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Infrared spectroscopy for biopharmaceutical protein analysis

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ABSTRACT

A mid-infrared transmission spectroscopic method with the possibility of high sample throughput was developed and validated on the basis of GMP requirements for protein therapeutics. In comparison with the method predominantly used, i.e. the surface-sensitive attenuated total reflection (ATR), we have shown that transmission measurements in solution possess several advantages, such as quantitative analysis on the basis of Lambert–Beer's law, determination of identity and purity based on specifically developed identity criteria, characterization of protein structure and structural changes including aggregation in solution and study of ligand binding to the protein. The usefulness of this method is exemplified by the characterization of the drug substance of ONCOHIST® (recombinant human histone H1.3 and bis-Met-histone H1.3) and its interaction with phosphate ions. Our conclusion is that transmission mid-infrared spectroscopy is a powerful tool for protein analysis in biotechnology and supplements the current analytical techniques for biopharmaceutical quality control of therapeutic proteins.

1. Introduction

To ensure the quality and safety of a biopharmaceutical it is necessary to analyse the finished product by different methods for identity, purity and quantity. Especially during the development of new biopharmaceuticals, the objective is to collect a maximum of information, covering all accessible structural and process parameters. Currently most biopharmaceutical protein characterizations rely on different HPLC techniques, electrophoresis, and spectroscopic techniques [1]. Secondary/tertiary structure analysis is routinely done by X-ray, CD, fluorescence, NMR and FTIR spectroscopy. In particular the latter is applicable to samples in solution and in solid state, can be applied in numerous modes and has a very high sensitivity. Besides the possibility to study a protein's secondary structure with FTIR, its potential for detecting impurities is also high, for example the detection of purification-related buffer residues, detergents or protein impurities. In case of measuring protein solutions in transmission, the possibility for *direct* protein quantification exists by using Lambert–Beer's law. Protein quantification can cause problems with *indirect* colorimetric or spectrophotometric assays or when working with small proteins or peptides with fewer or no aromatic side chains and consequently a small UV signal at ~280 nm.

Application of mid-infrared spectroscopy in the field of biotechnology has mainly been limited to the analysis of protein secondary structure. Different approaches exist in the field, which all rely on the fine structure analysis of the protein's peptide bond vibrations, namely the Amide-I, -II and -III absorption bands [2–6]. The Amide-I band of a protein, which is most widely used for secondary structure analysis, is quite dependent on the protein's overall conformation. Numerous amino acid side chains also absorb in the same range and disturb the analysis [7]. The absorption bands of the different secondary structure elements are very broad and overlap to a great extent, which further complicates the evaluation. The assignment of a defined IR region to a single secondary structure element is a simplification, which is not applicable in every case. Even α-helices of different length can have various band positions [8]. Therefore the theoretical basis for secondary structure analysis with IR is not yet fully understood and suffers from different shortcomings. But the monitoring of the change of secondary structure, without exact interpretation, between samples of different production stages, during stability studies or for the reason of batch comparison is a very useful application of IR spectroscopy, disregarding the mentioned theoretical problems.

The study presented below explores the potential of IR spectroscopy for biopharmaceutical quality control by measuring *transmission* spectra of proteins in aqueous buffers. Due to the high absorptions of water at the bands of interest for a protein (e.g. 1650 cm^{-1}, Amide-I) many authors have preferred the surface-sensitive method ATR (attenuated total reflection spectroscopy) in order to avoid elaborate and time-consuming H/D exchange or the complications by using a very thin liquid layer cell (5–10 μm). However the transmission mode has distinct advantages compared to surface-sensitive analytical methods like ATR: in ATR spectroscopy the IR signal is mostly determined by the first few protein layers on

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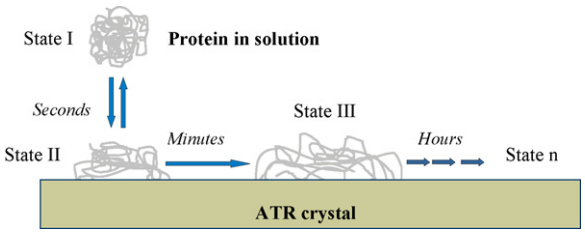

Fig. 1. Model of the irreversible protein adsorption onto an ATR crystal for dissolved proteins [9]. After the protein adsorbs onto the solid state it normally changes its secondary structure during long lasting transformation processes.

the interface between crystal surface and solution. In surface biochemistry the rule of thumb is, that 'every protein adsorbs on every surface' [9]. Considering the size and amphiphilic nature of proteins this is not surprising. In most cases the protein adsorption is irreversible and associated with extensive and long lasting (in the range of hours) protein secondary structure changes (Fig. 1) [9]. These processes can have a large impact on the ATR spectrum of a protein, resulting in erroneous conclusions. For example the secondary structure analysis can be disturbed, because the measured IR spectrum corresponds mainly to the adsorbed species with modified conformation and 'permanently changing' secondary structure.

During ATR analysis of different proteins, we could confirm the model of irreversible protein adsorption on the surface of ATR crystals exposed to protein solutions (unpublished own results).

Besides avoiding problems with protein secondary structure changes in transmission analysis compared to ATR, the second advantage of transmission measurements is the possibility of *direct* protein quantification on the basis of Lambert–Beer's Law; whereas no strict correlating law exists in case of ATR. The third advantage of transmission spectroscopy is a practical one: the lack of disturbing protein-surface interactions allows a high sample throughput without meticulous cleaning of the cell after each measurement.

A fundamental question by application of IR spectroscopy to biotechnology is, how the huge information, contained in a protein IR spectrum, can be properly extracted. Every protein spectrum is dominated by broad and intensive peptide bond vibrations of the backbone and numerous side chain vibrations. The polymeric nature of proteins built up from amino acids leads to a large overlap und summation of closely related IR absorption bands. Therefore, at first glance one could expect that the IR spectra of different proteins are similar to the extent that the identification of a particular protein by this method would seem very difficult if not impossible. One goal of this study was to explore the potential of transmission IR spectroscopy to contribute to the identification of a protein by analysing its individual infrared spectrum.

The subject of the present study was the drug substance of ONCOHIST®, a protein drug developed for the treatment of malignant diseases. This drug consists of an aqueous solution of recombinant human histone H1.3 and recombinant human N-bis-Met-H1.3. A first clinical trial Phase I/II with relapsed or refractory acute-myeloic-leukaemia (AML) patients has shown, that the protein is very well tolerated without side-effects and first signs of efficacy were observed [10].

2. Experimental

2.1. Spectra acquisition

All spectra were measured with a Bruker Tensor 27 FTIR spectrometer (Karlsruhe, Germany) with the Software Opus 5.5 and a HgCdTe detector. A resolution of 4 cm^{-1} and 25 scans per measurement were taken in the range between 3000 cm^{-1} and 1000 cm^{-1}. The flow-through liquid transmission IR cell was equipped with CaF_2 windows of 4 mm thickness, had an optical path length of 7.4 μm and an aperture of 5 mm (Microbiolytics GmbH, Freiburg Germany). The whole cell was temperature controlled, all measurements were done at 25 °C. For every measured sample, a spectrum of the pure buffer, in which the sample was dissolved, was measured as reference or background single channel spectrum for calculating the absorption spectrum of the sample.

2.2. Samples

Throughout the study the drug substance ONCOHIST® was used as solution in 0.9% NaCl for infusion (Ph. Eur.) at different concentrations, exceptions are mentioned separately. All samples were measured in duplicate while the instrument was flushed with gaseous nitrogen at a flow-rate of approximately 160 l/h. The sample volume necessary for one measurement was 20 μl. For the study of protein specificity 13 arbitrarily selected, proteins were measured in 0.9% NaCl at a concentration of 10.0 mg/ml: BSA (Sigma, A-9085), Anti-human IgG (Beckman Coulter, PNIM 0837), Chymotrypsin (Calbiochem, 230834), Cytochrome *c* (Merck, 1.24804), Bovine Insulin Chain A (Sigma, I-2254), Bovine Insulin Chain B (Sigma, I-6380), β-Lactoglobulin (Sigma, L-4520), Lysozyme (Sigma, L-6876), α-2-Macroglobulin (Dako Cytomation, A0033), Papain (Merck, 1.07144.0025), Transferrin (Behringwerke, 1227/34), Trypsin-Inhibitor (Serva, 37329), Urease (Boehringer Mannheim 1070225).

2.3. Spectra evaluation

If necessary a buffer correction before analysis was done by adjusting a defined Amide-I to Amide-II ratio. This ratio was quite sensitive to small differences between sample buffer and the corresponding background buffer. The correction was done by defining the Amide-I/Amide-II ratio of two ONCOHIST® standard spectra as reference values and by adjusting all other spectra between these two given limits by subtraction or addition of the background buffer spectrum. This kind of buffer correction is only applicable for very similar protein samples as it is the case in this study.

2.3.1. Comparison of second derivative spectra

Second derivative spectra are useful for resolution enhancement and determining band positions or shoulders. The software OPUS 5.5 offers the possibility for automated comparison of second derivative spectra by a mathematical algorithm. The results are quoted as a coefficient of correlation in percentage between 0% and 100%, where 100% means identical spectra. The comparison was always done at a fixed ONCOHIST® concentration of 10.0 mg/ml between the test and the standard spectrum in the range between 3000 cm^{-1} and 1000 cm^{-1}. As standard spectrum we defined the spectrum of an ONCOHIST® batch of the highest attained quality.

2.3.2. Secondary structure analysis

The prediction of secondary structure elements α-helix and β-sheet were done with the methods supplied by the Confocheck™ system (Bruker Karlsruhe, Germany). These methods calculate the secondary structure with a multivariate partial-least-squares algorithm (PLS) on the basis of a calibration data set of 45 different proteins.

2.3.3. Difference spectra

A very sensitive method to compare two spectra is the generation of a difference spectrum between them with preceding normalization of spectra. To this end is reasonable to define an ONCOHIST® standard spectrum and subtract it from the spectrum of interest. This new spectrum is then termed the difference spectrum of the sample. Afterwards the difference spectra of various