Biotechnology Advances 77 (2024) 108472

Contents lists available at ScienceDirect

Biotechnology Advances

journal homepage: www.elsevier.com/locate/biotechadv

Harnessing Raman spectroscopy for cell therapy bioprocessing

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ARTICLE INFO

Keywords:
Cell therapy manufacturing
Process analytical technology
Bioprocess control
Critical process parameters
Critical quality attributes
Bioreactors

ABSTRACT

Cell therapy manufacturing requires precise monitoring of critical parameters to ensure product quality, consistency and to facilitate the implementation of cost-effective processes. While conventional analytical methods offer limited real-time insights, integration of process analytical technology tools such as Raman spectroscopy in bioprocessing has the potential to drive efficiency and reliability during the manufacture of cell-based therapies while meeting stringent regulatory requirements. The non-destructive nature of Raman spectroscopy, combined with its ability to be integrated on-line with scalable platforms, allows for continuous data acquisition, enabling real-time correlations between process parameters and critical quality attributes.

Herein, we review the role of Raman spectroscopy in cell therapy bioprocessing and discuss how simultaneous measurement of distinct parameters and attributes, such as cell density, viability, metabolites and cell identity biomarkers can streamline on-line monitoring and facilitate adaptive process control. This, in turn, enhances productivity and mitigates process-related risks. We focus on recent advances integrating Raman spectroscopy across various manufacturing stages, from optimizing culture media feeds to monitoring bioprocess dynamics, covering downstream applications such as detection of co-isolated contaminating cells, cryopreservation, and quality control of the drug product. Finally, we discuss the potential of Raman spectroscopy to revolutionize current practices and accelerate the development of advanced therapy medicinal products.

1. Raman spectroscopy in bioprocessing

Cell therapies have shown great potential in the treatment of many currently intractable diseases, and recent years have witnessed an accelerated growth in their use in clinical trials and in the pharmaceutical marketplace. To meet the high demand for clinically-relevant cell doses at affordable costs and the need for safe products, scalable, tightly monitored, and controlled bioprocesses are required.

Allogeneic and “off-the-shelf” cell therapies demand the development of bioprocesses where one batch is usually linked to the production of several doses for patient treatment. Given the high costs associated to production batches, it is critical that cell manufacturing risks are minimized. On the other hand, in autologous cell therapies, the biological variability inherent to the use of variable starting material needs to be managed. To ensure that both allogeneic and autologous strategies successfully reach clinical application and are commercially viable, it is fundamental that the cell therapy industry adopts quality risk management strategies and bioprocesses that are robust to the biological variability of the input material. This can be in part accomplished through real-time monitoring and process control.

Real-time monitoring of cell culture enabling rapid feedback to perturbations has been restricted to parameters such as dissolved oxygen, pH and temperature. Other parameters, such as cell density, cell viability, metabolites and by-products concentration, and presence of biomarkers of interest are usually evaluated offline following manual sampling. Nonetheless, it is possible to analyze several of these process parameters in real-time and automatically while avoiding open operations and sample wasting using Process Analytical Technology (PAT) tools such as Raman spectroscopy (Matuszczyk et al., 2023), Near Infra-Red (NIR) spectroscopy (Kozma et al., 2019), acoustic sensors – e.g., based on ultrasonic pulsed Doppler (Akbari et al., 2022) -, dielectric spectroscopy – e.g., Incyte capacitance sensor (Isidro et al., 2021) -, Nuclear Magnetic Resonance (NMR) spectroscopy (Urzì et al., 2022), and Fourier Transform Infrared (FTIR) spectroscopy (Marienberg et al., 2024).

By offering a non-destructive approach to measure multiple critical process parameters (CPP) while allowing fast and continuous data acquisition, these techniques can accelerate biopharmaceutical

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https://doi.org/10.1016/j.biotechadv.2024.108472
Received 31 July 2024; Received in revised form 6 October 2024; Accepted 23 October 2024
Available online 28 October 2024

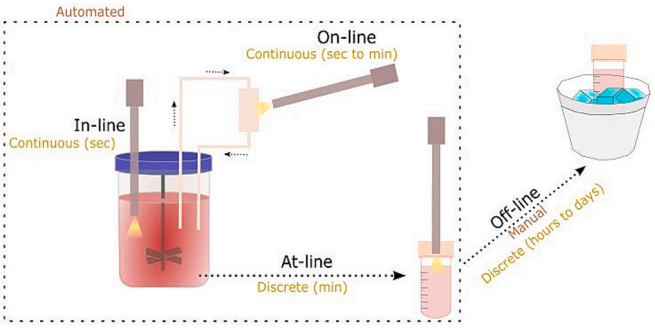

Fig. 1. Implementation variants of Raman spectroscopy and other PAT tools. Raman spectroscopy probes can operate as in-line or on-line sensors, which, contrary to off-line analysis, do not require samples to be withdrawn and enable real-time monitoring and process control. While in-line Raman sensors allow spectra collection in the process stream, at-line Raman is collected at a location close to the production process.

development. PAT tools have been widely adopted, for instance, in the antibody industry (using CHO cells) to monitor and identify variations in bioprocesses.

Particularly relevant to the cell therapy field is the ability of process monitoring supported by PAT tools to relate process parameters with critical quality attributes (CQA), identifying descriptors that could be used to not only increase productivity but also manage process risks therefore minimizing batch-to-batch variability on manufacturing processes. Indeed, increased throughput, reduced need of sampling handling by the operator and higher control over bioprocesses can be enabled by applying real-time (on-line) or near real-time (at-line) PAT tools (Fig. 1) which, ultimately, can reduce the probability of batch failure.

Given its low water interferences, particularly when compared with FTIR, Raman spectroscopy is seen as a versatile spectroscopic technique to monitor multiple process output parameters during cell culture. The utility of Raman spectroscopy-based methods in the biopharmaceutical industry has already been demonstrated in different stages of manufacturing (Fig. 2) and it is envisioned that its application will broaden in the upcoming years. While not exhaustive, the list of applications successfully using Raman spectroscopy in the manufacture of biologics includes quantification of chemical components, such as amino acids, in cell culture medium (Costa et al., 2023; Li et al., 2010), monitoring bioprocesses from small to larger bioreactors (Li et al., 2013), identification of protein aggregation during downstream processing (Zhang et al., 2019), assessment of product concentration during purification steps (Yilmaz et al., 2020) and identification of intracellular ice formed during cryopreservation (Dong et al., 2010; Li et al., 2020a; Pollock et al., 2016; Yu et al., 2021). Although studies reporting the use of Raman spectroscopy in cell therapies are limited, this review highlights several advances in Raman applications for biopharmaceutical manufacturing, which hold significant potential for the development of next-generation cell therapies.

Integration of Raman spectroscopy in cell bioprocessing can be essential to track key phenotypic markers, viability or even endotoxin levels in real-time and could therefore contribute to assess lot consistency and the phenotypic uniformity of a manufactured batch of cells or cell-derived products. By providing rapid insights into product quality, Raman spectroscopy allow process parameters to be dynamically adjusted, mitigating trial and error during process development, and minimizing the need for time-consuming off-line analyses. This can, not only streamline process optimization, but also significantly lower manufacturing costs and minimize batch failure by facilitating rapid quality control release of manufactured batches. Coupled with regulatory requirements, these benefits are driving a higher adoption level of PAT and, particularly, of Raman spectroscopy in the biopharmaceutical industry. Indeed, although it has been since the early 1990s that Raman spectroscopy has been increasingly applied to investigate the biochemical properties of living cells (Puppels et al., 1990), the Food and Drug Administration (FDA) PAT initiative released in 2004 has encouraged the use of on-line PAT tools such as Raman spectroscopy to understand and control the manufacturing of biopharmaceuticals (Center for Drug Evaluation and Research, 2004).

Being increasingly demanded by regulators, efforts are performed to

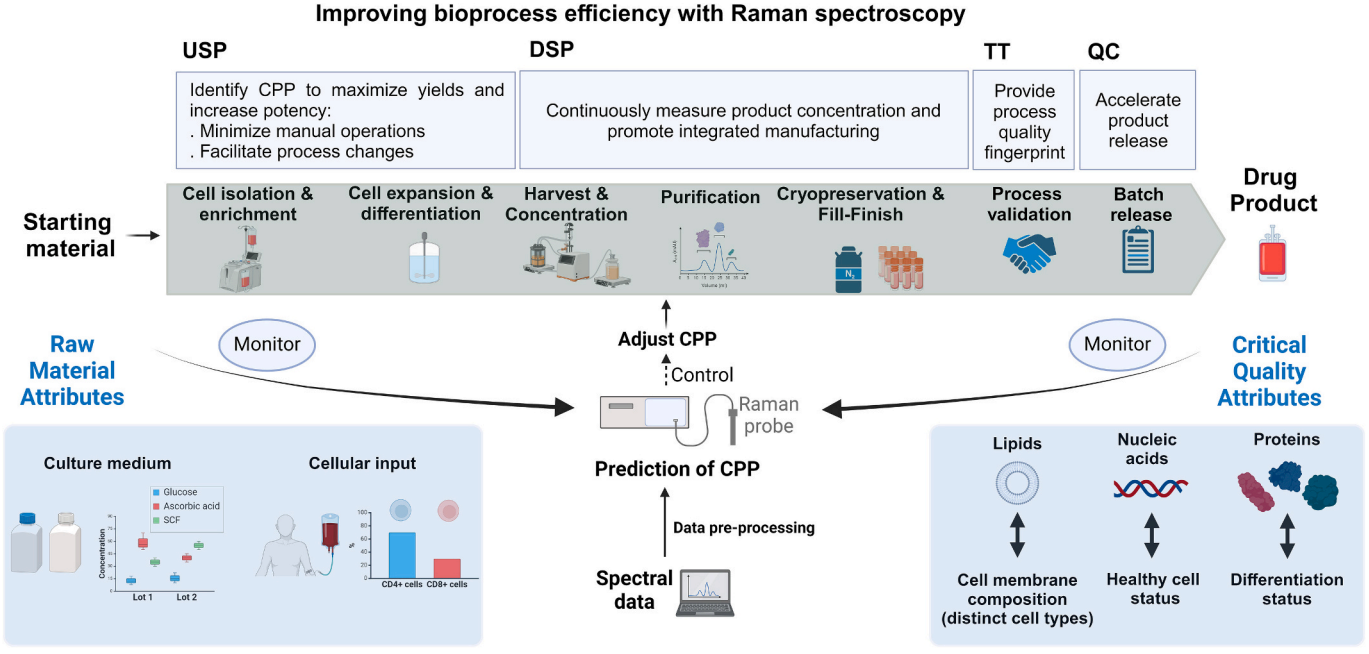

Fig. 2. Application of Raman spectroscopy across the entire lifecycle of cell therapy manufacturing, from upstream applications to downstream processing and formulation steps, including Tech Transfer (TT) activities and QC. By providing real-time information on both raw material attributes and critical quality attributes of the drug product, Raman spectroscopy can be used to adjust critical process parameters (CPP) aiming at increased bioprocess efficiency and at reaching the ultimate goal of real-time product release.

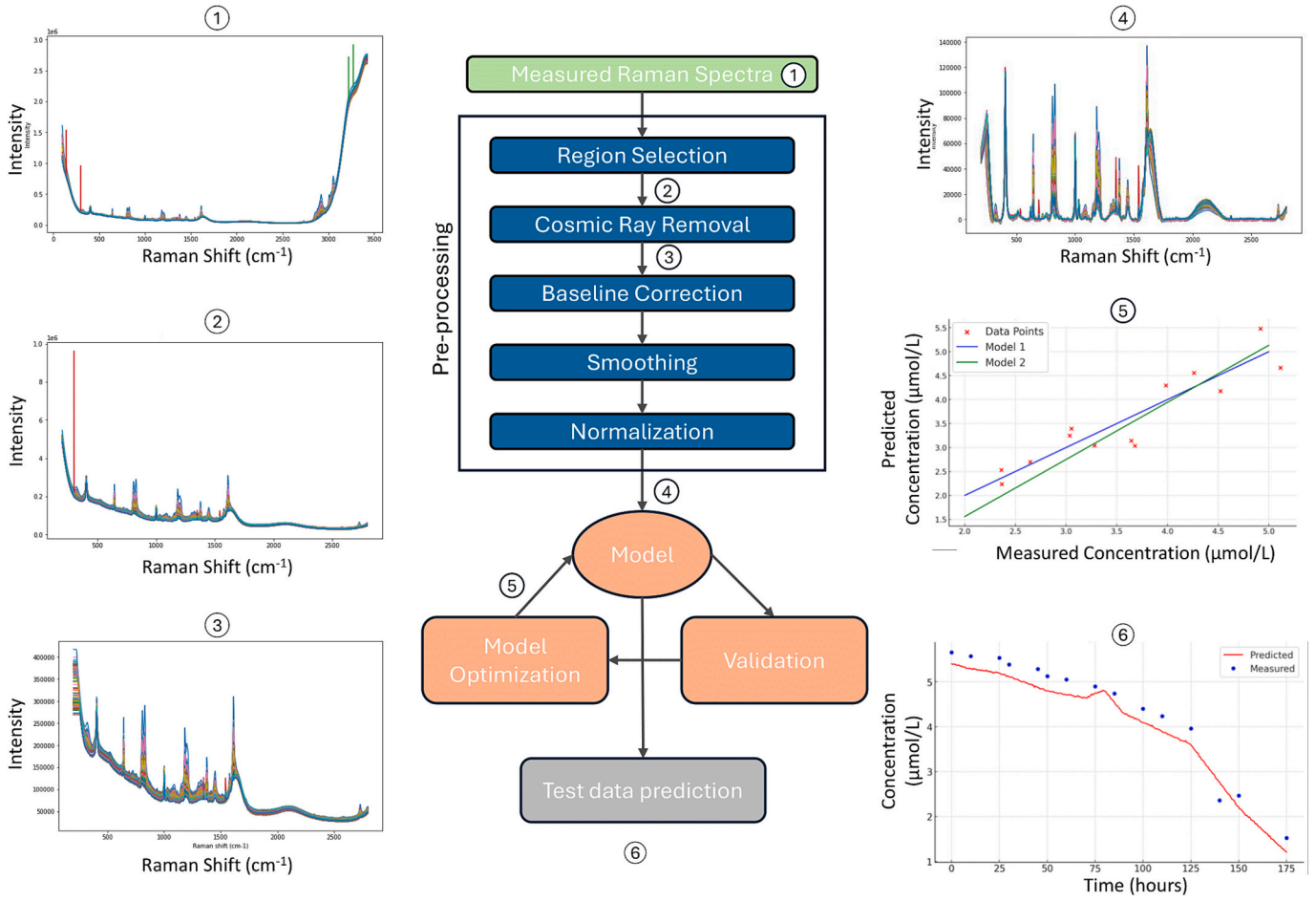

Fig. 3. Workflow from Raman spectra acquisition to predict key bioprocess variables. Spectral pre-processing improves the signal-to-noise ratio and prepares the data for analysis using baseline correction and cosmic ray removal algorithms. After pre-processing, a multivariate prediction model is developed, optimized, and validated, allowing for simultaneous prediction of variables based on spectra acquisition. This process highlights the steps required to achieve useful predictions and showcases the potential for Raman spectroscopy as a monitoring and decision-aiding tool for bioprocesses.

increase the robustness and standardization of bioprocessing alongside with digital record of manufacturing, aspects that are tightly associated with automation. Since Raman spectroscopy-based methods are amenable to automated operation and, importantly, are not harmful to live cells if an adequate power density, wavelength and duration of exposure are used, they can significantly contribute to implement bioprocess control and intensification through the development of predictive and feedback control strategies. Raman spectroscopy data can be used to support key decisions during manufacturing workflows, namely to adjust feed rates throughout a bioprocess to optimal levels, identify critical time points for steps such as cell transduction, harvest or to supplement cell culture medium with biological cues triggering cell differentiation. Altogether, this could contribute to increase product quality and maximize yields and, ultimately, increase the accessibility of cell therapies to patients.

2. Principles of Raman spectroscopy

Raman spectroscopy, first described in 1928 by the Indian physicist C. V. Raman (Raman and Krishnan, 1928), is a non-invasive, label-free, and sensitive optical technique used to identify characteristic fingerprints of molecules. This versatile method is employed in various fields such as chemistry, materials science, and physics for the analysis and characterization of a broad range of substances. In the field of biology, Raman spectroscopy is particularly valuable for analyzing and identifying biomolecules, often without requiring sample preparation.

The application of Raman spectroscopy to biological samples results in signals generated by lipids, proteins, nucleic acids, and metabolites, based on the inelastic scattering of a monochromatic laser at distinct wavelengths. When a photon interacts with a molecule, its energy can temporarily increase, and as the molecule relaxes, it releases a different quantity of energy relative to the incident photon – a phenomenon known as Raman scattering. The small changes in frequency between the absorbed and emitted photons reflect characteristic molecular vibrations corresponding to specific chemical bonds, as the energy required to excite a particular molecular vibration is unique to the functional groups of a given molecule. This energy consists of the difference in energy between the incident and scattered photon. Since each molecule has a unique set of molecular vibrations, detection of the scattered photons results in a series of peaks that reflect the sample composition. Importantly, the inelastic scattering of photons is proportional to the number of a given chemical bond, therefore allowing quantitative analysis of different compounds present in a sample. Raman spectroscopy is, however, a low-probability physical process as only 1 in approximately 10^8 photons are subjected to Raman scattering and, therefore, this technique is limited in its ability to measure low concentrations of a substance (Huser and Chan, 2015). Besides the analyte concentration, the intensity of Raman scattering is also impacted by the polarizability of molecules. Indeed, in a study where samples collected from a bioreactor were analyzed by Raman spectroscopy, NIR and 2D-fluorescence techniques, 2D-fluorescence presented a higher sensitivity for ammonium (but not for glucose and lactate). This resulted from

the fact that ammonium was present at low concentration and also shows lower polarizability compared to glucose and lactate (Rowland-Jones et al., 2017). Nonetheless, Raman techniques such as surface-enhanced Raman spectroscopy (SERS) (Goel et al., 2024), coherent anti-Stokes Raman scattering (CARS) (Li et al., 2020b) and stimulated Raman scattering (SRS) (Yu et al., 2024) can significantly enhance Raman sensitivity. In SERS, for instance, antibodies, linked to gold nanoparticles, specifically recognize cell surface markers with a sensitivity up to one in one million cells (Szaniawska and Kudelski, 2021), while, in comparison, current flow cytometry assays can reliably detect 0.01 % of abnormal cells (Craig and Foon, 2008). By combining the chemical selectivity of the Raman spectrum with the spatial resolution of scanning tunnelling microscope (STM) and atomic force microscopy (AFM), tip-enhanced Raman Spectroscopy (TERS) demonstrates sensitivity to single molecules (Cao and Sun, 2022).

Although a broad range of excitation light wavelength, from infrared to deep ultraviolet, could be explored in Raman spectroscopy, for most biomedical applications involving cells, long wavelength light (785 nm, 633 nm) is usually explored due to its low phototoxicity. The trade-off between photodamage risk and sensitivity while minimizing fluorescence interference results in the use of the near infrared region for continuous monitoring of live cells without disturbing physiological properties.

3. Building Raman spectroscopy analyte prediction models

Pre-processing and analysis of Raman spectra involve several critical steps, ranging from data acquisition to model development (Fig. 3). Researchers can implement pre-processing pipelines effectively with the help of various software and libraries. Numerous free and open-source Python packages, such as Rampy, PySpectra, and PyChemometrics, are available for spectral data analysis. Additionally, commercial software options like BWSpec by B&W Tek, LabSpec 6 by Horiba Scientific, and SIMCA by Sartorius offer robust functionalities that streamline the pre-processing and analysis of spectral data. In this section, we provide an overview of the typical pipeline required to build Raman predictive models.

3.1. Data acquisition

Commonly used analyzers for bioprocesses include the RamanRxn2™ from Endress+Hauser (Baradez et al., 2018; Berry et al., 2015; Rafferty et al., 2020a), Viserion® 785 nm Analyzer from Indatech (Hagedorn et al., 2023), BioPAT®Spectro and compatible probes, such as the HyperFluxPRO Raman spectrometer from Tornado Spectral Systems (Rowland-Jones et al., 2021).

Relevant settings for Raman spectra acquisition include the excitation wavelength, exposure time, spectral resolution, and the number of accumulations. Choosing the appropriate excitation wavelength (e.g., 532 nm, 633 nm, 785 nm, or 1064 nm) balances signal intensity and fluorescence. 785 nm is typically chosen for bioprocessing purposes. Exposure time, ranging from milliseconds to minutes, affects the signal-to-noise ratio and total acquisition time. Spectral resolution (typically between 4 cm^{-1} and 10 cm^{-1}, influenced by the spectrometer's slit width and grating) determines the ability to differentiate closely spaced spectral lines. Also, the accumulation refers to the number of scans that are averaged to improve signal to noise ratio. This increases acquisition time, so a balance must be reached according to how fast one expects the system to considerably change.

3.2. Region selection

Specific regions of the Raman spectrum correspond to different molecular vibrations and thus different chemical components. Selecting the right spectral region can enhance the detection and analysis of key bioprocessing parameters. The most commonly focused region is what is sometimes referred as "fingerprint region", located between 400 and 1800 cm^{-1}, where many distinct peaks corresponding to organic compounds, proteins, lipids, and nucleic acids can be found (Rafferty et al., 2020a).

3.3. Spike detection

Cosmic ray events (CRE) can introduce spurious spikes in Raman spectra, significantly distorting the data. These spikes, caused by high-energy particles striking the detector, obscure the true Raman signals. Effective detection and correction of these spikes are crucial to ensure the integrity of the spectral analysis. Methods such as wavelet transform and threshold-based algorithms are commonly employed to identify and remove these artifacts, preserving the accuracy of the Raman spectra and enabling reliable interpretation of the sample's properties (Li and Dai, 2011). Recently, machine learning-enhanced wavelet transforms have been applied for spike detection and noise filtering. Adaptive thresholding techniques – driven by unsupervised learning methods such as k-means clustering – dynamically adjusts to the unique noise characteristics of each spectrum.

3.4. Baseline correction

One of the major limitations of Raman spectroscopy techniques is linked to the low intensity of the Raman signal, which can be easily masked by noise (Smulko et al., 2014). Preprocessing and baseline normalization of Raman spectral data can contribute to improve the signal-to-noise ratio (Beier and Berger, 2009), with particular focus on mitigating fluorescence signals, a major challenge in cell culture due to the accumulation of organic metabolites (Matthews et al., 2018). While traditional methods like first- or second-order derivative, frequency domain filtering and polynomial fitting have been widely applied (Beumers et al., 2018; Cadusch et al., 2013; Lieber and Mahadevan-Jansen, 2003), more advanced approaches using deep learning, such as autoencoders – unsupervised neural networks – are now employed to automatically recognize and subtract fluorescence from Raman spectra, significantly enhancing baseline correction (Han et al., 2024). This allows the peaks of the Raman spectral signal to be distinguished amongst the diversity of molecular interactions present in the cell culture environment.

3.5. Smoothing

While spike detection reduces the effects of CRE and baseline correction reduces the effects of fluorescence, smoothing is the process used to reduce noise. Smoothing techniques help in minimizing this noise while preserving the true signal characteristics. One of the most commonly used smoothing methods is the Savitzky-Golay filter (Barton et al., 2018). The Savitzky-Golay filter applies a polynomial regression to a moving window of the spectral data, effectively smoothing the data by fitting successive polynomial curves and averaging them. Additionally, machine learning algorithms, such as convolutional neural networks (CNNs), can be applied to automatically identify and filter out noise while preserving relevant spectral features (Fuentes et al., 2023).

3.6. Normalization

Two commonly used normalization methods are Standard Normal Variate and Multiplicative Scatter Correction (Fearn et al., 2009). Standard Normal Variate works by transforming each spectrum to have a mean of zero and a standard deviation of one. Multiplicative Scatter Correction, on the other hand, adjusts the spectra by modelling and removing the scatter effects through a reference spectrum, typically the mean spectrum. Both methods are widely adopted for their effectiveness in improving the quality of Raman spectral data, facilitating more accurate analyses.

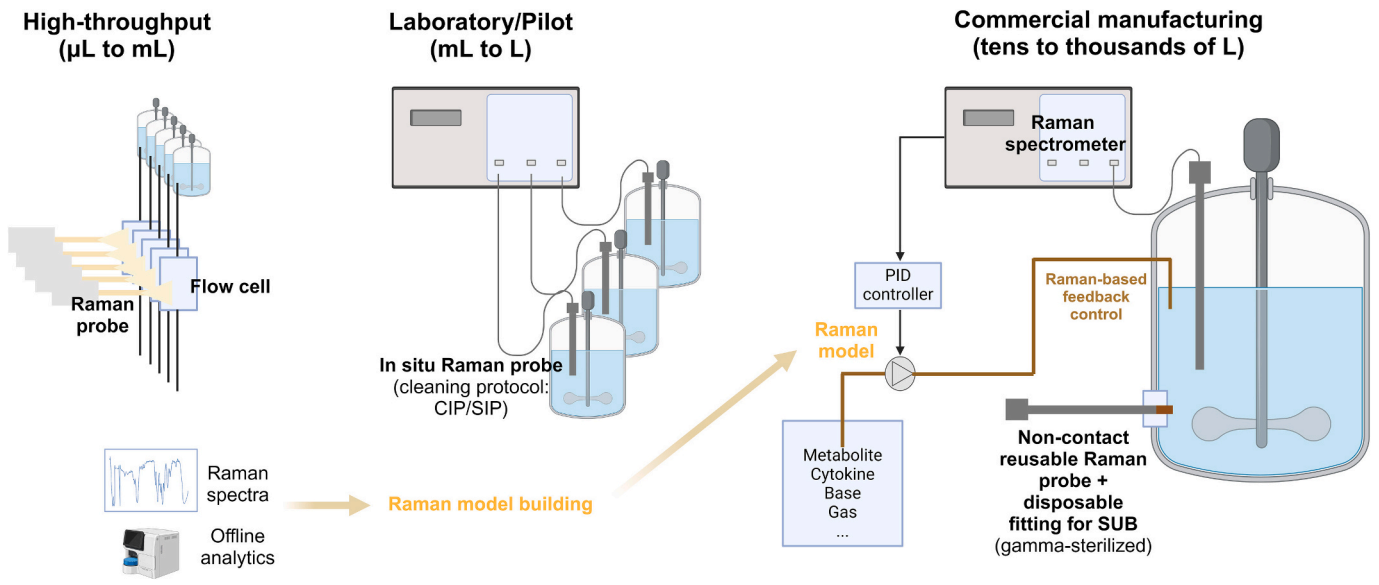

Fig. 4. Raman spectroscopy measurements performed across different bioreactor scales. Raman spectroscopy measurements in miniature bioreactors can be performed using integrated at-line systems (which have the advantage of allowing several conditions to be tested in parallel, therefore covering wider analyte concentration ranges, particularly if analytes are spiked directly in the bioreactors) while, typically, larger-scale process development bioreactors allow installation of in-line Raman probes in headplates, using autoclave procedures to sterilize the Raman sensor (following Clean in Place (CIP) and Sterilize in Place (SIP) procedures). Commercial manufacturing bioreactors can also use side ports that are often explored in single-use bioreactors to integrate gamma sterilized fittings of Raman probes. By aligning offline measurements with the measured Raman spectra, Raman models can be generated for different process parameters. The identified CPP are then kept within target ranges by using feedback controllers such as the proportional integral derivative (PID) controller based on in-line information provided by the continuously acquired Raman spectra.

3.7. Model development

Using Raman spectra to predict and monitor bioprocess variables in real-time requires establishing robust statistical models. Multivariate predictive models, such as partial least squares regression (PLSR) (Tulsyan et al., 2020), are employed to correlate spectral patterns (that are affected by cell debris, presence of bubbles, cell culture media components and cell by-products) with process parameters (Craven and Whelan, 2015). Other methods such as Ridge, XGBoost, or Neural Networks can be used, and their efficacy varies between different experimental variables and even types of analytes (Tanemura et al., 2023). Since deep neural networks (DNNs) and CNNs can handle large datasets and complex non-linear relationships without requiring manual feature selection, they are used to automatically extract relevant features from Raman spectra.

Hybrid Artificial Intelligence (AI) models that combine traditional methods (e.g., PLSR) with deep learning techniques offer an optimal balance between computational efficiency and predictive accuracy. Explainable AI techniques, such as SHAP (Shapley Additive exPlanations) and LIME (Local Interpretable Model-agnostic Explanations), can potentially enhance the transparency and interpretability of AI models in Raman spectroscopy (Contreras and Bocklitz, 2024).

Generating calibration data for robust Raman models can be time and resource intensive, as it should cover the entire operation space and changes in culture conditions can decrease the accuracy of the generated calibration models (Berry et al., 2015; Mehdizadeh et al., 2015). Integrating analyte variability into Raman calibration processes either requires spiking strategies or design of experiment studies that incorporate a wide range of analyte concentrations and demand a high number of runs to be performed (Santos et al., 2018). An alternative to in situ acquired Raman data that are obtained following insertion of Raman probes in bioreactor ports relies on flow cells installed on bypass loops integrated in perfused cell culture systems (Romann et al., 2022) (Fig. 4). To limit time/resource consuming process development runs to generate Raman models, strategies that would not require dedicated runs to obtain calibration data are needed. Recently, a method to build Raman calibration models without using culture data was developed (Hara et al., 2023). By using samples of artificially mixed analytes that did not involve cell culture to identify the Raman peak positions, intensities and baseline changes of each analyte, this approach results in a cost effective and less time-consuming method to develop robust calibration models for cell culture monitoring.

Transfer learning enables models pre-trained on one set of bioprocess conditions to rapidly adjust to new datasets with minimal retraining, therefore improving model scalability and reducing calibration costs. Despite its promise for bioprocess development and guiding novel manufacturing processes, its application remains underexplored and it does not overcome the challenge of requiring large amounts of data for initial model training (Helleckes et al., 2023; Kalatzis et al., 2023). To unlock the full potential of transfer learning, a broader approach to open-source data sharing is essential.

Remarkably, initiatives such as “MicrobioRaman”, a recently created open-access web-based repository for microbiological Raman spectroscopy data, if widely implemented by the cell therapy community, could contribute to strengthen collaboration amongst researchers and help generating more relevant and generalizable models (Lee et al., 2024).

4. Improving bioprocess control and efficiency using Raman spectroscopy

4.1. Upstream processing

Enhanced process understanding to predict cell CQAs online can be achieved by real-time monitoring of several cell culture parameters in combination with mathematical modelling (Dong et al., 2024; Tanemura et al., 2023). Identification of individual peaks of the Raman spectra enables simultaneous detection of several metabolites/amino acids, pH level in cell culture conditioned medium and cell concentration, viability and identity, allowing adaptive feeding strategies to be implemented. This can be critical during both upstream process development and in commercial manufacturing batches as, frequently, more than one factor is responsible to define the success of a batch production.

Table 1
Overview of examples of Raman spectroscopy studies focused on monitoring and upstream bioprocessing control of distinct cell types (stem, immune cells and CHO cells). The use of bioreactors is highlighted as well as the main findings of each study. AT-MSC – adipose tissue-derived mesenchymal stromal cells; BM-MSC – bone marrow-derived mesenchymal stromal cells; hESC – human embryonic stem cells; hiPSC – human induced pluripotent stem cells.

Application	Cell type		Monitoring type	Culture platform	Bioreactor operation	Operation mode	Feedback control implemented based on Raman data?	Key findings	Ref.
Cell identity	Stem and immune cells	AT-MSC, osteoblasts, adipocytes	CARS microscope	Static plates	No	NA		Successful non-invasive monitoring of mesenchymal stromal cells differentiation into osteoblasts and adipocytes was performed.	(Downes et al., 2011)
		BM-MSC	Raman microscope	Static plates				Successful non-invasive monitoring of mesenchymal stromal cells differentiation into chondrocytes was performed.	(Ravera et al., 2021)
		BM-MSC	Raman microscope	Static plates				Raman spectroscopy detected inter-individual differences between BM-MSC isolated from five distinct donors that were not highlighted by standard analytical methods investigating cell morphology, multilineage differentiation potential, phenotype, colony-forming capacity or proliferative potential.	(Kukolj et al., 2022)
		Neural stem cells	Raman microscope	Static plates				The differentiation status of neural stem cells was monitored by Raman spectroscopy.	(Geng et al., 2021)
		Dental pulp stem cell spheroids	Confocal Raman microscope	3D spheroids in static platform				Variations in 3D differentiation of stem cell spheroids was associated with the efficiency of diffusion of medium following correlation of cell-differentiation related peaks (hydroxyapatite, β-carotene and protein/cellular components) and water/medium Raman peaks.	(Kim et al., 2021)
		hESC-derived cardiomyocytes (CMs)	Raman microscope with environmental enclosure	Chambers of 3.5 cm diameter and 1.5 cm height				Raman spectroscopy has the potential to non-invasively monitor time-dependent molecular changes in hESC, particularly hESC-derived CMs (as spectral differences attributed to glycogen and myofibril bands can help distinguish CM from non-CMs).	(Pascut et al., 2011)
		hiPSC-derived erythropoietin producing cells	Raman microscope	Static plates				Three biomarkers (fatty acids, glycoproteins and DMSO), directly correlated with hiPSC-differentiation into erythropoietin producing cells, were successfully detected over the course of the cell differentiation.	(Ishigaki et al., 2022)
		B cells, T cells	Confocal Raman microscope	Static plates				Raman spectroscopy provided biochemical information that allowed to distinguish T- from B-	(Hobro et al., 2016)

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Table 1 (*continued*)

Application	Cell type	Monitoring type	Culture platform	Bioreactor operation	Operation mode	Feedback control implemented based on Raman data?	Key findings	Ref.
							cells and between individual T- and B-cell lines.	
	T cells, NK cells and dendritic cells	Confocal Raman microscope	Static plates				Wavelength-modulated Raman spectroscopy can be used for immune cell discrimination (namely $CD4^+$, $CD8^+$ T cells and $CD56^+$ NK cells as well as myeloid (mDC) and lymphoid/plasmacytoid (pDC) dendritic cell populations.	(Chen et al., 2015)
	hESC	Off-line	0.125 L shaker flasks or 0.25 L STB	Yes	Fed-batch	No	Changes in pluripotency of ESC can be detected in Raman spectra of spent media (being particularly reflected in the Raman bands associated with lipids).	(Hagedorn et al., 2023)
Metabolite (glucose)	AT-MSC	In-line	0.25 L STB		Fed-batch		Cell culture at low glucose levels, monitored using Raman probes in scalable STB, can improve EV manufacturing yields.	(Costa et al., 2023)
Metabolite (glucose, lactate, glutamine) and cell concentration	T cells	In-line	0.2 L STB		Fed-batch		Changes in Raman peak intensity were correlated with concentration and viability of T cells isolated from different donors.	(Baradez et al., 2018)
Metabolite (glucose, lactate), VCD, TCD, osmolality	CHO cells	In-line	3 L, 200 L, 2000 L STB		Fed-batch		Good prediction Raman models were reported for glucose, lactate and osmolality although, for viable and total cell densities, these components exhibited different performance depending on bioreactor scale.	(Berry et al., 2015)
Metabolites (glucose, glutamine, glutamate, lactate, ammonium), VCD, TCD, viability		In-line	500 L STB		Fed-batch		Raman spectroscopy was used to simultaneously monitor in-line the concentration of multiple culture parameters (metabolites and cell concentration) in large scale bioreactors.	(Abu-Absi et al., 2011)
Metabolites (glucose, lactate and ammonia)		In-line	1 L, 5 L and 2000 L STB		Fed-batch		Cubist outperformed PLS for modelling the Raman spectra of metabolites at 1 L and 5 L scale. It was used to predict the levels of glucose, lactate and ammonia at the 2000 L manufacturing scale bioreactor.	(Rafferty et al., 2020a)
Metabolites (glucose, lactate, glutamate, ammonium), VCD, TCD, product (mAbs) concentration		In-line	5 L and 10 L STB		Fed-batch		Accurate models were built using 5 L scale data and transferred to 10 L scale for glucose, lactate, ammonium, VCD, TCD but not for product (mAbs) or glutamate concentrations.	(Webster et al., 2018)
Amino acids (tyrosine, tryptophan, phenylalanine, methionine)		In-line	3 L STB		Fed-batch		Raman spectroscopy allowed to in-line monitor the	(Bhatia et al., 2018)

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Table 1 (*continued*)

Application	Cell type	Monitoring type	Culture platform	Bioreactor operation	Operation mode	Feedback control implemented based on Raman data?	Key findings	Ref.
							concentration of several amino acids in parallel.	
Metabolites (glucose, lactate) and VCD		In-line	1 L, 3 L and 500 L STB		Fed-batch		A good model fit was obtained, except for low concentrations of glucose and lactate where the model accuracy was limited.	(Mehdizadeh et al., 2015)
Metabolites (glucose, lactate and ammonium)		Off-line	10 L STB		Fed-batch		When mimicking the operational constraints specific to miniature bioreactors (i.e., low sampling volume and short acquisition times), Raman spectroscopy showed better correlation with off-line measurements of metabolites than NIR and 2D-fluorescence.	(Rowland-Jones et al., 2017)
Metabolites (glucose, lactate, ammonium, glutamine, glutamate) and product (mAbs) concentration		At-line and on-line	15 mL STB (ambr®15) and 50 L STB		Fed-batch		Miniature bioreactors, where experimental variation can be introduced through spiking, allow robust model generation at low costs. An integrated spectroscopy solution was implemented to facilitate technology continuity and model transfer between miniature and large-scale bioreactors.	(Rowland-Jones et al., 2021)
Metabolites (glucose, lactate), VCD, antibody concentration		Off-line and at-line	15 mL STB (ambr®15)		Fed-batch		A high-throughput Raman spectroscopy microscope was successfully applied to both USP and DSP operations.	(Goldrick et al., 2020)
Glycoprotein titer		Off-line	2 L, 100–200 L, 1000 L, 5000 L STB		Fed-batch		Raman spectroscopy was used to predict the glycoprotein yield in small scale batches up to 5000 L bioreactors.	(Li et al., 2013)
Amino acids, vitamins, VCD, viability, osmolarity, antibody titer, pH, ions		On-line	0.25 L STB		Fed-batch		Raman models were constructed for a wide range of analytes (not only detecting compounds with covalent bonds but also hydrogen ions, metal ions, oxygen, carbon dioxide) using spectra measured in a microfluidic channel.	(Tanemura et al., 2023)
Metabolites (glucose, lactate, glutamine, glutamate), mAb titer		On-line	0.25 L STB		Fed-batch		Multi-parallel mini bioreactors integrating flow cells enabling on-line Raman measurements (and reference measurements provided by a bioanalyzer) can generate robust models for nutrients, metabolites and product titer.	(Graf et al., 2022b)

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Table 1 (*continued*)

Application	Cell type	Monitoring type	Culture platform	Bioreactor operation	Operation mode	Feedback control implemented based on Raman data?	Key findings	Ref.
VCD, viability, viable cell volume and cell diameter		In-line	15,000 L		Fed-batch	Yes (based on capacitance values)	Raman spectroscopy supported capacitance data to deliver the feeding strategy.	(Rafferty et al., 2020c)
Metabolites (glucose, lactate, glutamate, glutamine), VCD, osmolality, ions (K^+, Na^+, $NH4^+$), pCO_2 and titer (IgG)		In-line	3 L STB		Perfusion	Yes (viable cell density control)	Raman spectroscopy constitutes a robust tool to monitor and control continuous manufacturing processes, successfully establishing an auto-control strategy for VCD.	(Chen et al., 2021)
Metabolite (glucose)		In-line	5 L STB		Fed-batch	Yes (targeting glucose)	Raman spectroscopy can be used to control culture conditions, namely maintaining glucose at low concentration and, consequently, reducing antibody glycation by over 50 %.	(Berry et al., 2016)
Metabolite (glucose)		In-line	5 L, 315 L STB		Fed-batch	Yes (targeting glucose)	Shifting the Raman excitation wavelength towards the near-infrared (993 nm, alternatively to the commonly used 785 nm excitation wavelength) allows reduction of autofluorescence in a mammalian cell culture process, contributing to establish a Raman adaptive feeding to maintain glucose at the target setpoint.	(Matthews et al., 2018)
Metabolites (glucose)		In-line flow cell included in the cell-free harvest stream of a perfusion process	Rocking motion (2 L and 20 L)		Perfusion	Yes (targeting glucose concentration)	In-line Raman spectroscopy enabled maintenance of stable glucose concentration (<±0.4 g/L) in a prolonged perfused-operated bioreactor.	(Graf et al., 2022a)
Metabolite (glucose)		On-line	0.25 L STB		Fed-batch	Yes (targeting glucose)	High throughput bioreactors were successfully controlled by a predictive Raman model for glucose.	(Sibley et al., 2020)
Metabolites (glucose), amino acids (arginine)		In-line	2 L STB		Fed-batch	Yes (targeting glucose and arginine concentration)	Higher cell proliferation and viability and, consequently, antibody production were achieved following feeding control strategies enabled by Raman data in comparison to the standard bolus fed culture.	(Domján et al., 2022)
Metabolites (glucose, lactate, ammonium), VCD and product concentration	Off-line or at-line	15 mL STB (ambr®15)		Fed-batch	Yes (targeting glucose concentration)	A DoE approach was applied using miniature bioreactors to cover the design space. The output of a Raman model focused on viable cell and glucose concentrations was	(Rowland-Jones and Jaques, 2019)	

(*continued on next page*)

Table 1 (*continued*)

Application	Cell type	Monitoring type	Culture platform	Bioreactor operation	Operation mode	Feedback control implemented based on Raman data?	Key findings	Ref.
						used to control feed rates.		
Metabolites (glucose, glutamine, lactate, ammonia, glutamate), TCD and VCD		In-line	3 L and 15 L STB	Fed-batch	Yes (targeting glucose concentration)	Raman calibration models were successfully transferred from 3 L to 15 L bioreactor scale.	(Whelan et al., 2012)	
Metabolite (glucose, glutamine, lactate and ammonia), VCD		On-line	15 L STB	Fed-batch	Yes (targeting glucose concentration)	Based on Raman data, efficient bioprocess control (targeting glucose concentration) was achieved in a 15 L bioreactor.	(Craven et al., 2014)	
Metabolites (glucose, lactate)	HEK293 cells	In-line	5 L, 200 L and 315 L STB	Fed-batch	Yes (targeting glucose and lactate concentration)	Closed loop control of glucose and lactate concentrations based on Raman data resulted in an 85 % improvement in the harvest titer.	(Matthews et al., 2016)	

Although the majority of the studies exploring Raman spectroscopy during upstream processing collect Raman spectra using in-line probes directly inserted in bioreactors, few reports have also explored Raman microscopy, particularly to extend the utility of Raman from measuring pH, metabolites, amino acids, cell viability and cell volume into analysis of biomarkers that could open the road to control the differentiation and reprogramming of stem and immune cells and to ensure the purity of the manufactured cell population for clinical applications (Table 1).

4.1.1. pH control

Control of the pH level is commonly implemented during the manufacture of biological products, with most bioreactors being equipped with electrochemical or optical pH probes that continuously monitor pH. However, undesirable deviations of pH can occur due to pH probe drift (Saucedo et al., 2011). Raman spectroscopy, coupled to PLS regression modelling, has shown its potential to perform in-line measurements of pH, therefore eliminating the need of off-line daily samples to correct potential pH probe drifts in bioreactor-operated cell cultures (Rafferty et al., 2020b).

Remarkably, SERS has also been applied to monitor the intracellular pH of single cells (Kneipp et al., 2010) as intracellular pH can reflect important physiological and pathological processes, such as tumour formation, inflammation and infection (Damaghi et al., 2013). Using Raman microimaging coupled with nanosensors enabling spatial pH sensing has also been applied to detect endothelial inflammation triggered by tumour necrosis factor-α (TNF-α) (Jaworska et al., 2015). These studies highlight the dual role of Raman spectroscopy to potentially both control pH during cell culture and as a sensor of cell state.

4.1.2. Metabolites

Real-time monitoring of metabolites such as amino acids is crucial in cell therapy bioprocessing, where shifts in metabolite levels can directly impact cell expansion, differentiation or secretion of target products. Traditional off-line methods often fail to provide a comprehensive view of metabolic states, hindering process control. On the contrary, Raman spectroscopy enables continuous assessment of metabolites, contributing to the consistency of the manufactured cell therapeutic product.

So far, the majority of the studies employing Raman spectroscopy tools during both bioprocess development and at commercial scale have focused on monitoring the concentration of metabolites (Table 1). Bhatia and colleagues, for instance, have showed that chemometric models applied to Raman spectroscopy could accurately quantify phenylalanine, tryptophan and tyrosine concentrations (Bhatia et al., 2018). Besides online monitoring, controlling the concentration of metabolites, both nutrients and metabolic by-products, can further tailor the use of nutrients by cells, increasing their metabolic efficiency (Wlaschin and Hu, 2006) and help guide cell fate decisions. Notably, incorporation of feedback control systems fbased on Raman measurements have contributed to optimize nutrient supply. Matthews et al., for instance, implemented a dual-metabolite feedback system to better control the metabolic profile of a conventional mammalian cell manufacturing process (Matthews et al., 2016). Raman measurements were performed to track glucose and lactate levels in a fed-batch HEK293 cell culture exploring small scale bench (5 L) and pilot (200 L and 315 L) bioreactors. Based on the real-time concentrations of glucose and lactate, an adaptive feeding of glucose was implemented to avoid lactate accumulation while limiting glucose depletion. When lactate levels reached the 4 g/L threshold, the Raman control halted the glucose feeds, promoting consumption of lactate by the mammalian cells until lactate reached the minimum setpoint and glucose feeding was reinitiated. The control of glucose and lactate concentrations in a closed-loop rendered an increase of 85 % on the protein production levels, only extending the process timeline by two days (Matthews et al., 2016). In other studies, target glucose and phenylalanine (Webster et al., 2021) or glucose and arginine (Domján et al., 2022) concentrations were maintained through feed-rate adjustments of a fed-batch culture of a CHO cell line guided by in-line Raman models. Besides avoiding daily offline sampling, this strategy decreased the feed addition of glucose and phenylalanine by 19 % and 27 %, respectively, comparing to manually adjusted feeds. The arginine-based dynamic feeding strategy also contributed to decrease by more than half the supplied feed medium, resulting in economically relevant gains, an approach that could be extended from therapeutic protein manufacturing into the cell therapy field. Indeed, Raman probes can be used to continuously track glucose concentration during culture of mesenchymal stem/stromal cells (MSC) in small-scale stirred-tank bioreactors (STB), facilitating the identification of optimal extracellular vesicle (EV) collection time points as lower glucose concentration has been suggested to maximize the number of EVs secreted per cell (Costa et al., 2023). This suggests that real-time prediction of metabolites using Raman spectroscopy can also be explored to biochemically tune cell culture and therefore attain higher control over the cell secretome.

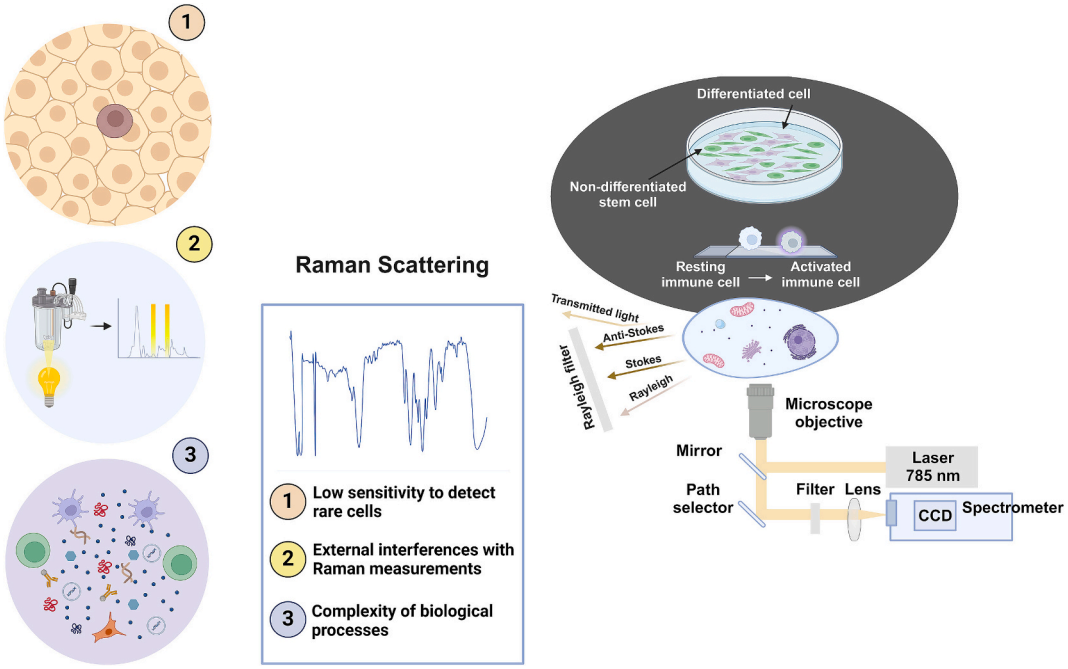

Fig. 5. Label-free Raman microscopy to monitor cell state, tracking processes such as stem cell differentiation and activation of immune cells. The photons emitted from a laser light (typically using excitation laser wavelengths in the near-infrared range (e.g., 785 nm) for eukaryotic cells) are differentially scattered according to the biochemical composition of a cell. The Raman spectrum results from Raman Stokes scattered photons that are recorded by the spectrometer while Rayleigh and anti-Stokes scattered light is filtered. Several challenges arise when using Raman spectroscopy in cell therapy bioprocesses. These include the low probability of Raman scattering events, which can hinder the detection of rare cells (1), external interferences, resulting in false peaks or mask peak identification (2) and the inherent complexity of cells and their surrounding microenvironment, making it difficult to isolate specific signals (3).

Integration of Raman spectroscopy during upstream cell culture processes contributes to increase the robustness of the manufacturing process by enabling real-time monitoring of critical culture parameters such as nutrient levels and cell health, for instance, facilitating a more precise control of feeding strategies and ensuring timely adjustments to the process. By facilitating the identification of critical time points to initiate/terminate process steps, Raman spectroscopy not only maximizes product yields but also contributes to maintain consistent product quality, thereby reducing the variability and risks associated with batch failure. This real-time and data-driven approach strengthens the overall process control, which is critical to reach scalable and reproducible manufacturing workflows.

4.1.3. *Cell concentration and viability*

Besides accurately monitoring pH, metabolite and nutrient production/consumption, Raman spectroscopy serves as a powerful tool for monitoring cell concentration and viability – key metrics in the production of cell therapies. Alterations in Raman peak intensities correlate with viable and total cell densities, allowing for real-time assessment of cell health and production levels. In a study performed with CHO cells using larger scale bioreactors (500 L), viable and total cell densities showed high level of agreement between measured and predicted values (Abu-Absi et al., 2011). In another study, Rafferty and colleagues proposed Raman spectroscopy as an in-line solution to model viable cell density (VCD), viable cell volume (VCV), viability and cell diameter in 15,000 L fed-batch bioreactors (Rafferty et al., 2020c). In the context of immunotherapies, Baradez and colleagues, besides developing chemometric models for glucose, lactate, ammonia, glutamine and glutamate during T cell culture in small-scale (200 mL) STB containing a Raman spectroscopy probe, demonstrated high correlation between Raman measurements and changes in cell numbers over time. Nonetheless, weaker correlation was observed for cell viability (Baradez et al., 2018). Although Raman bands can be used as surrogate markers to predict viability, differences in cell death and apoptotic mechanisms can lead to discrepancies amongst studies. Brauchle and colleagues, for instance, showed good correlation between Raman peak at 1375 cm^{-1} and apoptosis of sarcoma cell lines (Brauchle et al., 2014), while negative correlation was found in another study evaluating T cell death and apoptosis (Xiao et al., 2021). Detection of apoptosis is possible using Raman signatures that are observed during alterations of the cell composition typical of apoptotic processes, known to lead to an increase in lipid vesicles and degradation of nucleic acids (Rangan et al., 2018). Changes in peaks associated with DNA (at 782, 788 and 1095 cm^{-1}), for instance, have shown the applicability of Raman spectroscopy in determining the cell cycle stage and distinguishing live from dead cells (Notingher et al., 2003; Rangan et al., 2018; Uzunbajakava et al., 2003; Verrier et al., 2004). Interestingly, in a study performed with CHO cells, it was possible to detect cells entering apoptosis before they stained positive for annexin V (Brauchle et al., 2014). This ability to characterize cell populations non-destructively and in real time is invaluable for ensuring the quality and high yields of the manufactured cell products.

4.2. *Downstream processing*

Besides the pointed advantages of applying Raman spectroscopy during upstream processing, online monitoring of downstream steps is equally relevant to ensure that the cellular products retain their functionality throughout the entire manufacturing process. Even though Raman probes have been adopted in protein purification processes to ensure product consistency, their application in downstream monitoring of cell therapy products remains underexplored (Yilmaz et al., 2020). By providing insights into protein concentration, quality attributes such as protein aggregation and glycosylation (Goldrick et al., 2020; Wang et al., 2023; Zhang et al., 2019), early detection of membrane fouling during filtration (Tang et al., 2020), and structural conformational changes of proteins in chromatographic processes (Wang et al., 2020), Raman spectroscopy presents a powerful tool for enhancing the reliability and efficiency of DSP.

Although downstream processing (DSP) for cell-based therapies includes several steps such as harvesting cell or cell-derived products, washing, cell concentration or volume reduction, formulation, and fill and finish before cryopreservation, only few works have highlighted the potential of Raman spectroscopy in downstream process monitoring. In the following sections, we present some studies focused on using Raman spectroscopy as a non-destructive and fast tool to help identifying cell purity as well as to improve and determine the impact of cryopreservation methods on cell quality and viability.

4.2.1. Purity

In the cell therapy field, besides ensuring that cells retain high viability and proliferate to the required levels, manufacturers need to ensure that they are correctly differentiated and functional before DSP is initiated. Additionally, it is essential that downstream processing steps do not affect CQA. As continuous manufacturing is increasingly seen as the path to generate cell products more efficiently, the frontier between USP and DSP is becoming tighter. Ideally, the aim of manufacturing safe cell products, whose purity and function are tightly controlled, should be pursued during both USP and DSP, a requirement that is even more imperative in a scenario where cell or cell-secreted products are continuously harvested. In this regard, Raman spectroscopy can contribute to identify impurities during cell manufacturing and to select optimal time points to initiate DSP after cell expansion and differentiation.

Cell-based therapies require that both ex-vivo isolated and in vitro expanded cell populations are clearly identified and the potential co-isolation/co-culture of contaminating and undefined cell types is detected. Embryonic stem cells (ESC) and human induced pluripotent stem cells (hiPSC), for instance, are a relevant starting material to generate specialized cells for promising therapies. However, the presence of pluripotent stem cells in the final product poses risks, as these cells can lead to tumorigenesis (Sart et al., 2022). Exploring SRS, Nitta et al. demonstrated the effectiveness of Raman image-activated cell sorting to isolate hiPSC at a throughput of approximately 100 events per second, thereby enhancing purity by removing undesired cell types without the need for fluorescent labelling (Nitta et al., 2020). While this technique could be used for downstream characterization (e.g., RNA sequencing of selected cell populations), Raman-image activated sorting could also ensure compliance with regulatory requirements as the presence of undesired cell types is detected. Unlike techniques involving antibody labelling (magnetic bead isolation or fluorescence-activated cell sorting), the non-invasive nature of Raman-based sorting minimizes risks associated with traditional methods that can activate or modify the isolated cells. Furthermore, real-time assessment of distinct cell populations during DSP steps can significantly extend our understanding of the clinical potential of the manufactured cells. This topic will be discussed further in Section 4.3.

Overall, Raman probes can be very useful to support critical decisions during DSP and to streamline the QC of the manufactured cell and cell-derived products during the final steps of the manufacturing workflow.

4.2.2. Cryopreservation

Effective methods of cell cryopreservation allowing their long-term storage are critical for clinical and commercial applications. However, some cell types are challenging to be cryopreserved, limiting their off-the-shelf use. Raman spectroscopy can constitute a useful tool to gain insight into cells with poor freezing response as Raman signature reflects changes occurring during and after freezing and thawing protocols.

Low-temperature Raman spectroscopy has been widely applied to characterize the freezing response of different cell types. Louwagie and colleagues used this technique to determine the concentration of dimethyl sulfoxide (DMSO) at low temperatures, inside and outside the extracellular membrane of Jurkat cells, hiPSC and hiPSC-derived sensory neurons. The authors demonstrated that the concentration of DMSO in extracellular channels relatively to DMSO in the intracellular space (partitioning ratio) is influenced by the cooling rate, cell type, and DMSO concentrations, with partitioning ratios closer to 1 rendering higher post-thaw viabilities (Louwagie et al., 2023).

Low-temperature Raman spectroscopy has also been applied to study the impact of the cooling rate in hiPSC-derived neuronal cells at different stages of differentiation. The Raman data indicated that the D5 crest benefited from a cooling rate of −1 °C/min, contributing to minimize the loss of membrane integrity and cell adhesion (Li et al., 2021).

Although the current literature on Raman spectroscopy applied in cryopreservation is limited, its potential for developing optimized cryopreservation protocols tailored to specific cell types and differentiation stages is promising. Future work employing Raman probes could accelerate this development, providing fast and non-invasive analysis of cell function and potency following cryopreservation, ultimately ensuring that cell therapy products meet the stringent demands of clinical applications.

4.3. Quality control analytics

Despite the fact that Raman spectroscopy data sets have been used to develop generic Raman models that are independent of cell types (Mehdizadeh et al., 2015; Webster et al., 2018), predictive models can also be built to distinguish cell populations and monitor their differentiation state during bioprocessing (Fig. 5). This could be particularly useful if applied as a process quality control analytics to identify target cells during the manufacturing workflow or flag the presence of undesirable cell subpopulations in shorter turnaround times. As conventional methods, such as flow cytometry, are time consuming, they are ill suited to monitor and actively control cell differentiation. Therefore, the capability of Raman spectroscopy to monitor cell manufacturing processes in real-time facilitates process standardization, minimizing batch-to-batch variabilities and identifying discrepancies early in the manufacturing workflow.

4.3.1. Distinguishing cell types and differentiation status

In stem cell culture, whether the goal is to scale-up undifferentiated progenies or to guide their differentiation towards mature cell phenotypes (Abecasis et al., 2017; Correia et al., 2018; Cunha et al., 2017; Gomes-Alves et al., 2016; Serra et al., 2018), PAT tools like Raman spectroscopy can effectively monitor the cell pluripotency status. Early studies demonstrated Raman's ability to detect differentiation of first murine (Notingher et al., 2004a, 2004b) and later human (Chan et al., 2009; Chan and Lieu, 2009; Pascut et al., 2011) ESC into a more mature phenotype based on the intensity of the Raman peak of nucleic acids. Additionally, Raman spectroscopy allowed to distinguish atrial and ventricular cardiomyocytes derived from pluripotent stem cells (Brauchle et al., 2016) and native hESC from reprogrammed hiPSC (Parrotta et al., 2017). Despite the overall spectral profile being very similar, distinct Raman features were observed for both cell lines, particularly regarding the nucleic acid content with a higher amount being detected in hiPSC. Principal component analysis of Raman spectra has indicated the closer resemblance between hESC and hiPSC relatively to the differentiated progeny of hESC (Tan et al., 2012). Ghita and colleagues have also explored the use of Raman spectroscopy to distinguish neural stem cells (NSC)-derived glial cells from their undifferentiated progeny based on their distinct concentration of cytoplasmic RNA. While RNA could be detected in the cytoplasm of NSC, it was undetectable in the NSC-derived glial cells (Ghita et al., 2012).

The applicability of Raman spectroscopy in monitoring cell-state transition in pluripotent cells was also demonstrated in ESC undergoing reprogramming towards neural progenitor cells (Germond et al., 2020) or by Konorov and colleagues who followed the developmental trajectory of hESCs to insulin-positive cells throughout a 26-day differentiation protocol (Konorov et al., 2015). The stages of differentiation

over time were correlated with Raman signatures and characteristic ratios of Raman bands. In another study that compared four different label-free techniques, Raman spectroscopy rendered the highest accuracy (95 %) when applied to distinguish hESC from 7-day partially differentiated and fully differentiated hepatocytes (Tsikritsis et al., 2016). Several research groups have explored label-free Raman spectroscopy to identify differentiation states of hiPSC into erythropoietin (EPO)-producing cells, neurons and cardiomyocytes (Fujita et al., 2024; Hsu et al., 2020; Ishigaki et al., 2022; Li et al., 2021). Changes in the pluripotency state of stem cells are frequently associated to changes in bands of the Raman spectra for RNA and DNA markers, lipids and glycogen (Hagedorn et al., 2023; Moura et al., 2016; Rangan et al., 2020; Tan et al., 2012). Remarkably, single cell scRNA-Seq profiles have also been inferred from Raman images in a mouse iPSC reprogramming model (Kobayashi-Kirschvink et al., 2021), indicating the importance of Raman fingerprinting for predicting transcriptome.

Besides pluripotent stem cells, Raman spectra was shown to be a relevant tool to distinguish different cell types and differentiation status during the regeneration of nucleus pulposus (NP) tissue, particularly to distinguish amongst hMSC, differentiated NP cells and chondrocytes (Ehlicke et al., 2017). Raman has also been applied to monitor MSC differentiation towards osteogenic differentiation (Azrad et al., 2006; McManus et al., 2011), into a glial phenotype (Bautista-González et al., 2023) and to distinguish osteoblasts and adipocytes from their hAT MSC progeny by an increase in the Raman peak at 960 cm^{-1} (hydroxyapatite peak) or 2900 cm^{-1} (fatty acid peak), respectively (Suhito et al., 2018). Interestingly, the hydroxyapatite-specific Raman band, indicative of osteogenic differentiation of AT MSC, was detectable one week earlier than with the typical osteogenic assessment method of Alizarin Red S staining.

Since 3D cell culture is often explored to provide an improved microenvironment for cell differentiation, Raman spectra was evaluated in 3D hydrogels. Distinct Raman shifts in the range 1370–1390 cm^{-1}, previously assigned to glycosaminoglycans (GAGs) (Bonifacio et al., 2010), were observed between hMSC and the higher GAG content containing-chondrocytes and NP cells. Coherent anti-Stokes Raman scattering (CARS) microscopy has also provided a method to image mineralisation in osteoblasts and the presence of lipids in adipocytes, therefore distinguishing both these cell types from adipose tissue-derived stem cells (Downes et al., 2011).

Presence of fibroblastic contamination in MSC isolated from bone marrow (BM) has been identified using Raman spectroscopy, showing a distinctive spectral separation between both cell types when multivariate analysis of the obtained Raman spectra is applied (Pudlas et al., 2011). Phenotypic differences between human BM MSC and fibroblasts were postulated to result in differences in their Raman spectra baselines, attributed to their specific endogenous autofluorescence patterns. Indeed, and although the intensity of the autofluorescence could disguise some Raman peaks, distinct cell metabolic activity has been correlated with the background of the Raman spectra (Lieber and Kabeer, 2010). Of notice, the high proliferative ability associated to stem cells is usually accompanied by a high mitochondrial activity and, hence, increased autofluorescence (König et al., 2011).

Remarkably, several studies in the immunotherapy field have also shown that markers such as proteins, lipids, nucleic acids and carotenoids can be detected by Raman spectroscopy and used to distinguish T cells from B cells (Borek-Dorosz et al., 2022; Ichimura et al., 2016), to discriminate T cells from myeloid and epithelial cells (Guliev et al., 2023), and to reflect the distinguished identity of chemical bonds in $CD4^+$, $CD8^+$ T cells, NK cells and dendritic cells (Chen et al., 2015). Besides, Raman spectroscopy can also assume a particularly relevant role in enabling prediction of T cell activation status (Gavgiotaki et al., 2019; Ichimura et al., 2016) and T cell differentiation, contributing to monitoring shifts in functional subsets of T cell populations (Pavillon and Smith, 2023).

4.3.2. Single cell analysis

Beyond bulk measurements, Raman spectroscopy allows quantification of phenotypic diversity at the single cell level. For example, differences in the cytosol and nucleus of differentiated neuroblastoma and adipocyte mouse cell lines were captured at single cell level using Raman spectra (Ichimura et al., 2014). This technique has also captured differentiation stages in hepatocytes-derived progenitor like cells (Ma et al., 2021) and assessed the status of MSC differentiation into osteoblasts and adipocytes using Raman mapping, where the low signal-interference from the cell culture substrate (quartz vs slide glass, silicate glass and tissue culture plate tested in the experimental design) contributed to provide a reliable and non-destructive method for characterization of stem cell differentiation (Suhito et al., 2018). Remarkably, while standard biological assays (colony-forming capacity, multilineage differentiation potential, phenotype and morphology) could not distinguish amongst five different BM MSC donors, Raman spectroscopy identified inter-individual biochemical variations at the single cell level, suggesting that this PAT tool could be used to differentiate between donors (Kukolj et al., 2022).

Single cell Raman spectroscopy has also been successfully used to reflect changes in immune cells, being employed to assess macrophage activation state (Pavillon et al., 2018) and to track differentiation stages of long-term hematopoietic stem cells (HSC) into short-term HSC ((Ilin et al., 2015; Pastrana-Otero et al., 2020)) and the committed populations of lymphoid (B cells) and myeloid (granulocytes) cells (Ilin et al., 2015). Using label-free Raman spectroscopy microscale platforms can play a critical role when working with rare cell populations (such as the long-term HSC) as they could minimize the number of required cells for analytical assays while successfully tracking the fate decisions of individual cells. To this purpose, it is critical to develop Raman-compatible substrates with minimal background interference (Pastrana-Otero et al., 2020), as glass slides, for instance, generate a large Raman signal overlapping with cell-derived signals (Mikoliunaite et al., 2015).

In addition to unveiling cellular phenotypic characteristics, Raman has also been applied to identify the formation of ice at the single cell level during cryopreservation (Yu et al., 2021).

4.4. Raman spectroscopy applied to cell-derived products

4.4.1. Characterization of EVs secreted by distinct cell types

The therapeutic properties of cells are also largely mediated by their paracrine function, including by secreted EVs that act as signalling agents in intercellular communication. The capability of EVs to deliver proteins, nucleic acids and lipids has been explored in therapeutic applications, such as cardiac regeneration (Louro et al., 2024), immunotherapies (Besse et al., 2016; Katakowski and Chopp, 2016) and wound healing (Garima Sharma et al., 2023; Lu et al., 2022).

The potency of cell-derived EVs depends on the cell source, isolation method and cell culture conditions. These parameters are vital for understanding the complexity of EV preparations and overcoming barriers to the regulatory approval of cell-derived therapeutics. An effective EV characterization toolbox should include analytical techniques to assess the biochemical composition and cargo of EV batches, in addition to quantification of total particle numbers and protein concentration. Raman fingerprint allows biochemical information on proteins (1.650 cm^{-1} for amide I), lipids (2700–3200 cm^{-1}), and nucleic acids (720–820 cm^{-1}) to be obtained. Therefore, together with characterization of the size and concentration of EVs, quantification of specific protein, lipid and nucleic acid markers by Raman fingerprint has been proposed as a quality control of cell-derived conditioned medium (Giannasi et al., 2021).

The ability of Raman spectroscopy to detect differences in protein-to-lipid and nucleic acids-to-lipid ratio in stem cell-derived EV samples has been demonstrated by Gualerzi and colleagues (Gualerzi et al., 2019). Raman fingerprint revealed differences amongst distinct EV batches

following a 5 min acquisition protocol, quickly providing insights on EV composition, a critical parameter to help predicting EV functionality. Higher purity of EVs, indicated by well-defined peaks and reduced fluorescence background noise, was observed in Size Exclusion Chromatography (SEC)-isolated EVs in comparison to ultra centrifugation (UC)-processed samples (Gualerzi et al., 2019). A classification model built based on Raman data achieved 97 % accuracy in distinguishing non-EV from EV samples. Of notice, purer EV samples, obtained through sequential UC (2 x UC) procedures or a SEC protocol, exhibited distinct biochemical features detectable by Raman spectroscopy. In contrast, 1 x UC-isolated EVs displayed masked signatures due to co-isolated factors (Gualerzi et al., 2019). Nonetheless, the same study has indicated that not all EV preparations are likely to benefit from Raman spectroscopy analysis. For instance, EVs isolated using commercial precipitation kits containing polyethylene glycol (PEG) or those subjected to sucrose- or iodixanol density gradient purification often show masked fingerprints due to co-isolation of these compounds.

Distinct Raman fingerprints have also been attributed to EVs secreted by BM-, AT-derived MSC and human dermal fibroblasts. Particularly, the contribution of components of the lipid membrane (sphingomyelins, gangliosides and phosphatidylcholines) to the Raman spectra were identified (Gualerzi et al., 2017). Importantly, not only the composition, but also the purity and reproducibility of MSC-derived EVs preparations have been assessed by Raman spectroscopy (Carlomagno et al., 2021; Giannasi et al., 2021; Gualerzi et al., 2017).

Overall, Raman spectroscopy can be useful to find EV fingerprints and identify the presence of co-isolates while ensuring the integrity of the samples.

4.4.2. Quantification of viral titers

Lentiviruses, commonly produced using HEK293 cells, are essential in several cell-based therapies, particularly immunotherapies, due to their capacity to deliver genetic information for reprogramming cells to target specific antigens (e.g., to manufacture CAR-T cells). Accurately determining lentiviral titers during manufacturing is key for establishing adequate doses of effectively genetically modified cells. Although analytical methods such as ELISA and PCR are successful in their reliable quantification of virus titers, they are time consuming. Alternatively, Raman spectroscopy can be used to quickly measure viral titer (Morder et al., 2022). To enhance the Raman signal, SERS, which can also distinguish between adenovirus, HIV and rhinovirus particles based on their characteristic nucleic acid and amino acids fingerprints (Shanmukh et al., 2006), successfully quantified GFP-containing virus particles in formulation media. Raman spectroscopy has also demonstrated its suitability to monitor the production of rabies Virus-Like Particles (VLP) using a baculovirus/insect cell system. However, further optimization is required as the model demonstrated better prediction in fresh samples than in cryopreserved VLPs (Guardalini et al., 2024).

5. The role of Raman spectroscopy in bioprocess scale-up and intensification

Bioreactor-supported cultures are amenable to large-scale manufacturing of cell therapies. Unlike static cultures, which are limited in their ability to apply quality-driven approaches to promote bioprocess intensification, bioreactors offer tighter control over process parameters, thus contributing to increased process efficiency. Raman probes can withstand harsh clean-in-place (CIP) and sterilization-in-place (SIP) procedures and are compatible with bioreactors made of glass, stainless steel and single-use materials. However, the choice of Raman spectrometer and probe needs to align with the bioreactor design as well as the physical constraints imposed by CIP/SIP protocols. The optical interfaces and probe materials must ensure long-term durability without signal degradation due to harsh sterilization or cleaning chemicals.

Table 2
Potential role of Raman spectroscopy across different steps of cell therapy manufacturing.

Bioprocess step	Role of Raman spectroscopy in cell therapies	Limitations & considerations
USP and DSP - Cell isolation, expansion and/or enrichment	Validation that cell isolation, expansion and/or enrichment procedures result in cultures at the required level of purity (Raman activated cell sorting (RACS) and Raman optical tweezers could be relevant).	Need to ensure that Raman spectroscopy detects even low contamination levels of undesired cell populations. To constitute an added advantage, Raman spectroscopy needs to be able to assess cell identity and potency with the same accuracy as currently available methods. Given the weak intensity of spontaneous Raman scattering, RACS low detection speed and sorting throughput can limit its application to low volume samples. Additionally, the application of RACS in sterile, in-process environments such as bioreactors is technically challenging and needs to ensure that the sorting process does not affect cell functionality or ability to differentiate (iPSC, MSC, …). The lack of standardized protocols and limited regulatory guidelines hinder the validation of cell sorting supported by Raman tools in GMP settings. As the intensity of Raman signal is dependent on cell composition, some cell types might be difficult to accurately identify and/or more prone to laser-induced damage.
USP and DSP - Cell activation, expansion or differentiation	Accelerate process development, contributing, for instance, to identify optimal timing to stimulate immune cells, trigger differentiation of iPSC into target cell populations or initiate distinct downstream processing steps at key time points.	A large library of Raman spectra might be required to train the model, requiring significant investment in data collection and analytical tools.
	Allow adaptation to process variability introduced, for instance, by distinct donors or culture conditions (e.g., minimizing differences in raw material).	Need to establish a correlation between donor/reagent characteristics and quality of the drug product. Raman spectroscopy may lack the clinical data needed to gain acceptance within regulatory bodies.
DSP - cryopreservation	Validate the quality of long-term stored cell products.	There is still a limited number of studies using Raman spectroscopy to

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Table 2 (*continued*)

Bioprocess step	Role of Raman spectroscopy in cell therapies	Limitations & considerations
DSP – product formulation	Ensure that formulation of cell or cell-derived products do not impact viability and function.	evaluate the quality of cryopreserved or formulated cell products, correlating Raman signatures with cell potency after thawing.
		Raman spectroscopy needs to be sensitive enough to detect changes of Raman signatures caused by product formulation.
QC	Accelerate batch release by providing real-time and non-destructive analysis of cell and cell-derived products.	To assess the potency of cell and cell-derived products, a large library of spectral signatures might be required to cover the full range of potential product variability.
	Decrease manufacturing costs by enabling fast and non-destructive cell analysis.	Ensuring that Raman spectroscopy provides the same level of detailed information of currently implemented potency assays would be essential for regulatory approval.

Moreover, when integrating Raman spectrometers into bioprocess workflows, it is essential to ensure that the optical properties of the spectrometer, such as probe design and fiber-optic transmission (Pence et al., 2021), are compatible with the scale of the bioreactor. In addition, the spectrometer must provide reliable performance across various scales, from laboratory scale systems to large scale manufacturing bioreactors. It should maintain signal integrity despite variations in optical path lengths or background noise, which can change with process scale or analyte concentration as the bioprocess intensifies.

Several studies have explored Raman spectroscopy to monitor and optimize cell culture in dynamic platforms, being the STB the most commonly used bioreactor type (Table 1). If coupled with Raman spectroscopy's ability to continuously monitor bioreactor cultures, this PAT tool could control cell culture conditions to account for cell source variability and the complex biological processes that guide cell expansion, differentiation and secretion of cell derived products. Thus, Raman spectroscopy has the potential to be a valuable tool for bioprocess intensification.

Building predictive Raman models requires monitoring multiple runs with random variations or, ideally, planning process variations within a larger design space. Raman spectroscopy has been explored in small-scale bioreactors, such as the ambr®15 (Rowland-Jones et al., 2021) or the ambr®250 (Graf et al., 2022b; Sibley et al., 2020), which mimic cell culture at manufacturing scale. These small-scale systems are cost-effective tools for expanding the design space and developing Raman models. However, the reduced working volume of these small-scale bioreactors limits the number of offline measurements that can be performed, a factor that must be considered when developing Raman models.

Sibley and colleagues have described at-line integration of Raman spectroscopy in ambr®250 bioreactors to build a glucose model with data collected from 16 minibioreactors using a CHO cell line. Despite demonstrating the applicability of Raman tools at laboratory scale (Sibley et al., 2020), future efforts need to focus on demonstrating the scalability of the ambr®250-derived Raman models to manufacturing scale.

Raman spectroscopy has been used for real-time prediction and control of glucose concentration in larger-scale bioreactor platforms (Abu-Absi et al., 2011; Berry et al., 2015; Craven et al., 2014; Kozma et al., 2017). At the 500 L scale, Raman spectra has been correlated with viable and total cell density (TCD) as well as several metabolites (glucose, glutamine, glutamate, lactate, ammonium) using PLS modelling (Abu-Absi et al., 2011). Importantly, a study involving cell culture in production scales spanning bench (3 L) to pilot (200*L*) and clinical manufacturing (2000*L*) demonstrated the scalability of Raman-based models (Berry et al., 2015). However, some limitations were observed, such as differences in predicted concentrations of glutamate and ammonia across scales. Webster et al., for instance, developed good predictive models for glucose, lactate, ammonium and cell concentration across 5 L and 10 L scales but the predictive model was unable to accurately monitor changes in glutamate and product (mAbs) concentration during cell culture (Webster et al., 2018). The authors have pointed out that incomplete datasets from earlier culture time points used for model development could limit model transferability across scales. This highlights the importance of building comprehensive models with adequate design-space coverage to ensure better analyte concentration predictions when using Raman datasets for large-scale bioprocesses (Fig. 4).

In addition to predictive modelling, Raman spectroscopy could be a key player in establishing tightly controlled cell processes. Although Raman spectroscopy has primarily been applied to suspension cell cultures, future studies are expected to focus on adherent cultures supported by microcarriers. Raman spectroscopy's capability to monitor multiple process variables simultaneously enables more sophisticated feedback control strategies, including optimized feeding in continuous bioprocesses (Matthews et al., 2016), that can benefit from fine-tuning perfusion rates, further promoting bioprocess automation and intensification in the cell therapy field.

The huge potential for integration of Raman-based tools in scalable bioprocesses is closely related to their ability to support continuous manufacturing, favouring process automation and, ultimately, process intensification. Removing sample requirements and minimizing operator interventions to evaluate CQAs and guide process decisions can streamline a predictive manufacture approach. However, since Raman scattering is a low probability process, sensitive detection systems are required. This, in turn, increases the chances that signals originated outside the bioprocess (e.g., light impurities affecting typically used transparent glass and plastic containers in distinct bioprocessing steps) interferes with the Raman measurement. Moreover, as bioprocesses transition to continuous manufacturing, wear of the spectrometer components or small displacements during longer bioprocesses can impact the collected Raman spectra, resulting in false Raman peaks.

Additionally, regulatory agencies such as the FDA or EMA require manufacturers to demonstrate that process monitoring techniques can detect impurities at low levels. Since analyte concentrations below mg/mL are generally difficult to accurately detect in aqueous solutions (unless techniques such as SERS, for instance, are applied) (Thyr and Edvinsson, 2023), the use of Raman spectrometers as a primary monitoring tool is unlikely to be implemented in biomanufacturing until sensitivity limitations are resolved. Alternative methods (e.g., ELISA, qPCR, HPLC) may be preferred to detect low-level contaminants, slowing the adoption of Raman under GMP guidelines. Integrating Raman as a real-time PAT tool in a GMP environment is complex as it would involve extensive testing to ensure that variability in cell cultures and sensitivity limitations can be captured across multiple batches and operating conditions. Importantly, Raman spectroscopy generates complex datasets that, if Raman is applied as a real-time PAT tool to monitor continuous bioprocesses, would require robust data management systems to handle the large number of spectral data. Processing such volume of data can be challenging, particularly as GMP regulations require integrity, traceability and security to be maintained over time and that any changes to data (e.g., data smoothing, baseline correction) are recorded with audit trail.

Overall, and although some limitations still need to be overcome,

Raman spectroscopy is expected to play a critical role at distinct stages of production of next-generation cell therapies (Table 2).

6. Conclusions and future perspectives

Although Raman spectroscopy has been widely implemented in protein production workflows, the cell therapy industry has yet to adopt it on a routine basis. Recent studies have highlighted the advantages of integrating Raman tools in both upstream and downstream bioprocesses, enabling the control of CPPs by automating feedback control loops and facilitating continuous real-time batch release. Additionally, fulfilling the need for non-destructive phenotypic cell assays, Raman spectroscopy variants such as Raman microspectroscopy offer the chance to capture molecular features that reflect the biological phenotype of distinct cell populations. Besides, the amenability of Raman probes to be integrated in cell therapy processes at scale, allow Raman spectroscopy to in-line monitor CQAs, therefore replacing laborious off-line analytical techniques in a rapidly and non-invasively manner.

As the field moves from applying Raman sensors to increase process understanding towards their use for process control, several process shortcomings in cell therapy applications could be potentially overcome. Controlled feed additions informed upon Raman data can increase productivity and address challenges associated with batch-to-batch variability, optimizing the use of raw materials and adjusting to autologous manufacturing processes. Identification earlier in culture of batch failure, aid in selection of timings of key process decisions and circumventing the need for time-consuming analytical methods that are implemented for QC and batch release are examples where Raman spectroscopy can thrive as a key in-line PAT to control the manufacture of advanced therapy medicinal products.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was supported by Fundação para a Ciência e a Tecnologia (FCT) through project CARTool (DOI 10.54499/2022.07312.PTDC) and by the European Union's EIC Transition programme under Grant Agreement n° 101113067. This work was also funded by FCT/Ministério da Ciência, Tecnologia e Ensino Superior (FCT/MCTES, Portugal) through national funds to iNOVA4Health (UIDB/04462/2020 and UIDP/04462/2020) and the Associate Laboratory LS4FUTURE (LA/P/0087/2020).

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