Optical detection of microcystin produced by cyanobacteria

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Abstract. Microcystin (MC-LR) produced by cyanobacteria (blue-green algae) was detected in direct immunoassay with specific monoclonal antibody MC10E7 using an optical method of Total Internal Reflection Ellipsometry (TIRE). The minimal detected concentration of MC-LR of 0.1 ng/ml is a remarkable achievement for direct immunoassay against such low molecular weight analyte molecule. The study of binding kinetics of MC-LR to MC10E7 antibody allowed the evaluation of the association constant $K_A$ of about $10^8$ (l/Mol) typical for highly specific immune reactions. Concentration of MC-LR in aqueous solutions was reduced using an absorbent made of polyelectrolyte-coated microparticles functionalized with MC10E7 antibodies.

1. Introduction
Photosynthetic cyanobacteria (also known as blue-green algae) exist in any types of surface waters and depending on the environmental conditions may release a number of toxins microcystins [1, 2] which possess a serious threat to human, animals and generally to the environment. Microcystin-LR (MC-LR) is perhaps the most toxic from a large family of cyanotoxins; it is known to cause liver damage and also acts as carcinogen [3]. World Health Organization set the limit of 1µg/l for MC-LR in drinking water, and 0.04 µg/kg of body weight, based on liver pathology [4].

There are a number of analytical methods currently available for detection of microcystin-LR in water in a desired concentration range of 0.1-1µg/l, for example HPLC which requires expensive equipment and highly professional personal to carry out complex chromatography procedures and long time. The method of ELISA has been widely employed to monitor microcystin-LR at levels below 1µg/l [5] but it is also based on quite complex preparation procedures which require the use of expensive chemicals. Another method of protein phosphates inhibition assays (PPIA) [6] can provide the required sensitivity but may yield false positives if the enzyme is inhibited by other compounds present in the sample. The method of lateral flow dipstick (Microcystin Immuno Strip) based on colloidal gold nano-particles [7] was recently introduced for detection of MC-LR however its sensitivity is not sufficient. Surface plasmon resonance (SPR) has become a widely used analytical technique which lead to development of SPR based immunosensor for the determination of MC-LR [8], the required high sensitivity can be achieved using competitive immunoassay format. Furthermore, the Quartz Crystal Microbalance (QCM) method was developed in combination with sandwiched immunoassay amplified by Au nano-particles which reduces the detection limit for (MC-LR) down to 0.1 pg/l [9]. Another detection method using color-changeable polydiacetylene vesicle achieved the 1 pg/l detection level [10]. Relatively low molecular weight of MC-LR (995 m/mol) makes it difficult to detect using direct...
immunoassays in combination with conventional QCM and SPR based analytical methods; the sensitivity enhancement can be achieved using more complex and expensive assays, i.e. competitive or sandwich immunoassays. Therefore, the development of highly sensitive, reliable, and (at the same time) inexpensive and easy-to-use methods of detection of microcystin is of very high importance nowadays. The method of Total Internal Reflection Ellipsometry (TIRE) [11] is particularly attractive for the above task considering its high sensitivity and particular suitability for detection of low molecular weight analytes in direct immunoassay format [12]. This method has been used recently for detection of different toxins, i.e. mycotoxins (T2, aflatoxin, zearalenone) [13-15], alkylphenols (nonylphenol) [16] and herbicides (simazine and atrazine) [17] and achieved the detection of the above substances in sub-ppb range (0.1 ng/ml) in direct immunoassay format. In this work we tried to utilize the method of TIRE for detection microcystin LR.

2. Experimental details

2.1 Chemicals for immunoassay and samples preparation

Microcystin LR (MC-LR) was detected in direct immunoassay with specific monoclonal antibody MC10E7 raised against MC-LR in rabbit (the immune pair was acquired from Enzo Life Scientific). Other chemicals used were: systamine hydrochloride, (poly)allylamine hydrochloride (PAH) protein G, Tris-HCl and PBS buffers (all from Sigma Aldrich).

The antibodies were immobilized on the surface of gold coated microscopic glass slides which were prepared by consecutive thermal evaporation of layers of chromium (3 nm) and gold (25 nm) without breaking the vacuum of about 10^{-6} Torr in the Edwards E360A evaporation unit. The presence of thin Cr layer improves the adhesion of Au layer to glass. The surface of gold was charged negatively by immersing gold coated slides overnight into 100mM solution of mercapto-ethylsulfonate sodium salt (SH-(CH2)2-SO3^− Na^+ in methanol.

The following immobilization steps were performed by injection of required solutions into reaction cell. First, a layer of polycationic PAH was electrostatically adsorbed on the surface of gold from its 2 mg/ml aqueous solution in water. Secondly, protein G molecules, carrying net negatively charge at pH 8 in Tris-HCl buffer, were electrostatically adsorbed on the surface. Then, antibodies MC10E7 were attached to protein G, and therefore have their Fab-fragments oriented towards the solution.

Microcystin LR (MC-LR) is a hyrophobic molecule with molecular weight of 995D. In order to reduce the probability of formation of micelles of microcystin in aqueous solutions, the 100 µg/ml stock solution of MC-LR was prepared in a mixture of 10% of methanol and 90% of deionised water (MQ Elga). The series of samples of different concentrations of MC-LR from 0.1 ng/ml up to 10 µg/ml were prepared by multiple dilution of the stock solution in deionised water.

2.2. TIRE measurements.

Total Internal Reflection Ellipsometry (TIRE) measurements were performed using the experimental set-up described in detail earlier [12-17] which is based on J.A.Woollam spectroscopic ellipsometer M2000. As shown schematically in Fig. 1 (inset), the key element of the above set-up is a 68° glass prism in optical contact (via index matching fluid) with the gold coated glass slide. Such configuration allows coupling the light into the thin gold film at an angle close to the critical angle of total internal reflection at the glass/water interface, and therefore forcing the ellipsometer to operate in the surface plasmon resonance (SPR) mode. The main advantage of TIRE method, however, lies in the ability of recording spectra of two parameters $\Psi$ and $\Delta$ representing, respectively, the ratio of amplitudes of and the phase shift between p- and s- components of polarized light, as compared to one parameter of the reflected light intensity in conventional SPR. The modelling showed that the parameter of $\Delta$ is about 10 fold more sensitive than $\Psi$ to small changes in the refractive index and thickness of adsorbed layer on the surface of gold [12]. A PTFE reaction cell of 0.2ml in volume sealed against the gold layer has the inlet and outlet tubes enabling the injection of different solutions into the cell.
Two types of TIRE measurements were performed: (i) dynamic spectral measurements where a number of $\Psi(\lambda)$ and $\Delta(\lambda)$ spectra were recorded during the adsorption or binding of all molecules (the incubation time of 15 min was used for all adsorption or binding stages); (ii) single spectroscopic scans which performed in the same PBS buffer solution (pH 7.5) after washing our non-specifically bound material by purging of up to 6 ml of water (or PBS) buffer through the cell.

2.3. TIRE data fitting

Figure 1 shows typical $\Psi(\lambda)$ and $\Delta(\lambda)$ spectra recorded on bare gold coated glass slides. $\Psi(\lambda)$ spectrum resembles traditional SPR curve while $\Delta(\lambda)$ spectrum exhibits a characteristic phase drop from 270° to -90°. Because of higher sensitivity of $\Delta$, all further analysis is based on $\Delta(\lambda)$ spectra. The thickness evaluation was performed by the fitting of the obtained spectra of $\Psi(\lambda)$ and $\Delta(\lambda)$ using a least-square minimization software available in J.A. Woollam instruments [18]. Four-layer model containing (i) BK-7 glass, (ii) Cr/Au layer, (iii) adsorbed molecular layer, and iv) water was used for TIRE data fitting [12]. The effective values of the thickness ($d$) and effective dispersion functions of the refractive index $n(\lambda)$ and extinction coefficient of $k(\lambda)$ for a mixed Cr/Au layer were obtained by fitting the spectra recorded on the samples with bare surface of gold and then kept fixed at consecutive fittings on the same sample. All molecular layers adsorbed on the gold surface are considered to be transparent in the 370-1000nm spectral range of M2000 ellipsometer so that $k = 0$, and the dispersion function of the refractive index is described by Cauchy model [18]:

$$n = A + \frac{B}{\lambda^2} + \frac{C}{\lambda^4},$$  \hspace{1cm} (1)

Because the refractive indices of all amino acid-based bio-molecules are very close [19] we used the fixed values of $A=1.39$, $B=0.01$, and $C=0$ which yield $n = 1.42$ at 630 nm and therefore the effect of molecular adsorption is associated with the changes in the effective adsorption layer thickness only.

3. Experimental results and discussion.

3.1. TIRE study of the immune reaction between MC-LR and MC10E7

A typical set of $\Delta$ spectra in Figure 2 shows consistent spectral shift to high wavelengths stared from bare gold treated with SH-(CH$_2$)$_2$SO$_3$ Na$^+$ (curve a), then after electrostatic deposition of PAH layer (curve b), after electrostatic deposition of protein G (c), and after deposition of MC10E7 antibodies (d). The following group of curves (e - h) corresponds to sequential binding of MC-LR of different
concentrations (starting from the smallest 0.1 ng/ml up to 10 µg/ml). All deposition and binding stages (e–h) were performed by injecting corresponding solutions into TIRE cell. Intermediate rinsing was performed between adsorption (binding) steps by purging 2-3 ml of water or buffer through the cell. The dependence of the of the thickness increment of the molecular layers the concentration of MC-LR in shown in Figure 3 was obtained by fitting TIRE data. The lower limit of detection for MC-LR is between 0.1 ng and 1 ng/ml. The maximal thickness increment of about 1.2 nm is reasonable for non complete coverage by MC-LR molecules of about 3 nm in size. The obtained dependence in Fig. 3 can be used in future as a calibration curve for testing samples of water containing unknown concentration of MC-LR.

3.2. The study of MC-LR/MC10E7 binding kinetics.
Dynamic spectral measurements described earlier were performed in-situ during all adsorption and binding steps. Typical time dependencies of Ψ and Δ at particular selected wavelength (670 nm this time) during binding MC-LR to MC10E7 are shown in Figure 4a. As one can see the noise is much larger on Y curve which is quite obvious considering different angle spans 0.5° for Ψ and 2.5° for Δ. It illustrates once again much high sensitivity of the parameter Δ and justify the use of time dependences of Δ for further analysis. Figure 4a also shows that the binding reaction reaches saturation after 15 min of incubation.

Figure 3. Dependences of the molecular layer thickness increase vs concentration of MC-LR: (a) for untreated MC-LR solution, (b) for MC-LR solution treated with microparticles functionalized with MC10E7 antibodies.

Figure 4. (a) Typical time dependencies of Ψ and Δ during binding MC-LR from its 100 ng/ml aqueous solution to MC10E7 antibodies; (b) The evaluation of the association constant $K_A$ from the dependence $1/\tau$ vs. concentration of MC-LR.
Both curves in Figure 4a can be described by exponential dependences, and the characteristic time constant ($\tau$) can be found by curve fitting. Further analysis and the evaluation of the association or/and affinity constants is based on the solution of a first order differential equation for molecular adsorption on the surface [17]. It appeared that reciprocal of the time constant $1/\tau$ is linearly dependent of the concentration of adsorbed molecules ($C$).

\[ \frac{1}{\tau} = k_a C + k_d \]  

(2)

The values $k_a$ and $k_d$ being the rates of adsorption and de-sorption can be evaluated from the linear graph $1/\tau$ vs. $C$ in Figure 4b as the gradient and intercept, respectively. Then both the association and affinity constants can be found as:

\[ K_A = \frac{k_a}{k_d}, \quad K_D = \frac{1}{K_A} \]  

(3)

The analysis of the data presented in Fig. 6b yield the values in the range of $10^7 - 10^8$ l/mol (8.89.10$^7$ l/mol for the particular set of data presented) with the accuracy of 10-15%. The obtained values of $K_A$ are typical or highly specific immune reaction between MC-LR and MC10E7 antibodies.

3.3. Formation of functionalized polyelectrolyte microcapsules for MC-LR cleansing

Polyelectrolyte microcapsules were prepared by coating inorganic CaCO$_3$ template particles with the layers of polyanions (sodium poly-styrene sulfonate or PSS) and polycations (poly-allylamine hydrochloride or PAH). The obtained micro-particles were further functionalized with protein G and MC10E7 antibodies specific to MC-LR following the procedure described in [20]. Fluorescence microscopy image of the obtained micro-particles stained with SYTO-9 green fluorescent dye is shown in Figure 5 (a), SEM images of different magnifications are shown in Figure 5 (b, c).

Following the procedure developed earlier [20] micro-particles functionalized with MC10E7 antibodies were utilized for purification of solutions containing MC-LR. The solutions of MC-LR of different concentrations were mixed 1:1 with suspension of functionalized microparticles and incubated for up to 1 hour. Heavy particles sediment on the bottom of sample tubes leaving purified solution above.

UV-vis spectral measurements of MC-LR samples in Figure 6 show the reduction in the intensity of the main absorption band of MC-LR at about 240 nm after treatment with microparticles functionalized with MC10E7 antibodies. The effect of purification was also confirmed with TIRE measurements taken on MC-LR samples after treatment with functionalised capsules. As one can see in Figure 2, the data points of the thickness increment for the samples treated with microparticles (curve b) lie well below the original calibration curve (a).
4. Conclusions

The method of TIRE was proved to be capable of detection of Microcystin LR in low concentrations down to 0.1 ng/ml in direct immunoassay with specific monoclonal antibodies MC10E7. The above immune reaction is highly specific according to the results of the binding kinetics which yielded the values of the association constant in the range of $10^7$ - $10^8$ (l/mol). CaCO$_3$-based microparticles functionalized with MC10E7 antibodies are acting as absorbent for MC-LR in water solutions, and could be used in future for purification of contaminated samples. Further work will focus on detection of microcystin produced by cyanobacteria in real water samples.

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