

Whole Cell Biosensors For (Eco)Toxicity Screening: Alternatives For The Future

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ABSTRACT

As a response to the emerging environmental threats, different Regulatory Authorities have introduced tough new rules for the testing and approval of thousands of chemicals. Questions are raised towards the practicality of the new legislation such as the European REACH initiative as around 12 million laboratory animals need to be sacrificed. No in vitro toxicological procedure is available to cope with this demand so far. 'Classical reporters' are cumbersome and difficult to use for this high throughput screening. Whole Cell biosensors are ideally suited for these purposes, but straightforward measuring systems need to be developed. Crucial is that the "bio"-part needs large-scale modifications to be compatible with biosensor (nano)technology. Luminescing and fluorescing reporters allow easy registration with an optical sensor. It is possible to determine with a battery of stress related genes the Toxicological Response Profile (TRP) of individual compounds and wastes.

Keywords: whole cell biosensor, (eco)toxicology, environment, high throughput, toxicological response profile (TRP)

1 INTRODUCTION

Risk assessment usually relies on information gathered using surrogate species of a human being. On the one hand, new and tough legislation for the market approval of new chemicals requires higher species for toxicity testing, while on the other hand animal welfare and other organisations are lobbying to avoid unnecessary suffering of these animals and stimulate the use of alternative systems. Bacteria have long been used as a bioreporter of genotoxic testing (eg Ames test), however it has become clear that bacteria also have the potential for screening other toxic modes of action. Bioreporter development has become one of the most dynamic areas in (eco)toxicology. Hereby new (cellular) assays are developed, or existing assays redesigned.

Technological innovation for easier and more sensitive detection of different cellular reporters is co-evolving to make both parts compatible with each other.

2 CHEMICAL ANALYSIS VERSUS BIOREPORTER

Analytical chemical methods such as gas chromatography mass spectrometry (GC-MS) or liquid chromatography (LC)-MS achieve a selective detection and identification of target pollutants in the nM to ng/l range, although at considerable cost and effort. In parallel to the continuous development of increasingly more sophisticated analytical technologies for the detection of environmental pollutants, there is a high need for bioassays which report not only on the presence of a chemical but also on its bioavailability and its adverse biological effects. Recent trend analyses promise that additional improvements will make microbial biosensors and mainly *E. coli* the ideal tool for future environmental analysis [1]. Novel bioreporter development is based on in depth knowledge of *E. coli*'s transcriptional regulation and molecular signalling, microbial physiology, toxicological response and applying more sensitive detection methodologies leading to better performing bioreporters.

2.1 Bacterial Bioreporter

Most bacterial bioreporters do not perform particularly well at concentrations below 0.1mM. However the few existing examples of sensitive bioreporters demonstrate that they have a potential that is superior to chemical analysis: 1. Microorganisms are living beings, their 'measurements' reflect a bioavailable concentration rather than the total concentration that many chemical methods assess. 2. They are very cost-effective compared to analytical methods, as they reproduce by themselves. Owing to their low cost price, bacterial cells are well fitted for multianalyte approaches. 3. Bioreporter cells often detect groups of compounds rather than single analytes, which can be a useful for rapid screening for the presence

of major contaminant classes. 4. Bioreporters are also sensitive to metabolites of a toxic compound, information that is difficult to gather in an analytical analysis. 5. Bacterial reporter systems have good potential for equipment miniaturization. 6. *E. coli* has the potential for biosensing rather than only bioreporting. Biosensing offers the possibility for on line measurement and allows dose-response measurement, while bioreporting is just giving information about the presence or absence of a compound. 7. Reporter genes with easy measurable phenotypes form the basis of sensitive, quantitative and reproducible assays compatible with different sensor technologies. 8. An important demand posed on bioreporters is the time needed for detecting the signal. As bacterial bioreporters are living cells, there is probably an inherent incubation period needed to achieve a useful signal from the reporter. Most experimentally described bacterial bioreporter systems report incubation times of around 30 min to more than 5 h before recording the reporter signal. At shorter incubation times, the detection limit generally increases substantially. Theoretically the fastest response – in case of facilitated transport – could be expected within 2-5 min after exposure [2].

2.2 Cellular systems and *E. coli*

Transgenic cells - prokaryotic or eukaryotic- were introduced in environmental toxicology thanks to the introduction of biotechnology and genetic engineering. Bioreporters typically combine a promoter-operator, which acts as the sensing element, with reporter gene(s) coding for easily detectable proteins.

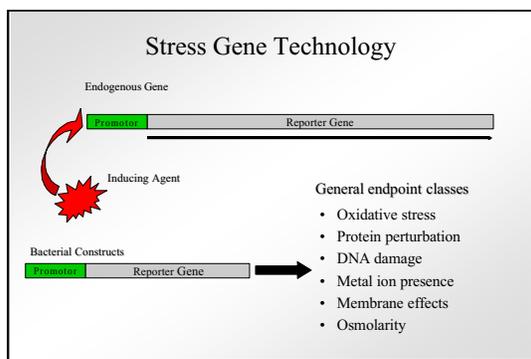


Figure 1: Bioreporters combine a sensitive promoter with a measurable element

Nowadays there is a high need to fit both reporter-novel and classical- with the newest detection technologies to obtain the required innovation to cope with the high demand of toxicological assays.

2.2.1 Specificity of the assay- Promoter

Specificity is determined by the spectrum of a regulatory protein driving the reporter gene by interacting with the promoter. Some general classes of promoters are well characterized and several classical reporter systems combined with these promoters have been described [3].

2.2.2 Reporters- Which, Where, When?

Several reporter genes have been isolated from a variety of naturally occurring organisms and have been categorized by the means of detection. In general, one can distinguish between substrate dependent and substrate independent reporters. The first produce a detectable signal after substrate addition, the latter produce a detectable signal from them selves. The latter are ideally suited for biosensor development thanks to their potential for high throughput and automatisation.

2.2.2.1 Substrate dependent reporter

Classical reporters based on β -galactosidase have already been used for a long time. Often visualising of the enzymatic activity depends on substrate addition, however, in many cases this system is not compatible with novel detection methods or is subject to interference (turbidity, colour). However recently new substrates have been designed elevating this problem, and enlarging the applicability scope.

Umbelliferone: Umbelliferone is a fluorogenic phenol. Esters and ethers of umbelliferone are non-fluorescent, whereas free umbelliferone is strongly fluorescent owing to the blue emission of the phenolate anion. Umbelliferyl and nitrophenyl esters, phosphates, sulphates and glycosides are classical fluorogenic and chromogenic substrates for the corresponding hydrolytic enzymes. This new type of substrate has caused a revival of the classical reporter systems. However, low penetration of this substrate often means that cell lysates still have to be prepared, hindering a high throughput design.

2.2.2.2 Substrate independent reporter

Luciferase: Both prokaryotic (*lux*) and eukaryotic (*luc*) luciferase have been cloned and used in *E. coli* for a bacterial reporter assay.

Luc- Firefly luciferase

Luciferase catalyses the oxidative carboxylation of beetle luciferin, emitting photons that can be measured. It is rapid and has a broad linear range. A major drawback is that the substrate luciferin has to be added to the bacterium. *E. coli* has only marginal membrane permeability, and therefore EDTA has to be added, which is suboptimal for the *E. coli* cells. Such system was used to detect benzene in polluted air; detection was based on a CCD- colour coded device [4]

Lux- bacterial luciferase

In contrast to eukaryotes, prokaryotes do have the possibility for non-substrate luminescence detection. Prokaryotes from the species in the genera *Vibrio*, *Photobacterium* and *Photorhabdus* activate a gene cluster to produce the enzymes required for bioluminescence [5]. *Vibrio fischeri* is encoding a lux operon consisting of 7 genes ICDABEG. The regulation is complex but well studied. A drawback for the development of marine *Vibrio fischeri* as a reporter is the requirement of high concentrations of salt, which may alter the chemistry and toxicity of samples. However the entire luxCDABE operons for *Vibrio fischeri* also gave strong signals when expressed in *E. coli*, making it ideal as a bioreporter.

Similar as with eukaryotic luciferase, exogenous substrate (here mostly decanal) can be added. The addition of the long-chain fatty acid aldehyde is reducing the energy cost for the light-generating reaction by 67%, but the process still remains energy consuming, and similar problems as with eukaryotic luciferin do occur like low membrane penetration and low solubility. This high-energy cost might cause a metabolic burden to cells expressing luciferase, and bacteria that have reduced metabolic activity (e.g. in the case of starvation, toxicity) may have too little energy to drive an efficient light response.

Fluorescent reporter

Fluorescent reporters are gaining a lot of popularity. GFP – green fluorescent protein- isolated from the pacific jellyfish *Aequoria victoria*, is far the best characterized. GFP is an intrinsically fluorescent protein. Applications using GFP have expanded greatly as a result of the availability of mutant *gfp* genes encoding variants with shifted excitation and emission spectra, faster maturation rate and optimized codon usage for expression in different hosts and new detection possibilities. A huge advantage is that GFP fluorescence can be measured without the need for exogenous substrates, cofactors or ATP. GFP is non-invasive and can be monitored as such in living cells. It is easy to use and does not require any exogenous substrate or cofactor.

GFP was the first fluorescent reporter isolated, however in the meantime some other proteins have been isolated as well. Dsred, the red fluorescent protein from *Discosoma* coral has another emission and fluorescent wavelength and can be used together with GFP. A combination of two reporter proteins is straightforward to have an indication of both general cellular toxicity and induced toxicity. In this combination one of the genes is cloned behind a constitutive promoter, and a decrease in fluorescence is indicative for general cellular toxicity.

The advantage of GFP is its stability, which means measurements can occur during a prolonged timeframe. However, this feature also means a drawback in the use of GFP as a biosensing reporter for on line measurements. A newly designed ‘destabilized’ GFP with a shorter half life does open nice perspectives for such applications [6].

2.3 Comparison of different ‘light reporters’

A comparison between eukaryotic luciferase luc and prokaryotic luciferase lux proves the advantages and drawbacks of each reporter gene. Eukaryotic luciferase has a very high quantum yield and a less expensive energy consumption with only one ATP per photon emitted. A comparison between bacterial lux and firefly luciferase shows a two- to threefold increase in bioluminescence and a much lower detection limit (1 fM) in a luc-based mercury-specific bioreporter than in an equivalent lux-based bioreporter [7]. On the other hand, the substrate for eukaryotic luciferase, D-luciferin, is much more difficult to donate to the bioreporter cells. D-luciferin penetrates the bacterial membrane only in its protonated form. Under pH-neutral conditions, therefore, D-luciferin transfers the membrane very slowly, and cell extracts might even have to be used to perform a bioreporter measurement.

GFP-based bioreporters are usually less sensitive than the same lux- or luc-tagged constructs. The reasons for this may be that low fluorescence signals cannot be differentiated from autofluorescence backgrounds and that enzymatic reporter proteins can essentially amplify the reaction by substrate conversion. In a study that compared the responses of fluorescent (GFP and Dsred proteins) and bioluminescent (luxCDABE and luc FF genes) *E. coli* plasmid-based bioreporters, bioluminescence had faster response times and lower detection limits than the fluorescence signals [8].

Conclusion: Reporter genes coding for proteins that produce colour changes, fluorescent molecules or bioluminescence have been discovered and can be detected by different detection methods. Some of these reporters are specially designed because of their compatibility with bio(nano)sensor technology. Alternatively some new substrates have been designed for ‘classical’ reporters, making these suitable to be measured with the newest detectors methods.

2.4 How to see the light

Bioreporter bioluminescence can be detected by different types of optical transducers, including photomultipliers, photodiodes photomultiplier tubes or imaged with photographic film, charge-coupled

device cameras and microchannel plates. In many of these devices light is collected and transferred to the transducer using lenses, fibre-optic cables or monitored directly by a detector.

2.5 Toxicological Response Profile (TRP)

A TRP is a stress gene assay that gives an insight into the dose response profile of the different types of stress (eg. mutagenic, membrane, oxidative, ...) that are caused by a chemical compound or an environmental sample. In the first dimension of the TRP, the different stress types are represented, while in the second dimension the dose is shown. The third dimension gives a measure of the reporter readout. The general picture gives an overview of the 'stress profile' of the tested sample, and enables to identify the most important stressors. Figure 2 shows the TRP of a newly designed chemical compound. Different combinations of stress promoter-reporters are required for an adequate risk assessment. The described properties of luminescent and fluorescent biosensor are ideal to be used for these measurements. The new European REACH legislation that will be approved in the coming months or years, requires that the toxicological profile of a chemical compound is determined thoroughly before it can be launched on the market. Therefore there is a high demand on the market for high throughput assays. This offers a huge challenge and opportunity for newly developed biosensors.

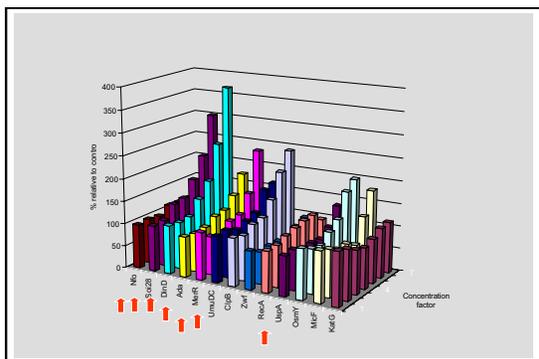


Figure 2: a TRP profile of a newly designed compound. The first dimension gives the different promoter-reporter combinations, the second dimension the dose, the third dimension the bioreporter readout

2.6 References

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