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Review

Engineering chimeric signaling proteins for microbial whole-cell biosensors: from design to deployment

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Microbial whole-cell biosensors (MWCBs) harness living cells to detect analytes and produce measurable outputs, enabling continuous, low-cost, and *in situ* sensing. Central to MWCB function are modular sensing architectures, which can be reprogrammed to respond to diverse signals. Particularly, two-component systems (TCSs) and allosteric transcription factors (aTFs) offer modular, engineerable frameworks for building chimeric proteins. Recent advances in domain swapping, fusion point selection, and protein engineering are expanding the versatility and specificity of these systems, presenting new opportunities for tailored and multiplexed detection. However, translating chimeric MWCBs into real-world applications still faces multiple hurdles. This review examines current strategies for engineering TCS- and aTF-based biosensors and outlines key opportunities and challenges for their deployment in applications such as diagnostics, environmental monitoring, and biomanufacturing.

Highlights

Chimeric signaling proteins are gaining traction in the development of microbial whole-cell biosensors (MWCBs), because they enable versatile combinations of input and output domains from various organisms.

Two-component systems and allosteric transcription factors are the most widely used architectures for developing responsive sensing elements in MWCBs.

Advances in domain swapping and chimeric fusion strategies are expanding MWCB capabilities, enabling detection of previously inaccessible or complex analytes.

Deployment of MWCBs in real-world settings requires advances in device integration, multiplexing, signal robustness, stability, and performance in context-specific and variable conditions.

Despite promising field-ready prototypes, implementation remains limited by biological complexity, technical constraints, regulatory ambiguity, and inconsistent terminology, among others.

Harnessing chimeric signaling proteins for microbial biosensors

Microbial whole-cell biosensors (MWCBs) use living cells with repurposed genetic components to detect specific analytes and produce quantifiable output signals. These systems harness the potential of natural sensing mechanisms, making them inherently responsive to the bioavailability of target analytes [1,2]. Unlike conventional analytical techniques, MWCBs require neither trained personnel nor specialized equipment nor sampling, enabling both on-site detection [3,4] and continuous monitoring [5].

By contrast to enzymatic and antibody-based sensors, which typically require purification steps, MWCBs function without downstream processing while still achieving micromolar to nanomolar sensitivity [6]. Microbes' natural adaptability allows them to survive in diverse and harsh environments by maintaining homeostasis thanks to their complex signaling pathways. Among other factors, this resilience contributes to their robustness, making MWCBs promising biosensor candidates for continuous monitoring in field applications. Furthermore, their ability to proliferate allows low-cost and scalable production, enhancing their accessibility and practicality [7].

Nature's vast biological diversity supports the engineering of MWCBs to detect a plethora of compounds. These biosensors hold promise in various domains: from screening overproducer strains and identifying novel bioactive compounds to monitoring pollution and enabling noninvasive diagnostics [8–11]. Importantly, real-time data from MWCB measurements provide a solid basis to construct digital twins of a range of processes, from bioreactors to large ecosystems. Combined with synthetic gene circuits, MWCBs can be multiplexed for the simultaneous detection of several

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inputs and endowed with biocomputing capabilities for cybergenetics [12,13], enabling real-time, on-site interventions in biomanufacturing [14] and theranostic applications [15].

Despite their potential, the lack of standardized pipelines and integrated MWCB prototypes hinders serious progress on making such sensors at scale. One way forward involves using chimeric signaling proteins to streamline biosensor design and evaluation.

Chimeric signaling proteins are created by fusing specific sensing domains with output domains that regulate gene expression. Given the vast natural reservoir of sensing domains and signaling cascades, countless combinations can be tailored to specific applications. Chimeras, whether derived from members of the same protein family or across families, have been successfully constructed by leveraging shared signal transduction mechanisms. Examples include **methyl-accepting chemotaxis proteins (MCPs)** [16] (see Glossary), **ABC transporters** [17], **G protein–coupled receptors** [18], and, notably, two-component systems (TCSs) and allosteric transcription factors (aTFs), which are the focus of this review.

Strategic domain swapping of sensing and signaling modules enables the construction of chimeric protein-based MWCBs, which offer advantages in terms of orthogonality, flexibility, and versatility compared with native systems. Incorporating insulated modules or domains from phylogenetically distant organisms can minimize crosstalk with host pathways and bypass limitations associated with conserved stress responses [19]. Additionally, putative ligand-binding domains with uncharacterized signaling pathways, commonly found in genomic and protein databases, can be paired with well-characterized transduction modules to unlock their biosensing potential [16,20]. Specificity can be enhanced by integrating sensing units with higher fidelity, and improved performance can be achieved by employing signal transduction cascades with broader operational and dynamic ranges [21]. Considering the ever-growing knowledge in this field, a modular platform could serve as a blueprint for rational chimeric protein design.

Bacteria rely on finely tuned molecular mechanisms to detect and respond to changing environmental conditions. Two of the most widely used sensing systems in synthetic biology are TCSs and aTFs. Both act as signal transducers that convert external or internal cues into changes in gene expression, enabling dynamic adaptation and regulation of cellular functions. Despite differing in architecture and complexity, both systems offer modular, engineerable frameworks for building synthetic biosensors. TCSs are typically used to detect analytes located outside the cell, whereas aTFs are employed mainly to sense intracellular analytes. Having two systems that fulfill similar functions through different mechanisms is particularly valuable when developing MWCBs for specific applications. For example, the use of a phosphate-sensing aTF may be unsuitable because of high intracellular phosphate levels, whereas a TCS may not be ideal for the selection of overproducing strains of intracellular metabolites. Optimizing both mechanisms, specifically in the form of chimeric proteins, is crucial to expand the range of options available for the development of novel MWCBs.

In this review, we provide an overview of chimeric TCSs and aTFs, with particular emphasis on fusion strategies and module choices. To support future biosensor engineering efforts, we outline commonly used components and approaches and offer comparative insights into design strategies. Finally, we discuss best practices for integrating MWCBs within deployment strategies, highlighting the current state of the art for real-world implementation.

Chimeric TCSs: the gold standard for extracellular sensing

TCSs are central to how bacteria (and some archaea, fungi, and plants) sense and respond to environmental changes. A typical TCS consists of two proteins: a transmembrane sensor histidine

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kinase (SHK) that detects extracytoplasmic signals and a cytoplasmatic response regulator (RR) that mediates the cellular response (Box 1, Figure 1).

Considering that conformational shifts and phosphorylation-dependent mechanisms are conserved among many membrane sensor–based signal transduction pathways, the modular structure of TCSs makes them excellent candidates for engineering these mechanisms. A common strategy involves domain swapping, replacing the sensor domain of an SHK with that from another TCS system to reprogram signal input. A similar rewiring strategy involves constructing chimeric TCSs by using the sensory domains of MCPs. Although many chimeric TCSs have been engineered to date (Table 1), one of the biggest challenges in their design lies in selecting the optimal fusion point to ensure functional signal transmission.

The first reported chimeric TCS, Taz, was constructed by fusing the sensing domain of the Tar MCP with the cytoplasmic signaling domain of *Escherichia coli*'s EnvZ/OmpR TCS [22]. Although Tar naturally responds to both aspartate and maltose, Taz was specific to aspartate, illustrating that TCS domain swapping can yield novel input–output behaviors. The fusion point was conveniently chosen at a shared NdeI restriction site near the **HAMP domain**'s C-terminus, incorporating 687 bp of EnvZ. Taz exhibited approximately sevenfold induction upon aspartate exposure. Another chimera, Tez1, was obtained by fusing the sensing and transmembrane domains of Tar with the full cytoplasmic domain of EnvZ [23]. Although Tez1 retained kinase activity, disruption of the transmembrane/linker junction caused an imbalance in the kinase/phosphatase ratio, resulting in a constitutively active phenotype. Adjusting the length of the transmembrane/linker junction through alanine insertions near the second transmembrane helix (TM2) restored the balance between kinase/phosphatase state and yielded an aspartate-responsive chimera [23], emphasizing the critical role of the transmembrane/linker junction and the sensitivity of linker architecture. Even though EnvZ and Tar linkers share a common helix-turn-helix structure, the findings of this study indicated that both helices need to originate from the same protein to obtain a functional chimera.

The EnvZ/OmpR TCS, because of its well-characterized structure, has frequently been repurposed as a signaling scaffold in chimeras. Fusions with various sensing units have enabled the detection of specific metabolites: RetS for mucin dependence for biocontainment [24], MalKZ

Box 1. How TCSs sense and respond

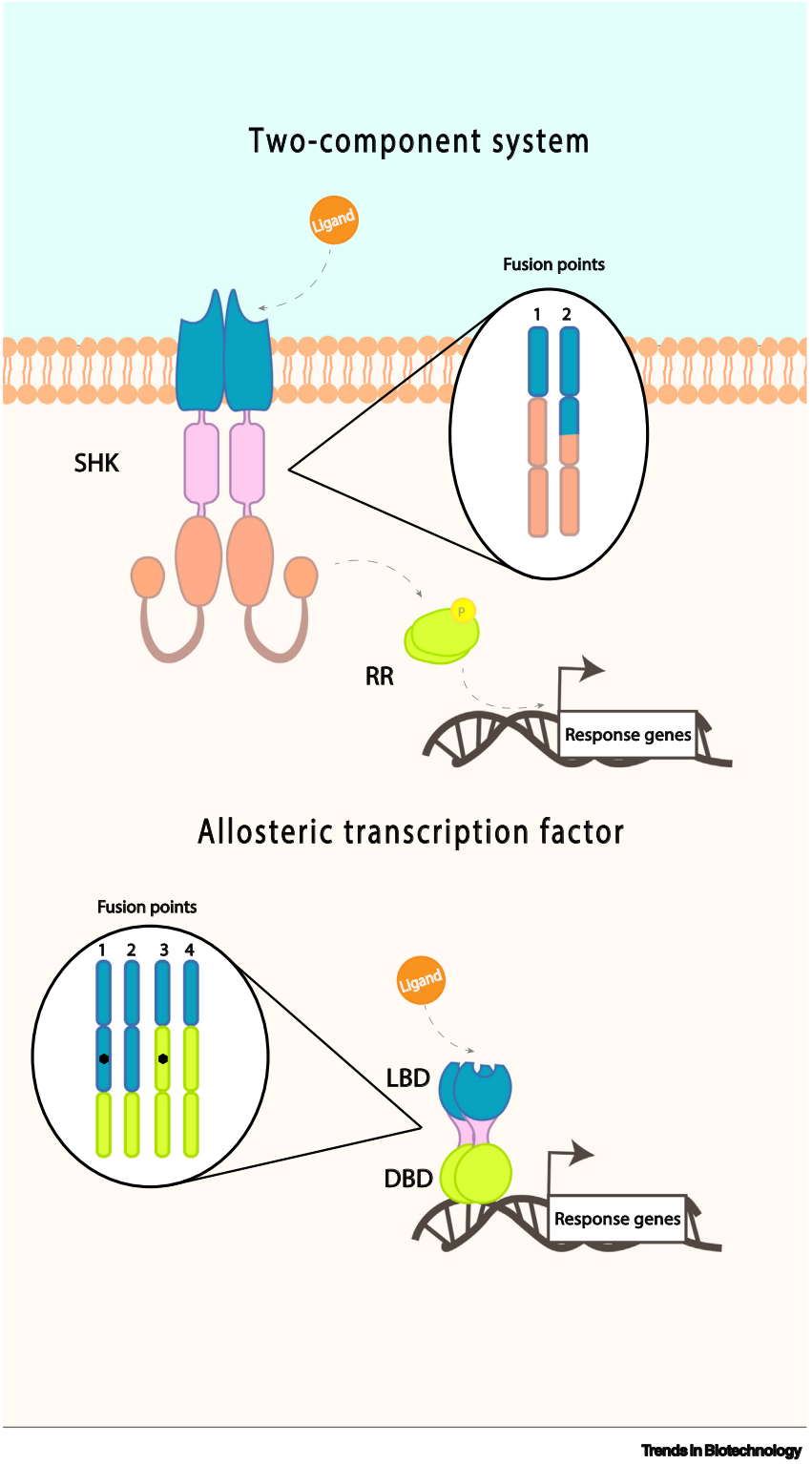
A canonical TCS consists of a transmembrane sensor histidine kinase (SHK) and its cognate response regulator (RR). Both SHKs and RRs are dimeric proteins that consist of two monomers. Perception of environmental stimuli induces conformational changes in the sensing domain of SHKs, which are transmitted through transmembrane helices and linker domains (e.g., GAF, PAS, HAMP) that typically share helical structures.

GAF and PAS linker domains found in some SHKs contribute to sensing of small molecules and various signals such as light, oxygen, and redox cofactors [31,75,76]. Many SHKs contain a HAMP domain composed of two α-helices. In dimeric form, these α-helices are arranged into a four-helix parallel coiled coil that displays a heptad repeat pattern [77]. The signaling helix is another element commonly found in SHK linkers that prevents unspecific activation of the catalytic domain by separating N-terminal sensory domains from C-terminal catalytic domains [78]. The linker domains relay the signal to the catalytic core. This core includes the dimerization and histidine phosphotransfer (DHp) domain, which facilitates dimer formation through helical bundle interactions, and the catalytic ATP-binding (CA) domain. In the presence of a stimulus, the CA domain phosphorylates a conserved histidine in the DHp domain, which subsequently transfers the phosphate to a conserved aspartate residue in the RR.

The phosphorylated RR binds to its target DNA sequence, regulating the transcription of downstream genes. Although some SHKs exhibit phosphatase activity, removing the phosphate group from the RR upon activation [36], many exhibit dual activity, acting as kinases in the presence of stimuli and phosphatases in its absence or vice versa. The dynamic balance between these opposing activities determines the level of phosphorylated RRs and thereby modulates the strength of gene expression [79]. This dual functionality enables rapid and precise control of transcription, allowing bacteria to fine-tune responses to fluctuating environmental conditions [79].

Glossary

ABC transporters: ATP-binding cassette transporters. A large family of membrane proteins that use the energy of ATP hydrolysis to transport various substrates across cellular membranes. In biosensing, ABC transporters can contribute to signal transduction by mediating ligand uptake or triggering conformational changes that initiate downstream responses.
BESSY: bioelectronic sensing system. A portable bioelectronic sensing platform designed for field-deployable detection of analytes using biological recognition elements coupled with electronic readouts. BESSY systems integrate engineered biological components, such as MWCBs, with transducers, enabling rapid and sensitive detection outside laboratory settings.
EMeRALD receptor platform: engineered modular receptors activated via ligand-induced dimerization. A synthetic biology framework for designing modular biosensors in bacteria. It uses ligand-induced dimerization of engineered receptor domains to trigger transcriptional responses.
Genetically modified organisms (GMOs): organisms whose genetic material has been altered using genetic engineering techniques. In the context of biosensing, GMOs are often used as chassis for constructing MWCBs.
G protein–coupled receptors: a large and diverse class of membrane proteins that detect external signals and activate intracellular pathways via G proteins. Although studied primarily in eukaryotes, efforts have been made to repurpose G protein–coupled receptors in microbial biosensors because of their high sensitivity and broad ligand range.
HAMP domain: histidine kinases, adenylyl cyclases, methyl-accepting proteins, and phosphatases. A conserved signal relay module found in transmembrane sensor proteins such as SHKs and MCPs. It transmits conformational changes from extracellular or periplasmic sensing domains to cytoplasmic output domains. Its role is critical in engineered chimeric systems.
IMBED: integrated molecular bioengineering and experimental design. A computational-experimental



framework that integrates structural biology, molecular modeling, and synthetic biology to design and optimize biosensor components. IMBED facilitates the rational engineering of signal transduction pathways by predicting domain compatibility and linker functionality.

Isopropyl β-D-1-thiogalactopyranoside (IPTG): a molecular mimic of allolactose, used as an inducer of the lac operon in bacterial systems. In biosensor studies, IPTG is commonly employed to induce expression of target genes, including synthetic receptor systems and reporter circuits.

Methyl-accepting chemotaxis proteins (MCPs): a class of transmembrane receptors used by bacteria to sense chemical gradients in their environment and mediate chemotaxis. MCPs are modular, with periplasmic ligand-binding domains and cytoplasmic signaling domains, making them attractive input modules for synthetic biosensors and chimeric TCSs.

PATCHY method: primer-aided truncation for the creation of hybrid proteins. A combinatorial method for generating libraries of chimeric proteins with diverse fusion points. It uses staggered PCR primers to create overlapping truncations of protein domains, followed by ligation and activity screening.

(See figure legend at the bottom of the next page.)

Table 1. Chimeric two-component systems[a]

Chimera	Sensing domain	TCS	Fusion point	Input stimuli	Refs
Taz	Tar	EnvZ/OmpR	HAMP	Aspartate	[23]
Tez1	Tar	EnvZ/OmpR	TM2	Aspartate	[23]
RetS-EnvZ	RetS (*Pseudomonas aeruginosa*)	EnvZ/OmpR	Not specified	Mucin	[24]
MalKZ	MalK	EnvZ/OmpR	Not specified	Maltose	[17]
AauSZ	AauS (*Pseudomonas putida*)	EnvZ/OmpR	Not specified	Acidic amino acids	[25]
MxaYZ	MxaY (*Paracoccus denitrificans*)	EnvZ/OmpR	Not specified	Methanol	[26]
DcuSZ	DcuS	EnvZ/OmpR	Not specified	Fumarate	[21]
MxcQZ	MxcQ (*Methylobacterium extroquens*)	EnvZ/OmpR	Not specified	Methanol	[28]
MxbDZ	MxbD (*Methylobacterium extroquens*)	EnvZ/OmpR	Not specified	Methanol	[28]
FlhSZ	FlhS (*Paracoccus denitrificans*)	EnvZ/OmpR	Not specified	Methanol	[29]
NrsS-NarX	NrsS *(Synechocystis sp.)*	NarX/NarL	HAMP	Nickel	[30]
RssA-NarX	RssA *(Serratia marcescens)*	NarX/NarL	HAMP	Iron	[30]
PcaY-NarX	PcaY (*Pseudomonas putida*)	NarX/NarL	HAMP	Aromatic amino acids	[16]
PcaY-NarQ	PcaY (*Pseudomonas putida*)	NarQ/NarL	HAMP	Aromatic amino acids	[16]
YF1	YtvA *(Bacillus subtilis)*	FixL/FixJ *(Bradyrhizobium japonicum)*	PAS	Blue light	[31]
HtlD-YF1	HtlD *(Haloarcula marismortui)*	FixL/FixJ *(Bradyrhizobium japonicum)*	PAS	Indole-3-aldehyde	[20]
PmrB-CusS	PmrB	CusS/CusR	TM2	Antimicrobial peptides	[32]
NarQ-NisK	NarQ	NisK/NisR *(Lactococcus lactis)*	TM2	Nitrate	[33]
AQ4	AHK4 (*Arabidopsis thaliana*)	PhoQ*/PhoP* (insulated pair)	PATCHY	Trans-zeatin	[19]
PctC-EnvZ	PctC (*Pseudomonas aeruginosa*)	PhoQ/PhoP	TM2	GABA	[35]
PctC-PhoQ	PctC (*Pseudomonas aeruginosa*)	PhoQ/PhoP	TM2 & PATCHY	GABA	[35]
DrF1	DrBphP *(Deinococcus radiodurans)*	FixL/FixJ *(Bradyrhizobium japonicum)*	PAS	Red light	[36]
DmF1	DmBphP *(Deinococcus maricopensis)*	FixL/FixJ *(Bradyrhizobium japonicum)*	PAS	Red light	[37]

[a]Nonexhaustive list of chimeric TCS examples. When not indicated, domains are native to *E. coli*. When indicated with an asterisk, domains are from unknown or not explicitly indicated organisms.

for malate [17], AausZ for acidic amino acids [25], and MxaYZ for methanol [26], the latter three used to screen overproducing strains. For example, to overcome weak output from the native fumarate-sensing DcuS/DcuR TCS, Ganesh *et al.* fused the DcuS sensor domain, including its conserved histidine, with EnvZ's signaling domain, generating a tunable fumarate-responsive output [21]. Performance was further enhanced using a positive feedback loop in which the cognate RR was expressed under the TCS promoter [27]. Interestingly, chimeras AauSZ and DcuSZ used 684 bp of EnvZ's C-terminal sequence, whereas MalKZ and MxaYZ used 687 bp, likely reflecting the design precedent set by Taz.

Figure 1. Mechanism of a two-component system (top) and an allosteric transcription factor (bottom). Top: The two-component system (TCS) consists of a dimeric sensor histidine kinase (SHK), whose specific domains are a sensing domain in dark blue, a linker domain in pink, and a cytoplasmic domain indicated in peach pink. The response regulator (RR), indicated in bright green, controls specific response genes when phosphorylated. Fusion points for chimeric TCSs are indicated in the close-up: (1) sensing domain from protein A and cytoplasmic domain from protein B, (2) fusion of linker domain between proteins A and B. Bottom: Allosteric transcription factors (aTFs) also form dimers and encompass a ligand binding domain (LBD) in dark blue, a linker domain in pink, and DNA-binding domain (DBD) in bright green that control the expression of specific response genes. Possible fusion points in the linker domain to construct chimeric aTFs are indicated in the close-up: (1) from LBD with mutated residue(s) indicated with a hexagon, (2) from LBD, (3) from DBD with mutated residue(s) indicated with hexagon, and (4) from DBD.

Fusion points have also been designed on the basis of structural data and sequence alignments. Methanol-sensing chimeras using MxcQ and MxbD domains were engineered on the basis of structural insights from EnvZ and alignments with each chemoreceptor, achieving detection limits of ~0.01% methanol [28]. Similarly, a FlhS-EnvZ chimera was built using a hydrophobic methionine residue as the optimal fusion point, guided by homology modeling [29].

For detection of aromatic amino acids, PcaY-NarX and PcaY-NarQ chimeras were created on the basis of HAMP domain alignments. The chimera containing NarQ's HAMP exhibited the best performance. Using the same fusion point, 17 additional chimeras with LBD domains from diverse protein families were constructed, 12 of which were functional, highlighting the selected fusion point's success rate for constructing MCP-TCS chimeras [16].

Chimeras have also been engineered within synthetic vesicles. Alignments of HAMP domains from SHKs such as NrsS, RssA, and VanS with the NarX/NarL TCS informed consistent fusion sites. Although NrsS- and RssA-NarX chimeras showed metal-specific responses to nickel and iron, respectively, their performance depended on membrane composition. By contrast, the VanS-NarX chimera exhibited constitutive activity, possibly because of HAMP destabilization [30]. These results illustrate that even identical fusion points can yield divergent behaviors, depending on the domains involved.

The conserved DIT motif within PAS domains has also served as an effective fusion point. Both the blue light–responsive YF1 [31] and the indole-3-aldehyde-sensing HtlD-YF1 [20] were constructed using this motif. However, because of the diverse signaling mechanisms of PAS domains, such as disorder/order transitions or conformation shifts, their general applicability remains uncertain.

Fusion at TM2 has also yielded functional chimeras. For instance, the CusS sensing domain was swapped with that of PmrB to link TCS signaling with antimicrobial peptide sensing. TM2 fusion preserved function, whereas HAMP fusion caused elevated baseline activity due to unwanted structural effects [32]. Similarly, swapping the periplasmic domain and transmembrane helices of NisK with those of nitrate-responsive NarQ altered the specificity of the NisK/NisR TCS [33].

The **PATCHY method** provides a combinatorial approach for chimeric TCS construction [34]. It employs staggered primers to generate truncated versions of domain-encoding DNA fragments that are assembled in a one-pot ligation reaction to construct a fusion protein library that contains different combinations of variable in size domains [34]. This method enabled the creation of the trans-zeatin biosensor AQ4 by fusing truncated AHK4 and PhoQ* SHKs. Screening identified a highly responsive, insulated chimera that upregulates transcription in response to trans-zeatin [19].

Zhao and colleagues used PctC as a sensing domain and combined it with five different TCSs to construct GABA (gamma-aminobutyric acid) biosensors [35]. The C-terminal of TM2 was initially selected as the fusion point. The PctC-PhoQ chimera displayed the best response; others were nonfunctional or weakly active. Subsequently, a PATCHY library combining the PctC sensing domain and the PhoQ DhP domain further revealed a high-performing variant lacking a HAMP domain.

Red/far-red light-sensing chimeras were also developed using sequence-guided design. Alignments between the YF1 (YtvA-FixL) chimera and the *Deinococcus radiodurans* bacteriophytochrome (DrBphP) led to a DrF1 chimera (DrBphP-FixL). Moreover, in a follow-up study, comparison of the bacteriophytochromes from the *D. radiodurans* (DrBphP) and *Deinococcus maricopensis* (DmBphP) sensing domains resulted in the development of DmF1 (DmBphP-FixL) [36,37], illustrating how prior

chimera designs can guide future constructs. The DmF1 study has successfully showcased approaches for the enhancement of signal sensitivity and inversion of the output signal, which can be employed for the optimization of other TCSs. Notably, linker length emerged as a crucial parameter in PATCHY-generated DmF1 and DrF1 libraries [37]. Variations in linker length modulated SHK conformation and TCS output. Functional variants showed linker lengths not commonly observed in naturally occurring SHKs, suggesting that fine-tuning linker length can enhance biosensor performance.

As outlined in this section, current design strategies for chimeric TCSs include combining a desired sensing domain with various TCS signal transduction domains, using previously explored fusion points, homology-based chimeric protein design, and the PATCHY method. Screening of functional variants predominantly involves testing individual colonies under induced and uninduced conditions and measuring fluorescence or colorimetric signals. Alternatively, transcription of downstream genes is quantified using qPCR or RT-qPCR. In some cases, promising variants are selected through iterative FACS cycles and population enrichment prior to individual construct evaluation [35].

Beyond chimera generation, several strategies have been employed to optimize signal output and screen chimeric TCSs for reduced basal activity, enhanced sensitivity, and improved dynamic range. Insulated TCSs with no crosstalk to native TCSs have been generated and incorporated into chimeras [19]. Additionally, variations in plasmid copy numbers, promoter strength, RBS sequences, and DNA-binding sites have been explored to fine-tune signal readouts [20,30,35]. Designs incorporating positive feedback loops to amplify RR levels [27] and genetic circuits integrating protease production have also been used to achieve tighter control over reporter accumulation [35].

Despite these advances, no universal strategy currently exists for building and screening functional chimeric TCSs. Fusion points that prove successful in one context may fail in another. Although linker design, domain compatibility, and structural stability are all critical for achieving desired properties, integrating all three simultaneously remains challenging due to the inherent complexity of protein–protein interactions and context-specific behavior. Nevertheless, cumulative knowledge from successful designs is laying the groundwork for a more predictive framework to guide engineering of chimeric TCSs. A chassis strain incorporating the aforementioned elements for enhanced signal output, together with high-throughput methods for chimera design and screening, could help overcome one of the major bottlenecks in this field.

Repurposed aTFs through domain swapping for intracellular sensing

An aTF is a regulatory protein that can bind DNA and modulate gene expression in response to the binding of a ligand at its allosteric site. A key feature of aTFs is their conserved structure with two functional domains: one binding to the ligand and one binding the DNA (Box 2, Figure 1). As in the case of the SHKs, domain swapping has emerged as an effective strategy to alter ligand specificity of aTFs. By retaining the native DNA-binding domain (DBD) and corresponding transcription factor binding site (TFBS) driving a reporter gene, the ligand-binding domain (LBD) can be exchanged to respond to alternative molecules. This section focuses on common strategies for domain swapping within the same aTF family, referred to here as intrafamily domain swapping.

The LacI/GalR family is a well-studied model for allosteric interaction and domain swapping (Table 2). Meinhard *et al.* demonstrated that by maintaining the DBD and hinge linker domain of LacI and fusing them with the LBDs from different homologous proteins, it was possible to shift specificity from **isopropyl β-d-1-thiogalactopyranoside (IPTG)** to various sugars. Additionally, mutations at nonconserved residues in the linker region, such as at the fusion point of the

Box 2. How aTFs sense and respond

aTFs are a subset of transcriptional regulatory proteins found across microorganisms, which operate via their own conserved mechanism. In response to specific intracellular stimuli, aTFs also undergo conformational changes that modulate the expression of the target genes and associated metabolic pathways. This regulation occurs by altering the protein's affinity for a specific DNA region, typically called the 'operator' or 'transcription factor binding site' (TFBS), located within the promoter.

Because of their central role, a wide variety of functions, such as housekeeping or response to environmental changes, and mechanisms are found in aTFs. Functioning as dimers, they work either as repressors, by blocking the RNA polymerase, or as activators, by recruiting the RNA polymerase [80]. Because of their diversity, aTFs are categorized into different families on the basis of their domain organization and DNA binding motifs, such as helix-turn-helix (HTH), the β ribbon, and the winged helix (WH) [81].

Despite their functional, structural, and mechanistic diversity, aTFs generally present a common architecture: a ligand-binding domain (LBD), also known as environmental sensing module (ESMs), regulatory domain (RD), or activator domain (AD) [40,80,82], which is connected via a hinge domain or linker region to a DNA-binding domain (DBD) that recognizes the TFBS and regulates gene expression. The hinge domain has been studied mainly within the LacI family and seems to have a fundamental role in the allosteric response of the aTF by determining the protein conformation [83]

In biosensor design, the aTF is often referred to as the 'detector module,' whereas the TFBS alongside the reporter gene is referred to as the 'effector module' [53]. This defined structure and their modularity makes aTFs excellent candidates for building interchangeable systems.

two domains, selectively improve some chimeras, highlighting both the importance but also the difficulty of finding patterns for these regions [38].

Building on this, Shis *et al.* used LacI chimeras in synthetic AND logic gates to minimize crosstalk [39]. Furthermore, LacI chimeras were used for the development of the 'Passcode' kill switch developed by Chan *et al.*, which incorporated in logic gates a combination of chimeras based on LacI and ScrR DBDs with CelR and GalR LBDs responsive to cellobiose and galactose, respectively, for biocontainment purposes [40].

Further characterization of domain swapping in the LacI/GalR family was conducted by Dimas and colleagues, who employed coevolutionary computational analyses to assess compatibility across homologs [41]. To validate the computational strategy, a library of 35 chimeric and five native proteins was constructed by combining five LBDs with eight well-characterized DBDs of the LacI/GalR family. Although some variants exhibited high basal GFP levels and low induction, 22 chimeras showed a fold induction greater than 10 with minimal background: ideal characteristics for biosensors. Notably, some hybrid repressors such as LacI-GalR and ScrR-GalR exhibited higher induction levels (8-fold and 200-fold, respectively) compared with the native systems, suggesting that domain swapping can enhance the dynamic range. ScrR-GalR and ScrR-CelR, along with native ScrR, have since been employed to optimize a two-stage ethanol bioproduction strategy in *Pseudomonas putida* by improving resource allocation during fermentation [42].

Ligand specificity was also altered in the TetR family through the construction of a TetR-MphR chimeric aTF, shifting responsiveness from anhydrotetracycline to erythromycin [43]. A 3D structural design strategy based on template modeling was used to guide chimera construction. Using the end of the α3-α4 helices of TetR as the DBD boundary, the researchers fused it to the LBD of MphR at homology-guided positions. This resulted in 15 chimeric proteins with different fusion points (TM44–58), among which TM52 showed the best performance, although still lower than that of native TetR. To further improve the dynamic range of TM52, a rational design approach was employed involving mutagenesis at amino acid positions 14, 54, and 55, critical for aTF responsiveness. However, these modifications did not yield the expected improvements. In a different study, TetR-Elk-1 and TetR-CREB, activated in response to the presence of MAPK ERK kinase

Table 2. Chimeric allosteric transcription factors[a,b]

DBD/TFBS	Fusion point	LBD	Original molecule	Change specificity	Refs
LacI/GalR					
LacI	LacI	FruR (CRA)	IPTG	Fructose-1-phosphate	[38]
LacI	LacI	AscG	IPTG	Unknown	[38]
LacI	LacI	CelR (*Thermobifida fusca*)	IPTG	Cellobiose	[38]
LacI	LacI	CytR	IPTG	Cytidine	[38]
LacI	LacI	GalS	IPTG	Galactose, Fucose	[38]
LacI	LacI	GalR	IPTG	Galactose, Fucose	[38]
LacI	LacI	PurR	IPTG	Hypoxanthine	[38]
LacI	LacI	RbsR	IPTG	Ribose	[38]
LacI	LacI	TreR	IPTG	Trehalose-6-phosphate	[38]
LacI	CelR	CelR	IPTG	Cellobiose	[40]
LacI	GalR	GalR	IPTG	Galactose	[40]
ScrR (*Klebsiella pneumoniae*)	LacI	LacI	Sucrose	IPTG	[40]
ScrR (*Klebsiella pneumoniae*)	CelR	CelR	Sucrose	Cellobiose	[40,42]
ScrR (*Klebsiella pneumoniae*)	GalR	GalR	Sucrose	Galactose	[40,42]
TetR					
TetR	Hybrid, multiple fusion points	MphR	Tetracycline	Erythromycin	[43]
TetR	Not specified	Elk-1	Tetracycline	MAPK ERK kinase 1	[44]
TetR	Not specified	cAMP-responsive element binding protein (CREB)	Tetracycline	Protein kinase A	[44]
LysR					
FdeR (*Herbaspirillum seropedicae*)	FdeR	NodD1 (*Sinorhizobium meliloti*)	Naringenin	Luteolin	[45]
FdeR (*Herbaspirillum seropedicae*)	FdeR mutated	NodD1 (*Sinorhizobium meliloti*)	Naringenin	Luteolin	[45]
FdeR (*Herbaspirillum seropedicae*)	NodD1	NodD1 (*Sinorhizobium meliloti*)	Naringenin	Luteolin	[45]
LuxR					
LasR (*Pseudomonas aeruginosa*)	Different fusion points, not specified	MupR (*Pseudomonas fluorescens*)	Quorum sensing	Mupirocin	[10]
MerR					
MerR (*Staphylococcus aureus*)	Not specified	ZntR	Mercury	Zinc	[46]
MerR (*Staphylococcus aureus*)	Not specified	CueR	Mercury	Copper	[46]

[a]Abbreviations: ERK, extracellular signal-regulated kinase; IPTG, isopropyl β-D-1-thiogalactopyranoside; MAPK, mitogen-activated protein kinase.
[b]Comprehensive list of chimeric aTFs. When not indicated, domains are native to *E. coli*. The work of Dimas *et al.* (2019) is not included because of its highly combinatorial nature with 35 different variants [41].

1 and protein kinase A, were designed to study cell signaling [44]. However, only the TetR-Elk-1 chimera indicates a greater induction than a traditional hybrid system used to report cell signaling. Moreover, although domain swapping within the TetR family was demonstrated, these results suggest that approaches beyond rational design may be necessary to achieve efficient biosensing.

The LysR family, one of the largest aTF families, has also been targeted for domain swapping. De Paepe and colleagues reengineered the FdeR detector module to alter ligand specificity from naringenin to luteolin [45]. This was achieved by combining the DBD of FdeR with the LBD of LysR-family NodD1 using three different hinge domains: native FdeR's and NodD1's natives

and a mutated FdeR variant (Pro92Asn; from a rigid to an uncharged amino acid). All chimeras demonstrated increased specificity toward luteolin compared with native FdeR. However, the chimera with the native FdeR hinge domain exhibited high basal expression, whereas the one with the NodD1 hinge indicated a tighter repression. The mutated FdeR hinge provided the best balance of specificity and low leakiness, underscoring the importance of hinge domain length and flexibility.

Domain swapping has also expanded to less commonly used aTF families, such as the LuxR and MerR families. Functional chimeras were created by combining the DBD of the well-characterized LasR/pLasI with the LBD of MupR, a LuxR-like transcription factor involved in mupirocin biosynthesis [10]. Although activation did not reach the level of native LasR, the ability to activate transcription in heterologous hosts allowed the discovery of novel biosynthetic gene clusters and new natural products.

In a separate effort, Ghataora and colleagues, aiming at bypassing the incompatibility of biological parts, developed MerR chimeras [46]. They combined MerR DBD with the LBDs of ZntR and CueR, responsive to mercury and copper, respectively. The MerR-ZntR chimera was constructed using sequence alignment and validated with structural homology. A second MerR-ZntR chimera containing an Ala29Glu mutation to maintain active residues was then constructed, showing improved reporter expression. Similarly, the MerR-CueR chimera with a targeted mutation demonstrated enhanced activation. These findings highlight the value of structural analysis and homology models when building hybrid proteins.

Previous examples focused on engineering the detector module to change ligand specificity. However, further improvements, such as minimizing crosstalk or improving dynamic range, can be achieved by modifying the effector module. For instance, transcriptional tuning via operator region redesign and translational control via RBS optimization have proved effective [42,43,45].

As previously mentioned, the examples presented here are all based on intrafamily domain swapping. Although chimeras between aTFs and other types of proteins have been demonstrated [47–51], to our current knowledge, successful interfamily domain swapping has yet to be reported.

Domain swapping within the detector module offers a strategy to harness aTFs with known ligands but with uncharacterized TFBS or modes of action. However, similarly to TCS, identifying optimal fusion points between LBD and DBD remains a major bottleneck. No universal pattern to build novel chimeric aTFs has emerged either, and current strategies rely on case-by-case design and experimentation, limiting speed and scalability of the approach. As an alternative to rational design, high-throughput and combinatorial approaches can be considered to guide the design and screening of improved chimeric aTFs. On the one hand, general screening methodologies, such as selecting the best biosensing mechanism by comparing induced and noninduced conditions, are similar to those described in the previous section for TCSs. On the other hand, high-throughput strategies have been used to identify optimal promoter and RBS combinations in native aTFs [52,53] and to explore linker libraries during the construction of chimeric aTFs [48]. One strategy commonly used to improve native aTFs that could be employed for the construction of chimeric aTF libraries is the coupling of random mutagenesis with a screening method, such as growth-couple selection, to identify sensors with enhanced dynamic range [54].

Moreover, a recurring theme across successful designs is the critical role of structural and protein alignment analysis coupled with computational tools, which are essential for guiding rational design and exploiting chimeric aTFs' full potential.

Real-world implementation of MWCBs: where are we?

MWCBs based on chimeric signaling proteins offer a versatile platform for constructing novel and on-demand biosensors, particularly for detecting ligands associated with poorly understood mechanisms. However, in the aforementioned examples, the application of chimeric MWCBs remains limited largely to laboratory use, primarily for studying molecular mechanisms of signal transduction or gene regulation or for optimizing microbial strains. The limited translation to real-world scenarios is likely due to the molecular and structural complexity of these systems, as well as the absence of standardized platforms for modular and scalable design.

Nonetheless, several promising examples demonstrate the potential of MWCBs for real-life deployment, especially in diagnostics and environmental monitoring. Mimee *et al.* engineered the probiotic *E. coli* Nissle strain to detect heme for diagnosing gastrointestinal bleeding in swine [9]. This biosensor was integrated into an ingestible microbioelectronic device **(IMBED)** equipped with a wireless transmission system and patented (WO2017008018A1) (Table 3). A later and smaller version of this prototype, optimized for ingestible size constraints, enabled detection of various biomarkers, including hydrogen peroxide, nitric oxide, tetrathionate, and thiosulfate [55]. Demonstrating that IMBED can be used with various genetic circuits, this example sets a major achievement for the integration of MWCBs with a prototype.

Table 3. Intellectual property and MWCBs[a]

Number	Filing year	Application	Information	Status	Refs
US5972638A	1997	Security	Microbial biosensor for detection of chemicals from explosive	Expired	[88]
WO2003102223A1	2003	Environmental monitoring	Microbial biosensor for arsenic.	IP right grant	[89]
CN102796693A	2012	Environmental monitoring	Microbial biosensor for arsenic bioavailability	Pending	[90]
WO2014012089A1	2013	Environmental monitoring	Microbial biosensor for organic and inorganic arsenic	Application filing	[91]
KR101898178B1	2016	Strain optimization	Microbial biosensor to measure the production of glutamate	Active	[92]
WO2016133830A1	2016	Environmental monitoring	Microbial microfluidic biosensor to monitor toxin level in water	Application pending	[93]
WO2017008018A1	2016	Medical diagnostics	Microbial biosensor for in vivo detection of internal bleeding	Application filing	[94]
WO2017049182A1	2016	Medical diagnostics	Microbial biosensor for nitrate detection and gut inflammation	Application filing	[95]
WO2017196983A1	2017	Screening for novel biologicals	Microbial biosensor for high-throughput detection of polyketides	Application filing	[96]
WO2019113075A2	2018	Sensing of hormones	Microbial biosensor based on transcription factor-FRET	Application filing	[97]
CN110283764A	2019	Strain optimization	Single-cell biosensor for detecting L-cysteine output of *E. coli*	Pending	[98]
CN111690647B	2020	Strain optimization	Biosensor for pyruvic acid to dynamic control of intracellular metabolic flow	Active	[99]
CN113005070A	2021	Security	Microbial biosensor with self-luminous operon for explosive molecules	Active	[100]
US20230404472A1	2022	Medical diagnostics	Wearable device containing microbial biosensor for pathogen detection	Abandoned	[101]
WO2023201243A1	2023	Medical diagnostics	Microbial biosensors that may include a promoter able to sense marker for gut inflammation	Application filing	[102]
CN116121285B	2023	Strain optimization	Biosensor for 2-pyrrolidone high yield strains	Active	[103]
US20240199704A1	2024	Genetic engineering	Microbial biosensor consisting of an operon to control different biochemical pathway	Active	[104]

[a]Nonexhaustive list of patents based on microbial biosensors. *Important note*: Certain parts of the patent text were not always available in English, and that filing year on the patent text does not always align with the year shown as part of the reference.

In the environmental domain, exoelectrogenic bacteria, capable of producing electrical current, have been used to construct the portable bioelectronic sensing system **BESSY** [56], one of the first portable devices incorporating MWCBs. Similarly, Atkinson *et al.* engineered *E. coli* to generate an electrical current in response to thiosulfate, a pollutant associated to algal blooms, and 4-hydroxytamoxifen, an endocrine disruptor [57].

Paper-based tests that provide a colorimetric readout using MWCBs have also been developed. Wu and colleagues designed a paper strip embedded with engineered bacteria for point-of-care detection of bacterial pathogens in water. In this system, bacteria regulate through a genetic circuit the expression of lycopene, a visible red pigment, upon sensing *N*-acylhomoserine lactone (AHL) [58]. A similar test was developed by Ma and coworkers for on-site detection of organophosphorus pesticides [59]. Chang *et al.* prototyped a colorimetric assay using their **EMeRALD receptor platform** (engineered modularized receptors activated via ligand-induced dimerization) to detect bile acids in patient serum [60].

In addition to colorimetric outputs, fluorescent or bioluminescent reporters are used to facilitate MWCB readouts. Wang *et al.* developed a microbial biosensor for 1,3-dinitrobenzene (1,3-DNB), a TNT (2,4,6-Trinitrotoluene) by-product associated with landmines, embedded in a luminescence-based detection system [61]. Chen *et al.* created a 3D printed living composite hydrogel containing engineered *E. coli* with fluorescent reporters to detect ionizing radiation [62]. Although lacking a fully developed prototype, it is worth mentioning that their study examined survival rate and integration within a functional matrix using scanning electron microscopy.

Robustness is one of the key characteristics that makes microorganisms stand out to construct novel biosensors. Although tests with environmental samples have been shown to maintain biosensing capability in laboratory settings [63,64] and in simulated conditions [65], performance in real-world applications remains largely unproved. Beyond the survival and stability of the biosensor organism, a key challenge is the specificity of detection in complex environments. In use-case scenarios, MWCBs may encounter diverse and potentially interfering signals, which can hinder accurate recognition of the target stimulus. Addressing this requires a better understanding of sensor cross-reactivity, background noise, and the influence of environmental matrices on sensing fidelity. One example that selects the sensing mechanism on the basis of the final application setting is the work of Robinson *et al.* that curated a library of inflammation-responsive TCS in *E. coli* and screened the optimal performer directly in mouse gut [66]. In this context, directing research efforts to engineering systems that naturally exist in the final application setting can harness the power of inherent robustness of cells.

One of the few MWCBs known to have undergone field testing is *E. coli* DH5α-2697, which expresses the transcription factor ArsR and the *luxCDABE* operon for luciferase activity under the control of its cognate promoter. This biosensor, named ARSOlux, was used in rural Bangladesh to assess groundwater arsenic levels [8]. A similar project involved a *Bacillus subtilis* arsenic biosensor engineered with three containment strategies (one biological, two physical), designed for deliberate yet contained release under UK regulatory oversight [67]. However, because of regulatory discrepancies, the project was ultimately abandoned. Although regulatory barriers persist, field testing with microbial sensors is gaining traction. A recent example is the biosensor developed by Chemla and colleagues, which incorporates hyperspectral reporters and holds promise for a wide range of applications [68].

Concluding remarks

TCSs and aTFs serve as ideal candidates for constructing chimeric signaling proteins because of their well-defined and modular structures. In this review, we have highlighted common practices used in their construction, with a primary focus on rational design approaches. However,

Outstanding questions

How can high-throughput methods be developed or adapted to accelerate the design-build-test-learn cycle for chimeric MWCBs?

To what extent can predictive computational tools and artificial intelligence–based protein design reduce the empirical burden in the engineering of synthetic MWCB chimeras?

How can we develop universal standards or semantic frameworks for naming biosensor components, fusion proteins, and genetic architectures to improve literature searches and data interoperability?

What are the most promising strategies for improving the genetic stability and long-term functionality of MWCBs outside of laboratory conditions?

How can the biological part of biosensors (bioware) be better integrated with hardware and software to enable real-time, field-ready sensing platforms?

What are the best strategies for designing MWCBs that are resilient to variable environmental conditions, including pH, temperature, and contaminant load?

What are the current technical and biological limits of multiplexing chimeric MWCBs, and how can crosstalk and signal interference be minimized in complex sensing environments?

How can regulatory frameworks evolve to accommodate emerging applications of physically contained **genetically modified organisms (GMOs)** used outside of laboratory settings (i.e., 'contained release')?

In what ways can MWCB-based sensing systems be made accessible, user-friendly, and safe-and-sustainable-by-design for nonexpert users, including regulators, clinicians, or communities affected by pollution?

What role could international collaboration play in establishing open databases, shared repositories, and harmonized standards for biosensor parts and performance benchmarks?

incorporating high-throughput methods [53,54,66] into chimeric TCS and aTF design and screening would be highly beneficial. A standardized pipeline for such approaches, enabling streamlined cloning, screening and characterization, remains a key outstanding need (see Outstanding questions).

Moreover, the design of chimeric signaling proteins, for both TCS and aTFs, should be complemented by computational approaches. These offer significant advantages in bioprospecting, for example, by aiding the identification of novel aTF ligand-binding domains using tools such as DeepTFactor [69] and Sensbio [70]. Computational approaches can also support mutagenesis prediction, RBS optimization, and gene copy number tuning, ultimately accelerating the design-build-test-learn cycle.

Although MWCBs and chimeric-based MWCBs hold great promise across various applications, several technical, biological, and regulatory challenges continue to hinder their real-world deployment (Box 3). These limitations underscore the need for targeted research that considers the intended application setting from the ideation stage onward. This includes integrating biosensor performance with considerations of safety, as done by Tang and coworkers [71], alongside microbial viability, genetic stability, and optimal storage conditions. Promising preservation strategies, such as spore formation [72], lyophilization [73], and hydrogel encapsulation [62,71,74], have already been explored but are yet to be evaluated in real-life scenarios.

Moving forward, the development of clear, actionable guidelines for the use of genetically modified microorganisms, including specific criteria for contained release and associated (bio)containment measures, will greatly facilitate the translation of MWCBs from lab to market. Realizing the full potential of MWCBs will demand coordinated contributions from engineers, (synthetic) biologists, microbiologists, data scientists, and regulators alike. By establishing a multidisciplinary pipeline that encompasses technical development, integration, validation, and regulation,

Box 3. Challenges of chimeric MWCBs

Despite their great potential, chimeric MWCBs face multiple hurdles that limit their broader implementation in real-world applications. The main obstacles can be grouped into the following areas:

- *Biological challenges:* The lack of a standardized platform for high-throughput design, assembly, and screening of biosensor components remains a major bottleneck. Both bioprospecting and iterative DBTL (design-build-test-learn) cycles are time- and resource-intensive, particularly when developing MWCBs for novel analytes in specific application settings.
- *Semantic challenges:* Although MWCB is the most accepted term, alternative nomenclature, such as living biosensor, genetically encoded biosensor, or bioreporter, remains in use. Furthermore, inconsistencies in naming biological parts (e.g., different names for DBD and LBD), chimeric proteins (e.g., hybrid, synthetic), or even the formatting of fusion constructs (e.g., new name, EnvZ-OmpR, EnvZOmpR, EnvZ:OmpR) hinder literature searches and comparative analyses.
- *Technical limitations:* Although MWCBs enable real-time *in situ* sensing, the development of portable devices capable of continuous monitoring, whether through optical or electrochemical outputs, is still in its infancy. Although some promising prototypes exist, most have been validated only under laboratory conditions.
- *Integration issues:* Studies combining MWCBs with electronic or optical systems are too scarce to provide clear design guidelines. Although stability of biosensing mechanism over time was considered for some applications [5,74,84], aside from a few efforts [62,64,65,71,72,85], there is a lack of comprehensive research into the genetic stability, viability, long-term robustness, and storage of MCWBs integrated with their respective devices.
- *Regulatory challenges:* Current regulations in the EU distinguish between contained use (Directive 2009/41/EC) [86] and deliberate release (Directive 2001/18/EC) [87], leaving a gap around 'contained release' [67]: that is, the use of physically contained GMOs outside traditional laboratory settings. This regulatory gray area affects many MWCB applications. Moreover, regional variations and ambiguities can prolong assessment timelines and complicate approvals.
- *Other considerations:* Key parameters such as shelf life and microbial escape frequency are often overlooked. The absence of evaluation guidelines for these factors impedes commercialization efforts.

MWCBs can be robustly deployed across a wide range of real-world applications. In particular, chimeric MWCBs, because of their modularity and adaptability, could become the cornerstone of next-generation biosensing platforms, provided they are developed with a mindset that bridges molecular design and deployment reality.

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Declaration of interests

V.A.P.M.d.S. has interests in LifeGlimmer GmbH. The other authors have no interests to declare.

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