

# Harnessing the Potential of Biological Recognition Elements for Water Pollution Monitoring

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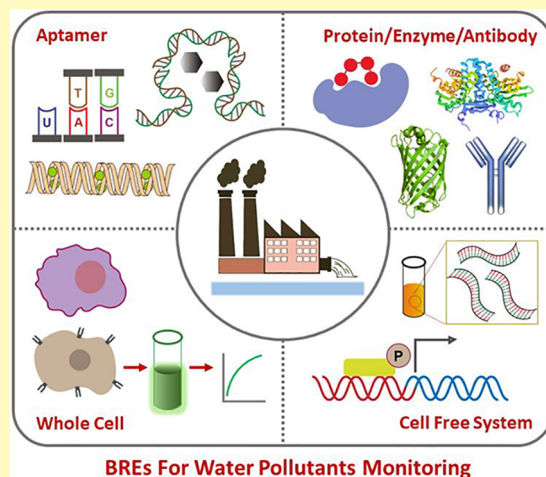
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**ABSTRACT:** Environmental monitoring of pollutants is an imperative first step to remove the genotoxic, embryotoxic, and carcinogenic toxins. Various biological sensing elements such as proteins, aptamers, whole cells, etc., have been used to track down major pollutants, including heavy metals, aromatic pollutants, pathogenic microorganisms, and pesticides in both environmental samples and drinking water, demonstrating their potential in a true sense. The intermixed use of nanomaterials, electronics, and microfluidic systems has further improved the design and enabled robust on-site detection with enhanced sensitivity. Through this perspective, we shed light on the advances in the field and entail recent efforts to optimize these systems for real-time, online sensing and on-site field monitoring.



**KEYWORDS:** biosensor, environmental monitoring, biological recognition elements (BREs), xenobiotics, electrochemical and optical biosensors, nanomaterials, microfluidic systems

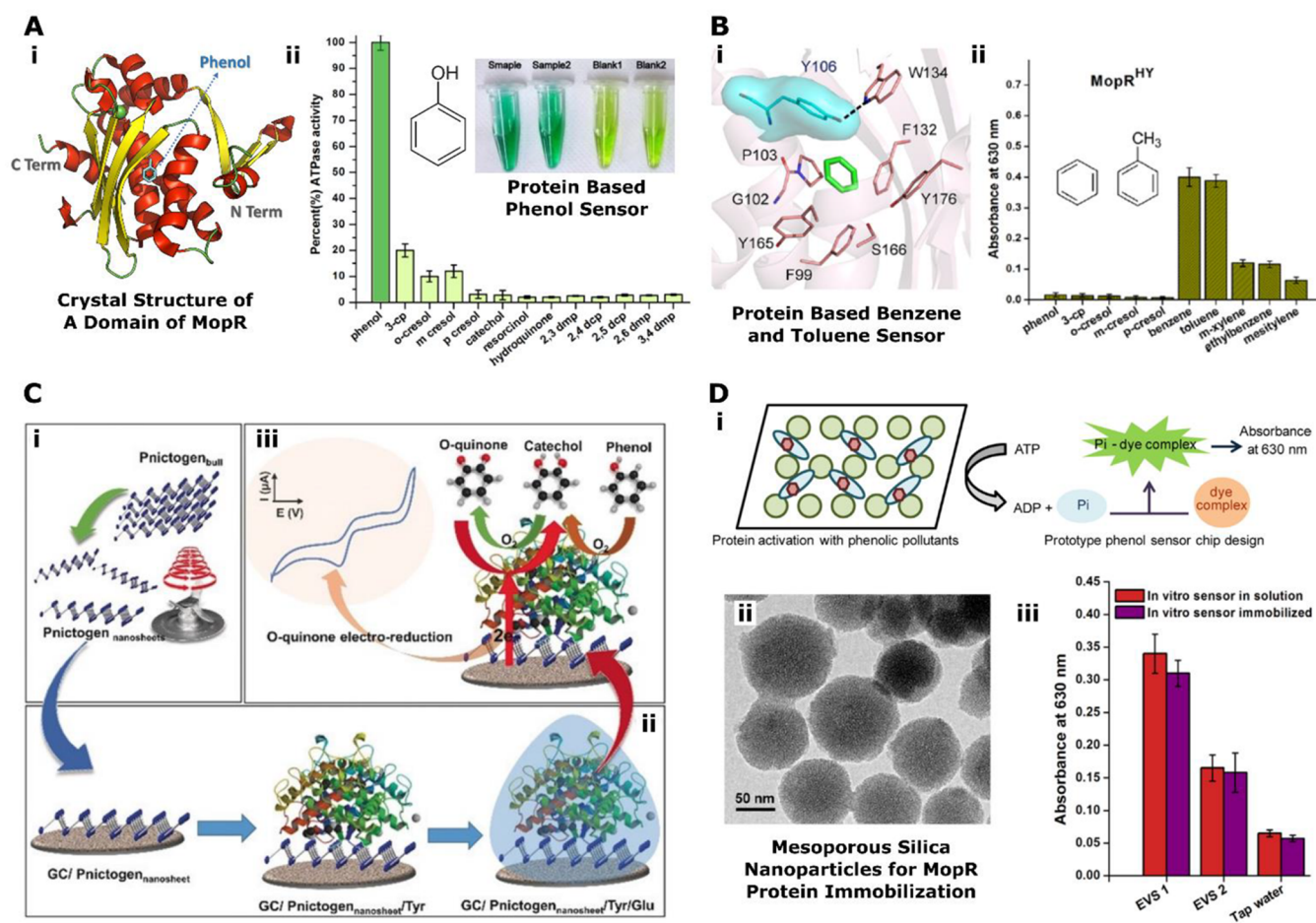
Environmental monitoring related to water contamination has been a global priority due to the crucial juxtaposition between human health and socio-economic development.<sup>1,2</sup> The increase in the momentum of urbanization and industrialization has led to an unregulated release of industrial effluents such as organic aromatic compounds, heavy metals, pesticides, and pharmaceuticals into the surroundings, resulting in a growing demand to combat this uncontrolled pollution. For instance, UNICEF has reported >140 million people drink arsenic-contaminated water every day, which can eventually lead to skin lesions and cancer. To accurately assess the level of xenobiotics in drinking water sources, standardized lab-based techniques such as atomic absorption spectrometry, mass spectrometry (MS), inductively coupled plasma MS, atomic emission spectrometry, and high-performance liquid chromatography have been routinely used.<sup>3</sup> However, the current need is to develop alternative detection methods that do not involve multistep sample preparation and complex analytical procedures such that they can be used for on-site and real-time measurements.<sup>4</sup> A similar scenario exists where, due to lack of active functional groups, detection of carcinogenic aromatics including benzene, toluene, ethylbenzene, and xylene (BTEX) in drinking water sources remains a challenge. Hence, the need for more sensitive, cost-effective, rapid, easy to operate, and

portable technologies to effectively monitor environmental xenobiotics has been an ongoing challenge.

As an alternative to conventional technologies, biosensors that exploit nature's sensory machinery were introduced. The development of the first successful biosensor dates back to 1962 by Clark and Lyons<sup>5</sup> where they had demonstrated methods to quantify glucose in a biological sample. In the last 20 years, the field has transcended significantly to almost every sphere of science.<sup>6</sup> The present biosensor market is valued at \$25.5 billion; it is expected to expand with a CAGR of 7.5% and is projected to reach \$36.7 billion by 2026.<sup>7</sup> Biosensors typically harbor biological recognition elements (BREs) capable of transducing highly sensitive binding signals arising from the interaction between analytes and BREs. Traditionally, biosensors have distinct modules consisting of (1) a sensor module that detects one or more environmental conditions as

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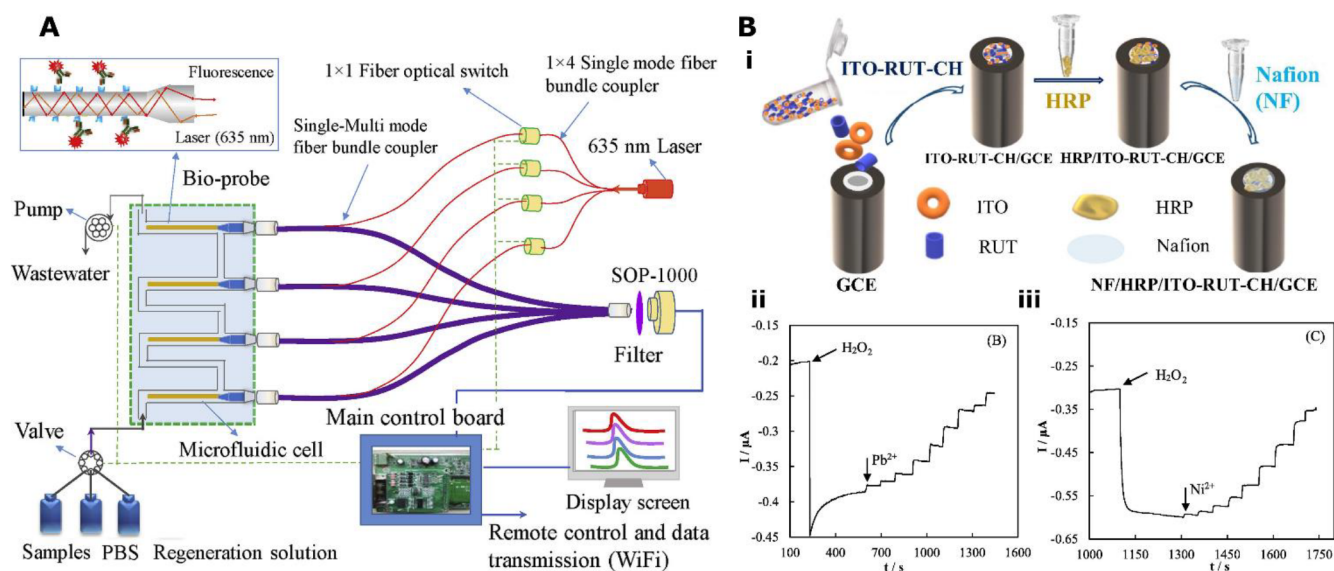


**Figure 1.** Examples of protein-based biosensors for monitoring aromatic xenobiotics. (A)-(i) Crystal structure of phenol biosensing domain of MopR protein. (A)-(ii) Colorimetric ATPase assay response showing a selective response of native MopR toward phenol. Reproduced from ref 22. Copyright 2018 American Chemical Society. (B)-(i) Mutation (H106Y) in native ligand-binding pocket of MopR to accommodate benzene moiety. (B)-(ii) Colorimetric response exhibiting specificity of the MopR<sup>HY</sup> biosensor toward benzene and toluene. Reproduced from Ray et al.<sup>24</sup> and arranged to fit the figure. Copyright 2018 American Chemical Society. (C) Tyrosinase protein immobilization on pnicitogen-based substrate and fabrication of electrochemical phenol biosensor. The schematic is taken from ref 26 with permission. Copyright 2018 John Wiley and Sons. (D) Mesoporous silica nanoparticles as a substrate for protein immobilization and phenol sensing in real environmental samples. (D)-(i) Schematic representation of the biosensor operation. (D)-(ii) Morphology of mesoporous silica nanoparticles used for sensor fabrication. (D)-(iii) Activity of the protein-coated strips toward phenol detection in real environmental samples (EVS1 and EVS2). Reproduced from ref 22. Copyright 2018 American Chemical Society.

inputs, (2) a processing module that performs calculations using the input signals and converts to a perceptible signal, and (3) an output module that produces a detectable and quantifiable signal. The sensing module is the crucial unit responsible for recognizing the pollutant and is the key element that gives unique specificity toward the xenobiotic of concern to be detected. Therefore, a large body of research has been focused on developing effective sensing modules for environmental monitoring. The coupled processing module to the BRE unit is generally either optical<sup>8</sup> or electrochemical.<sup>9</sup> The aim here is to amplify and maximize standard parameters such as selectivity, sensitivity, the limit of detection, and dynamic range. For instance, a typical electrochemical biosensor uses a BRE which can be a protein, DNA, or whole cells, for the analyte detection/binding purpose. Such an interaction further gives rise to a change in sensor surface properties like potential/capacitance arising from any binding or redox reaction, monitored directly through deviation in output current or voltage. Here, electrochemical techniques such as traditional potentiometry and amperometry<sup>10</sup> and

miniature device units such as a modern field-effect transistor<sup>11</sup> and organic electrochemical transistor<sup>12</sup> are linked to the BRE to create an effectual electrochemical sensor output. On the other hand, in optical methods, the output signal is deciphered through optical properties such as absorbance, fluorescence, or luminescence<sup>8</sup> produced due to a reaction or binding at the BRE unit, which is then followed spectrophotometrically for a detectable change as a function of pollutants. Frequently, combinations of different nanomaterials are additionally used to facilitate the immobilization of the BREs, which often impacts the stability of the BRE as well as aids in the amplification of the signal.<sup>13</sup> In essence, irrespective of the operational differences involved with these methods, different BREs (such as aptamers, proteins, whole cells, etc.) are embedded within both types of biosensors as their core detection unit.

In this perspective, we highlight the current status of biosensor-based techniques for environmental water toxicant monitoring. Here, we discuss both standard biosensor technologies such as enzyme and nucleotide-based and, in



**Figure 2.** Subsidiary device and detection modules integrated with BREs for biosensing. (A) Design of automated fluorescence microarray biosensing platform (FMB) for early detection of water contaminants such as bisphenol A, atrazine, APTEs, etc. Reproduced from ref 31 with permission. Copyright 2020 Elsevier. (B) Novel nanocomposite matrix (ITO-RUT-CH) for protein immobilization and heavy metal sensing (Pb<sup>2+</sup>, Ni<sup>2+</sup>, etc.) through electrochemical methods. (B)-(i) Schematic representation of the device fabrication steps. (B)-(ii,iii) Chronoamperometric responses of Pb<sup>2+</sup> and Ni<sup>2+</sup> demonstrating specific current response for the pollutants. Adapted with permission from ref 37. Copyright 2020 Elsevier.

addition, emerging methodologies such as synthetic biology, cell-free systems, etc. Further, their interwoven combinations with polymers and novel nanomaterials, harboring the potential to improve the performance of traditional biosensors, are highlighted. In particular, we have illustrated the role of such BREs and allied modern strategies for detection of aromatic water pollutants such as phenol, catechol, benzene, etc., heavy metal pollutants like arsenic, mercury, and lead, as well as for detection of fluoride. In addition, biosensors for detection of microorganisms as well as pesticides have also been discussed. Challenges and future outlook associated with biosensor research for xenobiotics monitoring are also emphasized. Overall, we elaborate on some of the popular and upcoming BRE technologies that have revolutionized sensing applications in environmental water monitoring.

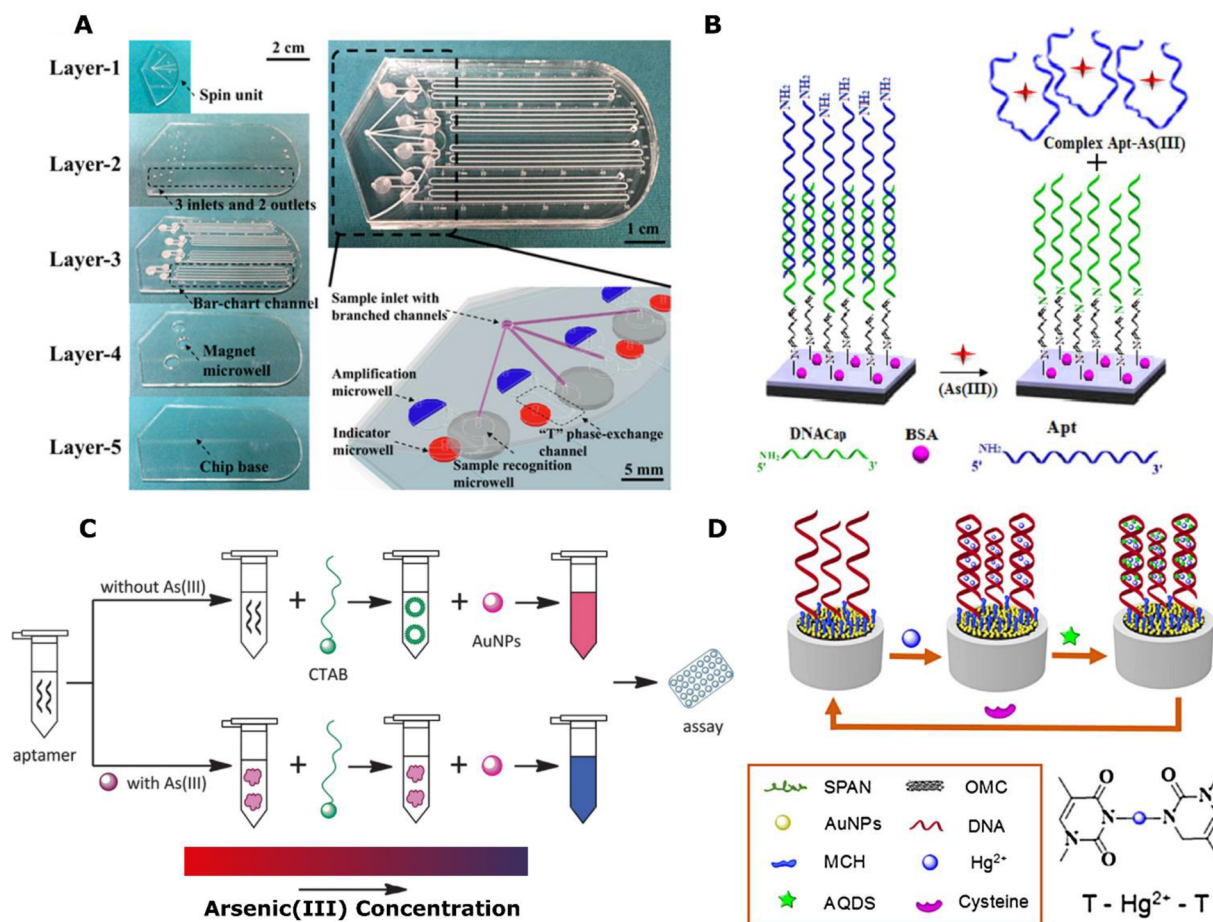
### ■ PROTEIN-BASED XENOBIOTIC SENSORS

In the field of biosensing, protein-based sensors were the first to be developed.<sup>5</sup> For decades, the scientific community has been using these molecular machines in an *in vitro* format combining biological reactions with electrochemical and colorimetric outputs.<sup>14–16</sup> The glucose oxidase-based glucose sensor is a classic example of an effectively applied sensor technology for healthcare monitoring.<sup>17</sup> As an example, in the field of environmental monitoring, aromatic pollutant sensing via tyrosinase<sup>15</sup> and laccase<sup>18</sup> have been used to detect phenolic pollutants, mostly effluents from dye and tannery industries. These enzymes are great generic sensors and work on the principle of detection of the phenolic OH group on a chemical moiety. For example, tyrosinase is a metalloprotein containing binuclear copper which catalyzes the oxidation and hydroxylation of mono and diphenols.<sup>19</sup> Although very successful, the major drawback of tyrosinases and laccases was the lack of specificity and is therefore marred by cross-reactivity and selectivity issues. To tackle this scientific challenge, Ray and co-workers exploited the natural phenol

sensor protein found in *Pseudomonas sp.* and *Acinetobacter sp.*<sup>20</sup> These bacteria can grow in a toxic aromatic environment harboring special genetic cassettes that allow them to sense and degrade xenobiotics such as phenol, 2,3-dimethylphenol, benzene, etc. and use them as a carbon source by converting them into TCA cycle intermediates.<sup>21</sup> However, efforts to make *in vitro* protein sensors for this class of enzymes were largely unsuccessful, as none of these proteins could be isolated in a soluble form. After much manipulation of the original protein, Anand and co-workers have been able to engineer a protein-based biosensor by using a fragment of the natural phenol sensor protein of MopR from *Acinetobacter calcoaceticus* that is not only soluble but stable to 70 °C,<sup>22</sup> making it a robust environmental sensor. Briefly, the sensor design consists of a fragment of MopR that has an N-terminal phenol sensor domain followed by an ATP hydrolysis domain whose activity is regulated via phenol in a concentration-dependent fashion (Figure 1A-ii).<sup>22</sup> A malachite green-based colorimetric sensing protocol was developed that selectively detected phenol with a limit of detection (LOD) of 0.1 μM. The MopR protein was further engineered using the X-ray structure of its phenol sensor domain and selective protein-based sensors for a spectrum of aromatic xenobiotics, for which soluble proteins could not be extracted, were developed<sup>23,24</sup> (Figure 1B). Here, the structure-guided design was vital in multiplexing the MopR sensor platform to detect a plethora of organic BTEX group of pollutants. Subtle mutations in the pocket scaffold were incorporated to exclusively recognize a specific ligand of choice.

Fast response and accurate molecular interactions of different proteins have resulted in many modern, sophisticated, real-time biosensors, but one persisting lacuna is the marginal thermostability of proteins that leads to their easy denaturation, limiting their application for environmental monitoring. To circumvent this issue, the design of suitable anchoring substrates or immobilization platforms responsible for the





**Figure 3.** Aptamer-based biosensors for tracking of water pollutants. (A) Microfluidics device for rapid detection of pathogenic microorganisms employing aptamer modified magnetic beads. Reproduced from ref 43. Copyright 2018 American Chemical Society. (B) Chitosan-Nafion-based platform for the immobilization of arsenic binding aptamers for the detection of As(III). Adapted with permission from ref 46. Copyright 2019 Elsevier. (C) Colorimetric biosensor for As(III) detection using surfactant-based aggregation of gold nanoparticles. Reproduced with permission from ref 54. Copyright 2020 Elsevier. (D) Reusable electrochemical biosensor based on the conformational change of Hg<sup>2+</sup> binding thymine-rich ssDNA (aptamer). Reproduced from ref 52. Copyright 2017 Elsevier.

protection and activity maintenance of enzymes has been undertaken. For instance, to enhance the stability and durability of laccase electrochemical phenol sensors, these enzymes have been immobilized on graphite electrodes using glutaraldehyde-based covalent linkage.<sup>14</sup> Similarly, in an amperometric tyrosinase sensor, the enzyme was immobilized on a sol-gel silicate/Nafion composite film coated glassy carbon electrode with the capability of detecting catechol concentration of 0.35 mM.<sup>25</sup> Subsequently, using the ZnO nanoparticle matrix, which provides a favorable microenvironment in terms of isoelectric point retaining the activity, a sensitive cyclic voltametric (CV) based phenol biosensor with an LOD of 50 nM was designed for the same protein.<sup>15</sup> More recently, Mayorga-Martinez et al.<sup>26</sup> have demonstrated that pinctogen can also be used as novel materials for enzyme immobilization. They used a pinctogen nanosheet on a glassy carbon electrode as a matrix for the tyrosinase enzyme (Figure 1C), and via chronoamperometry, the detection limit of 500 nM was achieved.

Using silica nanoparticles<sup>27,28</sup> (such as KCC and mesoporous silica) for protein adsorption has also been proven as a successful immobilization approach. The hydroxyl groups on the silica surface, porous nature for adsorption, and overall biocompatibility has made them essential not only in the field

of drug delivery but also for biosensor fabrication.<sup>29</sup> The previously mentioned MopR sensor has also been successfully immobilized on such a mesoporous silica support, and this approach has been demonstrated to work effectively on wastewater samples from polluted sites without loss of sensitivity or selectivity (Figure 1D).<sup>22</sup> Another aspect of immobilization is to enable the portability of the protein sensor unit such that miniaturization can be achieved. Zhao et al.<sup>30</sup> have shown that it is possible to monitor *p*-cresol in water samples in near real-time by employing a screen-printed carbon electrode (SPCE) which is surface modified with laccase immobilized carbon nanotubes. Through a CV based test, the sensor reached a LOD of 0.46 μM for *p*-cresol detection in wastewater. An automated fluorescence microarray biosensing platform (FMB) is another type of modern portable sensor unit that can be used for early warning of pollutants. A recent article by Long et al.<sup>31</sup> has shown that an optical fiber-based design can multiplex FMBs, and contaminants such as atrazine and bisphenol can be detected rapidly on-site (Figure 2A).

Besides aromatic xenobiotics, protein-based heavy metal sensors are also prevalent in the literature. A plethora of sensors for detecting arsenic, lead, cadmium, etc., have been reported.<sup>32,33</sup> The trivalent arsenicals are thiophilic metalloids



and hence, in several cases, can be detected using proteins by exploiting their strong coordination with cysteines. For instance, As(III) detection is enabled via an amperometric biosensor designed to study the inhibition of immobilized acetylcholinesterase on SPCEs.<sup>34</sup> In this case, the thiocholine oxidation current decreases proportionally to the concentration of As(III). The limit of detection achieved for arsenic was  $1.1 \times 10^{-8}$  M. Apart from this, although several other enzymes such as arsenite oxidase<sup>35</sup> and acid phosphatase<sup>36</sup> have also been used to develop arsenic biosensors, they either suffer from low specificity and stability or have a detection limit much higher than the WHO/EPA limit. More recently, better sensitivity has been achieved by intelligently designed indium tin oxide nanoparticles for increased electron transfer and Ru(III) hexamine trichloride for mediating electrocatalytic activity, creating an immobilization matrix for HRP enzyme<sup>37</sup> (Figure 2B). Here, via this design, the author has been able to push the detection limit of heavy metals such as lead, nickel, and cadmium toward an ultralow LOD of 8, 3, and 1 nM, respectively.

### ■ USE OF APTAMERS AS BRES FOR ENVIRONMENTAL MONITORING

One of the most widely utilized BRES for environmental monitoring is an aptamer.<sup>38</sup> Aptamers are unique single-stranded DNA or RNA molecules (ssDNA or ssRNA) that possess the characteristic of high affinity for a specific analyte and enable recognition via structural scaffold formation.<sup>39</sup> The SELEX technology,<sup>40</sup> which has perfected the calibrated design of DNA-based biorecognition elements targeted toward different analytes, has accelerated the development in this field.<sup>41</sup> SELEX has made it possible to design aptamers that can even specifically target different whole-cell markers, and using this approach, numerous microorganisms can be monitored directly.<sup>42</sup> For example, bacterial species like *Salmonella enterica*, *Escherichia coli*, and *Listeria monocytogenes* can be harmful and are regarded as significant environmental threats. Promising research by Wei X. et al.<sup>43</sup> showed that instrument-free, direct point-of-care testing of environmental pollutants of the aforementioned microorganisms is possible by using distinct aptamers targeted for each species (Figure 3A).

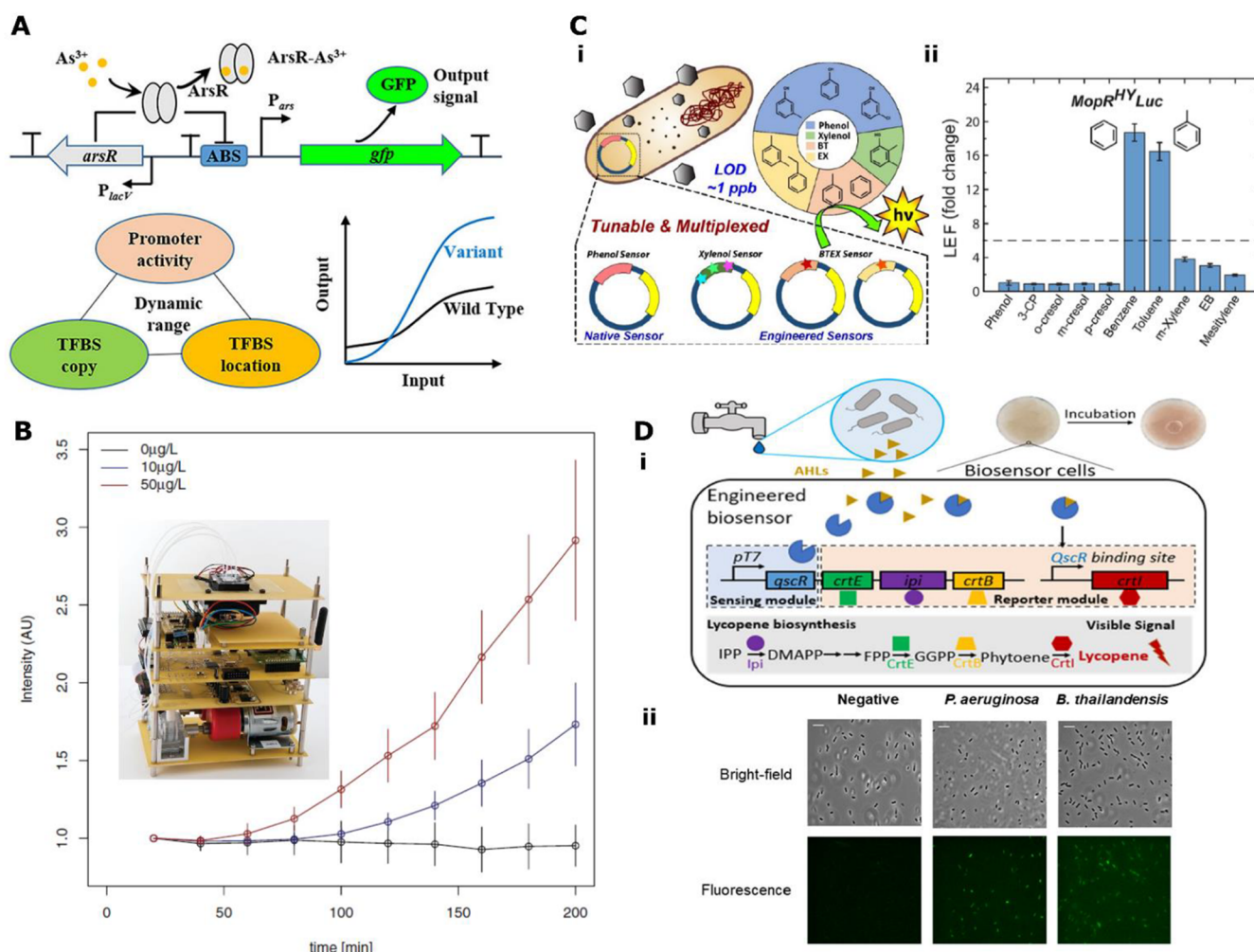
Another format in which aptamers have been widely utilized is electrochemical-based aptasensor technology. Here, electrochemical techniques like electrochemical impedance spectroscopy (EIS), differential pulse voltammetry, and CV are utilized for signal enhancement.<sup>44</sup> For instance, the aptamer-based sensor with signal amplification mediated by hybridization chain reaction was employed by Haidong Gu et al.<sup>45</sup> for detection of As(III) with a reported LOD of 270 pM is noteworthy. Here, the working protocol of the biosensor is based on alteration of the native/control device surface properties by As(III). In the presence of As(III), a specific aptamer binds to a ssDNA sequence present on the gold electrode, inducing a structural change in the aptamer scaffold via the formation of a hairpin configuration. The effective difference of the charge transfer resistance ( $R_{ct}$ ) in the absence and presence of As(III) is monitored via EIS. Further, the amalgamation of different nanomaterials helps in the diversification of aptamer sensing systems providing high specificity and sensitivity. For instance, as shown by Baghbaderani and Noorbakhsh<sup>46</sup> the use of polymer doped surfaces like chitosan-nafion (Chit-Naf) can potentially increase the conductivity and signal amplification of the

arsenic sensor designed by them (Figure 3B). The authors concluded that Chit-Naf functionalization on the glassy carbon electrode (GCE) increases the electron transfer kinetics. When the GCE-Chit-Naf-aptamer biosensor is subjected to EIS it can detect As(III) as low as 74 pM.

In the optical sensing regime of aptamer-based heavy metal sensing, gold nanoparticles (GNPs) are widely used. In general, GNPs are of particular interest to sensor researchers due to their interesting optical and distance-dependent properties, exhibiting a strong absorption band in the visible region.<sup>47</sup> The ease of aptamer immobilization on the GNPs, mainly through thiol moiety ( $-SH$ ), has an accelerating contribution to aptamer-based biosensor development. Yuan-gen Wu et al.<sup>48</sup> used Ars-3 aptamer and GNPs to detect As(III) through cationic surfactant chemistry. In this system with the enhancement of As(III), the sensor exhibits a visible blue color due to GNP aggregation (Figure 3C). The resultant biosensor exhibits high sensitivity, delivering a LOD of  $\sim 8$  nM. Bisphenol A (BPA), a known environmental contaminant<sup>49</sup> used in the industry associated with the production of polysulfone resin, polycarbonate, etc., and a well-studied endocrine disruptor, has also been detected using aptamer GNP aggregation based colorimetric assay. Using this methodology, Ragavan et al.<sup>50</sup> developed an aptamer-based nanobio probe where quenching of fluorescently labeled aptamers upon BPA binding was exploited to detect BPA down to 0.1 nM. This design was also demonstrated to perform equally efficiently in human urine samples strengthening the utility of the approach.

Another heavy metal that has benefited from aptamer-based technology is mercury. The property of  $Hg^{2+}$  to specifically bind to thymine bases and form mismatched T- $Hg^{2+}$ -T pairs, which are more stable than the Watson-Crick (A-T) pairing,<sup>51</sup> has been exploited for the design of mercury biosensors. Jingjing Zhua and co-workers<sup>52</sup> demonstrated that thymine-rich DNA when immobilized on GCE and suitably modified with self-doped polyanilines and ordered mesoporous carbon was an optimal setup for detection of mercury. Moreover, a DNA-based  $Hg^{2+}$  biosensor, when complemented with GNPs in an electrochemical configuration, can reach an ultrasensitive limit of  $\sim 0.6$  fM (Figure 3D). The added advantage of this design was its remarkable reusability which can be evoked by cysteine treatment. In parallel, other BRE scaffolds consisting of DNA dual cycle based organic-inorganic hybrid nanoflowers<sup>48</sup> and other aptamer-based force microscopy<sup>53</sup> methods have also been employed to track  $Hg^{2+}$ . These techniques have been proven to be efficient and yielded detection as low as 0.19 fM and 10 pM, respectively.

Overall, it can be concluded that aptamer-based biosensors exhibit a great sensitivity profile; however, some of the drawbacks of this technology are heterogeneity in performance implying a dire need for their detailed characterization before setting a standard benchmark. Other issues related to stability and portability are immediate anticipated improvements in the field. DNA, although being one of the robust biomolecules, may interact with other species in complex polluted samples consisting of other nontarget elements/compounds, which is the primary concern considering real environmental samples.<sup>6</sup>



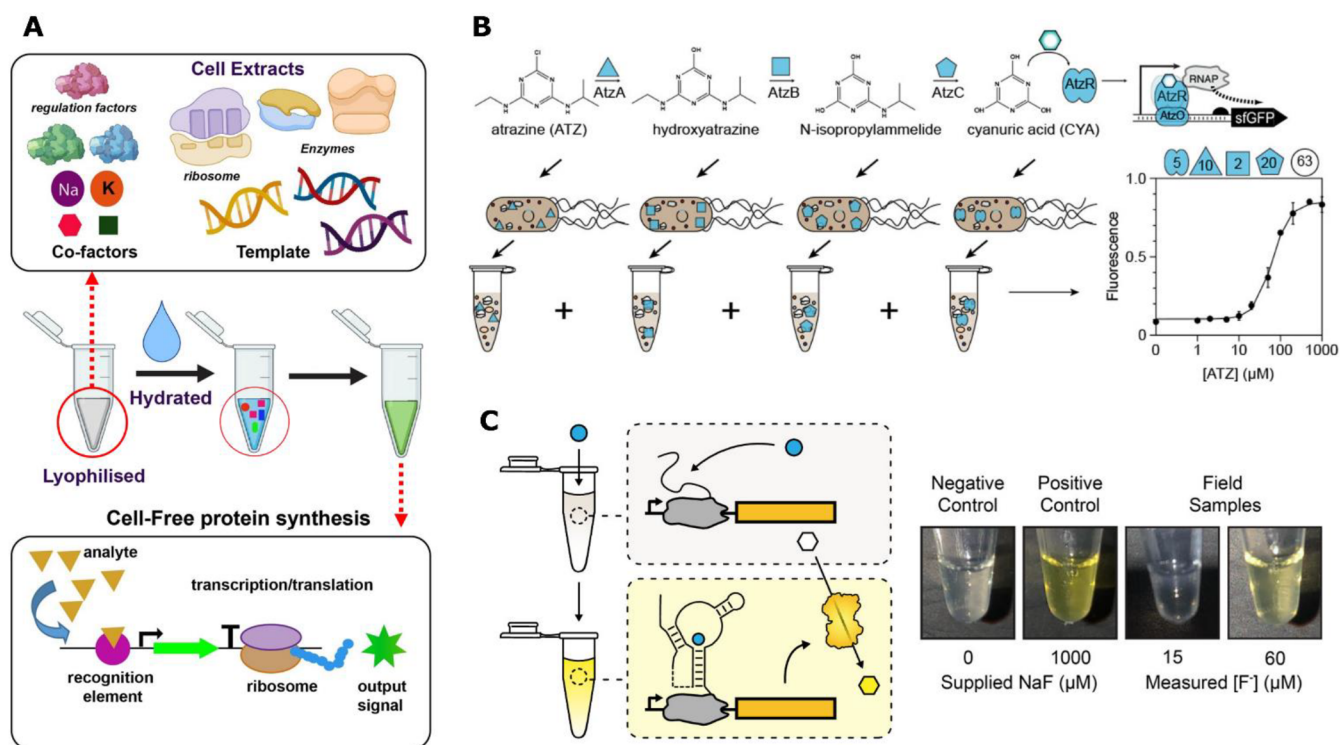
**Figure 4.** Various applications of whole-cell biosensors. (A) Schematic design of a whole-cell As(III) biosensor by tuning the transcription factor and RNA polymerase binding sites for enhancing the output signal-to-noise ratio. Reproduced from ref 57. Copyright 2019 American Chemical Society. (B) Portable device-based prototype for the detection of As(III) in drinking water. Adapted with permission from ref 58. Copyright 2014 AIP Publishing. (C)-(i) Schematic of whole-cell biosensor for monitoring aromatic xenobiotics (benzene, toluene) using luciferase as a reporter gene. (C)-(ii) Biosensor response showcasing the selective binding of benzene and toluene. Reproduced from ref 59. Copyright 2021 American Chemical Society. (D) Point-of-care detection of waterborne pathogens (*P. aeruginosa*, *B. thailandensis*) through whole-cell biosensors using quorum sensing as the signal. Reproduced from ref 60. Copyright 2021 American Chemical Society.

## ■ SYNTHETIC BIOLOGY TUNED WHOLE CELL-BASED BIOSENSORS: A SENSITIVE AND ROBUST APPROACH

The inherent property of the microorganisms to grow naturally, their ability to directly interact and take up pollutants, has given the whole-cell biosensor (WCB) an edge over the others. One of the main advantages of whole-cell systems is that an engineered genetic circuitry is introduced into the cells via synthetic biology approaches that use specific promoters and transcription elements that are already tuned by nature to respond to a particular pollutant.<sup>55</sup> Thus, the starting sensor unit has already been perfected by evolutionary rigor, making these systems highly sensitive, selective, and specially tuned to detect pollutants within drinking water limits. Once the design is optimized, these biosensors can be quickly produced in large quantities with limited expense making them one of the most affordable sensors. Moreover, WCBs have been very effective, as they can detect bioavailable compounds directly from their environment without complex sample preparation.<sup>56</sup> In a typical sensor based on synthetic biology

principles, there is a control unit such as a transcription factor which switches on/off expression, that can be activated in a particular condition of interest which in turn then initiates translation of downstream reporter genes such as green fluorescent protein (GFP), luciferase, or other colorimetric/fluorometric detection biological units. For environmental monitoring of heavy metals such as arsenic and mercury, this technique superseded traditional high-end chemical analysis methods.

For arsenic, early WCBs exploited the arsenic resistance operon *arsRBC* of *E. coli* K12 genome as a sensing element.<sup>61</sup> The natural system was marred by limited sensitivity and dynamic range. Hence, Bang-Ce Ye's group manipulated the promoter sequence and incorporated several arsenic binding sites (ABS) in tandem to improve selectivity and reduce background.<sup>57</sup> This sensitivity related to the translation efficiency of the system was enhanced by tuning the ribosome binding sequence (RBS) (Figure 4A). Incorporation of synthetic transcriptional amplifier circuits between the sensor and the reporter module is now commonly achieved in



**Figure 5.** Potential application of different cell-free biosensors. (A) Schematic of the working principle of a cell-free biosensor. The biosensor mixture can be hydrated when required, and in the presence of a specific pollutant, it shows a detectable response. (B) Cell-free atrazine biosensor using *in vitro* atrazine-to-cyanuric acid metabolic pathway reconstitution. Image is reproduced from ref 65. Copyright 2020 American Chemical Society. (C) Riboswitch-based cell-free biosensor for the on-site detection of fluoride in environmental samples. The reporter is an enzyme that converts the colorless substrate into a yellow-coproduct in a dose-dependent manner. Reproduced from ref 66. Copyright 2020 American Chemical Society.

engineered sensor modules where the number of transcription factor binding sites and promoter activity are optimized to effectively improve the signal-to-noise value of a biosensor.<sup>62</sup> By tuning intracellular sensory receptor densities, engineering a multilayered transcriptional amplifier that could sequentially boost the output expression levels, the detection limit for both arsenic and mercury was improved by 5000- and 750-fold, respectively, as shown by Wan et al.<sup>63</sup> The real challenge toward making a biosensor module portable is to maintain the structural and functional integrity unaltered. The authors were able to achieve this feat, and it was possible to design a portable version, where the imaging and detection were made possible through a smartphone camera reaching a limit of detection  $\sim 13.5$  nM for arsenic detection. Earlier, Gudlavalleti et al.<sup>64</sup> were also able to devise an EGFP based WCB for a portable fluorometric device that can effectively monitor arsenic in groundwater within a dynamic range of 0.06–1.35  $\mu\text{M}$  using a compact printed circuit board connected to the cuvette chamber for direct detection purposes. A similar low-cost flow-based microfluidic chip containing whole *E. coli* cells entrapped in agar as a biorecognition module was fabricated by Truffer et al.,<sup>58</sup> which can operate autonomously, commanding the measurements and can also transmit the As(III) toxicity data over GSM networks (Figure 4B).

More recently, as a next step forward, Anand and co-workers have combined structural biology approaches with synthetic biology to design a series of tunable biosensors for targeting a plethora of aromatic xenobiotics such as the benzene, toluene, phenol, xlenols, etc., category of compounds.<sup>59</sup> They used the natural genetic circuitry present in *A. calcoaceticus* that harbors

the phenol sensing ability to elicit a response. Such sensors have also been designed earlier for monitoring benzene and 2,3-dimethylphenol.<sup>22,24</sup> However, Anand and co-workers introduced a novelty in design by combining structure guide design with synthetic biology to tweak the sensor specificity. In this regard, this approach can be extended to sensors for xenobiotics for which sensing systems are not available in nature or not yet discovered.<sup>59</sup> Using this approach, the phenol sensor was converted into a plethora of selective sensors such as xlenols, benzene, and toluene, and even a highly selective sensor for ethylbenzene for which no known genetic sensing system exists in nature was constructed (Figure 4C). The strength of the methodology lies in the fact that the engineered sensors exhibit a LOD as low as  $\sim 1$  ppb without any compromise in either selectivity or sensitivity. Direct detection of these aromatic xenobiotics that are highly toxic could be achieved without any preconcentration steps, within drinking water limits.

Almost 10% of the diseases occurring worldwide are attributed to unsafe potable water, and several of these diseases are a consequence of dangerous pathogens that contaminate these sources. Therefore, global surveillance of waterborne pathogens that cause deadly infections via precise monitoring of microbial contamination is becoming critical. By exploiting quorum sensing receptors commonly found in bacteria such as *ahl*, *agr*, *cqs*-lux, and several others, pathogenic detection using WCBs is a common strategy to develop sensors for detecting pathogens. For instance, Wu et al. created a point-of-care WCB capable of detecting *Pseudomonas aeruginosa* and *Burkholderia pseudomallei*.<sup>60</sup> The biosensor developed



by them is based on the QsCR quorum sensing system found in *P. aeruginosa* which detects the quorum sensing ligand N-acylhomoserine lactone (AHL). The final output is a colorimetric lycopene-based red color readout that measures AHL levels (Figure 4D).

Thus, WCBs discussed here have great potential for environmental pollution monitoring. The next level in the field of WCBs has now been achieved, where researchers have now combined WCBs with multiplexed microfluidics-based devices,<sup>67</sup> which can effectively reduce the delay in signal processing and help in simultaneous near real-time pollutant monitoring. Since these devices are easy to operate, highly sensitive, and selective, and can be made into cheap portable devices, the technology is gaining popularity. This is the future where WCBs sensor technology is heading.

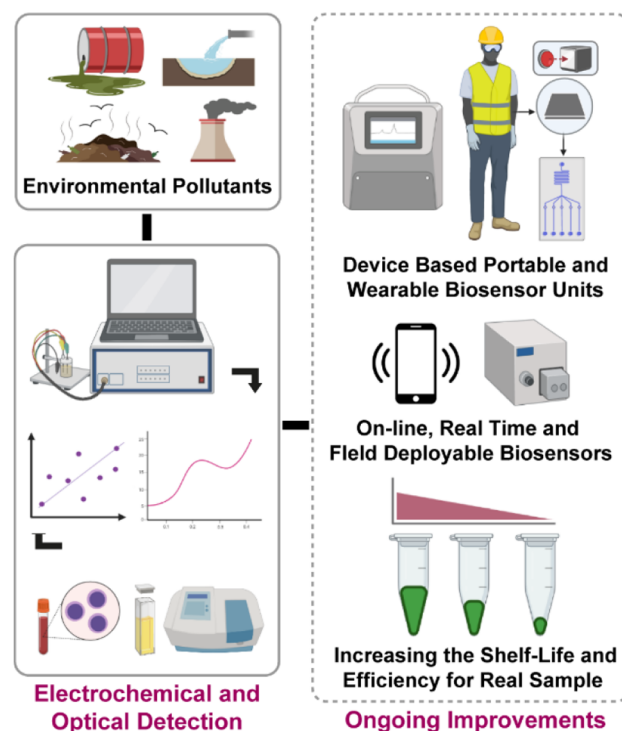
### CELL-FREE SYSTEMS AS BIOSENSORS

One of the major drawbacks and concerns associated with WCBs is that they are living systems, and since several of these systems undergo extensive genetic manipulations, there is always an element of unknown threat associated with the escape of these living moieties into the environment. In recent years, synthetic biology has leaped forward, and to circumvent the above problem, the next generation of sensors has evolved where cell-free biosensor units can partially replace WCBs.<sup>68,69</sup> Here, synthetic biology is carried out in a test tube, eliminating biohazard-related issues. Concerns about biocontamination are reduced, mutation and growth-related restrain dissipates, and membrane transport limitation issues are also not applicable here. The impressive progress in the field has empowered scientists to program and manipulate genetic circuits desirably resulting in a broad class of highly sensitive biosensors.<sup>70</sup> Like WCBs, the cell-free sensor is premised on a genetic transcription circuit wherein the transcription product is a reporter element and the allosteric transcription factors (aTFs) tightly regulate the transcription event itself (schematically shown in Figure 5A). However, isolated transcription or translational machinery is added in an *in vitro* environment with stringent molecular checkpoints, feedback control, and inductive mechanisms to facilitate the WCB environment within a test tube. An *in vitro* tunable modular configuration can be intelligently designed by exploiting molecular properties such as a standard RNA transcript as output, activation/inactivation of transcription via aTFs using sensor analytes, or regulating the translation by toehold switch, etc. A few examples where cell-free sensors have created a strong foothold in the field of environmental pollution monitoring are toward accessing herbicides like atrazine;<sup>65</sup> heavy metals like mercury,<sup>71</sup> lead, and copper<sup>72</sup> and nonmetal toxicants such as fluoride; even antibiotics including tetracycline and erythromycin.<sup>72</sup> Furthermore, it has been demonstrated that it is also possible to translate the system to a portable environmental sensor by freeze-drying the entire biosensor mixture required for efficient cell-free detection and later hydrating it for real-time, on-site detection in water samples. For instance, the atrazine<sup>65</sup> and fluoride<sup>66</sup> cell-free detection sensors developed by Julius B. Lucks and co-workers use the pollutant-controlled expression of sGFP and fluoride responsive riboswitch respectively as the detection method (Figure 5B,C). As proof of principle, a 3D-printed LED-based prototype device has been developed by Jung et al. to demonstrate utility for on-field applications.<sup>72</sup> Very recently, Nguyen et al.<sup>73</sup> pushed the boundary of cell-free sensor

research even further by exhibiting their cutting-edge application toward fabricating portable, sensitive, wearable biosensors which can operate in real-time and are capable of tracking several healthcare markers. Though the field of cell-free biosensors is in its nascent stage, owing to their re-engineering capabilities on the genetic level, it possesses immense potential in the future. A few shortcomings which need innovative solutions and can be nontrivial rate-limiting toward further development are the cumbersome process of initial protein preparation and optimization, reduction of activity upon storage, the *in situ* detection specificity, sensitivity, and multiplexing capabilities. Attention is already being given to such issues by several research groups and enhancing performance standards of future cell-free sensors is underway.

### CHALLENGES, OPPORTUNITIES, AND FUTURE OUTLOOK

Water is the cornerstone of sustenance, and the global biosensor market for water pollution, testing, analysis, and instrumentation is valued at almost \$25.5 billion in 2021.<sup>7</sup> Considering the vast investment in the endeavor, to upgrade environmental monitoring efforts, modern biosensors have been introduced. The development of effective biosensors has a myriad of aspects involved, such as the selection of biological elements, correction of transduced signal and amplification, optimization of supporting materials such as immobilization platforms, and finally, the design of fast and sensitive detection strategies. Therein lie distinct challenges and improvement opportunities involved with each of the steps (Figure 6). It has to be realized that while the standard techniques are extremely



**Figure 6.** Future outlook associated with the field of environmental monitoring. As the forthcoming development, focus is being given to fabrication of portable devices, enhancing the shelf life of the biosensor scaffold, and real-time field implementation.

precise and provide consistent output, accurate detection around the permissible limit can be sufficient for the majority of the pollutants in a real-world context. Instead, in our perspective, the community should put more effort toward optimizing the shelf-life, selectivity, and portability of a biosensor for diverse environmental conditions.

The first biosensor module discussed here was *in vitro* protein-based sensors. The major drawback of these protein-based biosensors which came to light is protein stability and the lack of an optimal target protein for every plausible analyte. For this purpose, directed evolution<sup>74</sup> is an emerging technique, as it enables alteration of binding specificity, amplifying the binding of the already known interacting pollutants, enhancing the selectivity, etc.<sup>75</sup> The technique is equipped with powerful high-throughput screening strategies where through random mutations, the search for the appropriate scaffold can be undertaken.<sup>74</sup> Different computational biology and bioinformatics-based tools (such as Rosetta,<sup>76</sup> I-TASSER<sup>77</sup>) also hold the potential to guide and improve the design of different biosensors.

In parallel, tools like artificial intelligence, machine learning (ML) can be the next significant improvement in revolutionizing sensing technologies for effective environmental monitoring. These fields harness the immense potential to deconvolute multidimensional information, which can be a requirement for processing and analyzing bulk amount biosensor readout data sets. The ascending importance of ML-based recommendation and analysis tools is already quite visible in chemometrics, healthcare-related biosensors, nanobiosensors, as well as synthetic biology.<sup>78</sup> As a stepping stone in environmental monitoring, Graham et al.<sup>79</sup> have already employed a deep neural network to analyze a large amount of gene expression data and trace levels of heavy metals in urban water and mine spill samples. Apart from this approach, single-molecule biosensors are also emerging as the next-generation sensors to detect low levels of pollutants. Currently, this is primarily restricted to drug discovery and medicine, as single-molecule techniques provide superior detection limits.<sup>80,81</sup> However, it can be envisioned soon that this ultrasensitive detection can be exploited to analyze environmental xenobiotics where accurate detection to very low levels is a prerequisite.

Apart from future possibilities for BREs there is also an immense need to develop methodologies to tackle both detection and remediation of modern pollutants such as microplastics (MPs)<sup>82</sup> and e-waste.<sup>83</sup> Both these synthetic products pose a substantial threat, as they are reported in all major aquatic habitats. In recent times, there has been some progress in plastic bioremediation by using a variety of microorganisms such as different strains of *Pseudomonas sp.* and *Bacillus sp.*,<sup>84</sup> but there is a long road ahead. The challenges associated are nontrivial due to the heterogeneous size and composition of different types of microplastics.<sup>85</sup> Similarly, advances in technology have led to astounding accumulation of e-waste/semiconductor waste. Here, a related problem is leaching of heavy metals such as mercury, lithium, lead, and barium that leads to additional toxicity as they accumulate in soil, open water, and even groundwater.<sup>83</sup> Therefore, establishing future technologies addressing both these pollutants will really be helpful to society.

It is an uphill task to translate biosensors for commercialization due to the immense trial and error involved in real-sample performance optimization and quality control. Another hardship for the commercialization of biosensors is maintain-

ing their consistent reproducibility due to the interdisciplinary approach of fabrication frequently involving an assembly of BREs, nanomaterials, polymers, etc. The present biosensor research is still at its nascent stage. However, there is hope as many biosensor prototypes are already being made portable and upgraded for real-time field usage.<sup>86,87</sup> In recent years, several cutting-edge techniques, including printing technologies (involving deposition of dielectric, semiconductor materials), smart supports in combination with nanomaterials (such as graphene, carbon nanotubes, etc.), have contributed toward remarkable progress in making futuristic electrochemical environmental biosensors.<sup>88</sup> In conclusion, as a consequence of constant efforts to address the lacuna in the field, efforts to focus on increasing the robustness of the biosensor design and miniaturizing the sensor module have led to many environmental biosensors being translated to a portable, on-site scale, delivering a flagship-level performance.<sup>13,87</sup>

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### Author Contributions

The manuscript was written through the equal contributions of all authors. All authors have given approval to the final version of the manuscript.

### Notes

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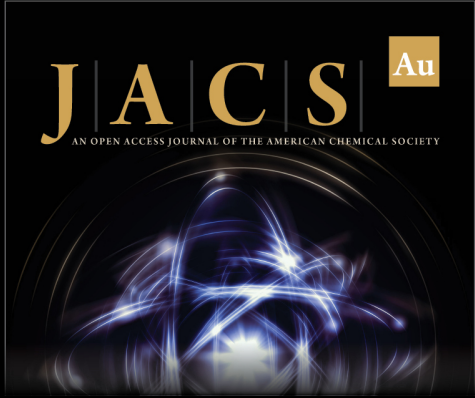
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