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Applications of Raman Spectroscopy in Biopharmaceutical Manufacturing: A Short Review

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Kevin Buckley and Alan G. Ryder

Abstract

The production of active pharmaceutical ingredients (APIs) is currently undergoing its biggest transformation in a century. The changes are based on the rapid and dramatic introduction of protein- and macromolecule-based drugs (collectively known as biopharmaceuticals) and can be traced back to the huge investment in biomedical science (in particular in genomics and proteomics) that has been ongoing since the 1970s. Biopharmaceuticals (or biologics) are manufactured using biological-expression systems (such as mammalian, bacterial, insect cells, etc.) and have spawned a large (>€35 billion sales annually in Europe) and growing biopharmaceutical industry (BioPharma). The structural and chemical complexity of biologics, combined with the intricacy of cell-based manufacturing, imposes a huge analytical burden to correctly characterize and quantify both processes (upstream) and products (downstream). In small molecule manufacturing, advances in analytical and computational methods have been extensively exploited to generate process analytical technologies (PAT) that are now used for routine process control, leading to more efficient processes and safer medicines. In the analytical domain, biologic manufacturing is considerably behind and there is both a huge scope and need to produce relevant PAT tools with which to better control processes, and better characterize product macromolecules. Raman spectroscopy, a vibrational spectroscopy with a number of useful properties (nondestructive, non-contact, robustness) has significant potential advantages in BioPharma. Key among them are intrinsically high molecular specificity, the ability to measure in water, the requirement for minimal (or no) sample pre-treatment, the flexibility of sampling configurations, and suitability for automation. Here, we review and discuss a representative selection of the more important Raman applications in BioPharma (with particular emphasis on mammalian cell culture). The review shows that the properties of Raman have been successfully exploited to deliver unique and useful analytical solutions, particularly for online process monitoring. However, it also shows that its inherent susceptibility to fluorescence interference and the weakness of the Raman effect mean that it can never be a panacea. In particular, Raman-based methods are intrinsically limited by the chemical complexity and wide analyte-concentration-profiles of cell culture media/bioprocessing broths which limit their use for quantitative analysis. Nevertheless, with appropriate foreknowledge of these limitations and good experimental design, robust analytical methods can be produced. In addition, new technological developments such as time-resolved detectors, advanced lasers, and plasmonics offer potential of new Raman-based methods to resolve existing limitations and/or provide new analytical insights.

Keywords

Raman spectroscopy, biopharmaceutical manufacturing, cell culture media, chemometrics, online monitoring, proteins

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Introduction

In the 1970s, scientists began to produce protein-based therapeutic agents, or biopharmaceuticals (biologics), using recombinant DNA technology and cell-culture-based production processes. Some of these biopharmaceuticals, like insulin, human growth hormone, and erythropoietin, were shown to be more potent than traditional small-molecule drugs and their arrival motivated a huge global investment

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in biomedical research to produce new biologics.^{1,2} In the decades since, this research has borne fruit and the BioPharma sector has become a behemoth that has produced “blockbuster” drugs with multi-billion-euro sales, e.g., Herceptin (trastuzumab), Mylotarg (gemtuzumab ozogamicin), and Erbitux (cetuximab). In the present decade, the rate of sales-growth for biological medicines is more than four times that of the pharmaceutical market as a whole.^{1,3}

Biologics are made by living cells and thus are the result of complicated and interconnected biochemical pathways. In order to keep the drug-producing cells healthy and the manufacturing process consistent, optimized industrial processes and carefully engineered cell culture media have to be employed; if the production process is not working correctly, then the final product (usually a protein macromolecule, and most commonly a monoclonal antibody [MAb]) may not have the required primary, secondary, tertiary, or quaternary structure required for therapeutic action. If the biologic is glycosylated, then an additional layer of complexity is added. Monitoring, measuring, and validating each stage in a Biologic production process thus involves physical, chemical, and molecular complexity and can present immense analytical challenges.²

As the BioPharma sector has evolved, and production has moved from small-scale, university-research labs to large kiloliter-scale manufacturing facilities, regulatory requirements and demands for accurate analytical data have mushroomed. Regulatory agencies like the U.S. Food and Drug Administration (FDA) and European Medicines Agency (EMA) have directed the biopharmaceutical industry to improve understanding and control of the bioprocesses so that safer and more consistent biologics can be delivered to the patient. These regulatory demands have led to the adoption of smarter process-management systems (such as Quality by Design [QbD]⁴ and process analytical technology [PAT]^{5,6}) and to increased need for new/novel analytical technologies.⁷ Governments and lawmakers are also stakeholders because they want to see efficiencies in manufacturing that could reduce the cost of these therapies.

Raman spectroscopy is a vibrational spectroscopy with a number of useful properties (nondestructive, non-contact, high molecular-specificity, and robustness) that make it particularly suited for PAT applications in which molecular information (composition and variance) is required. Here, we review some of the most important applications of Raman to the manufacturing and analysis of biopharmaceuticals. This focal point article covers all aspects of the biomanufacturing process, from the identity/variance testing of raw materials and cell culture media, to online and offline monitoring of the bioprocess, characterization of the macromolecule product, and the final analysis of the drug formulations (Figure 1). This review also shows that in many cases, advances in Raman applications from other sectors (e.g., polymer science, forensics, material science)

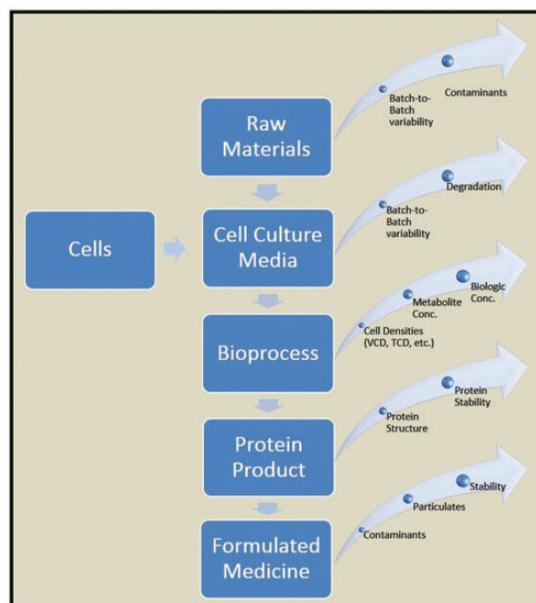


Figure 1. The biopharmaceutical production process illustrating the key steps in the process (blue boxes), and the areas in which Raman spectroscopy has been, and is being, applied (arrows).

can be successfully and usefully translated into, and applied to the BioPharma domain without too much difficulty. However, for certain applications, especially where chemical complexity, low analyte concentration, and/or intrinsic fluorescence are encountered, Raman is unsuitable and does not offer an improvement on existing techniques.

This article is not intended as a comprehensive review of account of every paper published in the Raman and BioPharma fields, rather it focuses, in our view, on the most important issues and topics. We hope that this article will serve as both a useful overview of the diversity and potential of Raman spectroscopy techniques for non-spectroscopists working in BioPharma, and as a useful introduction to the complexities of Raman analysis in BioPharma for spectroscopists.

Analytical Technologies in the Biopharmaceutical Industry

Effective elemental, molecular, and microbiological analyses are critical at every step of the BioPharma production process, from the characterization of raw materials and preparation of cell culture media, to real-time bioprocess monitoring, and the analysis of the macromolecule product.

Established Analytical Techniques. Separations-based chromatographic techniques such as high performance liquid chromatography (HPLC) and ultra-high performance liquid chromatography (UHPLC) are the dominant analytical tools for molecular analysis in BioPharma. These analyses generally involve the dissolution of the sample in the liquid

mobile phase, the passing of the mobile-phase solution through a column that contains a stationary phase, and the measurement of the differential interaction between the two (i.e., sample molecules in the mobile phase become separated in time and/or space because they interact with the stationary phase in different ways). Chromatography is a very mature technology, is widely adopted, and has been reviewed in great detail elsewhere.^{8–11}

BioPharma's other workhorse technique for chemical/compositional analysis is mass spectrometry (MS), a technique that involves the creation of ionized fragments of the target-molecule and the separation of the ions according to their mass-to-charge ratio.^{12–14} Mass spectrometry techniques are often coupled with chromatographic separation techniques producing methods that have very high analyte resolution which are often the best means of characterizing complex biological samples in detail. Again, these combined MS/chromatography technologies are widely adopted and are the subject of much scientific literature (e.g., HPLC-MS,^{15,16} UHPLC-MS,^{17,18} CE-MS^{19,20}).

Chromatographic and MS-based techniques are well understood, very effective, and widely used; they do, however, have several significant drawbacks. First, and foremost, is the fact they often require extensive sample preparation, this slows the measurement process and can introduce significant costs (for labor, solvents, vials, columns, etc.). Sample preparation can be particularly problematic for very complex sample matrices like cell culture media and bioprocess-broths. In addition, different chromatography-based methodologies are required for each type of analyte, e.g., one for sugars, one for amino acids, etc. Consequently, a number of dedicated measurement processes (with different columns, buffers, SOPs, etc.) may have to be developed and implemented in order to undertake comprehensive molecular characterization. Even when the measurement processes are perfected, the analyst has to be aware of column aging, which commonly leads to changes in peak position/shape and can make chemometric/automated data analysis more complicated. Finally, the huge volume of information generated by the hyphenated analytical methods like LC-MS can cause difficulties in interpretation and analysis, often dedicated scientific staff are required for the purpose.²¹ These issues, when combined with high capital acquisition costs, can result in relatively expensive unit test costs and thus these methods are not feasible or practical for all applications. These issues associated with chromatography/MS-based analytical techniques (which are often the gold-standard and reference methods) have generated a need to develop new techniques for the analysis of BioPharma materials. Ideally, these new analytical approaches should be faster, less expensive, and less time-consuming to implement; they should require minimal sample preparation or, better still, no sample preparation. An ideal technique should also be fully automatable, thus reducing the

requirement for scientific staff to be on hand for analysis/interpretation.⁵

Spectroscopy in BioPharma

Analytical optical spectroscopy involves the utilization of light-matter interactions to probe and interrogate molecules and materials to generate information for qualitative or quantitative analysis. It is a very large and diverse domain encompassing core elements of science, engineering, and mathematics. The two classes of optical spectroscopy that have most relevance to the BioPharma field are the electronic and vibrational spectroscopies.²² These offer many advantages over other analytical techniques in that samples can be probed without destruction/contact, the systems often have lower instrumentation/capital costs, and, in many cases, the analyses can be made in situ in the process. Furthermore, many optical spectroscopies are well suited to automation and can be set up to work with minimal (or no) human intervention. These inherent features of electronic/vibrational spectroscopy offer the potential for faster, easier measurements, and thus lower unit-test costs.

Electronic Spectroscopies. Ultraviolet-visible (UV-Vis) absorption spectroscopy, which covers the spectral range of 200–800 nm, interrogates changes in a molecule's electronic energy state. Spectra of liquid samples at ambient temperatures tend to have broad featureless bands with poor molecular specificity. However, the molar absorption coefficients of many important molecules are large and they absorb light efficiently and this absorption can be easily measured using inexpensive spectrometers. Absorption measurements, when combined with the Beer–Lambert law, enable simple quantitative measurements typically down to the micro-molar range for a wide range of molecules. The technique is probably most widely used for protein quantification by simple A_{280} (absorbance at 280 nm) measurements.²³ The specificity of UV-Vis spectroscopy can be enhanced if circularly polarized light is used; in this case the intrinsic properties of a target molecule, as well as its orientation in space (which in turn will be affected by temperature, concentration, solvation-state, etc.), cause the left-handed and right-handed light to have different absorption coefficients. Circular dichroism (CD) spectroscopy,²⁴ as this form of UV absorption spectroscopy is known, is commonly used for the secondary/tertiary structure analysis of proteins.^{25,26}

In some cases, after a molecule absorbs a photon of appropriate energy and is in an excited singlet state, it can return to the ground singlet state via the emission of a photon in a process known as fluorescence.²⁷ Many important biological molecules are fluorescent (e.g., phenylalanine, tryptophan, tyrosine, riboflavin, etc.) and thus nearly all cell culture media and biopharmaceuticals contain fluorophores. The fluorescence process can be very efficient, with high quantum yields. This gives

fluorescence-based techniques high sensitivity and good signal-to-noise (S/N) ratios; routine assays that use conventional spectrometers can have limit of detection (LoD) down in the nanomolar concentration range.^{28,29} This sensitivity has been exploited to produce methods with single molecule detection such as fluorescence correlation spectroscopy (FCS) for the study of proteins.³⁰

For complex biological molecules and samples there can be multiple fluorophores and other photophysically active molecules present, and all are involved in fluorescence emission. This means that there are multiple processes such as energy transfer, static quenching, and dynamic quenching which affect the wavelength and intensity of the emitted light.²⁷ The complete fluorescence emission space can be recorded with an excitation emission matrix (EEM) measurement and this represents a unique “molecular fingerprint” of the sample. EEM has been combined with chemometrics to provide a very effective and useful tool for the qualitative characterization of nearly all the liquid materials that are encountered in biopharmaceutical manufacturing: analyses of cell culture media;^{28,31–34} biogenic raw materials;³⁵ bioprocess broths;³⁶ and proteins.³⁷ This is just a sample of the fluorescence-based applications in BioPharma; there are also many applications utilizing fluorescence microscopy and plate-readers for both research and routine analytical tasks, and these are outside the scope of this review.

Vibrational Spectroscopy: Mid-Infrared (MIR) and Near-Infrared (NIR). Absorption of IR light in the mid-IR region (approximately 2.5–50 μm) by molecules leads to the generation of unique spectra.²² MIR absorption spectroscopy provides a molecularly specific analytical technique that has been used for a variety of BioPharma applications including raw material analysis, bioprocess monitoring,^{38,39} and the characterization of formulated drug product.^{40–42} NIR absorption spectroscopy is a near analogue that utilizes shorter-wavelength/higher-energy light (800–2500 nm).⁴³ NIR spectroscopy measures combinations and overtones of molecular vibrations and this tends to produce spectra (for solids and liquids) that have bands that are broad and overlapped. This means that NIR does not generate the same level of molecular specificity compared with mid-IR or Raman spectroscopy. However, NIR does have other advantages that make it attractive for complex materials analysis and process monitoring.^{44–47} First, the wavelength range allows for the use of simple light sources (e.g., tungsten lamps) and relatively inexpensive glass-based optical components, fibers, and sampling systems. Second, the lower molar absorptivity of most molecules in the NIR region allows for longer sample path-lengths/more homogeneous sampling, and can facilitate sampling through relatively thick container walls.^{45,46} In BioPharma, NIR's main application tends to focus on either raw material testing,⁶ or process monitoring applications.^{44–46,48–51}

Raman Spectroscopy

When monochromatic light is incident on a molecule, the vast majority of the photons that undergo scattering are scattered elastically (Rayleigh scattering). However, a tiny proportion, (~ 1 in 10^9 or 10^{10}) of the photons are inelastically scattered.⁵² During one of these “Raman” scattering events, a photon that loses energy can excite a molecular vibration from the ground state to the first excited state (this event is labelled Stokes scattering), or a photon can gain energy by de-exciting the molecule from an excited vibrational state to the ground state (this event is labelled anti-Stokes scattering).^a The Raman scattered photons carry information about its molecular structure and Raman spectra are complementary to, but intrinsically different from, those obtained from mid-IR absorption spectroscopy. The differences between Raman and mid-IR absorption spectra are due to the different selection rules governing the phenomena (in short, IR absorption spectroscopy is associated with changes in the dipole moment during molecular vibrations, whereas Raman scattering involves a change in polarizability during the molecular motion).^{53,54}

In practice, the different selection rules mean that Raman scattering from polar molecules is less efficient and thus the Raman spectrum of water is relatively weak (compared to mid-IR absorption). The relative weakness of the Raman water bands enables the collection of good quality Raman spectra from analytes in aqueous environments or in aqueous solutions; this facilitates bioprocess monitoring, cell culture media analysis,^{55,56} and protein analysis in solution. Since Raman spectra (and IR absorption spectra) are ultimately derived from the sample's molecular structure and bonding, their spectra can be said to be “molecular fingerprints.” This inherent molecular/chemical specificity means that these methods are well suited to molecular identification tasks, measuring gross changes in composition, and measuring changes in some physical properties (e.g., polymorphism).

There are many variants of Raman spectroscopy and they each exploit the Raman scattering phenomenon in different ways (Figure 2). The choice of variant to use for a particular measurement will depend on inherent factors such as the complexity of the sample and/or the concentrations of the target analytes. For example, in biopharmaceutical cell culture media, concentrations can range from g/L for glucose to mg/L for amino acids and $\mu\text{g/L}$ for vitamins and growth promoters. At the higher concentration ranges (g/L), conventional Raman spectroscopy is suitable and has demonstrated efficacy, but at the lower ranges (mg/L) one has to employ alternative methods that are capable of amplifying analyte signals sufficiently to enable detection and analysis.

Conventional Raman Spectroscopy (CRS). CRS is the Raman technique most widely applied to BioPharma analyses. CRS

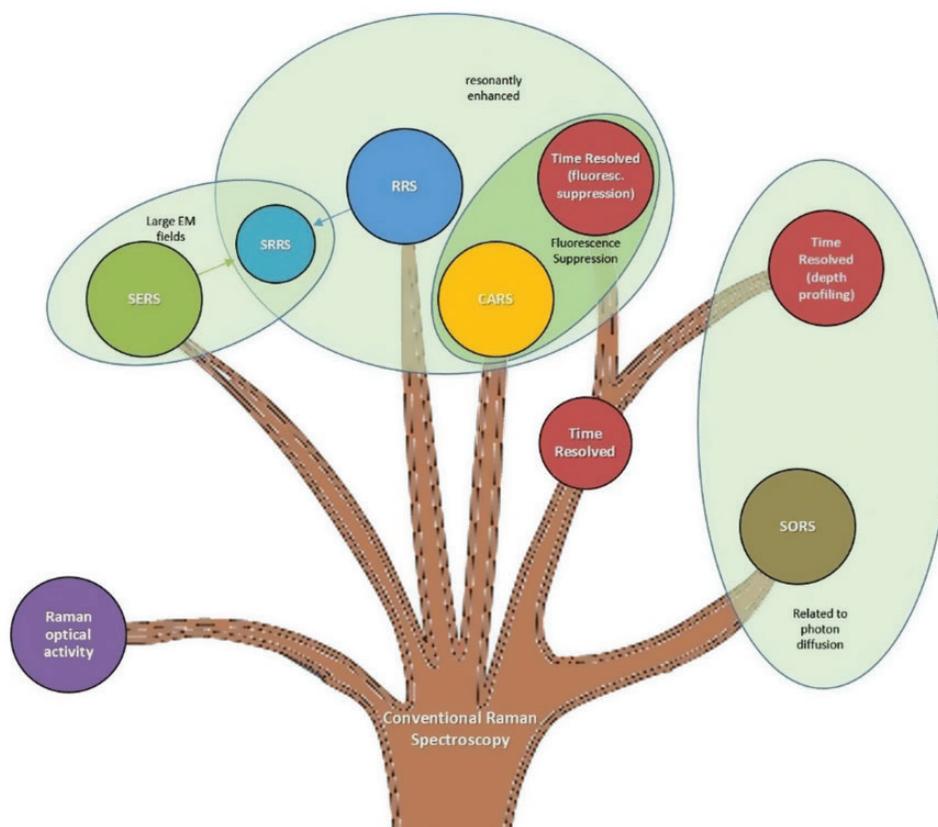


Figure 2. The Raman “family tree” of techniques. Relatively simple conventional Raman spectroscopy (CRS) is the root of the more complex surface-enhanced, resonantly enhanced, chirally, time, and spatially resolved techniques. SERS = surface-enhanced Raman scattering; CARS = coherent anti-Stokes Raman spectroscopy; RRS = resonance enhanced Raman scattering; SORS = spatially offset Raman spectroscopy.

involves the illumination of the sample with a monochromatic light source (usually a continuous-wave laser with a wavelength in the 400–1000 nm range) and the collection of the inelastically scattered photons. Since CRS is usually measured in the visible to NIR spectral region, conventional glass and quartz based optics can be used to get the light to and from the sample and a wide variety of sampling configurations can be facilitated (e.g., fiber optic probes, microscopes, high throughput platforms, etc.).⁵² Micro-Raman spectroscopy can reach spatial resolutions of $<1\ \mu\text{m}$ (as opposed to 10–20 μm for conventional IR absorption spectroscopy) meaning it can be used to analyze very small particles (e.g., polymer fibers and fragments). CRS is a mature and widely adopted technique that is particularly suited to being integrated into the manufacturing systems that require real-time quantitative and qualitative chemical information.⁵⁷

As noted above, only a very small fraction of scattering events results in the creation of a Raman scattered photon and thus the Raman signal is intrinsically weak and is many orders of magnitude less intense than the Rayleigh scattered light (and also many orders of magnitude less efficient

than the IR absorption process).^b In the BioPharma domain, many analytes of interest are present in compositionally complex solutions at sub-mM concentrations and thus they may not be observable by CRS. Raman signal intensity can be increased by using higher power excitation lasers but this is not always possible, e.g., sample-burning can occur for colored solid samples. Increasing the Raman signal by using longer integration times or by using more efficient detection-optics can also help, but in many cases the sample, and the context of the test environment, will limit what can be done.^{52,58,59} The weakness of the Raman scattering process therefore is one of the key considerations when applying a Raman-based solution, and invariably requires careful experimental design and planning.

The second major drawback with CRS in BioPharma is fluorescence. If the incident photons excite sample molecules into higher electronic states, they can sometimes decay back to the ground state with the emission of a lower energy photon. This emitted light can completely obscure the Raman signal because the fluorescence generally occurs in same wavelength range as the Raman anti-Stokes signal, and fluorescence has a much higher quantum

yield (by orders of magnitude) than Raman.²⁷ Biogenic samples such as cell culture media or bioreactor broths frequently contain many fluorophores and thus interference of this kind can be particularly problematic. The simplest solution to removing fluorescence interference is to use longer wavelength excitation sources (785, 830, and 1064 nm being most common) in order to avoid the initial electronic absorption. Unfortunately, this solution also imposes a penalty by reducing the (already weak) Raman signal because Raman scattering efficiency is proportional to the fourth power of the excitation frequency.^{31,32,35,36,60}

Fluorescence interference can also be reduced by the use of time-gating or modulation-based instrumentation. For example, the use of fast pulsed excitation and time-gating of the detection system can be used to eliminate fluorescence, because Raman scattering is an instantaneous effect (<fs) whereas fluorescence requires some time (>1–200 ps) to occur. Thus, if one can switch on and off the detector (or a filter) at a high enough temporal resolution, one can prevent the fluorescence signal being recorded by the detector. This approach was demonstrated more than a decade ago but the instrumentation was expensive, complicated, and not commercialized^{61,62} (diode arrays had been mooted and demonstrated as a potential solution to the problem in the 1980s but suffered from technology limitations at the time⁶³). Recent advances in solid-state, multi-channel avalanche photodiode detector design have now facilitated the implementation of time-gated Raman spectroscopy by providing a much less expensive detection system.⁶⁴ The first commercial time-resolved Raman instruments with 532 nm excitation have recently become available from a Finnish company, Timegate Instruments (<http://www.timegate.fi/>).

The fundamental advantage of time-gating methods for dealing with fluorescence is that they prevent the fluorescence photons from being detected in the first place, and thus any fluorescence shot noise contribution does not get mixed with the Raman signal and degrade sensitivity. Many of the other methods for dealing with fluorescence, such as modulated Raman spectroscopy,⁶⁵ shifted excitation Raman difference spectroscopy (SERDS),⁶⁶ and subtracted shifted Raman spectroscopy (SSRS),⁶⁷ are intrinsically limited by the fact that fluorescence and Raman photons are measured simultaneously, and thus shot noise contributions cannot be separated. Mathematical and chemometric treatment of fluorescence background signal also suffer from the fact that the background shot noise cannot be removed. This is important for the Raman analysis of very complex materials like cell culture media where the Raman signals of many analytes of interest can be very weak, with LoDs in the high mM range.

Surface-Enhanced Raman Spectroscopy (SERS). Nanostructured metal surfaces can have interesting and useful optical properties, and when they are illuminated with light of

an appropriate wavelength they can, in some situations, develop very large electromagnetic fields in small, localized regions. When these (typically gold or silver) systems are used for Raman measurements, sample molecules that enter the strong-field regions generate more Raman scattered photons. The enhancements (over conventional Raman) reported for this SERS are typically in the range 10^3 – 10^6 , but in certain circumstances they can be greater, and experiments with single-molecule detection have been reported.^{68–71} SERS enhancement has the added advantage of effectively “quenching” analyte molecule fluorescence because it increases the Raman signal to a much greater degree than it does the fluorescence signal.⁷²

SERS has the potential to overcome many of the analytical challenges in biopharmaceutical manufacturing but despite its effectiveness (there are more than 13 000 SERS papers in the literature^c and more than 1000 patents in the UPTO database), its adoption in highly regulated environments has been very slow. There a number of reasons for the slow uptake; first, it can be difficult to get reproducible results, especially with chemically complex biogenic samples (large variations can be caused by the different surface populations, different signal enhancements, and variable rates of aggregation). Second, it is not always clear which specific substrates and excitation-wavelengths combinations work best and thousands of different substrates and measurement strategies have been reported. Third, SERS is very sensitive to contamination, and even trace amounts of SERS active contaminants can cause massive and indecipherable (to the analyst) spectral changes. Fourth, the SERS spectra of complex materials are often dominated by a single analyte (such as adenine which has a very strong SERS enhancement).³⁵ In our laboratory we have found that the implementation of SERS has required a very significant amount of validation and cleaning work which made the technique very complex and time-consuming.^{35,73}

In addition to the analytical challenges there are also practical/cost barriers to adoption which will need to be overcome before SERS can be widely adopted; for example, the individual cost of the substrates can be prohibitive, it is common for commercially available substrates to cost >€10 each (indeed, this author has been quoted prices of >€100 per substrate). The substrate cost makes the collection of multiple replicate measurements (necessary for robust chemometric modeling) impractical, especially if you are investigating/measuring media variance, or high-throughput screening, and/or large numbers of samples. For example even the small-scale yeastolate study described below required 66 sample measurements, and this did not include setup and validation measurements.^d Ultimately, SERS will not be a competitive analytical method for Biopharma applications until the cost of high quality, reproducible, and traceable SERS substrates drops

to a more sustainable level, probably in the range of a few cents per substrate.

Resonance Raman Spectroscopy (RRS). Resonance Raman spectroscopy involves purposely selecting excitation photons whose energy are similar to the energy gap between the electronic orbitals of a specific target molecule.⁷⁴ By designing the system in this way, one creates a resonance effect and increases the probability that the photon will inelastically scatter from the molecule. Typical RRS enhancements of $\sim 10^4$ lead to the significant lowering of LoDs for specific analytes to <0.1 mM, a range that is more relevant to BioPharma. The fact that only a single specific molecule/analyte is being targeted can be an advantage or a disadvantage depending on the analytical requirement.^{75–77} RRS can be also be performed on nanostructured metal surfaces (giving a combined RRS/SERS enhancement [SERRS])⁷⁸ or with higher-energy UV photons in a technique known as UV resonance Raman spectroscopy [UVRRS].

In UVRRS, the deep UV ([DUV] $\lambda < 250$ nm) excitation light induces resonance effects with higher-energy electronic orbitals. Sensitivity is enhanced for two reasons: first, the Raman bands from molecules with chromophores are selectively enhanced (as described above),⁷⁷ and second, the intrinsic Raman scattering intensity is dependent on the fourth power of the frequency of the excitation light.⁷⁴ Many biologically important molecules such as amino acids,^{79–81} peptides,^{82,83} and nucleotides,^{84,85} produce highly selective resonance Raman effects. This allows for the characterization of complex materials relevant to BioPharma such as whole viruses and bacteria,^{86–88} proteins, peptides, and bioprocess broths.⁸⁹ Another advantage of UVRRS, which contributes to increased sensitivity, is the avoidance of fluorescence when using DUV excitation. This is due to two factors: first, there are comparatively few fluorophores which both absorb and emit in this DUV region,²⁷ and second, if fluorophores are excited, the Raman signal tends to occupy a spectral region free from the more Stokes shifted fluorescence.^e

UVRRS thus offers increased sensitivity, chemical specificity, and fluorescence minimization but the spectrometers, Rayleigh rejection filters,^{90–93} and efficient DUV lasers needed for UVRRS are expensive and/or complicated (e.g. the laser excitation source can contribute $>50\%$ of system costs, compared to $\sim 10\%$ for CRS).⁹⁴ These demands have prevented its widespread adoption outside of academia, however, the component technologies are advancing all the time and the rapid pace of photonics developments in the past decade suggests that the relevant technological issues can be solved (particularly if instrument manufacturers can leverage technologies being developed for semiconductor manufacture using extreme UV).

Raman Optical Activity (ROA). Circular dichroism spectroscopy can also be performed using IR radiation and the

resultant vibrational circular dichroism (VCD) spectroscopy has recently emerged as a potentially useful spectroscopic technique for macromolecule structural and stability analysis.⁹⁵ The equivalent Raman method, ROA can provide very useful structural information: in the small molecule domain providing data about chirality, and for biomolecules additional information about 3D structure.⁹⁶ ROA has been applied to the studying the effect of crowding on protein structure⁹⁷ and thermal stress effects on IgG type proteins.⁹⁸ Again, the weakness of the Raman effect means ROA is not a fast technique and long sample acquisition times (of up to 48 h or 72 h) have been reported.^{97,98} As well as the chiral resolution ROA offers, many of the benefits of CRS, such as low background signals in aqueous environments and the ability to measure close to the Rayleigh line (<100 cm^{-1}) are applicable.

Chemometric Analysis of Raman Data

In Raman spectroscopy, sample complexity begets spectral complexity, and in BioPharma the unambiguous assignment of bands to specific analytes is often difficult or impossible. Assignment is further hindered by the fact that many analytes of interest in solution are present at low concentrations (<1 mM) and their signal is overwhelmed by other high concentration compounds like water and glucose in cell culture media. For cell culture media in the solid state, spectral complexity is also an issue because of the complex formulations which leads to extensive band overlap which can make the identification and quantification of specific components difficult. These difficulties mean that it is often necessary to employ advanced mathematical and statistical tools, or “chemometrics,” in order to perform accurate and robust analysis.^{99–105} Chemometrics covers a vast array of methods and applications including experimental design, spectral pre-processing, qualitative/quantitative analysis of variance, and quantitative analysis of specific components in samples.^{106,107} In the context of Raman spectroscopic analysis of complex BioPharma samples, chemometrics can, for convenience, be categorized into six discrete activities:

1. Spectral variance analysis: The objective here is to assess the spectral variance using principal component analysis (PCA) or robust PCA (ROBPCA),^{108,109} in order to understand the factors that influence the appearance of the spectra. At this stage, it is important to unambiguously determine if spectral variations are due to instrumental factors, or are due to the intrinsic chemical/compositional changes under investigation, or are due to physical changes in the sample (due to handling/storage issues). This step is critical in assessing data reproducibility and often needs to be combined with iterative experimental design changes to minimize

- unwanted instrumental/sample handling variance. Once the measurement method has been understood and optimized, then reproducible and accurate Raman data should be generated. It is important to note that with many of the compositionally complex samples in BioPharma the spectral changes/differences are often very small and measurement error can be a very significant problem if not identified. Furthermore, this step is required to identify the specific sources of noise/offset, etc. in advance of the implementation of the appropriate pre-processing methods.
2. Data pre-treatment: The second step involves the removal of interfering signals so as to highlight analyte specific bands and/or changes due to variation in composition. This stage generally involves techniques like cosmic ray artefact (CRA) removal,¹¹⁰ baseline correction,¹¹¹ water signal removal, normalization, and first derivative transforms.^{58,59} This stage can be difficult when the Raman analyte spectra exhibit poor S/N ratios (e.g., minor components in more complex media and broth samples) and it can be very time-consuming. A secondary consideration is to ensure that no useful information is accidentally eliminated during pre-processing. A cyclic iterative process between steps 1 and 2 is often needed to ensure that the best quality, and most robust data sets are generated for subsequent chemometric analysis.
 3. Sample variance analysis: This third stage generally involves building and validating a variance/quality-assessment model that is based on PCA, ROBPCA, or equivalent methods which uses extraneous information about the process/samples. These PCA/Quality models provide information (such as the scores from each principal component) that can be used to understand how different samples relate to each other (and, in some cases, identify specific chemical changes). At this stage in the data analysis process, it is often beneficial to try to increase model sensitivity for a specific analyte by removing any excess (unrelated) spectral variables. As with all other aspects of chemometrics, there are many different algorithms that can be used for variable selection,^{112–115} in our group ant colony optimization (ACO)^{116–118} has been used most often.
 4. Classification: When PCA models are used for statistical process-monitoring or quality-control purposes, two important statistics, the Hotelling's T^2 and Q residual, are often calculated. T^2 versus Q plots are a simple way to provide a quantitative measure of variance, which in turns enables a rapid classification of sample quality. By combining these PCA models with "Soft Independent Modeling of Class Analogy" (SIMCA) and Raman spectroscopy, automated ID methods can be developed for quality control (QC) of incoming media components, or the prepared cell culture media.⁵⁸
 5. Spectral deconvolution: This involves following any identified changes in molecular composition using curve resolution methods such as multivariate curve resolution (MCR)^{103,119,120} and/or parallel factor analysis (PARAFAC).¹²¹ Both methods are commonly applied to the analysis of Raman data and can be used for both reaction monitoring (most significantly for small molecule synthesis) and structural analysis of proteins. They have not, however, been widely applied to complex biogenic samples (media and broths) because there are usually too many components present to provide unique and robust solutions.
 6. Quantitative regression analysis: This involves the development of quantitative models for prediction of important process or product parameters (e.g. the concentration of a specific analyte). The most widely used regression based method for quantitative analysis of Raman data is partial least squares (PLS)^{99,100,122–124} and derivatives thereof.
- In general, the major objectives of the chemometric modeling of Raman data in BioPharma are either: sample variance/quality analysis, quantitative correlation of spectral change with process parameters, or the quantification of specific analytes in the media or bioreactor. One bottleneck encountered during the development of chemometric models is the exploration of many different combinations of pre-processing and variable selection techniques in order to find the best data for which to build a PCA or PLS model. This process can be very inefficient and even more so when the data being analyzed are the complex (and often noisy) Raman spectra associated with media and broths. In general, it is better to focus on a small set of well-known and proven chemometric techniques for data analysis and avoid the temptation to investigate every new algorithm in the literature. In our experience, if it does not work with tried, tested, and established methods then it usually means one of three things: the data were badly collected and are erroneous; the data were too noisy; or the analyte signal is intrinsically too weak to measure.
- The cost of computing power and data storage is continuing to decrease very significantly and this, coupled with the availability of automated chemometric software platforms, now makes it feasible to implement chemometric methods in situ, and in real time. This means sophisticated spectral analysis can be deployed at all stages in the upstream manufacturing process from materials ID and acceptance testing, to cell culture media preparation, and to online monitoring of the bioreactors. When combined with the digital outputs of increasingly more compact and robust optical spectrometers makes the deployment of in-line or at-line quantitative analyses based on spectroscopic measurements a viable, practical solution in biopharmaceutical manufacturing, as will be shown below.

Applications

In the past two decades, Raman spectroscopy has been used in a wide range of industrial applications like material ID testing and reaction/process monitoring.^{52,54} Many of these applications are now very mature and can be easily deployed into different settings; for example, the application of Raman spectroscopy for polymer and fiber analysis^{125,126} and knowledge in this area can be used in the identification of foreign bodies in APIs.¹²⁷

In the BioPharma domain studies have shown or demonstrated the utility of Raman-based methods at all stages of the manufacturing process, from the analysis of raw materials and cell culture media,^{35,58,59} to bioprocess monitoring,¹¹⁸ and macromolecule characterization.^{128–130} For each application and, more specifically, for each sample type, Raman spectroscopy has distinct strengths and weaknesses which need to be carefully considered. In addition, for Raman spectroscopy (or any other rapid analytical method) to supplant or replace the established analytical technologies in the BioPharma field, it is necessary to rigorously benchmark new measurements against the gold-standard reference methods. From a rigorous scientific standpoint, the two approaches should be run side-by-side on the same samples. This is not a trivial undertaking as many of the biogenic samples generated by BioPharma are biologically, chemically, and physically unstable and even apparently minor differences in sample handling (e.g., stirred not shaken) can, and will, cause major differences in measurements for all techniques.^{28,32,34–36,58,59,73,118,131}

Sample Handling

A very important issue in BioPharma concerns the physical nature of the sample and whether one is testing solids or liquids. Solid-state cell culture media, for example, are complex powders (e.g., media, hydrolysates, etc.) which are very hygroscopic. Uncontrolled water adsorption from the atmosphere can occur and this will cause problems with variable baselines and erratic signals. Thus, one has to exercise caution when dealing with solid-state media. Another issue concerns the fact that some solid-state media are colored (tan or brown powders are common) and can absorb the excitation light very effectively, thus leading to problems with sample dehydration and/or burning.

When dealing with the complex liquid BioPharma samples (media, reactor broths, etc.), there are several important experimental factors that need to be carefully considered in order to obtain good quality, reproducible Raman spectra. One problem we encountered when analyzing liquid media and bioreactor broths in this laboratory was the type of container used to hold liquid samples. The use of conventional plastic multi-well plates was not recommended because they contributed spectral bands which could not be eliminated easily from the already

weak and complex sample spectra (likewise with the use of microscope slides). To rectify this issue we designed and fabricated electro-polished stainless steel 96 multi-well plates which produced better quality (more reproducible) spectra and were impervious to corrosion^f by acidic raw materials.⁵⁹ A second consideration is that only small numbers of wells should be filled at a time because evaporation losses of water cause large spectral changes which adversely affect data analysis. It should also be noted that concentration changes in media formulations are a desired measurable characteristic and so one needs to prevent evaporative losses. A third consideration is that in many cases samples are harvested/obtained and then stored frozen (−20/−70 °C) prior to testing at a later date.^g Freezing is done to preserve molecular composition, but when being defrosted for analysis, care has to be taken to ensure that samples are completely defrosted and all components have re-dissolved. Sometimes, incomplete defrosting can result in nanocrystal suspensions of components (often amino acids) being formed which then generate very strong Raman signals compared to the dissolved components.

Raw Materials and Cell Culture Media Analysis

The cell culture media used in biopharmaceutical processes must provide the cells with nutrients and energy, and must maintain the conditions inside the bioreactor (e.g., pH, osmolality, etc.) so as to generate optimal yield of the product protein. As a result, cell culture media usually comprise complex chemical mixtures of inorganic salts, sugars,⁵⁹ amino acids, vitamins, growth factors, and buffers (Table I). The exact composition of a specific culture medium varies significantly depending on the manufacturer, its function, and on the cell line which it is to support (e.g., a basal nutrient medium for one cell line would differ in

Table I. Typical concentration ranges of the most significant analytes in freshly prepared cell culture media. The figures come from the technical descriptions of Sigma Aldrich's "Basal Media Eagle medium," its "Nutrient Mixture F-10 Ham Formulation," its "Ames' Medium Formulation," and its "Glasgow Minimum Essential Medium Formulation." The values are representative of many commonly used media used for mammalian cell culture.

Component	Typical concentration ranges
Water	>900 g/L (>50 M)
Inorganic salts (e.g., NaCl, NaHCO ₃ , etc.)	7.5–10.0 g/L (mM)
Sugars	1.0–5.0 g/L (mM)
Amino acids	0.1–0.8 g/L (mM)
Vitamins	0.006–0.05 g/L (μM to mM)

composition from the feed medium for that, or for any other, cell line). In addition, some bioprocesses also require the addition of complex biological derived additives, such as fetal bovine serum (FBS), proteins (e.g., bovine serum albumin), or hydrolysates (e.g., soy¹³² or yeast).

Under current good manufacturing practice (CGMP) regulations, all batches of raw materials (active or inactive components) should be first identity-tested^h prior to the material's use in API/biologic manufacturing.¹⁰¹ To further increase process understanding and control (and therefore product quality), material variance, quality testing, and impurity profiling should also be carried out. A typical example of variance testing is the comparison of different lots of complex cell culture media or of different lots of its components (such as hydrolysates). Raman can be very useful (due to speed of analysis and minimal sample handling) for this application because the cost of traditional testing (using separations based techniques) can be prohibitive. Stability testing is also important, as media are susceptible to a variety of chemical and biological degradation pathways that can adversely affect their performance, for example, riboflavin containing media are photosensitive and the breakdown products are cytotoxic.^{34,133,134} If the testing enables the identification of unsuitable raw materials, or compromised media, then this knowledge can be used to make the manufacturing process more efficient.

Another important aspect of testing at this stage is identifying the presence of real correlations between the measured spectral variance of the raw materials/media lots and with critical process parameters or a critical quality attributes. It is known that media quality can have a big impact on final product yield and quality, and also that a specific (raw material) component of the media can sometimes affect process outcomes. Unfortunately, the compositional complexity of these materials means that routine comprehensive testing for the identification of the specific analytes impacting on process performance or product quality (using chromatographic and MS-based techniques) can be a very difficult, time-consuming, and expensive procedure that it is often impractical (or impossible) to implement.^{58,135} If faster, easier compositional analysis could be carried out, then media quality could be better controlled and more efficient, and consistent manufacturing processes would result.^{5,6,136,137}

Raman Spectroscopy of Cell Culture Media Raw Materials. In recombinant protein production, multiple process parameters affect product yield and quality (e.g., feed quality, feeding strategy, inoculum age, harvest point, etc.). However, once a process has made it to the large-scale bioreactor manufacturing stage, most of the operational parameters will have been fixed or will be subject to effective monitoring/control. Nevertheless, there will be certain parameters that are difficult to monitor and that can dramatically affect the outcome of the bioprocess, one

of those is the batch-to-batch variability of the raw materials and feed media. In 2010, Li et al. showed that Raman spectroscopy and chemometric data analysis could be used to characterize complex cell-media components from a commercial recombinant-protein manufacturing process.⁵⁸ Industry partners supplied multiple lots of five different chemically defined (CD) media components that are usually blended together to produce a feed medium for a Chinese hamster ovary (CHO)-cell-based culture. These components (in the form of aqueous solutions) were analyzed using CRS (785 nm excitation) and both PCA and SIMCA were used to assess their compositions and quality (Figure 4). The results showed that the materials could be easily discriminated and identified and that the gross lot-to-lot variance in incoming batches could be assessed. The results also highlighted some of the main difficulties associated with CRS: the analyte signals were very weak relative to the water signal (Figure 3) and required the use of a water-spectrum elimination step in the data analysis (Figure 4). In addition, the spectra themselves were very sensitive to environmental and physical factors (e.g., light, evaporation, freeze-thaw processes, microbial growth, and chemical reactions). These phenomena affected the spectra and complicated the chemometric and band analyses (e.g., some of the water-eliminated spectra showed baseline artefacts at 1400 cm^{-1} , Figure 4c).⁵⁸

SERS can be used to increase the S/N ratio of some analyte bands in complex aqueous media. In 2011, Wen et al. demonstrated that it could be used as a potential quality-control method for certain cell culture media components. The study was focused on the detection of an adulterant, melamine,ⁱ in biopharmaceutical raw materials (urea, sucrose, arginine, and histidine) and in a commercially available cell culture medium (a medium that comprised 20 amino acids). SERS is most effective for probing molecules with functional groups that contain sulphur and nitrogen (these can most efficiently couple with the Au and Ag surfaces) and the study showed that melamine could be detected at trace levels in some raw materials ("10 ppb in raw material dissolved in 30:70% water/acetonitrile [...] equivalent to 0.5 ppm in solid raw material"). It proved impossible, however, to resolve melamine in histidine or in the prepared cell culture medium; the authors concluded that detecting "melamine in cell culture media could be difficult by SERS as it is composed of many ingredients plus potential fluorescence interferences."¹³⁸ This is one of the critical issues associated with SERS analysis of complex liquids, only certain analytes will be adsorbed and the spectra will be dominated by their signal.

Raman Spectroscopy of Biogenic Media Components, Hydrolysates. For some legacy media and bioprocesses, the medium is supplemented with (or is composed of) complex hydrolysates that are the result of the chemical breakdown of biological feedstock such as meat, yeast, or soy.

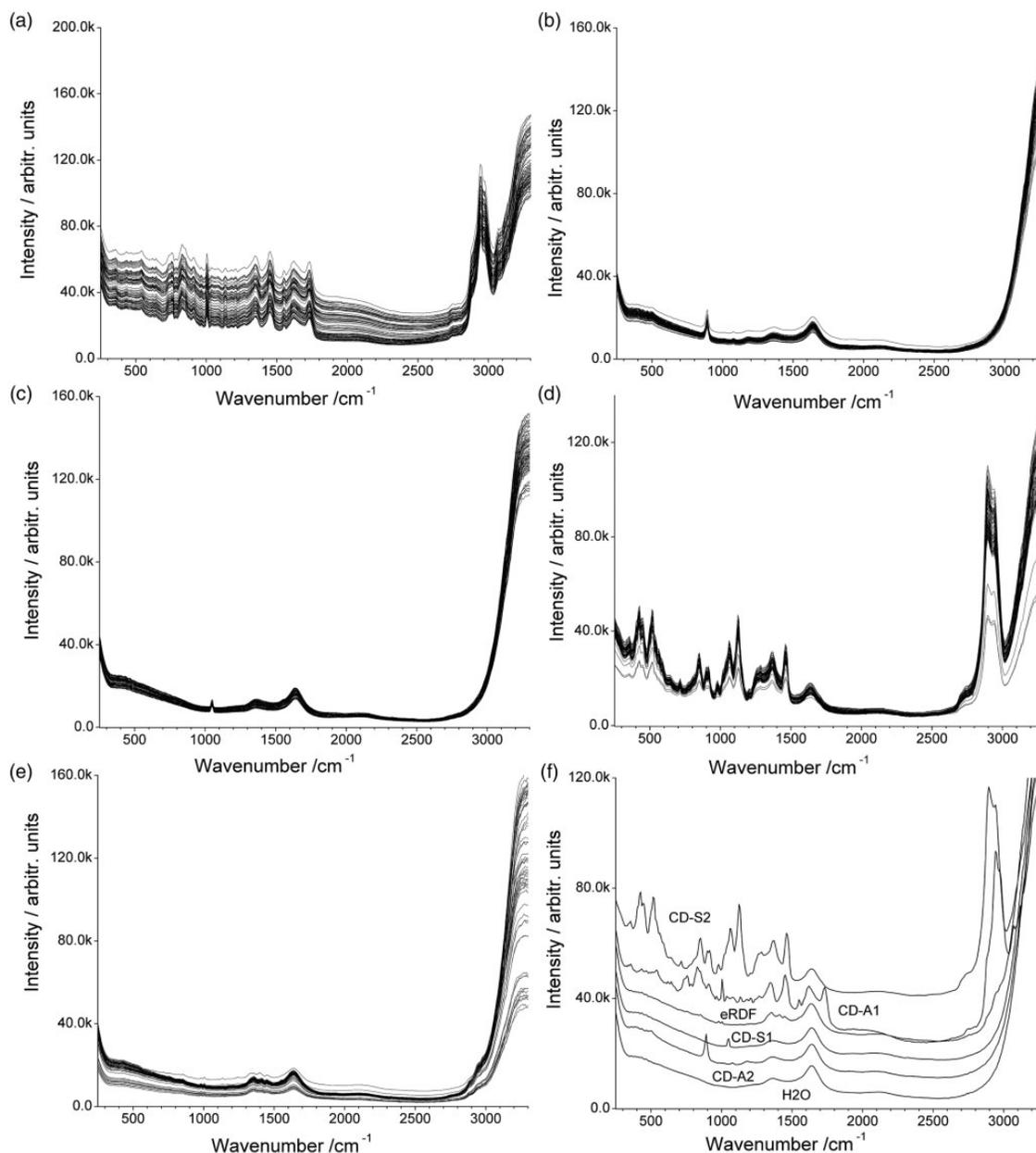


Figure 3. The raw Raman spectra of: (a) 93 CD-A1 (Chemically Defined Acid I mixture) measurements, (b) 93 CD-A2 (Chemically Defined Acid II mixture) measurements, (c) 84 CD-S1 (Chemically Defined Salt I mixture) measurements, (d) 90 CD-S2 (Chemically Defined Salt II mixture) measurements, (e) 63 eRDF (enhanced RDF medium,¹³⁹) measurements, and (f) spectral comparison of the five solutions. All the data were collected over a one-month period. Reproduced with permission from Wiley.⁵⁸

The hydrolysis breakdown products are a complex mixture of everything from carbohydrates, to amino acids, peptides, and a host of other often uncategorized molecules and minerals. Hydrolysates are used in media because many different cell types thrive on them.^{132,140–143}

Soy hydrolysate is one such chemically complex raw material that is derived from soybeans. Its composition is not chemically defined and lot-to-lot composition variability can affect rates of cell growth and protein production. In 2012, four different spectroscopic techniques (NIR, 2D

fluorescence, X-ray fluorescence, and CRS) were evaluated to investigate the compositional variability in a commercially available soy hydrolysate. It was suspected that hydrolysate variability was linked to process performance. The study goal was to see if it was feasible to predict process parameters such as integrated viable cell density (IVCD) and immunoglobulin G (IgG) yield from the hydrolysate spectral data. The different techniques were compared separately in terms of their prediction capability and then ensemble partial least squares (EPLS) was employed to

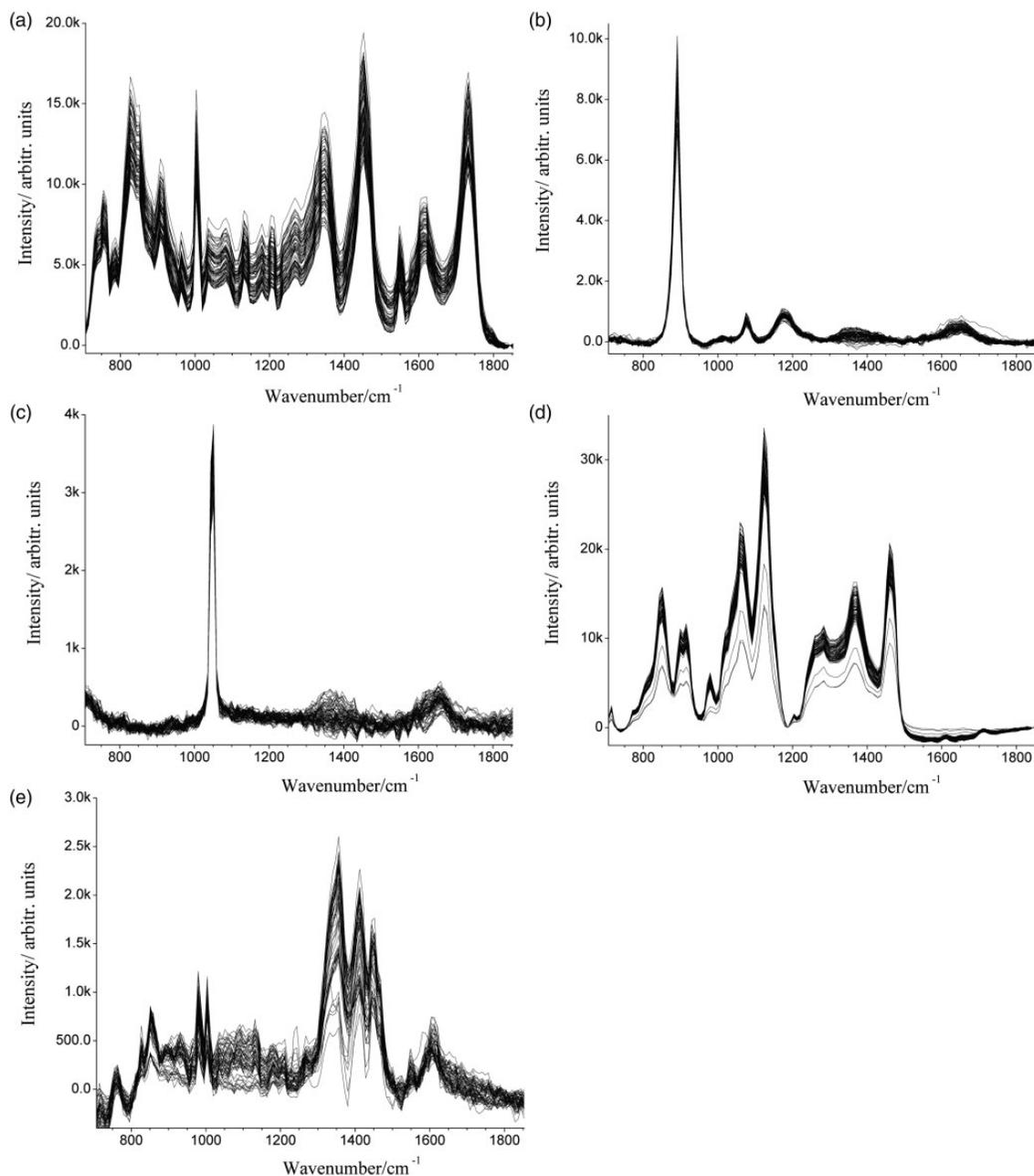


Figure 4. The overlaid water-eliminated Raman spectra of the different chemically defined media components shown in Figure 3, (i.e., (a) CD-A1, (b) CD-A2, (c) CD-S1, (d) CD-S2, and (e) eRDF). The dissolved solids were estimated to be in the 0.5–2% range.⁵⁸ Reproduced with permission from Wiley.

combine all the spectral data sets and produce a more accurate estimation of raw material properties. The Raman spectra had to be collected from aqueous solutions (10 g/L) because the solid material was too fluorescent (authors did not specify the excitation wavelength used). The published (pre-processed) spectra show some sharp peaks that resemble Raman artefacts usually associated with the sapphire window of an immersion probe (this is a common issue). The published analyte bands were

strongest in the 1200–1400 cm^{-1} region and the results showed that Raman spectroscopy on its own was good at predicting the aforementioned process parameters. In addition, a model combining all the analytical data was presented; it gave the best prediction accuracy and demonstrated the synergetic effects of data fusion in characterizing the raw material quality.¹⁴⁴ The key outcome was that process performance could be predicted on the basis of the measured spectral variance of the media. A similar

type of correlation between feed-media variance, as measured by spectroscopic means and process yield/performance had previously been demonstrated by our group using fluorescence EEM.¹ In that study, Raman analysis of the same samples did not show any correlation, indicating that the most important components related to process performance were present at too low a concentration to be detected by CRS.^{31,58,59,118}

Yeastolate is another complex hydrolysate that is sometimes used as a raw material in industrial cell culture processes.^{145,146} It is produced by autolysis of yeast and contains many of the amino acids, peptides, vitamins, growth factors, trace elements, and energy sources (such as carbohydrates) that are needed by cells. Like soy hydrolysate, its composition is not well defined and compositional variability (which can be due to variability in the yeast, the hydrolysis process, and/or drying processes) can affect rates of cell growth and protein production. Conventional Raman spectroscopy is not suitable for the analysis of most types of yeastolate because of its very strong fluorescence.³⁵ The use of longer excitation wavelength (993, 1064 nm) light can reduce the effect, but the Raman signal tends to be weak and adversely affected by the considerable shot noise from the background signal. This makes it very difficult to observe clearly the subtle compositional changes which may be important in a biopharmaceutical manufacturing context. In 2012, SERS was evaluated as a means of overcoming these problems. The study analyzed yeastolate solutions using a silver colloid substrate showed that SERS could be used to detect small variations in composition that were associated with differing manufacture/source. The spectra were dominated by adenine and they changed dramatically as the yeastolate was degraded by microbial growth. The data did, however, show that SERS held promise for implementation as a rapid screening tool for lot-to-lot consistency.³⁵

Raman Spectroscopy of Cell Culture Media. Cell culture media and aqueous solutions of raw materials are often pre-prepared in batches and stored under sterile conditions for periods of days or weeks before use. Cell culture media tend to be complex chemical mixtures with typically less than 10% dissolved solids, of which glucose is often present at the highest concentration. Media are inherently unstable in the liquid state, and despite being stored at low temperature (typically 2–8 °C), significant compositional variation can appear over time (even when stored sterile, in the dark^k).^{34,133} Recently, the possibility of monitoring cell culture media degradation using Raman spectroscopy was explored. It was shown that CRS was unable to detect the small chemical changes associated with the degradation but that SERS could detect some changes and in particular the variances associated with the cysteine/cystine concentration.⁷³ The SERS substrate used in the study was a simple silver colloid, which offered a reasonable level of

reproducibility for this type of complex sample; considerable effort had to be expended, however, to ensure reproducible substrates and data.^{35,73}

Bioprocess Analysis and Monitoring

There are two dominant modes of operation for mammalian cell culture based bioreactors: fed-batch and perfusion.¹⁴⁷ In fed-batch culture, the inoculum and their various media are charged to the bioreactor at the beginning of the process and there is a gradual addition of a fresh volume of selected nutrients (feed media) during the growth–culture cycle. The culture is subsequently harvested and the product recovered. In perfusion culture, a continuous supply of fresh media is fed into the bioreactor and the spent medium is constantly removed. In both cases, the progression of the process results in the cell/medium suspension becoming more chemically/physically complex as it gets infused with the various substances produced (metabolites, cell debris, host-cell proteins [HCP], DNA, and biologic product). Efficient molecular analysis and monitoring of this complex broth poses several major analytical challenges because the analytes of interest span a wide range of molecular types and concentration-ranges, have varying structural complexities, and are obscured by various matrix effects. Raman offers considerable benefits here as it can provide continuous, online quantification of the higher-concentration analytes in the culture.

Offline Raman Analysis. Offline Raman analysis of bioreactor broth requires a sterile sampling loop (using an extraction port for instance) and, in most cases, human intervention. Sample extraction can be a cumbersome process but ports are often built into bioreactors and samples are often drawn for other reasons so are already available for Raman analysis. In 1997, workers from Florida State University and Abbott Laboratories showed that CRS could be used to quantify glucose, glutamine, lactate, and ammonia in liquid bioreactor broths that had been extracted from a bioreactor. The cells were removed by centrifugation and the (spent) culture medium analyzed with 514.5 and 785.0 nm excitation sources. They were able to detect several analytes at typical bioreactor concentrations (for example, glucose was measured in the 0.6–27.5 mM range with 514 nm excitation). The lower concentration limits in these experiments were set by intrinsic fluorescence; the use of 785 nm excitation reduced the effect and the authors concluded that further experimental/instrumental refinements (e.g., better NIR detectors and optics) could improve the results.¹⁴⁸ A similar approach was used in 2008 when CRS analysis (785 nm excitation) of a different palm-oil-producing plant cell bioprocess¹⁴⁹ enabled accurate quantification of several analytes. These analytes were present at reasonably high (g/L)

concentrations and the results obtained were in good agreement with HPLC reference measurements.

In 2011, workers from East Carolina University and Biogen Inc. reported a Raman monitoring study using sample aliquots taken from an industrial-scale (200 L) CHO cell culture process using a proprietary cell culture medium over an 18-day period. Once again, good-quality quantitative results were obtained for components like glucose, glutamine, lactate, and ammonium, which were present at relatively high concentrations (>2 mM). In addition to direct measurements of specific molecular analytes, they showed correlations between the Raman data and the viable cell density (VCD) in the broth, and between Raman data and the osmolality of the broth (i.e., the concentration of osmotic pressure contributing solutes in the broth). Due, presumably, to the proprietary nature of the medium, the report did not contain any spectra and did not outline the specific Raman features that were used to construct the chemometric models.¹⁵⁰

Thus, by the start of the present decade it was well established that CRS could accurately quantify multiple analytes as well as certain physical parameters within the bioreactor, which could be used for process control. The ultimate success, however, of a biopharmaceutical production process is determined by both the yield and quality of the biologic. This, when combined with the fact that many of mammalian cell culture processes in biologic manufacturing are relatively long processes (20–30 days are common for fed batch whereas perfusion based processes can run for up to 90 days), has led to a need for predictive modeling of bioprocesses. In 2013, a comprehensive Raman study was published in which we investigated the feasibility of predicting the final protein yield from a chemometric analysis of Raman data from bioreactor broth samples extracted at different stages of the bioprocess.¹¹⁸ In the study, aliquots of broth from a CHO based manufacturing process were collected at the 12 different time points and across 37 separate production runs. The 12 time points spanned the complete range of fed-batch bioprocess from a 2 L shake flask up to the 5000 L scale final production bioreactor. The samples had the cells and solid debris removed by centrifugation/filtration and were examined using CRS with 785 nm excitation (the same sample set was also analyzed by fluorescence EEM).³⁶ The spectral data set, which contained variation due to changes in molecular composition as the process progressed (12 steps) and the variation between the 37 production processes, was extensively analyzed using chemometric modeling to see if it was feasible to predict final protein yield. The chemometric models developed were able to predict the recombinant protein yield accurately (relative error of prediction between 2.1% and 3.3%). However, because of the compositional complexity, weakness of the signals, and band overlap, it was not possible to say conclusively which specific molecular component was most correlated with

yield.¹¹⁸ Our suspicion was that the cell culture media variance was the key factor, and we proposed that predictive process modeling using the Raman analysis of the cell culture media alone, could be possible.⁵⁸ Unfortunately, when investigated, we found that the Raman spectroscopy of the feed media was uninformative (fluorescence EEM measurements did, however, produce accurate predictive models for the process).³¹

The results from the various CRS studies described above suggest that for most relevant BioPharma analytes, the LoD (with visible or NIR excitation) is between 0.50 mM and 2.0 mM (Table 2). As has been pointed out, there are two reasonable ways of going beyond that: (1) use an enhancement technique to amplify the analyte signal; or (2) increase the Raman scattering efficiency by decreasing the excitation wavelength.¹ In 2013, the Goodacre group at the University of Manchester did both and demonstrated that UVRRS (244 nm excitation) could be used to generate high quality spectra (i.e., with higher S/N ratios) of specific analytes from bioreactor broths (Figure 5). They collected UVRR spectra from samples solutions clarified by centrifugation, taken from three processes: two from glutamine synthetase (GS)-CHO cell lines that produced two different recombinant antibodies (designated LB01 & 60B1) and another GS-CHO cell line (CL8) that lacked the sequences for antibody production. The spectra showed contributions from glucose, lactate, and the antibody product.⁸⁹ Using chemometric methods, they were able to build PLS models with this UVRRS data for antibody, glucose, and lactic acid concentrations. The antibody concentration models spanned a range of ~50–600 mg/L for the two cell lines, and reference measurements (ELISA and HPLC) showed they had errors of 11% and 8%. Two-dimensional correlation analysis and variable importance in projection (VIP) plots showed distinct differences between the antibody producing cell line and the non-producing CL8 line, but it was not possible to determine, unambiguously, if the spectral changes were due to the antibody product.

The UVRR study of bioreactor broths illustrated one of the major problems associated with Raman spectroscopy in BioPharma. Even though the UVRR spectra of the pure components can look very different (Figure 5a–c), the measured spectra from a complex broth will contain contributions from all the molecular components in the sample volume. This means that the spectra from different bioprocesses can look very similar (e.g., the UVRR spectra of supernatant liquid collected from the different cell lines in Figure 5d).⁸⁹ It is often difficult to unscramble the data from the various chemical components (metabolites, lipids, product protein, HCP, nucleotides, etc.) and the protein bands (if present) will be extensively overlapped with amino acid bands from the media; this all increase the difficulty of the analysis.

Until 2015, studies describing Raman bioprocess monitoring involved the use of implicit models, where reference

Table 2. Some of the analyte quantification limits that have been reported for different Raman studies.

Analyte	Limits of detection	Conc. range tested.	Accuracies
Glucose	~2 mM ¹⁵¹ 20 mM ⁵⁵	0.6–27.5 mM ¹⁴⁸ ~2.0–40.0 mM ¹⁵¹ 2.01–6.22 g/L ¹⁵⁹ trace ~14 g/L ¹⁶³ ~0.5–4.5 g/L ¹⁶⁴ ~10.0–20.0 mM ¹⁶⁶	R ² – 0.85, ¹⁵⁰ 0.99, ¹⁵¹ 0.855, ¹⁵⁹ 0.912, ⁵⁶ 0.903 ¹⁶³ RMSECV – 0.30 g/L, ¹⁵⁰ 0.89 g/L, ¹⁶³ 0.33 g/L ¹⁶⁴ RMSEP – 0.53 g/L, ¹⁵⁰ 0.91 g/L, ¹⁶⁸ 0.43 g/L ¹⁶⁴
Lactate	~3 mM ¹⁵¹ 22 mM ⁵⁵	0.5–15.0 mM ¹⁴⁸ ~3.0–20.0 mM ¹⁵¹ 0.23–5.21 g/L ¹⁵⁹ ~0.25–2.5 g/L ¹⁶³ ~0.5–2.0 g/L ¹⁶⁴ ~20.0–100.0 mM ¹⁶⁶	R ² – 0.87, ¹⁵⁰ 0.98, ¹⁵¹ 0.966, ¹⁵⁹ 0.990, ⁵⁶ 0.946 ¹⁶³ RMSECV – 0.17 g/L, ¹⁵⁰ 0.14 g/L, ¹⁶³ 0.20 g/L ¹⁶⁴ RMSEP – 0.32 g/L, ¹⁵⁰ 0.16 g/L, ¹⁶³ 0.26 g/L ¹⁶⁴
Glutamine	0.66–4.26 mM ¹⁵⁹	0.2–15.0 mM ¹⁴⁸ ~2.5–4.0 mM ¹⁶⁶	R ² – 0.96, ¹⁵⁰ 0.686, ¹⁵⁹ 0.925 ⁵⁶ RMSECV – 0.19 mmol/L ¹⁵⁰ RMSEP – 0.67 mmol/L ¹⁵⁰
Glutamate		2.21–5.72 mM ¹⁵⁹ ~1–6 mM ¹⁶³	R ² – 0.83, ¹⁵⁰ 0.855, ¹⁵⁹ 0.959, ⁵⁶ 0.833 ¹⁶³ RMSECV – 0.27 mmol/L, ¹⁵⁰ 0.12 mM ¹⁶³ RMSEP – 0.85 mmol/L, ¹⁵⁰ 0.32 mM ¹⁶³
Ammonia		1.0–20.0 mM ¹⁴⁸ 2.01–8.51 mM ¹⁵⁹ ~1–8 mM ¹⁶³ ~1.0–4.0 mM ¹⁶⁶	R ² – 0.96, ¹⁵⁰ 0.909, ¹⁵⁹ 0.958, ⁵⁶ 0.890 ¹⁶³ RMSECV – 0.43 mmol/L, ¹⁵⁰ 0.29 mM ¹⁶³ RMSEP – 1.13 mmol/L, ¹⁵⁰ 0.42 mM ¹⁶³
Phenylalanine	1.1 mM ⁵⁵		
Formate	14 mM ⁵⁵		
Acetate	7 mM ⁵⁵		

The data illustrate the typical limits of detection (LoDs) that can be achieved with CRS in an industrial BioPharma environment. Limits of quantification can be estimated to be at least 3× the LoD, which means that typically the lower LoQ for CRS is in the 1–3 mM range, at best. RMSECV = root mean square error of cross validation, RMSEP = root mean square error of prediction.

Table 3. Summary of some important practical aspects of the different Raman techniques.

Technique	Unique Advantages	Limitations
CRS	Conceptually/instrumentally simple Highly reproducible Mature technology Many commercially available systems/manufacturers	Weak effect Susceptible to fluorescence interference
SERS	Can use simpler, lower specification Raman spectrometers High sensitivity, theoretically down to single molecule	Poor reproducibility Expensive unit substrate costs Complicated analyte-substrate dynamics for biogenic materials
RRS	High chemical specificity Good sensitivity	Light can degrade the sample Can be complicated/expensive
SERRS	High chemical specificity High sensitivity, theoretically down to single molecule	Can be hard to get good reproducibility Substrates can be a relatively expensive consumable Complicated analyte-substrate dynamics for biogenic materials
UVRRS	High chemical specificity Good sensitivity	Hardware is in general complicated and expensive Photo-damage a serious issue and has to be managed carefully Resolution is generally poorer than CRS

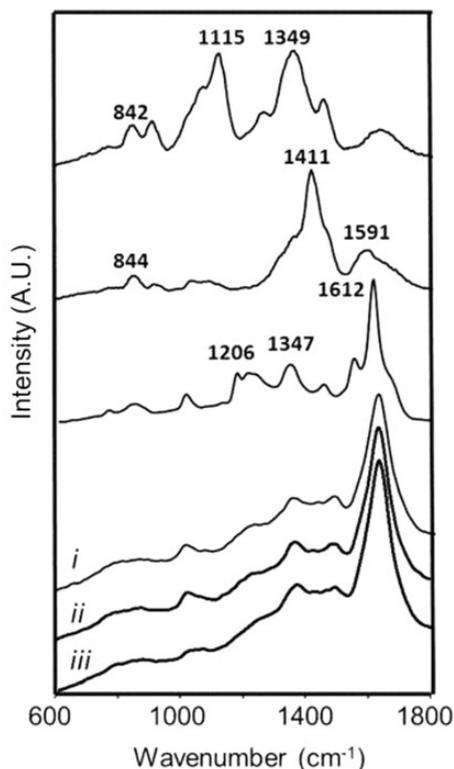


Figure 5. Ultraviolet resonance Raman spectra of (a) glucose, (b) lactate, (c) IgG4 antibody, and (d) supernatant harvested on day 5: (i) antibody-producing cell line LB01, (ii) antibody-producing cell line 60B1, (iii) cell line lacking the antibody sequences CL8.⁸⁹ Reproduced with permission from the Royal Society of Chemistry.

measurements were performed with a training sample set and a model was then constructed. The main problem with this approach was that the training samples were system specific and only model the particular experiment under investigation. A recent study attempted to demonstrate the feasibility of using an explicit model-based classical least squares (CLS) algorithm and a spectral-library of media components.¹⁵¹ With this approach, known analyte spectra were fitted to the measured spectra of the sample liquid (supernatant extracted from a cell culture) and an attempt was made to measure the concentrations of glucose and lactate. A high degree of precision was reported (glucose: $R^2=0.98$, relative standard error = 3.5%; lactate: $R^2=0.99$, relative standard error = 5.2%) but the predictions of lactate or glucose concentration were not in agreement with the reference HPLC measurements. The deviations in the concentrations were significant (too large to be useful) and illustrated the problems associated with the spectral fitting of complex/weak Raman spectra that contain contributions from unidentified chemical species. CLS^{57,152} may not be the best method to employ either, as it cannot deal adequately with the Raman signals from unknown contributors. The authors did, however, make a very good point that the implementation of the

Raman measurement method was much more cost and time effective than the HPLC reference method.

In-line Raman Monitoring and Bioprocess Control. Offline Raman studies of bioreactor broth aliquots have shown that analyte quantification (at an accuracy suitable for bioprocess monitoring) is possible. The process of sample extraction, however, and the handling of the material is not ideal, because of both the time delay and breaching of bioreactor integrity. In-line Raman monitoring of the bioprocess using an immersion probe^m (or through an appropriate sight glass) would negate these problems and would be preferable because the Raman data output could be (almost) instantaneous, data could be collected continuously, and the system could be fully automated. The efficacy of Raman spectroscopy for the non-invasive, in-line monitoring of different processes was first demonstrated in the 1980s and 1990s. Much of the early works focused on the production of simpler molecules, such as yeast-based fermentations for the production of ethanol,^{153–155} and other products such as plant hormones¹⁵⁶ and a carotenoid antioxidant.¹⁵⁷ In most of these studies, the product molecule produced a reasonably strong Raman signal that had bands distinct from the (broad, less well defined) background. In these applications, there was generally less focus on the monitoring of specific media or metabolite components; instead the aim was to quantify the concentration of the product molecule.

In 2004, Lee et al. demonstrated the use of an in-line Raman probe to monitor bioreactors which contained phenylalanine-producing strains of *Escherichia coli*. They showed that number of different molecular analytes (including glucose, phenylalanine, acetate, formate, and lactate; Figure 6) could be quantified and that the measured concentrations compared well with reference HPLC measurements (Table 2).⁵⁵ In general, the liquid in a bioreactor progresses from translucent to increasingly opaque as the cells multiply, by-products accumulate, and light-scattering increases; concomitant with these changes are increases in the complexity of the molecular-composition/Raman spectra.¹⁵⁸ In the Lee et al. paper, the attenuation due to light scattering (from air bubbles and biomass) was corrected for using a normalization method based on the use of water concentration as an internal standard. This reduced the problem but the authors suggested it was still the limiting factor for their measurement accuracy; they also suggested that with improved physical modeling and better window materials, the chemical species present in relatively high concentrations could be measured very accurately and that “the detection limits and sensitivity should approach the shot-noise limited values, which were in the range of 0.1 mM for reasonable excitation power and integration times.”⁵⁵

In 2011, Abu-Absi et al. reported similar results for the analysis of an industrial-scale mammalian cell culture bioprocess. Their study involved the use of multiple Raman

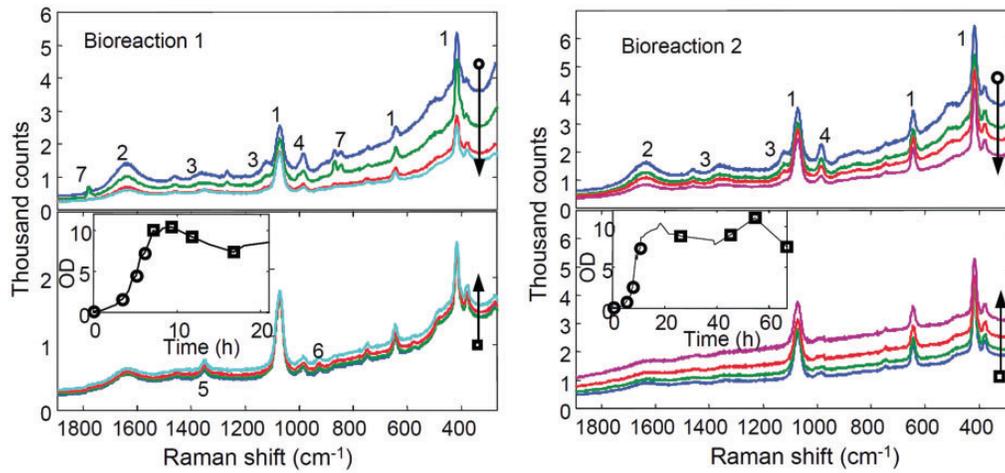


Figure 6. Raman data collected at selected times with corresponding optical density curves for two different *E. coli* bioprocesses, arrows indicates increasing time and numbered spectral features correspond to: (1) sapphire; (2) water; (3) glucose; (4) medium sulfate; (5) formate; (6) acetate; (7) room lights. Sapphire is often used as the window material for immersion probes and commonly the Raman spectrum of sapphire contaminates the signal. Reproduced with permission from Elsevier.⁵⁵

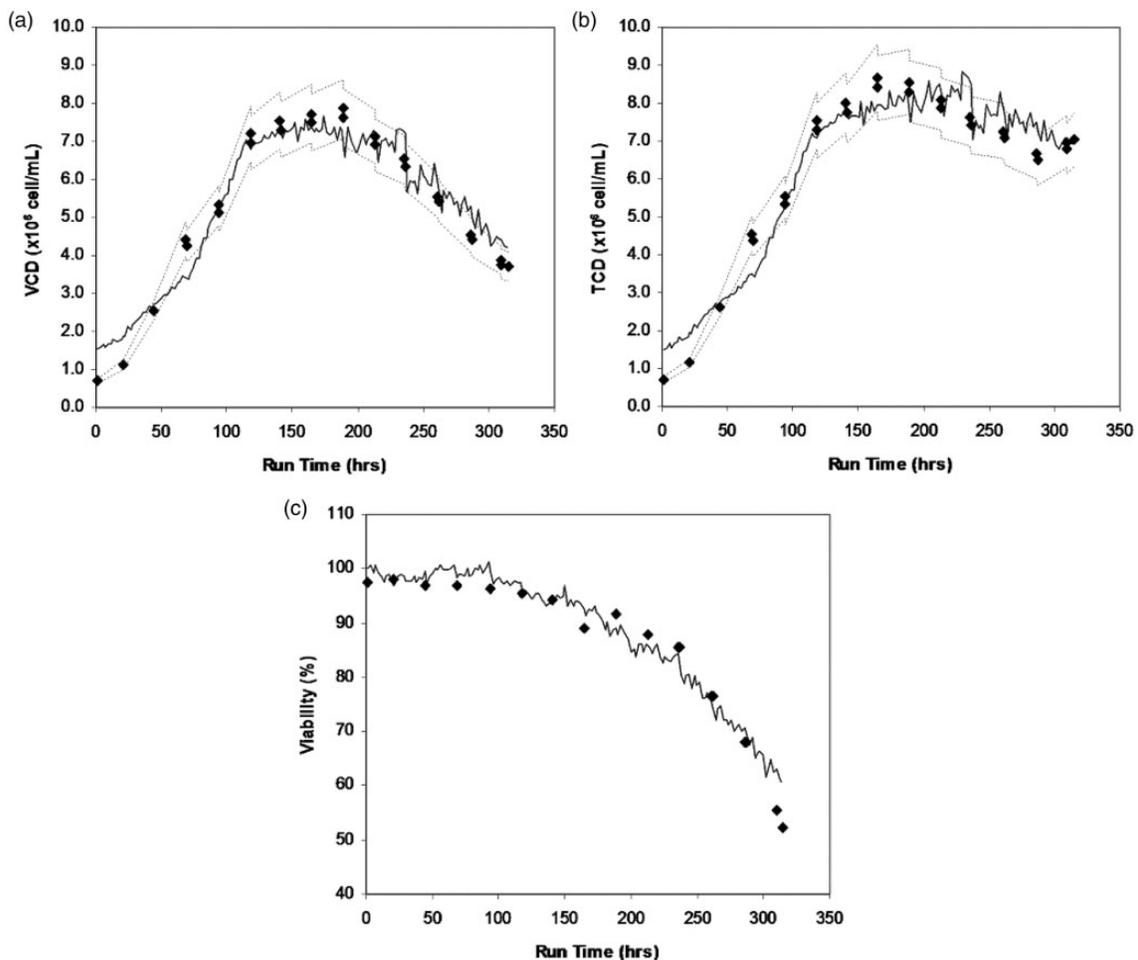


Figure 7. Comparison of (a) viable cell counts, (b) total cell counts, and (c) viabilities using a Cedex cell counter (solid diamonds) and predictions from Raman spectra for cell counts (solid lines). Dashed lines indicate the standard deviation for the reference methods. Predicted viabilities were calculated from predicted-viable-cell and total-cell densities so no errors were reported. Reproduced with permission from Wiley.¹⁵⁹

immersion probes into medium scale bioreactors (500 L) and the comparison of the collected Raman data with off-line validation measurements. The results presented showed good agreement between the in-line and offline measurements for lactate, ammonia, glucose, glutamate, and glutamine quantification (Table 2).¹⁵⁹ In addition to quantifying analytes with Raman, Abu-Absi et al. used a Cedex automated cell counting instrument to record reference-metrics for the actual performance of the bioprocess, total cell density (TCD), and the viable-cell density (or VCD)¹⁶⁰. They then explored correlations between TCD, VCD, and Raman measurements; the results were impressive (Figure 6), but the relevant Raman spectra were not reproduced and the spectral bands/features used to measure the correlations were not identified.¹⁵⁹

In 2012, another group reported on the application of Raman spectroscopy to in-line monitoring and the simultaneous quantitative determination of glucose, glutamine, lactate, ammonia, glutamate, TCD, and VCD in 3 L and

15 L bioreactors (Figure 8). Spectral pre-processing and PLS-based regression were used to correlate spectral data with offline reference data. Separate PLS calibration models were developed for each analyte at the 3 L lab scale before assessing its transferability to the same bioprocess conducted at the 15 L pilot scale. PLS calibration models were successfully developed for all analytes (but not VCD) and the models were transferred to the 15 L scale.⁵⁶

It can be safely said that this type of routine process monitoring is now well established. However, care does need to be taken with the probe–bioreactor interface and alignment to ensure reproducible data.¹⁶¹ The same researchers also investigated strategies for dealing with unsynchronized bioprocess monitoring Raman data sets that were being used for multivariate statistical process control (MSPC).¹⁶²

As mentioned above, many of the reported bioprocess monitoring studies have involved the use of system-specific sample-sets to train the model, and the use of reference measurements, often with low sample numbers. Recently a group from Biogen Inc. reported a different approach in which they combined online Raman data collected from both small-scale and larger-scale CHO cell culture studies (from 5 L to 2000 L; Figure 9) and used all the data to build robust, generic chemometric models. These models were then used to predict various nutrient, metabolite, and cell growth parameters (Figure 10). One of the aims of this research was to improve the characterization of scale-up processes and to improve the ability to predict process performance in (large-scale) commercial manufacturing based on data from the smallest (least expensive and most flexible) bioreactors models possible.¹⁶³ Shortly after this, a group from Pfizer reported on the use of Raman for the in-line measurement of glucose, lactate, and VCD in a fed-batch mammalian (CHO) cell culture process. The models in the study also used training sample sets but were reported to be generic in the sense that they were scale and process independent, and thus could be used at multiple scales and for various processes (i.e., the methodology worked even if the target cell line had not been used in the initial calibration data set).¹⁶⁴

Since the first offline Raman analysis of spent cell culture media in the late 1990s, the technology has progressed to a point where the bioreactor broth is now analyzed in real time,^{159,163} and the yield of a bioprocess could be predicted.^{118,165} In 2014, Craven et al. demonstrated the next logical step, a Raman analysis that could be used to control a bioprocess; the “nonlinear model predictive controller” (NMPC) used online Raman measurements and a closed-loop feedback control system to maintain the glucose concentration at 11 mM inside a CHO bioprocess (15 L) bioreactor (Figure 11).¹⁶⁶ A similar approach was recently published for the control of lactate concentration during culture.¹⁶⁷

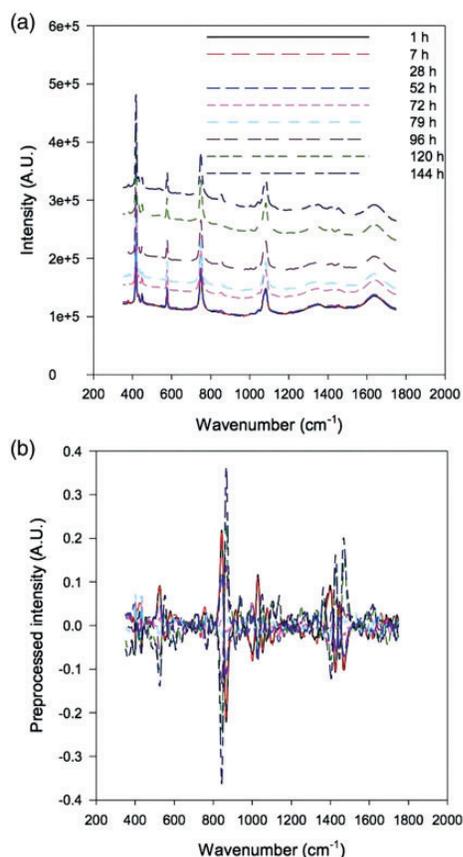


Figure 8. The panel at the top shows the raw Raman spectra over the course of a (3 L) validation run and panel at the bottom shows the processed spectra. It is important to note that the strong sharp Raman bands all arise from the sapphire window signal and that the analyte bands are around an order of magnitude weaker. Reproduced with permission from Wiley.⁵⁶

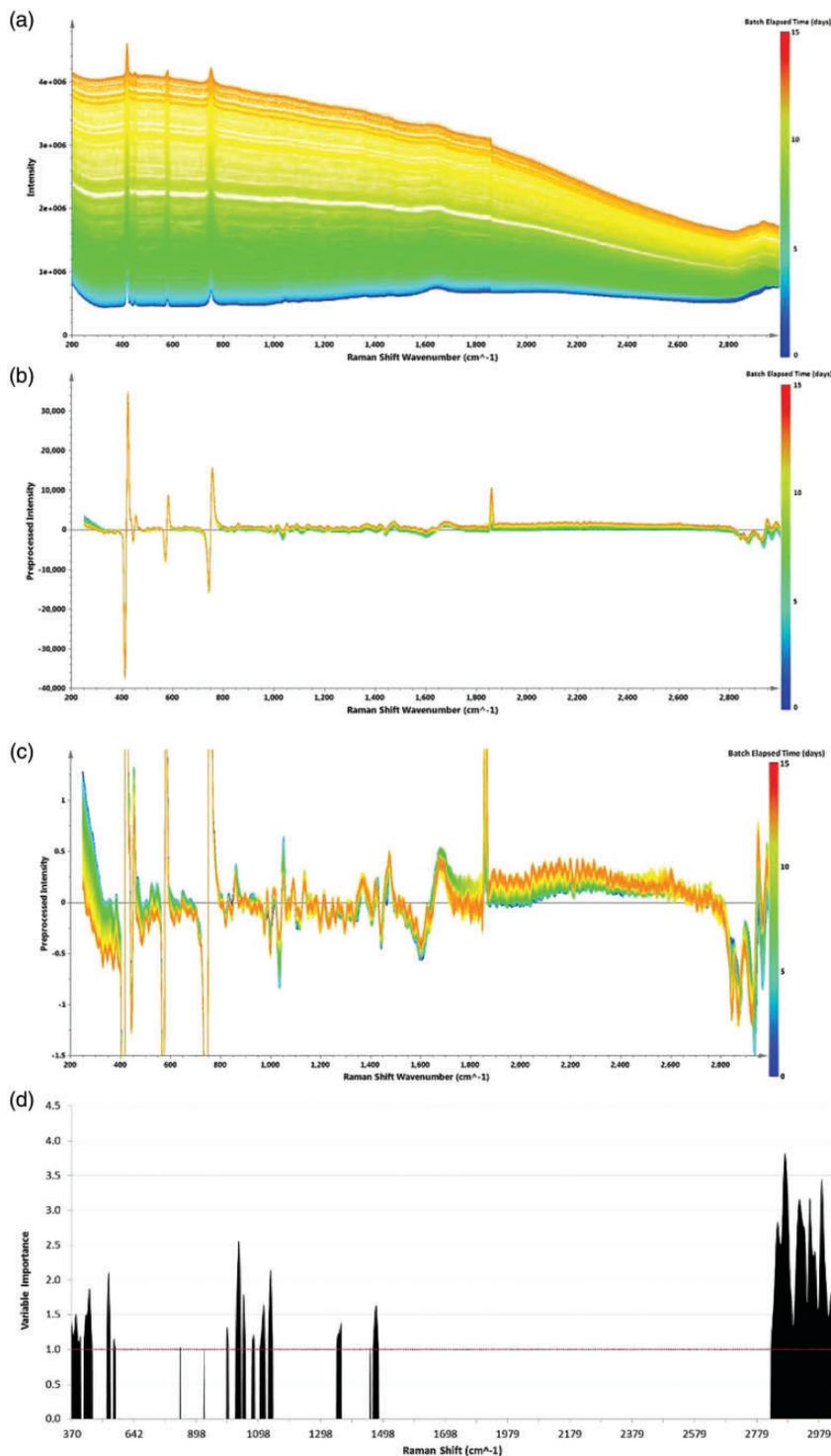


Figure 9. Color-coded time-course spectra (blue = earliest, red = latest) from a single run of a manufacturing scale (2000 L) bio-reactor: (a) raw spectra, (b) pre-processed spectra with first derivative only, (c) pre-processed spectra with first derivative, Savitzky–Golay and standard normal variate (SNV) and zoomed in along the y-axis, and (d) results of VIP algorithm/PLS model that predicts glucose concentration. The algorithm/model was built with the data from the other runs (see paper). The spectra show some of the common problems associated with Raman data collected from in-reactor probes, namely the sapphire bands; the background signal from fluorescence and the scatter. The increase in noise above $\sim 2800\text{ cm}^{-1}$ that is caused by the drop in CCD quantum efficiency above 1000 nm. Reproduced with permission from Wiley.¹⁶³

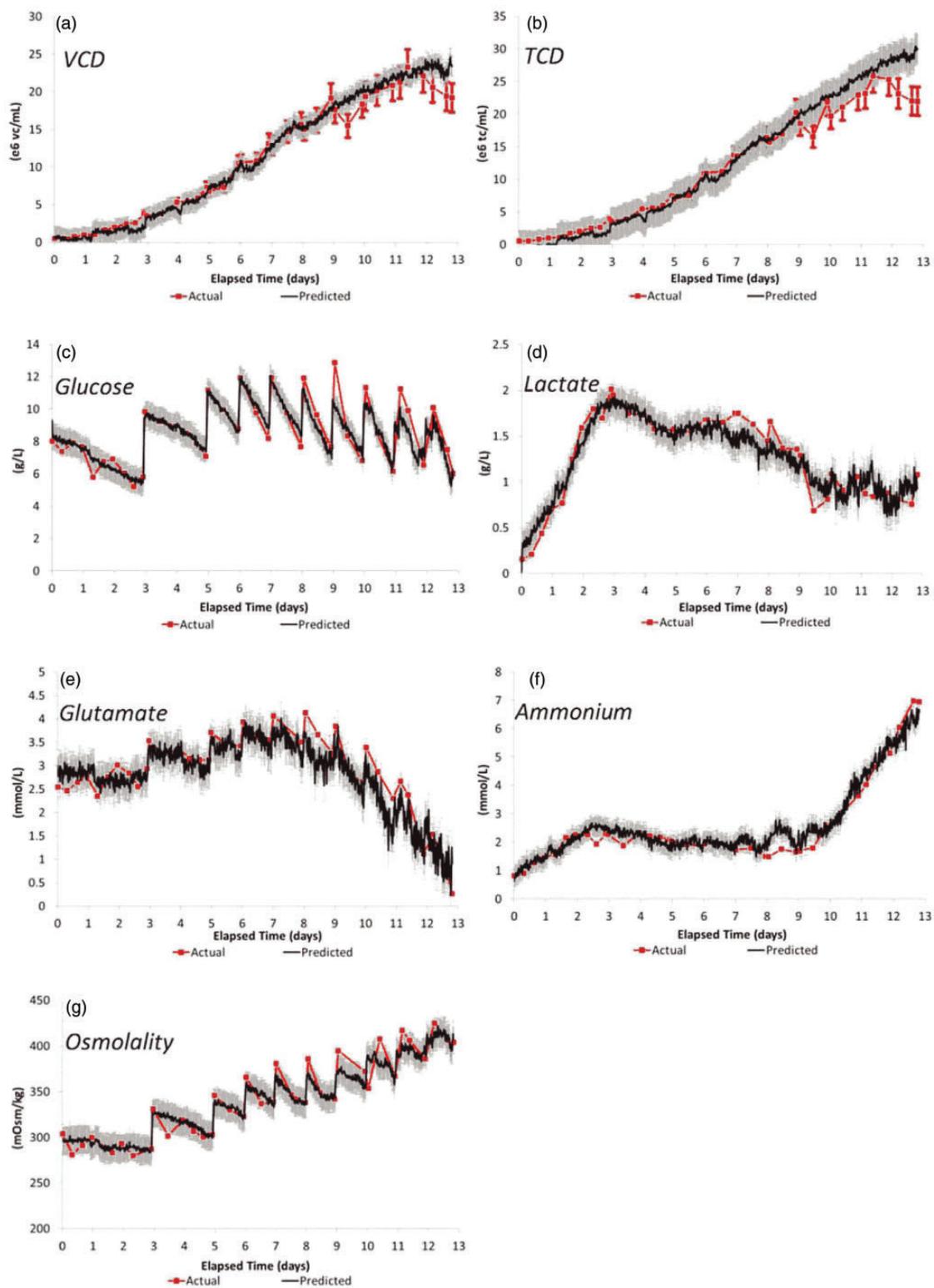


Figure 10. The various PLS models were able to make good predictions for a manufacturing scale (2000 L) run: (a) VCD, (b) TCD, (c) glucose, (d) lactate, (e) glutamate, (f) ammonium, and (g) osmolality. Reproduced with permission from Wiley.¹⁶³

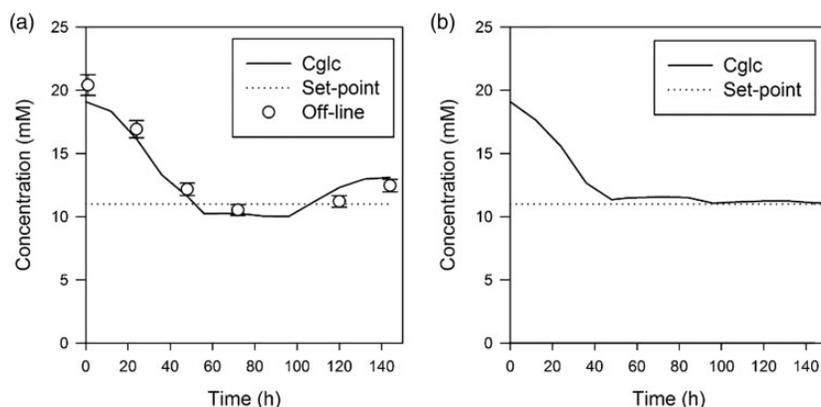


Figure 11. The right-hand panel shows real Raman measurements of glucose concentration (Cglc) under the control of the NMPC and the left-hand panel shows simulated glucose concentration (Cglc) under the control of the NMPC. The system is trying to close on the 11 mM set point. Reproduced with permission from Elsevier.¹⁶⁶

More recently, Raman spectroscopy has been used for in-reactor glucose measurements to provide feedback control of glucose concentration to minimize end-product glycation.¹¹ In that study a broth comprising a CHO cell line growing in a chemically defined medium, was monitored with a conventional 785 nm Raman spectrometer with an in-reactor probe, and standard chemometric data analysis techniques. The authors demonstrated that this Raman-based approach to controlling glucose concentration (at a specified low value) could reduce glycation of their protein by ~50%.¹⁶⁸

From both practical and theoretical standpoints, offline and in-line monitoring of bioprocesses are extremely challenging. No single analytical method can adequately measure all of the required molecular parameters (analyte concentrations, process trajectories, protein quality, etc.) but Raman spectroscopy can address several of the needs well. Raman has shown efficacy for measuring the depletion of glucose (and other specific nutrients), the increase in the concentration of (potentially harmful) metabolites like ammonia, and the changes in cell density and protein content. Offline analysis is useful because it avoids issues with probe fouling and allows for optimal optical sampling of the sample (thus yielding better quality Raman data). Raman also provides for near-simultaneous monitoring of multiple bioreactors which is beneficial if one is conducting a large-scale study and there are many small bioreactors involved (analysis by HPLC or other methods may be impractical or prohibitively expensive in this situation). In-line monitoring has shown utility for collecting continuous and instantaneous data (when compared to the long duration of a typical mammalian cell culture, ten or more days), and both forms of bioprocesses monitoring have demonstrated that they can provide useful information on the scale-up of fed-batch processes (there is usually a gradual scale-up from laboratory bioreactors to full kilo-liter units).^{31,118,169}

Analysis of Biopharmaceutical Products

In general, the analysis of biologic drugs is much more challenging than the analysis of small molecule APIs because of the sheer complexity of the protein structure (secondary and tertiary structure, the nature of the side-chains, the degree of glycosylation, etc.). In addition, other structure-related parameters, such as the degree of aggregation and the thermal/chemical stability of the biologic in different environments, can affect how the medicine will function.¹⁷⁰ Accurate measurement of these factors has become even more important as the patents on the first generation of biologics expire and biosimilar drugs are brought to market.¹⁷¹

Raman Analysis of Biologic Structure. Raman analysis of proteins has been practiced since the 1970s,^{172,173} and the technique has progressed significantly (in particular with advances in instrumentation). CRS and resonance Raman can be used to easily discriminate different proteins,¹⁷⁴ on the basis of molecular structure and the technique can be implemented on both solid-state and solution samples. While no single analytical technique is capable of complete protein characterization, Raman spectroscopy can tackle several important problems. A number of reviews of these applications have been published and only a flavor of the literature will be given here.^{25,130,175}

The relationship between protein secondary structure and spectral band profiles^o has been the subject of intense research and is reviewed in detail elsewhere.^{129,176} UVRRS in particular is well suited to protein studies because the amide-band vibrations, which are linked to structure, can be enhanced.¹⁷⁶ In 2012, the utility of UVRRS as a tool for fast biopharmaceutical characterization of proteins was investigated and the secondary structure of the salmon calcitonin (sCT) protein was probed. Protein solutions of different concentrations were investigated (0.5–200 mg/mL)

and the sCT was found to have unordered regions, β -sheets, and minor α -helical content. At 200 mg/mL, the sCT solution turned from a transparent solution to a turbid gel and the amide I band characteristic of β -sheet (1677 cm^{-1}) increased.¹⁷⁷ The same year workers at the Division of Pharmaceutical Analysis of the United States FDA also evaluated the use of URRS for quality control of formulated therapeutic proteins. They studied two forms of insulin (insulin lispro and insulin glargine) that have slightly differing primary protein structures. The study showed that these primary structure variations were manifested as differences in secondary structure and that this could be detected by URRS, the authors concluded that the method could be used as a direct characterization tool for formulation quality control.¹⁷⁸

It has also been demonstrated that Raman spectroscopy can be used to probe protein side chain structure. For example, specific well characterized bands can reveal the presence of certain amino acids (e.g., the cysteine sulfhydryl group, SH, gives rise to a band in the $2500\text{--}2600\text{ cm}^{-1}$ region), the nature of the molecule's hydrogen-bonding (ratio of the tyrosine bands at $\sim 830\text{ cm}^{-1}$ and $\sim 850\text{ cm}^{-1}$), and the degree of methionine oxidation (bands at 704 cm^{-1} and 1010 cm^{-1}).¹²⁹ In one 2007 study, workers from Amgen Inc. reported a study in which different forms of Raman spectroscopy were used to characterize the cysteine side chains of a specific biopharmaceutical molecule, recombinant human interleukin-1 receptor antagonist (rhIL-1ra). Raman was able to prove that rhIL-1ra in solutions and the solid-state was different, and that there was no di-sulfide bond in solutions of rhIL-1ra. Analysis of the Raman data also ruled out the possibility of cation-p interaction involving tryptophan in rhIL-1ra. These cation-p interactions in proteins have been recognized as playing an important role in protein structural stability and in protein-protein interaction between ligand and receptors.¹⁷⁹

Protein Glycosylation. Glycosylation is a critical product quality attribute in biopharmaceutical and the accurate measuring and characterization of glycosylation is an important analytical challenge.¹³ Most of the measuring/monitoring of post-translational modifications (e.g., the differentiation of glycosylated forms¹³ of native proteins) in BioPharma is MS-based and there is not a very extensive literature related to spectroscopic methods. Raman has been shown to be capable of distinguishing, the (relatively simple) changes associated with glycation of hemoglobin (Hb),¹⁸⁰ when pre-concentration sample preparation (drop coating deposition) and standard chemometric data analysis are employed. For Hb and its glycated analogue, HbA1c, there are clear spectral differences (visible even in noisy spectra) and it is possible to both classify protein forms using PCA with high accuracy. Quantification of binary Hb/HbA1c mixtures has also been demonstrated with reasonable accuracy.

Recently, Brewster et al. used a simple protein model system, the bovine pancreatic Ribonuclease proteins, RNaseA (non-glycosylated) and RNaseB (glycosylated) to investigate whether Raman spectroscopy (785 nm excitation) could be a useful tool for (first) discriminating and (second) probing the structure of glycoproteins. The proteins could be discriminated and the discriminatory bands included vibrations associated with both structural changes and bands that originate from the glycan component.^{170,181} More recently again, it was demonstrated that tip-enhanced Raman spectroscopy (TERS) can be used to distinguish between native and glycosylated forms of bovine pancreatic ribonuclease (RNase). In the work, the authors describe the difficulty in implementing TERS and explain that its variability means it is unlikely to be suitable for routine glycosylation analysis in an industrial laboratory.¹⁸² Finally, the combination of TERS, ROA, and CD spectroscopies was used successfully to study some aspects of the structure and glycosylation of mucin proteins.¹⁸³

Unfortunately, the implementation of a Raman spectroscopy-based method capable of displacing MS-based methods for MAb glycosylation analysis is likely to prove impossible for the time-being. This is because industry has a need for very comprehensive glycan and glycoform characterization to meet regulatory requirements and the sheer complexity and variability of glycosylation in terms of sites, branching, micro-heterogeneity, and macro-heterogeneity, produce very complex Raman spectra that are intrinsically difficult to analyze.

Protein Stability and Aggregation. Protein aggregation is a serious issue in biopharmaceutical manufacturing and has to be strictly controlled/minimized to ensure that the biologic drug works as licensed.¹⁸⁴⁻¹⁸⁷ Aggregation can occur at all stages of production, purification, formulation, and storage because the biologic passes through a wide variety of chemical and physical processing steps, all of which can have adverse effects on the molecule. Changes in protein conformation can also lead to fibrillation, where misfolded proteins form large linear aggregates or amyloid fibrils.⁹⁴ It is therefore very important to monitor the protein so as to ensure that no unwanted structural changes or aggregation have been induced.^{129,188} Raman spectroscopy offers some useful characterization of the larger aggregates which can be analyzed using confocal microscopy-based instruments. For soluble oligomers and sub-visible aggregates, Raman can also be used as long as there is some discernible change in the spectra due to the aggregation process.

In 2003, CRS was used to probe the fibrillation of insulin, a process that needs to be minimized. Raman spectroscopy enabled elucidation of the fibrillation mechanism and revealed chemical/structural information that would have been difficult to obtain with any other technique.^{130,189} In 2013, the Goodacre group used 2D correlation analysis of CRS data (780 nm excitation) to probe the stability of

ribonuclease proteins. They showed it was possible to compare the unfolding profiles of the proteins and to show very subtle protein conformational changes that could not be observed with a spectrofluorophotometer.^{170,190} The following year, they demonstrated that CRS combined with 2D perturbation-correlation moving windows (PCMW) data analysis could be used to characterize conformational transitions in antibodies. Five antibodies with varying propensity to aggregate were compared and spectra were collected at a range of temperatures (56–78 °C). The PCMW analysis method allowed distinct differences in conformational changes related to protein stability, as measured by CRS to be determined thus providing greater understanding of the aggregation mechanisms of problematic antibody variants.¹⁸⁷ A recent book chapter¹⁹¹ describes the use of UVRRS methods for observing and monitoring protein fibrillation. These include hydrogen-deuterium exchange experiments to study β -structures.¹⁹²

Protein Formulations and Identity Testing. Biologics in their final form are often produced as either concentrated solutions or lyophilized powders, and these are very good sample types for Raman spectroscopy (relatively simple compositions and high concentrations). As with small molecule API formulation analysis, the key objectives in this situation are: process monitoring, measurements of API and excipients concentrations, content uniformity, impurity/contaminant analysis, and formulation/drug stability. All of these can, to a certain degree, be achieved with Raman spectroscopy. In 2004, Sane et al. demonstrated that Raman spectroscopy (Fourier transform [FT], using 1064 nm excitation) could be used to characterize drying-induced structural changes in a therapeutic recombinant humanized monoclonal antibody (rhuMAb). Fitting of subtle features of the amide I Raman band revealed information about the rhuMAb stability, and the degree of structural perturbation immediately after spray-drying correlated well with the antibody's actual rate of aggregation over (simulated) long-term storage. The results demonstrated that Raman analysis of the amide I band could be a quick and reliable way to screen excipients and their concentrations during lyophilized or spray dried formulation development.¹⁹³

A few years later, De Beer et al. demonstrated that Raman could be used for the in-line and real-time monitoring of freeze-drying. A Raman probe was inserted into the freeze-dryer chamber to monitor changes in a mannitol solution (mannitol being a widely used excipient in freeze-dried pharmaceutical formulations). The spectra were analyzed using chemometric techniques, and information was retrieved about: (1) the mannitol solid state throughout the entire process; (2) the endpoint of freezing (endpoint of mannitol crystallization); and (3) several physical and chemical phenomena occurring during the process (onset of ice nucleation, onset of mannitol crystallization, etc.).¹⁹⁴ More recently, Raman spectroscopy has been used to monitor

the secondary structure of proteins in frozen storage. A set of reference proteins with different amounts of α -helix and β -sheet were probed during frozen storage and thawed storage. Some of the proteins were observed to unfold partially, some these unfolding events were reversed by thawing and others were not.¹⁹⁵ Other recent studies by Ota et al.,^{196,197} demonstrated that CRS using visible excitation can be used to easily analyze with a relatively high level of detail, high protein concentration solutions (up to ~300 mg/mL) and that CRS combined with straightforward chemometric (PCA and MCR) analysis could be used to follow the evolution of protein–water and protein–protein interactions, to assess protein stability, and to monitor the chemical changes in high concentration solutions.

These studies illustrate how the fundamental strengths of Raman (minimal sample handling, molecular specificity, online/non-contact) can be exploited to produce very useful information for protein formulation.¹⁹⁸ Recent developments in filter technologies which enable Raman measurements in the $<100\text{ cm}^{-1}$ spectral region will allow the retrieval of detailed information about diagnostic low frequency vibrations.¹⁹⁹ This spectral region is useful for probing solid-state properties (such as polymorphism) and or analyzing protein stability.²⁰⁰ In summary, protein formulation analysis represents a very promising application area for Raman spectroscopy, that will deliver a variety of robust analytical methods suitable for industrial use.

Raman Analysis of Particulates and Contaminants

During biologic manufacturing and formulation, unknown foreign particulates can appear in the manufacturing vessels, delivery vials, or in the drug products themselves, raising questions about the safety of the medicines. Raman spectroscopy, with its ability to be easily coupled with confocal microscopes, enables the measurement and analysis of individual particles down to sizes of $\sim 1\ \mu\text{m}$, is an ideal technique for identification particularly for polymer fragments arising from the pipes, valves, bags, filters, etc. used for liquid handling.

Raman has shown utility for the identification, in situ, of foreign particulates that have formed (in aqueous solutions) inside glass containers; of droplet-like particles inside pre-filled glass syringes, of fibrous particles inside glass test tubes, and of specks of an unknown white substance inside glass vials.^{201,202} Recently, CRS has also been used to identify sub-visible particles in biopharmaceutical formulations (the spectra were used to elucidate the particle-formation mechanism in a test system)²⁰³ and an automated Raman system has also been deployed to identify silicone-oil induced aggregation of proteins in drug solutions (it was particularly suited to differentiating between protein, protein/silicon particles, and silicone particles).²⁰⁴ When coupled with spectral databases of all the materials present in the manufacturing processes and robust search

algorithms, Raman can provide a convenient and rapid tool for contaminant material ID. The method can be further enhanced when combined with digital image analysis (e.g., the Morphologi system from Malvern, ParticleFinder from Horiba Scientific) as it provides a forensic tool of immense capability for trouble shooting in both upstream and downstream operations.

Biologic medicines can also contain biological contaminants (like HCPs, residual DNA/RNA, metals, and molecules) that have the potential to cause adverse patient outcomes. Analysis and quantification of these contaminants is another potential application of Raman spectroscopy but for many biologics the LoD requirements for residual HCP, DNA,²⁰⁵ RNA, contaminants are in the ppm concentration ranges and this would make CRS unsuitable for routine release testing. It has recently been demonstrated that UVRRS can be used to monitor biopharmaceuticals and detect, at reasonably low concentrations, some specific contaminants. UVRRS has been able to detect small amounts of residual organic solvent inside starch-peptide conjugate solutions (containing only 40 µg/mL of peptide)¹⁷⁷ and to monitor residual DNA/RNA in clarified recombinant proteins.²⁰⁶ The latter study was very significant because host-cell DNA/RNA have the potential to induce serious adverse clinical effects^P and have been the subject of much scientific research.²⁰⁵

Strengths and Weaknesses of Raman Spectroscopy

Reviewing the literature, and being mindful of our own experiences, has brought into focus the important role of understanding the strengths and weaknesses of Raman and how they are particularly relevant to biopharmaceutical manufacturing domain. Successful implementation of Raman solutions always requires a very careful assessment of the chemical, physical, and instrumental factors that govern each and every measurement.

Strengths. Many of the chemical analyses described above can be, and have been, performed using other analytical technologies. The important drivers for the use of Raman are its inherently high molecular specificity, its ability to perform non-contact/nondestructive testing, and its ability to probe samples in aqueous environments. Raman instrumentation is very flexible in that it can be easily integrated into many manufacturing systems; measurements can be carried out remotely using fiber optic probes (with variable excitation geometries, variable measurement distances, etc.), small particles/samples can be probed, and there is potential to automate both the signal collection and data analysis.^{207,208} Non-invasive Raman measurements through glass²⁰⁷ and plastic, or in turbid media^{209,210} are also possible.

In summary, Raman has the potential to be faster, less labor-intensive, and less expensive (low unit test cost) than competing techniques. It can also offer very good

reproducibility; for example, spectrometer and laser performance degradation is very slow compared to, for instance, the aging of a chromatography column.²⁰⁷ In two recent solid-state analysis studies we collected nearly 1 million spectra,²¹¹ and ~9 million Raman spectra (as yet unpublished results) in experiments over a timeframe of three to six months. In both cases, the same samples were concurrently analyzed using HPLC, which proved to be both more difficult to implement and was less reproducible on month-to-month timeframes. Overall, Raman spectroscopy was intrinsically a much more stable measurement technique once a good experimental design and appropriate sample handling procedures were implemented.

Some of the specialized variants of Raman spectroscopy such as ROA and UVRRS provide additional unique analytical advantages, particularly as tools for the study of various aspects of protein structure in solution.^{81,96,97} These Raman-based approaches have clear advantages compared to other analytical methods such as Fourier IR, X-Ray crystallography, or NMR (e.g. protein structural analysis can be carried out both in the solid-state and in solution using Raman spectroscopy).

Weaknesses. The biggest problem with Raman spectroscopy in general, and CRS in particular, is the inherent weakness of the Raman scattering process, which ultimately limits the sensitivity of the technique. While some techniques like SERS, SERRS, and UVRRS can improve sensitivity, they are either not sufficiently robust or cost effective for routine analytical applications. The use of UVRRS to increase the efficiency of the Raman scattering process is also problematic from the fact that deep-UV light can cause serious photo damage, however, the use of specialist sample holders such as flow cells can somewhat minimize these effects.²⁰⁷

The second major issue which particularly affects CRS, is its susceptibility to fluorescence inference when using visible excitation wavelengths for the analysis of liquid and solid samples, or when using NIR wavelengths to analyze complex solid-state materials like media. Both of these problems are particularly significant in the biopharmaceutical domain where many samples are aqueous solutions, with low concentration analytes, that contain multiple fluorophores.

The other major, complicating factor in the use of Raman spectroscopy for biopharma is the fact that many of the aqueous solution samples (i.e. the cell culture media and spent bioreactor broths), and the biogenic molecules themselves, can have extremely complex compositions/structure. This produces very complex Raman spectra (e.g., Figure 3 and Figure 4) which comprise many overlapping bands from the different chemical species, often with very poor SNR.²⁰⁷ This makes unambiguous assignment of bands to molecular species and vibrational modes an often difficult proposition.

Finally, there are two other issues which have a negative impact on the use of Raman spectroscopy in Biopharma. First, the cost of instrumentation is a major factor as high quality Raman systems are often significantly more expensive than equivalent FT-IR, NIR, UV-Vis, and fluorescence spectrometers. This can be attributed largely to expensive individual component costs, such as low noise CCD detectors, high stability narrow linewidth lasers, and optics. Another serious consequence of this cost factor is that it can make Raman instrumentation too expensive for many undergraduate teaching laboratories. This leads to a secondary problem that Raman spectroscopy does not have the same level of penetration into undergraduate practical courses and thus a majority of science students (particularly life science students who may end up working in BioPharma) do not have any practical first-hand experience of Raman spectroscopy unlike UV-Vis, FT-IR, and separation techniques like HPLC. This lack of familiarity naturally leads to these other techniques being considered first when addressing analytical challenges, even when Raman might be the optimal method.

Conclusions and Future Directions

In the past two decades, Raman spectroscopy has become an important analytical tool in biopharmaceutical manufacturing and it has been shown to have value at all stages of production from the earliest stages of the manufacturing processes (e.g., identity and variance testing of raw materials and cell culture media), to online and offline monitoring of bioprocesses, and the final characterization of biologics. With appropriate foreknowledge of Raman's limitations and good experimental design, robust analytical methods can be produced and implemented in an industrial context.

The biopharmaceutical industry is highly regulated and changes to both manufacturing and analytical systems/practices can be associated with large administrative burdens. Despite the inherent (and necessary) conservatism of the industry, the analytical strengths of Raman spectroscopy make it likely that, with time, it will be adapted much more widely. It is also likely that some of the practical weaknesses of the technique will be reduced or eliminated by advances in technology and academic research. Some of the likely improvements will be in:

- **Core Instrumentation.** Instruments will become less expensive, more robust, have higher throughput, smaller, easier-to-use, and more automated as the associated technologies (photonics, electronics, and integration) continue to advance. As system cost decreases, we should also see an increased adoption of Raman spectroscopy in undergraduate teaching laboratories. This should increase the exposed user base which in turn will drive increased acceptance of Raman techniques in industry.
- **Better Sampling.** Variants of CRS such as transmission Raman,^{208,212} which delivers better sampling statistics and spatially offset Raman spectroscopy (SORS)^{209,213–215} that allows spectra to be collected through opaque materials, are making big inroads in the small molecule API sphere. These techniques will be translated into specific applications in BioPharma.
- **Chemometrics and Automation.** The issues of sample complexity may also be ameliorated by the development of new chemometric techniques. This, when coupled with decreasing computing costs, and software engineering should lead to more fully automated Raman-based analytical methods for continuous monitoring of both upstream and downstream processes.
- **Technique Maturation.** Research methods like UVRRS and SERS may also mature and become more cost-effective and widely used in time. Their unique capabilities may find applications for protein analysis and characterization, and low analyte concentration assays respectively. Raman optical activity will also continue to become more widely adopted and used for protein characterization particularly in R&D and downstream processing environments.

In conclusion, Raman spectroscopy in BioPharma has a promising future and in online bioprocess monitoring it can be already be considered an established, mature methodology. Several other CRS-based applications that provide unique solutions to BioPharma problems (such as contaminant/particle and formulation analysis) should become more widely established in the near future. Other methods, such as ROA, SERS, UVRRS, will continue to produce much interesting academic research but are further from adoption in BioPharma manufacturing as routine analytical techniques.

Conflict of Interest

The authors report there are no conflicts of interest.

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Notes

- a. For all conventional Raman measurements in the biopharmaceutical domain, which are typically made at room temperature, the Stokes scattering will always be much stronger than the anti-Stokes scattering.
- b. The analysis of low density samples such as gases typically requires the use of multi pass cells to generate usable levels of signal.⁵⁴

- c. Scopus search for [“(surface enhanced Raman”) and (SERS)] on 6 October 2016.
- d. Reuse of substrates is not feasible in a working, regulated analytical laboratory because of the potential for contamination and the need for rigorous, validated cleaning methods.
- e. For example, an excitation wavelength of 210 nm ($47\,619\text{ cm}^{-1}$) will produce a Raman spectrum out to $\sim 229\text{ nm}$ ($43\,619\text{ cm}^{-1}$) at most, which is often before any Stokes-shifted fluorescence emission begins to appear.
- f. An aluminium multi-well plate was also fabricated and evaluated, but its corrosion resistance was poor. For example, with some of the more acidic liquid media components we noticed hydrogen evolution from the wells.
- g. This is especially the case when samples are being supplied from external sites or are being harvested at times when the analytical technique may not be available.
- h. It is recommended that more than one test be used to confirm identity.¹⁰¹ In small molecule API CGMP, the analysis is relatively simple a component's identity includes its chemical structure, its physical form (e.g., polymorph, solvate, and appearance) and, if appropriate, its stereochemistry or immunochemistry. In biopharmaceutical manufacturing, single components are the exception and thus the process is much more complicated.
- i. Melamine is a nitrogen-rich molecule that is sometimes put in the food chain to deceive total nitrogen analysis and to artificially give the appearance of higher protein content. Total nitrogen analysis is carried out using the Kjeldahl method; an analysis is not molecularly specific and measures the sum of the organic nitrogen, ammonia, and ammonium.
- j. In that study, EEM exhibited higher sensitivity (due to the better S/N ratios) and it outperformed Raman spectroscopy.
- k. Photo-degradation of cell culture media can have even larger effects on media composition, particularly when riboflavin is present. The variation can have adverse effects on cell viability (and thus bioprocess performance), and can be easily observed and quantified using multi-dimensional fluorescence spectroscopy.
- l. There are other approaches: one could, for instance, remove the water to increase the analyte concentration (this would introduce a non-trivial sample-handling step, however, and would not be conducive to high accuracy).
- m. Bio-fouling of the in-line probe-tip is an issue which must also be considered as this degrades optical performance. This can take many forms including reduced signal intensities, increased background offsets, and fluorescence contamination.
- n. Glycation is the covalent addition of a sugar, often glucose to proteins, via non-enzymic processes. It is usually an unwanted process in biopharmaceutical processes and is often sensitive to the glucose concentration in the media. Glycosylation on the other hand refers to the carbohydrates which have been added via enzymic processes in the cell and is a critical quality attribute (CQA) as it affects bioavailability.
- o. Examples of these bands would include those associated with the coupled modes of the polypeptide backbone, namely the Amide I band at $\sim 1650\text{ cm}^{-1}$ (related to the C=O stretching of the peptide carbonyl groups) and the Amide III band which originates from coupled C-N stretching and N-H bending motions and appears in the 1200–

1400 cm^{-1} range. Amide I band's position and shape is very sensitive to changes in protein secondary and tertiary structure because the peptide carbonyl groups from which it originates act as acceptors of hydrogen bonds within ordered secondary structure.

- p. The WHO's guidelines recommend that a dose of biologic medicine should contain $\leq 10\text{ ngr DNA/per dose}$.

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