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Chapter 4

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1. Introduction

Water blooms of toxic cyanobacteria (blue-green algae) represent a serious problem because of the potent toxins that can be released by these algae (Dawson, 1998). Toxin-producing microalgae species have a negative influence on the environment, food safety and health (de Figueiredo et al., 2004). Microcystins (MCs) are a group of cyanobacterial toxins that are mainly produced by microcystis, which appear in lake, ponds, reservoirs and rivers with low turbidity flow regimes. More than 80 structural variants of MCs are known, and each one shows very different toxicity levels. These toxins are cyclic heptapeptides with the general structure cyclo-(d-Ala-X-d-MeAsp-Y-Adda-Adda-d-Glu-N-methyldehydro-Ala), where X and Y represent variable l-amino acid residues. The amino acid Adda (3-amino-9-methoxy-2, 6, 8-trimethyl-10-phenyldeca-4, 6-dienoic acid) is considered to be responsible for the MC hepatotoxicity (Dawson, 1998). MC-LR (L and R designating leucine and arginine, respectively) was the first MC chemically identified, and it is the most toxic and most frequently found.

After ingestion, MC can penetrate into hepatocytes. Within the hepatocytes, MCs irreversibly inhibit protein phosphatases type 2A (PP2A) and 1 (PP1) (Dawson, 1998). External signs of poisoning, which include weakness, pallor, heavy breathing, vomiting and diarrhea, are then observed. MCs are potent tumor promoters, causing disruption of liver structure and function, haemorrhaging into the liver and death by respiratory arrest (Codd, 2000). Several cases of animal and human intoxication due to MCs have been reported. For example, in 1996, patients at a Brazilian hemodialysis center using municipal water contaminated with cyanotoxins were exposed to lethal levels of MCs. One hundred of the 131 patients developed acute liver failure and 52 of these victims are died due to hepatotoxin poisoning (Pouria et al., 1998; Jochimsen et al., 1998). In 2009, water pollution in Yancheng China affected the water supply system, which was closed for three days.

The toxicity and ubiquity of MCs necessitate the development of fast, sensitive and reliable methods to detect them. To guarantee water quality and to minimize the potential risk to human health, the World Health Organization (WHO) has recommended a maximum level of 1 $\mu g\ L^{-1}$ of MC-LR in drinking water (WHO, 1998). Accordingly, detection systems must be sensitive to MC concentrations below the limit established by the WHO. The simplest screening method is the mouse bioassay, which suffers from low sensitivity, specificity and ethical problems due to animal experimentation. In vitro cytotoxicity assays, based on morphological changes in cells after exposure to toxins, have been developed to provide a substitute for the mouse bioassay (Boaru, Dragos & Schirmer, 2006; Chong et al., 2000). These assays are easy to perform and economical but they are also subjective, time-consuming and confusing results may appear in the presence of toxin mixtures (Campas et al., 2007). Other cytotoxicity tests are based on the simple and sensitive analysis of the toxin effect on cells by measuring changes in O2 consumption by optical oxygen sensing technique (Jasionek et al., 2010). MCs are routinely analyzed using high-performance liquid chromatography (HPLC) coupled to mass spectrometry. These techniques allow highly selective identification and sensitive quantification of the different toxins present in a sample. However, they require expensive equipment, complex procedures, lengthy analysis times and trained personnel (Sangolkar, Maske & Chakrabarti, 2006; McElhiney & Lawton, 2005). An alternative and interesting approach is the use of biosensor for rapid, easy and sensitive detection of the toxin. A biosensor is a device composed of two intimately associated elements (Figure 1):

- A bioreceptor, that is an immobilized sensitive biological element (e.g. enzyme, DNA probe, antibody) recognizing the analyte (e.g. enzyme substrate, complementary DNA, antigen).
- A transducer, that is used to convert the (bio)chemical signal resulting from the interaction of the analyte with the bioreceptor into an electronic one. The intensity of generated signal is directly or inversely proportional to the analyte concentration. Biosensors can be based on electrochemical, gravimetric, calorimetric or optical detection. Electrochemical transducers (Thévenot et al., 1999) are classically used to develop biosensors (Ronkanein, Halsall & Heineman, 2010). These systems offer some advantages such as low cost, simple design or small dimensions.

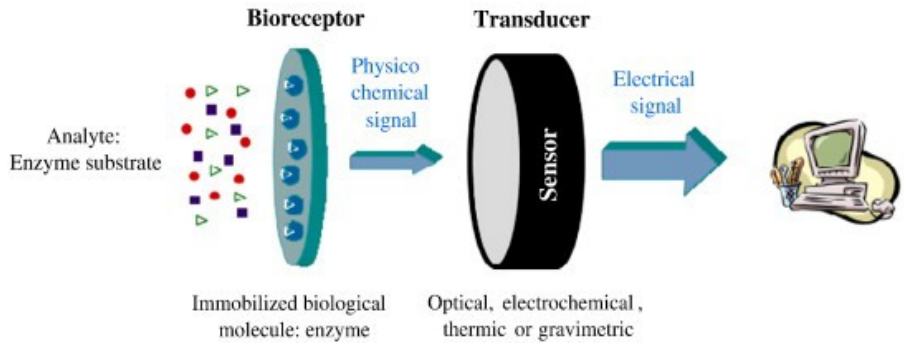

Figure 1. Scheme of a biosensor (Sassolas, Blum & Leca-Bouvier, 2012)

This review presents a state-of-the-art in electrochemical biosensors for the detection of microcystins. To clearly report the last advances, biosensors have been classified according to the immobilized recognition element. New trends in the field of microcystin analysis are also reviewed. Aptamers are shown as good candidates to replace the conventional antibodies and, thus, to be the biorecognition elements in more robust and stable biosensors for the detection of microcystin. Recent reports on the properties of nanomaterials show nanoparticles and nanotubes as promising tools to improve the efficiency of biosensors for the detection of microcystins.

2. Enzyme-inhibition based biosensors

2.1. Protein phosphatases

Enzyme inhibition-based methods have been widely used for the detection of MC. The main enzymatic method is based on the inhibition of protein phosphatases by the toxin. The enzyme inhibition can be detected by several methods such as colorimetry (Sassolas et al., 2011), fluorescence (Fontal et al., 1999) or electrochemistry (Campas et al., 2007; Campas et al., 2005; Szydlowska et al., 2006; Campas, Olteanu & Marty, 2008).

The commercially availability of protein phosphatases which avoids laborious purification makes the approach very attractive. Several assays and biosensors have been developed using PP2As purchased from Millipore (New York, USA). This enzyme is isolated as the heterodimer of 60 kDa (A) and 36 kDa (C) subunits from red blood cells. A French company called GTP Technology

produced (by genetic engineering) PP2A that consists of a 39 kDa (tag included) human catalytic (C) subunit of the α-isophorm isolated from SF9 insect cells infected by baculovirus. Recently, PP2A from ZEU Immunotec (isolated from red blood cells) and a recombinant PP1 from CRITT (Toulouse, France) were also used for the development of colorimetric PP inhibition assay (Sassolas et al., 2011). Recently, the inhibition of three protein phosphatases (PP2As from ZEU Immunotec and GTP Technology, and PP1 from CRITT) by three MCs (LR, YR, RR) was investigated. It was demonstrated that the inhibition type in all cases was non-competitive (Covaci, Sassolas, Alonso, Munoz, Radu & Marty, 2012). The sensitivity of enzymes to MC can be drastically different. Thus, the choice of the enzyme is crucial to the performance of the system. A comparative study demonstrated that PP2A from ZEU Immunotec is more sensitive to MC than the other enzymes (Sassolas et al., 2011).

This enzymatic approach is limited due to the poor enzyme stability. To overcome this problem, enzymes can be immobilized. The choice of the immobilization technique is crucial for the performance of assays and biosensors.

2.2. Electrochemical enzyme-based biosensors for the detection of MCs

Marty's group developed electrochemical protein phosphatase-based biosensors for the detection of microcystin-LR (Campas et al., 2007; Szydlowska et al., 2006). The enzyme from Millipore was immobilized within a photopolymer formed on a screen-printed working electrode. The electrochemical measurement of the enzymatic activity was achieved using appropriate substrates electrochemically active after dephosphorylation by the enzyme. The enzymatic activity of PP2A is inhibited by the presence of MC, and hence the current intensity produced by the oxidation of electroactive product decreases proportionally to the toxin concentration. Several substrates were tested: catechol monophosphate, α-naphthyl phosphate and p-aminophenyl phosphate.

Real samples of cyanobacterial blooms from the Tarn River (Midi Pyrénées, France) have been analyzed using the developed amperometric biosensor (Campas et al., 2007). Electrochemical results were compared to those obtained by a conventional colorimetric protein phosphatase inhibition assay and HPLC. Despite the restricted sensitivity of the biosensor, potentially due to the electrode fouling by some cell extracts components, the applicability of the electrochemical system to rapidly assess the environmental and health risk due to MCs was demonstrated.

A signal amplification strategy based on enzymatic recycling was used to improve the sensitivity of the previously described biosensor (Campas et al., 2008). In this work, PP2A from Millipore was immobilized within a photopolymer. The detection principle was based on the dephosphorylation of non-electroactive p-aminophenyl phosphate (p-APP) by PP2A and the ability of diaphorase and NADH oxidase to recycle p-aminophenol in order to amplify the electrochemical signal arising from its oxidation (Figure 2). The amplification system allowed to improve the sensitivity of the biosensor. This strategy decreased the detection limit from 37.75 $\mu g.L^{-1}$ to 0.05 $\mu g.L^{-1}$ and enlarged the linear range by more than four orders of magnitude. The application of the amplification system to MC detection with a PP2A inhibition-based biosensor has resulted in a 755-fold lower detection limit, making the biosensor useful as a reliable screening tool to assess the water quality.

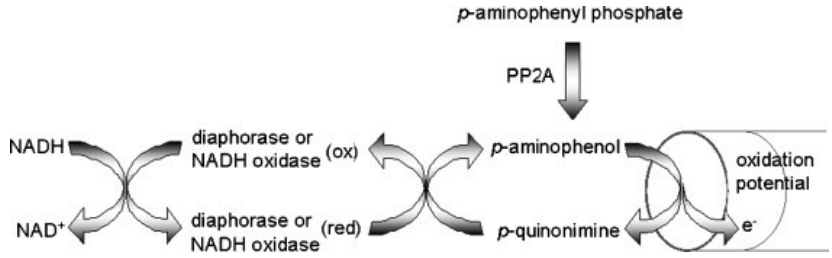

Figure 2. Scheme of the enzymatic signal amplification for the detection of the PP2A activity (Campas et al., 2008). The detection principle is based on the dephosphorylation of non-electroactive p-APP by PP2A, the oxidation of the corresponding electroactive p-aminophenol to p-iminoquinone (p-IQ) on the electrode surface and the regeneration of p-IQ by diaphorase, which requires NADH as substrate

3. Immunosensors

3.1. Principle

Immunosensors are characterized by the highly selective affinity interactions between antibodies (Ab) or antigens (Ag) immobilized on the transducer surface and their specific analytes, Ag or Ab respectively. Electrochemical immunosensors are obtained through the immobilization of the recognition element (Ag or Ab) on the electrode surface (Ricci, Adometto & Palleschi, 2012).

Different formats can be used to develop an electrochemical immunosensor. Due its small size (MW 900-1100 $g.mol^{-1}$), sandwich assays are not possible for MC detection. Two approaches could be considered when dealing with competitive immunosensors. A first one in which immobilized antibodies react with free antigens in competition with labeled antigens (Campas & Marty, 2007). A second one, using immobilized antigens and labeled antibodies, is generally preferred and prevents all the problems related to antibody immobilization (loss of affinity, orientation of the immobilized protein) (Ricci et al., 2012). Electrochemical detection of immunoreactions can also be carried out directly without label. In this case, the immunosensor measures changes in electronics or interfacial properties due to the Ag/Ab complex formation on the electrode surface.

3.2. Label-based electrochemical immunosensors

Marty's group developed the first immunosensor for the MC analysis (Campas & Marty, 2007). This amperometric immunosensor was based on the affinity between the cyanotoxin and the corresponding monoclonal or polyclonal Abs. In this work, Abs were immobilized on a SPE. The toxin present in a sample and MC-HRP conjugate competed for binding to the immobilized anti-MC Ab. The electrochemical detection required the use of 5-methyl-phenazinium methyl sulfate (MPMS) as a redox mediator in order to provide electrical contact between enzymatic label and the electrode surface. The more MC present in the sample, the less the electrochemical signal measured. The monoclonal Ab system provides lower detection limit. In this case, the MC-LR could be detected between 0.1 ng/L^{-1} and 100 $\mu g/L^{-1}$. Although the monoclonal Ab sensor was sensitive, this system had a low reproducibility and, consequently was not enough reliable. Despite the potential matrix electrochemical effects, the analysis of algal samples with both immunosensors and the comparison of the results with those obtained with a colorimetric

enzymatic assay and HPLC validate the applicability of the developed devices as screening tools for fast and reliable cyanotoxin detection.

In the last few years, carbon nanomaterials have been used in sensitive detection of various analytes. Recently, single-walled carbon nanohorns (SWNHs) were used for the development of an electrochemical immunosensor for rapid detection of MC-LR (Zhang et al., 2010). SWNHs are spherical aggregates of thousands of graphitic tubule closed ends with cone shaped horns. The functionalization of SWNHs was performed by covalently binding MC-LR to the carboxylic group on the cone-shaped tips of SWNHs. Competition of HRP-labeled MC-LR Ab for free and immobilized toxin was subsequently performed. This immunosensor exhibited a wide linear response to MC-LR ranging from 0.05 $\mu g.L^{-1}$ to 20 $\mu g.L^{-1}$ with a detection limit of 0.03 $\mu g.L^{-1}$. This method showed good accuracy and reproducibility. This immunosensor was used for the analysis of polluted water samples and the results are in good agreement with those obtained with HPLC.

However, these methods require an enzyme label. The analyses are rather complicated with relatively high cost.

3.3. Label-free immunosensors

The development of label-free immunosensors represents an attractive approach for detecting affinity interactions. These systems exploit the unique properties of nanomaterials. To further enhance capability of a biosensor, immobilization using nanomaterials is of considerable interest. The field of environmental diagnostics has been interested in using NPs for analyzing toxins (Wang et al., 2010). Metal nanoparticles are generally defined as isolable particles between 1 and 50 nm in size, that are prevented from agglomerating by protecting shells. Owing to their small size, nanoparticles have physical, electronic and chemical properties that are different from those of bulk metals. Such properties strongly depend on the number and kind of atoms that make up the particle (Wang, 2005a). Nanoparticles have been exploited as biomolecule immobilization supports because of their large surface-to-volume ratio, high surface reaction activity for biomolecule loading and high catalytic activity. On the one hand, a high number of biomolecules can be immobilized on NPs, retaining their biological activity. On the other hand, electron transfer between biomolecules and electrode surfaces is promoted (Campas, Garibo & Prieto-Simon, 2012). The properties of NPs have been exploited for the development of sensitive immunosensors. For instance, a label-free capacitive immunosensor for the detection of MC-LR was developed (Loyprasert et al., 2008). Anti-MC-LR Ab was immobilized on silver NPs bound to a self-assembled thiourea monolayer formed on the working electrode. NPs were incorporated into modified electrodes to enhance response and achieve a more sensitive system. Capacitive immunosensors measure the changes in dielectric properties when an Ag/Ab complex is formed on the surface of an electrode. Under optimum conditions, the detection limit was 7 $pg.L^{-1}$. The immobilized anti-MC-LR Ab on self-assembled thiourea monolayer incorporated with silver nanoparticles was stable and good reproducibility of the signal could be obtained up to 43 times with a R.S.D. of 2.1 %. The immunosensor was applied to analyze MC-LR in water samples and the results were in good agreement with those obtained by HPLC. A label-free impedimetric immunosensor for the detection of MC-LR was also developed by immobilizing Ab on gold Nps/L-cysteine coated electrode (Sun, et al., 2010). Under optimal conditions, MC-LR could be determined with a detection limit of 18.2 $ng.L^{-1}$. Moreover, the immunosensor exhibited a long-term stability and good reproducibility of the signal could be obtained up to 42 times with a R.S.D. of 3.58 %. The same strategy was also used to develop a

label-free amperometric immunosensor for the detection of MC-LR in water (Tong et al., 2011). In this case, the detection limit was 20 $ng.L^{-1}$.

The unique chemical and physical properties of carbon nanotubes (CNTs) have paved the way to new electrochemical biosensors (Wang, 2005b; Viswanathan & Radecki, 2008; Balasubramanian & Burghard, 2006). CNTs can be described as a graphite sheet rolled up into a nanoscale-tube (Single-wall carbon nanotubes, SWCNT) or with additional graphene tubes around the core of a SWCNT (multi-wall CNTs, MWCNTs) (Trojanowicz, 2006). A SWCNT-coated paper was developed for the detection of MC-LR (Wang et al., 2009). First, Ab was dispersed together with SWCNTs. Then, the dispersion was used to dip-coat the paper rendering it conductive. The obtained SWCNT-coated paper was used as working electrode. The interaction MC-LR/Ab induced a change in conductivity of SWCNT-coated paper, which was used to detect the toxin in the water. The detection limit was found to be 0.6 $\mu g.L^{-1}$.

4. DNA sensors

The use of DNA recognition layers has been extensively explored in the field of analytical chemistry due to their wide range of physical, chemical and biological activities (McGown et al., 1995). An oligonucleotide with a known sequence of bases or a fragment of DNA is used as sensing element in DNA biosensors (Sassolas, Leca-Bouvier, & Blum, 2008). The DNA biosensors (also called genosensors) are either based on the hybridization of complementary strands of DNA or could be used as highly specific receptor for many target molecules (Singh et al., 2012). Given the high affinity and specificity for the target molecules, DNA biosensors have potential applications in a variety of detection and diagnostic systems and can thus be considered as a valid alternative to antibodies or other bio-receptors, for the development of biosensors. DNA offers many advantages compared to antibody in the design of biosensors. In contrast to antibody, conformational changes of DNA can also be explored to monitor molecular recognition event. The handling of biosensors based on DNA is much easy than that of immunosensors, since proteic nature of antibody limits their application to physiological conditions. The stability of DNA biosensor is higher than that of antibody based devices. DNA biosensors can stand to high temperature, low pH and high level of organic solvent that denatures antibodies. Furthermore, immobilized DNA can be regenerated easily by changing the temperature or pH or by the addition of chaotropic agent in case of DNA biosensors. Other advantage is the easy conjugation of the labelled molecules to DNA and also easy immobilization of DNA on different transducer surfaces in the design of biosensors. Furthermore, the small size and versatility of DNA strands make them suitable to immobilize in high-density monolayer onto the electrode surface, which is of vital importance to miniaturise the biosensors (Wang et al., 1997; Bagni, et al., 2006).

The detection techniques play very important role in the design of biosensors, and should be selected according to the requirement of particular application. Among the various sensing device designed so far, electrochemical DNA biosensors have attracted more attention due to their high sensitivity and rapid response. In addition electrochemical techniques are suitable for miniaturization and have the potential to simplify the nucleic acid analysis using low cost electronics.

Yan *el al*. (2001) developed an electrochemical biosensor for voltammetric detection of gene sequence related to genera of cynobacteria, *Microcystis* spp. The sensor was based on the immobilization of a complementary 17-mer DNA probe on a gold electrode through specific

adsorption. The target gene in the solution was determined by the use of methylene blue and ruthenium bipyridine as electrochemical indicators. $Ru(bpy)_3^{2+}$ interacts with guanines of ssDNA whereas the formation of the double helix precludes the collision of $Ru(bpy)_3^{2+}$ with guanine bases. When $Ru(bpy)_3^{2+}$ oxidizes guanine, a high catalytic cuurent is measured after hybridization. Methylene blue is used as an electrochemical intercalator to monitor the DNA hybridization because ssDNA and dsDNA have different affinities for it (Sassolas, Leca-Bouvier et al., 2008). The anodic peak currents of $Ru(bpy)_3^{2+}$ were linearly related to the concentration of the target oligonucleotide sequence in the range The detection limit of this approach was 9.0×10^{-11} M (Yan et al., 2001). Similarly, Erdem et al. (2002) described an electrochemical biosensor for the voltammetric detection of DNA sequences related to *Microcystis* spp. A specific DNA probe was designed and immobilized on the carbon paste electrode. Differential pulse voltammetry (DPV) and cyclic voltammetry (CV) in the presence of methylene blue and tris ruthenium were used to evaluate the hybridization process between the target sequence and the immobilized DNA probe. The system was used to detect Microcystis spp from real tap water and river water (Erdem et al., 2002). Recently, Lan et al. (2010) developed a disposable electrochemical DNA biosensor for in situ determination of *Microcystis* spp. The DNA probe, complementary to target DNA, was immobilized on the screen printed carbon electrode (SPCE) surface by the use of gold nanoparticles (AuNPs). The AuNPs were used to enhance the immobilization process and subsequently to increase the system sensitivity. Methylene blue was used as redox indicator and different immobilization steps were characterized by CV and DPV (Lan et al., 2010). Although electrochemical DNA biosensors based on the use of redox indicator provided reliable and precise information in mutation detections, redox probes require high potential that are prone to interferences and often destroy the hybrid double strand structure. To overcome this problem, an electrochemical DNA biosensor based on the concept of metal-enhanced detection for the determination of *Microcystis* spp was developed (Owino, Mwilu & Sadik, 2007). The biosensor was constructed by immobilizing 17-mer DNA probe on a gold electrode via avidin-biotin chemistry. Electrochemical reduction and oxidation of DNA-captured Ag^+ ions provided the detection signal with a detection limit of 7×10^{-9} M.

5. Aptamer-based biosensors

Aptamers, a new class of molecules, have been appeared as promising recognition tools for analytical applications. Aptamers are short single stranded oligonucleotides, either DNA or RNA that fold into well-defined 3D structures and bind to their ligand by complementary shape interactions, with antibody-like binding ability. They are engineered through an *in vitro* selection procedure, also called SELEX (Systematic Evolution of Ligands by EXponential enrichment), which was first reported in 1990 (Ellington & Szostak, 1990; Tuerk & Gold, 1990). The SELEX process is a technique for screening very large combinatorial libraries of oligonucleotides by an iterative process of *in vitro* selection and amplification. In the screening process, a random sequence oligonucleotide library is incubated with the target of interest. Sequences that bind to the target are separated from the unbound species using a suitable partitioning method, and then, the sequence of these candidates is amplified using polymerase chain reaction (PCR). This population of selected sequences represents a mixture of oligomers with variable affinity towards the target analyte. The single-strand population obtained after the purification step is incubated with a fresh sample of the target for the next round of selection. Iteration of the above protocol results in the isolation of a pool of nucleotide sequences displaying sequential motifs, which after 8-15 iterative SELEX runs converge to one or a few binding sequences. Once

the sequence is identified, an aptamer is produced by chemical synthesis (Stoltenburg, Reinemann & Strehlitz, 2007).

Aptamers hold significant advantages over other bioreceptor molecules. As they are chemically synthesized, their production does not require the use of animal and is therefore less expensive and tedious. Aptamers can be also easily labeled with a wide range of reporter molecules such as fluorescent dyes, enzymes, biotin, or aminated compounds, enabling the design of a variety of detection methods. Due to its many advantages, numerous aptamer-based biosensors have been developed for the detection of a wide range of targets (Sassolas, Blum & Leca-Bouvier, 2011; Sassolas, Blum & Leca-Bouvier, 2008; Hianik & Wang, 2009).

The selection of an appropriated aptamer, and its use in the development of aptasensors could offer various advantages over the existing methods for microcystin detection. A surface Plasmon resonance (SPR) aptasensor based on anti-microcystin DNA aptamer was developed by Nakamura et al. in 2001. The sensitivity of the aptasensor was not as high compared with the methods reported previously, suggesting further improvement in the SELEX process for microcystin molecules (Nakamura et al., 2001). Recently, a RNA aptamer specific for microcystin-LR has also been selected (Gu & Famulok, 2004; Hu et al., 2012). Currently, the potential of aptamers for the microcystin detection has not still been exploited but electrochemical aptamer-based biosensors could be an alternative to the conventional methods of toxin analysis.

6. Conclusion

Biosensors are good candidates for the environmental monitoring. They exploit the remarkable specificity of recognition elements to design efficient analytical tools that can detect the presence of microcystins in water. Several configurations that depend on the type of recognition elements and the immobilization technique can be envisaged for the development of biosensors. The biological elements used for toxin analysis are classically enzymes and antibodies. In the last decade, aptamers have been used as new molecular recognition elements to develop biosensors. However, unique properties of aptamers have not been yet exploited for the microcystin analysis. We believe that these recognition elements could be used, in the near future, for the development of efficient electrochemical aptasensors allowing the detection of microcystins.

Nanotechnology is playing an important role in the development of efficient biosensors for the toxin detection. Different types of nanomaterials (e.g. nanoparticles and nanotubes) with different properties have been used. They offer exciting new opportunities to improve the performance of electrochemical biosensors for the detection of microcystins.

The use of biosensors in environmental field is still limited in comparison to medical applications. Most commercial biosensors are for medical applications, whereas only few are adapted for the environmental monitoring. Even if some commercial kits are available for the analysis of DSP toxins, there is still a challenge to develop improved and more reliable devices allowing the analysis of microcystins in water.

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