

Strategies of Immobilizing Cells in Whole-cell Microbial Biosensor Devices Targeted for Analytical Field Applications

Nadine LOBSIGER and Wendelin J. STARK[†]

*ETH Zürich, Department of Chemical- and Bioengineering, Functional Materials Laboratory,
Vladimir-Prelog-Weg 1, 8093 Zürich, Switzerland*

This review summarizes the development of whole-cell biosensors with a special focus on device development and cell immobilization. Integration of biosensor functions in a device will pave the way for field applications in remote areas and resource-limited settings. Firstly, an introduction to the field of whole-cell biosensors is provided, followed by examples of genetic engineering of cells in order to fulfill sensor functions. A framework of requirements to enable future field applications of biosensors is elaborated. A special focus is on different cell immobilization techniques ranging from polymers, to microfluidic devices, immobilization on paper and combinations of these methods. Looking at globally successfully implemented point of care devices such as a home pregnancy test or a blood glucose meter, we conclude the review with thoughts on long-term stability, portability, ease of use and user safety design guidelines for whole-cell biosensor devices.

Keywords Whole-cell biosensor, field application, biosensor device, bioreporter, cell immobilization, storage

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1 Introduction

Chemical and physical analyses are the standard tools for monitoring the presence of chemicals in the environment or in

medicine. For example, in the analysis of drinking, waste, and surface waters, various parameters such as pH, conductivity and dissolved oxygen are routinely measured. Concerns are increasing about the pollution of bodies of water by heavy metals, pharmaceuticals and pesticides, and the effects on



Nadine LOBSIGER is a biotechnologist who received her master's degree from ETH Zürich. Her Ph.D. thesis in chemical engineering focuses on the development of a field-applicable whole-cell biosensor platform. Recently, a proof of concept combining sensor bacteria in a dormant state, a polymeric growth matrix and diffusion-based sensor quantification has been presented. The platform is currently expanded to different sensor organisms enabling the detection of relevant environmental pollutants.



Wendelin Jan STARK is a full professor at ETH Zürich. His research combines materials with specific functions for medical or industrial use. He has developed nanoparticles for environmental applications, that is, to increase energy efficiency in water and solvent purification, to enable large scale remediation or for more environment-friendly catalytic processes. He has pioneered inherent safety aspects in nanoparticle applications and toxicology during industrial product design.

[†] To whom correspondence should be addressed.
E-mail: wendelin.stark@chem.ethz.ch

human health and wildlife even at very low levels. This is driving the need for cost-effective, remotely applicable and user-friendly analytical methods. Chemical analyses, such as liquid chromatography-mass spectrometry (LC-MS), are powerful tools to identify and specifically quantify complex compounds only present in very low concentrations.

These methods allow for highly accurate and sensitive determination of sample composition with the disadvantage that they are costly, complex and require specialized facilities. Additionally, effects on the bioavailability of polluting chemicals and effects on living systems are not detected.¹ To address these issues, the use of different biological systems, such as multicellular organisms, microorganisms, enzymes, antibodies and subcellular components, has been proposed. It has been reported that higher and lower organisms such as fish, invertebrates, algal cells and plants have been used in biomonitoring.² Biomonitoring is the discipline where living organisms or their responses are used to determine changes in an environment such as water, air or soil. Because of their easy cultivation and short generation times, microbes have often been used as biomonitors. In microbial toxicological bioassays, the response to adverse environmental conditions is rapid and readily detectable. Possible responses are cell death, slowed cell growth or changes in cell metabolism exhibited by changed oxygen uptake. Eltzov *et al.*³ described biomonitors as excellent tools to detect total effects of compounds and acute but not chronic toxicity. Similarly Cui *et al.*⁴ reported exposure experiments using marine species to be time consuming, laborious and not suitable for rapid emergency response in cases of contamination.

In whole-cell biosensors, a target compound interacts with the cellular biorecognition element and a physico-chemical transducer converts the interaction into a measurable signal (Fig. 1). The electrical or optical signal is subsequently converted into a readout or display available to the user. For biosensors producing an optically measurable output, two different systems are known: “lights-on” and “lights-off”.⁵ “Lights-off” bioreporter assays are based on the same principle as the aforementioned microbial toxicological bioassays. The effects of cell exposure to a chemical resulting in cellular damage or disruption of the metabolism is exploited. This correlation is visualized as the cell is either naturally emitting light or has been genetically engineered to continuously (constitutively) do so and a decrease in light intensity after chemical exposure is observed. Apart from light, other signs of cellular activity (*e.g.* respiration, motility and growth inhibition) can be observed and used to estimate sample toxicity by comparing the degree of inhibition of a “normally on” activity.^{1,6,7}

The basic engineering principle of “lights-on” biosensor systems is a transcriptional fusion of an inducible promoter (tailored to a given analyte) with a reporter gene. Upon stimulation a reporter protein such as luciferase, β -galactosidase or green fluorescent protein (GFP) is produced. The promoter gene is quantitatively induced by the target analyte and therefore the fluorescence intensity of for example the GFP can be correlated with the analyte concentration.⁸ The presence of reporter proteins can be monitored either by naked eye or devices capable of monitoring fluorescence/luminescence. Advances in genetic engineering have enabled researchers to set up toolboxes to easily fuse DNA response elements with reporter genes to construct novel biosensors.^{9,10} Biosensor cells can be engineered to respond to analytes such as ions, small molecules, nucleic acids and proteins in a dose-dependent manner.¹¹ Biosensors have been described for many different applications ranging from environmental pollution monitoring to medical

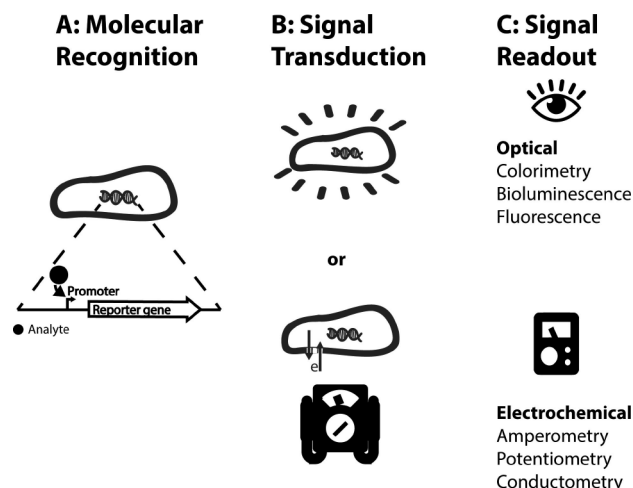


Fig. 1 Schematic representation of the basic mode of action for a whole-cell biosensor. A) Sensor cells are genetically engineered in order to respond to a stimulant and undergo a process of molecular recognition. B) Within the cell, the signal is processed and transduced. Transduction can exemplarily happen as the development of a reporter protein causing fluorescence or bioluminescence, or electrochemically by enabling electron transport due to activation of membrane channels or enzymes. C) The generated signal can be analyzed by either optical or electrochemical methods.

scenarios and analysis of food contaminants.^{4,12-17}

Despite the efforts to genetically engineer microbes to increase sensitivity to stimuli, it remains a challenge. On a genetic level, the sensitivity to a pollutant can be improved by for example employing genetic positive feedback amplifiers. As environmental samples provide significantly more complex matrices for analysis, field-applicable whole-cell biosensors always need to be assessed for cross-sensitivity between matrix components. Finally, the true value of a biosensor can only be assessed by comparing its results to the results of chemical analytics of the same sample. To conclude, we recommend efforts to employ whole-cell microbial biosensors as a first line, cost-effective bioactivity monitoring for environmental contaminants in remote areas and resource-limited settings. Upon obtaining positive results of the biosensor test, the samples are sent to a centralized laboratory facility for more thorough analysis by LC-MS/MS.

In this review we present criteria for guiding the design of whole-cell bacterial biosensor systems applicable in real-world scenarios. First, the design of whole-cell biosensors by the means of genetic engineering is described. Secondly, current laboratory applications of whole-cell biosensors and emerging field use are covered. In the following section, the framework for applying biosensors in the field is established by defining criteria necessary for the transition from the laboratory to the field. Subsequently, the different approaches described in recent literature to tackle the challenge of immobilizing biosensor cells in order to achieve large-scale field application of whole-cell microbial biosensors are compared and future steps are presented. Lastly, two globally successful examples of point of care analytical devices are presented, key design criteria are extracted and applied to formulate design guidelines for the commercialization of whole-cell biosensors.

1-1 Genetic engineering of whole-cell biosensors by the means of synthetic biology

The discipline of synthetic biology deals with the engineering of complex artificial biological systems and ultimately aims at achieving predictability and reliability of these systems. The challenges arising from the inherent complexity of biological systems can be addressed using engineering strategies of standardization, decoupling and abstraction.^{18,19} Organisms are broken down into a hierarchy of composable parts, which can subsequently be assembled in a biological milieu. In this process, synthetic biological components are created by adding, removing or changing genes and proteins.¹⁹ By coupling gene regulatory networks with biosensor modules and biological response systems, new functions can be obtained. A plethora of research has been conducted on the sensor applications of many different organisms from fungi, algae, yeast and bacteria to tissue culture cells. Synthetic biologists aim at moving engineered biomolecular networks into the application stage and progress can be observed, for example, in biosensing, therapeutics and the production of biofuels and pharmaceuticals.¹⁸

Numerous standardized parts for the genetic engineering of *Escherichia coli* exist.²⁰ The international Genetically Engineered Machine Competition (iGEM) is providing an extremely valuable contribution to the development of a library of standardized biological parts known as BioBricks.²¹ These are building blocks used to design and assemble larger synthetic biological circuits from individual parts. Mascher *et al.*^{9,10} created the Bacillus BioBrick Box to enable standardized work with the Gram-positive, spore-forming model organism *Bacillus subtilis*. They have established well-characterized genetic building blocks as a foundation for the convenient and reproducible assembly of novel genetic modules and devices. *Saccharomyces cerevisiae* is an increasingly attractive host for synthetic biology. Dueber *et al.*²² present a versatile engineering platform for yeast, which contains a rapid, modular assembly method and a basic set of characterized parts. Advantages of yeast in a biosensor are established procedures for longterm storage in a dried state, an extensive genetic toolkit and a non-threatening public perception.²³

1-2 Current laboratory applications of whole-cell biosensors and emerging field use

Academic literature has thoroughly transformed the field of whole-cell biosensors in laboratory settings and many different aspects of whole-cell biosensors have been discussed in the past three decades. In the following, we briefly discuss some of the relevant work and provide a general overview of the biosensor field. After King *et al.*²⁴ described an early sensor for the detection of naphthalene using genetically engineered cells, Bousse *et al.*²⁴ provided a brief review of the field in its early years. Questions such as why biosensors are chosen and how to extract information from cells were addressed. Since then, the field has seen vast growth. Many whole-cell-based biosensors have been developed and Gu *et al.*² gave a comprehensive review of different reporter systems, sensitivity, cell immobilization and discussed biosensor applications in different environmental media. In 2004, van der Meer *et al.*²⁵ closely investigated the detection chain of bacterial bioreporters. The goal was to provide guidelines on how to improve existing designs of sensing and regulatory proteins in order to improve detection limits and increase the speed of detection. Analyte transport, sensitivity, selectivity, response rate and other theoretical aspects of the reporter performance have been described. Su *et al.*⁸ provided insight on the progress in electrochemical and optical microbial biosensor research and

discussed strategies for the design of future microbial biosensors. More recently, Verma and Kaur²⁶ have described trends on biosensing systems for heavy metal detection. The authors describe enzymatic, nonenzymatic as well as whole-cell based biosensors and elucidate why biosensors are considered promising tools for heavy metal detection and provide future perspectives.

In summary, a vast body of literature is available, extensively covering various aspects of whole-cell microbial biosensors ranging from their early development to improvements of sensor constructs, novel detection strategies and proposed field applications. In order to incorporate the sensing capabilities of microorganisms into a universally applicable biosensor platform, several design requirements have been identified and are described in the following section.

2 Setting the Frame for Development of Biosensors Applicable in the Field

In order to determine design guidelines for field-applicable whole-cell biosensor devices, we have analyzed analytic, non-cell-based devices, which are used globally. The enabling factors for such widespread application were determined and applied to develop design requirements to transition from laboratory-based biosensor systems to a field-applicable and commercially available biosensor platform. The first example of a non-cell-based, globally used device is the home pregnancy test. It is a lateral flow device made from paper to detect the presence of the hormone human chorionic gonadotropin (hCG) in urine. There is no need for specialized and costly equipment, only a small volume of liquid sample is required and the readout of the result is unambiguous. In summary, it is a robust and simple method to detect pregnancy with a shelf life of 2 – 3 years at room temperature.

Blood glucose meters used by diabetics, are an example where a paper-based system is combined with an electronic device enabling readout and providing a quantitative output. Key characteristics of a glucose meter are the short time until a result is obtained (3 – 5 s), high accuracy, portability and the ease of use. Based on these two examples, we defined several requirements to move from laboratory-based biosensor systems to a field-applicable and commercially available biosensor platform. Three levels can be distinguished: i) organism, ii) fabrication of device and iii) analysis/readout (Fig. 2).

On the level of the organism, care must be taken to ensure that a non-pathogenic, genetically well-characterized strain of bacteria is used. Preferably, it should be a model strain for molecular cloning, making genetic modifications of sensor/signal entities easier. In terms of viability and maintenance of culture, the organism of choice should be robust. Robust means that the organism should not have a too specific range of conditions to grow, enabling combination with complex environmental samples. An example of such an organism is the Gram-negative, *Escherichia coli*. In the optimal case, the organism is able to form a dormant state, for example *Bacillus subtilis* or *Saccharomyces cerevisiae* forming spores, enabling facilitated sensor material production and storage.^{1,27} Physicochemical parameters such as medium, temperature and pH also affect cellular responses and therefore the culture and assay methods need to be standardized.²⁸ A field-applicable whole-cell biosensor needs to comply with strict biosafety rules. Biosafety measures are designed to ensure the safety of people handling potentially hazardous materials of chemical as well as biological nature.²⁹ During distribution and handling, genetically

Field-applicable whole-cell biosensor device

A: Organism and Biosafety

Laboratory Scale: 10 - 1000 devices



B: Fabrication

Industrial Scale: 10'000 - 100'000 devices



C: Storage/ Handling/ Readout



Fig. 2 Schematic overview of requirements of a whole-cell biosensor in order to move from a laboratory to a field application. Three levels can be distinguished. A) The sensor organism, the ease to genetically modify it and its compatibility with biosensor fabrication and corresponding biosafety levels need to be considered. On a laboratory scale, the organism and its sensor applications are characterized and usually prototypes of an integrated device are built. The process of device fabrication is rarely compatible with large scale production. B) On a second level, we consider the fabrication process of a device including compatibility with bacterial life requirements, scalability and cost of the manufacturing process. These considerations are based on production of sensor devices on an industrial scale of 10000 – 100000 units. C) The third level addresses questions of storage until field use, handling and readout procedure for an untrained user in the field.

modified organisms need to be safely contained, protecting the user and environment. After the analysis has been completed, the design of the sensor device needs to allow for inactivation of the biological components and subsequent safe disposal.

Requirements for the fabrication of biosensor devices are compliance with the viability parameters of the microorganism. Therefore, the use of harsh solvents, increased temperatures and harmful radiation must be avoided. The biosensor devices are envisioned to be of use in remote, resource-limited areas and therefore manufacturing costs are a constraint to fabrication. There is a need for cheap, biocompatible materials and processes compatible with mass production to cut costs. In terms of biosensor device storage, the goal is long-term stability under different environmental conditions, enabling decentralized field usage.

Once organism, fabrication and storage requirements are met, the analysis and readout procedures need to be established. Given the envisioned field application, only simple or no sample pretreatment is desired. Furthermore, the device needs to be field-portable and enable on-site evaluation. As the sensor material is to be used outside of a professional laboratory, another key point is the ease of handling for an untrained user. The ease of handling is also important as there is a need for the analysis of large numbers of samples. The time until a result is

obtained is another critical factor affecting the potential for field use.³⁰ In summary, there is a need for an immediate and quantitative result (after an extended period of storage) with a minimal number of handling steps for an untrained user in a resource-limited setting.

3 Cell Immobilization: A Necessity for the Field Applicability of Whole-cell Biosensors

In the following section, the focus is on research showing advances in translating biosensor technologies towards field applications. In order to enable safe cell handling and extended storage, the sensor cells need to be immobilized. Cell immobilization techniques, as presented in recent biosensor literature, were extracted and are presented. An overview of the workflow of cell immobilization is shown in Fig. 3.

3-1 Live cells *versus* spores

The choice of immobilization and storage technique is determined by the biological constitution of the organism. Many whole-cell biosensors described in literature are based on Gram-negative bacteria, which cannot form spores.^{16,31–33} For such organisms, the major challenge is to extend the shelf life by maintaining live cultures at low temperatures or (freeze-) drying sensor cells with protectants. The survival rates after freeze-drying depend on the microbial species and factors such as culture media, growth phase before lyophilization and lyoprotectants.³⁴

Spore-based sensing systems have been shown to retain analytical performance well after 6 – 8 months of storage at room temperature in terms of reproducibility and detection limit.³⁵ Sporulation enables the biosensor engineer a wider range of conditions in terms of temperature, matrix composition and potentially harmful irradiation used in the immobilization process.

3-2 Cell immobilization techniques

In its simplest form, a whole-cell biosensor is a genetically modified reporter organism in suspension culture, incubated in nutrient medium supplemented with the aqueous sample promoting reporter signal development. An analytical device is used to convert the reporter signal to a quantitative measure with the help of a calibration series of known analyte concentrations. Safe and easy use for an untrained user is described as one of the challenges in order to develop a field-applicable whole-cell biosensor (Fig. 2). Safe handling involves minimizing the steps where liquid bacterial cultures are handled, as this process poses an increased risk for contaminations and spills. To this end, different cell immobilization strategies have been developed. Su *et al.*⁸ described the immobilization of microorganisms either by physical methods, such as adsorption or entrapment, or chemical methods, such as covalent binding and cross-linking. Covalent methods affect cell viability and function whereas physical adsorption shows poor stability as microbes desorb. An immobilization procedure should preserve cell functionality and viability as the matrix is optimally formed under neutral pH conditions and at room temperature. The immobilization matrix must provide mechanical stability to prevent cell leakage but still ensure efficient access of analyte molecules by diffusion.^{36,37} In the following section we first highlight efforts to immobilize cells in polymeric matrices, followed by immobilization strategies involving microfluidic devices, paper matrices and combinations thereof.

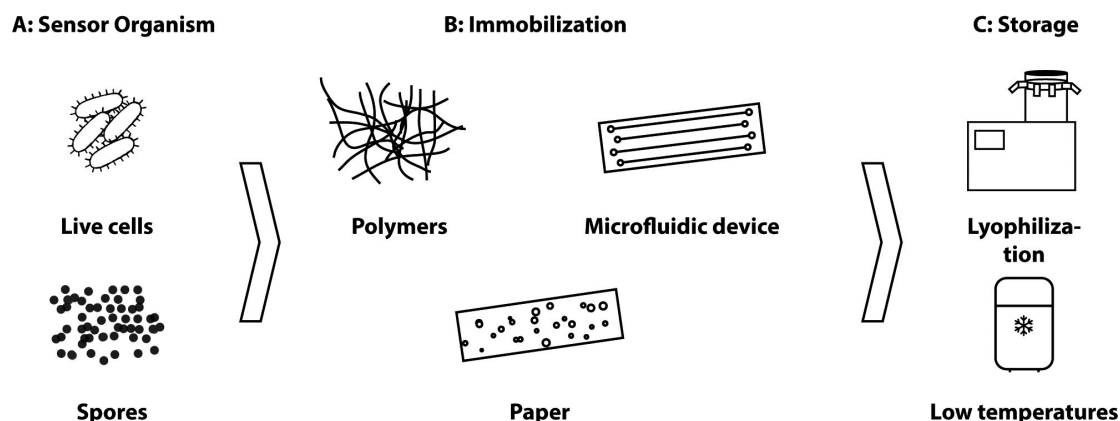


Fig. 3 Schematic highlighting different concepts for cell immobilization. A) The ability of the selected organism to undergo sporulation is a first criterion guiding the decision regarding which immobilization and storage technique to use. B) Many different methods for cell immobilization are known, ranging from immobilization in polymers, in a microfluidic device, on paper and on an electrode and combinations thereof. A wide variety of polymers featuring good cell viability, diffusion and mechanical stability have been described.^{3,33,34,37,39,42,60-64} Different approaches to employ microfluidic platforms to immobilize, store and visualize signal output of cells have been employed.^{31,46,50,51,65} Additionally, immobilization on different kinds of filter paper, either only by adsorption or in combination with a polymeric matrix, have been described.^{52,54,66} Last but not least, different immobilization strategies involving electrodes have been explored.^{37,40,44,60,64,67}

3-3 Polymers

Cross-linked hydrophilic polymers retaining large amounts of water in their 3D networks are known as hydrogels.³⁸ The aqueous environment is highly beneficial for cell growth and therefore they have been widely employed in cell immobilization. Common examples are agarose and alginate, which are described in the following section.

3-3-1 Agarose

Bacterial, as well as mammalian sensor cells can be immobilized in agarose, a polysaccharide polymer matrix, by temperature-induced gelation. For example, a smartphone-based bioluminescence whole-cell toxicity biosensor employing genetically engineered human embryonic kidney cells has been developed. So-called “sentinel cells” constitutively expressing a green-emitting luciferase were entrapped in agarose within the wells of a 3D printed cartridge. The cartridge was compatible with 3D printed smartphone adaptors to enable an aligned optical interface between the smartphone camera and the cell cartridge for signal acquisition. The device allows the performance of toxicity assays within 30 min with the results comprehensibly presented in a smartphone application. Storage within the agarose matrix has been shown to be 6 days at 4°C due to poor time stability of the sentinel cells, limiting the application in decentralized locations.³⁹

The immobilization of mid-exponential phase grown *E. coli* or *S. cerevisiae* cells in a mixed immobilization matrix composed of agarose, polyvinylpyrrolidone (PVP) and collagen has been described. The cell-matrix mixture has been immobilized on a customized ready-to-use disposable microwell array biosensor cartridge based on a polystyrene microtiter plate. The cartridge was connected to a CCD sensor through a fiber optic taper and a portable biosensing device was created. Storage has been demonstrated for 35 days at 4°C.⁴⁰ Mora *et al.*⁴¹ presented a programmable living material based on *E. coli* cells immobilized in an agarose matrix. Employing a fluorescence-based reporter construct, disease-relevant oligosaccharides have been quantified. Storability has been assessed and confirmed over a period of seven days.

3-3-2 Lysogeny Broth (LB) agar

Similar to agarose, LB agar has been employed as immobilization matrix. Agar is a mixture of agarose and smaller agaropeptin molecules in water containing the nutrients of the LB medium. Based on this matrix a bacterial biosensor to detect *Pseudomonas aeruginosa* infections in patients suffering from otitis externa has been described. The sensor is based on cells plated on a LB-agar matrix within the cap of an Eppendorf tube. *P. aeruginosa* produce the volatile 2-aminoacetophenone (2-AA), inducing biosensor luminescence. The luminescence is measured either in a Photo Multiplier Tube (PMT) based plate reader or by a PMT-based compact biosensor device. Activation of the biosensor after five weeks of storage at 4°C has been demonstrated.⁴²

3-3-3 Alginate

Alginate is an anionic polysaccharide, which can be cross-linked *via* the interaction between the carboxylic acid groups of the alginate and divalent ions such as Ca^{2+} in a mild and biocompatible process.⁴³ It forms an open lattice structure providing a hydrated environment for cells and the high porosity facilitates diffusion.

A prototype of a biosensor, the “BioPen”, entraps bioluminescent bacteria within calcium alginate pads. The pads were produced by dripping an alginate/bacteria solution into a CaCl_2 bath inducing the spontaneous formation of spherical pads. The pads were placed just above a complementary metal-oxide-semiconductor (CMOS) sensor surface to monitor cell light activity. The authors expected to achieve a fully integrated, field-operable and disposable system.^{3,33} Leakage of cells from alginate matrices has been observed due to its open lattice structure. In order to address this issue, the mixing with photocross linkable polymers has been described. In a recent study, the swelling properties, viability, bacteria escape, and functionality of the encapsulated reporter *E. coli* from alginate-based hydrogel microbeads were investigated. The cells were engineered to carry a bioluminescent sensor construct to detect other bacteria *via* their secreted autoinducers. Successful storage within the alginate-based hydrogel microbeads for 35 days

at 4°C was confirmed.⁴⁴ Another good example employing alginate immobilization is the improvement of LumiSense, a CCD-based lens-free optical analyzer for the detection of antibiotic residues in food samples based on luminescence.⁴⁵ LumiSense is a system composed of microfluidic sample chips, a bacterial incubation chip (BacChip), and a linear CCD as photodetection component. In this study, ciprofloxacin was used as a model antibiotic and it was detected by an antibiotic-responsive *E. coli* reporter strain showing a dose-dependent bioluminescence signal. Bacterial sensor cells were immobilized in alginate acid, transferred into the wells of the BacChip and left to solidify in a CaCl₂-LB bath.⁴⁶

3-4 Immobilization in a microfluidic device

The use of poly(dimethylsiloxane) (PDMS) as a platform for microfluidic systems is widely known. Fluid flow and mixing in microchannels can be controlled and biological procedures can be miniaturized.⁴⁷ There exist many examples where molecules and cells have been immobilized, manipulated and transported in a controlled manner within a microfluidic device.⁴⁸ The use of microfluidic devices for cell culture, even in 3D within a hydrogel, and subsequent cell-based assays have been described for bacterial, as well as mammalian cells.⁴⁹

3-4-1 Combination of microfluidics and agarose

An example for a microfluidic biosensor system is Lumisens3. The system consists of four modules: (1) module for sample and waste handling, (2) module for media and liquid storage, (3) module for culturing biosensor cells and (4) module for the measurement and the fluidics distribution system for liquid transport between the modules. Bacteria have been immobilized in an agarose matrix and a CCD camera was employed to monitor bioluminescence. The system was only limited by the short shelf-life but otherwise enables the incorporation of any bacterial cells, which have been modified to translate the concentration of an analyte into a dose-dependent luminescent signal.³¹ LumiSense has, for example, been used with a luminescence-based whole-cell mercury biosensor. Another example is the case of genetically modified *Saccharomyces cerevisiae* entrapped in low melt agarose within a microfluidic device to detect diclofenac in wastewater. Upon stimulation with diclofenac, the cells produce a fluorescent protein and the intensity of the protein is detected by fluorescence microscopy. The agarose entrapment allows safe enclosure of the sensor cells, prevents falsified fluorescence results due to cell loss, while at the same time providing access to nutrients and analytes.⁵⁰

3-4-2 Combination of microfluidics and spores

Spore-based whole-cell sensing systems have been incorporated into centrifugal compact disk (CD) microfluidic platforms. The device by Daunert *et al.*⁵¹ is a circular disk made of poly(methyl methacrylate) (PMMA), which comprises six identical structures microfabricated using CNC machining. Within the platform, spores of the bacterium *B. subtilis* were stored in reverse osmosis purified water until use. The spores were engineered for the detection of arsenite and zinc. Germination efficiency of the spores as well as the ability of the spore-derived sensing cells to detect the target analytes on the CD were evaluated. A quantitative response to the analyte was obtained in 2.5 – 3 h. The combination of spore-based sensing systems with miniaturized microfluidic platforms paves the way for compact and portable sensing devices suitable for on-site applications.

3-5 Immobilization on paper

A semi quantitative *E. coli* lacZ-based biosensor strip test for

the detection of arsenite in an aqueous sample has been developed. Sensor cells in a mix of drying protectants (peptone, meat extract, gelatin, sodium ascorbate, raffinose and sodium glutamate) were dried on a paper matrix. For analysis, the paper strips were incubated with the arsenite-containing sample, the substrate X-Gal added and the development of blue spots observed. The intensity of the spots was proportional to the arsenite concentration. Storage of the cells dried on the paper matrix was demonstrated for two months at –20, 4, or 30°C without noticeable loss of immediate induction in the assay. One drawback of the immobilization by drying cells on paper is that a proportion of the cells resuspended from the strip into the test solution.⁵²

A paper-based analytical device (BioPAD) sensitive to antibiotics in the tetracycline family has been developed. With such a device, questions of pharmaceutical quality as well as antibiotic contamination in liquids can be addressed. Two different test designs for analyte exposure, either a lateral flow design or a stationary system, have been proposed. Lateral flow devices were fabricated by wax-printing, subsequent baking to form hydrophobic barriers and spotting of reagents.⁵³ Stationary or “spot” BioPADs were fabricated similarly. Yeast cells were suspended in sodium alginate containing trehalose. The yeast-hydrogel slurry was spotted onto PADs and cross-linked by immersion in a CaCl₂ solution. Subsequently the papers were air dried at room temperature before use in testing. Colorimetric analysis of the BioPAD signal was achieved with a conventional scanner and the mean intensities of test regions were measured. The sensor has been shown to be viable for at least 415 days when stored at 4°C.²³

A spore-based biosensor system called Sposensor envisioned a one-step incubation/detection process in order to meet the constraints of on-site analysis. Spores of *B. subtilis* were encapsulated in agar and subsequently spotted on autoclaved Whatman paper discs, detecting two different compounds, a metal (Zn²⁺) and bacitracin, which is a peptidic antibiotic.⁵⁴

Based on a cellulose nitrate membrane, a fluorescence-based fiber optic toxicity biosensor has been developed. The bacteria were immobilized on the membrane by filtration, smeared with a layer of alginate solution, immersed in a calcium chloride solution and punched into sensor discs. The fluorescence response of the immobilized *E. coli* cells was measured with a fiber-optic fluorescence spectrophotometer by pointing the probe's distal end directly above the microbial membrane. The fluorescent response could reliably be observed for a duration of five weeks when stored at 4°C.⁵⁵

3-6 Lyophilization

Lyophilization, also known as freeze-drying, is a low temperature dehydration process. Water from a frozen sample is sublimed under vacuum and the original shape mostly preserved.

For example, *E. coli* carrying an arsenite-responsive element have been lyophilized and the performance of the lyophilized biosensor after seven months of storage has been evaluated and proven to be maintained. The cells were frozen at –80°C, lyophilized for 24 h and subsequently stored under nitrogen atmosphere at 4°C or room temperature until use.⁵⁶

Another example are cells for the detection of phenols as they are commonly found in wastewater. The biosensor device consists of three parts, a freeze-dried biosensing strain in a vial, a small light-proof test chamber, and an optic fiber connected between the sample chamber and a luminometer. The inducible strains show an increase in bioluminescence under specific stressful conditions, such as membrane-, protein-, DNA-, and oxidative-stress. The toxicity of a sample could be detected by

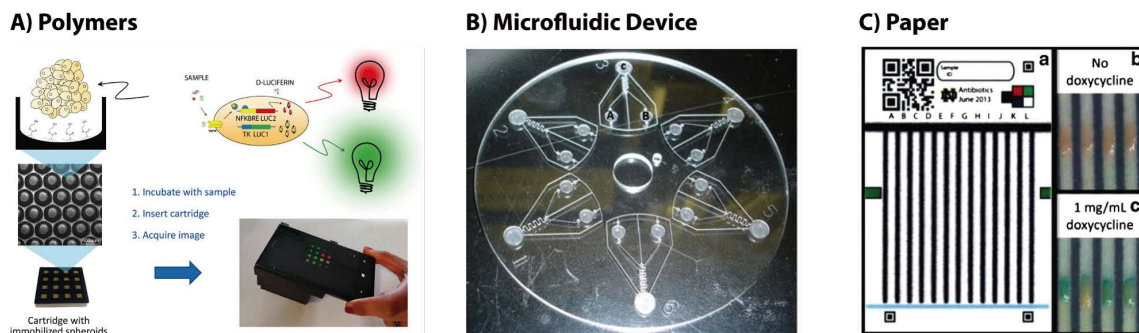


Fig. 4 Selected examples of devices containing biosensor cells highlighting the different immobilization methods. A) In gelatin immobilized mammalian cells genetically engineered to express red- and green-emitting luciferases as inflammation and viability reporters in a smartphone-compatible, 3D-printed cartridge.⁶⁸ B) Compact disk microfluidic platform on a PMMA disc. Labels on the disc denote the reagent reservoirs (A, B) employed for analyte standard solutions/samples and spore suspensions and the detection chamber (C).⁵¹ C) The BioPAD lateral flow test platform with cells immobilized on a paper matrix. Test regions containing yeast exposed to media lack blue pigment and tests exposed to media with the target analyte doxycycline appear blue.²³

measuring the bioluminescence 30 min after sample addition to the freeze-dried strains.⁵⁷ The most advanced demonstration of lyophilized bacterial biosensor cells is a field campaign in six villages across Bangladesh. *E. coli* biosensor cells that had been lyophilized in vials were stored at 4°C for 6 weeks. Subsequently the biosensor vials were used for field measurements of arsenic in groundwater from tube wells.¹⁷

3-7 Storage at low temperatures

For both *E. coli* and *B. subtilis* optimum growth temperature has been determined to be between 30 and 37°C. When both species are kept at lower temperatures, for example in a refrigerator, bacterial growth is slowed down and cultures can be maintained for extended periods. In a laboratory setting, continuously maintaining a cold chain is unproblematic as refrigerators and constant electricity supply are available. In field applications, the cold chain is challenging but a necessary requirement to ensure cell viability and hence accurate and reproducible test results. As described for the BioPAD, responsiveness to the analyte was shown for at least six months when stored at 4°C.²³ The toxicity of several hazardous heavy metal ions were monitored by GFP-expressing *E. coli*. It was found that 100% of the sensor stability was maintained for the first three weeks of storage at 4°C.⁵⁵

4 Conclusion and Design Guidelines for Future Whole-cell Biosensor Platforms

We have analyzed and compared different cell immobilization techniques. In order to minimize the number of handling steps in a potential field application, cells can be immobilized by entrapping within a polymer matrix, preserved in the chambers of microfluidic devices, simply adsorbed onto paper by combining these methods in different ways. The first challenge is to decide whether to maintain the physiologically active state of the biosensor cells until use or exploit the ability of certain organisms to form spores, which naturally survive even under harsh conditions. Cell immobilization in polymeric matrices enables optimal culture conditions in an aqueous environment and facilitates cell handling when designed to have suitable mechanical properties. Despite these advantages, the sensitivity

of cells to polymer crosslinking procedures need to be considered. Incorporating cells within microfluidic devices has the advantage of decreasing the number of handling steps and automating mixing processes. In traditional microfluidic systems, liquids are transported and mixed by pumps, requiring electricity, space, *etc.* More recent developments show the replacement of pumps by centrifugal disc systems. Truly paper-based whole-cell biosensor devices without additional cell immobilization matrix have proven difficult for the application because cells easily desorb.

In the section where we defined the framework for field-applicable whole-cell biosensors, the example of a pregnancy test and the blood glucose meter have been discussed. Looking at these two examples, some requirements for analytic devices to be universally applicable were identified. The requirements for the specific case of whole-cell biosensors are depicted in Fig. 2 and can be summarized on three levels: organism and biosafety, device fabrication and storage, handling and readout. Figure 4 shows a summary highlighting three examples where the different cell immobilization techniques have successfully been implemented in the form of a device. Each of the immobilization techniques has been described in more detail in the previous sections.

Examining biosensors from a user-oriented perspective, we have elaborated some guidelines, which could drive future biosensor device design. In order to meet the requirement for extended shelf life, the use of spores is favorable, as they are less influenced by environmental conditions.^{35,58} Envisioning the use of whole-cell biosensor devices in remote areas with limited access to resources, the goal is to develop a device without complex electronic circuitry.^{23,54} With smartphone ownership rates in emerging and developing nations rising at an extraordinary rate, climbing from a median of 21% in 2013 to 37% in 2015, one could imagine smartphones to replace expensive and often bulky analytic equipment.⁵⁹ Phones could be used to convert the optical information from a chemical reaction, producing colorimetric or fluorescent output, into quantitative data and thereby providing a solution for a point of care test. In order to reduce the need for big and energy-intense equipment such as pumps in microfluidic devices, inspiration can be drawn from lateral flow devices, where migration of fluid happens without pumps.

Considering whole-cell biosensors from a product design perspective, the goal is to safely contain cells within a sensor platform, allowing them to process a signal from the outside but preventing the release of the genetically modified organisms to the environment by physical containment in a nanoporous container. The requirements of such a sensor platform can be met by combining established biotechnological platforms with modern engineering and cutting edge product development tools resulting in integrated systems. Optimally designed, such systems avoid laborious sample preparation and difficult signal readout strategies, making them less capital and labor intensive than conventional analytical methods. The final product is envisioned to be a small, portable, field-ready system, which can be produced at very low cost and, due to its simplicity, operated by an untrained user.

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