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Staying alive! Sensors used for monitoring cell health in bioreactors

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Abstract

Current and next generation sensors such as pH, dissolved oxygen (dO) and temperature sensors that will help drive the use of single-use bioreactors in industry are reviewed. The current trend in bioreactor use is shifting from the traditional fixed bioreactors to the use of single-use bioreactors (SUBs). However as the shift in paradigm occurs there is now a greater need for sensor technology to play 'catch up' with the innovation of bioreactor technology. Many of the sensors still in use today rely on technology created in the 1960's such as the Clark-type dissolved oxygen sensor or glass pH electrodes. This is due to the strict requirements of sensors to monitor bioprocesses resulting in the use of traditional well understood methods, making it difficult to incorporate new sensor technology into industry. A number of advances in sensor technology have been achieved in recent years, a few of these advances and future research will also be discussed in this review.

Graphical Abstract

Cutting Edge Capsule Technology to be used in bioreactor environment monitoring

Keywords: Bioreactor, electrochemical sensors, optical sensors, capsule technology, pH, dissolved oxygen, temperature, microfabrication, Process analytical technology

1. Introduction

Bioprocesses have been used for thousands of years through fermentations to produce alcohol, cheese and more. However, it was not until Louis Pasteur's fermentation and germ theory in 1857, that this process was understood. Since 1916, with the first ever industrial production of acetone by Chaim Weizmann who is considered the father of Industrial fermentation, large scale stainless steel bioreactors have been the mainstay of the bioprocessing industry. One of the biggest milestones achieved in bioprocessing occurred in the 1980's with the first production of recombinant human insulin. The insulin was produced by splicing the human genomic sequence for Insulin into *Escherichia coli* bacterial cells. These cells are grown by fermentation in bioreactors to produce proinsulin, then my means of enzymatic cleaving human insulin was obtained [1]. These bioreactors can be seen in Figure 1.

High demand for these bioprocess derived products over the years led to using large stainless steel bioreactors to meet the demand, some of which reach volumes of around 10,000 L [2] and in some cases biopharmaceutical industries have used multiple 20,000 L bioreactors. [3].

Today, increases in product titre has greatly increased production. Since 1986 a large number of recombinant monoclonal products have been brought to market such as Rituxan, Herceptin and Remicade. As such, the ongoing need to change facilities to allow for the production of multiple products has never been greater. This need for product flexibility and advancements in product titres is causing a shift from the classic stainless steel bioreactors [4]

Therefore a move to more affordable and scalable single-use bioreactors is occurring in industry. Single-use bioreactors (SUB) offer a more environmentally friendly, easier to handle alternative with less chance of cross-contamination that can occur with current multiple use technology. [5] [6]. Many of these disposable bioreactors can reach volumes of up to 2000 L [6]. Many sensors currently in use are those that have been used in the large-scale bioreactors of the past, some of these sensors still rely on technology that was developed in the 1960's with regard to the Clark-type dO sensor or the glass pH electrode. This old sensor technology is a limiting factor for disposable bioreactors as they are often large, fragile, and are multiple use, which is in contrast to the nature of single-use bioreactors, potentially creating a source of contamination, thus making them ineffective for SUB usage. Biotechnology companies are also concerned with the fixed nature of probe technology, as it is thought that 'hot spots' may arise within the bioreactor. A greater emphasis has been placed on these concerns in recent years as the need for greater titres from smaller single-use bioreactors is required. Current probe technology only provides information on the properties of the media that passes by the probe sensor surface per unit time, which does not give a full representation of the complete bioreactor properties.

In this review, current trends in bioreactor design, sensors and usage will be examined, as well as the exploration of next-generation bioreactor technology. A comparison of current sensors, with their advantages and limitations, will be outlined particularly in the area of pH, dissolved oxygen and temperature sensing. Other parameters that are often monitored during bioprocessing include product concentrations, substrate concentrations and metabolites, cell densities and biomass, however a number of these parameters are monitored offline and are outside the scope of this review.

The review will detail next generation sensors and new possible direction in which sensor technology could move. New novel technology is required that will give accurate, cheap and reliable data online and in real time. Advancements in electrochemical sensors such as miniaturisation and new multiparameteric devices and the growing trend of biosensors are driving the development of exciting new technology that aims to bring bioreactor sensors into the 21st century, breaking the grip of the old well known sensors.

2. Current bioreactors and the advent of Single-Use Bioreactors

There are a number of different bioreactor types that are utilised in the biotechnology industry. However the most prevalent and widely regarded of these is the stirred tank bioreactor. This bioreactor has been in use for a number of years, its simplicity and ease of scale up has made it the industry standard. In this reactor mixing of the bulk media and cells is completed by means of an impellor which ensures that homogeneity is maintained throughout the reactor. However, a balance between homogeneity and shear forces caused by the impellors must be found to avoid damaging the cells, which can result in a large product loss [7] This puts a greater emphasis on probe technology, to allow operators to understand the exact parameters within the bioreactor.

Fig 1 Example of 1000 L Bioreactor (Left) and a 50 L bioreactor (Right)

Airlift bioreactors may not be the industry choice but are gaining favour in some companies due to their energy efficiency and due to their milder hydrodynamic environment. This reactor moves the cells and media around by pumping air bubbles through the reactor, thus churning the media. The disadvantage of this type of bioreactor is that it is only ideal for aerobic processes and also has a high dissolved oxygen due to its sparging method. [8]

Recently single use bioreactors (SUB) have been developed and applied in the biopharmaceutical sector. Single use technologies (SUT) were first introduced into the pharmaceutical industry in an effort to avoid high-cost cleaning and validation requirements while simultaneously reducing risks associated with cross-contamination. Although initially confined to items such as tubing and buffer containers, the application of SUT to bioreactor design has been gaining favour over the last number of years due to a number of factors. SUBs do not require the vigorous cleaning and sterilisation processes between batches that are necessary when using multiple-use stainless steel bioreactors. Hence the need for costly cleaning validation, laborious and time-consuming cleaning and sterilisation between runs and extensive utilities for clean-in-place and steam-in-place processes is eliminated. Despite the disposable nature of SUBs, the environmental benefits of a reduction in energy demands associated with cleaning have been reportedly shown to out-weigh the environmental impact of increased solid waste generation. [9] In addition, recent advancements in specific productivity achievable with recombinant protein-producing cell lines has led to a reduction in cultivation volume necessary to achieve required batch yields, and hence reduction in bioreactor size necessary for production. As a consequence the large-scale stainless steel (<20,000 L) bioreactors could now be replaced by much smaller volume bioreactors, potentially allowing SUBs to be used in biopharmaceutical production. Use of smaller SUBs will in turn reduce capital investment costs associated with set-up of a stainless steel bioreactor facility, as smaller, less complex and more flexible facilities are required. [9]

A variety of SUBs are currently available, each consisting of a cultivation tank composed from polymer(s), including WAVE bioreactors, orbitally shaken bioreactors, pneumatically mixed bioreactors and stirred tank SUBs. The WAVE bioreactor was the first to be introduced and featured a bag, partially filled with cell culture, mounted on a rocking apparatus that served to agitate the culture and allow for gas transfer from the head-space above the cell culture. [6] Orbitally shaken SUBs also feature a bag partially filled with cell culture, however unlike the WAVE bioreactor, culture in an orbitally shaken SUB is agitated by rotating the SUB around a central axis to allow for mixing and gas transfer. Pneumatically mixed SUBs and stirred tank SUBs function similarly to airlift- and stirred tank-bioreactors, respectively. Regardless of the range of SUBs available, stirred tank SUBs are currently the only production-scale SUBs available and are obtainable in up to 2000 L working volume.

Despite the many advantages of using SUBs there are also a number of challenges that need to be addressed to enable the widespread adoption of SUBs in industry. One major concern is the current

lack of studies regarding the interaction of polymers from which SUBs are composed and components of the cell culture contained within the SUBs. In addition, there is a lack of standardised analytical protocols for determination of chemical compounds that may potentially migrate from SUBs under normal (leachables) or exaggerated (extractables) process conditions. [10]

In addition size limitations of currently available SUBs, lack of regulatory guidance regarding validation of single-use systems and a resistance of companies to change currently validated production processes have slowed the application of SUBs in GMP manufacturing. [11]

Although disposable bioreactors have been in use since 1996, innovation in related instrumentation including sensor technology has been slow to adapt to disposable bioreactors. Many of the sensors currently used with SUBs are those that have been used in a full scale stainless steel reactor for many years and thus, are large and fragile. In many cases these probes are multiple-use which could cause problems with cross-contamination.

However, as companies continue to seek more cost efficient production processes with greater flexibility and decreased investment costs it is likely that SUBs will not only dominate small- and mid-scale bioprocessing (*e.g.* research and development and clinical-scale sectors), but will also emerge as leading technology in mainstream commercial manufacturing.

3. PAT and the Role of Sensor Technology

Regulatory agencies have encouraged manufacturers of pharmaceuticals and biopharmaceuticals to innovate in the area of process monitoring to enable greater process control and hence, ensure sufficient product quality. In 2003, the U.S. Food and Drug Administration first introduced a process analytical technology (PAT) framework which outlined a strategy for designing, analysing and controlling manufacturing processes through continuous and timely measurements of critical process parameters (*i.e.* parameters within which a process must be restricted to ensure acceptable product quality) and characteristics that ensure a product is safe and efficacious (critical quality attributes). [12] This PAT framework also forms part of the 'Quality by Design' (QbD) paradigm, introduced by the International Conference on Harmonisation (ICH). [13-15] The QbD paradigm moves away from the traditional rigid 'three batch validation' approach to process validation towards continuous improvement of the manufacturing process over the life cycle of a product. This continuous improvement approach may be facilitated by increased process knowledge gained by continuous process monitoring of critical process parameters (CPPs) using PAT tools and accumulated scientific understanding of the relationship of CPPs to the products' critical quality attributes (CQAs). Ultimately, ensuring a biopharmaceutical process remains within the limits of the CPPs may enable real-time release of drug products, as their quality is assured during manufacturing using PAT tools.

Since increased process control is recognised as leading to better quality drug products, improvements and modernisation of PAT tools used to monitor and control production processes (*e.g.* sensor technology) are being explored. In the bioprocessing industry, CQAs that are frequently monitored include temperature, pH, dissolved oxygen, cell density, viable cell density nutrient media content and metabolite concentration. The bulk of PAT tools currently in use are sensor-based technology including probes that measure temperature, pH, dissolved gases (O_2, CO_2) and total organic carbon, and spectroscopy probes such as near infrared (NIR), mid-infrared (MIR) and Raman spectroscopy probes. Commonly used sensors that measure specific process parameters *e.g.* pH, temperature, dissolved oxygen, are discussed in detail in the next section.

NIR and MIR are employed to screen basal medium powders used in mammalian cell culture however, a strong interference from water can make applications during upstream processing difficult. Raman spectroscopy has been applied for in-line monitoring of nutrient components of culture media and metabolite waste products (glutamine, glucose, glutamate, lactate and ammonium) and also viable and total cell density. [16] Raman spectroscopy has also been evaluated as a tool for monitoring of glycoprotein product yield in fed-batch fermentation of Chinese hamster ovary production cells. [17] Dielectric probes positioned within a bioreactor have been used to monitor bulk capacitance of mammalian cell cultures and have shown sensitivity to early apoptotic changes in

cells, thereby showing great potential for use as a PAT tool for monitoring physiological changes in cells. [18]

Probe-based instrumentation has the advantage of providing real-time information without adversely impacting drug product material. However, such analyses often lack specificity and are unable to provide quantitative information. Consequently, efforts have been made to harness the selectivity of chromatographic methods for PAT to enable at-line or on-line process monitoring. Systems, such as Waters PATROL UPLC™ process analyser, have been implemented for monitoring of drug product and impurity concentrations during upstream processing of biopharmaceutical products [19]

Despite recent efforts outlined to develop PAT tools for bioprocessing, increased innovation is needed to permit the development of new accurate, specific and robust PAT instrumentation. Only then can the true goal of PAT be realised, that is to build quality into the product eliminating the need for end-process release testing of drug products.

This is especially true for SUT applications as new innovative bioreactor technology must be matched with novel disposable sensor technology to realise the potential of SUT in the bioprocessing industry

3.1. Commonly used sensors

The bioprocessing industry has been slow to implement process analytical technology, this could be due to the belief that protein production is more an art form than a science [20]. Or another possibility is that due to the high requirements of monitoring systems in the bioprocessing industry it is preferred to choose tried and tested detection methods, which are now becoming increasingly outdated with the advent of disposable bioreactors. This has made it difficult for PAT to be implemented [21]. This review will focus on the 3 main sensor parameters that are required for all biopharmaceutical processes, pH, dissolved oxygen and temperature.

3.1.1. pH

pH is one of the 3 crucial parameters that must be closely monitored and controlled to prevent cell apoptosis from occurring. pH for optimal cell growth is usually about 7.6 for most animal cells but fluctuations do occur during the cell cycle with pH's reaching levels of 7.0 in some cases. [22]. Cell culture media normally contains buffer agents and sodium bicarbonates to keep pH within optimal working parameters. Combined with CO_2 sparging to reduce pH and base to increase it. It is also noted that optimal pH changes over the course of a bioprocess. It must also be noted that even a small change of 0.1 pH units from the optimum can have a large impact on cell viability and concentration.

3.1.2. Electrochemical Sensors

Electrochemical sensors have been around since 1906 when Cramer reported changes in electrical potential across a glass membrane as a result of pH changes [23]. Glass electrodes have been the industry standard due to their high reproducibility, long life and have an ideal Nernstian response. It is because these sensors are so well understood and can be calibrated to a wide range of pH that there has been a reluctance to use new to the market sensors. The electrochemical pH electrode is known as an ion-selective electrode (ISE) which is a broad sub-category of potentiometry, an electrochemical method of measuring the potential between electrodes with no current flowing. The device consists of a thin membrane, this is commonly glass, which houses a silver/silver chloride electrode, known as the indicator electrode. This is immersed in a solution with a constant Cl- concentration, such as a known concentration of HCl. The indicator electrode measures potential differences between the internal solution and the analyte across the solid state membrane, this is compared against the reference electrode. A reference electrode is required in order to have a baseline or known value for which to compare the test pH value to. The reference electrode is commonly a silver wire coated with silver chloride encased in a plastic or glass tube which is filled with an understood electrolye such as KCl, this electrode is kept separate from the analyte. Modern pH meters incorporate the reference electrode into the probe body and as a result they can be rather bulky. [24] [25] as seen in Figure 2.

While these sensors are well understood and have a wide range of detection, they are quite large and bulky and due to their glass construction they can be fragile. These sensors tend to be unstable in alkaline solutions or at temperatures greater than 100^{o}C. This could cause issues or damage to the sensor during some sterilisation processes where high temperatures in excess of 100^{o}C are normally employed. They are rather sluggish in obtaining a pH value and due to their fragile nature are not ideal, but are still in use, in disposable bioreactors.

Fig 2: Commonly used electrochemical glass pH probe (mettler-toledo)

3.1.3. Optical pH Sensors

Optical pH sensors have been gaining favour due to their smaller size and cheap construction. Optical pH sensors can be broadly categorised as absorbance based or fluorescence based and work by measuring the absorption or fluorescence of a pH indicator dye, which is normally a weak acid or base, which can be either in the bulk media or bound to the sensor surface. The fluorescence or absorption depends on the protonation/deprotonation of the dye. An example of the dyes used can be broken down into absorption dyes, such as phenol red [26], bromocresol green and phenolphthalein [27] and for fluorescent dyes such as; 8-hydroxy-1,3,6-pyrenetrisulfonic acid trisodium salt [28] and fluorescein. The absorption or fluorescence of these dyes are modified by the change of concentration of the hydrogen ions in the solution. The difference in measuring techniques of optical and electrochemical sensors is that electrochemical sensors directly monitor the activity of H_3O^+ ions whereas optical sensors measure the concentration of the pH indicator. [26] [29]. The advantages of an optical sensor over the electrochemical is that it is minimally invasive, provides continual measurements and does not require a separate reference electrode, thus miniaturisation is less of a problem [30] [21]. This allows for the use of patch type systems, ass seen in Figure 3. One of the biggest concerns with these types of sensors is a process known as photobleaching. Photobleaching is the chemical alteration of the indicator dye, be it a fluorophore or a colorimetric dye, so that it is unable to fluoresce due to the destruction of covalent or non-covalent bonds due to non-specific binding caused by excitation light. This results in inaccuracy over time. [31]. The main trends in optical sensor development indicate a move towards miniaturisation, lowering the cost and increasing the mass-producibility of the sensors but creating new production methods. One such device is the optical pH patch, which incorporates the pH sensor onto a sticky disk that is stuck on the bioreactor surface. Another area of development that will assist in the use of disposable bioreactors is in the production on single use optical sensors, this will lend themselves greatly to the single use of disposable bioreactors [32]

Measurement accuracy between optical and electrochemical sensors has been shown to be comparable. A study was carried out comparing an electrochemical sensor to an optical sensors, it was shown that throughout the experiment that the optical sensor underestimated the pH level with the correlation between the electrochemical and the optical probes was 99.7%. 60% of the time the optical probe was within 0.05 pH units and within 0.1pH units the rest of the time [33]. Considering

that tests have shown that a change of as little as 0.15 pH units can result in a reduction of protein expression levels, so it is highly important that these probes are highly accurate. [34]

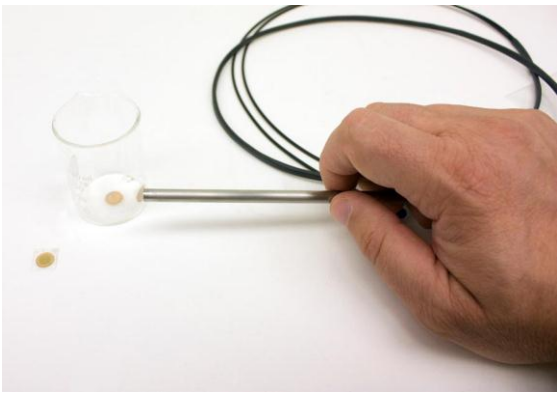

Fig 3: Example of the optical pH 'patch' that can adhere to the inside of a container, while the colour change is observed via an external fibre optic probe (Oceanoptics)

3.1.4. Dissolved Oxygen

Dissolved oxygen (dO) is another key parameter that must be closely monitored and optimised in cell production in a bioreactor. This is crucial for aerobic cell types such as Chinese Hamster Ovary (CHO) cells, being mammalian cells oxygen is key to their survival. The main aspect that makes dO crucial is its effect on glycosylation, many therapeutic proteins undergo post-translational modification, and these modifications are crucial to the efficacy of the protein but can also effect the proteins pharmacodynamics [35]. Therefore it is crucial that the cells oxygen requirements are met. [36] . High dissolved oxygen levels can cause the production of super-oxides or peroxides which have a detrimental effect on the cell membrane of the cells or cause DNA break down. [37]

3.1.5. Electrochemical dissolved Oxygen

Electrochemical dissolved oxygen sensors have been in use since 1953 with the creation of the Clark-type dissolved oxygen sensor. The Clark-type works by having a platinum cathode and a sliver/ silver-chloride reference anode, which is in a known sample of KCl. These electrodes are separated from the sample by means of a gas-permeable polyethylene membrane [38]. Using voltammetry methods the current is measured at a constant voltage, the oxygen in the solution permeates the membrane and is reduced on the cathode. This reaction gives a current flow that is proportional to the concentration of the dO in the solution. [39] [40].

Modern electrochemical dissolved oxygen sensors are also known as galvanic sensors. These sensors follow the same principles as the original Clark-type sensors in that they use two dissimilar metal electrodes. However, this is now typically silver and lead or a silver anode and zinc cathode and contain an oxygen permeable membrane. The metallic lead electrodes are consumed in the process while the silver electrode is attacked by trace acid gases, thus the sensor has a limited life-time, normally of several months. [41]. One of the limitations of the Clark sensor is that the solution must be in constant movement past the electrode, this is due to the oxygen at the site of the electrode being consumed in the detection process. Another disadvantage is that the response times can be rather long due to the oxygen having to diffuse through the membrane, this has been limited somewhat by new miniaturised sensors that have a very thin membrane. The shelf-time of Clark sensors are rather limited to about 6-12 months of use and amount 2 years in storage due to the degradation of the membrane. [42]. One of the main advantages of the Clark type sensor is that there is less interference due to the gas permeable membrane preventing most of the sample from diffusing to the cathode or anode. [39] . Another advantage of the Clark type sensorthat they can be easily miniaturised, this has been helped greatly with the ability to micro-machine sensors, particularly with micro total analysis technology (µTAS). The miniaturisation of these sensors does affect the accuracy, however these sensors had a discrepancy between a commercial blood analyser and the miniaturised Clark-sensor of approximately 6-8%. [41]. Clark-sensors are also susceptible to aging as the electrode sites become depleted

3.1.6. Optical dissolved oxygen sensors

Optical dissolved oxygen sensors have been in use since the discovery that fluorescence is quenched in the presence of oxygen [43] and its subsequent introduction into medical use by [44]. A number of studies in the area of detecting dissolved oxygen using quenching methods have been carried out over the years. An initial study looked at the oxygen quenching of luminescent ruthenium-(11) diimine complexes in silicone rubber. While McDonagh *et al* showed a method ruggisdising the ruthenium dO sensor by immobilising the complex on a sol-gel [45-47] however these studies operate under similar principles.

Optical dissolved oxygen sensors consist of a luminescent probe molecule which has a high quantum yield of luminescence and a long luminescence lifetime which are encapsulated in a gas-permeable, ion-impermeable rubber in the optical fibre tip. The luminescent molecules are then irradiated with an excitation beam, usually using blue LEDs to provide the excitation beam, a red fluorescence is emitted with long-lived relaxation. In the presence of oxygen the fluorescence is reversibly and quantitatively quenched according to the Stern-Volmer equation, therefore the fluorescence is limited correlating to oxygen concentration. [48]. The main advantage of optical sensors is that they are non-destructive to the sample, unlike electrochemical methods that use-up oxygen at the site for detection. Optical sensors also have a long shelf life compared to the short 6 month shelf life of electrochemical devices, owing their use to long term fermentations and bioprocessing. However, like the pH optical sensors they are also susceptible to photobleaching, although this is lessened due to the fluorescent dye being imbedded into a silicone rubber. The response time is generally longer for optical probes due to this membrane. Electrochemical sensors generally perform best at higher oxygen levels whereas optical sensors perform best at lower oxygen levels. One of the methods is to place the dissolved oxygen sensors at the inlet and outlet of the bioreactor to monitor cell oxygen uptake as a means of monitoring cell viability [49]. However, as effective this approach may be, it does not supply accurate holistic information of the dissolved oxygen used by the cells.

3.1.7. Temperature

Table 1: Comparison of resistance thermometer and thermocouple usage

Use Platinum resistance thermometer (PRT) if:	Use Thermocouple if:
Operating temperature: between −200 to 500 °C	**Operating Temperature:** Between −180 to 2,320 °C, so temps greater than 500 °C use thermocouple.
Accuracy: Use PRT if High of accuracy degree required	**Accuracy:** If allowance of tolerance of 2 °C, then thermocouple will serve purpose
Response times: Faster than thermocouples	**Response times**: Slower than PRT
Size: generally larger that thermocouples	**Size:** Can be made smaller than PRT

Temperature monitoring is crucial to ensure optimal cell viability and product yield during bioprocessing. For mammalian cells the optimal temperature for production has been understood for a number of years to be around 37^{o}C, this is due to the body temperature from which these mammalian cells are obtained. However recent studies have shown that lower temperature in the

range of 30-35^{o}C could yield high productions of some protein types. It had been noticed that sub 37^{o}C temperatures for cultures would supress cell growth and glucose usage but the level of cellular activity, and thus product production, was unchanged for extended periods [50]. Other studies showed that similar occurred with CHO cells when producing a recombinant protein at 32^{o}C, or at 30^{o}C [51]. It was also shown that temperatures over 37^{o}c caused a great loss in cell viability and cellular production. [52] Therefore, it is clear that temperature sensors must operate accurately in a range of 30-40^{o}c as the process temperature will change over time. Due to this small margin these sensors must be pinpoint accurate to avoid loss in cell viability.

There are a number of different methods in which temperature can be measured within a bioreactor. Thermocouples are cheap and are amongst the most rugged of the temperature sensors used in bioreactors. However these types of sensors are not as sensitive as other temperature sensors on the market, as such they have been relegated for use in bioprocesses where temperature fluctuations do not cause a great impact. Thermocouples were first developed in 1821 where it was seen that a conductor produces a voltage when subjected to a temperature gradient. [53]. Modern bioreactors, especially those that require very accurate temperature measurement now use platinum resistance temperature detectors. These thermometer elements are constructed of a length of wire wrapped around a glass/ceramic core and housed in a metal sheath to provide protection. Resistance thermometers work by measuring the resistance of the element with temperature changes, the relationship is defined as the amount of resistance change of the sensor per degree of temperature change. There are a number of elements that can be used for the length of wire, such as zinc and copper, however platinum has proved the most useful. [54]. Many studies use Pt resistance thermometers for example [55-58]. The choice of the correct thermometer for the planned process is crucial to obtain optimal cell growth and production. For bioprocessing involving mammalian cells the thermometer must be accurate, have a high level of repeatability and operate around the temperatures of 30-37 ^{o}C, therefore the platinum resistance thermometer is the obvious choice. However for other bioprocesses the accuracy is of less importance and for this a thermocouple is sufficient to the task. The choice of thermometer is often carried out based on a number of principles, which can be seen in Table 1

3.1.8. Summary of electrochemical vs. Optical

The choice between optical and chemical sensors for bioreactors has become difficult in recent years. Optical sensors have seen much advancement over the years, from fibre optics that detect changes in an indicator in media to self-contained sensors with the indicator bound to the sensor surface. However the biopharmaceutical industry has been slow to implement this new technology, instead preferring to stick with the tried and true electrochemical methods. This is either because of the capitol cost required to implement the new technology that would require new instrumentation or that the industry are comfortable and confident in traditional sensors.

Electrochemical sensors tend to be more accurate, do not have issues with photobleaching or use fluorophores that may cause issues with the sensitive cells found in bioprocessing and have faster response times [33]. However their size and fragile construction and lack of innovation over the years has been a large limiting factor for these devices. However, advancements in the electrochemical sensor methods could prove to further cement their use in industry. New microfabrication methods are allowing for the construction of smaller and multi-parametric devices are currently being researched, these devices could streamline and provide the industry with new options that can be implemented with their currently in place instrumentation [59].

Table 2 outlines the advantages and disadvantages of current optical and electrochemical sensors. With the rapid advancements in this area in the last number of years with the aim of reducing disadvantages for both sensor types.

Electrochemical Sensors	
Advantages	**Disadvantages**

Table 2 Advantages and disadvantages of current electrochemical and optical s

Well understood, been industry standard for many years	Current sensors are generally fragile, made of glass
Low production cost	Difficulty in miniaturisation
Faster response times	Susceptible to electronic interference and aggressive analytes
Wide range of measurements possible	
Suitable for disposable technology	

Optical Sensors

Advantages	**Disadvantages**
Ease of miniaturisation	Susceptible to photobleaching or leaching of detection dye/fluorophore
Separate reference electrode not required	Temperature dependant
Minimally invasive and provides continual measurements	Slower response times compared to chemical devices
Suited to mass-production	

4. Future Sensors

4.1. Recent sensor developments

There have been a number of developments in sensors in recent years that look to 'modify' or alter the existing technology in order to improve certain aspects such as the sensitivity, selectivity or biocompatibility of the current sensor technology. This can be done in a number of different ways such as chemical sensing, biomodification and fabrication method.

One such improvement in the area of chemical modification is with the use of polymers instead of a glass electrode for pH detection. Herlem et al, [60] proposes a smooth Pt electrode coated with linear polyethylenimine, which contains a number of amino groups on the surface that were sensitive to H^+ concentration. Unlike previous polymers used, the polyethylenimine exhibited a uniform thickness with few defects which acted as a transducer of the electrode potential versus the pH value in the aqueous media. The study was unable to conclusively determine the mechanism by which the polyethylenimine responds to pH changes. One possible mechanism that was put forward was that there is an affinity of the amino groups of the polymer to the protons in the solution. The reaction of H^+ with amino groups creates a local charge density excess at the electrode surface. The potentiometric response can be considered as a behaviour controlled by a surface reaction. It was noted that the potentiometric response to pH was linear reversible and stable providing a good use in miniaturised analytical sensors, which can be more robust than the standard glass electrodes.

Further developments in the area of dissolved oxygen have yielded a number of advancements in the area. This includes chemical modification designed to make sensing technology more sensitive and selective for dissolved oxygen detection but also in new micro and nanofabrication methods.

One such nano/micro fabrication method was developed in Tyndall National Institute to use nanoporus gold that allows for the detection of dissolved oxygen. Twomey et al [61], utilised a nanoporus gold microdisc array in a three electrode cell for dissolved oxygen sensing. The work was originally carried out to provide a miniaturised, low-cost dissolved oxygen sensor that could be used to test water quality for communities in rural India.

The three electrode system, comprised of the gold working electrode, the platinum counter and Ag/AgCl reference electrode was fabricated on a silicon wafer using standard deposition, etching and e-beam lithographic methods. Photolithographic masks were used in order to assist in the etching process, this allows only specific areas of the device to be etched based on the mask allowing for accurate creation of the device design. This method was used for the gold working, Pt counter and Ag

reference. However further chemical modification of the reference was required to form a Ag/Cl layer. After this the microdisk array was modified with nanoporus gold by elecodeposition of a gold-silver alloy. Cyclic voltammetry and Energy-dispersive X-ray (EDX) analysis was used to determine the successful deposition of the Nanoporus gold. Figure 4 shows SEM images of the successful deposition of the nanoporus gold on the electrode surface.

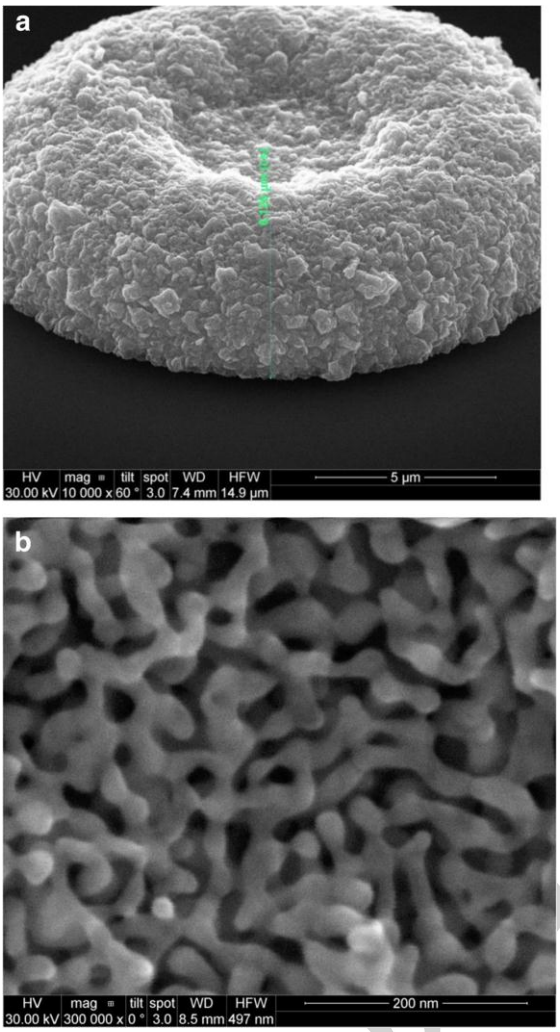

Fig 4 Gold microdisc array modified with nanoporous gold

Dissolved oxygen testing was carried out using cyclic voltammetry between −0.8 and 0 V. A Dryden Aqua probe was used as a commercial standard to compare against and to determine dissolved oxygen levels. Oxygen was pumped into the water samples in order to vary the dissolved oxygen levels. The results showed that the Nanoporus gold obtained faster response times compared to the commercial probe, 20s vs 40s. It was theorised that this was likely due to the oxygen having to diffuse through a membrane for the commercial sensor. An unmodified device was also tested, results showed that the nanoporus gold had a greater correlation r^2=0.925, compared to the unmodified device, r^2=0.870. It was also noted that the nanoporus gold had a greater signal to noise ratio compared to the unmodified microdisc array.

Nanoporus gold for dissolved oxygen detection is only in the early stages of testing but is showing promise over older sensors that still rely on membranes in order to selectively detect dissolved oxygen, thus slowing down the detection times and increasing the size of the probes used.

4.2. Capsule and sensing chip Technology

The use of capsule technology is a novel idea designed to overcome this problem. The capsule works by having a multiparametric sensing chip housed in a biocompatible capsule housing, often made of a

polymer. A number of these small devices can be placed in a bioreactor which will move throughout the bioreactor due to the mechanical forces of the impellors. This will allow for measurements at every point of the bioreactor, giving accurate representations of the homogeneity of key parameters such as pH, dissolved oxygen and temperature. Having many of these devices can be placed in the reactor to increase resolution depending on the reactor sizeOne such project is the use of an Autonomous Sensor capsule for use in a photobioreactor. A photobioreactor is a type of bioreactor that uses a light source to grow phototrophic, i.e. use photosynthesis to produce biomass from light and CO_2. Some of these microorganisms include cyanobacteria, purple bacteria and micro/macro-algae. Recirculation is carried out using pumps or an airlift system but it was noted that in the main tubes of the reactor that poor mixing was resulting in pH and O_2 which had an impact on the growth of algae.Todtenberg, N. [62] has developed a capsule that has the capability to detect and measure biochemical parameters within a photo-bioreactor by moving with the flow of media. The capsule then transfers the information wirelessly to an external receiver. The sensor consists of a waterproof top cover that protects the electronics from the liquid media and a permeable bottom cover that allows the sensor to have direct contact with the environment. The sensor itself consists of the biochemical sensor which was designed to detect potassium, sodium glucose, pH and conductivity. The microcontroller which initiates control and data-acquisition, the transceiver which coupled with the antenna sends the information wirelessly using radio signals to an external receiver. It was noted that the packet error rate of data transferred had a difference of 24% compared with a static air setup, it is hoped that this can be improved with further studies planned.

A similar project in the area of capsule technology is the Process Analytical Technology Capsule (PATsule™). This device aims to detect pH, temperature and dissolved oxygen in disposable bioreactors. This project will be discussed in the next section.

4.3. Case study of Novel technology: PATsule

The PATsule is a novel capsule for real-time, multi-parametric monitoring of critical parameters for biopharmaceutical production is proposed. The unique selling point of the technology is its ability to provide continuous, remote monitoring of a bioreactor environment in both a temporal and spatial manner. A key desire within the biopharmaceutical industry is the availability of process analytical technology to facilitate more complete monitoring of mammalian cell culture and ultimately better control of process scale bioreactors. While solutions currently exist for monitoring key process parameters such as pH, dissolved oxygen (dO), temperature and certain media components using electrochemical or optical sensing devices, current state-of-the-art technology is limited. This limitation arises as commonly used sensor probes are generally fixed at one position following insertion through a port on the bioreactor. This lack of resolution within the bulk media could cast doubts on the homogeneity within the media, this may result in altered cellular viability, productivity or expression of variants of the therapeutic protein with reduced product critical quality attributes.

Fig 5 Concept of the PATsule technology and how it can be incorporated into a bioreactor

The proposed capsule incorporates autonomous sensing technology that probes the bioreactor environment, gathering multiparametric data on pH, temperature and dO and transmitting that information wirelessly to an external system control and data analysis (SCADA) computer, the concept can be seen in Figure 6. A significant advantage of the proposed technology is that the capsule is small and buoyant facilitating its ability to move around the media bulk within the bioreactor through the mixing action of the impellers. The technology has the potential to be highly disruptive, multiple capsules can be added to one large scale reactor to improve the resolution of the measurements and allowing for more in depth process monitoring and control than is currently achievable using available state-of-the-art technology. It will potentially be a high value added activity, which will facilitate enhanced process efficiency.

PATsule builds on a previously developed capsule technology [63-65] the DIACAPS [66-69]. DIACAPS is a capsule, which monitors the environment in the gut and provides a detailed analysis of the gut conditions. The capsule's role is to accurately detect intestinal diseases including inflammatory bowel disease (IBD) and Crohns Disease. The capsule uses a combination of a powerful fluid analysis sensor termed the 'electronic tongue' [70-72], which was used to analyse complex gut solutions. The main objective is to obtain a more accurate clinical diagnosis of intestinal diseases through use of these novel sensing methods.

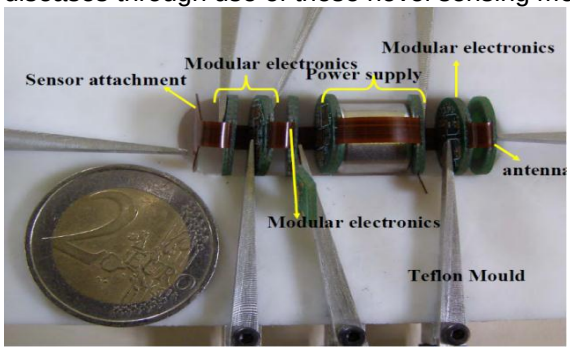

Fig 6: This figure shows the small construction of swalloable capsule, this technology is invaluable to the PATsule project

The instrumentation developed for DIACAPS is highly miniaturised, low power, and battery operated. The technology illustrates a move from the more traditional bench-top based analysis systems [71, 73]. The transducer includes a working, counter and reference electrode on chip; this is the site at which the sensing reaction takes place. The sensor is then capable of many electrochemical techniques including cyclic voltammetry and differential pulse techniques to be processed *via* a microcontroller; this is then transferred wirelessly using industry standard frequencies to an external PC for analysis. The capsule, powered by a single lithium-ion cell , is encapsulated in polyether ether ketone (PEEK), a biocompatible material to measure 12 mm in diameter and 28 mm in length [66]. PEEK is a robust material and it has been used as the packaging material for an e-tongue operating in a food application, where it successfully underwent eight months without malfunction and maintenance [71, 72, 74].

Semiconductor processing methods are applied to fabricate the sensor into a planar, miniaturised chip format. Use of these methods allows careful control of the materials deposition (down to nanometre dimensions) and large scale batch fabrication (large number of sensor chips on one wafer and multiple wafers processed on one fabrication run). There are many examples in the literature on development of sensor chips using these microelectronic based fabrication methods [70, 75-79]. Here the focus is on the development of electrochemical sensors. The dissolved oxygen sensor, discussed in section 4.1.,which incorporates the nanoporous gold surface could be integrated into the capsule. The three electrode cell is manufactured using semiconductor processing techniques where large numbers of devices are stepped across a wafer, and many wafers can be processed in one run. This leads to large economies of scale, resulting in very cheap individual chips. The nano-porous gold layer is fabricated in a wet chemistry lab by electrodepositing AuAg alloy in the recessed gold microdiscs followed by dealloying in nitric acid.

Electrochemical methods are of particular interest for in-situ bioreactor applications as operating procedures remain simple and are without or with limited sample pre-treatment, which is essential for their implementation outside the laboratory. Miniaturised electrochemical sensors offer a number of

advantages over macroscopic electrodes including (i) improved mass transport and hence increased sensitivity (due to the hemispherical diffusion); (ii) improved signal to noise ratio; (iii) reduced *iR* drop [80]. Thus, microfabricated electrochemical sensors can provide sufficient sensitivity and achieve required limits of detection for practical applications due to improved mass transfer and signal to noise ratio.

Recent advances in electronics and semiconductor processing technologies have enabled the design of portable, small size, low power electrochemical sensing systems. These systems are capable not only of performing electrochemical measurements with different techniques but also autonomous electrode electrochemical cleaning followed by multi analyte quantification without end-user intervention [81]. Ogurtsov et al [81] , developed a multiparameteric sensing system that slotted into a robotic fish which was designed to swim around in a working port in Gijon, Northern Spain and probe the water conditions looking for key pollution parameters. The techniques learned from developing a long-term sensor capable of operating in sea-water for extended periods of time will be of great benefit for the PATsule™ project. Many cell medias contain a number of salt based buffers in order to maintain a pH balance throughout the process, these high salt levels can cause problems for the long-term stability of sensors. Seawater is inherently more unpredictable than modern day chemically defined cell media, therefore it would be easier to identify any electrochemically species within the media that could cause interference, this would not be possible with seawater. The materials used in the fish also needed to be of the highest standard as it would be operating in the environment, therefore biocompatible material is crucial, this is also true for sensor design in bioreactors.

Specialised packaging methods are applied in the packaging of the final capsule technology [68, 71, 82-84]. Jesudoss achieved the first level packaging of the sensor in a swallowable capsule by Flip Chip Over Hole (FCOH) methods using anisotropic conductive adhesive (ACA) which not only provided the electrical interconnection but simultaneously sealed the interconnect area and the underlying electronics from the sensor area in a capsule application, FCOH interconnection involves attaching a sensor chip's bond pads face down on to a substrate with an opening in it. This allows interaction between a sensor dye and the medium to be sensed. FCOH is particularly suitable for low I/O count applications such as few I/O sensors because it provides [82]:

- Ruggedized connections;
- Low processing temperature requirements (which results in low thermal stress during processing);
- Dual function, ACA interconnect providing both suitable electrical conduction and liquid insulation around the substrate hole perimeter;
- Mask free process; potentially no post clean step.

An appropriate packaging method is critical for sensors being developed for bioreactors. This is especially true for PATsule™ as it will be completely submerged below the media. Effective packaging is required to not only protect the sensitive electronics but also to protect cells in solution. The packaging material must be biocompatible to ensure that no damage to the delicate cells will occur, in some cases additional protective coatings are required in order to prevent the cells from adhering to the sensor surface.

A similar approach to the DIACAPS capsule technology was utilised in the development of PATsule™. The capsule technology incorporates a miniaturised sensing system which can carry out chemical measurements, on-chip analysis to extract the information from the chemical measurement, and finally wireless transmission of the information to an outer SCADA system. Robust sensor packaging materials were investigated e.g. PEEK to surround the electronics and protect from the sterilization routines applied in the bioreactor. . Another packaging option is the use of Parylene C, which has been used in capsule construction [85] and intraocular implants [86]. This material offers many favourable features including biocompatibility, high mechanical strength, low water permeability and is vapour deposited at room temperature, It has been used as an outer coating of a capsule which has inner polycarbonate construction. This final step ensured the biocompatibility of the final device and the room temperature deposition ensured that there was no mechanical or thermal stress on the inner components [85].

PATsule provides multi-parametric information on a number of sensing parameters as it moves through the bioreactor being aided by the impeller movement. It incorporates recent developments in capsule technology and will combine multi-disciplinary R&D efforts in sensor chip design, miniaturised and lower power instrumentation, wireless communication and robust packaging.

5. Conclusion

As this review has outlined there is a great need for sensors to 'catch-up' with the advancements in bioprocessing technology and methods which have been discovered in recent years. These new bioprocessing methods have increased gram per litre production in some cases by 100-fold over similar processes in the 1980's. However this increase in product production calls upon more accurate, smaller and disposable technology to truly obtain the maximum production levels. These higher titres have also called into question the ability of current probe technology to report on the homogeneity of the bioreactor as the sensors can only detect what passes by the sensor surface per unit time. If homogeneity can be identified and confirmed, then the prevention of parameter 'hot spots' throughout the reactor can be eliminated then even higher product production could be achieved. However, there has been reluctance in industry to adopt new methods due to cost, as many of these large bioreactors have instrumentation fitted it would require a large capital investment to implement the new technology. The bioprocessing industry is also very strict in the instrumentation used and as such there is a reluctance to change. Many bioprocessing plants prefer to implement tried and tested parameter sensors such as the Clark dissolved oxygen sensor or glass pH probes, which rely on technology from the 1960's. And thus it is difficult for new technology to get to market. Therefore it is necessary to develop technology that can be implemented using the instrumentation already in place. It is clear that electrochemical detection methods would be the ideal answer to this, as the use of electrochemical sensors are the industry standard thus the instrumentation is already implemented. This technology should also be able to report on the parameters throughout the bioreactor not just that obtained at a single probe surface.

This review shows that there are a number of new technologies being developed which could answer many of the questions and requirements of the bioprocessing industry. Capsule technology is an area that shows some real promise. This technology uses a multi-parametric sensor chip that is combined with a capsule, allowing it to move throughout a bioreactor with the mechanical motion of the bioreactor impellors. A number of these devices can be deployed within a bioreactor to increase coverage and resolution of the parameter data throughout the bioreactor. The electrochemical nature of this device means that it could link in with existing instrumentation to allow for easier integration into industry. Breaking the paradigm and culture of bioprocessing remains the largest hurdle for new technology to over-come. However industry is beginning to realise that to move forward the older technology must be set aside. This can be seen through the use of disposable bioreactors, new bioreactor types such as the airlift bioreactor and through the slow integration of new sensor types like the 'sensor patch' and optical sensors. We would like to acknowledge EI () for funding this work.

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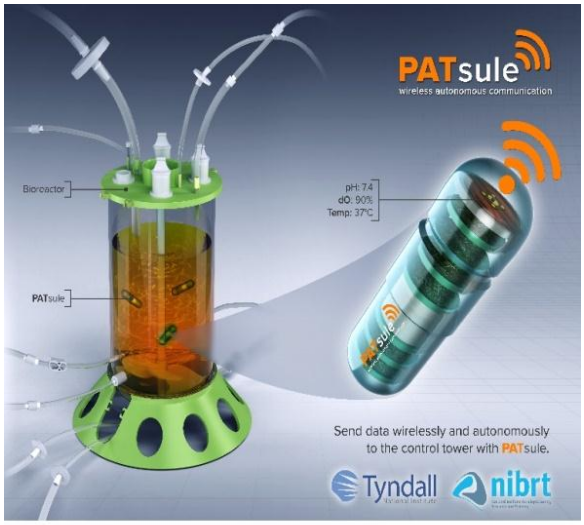
Highlights:

- Current sensor technology used in Process Analytical Technology (PAT)
- Review of next generation sensors
- Case study: PATsule technology



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Graphical Abstract