Fluorescent Nanosensors

Real-Time Biochemical Measurement for Cell and Gene Therapies

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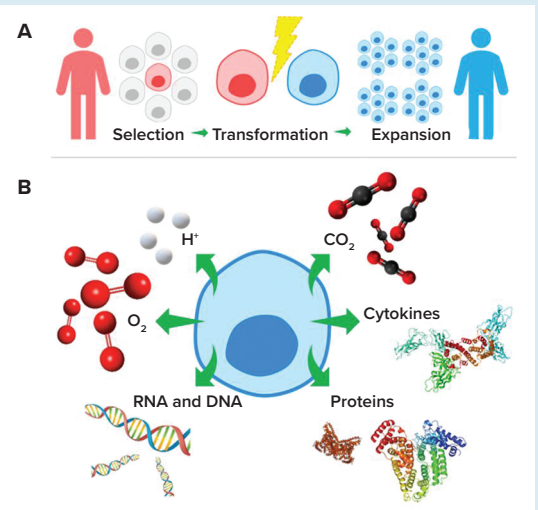
Cell and gene therapies are destined to transform the methods by which global healthcare challenges are approached and overcome (**1**). The US Food and Drug Administration is reviewing and approving an increasing number of cell and gene therapy products (**2**), and biopharmaceutical developers are dedicating immense resources to realizing the enormous potential of these therapeutics. Therefore, technologies that facilitate their effective and efficient manufacture will accelerate cell and gene therapies' transition from medicines of the future to medicines of the present (**3**).

Cell therapies are administered as live cells from a patient (autologous) or donor (allogeneic) for therapeutic benefit (e.g., blood transfusions and stem-cell therapy). *Gene therapies* can be considered as the addition, silencing, correction, or reprogramming of genetic material through application of vectors for therapeutic benefit (e.g., Luxturna voretigene neparvovec-rzyl from Spark Therapeutics for inherited retinal RPE65 gene disease) (**4**).

Cell and gene therapies require extracting and selecting autologous or allogeneic cells, then transforming them to a final product that is therapeutically advantageous (Figure 1A). Transformed material is expanded ex vivo and readministered for therapeutic benefit. Chimeric antigen receptor (CAR) T-cell therapies are examples of cell and gene therapies. They are developed by reprograming immune cells to target cancer cells. The FDA has approved two CAR T-cell therapies: Kymriah (tisagenlecleucel) from Novartis and Yescarta (axicabtagene ciloleucel) from Kite Pharma for the treatment of B-cell acute lymphoblastic leukemia and relapsed or refractory large B-cell lymphoma, respectively (**5, 6**).

Bioreactors provide the growth conditions required to increase cells and vectors to quantities sufficient for therapeutic doses. The vessels provide a sterile environment for eukaryotic or microbial culture conditions (e.g., temperature, aerobic/anaerobic, and pH), with the addition of nutrients (e.g., carbohydrates, proteins, and lipids) and growth factors (e.g., cytokines), which typically are monitored extracellularly (**7**). Extracellular measurements provide an excellent indication of cell-culture inputs and outputs. However, they do not provide a direct indication of subcellular processes, which are where many biochemical changes important for effective cell culture occur (Figure 1B). Therefore, the introduction of tools that permit real-time analytics of subcellular biochemical processes could play a significant role in the optimization of cell and gene therapy manufacturing and facilitate the transition of these products to mainstream therapeutics.

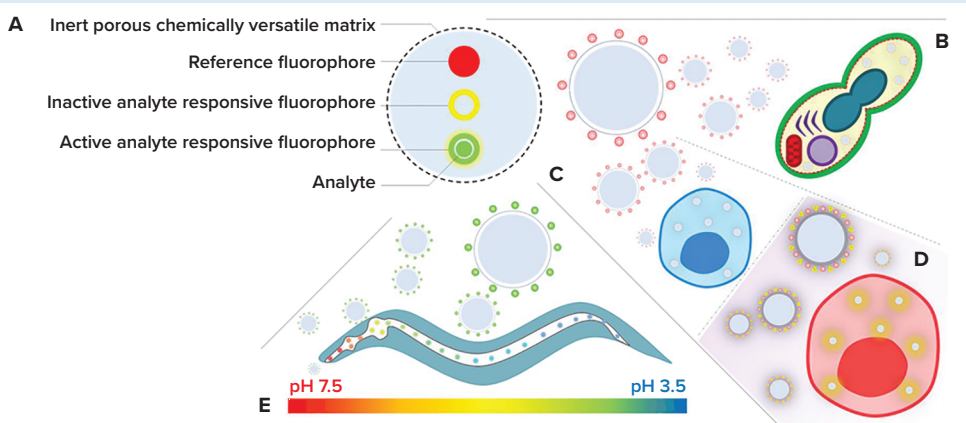
Figure 1: (A) Cell and gene therapy development pathway from patient/donor (red) to recipient (blue); (B) subcellular molecules and ions important for effective cell and gene therapy cultures



Background

Fluorescent nanosensors are emerging technologies that permit silent measurement of key biochemical parameters to high spatial and temporal resolution (**8**). Nanosensors can be manufactured to a range of

Figure 2: (A) Ratiometric fluorescent nanosensors; (B) delivery to *Saccharomyces cerevisiae* (**26**) and human mesenchymal stem cells (hMSCs) (**27**) for (C) determining intracellular processing of silica magnetic iron nanoparticles and (D) photo induced phenotypic changes (**28**) as well as (E) pharyngeal and intestinal pH mapping in *Caenorhabditis elegans*



sizes, from 10 nm to 1,000 nm in diameter (**9**). They are manufactured from inert biocompatible materials (**10**) that bind to or encapsulate fluorophores (Figure 2A).

Fluorescent nanosensors typically consist of two or more different fluorophores that are reference and analyte-sensitive entities. A *reference fluorophore* produces a stable fluorescence emission upon excitation, which is insensitive to an analyte of interest. An *analyte-sensitive fluorophore* exhibits a dynamic fluorescence emission dependent on the amount of target. Multiple targets can be detected by incorporating more than one analyte-sensitive fluorophore (**11**). The combination of reference and analyte fluorophores enables accurate ratiometric measurements (**12**).

Fluorescence techniques have a greater sensitivity than do other spectroscopic methods. Therefore, nanosensors can be delivered at subharmful concentrations and accurately report subcellular biochemical processes through the control of input excitation only (**13**). Fluorescent nanosensors have been reported for the "A to Z" of analytes, from adenosine triphosphate (ATP) to zinc, and research in this exciting field continues to advance (**14–16**).

My group's research focuses on nanosensors relevant to cell and gene therapies. They are for pH (H^+) (**12**) and molecular oxygen (O_2) (**17**) and manufactured from inert polyacrylamide matrices. These nanoparticles (~50 nm in diameter) have been engineered to traverse biological membranes to observe and induce biochemical changes. Our research also has been extended to observe whole-organism bioprocesses.

Subcellular Analytics

Noninvasive targeted subcellular delivery can be considered as the zenith of formulation sciences. That is because cell membranes act as physical barriers, and programed cellular inertia prevents entry of unknown systems. Research has focused on reducing the effect of membrane barriers (**18**) using heat shock (**19**), surfactant treatment (**20**), electroporation (**21**), and changes in osmolarity (**22**). Other methods include accessing known cell-receptor internalization (e.g., clathrin mediated) (**23**) and using forced entry (e.g., gene-gun delivery) (**24**). As such, delivery of diagnostics tools such as fluorescent nanosensors has been challenging.

In my group's strategy, we functionalized fluorescent nanosensors matrices with cationic molecules (Figure 2B–D). Cationic molecules increase the zeta potential of nanoparticle surfaces, which has been hypothesized to facilitate nanoparticle penetration between hydrophobic moieties of lipid bilayers and cause membrane disruption at high concentrations (**25**). When we control the amount of positive functionalized monomer and nanoparticle concentrations, nanosensors can traverse multiple biological membranes and silently report on key biochemical changes. In separate studies, we have demonstrated how fluorescent nanosensors can be used to characterize subcellular environments for *Saccharomyces cerevisiae* (**26**) and human mesenchymal stem cells (hMSCs) (**27, 28**).

S. cerevisiae has been implemented as an animal-free alternative to using adenoassociated viruses (AAVs) as vectors for gene therapies (**29**). *S. cerevisiae* comprises both a cell wall and membrane. The cell wall acts as an additional physical barrier to

nanoparticle delivery. Our research has shown that by engineering polyacrylamide-based nanoparticles with the cationic molecule acrylamidopropyl trimethyl ammonium hydrochloride (ACTA), we demonstrated significant improvements in subcellular delivery without obvious changes to *S. cerevisiae* viability when compared with unfunctionalized particles (**26**).

We also have shown that fluorescent nanosensors can be used to study the influence of adding carbon sources in the form of sugars such as glucose on intracellular pH. Addition of glucose to *S. cerevisiae* results in an initial decrease in subcellular pH (after 10 minutes). Subcellular pH recovers to preglucose dose levels after 30 minutes. This research could be translated readily to improve AAV development and manufacturing by optimizing carbon-dose cycles and media replenishment.

hMSCs have been the focus of immense clinical research for their application in cell therapies and regenerative medicine (**30**). They can differentiate into multiple cell types and replenish their own stem-cell stores (**31**). Because of their ease of extraction, isolation, and expansion, hMSCs have been used in clinics for treating blood and bone marrow cancers. Through advances in their biomanufacturing, their full potential could be realized (**32**). Research we have conducted has shown how pH-sensitive nanosensors can be used to monitor the degradation of tools used to facilitate targeted delivery of hMSCs (**27**) and how light-induced irradiation of internalized nanoparticles could be used to induce biochemical changes and consequential cell differentiation (**28**).

Silica magnetic-iron nanoparticles (SiMAGs) have been studied for their potential in direct hMSC therapy by using magnetism to facilitate targeted therapy (**33**). However, the challenges with this strategy are understanding how SiMAGs are processed subcellularly and determining whether they permit long-term magnetic manipulation. Our research investigated whether pH-sensitive fluorescent nanosensors could address those challenges when combined with state-of-the-art technologies such as super-resolution fluorescence microscopy and multicolor flow-cytometric analysis (Figure 2C) (**27**). Super-resolution fluorescence microscopy confirmed that pH-sensitive nanosensors are colocalized with SiMAGs in lysosomal–endosomal spaces and permit long-term measurement. Flow cytometry was implemented to determine qualitatively and quantitatively the influence of SiMAGs on intracellular pH for seven days. Our analyses suggest that SiMAGs reduce intracellular pH over four days (from pH 5.43 ± 0.06 to 4.81 ± 0.14), which corresponds to SiMAG subcellular processing. The pH recovers to pre-SiMAG dosage levels on day 6 (pH 5.33 ± 0.17). Our studies suggest that SiMAG lysosomal–endosomal pathway exposure degrades both fluorescent silica coatings and iron cores, reducing cell loading by no more than 50% over seven days. That reduction in subcellular SiMAG concentration still would allow long-term magnetic manipulation of hMSCs.

Controlled changes in biochemical processes that produce predifferentiation of hMSCs in vitro afford the prospect of improvements in regenerative efficacy in vivo (**34**). Our research has shown that controlled changes in subcellular hMSC biochemical processes can be achieved through light-induced generation of reactive oxygen species (ROS), using photoactivatable porphyrins attached to polyacrylamide nanoparticles (Figure 2D) (**28**). Through control of either the concentration of photoactivatable porphyrins or number of light irradiations, the amount of ROS produced can be regulated. We used a newly synthesized fluorophore to identify ROS generation events through increases in emission intensity. Cytokine analyses, with flow cytometric analysis, indicated light-irradiation–induced controlled apoptotic cell death, rather than uncontrolled necrotic cell death. Our experiments contribute to the development of light-induced differentiation of hMSCs for clinical translation in vitro and for whole-organism analyses.

Whole-Organism Analyses

The potential of fluorescent nanosensors can be demonstrated when they are used to determine real-time in situ analytics for complex bioprocesses in living organisms such as *Caenorhabditis elegans* (Figure 2E). *C. elegans* is a free-living soil nematode and the most completely understood animal on the planet in terms of genetics, neurology, and cell survival. Its application as a model in the study of complex biochemical processes has gathered significant momentum. That is because *C. elegans* is easy to culture (feeds on bacterial lawns on agar plates), has a short lifecycle (egg to adult in three days), is optically transparent (permits optical visualization of anatomical events), and has freely available mutants that could function as experimental controls. In addition, because *C. elegans* nematodes are invertebrates, they do not require ethical approval to conduct research. Therefore, they are an extremely useful model

organism in research that combines complex biology such as cell and gene therapy and bioanalytical tools such as fluorescent nanosensors (**35**).

Our research has shown that pH-sensitive nanosensors can be delivered to *C. elegans* pharyngeal and intestinal lumen to make measurements (**36**). *C. elegans* can be fed nanosensors continually at relatively high concentrations for extended periods in the absence of significant toxicity. Furthermore, nanosensors remain in the *C. elegans* pharyngeal and intestinal lumen in excess of 24 hours after the challenge has been removed. These findings highlight the inert nature of fluorescent nanosensors and their potential to silently report biochemical changes for extended periods. Measurements of pharyngeal and intestinal pH show that *C. elegans* acidifies ingested matter such that there is pH gradient from the anterior of the pharynx (5.96 ± 0.31) to the posterior of the intestine (3.59 ± 0.09). Using high-speed fluorescence microscopy and temperature control to reduce the feeding rate, studies have shown that dynamic acidification of intestinal contents can be mapped in real time. These findings suggest that, under optimized conditions, fluorescent nanosensors could be used to monitor the efficacy of cell and gene therapies in situ.

Future Perspective

Fluorescent nanosensors are an extremely powerful tool and have been used to perform inert, long-term high spatial and high temporal measurements in a number of model organisms (**8**). However, it is important to note that the applications of fluorescent nanosensors to cell and gene therapies are in the early stages, and the true potential is yet to be determined. The tangible benefits of this technology to cell and gene therapies will be realized when measurements of key subcellular molecules and ions demonstrate advantages over extracellular measurement technologies that are essential to biomanufacturing (e.g., time lags and analysis of biochemical processes that occur only subcellularly).

Nevertheless, fluorescent nanosensors' enhanced measurement capabilities indicate a strong potential to characterize complex biochemical processes upstream, downstream, and in situ for cell and gene therapy manufacture. Furthermore, through the introduction of a new wave of analytical biosensors (**37**) — which have biodegradable polymeric matrices (**38**) — advanced production methods (**39**), and coupled innovative analytical instruments, fluorescent nanosensors are well positioned to enrich biomanufacturing for cell and gene therapies.

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