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PAPER

Development of electrochemical based sandwich enzyme linked immunosensor for *Cryptosporidium parvum* detection in drinking water†

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Cryptosporidium parvum is one of the most important biological contaminants in drinking water and generates significant risks to public health. Due to low infectious dose of *C. parvum*, remarkably sensitive detection methods are required for water and food industry analysis. This present study describes a simple, sensitive, enzyme amplified sandwich form of an electrochemical immunosensor using dual labeled gold nanoparticles (alkaline phosphatase and anti-oocysts monoclonal antibody) in indium tin oxide (ITO) as an electrode to detect *C. parvum*. The biosensor was fabricated by immobilizing the anti-oocysts McAb on a gold nanoparticle functionalized ITO electrode, followed by the corresponding capture of analytes and dual labeled gold nanoparticle probe to detect the *C. parvum* target. The outcome shows the sensitivity of electrochemical immune sensor enhanced by gold nanoparticles with a limit of detection of 3 oocysts/mL in a minimal processing period. Our results demonstrated the sensitivity of the new approach compared to the customary method and the immunosensors showed acceptable precision, reproducibility, stability, and could be readily applied to multi analyte determination for environmental monitoring.

Introduction

Cryptosporidium parvum is a key significant diarrhoea-causing parasitic protozoan found both in humans and animals.[1] Approximately 30% of adults of eminent-rich countries and virtually all of adults in resource-poor countries have serological evidence of prior infection with this organism.[2] In general very low numbers of oocysts are found in environmental water, however it is suggested that an infectious dose is as low as 30 oocysts. Due to the low infectious dose of *C. parvum* extremely sensitive detection methods are required for water and food analysis. Presently DNA based methods are used to detect oocysts in environmental samples, especially PCR based detection methods demonstrated a sensitive and specific detection of *C. parvum* in clinical specimens and environmental samples.[3–6] The electrochemical based sensor measurements were also performed for *C. parvum* detection.[7,8] Wang[9] *et al.*, (1997) reported the use of electrochemical DNA hybridization based *Cryptosporidium* detection in environmental water samples. Similarly, a electrochemical and surface plasmon resonance based biosensor was also developed for *C. parvum* detection and

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Environmental impact

Cryptosporidium parvum is a key significant diarrhoea-causing parasitic protozoan found both in humans and animals. Due to the low infectious dose of *C. parvum*, extremely sensitive detection methods are required for water and food industry analysis. Presently DNA and immuno assay based methods are in use to detect oocysts in the environmental samples like water *etc.* Though, these methods have the advantages of being simple, time saving, easily automated, and also can avoid a strict stripping procedure, the sensitivity and feasibility of these protocol needs to be further improved. Hither we have developed a simple antibody and alkaline phosphatase (ALP) labeled gold nanoparticle based electrochemical immunosensor for *C. parvum* detection in environmental samples. Our results demonstrated the sensitivity of this new approach to identify and monitor *C. parvum* in environmental samples at an earlier stage.

showed a high specificity and sensitivity.[10,11] Likewise, a piezo-electric-excited millimetre-sized cantilever (PEMC) based biosensor was used to detect a *G. lamblia* limit from 1 to 10 cells mL^{-1} in water samples.[12]

Several studies of nanomaterial based electrochemical immunosensors have been investigated in recent years.[13–17] However, gold nanoparticles based (AuNP) bioanalytical methods have many beneficial applications which have influenced the development of their remarkable industrial and engineering usage in biotechnological systems. AuNP based electrochemical biosensors emerged as an important analytical diagnostic tool converging the demands for increased sensitivity, early detection in a limited time and controlling disease outbreaks at the single cell and molecular level. AuNP based electrochemical immunosensors have been reported elsewhere,[18–23] though sandwich type enzyme linked antibody and DNA based electrochemical biosensors are considered as effective and sensitive analytical tools to detect biomolecules.[24] Several enzymes such as horseradish peroxidase, alkaline phosphatase (ALP), and glucose oxidase have been widely used as signal amplifiers in immunosensors for the ultra sensitive detection of biomolecules.[25–28] Due to its high catalytic activity and formation of electro-active *p*-nitro phenol (*p*-NP), hydrolysis of phosphate ester *p*-nitro phenol phosphate (*p*-NPP) by alkaline phosphatase has been given significant attention in immunosensor development. Zhengzhi[29] *et al.*, reported a sandwich based ALP labeled nano sphere detection of α-tumor necrosis factor (αTNF). Similarly, Jagotamoy[30] *et al.*, showed a signal amplification method using a *p*-aminophenol redox cyclic by a hydrazine-based ALP-labeled antibody, which evidently optimized the sensitivity and linear range. Electrochemical based IgG and DNA detection was also developed using ALP labeled biomolecules.[31–33]

While all the existing analytical methods could detect a small number of oocysts, they still possess various difficulties including assay time consumption, cost overrun, complicated equipment and lack of expertise. To overcome such difficulties, enormous efforts have been made in recent years to develop sensitive and specific detection methods for related issues. In this paper, we attempted to address these hypothetical issues by developing a simple AuNP based electrochemical immunosensor to detect *C. parvum* in drinking water samples. The developed electrochemical biosensor was fabricated using a "sandwich" detection strategy involving captured anti-oocysts monoclonal antibodies (McAb) and a dual labeled AuNP probe (ALP and anti-cysts McAb) introduced through sandwich hybridization. The detection sensitivity was enhanced by AuNP which enabled the carrying of a higher number of ALP molecules per immuno reaction. The electrochemical signal generated by electro-active molecules produced through a catalytic enzyme reaction in electrolyte solution and demonstrated the eminent sensitivity of this new approach compared with customary method.

Materials and methods

Materials

Parasitic *Cryptosporidium parvum* at a concentration of 1×10^5 oocysts/mL and anti-oocysts monoclonal antibody of 1/20–1/2000 unit were purchased from BTF Microbiology, Australia. Alkaline phosphatase of 10 DEA units/mg, chloroauric acid, tri-sodium citrate, *para*-nitro phenyl phosphate (*p*-NPP) and ITO electrode coated glass slides were purchased from Sigma-Aldrich. Chemicals used were of analytical grade and were applied without any further purification. The electrochemical studies were performed under an AutolabPGStat 12 electrochemical analyzer in a three-electrode cell using a platinum wire as the counter electrode and Ag/AgCl (3M NaCl) as the reference electrode. The cyclic voltammogram (CV) potential ranged from 0 to 0.4 V with a scan rate of 50 mV s^{-1} and the electrochemical impedance spectroscopy (EIS) measurements were recorded with in the frequency range of 0.01 Hz to 100 kHz at 0.24 V.

Synthesis of AuNP

The AuNP was prepared using a standard citrate reduction method.[34] In brief, 100 mL of 1 mM aqueous $HAuCl_4$ was heated under refluxing conditions at 100 °C and 10 mL of 38.8 mM tri-sodium citrate was added and kept at 100 °C for 15 min. At the end point, indicated by a wine red color, the mixture was continuously stirred whilst cooling at room temperature. The product of the AuNP suspension was finally adjusted to pH 7 with 1 mM sodium carbonate buffer and analyzed using UV-vis spectroscopy (Bio Tek, Synergy HT, Gen 5 Software) and a high resolution-transmission electron microscope (HR-TEM) (JEOL 3010 instrument with a UHR polepiece)

Preparation of dual labeled AuNP probe

The optimum assay condition for the preparation of the dual labeled AuNP was performed using a salt induced aggregation test[35] and the probe was synthesized under optimal conditions. The dual labeled gold nanoparticles were prepared according to a modified procedure.[36] Briefly, 1 mL of AuNP (0.75 A_{520} units/mL at pH 7) was mixed with 100 μL of primary anti-oocysts McAb (10 μg mL^{-1}) and stirred gently for 1 h at room temperature. 100 μL of ALP (200 μg mL^{-1}) was then added and kept at room temperature for 1 h. The conjugate was then centrifuged, the supernatant discarded and the pellet was dispersed in 1 mL of Tris buffer (0.1 M, pH 7.6) containing 0.5% BSA. The mixture was centrifuged again and the supernatant was removed and the pellet was adjusted to 200 μL of preservative buffer (PBS, pH 7.6, 0.5% BSA).

Fabrication of ITO electrode for *C. parvum* detection

For the working electrode, the ITO coated glass plates were subjected to sequential modification on the surface of electrode prior to use with dual labeled gold nano probe. The ITO plates were cleaned thoroughly with a ultra-sonication process using acetone followed by water. SAM of APTMS on the pre-cleaned ITO plates was prepared as reported earlier.[35] In brief, ITO plates were immersed in a solution of 1 : 1 : 5 (v/v) H_2O_2–NH_4OH–H_2O for 30 min at 80 °C for hydrolysis and rinsed thoroughly with de-ionized water. They were subsequently dried out and immersed with 2% of (v/v) 3-aminopropyl trimethoxy-silane–methanol for 20 h and freshly prepared colloidal gold for 15 h at room temperature. A formation of sub-monolayer AuNP was then incubated with 200 μL of (5 μg mL^{-1}) anti-oocysts McAb for 1 h and blocked with 3% BSA in phosphate buffer saline for 1 h at room temperature.

Electrochemical detection of *C. parvum*

The immunoassay protocol, shown in Scheme 1, is as follows; the fabricated electrochemical immunosensor was incubated with the target *C. parvum* antigen at room temperature for 30 min and washed with PBS buffer. It was maintained in 200 μL (10 μg mL^{-1} anti-oocysts McAb and 200 μg mL^{-1} ALP) of the dual labeled AuNP probe at room temperature for 30 min. The immuno reaction between the AuNP probe and the oocysts brought them onto the electrode surface, which was followed by washing the immunosensors with water to eliminate the non-specific conjugate. The modified electrode was then incubated with a 3 mM *p*-NPP solution (0.1 M Tris-HCl pH 9.0) for 10 min and a differential pulse voltammetry (DPV) scan was recorded at 0 to 0.4 V.

Water sample collection and field evaluation study

10 L samples of drinking water were collected randomly from ten different zones of Chennai, India. Each sample of 10 L was then concentrated to 10 mL *via* a U-APB method and it was used for analysis throughout this study.[37] The total genomic DNA was extracted using a CTAB[38] method to detect the *C. parvum* occurrence and which was subjected to PCR analysis designed as a 18S rRNA gene with the reverse and forward primer of CGTTAACGGAATTAACCAGAC and AGTGCTTAAAG CAGGCAACTG. The product of 556 bp amplification concedes were solved using 1% agarose gel in the presence of ethidium bromide and visualized under a Bio-Rad gel documentation system (Molecular Imager® GelDoc™ XR). The efficiency and specificity of the AuNP based electrochemical immunosensor assay was compared and validated with a conventional PCR method and fluorescence microscopic[39] analysis using the concentrated water samples.

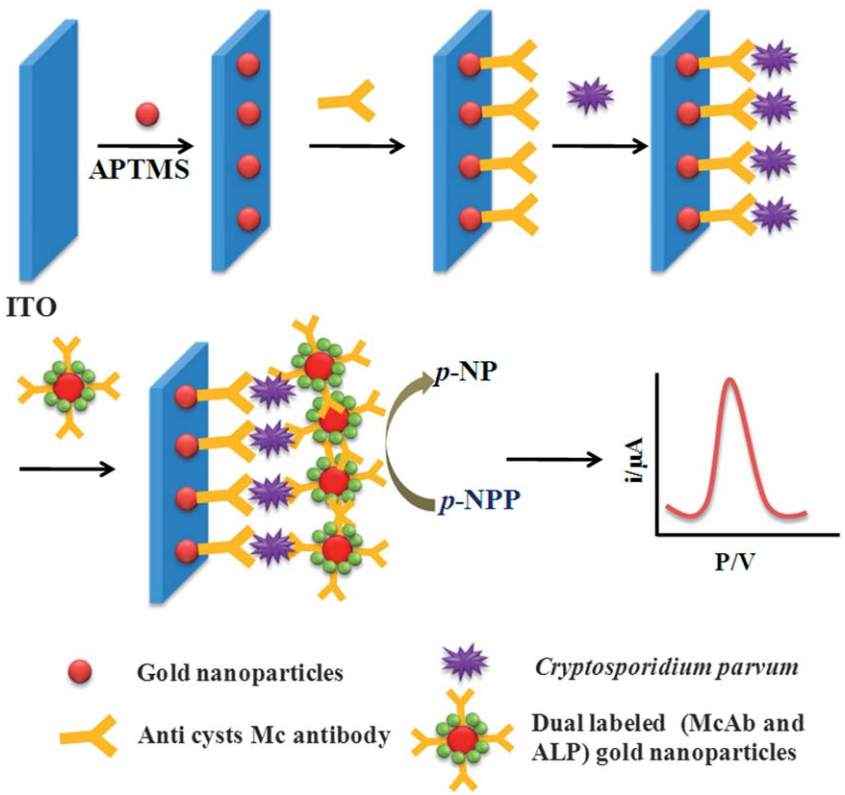
Results and discussion

Principle of dual labeled AuNP based electrochemical immunosensor

The principle of the dual labeled AuNP (ALP–AuNP–Anti-cysts McAb) based electrochemical immunosensor method is based on the sandwich of an enzyme linked immuno assay and an enzyme catalytic reaction. The developed method proved that the signal intensity was amplified by manifolds. The gold nanoparticle bound with primary anti-oocysts McAb and more ALP molecule offers a significant amplification for the catalytic reaction, which improves the pathogen detection. The electrochemical signal of the electro-active species *p*-nitro phenol (*p*-NP), on hydrolysis of *p*-NPP by enzyme ALP, was measured by differential pulse voltammetry (DPV). The AuNP acted as an anchorage platform for both the antibody and the enzyme ALP and is clearly shown in Scheme 1.

Characterization of dual labeled AuNP probe

The prepared AuNP and dual labeled AuNP were analyzed using a UV-vis spectrophotometer and HR-TEM. As shown in Fig. 1 the UV–vis spectra of the typical nanoscale plasmon resonance band revealed that the absorption maximum of gold



Scheme 1 The schematic diagram represents the newly developed electrochemical immunosensor for *C. parvum* detection using a dual labeled AuNP probe.

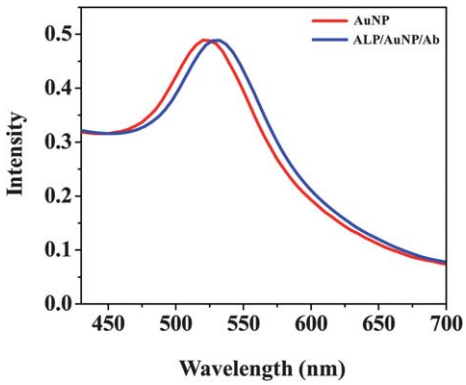


Fig. 1 UV Spectral analysis of AuNP (518 nm) and dual labeled AuNP probe (524 nm).

nanoparticles at 518 nm was shifted to 524 nm upon coupling of the antibody and ALP. Similar criterion of plasmon resonance peak shift was used as evidence for coupling.[40] The HR-TEM images displayed the colloid AuNP and nanoparticle conjugates, and indicated that an average diameter of 16 ± 0.2 nm AuNP increased to an average diameter of 18 ± 0.2 nm due to the antibody and ALP coupled nano conjugate which also shows the uniformity in the dispersion. (Data shown in ESI Fig. S1†). Under high magnification, we observed grayish halos around the modified nanoparticles, indication of coupled biomolecules on the surface nanoparticles.[41] Subsequently, the salt induced aggregation test was performed to determine the optimum conditions of the assay; the maximum number of protein conjugates on the surface of the AuNP were found at pH 7. Similarly, the optimum quantity used of anti-oocysts McAb and ALP to saturate the gold nanoparticles also determined. As shown in Fig. S2,† when 20 μg mL^{-1} of anti-oocysts McAb was used it resulted in a 100% saturation of the gold nanoparticles surface, for the conjugation 50% of anti oocysts McAb was used and examined. Simultaneously the uncoated gold nanoparticles surface coated with ALP enzyme were also examined. It shows that 200 μg mL^{-1} of ALP was needed to completely saturate the gold nanoparticle surface.

Electro impedance spectroscopic characterization of modified electrodes

The immobilization of APTMS, AuNP, anti-oocyst McAb, oocysts and dual labeled AuNP on the ITO electrodes were characterized using an electrochemical impedance spectroscopy (EIS) technique and different stages of the electrode responses are shown in Fig. 2 as Nyquist plots. The obtained Nyquist plots fitted perfectly by a Randle equivalent circuit, revealing that R_{ct} is the suitable signal for assessing the interfacial properties of the prepared immunosensor during all of the assembly procedures. The bare ITO electrode shows a low interfacial charge transfer resistance (R_{ct}) value of 366 Ω. After the immobilization of APTMS, the R_{ct} value increased to 490 Ω, this recognized the self assembled APTMS layer which perturbed the interfacial electron transfer rate formed on the electrode surface. Following the AuNP immobilization on the APTMS–ITO electrode it shows a noticeably decrease in the R_{ct} value (403 Ω), this signifies the formation of the conducting layer on the electrode surface.

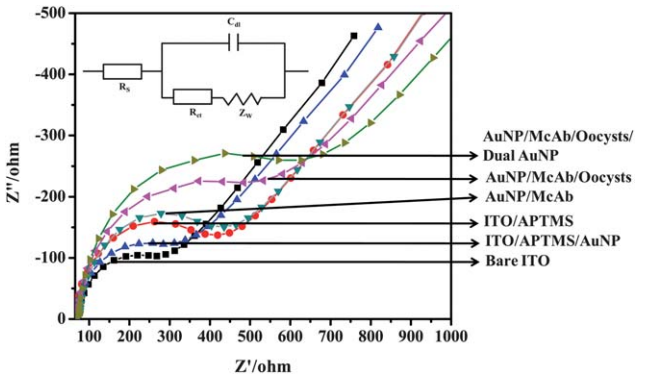


Fig. 2 Electrochemical impedance spectroscopic analysis of bare ITO, APTMS, AuNP, anti-oocysts McAb, oocysts, and dual labeled AuNP modified ITO electrode. The inset is the equivalent circuit applied to fit the impedance spectra in the presence of the redox probe of $Fe(CN)_6^{3-/4-}$.

Further immobilization of anti-oocysts McAb on the electrode surface inhibited the electron transfer and R_{ct} value increased (525 Ω) significantly. This may be due to antibodies bound towards the AuNP surface and the diameter of the Nyquist plot's semicircle increased[42] consequently. A similar pattern appeared in the R_{ct} value and increased further when *C. parvum* (717 Ω) and dual labeled AuNP probe (790 Ω) were immobilized on the electrode surface. The depicted CV measurements (Fig. S3†) effectively correlated with the EIS measurements obtained and substantiate that varied species were immobilized on the surface of the AuNP modified ITO electrode. However, the EIS spectra appeared due to physical adherence of other molecules on the electrode surface decreases the sensitivity of the immunosensor. In this context, differential pulse voltammetry (DPV) analysis was used to detect the pathogen *C. parvum*. DPV is an efficient electrochemical technique, and features a high specificity and sensitivity detection method for biomolecules.

Optimization of assay conditions for *C. parvum* detection

The sensitivity of the electrochemical immunosensor was based on the concentration of anti-oocysts McAb used in the assay. Various concentrations of 10, 5, 2.5 and 1.25 μg mL^{-1} of anti-oocysts McAb conjugated dual labeled AuNP were analyzed and

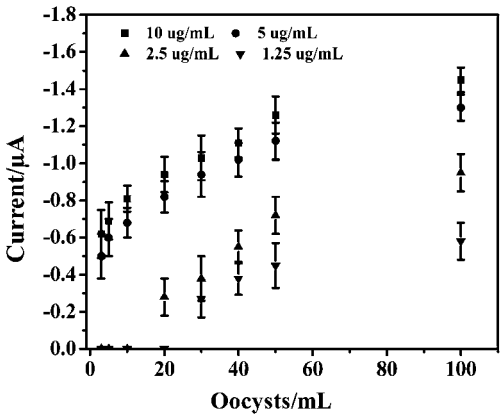


Fig. 3 Optimization of anti-oocysts McAb concentration for *C. parvum* detection. The values represent an average of triplicates ±SD ($n = 3$).

the optimum assay concentration found. Each concentration of the above mentioned nano conjugates were assayed using various concentration of 3–100 oocysts/mL and a constant concentration of 5 μg mL^{-1} of captured antibody was analyzed with differential pulse voltammetry (DPV). As shown in Fig. 3, the DPV signal correlated with the concentration of nano conjugates used in the assay. An anti-oocysts McAb nano conjugate concentration of 10 and 5 μg mL^{-1} could detect the least concentration of 3 oocysts/mL, whereas 20 and 30 oocysts/mL were detected at the very low concentration of 2.5 and 1.25 μg mL^{-1} nano conjugates respectively. It was clearly revealed that 5 μg mL^{-1} of anti-oocysts McAb conjugates dual labeled AuNP was adequate to detect the lowest number of oocysts.

Specificity and sensitivity of immunosensor assay for *C. parvum* detection

The efficiency, specificity and limit of detection were analyzed using our newly developed electrochemical immunosensor for *C. parvum* detection. The DPV responses for the detection of various concentrations of *C. parvum* using dual labeled AuNP probes is shown in Fig. 4A. The DPV signal increased in the presence of oocysts and the linear relationship between peak current *versus* concentration of oocysts was noticed (Fig. 4B). Whereas there was no DPV signal observed in the blank AuNP + ALP, which indicated that the appearance of a signal was not due to the non specific and physical adherence of dual labeled nano conjugates on the electrode surface. The limit of detection was found to be 3 oocysts/mL and linear responses were above the range of 5–100 oocysts/mL, which equalled a similar detection value for *G. lamblia* in water samples using a piezoelectric-excited millimetre-sized cantilever based biosensor (PEMC).[12] In addition, the developed electrochemical immunosensor was validated with a few other waterborne pathogens and it was revealed that the AuNP based electrochemical immunosensor was highly specific to *C. parvum* and no false positive or non-specific pathogens were detected. The obtained results comprised a good coherence with the PCR method (Fig. 5). As a consequence, the DPV signal was not due to non-specific bound of modified AuNP on electrode surface and it was clearly suggested that the dual labeled AuNP probe-based electrochemical immunosensors is highly sensitive and specific to *C. parvum*.

The reproducibility of electrochemical immunosensor was evaluated using a series of 5 electrochemical immuno assays performed under optimized conditions to detect 100 and 10 oocysts/mL. The relative standard deviation of the assay is 4.1% and 2.7% respectively; this suggested that the assay is reproducible in the optimized conditions.

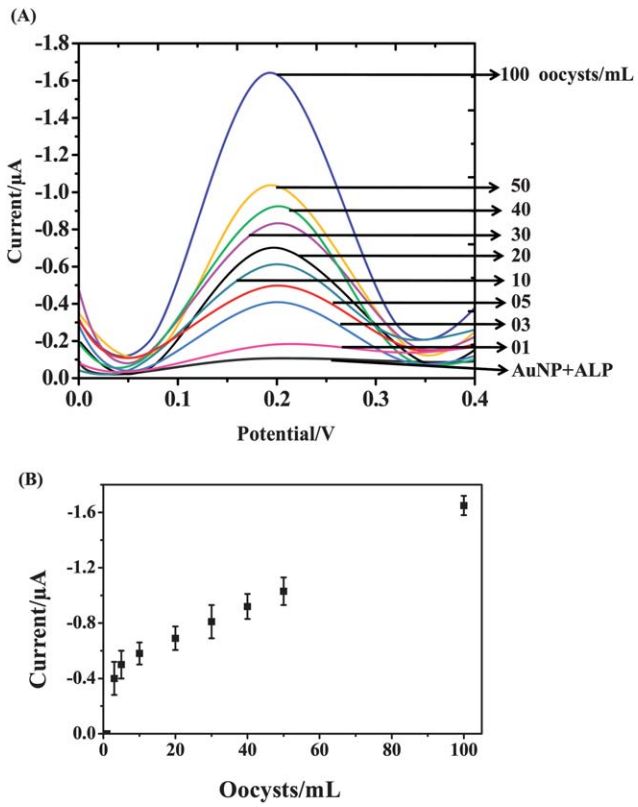


Fig. 4 Differential pulse voltammetry analysis of electrochemical immunosensors using dual labeled AuNP for *C. parvum* detection. (A) Various concentrations of oocysts used from top to bottom of 1 to 100 oocysts/mL (electrolyte: 0.1 M Tris-HCl pH 9.4 solution containing 3 mM *p*-NPP and incubated for 10 min) at a scan range from 0 to 0.4 V. (B) Corresponding calibration plots of peak current *versus* the concentration of oocysts using dual labeled AuNP. The values represent an average of triplicates ±SD ($n = 3$).

Application of electrochemical immunosensor analysis in environmental water samples

The developed method was investigated with real time drinking water samples, which were collected randomly from ten different places and subjected to AuNP based electrochemical

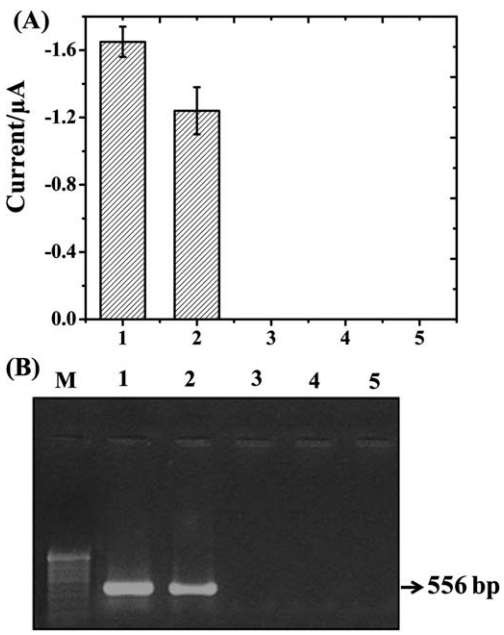


Fig. 5 Evaluation specificity of dual labeled AuNP based electrochemical immunosensor (A) PCR, (B) M-marker; 1) *C. parvum*; 2) *C. parvum* + *Shigella*; 3) *E. coli*; 4) *Vibriocholerae*; 5) *Salmonella*; The concentration of used microorganisms were 100 cells/mL. The values represent an average of triplicates ±SD ($n = 3$).

Table 1 Analysis of drinking water samples using AuNP based electrochemical immunosensor and compared with a fluorescence microscope. The presented values are an average of five results ($n = 5$)

	Oocysts/mL		
Sample No.	AuNP based electrochemical sensor	Fluorescence microscope	RSD (%)
Control (100 oocysts/mL)	97	98	1.4
1	27	28	2.5
2	57	59	2.4
3	33	31	4.4
4	115	111	2.5
5	41	43	3.3
6	23	22	3.1
7	47	44	4.6
8	107	110	1.9
9	12	13	5.6
10	18	17	4.0

immunosensor and fluorescence microscopic analysis. The obtained analytical results by both the methods are listed in Table 1. Relative standard deviation (RSD) of analysis was less than 6%. As a result, the developed dual labeled AuNP based electrochemical immunoassay possibly provide a promising alternative tool for monitoring *C. parvum* in environmental samples for early diagnostics.

Conclusion

A novel enhanced electrochemical immunosensor assay was developed using a dual labeled AuNP probe to detect the parasite *C. parvum* at very low concentration levels. It was demonstrated that AuNP could possibly enhance the performance of the electrochemical immunosensor and achieve higher sensitivity and specificity, especially with a 3 oocysts/mL detection limit. This is a significant consideration in view of fact that the infectious dose ranges from only 1 number up to 30 oocysts. Moreover, *C. parvum* in environmental water samples was also detected effectively using the enhanced electrochemical immunosensor method. In conclusion, the designed dual labeled gold nanoparticles based electrochemical immunosensor stands for high specificity, easy operation, low cost and sensitive assessment for the rapid and reproducibility of monitoring the *C. parvum* in the environmental samples at earlier stage.

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References

1 R. Fayer, *Vet. Parasitol.*, 2004, **126**, 37–56.
2 J. J. David, C. G. Alejandro, E. W. A. Shannon, Clinton White, Jr. and K. Rebecca Richards, *J. Clin. Microbiol.*, 2002, **47**, 4060–4066.
3 L. Xiao, K. Alderisio, J. Limor, M. Royer and A. A. Lal, *Appl. Environ. Microbiol.*, 2000, **66**, 5492–5498.
4 C. Amar, S. Pedraza-Diaz and J. McLauchlin, *J. Clin. Microbiol.*, 2001, **39**, 401–403.
5 G. D. Sturbaum, C. Reed, P. J. Hoover, B. H. Jost, M. M. Marshall and C. R. Sterling, *Appl. Environ. Microbiol.*, 2001, **67**, 2665–2668.
6 J. R. Limor, A. A. Lal and L. Xiao, *J. Clin. Microbiol.*, 2002, **40**, 2335–2338.
7 P. Z. Aguilar and I. Fritsch, *Anal. Chem.*, 2003, **75**, 3890–3897.
8 T. Houssina, J. Follet, A. Follet, E. Dei-Casa and V. Seneza, *Biosens. Bioelectron.*, 2010, **25**, 1122–1129.
9 J. Wang, G. Rivas, C. Parrado, X. Cai and M. N. Flair, *Talanta*, 1997, **44**, 2003–2010.
10 C. D. Kang, S. W. Lee, T. Hyun Park and S. Jun Sim, *Enzyme Microb. Technol.*, 2006, **39**, 387–390.
11 C. D. Kang, C. Cao, J. Lee, I. S. Choid, B. Woo Kim and S. Jun Sim, *Water Res.*, 2008, **42**, 1693–1699.
12 S. Xu and R. Mutharasan, *Environ. Sci. Technol.*, 2010, **44**, 1736–1741.
13 B. S. Munge, J. Fisher, L. N. Millord, C. E. Krause, R. S. Dowd and J. F. Rusling, *Analyst*, 2010, **135**, 1345–50.
14 M. Yang, H. Li, A. Javadi and S. Gong, *Biomaterials*, 2010, **31**, 3281–6.
15 G. Liu, J. Wang, R. Barry, C. Petersen, C. Timchalk, P. L. Gassman and Y. Lin, *Chem.–Eur. J.*, 2008, **14**, 9951–9.
16 Y. YeoHeung, B. Adam, R. William, Heinemanb, H. Brian Halsall, N. S. Vesselin, D. Zhongyun, P. Sarah, B. Michael, J. Abdul, T. Yi, K. Y. W. Danny, B. Amit and M. J. Schulz, *Sens. Actuators, B*, 2007, **123**, 177–182.
17 S. Pozzi Mucellibd, M. Zamunera, M. Tormenc, G. Stantab and P. Ugoa, *Biosens. Bioelectron.*, 2008, **23**, 1900–1903.
18 D. J. Georganopoulou, L. Chang, J. M. Nam, C. S. Thaxton, E. J. Mufson, W. L. Klein and C. A. Mirkin, *Proc. Natl. Acad. Sci. U. S. A.*, 2005, **102**, 2273–2276.
19 L. Fang, Z. Lü, H. Wei and E. Wang, *Anal. Chim. Acta*, 2008, **628**, 80–86.
20 Y. C. Zhang and A. Heller, *Anal. Chem.*, 2005, **77**, 7758–7762.
21 C. Leng, G. Lai, F. Yan and H. Ju, *Anal. Chim. Acta*, 2010, **666**, 97–101.
22 G. Shen and Y. Zhang, *Anal. Chim. Acta*, 2010, **674**, 27–31.
23 V. Mani, B. V. Chikkaveeraiah, V. Patel, J. S. Gutkind and J. F. Rusling, *ACS Nano*, 2009, **3**, 585–594.
24 D. Knopp, *Anal. Bioanal. Chem.*, 2006, **385**, 425–427.
25 X. Chen, H. Xie, Z. Seow and Z. Gao, *Biosens. Bioelectron.*, 2010, **25**, 1420–1426.
26 F. Patolsky, A. Lichtenstein and I. Willner, *Nat. Biotechnol.*, 2001, **19**, 253–257.
27 J. Hajdukiewicz, S. Boland, P. Kavanagh, A. Nowicka, Z. Stojek and D. Leech, *Electroanalysis*, 2010, **21**, 342–350.
28 J. Wang, A. Kawde, M. Musameh and G. Rivas, *Analyst*, 2002, **127**, 1279–1282.
29 Y. Zhengzhi, L. Yan, J. Li-Ping and Z. Jun-Jie, *Biosens. Bioelectron.*, 2010, **26**, 1890–1894.
30 J. Das, K. Jo, J. W. Lee and H. Yang, *Curr. Anal. Chem.*, 2007, **79**, 2790–2796.
31 A. C. Lee, Z. Y. Dai, B. W. Chen, H. Wu, J. Wang, A. Zhang, L. Zhang, T. M. Lim and Y. H. Lin, *Anal. Chem.*, 2008, **80**, 9402–9410.
32 A. Preechaworapun, T. A. Ivandini, A. Suzuki, A. Fujishima, O. Chailapakul and Y. Einaga, *Anal. Chem.*, 2008, **80**, 2077–2083.
33 C. Thiruppathiraja, S. Kamatchiammal, P. Adaikkappan, J. Santhosh and M. Alagar, *Anal. Biochem.*, 2011, **417**, 73–79.
34 K. C. Grabar, R. G. Freeman, M. B. Hommer and M. J. Natan, *Anal. Chem.*, 1995, **67**, 735–743.
35 A. Y. Mikawaa, S. A. Tagliavini Santosa, F. R. Kenfea, F. H. Da Silvac and P. I. Costa, *J. Virol. Methods*, 2009, **158**, 160–164.
36 C. Thiruppathiraja, S. Kamatchiammal, P. Adaikkappan and M. Alagar, *Biosens. Bioelectron.*, 2011, **26**, 4624–4627.
37 M. Anbazhagi, D. Loganathan, S. Tamilselvan, R. Jayabalou, S. Kamatchiammal and R. Kumar, *Clean: Soil, Air, Water*, 2007, **35**, 167–171.
38 G. Del Sel, G. Manfioletti and C. Schneider, *BioTechniques*, 1989, **7**, 514–520.
39 B. C. Ferari, G. Vesley, C. Weir, K. L. Williams and D. A. Veal, *Water Res.*, 1999, **33**(7), 1611–1617.
40 S. Kumar, J. Aaron and K. Sokolov, *Nat. Protoc.*, 2008, **3**, 314–320.
41 L. Yang, L. Yi, R. L. Raymond and Z. Xiangqun, *Biosens. Bioelectron.*, 2009, **24**, 2853–2857.
42 X. M. Li, P. Y. Fu, J. M. Liu and S. S. Zhang, *Anal. Chim. Acta*, 2010, **673**, 133–138.