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Review Article

ATR-FTIR spectroscopy and spectroscopic imaging for the analysis of biopharmaceuticals

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ABSTRACT

Attenuated Total Reflection Fourier Transform Infrared (ATR-FTIR) spectroscopy is a label-free, non-destructive technique that can be applied to a vast range of biological applications, from imaging cancer tissues and live cells, to determining protein content and protein secondary structure composition. This review summarises the recent advances in applications of ATR-FTIR spectroscopy to biopharmaceuticals, the application of this technique to biosimilars, and the current uses of FTIR spectroscopy in biopharmaceutical production. We discuss the use of ATR-FTIR spectroscopic imaging to investigate biopharmaceuticals, and finally, give an outlook on the possible future developments and applications of ATR-FTIR spectroscopy and spectroscopic imaging to this field. Throughout the review comparisons will be made between FTIR spectroscopy and alternative analytical techniques, and areas will be identified where FTIR spectroscopy could perhaps offer a better alternative in future studies. This review focuses on the most recent advances in the field of using ATR-FTIR spectroscopy and spectroscopic imaging to characterise and evaluate biopharmaceuticals, both in industrial and academic research based environments.

Contents

1. Introduction

Biopharmaceuticals, also commonly known as biologics, are drugs derived from biological organisms. They are utilised in treating or preventing a range of diseases such as arthritis, diabetes, and some cancers [4,5]. Biopharmaceuticals have a huge diversity of function due to their variation in chemical composition and their conformational flexibility. Biopharmaceuticals are highly selective and have a low non-specific toxicity, but their complexity promotes significant challenges in production. This is due to their multiple routes of administration to patient (e.g. oral, pulmonary, transdermal), and variation in pharmacokinetic parameters, such as half-life ($t_{1/2}$), protein binding, and bioavailability. Their comparatively large size means it is unusual for biopharmaceuticals, even small antibody fragments, to cross the blood brain barrier (BBB) [9]. Although this can limit their application to brain disorders, it also means there is reduced risk of unwanted side effects.

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Monoclonal antibodies (mAbs) (Fig. 1) are the most common biopharmaceuticals, and account for more than half of total biopharmaceutical product sales. Their major benefits are their ability to identify and bind to cell surface targets with very high specificity, and their capacity to be isolated effectively through changes in salt concentration and pH [10]. Alterations in the environment of biopharmaceuticals are common in production, such as the implementation of extreme changes in pH during cleaning-in-place (CIP) protocols, which results in effective separation of mAbs due to their intrinsic resistance to pH changes [11]. The hydrophobic interaction chromatography (HIC) step, also works through altering the environment of the protein, the sample is loaded onto the column at high salt concentrations, and is eluted at lower salt concentrations [12].

The complexity of biopharmaceutical manufacture is two-fold. Firstly, these large biopharmaceutical drugs are unpredictable due to their sensitivity and propensity to aggregate. Secondly, their manufacture is challenging due to the development and optimisation of the industrial multi-level production pathway. Current techniques in this pathway such as mass spectrometry (MS) can be expensive to run, and others such as nuclear magnetic resonance (NMR) have stringent sample pre-requisites for optimum results, which include but are not limited to, a pH range of 4–7, the use of a deuterated solvent, and the absence of excipients. This complex biopharmaceutical production pathway therefore requires a technique which can be applied to multiple uses, is inexpensive to run, and requires minimal to no sample preparation. FTIR spectroscopy is a label-free, non-destructive technique, which can be used to analyse biological systems, as previously outlined in a number of comprehensive review papers [13,14]. FTIR spectroscopy can be used to observe key characteristics of biopharmaceuticals such as denaturation and aggregation, prediction of metabolites, concentration, and to quantify levels of phosphorylation and glycosylation. These characteristics of FTIR spectroscopy are critical to providing insights into biotherapeutic behaviour and stability, and can also be used to optimise the production process through acting as a quality control measure.

FTIR spectroscopic imaging uses a focal plane array (FPA) detector instead of a traditional single element detector. The FPA typically consists of 64 × 64 or 128 × 128 pixels, and collects an interactive image containing thousands of spectra. FTIR spectroscopy and spectroscopic imaging have a variety of setups and approaches, offering the capability to investigate biopharmaceutical structural integrity, which is paramount to producing a reliable and safe product. The applications of FTIR spectroscopy and spectroscopic imaging to the manufacture of biopharmaceuticals are outlined and critically discussed in the following review.

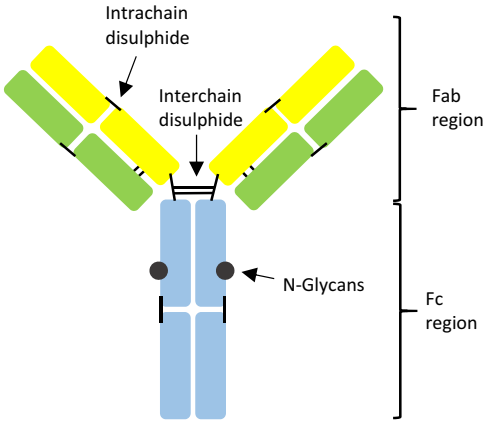

Fig. 1. Schematic of a mAb showing the Fab and Fc regions, disulphide bonds and N-glycan positions.

2. Biopharmaceuticals

In recent years there has been rising demand for effective biopharmaceutical drugs; in 2019, 8 of the 48 drugs approved by the FDA were for antibodies or antibody-drug conjugates [15]. However, the fast commercialisation of biopharmaceuticals in comparison to small molecule drugs remains a challenge, partly due to their relative chemical and physical instabilities. Analytical techniques are therefore required for characterisation of biopharmaceuticals, to ensure the effective monitoring of their stability and efficacy throughout production and delivery to the patient. This characterisation commonly involves the use of techniques such as liquid chromatography tandem mass spectrometry (LC-MS-MS) to monitor changes in mass [16], high pressure liquid chromatography (HPLC) to determine impurity profiles of samples [17], and infrared spectroscopy (IR) to identify impurities and compare biosimilars to the original, 'reference drugs' [8].

Most studies where FTIR spectroscopic analysis has been utilised to investigate biopharmaceuticals are focussed on mAbs as opposed to other biopharmaceuticals due to their highly specific, comparatively stable nature [10,18,19]. This specificity is determined by the chemical composition, physical forces, and molecular structure at the fragment antigen-binding (Fab) region (Fig. 1). The umbrella term of mAbs also includes derivative antibodies, such as bispecific antibodies (bsAbs), antibody-drug conjugates, radiolabelled antibody conjugates, antigen-binding fragment Fab, and Fc-fusion proteins [20]. Some of the more unusual biopharmaceuticals such as Fc fusion proteins, PEGylated proteins and antibody drug conjugates (ADCs), viruses, and virus like particles (VLPs), pose unique problems and challenges which require stringent monitoring of their higher order structures. For example, during production, VLP-based vaccines encounter problems due to their lack of a viral genome (essential for the formation of a virus) causing instability in downstream processing [21].

The high demand for mAbs is well-founded, and despite the challenges associated with industrial grade production of homogeneous and efficacious product, they have been used in a number of novel applications [22,23]. Research has suggested mAbs, when combined with BBB peptide shuttles, could penetrate the BBB, and deliver an effective treatment for brain metastases [9]. This could occur through the use of antibody fragments to reduce molecular weight, or the incorporation of cell penetrating peptides (CPPs) into mAbs to deliver large cargoes across cell membranes, and even the BBB [24]. Another created an affordable immunoassay using 4C9C9 and 4C9E11 mAbs in a sandwich-type UMELISA® assay to identify cystic fibrosis in newborns, specifically for use in Cuba and other Latin American countries [22].

Irrespective of the benefits, producing biopharmaceutical drugs has some significant drawbacks. Production requires substantial costs in terms of time, monetary investment, and protocol optimisation. An unfortunate consequence of this is a high cost to the patients. One course of biotherapeutics for arthritis costs around USD $20,000 annually [25], and for Crohn's disease it can exceed USD $44,000 annually [26]. Although this cost can be reduced by the use of biosimilars, it is still essential that efficiency in the process is retained through the minimisation of loss of product. The structural and functional integrity of the drug product must also be preserved throughout the varied conditions required for production, including isolation and storage, and delivery to the patient [27].

Selected biopharmaceuticals such as therapeutic antibodies and antibody fragments are prone to significant aggregation and misfolding during production and delivery [28]. To make both production and delivery more effective, it is essential to be able to observe, and fully understand the mechanisms by which aggregation and/or unfolding occur. Certain points have been identified in the production pathway where therapeutic proteins are more prone to aggregation or unfolding. These points are generally where the proteins are placed under stress conditions such as thermal [29], oxidative [30], and mechanical stress

[31], repetitive freeze thaw conditions [29], and/or interface agitation [32]. It is therefore ideal that data is collected at each biopharmaceutical process stage, and is utilised to make a real-time decision in order to produce high quality products. Diversity in production pathways mean a variety of monitoring techniques are employed at each phase to ensure safety and efficacy of final product. In-line, on-line, and off-line measurements (Fig. 2), summarised by Holzer et al. [33] all have their merits, but in-line measurements such as NIR spectroscopy [34] and ATR-FTIR spectroscopy can be combined with other techniques to offer real-time decision making [35]. For example in-line ATR-FTIR and NIR spectroscopy can be incorporated into a feedback loop to reduce the presence of contaminants or aggregates, therefore reducing the time and monetary costs of production.

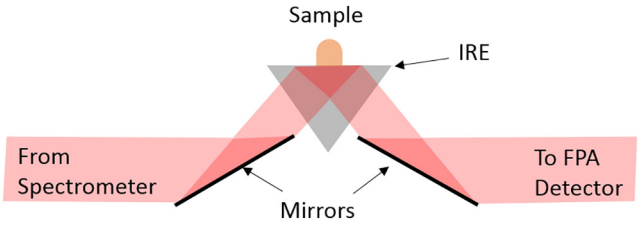

Fig. 3. ATR-FTIR spectroscopic set up demonstrating IR light being directed from the spectrometer to the accessory mirrors, towards the IRE, where it will interact with the sample on the surface of the IRE to a penetration depth of 0.5–5 μm, and will then be directed to the FPA detector.

3. FTIR spectroscopy and spectroscopic imaging

FTIR spectroscopy offers fast data acquisition with minimal sample preparation, and has limited sample volume requirements. There are abundant review articles outlining FTIR spectroscopy and spectroscopic imaging [13,36], and the successful application of FTIR spectroscopy to proteins in general [37] [38]. As these review papers discuss, the capabilities of FTIR spectroscopy can be enhanced with different accessories to cater to specific requirements, including micro and macro imaging, ATR, transflection, and transmission modes. ATR-FTIR spectroscopy measures samples at a depth of penetration of 0.5–5 μm from the surface of the internal reflection element (IRE). Despite the fact that FTIR spectroscopy in transmission measures the whole thickness of a sample, measured thickness for aqueous solutions is generally limited to 6 μm due to the strong absorbance of water [39]. Sample preparation using this transmission mode of measurement can also be laborious. Therefore, the most commonly used technique when studying solutions of proteins is ATR-FTIR spectroscopy (Fig. 3), which usually employs a diamond, Ge, Si or ZnSe IRE, and collects information from the layer of the sample adjacent to the surface of the IRE. Recent research has identified that IgG aggregates distribute unevenly close to the surface of the ZnSe IRE using a high-throughput ATR-FTIR spectroscopic imaging approach [6]. Thus, appropriate spectral processing must be undertaken when conducting measurements of protein samples to take this into account.

Current techniques used in biopharmaceutical processing are typically suited to only monitoring a few sample characteristics simultaneously, require lengthy sample preparation, or are costly. For example, mass spectrometry (MS), is usually focussed at the molecular level to monitor post translational modifications (PTMs), and for the identification of numerous proteins in a protein mixture, but it is costly to run [40]. UV spectroscopy is utilised in a variety of set ups to elucidate protein-protein interactions through monitoring changes in UV absorbance using delta absorbance (ΔAbs), but is used primarily for protein concentration measurements [41–43]. A recently revived technique, analytical ultracentrifugation, obtains high resolution data about molecule size of the sample in liquid state, allowing information to be collected about the aggregation status of the protein [44]. Although these techniques can all be applied to investigate protein structure and function, and to determine the feasibility of biopharmaceuticals in the production pathway, they all have their downsides. FTIR spectroscopy addresses most, if not all of these concerns, through its high throughput capabilities, inexpensive and easy sample preparation, and its range of technical applications.

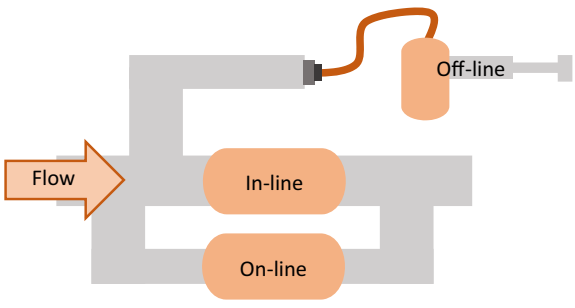

Fig. 2. Schematic showing different sampling points in a biopharmaceutical production pathway as follows: off-line, when a portion of sample is taken away for further analysis, in-line, when all of the flowing sample is analysed, and on-line, when a portion of the sample is analysed and returned to the system in a closed loop.

Although multifaceted and useful, FTIR spectroscopy requires appropriate spectral processing to ensure reliability and to optimise results. This is carried out straight after data collection and is primarily used to remove or reduce unwanted signals in spectra. Incorrect usage of these processing steps can have a serious impact on the reliability of data [45,46]. Post data collection spectral processing such as second derivative analysis is commonly used for quantification of proteins, and can combat the slightly changing backgrounds between different samples, as well as to accentuate spectral features [47–50]. Researchers have proposed slightly altered techniques such as simultaneous fitting of absorption and second derivative spectra [51], and have conducted comparability studies on the most effective second derivative techniques [52]. The correct usage of spectral processing techniques is essential in order to exploit the full power of ATR-FTIR spectroscopy in biopharmaceutical monitoring and processing. The choice and composition of biopharmaceutical buffers is also paramount to ensuring bio stability [53], and has a large effect on spectral quality, therefore sufficient corrections must be made to final spectra in order to account for this, and to obtain the most reliable information.

4. ATR-FTIR spectroscopy to characterise biopharmaceuticals

It is well known that protein secondary structural changes can be detected using FTIR spectroscopy [29,54–56]. Amide spectral bands are most commonly used for this protein characterisation, particularly the Amide I (1650 cm^{-1}) and Amide II (1545 cm^{-1}) bands [47,57,58]. The Amide I band between 1600 and 1700 cm^{-1} mostly consists of C═O stretching vibrations and C—N groups, and its peak position is determined by the backbone conformation and hydrogen bonding pattern, whilst the Amide II band between 1510 and 1580 cm^{-1} consists primarily of N—H bending, but also C—N, and C—C stretching vibrations [59]. Due to the strong overlap of the bending mode water band (1635 cm^{-1}) with Amide I, it has been proposed that other bands such as Amide III (1300–1200 cm^{-1}), could be used to discern secondary structure [49]. This could be beneficial due to the lack of overlap with the bending mode water band, and the supposed improved resolving nature of individual bands [60,61]. However, these claims are disputed by Goormaghtigh et al., who suggest that Amide III bands are irrelevant when you have obtained information from the 1545 cm^{-1} Amide II band [49]. Others imply that the Amide III band is significantly affected by side chains, and will be altered by the amino acid composition [62]. Therefore, the majority of research still uses Amide I and II bands for effective analysis and structure elucidation.

FTIR spectroscopy, combined with recent advances in data processing and chemometrics, is an ideal tool to investigate the stability and suitability of biopharmaceuticals. If proteins are of a high concentration (>30 mg/ml), which is typical in biopharmaceutical production, amide bands can more easily be seen, however lower concentrations also yield good Amide I and II spectra (Fig. 4) [63]. Macro ATR-FTIR spectroscopy is commonly used due to the ease of sample preparation and data collection. It is therefore well suited as an in-line technique to the analysis of biopharmaceuticals both during and post production, for example, the monitoring of protein concentration and secondary structure, as well as providing insights into PTMs (Fig. 5).

ATR-FTIR spectroscopy has many uses in biopharmaceutical processing, some examples being the effective characterisation of biomaterials [64], the monitoring of monoclonal antibody purification [3], and biopharmaceutical bioactivity under stress conditions [65]. Other uses include the investigation of structural stability of biopharmaceuticals such as Bevacizumab® [65], Humatrope® [66], and Humalog® [67]. ATR-FTIR spectroscopy has been used to study protein adsorption on ZnSe IRE [68], and in-column affinity chromatography purification of monoclonal antibodies, where ATR-FTIR spectroscopy was used to demonstrate changes in protein content at various stages of purification, such as after culture fluid binding (Fig. 6) [3].

ATR-FTIR spectroscopy is able to monitor a wide range of PTMs. Biochemical variability can include deamidation, phosphorylation, methylation, and acetylation [69], but glycosylation is the most common PTM in biopharmaceuticals, and has a significant impact on their efficacy and safety [70]. PTMs are still commonly analysed in biopharmaceutical processing using MS [71], and more recently, NMR [72], but these methods are respectively expensive to run and better suited to low concentration samples. One recent paper has successfully characterised glycosylation in biotherapeutic monoclonal antibodies with ATR-FTIR spectroscopy using 0.5 μl of dried IgG on a diamond IRE, and the multivariate tools, principal component analysis (PCA), and multivariate analysis of variance (MANOVA). Here, the authors obtained valuable information from only the infrared spectrum of the glycoprotein, with no labelling or separation required to determine an accurate fingerprint of the glycosylation profile (Fig. 5) [2]. Previous reports have shown contrasting relationships between protein synthesis rates and glycosylation of different proteins [73], however, glycosylation of the final product is certainly influenced by the host cell line, the cell culture and the purification process [70].

Goldsztein et al. have used FTIR spectroscopy for identifying phosphorylation of gastric ATPase. This research managed to observe the binding of a single phosphate on ATPase using a combination of ATR-FTIR spectroscopy with biospecific interaction analysis (BIA)-ATR biosensors, which allows the surface of the IRE to adsorb to a biomembrane [74]. ATR-FTIR spectroscopy has been used to investigate the secondary structure of denatured bovine serum albumin (BSA) alone and in a mixture with a native protein solid, which could be applied to investigate the solid-state formulation development process more thoroughly [75]. ATR-FTIR spectroscopy has also been used extensively to study proteins, in particular biopharmaceuticals, and exciting research has shown the potential applications of it to investigate and monitor changes effectively in PTMs and secondary structures. Due to the extensive applications of FTIR spectroscopy, it is surprising that it is still underutilised in biopharmaceutical production, however the wealth of data and robustness of this technique should ensure it is included in future innovative analyses.

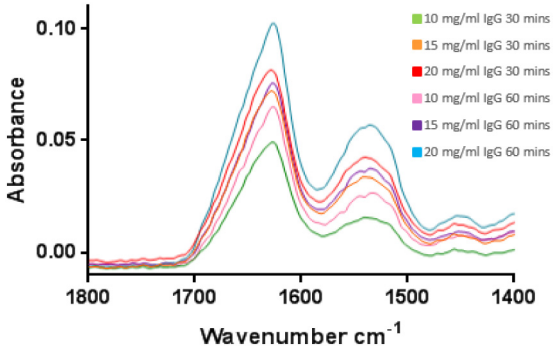

Fig. 4. Spectra showing increase in concentration of Amide I and II bands of IgG protein at two temperatures using ATR-FTIR spectroscopy. Red, orange and green spectra demonstrate 20, 15 and 10 mg/ml IgG at 30 min of heating at 60 °C, and blue, purple and pink spectra demonstrate 20, 15 and 10 mg/ml with heating of 60 min at 60 °C.

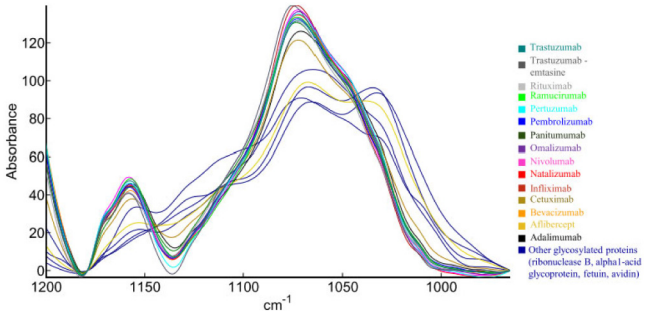

Fig. 5. FTIR spectra of glycoproteins, displaying different spectral signatures, due to the sensitivity of spectra to minor changes in glycan and monosaccharide composition [2]. (Reprinted with permission from Derenne, A.; Derfoufi, K. M.; Cowper, B.; Delporte, C.; Goormaghtigh, E., FTIR spectroscopy as an analytical tool to compare glycosylation in therapeutic monoclonal antibodies. *Analytica Chimica Acta* **2020**).

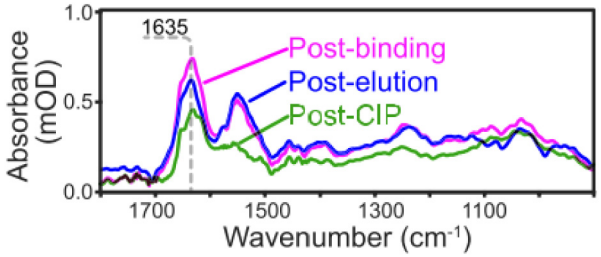

Fig. 6. Extract of a figure demonstrating the changes in Amide bands at various stages of the cleaning in place (CIP) cycle. This spectrum demonstrates the Amide I (1600–1700 cm^{-1}), Amide II 1510–1580 cm^{-1} and Amide III (1300–1200 cm^{-1}) bands, commonly used to monitor protein structure and PTMs [3].
(Reprinted with permission from Boulet-Audet, M.; Kazarian, S. G.; Byrne, B., In-column ATR-FTIR spectroscopy to monitor affinity chromatography purification of monoclonal antibodies. *Sci Rep* **2016,** *6*, 30526).

5. FTIR spectroscopy and biosimilars

A biosimilar can be defined as a biopharmaceutical which has no clinically significant differences from the reference product in terms of quality, safety, and efficacy [76]. The European Medicines Agency (EMA) was the first to approve a biosimilar (Somatropin) in 2006, and the first monoclonal antibody biosimilar (Infliximab) in 2013, whereas the first biosimilar (Zarxio) was not approved by the FDA until 2015 [77]. Biosimilars are driving cost effective manufacture of biopharmaceuticals as a whole, and as the patents on high income yielding original biopharmaceuticals come to an end, a number of which will expire this year [78], there is increasing demand for the development and manufacture of these biosimilars.

Although they are not expected to be identical, maximum similarity between reference product and biosimilar is desired, and any differences must be justified to meet safety and efficacy requirements. The lack of public information on reference biologics makes biosimilars

difficult to produce. In contrast to small molecule drugs, when biosimilars are reproduced there will be changes in the manufacturing process due to their complex structure and lengthy processing parameters which might affect the chemical properties, efficacy, and stability of the product. Two of the most prominent causes for concern in the production of biosimilars are their variable potency and immunogenicity [79]. There is also conflicting research into the effects of switching patient prescription drugs from original biopharmaceuticals to biosimilars, a potentially problematic practice [80,81]. However, one review paper believes these concerns are unfounded, and that the possible costs outweigh the benefits [82], and another reviewed 90 studies and found little differences reported between reference drugs and biosimilars [83]. This proposed interchangeability was thought to be problematic due to the difficulties in ensuring the product does not invoke different immune responses than the reference product. Recently, the FDA has approved the introduction of 'interchangeable products', which require additional information outlined by the Biologics Price Competition and Innovation Act. These interchangeable products are tested to ensure they produce the same clinical result as the reference product, and these drugs can therefore be dispensed by a pharmacy, even with a prescription for the reference product, and without the need for a separate prescription from the healthcare prescriber [84].

When properly utilised, FTIR spectroscopy can replace some other, more traditional methods [85]. Although circular dichroism (CD) can be used to estimate secondary structure, it is unsuitable for highly concentrated samples, or samples containing other highly absorbing molecules such as excipients [63]. In contrast, FTIR spectroscopy, particularly ATR-FTIR spectroscopy, caters to these requirements. For example, ATR-FTIR spectroscopy was used to assure that the secondary structure of the biosimilar Ristova® was similar to that of the original biologic [86]. In addition, using FTIR spectroscopy, quality control to a high standard has been carried out on Humalog®, an approved biosimilar, to investigate stability at varying temperatures [67].

It is common to use FTIR spectroscopy for secondary structure elucidation, however tertiary structures which are frequently explored using either fluorescence spectroscopy [87] or NMR [88], can also be characterised, and more quickly, by using FTIR spectroscopy. It is well documented that FTIR spectroscopy has been frequently used to clarify higher order, tertiary, structures of biosimilars [19,57,89]. However one conflicting study compared a reference biopharmaceutical with its respective biosimilar using FTIR spectroscopy, and did not observe differences between the original drug and the biosimilar. Though they simply compared the Amide I and Amide II band shape with no further spectral analysis, which could have conclusively identified any differences [90]. Methionine oxidation is a significant degradation pathway in biopharmaceuticals, and has an important impact on biosimilar stability and efficacy. This is exacerbated by the presence of phosphate buffer [91], and is typically investigated using liquid chromatography with mass spectrometry (LC-MS), although this is time consuming. FTIR spectroscopy has been used to successfully quantify oxidation [92], and measure subtle spectral changes. This study demonstrated the power of FTIR spectroscopy to provide detailed analysis of biosimilars,

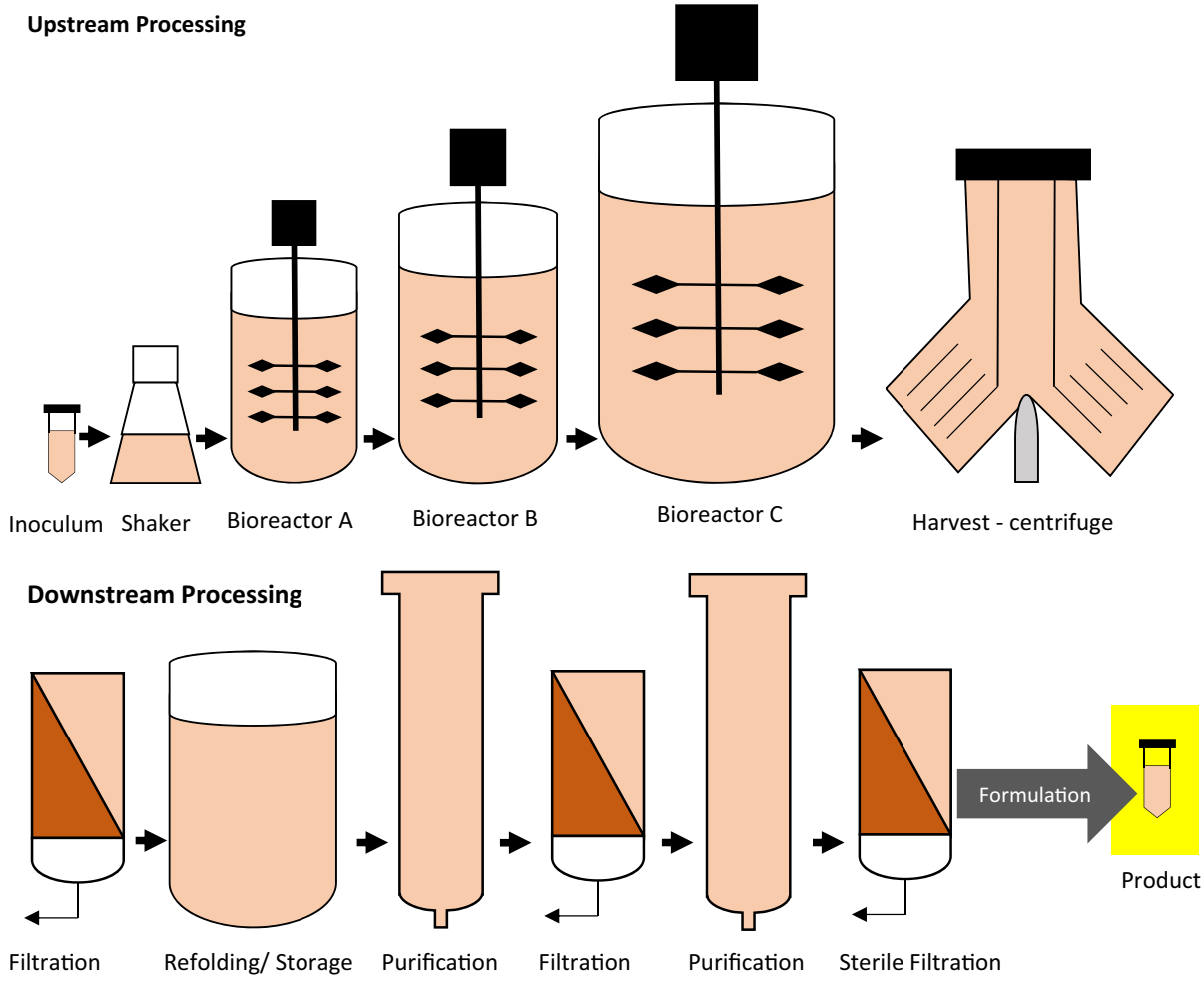

Fig. 7. Schematic of upstream and downstream processes commonly used in biopharmaceutical production, specifically for the production of mAbs.

and other interchangeable products compared to the original licensed biotherapeutic.

6. FTIR spectroscopy in biopharmaceutical production

Process analytical technology (PAT), has been used since 2004 to enhance the understanding and control of processes throughout product development and manufacturing [93], and has been previously outlined in a comprehensive review [94]. In biopharmaceutical production, processes are largely based on historical data, it is therefore important that analytical methods such as ATR-FTIR spectroscopy are employed in-line to ensure rapid responses to processing requirements. Research has shown that a reduction in the costs of products available to market, possibly through the use of efficient, expedited processes, would result in increased investment in R&D [95]. Previously most sampling techniques were off-line, meaning samples were extracted and measured with a time-delay, resulting in inefficiencies. FTIR spectroscopy offers non-invasive, in-line, and instantaneous measuring of numerous critical processing parameters (CPPs). For example, Grosshans et al. used in-line FTIR spectroscopy, along with partial least squares (PLS) analysis, as an effective process analytical tool (PAT) for preparative protein chromatography, they found it to be useful to distinguish and selectively identify proteins based on their secondary structure [96].

Traditional manufacturing of biopharmaceuticals is typically divided into two sections: upstream processing (USP), which includes cell culture and harvesting, and downstream processing (DSP), consisting of multiple steps of chromatography, filtration and diafiltration, and finally product formulation (Fig. 7). Upstream techniques in processing of biopharmaceuticals experience high levels of monitoring and feedback when compared to downstream techniques [97–99]. For example, in-line applications of ATR-FTIR spectroscopy include upstream process monitoring of glucose and ethanol in different cell lines, observing changes in α-glucose spectra with time, and the possibility of simplified calibration techniques using a spectral library [100,101]. FTIR spectroscopy is also commonly combined with a flow through ATR capability to monitor ethanol fermentation [102].

FTIR spectroscopy can also be utilised in a wide range of downstream applications in biopharmaceutical production such as purification, where the sample is placed under high stress levels due to fluctuations in shear and pH. FTIR spectroscopy has been successfully used for an in-line estimation of the degree of PEGlycation of eluting sample from a chromatography column in near real-time [103]. FTIR spectroscopy was applied in-line to the bioreactor cell culture process, and correlated with offline measurements, for real time monitoring of bioreactor mAb IgG3 cell culture process dynamics. The concentration profiles of four key cell culture metabolites (glucose, glutamine, lactate and ammonia) were predicted using multivariate calibration models [18].

MAb binding propensity during the protein A capture step has been monitored using FTIR spectroscopy [3]. When mAbs are in the purification step of manufacture, protein A resin experiences a decrease in binding capacity and product recovery. FTIR spectroscopy can therefore be used to monitor changes in resin composition via a continuous feedback loop to eventually make purification more effective. Some recent research argues that fluorescence is better for this downstream application, due to its ability to monitor changes in situ [104]. However others have already shown this is not only possible, but easier, with ATR-FTIR spectroscopy [105].

Lin et al. suggests that FTIR spectroscopy can be used effectively for monoclonal antibody comparability studies, but for higher order structure identification only, such as secondary or tertiary structure, or for product characterisation. They came to this conclusion through the comparison of size exclusion and ion exchange chromatographies (SEC and IEC) with CD and FTIR using mAbs samples of varying pHs and concentrations [1], and by carrying out second derivative analysis of proteins (Fig. 8). Another paper used FTIR spectroscopy for secondary structure classification of mAbs subjected to environmental stresses

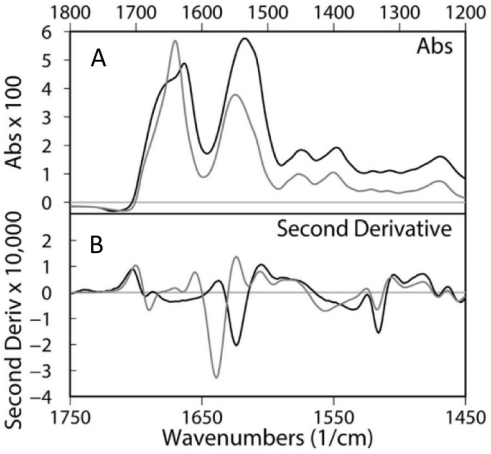

Fig. 8. (A) Absorbance of Amide I and II band of stressed mAb after heating to 90 degrees compared with an unheated control at room temperature (B) Respective second derivative spectra for each spectrum [1].
(Reprinted with permission from Lin, J. C.; Glover, Z. K.; Sreedhara, A., Assessing the Utility of Circular Dichroism and FTIR Spectroscopy in Monoclonal-Antibody Comparability Studies. *J Pharm Sci* **2015,** *104* (12), 4459–4466).

[19]. Here, FTIR spectroscopy was interestingly used to prove the apparently negligible effect small alterations in tertiary structure had on overall long-term stability and bioactivity [106]. Despite this research indicating the use of FTIR spectroscopy solely for higher order structure identification, it has been shown conclusively that FTIR spectroscopy could be applied to investigating the primary structure of biopharmaceuticals, such as PTM changes [74].

MAbs are the most common class of biopharmaceuticals, and are mostly favoured due to their reduced immunogenicity owing to the humanisation of murine mAbs, which greatly improves their in vivo tolerability [107]. Immunogenicity can also be affected by genetics, impaired immunity of a patient, and administration of and impurities within a drug [108]. Typically mAbs are produced using the upstream processes of cell culture; centrifugation, and depth and membrane filtration, and the downstream processes of protein A chromatography; low pH viral activation, cation exchange chromatography, anion exchange chromatography, viral filtration, and ultrafiltration/diafiltration [109]. MAb production supported by the in-line capabilities of ATR-FTIR spectroscopy complement the trend of moving from batch to continuous processes for biopharmaceutical processing in order to reduce costs, and improve quality of drug and flexibility of processes [110].

Aside from detecting protein presence, FTIR spectroscopy can also detect impurities in solution, such as detergents, buffer residues, and protein contaminants in manufacturing (Fig. 9), not detectable by reversed phase HPLC electrospray ionisation MS (RP-HPLC-ESI-MS) or SDS-PAGE. In fact it is predicted Triton X-100, the detergent seen in Fig. 9(B), could not be seen by any other method [8,111], a significant advantage when compared to CD. This detection of impurities is possible through the comparison of sample FTIR spectra with standard FTIR spectra.

Another recent study has developed a reusable immuno-infrared sensor, which uses a functionalised germanium ATR crystal to analyse human blood and CSF fluid, and subsequently detect biomarkers for Alzheimer's disease (AD). This technique reduces time and cost of identification, and could be applied to identify impurities in biopharmaceutical processing [112]. Some reviews have proposed other techniques such as size exclusion chromatography (SEC) and mass spectrometry (MS) for this application [113], however, these alternative techniques are more time consuming and costly. One paper limited the application of FTIR spectroscopy in biopharmaceutical production to only identifying higher order structural changes such as aggregate levels in purification steps [114]. In this review paper we have presented the full

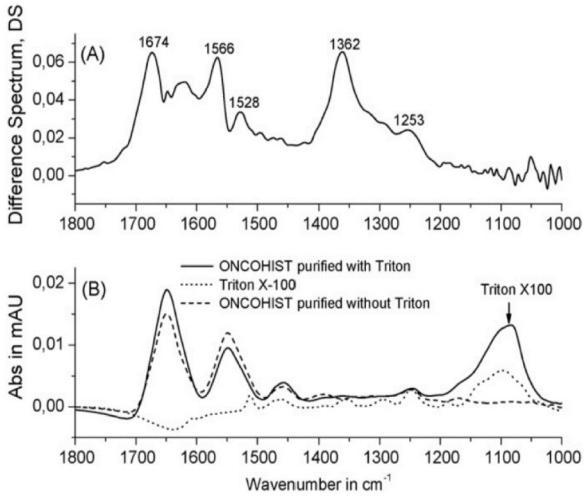

Fig. 9. (A) FTIR spectrum of ONCOHIST® after small changes in the production process led to significant deviations from baseline difference spectrum (B) FTIR spectrum showing unexpected and unwanted adsorption of Triton X-100, a detergent [8].
(Reprinted with permission from Gross, P. C.; Zeppezauer, M., Infrared spectroscopy for biopharmaceutical protein analysis. *J Pharm Biomed Anal* **2010,** *53* (1), 29–36).

potential of FTIR spectroscopy to identify changes of biopharmaceuticals under a range of conditions, and at primary, secondary, and tertiary structural levels.

7. ATR-FTIR spectroscopic imaging applied to biopharmaceuticals

FTIR spectroscopic imaging is especially useful for studying complex and heterogeneous samples, as it produces a chemical image of the sample or system being investigated, and offers the opportunity to collect dynamic images. Macro ATR-FTIR spectroscopic imaging consists of a large sample compartment containing an ATR accessory attached to a spectrometer, whilst micro ATR-FTIR spectroscopic imaging involves the use of an infrared microscope with an ATR objective. The first paper to study aggregation of IgG by macro ATR-FTIR imaging investigated secondary structural changes of 1 mg/ml IgG4 solution under thermal stress and found IgG4 is more stable at high pH or at low or high salt concentrations [115]. The use of macro ATR-FTIR spectroscopic imaging and depth profiling is particularly interesting as it could be used to visualise biopharmaceutical therapeutic release systems. Macro ATR-FTIR spectroscopic imaging of biomaterials has increased in popularity in recent years. Research using this technique has demonstrated the ability of a germanium ATR IRE crystal to monitor distribution within materials, and the presence and states of live cells [116], and it has also been used to investigate cancerous tissue samples and bio fluids [117]. As previously seen, macro ATR-FTIR spectroscopic imaging can be applied to depth profiling studies (Fig. 10) [6].

Micro ATR-FTIR spectroscopic imaging has previously been used to investigate protein crystallisation [118], and aggregates in live cells [119], as well as to probe protein hydration in living cells [120]. Terahertz time-domain spectroscopy (THz-TDS) in the far IR region was used to investigate BSA protein solution at pHs ranging from 2.5–10, this paper found different conformations of BSA have varying impact on the dynamics of surrounding water molecules [121]. Micro ATR-FTIR imaging has recently been used for depth profiling of prostate cancer tissue in the z-direction, at varying angles of incidence, to study embedded components using a created 3D model [122]. Micro FTIR spectroscopic imaging has also been applied to determine the structure of protein-based silk fibroin/polyethylene oxide (SF/PEO) polymer blends [7], where conformation transitions of random coil and/or α-helical conformation were shown in the SF-rich domains, to β-sheet conformation in PEO-rich matrix (Fig. 11). This research used FTIR and X-ray microscopy spectroscopic imaging (STXM) as complementary techniques, and found they are useful to study phase behaviour and molecular conformation of protein based polymers. This could be applied to the biopharmaceutical industry through the use of therapeutic delivery systems [123].

Macro ATR-FTIR spectroscopic imaging has been used to monitor affinity chromatography purification of monoclonal antibodies [3], a particularly useful investigatory method which looks at the binding capacity of resin. This is important due to the high financial cost of replacing protein A resin [124]. Macro ATR-FTIR spectroscopic imaging has been successfully applied to cleaning procedures of a fouling layer on membranes such as polypropylene (PP), and polytetrafluoroethylene (PTFE) [125], the small penetration depth of ATR is well suited to this application due to the thin nature of membranes. It is predicted that the membrane filtration market will be worth $8.6 billion USD by 2024 [126], therefore the identification of FTIR as a valid monitoring technique of membranes could have significant positive impacts on the acceleration of this market due to the ease of data collection.

Microfluidics and FTIR spectroscopic imaging have previously been combined to investigate pharmaceuticals (or small molecule drugs) such as ibuprofen [127]. In this study ibuprofen was studied at neutral and acidic conditions, and the structural change from compacted powder to crystalline form was characterised. Macro ATR-FTIR imaging allowed the phase change of different areas in the tablets to be observed simultaneously. Another application for macro ATR-FTIR spectroscopic imaging is the monitoring and quantification of lysozyme within water droplets studied under dynamic flow [128]. Building on this research, protein solutions under flow and ATR-FTIR spectroscopic imaging could be applied to biopharmaceutical processing. One review has found that microfluidics, in tandem with chromatography and MS is effective in characterising the stability of therapeutic proteins [129]. These experiments could be adapted to work with macro ATR-FTIR spectroscopic imaging, and provide an alternative low cost, fast acquisition time, and high throughput tool.

8. Future perspectives

This review has highlighted the use of ATR-FTIR spectroscopy for the investigation of efficient production of biopharmaceuticals. Although ATR-FTIR spectroscopy is already in situ at a number of points in the production pathway, there is real potential for it to be utilised more effectively. ATR-FTIR spectroscopy will shorten measurement times [6] (compared to other analytical techniques such as HPLC), and increase the effectiveness of measurements through improved signal to noise ratio, and the increased number of parameters and characteristics of the biopharmaceuticals which can be observed from a single spectral measurement. Macro ATR-FTIR spectroscopic imaging has yet to fulfil its potential in biopharmaceutical analysis. In the future it could be used to study biopharmaceuticals under flowing conditions, in a scaled down approach. An example of this would be the study of live cells in the first stages of USP, and the study of PTMs, along with higher order structures, in a single measurement. Macro ATR-FTIR spectroscopic imaging could also be applied to investigate the level and distribution of aggregation in DSP, which would be ideal as an in-line technique, reducing the margin of error that comes with off-line measurements.

The true power of biopharmaceuticals, and monoclonal antibodies in particular, is being realised in the worldwide battle against SARS-CoV-2. Some companies are using single cell sequencing to structure the library of antibodies from SARS-CoV-2 patients, and then using polyclonal therapies for treatment [130]. Another company is harnessing the power of monoclonal antibodies to develop a therapy for treatment, and is considering various mAbs which could be repurposed for Covid-19 treatment, such as those which treat rheumatoid arthritis or some cancers [131]. Indeed some researchers think they have already found an effective treatment using monoclonal antibodies [132]. The fast development of monoclonal antibody therapies has previously been effective,

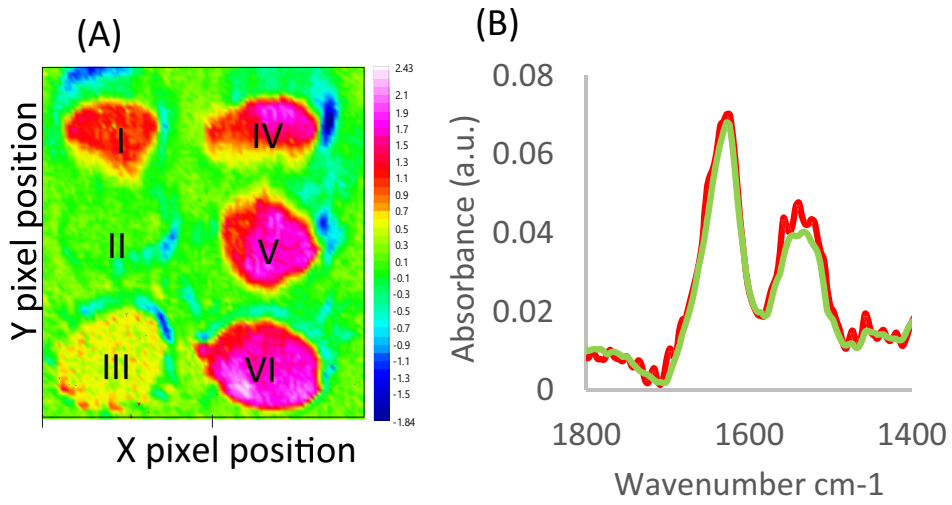

Fig. 10. (A) Integrated absorbance of Amide I (1600–1700 cm^{-1}) plotted as images, where I = 5 mg/ml, II = empty well, III = buffer, IV = 10 mg/ml, V = 15 mg/ml, and VI = 20 mg/ml. All protein has been heated to 60 °C for 0.5 h. The unit of the scale bar is integrated absorbance in cm^{-1} (B) Spectra before (red) and after (green) water vapour subtraction at 45° set angle of incidence. Spatial resolution of these images is 44 μm [6].
(Reprinted with permission from Tiernan, H.; Byrne, B.; Kazarian, S. G., Insight into Heterogeneous Distribution of Protein Aggregates at the Surface Layer Using Attenuated Total Reflection-Fourier Transform Infrared Spectroscopic Imaging. *Anal Chem* **2020,** *92* (7), 4760–4764).

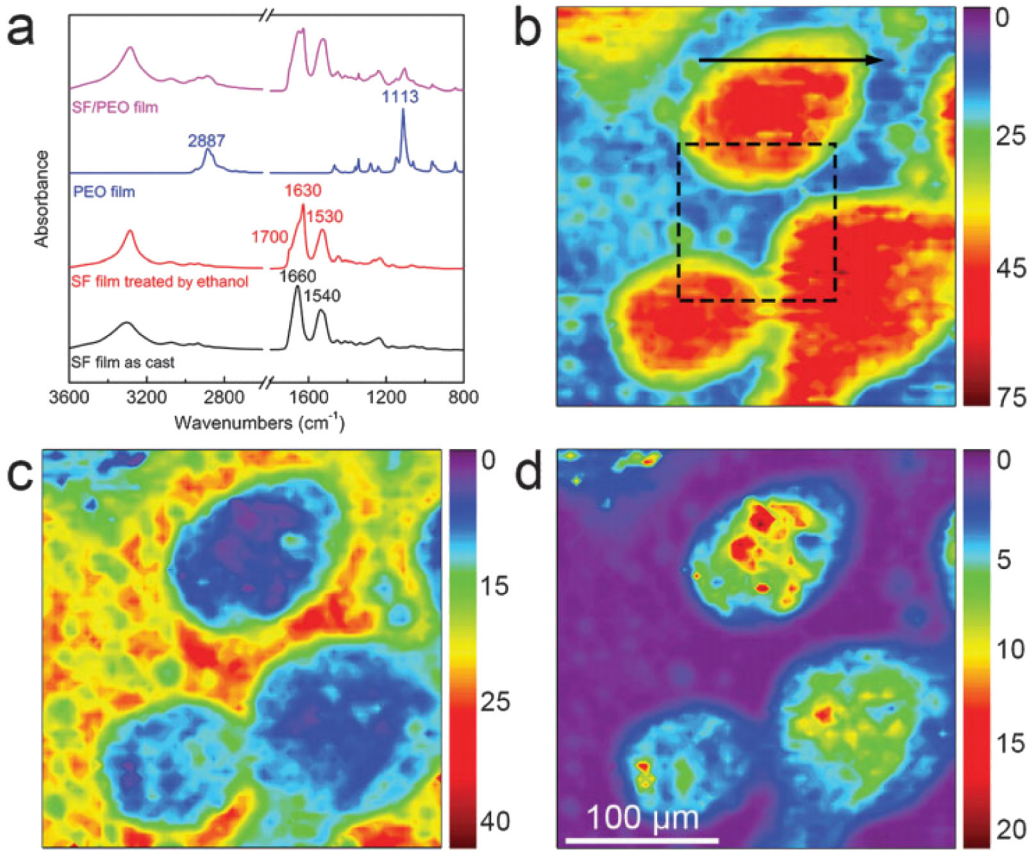

Fig. 11. FTIR spectra and FTIR images of the SF/PEO blend. (a) FTIR spectra of SF film as a cast, SF film treated with 70% ethanol solution, pristine PEO film, and SF/PEO blend film; (b) SF-specific FTIR image of the SF/PEO blend; (c) PEO-specific image of the SF/PEO blend; (d) ASF/APEO image of the SF/PEO blend. The scale bar in (b), (c), and (d) is the same [7].
(Reprinted with permission from Ling, S.; Qi, Z.; Watts, B.; Shao, Z.; Chen, X., Structural determination of protein-based polymer blends with a promising tool: combination of FTIR and STXM spectroscopic imaging. *Phys Chem Chem Phys* **2014,** *16* (17), 7741–8).

for example in the Ebola epidemic in West Africa. Despite the disadvantages of monoclonal antibodies, such as transport and delivery difficulties, they offer a promising solution in the weeks, months, and years to come [133]. If these proposed monoclonal antibodies therapies are taken to production, ATR-FTIR spectroscopy will almost certainly have a role to play in stability testing, but the capabilities of ATR-FTIR

spectroscopy and spectroscopic imaging reach much further. It is hoped that future research will utilise these techniques to the limit of their capabilities, to improve efficiency and sample yield.

Declaration of competing interest

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

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