

Review

Electrochemical biosensing of algal toxins in water: The current state-of-art

Wei Zhang, Mike B. Dixon, Christopher P Saint, Kar Seng Teng, and Hiroaki Furumai

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4 2 state-of-art
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6 3 Wei Zhang^{1,3,4*}, Mike B. Dixon², Christopher Saint³, Kar Seng Teng⁴ and Hiroaki Furumai¹
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8 4 1. Research Centre for Water Environment Technology, Department of Urban Engineering, The
9 5 University of Tokyo, Tokyo 113-0033, Japan.
10 6 2. MDD Consulting, Kensington Calgary, Alberta, Canada.
11 7 3. School of Natural and Built Environments, University of South Australia, Mawson Lakes, South
12 8 Australia 5095, Australia.
13 9 4. College of Engineering, Swansea University, Bay Campus, Swansea SA1 8EN, UK.
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16 11 *Corresponding author: zhangwei@env.t.u-tokyo.ac.jp
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18 13 **Abstract**

19 14 Due to increasing stringency of water legislation and extreme consequences that failure to detect some
20 15 contaminants in water can involve, there has been a strong interest in developing electrochemical
21 16 biosensors for algal toxins detection during last decade, evidenced by literature increasing from 2
22 17 journal papers pre-2009 to 24 between 2009 and 2018. In this context, this review has summarised
23 18 recent progress of successful algal toxin detection in water using electrochemical biosensing
24 19 techniques. Satisfactory detection recoveries using real environmental water samples, good sensor
25 20 repeatability and reproducibility have been achieved, along with some excellent limit-of-detection
26 21 (LOD) reported. Recent electrochemical biosensor literature in algal toxin detection are compared and
27 22 discussed to cover three major design components: 1) biorecognition elements, 2) electrochemical
28 23 read-out techniques, 3) sensor electrodes and signal amplification strategy. The recent development of
29 24 electrochemical biosensors has provided one more step further towards quick in-situ detection of algal
30 25 toxins in the contamination point of water source. In the end, we have also critically reviewed the
31 26 current challenges and research opportunities regarding electrochemical biosensors for algal toxin
32 27 detection that need to be addressed before they attain commercial viability.
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34 28 **Keywords** Electrochemical; Biosensor; Algal toxin; Enzymes; Nanomaterials.
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Drinking water security and their sufficient treatment is of paramount importance to the health of a community and the quality of life in any country [1, 2]. Episodes of harmful algal blooms (HAB) usually involving cyanobacteria or dinoflagellates, occur frequently in fresh or marine water bodies worldwide as a consequence of eutrophication resulting from anthropogenic activities, such as agricultural run-off, urban waste, and manufacturing of detergents and global warming [3, 4]. HAB often produce undesirable color, odor, and tastes but most importantly, harmful algal toxins, such as hepatotoxins (i.e. microcystins, cylindrospermopsin, and nodularin), neurotoxins (i.e. anatoxins, brevetoxins and saxitoxins), and dermatotoxins (i.e. lyngbyatoxins and lipopolysaccharides). It has been estimated that 50 to 70% of metabolites produced by HAB are harmful toxins (e.g. cyanotoxins) [5], which is a significant hazard for human health and the ecosystem in drinking water, recreational water, and aquaculture. Exposure to algal toxins occurs through drinking water, recreational activities, or by consuming foods, in which algal toxins has accumulated. The concentrations observed in various freshwater and marine water bodies range from undetectable at the ng/L to lower ng/L and can reach up to several hundreds of $\mu\text{g/L}$. For example, residents in the city of Toledo, USA could not use and drink tap water in the summers of 2013 and 2014 due to the presence of cyanotoxins detected in their drinking water supplies [6]. Following a significant HAB event, there is an urgent need to develop appropriate early alarming systems and establish when a water source is safe to use or to evaluate the level of treatment required to make a source safe.

Cyanobacteria toxins in water

Microcystins belong to a group of cyclic heptapeptides, which are potent and specific in inhibiting protein phosphatases 1 and 2A (PP1, PP2A) [7]. Acute or prolonged exposure to microcystins will cause liver damage, followed by a massive intrahepatic haemorrhage and probably leading to death. Microcystins are some of most frequently detected cyanotoxins in freshwater throughout the world. To date, 80 variants of microcystins have been isolated depending on the amino acids and identified from freshwater cyanobacteria genera, among which microcystin-LR (MC-LR, MW 995.19) is the most toxic variant [8], with a LD_{50} value of 43 $\mu\text{g/kg}$ for mouse bioassay [9]. MC-LR is mainly produced by *Microcystis*, but may also be produced by other genera, such as *Anabaena*, *Nostoc*, *Phormidium* and *Planktothrix*. It has been confirmed that microcystins were responsible for some poisonings of animals and humans where water sources contained toxic cyanobacteria blooms [10].

Nodularins (NOD, MW 824.98) are cyclic pentapeptides of a similar structure to MC-LR with strong hepatotoxic activity, however they are only produced by *Nodularia spumigena* in brackish water [11]. Although MC-LR and NOD are chemically and toxicologically very similar, NOD does not bind covalently to PP1, as in the case of MC-LR [12]. Only six variants of nodularins has been identified. Beyond protein inhibition, other adverse toxicological effects have been reported concerning MC-LR and NOD exposure, such as intracellular glutathione alteration, reactive oxygen species production and lipid peroxidation [13, 14]. The mouse bioassay study shows slightly less toxicity when compared with MC-LR with a LD_{50} value of 30 $\mu\text{g/kg}$.

Another major group of cyanotoxins are alkaloids, including cylindrospermopsins and anatoxins. Cylindrospermopsin (CYN, MW 415.43) is readily emerging as a globally important cyanobacterial freshwater toxin, since the human CYN poisoning accident in 1979 (Queensland, Australia) [15]. CYN is a water-soluble and heat-stable, produced by a variety of cyanobacteria species such as *Cylindrospermopsis raciborskii* [16] and *Anabaena bergii* [17]. CYN has two natural variants, deoxycylindrospermopsin (deoxy-CYN) and 7-epicylindrospermopsin (7-epi-CYN) [18], which have very similar structures (the position of the hydroxyl group being the only difference). A guideline value for safe water supply of 1 $\mu\text{g/L}$ of CYN has been recommended based on toxicity studies [19]. While hepatotoxicity is the main effect of CYN, it also affects lungs, kidneys, intestinal tract, stomach, and vascular and lymphatic systems. [20] Moreover, CYN is proved to be cytotoxic and genotoxic [21]. Unlike microcystins, CYN is typically extracellular in water, thus, the removal of the bacterial filaments during water treatment does not remove the toxin [22].

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Anatoxin-a (ATX) is the smallest potent neurotoxin (MW 165.23 Da) with a LD₅₀ value of 200 µg/kg in the mouse bioassay study, produced by a variety of cyanobacteria of *Anabena flos-aquae*, *Aphanizomenon flos-aquae* and *Oscillatoria* [23]. Anatoxin-a(s) (MW 252.21), a phosphate ester of N-hydroxyguanidine, is another *Anabaena flos-aquae* produced cyanotoxins, of which the toxicity is much higher, with a LD₅₀ value of 50 µg/kg in the mouse bioassay study [24]. Although they are structurally quite different, they are both potent quasi-irreversible inhibitors of acetylcholinesterase activity in the neuromuscular junctions, thus resulting in serious nervous dysfunction [25]. The exposure to anatoxin-a and anatoxin-a (s) commonly occurs through swallowing or drinking contaminated water and causes the symptoms of cardiac arrhythmia and respiratory paralysis. A mouse toxicity study has proposed a guideline value of 1 µg/L for anatoxin-a in drinking water [26].

Dinoflagellates toxins in water

HABs caused by dinoflagellates, often referred to as red tides, can also produce a range of neurotoxins. Among these, saxitoxins (STX, MW 299.29) are one of the most toxic non-protein substances with a LD₅₀ value of 10 µg/kg in mouse bioassay study produced by dinoflagellates species of *Alexandrium* spp. and *Gymnodinium* spp. They consist of a family of carbamate alkaloid neurotoxins that are nonsulfated, singly sulfated, or doubly sulfated. Saxitoxins selectively block the sodium channels on excitable cells in a high affinity specific receptor binding process and reduce the number of conducting Na⁺ channels [27]. They are rapidly absorbed through the gastrointestinal tract and can diffuse through the blood-brain barrier [28], resulting in a variety of neurological symptoms culminating in respiratory arrest and cardiovascular shock.

Brevetoxins (PbTx) are a group of potent lipid soluble cyclic polyether neurotoxins naturally produced by the dinoflagellate *Karenia brevis*. BTXs are tasteless, odourless, acids and heat stable (up to 300 °C). BTX-2 (MW 895.08) and -3 (MW 897.2) are the most predominant forms among ten BTXs that have been isolated and characterized from aerosols, field blooms and *Karenia brevis* cultures [29]. BTXs as depolarizing substances can open the voltage-gated sodium channels in nerve cell walls, leading to uncontrolled Na⁺ influx into the cell and subsequent neurologic poisoning [30].

The US EPA currently suggests three cyanotoxins on contaminant candidate list 3: namely, anatoxin-a, microcystin-LR, and cylindrospermopsin [31]. After its first fatal incident in Brazil [10], MC-LR was the first algal toxin assigned a provisional drinking water guideline value (i.e.1 µg/L) by the World Health Organization (WHO) in 1998 [32]. The WHO drinking water concentration limit for nodularins is 1.5 µg /L, which is extended from microcystins-LR. In spite of serious harmful effects, monitoring for any other cyanotoxins is not routinely performed in fresh water and WHO has not declared their drinking water guideline values due to inadequate toxicological data. However, few countries were expanding their monitoring programs and leading the way to developing provisional guidelines for other algal toxins. For example, Australia has suggested provisional drinking water guidelines of 3 µg/L, 3 µg/L, and 1–13 µg/L for anatoxin-a, saxitoxin and cylindrospermopsin, respectively [33].

Current status of developing HAB toxin monitoring technique in water

The development of reliable methods for monitoring HAB toxins in water resources is of great interest to determine the occurrence and to prevent human exposure. High-performance liquid chromatography/mass spectrometry (HPLC-MS) is an U.S. EPA approved analytical method for detection of a wide range of algal toxins, including microcystins [34], nodularins [35] and cylindrospermopsin [36], which are in common use with a low detection limit of 0.1-1 µg/L. In contrast, the analysis of saxitoxins [37], anatoxin-a, and anatoxin-a(s) [38,39] often require non-standard analytical techniques, such as capillary electrophoresis or chemical derivatization in combination with chromatographic techniques. Overall, well-established laboratory techniques, while being competent, often need complex instrumentation and procedures, highly-trained technicians,

1 long turn-around time, high processing cost, sample pretreatment, and are only practical in the
2 laboratory, not *in situ*. Currently, various commercial enzyme-linked immunosorbent assay (ELISA)
3 kits are also available to monitor and quantify HAB toxins in water samples [40], however,
4 concentrations determined by ELISA were systematically higher than concentrations determined by
5 LC-MS, which was attributed to matrix effects and cross-reactivity with other unidentified
6 derivatives.

7 Electrochemical sensors have become a mature discipline with some outstanding commercial success,
8 since they are suitable devices for *in situ* monitoring, due to their possible miniaturisation towards the
9 fabrication of an implantable biosensor, portability (e.g., hand-held) and automation (e.g. continuous
10 monitoring) [41]. Other advantages of electrochemical sensors include high specificity, low-detection
11 limits, relative freedom from matrix interference and low cost over other types of sensors [42]. A
12 recent analysis indicated that electrochemical biosensors dominate the biosensor industry contributing
13 to 71% of market share (USD 15.96 Billion) in 2016 and anticipated to exceed USD 21 billion by
14 2024 (*Biosensors Market Size by Technology, by Application, by End-user, Industry Analysis Report, Regional Outlook, Growth Potential, Price Trends, Competitive Market Share & Forecast, 2016-2024*).
15 Based on their application, the environmental biosensor segment is small but is growing fast (>
16 14% p.a.), partly spurred by increased stringency of water legislation, due to the extreme
17 consequences that failure to detect some contaminants in water can involve. In this context, there has
18 been significant progress over the last decade in developing highly sensitive and specific
19 electrochemical biosensors/immunosensors for environmental monitoring, especially for algal toxins
20 [43]. As the only type of algal toxin currently governed by WHO drinking water guidelines,
21 electrochemical sensor detection of MC-LR in water has received by far the most attention in the
22 literature (i.e. 18 out of 26 total studies surveyed in this review), which are summarised in Table 1.

Table 1 Reported studies of algal toxins detection in water using various electrochemical biosensors

Toxins	Electrodes	Signal labels	Blocking agent	Cross-linkers	Detection range (LOD)	Recovery (%)	Detection techniques	Electroactive probes	References
MC-LR	CNT	antibody	-	-	10 to 40 ng/L (0.6 ng/L)	94.05-101.32	Amperometry	-	[44]
MC-LR	GCE/GS	Ab ₁ and PtRu-Ab ₂	-	EDC-NHS	0.01 to 28 µg/L (9.63 ng/L)	99.5-102	Chronoamperometry	H ₂ O ₂	[45]
MC-LR	CNx-MWNTs/Au/GCE	Antibody	-	-	-	95.1-96.2	DPV	<i>o</i> -PD and H ₂ O ₂	[46]
MC-LR	Gold-GNPs	Monoclonal antibody	BSA	L-cysteine	0.05 to 15 µg/L (20 ng/L)	95.6-105	DPV	hydroquinone	[47]
MC-LR	Gold	5' thiolated aptamer	-	-	0.05 to 100 µg/L (18 ng/L)	91.2-113.7	EIS	[Fe(CN) ₆] ⁴⁻ /[Fe(CN) ₆] ³⁻	[48]
MC-LR Nodularin	Carbon/UltraBind membrane	IgG	Gelatin	Polyethersulfone	0 to 10 µg/L (0.5 µg/L)	-	Chronoamperometry	HRP and H ₂ O ₂	[49]
MC-LR	AuNPs/GCE	HRP-mAb	4-chloro-1-naphthol with the aid of H ₂ O ₂	Cysteamine/glutaraldehyde	0.01 to 100 µg/L (4 ng/L)	94.1-98.1	EIS	[Fe(CN) ₆] ⁴⁻ /[Fe(CN) ₆] ³⁻	[50]
MC-LR	CNFs/PEG/GCE	Ab ₁ and Au-Ab ₂	BSA	-	0.0025 to 5 µg/L (1.68 ng/L)	98-99.2	DPV	AuNPs in HCl	[51]
MC-LR	AuNP-polyDPB-G-AuNP/GCE	Polyclonal Ab	BSA	EDC-NHS	0.1 pg/L to 8 pg/L (0.037 pg/L)	96.3-105.8	DPV	-	[52]
MC-LR	Gold	5'-disulfide terminated Aptamers	-	Aminoethanethiol/NHS	0.01 to 10 µg/L (7.5-11.8 ng/L)	-	SWV	[Fe(CN) ₆] ⁴⁻ /[Fe(CN) ₆] ³⁻	[53]
MC-LR Nodularin-R	GCE/SDD-Co(II)/AgNPs	5' thiolated aptamer	6-mercapto-1-hexanol (MCH)	-	0.1 to 1.1 µg/L (0.04 µg/L)	94.3-115	CV	-	[54]
MC-LR	SWCNT/SiO ₂ /Gold	Monoclonal Ab	TW20	APTES-PBASE	1 to 1000 ng/L (0.6 ng/L)	84.7-124.2	Electrical resistance	-	[55]
MC-LR	CNT@Co-silicate	Multi-HRP-(Fe ₃ O ₄ @PDA-Au)-Ab	Skim milk	PDA, APTES-glutaraldehyde	0.005 to 50 µg/L (4 ng/L)	91.6-110.7	CV	HRP and H ₂ O ₂	[56]
MC-LR	GSPE	Ferrocene and	-	Physical adsorption	0.1 to 1000 ng/L	91.7	SWV	[Fe(CN) ₆] ⁴⁻	[57]

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		disulfide labelled aptamer			(1.9 ng/L)			$[\text{Fe}(\text{CN})_6]^{3-}$	
MC-LR	GSs-CS/CGCE	HRP-CNS-Ab	-	EDC-NHS	0.05 to 15 $\mu\text{g/L}$ (16 ng/L)	88-107.8	DPV	$[\text{Fe}(\text{CN})_6]^{4-}$ $[\text{Fe}(\text{CN})_6]^{3-}$	[58]
MC-LR	CNT	Monoclonal antibody	-	EDC-NHS	0.05 to 20 $\mu\text{g/L}$	-	EIS	$[\text{Fe}(\text{CN})_6]^{4-}$ $[\text{Fe}(\text{CN})_6]^{3-}$	[59]
MC-LR	GF	Monoclonal antibody	-	EDC-NHS	0.05 to 100 $\mu\text{g/L}$	92.7	EIS	$[\text{Fe}(\text{CN})_6]^{4-}$ $[\text{Fe}(\text{CN})_6]^{3-}$	[60]
MC-LR	GFC	Monoclonal antibody	-	EDC-NHS	0.005 to 10 $\mu\text{g/L}$	93.5-98.2	EIS	$[\text{Fe}(\text{CN})_6]^{4-}$ $[\text{Fe}(\text{CN})_6]^{3-}$	[61]
Cylindrosp ermopsin	Gold	Disulfide-modified aptamer	6-mercapto-1-hexanol (MCH)	-	0.1 to 80 $\mu\text{g/L}$ (0.1 $\mu\text{g/L}$)	-	EIS	$[\text{Fe}(\text{CN})_6]^{4-}$ $[\text{Fe}(\text{CN})_6]^{3-}$	[62]
Cylindrosp ermopsin	Thionine-graphene	Aptamer	-	glutaraldehyde	0.39 to 78 $\mu\text{g/L}$ (0.117 $\mu\text{g/L}$)	-	EIS	$[\text{Fe}(\text{CN})_6]^{4-}$ $[\text{Fe}(\text{CN})_6]^{3-}$	[63]
Anatoxin-a(s)	7,7,8,8-tetracyanoquinodimethane (TCNQ)-graphite	Acetylcholinesterase (AChE)	-	-	1 to 10 $\mu\text{g/L}$ (1 $\mu\text{g/L}$)	95.8-103.2	Amperometry	Actylthiocholine chloride	[64]
Anatoxin-a	Gold	Aptamer	6-mercapto-1-hexanol (MCH)	Cysteamine/Sodium borohydride	1 to 100 $\mu\text{g/L}$ (0.5 $\mu\text{g/L}$)	94.8-108.6	EIS in	$[\text{Fe}(\text{CN})_6]^{4-}$ $[\text{Fe}(\text{CN})_6]^{3-}$	[65]
Saxitoxin	MWCNT/Au	3'-amino-modified aptamer	ODT	EDC-NHS	0.9 to 30 $\mu\text{g/L}$ (0.38 $\mu\text{g/L}$)	63-20.5	DPV	MB	[66]
Saxitoxin	Cu/GNS/lipid layer	Antibody	-	-	1 to 1000 $\mu\text{g/L}$ (1 $\mu\text{g/L}$)	92-103	Voltage	-	[67]
Saxitoxin and bevetoxin-3	Hydrogen peroxide/Immobilized on AV membrane	Glucose oxidase-labeled antibody	BSA	Succinic anhydride/EDC and glutaraldehyde	(15 $\mu\text{g/L}$)	-	Amperometry	Glucose	[68]
Brevetoxin-2	Gold	Aptamer	ethanolamine	Cysteamine/1,4-phenylene diisocyanate (PDIC)	0.01 to 2000 $\mu\text{g/L}$ (106 ng/L)	-	EIS	$[\text{Fe}(\text{CN})_6]^{4-}$ $[\text{Fe}(\text{CN})_6]^{3-}$	[69]

Electrochemical biosensing of algal toxins: principle and applications

Electrochemical sensing has become one of the most promising analytical techniques because of the high sensitivity, ease of miniaturization, low-cost and relative simplicity [70]. Developing electrochemical biosensors for algal toxins detection usually consist of three major components: biorecognition elements, signal transduction (i.e. electrochemical read-out) mechanism, and sensor electrodes.

Biorecognition elements

To offer detection selectivity and specificity, biorecognition receptors are essential for electrochemical biosensors, including anti-bodies (Ab), DNA/aptamers, carbohydrates, and antimicrobial peptides [71]. So far, antibodies are the most reported receptors in electrochemical biosensing of algal toxins, because they are readily available and provide highly specific molecular recognition without any preconcentration or pretreatment. Indeed, due to their large size (MW ~150k Da), the electrochemical sensing depending on conformation changes of antigen/antibody conjugation is more suitable for the detection of large analytes. In the case of algal toxins (<1k Da), many studies adopted a competitive or displacement immunoassay design, in which sensor electrode surfaces were bound with algal toxins first and then incubated in solutions of a fixed amount of antibodies and algal toxins of various concentrations [55, 51, 50, 58, 56, 59, 60]. As a result, algal toxins in sample solution would compete with algal toxins immobilized on the electrode to interact with antibodies. Electrochemical responses (i.e. redox peak currents or electron transfer resistance) upon this biorecognition event are much higher than a direct immunoassay procedure (i.e. antibodies onto the electrode surfaces first and then algal toxins binding to them), leading to better biosensing sensitivity (i.e. response per analyte concentration change).

Aptamers belong to a group of synthetic small single-stranded (ss) DNA/RNA molecules that recognizes various target molecules, including cells, proteins, peptides, and amino acids [72]. Compared with antibodies, aptamers are of much smaller size, higher affinity, specificity and stability with target molecules under a variety of conditions. Aptamers are normally prepared with terminal moieties such as thiol [48, 54], amino [66], ferrocene [57] and disulphide groups [53, 62], which can readily bind to various electrode surfaces. In case of gold surface binding, 6-mercapto-1-hexanol (MCH) can be used to remove weakly adsorbed aptamer nucleotides from gold electrode surface in order to free up their secondary structure (e.g. stem and loop segments) for more access to target detection (i.e. algal toxins) [73]. The amount of aptamer loaded onto the sensor electrode surface during or before a biorecognition event should be controlled or optimized, since cross-hybridization may occur between neighbouring aptamers resulting in unnecessary interfering read-out signals. A few studies showed that increasing Mg^{2+} concentration enhanced the binding affinity of three algal toxins (i.e. MC-LR, CYN and PbTx-2) to their aptamers until it plateaued, while Na^{+} had insignificant effects [57, 62, 69]. It is speculated that aptamers conformation can be controlled through the interaction with Mg^{2+} , which in turn stabilizes their secondary structure for easy recognition of its target [74]. Low pH (<3.5) was also found to affect the conformation of DNA and thus reduce their binding affinity to aptamer [57, 69]. In light of these findings, pH and Mg^{2+} concentration of water samples should be pre-conditioned to avoid any possible interference with algal toxin/aptamer binding. It is noteworthy that aptamers as bio-recognition materials for algal toxin electrochemical detection currently offer the best promise for commercialization in terms of the storage stability, durability and quick response time of the sensor.

There are also some less conventional biorecognition receptors reported for toxin detection in the literature, such as peptides [75,76] and cells [77]. In the case of algal toxin, cultured frontal cortex neuronal networks - a cell recognition type receptor, were grown over microelectrode arrays for detection of STX and PdTx-3 with action potentials or spikes as electrochemical response signal. LOD of 0.031 and 0.33 $\mu g/L$ were achieved for STX and PdTx-3 in buffer solutions, while in 25-fold-diluted seawater they increased to 0.076 and 0.48 $\mu g/L$, respectively [78].

To facilitate the biomolecules (i.e. algal toxins, antibodies or aptamers) binding to the electrode surface, various types of cross-linkers are frequently employed. Cysteamine with one amine and thiol group is useful for binding a gold or silver electrode surface with a carboxyl group, another amine group (e.g. MC-LR) when coupled with glutaraldehyde [50], hydroxyl group (e.g. PbTx-2) when coupled with 1,4-phenylene diisocyanate (PDIC) [69], and carbonyl group (e.g. ATX) when coupled with sodium borohydride to form a Schiff-base bond [65]. Glutaraldehyde with two carbonyl groups is used for binding an amine group (e.g. MC-LR) and with assistance of (3-Aminopropyl) triethoxysilane (APTES) it was used to conjugate MC-LR antibody with a hydroxyl group containing CNT electrode [56]. 1-Pyrenebutanoic acid succinimidyl ester (PBASE) can facilitate conjugation with graphene and CNT electrodes through π - π stacking, and its succinimidyl ester group can covalently bind with an amine group (e.g. MC-LR) [55]. Polydopamine (PDA) is a natural pigment and major component of animal melanin, which would undergo spontaneous self-polymerization of dopamine in the presence of oxygen under alkaline conditions, providing excellent adhesion to most electrode surfaces. Upon adhesion, its catechol group can then covalently bind with amino groups or thiol groups of any biomolecules via Schiff base reaction or Michael addition [79], while the non-covalent immobilization was also found through hydrogen bonding [80], metal coordination or chelating and π - π interaction [81]. EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) and NHS (N-hydroxysuccinimide) are frequently used to assist the covalent conjugation between the thiol-group or amine-group of biomolecules and carboxyl group containing electrodes [82]. NHS or its water soluble sulfo-NHS was added to increase the stability of the formed amine-reactive intermediate by converting it to an amine-reactive sulfo-NHS ester and thus prevent its rapid hydrolysis in aqueous solutions. They are particularly useful for electrochemical detection process involving interfacial electron-charge transfer, since they introduce no spacer molecules (hence the name 'zero-length cross-linker') that are non-conductive [45, 52, 58, 59, 60, 66]. Nevertheless, it is noteworthy that cross-linkers induced coupling could potentially be hydrolysed during storage or reaction, which may lead to a low efficiency of biocompatibility [83].

Some studies simply resorted to physical adsorption for immobilizing algal toxins or their antibodies to the electrodes [57], although the stability of such binding (e.g. long-term storage) should be subject to further scrutiny. In addition, commercially available affinity membrane (UltraBind US-800) (pre-activated by polyethersulfone to generate aldehyde group) was also employed for binding biomolecules (e.g. MC-LR antibody) on a screen-printed carbon electrode [49]. Bratakou et al. reported a lipid membrane self-polymerised on a glass fibre filter for immobilization of STX antibody upon physical absorption [67].

Prior to a biorecognition event, applying blocking agents is also essential to obtain higher sensitivity by minimizing non-specific binding of analytes to sensor electrodes. BSA [47, 51, 52, 68] and ethanolamine [69] were used to block the unbound carboxyl sites, while 6-mercapto-1-hexanol (MCH) [62, 65], L-cysteine [47] and octadecanethiol (ODT) [62] formed self-assembled monolayers (SAM) on a gold surface and restricted its access to further binding. However, the coverage of blocking agents or SAM layers on the sensor electrode can be difficult to characterize or quantify. At the moment, some authors suggest that the amount of SAM layers can be estimated by measuring their one-electron reductive desorption from sensor electrodes in CV analysis (i.e. integration of first reduction peak) [84].

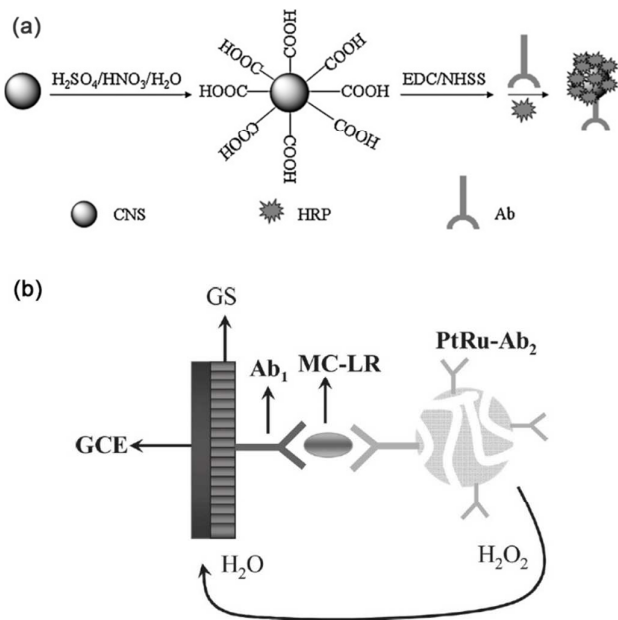


Figure 1 Preparation schematics of carbon nanosphere (CNS) (a) (Reproduced with permission from ref 58, Copyright 2013 Elsevier) and PtRu (b) as carrier agents in sandwich type electrochemical biosensors (Reproduced with permission from ref 45, Copyright 2011 Wiley).

The biorecognition process can also be improved by a sandwich type design (see Figure 1), where an enzyme-antibody or double antibodies Ab_1 - Ab_2 can be configured on a carrier agent. Nanomaterials such as carbon nanospheres (CNS) with larger surface area and good biocompatibility were demonstrated as good carrier agents to increase the loading of biomolecules (i.e. enzymes and MC-LR antibody) [58]. Wei et al. provided a double antibody type sandwich electrochemical biosensor using nano-metal alloys PtRu as the carrier agent to detect MC-LR [45]. Fe_3O_4 nanoclusters are another promising candidate as carrier agents for this type of biosensor due to easy magnetic collection without any impurities, which has been demonstrated in electrochemical detection of MC-LR [56].

Electrochemical read-out techniques

Electrochemical read-out techniques typically involve sensor electrode surface-bound receptors that can change its responses to electroactive probe species in the solution, leading to measurable currents (i.e. voltammetry and amperometry), potential changes (i.e. potentiometry), or charge accumulation resulting changes in capacitance or impedance measured by impedance spectroscopy or electric-field effects upon a specific biorecognition event. For easy interpretation and comparison, most of the electrochemical techniques used for algal toxin detection reported in the literature can be broadly classified into two categories: 1) voltammetry or amperometry, and 2) electrochemical impedance spectroscopy, of which typical response plots are shown in Figure 2, respectively.

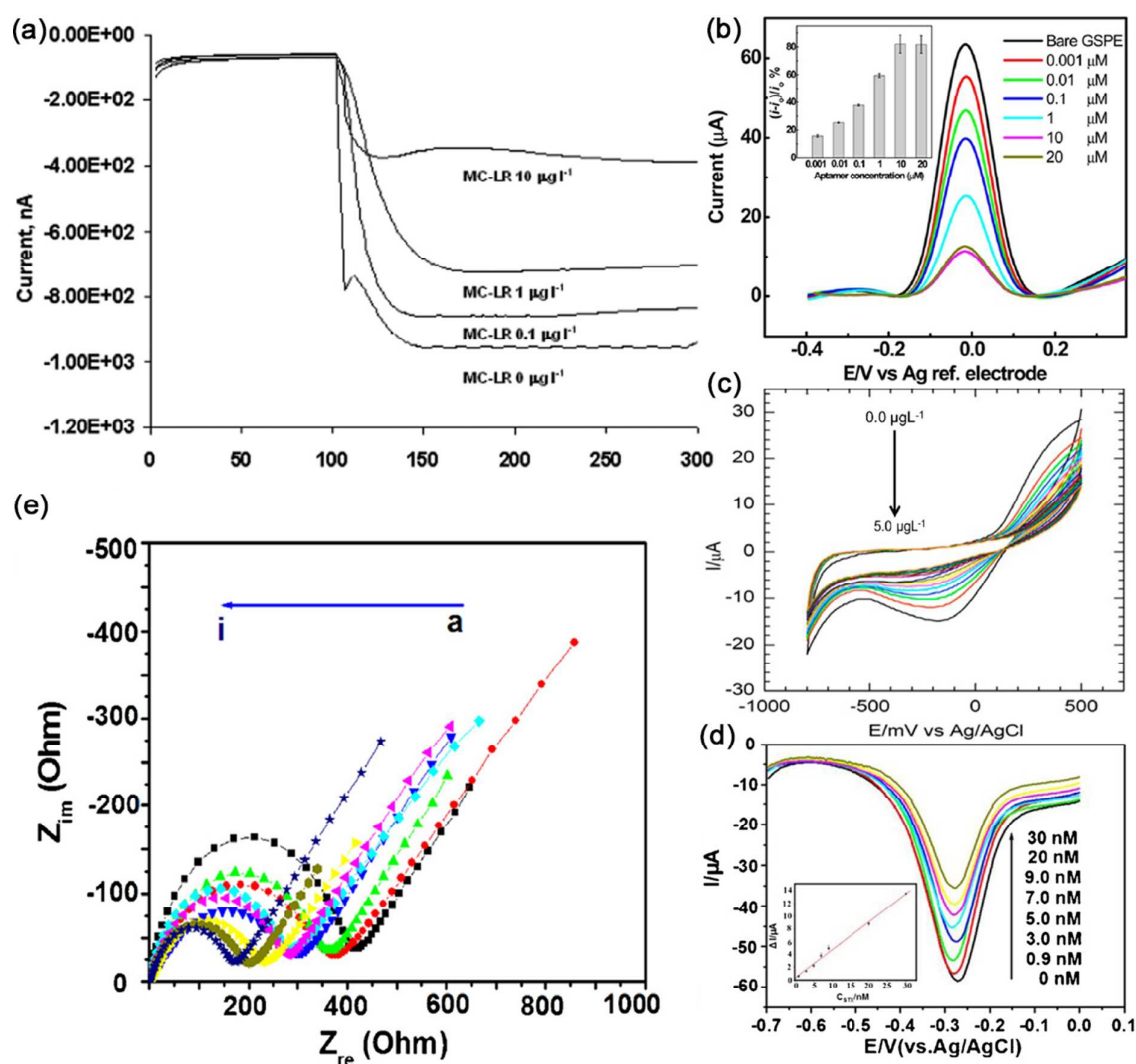


Figure 2 Typical biosensor responses to algal toxin in different concentrations using various electrochemical detection techniques: (a) Amperometry (Reproduced with permission from ref 49, Copyright 2012 American Chemical Society), (b) SWV (Reproduced with permission from ref 57, Copyright 2014 American Chemical Society), (c) CV (Reproduced with permission from ref 54, Copyright 2016 MDPI), (d) DPV (Reproduced with permission from ref 66, Copyright 2016 Springer), and (e) EIS, where a to i represent 0.01, 0.05, 0.1, 0.25, 0.75, 1, 10, 50, and 100 µg/L MC-LR (Reproduced with permission from ref 50, Copyright 2016 Elsevier).

Voltammetry or amperometry is a major electrochemical technique used for algal toxins detection in water. Based on the waveform in which the potential is varied as a function of time, it can be further divided into cyclic voltammetry (CV) [54, 56], square wave voltammetry (SWV) [53, 57] and differential pulse voltammetry (DPV) [46, 47, 51, 52, 58, 66]. Voltammetry method concept generally relies on an electroactive redox probe compounds present in solution that is denied access to the electrode surface upon binding of the target analyte (e.g. algal toxins) to a surface-confined receptor (e.g. antibodies). This denial of access could be the result of steric hindrance due to antigen-antibody complex formation resulting in increased mass transfer resistance of the redox probes. This coupled with the insulating nature of the formed biocomplex layer on the electrode surface generally cause decrease in the corresponding redox currents that can be measured as a function of target analyte concentration. A range of aqueous electroactive redox probes have been applied to facilitate voltammetry detection of algal toxins, including methylene blue [66], hydroquinone [47], Au/AuCl₄⁻ [51], [Ru(NH₃)₆]³⁺ [53], and [Fe(CN)₆]^{3-/4-} [52, 57]. Without using any electroactive probes/mediators

in the solution, one study demonstrated that the linear range of MC-LR concentration to CV current response was very limited (i.e. 0.1 to 1.1 $\mu\text{g/L}$) before it began to plateau [54]. It should also be noted that in the case of voltammetry sensor using negatively charged aptamers, their electrostatic interaction with electroactive probes chosen (i.e. repulsion or attraction) could play more important role than steric/conformational changes into locked structure upon binding with algal toxins. For example, when using negatively charged $[\text{Fe}(\text{CN})_6]^{3-/4-}$ as electroactive probes, it was observed that redox current increased with the amount of MC-LR binding to aptamers [57]. On the other hand, one study reported the opposite for STX when positively charged MB was used as probes [66]. In some cases, the voltammetry detection process can be further assisted by coupling redox probes with different enzymes as mediators, e.g. H_2O_2 with horseradish peroxidase (HRP) and *o*-phenylenediamine (*o*-PD) [46], H_2O_2 with HRP [56, 58], acetylthiocholine chloride with acetylcholinesterase (AChE) [60], and glucose with glucose oxidase [68].

Chronoamperometry is another subclass of amperometry techniques, which has also been employed in a couple of studies on electrochemical detection of algal toxins using electro-active probes such as H_2O_2 [42], ABTS (2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) and H_2O_2 with HRP/hydroquinone as mediators [46]. Since the current is measured as a function of time at a fixed voltage, the key to sensitive amperometry detection of target biomolecule markers (i.e. algal toxins) in the solution is to optimize the concentration and redox potential of redox-active probe compounds for a maximum target analyte signal and minimal background response.

Electrochemical impedance spectroscopy (EIS) is another frequently used methods in electrochemical sensor for monitoring the interaction between antibodies and biomolecules at an electrode surface, which impart excellent advantages, such as high sensitivity, ease of performance and use of simple equipment [85]. Consequently, EIS has attracted a lot of attention to establish bioelectronic devices for detection and quantification of algal toxins [48, 50, 59, 60, 62, 63, 65, 69]. These sensors measure changes in electrode impedance in a reaction solution when target biochemical molecules (i.e. algal toxins) are captured by probes (e.g. antibodies) attached on the electrode surface. Changes in electrochemical impedance are due to changes in capacitance or charge-transfer resistance (or combination of both) on the interface between electrode surface and bulk. Electrode impedance variations are highly dependent on the surface coverage of the electrodes by target biomolecules. The formation of such bio-affinity complexes commonly leads to an insulating layer that retards the interfacial electron transfer kinetics between the redox probe and the electrode and increases the electron-transfer resistance. A small alternating current (AC) current voltage (i.e. in the order of millivolts) is essential in EIS set-up to avoid denaturing of large biomolecules. The ferric/ferrous cyanide ion pair $[\text{Fe}(\text{CN})_6]^{3-/4-}$ is the most applied electro-active redox probe for EIS measurement of algal toxins in water so far. Typical EIS spectra consist of a semicircle portion at higher frequencies corresponding to the electron-transfer-limited process, of which the diameter equals the electron-transfer resistance, and a linear portion at lower frequencies represents the diffusion-limited process (see Figure 1e). Sensitivity of EIS techniques is higher compared to other voltammetry-based techniques (e.g. CV and DPV) and amperometry, since EIS can effectively measure small changes upon biorecognition or binding events on the surface of the electrodes [86, 87] and the response time is significantly faster [88, 89]. Enzyme assisted biocatalytic precipitation (BCP) can also be used to further enhance the EIS detection sensitivity [90, 91]. For example, the 4-chloro-1-naphthol (4-CN) with HRP labelled antibody and H_2O_2 was used to generate insulating precipitates (i.e. benzo-4-chlorohexadienone) on biosensor electrode surface and thus restrict the access of an electroactive redox probe (i.e. $[\text{Fe}(\text{CN})_6]^{3-/4-}$) [50]. Using fixed frequency (usually less than 1 Hz) to measure impedance can lead to even faster analysis, and possible real-time monitoring tools. Contrary to EIS-based biosensors using antibodies with negatively charged $[\text{Fe}(\text{CN})_6]^{3-/4-}$ as redox probe, many aptamer sensor studies observed that the aptamer-algal toxin interaction actually lead to a decrease (rather than the common increase) in the electron transfer resistance [48, 62, 65], of which the mechanism is illustrated in Figure 3.

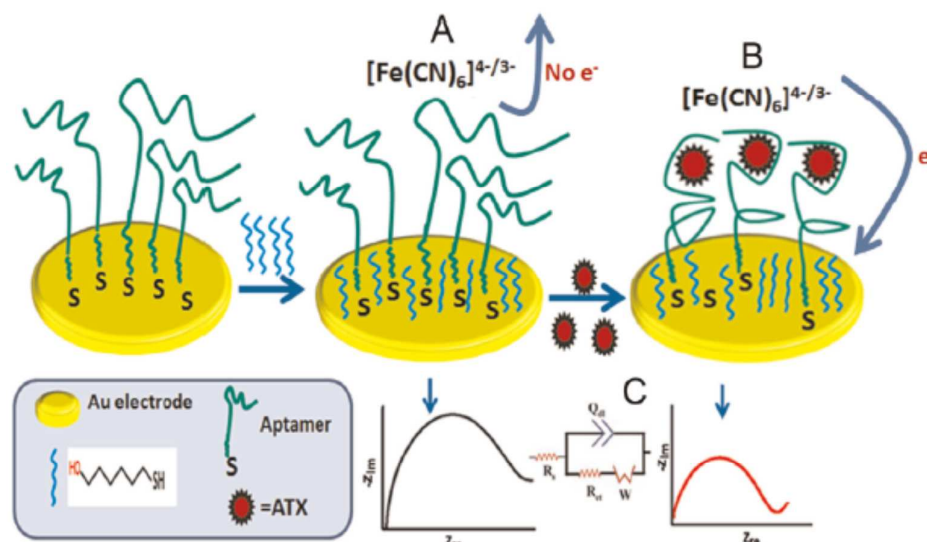


Figure 3 Fabrication of the label-free impedimetric aptasensor of anatoxin-a. Without toxin, the negatively-charged $[\text{Fe}(\text{CN})_6]^{4-/3-}$ redox probe is repelled from the surface and its redox reaction is hindered (A). Upon toxin recognition to the aptasensor, the aptamer induced switching results in compact structure, allowing accessibility of the $[\text{Fe}(\text{CN})_6]^{4-/3-}$ marker to the surface and the resistance to electron transfer is decreased (B). Equivalent circuit $R_s(Q_{dl}[R_{et}W])$ is used to fit the frequency scans along with an impedance spectra (C). Figure adopted with permission from ref 65, Copyright 2015 Elsevier.

There are also a couple of studies reported on less usual electrochemical methods for algal toxin detection. Direct measurement of field-effect transistors (FET) electrical resistance variations upon MC-LR and antibody binding to its surface was carried out using the slopes of I-V curves between +0.1 V and -0.1 V [55]. In another study, potential difference between working and reference electrodes was directly measured as response to STX concentration changes [67]. This potential change was brought about by induced lipid crystallinity when STX bind to its antibody pre-coated on lipid film layer (see Figure 4). This crystal phase alteration can lead to electrostatics interactions [92], dynamic conformational and fluidity changes of the lipid film [93].

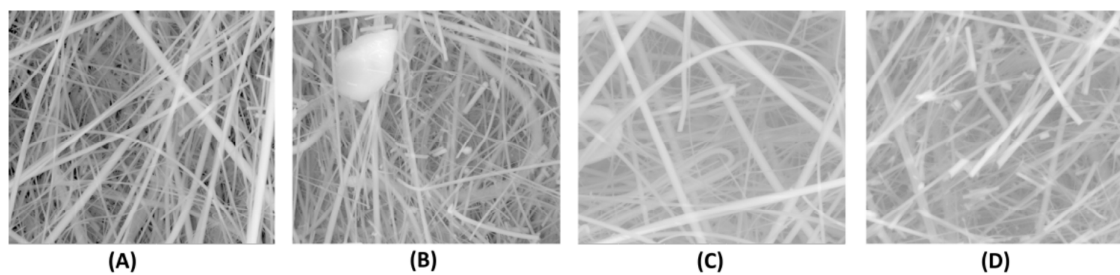
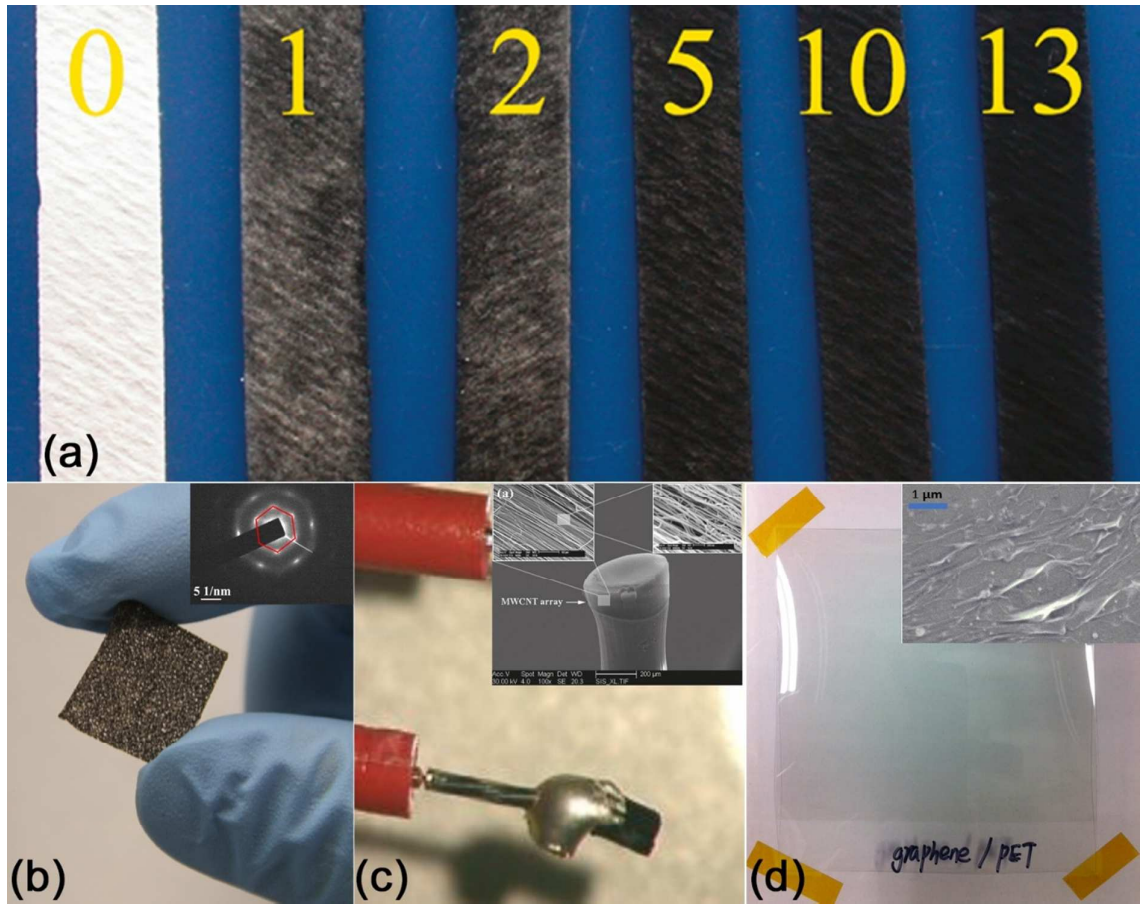


Figure 4 SEM images of (A) polymer without the lipid, (B) polymer with the lipid, (C) polymer with the lipid after the incorporation of Anti-STX, and (D) same as (C) but a drop of toxin is placed on the filter. Magnification is 2000 x (nm). Figure adopted with permission from ref 67, Copyright 2017 Wiley.

Sensor electrodes and signal amplification

Conventional sensor electrodes reported for electrochemical detection of algal toxins include glass carbon electrodes (GCE) [45, 46, 50, 51, 52, 54, 58] and gold [48, 53, 55, 57, 62, 65, 69,] prepared using screen printing techniques. More recently, signal amplification by utilizing various nanomaterials are regarded as an ideal strategy to produce highly sensitive sensor electrodes during the electrochemical sensing process resulting from their unique structural and electronic features. Facile surface modification can be achieved, giving many opportunities for noncovalent and covalent

1 immobilization of small or large biomolecules, which has been widely employed in clinical
2 diagnostics, environmental control and other fields [94].



3
4 **Figure 5** Photographs of various nanomaterials electrodes for electrochemical biosensing of algal
5 toxins in water: (a) SWNT on filtration paper, where the numbers represent SWNT deposition cycles
6 (Reproduced with permission from ref 44, Copyright 2009 American Chemical Society), (b)
7 Graphene foam (GF) (Reproduced with permission from ref 60, Copyright 2017 Elsevier), (c) MWNT
8 (Reproduced with permission from ref 59, Copyright 2013 Wiley), and (d) Graphene film/PET
9 composite (GFC) (Reproduced with permission from ref 61, Copyright 2018 Nature).

10 To facilitate efficient signal amplification, there are several studies using carbon nanotubes (CNT) to
11 fabricate the sensor electrodes. As one-dimensional (1D) materials, CNTs offer better sensitivity and
12 faster response speed toward the change in the surface microenvironment of the transfer channels
13 derived from the adsorption/modification of extraneous molecules. Earliest example of using CNTs as
14 electrodes for MC-LR electrochemical detection was demonstrated by Wang et al. in 2009, in which
15 simple disposable sensor strips were fabricated by filtering single-walled carbon nanotube (SWNT)
16 out on normal filtration paper (see Figure 5a); amperometry measurement by directly oxidizing target
17 toxins (i.e. MC-LR) without using any receptors such as antibodies was carried out [44]. Although
18 decent LOD was obtained, one would expect that it would give rise to serious cross-reactivity issues
19 with other algal toxins present in water. Since then, Han et al. has demonstrated a multi-walled carbon
20 nanotubes (MWNT)-based sensor electrode for MC-LR detection using EIS technique (see Figure 5c)
21 [59]. Tan et al. demonstrated SWNT enabled electrochemical detection of MC-LR by measuring
22 electrical resistance variations [55]. Zhang et al. reported a nitrogen-doped multi-walled carbon
23 nanotube (CNx-MWNT) based electrode, of which doping process was claimed to provide more
24 surface-active sites and better biocompatibility [46]. Gan et al. provided a study on CNT electrode
25 modified with core-shell hierarchical three-dimensional villiform-like nanostructures (CNT@Co
26 silicate) [56]. Hou et al. constructed a MWCNT modified gold sensor electrode for electrochemical

detection of saxitoxin [66]. In addition to CNT, Zhang et al. showcased an electrochemical sensor based on carbon nanofiber (CNF) [51] for MC-LR detection in water. Compared with CNTs, CNFs possess much larger functionalized surface area and more edge sites on the outer wall [95], and thus they are more suitable for immobilization and stability of biomolecules [96].

The unique physical and electrochemical properties (e.g., high electrical conductivity, ease of functionalization, high electrochemically active surface area, and broad range of working potentials in aqueous solutions) of graphene would make it a candidate material for developing novel and fit-for-purpose electrochemical biosensors/immunosensors as alternatives to the time-consuming, expensive, non-portable and often skills-demanding conventional methods of analysis involved in water quality assessment [97, 98]. Many preparation methods have been reported to produce high quality graphene nanoplatelets, nanoflakes or even single graphene sheets, e.g. mechanical exfoliation of graphite and chemical reduction of graphene oxide. So far, there are a few studies reporting on graphene enabled electrochemical chemical sensors for algal toxin detection, including commercially available graphene-modified screen printed carbon electrodes (GSPE) (Dropsens, Inc. 110GPH) [57], in-situ electrodeposition and reduction of graphene oxides [52, 58] and drop-casting of graphene dispersion on electrode surfaces [45, 67]. Zhao et al. even conducted a direct comparison study between chitosan-coupled graphene and CNT biosensor electrodes in a controlled experiment of MC-LR electrochemical detection, where the former achieved 2.3 times higher DPV signal than the latter [58]. However, these graphene preparation methods are usually a labor intensive, low yield, non-scalable process and produce very dispersive forms of graphene. Therefore, special emphasis has been placed to find economic and scalable routes of large-sized monolithic graphene materials fabrication, which are more suitable for commercially viable devices in this direction. Recently, a method using modified chemical vapour deposition (CVD) to grow large-sized monolithic graphene has been developed, such as macroporous foam-like 3D graphene network [99] and graphene/polymer sheets featuring multilayer graphene deposition (i.e. the roll-to-roll method) [100]. In this context, Zhang et al. have been amongst the first to demonstrate MC-LR electrochemical sensors using these two types of graphene-enabled electrodes (see Figure 5b and d) [60, 61]. Coupled with EIS, broad linear detection range of MC-LR was achieved between 100 and 0.005 $\mu\text{g/L}$. In addition, the scalability and the processibility of CVD provides one step further towards developing practical graphene-based biosensors in large scale as the resulting graphene material is free standing, scalable and macroscopic with easily tuneable size rather than previously reported two dimensional nanoflakes or their agglomerates often in fine powder form.

Usage of nano-sized noble metals to modify sensor electrode for electrochemical algal toxin detection have also been attempted to facilitate surface electron transfer due to their high conductivity and large specific surface area, such as gold nanoparticles (AuNP) [47, 50]. When immobilized on the sensor electrodes, AuNPs can firmly bind antigens/antibodies through hydrophobic, electrostatic interactions or covalent bond formation with the help of cross-linkers [101, 102]. Bilibana et al. investigated a nanocomposite sensor fabricated by electro-synthesis of silver nanoparticles (AgNP) on the surface of cobalt(II) salicylaldimine metallodendrimer (SDD-Co(II)) dendrimer for electrochemical detection of MC-LR [51]. Dendrimers belong to a group of well-ordered and symmetrical polymers with a core, self-replicating branching units and peripheral surface group at different positions. Using them as electrode substrates was shown to provide additional functional groups and internal cavities leading to much improved sensitivity [103]. Furthermore, potential synergistic effects were also demonstrated when combined with other nanomaterials, such as graphene [52] or CNT [43]. For example, Li et al. electrodeposited graphene alternatively with AuNPs and 2, 5-Di(2-hienyl)-1-pyrrole-1-(p-benzoic acid) (DPB) for multiple cycles (i.e. 20 times) and obtained the lowest LOD (i.e. 0.037 pg/L) for MC-LR among all the studies [52].

Current challenges and future opportunities

A sensitive, specific, simple, and rapid method for monitoring algal toxins in water could help to prevent human exposure especially in a contamination event. Over the last decade, significant progress has been made towards developing commercial electrochemical biosensor for algal toxins detection, of which great sensitivity and fast response were achieved by the nanomaterials related

signal transduction upon a biorecognition event. According to the 2005 ICH guidelines for analytical validation Q2 (R1), apparent recoveries of 80-115 % are acceptable. In this context, most of electrochemical biosensing studies for algal toxins was reported to demonstrate satisfactory recoveries using real environmental water samples as well as good repeatability and reproducibility. Coupled with some excellent LODs reported, developed electrochemical biosensor in these works has provided one more step towards quick in-situ detection of algal toxins in the contamination point of water source. Current challenges and opportunities regarding the electrochemical biosensor for algal toxins that needed to be addressed before they attain the commercial viability include:

- 1) There are no reported studies on developing electrochemical biosensor for algal demertoxins detection, including lyngbyatoxins, aplysiatoxins and lipopolysaccharides. Some pioneering work in this particular area is much needed.
- 2) Using of conductive polymer as binder or underlay (e.g. polythiophene, polyaniline and polypyrrole) could potentially enhance the signal and sensitivity. Only study carried out was using 2,5-Di(2-hienyl)-1-pyrrole-1- (p-benzoicacid) (DPB) and reported with a much improved LOD for MC-LR [52]. However, they may suffer from possible conductivity loss issue (or aging effect) over long time storage [104]. With fast progress made in this field every year, opportunities may rise to overcome this issue in the near future.
- 3) Most of works reported in electrochemical biosensing of algal toxins are disposable biosensor. On the other hand, regeneration of used sensor electrode can be carried out by using different chaotropic agents [105], which have been demonstrated in some of reported studies, including urea [47] and glycine-HCl [51, 52, 68] solutions. However, their effectiveness and potential side effects on biomolecules immobilization have not been assessed across the studies systematically.
- 4) Response time of electrochemical biosensors for algal toxin detection were not widely reported and discussed in most of studies. In one immunosensor study, an optimal 60 min response time was recommended using HRP enzyme assisted CV detection of MC-LR [56]. In another immunosensors design, a much-reduced response time of 5-20 min was reported using voltage changes as electrochemical signal for saxitoxin detection [67]. This necessitates more feasibility and comparison studies across the different biosensor designs systematically, since less 20 min of response time is the rule-of-thumb requirement for any realistic in-situ monitoring or being commercially meaningful.
- 5) Recently, the use of microfluidics chips (MFCs) have gained in popularity for pathogen or toxin detection. Since MFCs-enabled reaction chambers are usually on the micro- or nano-scale, both the volume and the distance of diffusion inside the microchannels are significantly reduced, and so are the analysis time and reagent consumption. This has proved to be cost effective due to the ease of automation, integration (i.e. sample preparation, amplification, and signal detection), miniaturization, and multiplexing. There are few studies reported on using MFCs to facilitate algal toxin sensing, such as crystal microbalance (QCM) sensor [106] and immunosensor [107]. One can expect that integration of MFCs into electrochemical biosensing protocols of algal toxins would certainly be advantageous based on the same principle.
- 6) Until now, most of electrochemical sensors reported in literature utilized Faradaic process, that is, after incubation in analyte solutions for biorecognition (i.e. antigen/antibody binding), biosensors are then transferred to a secondary solution containing electroactive species (e.g. $\text{Fe}(\text{CN})_6^{3-/4-}$) for electrochemical signal measurement. This changing of solutions make Faradaic process based electrochemical detection impractical for continuous monitoring in-situ. On the other hand, non-Faradaic process could potentially allow continuous in-situ monitoring since no electroactive redox species are required in the analyte solution. In a typical non-Faradaic EIS measurement, phase offset or shift (degree) between the input voltage and output current is commonly used as electrochemical sensing signal. This is an electrode surface area independent signal that directly reflect change in interfacial capacitance of sensor electrode. In other words, any physical change (i.e. a biorecognition event) occurred on the electrode interface will likewise generate a detectable change in phase shift over a range of frequencies [108,109]. So far, non-faradaic EIS technique has been reported to detect different diseases with satisfactory performances, such as diabetes, cancer

and cardiovascular diseases [110, 111, 112, 113]. It could be a valuable tool in developing EIS-based algal toxin biosensor as well. For this type of EIS biosensors, a full coverage of insulating self-assembly monolayer (SAM) on the surface of sensor electrodes is essential and need to be meticulously carried out.

- 7) In the last decade, synthetic receptors produced by various surface-imprinting technologies, such as self-assembly, moulding, and stamping methods, have become a promising area of research in specific biomolecules or bioparticles detection [114]. A typical surface-imprinting process involves two essential steps: firstly, target molecules are imbedded as template into formed SAM substrate and secondly, these molecule templates are extracted from SAM surface to create microcavities, which can offer highly specific target molecule rebinding through intermolecular interactions like hydrogen bonds, dipole–dipole and ionic interactions between the template molecule and functional groups present in SAM. As alternatives to natural receptors, such as antibodies or aptamers, synthetic receptors made from polymers are more chemically and thermally robust, and thus can offer multiple advantages, such as on-line continuous monitoring, easy electrode regeneration, and unlimited shelf-life at room temperature without concerns of receptor deterioration. Coupled with synthetic receptors, amperometry, potentiometry and EIS methods have been shown to detect biomolecules with decent LODs down to ng/mL level, including protein glycoprotein (gp51) [115], carcinoembryonic antigen (CEA) [116], human ferritin and human papillomavirus derived E7 protein [117]. At the moment, the main challenge of applying synthetic receptors in electrochemical detection of algal toxins could be low sensitivity of electrochemical response and increased difficulty of imprinting template molecules extraction, considering exceptionally small size of algal toxin molecules (less than 1k Da) and thickness of imprinted SAM layers. However, obvious advantages of synthetic receptors cannot afford to be overlooked, and it is certainly worthy of more concerted efforts in this direction.

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Vocabulary

Harmful algal blooms (HAB), colonies of algae grow out of control due to an overabundance of nutrients (i.e. nutrient and phosphorous) present in the water. HABs can severely lower oxygen levels, killing marine life, and release algal toxins in natural waters; Electrochemical impedance spectroscopy (EIS), is a frequently used method for monitoring change of electrochemical response upon a bio-recognition event at an electrode surface with high sensitivity; Aptamers is a group of bio-recognition materials for cells, proteins, peptides, and amino acids, which is synthesized from small single-stranded (ss) DNA/RNA molecules. Aptamer-based biosensors offer the best promise for commercialization in terms of the storage stability, durability and quick response time; Self-assembled monolayers (SAM), is formed by the chemisorption of organic molecules onto a substrate from either the vapor or liquid phase followed by a slow and orderly organization into thin monolayer deposit. SAMs has wide applications in electrochemistry and electronics as effective insulators or blockers; Microfluidics chips (MFCs), feature a set of micro-channels etched or moulded into a substrate (glass, silicon or polymer). These features enable MFCs to create biosensors that are quick, efficient, and easy to manipulate as well as portable.

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