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DEVELOPMENT OF LATERAL FLOW ASSAY BASED ON SANDWICH ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR THE DETECTION OF CIGUATOXINS IN FISH SAMPLES

MASTER´S DEGREE THESIS

Supervised by Dr. Ciara K. O'Sullivan and Dr. Miriam Jauset Rubio

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Development Of Lateral Flow Assay Based On Sandwich Enzyme-Linked Immunosorbent Assay (ELISA) For The Detection Of Ciguatoxins In Fish Samples

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Abstract. Ciguatera fish poisoning (CFP) is caused by the ingestion of fish contaminated with a marine toxin called ciguatoxin (CTXs). Annually, CFP cases are reported with an estimation of 50,000 and this value will likely increase due to globalization of trade, where contaminated fish disseminated in different areas along with global warming issues will further endanger the public. Avoidance in the consumption of the ciguateric fish is difficult due to the fact that it has the same appearance, smell and taste with the non-toxic fish and cannot be inactivated by freezing or cooking. Moreover, currently available methods such as mouse bioassay, analytical and immunoassay methods fall short in addressing reliability, sensitivity and simplicity for detecting CTXs at the point-of-need at fisheries. Therefore, a reliable, sensitive, and portable assay for routine detection of CTXs is urgently needed. Lateral flow assay (LFA), a commonly implemented assay has gained wide interest in the market due to its simplicity, reliability and accuracy to detect variety of target analytes. Nevertheless, a drawback of this assay can sensitivity in which the format and immunoreagents used plays an important role. In this work, we utilised different nanoparticles such as carbon nanoparticles, gold nanoparticles and latex microspheres as reporter labels and compared their performances. In addition, rabbit IgG was used as a sample target and antibodies against rabbit IgG were utilised as a proof of concept due to the small amounts of CTXs available. Among the particles studied, carbon nanoparticles demonstrated to give the most sensitive results and were then applied to ciguatoxin detection. We successfully developed a LFA based on sandwich ELISA, which can simultaneously detect the four major ciguatoxin congeners (CTX1B, 54-deoxyCTX1B, CTX3C and 51-hydroxyCTX3C) by mixing the 3G8 and 10C9 monoclonal antibodies as capture probe and the 8H4 antibody conjugated to carbon nanoparticle as a reporter label. The assay was tested using standard samples of CTX1B, and then real samples containing ciguatoxins extracted from fish were applied with a detection range up to 35.66 pg/g of fish sample.

INTRODUCTION

Ciguatera fish poisoning (CFP) is a type of food poisoning, which infects humans through the consumption of coral reef fish containing ciguatoxin. The causative neurotoxin, ciguatoxin (CTX), is a lipophilic polyether toxin originated from marine dinoflagellate of the genus *Gambierdiscus* and *Fukuyoa* [1]. These dinoflagellates adhere to the surface of dead corals where bioaccumulation takes place when coral reefs containing ciguatoxin are eaten by herbivorous fish followed by carnivorous fish. Subsequently, the toxic fish is consumed by humans, which presents itself as gastrointestinal symptoms such as abdominal pain and diarrhoea, followed by the manifestation of neurological effect such as paraesthesia and cold or hot allodynia (a reversal sensation), and rare cases of cardiovascular symptoms such as bradycardia with hypotension [2]. To date, the number of individuals who suffer from CFP is estimated to be approximately 50,000 worldwide yearly and is likely to be higher since there are under-reported cases of CFP due to the unfamiliarity of the disease and misdiagnosis [3,4]. The incidence of CFP is higher in tropical and subtropical areas particularly in the Caribbean Sea, as well as the Indian Ocean and Pacific Ocean [5]. However, a dramatic increase of toxic reef fish has emerged in different areas. For instance, in Europe, an outbreak of CFP was reported in the Canary Islands (Spain) in 2004 [6] and in the Selvagens Islands (Portugal) in 2008 [7]. Prior research substantiates the belief that the increasing cases of CFP is due to the globalisation of trade, where ciguateric fish are imported in different areas and along with global warming issues could lead to damage human health, fishery resources, and economies of the affected areas [8]. An avoidance of the consumption of ciguateric fish is difficult, as the ciguateric fish has the same appearance, smell and taste as non-toxic fish and neither cooking nor freezing can inactivate CTX. [4].

Prior investigations have implemented diverse methods to detect CTX in fish samples including the commonly used mouse bioassay, radioligand binding [9], high-performance liquid chromatography [10], mass spectrometry [11], liquid chromatography-coupled to tandem mass spectrometry (LC-MS/MS) analysis [12] and the sodium channel assay [13,14]. In addition, Hokama *et al.* [15] reported the use of a membrane immunobead assay (MIA) utilising antibodies against ciguatoxin for qualitative testing, although cross-reactivity with other marine toxins such as okadaic acid was observed [16]. Nevertheless, these methods fail to demonstrate simplicity, specificity, sensitivity and cost-effectiveness for rapid and routine screening of fish samples at the point-of-need at the fishery sites.

Currently, lateral flow assays (LFA) is the preferred method for the detection of a wide variety of targets including toxins, drugs, proteins, nucleic acid etc. LFA is a paper-based method, which is a portable one-use device that is inexpensive and easy to operate and can be used for the

detection and quantification of the target. Interest in LFAs for cost-effective detection at the point-of-need has increased exponentially since the first pregnancy test kit was launched in 1984. This test utilised a sandwich immunoassay to detect human chorionic gonadotropin (hCG) in urine [17]. This assay format is now commonly implemented for the detection of various targets in different areas such as biomedicine, food, and environmental health and safety [18]. This portable device provides rapid detection of the target giving either qualitative, semi-quantitative or quantitative results within 5-30 minutes following sample introduction. However, a challenging problem in LFA is the sensitivity of the assay where the limit of detection varies depending on the immunoreagents used. One approach to overcome this problem involves the use and proper optimisation of the different assay parameters including antibodies, conjugate labels, as well as the assay format used in LFA. The work reported here explores the development of a portable LFA based on sandwich ELISA for the rapid and sensitive detection of CTX in real fish samples. Optimisation of different reporter particles such as latex microspheres, carbon and gold nanoparticles conjugated with monoclonal antibodies were carried out in order to improve the sensitivity of the assay. The assay is based on a sandwich LFA utilising mixtures of monoclonal antibodies (3G8 and 10C9) as a capture probe and the 8H4 antibody as a reporter probe mimicking the ELISA reported by Tsumuraya *et al.* [19]. These antibodies were generated against synthetic haptens conjugated to the keyhole limpet hemocyanin (KLH) carrier protein injected into a mouse animal host. The antibodies generated were demonstrated to have a high affinity and specificity to CTX with no cross-reactivity to other marine related toxins such as okadaic acid, brevetoxin and maitotoxin [20–26]. In this work, rabbit IgG was used as a model target and antibodies against rabbit IgG were used for optimisation of assay parameters, due to the limited amounts of antibodies and CTX available. Finally, the optimum parameters were applied to a LFA using the anti-CTX antibodies, and the LFA applied to the assay of real samples.

MATERIALS AND METHODS

Phosphate buffered saline (PBS, 10 mM phosphate, 138 mM NaCl, 2.7 mM KCl, pH 7.4), Tween 20, Microtiter plate Nunc-immuno plate F96 Maxisorp, MES, latex sphere particles, boric acid, bovine serum albumin (BSA), 1-(3-Dimethylaminopropyl) 3-ethylcarbodiimide hydrochloride (EDC) were purchased from Fischer Scientific (Spain). Carbonate-bicarbonate buffer, sodium tetraborate decahydrate, skimmed milk powder, sulphuric acid, 3,3',5,5'-Tetramethylbenzidine (TMB), sodium azide, N-hydroxysuccinimide (NHS), ethanolamine, polyclonal anti-rabbit IgG Fc Specific produced in goat, IgG purified from rabbit serum, monoclonal anti-rabbit IgG antibody produced in mouse, anti-mouse IgG-peroxidase, protein A from Staphylococcus aureus and carbon nanopowder were purchased from Sigma-Aldrich (Spain). Monoclonal antibodies (3G8, 10C9 and 8H4) against ciguatoxins (CTXs) were kindly provided by Dr. Takeshi Tsumuraya. Ciguatoxin-1B (CTX1B) and ciguatoxins extracted from fish sample following the reported protocol by Diogène *et al.* [27] were kindly provided by Institut de Recerca I Tecnologia Agroalimentària (IRTA), Sant Carles de la Ràpita, Spain. Ultrapure water from Milli-Q systems (Millipore) with 18.2 MΩcm resistivity was used to prepare all the reagents.

Enzyme-linked Immunosorbent Assay (ELISA)

Each well of 96-well microtiter plate was coated with 50 µL of 20 µg/mL of polyclonal Fc Specific anti-Rabbit IgG produced in goat in 50 mM carbonate-bicarbonate buffer pH 9.6 and incubated for 30 minutes at room temperature. After washing the plate three times with the washing buffer (10 mM phosphate, 138 mM NaCl, 2.7 mM KCl, 0.05% v/v Tween 20, pH 7.4), 200 µL/well of the blocking buffer (5% Skim milk in PBS-tween 20, pH 7.4) was added to each well and incubated for 30 minutes at room temperature. The blocking buffer was removed and different concentrations of rabbit IgG diluted in 1 to 2 starting from 12 µg/mL to 0 µg/mL were added to the ELISA plate and incubated for 30 minutes at room temperature. The supernatant was then removed from each well and the plate was washed three times with washing buffer (200 µL/well). 50 µL/well of the monoclonal anti-Rabbit IgG produced in mouse was added and incubated for another 30 minutes, followed by a thorough washing and finally addition of the secondary labelled anti-mouse IgG-Peroxidase diluted in PBS buffer. Following five thorough washes (200 µL/well), 50 µL/well of TMB was added to each well, and after 5 minutes, the reaction was stopped using 1M sulphuric acid. The plate was measured at 450 nm wavelength using SpectraMax 120 PC Machine. The data was plotted in the GraphPad Prism 6.0 Program to obtain the calibration curve and calculate the limit of detection of the assay by using the formula blank + 3 x STDEV of blank and this value was interpolated on the curve. Duplicate measurements were performed for each concentration.

Preparation of different conjugates

Carbon conjugate: Carbon nanoparticles were bound to antibodies via adsorption following the protocol of Noguera *et al*. [28] with some modifications. Briefly, 10 mg of carbon nanopowder was suspended in 1 mL of Milli-Q water and sonicated for 5 minutes (Bransonic 2510E-MT Ultrasound bath, Barcelona, Spain). The resulting 1% (w/v) carbon suspension was ten-fold diluted in 5 mM Borate buffer pH 8.8 resulting in a final concentration of 0.1% w/v carbon and was again sonicated for 10 minutes. Subsequently, 350 µg/mL of monoclonal anti-Rabbit IgG was added to 300 µL of 0.1% w/v carbon suspension and incubated overnight in the Dynal MX1 sample mixer at room temperature. Following this the suspension was centrifuged (Eppendorf Centrifuge 5417 R) at 13,636*x*g for 15 minutes and the supernatant was then discarded and the pellet was resuspended and washed with washing buffer (5 mM Borate buffer, 1% (w/v) BSA, 0.02 % w/v NaN_3) by centrifugation. The process of washing was repeated three times. After the final wash, the pellet was resuspended in

300 µL of storage buffer (100 mM Borate buffer, 1% (w/v) BSA, 0.02 w/v NaN_3) and stored at 4°C. The resulting carbon suspension contained 0.1% (w/v) carbon conjugate.

Gold conjugate: Gold nanoparticles were synthesised following the protocol of Jauset *et al*. [29] Gold nanoparticles were bound to antibodies via adsorption following the protocol of Dong *et al.* [30] with some minor modifications. Monoclonal anti-Rabbit IgG (25 µg/mL) was added to 1 mL of gold nanoparticles (AuNPs) and 133 µL of 15 mM borate buffer pH 8.5. The solution was then incubated for 30 minutes in the Dynal MX1 sample mixer at room temperature. Following incubation, 100 µL of 10 mg/mL of BSA in PBS was added to the solution to block the AuNP surface and then incubated for 15 minutes at room temperature. Subsequently, the gold conjugate was washed with 1 mL of 1 mg/mL BSA in PBS by centrifugation using 10,000 rpm for 20 minutes. The process of washing was repeated twice. After the final wash, the pellet was resuspended in 1 mL of PBS.

Latex with monoclonal antibody conjugate: The latex particles were bound to antibodies via covalent binding following the manufacturer´s instructions. In 1.5 mL eppendorf tubes, 50 µL of 500 mM MES buffer pH 6.1, 150 µL of Milli-Q water, 50 µL of 4% latex spheres, 125 µL of NHS and 125 µL of EDC was added. The mixture was then incubated for 30 minutes at room temperature in the Dynal MX1 sample mixer. The solution was then washed with 50 mM MES buffer pH 6.1 by centrifugation at 14,000 rpm for 7 minutes. After the supernatant was discarded, the pellet was resuspended with 50 mM of MES buffer, pH 6.1. The process of washing was repeated twice. Monoclonal anti-rabbit IgG (200 µg/mL) was added and the mixture was incubated in the Dynal MX1 sample mixer for 1 hour at room temperature. The solution was washed twice by centrifugation. The latex particle surface was blocked with 1M of ethanolamine in PBS for 1 hour at room temperature in the Dynal MX1 sample mixer. The solution was then washed twice by centrifugation. Finally, the pellet was resuspended in PBS and the solution was stored at 4°C.

Latex with protein A conjugate: The latex particles were bound to Protein A via covalent binding following the manufacturer´s instructions. In 1.5 mL eppendorf tubes, 50 µL of 500 mM MES buffer pH 6.1, 150 µL of Milli-Q water, 50 µL of 4% latex spheres, 125 µL of NHS and 125 µL of EDC was added. The mixture was then incubated for 30 minutes at room temperature in the Dynal MX1 sample mixer. The solution was washed with 50 mM MES buffer pH 6.1 by centrifugation at 14,000 rpm for 7 minutes. After the supernatant was discarded, the pellet was resuspended with 50 mM of MES buffer pH 6.1. The process of washing was repeated twice. Protein A from *Staphylococcus aureus* (5 µg/mL) was added. The mixture was incubated for 1 hour at room temperature. The solution was washed twice by centrifugation. The latex particles surface was blocked with 1M of ethanolamine in PBS for 1 hour at room temperature. The solution was washed twice by centrifugation. Finally, the pellet was resuspended in PBS and the solution was stored at 4°C.

Latex-protein A with monoclonal antibody conjugate: The latex particles were bound to Protein A via covalent binding following the manufacturer´s instructions. In 1.5 mL eppendorf tubes, 50 µL of 500 mM MES buffer pH 6.1, 150 µL of Milli-Q water, 50 µL of 4% latex spheres, 125 µL of NHS and 125 µL of EDC was added. The mixture was then incubated for 30 minutes at room temperature in the Dynal MX1 sample mixer. The solution was washed with 50 mM MES buffer pH 6.1 by centrifugation at 14,000 rpm for 7 minutes. After the supernatant was discarded, the pellet was resuspended with 50 mM of MES buffer pH 6.1. The process of washing was repeated twice. Protein A from *Staphylococcus aureus* (5 µg/mL) was added. The mixture was incubated for 1 hour at room temperature. The solution was washed twice by centrifugation. The latex particles surface was blocked with 1M of ethanolamine in PBS for 1 hour at room temperature. The solution was washed twice by centrifugation. Monoclonal anti-rabbit IgG (100 µg/mL) was added and incubated for 1 hour at room temperature. The solution was washed twice by centrifugation. Finally, the pellet was resuspended in PBS and the solution was stored at 4°C.

Characterization of different nanoparticles (gold, carbon and latex) using Malvern Zeta sizer Ultra

Sample preparation for Zeta sizer and Zeta potential analysis was prepared as follows: For latex microspheres, 1% of latex microspheres was prepared in 50 mM MES buffer pH 6.1 and 10 µL of the latex solution was added to 990 µL of 10 mM PBS, pH 7.4. For carbon nanoparticles, 0.1% w/v of carbon nanoparticles was prepared by diluting ten-fold 10 mg/ml of carbon nanopowder in 5 mM borate buffer pH 8.8. Subsequently 50 µL of the 0.1% w/v carbon suspension was added to 950 µL of 10 mM PBS pH 7.4. Finally, 50 µL gold nanoparticles were directly diluted in 950 µL of water as a dispersant. For the conjugates, 50 µL of each conjugate (carbon, latex-mAb, latex-protein A and latex-protein A-mAb) were added to 950 µL of 10 mM PBS pH 7.4 whilst for the gold conjugate water was used as a sample dispersant.

The particles were analysed using a Malvern Zeta Sizer Ultra at the SCRiT centre in Universitat Rovira i Virgili Campus Sescelades and analysing both particle size and zeta potential for each sample.

Lateral flow assay

Preparation and assembly of the test strip: The test and control lines were prepared by drawing lines with a pipette tip containing 1 mg/mL of polyclonal anti-rabbit IgG antibody and anti-mouse IgG antibody in 10 mM PBS buffer pH 7.4 at the test and control lines, respectively. Subsequently, the membrane was allowed to dry at 22ºC for at least 1 hour, followed by blocking with 1% w/v skimmed milk powder and 0.1% v/v empigen detergent in 10 mM carbonate-bicarbonate buffer pH 9.6 for 15 minutes, under shaking conditions. The membrane was left to dry, again at 22ºC for approximately 2 hours and then stored at 4ºC until use.

DCN backing card with 0.015´´ thick was used as a backing support of the test strip. Whatman/GE FF80HP, Millipore HF90, Millipore HF120, Millipore HF135, Whatman /GE FF170HP, Millipore, HF180 were pasted in the centre of the backing pad and Millipore C048 Absorbent pad was pasted in the upper part of the test strip overlapping of 2 mm into the membrane. The test strips were manually cut in strips of 4 mm width.

Rabbit IgG detection: For each conjugate, 20 µL was added to 10 µL of the sample target (rabbit IgG) in PBS-Tween 20 at a range of concentrations of rabbit IgG (6.66 µg/mL, 3.33 µg/mL, 1.67 µg/mL, 0.83 µg/mL, 0.42 µg/mL, 0.21 µg/mL, 0.10 µg/mL, 0.05 µg/mL, 0.03 µg/mL, 0.01 µg/mL, 0.007 µg/mL and 0 µg/mL). Following incubation of the mixtures for 30 minutes at room temperature, they were applied to the Whatman/GE FF80HP, Millipore HF135, Whatman/GE FF170HP membranes for each of the carbon, latex and gold conjugates, respectively. The band formed at the test line and control line were imaged using a mobile camera and the intensity of the band was calculated using ImageJ software. The data obtained was plotted using the GraphPad Prism Version 6.0 Program to obtain the calibration curve and calculate the limit of detection of the assay as explained above. Triplicate measurements were performed for each concentration.

Ciguatoxin detection: 1 mg/mL of anti-mouse IgG was immobilised on the membrane at the control line and a mixture of monoclonal 3G8 and 10C9 antibodies ratio 1:1 to a final concentration of 3 mg/mL were used as capture antibodies for the test line. The carbon nanoparticles were conjugated with monoclonal 8H4 via adsorption following the procedure explained above. Subsequently, 10 µL of ciguatoxin standards (CTX1B) prepared in PBS buffer were added to 20 µL of the conjugate. Following pre-incubation of the sample for 30 minutes, the mixture was applied to the Whatman/GE FF80HP membrane. The bands formed at the test and control line were imaged using a mobile phone camera. Triplicate measurements were performed for each concentration. For the fish extracts from real fish samples, 40 µL of conjugate and 20 µL of sample were used in order to increase the signal achieved.

RESULTS AND DISCUSSION

Rabbit IgG detection as a model target

Enzyme-linked Immunosorbent Assay (ELISA)

In order to mimic the final approach for the ciguatoxins detection based on a sandwich assay, a polyclonal antibody and a monoclonal antibody against rabbit IgG as capture and reporter probe respectively were used as a proof-of-concept (Figure 1a).

The assay confirmed the functionalities of the antibodies, which were specific to the target as the signal obtained was directly proportional to the amount of target used. In addition, no cross-reactivity between capture and reporter antibodies were observed in the absence of target, and no non-specific binding when no capture antibody was functionalised on the plate (no coating) was observed, which means that the plate was correctly blocked. Finally, the LOD was determined (0.23 µg/mL) in order to obtain a reference method to compare with the lateral flow assay to be developed (Figure 1b).

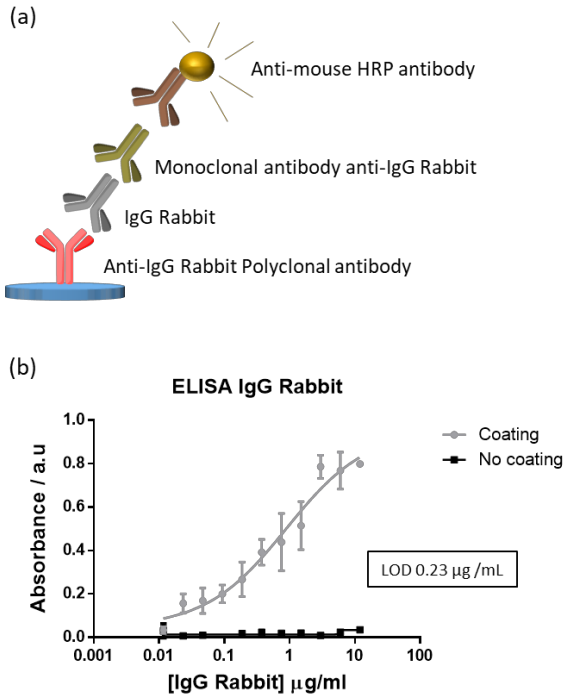

Figure 1. ELISA sandwich assay for IgG detection: (a) Schematic representation of the assay; (b) Calibration curve to calculate the LOD.

Lateral flow assay

The successful development of LFA varies depending on several factors such as the format and the reagents applied in the assay and should be taken into account since they can cause some problems related to the specificity and sensitivity of the assay [31]. In this work, we explored some of these essential components such as the labels for detection and different types of membranes in order to develop the most sensitive and specific LFA.

Characterisation of detection labels

Various types of reporter labels can be used for the visualisation of a signal. The most commonly used are colloidal gold nanoparticles (AuNPs) since they are easy to synthesise and manipulate, stable over time, size-tuneable, biocompatible and possess an intense red colour easy to be detected visually by the naked eye, or using strip readers to achieve better detection limits [32]. Alternative labels used include fluorescent particles such as Quantum Dots (QD), and whilst it is reported that QDs demonstrates better sensitivity than AuNPs, they are often composed of toxic materials and require a fluorescent strip reader for their detection [33]. Latex beads (LB) or polystyrene nanoparticles are also used by several companies due to their relative inexpensiveness whilst having similar

behaviour to AuNPs, with similar or better limits of detection [34]. Finally, there has been a recent increase in the use of carbon nanoparticles (CNPs), also known as carbon black, as labels in LFAs. CNPs are strongly dark coloured nanoparticles that exhibit higher contrast against the background than AuNPs, improving their limit of detection by a factor of ten [35].

In this work, different nanoparticles including AuNPs, CNPs and carboxyl latex microspheres (CLMs) were used to compare and determine the most specific and sensitive label in an LFA for the detection of CTXs. Whilst, CLMs and CNPs were commercially available, with a size of 390 nm and <100 nm respectively, AuNPs, were home-made with a size of 15-30 nm characterised by UV-Vis spectrophotometry (data not shown).

Herein, two different parameters were explored (i) the size of the particles and (ii) the functionalisation method of the monoclonal antibody (mAb) to the particles (adsorption or covalent binding). Four different conjugates were prepared. Both AuNPs and CNPs were functionalised with the mAb against Rabbit IgG via hydrophobic or ionic interactions with no control on a correct orientation of the antibodies on the surface. In the case of CLMs, covalent binding via carbodiimide coupling was carried out. In covalent binding, two kinds of conjugate were prepared: (i) direct immobilisation of the mAb to the particles, where the amine group of mAb bound to the carboxyl group of nanoparticles obtaining more stable conjugate than the adsorption process; (ii) CLMs are functionalised first via covalent binding with protein A followed by affinity interaction with the Fc portion of the mAb. Protein A has a strong affinity to the Fc portion of monoclonal antibodies resulting in an improved orientation for antigen binding and more stable conjugate (Figure 2).

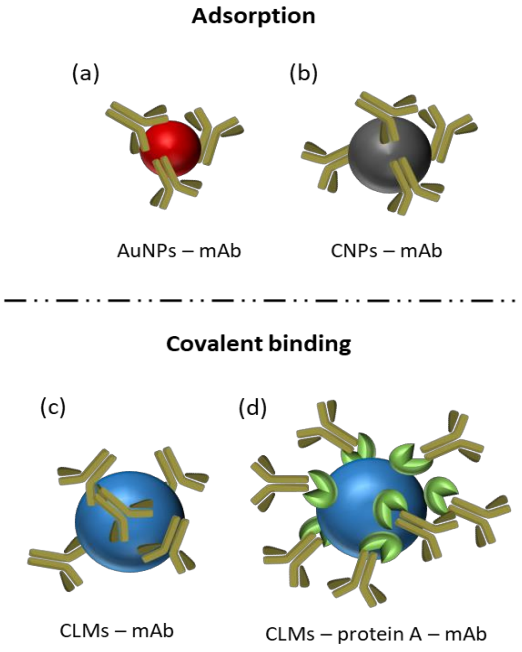

Figure 2. Schematic representation of the conjugation of nanoparticles to mAb: (a) AuNPs – mAb; (b) CNPs – mAb; (c) CLMs – mAb; (d) CLMs – Protein A – mAb.

To confirm the successful conjugation of antibodies to the surface of the particles, characterisation was carried out using a Malvern Zeta Sizer, which permits simultaneous analysis of particle size and zeta potential. The nanoparticles were compared before (without antibody) and after conjugation (with the antibody).

Zeta sizer analysis permitted an estimation of the size of the particles before and after conjugation, and to compare with the reported size indicated by the manufacturers. In figure 3, the peak size of AuNPs and CMLs (Figure 3b, c, d) were 37 nm and 420 nm, respectively, and thus close to the expected sizes. However, the peak size of CNPs (Figure 3a) was 890 nm, a huge difference as compared to the size according to the manufacturer's data sheet. It was assumed that this may be due to the aggregation of the particles. Therefore, sonication of the particles before characterisation is required. Furthermore, the particles were evaluated following conjugation with antibodies. As can be seen in Figure 3 (a, b, c, d), an increase in the sizes were evident indicating the presence of mAb on the surface of the particles, indicating that the conjugation was successful.

Zeta potential analysis was also performed to confirm the successful conjugation of the antibody on the surface of the particle. In figure 4, the data illustrates that particles had a high negatively charge (-42.2 mV for CLMs, -26.4 mV for CNPs and -18.5 mV for AuNPS) when no antibodies were conjugated to the surface of the particles. Whilst, a slightly decrease negatively charge was evident when successful conjugation is achieved. (for CMLs results: -17.4 mV, -33.2 mV, and -22.5 mV for CLMs – mAb, CLMs – protein A and CMLs – protein A – mAb respectively. For CNPs – mAb -16.3 mV and AunNPs – mAb -10.9 mV).

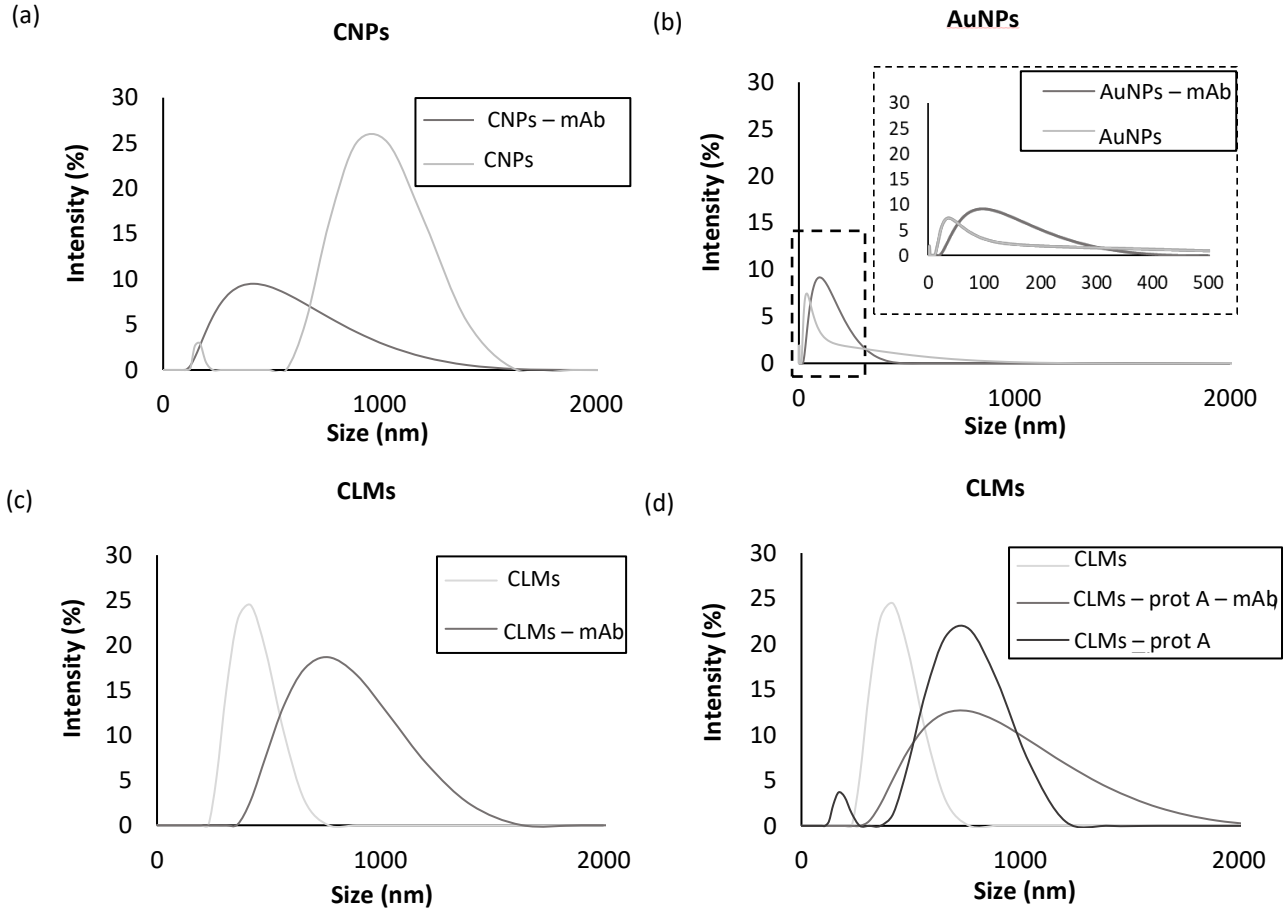

Figure 3. Zeta sizer comparing the different particles before and after conjugation with the anti-rabbit IgG monoclonal antibody: (a) CNPs; (b) AuNPs; (c) CLMs; (d) CLMs with protein A.

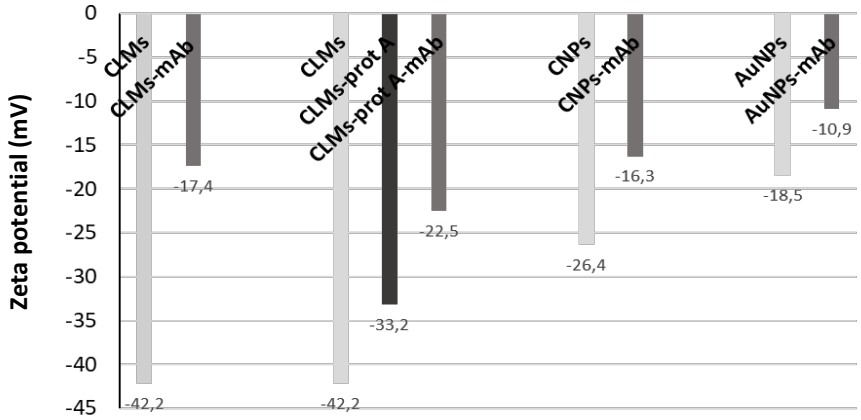

Figure 4. Zeta potential comparing the different particles before and after conjugation with the anti-rabbit IgG monoclonal antibody.

Membrane

Following the demonstration of successful conjugation, the conjugates were evaluated using different membranes in order to determine the most suitable membrane for each particle. The membranes used were all composed of nitrocellulose, the most commonly used material in LFAs since it offers some advantages such as low cost, true capillary flow characteristics, high protein-binding capacity, relative ease of handling and availability of the product with varying flow rates and surfactants [36], which directly affect the assay sensitivity. For instance, a rapid flow rate can result in a false negative due to an insufficient reaction time, whilst a low flow rate can lead to false-positive results due to slow reaction times [37]. For this reason, multiple membrane types should be evaluated during the development of LFA.

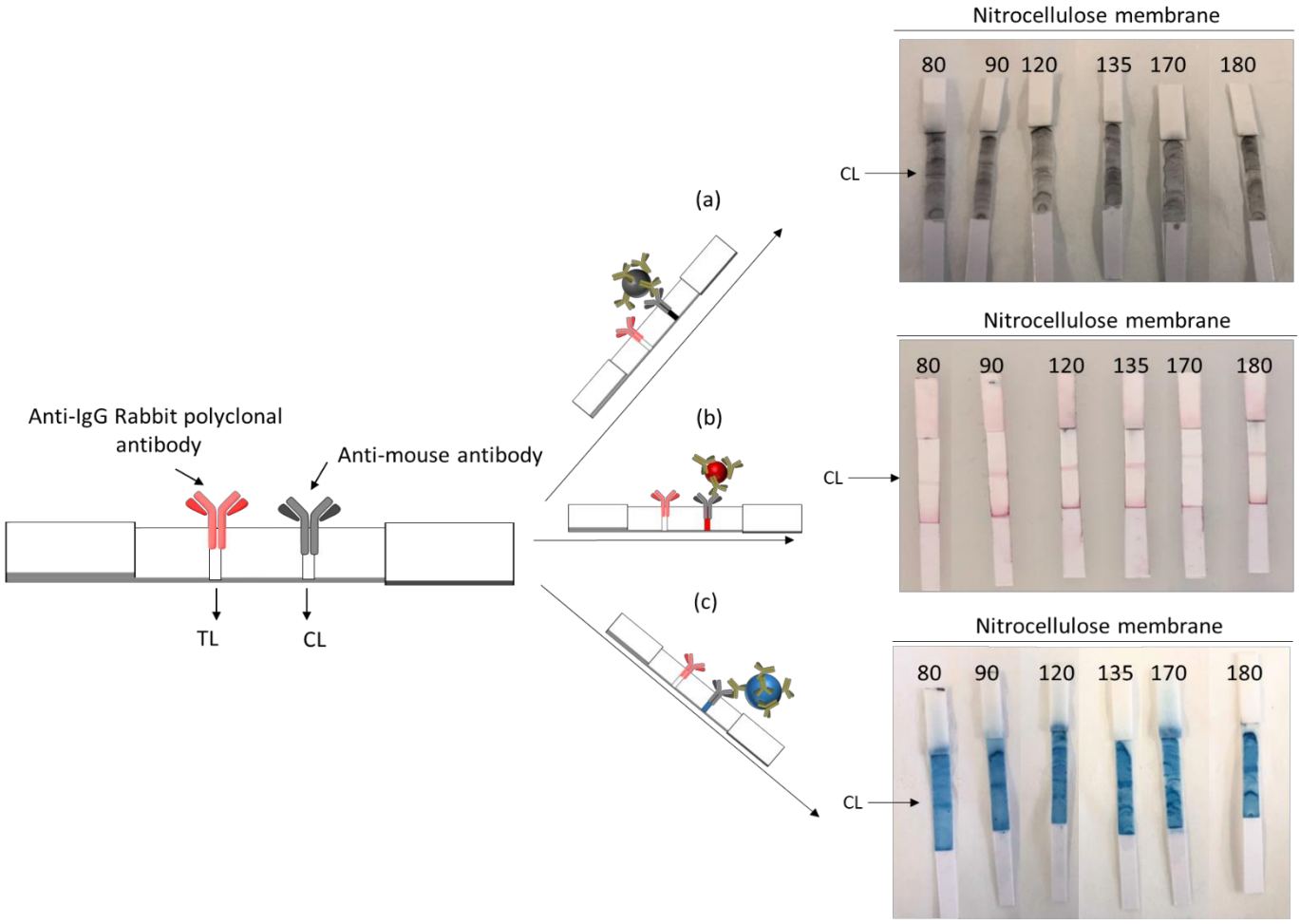

Figure 5. Checking different pore size of membrane to define the optimal conditions for each conjugate: (a) CNPs; (b) AuNPs; (c) CLMs.

The assay basically consisted of the addition of 20 µl of conjugate pre-mixed with 10 µl of 10 mM PBS buffer pH 7.4 and added on the strip as represented in Figure 5. This ratio was previously optimised (data not shown). In the case of CNPs and CLMs, a higher background was observed as compared to AuNPs. Moreover, a difference in the signal using different membranes was evident. Thus, membranes with a flow rate of 80 s/4cm, 135 s/4cm and 170 s/4cm were selected for the detection of rabbit IgG using each of CNPs, CLMs and AuNPs conjugates, respectively.

Additionally, different parameters such as amount of mAb and nanoparticles, incubation time and buffers used during the conjugation were also optimised. In this optimisation process, a decrease in the membrane background for both the CNPs and CLMs were observed. The optimised conjugates were used to detect different concentrations of rabbit IgG to evaluate their sensitivity and specificity. CNPs and AuNPs demonstrate the same behaviour with a good ratio between signal and background and without non-specific binding (Figure 6a, b). However, CLMs, in both cases (directly functionalised with mAb and with protein A and mAb), a higher background and non-specific binding were observed when no sample (rabbit IgG) was added (Figure 6c, d). This problem can be explained by two different issues; (i) the large size of the particles altering the flow rate that leads to false positive results and (ii) the particles are not completely blocked or the blocking agent used (ethanolamine) was not the adequate for this type of assay. Thus, for CLMs, further optimisation process is needed and work with the CLMs was not pursued further.

CNPs and AuNPs were further evaluated and a calibration curve using rabbit IgG was performed for each of them in order to calculate the LOD. A smartphone camera coupled to Image J software, which permits measurement of the intensity of the bands achieved, was used as alternative method to a strip reader device, taking advantage of its high portability, low cost, and ease of use to convert the qualitative LFA to a semi-quantitative device. As a result, the data demonstrated that CNPs are slightly more sensitive than AuNPs with a LOD of 0.069 µg /mL as compared to 0.101 µg /mL of AuNPs – mAb and a better curve fitting was achieved (Figure 7).

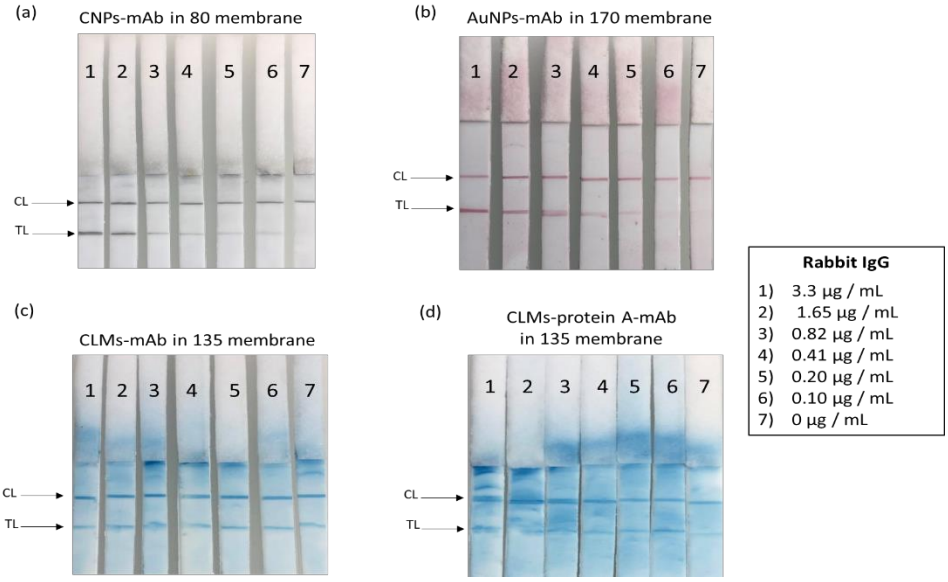

Figure 6. Testing different concentrations of Rabbit IgG with the optimised conjugates: (a) CNPs; (b) AuNPs; (c) CLMs; (d) CLMs with protein A.

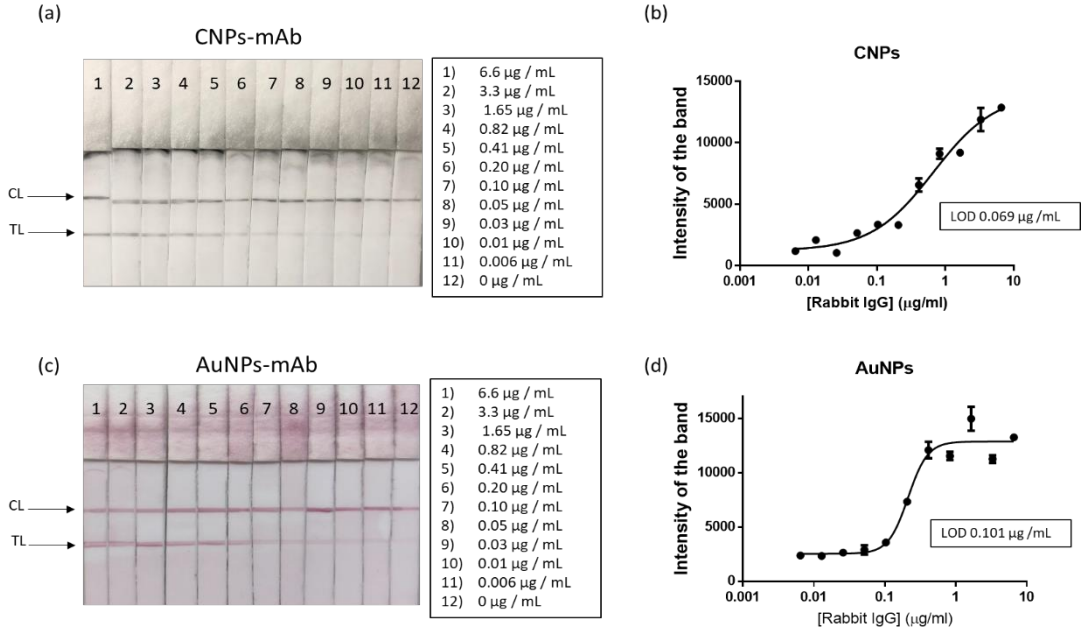

Figure 7. Calibration curves: (a) Visual detection of Rabbit IgG using CNPs; (b) Limit of detection of Rabbit IgG using CNPs and GraphPad Prism software; (c) Visual detection of Rabbit IgG using AuNPs; (d) Limit of detection of Rabbit IgG using AuNPs and GraphPad Prism software.

Ciguatoxins (CTXs) detection using lateral flow assays (LFA)

In 2014, Tsumuraya *et al*. reported the preparation of anti-ciguatoxin monoclonal antibodies using synthetic haptens and demonstrated their functionalities using ELISA [26]. Four antibodies were prepared to bind to the four major CTXs congeners with high affinity: 10C9 binds to the left wing of CTX3C and 51-hydroxyCTX3C, 3G8 binds to the left wing of CTX1B and 54-deoxyCTX1B, 3D11 binds to the right wing of CTX3C and 54-deoxyCTX1B and 8H4 binds to the right wing of CTX1B and 51-hydroxyCTX3C. Recently, a fluorescent sandwich ELISA was published by the same author, capable of detecting any of the four CTX congeners in a single operation. The assay is based on coating the plate with a mixture of two mAbs (10C9 and 3G8) and using ALP-linked to 8H4 mAb as reporter probe, demonstrating that the 8H4 mAb can also detect CTX3C and 54-deoxyCTX1B with a detection limit of less than 1 pg/mL [19].

Based on this sandwich assay, the LFA was developed. Briefly, for the test line, a mixture with a ratio 1:1 of 10C9 and 3G8 mAbs was immobilised in the membrane, whilst anti-mouse IgG was immobilised in the control line. Among those three particles mentioned above, CNPs was

demonstrated to be the most sensitive label for use in LFA. Thus, CNPs were chosen as reporter label and were conjugated with the 8H4 mAb for use in the LFA for ciguatoxin detection (Figure 8a).

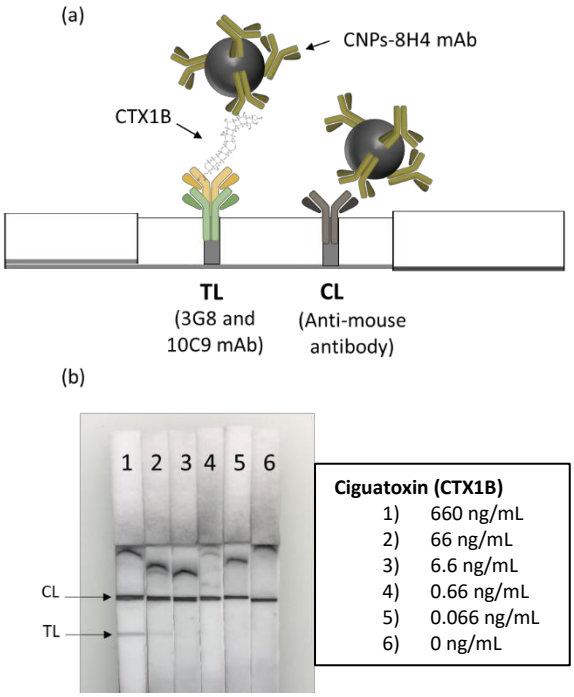

Figure 8. Ciguatoxin detection by LFA: (a) Schematic representation of lateral flow assay; (b) Visual detection of Ciguatoxin (CTX1B).

Different concentrations of CTX1B were evaluated and the assay was capable to detect concentrations from 660 ng/mL up to 6.6 ng/mL by the naked eye (Figure 8b). This detection limit is similar to those achieved in the sandwich ELISA as reported by Tsumuraya *et al*. when the 8H4 mAb was conjugated with HRP [19]. However, whilst the reported detection limit is not enough to detect CTX1B alone considering the FDA guidance level of 0.01 ppb in fish (10 pg/g or 10 pg/mL), it may still be useful for the detection of all four congeners, and it was hypothesised that a better detection limit could be achieved since a mixture of mAbs against CTXs are immobilised in the membrane (test line) and can detect four congeners of CTXs (CTX1B, 54-deoxyCTX1B, CTX3C and 51-hydroxyCTX3) in a single operation without the need to differentiate each congener.

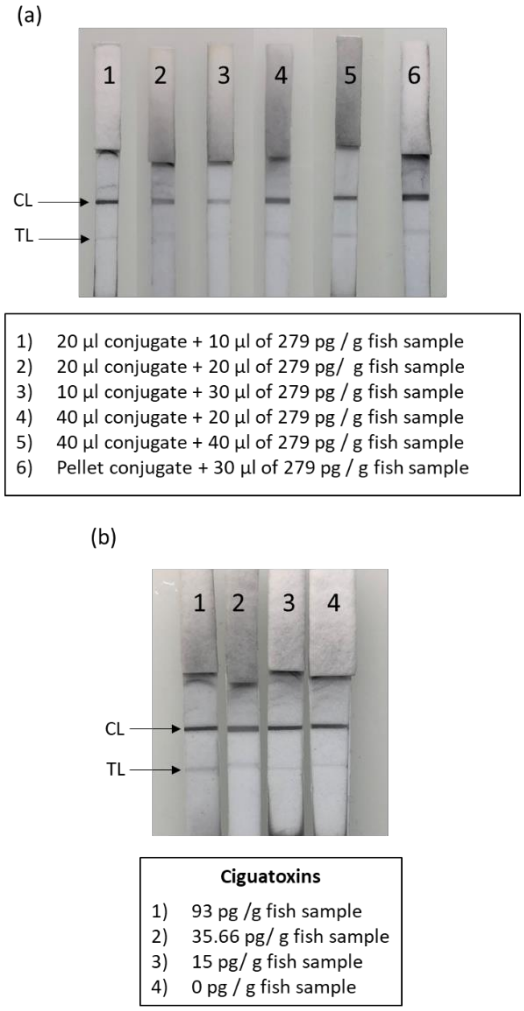

Figure 9. LFA for the detection of extracted CTXs from fish sample: (a) Optimisation of amount of conjugate; (b) Visual detection of CTXs.

Different concentrations of the sample and conjugates were evaluated in order to enhance the signal in the test line. As shown in Figure 9a, the most intense signal was observed using 40 µl of the conjugate and 20 µl of the fish sample. Therefore, this ratio was used to test three different concentrations of CTXs extracted from fish: 93 pg/g, 35.66 pg/g and 15 pg/g (Figure 9b).

As a result, the assay was demonstrated to detect concentrations of CTX of 35.66 pg/g. Comparing to the FDA guidance level (10 pg/g), a little further optimisation is required and other strategies will be explored in order to improve this LOD.

CONCLUSIONS

Rabbit IgG was used as a proof of concept to evaluate four different conjugates using three different nanoparticles (AuNPs, CNPs and CLMs) in terms of sensitivity and specificity. Results demonstrated that CNPs are the most sensitive label for use in LFA. These particles were then applied to the development of the first LFA based on sandwich ELISA for the simultaneous detection of the four major CTXs congeners (CTX1B, 54-deoxyCTX1B, CTX3C and 51-hydroxyCTX3). The developed assay is faster compared

to the developed ELISA and can be useful for rapid screening of ciguatoxins. The assay demonstrated detection of CTX extracted from real samples at 35.66 pg/g, a value close to the FDA guidance level (10 pg/g) and a little further optimisation process is still needed to achieve the same or lower value to the FDA guidance level and work is ongoing to achieve this.

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