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Review Article

Synthetic biology techniques to tackle heavy metal pollution and poisoning

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ABSTRACT

The requirement for natural resources and energy increases continually with the increase in population. An inevitable result of this is soil, water, and air pollution with diverse pollutants, including heavy metals. Synthetic Biology involves using modular, interchangeable biological parts, devices in standard chassis or whole organisms to achieve a programmed result that can be quantified and optimized till it meets the required efficiency. This makes synthetic biology techniques very popular to tackle pressing global issues such as heavy metal poisoning. This review aimed to highlight various advancements as well as benefits, risks, and problems in synthetic biology techniques for detection, bioaccumulation, and biosorption of various heavy metals using engineered organisms. We found that while such an approach is cost-effective, accessible, and efficient, there are several inherent technological and ethical issues including but not limited to metabolic burden and consequences of use of genetically modified organisms respectively. Overcoming these hurdles will probably take time and innumerable conversations, and should be done through education and a culture of responsible research, rather than enforcing restrictions on the development of synthetic biology.

1. Introduction

Heavy metals are metals, including lead, arsenic, mercury, cadmium, iron, etc., that have high atomic numbers, densities, and weights. They enter water bodies, soil, and air through a variety of sources, both anthropogenic and natural (volcanic activity, etc). Heavy metals are utilised for a variety of applications from anti-septics, electrical and electronic equipment, cars, ovens, etc. to industrial applications like the chemical and mining sectors. Unfortunately, inadequate disposal practices and protection cause major issues that can (and have in the past) result in disasters. These metals can enter the body through inhalation, skin absorption, or ingestion and induce symptoms such as ataxia, decreased motor functioning, dyspnoea, multiple organ failure, and even death while also having the potential of causing harm to future generations. Currently, the cures available are not only inefficient and expensiv e but can also cause many gruesome side effects. The advent of synthetic biology has allowed researchers to utilize engineering techniques and biological knowledge to come up with solutions to important global and local issues. This technology has also provided the opportunity to develop cost-effective, specific, and efficient methods of detection, bioremediation, and therapeutics which are also environmentally sustainable. This review has discussed the various novel techniques developed using synthetic biology to combat heavy metal poisoning and pollution to highlight the need, advancements, and challenges of the approach.

2. Heavy metal bioremediation and resistance in nature

Redesigning biological parts present in nature for useful purposes is the essence of synthetic biology (SynBio). Natural biological methods to tolerate and remediate heavy metals can provide clues to engineer a good system to tackle heavy metal poisoning and pollution. Concerning heavy metal bioremediation and resistance, organisms have evolved innumerable biological parts such as genes/gene-systems, proteins, and other mechanisms to survive and thrive in heavy metal-rich environments (Fig. 1).

2.1. Operons

In bacteria, operons are a genetic regulatory system wherein genes

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encoding functionally similar proteins are organized along with the DNA. One of the mechanisms for heavy metal bioremediation utilized by bacteria is an operon system. There are several operon systems pertaining to various heavy metals such as the *mer* operon for mercury, the *ars* operon for arsenic, the *czc* operon for cobalt, zinc and cadmium, the lead operon for lead and so on. These systems translate to form various structural proteins, transport proteins, regulatory proteins, and enzymes. In the presence of heavy metal, the activating regulatory protein linked to the promoter region will allow the DNA polymerase to bind to the promoter by binding to metal instead. The operon will then transcribe efflux systems, conversion systems, multiple types of resistance systems, etc. Efflux systems are one of the standard methods of heavy metal extrusion and resistance in many bacteria [1,2].

2.2. Metallothioneins (MTs) and phytochelatins (PCs): [1]

Bacteria, fungi, plants, and eukaryotic species all have MT proteins. They have low molecular weight (6–7 kDa) and 20 cysteines among the 60+ amino acid residues. Metal ion sequestration and dispersion are two functions attributed to MTs [3]. In cells exposed to high levels of zinc, cadmium, mercury, copper, cobalt, chromium, or nickel, the abundance of smtA metallothionein transcripts increases [3]. In comparison to most other known MTs, ciliate MTs have an unusually high molecular mass and length. They have a high amount of Cys residues, allowing them to bind more metal ions than other MTs. Moreover, they uniquely induce fast and robust gene expression [3]. A variety of soil and water-dwelling microorganisms can convert inorganic and organic lead compounds into volatile forms, reducing their toxicity. Components such as siderophores, insoluble phosphates, and others can also confer lead resistance [4].

2.3. Others

Many anions, such as chlorides and phosphates, have been reported to react with lead(II) ions and generate insoluble precipitates. It can also precipitate by forming complexes with cysteine, succinic acid, and other amino acids. By sequestering free lead ions as phosphate salts outside and inside the cell, the microbe can reduce the concentration of free lead ions [4]. One of the primary mechanisms bacteria use to counteract lead exposure is restricting mobility within the cell itself. For the entry of lead (II) ions, the cell wall serves as a natural barrier. Peptidoglycan-teichoic-teichuronic acids, lipopolysaccharides, hydroxyl, carboxyl groups, amides, sulfonamides, extracellular polymers such as proteins, nucleic acids, polysaccharides, uronic acids, and humic acids are all examples of lead binding molecules [5].

3. Synthetic biology techniques to mitigate heavy metal poisoning

The fabrication of these biological parts and circuits with the help of SynBio has paved the path to solving some of the world's most pressing issues like heavy metal poisoning. The uniqueness in solving a particular problem using genetic engineering techniques has opened doors for an efficient solution and future researchers to build on the same problem more effectively. Heavy metal contamination in drinking water has inspired researchers worldwide to develop different perspectives on dealing with the issue. This section has focused on some exciting approaches researchers have carried out to solve heavy metal poisoning using SynBio techniques.

3.1. Detection

3.1.1. Mercury

The most common heavy metal pollutants are arsenic, mercury, lead, copper, and cadmium which contribute to many diseases and ecological harm. Many microbial biosensors have been devised using SynBio techniques to detect heavy metals in polluted sources as they are quick, efficient, and specific. Detection of such heavy metals poses different challenges, and thus, researchers have devised novel approaches to engineer a product capable of tackling such a significant concern. Several studies have used a variety of genes and reporter methods to ensure high sensitivity, higher efficiency in different types of polluted samples, and cost-effective use. For example, a study used *Pmer/merR-lucGR* genetic circuit to induce luciferin-mediated bioluminescence and was reported to have a detection range of 100 nM-10 μM (*E. coli*) and 100 nM-1 μM (*P. fluorescens*) [5]. To enable mercury detection in soil samples, another study used a *pmerRBPmerlux* genetic circuit where a bioluminescent immobilized bacterium, *Escherichia coli* MC106, contains the circuit and a rhamnolipid biosurfactant, aiding in boosting the rate at which mercury was released from the soil into the water [6].

Since mercury biosensors are only sensitive to intracellular mercury, a study using *Pmer - MerATPER* compared uptake rates in strains with working transport systems versus strains with deletion of important transporters genes. *MerA* reduces Hg(II) intracellularly, providing a

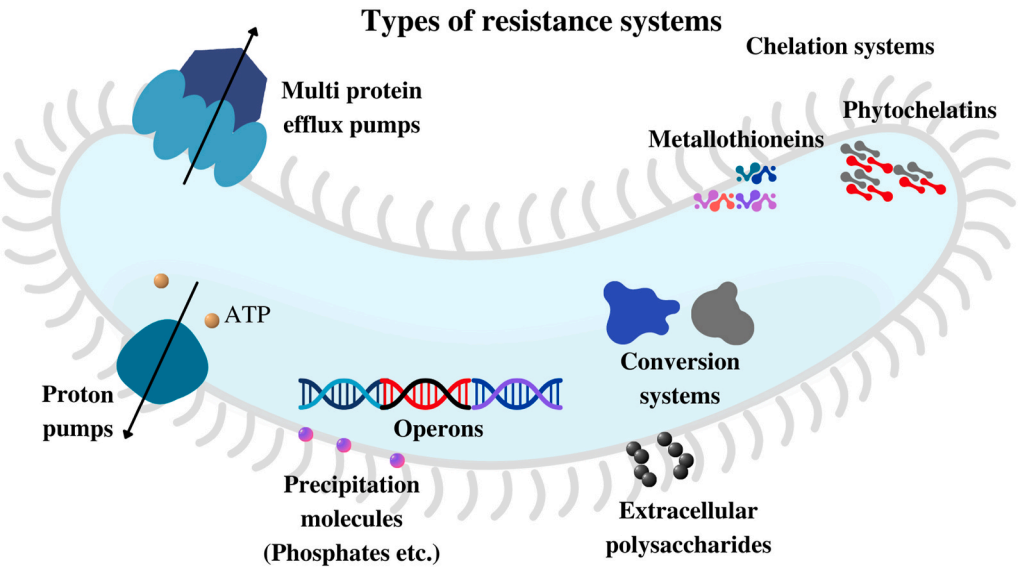

Fig. 1. Types of resistance systems.

quantitative measure of Hg(II) bioavailability [7]. In an alternative study using *MerR-efe*, the MerR protein was employed to trap mercury ions and then bind to the *efe* gene promoter to start the production of the ethylene (C2H4)-forming enzyme that generated the gas, which was detected using a gas sensor to enable on-site rapid detection of mercury in soil [8].

Another study developed a whole-cell and cell-free system containing genetically modified plasmids with merR with emerald green fluorescent protein (EmGFP) and firefly luciferase (LucFF) genes introduced separately as reporters for the detection of mercury [9]. They found that the detection limit of both plasmids in both the cell-free and whole-cell systems were the same (1 ppb). However, they suggested using the cell-free system as it was found to be more adaptable to the environment such as a change in pH and quenching effect of an excess of Hg [9].

3.1.2. Arsenic

Many important studies have been undertaken to enable efficient detection of Arsenic. Since many biosensors which were developed had low sensitivity, a research developed a biosensor, highly sensitive to mg/L arsenite by modifying the 5' untranslated region length and placing an auxiliary binding site for ArsR thereby enabling an excellent signal-to-noise ratio [10,11]. Wan X. et al. further enhanced the sensitivity of whole-cell arsenic sensors using a modular cascaded signal amplifying methodology [12]. Another studied further improved the limit of detection of arsenic using a mutant of ArsR isolated with high-throughput screening from an error-PCR library [13]. However, the study found that using bacterial biosensors to monitor arsenic was not practical as there were many problems related to cell stability and viability outside laboratory conditions. Hence they developed another cell-free system using an evolved mutant of ArsR that enabled efficient, sensitive detection of Arsenic with a limit of 3.65 μg/L which is within the limit given by WHO [14].

Additionally, another research [15] designed and reported *Pars/arsR-phiYFP* biosensor had a good response to expression of *phiYFP* and, arsenic according to the results of the experiments. The generation of yellow fluorescence in strain WCB-11 was time and dose-dependent when exposed to As3+ and As5+, with detection ranges of up to 8 mol/L arsenite and 25 mol/L arsenate [15]. Like the *Pmer/merR-lucGR* genetic circuit, *Pars/arsR-lucGR* this biosensor was reported to have a detection range of 10 nM - 1 μM (*E. coli*) and 10 nM - 10 μM (*P. fluorescens*).

Another study used the *luxCDABE/arsR/luxAB* system with bioluminescence reduction as the output and allowed for easy usability and quick and cost-effective analysis of pollutant bioavailability. Arsenate has a detection range of 500–2000 g/L, while Arsenite has a range of 11000–56000 g/L [16]. With a detectability of 0.5–500 g/L of arsenite, *arsR/crtI* biosensor was reported to change colour to a red pigment on exposure to arsenite, and the change was visible to the human eye after 24 h without further interventions [17]. A paper that tested *Pars/arsR-gfp* circuit reported the lowest measurable concentrations for As (V), As(III), and Sb(III) during a 2-h exposure using the biosensor were 0.4, 1, and 0.75 microM, respectively, and 0.1 microM for all three metal ions after an 8-h induction period [5]. Alternatively, a research designed and tested a *Pars/arsR-lacZ* biosensor. Unlike prior systems, this biosensor would output a pH change, with urease increasing pH in the absence of β-galactosidase (LacZ) and arsenate decreasing pH in the presence of arsenate. Then, using a pH electrode or a pH indicator solution allows for quick and low-cost detection. It was reported to have a distinct response to arsenate levels as low as 5 ppb arsenic, much below 10 ppb arsenic (WHO recommended limit) [18,19].

3.1.3. Lead

There have been multiple studies regarding lead biosensors with a variable limit of detection and specificity. In a study that tested the efficacy of *promoter-pbrR-GFP,* the lead biosensor genetic component was cloned onto a broad host range low-copy number plasmid and reported high sensitivity, efficiency, and specificity in numerous bacteria, including *Enterobacter, Pseudomonas*, and *Shewanella* [20]. Several factors affected the response time including microbial growth rate and lead concentration. Moreover, it was reported in Ref. [21] that in the presence of additional metals such as cadmium, zinc, nickel, and tin, the pGL3-luc/pbr biosensor can detect lead concentrations between 1 and 100 M with no discernible signal from the other metals.

Alternatively, a study using *B. subtilis* and *S. aureus* luminescent biosensors detected 0.01 of a Pb compound after an approximate exposure of 2 h. However, these biosensors were not completely specific to lead [22]. Another study using *A. eutrophus* as chassis detected ~331 μg/mL of an unspecified Pb compound with high specificity which could be attributed to the concentration of Pb compound or the medium used [21]. The detection limit limitations could be due to reduced cell growth due to unavailability of nutrients, plasmid copy number, and so on [20].

Another research designed six genetic circuits to improve the whole-cell biosensor capability for the detection of Pb. They incorporated positive feedback loops and re-configured the elements associated with regulation and discovered that positive feedback loops and configuration affected the sensitivity and effectiveness by 1.5-2-fold and 10-fold respectively. They suggested the same as a suitable method to improve lead biosensor performance [23].

3.1.4. Cadmium and copper

Yan Guo et al. effectively built single-, dual-, and triple-signal output Cd(II) biosensors employing artificial translationally coupled cad operons and measured sensitivity, selectivity, and responsiveness toward cadmium and mercury ions. The three biosensors' reporter signals all rose within the range of 0.1–3.125 M Cd (II). Cd(II) elicited high responses in three biosensors. In the same study, innovative Cd(II) biosensing was combined with bioadsorptive artificial cad operons. Based on the revised heavy metal resistance operons, this work demonstrated one approach to achieve numerous signal outputs and bioadsorption [24]. Another work used *CadR* and *CadC* as independent metal sensory components and *mCherry* and *eGFP* as fluorescent reporters in a single genetic construct to produce a dual-sensing bacterial bioreporter system for detecting bioavailable Cd. The amount of double-color fluorescence produced was directly proportional to the cadmium exposure concentration, making it a functional quantitative biosensor for detecting bioavailable cadmium [25].

Another study created a GFP-based bacterial biosensor *E. coli* DH5alpha (pVLCD1) where the expression of GFP was dependent on the control of cadC gene and cad promoter of *S. aureus* pI258 plasmid. With 2 h exposure, DH5alpha (pVLCD1) mostly responded to Cd(II), Sb(III), and Pb(II), with the detection limit concentrations being 0.1 nmol/L, 0.1 nmol/L, and 10 nmol/L. The biosensor was put to the test in the field, measuring the heavy metals' relative bioavailability in soil samples and contaminated sediments.

Alternate research demonstrated improvement of a whole-cell sensor for cadmium detection using a toggled circuit with PcadR(*P. putida* 06909 regulatory promoter)-cadR promoter-lacI-gfp-Ptac-cadR. They reported that the detection limit was reduced 20 times and the background fluorescence reduced in the toggled circuit. The specificity to cadmium(II) was also reported to be high with no response from other heavy metals such as mercury, lead, copper, and so on [26]. To test the best performing biosensor combination, a study designed 30 whole-cell cadmium biosensors and selected WCB KT-5-R with *P. putida* KT2440 as the host with gene circuit of CadR and mCherry. A positive feedback amplification module and increased reporter gene dosage were implemented to increase efficiency. With a detection limit of 0.01 M, the WCB with the T7RNAP amplification module, p2T7RNAPmut-68, exhibited high specificity and enhanced cadmium tolerance [27].

A recent study indicated that a genetically modified *E. coli* Rosetta microbial fuel biosensor (MFC) expressed *OprF* and *ribB* with promoters *Pt7* and *PcusC*, which could synthesize porin and sense Cu^{2+} in water [28]. In the presence of Cu^{2+} in water, *PcusC* was activated, thereby

promoting the synthesis of riboflavin [28]. Riboflavin was released into the extracellular membrane with the help of the *OprF* encoded porin and increased voltage production of MFC [28]. The results demonstrated that a linear relationship between Cu^{2+} and voltage generation of the MFC biosensor was established at Cu^{2+} concentrations of 0.1–0.5 mM, indicating that this study proves to be an innovative technology for detecting Cu2+ in drinking water [28].

There are several other heavy metal pollutants, including zinc, chromium, cobalt, and so on, which can cause a variety of problems to the environment and human health. Several synthetic biology detection methods are using various reporters to detect these heavy metals, including using sensitive promoters, binding proteins, and so on (Fig. 2).

3.2. Bioremediation - biosorption and bioaccumulation

The terms "biosorption" and "bioaccumulation" are not interchangeable. Chelation, physical interactions (electrostatic forces), complexation, or chemical interactions (ion or proton displacement) are all used in biosorption to bind particles to a biological substrate. Conversely, bioaccumulation is a metabolically active process in which bacteria use importer complexes to construct a translocation channel through the lipid bilayer to absorb heavy metals into their intracellular space [29]. In addition, researchers have also genetically modified organisms to display recombinant metal-binding peptides and proteins on the cell surface, improving specificity and metal-binding capacity.

Biosorption, like several adsorption-based traditional approaches, is susceptible to ionic strength and pH changes found in heterogeneous wastewater effluents. Biosorbents also have a short lifespan because they frequently use degraded biomass, and fouling renders the binding sites inaccessible [29]. In contrast, bioaccumulation requires a living host cell which on its application can impose several challenges such as aeration levels to accommodate the needs of anaerobic and aerobic microbes, nutritional requirements for growth and proliferation of the organisms, decreased cell viability, etc [29].

Using synthetic biology, researchers have utilized these concepts and engineered genetic elements for various chassis to remediate heavy metal pollution. Cloning eukaryotic MTs in bacteria for intracellular expression was one of the first efforts in the genetic engineering of biosorbents. In one study, cytoplasmic synthesis of human MT coupled to araB in *E. coli* resulted in a 3–5-times increase in bioaccumulation [30]. Another research found a 15–20-times rise in cadmium(II) binding in an *E. coli* strain that produces MT coupled to the outer membrane maltose protein (LamB) compared to its wild-type equivalent [30]. In addition, cloning pea or yeast MTs linked to glutathione S-transferase in *E. coli* and combining them with a nickel transporter from *H. pylori* resulted in a 3-times increase in bioaccumulation compared to cells expressing MT but not the transporter [31]. In several experiments, phytochelatin analogs on the bacterial surface increased Cd2+ and Hg2+ bioaccumulation by 12-times and almost 20-times, respectively [32]. Additionally, several studies have been conducted on precipitation, enzymatic transformations, phosphate precipitation, and so on [33].

The initial steps to remove metals from the environment lie in the detection of their presence either in water samples or contaminated soil [34]. This is best achieved through biosensors as they offer a more sustainable way to carry out the analysis in-situ [34]. They are engineered to be sensitive to a lower concentration of metals and can be incorporated into extensive gene circuits that can be used to capture metals [34]. Recently, a novel study described how a metal-tolerant bacterium, *R.metallidurans* CH34, was engineered by expressing mouse MT on its surface for metal biosorption [34]. By introducing this modified bacterium into contaminated soil, it immobilized cadmium in situ, thereby protecting the plants from heavy metal [34].

An interesting study using *E. coli* combined MerR, mer genes involved in absorption, and extracellular protein nanofiber (curli). In the presence of mercury, these nanofibers form a biofilm which provide a large mercury absorption surface area as well as reduce the toxicity of mercury ions accrued intracellularly [35]. This circuit was also reported to have a relevant detection limit. However, this technology cannot function with *E. coli* and needs mercury-resistant species which may pose ethical and regulatory issues. Another study was also conducted for arsenic where two gene circuits to detect and bioremediate arsenic were developed. Using S. epidermidis as a host grown on a nylon mesh, the circuit would produce fluorescence when arsenic ions are present and

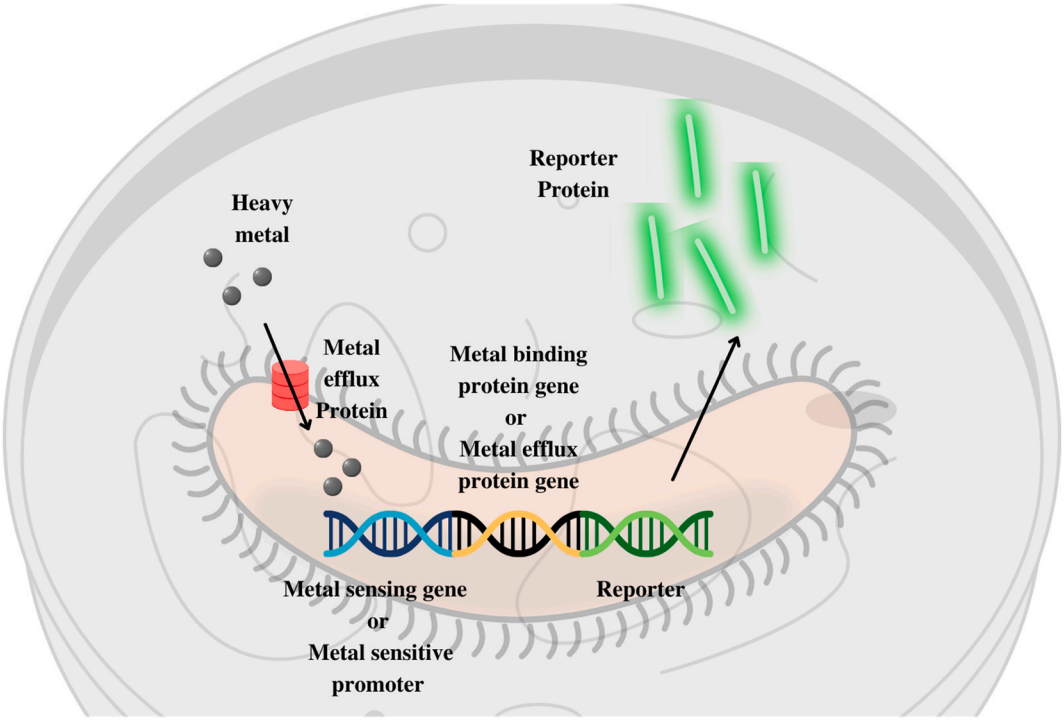

Fig. 2. General mechanism of a detection system.

bioremediate them [36].

To tackle cadmium pollution, several studies have utilized engineered microbes that enable efficient bioaccumulation. A study using engineered *E. Coli*(M4) expressing an MT and a cadmium transport system reported that the M4 grew in the presence of cadmium and showed resistance to it. Compared to the original host bacterial cells' Cd2+ uptake capacity, M4's Cd2+ accumulation was increased by more than one-fold. M4 showed a good binding capacity to Cd2+ in a pH range of 4–8. Certain compounds can be a limiting step to phytochelatins, as shown in the study where a *Thlaspi caerulescens* phytochelatin synthase gene (TcPCS1), two glutathione synthesis genes (gshA and gshB), a heavy metal ATPase gene (TcHMA3), and a serine acetyltransferase gene (cysE), were all transformed into *E. coli* BL21 [37]. The altered bacterium's Cd tolerance and accumulation was much greater than the initial bacteria. Furthermore, bacteria containing cysE, TcPCS1, gshB, and gshA, had better Cd resistance than bacteria containing cysE, gshA, and TcPCS1. This observation proved that gshB was involved in glutathione synthesis and that the glutathione synthase-catalyzed reaction was the limiting step in the production of phytochelatins [38].

There have also been several studies on copper, zinc, lead and so on that face similar limitations of cytotoxicity, cell viability, low sensitivity and specificity [39]. Artificial organelles are one of the methods of reducing the toxicity as demonstrated by a study where uptake was increased after *E. coli* polyphosphate kinase encapsulation [40].

These studies have identified various needs and challenges in the detection and elimination of heavy metal pollution and poisoning, such as organism limits, technological limits, cost, and usage limits. They have approached the problem in a variety of ways in order to provide an optimal solution that can be commercialized. Most of these studies employ plasmids to incorporate the genetic circuit and, in turn implement their solution. However, one of the most overlooked problems of synthetic biology is that a lot many times, the construct does not behave as intended. Using a living organism as a vector implies a high variability in the implementation and production of intended substances. Moreover, plasmid loss over time implies that these systems would be effective only for a couple of minutes to days. The metabolic burden on the organisms is not completely understood; hence, it is a difficult challenge to overcome [41]. However, such studies only further advancement towards understanding and analyzing the effectiveness and scope of SynBio constructs to tackle global issues and contribute to improvements and standardization. Improvements in technology might finally lead to the commercial implementation of SynBio organisms outside the lab environment, provided the ethical challenges are discussed.

4. Future perspectives

These novel applications of synthetic biology are practical in numerous ways and much more efficient and environmentally friendly in the general sense than other remediation and detection methods. However, several problems are plaguing the commercial use of such genetically modified organisms (GMOs). One of the significant problems in transferring these biosensors from lab scale to commercial scale is due to limitations of the technology itself and the ethical aspects of GMOs. The limitations of technology include the change in sensitivity depending on the strains used, variable response rate, detection limited to single or a few metals at a time when the source usually will contain several, competition between wild type and the genetically modified organism, lower sample turnover due to need for containment, poor response depending on the environment, reporter inefficiencies, need for optimal conditions to grow the organisms and so on. These technological limitations are slowly being combated using various methods, including cell-free systems, paper-based systems, portable devices, and so on as exemplified by the various methods described in the previous section. CRISPR-based devices could eliminate the need for metabolically intensive plasmids and help the systems work more efficiently. Using more efficient, sensitive, and specific reporter genes or reporting methods that can handle changes in environmental conditions can also help in accelerating commercialization.

The ethical limitations include the controversial nature of synthetic biology and genetically modified organisms, the spread of the artificial organisms in the natural ecology, horizontal gene transfer of unwanted genes which could lead to the accumulation of such organisms etc, which are all collectively biosafety and biosecurity threats. Hence understanding the biosafety and biosecurity aspects of synthetic biology is extremely important for commercializing such biological devices. Safe, secure, and responsible biotechnology research, and the implementation of its products, require combined efforts from multiple stakeholders, including scientists, regulators, and policymakers. Being honest about the risks will not only lead to more ideas for handling them but will also turn the conversation to synthetic biology's immense potential for not only combating heavy metal poisoning but also overall global development. Overcoming these hurdles will probably take time and innumerable conversations. The development of such technologies that have a dual-use concern should not be restricted at the research stage.

5. Conclusion

Heavy metal pollution and poisoning require urgent attention, and SynBio has much promise to combat the issue. As discussed in the review, there have been numerous approaches to detect, bioaccumulate, bioremediate heavy metals from the body and the environment, from microbial biosensors to probiotics. While the potential of SynBio is limitless, the implementation of its products might require more discussion as its implications are unknown. The development of technology should be in tandem with the development of biosafety and biosecurity. Robust risk assessment frameworks should be developed and followed. However, addressing the ambiguity and potential for harm of such SynBio products should be done through education and a culture of responsible research, rather than enforcing restrictions on its development. The SynBio community should educate not only themselves but also the stakeholders and create an environment of open dialogue. Being honest about the risks will not only lead to more ideas for handling them but will also turn the conversation to biotechnology's immense potential for global development.

Ethics approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Consent to participate

The authors agree to participate.

Consent for publication

The authors agree to submit the article.

CRediT authorship contribution statement

Adithi Somayaji: Formal analysis, Investigation, Data curation, Visualization, Writing – original draft, Writing – review & editing. Soumodeep Sarkar: Methodology, Investigation, Data curation, Writing–original draft. Shravan Balasubramaniam: Formal analysis, Writing–original draft. Ritu Raval: Conceptualization, Supervision, Data curation, Visualization, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

the work reported in this paper.

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Abbreviation

SynBio	Synthetic Biology
MTs	Metallothionein
PCs	Phytochelatins
iGEM	International Genetically Engineered Machine
GMOs	Genetically Modified Organisms

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