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Enhancing cell and gene therapy manufacture through the application of advanced fluorescent optical sensors (Review)

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Cell and gene therapies (CGTs) are examples of future therapeutics that can be used to cure or alleviate the symptoms of disease, by repairing damaged tissue or reprogramming defective genetic information. However, despite the recent advancements in clinical trial outcomes, the path to wide-scale adoption of CGTs remains challenging, such that the emergence of a "blockbuster" therapy has so far proved elusive. Manufacturing solutions for these therapies require the application of scalable and replicable cell manufacturing techniques, which differ markedly from the existing pharmaceutical incumbent. Attempts to adopt this pharmaceutical model for CGT manufacture have largely proved unsuccessful. The most significant challenges facing CGT manufacturing are process analytical testing and quality control. These procedures would greatly benefit from improved sensory technologies that allow direct measurement of critical quality attributes, such as *p*H, oxygen, lactate and glucose. In turn, this would make manufacturing more robust, replicable and standardized. In this review, the present-day state and prospects of CGT manufacturing are discussed. In particular, the authors highlight the role of fluorescent optical sensors, focusing on their strengths and weaknesses, for CGT manufacture. The review concludes by discussing how the integration of CGT manufacture and fluorescent optical sensors could augment future bioprocessing approaches.
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I. INTRODUCTION

The last four decades have seen enormous strides in our ability to effectively culture mammalian cells, which is an essential requirement for the development of innovative biotechnology.[1] Cell culture is now the established method for producing proteins, as it permits the production of large, functionally modified and glycosylated macromolecules.[2] In addition, cell culture has also become essential for the synthesis of viral vectors for gene therapies.[3] Application of these important cell and gene therapies (CGTs) has required significant advancement in both manufacturing capacity and sensory bioprocessing technology.[4,5]

While this capacity has proved exceptionally capable for large-scale manufacture of pharmaceuticals and biologics, future CGTs and tissue engineered therapies (TETs) are significantly more challenging to manufacture. This is because unlike traditional biotechnology products, manufactured by and purified from bacteria and yeast, CGTs and TETs are dependent on the preliminary production of complex biological machinery, in the form of biological materials and distinct cells lines.[6] As a result, this produces challenges in determining the complexities that can arise from seemingly innocuous changes in the culture process, as well as downstream purification of specific cell populations. For this reason, it is difficult to achieve consistent cellular products with only the insights provided by existing sensory technologies, as they lack the sensitivity to detect key process parameters for efficient and reproducible CGT manufacture. Therefore, the efficient manufacture of CGTs will require a paradigm shift in both manufacturing and sensory bioprocessing technologies to become commercially viable.

A. Emerging cell and gene therapy manufacturing paradigms

Cell culture has progressed since its early beginnings as a tool to drive *in vitro* understanding in the field of biology.[7] The first commercial therapeutic application of cells was as viral hosts.[8] This permitted viruses to replicate in a nonhuman host, which in turn could be used to manufacture inactivated or weakened viruses for vaccine production.[9] The next notable step was the development of cell lines.[9] These workhorse cells share some basic biological attributes with cancerous cells, which rapidly and continually divide, making them excellent for producing large amounts of identical cells rapidly. Adoption of Chinese hamster ovary cells in suspension took this a step further, such that mammalian cells could be manufactured using the tried and tested methods of routine microbial culture.[9] Editing of cellular genetic makeup also

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allowed for the optimization of subcellular machinery to promote stable and efficient biologic medicine production.[8] Following the success of cell-derived biotechnology products, methods to create replacement cells or tissues for cellular therapy have been ongoing. The development of a blockbuster technology that can revolutionize the field, by acting as a template to model subsequent regenerative medicine based therapeutics, is still absent from the field.

In order to account for the different cell types and production requirements, commercial scale cell culture can take a number of forms. Commercial cell culture includes both suspension and surfaces adherent methodologies, which are highlighted in Fig. 1. While small-scale culture of cells is appropriate for *in vitro* studies, it is not suitable for mass production of biotechnology products for clinical use.[10] Therefore, for large scale production, highly efficient bioreactors, of up to 20 000 litres, have been developed.[11]

Adherent cells, which require a surface to attach and develop, are more challenging for scale up. This is due to the barriers presented by the removal of cells from surfaces and downstream purification and processing.[10] An interim solution, which has permitted limited scale-up of adherent cell cultures has been through the application of roller bottles and disk propagators.[9] However, a major breakthrough for adherent cell scale-up applications was the use of fibers or microcarriers as attachment surfaces.[14]

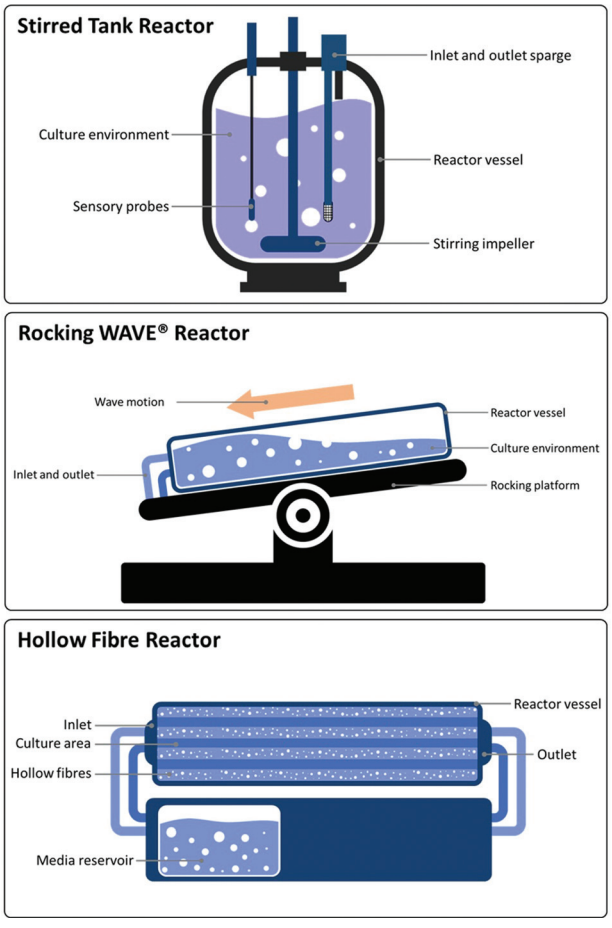

FIG. 1. Schematic diagrams of scalable bioreactor platforms. Stirred tank reactors (Ref. 12) and rocking reactors (Ref. 13) are both examples of suspension culture systems where the product is grown in a constantly agitated environment. Conversely, the hollow fiber reactors have a static perfused bed of porous fibers that allow for very high surface areas. The stirred tank reactor, when compared to the other two culture methods, permits the greatest scope for large scale up applications.

Fiber and microcarrier reactors can provide very large surface areas for cells to attach and grow.[10] Microcarriers are especially promising, as surface area increases can be readily achieved through the addition of more microcarriers to the system.[15] Furthermore, porous microcarriers permit further increases in surface area.[11] As the bioreactor increases in size, it has the potential to become much more productive. However, as size increases and culture densities are transformed, the culture environment must be optimized.[11]

In order to determine the optimal environment and processing of CGT cultures, a large degree of online sensory information is required. This is because mammalian cells continually require effective gas exchange and a regular replenishment of a highly complex culture media with specific nutrient levels. Therefore, as the volume of the bioreactors increase the need for sensory input also increases, as they are prone to the development of dead-spots, which are regions with suboptimal gas and media perfusion.[16] Examples of critical quality attributes (CQAs) that would benefit from sensors and enhance bioreactor performance include: small molecules and ions (*p*H, oxygen, and carbon dioxide), metabolites (glucose, lactate, and ammonia), and large macromolecules (enzymes, growth factors, and cytokines).[17–19]

B. Importance of measurement and sensors in bioprocessing

Quantification of essential molecules and ions has underpinned our knowledge of biological processes.[20] The purpose of sensors is to specifically detect analytes in their surroundings, transduce a signal to a detector, which is then quantified and interpreted as a measurement. Therefore, the importance of sensory technologies for understanding biological processes and how they contribute to cell function cannot be understated.[21] An ideal sensor should be noninvasive, so that it does not perturb the system or generate measurement artifacts, highly sensitive and selective to the analyte of interest, whilst providing high spatial and temporal resolution (Fig. 2).[22]

In order to establish enhanced bioprocessing control methods, it is an absolute necessity to have suitable monitoring (offline and online) for the culture environment.[23] Offline monitoring is conducted by extracting samples from the culture environment, which are taken to analytical equipment for examination. This allows for a greater range of measurements to be performed that cannot be conducted *in situ*. However, the validity of these measurements is limited if the samples are liable to degradation, when removed from their immediate surroundings and if the measurement itself is influenced through interaction with external parameters (*e.g.*, oxygen levels[15,24]). Conversely, online monitoring allows real-time

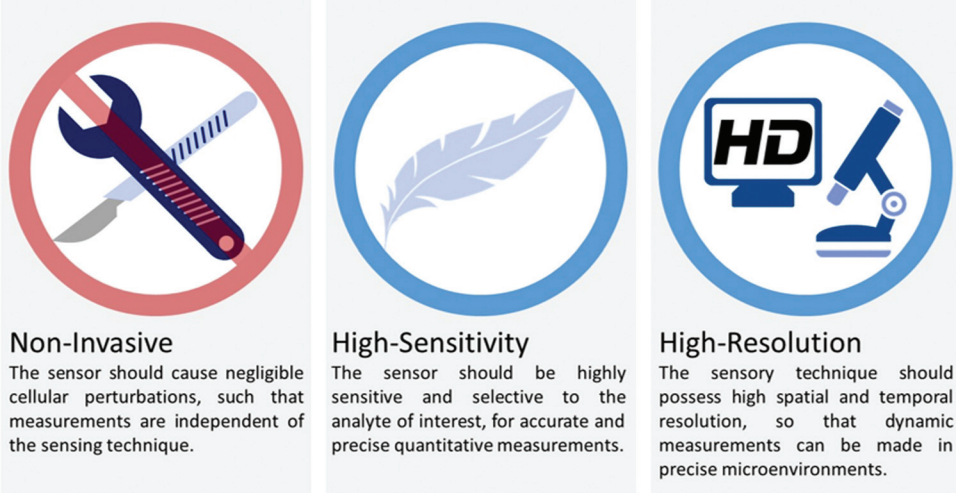

FIG. 2. Desirable sensor characteristics.

quantification of analytes of interest.[17] This will yield live information during bioprocessing, which will be essential for the early detection of deviations from method parameters and enable implementation of countermeasures.

C. The current state-of-art of sensory technology

Early bioprocessing was far removed from the scientifically informed engineered approaches we have today. Fermentation processes were more akin to an art form, controlled by skilled operators visually inspecting cultures.[25] Since that time, bioprocessing has advanced significantly, particularly on the back of high powered computing, automation and advanced sensory systems.[25]

Measurement systems developed for industrial scale bioprocessing of bacteria or yeasts are insufficient for providing the level of characterization required for effective control of complex cellular manufacturing environments.[4,5] This is because, traditionally these measurement systems were dependent on electrochemical sensors, which were limited by a narrow temperature window of operation, were subject to measurement inaccuracies, due to interfering gasses, and had short lifetimes, as they were gradually degraded through use.[25–27]

Mass spectrometry yields information rich datasets when utilized to optimize bioprocessing.[28] This technique has made its way into routine use in production processes and has been a major addition to the CGT research and development, despite the high initial costs of setup.[25] The wealth of data provided by this technique has facilitated the bioprocessing of the existing generation of biologics immensely and a similar advance in monitoring technology could augment CGT manufacturing.

More recently optical sensors have been widely applied to commercial bioreactor platforms, due to their high sensitivity and specificity for analytes of interest.[29–31] For example optical systems have been used to determine optical density, cell number and gas composition[32] as well as quantifying biochemical parameters, such as *p*H, oxygen, and carbon dioxide.[33]

II. ADVANCED FLUORESCENT OPTICAL SENSORS

Governments, pharmaceutical companies and universities annually invest large sums of money into the development of new technologies to measure biological systems both at the large and small scale.[34,35]

The development of fluorescent optical sensors is an exciting field of research involving highly multidisciplinary teams.[36] Existing probes are diverse in their formulation, with measurement applications ranging from the characterization of small molecules and ions to large macromolecules. Due to their noninvasive nature and relatively high sensitivity and specificity fluorescence based optical sensors, such as fiber optics, free fluorophores and fluorescent nanosensors are examples of excellent measurement technologies for characterizing biological environments.[37]

A. Fiber optic sensors

Fiber optic sensors usually comprise of a transparent core enveloped by a protective coating.[38] Light used for excitation is totally internally reflected to a fluorophore-based sensor at the tip of the optode. In turn, this sensor reports the external stimulus as a signal back to a spectroscopic detector at a different wavelngth. The protective coat surrounding the optode prevents unwanted loss of fluorophore in biological systems, which could reduce sensitivity or generate measurement artifacts. Coupled with established detection systems, such as fluorescence and confocal microscopy, correctly calibrated pulled optical fibers can characterize analyte concentrations through changes in fluorescence. This was effectively demonstrated for glucose oxidase by Portaccio *et al.*, who performed temporal glucose measurements with an optical fiber, coupled to a fluorescence spectrophotometer.[39]

The major drawback of utilizing fiber optic sensors for cellular measurements is the substantial damage that can be caused when fiber optic tips are inserted into fragile biological structures as well as the limitations associated with point-to-point measurements. Cellular damage can be caused by the initial puncture force and the volume occupied by the fiber optic tip within the cell. Both can cause perturbations in cell function, which inadvertently changes the system being examined. Therefore, to minimize the cellular perturbations, tip dimensions of less than 50 nm diameter have been achieved by laser heated optical fiber pulling.[40] Furthermore, operators develop a high level of skill to ensure the insertion does not cause excessive damage when probes are positioned in delicate structures such as subcellular microenvironments.[41]

The point-to-point measurements made by fiber optics probes are unable to fully characterize large volumes in technologies such as bioreactors. This is because bioreactors are continually evolving and developing regions of distinct biochemical heterogeneity for which fiber optic based sensors are unable to characterize, which can be referred to as measurement dark zones. Therefore, to overcome this limitation fluorophore and fluorescent nanosensors can be distributed throughout a volume to determine its properties.

B. Free fluorophores

Fluorescent molecules that are able to freely move within a system have been widely implemented for both quantitative and qualitative imaging. Individual molecules of fluorophores usually produce a fast and bright response. In addition, due to their relatively low molecular weight (typically <1000 MW) these molecules impose minimal physical perturbations when successfully delivered to cells.[42] However, there are drawbacks with fluorescent molecules as signal transducers. Cellular delivery can be challenging as molecules may permeate the cell membrane unassisted.[43] Furthermore, there could be interference from cellular components that generate measurement artifacts[44] and subsequently nonratiometric measurements.[45] Finally, isolated molecules of fluorescent sensors spread throughout a biological system may bleach to a level below detectable background limits more rapidly than packed clusters of sensors in one location (Fig. 3).[46]

The delivery of free fluorophores to living cells is challenging primarily because of the natural obstruction provided by the cell membrane. Certain fluorophores readily traverse cell membranes. However, this ease of transport across cell membranes is also their weakness, allowing them to readily leach out or into other subcellular spaces. This was demonstrated by Rink *et al.* who found 6-carboxyfluorescein leached by up to 40% after 10 min when applied to lymphocytes.[47]

To overcome challenges of the cellular delivery of fluorophores, they can be chemically modified or alternatively purchased with chemical moieties, *e.g.*, acetoxymethyl and acetate esters, to enhance their cellular uptake.[48] It is important to note, it is not always possible to chemically modify all fluorophores this way without affecting the sensory capabilities. Furthermore, the fluorophores which are successfully taken up by cells are able to freely interact with cellular components. Free interaction with biological components can also hinder sensing capabilities and unintentionally bind to cellular material such as proteins[49] or initiate cellular toxicity.[50] These interactions can also quench fluorescence or present as measurement artifacts.

When fluorophores are successfully delivered to a cell it is challenging to ascertain uptake levels of the fluorophore.

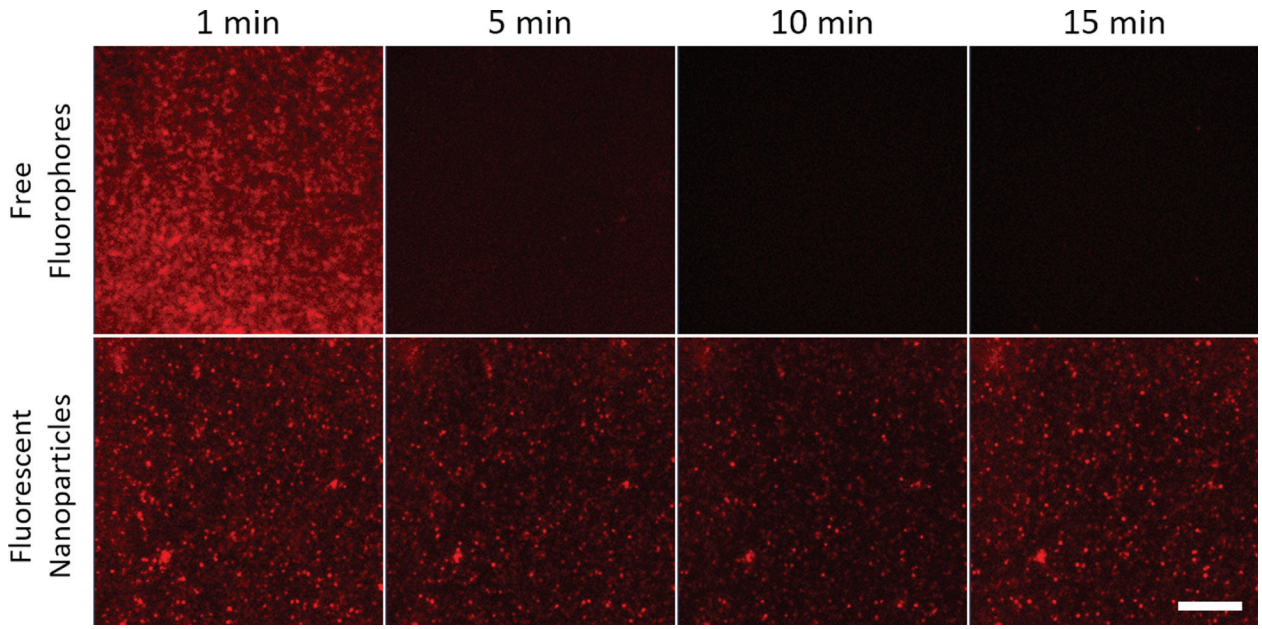

FIG. 3. Free fluorophore and polymeric nanosensors trapped in a polymer matrix demonstrating bleaching over time. In brief, ~50 nm polyacrylamide nanoparticles containing the dye 5-carboxytetramethylrhodamine were encapsulated in a translucent polymer matrix. These matrices were imaged on a Zeiss Elyra super resolution fluorescence microscope, with stochastic optical reconstruction microscopy. Images were recorded for 15 min continuously. Scale bar = 5 μm.

This is a critical drawback for making quantitative measurements with free fluorophores. To overcome this drawback, ratiometric fluorophores can be used, which emit a secondary reference signal at a different wavelength, independent of analyte concentration. By obtaining a ratio of indicator signal to reference signal, accurate ratiometric measurements can be made, which are independent of fluorophore concentration, fluctuations in excitation energy, as well as detector sensitivity and light scattering.[38] Thus, ratiometric fluorophores are well suited at making accurate quantitative measurements. However, very few fluorophores are intrinsically ratiometric,[48] yet it is possible to chemically modify fluorophores to convey ratiometric properties. This can however be at the expense of the sensory capacity of the fluorophore, which could be affected by the modification process. It is important to note the simultaneous delivery of a secondary reference fluorophore, in addition to the analyte sensitive fluorophore will not produce ratiometric measurements. This is because the observer cannot be certain if the secondary fluorophore occupies the same spatial coordinate as the indicator fluorophore, in identical ratios when distributed at a range of locations throughout a cell.

C. Polymeric fluorescent nanosensors

Polymeric fluorescent nanosensors are probes comprising of an inert matrix with nanometer-sized dimensions that selectively respond to specific analytes in their surroundings to transduce fluorescence signals to a detector.[51] Due to their small size, high signal-to-noise ratio and versatile inert matrix, fluorescent nanosensors can be thought of as powerful tools that represent an advance in optical sensor based technologies. Fluorescent nanosensors combine the benefits of both fiber optic sensors and free fluorophores, while overcoming some of their inherent weaknesses.

The key differentiator of fluorescent nanosensors that sets them apart from both fiber optic sensors and free fluorophores is the inert, chemically versatile nanosensor matrix, which serves to both enhance sensing capabilities and augment cellular delivery (Fig. 4). Sensors can be modified with key chemical entities to modify their interaction with external environments. Each modification can elicit a range of actions. These include enhancement of sensing capabilities through the attachment of different analyte sensitive fluorophores or extended circulation lifetime through polyethylene glycol coating and subsequent reduction of affinity to proteins and cell membranes.[52] Internally, sensors consist of a ratiometric reference dye, which facilitates both tracking of the sensor and ratiometric measurements.[53] Additionally, there must be a sensor dye which reacts to the analyte of interest.

Fluorescent nanosensors are composed of a porous biofriendly polymeric matrix, such as polyacrylamide,[54] silica sol-gel,[52] polystyrene derivatives,[55] and poly(methyl methacrylate),[56] and usually present with dimensions that are less than pulled optical fibers, but greater than free fluorophores. The large surface area of the nanosensor permits a small volume of sensing elements to interact with a large number of analytes. Due to the versatility of the nanosensor matrix, a large number of sensing elements can be incorporated into a very small volume, such that ratiometric nanosensors, or nanosensors sensitive to more than one analyte, can be fabricated which possess high signal to noise ratios. Typically, fluorescent nanosensors consist of two types of fluorophore, an indicator and a reference. The indicator functions as a transducer, which produces a signal corresponding to the concentration of the analyte of interest. In contrast, the reference fluorophore is insensitive to changes in analyte concentration, producing a constant signal at a wavelength different to the indicator

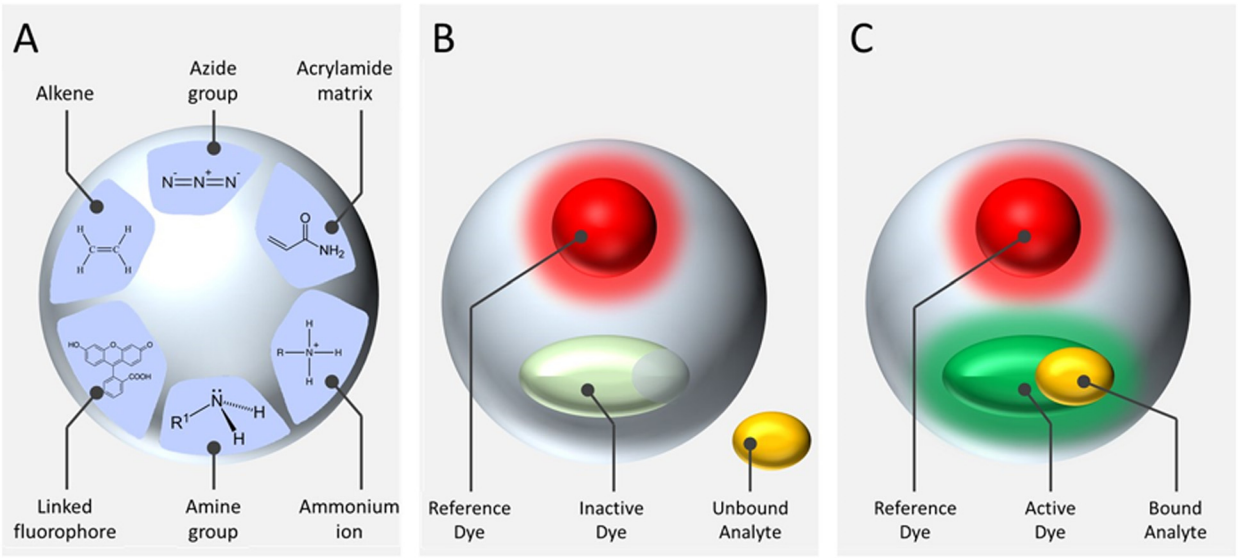

FIG. 4. Schematic diagrams of nanosensors. (a) Sensor surfaces can be chemically tailored to enhance sensing capabilities or to augment cellular delivery. (b) The reference dye is always active allowing for the nanosensors to be spatially located. (c) In the presence of the analyte of interest, activation occurs, and both the reference and the sensory dye are active allowing measurements to be acquired.

fluorophore. Therefore, incorporation of indicator fluorophores and reference fluorophores in a nanosensor matrix permits accurate ratiometric measurements to be made from a single spatial coordinate. Similarly, if more than one indicator fluorophore is incorporated into the matrix; fluorescent nanosensors could be used to make simultaneous measurements of multiple analytes of interest.

There are a diverse range of available fluorescent sensing elements available for use. Similarly, there are many potential matrices in which to encapsulate and protect these sensory elements. This wide availability has provided fertile ground for development of fluorescent nanosensors by a number of research groups around the world. Fluorescent sensors have been developed for physical properties such as temperature,[57–60] biological molecules such as proteins,[61,62] nucleic acids[63] and adenosine triphosphate,[64,65] as well as a large number of chemical entities including magnesium,[43] mercury,[66] potassium,[67] reactive oxygen species,[68,69] sodium,[70,71] zinc,[72] calcium,[73,74] copper,[75] chloride,[76] glucose,[77] iron,[78] lead,[79] hydrogen ions (*p*H),[54,80–82] and molecular oxygen.[83–85]

D. Complex molecule sensors

Complex molecule sensors (CMSs) or affinity sensors are similar to immunocytochemistry type assays in their ability to detect specific molecules. CMS systems rely on specific molecules able to bind both reversibly and specifically to the analyte of interest.[86] The major advantage for CMSs is that they allow continuous monitoring of biomolecules rather than one-off, end-point insights into a process. This is due to the fact that CMSs, unlike antibody based immunocytochemistry, bind reversibly to the molecule of interest and thus can be reused.

A good example of a class of CMSs are fluorescence resonance energy transfer (FRET) based sensing elements, which are usually composed of an analyte sensitive macromolecule, such as a proteins or nucleic acids (*e.g.*, aptamers), that are conjugated to two different fluorophores. The primary fluorophore acts as a FRET donor that emits fluorescence at a wavelength which excites fluorescence in a secondary fluorophore, a FRET acceptor. When the macromolecule senses an analyte of interest it undergoes a conformational change which can bring together or separate the two fluorophores. When the two fluorophores are extremely close together, typically with a Förster distance of less than 10 nm, the primary fluorophore excites the secondary fluorophore to express fluorescence that can be quantified. The combination of the two fluorophores in this manner makes FRET based sensors intrinsically ratiometric. Zhang and Wang have reported nanosensors that utilize cadmium-selenide-zinc sulfide (CdSe-ZnS) quantum dots and Cy5, which function as a FRET donor and acceptor, respectively, over a Förster distance of 69.4 Å (or 6.94 nm), to sense low concentrations of nucleic acids.[87] For further information regarding quantum dot based nanosensors for molecular sensing in biological systems please refer to an in depth review by Zhang *et al.*[88]

III. DISCUSSION

There is considerable potential for emerging fluorescence optical sensory probes to contribute to process control of CGT factories of the future. In order to facilitate these changes sensory systems that accurately detect and allow subsequent control of population heterogeneity and quality must ultimately be selected from the scientific advances and translated to commercial scale.[89]

For fluorescent optical sensors, in particular, fluorescent nanosensors, to acquire broad utility as a medical diagnostic tools it is important that their signals can be accurately obtained from thick and turbid samples, such as tissue and organs. Currently, it is challenging to obtain fluorescent signals at depths using high powered objective lenses, due to their short working distance, while turbid samples are known to scatter light such that accurate signal reconstruction is challenging. Ruan *et al.* have attempted to overcome some of these challenges by using pulsed ultrasound to modulate the scattered light from thick and turbid samples. However, at present, this approach is unable to reach the resolutions of conventional fluorescence microscopy.[90] Nevertheless, when fluorescence microscopy is combined with powerful tools such as fluorescent nanosensors, vast amounts of information can still be derived from individual cells that are thin and optically transparent.

Optical microscopy, in particular, fluorescence microscopy, has elucidated a vast amount of detail about the inner workings of cells. This is largely due to the high spatial and temporal resolution of fluorescence microscopy as a detection technique and the high sensitivity of probes that can be delivered to cells in a noninvasive manner. High numerical aperture objective lenses and conventional charged couple device or complementary metal–oxide–semiconductor cameras have a diffraction limit of approximately 200 nm. With the introduction of super-resolution fluorescence based techniques, this spatial and temporal resolution has further improved permitting the tracking of single molecules in biological systems.

Optical techniques will always be limited by distance from the detector. Thus, in order to contribute to highly scalable culture environments, optical techniques can benefit from fluorescent probe sensors that are able to provide insight from distances far removed from the detector, if implemented effectively. For example, real-time measurement of key biomanufacturing parameters from fluorescent nanosensors embedded within a packed bed bioreactor, could be considered as a method to overcome the limitations of traditional probe based sensors.

IV. CONCLUSION

The use of electrodes for determining culture environments is still common practice. Although, scalable CGT manufacture has evolved, it has become clear that there is an ever-increasing gulf between the process biology requiring observation and the technology available to study it. Chemical based optical sensors offer an exciting measurement solution to meet

these current challenges in acquisition. When compared to existing offline methods, the data these physiochemical sensors make available may seem limited. However, the ability to collect in real-time culture metrics beyond those typically available with electrode-based technology is a major step forward that will facilitate increasingly efficient processes with a greater degree of control and provenance.

For emerging CGTs, this advance in measurement technologies offers a significant insight into the complex metabolic processes that ultimately define the CQAs of the product. The key to unlocking these bioprocessing advances will be responsive and sensitive biosensors that are able to detect increasingly complex process parameters in efficient and scalable CGT manufacturing environments. To this end, strategies for translating lab-scale sensory technologies to production-scale should be pursued.

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