microfabricated gold (Au) electrode provided a reproducible and well-ordered layer for immobilization anti-Cryptosporidium antibody. EIS was used for measuring the change in the sensor film resistance and electron charge-transfer permittivity due to the formation of the Cryptosporidium-antibody complex. The proposed chip-based biosensor has comparable sensitivity, efficiency, and detection limits to other biosensors reported in the literature. However, the sensor developed here does not need expensive aptamers to enhance selectivity. Moreover, the sensor developed here does not require expensive substrates or complicated fabrication (such as gold nanoparticle-modified screen-printed carbon electrode) to enhance sensitivity. Thus, the proposed on-chip sensing platform has a great potential to be used as an inexpensive, flexible, portable, and reliable sensing platform for the early detection of Cryptosporidium in water samples.
Materials

Purified Cryptosporidium parvum oocysts were purchased from Waterborne, Inc., New Orleans, LA, the USA, at a concentration of 10^6 oocysts/mL. The oocysts were kept in 0.1 M phosphate-buffered saline (PBS), pH 7.4 at 4 °C. Cryptosporidium oocyst-specific antibody, i.e., IgG3 subclass monoclonal mouse, was purchased from ABD Serotec (Currently Biorad, Burlington, Ontario, Canada). Protein/G/thiol was purchased from protein MOD (Madison, Wisconsin, USA). Bovine serum albumin (BSA), sodium phosphate dibasic, and sodium phosphate monobasic monohydrate were purchased from Sigma (Sigma-Aldrich, city, state, Canada). All other reagents and solvents were of analytical grade, and ultrapure water was used throughout the experiments.

Experimental methods

Sensor fabrication. The fabrication of the chip-based electrochemical immunosensor was performed using the steps shown in Fig. 1a. A piranha solution was used to clean all glass slides, followed by an oxygen plasma treatment for 10 min. Chromium and gold layers with thicknesses of 50 nm and 250 nm, respectively, were sputtered (Angstrom Engineering) in an argon atmosphere on a glass substrate. The sensing platform consisting of the working electrode (WE with 2 mm in diameter) and auxiliary electrode (AE) was patterned using the standard lithography process (Fig. 1b). An external Ag/AgCl reference electrode was used for the measurement.

On-chip immunosensor preparation. Prior to surface modification, the fabricated electrodes were immersed in piranha solution of (H_2SO_4-H_2O_2 3:1 (v/v)) for 30 s and rinsed with ultrapure water. All electrodes were subsequently cleaned electrochemically by cycling them in a 0.5 M KOH (basic) solution in the range of −2 V to 0 V versus the reference electrode (Ag/AgCl), followed by cycling them in a 0.5 M H_2SO_4 (acidic) solution in the range of 0 to +1.5 V separately. Plasma oxygen was then used to treat the fabricated gold electrodes. For the formation of SAM, the cleaned electrodes were immediately incubated with recombinant protein/G/thiol at 4 °C for 48 h (Fig. 2). The recombinant protein/G/thiol monolayer served as the linker between the Au microfabricated gold WE and the anti-Cryptosporidium antibodies. The modified sensing electrodes were then thoroughly rinsed with ultrapure water followed by phosphate buffer solution (PBS) (to wash away unbound recombinant Protein/G/thiol) and blown dry by N_2 (g). The sensor/SAM was incubated with 3 μL of 100 μg/mL specific anti-Cryptosporidium antibodies (IgG3) at 4 °C for 24 h. The sensors/SAM-anti-Cryptosporidium antibodies were rinsed again with PBS (to wash away unbound antibodies) and blown dry. The modified microfabricated Au electrode surfaces were then blocked by incubation with 100 mM BSA at 4 °C for 2 h. Lastly, the electrodes were washed several times with PBS to remove excess BSA and blown dry. The prepared electrodes (Au-thiolated protein/G/Abs/BSA) were stored in dry conditions at 4 °C.

Electrochemical measurements of SAM and antibody immobilization. The modification of the on-chip fabricated electrodes with SAM and antibody immobilization was checked using different electrochemical methods (CV, SWV, and EIS). In a typical electrochemical experimental setup, the modified anti-Cryptosporidium antibody microfabricated Au electrode (2 mm) and AE were used as the working electrode and auxiliary electrode, respectively. An external Ag/AgCl/3.0 M KCl electrode was used as the reference electrode. A salt bridge, filled with agar and 1 M KNO_3 aqueous solution, was used to reduce chloride ion diffusion into the electrolyte solution. Electrochemical measurements were recorded using the VersaSTAT 4 electrochemical station (Princeton Applied Research) in the bare gold, SAM, and antibodies in the three-electrode setup. All electrochemical measurements were started at the open circuit potential (OCP) and were performed in the presence of an electrolyte consisting of a 5 mM [Fe(CN)_6]^{3-/-4-} aqueous solution with 1 M NaClO_4 (as the supporting electrolyte). To be consistent, an open circuit potential was used in all electrochemical measurements (Fig. 3).

The CV measurements started with a 30 s quiet time followed by a positive scan polarity, which was swept between the potential range of −200 mV to +600 mV (against Ag/AgCl) at a scan rate of 50 mV/s mV/s. The SWV measurements were conducted in the potential range of −100 mV to 600 mV with an amplitude of 25 mV.
and a 2 s quiet time. The EIS measurements were performed in the frequency range of 0.1 Hz–100 kHz with a 5 mV amplitude and started with a 30 s quiet time.

**Contact angle measurements.** Contact angle measurements were conducted to further verify the immobilization of the antibodies onto the electrodes. In essence, the contact angle measurement was conducted (as explained previously in3,4) to ensure the presence of the SAM and antibodies on the surface based on the changes in the surface properties from hydrophilic to superhydrophilic (Fig. 4). The procedure involved preparing two substrates: one with chromium and gold layers (referred to as blank) and the other with the SAM and immobilized antibodies on the chromium and gold layers (referred to as test). A water droplet was dispensed on the two surfaces using a syringe pump. A needle was used to dispense the water droplet. The needle was coated with a hydrophobic material to avoid the needle's effect on the contact angle measurements. A side view image was taken and measured.

**Sample preparation and EIS measurement.** A series of dilutions of *Cryptosporidium* samples were prepared in the PBS buffer. Typical dilutions were 300, 200, 100, 50, 30, 20, and 0 oocysts in a final volume of 5 µL. The modified SAM/anti-*Cryptosporidium* antibodies microfabricated Au WE were incubated with different *Cryptosporidium* concentrations in a final volume of 5 µL for 20 min at room temperature (23 °C). Subsequently, all electrodes were washed with PBS to remove any loosely bound and unbound *Cryptosporidium* from the WE.
surface. The samples were blown dry in the air, followed by measurements. The EIS measurements were carried out using the VersaSTAT 4 electrochemical station (Princeton Applied Research) before and after the incubation with different concentrations of *Cryptosporidium* oocysts. All measurements were repeated three times to confirm the detection limit and conduct a statistical error analysis (Fig. 5). The blank electrodes (Au-thiolated protein/G/BSA) were prepared using the same procedure shown in Fig. 2, excluding the antibody step. The results were evaluated using the Randles equivalent circuit in the ZView software. The equivalent circuit consisted of the double-layer capacitance at the electrode surface ($C_{dl}$), the solution resistance ($R_s$), Warburg impedance ($Z_w$) and charge transfer resistance ($R_{CT}$). The real ($Z'$) and imaginary ($Z''$) parts of the impedance were plotted in Nyquist diagrams. The charge transfer resistance occurs in the kinetically-controlled high-frequency region; whereas, the Warburg impedance was observed in the diffusion-controlled low-frequency region of the binding of *Cryptosporidium* oocyst to the modified Au electrode results in increasing $R_{CT}$'s value, which appears as a larger semicircle in the plot. The changes in the $R_{CT}$ between blank and after capturing *Cryptosporidium* were compared. The equation of $\% \Delta R_{CT} = (R_{CT(with Cryptosporidium)} - R_{CT(blank)}) / R_{CT(blank)} \times 100$ was used to calculate the relative change of the charge transfer resistance ($\Delta R_{CT} (%)$) as a function of the *Cryptosporidium* concentration (the six different cell concentrations used here range from 20 to 300 cells/5 µL).
Calibration curve and limit of detection (LOD). To assess the applicability of the anti-\textit{Cryptosporidium} antibodies-modified microfabricated sensor for detecting \textit{Cryptosporidium}, the calibration curve was constructed by plotting $R_{CT}$ versus the number of the \textit{Cryptosporidium} cells. The limit of detection (LOD) was calculated using the following expression: $LOD = 3SD_b/m$, where $SD_b$ is the standard deviation of the blank and $m$ is the calibration curve slope\textsuperscript{40,41}.

Results and discussion

Preparation and optimization of biosensing layer. The CV, SWV and EIS result in Fig. 6a,b show that there is a decrease in the charge transfer (and hence a decrease in the current and an increase in the impedance) after modifying the electrode surface with protein/G/thiol, after immobilization of antibodies, and after blocking with BSA. The current decrease confirms the formation of SAM, immobilization of the antibodies and the surface blocking step. This is due to the increase in the surface monolayer thickness after each modification step, increasing the separation between the oxidation and reduction peaks of the $[\text{Fe(CN)}_6]^{3-/4-}$. Furthermore, the current decrease confirms an increase in the film resistance and electron charge-transfer permittivity, further confirmed using EIS as shown in Fig. 6c. The data clearly show a significant increase in the $R_{CT}$ values form $\pm 20.42 \pm 0.5 \Omega$ (bare gold) to $3187 \pm 3.2 \Omega$ (anti-\textit{Cryptosporidium} antibodies modified surface). The increase in $R_{CT}$ is due to the formation of an electrically inactive layer (causing the isolation and consequently preventing the charge transfer to the electrode) after each modification step.

During fabricating and preparing the on-chip immunosensor, the concentration of the biological recognition element (antibodies) and incubation time on the surface was of great importance for optimizing the sensor's analytical performance. Such optimization will increase the amount of the molecular recognition element available on the surface for capturing the \textit{Cryptosporidium} oocysts. For this purpose, different concentrations of antibodies (20, 30, 50, 100, 200 and 300 $\mu$g/mL) were immobilized on the sensor surface to determine the

Figure 6. Characterizations of the on-chip label-free immunosensor for the detection of \textit{Cryptosporidium}. (a) CV, (b) SWV, and (c) EIS for the bare Au electrode, added protein/G/thiol, immobilized anti-\textit{Cryptosporidium} antibodies, and added BSA. The measurements were conducted using the microfabricated Au working electrode, microfabricated Au auxiliary electrode and an external Ag/AgCl reference electrode. All electrochemical measurements were started at the OCP and were performed in the presence of an electrolyte which consisted of a 5 mM $[\text{Fe(CN)}_6]^{3-/4-}$ aqueous solution with 1 M NaClO\textsubscript{4} as the supporting electrolyte. The CV measurements started with a 30 s quiet time followed by a positive scan polarity which swept between the potential range of $-200$ mV to $+600$ mV (against Ag/AgCl) at a scan rate of 50 mV/s. SWV measurements were conducted in the potential range of $-100$ mV to $600$ mV with an amplitude of 25 mV and a 2 s quiet time. EIS measurements were performed in the frequency range of 0.1 Hz–100 kHz with a 5 mV amplitude and started with a 30 s quiet time.
RCT influences the impedance measurements (∆RCT). The optimum analytical performance happens using 100 µg/mL of antibodies (chosen here for all experiments).

The real (Z′) and imaginary (Z′′) parts of impedance were plotted in Nyquist diagrams (Fig. 7b) and the sensor’s analytical performance. Figure 7b shows the incubation time’s influence on increasing the amount of antibodies available for capturing the target pathogen. It is clear that ∆RCT increases as the antibody’s incubation time increases, reaching a plateau after 24 h. Thus, 24 h was used as the optimal incubation time for the antibodies in all subsequent experiments.

**Surface characterization.** Contact angle measurements were employed to further confirm the surface’s functionalization by SAM and antibodies (see Fig. 4). Contact angle measurements, measuring the advancing and receding contact angles, reveal the surface’s level of wettability. In essence, the formation of SAM and the immobilization of antibodies result in a significant increase in the surface’s wettability. The measured advancing and receding contact angles for the bare gold were 88° and 42° (± 1°), respectively, and 53° and 3° for the SAM-antibodies modified surface, respectively. The decrease in the advancing and receding contact angle values confirms the formation of SAM-antibodies film on the Au surface. Furthermore, the results confirm the formation of SAM-antibodies film on the Au surface.

**Electrochemical detection of Cryptosporidium oocysts.** After confirming surface modification through CV, SWV, and EIS, the interaction of the anti-Cryptosporidium antibodies modified sensor with Cryptosporidium was studied. The binding of Cryptosporidium oocysts was monitored electrochemically in the presence of 5 mM [Fe(CN)₆]³⁻/⁴⁻ (as described in “Sample preparation and EIS measurement”). Briefly, EIS measurements were obtained first for the anti-Cryptosporidium antibodies modified microfabricated Au electrode (referred to as blank), and then after incubating the sensor with different concentrations of Cryptosporidium oocysts (referred to as test). The measured values are correlated to the number of the captured oocysts. Such changes are directly related to the concentration of the anti-Cryptosporidium antibodies.

To assess the extent of non-specific binding, a blank sensor was modified similarly to the test sensor (as explained in “Sample preparation and EIS measurement”), excluding the step taken for the antibodies immobilization. The real (Z’) and imaginary (Z’’) parts of impedance were plotted in Nyquist diagrams (Fig. 7b) and analyzed by the Randles’ equivalent circuit, which best models the EIS spectra using the ZView software. In essence, EIS measures resistance, which increases due to a decrease in the charge transfer and vice versa. Figure 8a shows a dramatic increase in the impedance and decreased charge transfer due to Cryptosporidium oocysts’ binding to the immobilized anti-Cryptosporidium antibodies. Such changes are directly related to the Cryptosporidium oocysts’ concentration; in a very high concentration of 200 oocysts, the RCT increase is not as significant as other concentrations. This could be due to the saturation of the sensor. The average values, statistical error, and the lowest detection limit were also calculated. The maximum change in the response due to the presence of different cell densities was observed. The maximum value of the relative change in RCT for each number of the captured oocysts was used to generate the calibration curve (Fig. 8c). The curve shows a linear range of up to 200 cells/5 µL and a LOD of approximately 20 cells/5 µL. The results confirm the on-chip microfabricated sensing platform’s effectiveness as a label-free, cost-effective and sensitive sensing tool.
that the possibility of non-specific binding is not significant. Hence, the developed sensor has a high specificity and selectivity towards Cryptosporidium. According to the results of the optimized sensor and calibration curve with error bars (see Fig. 8c), the limit of detection of the sensor for S/N = 3 was found to be 20 cells of Cryptosporidium in 5 μL of the sample. Moreover, the maximum relative standard deviation (RSD) of 2.8% at the Cryptosporidium concentration of 20 cells/5 μL (4 cells/μL) and 2.3 at the concentration of 200 cells/5 μL (40 cells/μL) confirmed the excellent reproducibility and repeatability of the developed sensor.

For testing the cross-reactivity, the modified sensor with anti-Cryptosporidium antibodies was incubated with four different concentrations of E. coli (1000, 2000, 3000, and 4000 CFU/mL). Figure 9a shows a negligible increase in the impedance and the ΔR<sub>CT</sub> (Fig. 9b). This increase confirms that there is low interaction with E. coli (which could be due to non-specific adsorption) and the sensor's high selectivity towards Cryptosporidium. Other non-ionic polymers or blocking agents should be tried to overcome this challenge.

Table 1 shows a comparison of the electrochemical sensors reported in the literature to date for detecting Cryptosporidium with the developed sensing platform in terms of detection principle, advantages, and disadvantages.

Conclusions
A simple, easy to fabricate, and cost-effective label-free on-chip-based EIS-based immunosensor was developed for the sensitive and rapid detection of Cryptosporidium. The specific anti-Cryptosporidium antibodies were immobilized on the on-chip-based microfabricated sensor via thiolated protein/G. The EIS measurements were conducted in the presence of the antibodies (test) and the absence of antibodies (blank). The detection limits, sensitivity and selectivity of the sensing platform were obtained. The results showed a linear detection range for Cryptosporidium concentrations of 20 cells/5 μL to 200 cells/μL with a detection limit of 20 cells/5 μL. The specificity and selectivity tests proved the developed sensing platform's high selectivity toward Cryptosporidium, demonstrating the developed microfabricated sensor's applicability for detecting Cryptosporidium. Furthermore, a sample volume of 5 μL was used for the measurement using the developed on-chip sensor. This reduces the sample and reagents volume and cost required for a single measurement.

Furthermore, the developed sensor in this research has a great potential to be integrated into a device to capture, separate, and concentrate Cryptosporidium. Such a device has been previously developed in the past.\(^{45}\)
This integration will allow for the portable, personalized and POC detection of *Cryptosporidium*. This integration will eliminate the need for trained technicians and specialized laboratories. Moreover, sample treatment by UV or sonication is not needed while using this sensor to detect the whole oocyst.

The developed proof-of-concept of our on-chip electrochemical biosensor is a stepping stone towards creating accessible and ubiquitous pathogen detection methods that could potentially be used in resource-limited settings.

**Table 1.** Comparative evaluation of the electrochemical sensors previously developed for the detection of *Cryptosporidium* with the sensor developed in this research.

| Detection principle | Detection limit | Advantages | Disadvantages | Ref |
|---------------------|----------------|------------|---------------|-----|
| Square wave voltammetry (SWV—change in current due to the presence of oocysts) | 4 cells/µL | Small sample volume (100 µL) High selectivity and sensitivity Low power requirement Robust and easy to miniaturize | Complicated fabrication (use of gold nanoparticles to enhance sensitivity) Use of expensive and non-commercial DNA aptamers | 1 |
| Differential pulse voltammetry (DPV—change in current due to the presence of oocysts) | 0.003 oocysts/µL (3 oocysts/mL) | Short-analysis time High stability Low power requirement | Complicated fabrication (use of gold nanoparticles to enhance sensitivity) Time-consuming Involve many preparation steps Require expensive labels Not suitable for on-site detection | 42 |
| Amperometry (change in current at a fixed potential due to the presence of oocysts) | 0.25 oocyst/µL (1 oocyst/4 µL) | Excellent detection limit (able to detect the amplified mRNA as low as a single oocyst) Low power requirement | Complicated fabrication (use of gold nanoparticles to enhance sensitivity) Involve many preparation steps A magnet is required for analyte pre-concentration and to capture the bead/liposome complex Time-consuming labelling and preparation steps, including amplification of mRNA before detection Unsuitable for on-site detection and fast-decision-making process | 43 |
| Electrochemical impedance spectroscopy (EIS—measuring the change of conductivity upon the release of ions from *Cryptosporidium*) | > 10 cells/µL | Label/PCR-free Easy fabrication Small sample volume (110 µL) Cost-effective | Low selectivity Interference with the sample conductivity Generation of inaccurate results A low conductive sample is required for producing reliable and accurate results Sample pre-treatment is required before measurements Time-consuming Unsuitable for on-site detection | 44 |
| Electrochemical impedance spectroscopy (EIS—measuring the change of the sensor film resistance and electron charge-transfer permittivity due to the formation of the *Cryptosporidium*-antibody complex.) | 4 cells/µL | Small sample volume (5 µL) Easy fabrication (no modification with gold nanoparticles to enhance the sensitivity, as reported previously in the literature) Rapid response Cost-effective (> 1 US dollar) Suitable for on-site detection High sensitivity and selectivity Label/PCR-free Low power requirement | Non-specific adsorption. (To overcome this challenge, other non-ionic polymers or blocking agents should be tried) | This work |

Figure 9. EIS response of the microfabricated immunosensor to the presence of *E. coli*. (a) cross-reactivity interaction of four different concentrations of *E. coli* with the modified sensor, (b) calibration curve.
Future research efforts are required to test the developed sensor with environmental water samples to confirm the sensor's applicability to replace the fluorescence-based microscopy detection part in the EPA 1623 method. Future studies are also required to test the sensor with other potential interferants and study the capability of the sensor to be extended to detect other biomarkers of interest for other environmental and biomedical applications.

Received: 25 November 2021; Accepted: 8 March 2022
Published online: 28 April 2022

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**Author contributions**

G.S.L. originated the idea and designed the experiments, performed the experiments, analyzed the data, selected reagents/materials/analysis tools, wrote the manuscript, prepared figures and tables, and reviewed drafts of the manuscript. H.N. and M.H. commented on the design of the experiment, reviewed the manuscript and oversaw the project.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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