

A fluorescent immunochromatographic test using immunoliposomes for detecting microcystins and nodularins

Nathalie Khreich · Patricia Lamourette ·
Bernard Lagoutte · Cyril Ronco · Xavier Franck ·
Christophe Créminon · Hervé Volland

Received: 28 September 2009 / Revised: 24 November 2009 / Accepted: 24 November 2009 / Published online: 22 December 2009
© Springer-Verlag 2009

Abstract Microcystins (MCs), a group of cyclic heptapeptides produced by common cyanobacteria (blue green algae), cause both acute and chronic toxicity. Due to their toxicity, constant monitoring in drinking water, recreational waters as well as other potential exposure through ingestion of contaminated sea food, is very important. In this context, an immunochromatographic test (ICT) using a monoclonal antibody labeled with fluorescent liposomes (immunoliposomes) as tracer was developed, allowing a rapid and simple detection of a large number of MC and nodularin variants in field samples. The present ICT using immunoliposomes proved to be ten times more sensitive than the ICT using colloidal gold for labeling. To achieve quantitative measure-

ment, this ICT was improved by including a stable signal on the control band allowing the expression of the results as a ratio of the fluorescence signals of the specific band versus the control band (SB/CB). Very low concentrations of MC-LR were detected in the analysis buffer (0.06 ng/ml), well below the guideline value of 1 ng/ml proposed by the World Health Organization (WHO), with a dynamic range from 0.06 to 1.5 ng/ml of MC-LR. This method was also validated using a hand-held commercial fluorometer (from ESE[®]), providing the same performances obtained via the analysis station (from Kodak[®]) used in our laboratory. Repeatability tests performed with both devices showed good accuracy (CV < 13%). Furthermore, quantification of MCs in natural samples (water bloom and *Microcystis* culture) was achieved using ICT, leading to similar results obtained via an EIA previously described. All these results demonstrate that this new fluorescent ICT could be used not only as a sensitive detection tool but also to quantify MCs in field samples.

N. Khreich · P. Lamourette · C. Créminon · H. Volland
CEA, iBiTec-S, Service de Pharmacologie et d'immunoanalyse,
Laboratoire d'Etudes et de Recherche en Immunoanalyse,
91191 Gif sur Yvette, France

B. Lagoutte
CEA, iBiTec-S, CNRS URA 2096,
91191 Gif sur Yvette, France

C. Ronco · X. Franck
Equipe de Chimie Bio-Organique,
COBRA-CNRS UMR 6014 & FR 3038 Rue Tesnière,
76130 Mont Saint Aignan, France

C. Ronco
IRCOF, Université de Rouen,
Rue Tesnière,
76130 Mont Saint Aignan, France

H. Volland (✉)
Laboratoire d'Etudes et de Recherche en Immunoanalyse,
CEA Saclay-Bât. 136,
DSV/iBiTec-S/SPI,
91191 Gif sur Yvette, France
e-mail: herve.volland@cea.fr

Keywords Microcystins · Nodularins · Monoclonal antibody · Immunoliposomes · Immunochromatographic test · Enzyme immunoassay

Abbreviations

ICT immunochromatographic test
EIA enzyme immunoassay
MCs microcystins
CR cross-reactivity

Introduction

Massive water blooms of cyanobacteria occur in many freshwater lakes, slow-running rivers, and shallow coastal

water due to anthropogenic eutrophication. In case of excessive growth (such as bloom), these bacteria can produce inherent toxins in quantities causing toxicity for humans and animals. The toxins of cyanobacteria are a very diverse group of compounds with variable toxicological characteristics [1]. The most widely spread and the most often reported toxins are hepatotoxic cyclic oligopeptides—microcystins and nodularins. Microcystins (MCs) are cyclic heptapeptide cyanotoxins produced by members of several cyanobacteria genera (there are more than 60 variants) including *Microcystins*, *Anabaena*, *Nostoc*, and *Planktothrix*. Nodularins are cyclic pentapeptides produced by *Nodularia spumigena* [2, 3]. MCs contain five invariant amino acids as well as two variant amino acids at positions 2 (X) and 4 (Z). The toxins are named according to these two variable L-amino acids (see Fig. 1 for structure). One of the invariant amino acids is a unique β amino acid, common to all MCs and nodularins, abbreviated as Adda (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid; Fig. 1). The toxicity of these cyanobacteria is mostly attributed to the Adda group. They act by inhibiting several eukaryotic protein phosphatases which are essential for many cell regulatory processes such as growth, protein synthesis, glycogen metabolism, and muscle contraction [4, 5]. Acute exposure to these toxins leads to severe and sometimes lethal

liver damage [6]. In addition, long-term exposure to low concentrations of MCs appears also to be involved in tumor promotion [7]. Since cyanobacteria can occur in drinking water reservoirs and recreational lakes, further inducing severe health problems, the development of fast and accurate analytical methods to detect MCs is required. On the basis of toxicity data, the World Health Organization (WHO) has set a provisional guideline value of $1\ \mu\text{g/L}$ for MC-LR ($1\ \text{pmol/ml}$), the most commonly occurring MC, in drinking water (free and cell-bound MCs). Several methods have been developed through the years, including animal bioassays [8], protein phosphatase-inhibition assay [9], HPLC methods combined with UV detection [10], mass spectrometry LC/MS [11, 12], and ELISA tests using a monoclonal or polyclonal antibody [13–16]. Even though each of these detection methods presents its own advantages, their main drawback remains the time-consuming procedures, extensive sample processing prior to analysis, expensive equipment, and the need for qualified experts. One of the current trends for developing an onsite toxin test based on immunodetection relies on the immunochromatographic test (ICT) because of its low cost, simplicity, and rapid screening capacity. A colorimetric ICT using colloidal gold as a tracer recently developed by Pyo et al. [17] can detect $1\ \text{ng/ml}$ of MC-LR. Kim et al. [18] previously developed a fluorescent ICT using

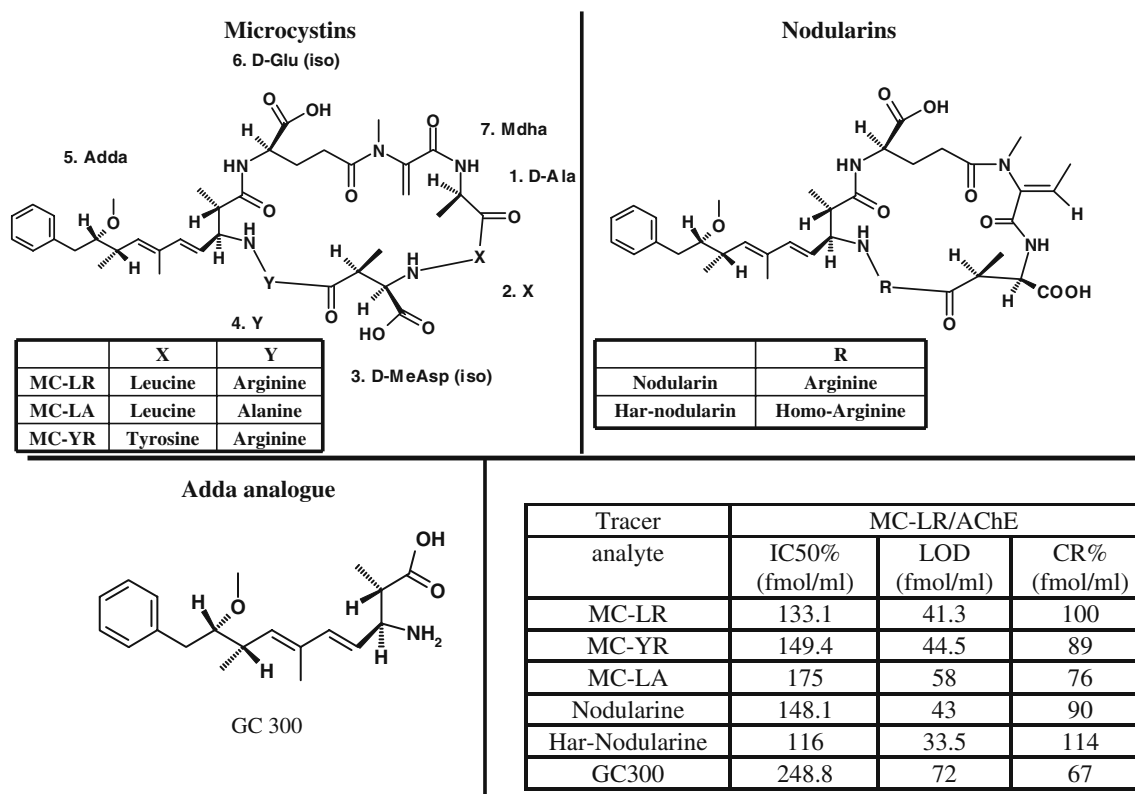


Fig. 1 Structures of the microcystins, nodularins, and Adda analog used in this study. *Insert:* evaluation of the specificity of the mAb MC178 via EIA using MC-LR-AChE tracer

a fluorophore-labeled antibody as a tracer, providing a sensitivity close to 0.05 ng/ml of MC-LR.

In the present study, immunoliposomes encapsulating SulfoRhodamine B (SRB) were prepared to develop a sensitive fluorescent strip assay using a monoclonal antibody of broad specificity for detecting various MCs and nodularins. To apply this test to field samples, an easy-to-use and portable fluorometer allowing reading of the strips was also evaluated. Environmental and cyanobacterial culture samples were successfully analyzed via this ICT and the results were further confirmed using a sensitive competitive enzyme immunoassay (EIA) and LC-MS/MS.

Materials and methods

Reagents

Unless otherwise mentioned, all chemicals were from Sigma, (Saint Louis, MO, USA). Sephadex G25 was from GE Healthcare Bio-Sciences Corporation (Piscataway, NJ, USA). The nitrocellulose membranes (Prima 40), the sample, and absorbent pads (standard 14 and Cellulose grade 470, respectively) were from Whatman (Dassel, Germany).

Microcystins (MCs) were from Sigma. Synthetic Adda analog GC-300 was kindly provided by Prof Renard (Université de Rouen, IRCOF, France). Nodularin and Har-nodularin were purified by HPLC from *Nodularia* culture (Pasteur Collection, PCC 7804) and characterized by mass spectrometry.

MCs containing samples (standard or analogs, bloom samples or cell cultures) require handling precautions, due to the toxicity of MCs. Appropriate laboratory attire should be worn, including a lab coat, gloves, and safety glasses. MC-contaminated materials were inactivated by immersion in bleach solution for 24 h.

The production and selection of monoclonal anti-microcystin MC178 mAb used for this study were described previously [19].

Preparation of MC-LR-coupled BSA

H₂N-MC-LR was prepared according to the procedure of Moorhead et al. [20]. H₂N-MC-LR was conjugated to *N*-succinimidyl *S*-acetylthioacetic acid (SATA) and coupled to BSA using *N*-succinimidyl-4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate (SMCC) in a molar ratio SH/maleimide = 4 as previously described (Khreich et al. [19]). MC-LR-coupled BSA was purified using a G25 Sephadex column with sodium phosphate buffer 0.1 M pH 7.4 + 0.15 M NaCl (PBS). The absorbance of the collected

fractions was measured at 280 nm. The concentration of MC-LR-coupled BSA was evaluated using a BCA protein assay kit.

Preparation of Adda-coupled BSA

The analog of Adda named GC 300 (MW 331.46; see Fig. 1) was conjugated to *N*-succinimidyl *S*-acetylthiopropionic acid (SATP) via its NH₂ function and purified using a chromolith performance RP 18E (100-4.6 mm) column (Merck) as already detailed in Khreich et al. [19]. Collected fractions containing GC300-SATP were then pooled. After reaction with *N*-hydroxylamine to generate a free thiol function, G300-SH was coupled to BSA, previously reacted with SMCC, using a molar ratio GC300-SH/BSA-SMCC=50 for 3 h at 20°C with rotation. The mixture was purified by sieve molecular chromatography using a Sephadex G25 column allowing the separation of unreacted BSA-SMCC. The concentration of the adduct GC 300-BSA was evaluated using a BCA protein assay kit.

Labeling of anti-MC mAb and goat anti-rabbit immunoglobulin antibodies

Labeling of antibodies using either liposomes encapsulating SulfoRhodamine B (SRB) or colloidal gold particles uses previously described protocols [21]. Briefly, the colloidal gold solution was prepared by adding 4 ml of gold chloride and 1 ml of 1% sodium citrate solution to 40 ml of boiling water with constant shaking. After the appearance of a purple color, the preparation was allowed to cool to 20°C and stored at 4°C in the dark. 25 µg of anti-MC mAb were added to 1 ml of colloidal gold solution before mixing with 100 µl of 20 mM Borax buffer pH 9.3. The reaction mixture was incubated for 1 h at 20°C leading to the ionic adsorption of the antibodies on the surface of the colloidal gold particles. 100 µl of 20 mM Borax buffer pH 9.3 containing 1% BSA was then added and the mixture was centrifuged at 15,000×*g* for 50 min at 20°C. The supernatant was discarded and the pellet was suspended in 250 µl 2 mM Borax buffer pH 9.3 containing 1% BSA, sonicated for a few seconds and stored at 4°C in the dark.

The preparation of antibodies labeled with liposome encapsulating SRB involves first the preparation of the liposomes. These liposomes were prepared via a reversed-phase evaporation method [22, 23], using a lipid mixture of dipalmitoylphosphatidylcholine, cholesterol, dipalmitoylphosphatidylglycerol, and dipalmitoylphosphatidylethanolamine (5/5/0.5/0.25; molar ratio). One hundred micromoles of the lipid mixture were first dissolved in 10 ml of a solvent mixture of chloroform, isopropyl ether, and methanol (6/6/1; v/v/v), followed by a 1-min sonication at 45°C under nitrogen

before adding 1.5 ml of 150 mM SRB in 0.1 M potassium phosphate buffer pH 7.4. After sonication of the solution for 6 min at 45°C under nitrogen, the organic solvents were removed at 45°C using a rotary vacuum evaporator, resulting in a dark purple suspension of liposomes. Three milliliters of SRB solution were then added before 5 min sonication at 45°C under nitrogen. The liposome preparation was incubated in a 45°C water bath for 30 min before extrusion through polycarbonate filters of decreasing pore size (three times through a 1.2- μm polycarbonate filter (isoporeTM membrane, Millipore, Billerica, USA) followed by three times through a 0.4- μm filter). The resulting lipid dispersion is a homogenous formulation of vesicles whose mean diameter reflects the theoretical pore size of the last used filter. This solution (liposome diameter close to 0.4 μm) was centrifuged at 45,000 rpm for 30 min at 4°C (Optima L-90 K Ultracentrifuge, Beckman Coulter, Fullerton, CA, USA). The resulting pellet was suspended in PBS (0.1 M potassium phosphate buffer pH 7.4+NaCl 0.15 M) before performing size exclusion chromatography using a Sephadex G 25 column to remove any residual non-encapsulated dye. The liposome solution was centrifuged again to eliminate the excess buffer. The pellet was finally resuspended in 2 ml of PBS and stored at 4°C in the dark.

Then the liposomes were derivated with SATP. Seventeen microliters of SATP (10 mg/ml in DMF) were added to 300 μl of SRB-encapsulated liposomes for 1 h reaction at 20°C. After stopping the reaction with 150 μl of 1 M Tris/HCl buffer pH 9 for 15 min, purification was achieved via a Sephadex G25 column eluted with PBS, followed by a centrifugation at 45,000 rpm for 30 min at 4°C. The pellet of SATP-liposomes was suspended in 225 μl of PBS and the thiol groups were further deprotected by adding 25 μl of 1 M hydroxylamine at pH 7.

Before their conjugation with the liposomes, the MC178 mAb needs to be derivated with SMCC. To 2 mg of MC178 mAb were added a 20-molar excess of SMCC (~9 μl of a 10 mg/ml solution in DMF) for 1 h reaction at 20°C. After stopping the reaction with 150 μl of 1 M Tris/HCl buffer pH 8, the maleimido-mAbs were purified via size exclusion chromatography using a Sephadex G25 column eluted with PBS and monitored at 280 nm. The covalent coupling of thiolated liposomes to maleimido mAb was achieved by reacting 500 μg of SMCC-mAb with the solution of thiolated liposomes for 18 h at 4°C with agitation in the dark. The liposome-labeled antibodies were separated from unconjugated mAb via size exclusion chromatography using a Sepharose CL-4B column eluted with PBS. The liposome-mAb conjugates were collected and centrifuged at 45,000 rpm for 30 min at 4°C. The pellet was then dissolved in 500 μl of PBS containing 0.01% NaN_3 and stored at 4°C in the dark.

Preparation of the strips

The strip (0.5 cm width; 4.5 cm length), includes three components stuck to a backing card (sold with the nitrocellulose membrane) (a) a sample pad (0.5 cm length), (b) a nitrocellulose membrane (2.5 cm length), and (c) an absorption pad (1.5 cm length). The detection zone consists of a test line (to detect MCs) and a control line (to verify that the test works properly). During initial experiments, this detection zone involved goat anti-mouse antibodies immobilized as control line and MC-LR conjugated BSA as test line (500 $\mu\text{g}/\text{ml}$ and 5 $\mu\text{g}/\text{ml}$ in 10 mM sodium phosphate buffer pH 7.4 containing 0.15 M NaCl, respectively), both dispensed at 1 $\mu\text{l}/\text{cm}$ using an automatic dispenser (BioDot Airjet XYZ 3050, Irvine CA, USA). The final improved format of the strips involves rabbit antibodies immobilized as control line and GC300-BSA as test line, both at 10 $\mu\text{g}/\text{ml}$ in 10 mM sodium phosphate buffer pH 7.4 containing 0.15 M NaCl. After drying for 1 h at 40°C in an air oven, the membranes are treated with a blocking solution (10 mM potassium buffer containing 0.15 M NaCl and 0.5% BSA) for 30 min at 20°C. After three washes for 1 min each in ultra-pure water, the membranes were incubated for 20 min at 20°C in 10 mM potassium buffer containing 0.15 M NaCl and 0.1% Tween 20. Then, the membranes were dried for 15 min at 40°C before sticking the absorption and sample pads to the top and the bottom of the membranes, respectively. The membranes were finally cut into strips of 5 mm width using an automatic programmable cutter (Guillotine Cutting (CM4000), BioDot, Irvine, CA, USA).

The ICT

To allow the assay and parallel measurement of a large number of samples, 96-well microtiter plates were used for support. The assay was performed at room temperature in a total volume of 110 $\mu\text{l}/\text{well}$ (100 μl of the sample with 10 μl of colloidal gold or immunoliposome-conjugated MC178 or of a mix (*v/v*) of two tracers MC178 and goat anti-rabbit conjugated immunoliposomes for the final improved format of the test). Unless otherwise stated, all dilutions of tracers or samples were performed in the analysis buffer (0.1 M potassium phosphate buffer pH 7.4 containing 0.1% BSA, 0.15 M NaCl, 0.01% NaN_3 , and 0.5% Tween 20). After a 10-min reaction of the mixture with shaking at 20°C in the dark, the strip was inserted into the well. The capillary migration from the bottom end of the sample pad to the absorption pad (cellulose pad) in the upper position lasts about 15 min. At the end, the absorption and the sample pad were removed and the strip was dried for 5 min at 40°C in an air oven before fluorescence signal measurement.

Measurement method

The signal intensity of the test and control lines were qualitatively estimated when using colloidal gold and quantitatively measured for fluorescence for 1 min using a 2000MM Kodak Image Station (Carestream Molecular Imaging, New Haven CT, USA) set up at 535 nm and 600 nm for the excitation and emission, respectively. The strip pictures obtained were further analyzed using IMAGE J software (Java image processing program) to quantify the fluorescence signal (Rasband, W.S., IMAGE J, U.S. National Institutes of Health, Bethesda, MD, USA, <http://rsb.info.nih.gov/ij/>, 1997–2006). For the field application, a hand-held fluorometer from Embedded System Engineering (ESE, Stockach, Germany) set up at 520 and 600 nm for excitation and emission, respectively, was used to read the strips. The strip was placed in a plastic cassette designed to fit the holder of the reader and was read individually. Results obtained with the two readers were analyzed and compared for validation of the portable reader for the field measurement.

Standard curve in buffer

For fluorescence detection, the tracers, i.e., MC178 mAb and goat anti-rabbit antibodies conjugated to liposomes, were diluted 1/500 and 1/1,000, respectively. Serial dilutions of MC-LR (2–0.03 ng/ml) were prepared before analyzing in triplicate using the ICT while the nonspecific binding (buffer alone devoid of MC-LR) was evaluated on eight strips.

After fluorescence measurement on the Kodak station and signal quantification using IMAGE J software, the standard curve was obtained by plotting the MC-LR concentrations (logarithmic scale) versus the ratio SB/CB, corresponding to the fluorescence measured for the specific band (SB, on the test line) and the control band (CB). These fluorescence values were calculated as the average of the fluorescence intensity (total fluorescence intensity divided by the area of the band using the same area value for the specific and control band) after background subtraction.

When using the ESE fluorometer, the standard curve was also obtained by plotting the MC-LR concentrations against the fluorescence ratio SB/CB, values corresponding to the fluorescence intensity of the specific and control bands.

In both cases, a nonlinear regression (4 parameters function $Y = \text{minimal signal} + (\text{maximal signal} - \text{minimal signal}) / (1 + 10^{(\log IC_{50} - X) * \text{value of the slope}})$) was used to calculate the limit of detection (LOD) defined as the lowest concentration of MC-LR producing a signal corresponding to the mean of the eight SB/CB ratios of the negative control (0 ng/ml of MC-LR) minus 3 SD.

Repeatability test

The inter-assay variations (on the same day) were evaluated to assess the precision and the accuracy of the method by using eight strips, from the same production batch, for each concentration of MC-LR (1; 0.5; 0.25; 0.125; 0.065, and 0 ng/ml in the analysis buffer). For each concentration, the coefficient of variation (CV) and mean were calculated for CB signal, SB/CB ratio and measured concentrations. This study was performed using the Kodak station and the ESE fluorometer.

Competitive enzyme immunoassay

EIA was performed using 96-well microtiter plates coated with goat anti-mouse immunoglobulin antibodies (5 µg/ml), anti-MC mAb MC159 and acetylcholinesterase-labeled MC-LR as previously described [19].

Detection of MCs in true samples

Samples of cyanobacteria blooms were collected from the lake of Villerest (France). Samples of *Microcystis* culture were from the Pasteur collection (PCC 7820). MCs were extracted and purified from lyophilized samples as previously described in Khreich et al [19]. After extraction, 1 ml of the whole extract (dissolved in 2 ml of ultra-pure H₂O containing 0.1% formic acid and 10% of ACN) was dispatched in a tube containing 1 ml of lyophilized analysis buffer (for the ICT test) or 1 ml of lyophilized EIA buffer (0.1 M potassium phosphate buffer pH 7.4 containing 0.1% BSA, 0.15 M NaCl, and 0.01% NaN₃) for the competitive enzyme immunoassay. Serial dilutions of the whole extract (10⁻¹, 10⁻², 10⁻³, 10⁻⁴, and 10⁻⁵ in the corresponding buffer) were prepared and analyzed by ICT and EIA. For both methods, MC concentration was calculated using a standard curve included in the experiment (see above). Each concentration was tested in duplicate and the negative control (0 ng/ml) in quadruplicate.

Results

Development, characterization, and optimization of the fluorescent ICT

Preliminary experiments for selecting the anti-MC mAb

For a rapid method allowing efficient detection of MCs in water, antibodies presenting both high affinity and broad specificity were required. The first set of experiments, performed with the initial ICT format (see the “**Materials and methods**” section) involved different colloidal gold

tracers, prepared with previously characterized monoclonal antibodies with such properties [19]. Among the different monoclonal antibodies evaluated, MC178 mAb gave the best results. When analyzing its characteristics as shown by EIA experiment using MC-LR labeled with acetylcholinesterase as tracer, MC178 strongly recognized MC-LA, MC-YR, nodularin, and Har-nodularin ($CR\% > 76$) and also Adda analog GC300 ($CR > 67\%$; see Fig. 1).

Comparison of the performance of colloidal gold and immunoliposomes in the ICT

Since colloidal gold is considered as reference for ICT, MC178 mAb was labeled with both liposomes and colloidal gold to make a comparison. Serial dilutions of MC-LR were tested by the ICT (initial format) using both MC178 tracers (1/500 and 1/30 dilution for immunoliposomes and colloidal gold, respectively). For the colloidal gold tracer, the colorimetric signal intensity of the test and control lines was qualitatively eye-estimated, while for the immunoliposome tracer, the fluorescent signal intensity was measured using the Kodak station. As expected, since this ICT works in a competitive format, the signal obtained is inversely correlated with the concentration of MC-LR present in the sample. For gold ICT (Fig. 2b), the first concentration giving a lower signal than the signal of the negative sample was 0.8 ng/ml, while the signal for 0.2 and 0.4 ng/ml concentrations were not significantly different. When analyzing the fluorescent signal (Fig. 2a), 0.09 ng/ml showed a strong difference with the negative sample.

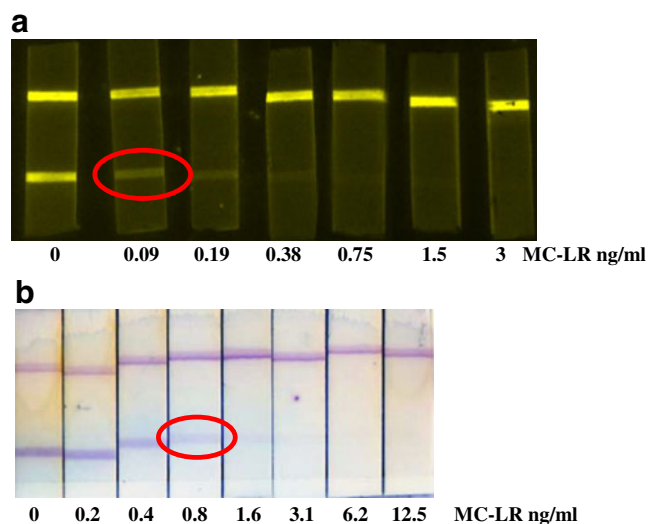


Fig. 2 Comparison of the performance of the two tracers in the ICT in detecting MC-LR: MC178 conjugated immunoliposomes (a picture obtained with Kodak station) and the MC178 conjugated colloidal gold (b image obtained with scanner). A serial dilution of MC-LR was prepared in the analysis buffer in each case. The strip format includes goat anti-mouse antibodies and MC-LR-BSA on the control and test line, respectively

Quantification of the fluorescence with IMAGE J software showed that immunoliposomes allowed the detection of MC-LR within a large range of concentrations from 0.09 to 1.5 ng/ml. When comparing the signal for the MC-LR samples versus the negative sample, a tenfold increase in sensitivity was observed for the immunoliposome tracer (0.09 ng/ml for the immunoliposomes versus 0.8 ng/ml for colloidal gold).

Optimization of the ICT

Standard curve

During early experiments using the initial format of ICT, the signal on the control line was related to the remaining tracer, i.e., not bound on the test line, which was further captured by the goat anti-mouse antibodies. Consequently, the signal observed for the control line may vary and depend on the analyte concentration. To obtain a stable signal of the control line acting as an internal standard independent of the analyte concentration, the corresponding goat anti-mouse immunoglobulin antibodies were replaced by rabbit polyclonal antibodies, while a new tracer, consisting of liposomes conjugated with goat anti-rabbit antibodies, was added and mixed with labeled MC178 mAb.

In this new improved format of ICT, competition occurs on the test line for the MC178 tracer binding between the analyte present in the sample and coated MC-LR-BSA, while the other tracer (liposomes conjugated to goat anti-rabbit antibodies) reacts with the rabbit antibodies of the control line. The intensity of the signal of the control band (CB) is thus independent of the MC concentration in the sample and remains stable. Since the fluorescent signal on the specific band (SB) is inversely proportional to the concentration of MCs present in the sample, the ratio SB/CB decreases when the concentration of MCs increases and presents a maximal value in the absence of MCs (for negative control). To achieve a large working range allowing easy calculations, the maximal value of SB/CB was set as above 1 by using an MC178 mAb tracer excess as compared with the goat anti-rabbit antibody tracer.

Since GC300 appears quite well recognized by MC178 mAb as shown by EIA experiments (see Fig. 1), the adduct GC300-BSA should be considered as an alternative for MC-LR-BSA initially used on the test line. Due to its chemical structure, GC 300 is easier to conjugate than MC-LR and could be obtained in large amount by synthesis. The sensitivity of the ICT using either GC300-BSA or MC-LR-BSA immobilized on the test line was evaluated by testing serial dilutions of MC-LR (2-0.03 ng/ml). After fluorescence measurement and analysis via IMAGE J software, two sigmoid curves showing a similar pattern were obtained as shown in Fig. 3. The sensitivity of the ICT

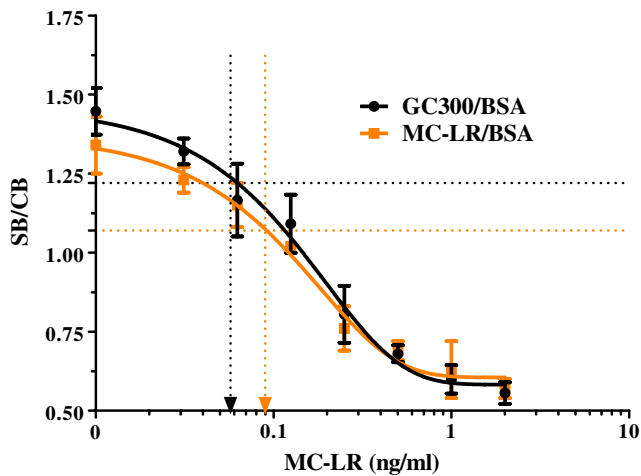


Fig. 3 Standard curves of MC-LR obtained with the tracer MC178 conjugated immunoliposomes using ICT with either MC-LR-BSA (filled square) or GC 300-BSA (filled circle) on the test line. LOD obtained with MC-LR was 0.06 and 0.09 ng/ml for GC300-BSA and MC-LR-BSA, respectively (fluorescence measurement performed for 1 min on the Kodak station). Each concentration was tested in triplicate and the negative control was evaluated on eight strips

using GC300-BSA appears to be slightly improved when compared with MC-LR-BSA, providing a LOD of 0.06 and 0.09 ng/ml, respectively. Hence, the combination of immunoliposome tracers with GC300-BSA and rabbit antibodies on the test and control line, respectively, allowed detection of MCs at very low concentrations, far below the guideline value of 1 ng/ml proposed by the WHO. This ICT method thus seems perfectly suitable for routine monitoring of drinking water.

Furthermore, MCs could be quantified within a large analytical range from 0.06 to 1 ng/ml, values corresponding to the LOD and the concentration producing the minimal signal (minimal SB/CB ratio), respectively (see Figs. 3 and 4).

Comparison of the performance of the Kodak station and the ESE fluorometer

Owing to its usefulness, the present ICT could be used for field sample testing, but the requirement of measuring

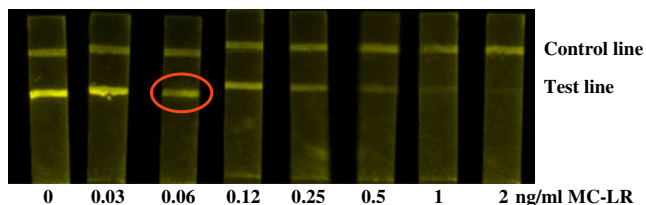


Fig. 4 Picture of strips corresponding to a calibration range of MC-LR obtained with the tracers MC178 and goat anti-rabbit antibodies conjugated immunoliposomes. GC300-BSA and the rabbit antibodies are immobilized (10 μg/ml) on the test and control line, respectively. The tracers are diluted 1/500 and 1/1,000 in the analysis buffer. Fluorescence measurement was made for 1 min on the Kodak station

fluorescence with a lab device (Kodak station) prevents any “in the field” application. Recently, progress in designing detection systems led to hand-held fluorometers, such as the one developed by ESE.

To test the efficiency of the ESE fluorometer for potential use “in the field”, the same standard curve was analyzed using either the Kodak station or the ESE fluorometer. As shown in Fig. 5, the instruments showed similar LOD values: 0.056 and 0.065 ng/ml for the Kodak station and ESE fluorometer, respectively. However, the ESE curve had a greater slope and a larger dynamic range (SB/CB) resulting in a better precision for the assay.

These results demonstrated that the ESE fluorometer allows efficient measurements and the combination of the present ICT and this hand-held device appears convenient for field sample testing.

Validation of the ICT

Repeatability test

Signal inter-assay variations were evaluated to assess the precision of the method by using 8 strips for each concentration of MC-LR. The coefficient of variation (CV) of the ratio SB/CB was calculated for each concentration, after background subtraction, and was always less than 13% for both the Kodak station and the ESE fluorometer (see Table 1). The reproducibility of the signal of the control band was also verified with both instruments. For the same concentration of MCs, the CVs of the control band signal were always less than 13% while when comparing the means obtained for the different concentrations, the CV was 10.7% with the Kodak station and

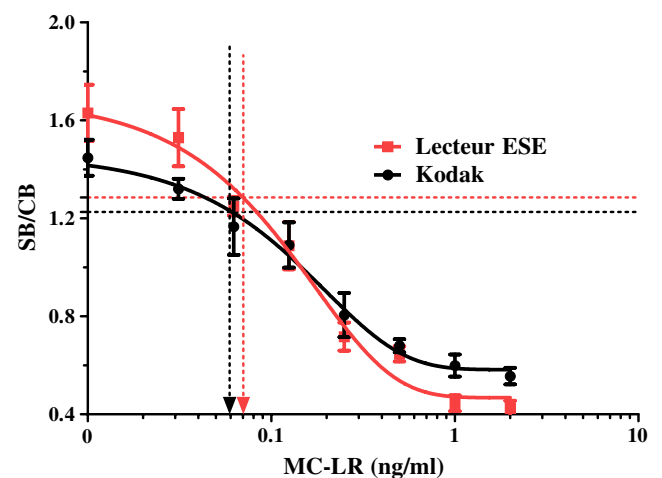


Fig. 5 Standard curves of MC-LR obtained by the ICT analyzed by the Kodak station (filled circle) and the ESE fluorometer (filled square). The ICT uses MC178-conjugated immunoliposomes as tracer, GC 300-BSA on the test line and rabbit antibodies on the control line

Table 1 Evaluation of the repeatability of the ICT using immunoliposomes as fluorescent tracers

Concentration MC-LR (pg/ml)	Kodak station		ESE fluorometer	
	SB/CB: mean (CV%)	Measured concentrations: mean (CV%)	SB/CB: mean (CV%)	Measured concentrations: mean (CV%)
1000	0.67 (2)	860 (10)	0.5 (7)	821 (37)
500	0.7 (4.5)	572 (22)	0.6 (7.3)	525 (26)
250	0.8 (4.8)	286 (23)	0.8 (4.7)	265 (13)
125	1.1 (6.9)	93 (22)	1.1 (11)	111 (13)
60	1.1 (13)	59 (72)	1.3 (10.5)	65.4 (38)
0	1.4 (10.2)		1.7 (11.4)	

Each concentration was tested on eight strips using the Kodak station or the ESE fluorometer

2.5% with the ESE fluorometer. These results show that the signal on the control band presents only minor changes, independent of the MC concentration, and thus can be used as an internal standard and as a tool to verify the conditions of the test. The ratio SB/CB allowed us then to set up the standard curve. The accuracy and the precision of the quantification were also checked (see Table 1). For both Kodak station and ESE fluorometer, we obtained a classical precision pattern for a competitive assay (higher CV for the higher and lower concentrations). Even if the measured CV appeared important, the mean presented a good correlation with the expected values, showing that the present ICT allows to quantify MCs in the sample.

Sample analysis

Comparison of immunoliposomes test strip and EIA

A parallel analysis using ICT and EIA was performed on different dilutions of the extracts from Villerest bloom and *Microcystis* culture; both samples including a large set of MCs, as previously identified by LC/MS [19]. In both tests, the quantification was achieved using a standard curve of MC-LR. As shown in Table 2, both ICT and EIA provide results in the same range with a correct correlation. However, ICT always underestimated the samples concentrations as compared to EIA (approximately 75% of the value). Even if both mAbs, i.e., MC178 and MC159 used for ICT and EIA, respectively, exhibit a broad specificity, further experiments should be performed to better investigate the observed

differences and further confirm the large specificity of the ICT. In spite of this drawback, these experiments document the possibility of using ICT not only as a detection tool but also to quantify MCs present in natural samples.

Discussion

Dye encapsulating liposomes were first introduced in test strip assays by Durst and colleagues and further used to detect toxic chemicals, toxins and bacteria, [24–28]. All these previous studies reported visually estimated signals, linked to the optical properties of the fluorescent dye in intact liposomes. These results thus took no advantage of the high sensitivity normally provided by the fluorescence measurement associated with the large amount of dye encapsulated in the liposomes. In fact, this high concentration of encapsulated dyes generated a quenching phenomenon resulting in the inhibition of the fluorescence. A procedure was recently developed in our lab to solve this problem. The strip was air-dried (for 5 min in the oven) to destroy the structure of the liposomes allowing release and local dispersion of SRB and further resulting in the recovery of the fluorescence. The localized fluorescent signal observed on the strips allowed a 15-fold increase in sensitivity as compared with visual detection of colloidal gold when applied to detect *Staphylococcus aureus* Enterotoxin B in a sandwich format [21]. In the present study, this procedure also leads to a tenfold enhancement of the sensitivity of the competitive ICT, taking colloidal gold as reference since this ICT label

Table 2 Samples analysis of Villerest bloom and *Microcystis* culture extract by ICT and EIA

Sample	Dilution in the analyzing buffer	Quantification by ICT (ng/ml)	Quantification by EIA (ng/ml)
Bloom of Villerest	1/10 ³	0.92	1.25
	1/10 ⁴	0.09	0.11
Culture of <i>Microcystis</i>	1/10 ⁶	0.14	0.18

The quantification is performed using a standard curve of MC-LR in both methods. Each concentration is tested in duplicate and the negative control is tested in quadruplicate

has been largely used for detecting infectious diseases, cancer, drug monitoring, or analyzing food safety [29]. Immunoliposomes encapsulating a fluorophore may thus be useful in both immunological formats, i.e., sandwich or competitive ICT.

On the other hand, detection of MCs is a rather difficult problem due to the huge diversity of these compounds, including more than 60 variants. Even if it is impossible to verify that all the MCs can be detected, the present ICT involves a monoclonal antibody, MC178, known to recognize efficiently numerous available MCs, due to its specificity towards the Adda residue common to all MCs. The present ICT detected different MCs present in natural samples and can thus be considered as an interesting tool for MC analysis.

The final format of the ICT includes a stable control band, useful as an internal control independent of the analyte concentration, and further allowing plotting of a standard curve using the ratio of the fluorescence signals of the specific band versus the control band (SB/CB). As characterized via this standard curve, the test using GC300-BSA-coated strips showed slightly better sensitivity (LOD=0.06 ng/ml) than with MC-LR/BSA (LOD=0.09 ng/ml). This probably results from the lower affinity of MC178 mAb towards GC300 in comparison with MC-LR, which makes the competition easier between the tracer and the MCs present in the sample. Thus, this new ICT has a very good sensitivity with an LOD well below the guideline value of 1 ng/ml set by the WHO. This result is very important because even if some authors reported that this guideline value is suitable by reference to the toxicity of MCs [30], others suggest a much lower guideline value, especially for long-term exposure in complex matrices [31].

The present ICT also compares favorably with similar recently published methods such as ICT using colloidal gold [17] allowing a detection of MC-LR close to 1 ng/ml and involving extraction of MCs via a supercritical fluid, or fluorescence ICT [32] for MC-LR with an LOD in spiked surface water close to 50 pg/ml using a laser fluorescence scanner.

Furthermore, the sensitivity reached with the ICT using immunoliposomes as tracer is comparable to that of recently reviewed biological detection methods for MCs [33, 34] and appears only six times less sensitive than the EIA recently developed in our laboratory [19].

Since the main purpose of ICT corresponds to “in the field” application, a simple and easy-to-use hand-held fluorometer (from ESE) was identified. Using the present ICT, this small device performs as well as the Kodak station, thus permitting on-the-spot reading of the strips. Preliminary experiments applied to natural samples allow comparison of the ICT with classic EIA. The methods give similar results, confirming the efficiency of the ICT and the possibility of quantifying MCs. The ESE fluorometer can

include a small calculation software and a pre-recorded standard curve from previously measured SB/CB ratios for known concentrations of MC-LR. The combination of the ICT with this precalibrated device may thus allow both direct detection and quantification of MCs (as MC-LR equivalent) during “in the field” testing. This is currently being evaluated in our lab.

On the other hand, total MC measurement raises another problem since MCs in a sample correspond to both extracellular and intracellular toxins. Quantification of the total MC concentration thus required an extraction, which should be performed prior to ICT after “in the field” sampling and possibly by unskilled people. We are currently investigating a rapid (less than 10 min) and easy method to extract MCs onsite. This method does not require instruments or heavy equipment and could be performed by untrained persons with minimum sample handling to reduce possible loss of analytes.

Conclusion

Due to the toxicity of cyanobacterial hepatotoxins, the best way to safeguard the health and wellbeing of humans and animals is the prevention of exposure to these toxins. Therefore, constant monitoring of drinking water, recreational waters as well as other potential exposure like contaminated sea food, is very important. In this study, a sensitive ICT using fluorescent immunoliposomes as tracers was developed, allowing rapid and simple detection of a large number of MC and nodularin variants. The fluorescent signal generated by these immunoliposomes can be measured and quantified using a small, transportable, and easy-to-use fluorometer. While colloidal gold provides a yes/no response indicating the presence of the toxin, the fluorescence ICT could be used not only as a sensitive detection tool but also as a quantification assay applied to field samples. In addition, a very simple method to extract cyanotoxins in the field is currently under evaluation in our lab. This step, associated with a reproducible standard curve memorized in the software of the fluorometer will complete our goal to create an ICT for field quantification of MCs. This method allows the sensitive detection of MC-LR (0.06 ng/ml) and was able to successfully detect and quantify MCs in bloom water samples and *Microcystis* culture extract. Therefore, this ICT fulfills all the criteria of a preliminary screening method prior to later quantification or further identification of MC variants in the sample using classical laboratory methods such as EIA or LC-MS/MS.

Acknowledgment The authors are greatly indebted to J.F. Humbert (INRA, Thonon) for providing the bloom samples. This work was supported by grants from the Commissariat à l’Energie Atomique (France).

References

1. Carmichael WW (1992) *J Appl Bacteriol* 72:445–459
2. Carmichael WW (1997) *Advances in botanical research incorporating advances in plant pathology—classic papers*. Academic, New York
3. Rinehart KL, Harada K, Namikoshi M, Chen C, Harvis CA, Munro MHG, Blunt JW, Mulligan PE, Beasley VR (1988) *J Am Chem Soc* 110:8557–8558
4. Runnegar M, Berndt N, Kong SM, Lee EYC, Zhang LF (1995) *Biochem Biophys Res Commun* 216:162–169
5. Mackintosh C, Beattie KA, Klumpp S, Cohen P, Codd GA (1990) *FEBS Lett* 264:187–192
6. Robinson NA, Matson CF, Pace JG (1991) *J Biochem Toxicol* 6:171–180
7. Nishiwakimatsushima R, Ohta T, Nishiwaki S, Sukanuma M, Kohyama K, Ishikawa T, Carmichael WW, Fujiki H (1992) *J Cancer Res Clin Oncol* 118:420–424
8. Torokne A, Vasdinnyi R, Asztalos BM (2007) *Environ Toxicol* 22:64–68
9. Heresztyn T, Nicholson BC (2001) *Water Res* 35:3049–3056
10. Lawton LA, Edwards C, Codd GA (1994) *Analyst* 119:1525–1530
11. Barco M, Rivera J, Caixach J (2002) *J Chromatogr A* 959:103–111
12. Zweigenbaum JA, Henion JD, Beattie KA, Codd GA, Poon GK (2000) *J Pharm Biomed Anal* 23:723–733
13. Nagata S, Soutome H, Tsutsumi T, Hasegawa A, Sekijima M, Sugamata M, Harada K, Sukanuma M, Ueno Y (1995) *Nat Toxins* 3:78–86
14. Mikhailov A, Harmala-Brasken AS, Meriluoto J, Sorokina Y, Dietrich D, Eriksson JE (2001) *Toxicol* 39:477–483
15. Zeck A, Eikenberg A, Weller MG, Niessner R (2001) *Anal Chim Acta* 441:1–13
16. Young FM, Metcalf JS, Meriluoto JA, Spoo L, Morrison LF, Codd GA (2006) *Toxicol* 48:295–306
17. Pyo D, Choi J, Hong J, Oo HH (2006) *Journal of Immunoassay & Immunochemistry* 27:291–302
18. Kim YM, Oh SW, Jeong SY, Pyo DJ, Choi EY (2003) *Environ Sci Technol* 37:1899–1904
19. Khreich N, Lamourette P, Renard PY, Clave G, Fenaille F, Creminon C, Volland H (2009) *Toxicol* 53:551–559
20. Moorhead G, Mackintosh RW, Morrice N, Gallagher T, Mackintosh C (1994) *FEBS Lett* 356:46–50
21. Khreich N, Lamourette P, Boutal H, Devilliers K, Creminon C, Volland H (2008) *Anal Biochem* 377:182–188
22. Szoka F, Olson F, Heath T, Vail W, Mayhew E, Papahadjopoulos D (1980) *Biochim Biophys Acta* 601:559–571
23. O'Connell JP, Campbell RL, Fleming BM, Mercolino TJ, Johnson MD, McLaurin DA (1985) *Clin Chem* 31:1424–1426
24. Siebert ST, Reeves SG, Durst RA (1993) *Anal Chim Acta* 282:297–305
25. Siebert STA, Reeves SG, Roberts MA, Durst RA (1995) *Anal Chim Acta* 311:309–318
26. Park S, Durst RA (2000) *Anal Biochem* 280:151–158
27. Ahn-Yoon S, DeCory TR, Baeumner AJ, Durst RA (2003) *Anal Chem* 75:2256–2261
28. Ahn-Yoon S, DeCory TR, Durst RA (2004) *Anal Bioanal Chem* 378:68–75
29. Peruski AH, Peruski LF Jr (2003) *Clin Diagn Lab Immunol* 10:506–513
30. Fawell JK, Mitchell RE, Everett DJ, Hill RE (1999) *Human Exp Toxicol* 18:162–167
31. Ernst B, Dietz L, Hoeger SJ, Dietrich DR (2005) *Environ Toxicol* 20:449–458
32. Kim YM, Oh SW, Jeong SY, Pyo DJ, Choi EY (2003) *Environ Sci Technol* 37:1899–1904
33. Msagati TAM, Siame BA, Shushu DD (2006) *Aquat Toxicol* 78:382–397
34. McElhiney J, Lawton LA (2005) *Toxicol Appl Pharmacol* 203:219–230