1 **Sense and sensitivity in bioprocessing – detecting cellular metabolites with biosensors**

2
3 Linda Dekker^{1,2} and Karen M Polizzi^{1,2,*}

4
5 Department of Life Sciences and Centre for Synthetic Biology and Innovation, Imperial College
6 London, London SW7 2AZ, United Kingdom

7
8 l.dekker@imperial.ac.uk

9 k.polizzi@imperial.ac.uk

10
11 *Corresponding author: k.polizzi@imperial.ac.uk, T: (44) 2075942851

12 13 14 **Abstract**

15 Biosensors use biological elements to detect or quantify an analyte of interest. In bioprocessing,
16 biosensors are employed to monitor key metabolites. There are two main types: fully biological
17 systems or biological recognition coupled with physical/chemical detection. New developments
18 in chemical biosensors include multiplexed detection using microfluidics. Synthetic biology can
19 be used to engineer new biological biosensors with improved characteristics. Although there
20 have been few biosensors developed for bioprocessing thus far, emerging trends can be
21 applied in the future. A range of new platform technologies will enable rapid engineering of new
22 biosensors based on transcriptional activation, riboswitches, and Förster Resonance Energy
23 Transfer. However, translation to industry remains a challenge and more research into the
24 robustness biosensors at scale is needed.

25 26 27 **Introduction**

28 Biosensors are a partially biological entity that can be used to monitor a parameter of interest or
29 target molecule [1] using biological recognition elements such as proteins or nucleic acids.
30 Biosensors come in different forms; some are fully biological, such as whole cells (biological
31 biosensor), and others utilise an *in vitro* enzymatic detection unit where information is processed
32 by a physical/chemical component (chemical or electrochemical biosensor). Biological
33 biosensors are genetically engineered cells or cell extracts that express a genetic mechanism
34 for sensing the compound of interest linked to an output, often a detectable reporter protein.
35 They tend to make use of natural sensing and signalling pathways in cells where the sensor
36 mechanism is transcriptional, translational or post-translational. A chemical biosensor produces
37 an electrical or optical signal proportional to the concentration of analyte usually via an
38 enzymatic catalysis step. Numerous biosensors have been constructed using synthetic biology
39 (reviewed in [2-6]).

40
41 Bioprocessing engineering entails translating biological science into manufacturing processes,
42 which involves many challenges in scale up and optimisation. Monitoring the manufacturing
43 process is vital to ensure product quality and reproducibility [7,8]. It requires both a systematic
44 investigation of the best operating parameters during process development and analysis of

cultures during production. Parameters such as the concentration of nutrients (e.g. glucose) and metabolites (e.g. lactate), reaction parameters such as pH, pressure, temperature, oxygen and CO₂ tension, and product parameters like cell density or the concentration of enzymes are typically monitored to help control the culture trajectory and ensure product quality (Figure 1, top left). Analytics are often employed during process development to better understand the relationship between external bioprocess conditions and cellular physiology. The latter is becoming increasingly important in the era of 'quality-by-design' initiatives proposed by regulators in the pharmaceutical and biopharmaceutical industries [8].

Synthetic biology has a lot to offer in the development of new biosensors for bioprocessing as it enables the rapid engineering of new biological biosensors with enhanced sensitivity, specificity, and robustness. However, the majority of biosensors currently used in the bioprocessing industries are chemical and this is also still an active area of research. In this review we discuss current trends in the development of biosensors, both biological and chemical, that can be applied to bioprocessing and critically discuss the improvements necessary to facilitate their adoption by industry. We will also review emerging trends in the development of biosensors using synthetic biology, particularly methodologies that can be exploited for developing new biosensors for small molecule metabolites. We omit biosensors for detecting cellular stress, as these have been recently reviewed elsewhere [9,10].

Current state-of-the-art practice in industry is to use primarily electrochemical sensors

Currently, in-bioreactor probes are used to monitor temperature, pH and dissolved oxygen (DO) concentration. pH and DO are generally measured using electrochemical probes or optical sensors and some contain integrated thermocouples to measure temperature [11]. For measuring key nutrients, waste metabolites, and some gases, the majority of biomanufacturers use automated amperometric biosensor devices [12], such as the BioProfile® Automated Chemistry Analyzers (Figure 1, bottom left), due to their ease of use, low cost per sample, and the ability to run a range of tests on a single sample. BioProfile® Analyzers use a combination of measurement technologies, including amperometric, potentiometric, and photometric sensors, to measure between 2 and 16 metabolites in a single sample depending on the model. In early models, high sample volumes were required (~1 mL), but the recently released BioProfile® FLEX2 Analyzer operates with a reduced sample volume of 265 µL, a 75% reduction in the required volume. Currently, the main limitation with BioProfile® Analyzers is that the linear range of measurement often does not span what is needed - the lower limit of detection of some metabolites (e.g. lactate) is in the mM range making it impossible to monitor these across the duration of the culture. In some cases, the upper limit of detection is such that the samples require dilution, leading to extra sample handling, decreasing throughput. Despite this, the BioProfile® FLEX2 Analyzer can measure 16 metabolites in an analysis time of 4.5 minutes.

Although there has been a lot of research activity in biosensors (both chemical and biological) in the past decades, there has been little translation of the research into industry. One of the main issues is that most research prototypes have not been subjected to industry scale

experimentation. Therefore, future research should prioritise the performance characterization of biosensors in bioreactors at scale. Due to the speed and efficiency of BioProfile[®] Analyzers, there has been little progress in the field of biosensors for bioprocessing; however, synthetic biology techniques offer many exciting opportunities for the development of new biosensors that are robust under industrial conditions.

Recently developed biosensors for bioprocessing

Regardless of the host organism or the product, a conserved set of metabolites is often of interest. These include glucose as the primary carbon source, lactate as the primary waste metabolite, and the overall pH of the bioreactor, which is related to the production of lactate and ammonia. In many processes amino acids may also be monitored, particularly in recombinant protein production, where deficiencies in amino acids can reduce yield. This section reviews the development of new chemical and biological biosensors for these metabolites (Figure 1, [top right](#)).

Glucose biosensors are typically electrochemical and exploit the glucose oxidase (GOx) enzyme, which catalyses the oxidation of glucose into gluconolactone and hydrogen peroxide. [Advancements in](#) electrochemical glucose biosensors [have](#) focussed on improving the electron exchange between glucose oxidase and the electrode (reviewed in [13], e.g. [14-16]). An algorithm with a feeding strategy that allows glucose to be maintained in a customisable range in a dynamic fed-batch culture has been designed to account for errors in biomass and glucose estimation in industrial CHO cell cultures [17]. This can be used in conjunction with currently available biosensors to ensure rapid implementation during technology transfer and scale up.

Lactate biosensors are often electrochemical biosensors relying on L-lactate dehydrogenase (LDH) and L-lactate oxidase (LOx) as biological recognition elements; however, many whole-cell biosensors also exist. Recently a whole-cell *Escherichia coli* lactate biosensor based on the transcriptional elements of the *lldPRD* operon was used to monitor lactate levels in mammalian cell culture [18]. The biosensor could measure lactate levels as low as 100 μ M in a variety of different cell culture media, making it useful for routine monitoring of cell culture trajectories and the lactate concentration detected correlated with other existing analytical methods. Overall, the biosensor had a lower limit of detection than the BioProfile[®] Analyzer and was less expensive per sample analysed than commercially available lactate oxidase assay kits.

Amino acids are products commonly produced in bioprocessing and are critical precursors in the production of recombinant proteins. Some amino acids (e.g. glutamine and glutamate) are detected by the Bioprofile[®] Analyzers, [while](#) others are detected by analytical methods such as HPLC. Amino acids are one group of metabolites where biological biosensors are commonly researched. For example, transcription-based biosensors have been reported for arginine [19], phenylalanine [20], [21], branched chain amino acids [22], and threonine [23], most of which were used in the context of screening for overproducing strains, but could be adopted for bioprocessing. Also, a series of Förster Resonance Energy Transfer (FRET)-based biosensors for lysine were created using the lysine/arginine/ornithine binding protein flanked by two

fluorescent proteins [24]. The sensors were cross validated with two other analytical assays and applied to monitoring the concentration of lysine in *Corynebacterium glutamicum* fermentations. The advantage of FRET sensors is that they are ratiometric, which increases the accuracy of quantification.

Optical biosensors are generally used for pH monitoring in small-scale bioreactors. They are cheap, do not require a separate reference sensor and can be easily miniaturized. Research has been undertaken to improve their long-term stability and expand the range of pH measured so they can be utilised in more applications [11]. Janzen et al. developed an acidic pH biosensor containing a fluorescent platinum-based reference dye (excitation at 505 nm, emission at 600 nm) that can conduct online measurements between pH 4.0 and 7.0. The operational stability was shown by online pH monitoring ($4.5 < \text{pH} < 5.8$) of the anaerobic batch production of acetone, butanol and ethanol with *Clostridium acetobutylicum* [25]. For biological biosensors, synthetic promoter variants of the *YGP1* promoter from *Saccharomyces cerevisiae* that are inducible at $\text{pH} \leq 3$ [26] could be useful in developing novel pH biosensors. In addition, a synthetic mammalian pH sensor and CO₂ transgene-control device was developed to monitor extracellular pH within the physiological range by rewiring the human proton-activated-cell-surface receptor TDAG8 to chimeric promoters. This pH biosensor, named CO₂ntrol, is controlled by gas-inducible gene expression and can be applied to biopharmaceutical manufacturing [27].

Multi-sensor platforms are smaller, simpler to use and more cost-effective than multiple single biosensors and are therefore a promising way to simultaneously monitor a wide range of parameters. A multi-sensor microfluidic system was developed for the parallel *in situ* monitoring of glucose, lactate, cell density and pH in bioprocessing using GOx and LOx amperometric enzyme sensors to monitor glucose and lactate, an impedance-based sensor to monitor cell density and a pH sensor based on capacitance-voltage measurements at an electrolyte-insulator-semiconductor structure. The biosensor was used to monitor cultures of *S. cerevisiae* and *Lactobacillus acidophilus* and cross validated by a commercial spectrophotometric assay (lactate/glucose), absorbance (cell density) and a commercial glass electrode (pH) with good correlation. The design of the chip is flexible, thus it also has the possibility of the integration of additional sensors [28].

Global trends in synthetic biology biosensors

Taking a broader view of the small molecule biosensors reported since 2014 (regardless of the intended application area), some clear trends emerge. First is that the majority are based on activation of transcription ([19-21,23,29-37]), which may reflect the fact that the genetic parts for such biosensors are more easily identified. For example, libraries of promoters driving GFP expression are available for *E. coli* [38] and can be used in combination with flow cytometry to identify candidate biosensors in high throughput (e.g.[21]). Alternatively, transcriptomic or proteomic datasets can be used to identify transcripts or proteins whose abundance change in response to the molecule of interest, e.g. a recently reported biosensor for threonine [23]. In addition, recent work has developed methodology for co-opting bacterial regulators for use in

other chassis, most notably yeast [19,32,35], paving the way for rapidly engineering new biosensors from previously reported genetic elements.

Within the transcription-based biosensors there is also a trend towards engineering synthetic promoters rather than utilising natural ones [19,20,29,31-33,35,39]. This can be used to manipulate key properties of the biosensor. Using an exemplar of an arsenic biosensor, Merulla and van der Meer demonstrated that the background signal can be reduced by engineering an additional repressor binding site upstream or downstream of the promoter [31]. As a consequence, the linear range of detection was also extended. While placing the additional site downstream was more effective at reducing background signal, the optimal placement of the site upstream resulted in higher reporter fluorescence, suggesting it should be considered when signal detection is the paramount issue.

A second emerging trend is generic strategies to develop biosensors for different analytes with a minimum amount of re-engineering by focusing on modular architectures. Examples can be found in all types of biological biosensors. For transcription-based biosensors, Younger et al demonstrated the development of an orthogonal metabolite biosensor based on a metabolite binding domain fused to a zinc finger DNA binding domain [33] using an example of the BCR-ABL1 zinc finger and the maltose binding protein (MBP). Given that MBP is an archetypal periplasmic binding protein, it should be possible to adapt this strategy to the entire family, many of which bind molecules that would be interesting metabolites for sensing in bioprocessing.

For eukaryotic chassis, Feng et al presented a generic strategy for the production of small molecule biosensors where a destabilised binding domain is fused with a reporter, a fluorescent protein, enzyme, or DNA binding and activation domains to create a transcription factor, with the latter creating the best sensor characteristics [34]. In the absence of the small molecule, the protein is actively degraded by cells due to its instability. However, the presence of the molecule stabilizes the protein, leading to a detectable signal. Interestingly, mutations in different domains of the chimeric transcription factor appeared to be additive and mutations in the DNA binding domain identified in the context of one biosensor had a similar effect in another biosensor. Therefore, biosensors can be modularly composed of different DNA binding, activation, and ligand binding domains.

For biosensors based on riboswitches, the engineering challenge is how to place the riboswitch in the correct DNA context for maximum functionality. Recent work demonstrated a **potentially generic**, modular way to tune the properties of poorly functioning or non-functional switches without the need for reengineering [40], which could result in significantly decreased effort to create new biosensors. Although the work was conducted with a tetracycline responsive switch in the context of creating a genetic selection method, it could be applied to the development of metabolite biosensors as a number of metabolite responsive riboswitches exist in Nature (reviewed in [41]).

Finally, one of the biggest challenges of developing FRET biosensors, finding proteins with an appropriate conformational change upon binding, was recently addressed [42] via a concept

called FREX (FRagment EXchange). In FREX, the fluorophores are located on two separate domains - the full-length protein with point mutations to abolish ligand binding and decrease stability and a fragment duplicated from the wild-type protein. In the presence of the molecule of interest, the fragment and the full length protein can interact, leading to detectable FRET. Although the fold changes with biological fluorophores were modest (1.3-1.8-fold), the affinity of the sensor is easily manipulated by using different fragments for the exchange. Once again, this represents a generic strategy that could be used to engineer new sensors for a variety of analytes.

Conclusions and future perspectives

The advantages of electrochemical biosensors include robustness, high sensitivity and excellent detection limits. They can also be used with small volumes and turbid samples in the presence of optically interfering absorbing/fluorescing compounds. Because of these advantages, electrochemical biosensors have been more readily translated into industry. However, using synthetic biology to develop novel biosensors for bioprocessing has great future potential. Biological biosensors display many advantages in terms of renewability and cost. They can also be used to directly gain access to intracellular concentrations without the need for destructive analysis (i.e. cell lysis) or inference from metabolic models. Since they are genetic, they can be designed directly into production organisms leading to enhanced capabilities. Rather than the production of a reporter protein, biological biosensors can be used to control the expression of an enzyme or metabolic factor to yield a self-regulating production strain. However, a remaining challenge is the adoption of new biosensors into industry. To ensure the success of biological biosensors in a bioprocessing environment, improvements are vital to shorten the analysis time, increase the shelf-life, and improve the scalability in industrial processes. For all types of sensors, multiplexing is increasingly necessary as more and more metabolite data is utilised in the development of bioprocesses. In this respect, microfluidic chips are promising for the development of multiplexed biosensors and can enable *in situ* measurements. In addition, bioinformatics and modelling can be used to mine new genetic or enzymatic detection elements and expand biosensing abilities through computer aided design of metabolic pathways (e.g. [43]). However, research should also focus on the effect of perturbations in the bioprocessing environments (e.g. low oxygen, fluctuating pH and temperature, inhomogeneities in bioreactors where mixing may not be complete) on biosensor function to facilitate adoption by industry.

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Figure legend:

Figure 1. **An overview of the application of biosensors in bioprocessing.** (Top left): Key metabolites that are currently monitored include glucose (primary carbon source), lactate (metabolic waste product), pH and key amino acids. (Bottom left): Currently industry uses instruments such as chemical analyzers (e.g. BioProfile® Analyser), but these have disadvantages in terms of cost, sensitivity, and sample volumes. They are also off-line and rely on an operator to perform the analysis. (Top right): Development of new biosensors is a very active area of research and examples include new whole cell, electrochemical, and FRET biosensors as well as microfluidic chip-based sensors. The major aims are to increase the sensitivity, specificity, and robustness, while decreasing costs. *In situ* or real-time monitoring would allow for a better understanding of the process and paves the way for process control. (Bottom right): **Areas where future improvement is needed include** the application of synthetic biology methods for engineering new biological biosensors with improved performance. What is currently impeding industrial translation is proof-of-concept of new designs under realistic conditions **to enable industrial scale-up of new technologies.**

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