View Online

Derivatisation of microcystin with a redox-active label for high-performance liquid chromatography/electrochemical detection

Kenneth Kam-Wing Lo,* Dominic Chun-Ming Ng, Jason Shing-Yip Lau, Rudolf Shiu-Sun Wu and Paul Kwan-Sing Lam*

Centre for Coastal Pollution and Conservation, City University of Hong Kong, Tat Chee Avenue, Kowloon, Hong Kong, P. R. China. E-mail: bhkenlo@cityu.edu.hk; Fax: +852 2788 7406; Tel: +852 2788 7231

Received 2nd July 2002, Accepted 16th September 2002
First published as an Advance Article on the web 6th December 2002

Published on 13 December 2002 on http://pubs.rsc.org | doi:10.1039/B206384K
Downloaded on 25 October 2010

Microcystins are a group of low molecular weight, cyclic peptide hepatotoxins. The most common detection and quantitation methods for these toxins are liquid chromatography with UV or mass spectrometric detections, phosphatase inhibition assays and enzyme-linked immunosorbent assays. In addition, derivatisation of these toxins with organic fluorophores followed by CE/laser induced fluorescence detection and HPLC/chemiluminescence detection; and with luminescent lanthanide chelates for competition assays have also been reported. However, the use of an electrochemical-active unit as a tag for microcystins has never been explored. Since the sulfhydryl group of 6-ferrocenylhexanethiol (**Fc-C6-SH**) can undergo a facile addition reaction with the α,β-unsaturated carbonyl group, this compound has been used as a redox-active labelling agent for a derivative of microcystins, microcystin-LR (**MC-LR**). The conjugate, **Fc-MC-LR**, has been isolated by high-performance liquid chromatography with electrochemical detection. The peak height-concentration curve was linear in the test range 20–400 ng of **MC-LR** (r value for linear regression >0.9987). The detection limit was determined to be *ca.* 18 ng **MC-LR** ($S/N = 3$). Meanwhile, the conjugate **Fc-MC-LR** has also been characterised by positive-ion electrospray-ionisation mass spectrometry. Electrochemical studies show that the adduct displays a reversible ferrocenium/ferrocene couple at *ca.* −0.040 V *vs.* SCE (scan rate = 50 mV s^{-1}) in 0.1 M aqueous ammonium acetate–acetonitrile (55:45 v/v).

Microcystins are a group of low molecular weight, cyclic peptide hepatotoxins produced by cyanobacterial species in eutrophic lakes and drinking water reservoirs.[1,2] To date, about sixty different types of microcystins have been characterised. Since these toxins represent significant hazards to humans, livestock and wildlife, their levels in the environment must be strictly monitored. There have been many reports on the detection and quantitation of this class of toxins. The most common one is liquid chromatography with UV or mass spectrometric detections.[3–6] Phosphatase inhibition assays[7,8] and enzyme-linked immunosorbent assays (ELISA)[9,10] are also effective quantitation procedures. In addition, derivatisation of these toxins with organic fluorophores followed by CE/laser induced fluorescence detection[11] and HPLC/chemiluminescence detection;[12] and with luminescent lanthanide chelates for competition assays have also been reported.[13] Compared to these analytical techniques, electrochemical detection of microcystins has been receiving much less attention, mainly because of the lack of a redox-active moiety on these toxin molecules. In this regard, differential pulse polarography has been used to study the binding of copper and zinc ions to microcystin derivatives.[14] The change in the electrochemical potential and decrease in the height of metal polarogram peaks have been correlated to the concentration of microcystins. Besides, the interactions between microcystins and mercury(II) ion, and other ions such as lead(II), have also been studied using anodic stripping voltammetry.[15] Meanwhile, direct electrochemical (EC) detection in the liquid chromatographic separation of microcystins, based on the oxidation of the arginine and tyrosine residues of these toxins, has also been examined.[16] Despite all these reports, to the best of our knowledge, the use of an electrochemical-active unit as a tag for microcystins has never been explored. In this paper, we report the utilisation of a redox-active compound, 6-ferrocenylhexanethiol (**Fc-C6-SH**), as a label for a derivative of these toxins, microcystin-LR (**MC-LR**). The reactivity of **Fc-C6-SH** towards the α,β-unsaturated ketone moiety has been demonstrated using methyl vinyl ketone (**MVK**) as the model substrate. The toxin-derivatisation procedure and HPLC/EC separation of the conjugate, **Fc-MC-LR**, together with its characterisation and electrochemical properties are also reported.

Experimental

Materials

All solvents for chemical synthesis were of analytical grade and used without further purification. Ammonium acetate (Junsei, Tokyo, Japan) was used as received. Water for aqueous buffers was purified to 18.2 MΩ cm on a Milli-Q apparatus (Millipore, USA). HPLC grade acetonitrile and trifluoroacetic acid (TFA) were purchased from RDH (Seelze, Germany). A commercial sample of **MC-LR** purchased from Calbiochem (La Jolla, CA, USA) was used as the standard.

Synthesis

The label **Fc-C6-SH** was prepared from modified literature methods.[17–19] To a dichloromethane (40 ml) solution of ferrocene (Acros, Geel, Belgium) (1.99 g, 10.7 mmol) was added 6-bromohexanoyl chloride (Acros, Geel, Belgium) (2.28 g, 10.7 mmol) dissolved in 5 ml CH_2Cl_2. Aluminium chloride (Acros,

 DOI: 10.1039/b206384k

This journal is © The Royal Society of Chemistry and the Centre National de la Recherche Scientifique 2003

View Online

Published on 13 December 2002 on http://pubs.rsc.org | doi:10.1039/B206384K
Downloaded on 25 October 2010

Geel, Belgium) (1.43 g, 10.7 mmol) was added to the mixture. The colour of the suspension changed from orange to purple and the suspension was stirred under an inert atmosphere of nitrogen for 4 h at room temperature. The reaction was then quenched by addition of 4 M HCl (100 ml). The aqueous layer was separated and extracted with CH_2Cl_2 (100 ml × 3). The organic layers were collected, dried over $MgSO_4$ and evaporated under vacuum to give a brown oil. The crude product, 6-bromohexanoylferrocene, was purified by column chromatography using silica gel as the stationary phase. The unreacted starting material was eluted with petroleum ether (40–60 °C) –diethyl ether (95:5) and the product with petroleum ether (40–60 °C)–diethyl ether (90:10). Yield: 2.50 g, 6.9 mmol, 64%. Positive-ion ESI-MS: $m/z = 363$, M^+. IR (KBr) ν/cm^{-1}: 1668 (s, C=O).

To a mixture of 6-bromohexanoylferrocene (1.50 g, 4.1 mmol), zinc (Sigma–Aldrich, St. Louis, MO, USA) (8.09 g, 123.8 mmol) and mercury(II) chloride (Sigma–Aldrich, St. Louis, MO, USA) (0.75 g, 2.8 mmol) in 20 ml toluene and 18 ml H_2O was slowly added 14 ml concentrated HCl (12 M) solution. The mixture was stirred at room temperature for 12 h, after which, the toluene layer was separated, washed with water (100 ml × 3), dried over $MgSO_4$ and evaporated under vacuum to give a brown oil. The crude product, 1-bromo-6-ferrocenylhexane, was purified by column chromatography on silica gel with *n*-hexane as the mobile phase. Yield = 0.78 g, 2.2 mmol, 54%. Positive-ion ESI-MS: $m/z =$ 348, M^+.

1-Bromo-6-ferrocenylhexane was then converted to **Fc-C6-SH**. A mixture of 1-bromo-6-ferrocenylhexane (0.73 g, 2.1 mmol) and thiourea (Junsei, Tokyo, Japan) (0.22 g, 2.9 mmol) in 8 ml ethanol was refluxed for 4 h under an inert atmosphere of nitrogen. Then, NaOH (0.01 g in 1.5 ml H_2O) was added and the solution was refluxed for another 2 h. The solution was then cooled to room temperature and acidified to pH 2 with 0.1 M HCl. The mixture was extracted with ethyl acetate (100 ml × 3) and the organic extracts were dried over $MgSO_4$ and evaporated under vacuum to give a brown oil. **Fc-C6-SH** was purified by column chromatography on silica gel using petroleum ether as the eluent. The product appeared as a yellow oil. Yield = 0.23 g, 0.8 mmol, 36%. ^{1}H NMR (300 MHz, $CDCl_3$, 298 K, relative to TMS) δ 4.09 (s, 5H, ferrocenyl H's), 4.04 (s, 4H, ferrocenyl H's), 2.52 (q, $J = 7.33$ Hz, 2H, CH_2-SH), 2.32 (t, $J = 7.33$ Hz, 2H, Fc-CH_2), 1.66–1.25 (m, 9H, Fc-CH$_2$CH_2CH_2CH_2CH_2CH$_2$SH). Positive-ion ESI-MS: $m/z =$ 302, M^+. IR (KBr) ν/cm^{-1}: 2561 (m, SH).

Isolation of MC-LR

Microcystis cell material was from a surface bloom collected from a farm pond in China. The purification was based on a modified reported procedure.[20] The dried cells (10 g) were mixed with 5% (v/v) aqueous acetic acid (200 ml) and the suspension was stirred at room temperature for 1 h. The mixture was centrifuged at 4620×*g* for 15 min and the supernatant was collected. The solid residue was extracted with 5% (v/v) aqueous acetic acid (200 ml × 2). The supernatant fractions were combined and applied to a reversed-phase silica gel (30 g) (Chromatorex, Fuji Silysia Chem. Ltd., Nagoya, Japan) column. The column was washed with H_2O (850 ml) and the toxins were eluted with methanol (400 ml). The fractions containing the toxins (monitored by positive-ion ESI-MS) were collected and concentrated to *ca.* 20 ml by rotary evaporation. To the solution was added normal-phase silica gel (230–400 mesh, silica gel 60, Merck, Darmstadt, Germany) (3.0 g) and the suspension was evaporated to dryness. The silica gel adsorbed with the toxins was then applied to a silica gel column (15 g) of the same stationary phase using chloroform–methanol–water (65:25:5) as the eluent. The fractions containing the toxins were collected. Positive-ion ESI-MS measurements revealed that the major microcystin derivative present was **MC-LR** ($m/z = 996$), while its counterparts **MC-YR** ($m/z = 1045$) and **MC-RR** ($m/z = 1038$) were also present in trace amounts. The toxin **MC-LR** was then further purified by HPLC. The HPLC system consisted of a Waters 600 pump (Milford, MA, USA) equipped with Rheodyne 7725i injector (Rohnert Park, CA, USA) with a 50-μl sample loop. The column was an Ultrasphere ODS column (250 mm × 4.6 mm, Beckman, Beckman, Fullerton, CA, USA). The gradient mobile phases consisted of (A) 30% (v/v) aqueous acetonitrile and 0.05% (v/v) TFA, and (B) 100% acetonitrile, with a linear gradient of 0–20% B from 0 to 20 min. The flow rate was 1 ml min^{-1}. UV detection was performed with a Waters 490E detector with the absorbance monitored at 238 nm. The fractions containing **MC-LR** were collected and the solvent removed under reduced pressure. The sample was then dissolved in ethanol (2 ml) to make up a stock solution. The concentration of this **MC-LR** stock solution was determined based on a calibration curve constructed by analysing commercially available **MC-LR** standards.

Reaction of MVK and Fc-C6-SH

A mixture of **Fc-C6-SH** (0.2 g, 0.7 mmol) and **MVK** (Aldrich, St. Louis, MO, USA) (39 mg, 0.6 mmol) in 5 ml CH_3CN was stirred at room temperature for 12 h. The solution was then loaded to a silica gel column with petroleum ether as the eluent. The fractions containing the product were collected and the solvent was removed by rotary evaporation to give **Fc-MVK** as a yellow oil. Yield = 169 mg, 0.5 mmol, 82%. ^{1}H NMR (300 MHz, C_6D_6, 298 K, relative to TMS) δ 4.04 (s, 5H, ferrocenyl H's), 4.00–3.98 (m, 4H, ferrocenyl H's), 2.61 [t, $J = 7.33$ Hz, 2H, SCH$_2$CH_2(CO)CH$_3$], 2.33 (t, $J = 7.03$ Hz, 2H, Fc-CH_2CH$_2$CH$_2$CH$_2$CH$_2$CH$_2$S), 2.25 (t, $J = 7.62$ Hz, 2H, Fc-CH$_2$CH$_2$CH$_2$CH$_2$CH$_2$CH_2S), 2.20 [t, $J = 7.33$ Hz, SCH_2CH$_2$(CO)CH$_3$], 1.60 [s, 3H, SCH$_2$CH$_2$(CO)CH_3], 1.55–1.21 (m, 8H, Fc-CH$_2$CH_2CH_2CH_2CH_2CH$_2$S). Positive-ion ESI-MS: $m/z = 372$, M^+. IR (KBr) ν/cm^{-1}: 1718 (s, C=O).

Labelling of MC-LR with Fc-C6-SH

Fc-C6-SH (10 μl) was added to 100 μl of a stock ethanolic solution of **MC-LR** (0.794 μg ml^{-1}). To the solution was added 5% (w/v) aqueous Na_2CO_3 solution (10 μl). The suspension was stirred at 60 °C for 4 h. The solid precipitated was removed by centrifugation. The supernatant was evaporated to dryness under reduced pressure. The solid residue was then resuspended in 600 μl water. The mixture was then centrifuged again to remove the solid residue. The supernatant was diluted with water to 1 ml. A 50-μl portion of this solution was applied to HPLC equipped with an EC detector for analysis.

HPLC/EC for Fc-MC-LR

The HPLC analysis of **Fc-MC-LR** was carried out using the Ultrasphere ODS column described above, under an isocratic condition. The mobile phase was 0.1 M aqueous ammonium acetate–acetonitrile (55:45 v/v) at a flow rate of 1.0 ml min^{-1}. . The HPLC system consisted of a Waters 515 HPLC pump (Milford, MA, USA) with a Rheodyne 7725i injector (Rohnert Park, CA, USA) equipped with a 50-μl sample loop. The detector was a Waters 464 pulsed electrochemical detector (Milford, MA, USA) equipped with a thin-layer flow cell (volume = 4 μl, gasket thickness = 0.04″). The working, counter and reference electrodes were a glassy carbon, stainless steel and Ag/AgCl electrode, respectively. The operation potential was +300 mV.

View Online

Published on 13 December 2002 on http://pubs.rsc.org | doi:10.1039/B206384K
Downloaded on 25 October 2010

Electrochemical studies of Fc-MVK and Fc-MC-LR

Cyclic voltammetry experiments were performed on a CH Instruments Electrochemical Workstation CHI750A (Austin, TX, USA). For **Fc-MVK**, the electrochemical experiments were carried out at room temperature with a two-compartment glass cell with a working volume of 500 μl. The working electrode was a glassy carbon electrode. A platinum gauze counter electrode was accommodated in the working electrode compartment. A silver/silver nitrate electrode was used as the reference electrode. The reference electrode compartment was connected to the working electrode compartment *via* a Luggin capillary. The solvent was acetonitrile with 0.1 M $^{n}Bu_4NPF_6$ (Sigma–Aldrich, St. Louis, MO, USA) as the supporting electrolyte. For **Fc-MC-LR**, a micro-volume voltammetry cell (Bioanalytical Systems, West Lafayette, IN, USA) with a working volume of 100 μl was used. A carbon paste working electrode, a platinum wire counter electrode and a silver/silver chloride reference electrode were used.

Other instrumentation

^{1}H NMR spectra were recorded on a Varian (Pal Alto, CA, USA) Mercury 300 MHz NMR spectrometer at 298 K. Positive-ion ESI mass spectra were recorded on a Perkin Elmer Sciex (Concord, ON, Canada) API 365 mass spectrometer. IR spectra were recorded on a Perkin Elmer (Shelton, CT, USA) 1600 series FT-IR spectrophotometer.

Results and discussion

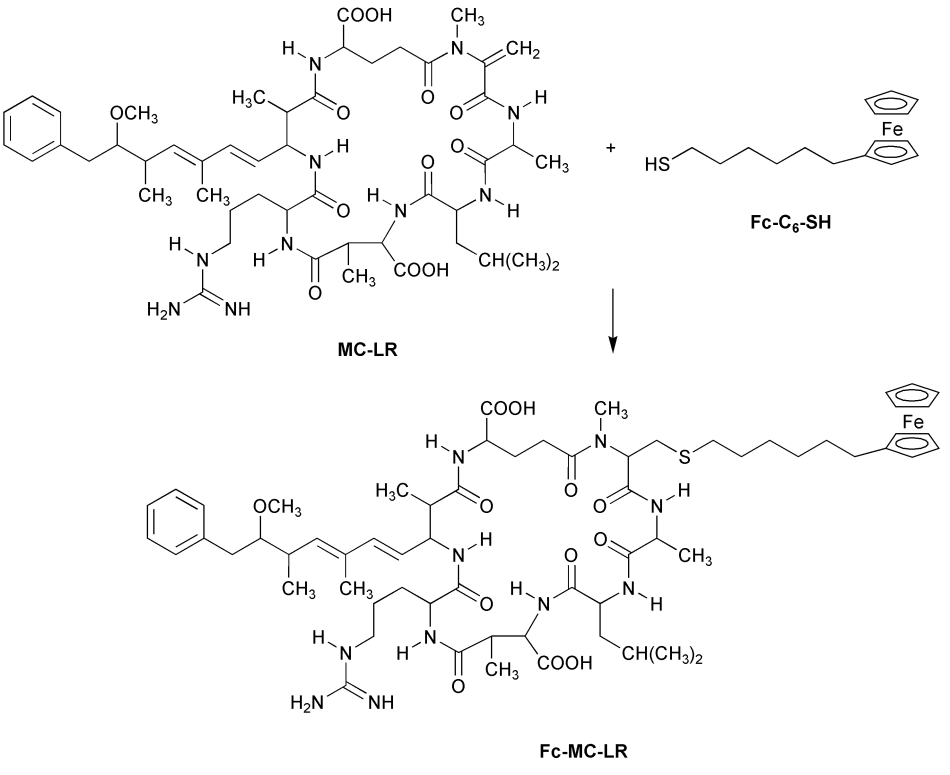
Design of redox-active label

The toxin **MC-LR** contains seven amino acid residues. It appears that the carboxylic acid groups can be readily functionalised, however, many studies have indicated that the carboxylic groups are inactive towards derivatisation due to steric reasons.[3,20] Since the toxin molecule contains an α,β-unsaturated carbonyl moiety, which is active towards addition reaction with a nucleophile, derivatisation procedures of microcystins in the literature have employed sulfhydryl-containing molecules such as cysteine,[11] glutathione[21] and 2-aminoethanethiol[22] to react with the toxin molecule, and to further derivatise the primary-amine containing adducts with amine-specific labels. However, these derivatisation procedures involve two labelling steps and have reduced specificity because the amine group commonly exists in biological samples compared to the characteristic α,β-unsaturated carbonyl group of these toxins. In the present work, our strategy is to incorporate a redox-active molecule specifically to the toxin molecule, most preferably *via* a one-step reaction. The simple molecule, **Fc-C6-SH**, is a very promising candidate based on the following reasons: (1) it contains an electrochemically active ferrocene group, which is well known to exhibit reversible electrochemistry; (2) the hexamethylene spacer can minimise any potential steric hindrance between the toxin molecule and the label; (3) the thiol moiety can undergo a Michael addition reaction with the α,β-unsaturated carbonyl group of the toxin molecule (Scheme 1). Using ferrocene as a redox-active tag for biomolecules such as DNA, peptides and proteins have been documented.[23–31] Electrochemical studies of ferrocenylalkanethiols immobilised on gold electrodes as self-assembled monolayers have also been investigated.[17,18] However, to the best of our knowledge, utilisation of **Fc-C6-SH** and related compounds as a derivatisation agent for biological molecules has never been explored.

The molecule **Fc-C6-SH** was synthesised based on modified literature procedures.[17–19] Ferrocene was first functionalised with a 6-bromohexanoyl substituent by the Friedel–Crafts acylation.[17,19] The carbonyl group was then reduced with Zn/Hg in toluene–aqueous HCl.[17,19] The bromine atom was finally converted to the thiol group by the reaction with thiourea.[17,18] The compound **Fc-C6-SH** was characterised by ^{1}H NMR, ESI-MS and IR.

Isolation of MC-LR

Microcystins for derivatisation described in this work were extracted from *Microcystis* cell material from a surface bloom



Scheme 1 Reaction scheme for the formation of **Fc-MC-LR** from **MC-LR** and **Fc-C6-SH**.

View Online

Published on 13 December 2002 on http://pubs.rsc.org | doi:10.1039/B206384K
Downloaded on 25 October 2010

collected from a farm pond in China and were purified according to a modified literature procedure.[20] Positive-ion ESI-MS measurements indicated that the major microcystin derivative present was **MC-LR**, which is also the most commonly found microcystin derivative in nature.[3,32] The extracts also contained the **MC-YR** and **MC-RR** derivatives in trace amounts. After a chromatographic separation on silica gel, the fractions enriched with **MC-LR** were collected and further purified by reversed-phase HPLC with UV detection. The toxin **MC-LR** was eluted at 19.74 min (Fig. 1). The presence of **MC-LR** was confirmed by positive-ion ESI-MS (Fig. 2). The fractions containing this microcystin derivative were then combined and the solvent removed under reduced pressure. The toxin was eventually dissolved in ethanol to make up a stock solution.

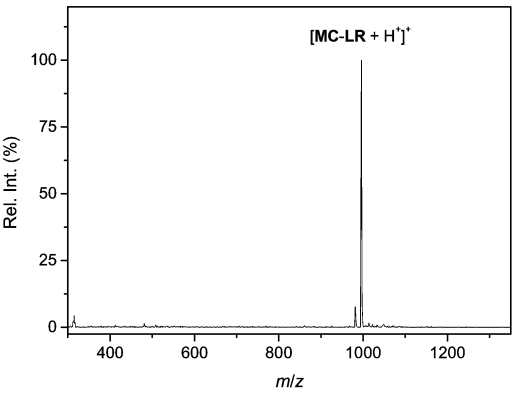


Fig. 2 Positive-ion ESI-MS of **MC-LR**.

Reaction of MVK and Fc-C6-SH

The reactivity of **Fc-C6-SH** towards organic molecules containing an α,β-unsaturated ketone moiety has been studied. **MVK** was chosen as the model substrate. The reaction of **Fc-C6-SH** and **MVK** was carried out in acetonitrile at room temperature for 12 h. The adduct **Fc-MVK**, obtained in a good yield after chromatographic purification, was characterised by ^{1}H-NMR, positive-ion ESI-MS and IR. In the ^{1}H NMR spectrum, the resonance signals for the vinyl protons of the starting material **MVK** were not observed at δ 6.27, 6.26 and 5.94. The S–H stretching absorption band at *ca.* 2561 cm^{-1} in the IR spectrum also disappeared. The formation of a thioether bond between the sulfhydryl group of **Fc-C6-SH** and **MVK** was indicated by the triplets at δ 2.61 [$SCH_2CH_2(C{=}O)CH_3$] and δ 2.20 [$SCH_2CH_2(C{=}O)CH_3$]. The positive-ion ESI measurement gave the adduct ion peak at $m/z = 372$.

Derivatisation of MC-LR with Fc-C6-SH

The derivatisation of **MC-LR** was carried out by reacting **MC-LR** with **Fc-C6-SH** in methanol–sodium carbonate solution at 60 °C for 4 h. The supernatant was isolated from the reaction mixture by centrifugation and then evaporated to dryness. The solid was redissolved into water for reversed-phase HPLC/EC analysis. The operation potential of the electrochemical detector was +300 mV *vs.* Ag/AgCl and the mobile phase was 0.1 M aqueous ammonium acetate–acetonitrile (55:45 v/v). At a flow rate of 1.0 ml min^{-1}, the adduct **Fc-MC-LR** was eluted at 9.56 min (Fig. 3). The fractions containing the labelled toxin were collected, the presence of which was confirmed by positive-ion ESI-MS. The mass spectrum (Fig. 4) showed two peaks at $m/z = 649$ and 1298, attributable to the molecular ions [**Fc-MC-LR** + 2H$^+$]$^{2+}$ and [**Fc-MC-LR** + H$^+$]$^+$, respectively. From the control derivatisation experiments in which no toxin samples were used, we found that the unreacted ferrocenyl derivatisation agent and its redox-active decomposed products were eluted at *ca.* 3.44 and 5.02 min, much earlier than **Fc-MC-LR**. Independent experiments show that the labelling method is very repeatable. The peak height-concentration curve was linear in the test range 20–400 ng **MC-LR** (r value for linear regression > 0.9987). The detection limit was determined to be *ca.* 18 ng **MC-LR** ($S/N = 3$). Since the environmentally relevant concentration of this toxin is in the μg l^{-1} scale, a pre-concentration step such as solid-phase extraction is required for analysis of real samples using the current method.[3] On the other hand, the detection limit of the current system is much lower than those of colorimetry methods (*ca.* 1 μg)[6] and visualisation reactions for the thin-layer chromatographic analysis (*ca.* 50–100 ng) of these toxins,[21] but is comparable to the typical detection limits of most HPLC/UV detection systems.[3,33,34] Meanwhile, this limit of detection is relatively high compared to those of other detection and quantitation methods for this class of toxins such as CE/MS (*ca.* 4 pg),[4] HPLC/MS detections (*ca.* 0.1 ng),[6] ELISA (*ca.* 1–150 pg),[8,9] and fluorescence and chemiluminescence detections (*ca.* 10–20 pg).[11,12] Nevertheless, the use of a ferrocene-containing labelling reagent for electrochemical detection of microcystins still has its merits because it is a relatively inexpensive method and the derivatisation procedure is simple, direct and specific, because the use of a linker molecule such

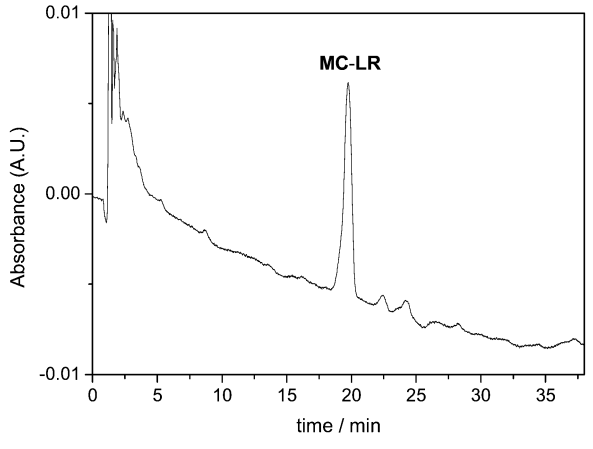


Fig. 1 HPLC purification of **MC-LR** with UV detection. Column: Ultrasphere ODS 250 mm × 4.6 mm. Mobile phase A: 30% (v/v) aqueous acetonitrile and 0.05% (v/v) TFA, B: 100% acetonitrile. Flow rate: 1 ml min^{-1}. Linear gradient: 0–20% B from 0 to 20 min. Detection: absorbance at 238 nm.

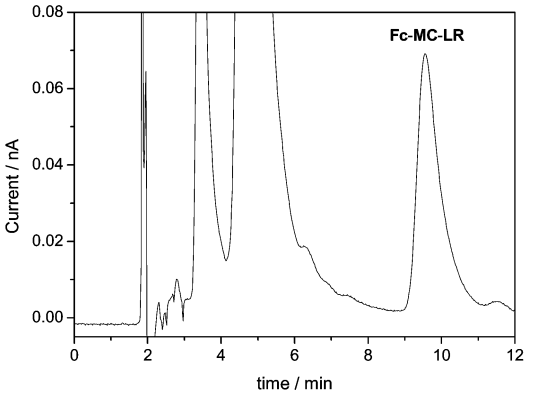


Fig. 3 HPLC separation of **Fc-MC-LR** with EC detection. Column: Ultrasphere ODS 250 × 4.6 mm. Mobile phase: 0.1 M aqueous ammonium acetate–acetonitrile (55:45 v/v). Flow rate: 1 ml min^{-1}. Isocratic elution. Detection: operation potential at +300 mV *vs.* Ag/AgCl, glassy carbon working electrode.

View Online

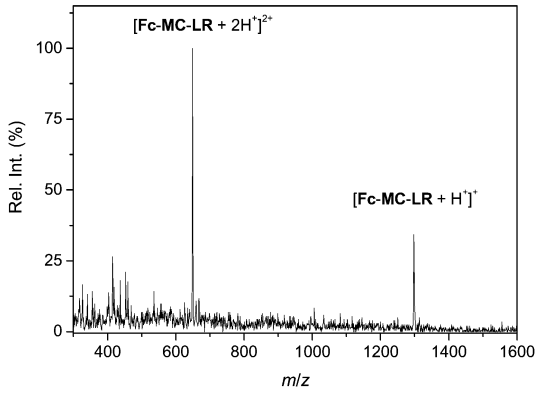


Fig. 4 Positive-ion ESI-MS of **Fc-MC-LR**.

as cysteine,[11] glutathione[21] or 2-aminoethanethiol[22] is not required. In addition, compared to phosphatase inhibition assays,[7,8] the current method also offers higher specificity originated from the specific reaction between the thiol group of **Fc-C6-SH** and the α,β-unsaturated carbonyl group of **MC-LR**.

Electrochemical properties of Fc-MVK and Fc-MC-LR

The electrochemical properties of both **Fc-MVK** and **Fc-MC-LR** have been studied by cyclic voltammetry. At a sweep rate, v, of 50 mV s^{-1}, **Fc-MVK** exhibits a reversible couple at *ca.* +0.333 V *vs.* SCE in CH_3CN (0.1 M $^nBu_4PF_6$) at 298 K, attributable to the ferrocenium/ferrocene couple (Fig. 5). The faradaic currents increase linearly with the square root of the sweep rates from 10 to 500 mV s^{-1}, suggestive of a diffusion controlled process.[35] Meanwhile, **Fc-MC-LR** also displays a reversible ferrocenium/ferrocene couple at *ca.* −0.040 V *vs.* SCE (v = 50 mV s^{-1}) in 0.1 M aqueous ammonium acetate–acetonitrile (55:45 v/v) (Fig. 6). The linear relationship between the faradaic currents and the square roots of the sweep rates (from 10 to 500 mV s^{-1}) indicates the diffusion-controlled nature of the electron transfer. It is interesting to note that native **MC-LR** is not redox-active due to the lack of an electrochemically active moiety. HPLC/EC detection of **MC-LR** with an operation potential at +1.20 V *vs.* Ag/AgCl has been reported.[16] While the linear range of this method (13–250 ng) is similar to that of our system, at such a relatively high potential, the arginine residue of the toxin is oxidised irreversibly. In contrary, the use of an electrochemically reversible ferrocene unit in the present work allows a much lower operation potential at which amino acids of the toxin molecules are stable, and hence higher detection specificity can be achieved.

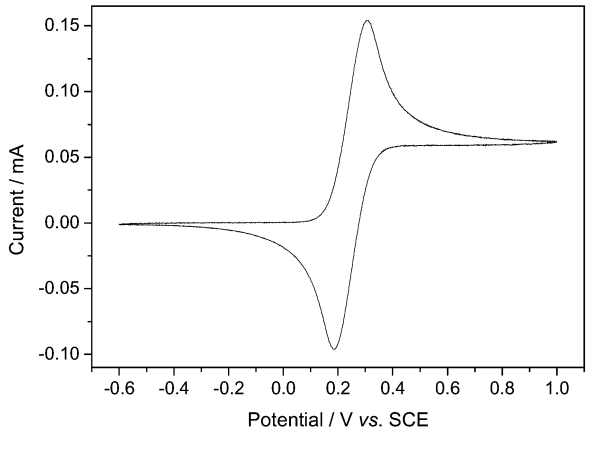


Fig. 5 Cyclic voltammogram of **Fc-MVK** in CH_3CN (0.1 M $^nBu_4PF_6$) at 298 K. Sweep rate: 50 mV s^{-1}. Glassy carbon working electrode.

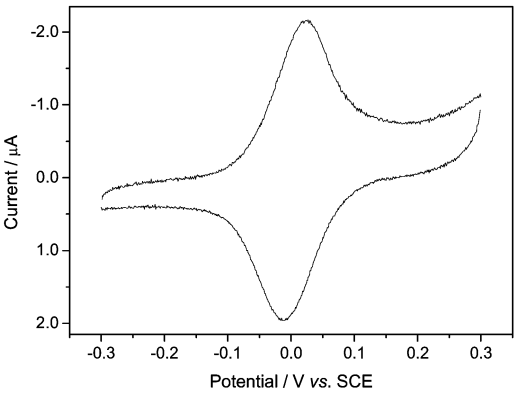


Fig. 6 Cyclic voltammogram of **Fc-MC-LR** in 0.1 M aqueous ammonium acetate–acetonitrile (55:45 v/v) at 298 K. Sweep rate: 50 mV s^{-1}. Carbon paste working electrode.

Conclusions

In summary, a redox-active label **Fc-C6-SH** has been designed as a derivatisation agent for the toxin microcystins. Since the sulfhydryl moiety of this compound is reactive towards the α,β-unsaturated ketone moiety, as revealed by the facile reaction with **MVK**, **Fc-C6-SH** has been used to derivatise a microcystin derivative, **MC-LR**. The ferrocene-toxin conjugate **Fc-MC-LR** has been analysed by HPLC coupled with EC detection. Its electrochemical properties have also been investigated. It has been shown that **Fc-C6-SH** is a versatile redox-active derivatisation reagent for **MC-LR**. Related studies using other derivatisation agents are underway.

Acknowledgements

We thank the Hong Kong Research Grants Council (project numbers 8730011 and CityU1103/00M) and the City University of Hong Kong (project number 7001283) for financial support. The blue-green algal sample was a gift from Ms. Xiaoyun Shen. J. S. Y. L. acknowledges the receipt of a postgraduate studentship and a Research Tuition Scholarship, both administered by the City University of Hong Kong.

References

1 K.-I. Harada, *Toxic Microcystis*, ed. M. F. Watanabe, K.-I. Harada, W. W. Carmichael and J. Fujiki, CRC Press, Boca Raton, FL, 1996, p. 103.
2 T. N. Duy, P. K. S. Lam, G. R. Shaw and D. W. Connell, *Rev. Environ. Contam. Toxicol.*, 2000, **163**, 113.
3 J. Meriluoto, *Anal. Chim. Acta*, 1997, **352**, 227.
4 K. P. Bateman, P. Thibault, D. J. Douglas and R. L. White, *J. Chromatogr., A*, 1995, **712**, 253.
5 H. S. Lee, C. K. Jeong, H. M. Lee, S. J. Choi, K. S. Do, K. Kim and Y. H. Kim, *J. Chromatogr., A*, 1999, **848**, 179.
6 K. Kaya, T. Sano, H. Inoue and H. Takagi, *Anal. Chim. Acta*, 2001, **450**, 73.
7 C. J. Ward, K. A. Beattie, E. Y. C. Lee and G. A. Codd, *FEMS Microbiol. Lett.*, 1997, **153**, 465.
8 O. I. Fontal, M. R. Vieytes, J. M. V. Baptista de Sousa, M. C. Louzao and L. M. Botana, *Anal. Biochem.*, 1999, **269**, 289.
9 J. McElhiney, L. A. Lawton and A. J. R. Porter, *FEMS Microbiol. Lett.*, 2000, **193**, 83.
10 A. Zeck, A. Eikenberg, M. G. Weller and R. Niessner, *Anal. Chim. Acta*, 2001, **441**, 1.

Published on 13 December 2002 on http://pubs.rsc.org | doi:10.1039/B206384K
Downloaded on 25 October 2010

View Online

11 P. C. H. Li, S. Hu and P. K. S. Lam, *Mar. Pollut. Bull.*, 1999, **39**, 250.
12 H. Murata, H. Shoji, M. Oshikata, K.-I. Harada, M. Suzuki, F. Kondo and H. Goto, *J. Chromatogr., A*, 1995, **693**, 263.
13 P. Mehto, M. Ankelo, A. Hinkkanen, A. Mikhailov, J. E. Eriksson, L. Spoof and J. Meriluoto, *Toxicon*, 2001, **39**, 831.
14 A. V. Humble, G. M. Gadd and G. A. Codd, *Water Res.*, 1997, **31**, 1679.
15 F. Yan, M. Ozsoz and O. A. Sadik, *Anal. Chim. Acta*, 2000, **409**, 247.
16 J. Meriluoto, B. Kincaid, M. R. Smyth and M. Wasberg, *J. Chromatogr., A*, 1998, **810**, 226.
17 K. Uosaki, Y. Sato and H. Kita, *Langmuir*, 1991, **7**, 1510.
18 A. S. Viana, L. M. Abrantes, G. Jin, S. Floate, R. J. Nichols and M. Kalaji, *Phys. Chem. Chem. Phys.*, 2001, **3**, 3411.
19 C. J. Yu, H. Wang, Y. Wan, H. Yowanto, J. C. Kim, L. H. Donilon, C. Tao, M. Strong and Y. Chong, *J. Org. Chem.*, 2001, **66**, 2937.
20 K.-I. Harada, M. Suzuki, A. M. Dahlem, V. R. Beasley, W. W. Carmichael and K. L. Rinehart, Jr., *Toxicon*, 1988, **26**, 433.
21 A. Pelander, I. Ojanperä, K. Lahti, K. Ninivaara and E. Vuori, *Water Res.*, 2000, **34**, 2643.
22 F. Kondo, Y. Ikai, H. Oka, M. Okumura, N. Ishikawa, K. Harada, K. Matsuura, H. Murata and M. Suzuki, *Chem. Res. Toxicol.*, 1992, **5**, 591.
23 G. Moorhead, R. W. MacKintosh, N. Morrice, T. Gallagher and C. MacKintosh, *FEBS Lett.*, 1994, **356**, 46.
24 T. Ihara, Y. Maruo, S. Takenaka and M. Takagi, *Nucleic Acids Res.*, 1996, **24**, 4273.
25 T. Ihara, M. Nakayama, M. Murata, K. Nakano and M. Maeda, *Chem. Commun.*, 1997, 1609.
26 K. Di Gleria, C. M. Halliwell, C. Jacob and H. A. O. Hill, *FEBS Lett.*, 1997, **400**, 155.
27 K. Di Gleria, D. P. Nickerson, H. A. O. Hill, L. L. Wong and V. Fülöp, *J. Am. Chem. Soc.*, 1998, **120**, 46.
28 K. K. W. Lo, L. L. Wong and H. A. O. Hill, *FEBS Lett.*, 1999, **451**, 342.
29 A. E. Beilstein and M. W. Grinstaff, *J. Organomet. Chem.*, 2001, **637**, 398.
30 C. J. Yu, Y. J. Wan, H. Yowanto, J. Li, C. L. Tao, M. D. James, C. L. Tan, G. F. Blackburn and T. J. Meade, *J. Am. Chem. Soc.*, 2001, **123**, 11 155.
31 K. K. W. Lo, J. S. Y. Lau, D. C. M. Ng and N. Zhu, *J. Chem. Soc., Dalton Trans.*, 2002, 1753.
32 L. A. Lawton and P. K. J. Robertson, *Chem. Soc. Rev.*, 1999, **28**, 217.
33 K.-I. Harada, K. Matsuura, M. Suzuki, H. Oka, M. F. Watanabe, S. Oishi, A. M. Dahlem, V. R. Beasley and W. W. Carmichael, *J. Chromatogr.*, 1988, **448**, 275.
34 L. A. Lawton, C. Edwards and G. A. Codd, *Analyst*, 1994, **119**, 1525.
35 A. J. Bard and L. R. Faulkner, *Electrochemical Methods, Fundamentals and Applications*, Wiley, New York, USA, 2nd edn., 2001.

Published on 13 December 2002 on http://pubs.rsc.org | doi:10.1039/B206384K
Downloaded on 25 October 2010