Biosensing strategies using recombinant luminescent proteins and their use for food and environmental analysis

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The first time María Cruz and we thought about writing a review like this was shortly before she passed away. Two years later, the harshness of life puts us in a different situation. We wanted to carry out this review in honor of María Cruz and the idea we had together, but never got to complete. We wrote this paper for her, us, those who were there, and those who came after her departure, hoping it would meet her demands and rigor.

Abstract

Progress in synthetic biology and nanotechnology plays at present a major role in the fabrication of sophisticated and miniaturized analytical devices that provide the means to tackle the need for new tools and methods for environmental and food safety. Significant research efforts have led to biosensing experimenting a remarkable growth with the development and application of recombinant luminescent proteins (RLPs) being at the core of this push. Integrating RLPs into biosensors has resulted in highly versatile detection platforms. These platforms include luminescent enzyme-linked immunosorbent assays (ELISAs), bioluminescence resonance energy transfer (BRET)-based sensors, and genetically encoded luminescent biosensors. Increased signal-to-noise ratios, rapid response times, and the ability to monitor dynamic biological processes in live cells are advantages inherent to the mentioned approaches. Furthermore, novel fusion proteins and optimized expression systems to improve their stability, brightness, and spectral properties have enhanced the performance and pertinence of luminescent biosensors in diverse fields. This review highlights recent progress in biosensing, showcasing their implementation for monitoring different contaminants commonly found in food and environmental samples. Future perspectives and potential challenges in these two areas of interest are also addressed, providing a comprehensive overview of the current state and a forecast of biosensing strategies to come using recombinant luminescent proteins.

Keywords: fluorescent proteins, luciferases, luminescent proteins, biosensors, biosensing, phage display technology, mycotoxins, allergens, antibiotics, pesticides.

1. Introduction

Food security and sustainable environmental management are fundamental elements that ensure the wellness of living beings [1]. Both aspects are prioritized in the 2030 Agenda for Sustainable Development as they directly concern the Sustainable Development Goals (SDGs), particularly those related to hunger, health, and climate action. The consumption of spoiled food and contaminated water facilitates the creation of a vicious cycle of disease and malnutrition. According to the World Health Organization (WHO), hundreds of diseases such as sepsis, diarrhea, allergies, autoimmune diseases and even cancer, can be linked to the intake of food and water polluted with natural toxins, microorganisms, viruses, parasites or chemicals of artificial origin. The incidence of these maladies is especially blistering in less favored countries, where children and elders are the most affected population groups [2]. Several factors, including poor hygienic practices, inadequate infrastructure, and limited food and environmental safety monitoring contribute to the higher risk of disease in these locations. It is also important to note that, beyond the health risk, the lack of controls also leads to severe economic losses.

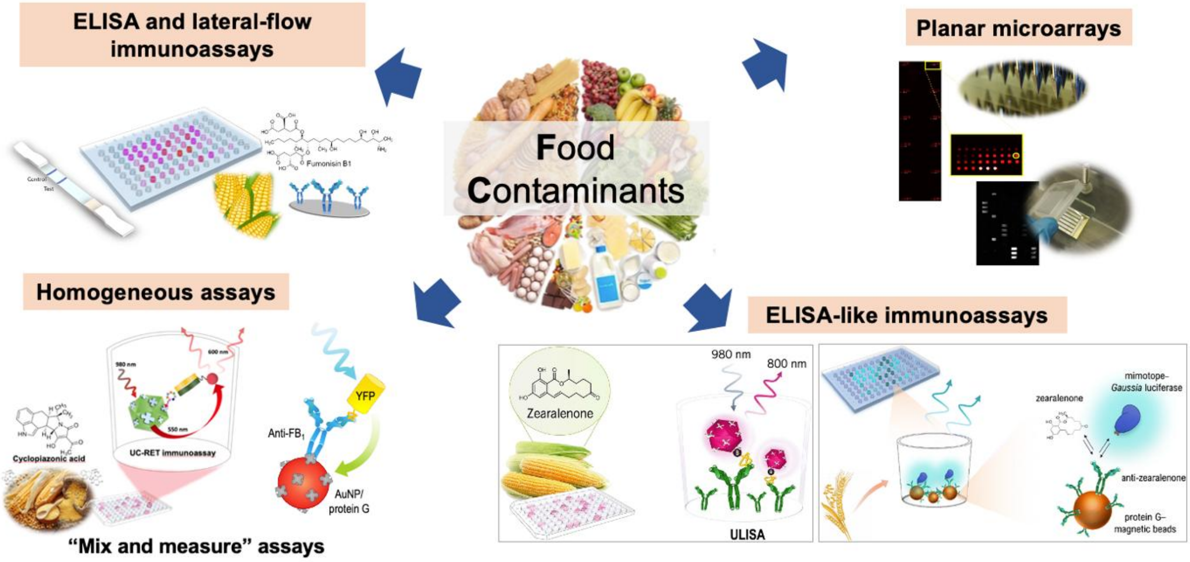

Figure 1. Some graphical examples of biosensors and biosensing platforms used for the analysis of contaminants in food and environmental samples.

Currently, most of the undesirable substances are determined by chromatographic separation techniques. However, agri-food and consumer sectors demand new detection strategies to comply with the increasingly strict international legal framework. Therefore, the commercialization of rapid, simple and inexpensive screening methods that can be used by non-specialized personnel and at the place where the food or the environmental alert occurs (silos, distribution chains, reservoirs, etc.) is highly desirable [3]. In this regard, biosensors and bioassays become essential tools. These devices are based on the specific recognition of the analyte by a receptor or recognition element of biological origin, and a physicochemical transducer that transforms the recognition event into a useful analytical signal [4]. Today, biosensing platforms and diagnostic devices have different designs and performances (**Fig. 1**). Although several factors determine their effectiveness, the receptor and transducer are crucial. Hence, these devices are often classified according to the type of receptor or recognition element they use, such as immunosensors based on antibodies or aptasensors based on aptamers. Progress in biosensing alongside advances in molecular biology and biotechnology has favored the development of new bioanalytical tools with improved features including sensitivity, multiplexing capability, response time and size. For instance, using proteins with exceptional optical properties revolutionized the existing analytical methods for controlling food and environmental contaminants [5].

Fluorescent proteins and luciferases are recombinant luminescent proteins (RLPs) which have become pivotal tools in modern bioscience. These proteins, renowned for their ability to emit light directly or catalyse chemiluminescent reactions, respectively, have deeply transformed not only the way researchers study biological processes in real-time but also biosensors. By harnessing the unique properties of fluorescent proteins and luciferases, scientists have created biosensing approaches that offer high sensitivity, specificity, and versatility in detecting, for example, contaminants within complex food and environmental samples. Consequently, the use of RLPs has enabled the development of sophisticated sensors for monitoring diverse, relevant targets ranging from small molecules to complex proteins and other macromolecules or pathogens [5, 6]. Traditional fluorescent proteins such as the well-known green fluorescent protein (GFP) and its derivatives, are used extensively due to their ability to emit visible light upon excitation with light of a specific wavelength range. Fluorescence allows visualization and tracking of molecular interactions and their concentration within living cells. Through genetic engineering, fluorescent proteins can be fused as optical probes to other proteins of interest such as antibodies, creating fusion constructs that change their fluorescence in response to the specific binding to their corresponding targets [7]. Furthermore, they can be fused to small peptides mimicking standard labelled antigens to be used in competitive assays [8]. Luciferases, on the other hand, are enzymes that catalyse the production of chemiluminescence (“bioluminescence”) through the oxidation of a substrate, typically luciferin. Chemiluminescence is highly sensitive and can be detected with high precision even at very low concentration of substrate [9]. This makes luciferase-based biosensors particularly useful for monitoring small molecules in various biological contexts. By coupling luciferases to specific binding domains or receptors, researchers can design biosensors that emit light in response to the presence of a target molecule, enabling quantitative analysis of molecular interactions and concentrations [10].

In this review, we discuss advances in biosensing for food safety and environmental control (**Table 1**) from the last five years based on recombinant luminescent proteins, an interesting type of biomolecules that boasts unlimited potential owing to their exquisite and diverse optical properties.

2. Background and perspectives on genetically encoded biosensing

2.1. Fundamental concepts on fluorescent proteins (FPs)

Fluorescent proteins (FPs) belong to a class of structurally homologous proteins that, from three amino acids in their polypeptide sequence, are capable of autocatalytically forming a luminophore that emits in the visible region of the electromagnetic spectrum (380-750 nm). Shimomura *et al.* [11] isolated the first member of the family in the 1960s, namely the green fluorescent protein (GFP), from the jellyfish *Aequorea victoria* (avGFP). From this precursor, many artificial variants have been developed and several other luminescent proteins have been isolated from living organisms such as corals, ascidians, sea pens, and anemones [12].

Regardless of their species or degree of manipulation, all FPs possess a size of approximately 25 kDa. They feature a barrel structure formed by 11 β-sheets linked by α-helices, with a diameter of approximately 2.4

nm and a height of 4.2 nm [12]. The correct folding of the barrel structure is required to form the autocatalytic chromophore with fluorescent properties. The chromophore is protected within the barrel, which increases its stability and isolates it from the medium, preventing deactivation by molecular oxygen or other reactive oxygen species. Specifically, in the case of GFP, the chromophore is formed from three adjacent amino acids, including serine 65 (Ser65), tyrosine 66 (Tyr66) and glycine 67 (Gly67). Following proper folding of the protein, cyclization occurs due to nucleophilic attack of the amino group of Gly67 on the carbonyl group of Ser65, followed by oxidation of the cyclic intermediate (**Fig. 2A-i**) Furthermore, site-directed mutagenesis of these amino acids allows obtaining variants that exhibit other colors [13]. For example, the substitution of Tyr66 by histidine or tryptophan yields blue (Azurite) and cyan (AmCyan) mutants, respectively. However, these modifications of avGFP cannot be used to access the entire palette of colors. In consequence, other fluorescent proteins of different origins are required for this purpose. For example, the red fluorescent protein (DsRed) is produced by the *Discosoma sp.* coral. Its synthesis requires an additional step compared to the maturation of avGFP which involves the extension of the π-conjugated system of the three amino acids that form the chromophore (**Fig. 2A-ii**) [14]. The discovery of this protein led to the development of fluorescent proteins that emit at longer wavelengths than their predecessors, with emission maxima of up to 655 nm. Mutations have been induced not only to vary protein colors, but also to increase brightness, improve folding and the associated folding time, avoid oligomerization, accelerate chromophore formation, or to improve photostability and pH resilience [15, 16].

Fluorescent proteins can be found in a wide variety of applications, being employed as universal genetically encoded fluorescent tags. In particular, they are often relied upon to monitor gene expression in bacteria, for live cell or organism imaging, or in FRET systems where different proteins are used as donors and acceptors, or for the development of bioassays and biosensors [15, 17–20].

2.2. Fundamental concepts on luciferases

Luciferases are bioluminescence catalysts and, unlike fluorescent proteins, they do not need excitation light to produce luminescence; instead, they require the presence of an appropriate substrate. The bioluminescent chemical reaction catalyzed by luciferases involves visible light generation upon oxidation of their substrates (luciferins). The most studied luciferases are produced by fireflies (Firefly luciferase or FLuc), sea pansies (*Renilla* luciferase), copepods (*Gaussia* luciferase), and the deep-sea shrimp *Oplophorus gracilirostris* (NanoLuc). The bioluminescent reaction also takes place in other living organisms such as jellyfishes, click beetles, bacteria and fungus species. The required substrate depends on the type of luciferase, as depicted in **Fig. 2B**. In addition to the substrate, some luciferases (e.g. firefly and click beetle luciferases) use adenosine triphosphate (ATP) and magnesium ions (Mg^{2+}) as cofactors.

The bioluminescence of the luciferase enzyme from the North American firefly *Photinus pyralis* (FLuc) has been thoroughly studied since the early 20th century. FLuc is a 62 kDa protein that reacts with D-luciferin in the presence of ATP, O_2, and Mg to yield light at approximately 560 nm [21]. It is worth noting that no redox cofactor (e.g. FAD or NADH) is required for the oxidation reaction to occur (**Fig. 2B-iii**). Other beetle luciferases, including firefly luciferases, can exhibit emission peaking at other wavelengths even if they use the same substrate (D-luciferin) and are highly homologous enzymes. The colour of the emitted light can vary from yellow-green (firefly) to orange (click beetle) and to red (railroad worms) depending on the chemical structure of the enzymatic oxidation product (or the relative contributions of different chemical forms). The precise mechanism that accounts for this dependence is under debate and yet to be validated by conclusive evidence [22].

In addition to FLuc purified by its natural source, recombinant wild-type and mutant variants have been obtained. The recombinant luciferase expressed from a cloned gene from *Photinus pyralis* is commercially available, with Promega Corp. QuantiLum® being a well-known product. Other synthetic luciferases showing enhanced properties for bioanalytical applications have been developed. The YY5 FLuc mutant with enhanced activity and thermostability [23], the orange-emitting luciferase BoLuc for high sensitivity bioassays, and the red-emitting luciferase BrLuc with multiplexing capability [24] are some recent examples. *Renilla* luciferase (RLuc) is a medium-sized protein (36 kDa) produced by *Renilla reniformis*. RLuc does not share amino acid sequence similarity to FLuc, uses coelenterazine as a substrate, and produces blue light (480 nm) independently from ATP (**Fig. 2B-iv**). Engineered *Renilla* luciferases with enhanced stability, high quantum yield and even a red-shifted spectrum have been created, namely RLuc8 [25] and SuperRLuc8 [26]. These variants further increase the use of *Renilla* luciferases as bioluminescent reporters in cellular and biomedical research, particularly for bioimaging applications. In contrast to FLuc and RLuc, which are intracellular proteins, GLuc from *Gaussia princeps* and NanoLuc are secreted luciferases, meaning that they can be detected directly in the cell culture medium within which they are expressed without cell lysis. GLuc is a natural small protein (20 kDa) that shows bioluminescence at 470 nm upon coelenterazine oxidation (**Fig. 2B-v**). NanoLuc is an engineered luciferase created in 2012 by Promega Corp. from the 19 kDa catalytic subunit of the *Oplophorus gracilirostris* luciferase. It uses a synthetic analogue of coelenterazine, furimazine, as the substrate (**Fig. 2B-vi**). The enzyme possesses high structural stability, improved luminescence intensity, and displays glow kinetics (emits blue light with a half-life > 2 h) with a specific activity approximately 150-fold higher than those of FLuc and RLuc [27]. The extreme brightness and small size of NanoLuc make it a perfect partner for protein fusion and expression, increasing its usefulness in live-cell imaging and biosensing applications.

Luciferase bioluminescence in biosensing strategies provides key advantages such as negligible background, broad dynamic range and simplified instrumentation demand as excitation light sources are not required. Enhanced sensitivity is achieved within complex sample matrices since the background signal is not affected by autofluorescence, in contrast to fluorescence-based biosensing methods.

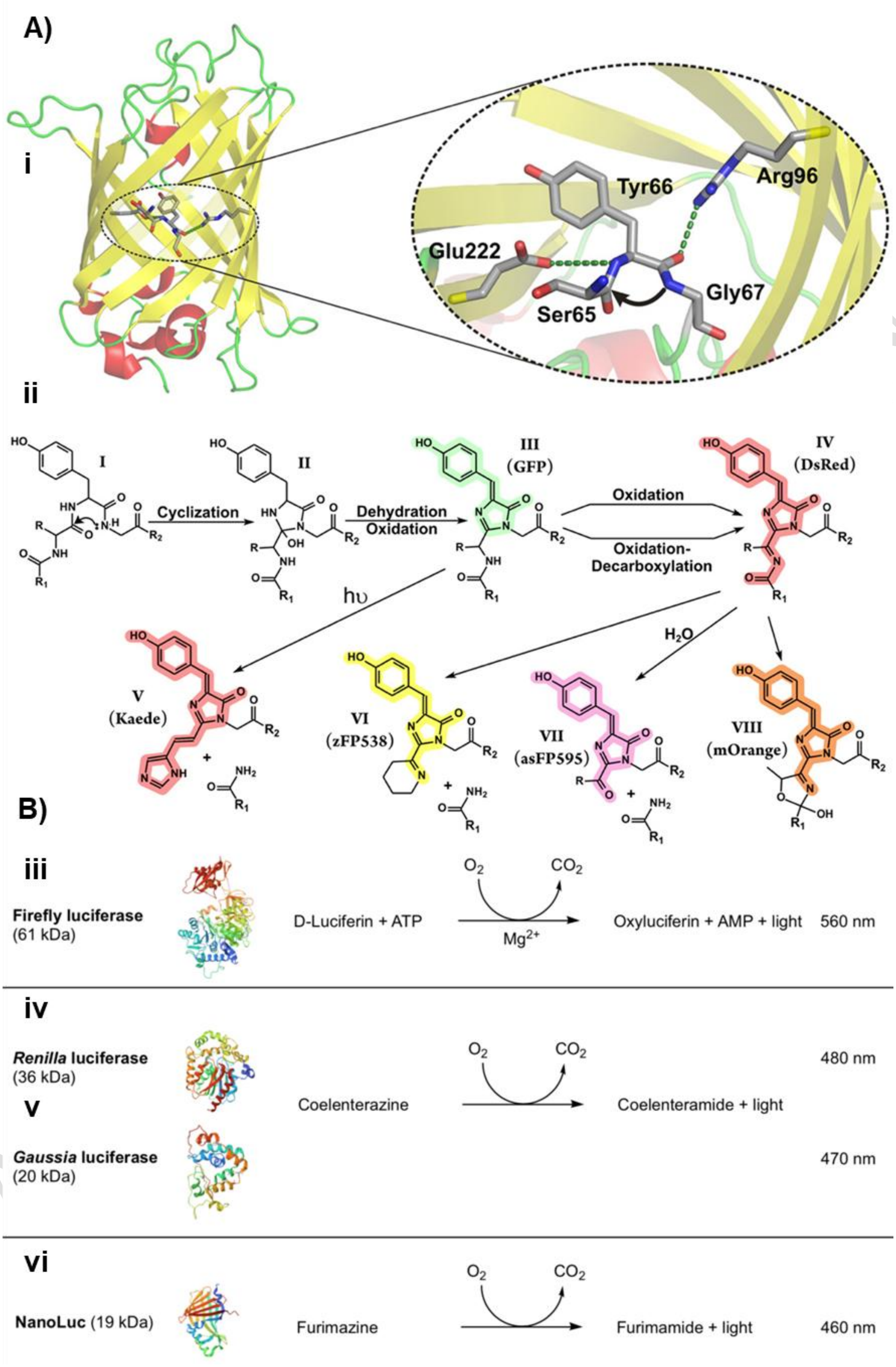

Figure 2. A) Post-translational reactions and representation of the chromophores of the GFP family proteins. i: Barrel-shaped structure of GFP with amino acids forming part of the autocatalytic chromophore. ii: Biosynthesis of the chromophores and diversity of their structures; π-electron conjugated systems are shown in the color of their emission. Reprinted (after adaptation) with permission from [28]. Copyright (2016) Elsevier. B) Bioluminescent reactions of common luciferase enzymes and their maximum emission wavelength. iii: Firefly luciferase from *Photinus pyralis* (FLuc), (PDB 1LCI [29]). iv: Renilla luciferase from *Renilla reniformis* (RLuc) (PBD 2PSJ [25]). v: Gaussia luciferase from *Gaussia princeps* (GLuc) (PBD 7D2O [30]). vi: NanoLuc luciferase from *Oplophorus gracilirostris* (NLuc) (PBD 5IBO [31]). Images from the RCSB PDB (RCSB.org).

2.3. Biosensing configurations using recombinant luminescent proteins

Genetically encoded luminescent biosensors can be classified according to different configurations and assay formats, i.e. the way in which the recognition element and the analyte interact, the detection methodology used to perform this interaction, and the nature of the recognition element or transduction system [5, 32, 33]. To simplify the classification, three main categories will be discussed: single RLP-based sensors, Förster and bioluminescent resonance energy transfer (FRET and BRET) biosensors, and protein-fragment complementation assays (PCA).

2.3.1. Single RLP sensors

In single FP approaches, the reaction to the stimulus for fluorescence intensity changes tends to be greater, thus yielding more sensitive responses. Single RLPs are typically easier to express because the codifying sequence is shorter [5]. This is particularly important when the proteins are fused to large recognition elements or are intended to be expressed *in vivo*. Moreover, multiplexed assays can be more effectively designed given the many luminescent proteins available and their different emission wavelengths [5, 6]. As a drawback, this format provides intensiometric signals that can be affected by small variations in the biosensing procedure, making them less robust than FRET/BRET assays.

Typically, single RLPs consist of three components: a circularly permuted fluorescent protein (cpFP), a ligand-binding domain, and ligands connecting the two domains. Conformational changes associated with the analyte-ligand interactions are transferred to the cpFP, changing the chromophore environment and, therefore, the fluorescent properties (intensity, lifetime, etc.) [34]. Nevertheless, developing biosensors with analyte-specific fluorescence response is challenging and laborious optimization and large-scale variant screening are required [35].

In vivo sensing using engineered whole cells or phages based on single RLP has been reported [36, 37]. In such sensors, the gene codifying the luminescent protein is expressed continuously; however, the emission intensity changes upon addition of the target analyte owing to a decrease of the bacterial survival rate (**Fig. 3A**) or to a rise of it, as in the case of phage-based assays for bacterial detection [38, 39]. These sensor types have been applied to food safety screening and environmental monitoring [40–42]. The expression of the luminescent protein gene can be controlled if it is fused to a promoter regulated by the analyte of interest (**Fig. 3B–3E**). Since luminescent protein expression is highly specific, this format has extensively used for clinical, environmental and food safety field [36, 41, 43, 44]. *In vitro* heterogeneous assays are also widespread. Luminescent proteins fused at their C- or N-terminal end to recognition elements such as mimetic peptides [45–48], nanobodies, and DNA-binding proteins [49] can be readily produced. Different assay platforms have been developed to remove unbound luminescent tracers, e.g., microtiter plates, magnetic particles or lateral flow assays (**Fig. 3E**).

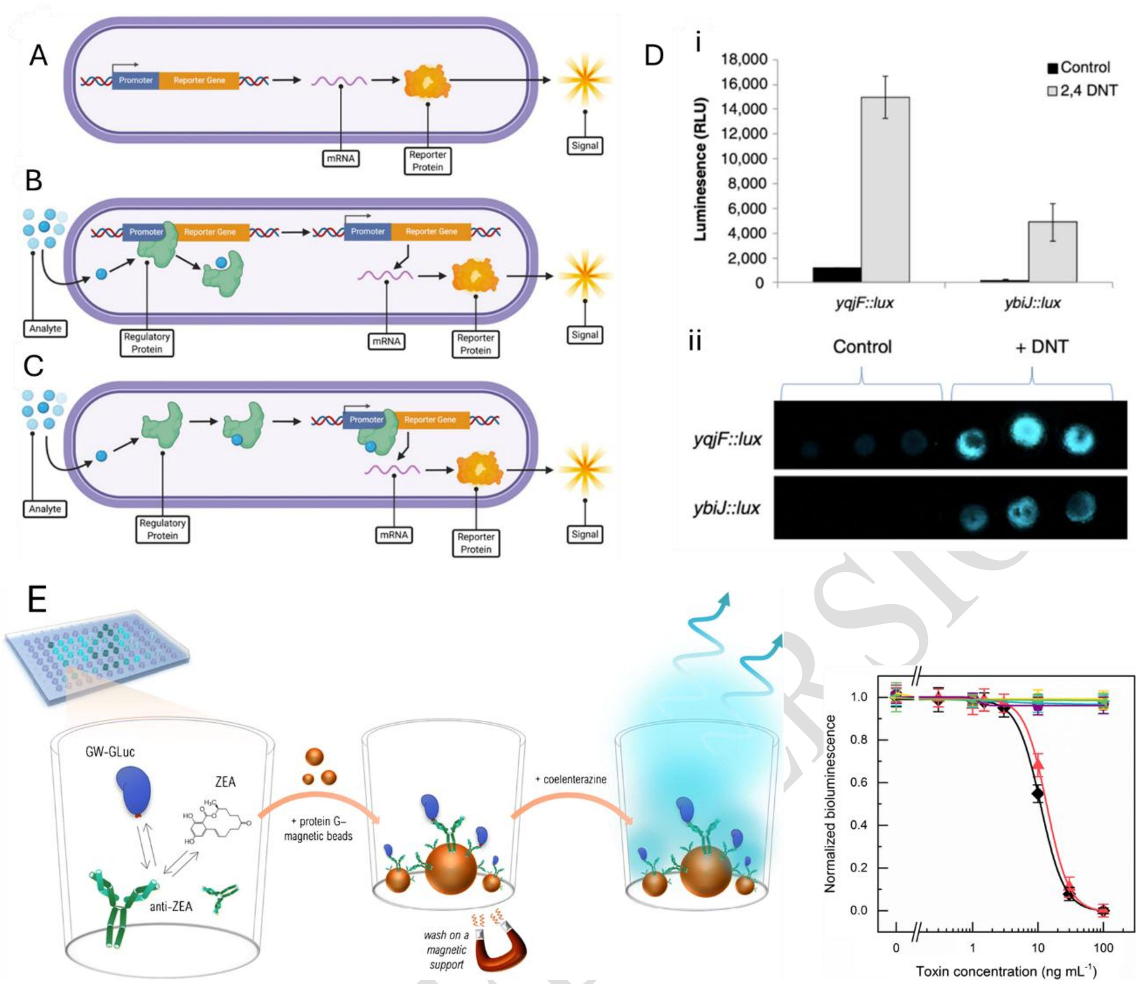

Figure 3. Microbial whole-cell biosensors (MWCB) sensing strategies. (**A**) No regulation of reporter expression. (**B**) Inducible MWCB with negative regulation of the reporter. The regulatory protein inhibits expression of the reporter via the promoter. Binding of the analyte to the regulatory protein releases it from the promoter, thus inducing expression of the reporter. (**C**) Inducible MWCB with positive regulation of the reporter. The regulatory protein-analyte complex binds specifically to the promoter, inducing expression of the reporter. (**D**) Detection of 2,4-dinitrotoluene (2,4-DNT) vapors by agar-immobilized cells carrying either a yqjF::luxCDABE or a ybiJ::luxCDABE fusion, in a closed chamber (i) or in soil (ii), following a 3.5-h exposure; (**E**) Bead-based bioluminescent immunosensor for different mycotoxins, namely ZEA (black diamonds), β-zearalenol (red triangles), fumonisins B$_1$ (yellow circles) and B$_2$ (blue triangles), deoxynivalenol (purple pentagons), and ochratoxin A (green down triangles). The total bioluminescence intensity of each well was measured after injecting native coelenterazine. Adapted reproduction with permission of references **[36]** and **[50]**. Copyright (2016) Elsevier and Copyright (2024) Springer Nature.

2.3.2. FRET/BRET bioassays

FRET is a technique based on the nonradiative energy transfer from a luminescent donor to a dye, nanoparticle or fluorescent protein acceptor [51, 52]. While contact is not required, the amount of energy transferred and, consequently, the acceptor emission intensity depends on the distance between the donor-acceptor pair. Usual working ranges in FRET are comprised within 1 and 10 nm. This effective distance largely depends on the relative orientation of the transition dipole moments of the donor and the acceptor, and on the spectral overlap between the donor emission and the acceptor excitation. Bioluminescence resonance energy transfer (BRET) is based on the same working principle than FRET; however, since the donor is a luciferase (substrate), it does not rely on external illumination. This independence makes BRET particularly well-suited for biosensing as it avoids the co-excitation of fluorescent molecules naturally present in complex matrices, and makes BRET-based schemes readily integratable in point-of-use devices [51, 53].

Despite the appealing properties of the FRET/BRET phenomena, platforms implementing them tend to be complex and often require extensive redesign and optimization to yield adequate results. Nevertheless, the ratiometric nature of the measurements allows for robust quantification of analytes independently of the photoluminescence signal intensity and the sample volume. An important consequence of the development of spectrally different luminescent proteins is the fact that both, donors and acceptors, can be used for working distances typically involved in biological assays and protein-protein interactions. The emission spectra of fluorescent proteins are usually broad but, by choosing the right pairs, even multiplexed analyses can be performed. For example, Suzuki *et al.* have reported five new spectral variants called "nano-lantern" (eNL), made by concatenation of NanoLuc with fluorescent proteins of diverse colors for multiplexed live-cell imaging [54]. Donor-acceptor pairs including a luminescent protein and a fluorescent nanoparticle or dye have also been reported [52, 53, 55].

Extensive literature on fusion proteins and FRET/BRET sensing featuring different configurations, such as intermolecular or intramolecular energy transfer and others (**Fig. 4**), is currently available. Some interesting sensing configurations using recombinant BRET biosensors are the LUCID (LUCiferase reporter of Intraluminal Deposition) [56] and the LUMABS (Luminescent Antibody Sensing Protein) [57] technologies for homogeneous immunoassays.

2.3.3. PCA bioassays

Protein-fragment complementation assays (PCAs) specifically split photoluminescent protein systems, allow bimolecular or trimolecular complementation that improves the ability of RLPs for protein labeling, and broaden their detection applications in biosensing [58–61]. Kerppola *et al.* proposed the concept of bimolecular fluorescence complementation (BiFC) by testing YFP [62]. BiFC is a technique that splits LPs into two non-luminescent polypeptide fragments, with complementation being assisted by interacting proteins fused to the different fragments [63]. The LP fragments reassemble as they approach to form the native protein and, consequently, recover the luminescence signal (**Fig. 4G**) [64]. BiFC has been developed for other fluorescent proteins such as GPF, GFP mutants and mCherry [60].

The original BiFC systems have some disadvantages as the reassembly is temperature sensitive and displays slow kinetics. Furthermore, the large size of the fragments interfere with the protein refolding and the solubility of the fusion protein [64]. To overcome these issues, a bipartite split FP system using GFP was proposed [65]. By splitting the protein into the smallest feasible fragment of GFP11 (residues 215-230) and a large fragment GFP1-10 (residues 1-214) (**Fig. 4H**), the intrinsic affinity between the two fragments was strong enough to ensure spontaneous binding without any assisting protein. In addition, the small fragment tag was easy to synthesize and had minimal effect on the folding efficiency and solubility of the original protein. The Waldo group [61] was also the first to design a tri-split GFP system (**Fig. 4I**) which, in this case, cannot self-assemble.

Among the split luciferase systems, we can find FLuc, Renilla RLuc, Gluc [58, 66] and NanoLuc including NanoBiT technology [67]. Recently a tripartite of NanoLuc has been successfully engineered (**Fig. 4J**) [68]. NanoBiT has proven to be a powerful system for sensing biological macromolecules such as antibodies [69], the

SARS-CoV-2 spike protein [70], and mycotoxins [71, 72]. Tripartite (**Fig. 4K**) applications of NanoLuc in biosensing can be found in ref. [59].

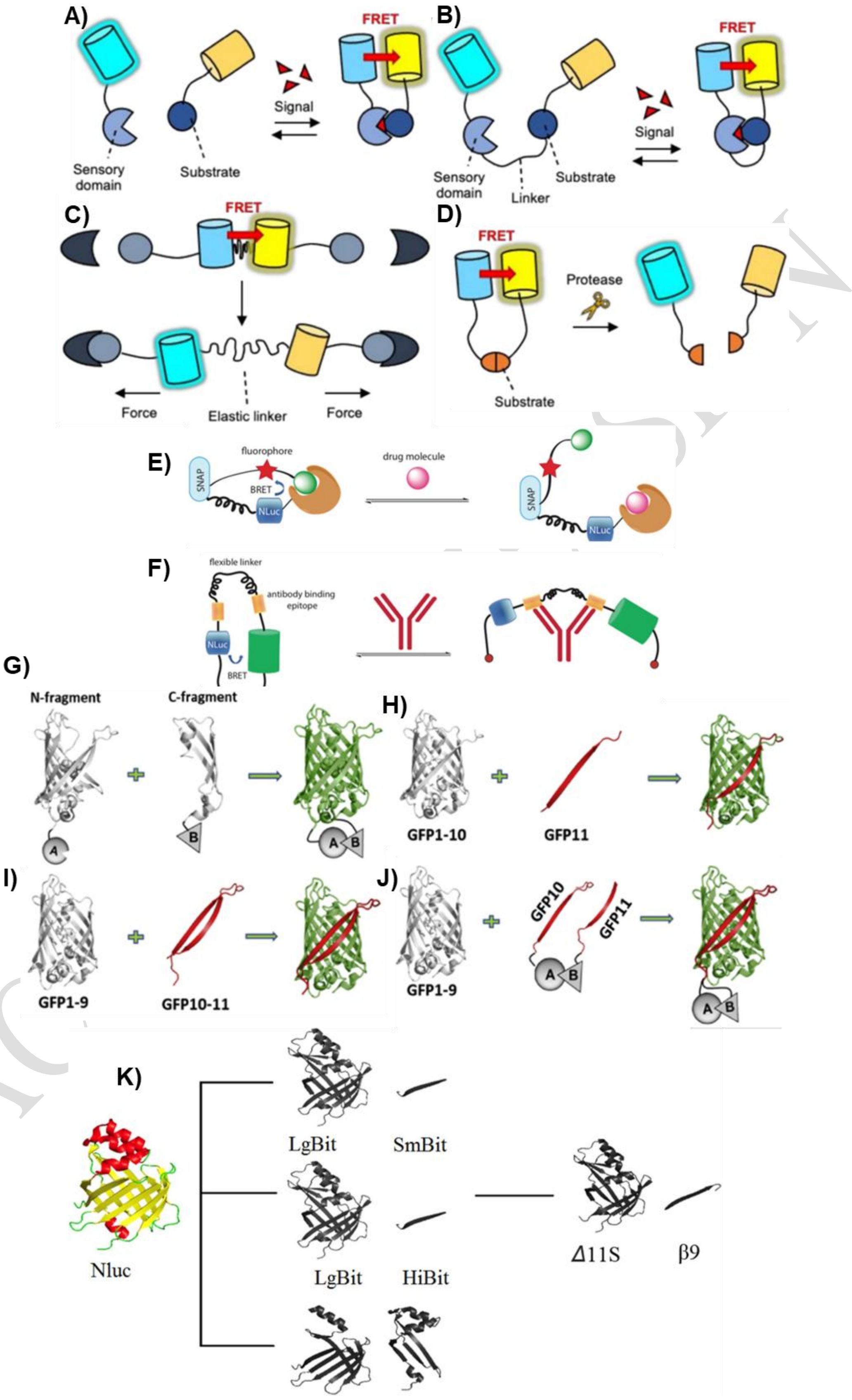

Figure 4. Designs of FRET/BRET biosensors. A) Representative design of an intermolecular FRET biosensor. The signal-induced interaction of the modified substrate and the sensory domain yields an increase of FRET between donor and acceptor FPs. B) Representative design of an intramolecular FRET biosensor; the signal-induced conformational change of the biosensor increases the FRET level. C) Design of a FRET-based tension sensor; its FRET level is designed to decrease by the applied tensional force. D) Design of a FRET-based protease sensor; the activated protease cleaves its substrate, resulting in lower FRET of the biosensor. Reprinted (adapted) with permission from [6]. Copyright (1996-2024) MDPI (Basel, Switzerland). E) LUCID technology developed by Johnsson and co-workers; BRET between NanoLuc and the fluorophore is disrupted by the presence of a drug molecule. F) LUMABS developed by Merkx and co-workers; BRET between NanoLuc and mNeonGreen is disrupted by the presence of an antibody. Adapted with permission from [85]. Copyright (2021) The Royal Society of Chemistry. G) Design principles of BiFC (H) and typical split GFP systems including (I) GFP1-10/11, (H) GFP1-9/10e11, and (J) GFP1-9/10/11. K) Split NanoLuc systems. Reprinted (adapted) with permission from [65] and [59]. Copyright (2021) The Royal Society of Chemistry and Copyright (2005) Nature Publishing Group, respectively.

3. Applications to food and environmental contaminants monitoring

3.1. Mycotoxins

Mycotoxins are a highly heterogeneous group of molecules that share four main features: they are produced by filamentous fungi, they are secondary metabolites, they are toxic to animals and humans, and they possess a low relative molecular mass (below 1000) [73]. They can cause diseases known as mycotoxicosis with a wide variety of effects, ranging from acute illnesses to chronic disorders. These contaminants are globally present in a multitude of crops and their occurrence is unavoidable. They are currently one of the most concerning food contaminants for global public health and are considered the most significant chronic food risk factor [74]. Moreover, they also have a significant economic impact, stemming from losses in sick livestock and contaminated crops. Many national and international institutions and organizations have recognized the toxic potential of mycotoxins and have established regulations for their control and maximum levels for those with the strongest impact. Currently, in the European Union, Regulation (EC) No. 2023/915 is in force [75]. Therefore, there is a high interest in the development and implementation of rapid analytical methods capable to guarantee food safety, regulatory compliance, early in-field detection of contaminants, and continuous monitoring of them throughout the food chain supply.

Ochratoxin A (OTA) is one of the most widespread mycotoxins worldwide and, consequently, numerous bioanalytical platforms are available for its detection, including RLP-based approaches. Since Liu *et al.* [7] isolated an OTA-selective VHH antibody (nanobody, Nb28) by phage display (general scheme in **Fig. 5A**), numerous immunoassays based on RLPs have been developed using this antibody and applied to the analysis of OTA in foodstuff**.** Wu *et al.* introduced a bioluminescent enzyme-linked immunosorbent assay (BLEIA) with the NanoLuc enzyme. In this approach, the nanobody (Nb28) and the reporter (NanoLuc) were conjugated post-translationally *via* the SpyTag/SpyCatcher bioconjugation system [76]. SpyCatcher was expressed as a 60-meric protein scaffold and the SpyTag peptide was produced with the Nb28 (Nb28-SpyTag) and NanoLuc (NanoLuc-SpyTag). The conjugation of multiple Nb28 and NanoLuc moieties led to an amplification of the analytical signal and a more sensitive BLEIA (LOD 0.113 ng mL^{-1}) compared to the previously described Nb28-NanoLuc RLP-based assay (LOD 3.7 ng mL^{-1}, **Fig. 5B**) [77]. Xie *et al.* described another bioluminescent immunoassay based on the NanoBiT system [18,19]. In their homogeneous complementation assay, they produced the recombinant Nb28-LgBiT protein, and the SmBiT subunit was chemically fused to OTA-bovine serum albumin (BSA) conjugate. This approach could detect OTA in just 5 min without washing steps and exhibited a LOD of 0.01 ng mL^{-1}, an IC$_{50}$ value of 0.31 ng mL^{-1} and a broad dynamic range (0.04–2.23 ng mL^{-1}). These analytical features were clearly superior to those obtained by the SpyCatcher-mediated scaffold assembly BLEIA.

Combined with quantum dots (QDs), Su *et al.* utilized the Nb28 nanobody fused to superfolded GFP (SGFP) to build a FRET-based biosensor [79]. FRET took place between Nb28-SGFP (donor) and OTA-RQDs (acceptor). Tang *et al.* previously reported a similar immunoassay based on FRET but, in this application, they chemically conjugated the Nb28 to QDs [80]. The approach proposed by Su *et al.* provided similar analytical characteristics (i.e. assay time and sensitivity) to that proposed by Tang *et al.*, but with the advantage that the donor was a recombinant protein (Nb28-SGFP) that could be obtained with high reproducibility, defined stoichiometric ratio (1:1 molar ratio), and produced from an unlimited source (*E.coli*). The Nb28-SGFP fusion protein yielded the lowest LOD (5 ag mL^{-1}) among those described with the Nb28 produced as a recombinant protein.

The detection of aflatoxin B$_1$ (AFB$_1$) has also been explored using RLPs. He *et al.* isolated a nanobody (Nb26) from a phage-displayed VHH library [81]. Nb26 was employed in various competitive lateral flow immunoassays (LFIA). In a first approach, a LFIA with fluorescent read-out was described for the analysis of AFB$_1$ in almond milk by Salvador *et al.* [82] using Nb26 fused to GFP (Nb26-GFP). The sensitivity of the LFIA was comparable to that of the 96-well plate-based format (19.3 vs 19.32 ng mL^{-1} IC$_{50}$), but the LFIA significantly reduced the assay time from 90 min to 5 min. Two LFIAs with different simultaneous reading capabilities were described and applied to maize samples by Zou *et al.* and Li *et al.* [83, 84]. They produced a recombinant protein with the Nb26 and the enhanced GFP (EGFP). The Nb26-EGFP fusion protein was modified with gold nanoflowers (AuNFs) or metal–carbon nanomaterials (Zn-CN) that significantly improved the detection limit of their fluorescent LFIA (0.0024 and 0.0094 ng mL^{-1}, respectively) compared to the assay reported by Salvador *et al.* (1.72 ng mL^{-1}).

Ren *et al.* [85] reported a BLEIA for the detection of AFB$_1$ using a novel anti-AFB$_1$ nanobody (G8) fused to NanoLuc. The assay LOD was 0.05 ng mL^{-1} and the IC$_{50}$ (0.41 ng mL^{-1}), almost 20 times lower than that of the classical two-step ELISA (8.14 ng mL^{-1}). In addition to the widely used nanobodies, RLPs based on single-chain fragment variable antibodies (scFv) have also been described for the analysis of AFB$_1$ in spiked maize samples. Rangnoi *et al.* reported the selection of scFv from a naive human phage-displayed scFv library [86]. The best scFv clone was fused to GFP (scFv-GFP) for its application to a competitive fluorescence-linked immunosorbent assay (FLISA). The fluorescence-based immunoassay featured a comparable sensitivity (26 ng mL^{-1}) to the ELISAs performed with the scFv-Fc (22 ng mL^{-1}) and the whole IgG (22 ng mL^{-1}), but not as low as that of scFv-AP ELISA format (8 ng mL^{-1}).

Recombinant antibodies have been investigated for the analysis of other mycotoxins, albeit to a lesser extent. Wang *et al.* obtained a nanobody (Nb3F9) selective against tenuazonic acid (TeA) [87]. They conjugated the nanobody with NanoLuc (Nb3F9-NanoLuc) and used the fusion protein for the detection of the analyte in rice, flour and apple juice by competitive BLEIA. The authors demonstrated the higher sensitivity of the one-step BLEIA (IC$_{50}$ 9.3 ng mL^{-1}) compared to the time-consuming ELISA performed with the Nb3F9 (IC$_{50}$ 54.8 ng mL^{-1}). Later on Wang *et al.* developed a more sensitive BLEIA for the detection of TeA in rice, flour, and bread samples [88]. In this case, a nanobody (Nb2D) was selected by phage display, but instead of acting as a recognition element it was used as an anti-idiotypic nanobody (AId-Nb) to compete with TeA for the binding sites of the specific monoclonal antibody (mAb) (**Fig. 5C**). The AId-Nb2D was engineered with NanoLuc (AId-Nb2D-NanoLuc) for the implementation of a direct competitive BLEIA (dcBLEIA), enabling IC_{50} of 6.5 ng mL^{-1}.

As a final example of RLP with antibodies, a sandwich-like assay was reported by Pradanas *et al*. for the analysis of HT-2 toxin in oat samples. In this approach, the authors used an anti-immune complex (anti-IC) scFv antibody previously selected by phage display [17]. The anti-IC scFv was conjugated to the mScarlet-I fluorescent protein *via* the SpyTag/SpyCatcher conjugation system. The sensitivity of the non-competitive FIA was superior to that of other reported methods, even when using the same pair of antibodies, with a LOD of 0.24 ng mL^{-1} and IC_{50} value of 4.8 ng mL^{-1}.

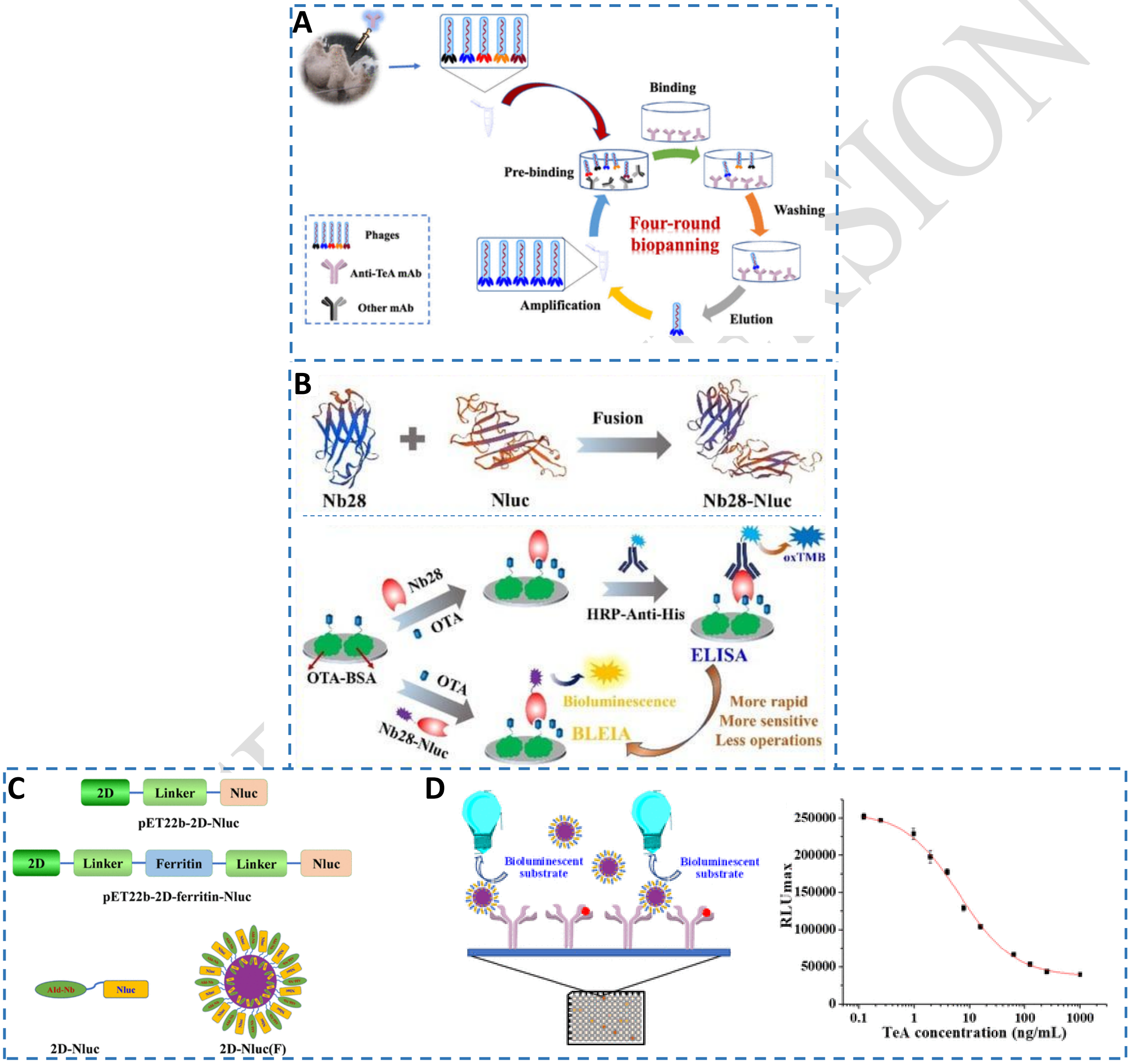

Figure 5. (A) General scheme for the selection of antibodies by phage display with the example for the isolation of Aid-Nb for tenuazonic acid (TeA) detection. (B) Scheme of the BLEIA based on the recombinant fusion protein Nb28-NanoLuc. (C) Vector construction and schematic structure of the ferritin-displayed Aid-Nb2D-NanoLuc. (D) Scheme of the competitive BLEIA for the detection of TeA. Adapted with permission from [77, 88]. Copyright (1996-2024) MDPI (Basel, CH). Copyright (2014) American Chemical Society.

In addition to recombinant antibodies, other biomimetic elements have been used to produce RLPs for mycotoxin analysis. Mimotopes are small peptides isolated by phage display that can mimic the behavior of an epitope. Similarly to AId-Nb, mimotopes can be used as competitors to avoid the use of toxin conjugates. A large number of mimotopes for various mycotoxins have been described; however, in few applications they have been produced as RLPs. Peltomaa *et al.* introduced an epitope-mimicking peptide of fumonisin B_1 [89]. The mimotope (A2) was conjugated to the yellow fluorescent protein (YFP) and the A2-YFP fusion protein was used to develop a competitive homogeneous quenching immunoassay with gold nanoparticles in spiked wheat samples (**Fig. 6**). The LOD was calculated to be 1.1 ng mL^{-1}, a 10-fold improvement over the previously reported method using the non-recombinant synthetic peptide. The IC_{50} value was 12.9 ng mL^{-1}, with a dynamic range from 7.3 to 22.6 ng mL^{-1}. Later on, Luque-Uria *et al.* used the same mimotope to produce RLPs with two luciferase enzymes, namely *Gaussia* luciferase (A2-Gluc) and nanoluciferase (A2-NanoLuc) [90]. The BLEIA based on A2-Gluc enhanced the sensitivity of the homogeneous quenching immunoassay (A2-YFP), with a LOD of 0.38 ng mL^{-1} and IC_{50} value of 1.3 ng mL^{-1}, although a narrow dynamic range (0.61 to 2.9 ng mL^{-1}) was observed. However, the A2-NanoLuc provided a BLEIA with sensitivity comparable to the quenching-based immunoassay (LOD 0.61 ng mL^{-1}, IC_{50} 13.5 ng mL^{-1}), but with a much wider dynamic range (1.91 to 95.2 ng mL^{-1}).

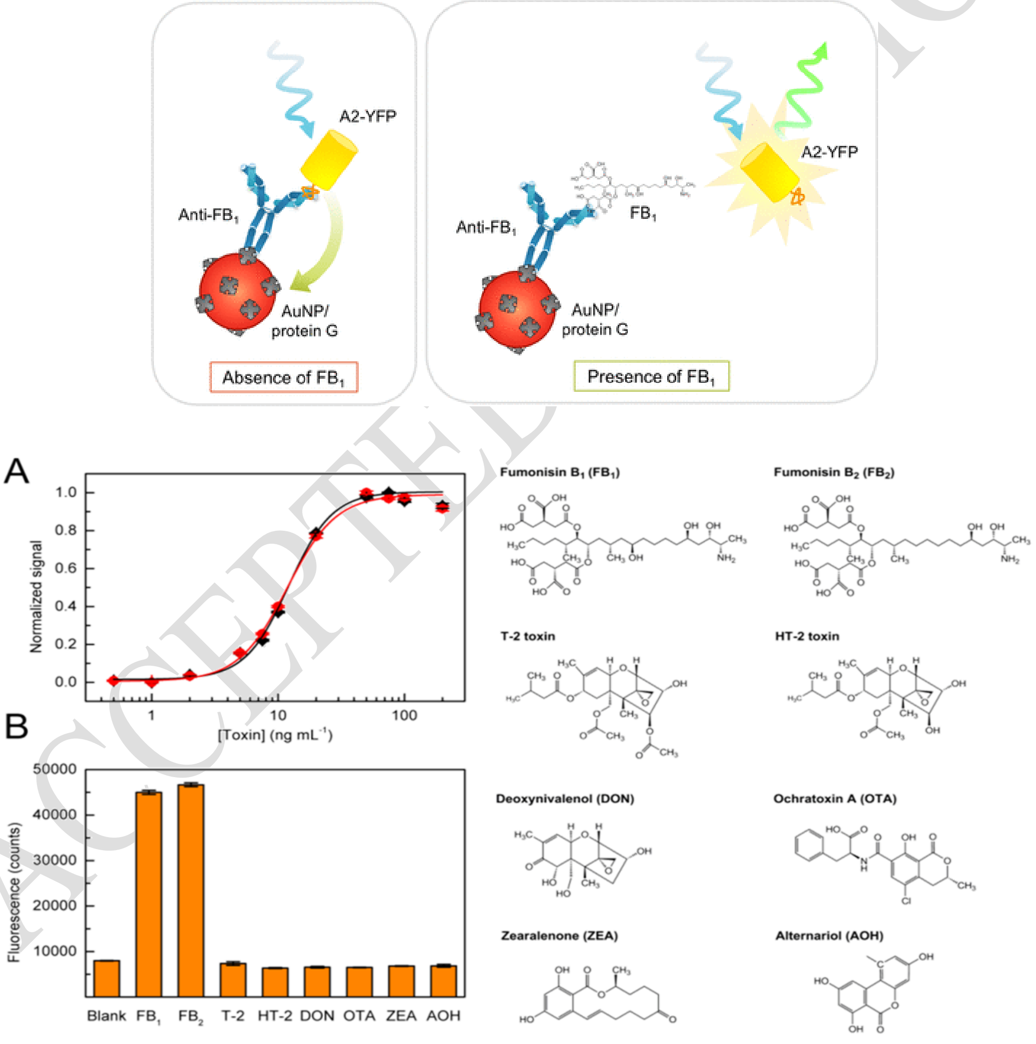

Figure 6. (Top) Scheme of the homogeneous quenching immunoassay based on the A2-YFP recombinant mimotope and gold nanoparticles (AuNPs) decorated with protein G. (Bottom) Specificity of the homogeneous assay. Fumonisins FB_1 and FB_2 showed almost identical calibration curves (**A**: FB_1, black line; FB_2, red line). Other mycotoxins did not give any response in the assay when tested at 100 ng mL^{-1} as the fluorescence signals (**B**) were comparable to the background signal of the blank without any toxin. The structures of the tested mycotoxins are depicted on the right side. Adapted with permission from [89]. Copyright (2018) American Chemical Society.

3.2. Allergens

Clinical cases related to allergic reactions have experimented a remarkable rise in number in recent years, with developed countries reporting increases of 5 to 10 % over the last two decades [91, 92]. Owing to climate change, the length of spring and summer periods have extended and, consequently, airborne seasonal allergens such as pollens have increased their presence in the atmosphere, now encompassing larger time windows [93, 94]. Furthermore, diet changes have been tied to a larger impact of food allergies and to the identification of new food-related allergens. The relevance of this public health issue resides fundamentally in the great distress induced in the daily life of diagnosed persons. This is particularly true in the case of food allergies as meticulous attention needs to be paid to the consumed products since mere traces originating from cross-contamination can lead to severe consequences such as anaphylaxis. With scientific evidence reporting a larger susceptibility of children to develop food allergies, it is expected that the trend of new cases related to these exacerbated immune reactions will keep its present increasing profile. Typically, allergens are proteins or glycoproteins with a large variety of sizes which can involve the production of different types of immunoglobulin E (IgE) in the body. Both analytes, the allergen and the specific IgE produced in response to it, are excellent targets for assays employing RLPs as signaling molecules, with a level of sensitivity adequate to rule out or correctly inform the consumer of the presence of hazardous compounds.

As reported by Goyard *et al.*, luciferases (nanoKAZ in their case) can be used to quantify IgE with exceptional LODs as low as pg mL^{-1} [95]. Experiments reported therein were performed on plasma samples from different donors, including that of an individual with a strong allergy to peanuts. The nanoKAZ demonstrated a high degree of selectivity towards binding to the Fc portion of the IgE specific for allergic reactions to peanuts. A luciferase immunoprecipitation assay was developed for the detection of the production of IgE against cats by Lin *et al.* [96]. These authors developed the fusion protein of Fel d1 marker for cat sensitization with Rluc1, which was expressed in mammalian 293-F cells for proper folding and glycosylation. The construct was tested in serum from healthy, allergic to cats, and allergic to other substance individuals. The good sensitivity of the approach guaranteed the successful exam of this common allergy using small quantities of the patient's blood (ca. 5 mL).

BRET has been employed to design elegant experiments aiming to identify the binding and kinetics of different drugs to histamine receptors (HR). Histamines are well-known mediators of the inflammatory symptoms characterizing allergic reactions (histamine 1) as well as other inflammatory diseases such as Parkinson's and epilepsy (histamine 3) or psoriasis and asthma (histamine 4). In a 2018 paper, Stoddart *et al.* fused NanoLuc to the *N*-terminus of the H1 receptor [97]. Cells expressing this NanoLuc-tagged HRs were then faced with pharmacophores conjugated to fluorophores capable of absorbing photons produced by the NanoLuc-mediated substrate reaction. Given the minimal distances required for BRET to take place, detection of light from boron-dipyrromethene (BODIPY) fluorophores fused to the pharmacophores implied binding between the reporter and the tagged histamine receptor. Based on this observation, the authors proposed a competitive assay to assess the binding affinities of antihistaminic drugs to the H1R tethered to NanoLuc. For those experiments in which drugs were included, the BRET signal declined, indicating preferred binding of the drug to the HRs instead of the reporter fluorescent probe. This idea may expand its application to a plethora of scenarios such as measuring affinities for other allergens or histamine receptors. The latter case was demonstrated as affinity of drugs for H3R and H4R was also reported in a subsequent publication [98]. In this reference, the authors replicated the strategy but now binding NanoLuc to H3R and H4R. Green- and red-tagged reporters were used to capture the photons produced by the NanoLuc-HR ensemble in competitive BRET assays.

3.3. Pesticides

Pesticide residues resulting from the use of plant protection products, and the related metabolites, can be found in feed crops and in foods of animal origin [99]. Their potential toxicity poses risks to public health and, therefore, sensing methods to detect trace amounts are required to ensure that the legal limits (maximum residue levels, MRLs) set by public authorities are not exceeded.

Watthaisong *et al*. developed a chemo-enzymatic cascade reaction for the bioluminescent detection of nitrophenols (NPs) and halogenated phenols (HPs), which are metabolites of pesticides, via the *de novo* synthesis of D-luciferin [100]. The reaction involves the denitration and dehalogenation of the toxicants to benzoquinone catalyzed by flavin-dependent monooxygenase (HadA) coupled to D-Cys condensation to produce D-luciferin. Subsequently, FLuc was used to generate the bioluminescence signal. 4-NP and 4-HPs were detected by their bioluminescence method at the ppb levels required by the U.S. Environmental Protection Agency (US-EPA) and the Agency for Toxic Substances and Disease Registry (ATSDR) for assessing the risk of overexposure to pesticides. Moreover, NPs and HPs act as biomarkers for exposure to pesticides, including organophosphates (Ops). The authors reported the determination of 4-NP and 4-chlorophenol (4-CP) in human serum and urine samples without pre-treatment (**Fig. 7**).

Nevertheless, this method does not detect pesticides in their original forms. Hence, the authors proposed an improved enzymatic cascade reaction for the one-pot synthesis of D-luciferin analogues from phenolic derivatives, called HELP (HadA Enzyme for Luciferin Preparation), and further used it to develop the LUMOS (Luminescence Measurement of Organophosphate and Derivatives) technology for in situ detection of Ops pesticides [101]. By coupling the enzymatic cascade reactions with the reactions of FLuc and Ops metabolizing enzymes, their LUMOS technology detected five aromatic Ops: parathion, methyl parathion, *O*-ethyl *O*-*p*-nitrophenyl phenylphosphonothionate, profenofos, and fenitrothion. The presence of these pesticides was directly confirmed in non-treated urine, blood serum and fruit samples. The method showed higher sensitivity (ppt level) than HPLC-MS and therefore covered the range regulated by the US-EPA, ATSDR, and the EU Commission.

A similar enzymatic cascade strategy was used by Silverman *et al*. [102] to detect atrazine (ATZ). The cell-free sensor mechanism for detection of this herbicide involves catabolism into cyanuric acid via a three-enzyme pathway, followed by activation of transcription by the transcription factor AtzR that recognizes an operon sequence within an engineered promoter. Detection is carried out upon expression of the superfolder green fluorescent protein (sfGFP) encoded in the reporter plasmid. This sensor has been used in tap and lake water samples and is able to discriminate, with only one hour of incubation, high concentrations of ATZ (10−100 μM), above the LOD established by the EPA (14 nM).

The biosensing capacity of fluorescent proteins has also been studied for the detection of pesticides. This is the case of the work by Saeed *et al.* [103] with the HriGFP protein from *Hydnophora rigida* for the detection of the methyl parathion (MP) organophosphate. After docking HriGFP with MP, a strong interaction with the amino group of Arg119, as well as hydrophobic interactions with 8 other residues, were found. The authors observed an increase in fluorescence intensity in HriGFP-producing cells as the concentration of MP used decreased, allowing the detection of the pesticide in the concentration range from 1.89 mM to 189 µM.

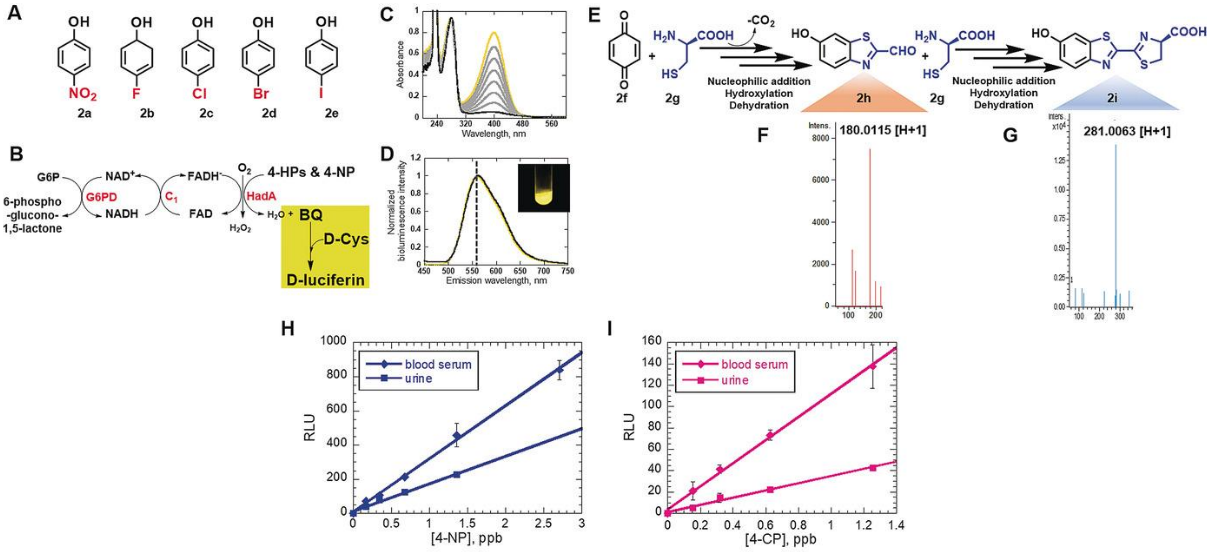

Figure 7. Biodetoxification and bioluminescent detection of hazardous phenols reported by Watthaisong et al. [100]. (A) The structures of 4-nitrophenol (4-NP, **2a**), 4-fluorophenol (4-FP, **2b**), 4-chlorophenol (4-CP, **2c**), 4-bromophenol (4-BrP, **2d**), and 4-iodophenol (4-IP, **2e**). (B) Overall reaction of the enzymatic cascade involving glucose-6-phosphate dehydrogenase (G6PD), flavin reductase (C1) and HadA for the bioconversion of halogenated phenols and nitrophenols to D-luciferin. Flavin reduction and NADH regeneration are required for the HadA-catalyzed biodetoxification of phenols. The chemical condensation of the benzoquinone (BQ) resulting from the HadA reaction and D-cysteine (D-Cys) produces D-luciferin. (C) Biodetoxification of 4-NP: The depletion of the phenol was monitored at 400 nm (start, yellow line; finish at 180 min, black line). (D) Bioluminescence due to the Fluc reaction with D-luciferin synthesized from the reported technology (black line) compared to commercially sourced D-luciferin (yellow line). (E) Chemical species detected from the condensation of BQ and 2 molecules of D-Cys using LC-ESI-QTOF-MS. Condensation of BQ (**2f**) and D-Cys (**2g**) generates the 6-hydroxybenzothiazole-2-carbaldehyde intermediate (**2h**). **2h** reacts with another molecule of D-Cys to generate D-luciferin (**2i**) as the final product. Key products **2h** and **2i** were identified by their mass spectra (F) and (G), respectively. Detection of pesticide biomarkers in biological samples by coupling the bioconversion of phenols to D-luciferin to the FLuc bioluminescent reaction; calibration plots for 4-NP (H) and 4-CP (I) in blood serum and urine samples. Reprinted from ref. [100] with permission Copyright (2019) John Wiley and Sons.

3.4. Antibiotics

Antimicrobials resistance (AMR) is deemed as one of the top threats to global public health and economic development by the World Health Organization (WHO). In short, AMR implies the evolution of microorganisms to protect themselves against therapeutic drugs, rendering them ineffective. From 2015, the WHO has published a total of five reports tied to its Global Antimicrobial Resistance and Use Surveillance System (GLASS) initiative, with the latest being released in 2022 [104]. In a possible scenario, common infections and minor injuries pose a lethal menace to life as bacteria cannot be terminated by antimicrobial medicines. Since the original report, AMR has seen its definition expanded to include viruses, fungi, and other parasites as they can cause infectious diseases in humans, animals and plants, and evolve to become resistant as bacteria can. We will focus on the use of RLPs for the detection of antibiotics, the original case of interest of GLASS and an area for which strong legislation has already been issued. To contextualize the relevance of the topic tackled herein, the EU already banned the use of antibiotics as additives to promote the growth of livestock as early as 2006. The main motivation to do so was that the indiscriminate use of antibiotics accelerated remarkably the development and prevalence of drug-resistant bacterial strains, thus increasing the possibility of outbreaks of bacterial infections. Even with the use of antibiotics limited to solely healing purposes, the legislation still calls for treated stock to be quarantined before yielding any foodstuff as AMR can build up from foodborne traces of drugs or directly from resistant bacteria moving up trophic levels. Inspired by the need to monitor the presence of antibiotics to prevent overconsumption, RPL-based sensing platforms have been designed for their application over a variety of products.

Bacteria themselves can be used as reporters of the presence of antibiotics in samples of interest. A common strategy for turning these organisms into a biosensor is transfecting them with plasmids encoding bioluminescent genes which will cause light emission upon the action of an antibiotic on the reporters. The 2018 paper by Kao *et al.* showcases the application of such an approach [105]. Therein, the authors developed a portable CCD analyzer which employed *E.coli* bacteria harboring the luxCDABE luminescent gene for the detection of ciprofloxacin in milk, eggs and chicken essence. Following exposure to ciprofloxacin, light could be collected in the system yielding LODs between 8 and 64 ng mL^{-1} with response times between 20 and 80 min. The authors continued their work in a later paper using the same sensing scheme but replacing the detector with a smartphone, yielding similar LODs for ciprofloxacin in whole milk [106]. Also targeting milk, *E.coli* expressing GFP was used for the detection of benzylpenicillin, ampicillin, gentamicin, tetracycline and neomycin with LODs ranging from 0.29 to 618.36 mg L^{-1} [107]. The luxCDABE reporter in *E.coli* was further used in a recent work as a probe to both detect and categorize residues in the concentration range of 125 to 1000 μg L^{-1} via machine learning models [108]. The Multiclass Decision Forest employed by Huang *et al.* enabled using a simple instrumental scheme for acquiring images of the antibiotic-induced bioluminescence of fifteen *E.coli* strains immobilized on agar gel. From data of which strand exhibited light emission, researchers were able to successfully classify 11 analytes from 5 different classes. Furthermore, the same gene was used for the screening of antibiotics targeting the Gram(−) *Pseudomonas aeruginosa* bacteria, which causes degradation of meats among other products [109]. In addition to foodstuff, RLPs have been used to assess the presence of antibiotics in other samples. For example, Miller *et al.* immobilized yeast on a paper-based device [110]. Yeast expressed the fluorescent protein yEmRFP, enabling the evaluation of the presence of doxycycline in physiological fluids such as human urine and bovine serum. Matsuura *et al.* performed the detection of aminoglycosides in whole blood/serum employing NanoLuc as the bioluminescent probe [111]. In their platform, cell-free NanoLuc-expressing reagents were embedded in the paper chip and frozen dry. The platform was rehydrated and incubated to yield NanoLuc in the absence of antibiotics, with lower emission intensity indicating increasing concentration of analytes.

Recent research aiming to detect antimicrobials in environmentally relevant samples showcases the potential of RLPs as a tool against a global threat and the many alternative pathways, ranging from sophisticated approaches to user-friendly devices, in which they can be implemented.

4. Conclusions

The future of sensing devices for food safety, environmental monitoring and clinical diagnosis is tied to several factors. Some of them have received significant attention in recent years, namely creating new recognition elements, using synthetic biology, or implementing further signal amplification strategies.

RLPs are increasingly exploited in a variety of biosensors and bioassays. Interdisciplinary collaborations of biologists, chemists and engineers aimed to transform traditional analytical devices into biosensing platforms with enhanced sensitivity and specificity without compromising simplicity and cost-effectiveness are, indeed, the main driving force behind this new paradigm. In this regard, RLPs cover a wide area of transduction possibilities through the simple manipulation of their genome, which allows their expression with well-defined optical properties and even fused to recognition elements in a stoichiometrically controlled manner. Additionally, artificial intelligence (AI) is bound to significantly transform the development of biosensors and biosensing assays, leading to previously unimaginable innovations. For example, machine learning models, such as deep learning networks, can design novel fluorescent proteins with improved brightness, stability, and spectral properties that might not be found through traditional experimental methods [112]. Nowadays, we see examples of AI algorithms enhancing receptor design, optimizing their target affinity/specificity, and improving high-throughput screening. As the integration of AI continues to evolve, it will undeniably unlock new exciting possibilities. An example of a critical trait to be fortified by means of recently available tools is stability. The preparation of highly stable RLP-based sensing platforms is mandatory if researchers strive for their suitability in scenarios where these analytical devices have typically limited use given the deterioration of their performance upon storage under non-optimal conditions; the latter are tedious to meet outside the tightly controlled laboratory environment.

One of the most promising applications of RLPs in biosensing is their combination with emerging materials and nanostructures (quantum and carbon dots, metallic, magnetic and upconversion nanoparticles, to name a few) to produce novel analytical devices that efficiently work in washings-free homogeneous bioassays (FRET, BRET, SET, etc.). The facile application of RLPs engineered into separate fragments, which can be reassembled during the assays to produce an analytical signal upon the target molecule binding (or release), should also be brought up. The popularity of this technology is expected to grow for the development of homogeneous bioassays to monitor any kind of hazardous substances over a broad number of samples of different origins.

While, actually, not every new development will make it into real-world applications and, in many cases, RLPs stability and cost production may not be feasibly improved to industry standards, RLPs have undeniably become very attractive and versatile optical probes that, in the long term, might replace traditional detection systems even for essential and demanding analytical tasks such as food quality and environmental surveillance.

Acknowledgments

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Table 1. Examples of RLP-based methods for the detection of food contaminants. The limit of detection (LOD) was based on three times the standard deviation of the blank unless marked with (*), where it was defined as the toxin concentration that induces a 10% decrease in the signal.

Analyte	Assay format	Recognition element	Recombinant luminescent protein	Measurement	LOD (ng mL^{-1})	Sample	Ref.
OTA	Competitive FRET (SGFP donor; QDs acceptor)	Nb	Nb-SGFP	FI (FRET)	0.005	Barley, oat, rice, wheat	[79]
OTA	Competitive BL IA	Nb	Nb-LgBiT	BL	0.01	Barley, oat, rice	[78]
OTA	Competitive BLEIA	Nb	Nb-NanoLuc	BL	3.7	Spiked coffee	[77]
OTA AFM_1	Competitive BLEIA	Nb	Nb-SpyTag NanoLuc-SpyTag SpyCatcher-mi3	BL	0.113 0.251	Barley, wheat, maize	[76]
AFB_1	Competitive LFIA	Nb	Nb-GFP	FL	1.72	Spiked almond milk	[82]
AFB_1	Competitive dual-modal LFIA	Nb	Nb-EGFP (+AuNFs)	Colorimetric (visual) / FI	1 (visual) 0.0024	Maize	[83]
AFB_1	Competitive trimodal LFIA	Nb	Multivalent Nb-EGFP (+Zn-CN)	Colorimetric/ FI/ Photothermal	0.0012 0.0094 0.252	Maize	[84]
AFB_1	Competitive BLEIA	Nb	Nb-NanoLuc	BL	0.05	Wheat, maize	[85]
AFB_1	Competitive ELISA/FLISA	scFv	scFv-AP scFv-GFP	Abs FL	1.5 13	Spiked maize	[86]
TeA	Competitive BLEIA	Nb	Nb-NanoLuc	BL	1.1	Spiked rice, flour, apple juice	[87]
TeA	Direct competitive BLEIA	AId-Nb	AId-Nb-NanoLuc	BL	0.7	Spiked rice, flour, bread	[88]

Table 1 (cont). Examples of RLP-based methods for the detection of food contaminants. The limit of detection (LOD) was based on three times the standard deviation of the blank unless marked with (*), where it was defined as the toxin concentration that induces a 10% decrease in the signal.

Analyte	**Assay format**	**Recognition element**	**Recombinant luminescent protein**	**Measurement**	**LOD (ng mL^{-1})**	**Sample**	**Ref.**
HT-2	Non-competitive FIA	anti-IC scFv	scFv-SpyCatcher SpyTag-mScarlet-I	FI	0.24	Oat	[17]
FB_1	Competitive homogeneous quenching IA	Mimotope	Mimotope-YFP	FI	1.1	Wheat	[89]
FB_1	Competitive BLEIA	Mimotope	Mimotope-NanoLuc Mimotope-Gluc	BL		Wheat	[90]
NP HP	Enzymatic cascade	HadA monooxygenase	FLuc	Luminescence spectrometer	0.04 (NP, serum) 0.05 (NP, urine) 0.19 (4-CP, serum) 0.12 (4-CP, urine)	Blood serum and urine	[100]
P MP EPN profenofos fenitrothion	Enzymatic cascade	OPs hydrolase	FLuc	Luminescence spectrometer	0.002$^{(a)}$ – 0.015$^{(a)}$	Apple, banana, guava, blood serum, urine	[101]
MP	Direct	RLP	HriGFP	Fluorometer	< 189 µM	Water	[103]
ATZ	Enzymatic cascade	TF	sfGFP	Microplate reader	20 µM	Water	[102]

Abbreviations: ELISA, enzyme-linked immunosorbent assay; Nb, nanobody; AP, alkaline phosphatase; Abs, absorption of light; PVDF, polyvinylidene fluoride; HRP, horseradish peroxidase; FIA, fluorescence immunoassay; FI, fluorescence intensity; IA, immunoassay; FRET, Förster resonance energy transfer; SGFP, superfolder green fluorescent protein; QDs, quantum dots; LgBiT, largeBit; BLEIA, bioluminescence enzyme-linked immunosorbent assay; NanoLuc*, nanoluciferase; LFIA, lateral flow immunoassay; EGFP, enhanced green fluorescent protein; AuNFs, gold nanoflowers; Zn-CN, zinc-organic carbon nanomaterial; MB-CLEIA, magnetic beads chemiluminescent enzyme-linked immunosorbent assay; CL, chemiluminescent; FLISA, fluorescence-linked immunosorbent assay; AId-Nb, anti-idiotypic nanobody; anti-IC, anti-immune complex: YFP, yellow fluorescent protein; NP, nitrophenols; HP, halogenated phenols; 4-CP, 4-chlorophenol; OPs, organophosphate pesticides; FLuc, firefly luciferase; LOD, limit of detection;* $^{(a)}$ *pg mL*$^{-1}$ *in buffer; P, parathion; MP, methyl parathion; EPN,* O*-ethyl* O*-p-nitrophenyl phenylphosphonothionate; AhR, aryl hydrocarbon receptor; ATZ, atrazine; RLP: Recombinant luminescent protein; TF: transcription factor*.

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