In Situ Protein Secondary Structure Determination in Ice: Raman Spectroscopy-Based Process Analytical Tool for Frozen Storage of Biopharmaceuticals

ULRICH ROESSL,[1,2] STEFAN LEITGEB,[1] SIGRID PIETERS,[3] THOMAS DE BEER,[4] BERND NIDETZKY[1,2]

[1]Research Center Pharmaceutical Engineering GmbH, Graz, Austria
[2]Institute for Biotechnology and Biochemical Engineering, Graz University of Technology, Graz, Austria
[3]Department of Analytical Chemistry and Pharmaceutical Technology, Free University of Brussels, Brussels, Belgium
[4]Laboratory of Pharmaceutical Process Analytical Technology, Ghent University, Ghent, Belgium

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ABSTRACT: A Raman spectroscopy-based method for *in situ* monitoring of secondary structural composition of proteins during frozen and thawed storage was developed. A set of reference proteins with different α-helix and β-sheet compositions was used for calibration and validation in a chemometric approach. Reference secondary structures were quantified with circular dichroism spectroscopy in the liquid state. Partial least squares regression models were established that enable estimation of secondary structure content from Raman spectra. Quantitative secondary structure determination in ice was accomplished for the first time and correlation with existing (qualitative) protein structural data from the frozen state was achieved. The method can be used in the presence of common stabilizing agents and is applicable in an industrial freezer setup. Raman spectroscopy represents a powerful, noninvasive, and flexibly applicable tool for protein stability monitoring during frozen storage. © 2014 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 103:2287–2295, 2014

Keywords: freezing/thawing; multivariate analysis; partial least squares; proteins; protein structure; Raman spectroscopy; stability; processing; unit operations

INTRODUCTION

Freezing bulk volumes of pharmaceutical protein solutions is a widely used, yet potentially critical unit operation. Increased shelf-life and flexibility in storage and logistics is opposed by the risk of inactivation and aggregation of considerable amounts of high-value protein drugs upon freezing and thawing (F/T).[1] Protein degradation can be triggered by cold denaturation, cryoconcentration of (co)solutes, and concomitant reduction of solubility as well as by exposure to surfaces of ice crystals or precipitated or crystallized excipients.[2] Given the large volumes of up to several hundred liters of concentrated protein product stored at industrial scale, any information about protein integrity inside a freeze container is highly desirable and would provide important information for F/T process design. Various analytical methods allow for an assessment of the impact of F/T processing on protein stability, however, only in the thawed state at the beginning and the very end of the process.[3–6]

In ice, monitoring of any stability parameter whatsoever was not possible because most established methods for probing protein conformational or colloidal stability require liquid and/or transparent solutions. For infrared (IR)-based methods, the contributions of frozen water is the prime factor disturbing structural analysis.[7] However, promising results were obtained by using IR microscopy.[8] Solid-state nuclear magnetic resonance might allow for protein structure assessment in ice, but lacks applicability as process analytical technology (PAT).[9,10] Also, small-angle neutron scattering (SANS) was employed to obtain protein structural information from the frozen state.[11] However, instrumental effort required for SANS studies is very high. Hydrogen/deuterium exchange mass spectrometry (HDX-MS) was used to study conformational changes of proteins in the frozen state.[12,13] The requirement of D_2O solvent and the slow isotopic exchange at low temperatures render HDX-MS impractical for broad application in monitoring of protein freezing processes. Fluorescence probes of protein conformation such as 8-anilino-1-naphthalenesulfonic acid (ANS) have provided qualitative or semiquantitative information about protein structural characteristics in the frozen state.[14] However, the presence of an extrinsic fluorophore in pharmaceutical protein formulations can be considered problematic. With ANS in particular, there is concern that interaction with the fluorophore is sufficient to induce changes in protein conformation.[15]

Raman spectroscopy is a noninvasive method with broad applicability to biochemical and pharmaceutical problems.[16,17] The extent of the Raman shift is specific for chemical bonds and their vibrational modes. Several regions in the Raman spectrum can be assigned to interactions of the laser light with protein backbone amides. They serve as indicators for the presence of secondary structural elements in proteins. Most

Abbreviations used: ADH, alcohol dehydrogenase; ANS, 8-anilino-1-naphthalenesulfonic acid; BSA, bovine serum albumin; CD, circular dichroism; F/T, freezing and thawing; HDX-MS, hydrogen/deuterium exchange mass spectrometry; IR, infrared; LDH, lactic dehydrogenase; PAT, process analytical technology; PLS, partial least squares; rhG–CSF, recombinant human granulocyte–colony-stimulating factor; rhIgG, recombinant human immunoglobulin G; RMSE, root-mean-square error.

Correspondence to: Bernd Nidetzky (Telephone: +43-316-873-8400; Fax: +43-316-873-8434; E-mail: bernd.nidetzky@tugraz.at)

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important ones are the amide I band (H-bonded C=O stretching) between 1600 and 1700 cm^{-1}, amide III band (N–H and C–H bending) at around 1230–1340 cm^{-1}, and C=C stretching band at 890–1060 cm^{-1}.[18,19] Raman spectroscopy is not disturbed by the presence of water and ice, and it can be easily applied for process surveillance. Even though Raman has been utilized for qualitative assignment of secondary structural elements in the frozen state,[20] the determination of α-helix or β-sheet content by *in situ* Raman spectroscopy was not demonstrated before.

Herein, we show that Raman spectroscopy can be extended to measure secondary protein structure composition in frozen solutions, and that it can be applied for stability monitoring of pharmaceutical proteins during F/T and frozen storage. For that purpose, relative α-helix and β-sheet content of 14 proteins were obtained with circular dichroism (CD) spectroscopy in the liquid state. Partial least squares (PLS) regression was then used to establish models for the prediction of α-helix and β-sheet content from Raman spectra.

MATERIALS AND METHODS

Materials

Chicken egg white lysozyme, bovine pancreatic trypsin type I, ribonuclease A and insulin, bovine serum albumin (BSA), bovine milk-derived β-lactoglobulin, porcine gastric mucosa pepsin, rabbit muscle L-lactic dehydrogenase (LDH), *Saccharomyces cerevisiae* alcohol dehydrogenase (ADH), and *Bacillus Subtilis* α-amylase were obtained from Sigma–Aldrich (St. Louis, Missouri). Recombinant human granulocyte–colony-stimulating factor (rhG–CSF) was kindly provided by Sandoz (Kundl, Austria). IgGs were donations from undisclosed sources. Chemicals and reagents used were from Carl Roth GmbH + Company KG (Karlsruhe, Germany).

Vivaspin ultrafiltration columns obtained from GE Healthcare (Little Chalfont, Buckinghamshire, UK; MWCO 3–10 kDa) were used for buffer exchange and sample concentration.

Sample Preparation

Protein powders were resuspended in suitable sample buffer (Table 1). Buffer exchange was performed twice, followed by sample concentration. Final protein concentration was determined by UV absorbance (DU 800 spectrophotometer, Beckman Coulter, Brea, California). Extinction coefficients for 280 nm were calculated with ExPASy Protparam,[21] if not provided by the supplier. Tris–HCl and acetate buffer (10 mM) were chosen to avoid large pH shifts during freezing.[22] In Table 1, protein names and origin, buffer type, pH, and concentrations of analyzed samples are displayed. After completion of CD and Raman measurements in the liquid state, samples were frozen in liquid nitrogen and stored at −20°C overnight, until recording of Raman spectra in the frozen state took place.

F/T cycle experiments were performed for 700 mL of IgG1-A (25 mg/mL). A stainless steel freeze container by Zeta Biopharma GmbH (Lieboch, Austria) was cooled by an external freeze controller (Tango Nuevo thermostat by Peter Huber Kaeltemaschinenbau GmbH, Offenburg, Germany). Each cycle lasted 4 h and contained a 2-h freezing step followed by a 2-h thawing step. During the freezing step, bulk temperatures of −30°C were reached by rapid cooling of the thermofluid down to the system minimum of −40°C. Solidification of the total volume was accomplished within 60 min. Thawing was accomplished by increasing thermofluid temperature to 20°C after the freezing step. The total bulk melted within 75 min. The Raman probe head, described below, was installed via a specialized container lid with an adapted fitting. The probe head was placed in a way that sampling occurred in a position exactly at half radius of the container and approximately at half of the fill height for 700 mL. The container was equipped with cooling coils mounted in its vertical axis. Thus, high protein concentrations could be expected in half-radial regions because of macroscopic cryoconcentration.[1] Heat stressing of recombinant human immunoglobulin G (rhIgG)1-A was performed for 10 min at 74°C in a volume of 1 mL on a Thermomixer Comfort by Eppendorf (Hamburg, Germany).

Table 1. Reference Proteins Used for CD and Raman Spectroscopic Analysis

Protein	Organism	Buffer (10 mM)	pH (20°C)	Concentration (mg/mL)
BSA	*Bos tauris*	Tris–HCl	7.5	30/70[a]
Insulin	*Bos tauris*	Tris–HCl	3.0[b]	36
Muscle LDH	*Oryctolagus cuniculus*	Tris–HCl	7.5	22[c]
rhG–CSF	*Homo sapiens*	Acetate	4.4	36/60[a]
Lysozyme	*Gallus gallus*	Tris–HCl	7.5	31/62[a]
ADH	*Saccharomyces cerevisiae*	Tris–HCl	8.6	25
α-Amylase	*Bacillus subtilis*	Acetate	5.0	25/34[a]
Ribonuclease A	*Bos tauris*	Tris–HCl	7.5	30/65[a]
Pepsin	*Sus scrofa*	Acetate	4.4	7[c]
β-Lactoglobulin	*Bos tauris*	Acetate	5.7	30
Trypsin	*Bos tauris*	Tris–HCl	7.5	20
rhIgG1-A	*Homo sapiens*	Acetate	5.7	30/69[a]
rhIgG1-B	*Homo sapiens*	Acetate	5.7	26
rhIgG2	*Homo sapiens*	Acetate	5.7	30/57[a]

[a]Two concentrations of native protein were examined when higher concentrations were reached easily by ultrafiltration.
[b]Sufficiently high concentrations of soluble insulin could only be reached by acidification using HCl.
[c]Higher concentrations were not reached using ultrafiltration; Raman spectra from the frozen state could not be analyzed because of the insufficient scattering yields.

CD Spectroscopy

Circular dichroism spectra of liquid samples were recorded with a Jasco J-715 spectropolarimeter (Tokyo, Japan) at room temperature between 190 and 260 nm. A cylindrical cuvette of 10 μm path length was used.

The respective protein concentration was as stated in Table 1. Spectra were recorded five times, averaged, and corrected for the appropriate buffer blank. The relative secondary structural composition was calculated using the CDSSTR algorithm implemented at the Dichroweb server.[23] The resulting fractions for *Helix1* and *Helix2*, *Strand1* and *Strand2*, as well as *Turns* and *Unordered* were each added up to single values for α-helix, β-sheet, and Others, respectively. Under Others, *Turns* and *Unordered* are added up.

Raman Spectroscopy

Raman spectra were recorded using a RamanRXN2™ Hybrid Analyzer by Kaiser Optical Systems (Ann Arbor, Michigan)

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