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Novel fluorescent biosensor for pathogenic toxins using cyclic polypeptide conjugates†

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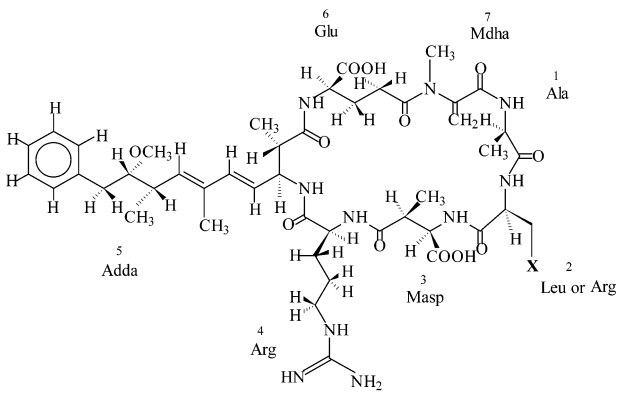
Received (in Columbia, MO, USA) 8th December 2003, Accepted 9th March 2004
First published as an Advance Article on the web 1st April 2004

This work describes a two-step conjugate synthesis of a new fluorescent analog of microcystin-LR and its subsequent utilization for the development of an optical biosensor for cyanobacteria toxins. The biosensor concept is based on the competitive binding between the native microcystin and its fluorescent analog at immobilized alkaline phosphatase enzymes.

Recently, there is a great deal of interests in developing novel approaches to counter the effect of toxins, chemicals and biological warfare agents. Such chemistry should enable the design of tools that can be used not only to detect, but also to effectively combat biochemical warfare. Despite the availability of numerous biodetection techniques, there are no biosensors that can detect, identify and accurately classify microcystin toxins. From studying the generation and identification of toxins derived from pathogenic bacteria (*via* the inhibition of phosphatase enzyme activity), we have developed a generic approach for covalent modification of microcystin using cysteine coupled with fluorescent isothiocyanate (MC-Cys-FITC) conjugate.

Microcystins are both effective and specific poisons having toxicities that are several orders of magnitude greater than most nerve agents.[1,2] A growing number of bacterial pathogens such as *Microcystis* have been identified as important food/water-borne pathogens.[3–5] The basic structure of microcystin (MC) is a cyclic heptapeptide and its variation gives rise to more than 50 types isolated and characterized to date.[6–10]

Figure 1 shows the structures of the two most extensively studied microcystins (*i.e.* microcystin-LR and microcystin-RR). Microcystin-LR accounts for nearly 90% of the total toxicity. Microcystins have a common moiety comprising five amino acids, 3-amino-9-methoxy-10-phenyl-2,6,8-trimethyldeca-4,6-dienoic acid (Adda), *N*-methyldehydroalanine (Mdha), D-alanine, β-linked D-*erythro*-β-methylaspartic acid, γ-linked D-glutamic acid, and two L-amino acids variants.[6–8] The synthesis of MC-Cys-FITC conjugate was developed in line with the basic action of microcystins as potent inhibitors of phosphatase enzymes.[9–11] The fact that microcystins inhibit protein phosphatase 1 and 2A enzymes (PP1 and PP2A) raised the possibility of a functional assay for microcystins and associated toxins. To our knowledge, a biosensor of this type has not been reported.[12] However, its usefulness should range from fundamental research to practical applications. In order to prepare the MC-Cys-FITC, a fluorescent active group has to be introduced into the molecules because intact MCs have no fluorophore. There are at least four possible sites for chemical modification of microcystin-LR, *i.e.* the guanidino group in the arginine residue, two carboxyl groups (in the methylaspartate and glutamate residues) and the C=C double bond in the *N*-methyldehydroalanine residue. The arginine residue is not the obvious choice for derivatization because it is not available in some species of *Microcystis*. Also, its location is too close to the Adda residue, which is essential for activity and toxicity of microcystins.[13–15] Microcystin-LR was reported to retain activity after saturation of the Mdha residue, thus suggesting that this site could provide a potential site for derivatization.[15,16] Hence, we have demonstrated the feasibility of a novel fluorescent biosensor for microcystins using chemical modification of MC-LR.[17,18]



Microcystin-LR X = $CH(CH_3)_2$
Microcystin-RR X = $CH_2CH_2NHC(=NH)NH_2$

Fig. 1 Structure of microcystins showing the common moiety composed of different amino acid variants.

MC-Cys-FITC conjugate was synthesized in two steps involving a Michael type addition of thiol available in cysteine to α,β-unsaturated carbonyl of Mdha in microcystin.[13] Characterization was achieved using reverse-phase high performance liquid chromatography (HPLC), fluorescence experiments, and kinetic studies. We examined the presence of the new conjugate by injecting a sample of the reaction mixture into the HPLC column. We observed the existence of the microcystin adduct with clearly distinguishable retention times (Fig. 2).

The native MC-LR eluted in 11.30 min while cysteine was detected in only 7.72 min. A new peak observed at 9.97 min demonstrates the existence of the MC-Cys adduct. Fluorescence experiments verified that the resulting conjugate is fluorescently active. The emission wavelength for FITC is 528 nm and the conjugate had an emission wavelength at 514 nm. No reproducible results were obtained when the carboxyl groups were used as the target for derivatization, probably due to steric hindrance around

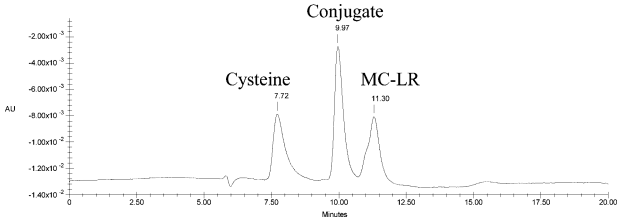


Fig. 2 High-performance liquid chromatogram of the reaction mixture of microcystin-LR with cysteine. Column: Zorbax C18, 150 × 4.6 mm. Mobile phase: MeOH/50 mM phosphate buffer (pH 3) (60 : 40). Flow rate: 1 mL min^{-1}. Detection: 238 nm.

† Electronic supplementary information (ESI) available: synthetic approach for microcystin conjugates. See http://www.rsc.org/suppdata/cc/b3/b316057b/

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DOI: 10.1039/b316057b

the functional carboxylic group.[13] The interaction of PP1 and MC-Cys-FITC was first tested in the solution phase. The emission of MC-Cys-FITC was recorded continuously for one hour at an interval of 30 seconds after 25 μl PP1 (0.1 unit) was added. Results show negligible but stable fluorescence intensity change over a 60-minute period. At a shorter response time (*i.e.* 10 min), the enzyme PP1 has no effect on the fluorescence of MC-Cys-FITC.

Prior to immobilization, the fluorescence of FITC-Cys-MC was also measured in the solution phase in the presence of phosphatase enzyme. It was diluted to different concentrations with the phosphatase assay buffer. The fluorescence of labeled MC-LR was measured using a quartz cuvette (1-cm light path). A least-squares fit to the data indicates that the signal responded linearly with correlation coefficients of $R^2 = 0.9983$; the linear response range in solution was between 0.5 and 15 pM or higher.

We designed and tested a new biosensor based on the competitive binding between labeled and unlabeled MC-LR. First, a certain amount of enzyme (*i.e.*, PP1) was immobilized onto optic fiber, and both MC-Cys-FITC conjugate and free unlabeled MC-LR in the matrices were allowed to compete for the limited binding sites provided by PP1. The resulting signal recorded was inversely related to the concentration of the unlabeled MC-LR. Figure 3 shows the fluorescence spectra upon addition of different concentrations of MC-LR.

It is evident that fluorescence decreases as the amount of unlabeled MC-LR increases, which agrees well with our prediction. The least-squares fit equation for the data is fluorescence units $y = -4.38 \times +500.77$, $R^2 = 0.9987$. Besides microcystins, phosphatase PP1 can also be inhibited by a structurally diverse group of natural toxins such as okadaic acid, nodularin, taunomycin, and calyculin A. It is crucial that the proposed competitive sensing approach works for the unlabeled microcystin-LR sample, and that the biosensor can resist interference when detecting the target toxins. Okadaic acid is used as an inhibitor of the serine threonine phosphatase PP2A ($ID_{50} = 0.2$ nM), and to a lesser degree, PP1 ($ID_{50} = 20$ nM).[19] When the biosensor was tested against okadaic acid, it was found to resist okadaic acid up to 48 nM. Thus a significant advantage of this fiber optic biosensor assay is the specificity for MC-LR. In summary, we have demonstrated the synthesis and successful deployment of MC-Cys-FITC for the detection of pathogenic toxins. A detection limit of 10 pM was obtained with response time *ca.* 10 min. HPLC with UV detection shows a 10 ng level sensitivity,[20] but our current biosensor exhibits 100 times greater sensitivity and enabled a rapid and selective analysis for microcystin. The reproducibility recorded for $n = 10$ measurements using the same sensor was ~2%, with operational lifetimes of several hours. This biosensor is not limited by the availability of antibody unlike conventional immunoassays. It may provide a generic approach to designing very high-affinity systems for toxins based on the ability of desired toxins or labels to inhibit protein phosphatase enzymes.

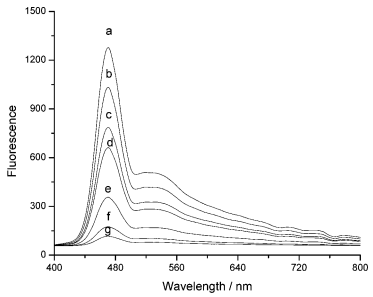

Fig. 3 Fluorescence of PP1-modified optic fiber upon addition of unlabeled MC-LR at different concentrations. a) 1 pM; b) 20 pM; c) 40 pM; d) 75 pM; e) 90 pM; f) 100 pM; and g) 120 pM.

We thank the National Science Foundation for financial support of this work (CHE-0210968).

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