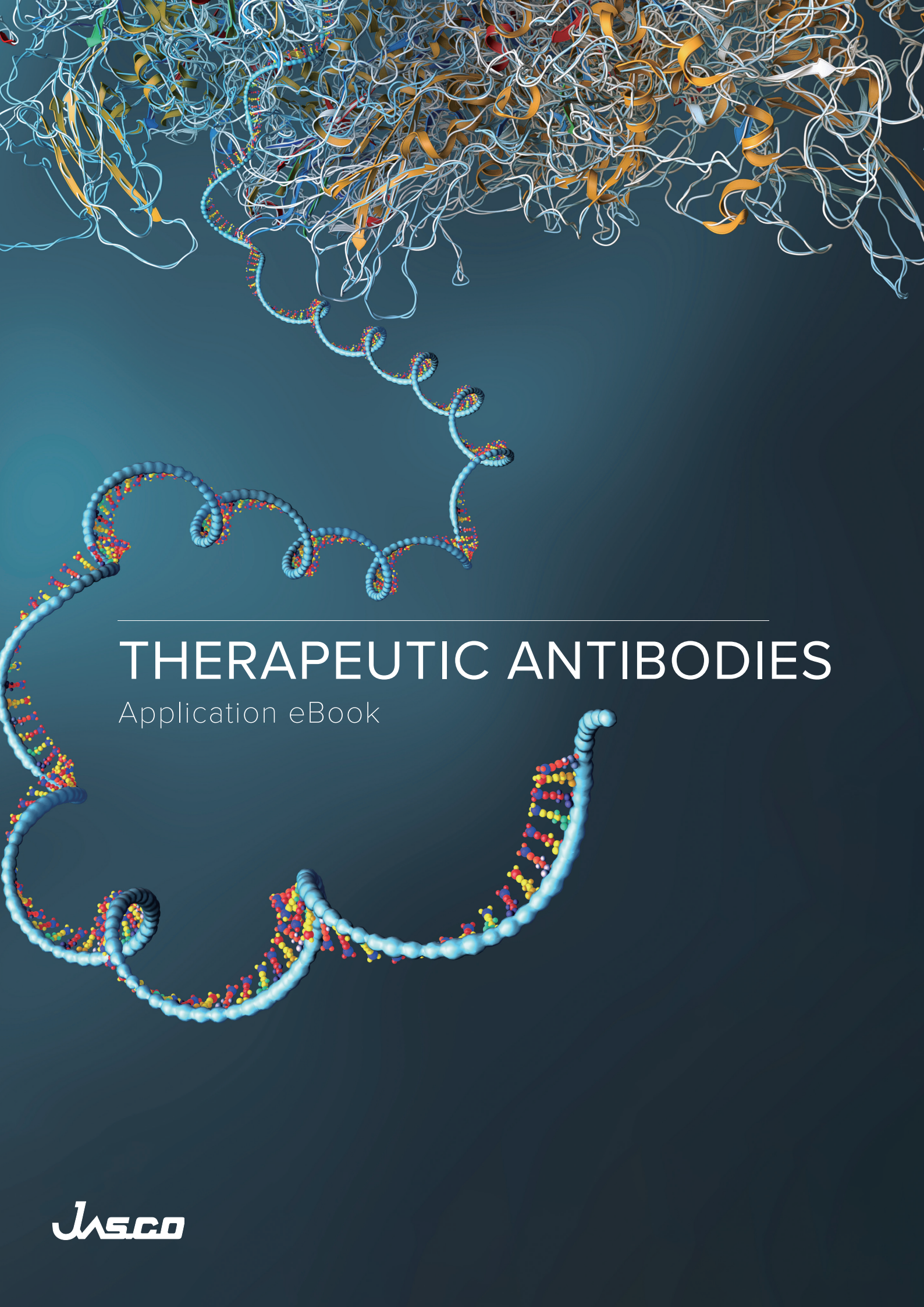




THERAPEUTIC ANTIBODIES

Application eBook



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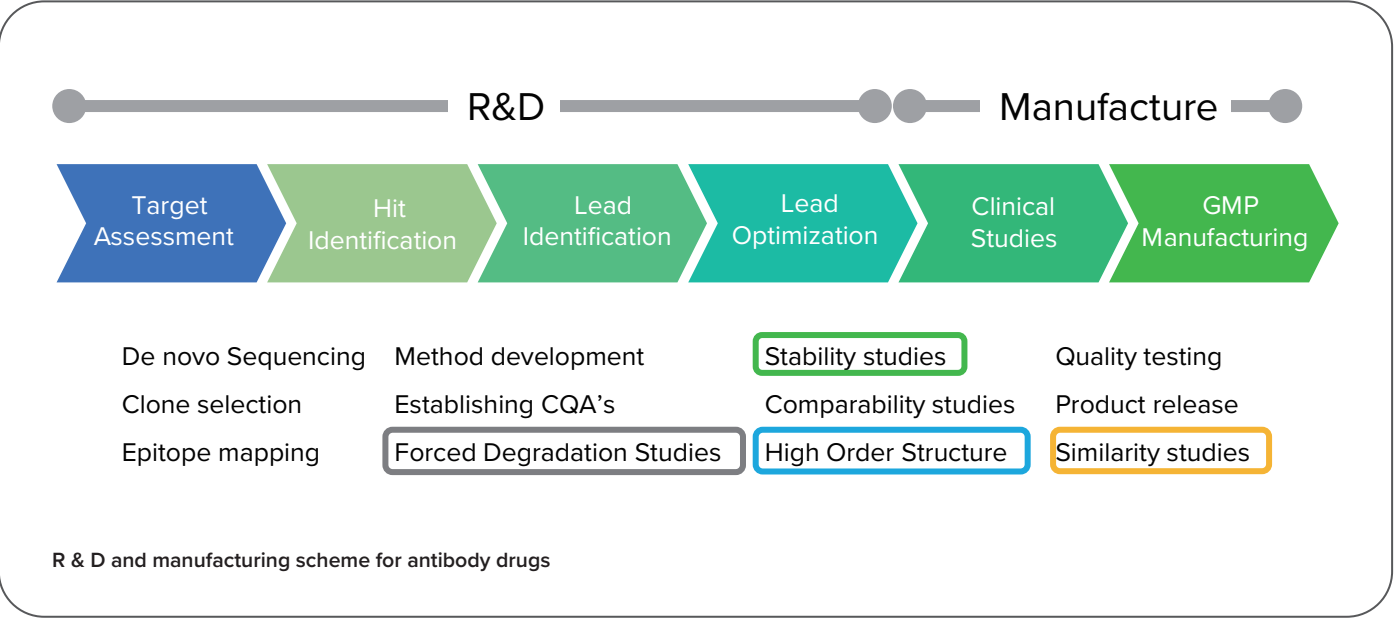
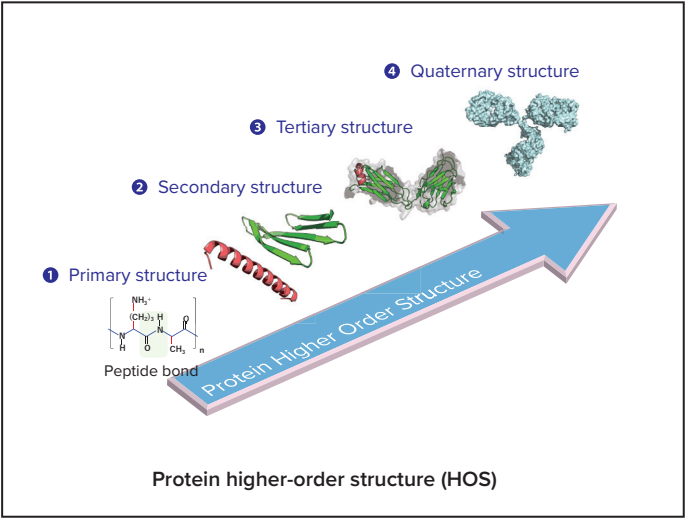
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Introduction

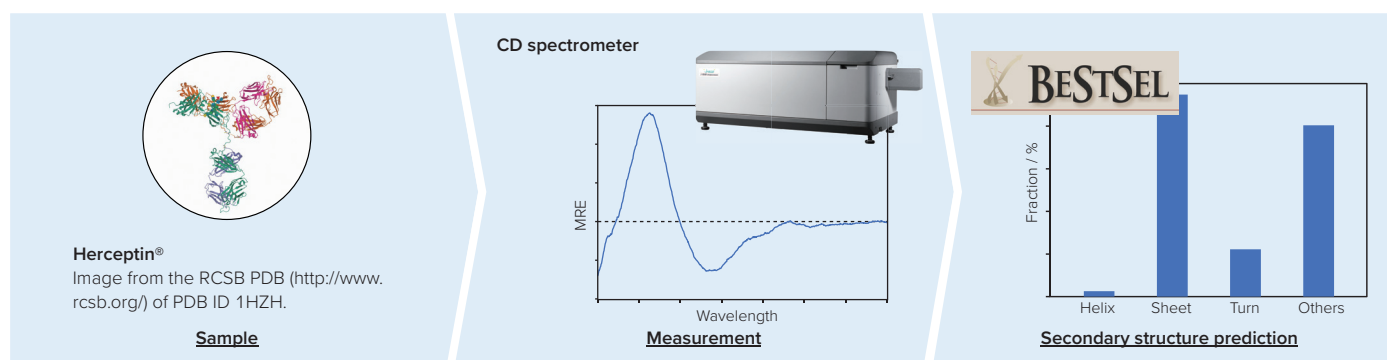
The market for therapeutic antibodies has been dramatically expanding over the past decades. Antibody drugs exhibit therapeutic effects, such as inhibiting the growth of malignant tumor cells and the activity of immune cells by binding antigens expressed in target cells with high affinity and specificity. Such antibody drugs are expected to be effective therapeutic agents for unmet medical needs.

The higher order structures (HOS) formed by therapeutic antibodies determines their efficacy. Because antibodies are unstable macromolecules, their HOS can be affected by various stimuli during the manufacturing process, resulting in reduced drug efficacy and impurities derived from the target substance. Therefore, HOS is considered to be a Critical Quality Attribute (CQA) and is subject to similarity studies, stability studies, and forced degradation studies in the R&D process from target assessment to lead optimization. In addition, it is monitored during the post-launch manufacturing process.

Antibody structures are hierarchically formed as primary, secondary, tertiary, and quaternary structures, and it is important to comprehensively evaluate each structure in the characterization of antibodies. This application eBook presents examples of evaluations using various analytical instruments, including Circular Dichroism (CD) spectrometers, Fourier-transform infrared (FTIR) spectrometers, Raman imaging microscopes and High Performance Liquid Chromatography (HPLC).



Secondary Structure Prediction for Herceptin® using CD Spectroscopy



Graphical abstract

Introduction

Higher-order structure (HOS) is a critical attribute that controls the function, safety and efficacy of therapeutic proteins.¹⁾ During the development of protein therapeutics, characterization of the HOS is required in order to achieve robust manufacturing processes, to develop stable formulations and to manufacture biosimilars identical to the innovator.²⁾ When investigating protein HOS, Circular Dichroism (CD) is the most well-known technique for studying protein secondary structure. This method is an easy, time-saving method, commonly used to monitor the effect of manufacturing, formulation and storage conditions on therapeutic proteins²⁾.

CD spectroscopy is a classic technique for the secondary structure prediction of proteins in solution. The secondary structure content is estimated based on CD signals in the far-UV region (170 to 250 nm), which are associated with electronic transitions involving peptide bonds.³⁾ The protein concentration required to detect the minimal CD signal in this range is low: approximately 0.1 mg/mL when using a 1 mm path length cuvette. This is a great advantage when the amount of protein is limited, which is commonly the case.

In this study, the secondary structure content of Herceptin® was analyzed using CD spectroscopy.

Keywords

Circular Dichroism (CD) spectroscopy, secondary structure prediction, BeStSel, Herceptin®, trastuzumab, therapeutic antibody, biopharmaceutical, higher-order structure (HOS)

Experimental

Sample

- Herceptin® (trastuzumab, Roche): powder dissolved in ultrapure water to 0.16 mg/mL

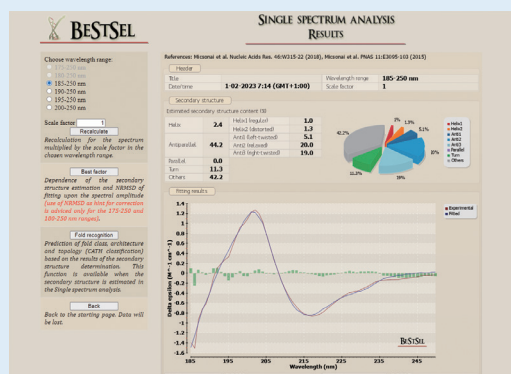
Measurement and analysis system

J-1500 CD spectrometer



- Accurate and efficient normalization of protein CD spectrum realized by simultaneous CD and Abs measurements.

BeStSel⁴⁾



- Secondary structure prediction method developed by Dr. József Kardos at Eötvös Loránd University.
- Highly accurate estimation of β -sheet content by dividing β -sheets into four classes.

Measurement Conditions

Bandwidth: 1.0 nm
D.I.T.: 2 sec.
Data pitch: 0.1 nm
Scanning speed: 50 nm/min.

Accumulation: 9
Optical path length: 1 mm
Sample volume: 200 μ L

Results

CD and absorbance spectra in the far-UV region were simultaneously obtained using the J-1500 (Figs. 1a and 1b). For secondary structure prediction, the measured CD spectrum is normalized using the absorbance or concentration to generate the Mean Residue Ellipticity (MRE) spectrum or the molar CD spectrum. Here, the measured CD spectrum was normalized using the absorbance at 205 nm to generate the MRE spectrum (Fig. 1c).^{3, 5)} The secondary structure content was then predicted using BeStSel (Fig. 1d).

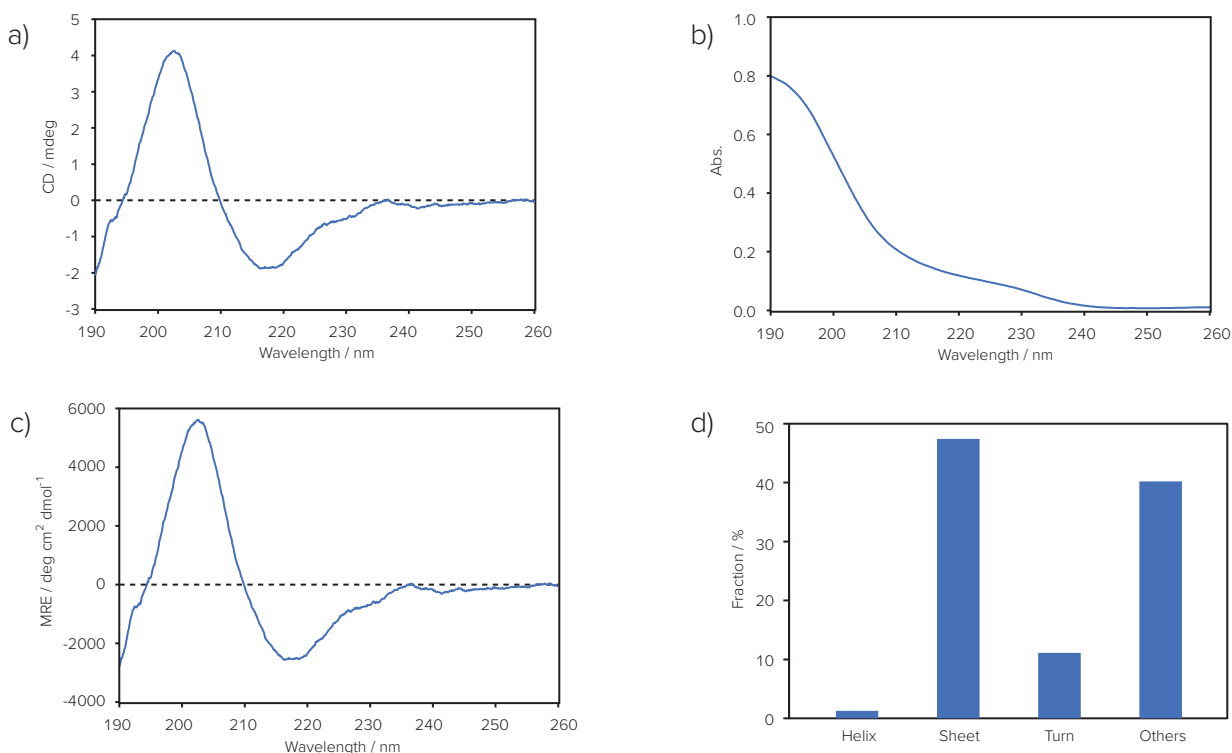


Fig. 1 Spectra and predicted secondary structure contents.
a) CD, b) absorbance, c) MRE spectra in far-UV region, and d) secondary structure contents.

Conclusions

The secondary structure of Herceptin® was easily predicted from the CD spectrum using BeStSel. CD spectroscopy is the strongest, fastest approach to determining protein secondary structure. The simplicity of this technique makes it applicable for efficient screening of protein solutions under different conditions, such as buffer composition or temperature to determine their effects on protein structural stability.

Additional Information

- Application: Highly efficient CD/CPL systems for accurate simultaneous CD/Abs and CPL/FL measurements #1.
<https://www.jasco-global.com/solutions/highly-efficient-cd-cpl-systems-for-accurate-simultaneous-cd-abs-and-cpl-fl-measurements-1/>
- Product information: CD spectrophotometer.
<https://www.jasco-global.com/product-spectroscopy/circular-dichroism/>
- BeStSel web server:
<https://bestsel.elte.hu/index.php>

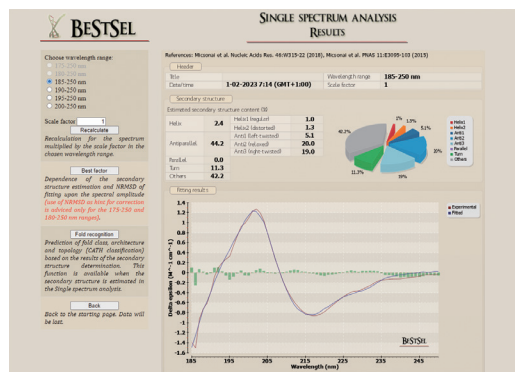
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About the Author

Ai Yamane is a spectroscopy application scientist at JASCO Corporation (Tokyo, Japan) specializing in CD, UV-vis, and fluorescence spectroscopy.

Secondary Structure Estimation by CD Spectroscopy - BeStSel



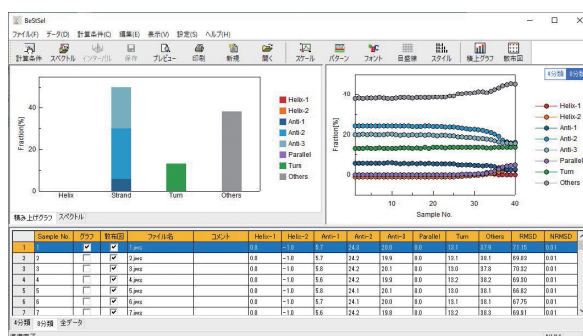
BeStSel web server:
<https://bestsel.elte.hu/index.php>

Recently, Micsonai et al. developed the BeStSel algorithm that accurately estimates the secondary structure composition from the CD spectrum by taking into account the parallel-antiparallel orientation of the β -strands and the twist of the antiparallel β -sheets. BeStSel has the following features: 1) high estimation accuracy for a wide range of proteins including β -structure-rich-proteins such as antibodies, 2) providing eight types of secondary structure information 3) a capability to predict the protein fold following the CATH classification and 4) an open web server.¹⁻⁵⁾

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- 2) Micsonai, A., Wien, F., Bulyáki, É., Kun, J., Moussong, É., Lee, Y. H., Goto, Y., Réfrégiers, M., & Kardos, J.: *Nucleic Acids Res.*, **46**, W315 (2018)
- 3) Micsonai, A., Bulyáki, É., & Kardos, J.: *Methods in molecular biology (Clifton, N.J.)*, **2199**, 175 (2021)
- 4) Micsonai, A., Moussong, É., Lee, Y. H., Murvai, N., Tantos, Á., Tóke, O., Réfrégiers, M., Wien, F., & Kardos, J.: *Front. mol. biosci.*, **9** (2022)
- 5) Micsonai, A., Moussong, É., Wien, F., Boros, E., Vadász, H., Murvai, N., Lee, Y. H., Molnár, T., Réfrégiers, M., Goto, Y., Tantos, Á., & Kardos, J.: *Nucleic Acids Res.*, **50**, W90 (2022)

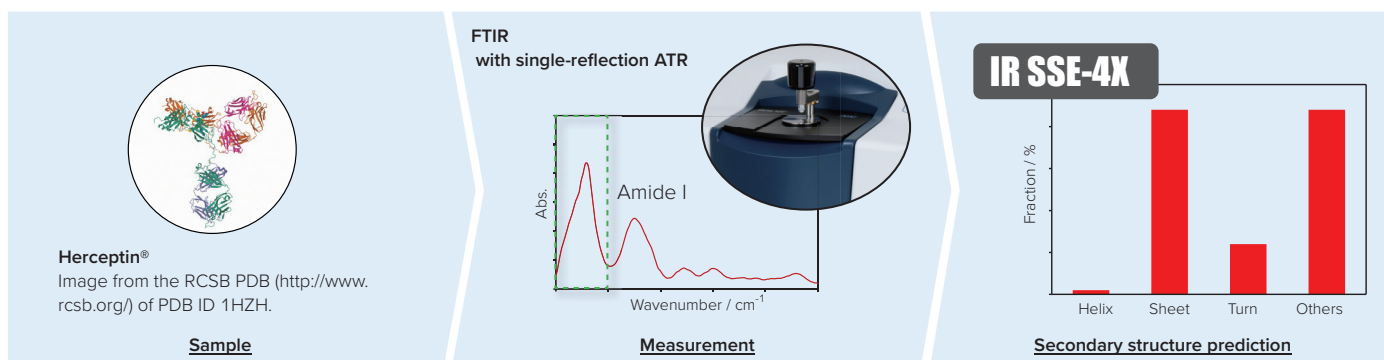
Regulatory Compliance - BeStSel CFR Program

While many academic researchers use the BeStSel web server, researchers in biopharma who need to work in a GxP environment have not been able to benefit from BeStSel. Spectra Manager™ 2.5 CFR BeStSel offers a control and analysis platform for CD spectrometers, which is compatible with GxP and satisfying computer system validation (CSV), electronic record/electronic signature (ER/ES) and data integrity (DI) for practicing ALCOA+ principles.



BeStSel program

Secondary Structure Prediction of IgG using IR Spectroscopy



Graphical abstract

Introduction

Like Circular Dichroism (CD) spectroscopy, infrared (IR) spectroscopy can be used to study protein secondary structure. IR is also a fast measuring method, but unlike CD, proteins in both highly concentrated solutions (up to several hundred mg/mL) and the solid state can be measured.

Protein secondary structure is estimated based on absorption associated with the amide I band (1700 to 1600 cm⁻¹), which is caused by C=O stretching vibrations of the protein backbone. It is known that the amide I band is sensitive to the β -sheet structure, which is convenient for monoclonal antibody (mAb) analysis. The higher concentration limits of IR spectroscopy make it apt for manufacturing conditions, where the formulation must be high concentration and Higher-order structure (HOS) evaluation is needed.^{1,2)}

In this study, the secondary structure content of a highly concentrated IgG solution was analyzed using FTIR spectroscopy.

Keywords

Fourier-transform infrared (FTIR) spectroscopy, Attenuated Total Reflectance (ATR), secondary structure prediction, IgG, monoclonal antibody (mAb), therapeutic antibody, biopharmaceutical, higher-order structure (HOS)

Experimental

Sample

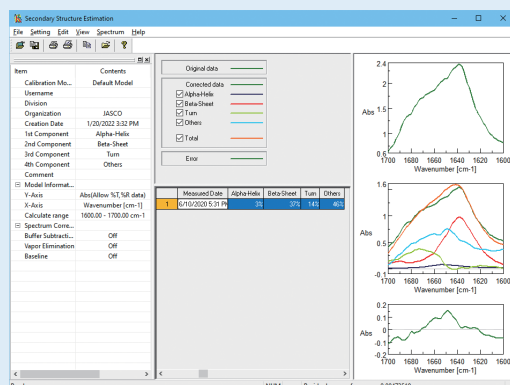
- Protein: IgG, from rabbit serum (Sigma Aldrich) 11.4 mg/mL and 116 mg/mL
- Buffer: 20 mM citric acid buffer (150 mM NaCl, pH 6.0)

FT/IR-4X FTIR spectrometer with ATR PRO 4X single-reflection ATR



- High S/N and resolution with small body size.
- Protein solution measurement with one drop of sample (approx. 10 μL) with ATR PRO 4X.

IR SSE-4X IR secondary structure estimation program



- Secondary structure prediction program developed by JASCO.
- Improved precision and agreement with CD spectroscopy by enhancement of reference model.

Measurement Conditions

Resolution: 4 cm^{-1}
Sample volume: 10 μL

Accumulation: 100 (2 min.)
 N_2 purge: Yes

Results

The IR spectrum of a 116 mg/mL IgG solution was measured using the FT/IR-4X with ATR PRO 4X (Fig. 1a). A relatively low-concentration IgG solution (11.4 mg/mL) was also measured for comparison. The secondary structure content for both solutions was predicted using the IR SSE-4X (Fig. 1b). The results indicated a change in the secondary structure as the concentration increased, including an increase in β -sheets and others as well as a decrease in turns.

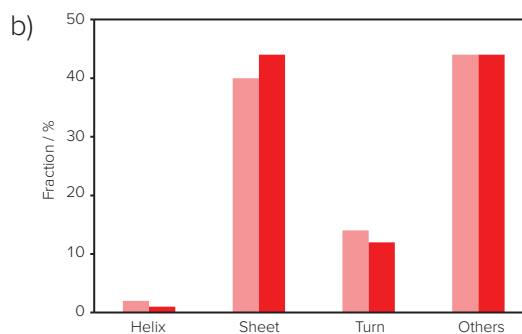
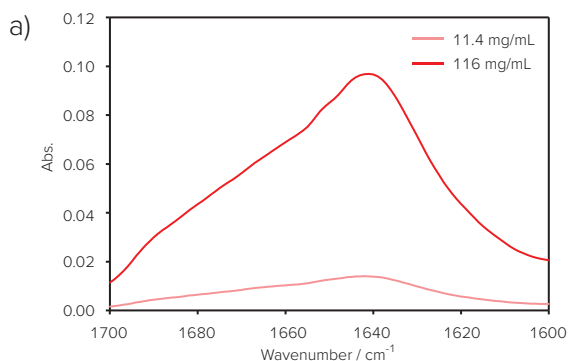


Fig. 1 IR spectrum and predicted secondary structure contents.

Conclusions

The secondary structure of IgG was easily analyzed using IR spectroscopy, an equivalently strong technique to CD spectroscopy for protein secondary structure prediction. It can be applied not only to protein solutions, but also suspensions and powders, as well as highly concentrated solutions. The sample amount needed for a solution is only 10 μL when using the ATR unit. Because of its versatility, IR spectroscopy allows secondary structure analysis of therapeutic proteins, which can be aggregated or highly concentrated.

For more information...

- Application: Analysis of the effects of low temperature on the secondary structure of VHH antibodies.
<https://www.jasco-global.com/solutions/analysis-of-the-effects-of-low-temperature-on-the-secondary-structure-of-vhh-antibodies/>
- Application: Protein secondary structure analysis in low concentration aqueous solutions.
<https://www.jasco-global.com/solutions/protein-secondary-structure-analysis-in-low-concentration-aqueous-solutions/>
- Product information: FTIR spectrometer.
<https://www.jasco-global.com/product-spectroscopy/ftir-spectrometer/>

References

- 1) S. A. Berkowitz, D. J. Houde: *"Biophysical Characterization of Proteins in Developing Biopharmaceuticals (Second Edition)"*. (2015), (Elsevier B. V., Amsterdam).
- 2) L. L. Liu, L. Wang, J. Zonderman, J. C. Rouse, H. Y. Kim: *J. Pharm. Sci.*, **109**, 3223 (2020).

About the Author

Ai Yamane is a spectroscopy application scientist at JASCO Corporation (Tokyo, Japan) specializing in CD, UV-vis, and fluorescence spectroscopy.

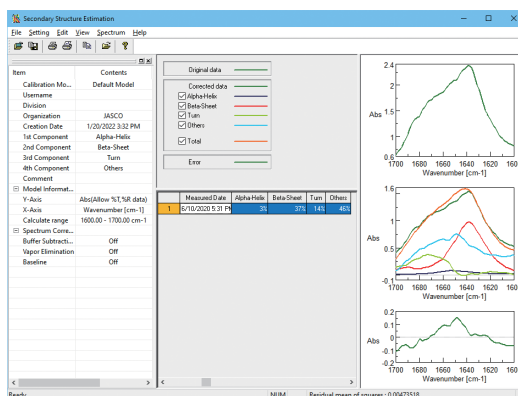
Secondary Structure Estimation by IR Spectroscopy

- IR SSE Program -

The IR SSE program estimates the secondary structure of a sample from a spectrum obtained using the multivariate analysis technique.

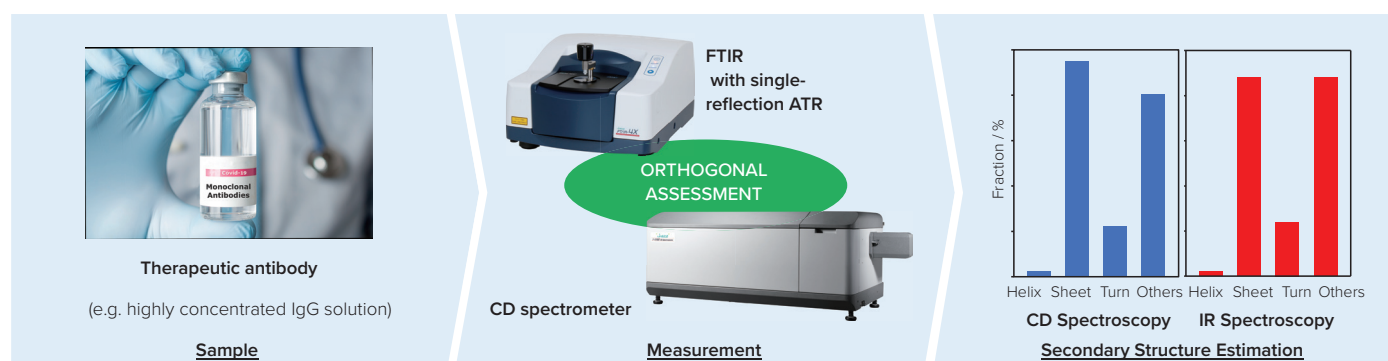
This program includes two types of standard models (standard calibration curves) created using the PCR and PLS multivariate analysis methods, based on the infrared spectra of 28 or 17 types of proteins contained in a database or published in the literature, respectively.

Instead of using the standard model, a custom calibration model created using the [Create SSE Calibration Model] program can also be used to estimate the secondary structure of sample proteins.



IR SSE-4X Secondary Structure Estimation program

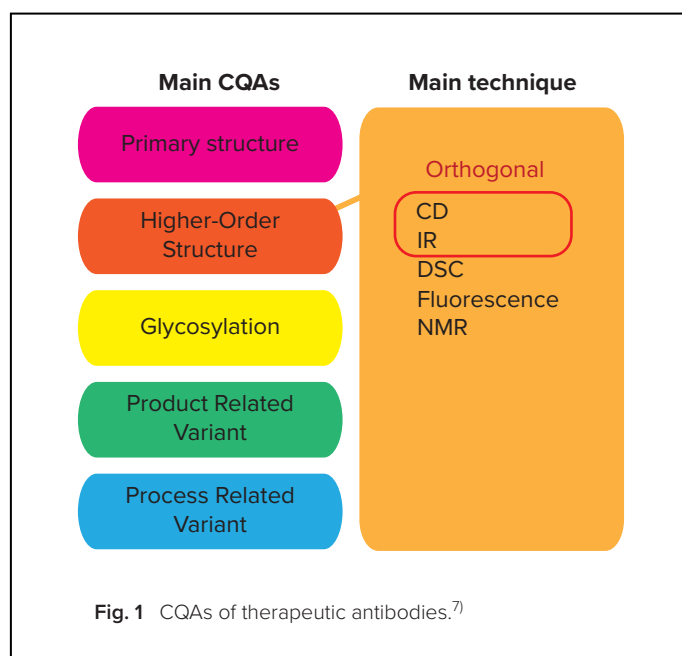
Orthogonal Assessment of Secondary Structure for IgG



Graphical abstract

Introduction

The market for therapeutic antibodies has dramatically expanded over the past decades since their first approval in 1986. As a result of this growth, therapeutics antibodies are a large part of new drugs approved in recent years.¹⁾ However, the physical characteristics of antibodies may change depending on their production and storage environments. These physical characteristics are referred to as critical quality attributes (CQAs) and may affect the safety and potency of antibody drugs.²⁾ Therefore, there is a need to assess the impact of production and storage conditions on the CQAs of antibodies, and this assessment should ideally be conducted at their prescribed concentration. Most therapeutic antibody drugs are prescribed at a relatively high concentration of up to 200 mg/mL³⁾; however, proteins are known to behave differently at high concentration compared to lower concentrations.³⁾

Fig. 1 CQAs of therapeutic antibodies.⁷⁾

The higher-order structure (HOS) of antibodies has an important influence on the CQAs because it directly affects physiological activity.⁴⁾ Circular Dichroism (CD) and infrared (IR) spectroscopy are the most popular techniques for secondary structure evaluation of proteins. CD spectroscopy is used to obtain secondary structural information for relatively low-concentration protein solutions with a maximum concentration of about 10 mg/mL. IR spectroscopy is used for secondary structure prediction for protein solutions with concentrations of up to 100 mg/mL, as well as protein suspensions or powders.

When assessing the physical properties of therapeutic antibodies, the importance of orthogonal assessment has been stressed.⁵⁾ In this approach, each physical characteristic is assessed by two or more techniques that are based on different principles. Orthogonal assessment is expected to improve the efficiency and lower the cost of clinical trials during biosimilar development.⁶⁾

Here, the secondary structure of IgG, the most common antibody used in antibody drugs, was assessed using CD and IR spectroscopy as orthogonal methods.

Keywords

Circular Dichroism (CD) spectroscopy, Fourier-transform infrared (FTIR) spectroscopy, Attenuated Total Reflectance (ATR), secondary structure prediction, BeStSel, IgG, monoclonal antibody (mAb), therapeutic antibody, biopharmaceutical, higher-order structure (HOS), Critical Quality Attribute (CQA), orthogonal assessment

Experimental

Sample

- Protein: IgG, from rabbit serum (Sigma Aldrich)
5.8 mg/mL, 11.4 mg/mL, 17.5 mg/mL and 23.1 mg/mL
- Buffer: 20 mM citric acid buffer (150 mM NaCl, pH 6.0)

Measurement and analysis system

J-1500 CD spectrometer

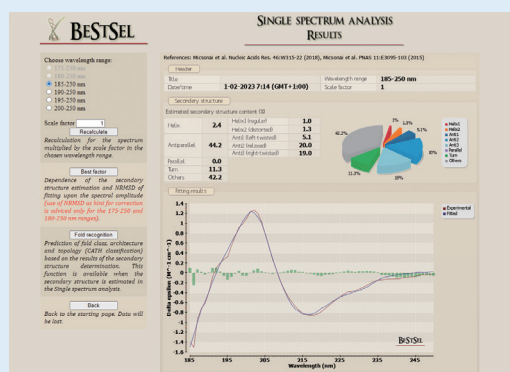


- Accurate and efficient normalization of protein CD spectrum obtained by simultaneous CD and Abs measurements.

0.01 mm path length demountable cuvette

- Very short path length enabling CD measurement of high-concentration protein solutions.
- Easy cleaning simply by wiping off the samples using lab tissues soaked with ultrapure water.

BeStSel⁽⁸⁾



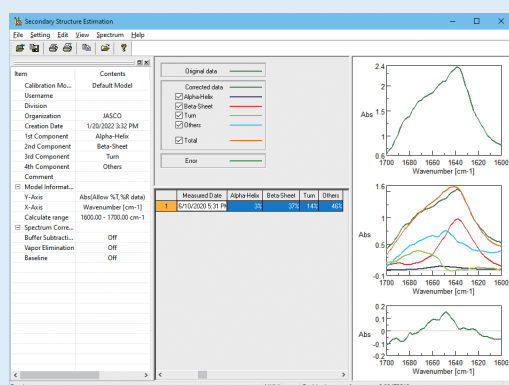
- Secondary structure prediction method developed by Dr. József Kardos at Eötvös Loránd University.
- Highly accurate estimation of β -sheet content by dividing β -sheets into four classes.

FT/IR-4X FTIR spectrometer with ATR PRO 4X single-reflection ATR



- High S/N and resolution with small body size.
- Protein solution measurement with one drop of sample (approx. 10 μ L) with ATR PRO 4X.

IR SSE-4X IR secondary structure estimation program



- Secondary structure prediction program developed by JASCO.
- Improved precision and agreement with CD spectroscopy by enhancement of reference model

Measurement Conditions

<Far-UVCD measurement>

Bandwidth: 1.0 nm
D.I.T.: 2 sec.
Data pitch: 0.1 nm
Scanning speed: 50 nm/min.

Accumulation: 4
Optical path length: 0.01 mm
Sample volume: 1.8 μL

<IR measurement>

Resolution: 4 cm^{-1}
Sample volume: 10 μL

Accumulation: 100 (2 min.)
 N_2 purge: Yes

Results

CD and absorbance spectra in the far-UV region were simultaneously obtained using the J-1500 (Figs. 2a and 2b). The CD signal and absorbance both increased with increasing sample concentration. For secondary structure prediction, the measured CD spectrum is normalized using the absorbance or concentration to generate the Mean Residue Ellipticity (MRE) spectrum or the molar CD spectrum. Here, the measured CD spectra were normalized using the absorbance at 205 nm to generate the MRE spectra (Fig. 2c).^{9,10)} The shape of the MRE spectra did not change despite the difference in concentration.

IR spectra were measured using the FT/IR-4X and ATR PRO 4X (Fig. 2d). Although the absorbance increased with increasing sample concentration, the amide I band (1700 to 1600 cm^{-1}) did not show significant changes in shape with concentration.

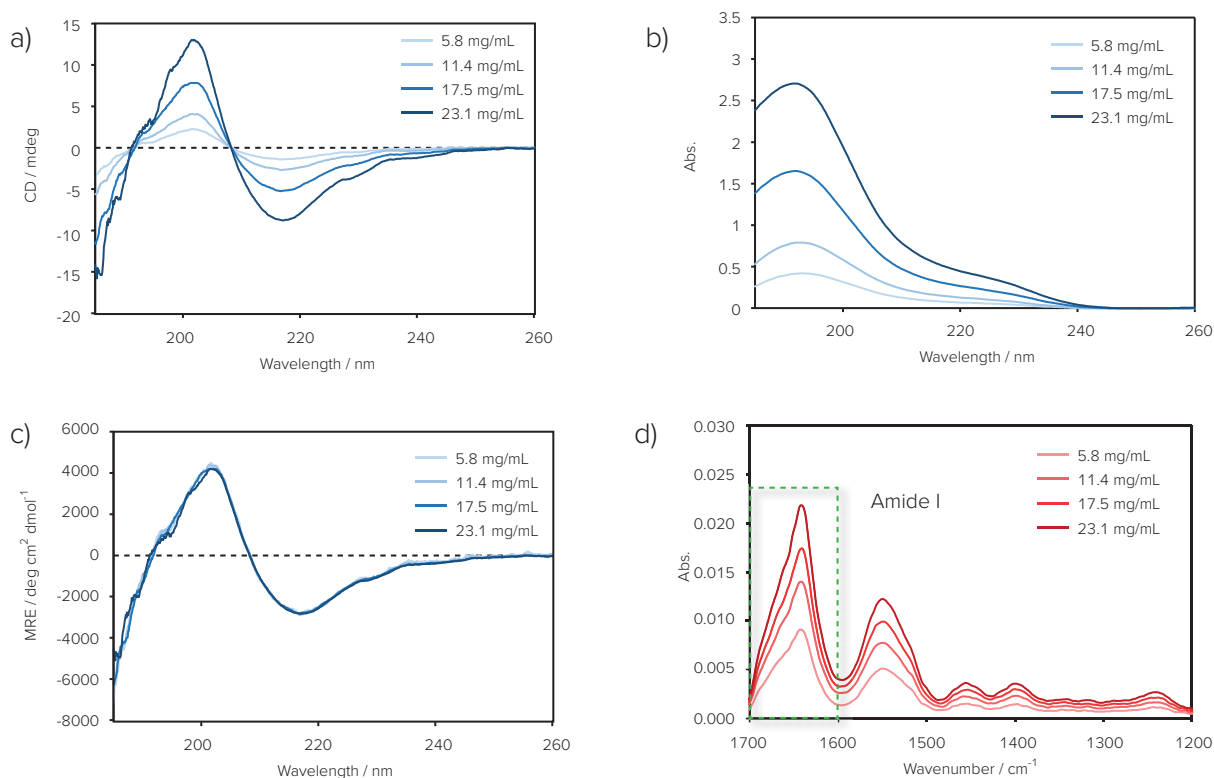


Fig. 2 CD and absorbance spectra in far-UV region and IR spectra of IgG solutions
a) CD, b) absorbance, c) MRE spectra in far-UV region, and d) IR spectra.

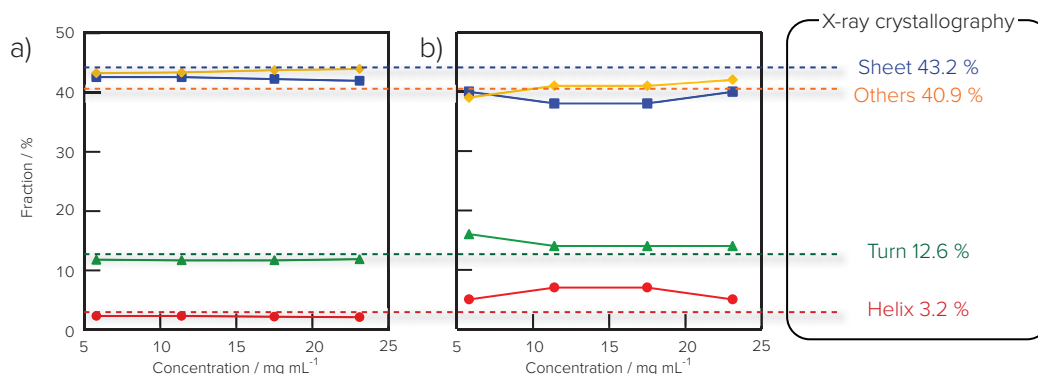


Fig. 3 Secondary structure prediction.
Secondary structure contents obtained using a) CD spectroscopy and b) IR spectrum.

Table 1. What should be checked for proper use of CD and IR spectroscopy

Item	CD (far-UV)	IR
Buffer and additives	Do not show excessive absorbance in the bare minimum wavelength for SSE (200 to 250 nm).	Do not show absorbance in amide I band (1700 to 1600 cm ⁻¹).
Sample state	• Solution (up to several tens mg/mL) • Only transparent, suspension not accepted.	• Solution (up to several hundreds mg/mL) • Suspension • Powder

The secondary structure content was predicted from MRE spectra using BeStSel (Fig. 3a) and from the amide I band using the IR SSE-4X (Fig. 3b). The secondary structure content for IgG (PDB ID: 1HZH) obtained by X-ray crystallography is shown in Fig. 3 for comparison.¹¹⁾ In this concentration range, the secondary structure content did not show a significant change in either the CD or IR secondary structure prediction. This agrees with the observation that the shape of the MRE (Fig. 2c) and IR (Fig. 2d) spectra did not depend on concentration. Also, the secondary structure contents obtained by CD and IR spectroscopy, as well as X-ray crystallography, are in good agreement.

Conclusions

The agreement between the CD and IR spectroscopy confirms the usefulness of these two techniques for orthogonal assessment of protein secondary structure. As shown in Table 1, the most appropriate choice of technique depends on the buffer and sample used. For instance, the amide I band in the IR spectrum derived from C=O stretching in proteins may overlap with the absorbance bands for certain buffers and additives. When this happens, accurate baseline subtraction is difficult, resulting in poor precision for secondary structure prediction. On the other hand, in the CD spectrum, the baseline can be accurately subtracted as long as the solvent does not show excessive absorbance. Therefore, for certain types of buffers and additives, CD is the better choice. However, CD measurements can only be performed for transparent solutions with concentrations of up to a few tens of mg/mL, even when using a very short path length. In contrast, IR measurements can be performed on protein solutions with concentrations of more than 100 mg/mL, as well as protein suspensions and powders. Accurate prediction of secondary structure can be achieved only when the appropriate technique is selected depending on the solvent type and sample state.

Both CD and IR spectroscopy are simple methods for HOS assessment. The present results confirm the applicability of both techniques in the development and quality control processes for therapeutic antibodies and biosimilars.

Additional Information

- Product information: CD spectrophotometer.
<https://www.jasco-global.com/product-spectroscopy/circular-dichroism/>
- Product information: FTIR spectrometer.
<https://www.jasco-global.com/product-spectroscopy/ftir-spectrometer/>
- BeStSel web server:
<https://bestsel.elte.hu/index.php>

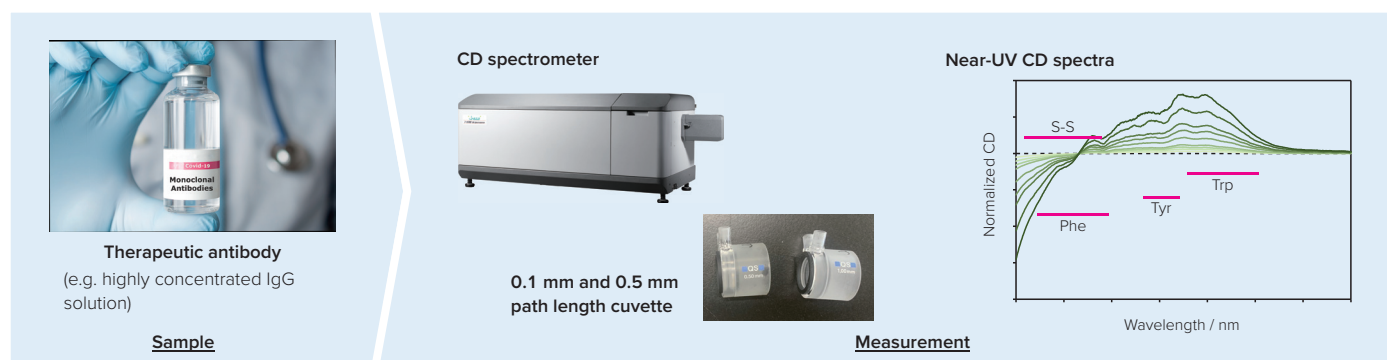
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About the Author

Ai Yamane is a spectroscopy application scientist at JASCO Corporation (Tokyo, Japan) specializing in CD, UV-vis, and fluorescence spectroscopy.

Near-UV CD Measurement of High-Concentrated IgG



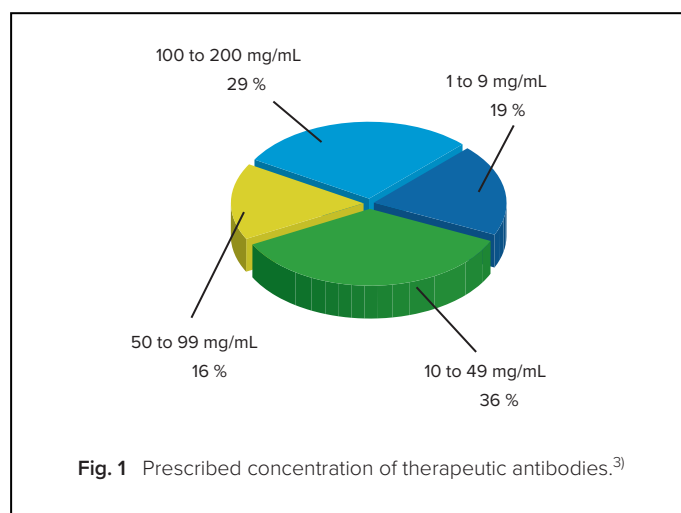
Graphical abstract

Introduction

Beyond secondary structure, Circular Dichroism (CD) spectroscopy is an excellent method to probe the tertiary structure of proteins. As previously mentioned, therapeutic antibody drugs are often prescribed at high concentration of up to 200 mg/mL (Fig. 1). Our previous example paired CD and infrared (IR) spectroscopy to bridge the low to high concentration gap for secondary structure evaluation. To evaluate the proteins physical characteristics and Critical Quality Attributes (CQAs) ¹⁾ on CD we can employ the near UV regions.

The near UV region of the CD spectrum is related to the tertiary structure of the antibody studied. The concentrations which can be studied in this region are allowed to be much higher because of the smaller molar extinction coefficient.

Here, near-UV CD spectra of IgG solutions with concentrations of more than 150 mg/mL were successfully obtained using a short path length cuvette. This allows one to study the possible effects of low and high concentrations of antibody on physiological activity.^{2,3)}



Keywords

Circular Dichroism (CD) spectroscopy, IgG, monoclonal antibody (mAb), therapeutic antibodies, higher-order structure (HOS), tertiary structure, critical quality attributes (CQAs), high-concentrated protein, short path length cuvette

Experimental

Sample

- Protein: IgG, from rabbit serum (Sigma Aldrich)
5.8, 11.4, 17.5, 23.1, 43.3, 61.7, 76.8, 114.9, and 156.2 mg/mL
- Buffer: 20 mM citric acid buffer (150 mM NaCl, pH 6.0)

J-1500 CD spectrometer



- Accurate and efficient normalization of protein CD spectrum obtained by simultaneous CD and Abs measurements.
- High-concentration protein measurement using short path length cuvette.

Measurement Conditions

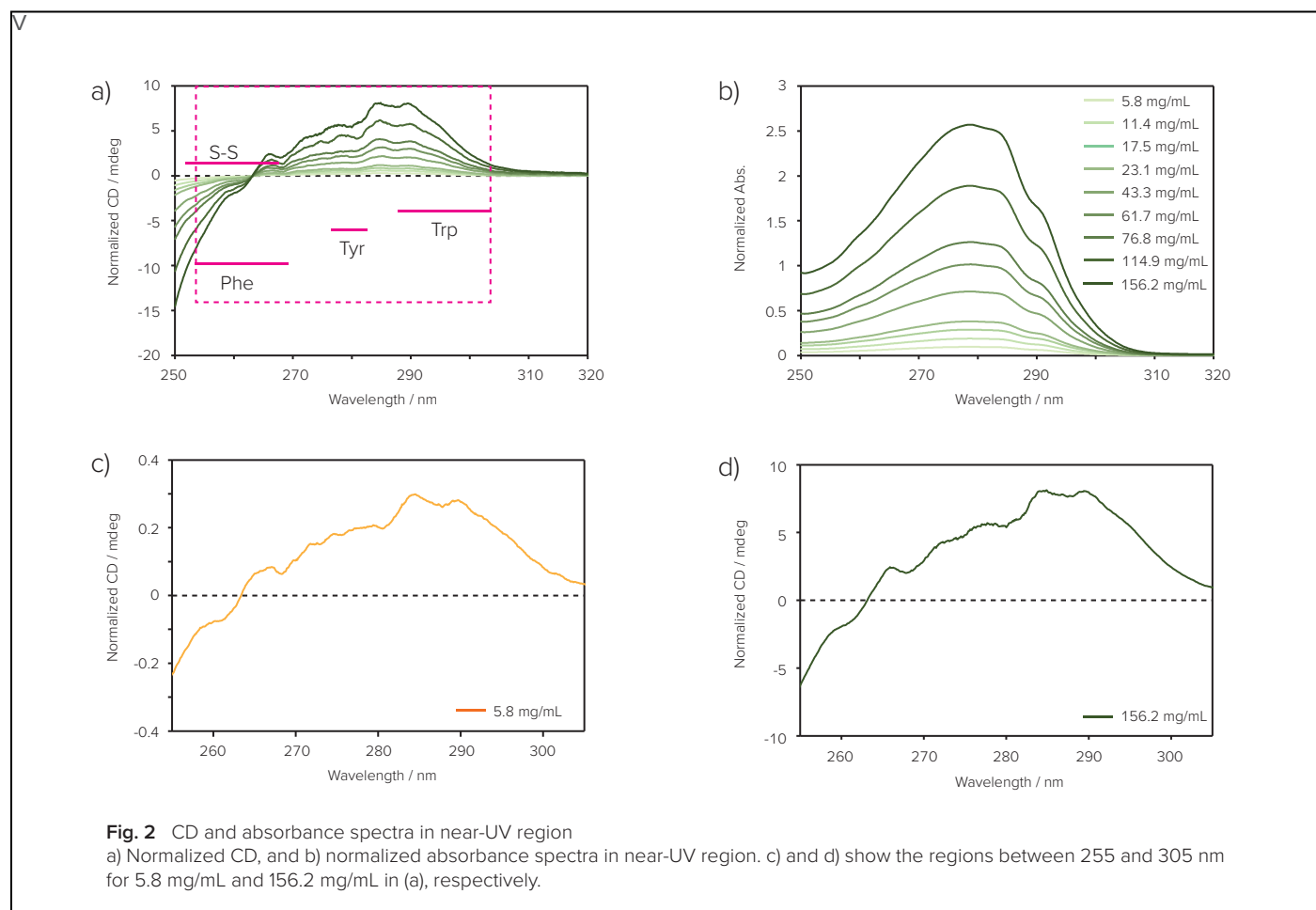
Bandwidth: 1.0 nm
D.I.T.: 4 sec.
Data pitch: 0.1 nm
Scanning speed: 20 nm/min.
Accumulation: 4 (9 for 156.2 mg/mL)

Optical path length:
1 mm (5.8 to 11.4 mg/mL)
0.5 mm (17.5 to 23.1 mg/mL)
0.1 mm (43.3 mg/mL or higher)

Sample volume:
280 μ L (5.8 to 11.4 mg/mL)
220 μ L (17.5 to 23.1 mg/mL)
22 μ L (43.3 mg/mL or higher)

Results

CD and absorbance spectra in the near-UV region were simultaneously obtained using the J-1500. IgG with a wide range of concentration was measured using an appropriate path length for each concentration. CD and absorbance spectra normalized based on the path length are shown in Figs. 2a and 2b.



Both the CD signal and the absorbance increased with increasing concentration. In the near-UV region, there are minor CD peaks between 255 and 305 nm derived from disulfide bonds and aromatic amino acids. These signals were clearly detected at both the minimum and maximum concentrations (Figs. 2c and 2d).

Conclusions

Selecting an appropriate path length allowed CD measurements of highly concentrated protein solutions (> 150 mg/mL) in the near-UV region, which has not been achieved before.

Additional Information

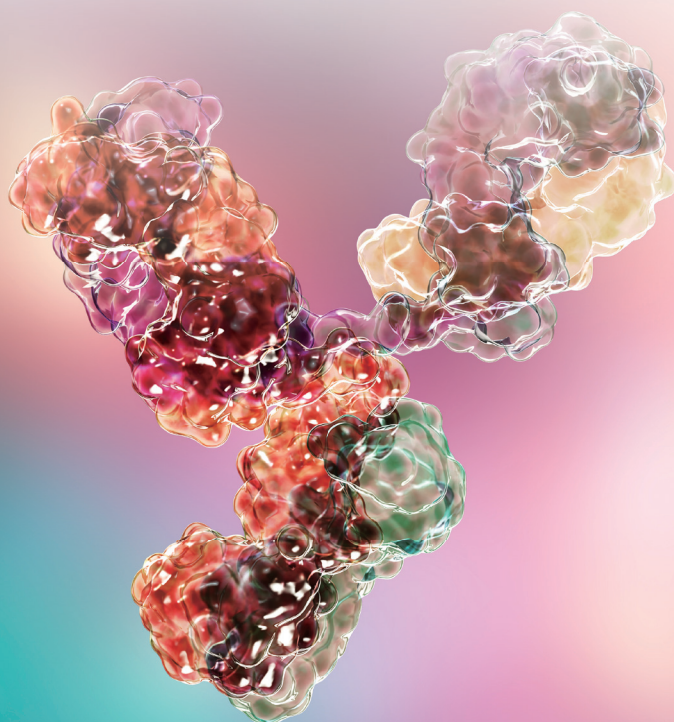
- Product information: CD spectrophotometer.
<https://www.jasco-global.com/product-spectroscopy/circular-dichroism/>

References

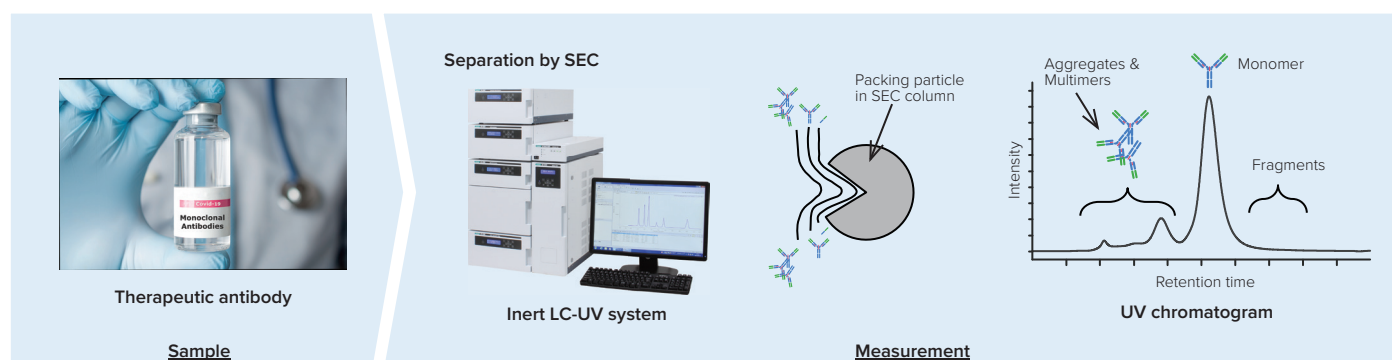
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- 2) European Medical Agency: “ICH Guideline Q11 on Development and Manufacture of Drug Substances (Chemical Entities and Biotechnological/Biological Entities)” < https://www.ema.europa.eu/en/documents/scientific-guideline/ich-guideline-q11-development-manufacture-drug-substances-chemical-entities-biotechnological/biological-entities_en.pdf>, (accessed 2022.07.28)
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About the Author

Ai Yamane is a spectroscopy application scientist at JASCO Corporation (Tokyo, Japan) specializing in CD, UV-vis, and fluorescence spectroscopy.



Assessment of Aggregates in Therapeutic Antibodies by SEC



Graphical abstract

Introduction

Since the first monoclonal antibody was approved by the U.S. Food and Drug Administration (FDA) in 1986, the market for therapeutic proteins has expanded rapidly, and antibody drugs have become a predominant treatment modality for various diseases over the past 25 years.¹⁾ The development of biopharmaceuticals, such as therapeutic antibodies, requires the evaluation of physicochemical, chemical, biological or microbiological properties that should be within the appropriate range to ensure their efficacy and safety. These properties are called Critical Quality Attributes (CQAs) and are analyzed by various analytical methods.^{2–4)}

During the manufacturing process, transportation and storage therapeutic antibodies may form dimer, trimer or higher order aggregates due to mechanical stress or environmental factors.^{5, 6)} These aggregates need to be evaluated as one of the CQAs because they may cause a decrease in drug efficacy and side effects. In 2014, the FDA provided guidance on the evaluation of the immunogenicity of therapeutic protein products (the ability of a drug to act as an antigen, produce anti-drug antibodies in the body and provoke an immune response). For protein aggregates contained in biopharmaceuticals, it is recommended to assess the validity of each analysis result by comparing the results using multiple methods with different measurement principles (orthogonal assessment).⁷⁾

Moreover, evaluation of physicochemical properties is considered to be important to show that the subsequent products (biosimilars) have similar quality, safety and efficacy to preceding biopharmaceuticals (innovators). Among the physicochemical properties, higher-order structure (HOS) is an essential element of characterization, which should include similarity assessment of primary to tertiary structure and quaternary structure, including aggregates, using appropriate analytical techniques, and again the Orthogonal Assessment for one property is recommended.^{8, 9)}

Aggregates are classified by their size into < 100 nm, 100 nm to 1 µm, 1 µm to 100 µm, and > 100 µm categories, and analytical methods are employed according to each size.^{10, 11)} Size Exclusion Chromatography (SEC) is often used as one of the qualitative and quantitative evaluation methods for aggregates of 50 nm or less.^{12, 13)}

In this article, antibody drugs with IgG-derived sequences in addition to IgG from human serum were measured using SEC to separate and detect monomer, multimers, and aggregates. The measured antibody drugs were innovator rituximab (MabThera®) and biosimilar (RIABNI™) for the treatment of non-Hodgkin lymphoma, and innovator trastuzumab (Herceptin®) for the treatment of breast cancer.

Keywords

High Performance Liquid Chromatography (HPLC), Size Exclusion Chromatography (SEC), TSKgel G3000SW_{XL}, UV detector, IgG, monoclonal antibody (mAb), MabThera®, RIABNI™, rituximab, Herceptin®, trastuzumab, therapeutic antibody, biosimilar, biopharmaceutical, higher-order structure (HOS), quaternary structure, Critical Quality Attribute (CQA), aggregate

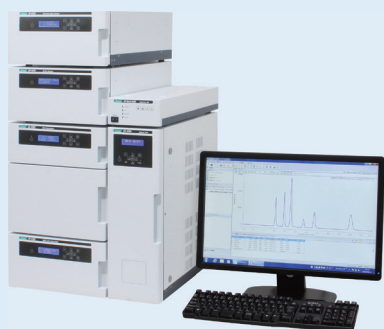
Experimental

Sample

- IgG, from human serum (Merck)
- MabThera® (rituximab, Roche) 100 mg/10 mL
- RIABNI™ (rituximab, AMGEN) 100 mg/10 mL
- Herceptin® (trastuzumab, Roche) 150 mg powder for concentrate for solution for infusion

Measurement and analysis system

LC-4000 HPLC system



<System configuration>

Pump: PU-4080i (with DG-4000-04)
Autosampler: AS-4050i (with TC-4000-1)
Column oven: CO-4060
UV detector: UV-4075 (with inert flow cell unit)
Chromatography data system:
ChromNAV Ver. 2
(with GPC calculation program)

Measurement Conditions

Column:	TSKgel G3000SW _{XL} (7.8 mmI.D. × 300 mmL, 5 μm)	Eluent:	0.2 mol/L sodium phosphate buffer (pH 6.7)
Flow rate:	0.8 mL/min.	Column Temp.:	25 °C
Wavelength:	220 nm	Injection volume:	10 μL
Standard:	Protein standard mix 15–600kDa (Merck) 30 mg/mL in water		

Results

Fig. 1 shows UV chromatograms of a blank (ultrapure water) and a protein standard sample. The column used in this experiment has an exclusion limit with globular proteins of 800 kDa and a measurement range of approximately 10 to 500 kDa. As shown in Fig. 2, a molecular weight calibration curve was created from the retention times and molecular weights of the four components, except for p-aminobenzoic acid, that eluted after the permeation limit (around 13.8 minutes). Based on this calibration curve, the molecular weight of each sample was calculated.

Fig. 3 shows a UV chromatogram of IgG derived from human serum. Table 1 shows the calculation results of the peak top molecular weight (Mp) of IgG derived from human serum and the area ratio of respective sections.

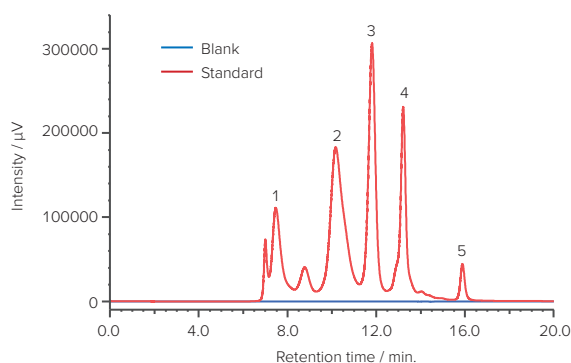


Fig. 1 UV chromatograms of a blank and a protein standard
1: Thyroglobulin (670 kDa), 2: γ -globulins (150 kDa), 3: Ovalbumin (44.3 kDa), 4: Ribonuclease A (13.7 kDa), 5: *p*-amino-benzoic acid (0.137 kDa)

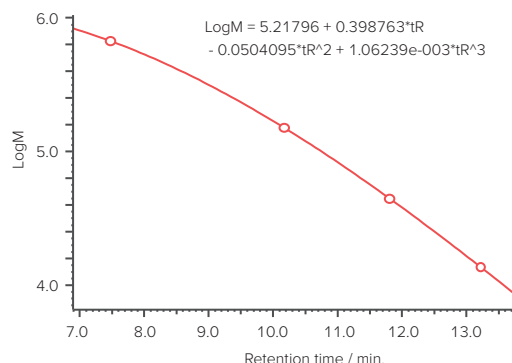


Fig. 2 Molecular weight calibration curve

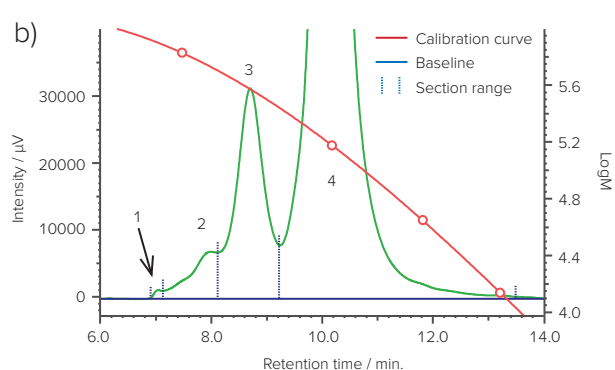
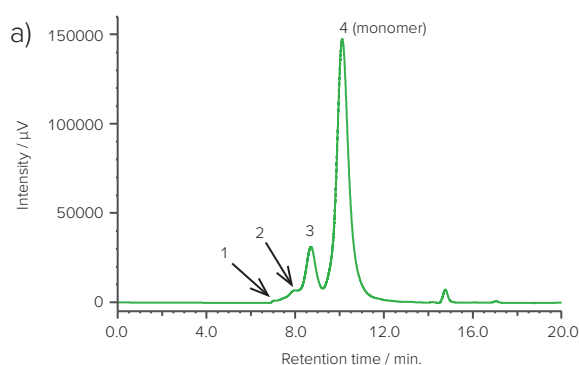


Fig. 3 UV chromatogram of IgG derived from human serum
a) Full scale, and b) enlarged view near the baseline at 6-14 min. (shown with molecular weight calibration curve)
Pretreatment: 1 mg of IgG derived human serum was dissolved in 1 mL of ultrapure water.

Table 1. Calculation results of peak top molecular weights and area ratios of IgG derived from human serum converted based on the results of standard sample

Peak No.	Section range [min.]	Baseline range [min.]	tR [min.]	Mp	Section area	Area %
1	6.873–7.120	6.000–18.000	7.043	788938	11988	0.16
2	7.120–8.107	6.000–18.000	7.987	535189	234438	3.04
3	8.107–9.243	6.000–18.000	8.703	371957	1160566	15.04
4	9.243–13.573	6.000–18.000	10.117	155903	6308812	81.76

Similarly, Figs 4 to 6 and Tables 2 to 4 show the UV chromatograms and calculation results of molecular weight for MabThera®, RIABNI™ and Herceptin®, respectively. The peaks that elute after the permeation limit in Figs 4 to 6 are considered to be the peaks derived from the additives contained in each antibody drug.

Comparison of the Mp of the monomer peaks (peak 3) in Tables 1 to 4 revealed that the molecular weight of each antibody drug was about 10 kDa smaller than that of IgG. The molecular weights of MabThera®, RIABNI™ and Herceptin® were calculated to be 145 kDa (published value: 145 kDa), 146 kDa (published value: 145 kDa) and 149 kDa (published value: 148 kDa), respectively. All the molecular weights were close to the published values.^{14–16)}

In addition, judging from their molecular weights (27 to 28 kDa), peak 4 eluted between 12 to 13 minutes in each sample except for IgG, which is thought to correspond to one light chain constituting each antibody drug eluted as a fragment peak.

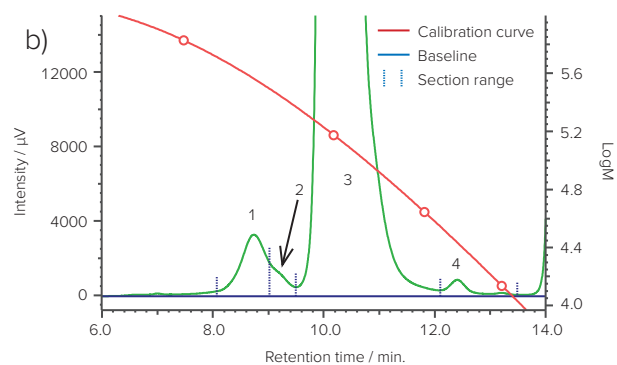
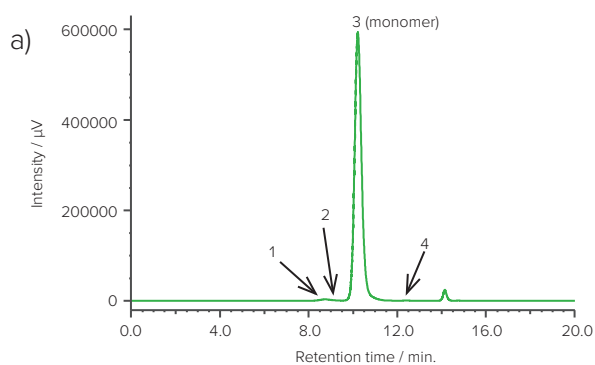


Fig. 4 UV chromatogram of MabThera®

a) Full scale, and b) enlarged view near the baseline at 6-14 min (shown with molecular weight calibration curve)
Pretreatment: 100 mg/10 mL of MabThera® was diluted with ultrapure water to 1 mg/mL.

Table 2. Calculation results of peak top molecular weights and area ratios of MabThera® converted based on the results of standard sample

Peak No.	Section range [min.]	Baseline range [min.]	tR [min.]	Mp	Section area	Area %
1	8.100–9.043	6.000–18.000	8.743	363886	105056	0.78
2	9.043–9.510	6.000–18.000	9.257	270669	28743	0.21
3	9.510–12.117	6.000–18.000	10.220	145271	13226663	98.80
4	12.117–13.570	6.000–18.000	12.407	27215	27055	0.20

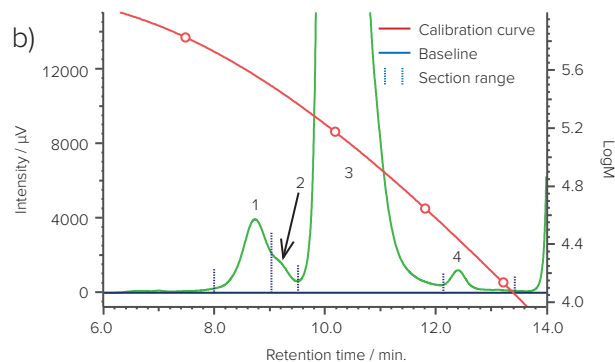
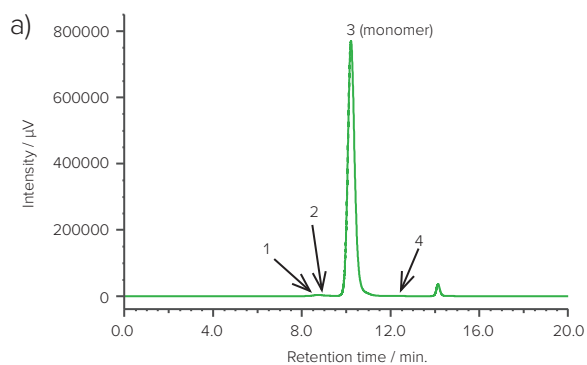


Fig. 5 UV chromatogram of RIABNI™

a) Full scale, and b) enlarged view near the baseline at 6-14 min (shown with molecular weight calibration curve)
Pretreatment: 100 mg/10 mL of RIABNI™ was diluted with ultrapure water to 1 mg/mL.

Table 3. Calculation results of peak top molecular weights and area ratios of RIABNI™ converted based on the results of standard sample

Peak No.	Section range [min]	Baseline range [min]	tR [min]	Mp	Section area	Area %
1	8.027–9.043	6.000–18.000	8.737	365223	125379	0.73
2	9.043–9.507	6.000–18.000	9.183	282810	36310	0.21
3	9.507–12.093	6.000–18.000	10.217	145605	17083631	98.86
4	12.093–13.630	6.000–18.000	12.403	27291	34582	0.20

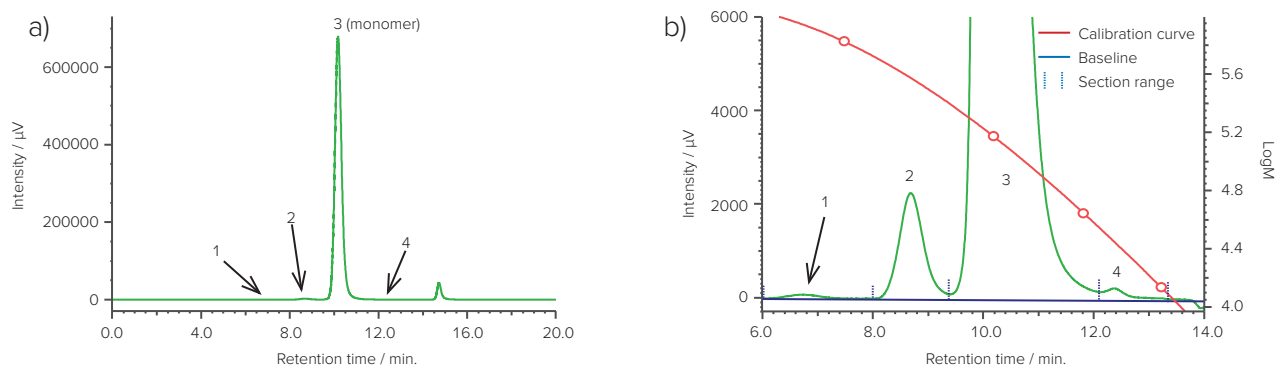


Fig. 6 UV chromatogram of Herceptin®
a) Full scale, and b) enlarged view near the baseline at 6-14 min. (shown with molecular weight calibration curve)
Pretreatment: 1 mg of Herceptin® was dissolved in 1 mL of ultrapure water.

Table 4. Calculation results of peak top molecular weights and area ratios of Herceptin® converted based on the results of standard sample

Peak No.	Section range [min]	Baseline range [min]	tR [min]	Mp	Section area	Area %
1	6.057–8.050	6.000–18.000	6.737	873721	3800	0.03
2	8.050–9.387	6.000–18.000	8.690	374673	70616	0.48
3	9.387–12.107	6.000–18.000	10.183	148974	14738698	99.45
4	12.107–13.373	6.000–18.000	12.37	28058	7586	0.05

Table 5. Comparison of area ratios of HMWS, monomer, and LMWS for each sample

MW classification \ Sample	HMWS	Monomer	LMWS
MabThera®	0.99 %	98.80 %	0.20 %
RIABNI™	0.94 %	98.86 %	0.20 %
Herceptin®	0.51 %	99.45 %	0.05 %

Table 5 shows the results of comparing the area ratios of monomer, High Molecular Weight Species (HMWS) containing aggregates that elute earlier than the monomer and Low Molecular Weight Species (LMWS) that elute later than the monomer in each sample. These results show that the innovator and biosimilar of rituximab (MabThera® and RIABNI™) exhibit very similar area ratios. Comparison with the data reported by Goyon et al. revealed that HMWS of MabThera® was 0.99% (literature value: 0.7%) and LMWS was 0.20% (literature value: 0.3%), both of which were close to their literature values. On the other hand, in the case of innovator trastuzumab (Herceptin®), the comparison value of LMWS is not available, but the HMWS was 0.51% (literature value: 0.4%), which is also very close to the literature value.¹⁷⁾

Conclusions

Through this experiment, it was confirmed that the measurement method using SEC can successfully separate and detect monomer and aggregates containing multimers in antibody drugs. In addition, it was also confirmed that this method is a powerful tool for evaluating the similarity of HOS, which is an important item of CQAs.

Additional Information

- Application: Analysis of Aggregates in Antibodies by SEC.
<https://www.jasco-global.com/solutions/analysis-of-aggregates-in-antibodies-by-sec/>
- Product information: HPLC.
<https://www.jasco-global.com/product-chromatography/integrated-hplc-system/>

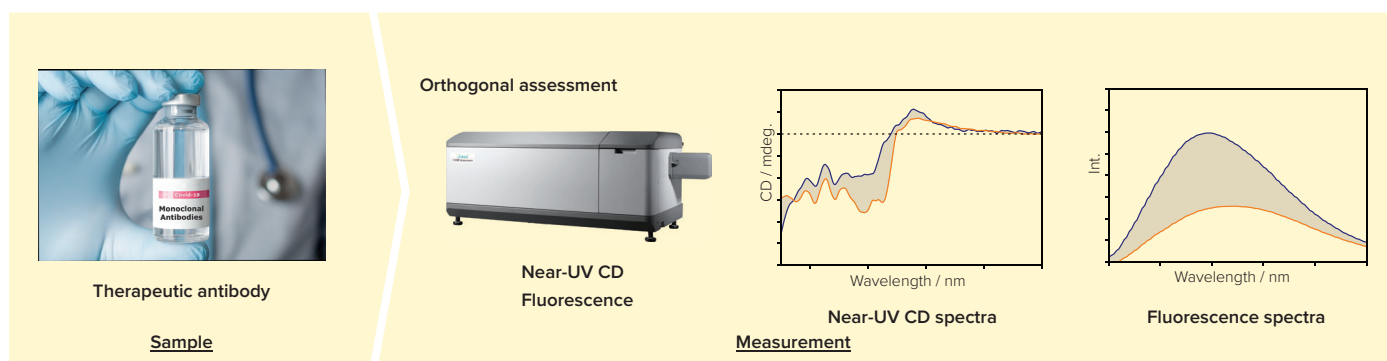
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About the Author

Satoe Iijima is a chromatography application scientist at JASCO Corporation (Tokyo, Japan) specializing in LC, LC/MS, SFC, and SFC/MS.

Orthogonal Tertiary Structure Similarity Assessment using Near-UV CD and Fluorescence Spectroscopy



Graphical abstract

Introduction

In recent years, there has been a rapid increase in the development, manufacturing and marketing of antibody drugs. Antibody drugs are expected to become therapeutics for malignant tumors and unmet medical needs because of their high specificity in targeting only antigens expressed on target cells.

ICH Q5E¹⁾ for biopharmaceuticals, including antibody drugs, specifies that the similarity of the higher-order structure (HOS) should be evaluated before and after changes in the manufacturing process. Circular Dichroism (CD) spectroscopy is widely used to evaluate the quality of antibody drugs because it can easily obtain information on the secondary and tertiary structure of antibodies.

Along with innovator antibody drugs, many biosimilar drugs with the same amino acid sequence as the innovator, but produced using different expressing cells, are now also being used. With the accelerated development of such biosimilars, there has been a growing focus on how to compare the HOS of innovators and biosimilars, and guidelines have been issued by the FDA,²⁾ EMA³⁾ and other institutions around the world.^{4,5)} These guidelines stress orthogonal assessment of the HOS of innovators and biosimilars using multiple methods based on different principles, increasing the importance of using multiple analytical instruments to measure a single critical quality characteristic.

The J-1500 CD spectrometer, in combination with the FMO-522 fluorescence monochromator and the FDT-538 fluorescence detector, can measure near-UV CD spectra and fluorescence spectra that provide information on protein tertiary structure, making it easy to perform orthogonal assessments. Here, we report the results of an orthogonal assessment of Herceptin® and MabThera® antibody drugs with different targets and formulation conditions, by measurement of near-UV CD and fluorescence spectra.

Keywords

Circular Dichroism (CD) spectroscopy, fluorescence spectroscopy, Herceptin®, MabThera®, trastuzumab, rituximab, therapeutic antibodies, biopharmaceutical, higher-order structure (HOS), tertiary structure, orthogonal assessment, inner-filter effects (IFE)

Experimental

Sample

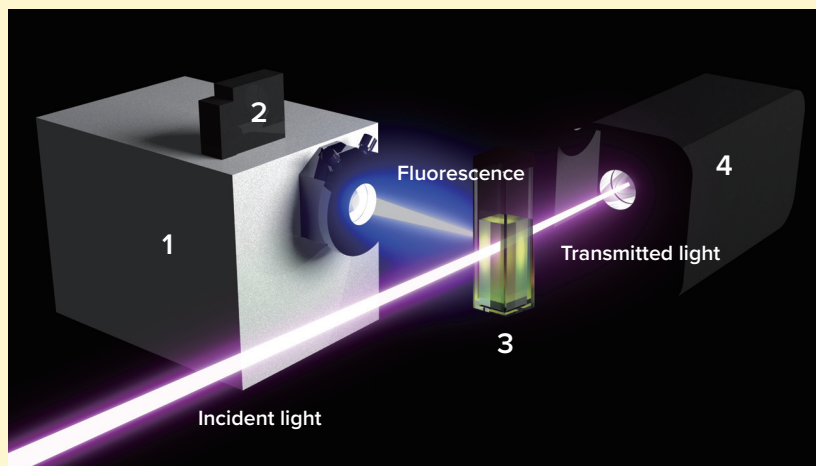
- Trastuzumab
Herceptin®(Roche): powder dissolved in ultrapure water to 10 mg/mL
Additives: trehalose hydrate 4.7 mg/mL, L-histidine hydrochloride hydrate 0.11 mg/mL, L-histidine 2.6×10^{-3} mg, polysorbate 2.1×10^{-2} mg/mL
- Rituximab
MabThera® (Roche) 100 mg/10 mL
Additives: sodium citrate dihydrate 7.4 mg/mL, sodium chloride 9.0 mg/mL, polysorbate 80 0.7 mg/mL

Measurement and analysis system

J-1500 CD spectrophotometer with FMO-522/FDT-538 fluorescence monochromator/detector



- Successive measurement for CD and fluorescence spectrum
- Orthogonal assessment for protein tertiary structure



Measurement overview
(1. FMO-522 monochromator, 2. FDT-538 detector, 3. Sample, 4. CD/UV detector)

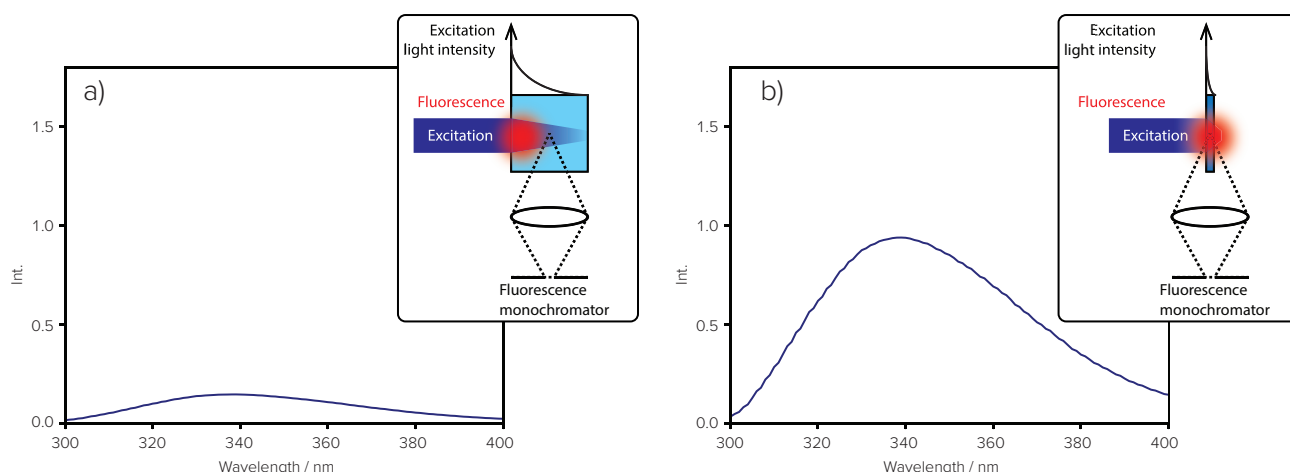


Fig. 1 Effect of cuvette shape on IFE reduction
a) 1 mg/mL Herceptin®, optical path length: 10 mm, b) 10 mg/mL Herceptin®, optical path length: 1 mm

Measurement Conditions

<Near-UV/CD measurement>

Bandwidth: 1.0 nm
D.I.T.: 4 sec.
Accumulation: 9

Scanning speed: 20 nm/min.
Data pitch: 0.1 nm
Optical path length: 1 mm

<Fluorescence measurement>

Excitation bandwidth: 2.0 nm
Excitation wavelength: 280 nm
Emission bandwidth: 10 nm
D.I.T.: 1 sec.

Accumulation: 4
Data pitch: 1 nm
Optical path length: 1 mm

Results

Confirmation and correction of inner filter effects

To measure the near-UV CD spectra of antibodies, an optical path length of 10 mm and a concentration of about 1 mg/mL are considered optimal. When measuring fluorescence spectra under these conditions, the excitation light is absorbed by the sample and does not reach the center of the cuvette, resulting in a significant decrease in fluorescence spectral intensity (Fig. 1a).

This phenomenon is called the Inner-Filter Effect (IFE). In this report, to suppress the IFE, the optical path length and sample concentration were set to 1 mm and 10 mg/mL, respectively (Fig. 1b), thereby reducing the IFE and achieving both near-UV CD and fluorescence spectrum measurement conditions.

Fig. 2 shows the concentration dependence of the fluorescence spectral intensity for Herceptin®. Fig. 2a shows the measured fluorescence spectrum, where it can be seen that a good signal-to-noise ratio was obtained. On the other hand, Fig. 2b shows the fluorescence intensity at 340 nm plotted against concentration and indicates that the linearity between the concentration and fluorescence intensity is impaired. This is thought to be due to the IFE, which could not be eliminated using the 1 mm optical path length cuvette alone. Therefore, IFE correction⁶⁾ was performed using Equation (1).

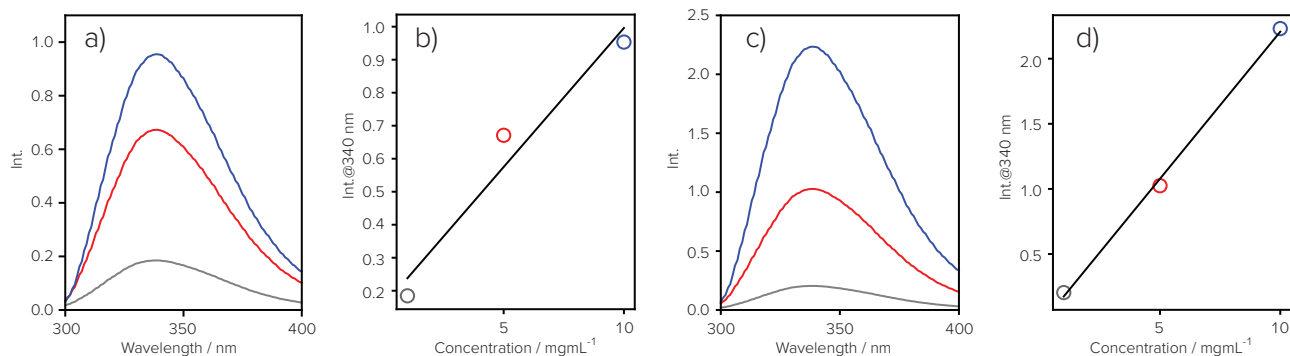


Fig. 2 Confirmation of IFE correction under near-UV CD and fluorescence spectral measurement conditions.
a) and b): Fluorescence spectra and intensity at 340 nm of Herceptin® c) and d): IFE-corrected fluorescence spectra and intensity at 340 nm of Herceptin® 1 mg/mL (gray), 5 mg/mL (red), and 10 mg/mL (blue)

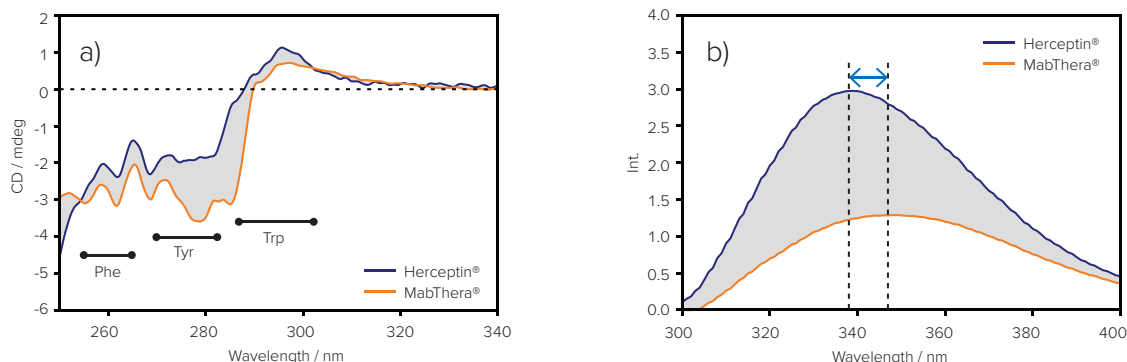


Fig. 3 Orthogonal assessment of tertiary structure of Herceptin® and MabThera®
a) Near-UV/CD spectrum, b) Fluorescence spectrum

$$F_{corr} = F_{obs} \cdot 10 \frac{Abs_{ex} + Abs_{em}}{2}$$

F_{corr} : Fluorescent intensity after correlation

F_{obs} : Measured fluorescent intensity

Abs_{ex}, Abs_{em} : Absorbance at excitation and emission wavelength

Abs_{ex} is the absorbance at 280 nm, which is the excitation wavelength when measuring the fluorescence spectrum, and therefore can be obtained from the absorption spectrum acquired by the CD spectrometer at the same time as the near-UV CD spectrum. Since Abs_{em} is the absorbance at the fluorescence wavelength, an absorption spectrum in the wavelength range of the fluorescence spectrum (300 to 400 nm) is required. However, since the measurement range for the near-UV CD spectrum is 250 to 340 nm, a separate measurement of the 300 to 400 nm absorption spectrum is required. In the case of proteins, the absorbance at 300 to 400 nm is extremely small, so Abs_{em} can be approximated as 0, and the information necessary for IFE correction can be obtained only by measuring the near-UV CD spectrum. The results of IFE correction of fluorescence spectra using this method show that the fluorescence intensity at 340 nm has a highly linear dependence on the concentration, with a correlation coefficient of 0.999 (Figs. 2c and 2d). Thus, the fluorescence spectrum can be corrected and a high-quality fluorescence spectrum can be obtained together with the CD spectrum.

Comparison of tertiary structures of antibody drugs

Near-UV/CD and fluorescence spectra of Herceptin® and MabThera® are shown in Fig. 3. The near-UV CD spectrum is normalized by the absorbance at 280 nm, and the fluorescence spectrum is normalized by the absorbance at 280 nm after IFE correction is performed, as for the near-UV CD spectrum.

In the near-UV CD spectra, there are significant differences in the spectral shape at 295 nm, 280 nm and near 260 nm for the two antibody drugs. These differences indicate different environments around the side chains of amino acids for tryptophan, tyrosine and phenylalanine, respectively, indicating that the two antibody drugs form different tertiary structures (Fig. 3a). The fluorescence spectra also show differences in peak position and intensity for the two antibody drugs (Fig. 3b). This reflects the difference in polarity around tryptophan, and as with the near-UV CD spectrum, indicates that the two antibody drugs form different tertiary structures. These results confirm the differences in the tertiary structure of Herceptin® and MabThera® by using two different measurement principles.

Conclusions

The J-1500 CD/fluorescence measurement system has made it possible to detect differences in the tertiary structure of Herceptin® and MabThera®, using orthogonal methods based on different principles. This system can detect even slight differences in tertiary structure with high sensitivity, making it effective for development and quality control of antibody drugs.

Additional Information

- Product information: CD spectrophotometer.
<https://www.jasco-global.com/product-spectroscopy/circular-dichroism/>

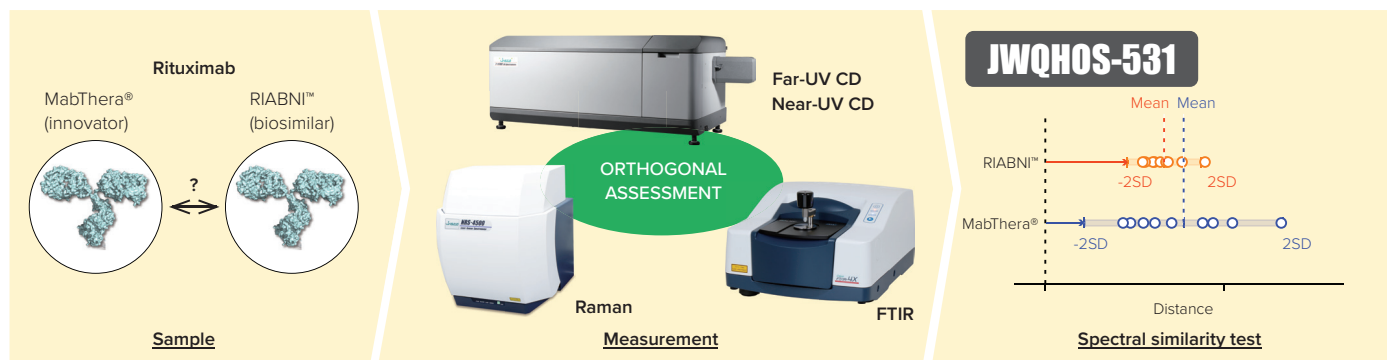
References

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About the Author

Taiji Oyama is a spectroscopy application scientist at JASCO Corporation (Tokyo, Japan) specializing in CD, IR, Raman, UV-vis, and fluorescence spectroscopy.

4-way Orthogonal HOS Similarity Assessment of Biosimilar Using Multi Spectroscopic Technique and Statistical Calculation



Graphical abstract

Introduction

The market for therapeutic antibodies has been dramatically expanding over the past decades. Antibody drugs exhibit therapeutic effects, such as inhibiting the growth of malignant tumor cells and the activity of immune cells by binding antigens expressed in target cells with high affinity and specificity. Such antibody drugs are expected to be effective therapeutic agents for unmet medical needs. Many antibody drugs are being used worldwide, and along with leading antibody drugs called innovators, many biosimilars, which have the same amino acid sequence as the innovators but are produced using different expression cells, are also beginning to be used.

Biosimilars tend to be thought of as having the same function and structure as the innovators because they have the same amino acid sequence; however, antibodies are subjected to various stimuli and post-translational modifications during the production process, which may result in a significant loss of function. The main factors are thermal stimulation and fragmentation by proteases; therefore, measuring changes in antibody structure due to stimulation or modification is an essential step in R&D and quality control of antibody drugs. Guidelines issued by the U.S. Food and Drug Administration (FDA), European Medicine Agency (EMA) and other regulatory agencies indicate the importance of measuring Critical Quality Attributes (CQAs) of therapeutic antibodies by analytical methods in the quality assessment of biosimilars.^{1–4)}

In addition, these guidelines state that a CQA should be evaluated using multiple analytical methods with different principles, i.e., orthogonal methods, rather than using a single analytical method.

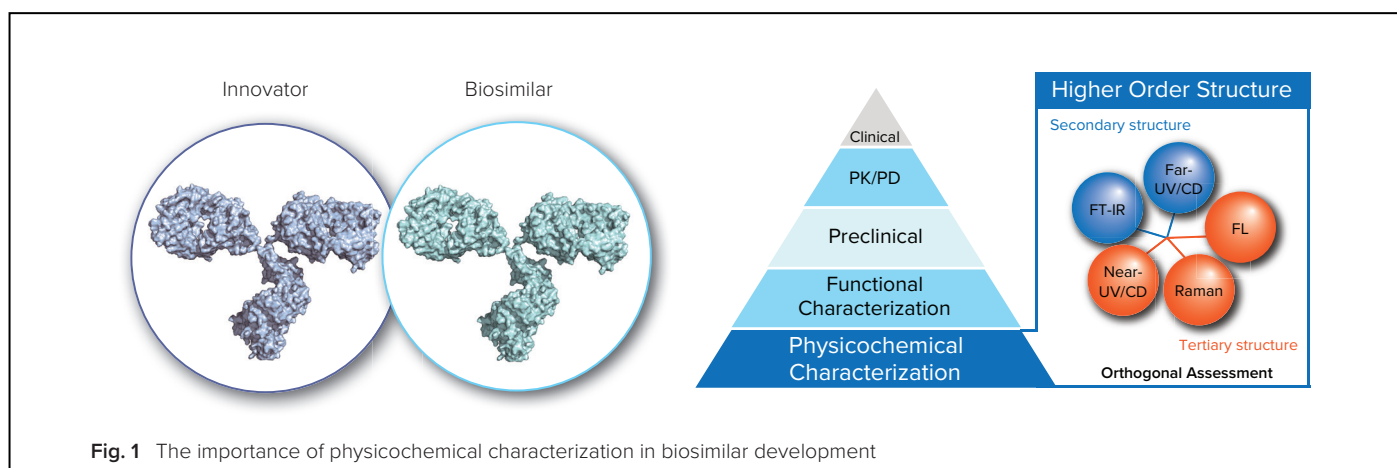


Fig. 1 The importance of physicochemical characterization in biosimilar development

As shown in Fig. 1, it is recommended to use far-UV CD and FTIR for the evaluation of secondary structure, and near-UV CD, Raman, fluorescence, or two or more of these methods for the analysis of tertiary structure.

Furthermore, there is the requirement for an objective assessment of structural similarity described in ICH-Q5E. The FDA's guidance places the most important Tier 1 method on objective and statistical evaluation of analytical similarity between biosimilars and their innovators. There is a growing demand for a process to objectively determine the similarity of spectra.^{5,6)}

Here we report the results of a higher-order structure (HOS) similarity assessment of Herceptin® (the innovator of Trastuzumab), MabThera® (the innovator of Rituximab) and RIABNI™ (a biosimilar to MabThera®) using the HTCD Plus high-throughput CD spectrometer, FT/IR-4X FTIR spectrometer, NRS-4500 Raman microscope and the qHOS program.

Keywords

Circular Dichroism (CD) spectroscopy, Fourier-transform infrared (FTIR) spectroscopy, Attenuated Total Reflectance (ATR), Raman spectroscopy, similarity assessment, Herceptin®, MabThera®, trastuzumab, rituximab, therapeutic antibody, biosimilar, biopharmaceutical, higher-order structure (HOS), secondary structure, tertiary structure, orthogonal assessment, high-throughput screening

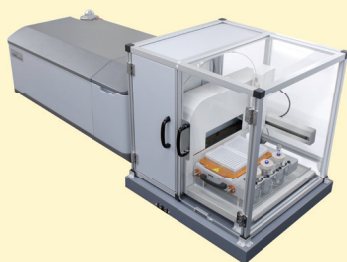
Experimental

Sample

- Trastuzumab
Herceptin® (Roche): powder dissolved in ultrapure water to 10 mg/mL
Additives: trehalose hydrate 4.7 mg/mL, L-histidine hydrochloride hydrate 0.11 mg/mL, L-histidine 7.4×10^{-2} mg/mL, polysorbate 2.1×10^{-2} mg/mL
- Rituximab
MabThera® (innovator, Roche) and RIABNI™ (biosimilar, AMGEN) 100 mg/10 mL
Additives: sodium citrate dihydrate 7.4 mg/mL, sodium chloride 9.0 mg/mL, sodium hydroxide 9.0 mg/mL, polysorbate 80 0.7 mg/mL

Measurement and analysis system

HTCD Plus high-throughput CD measurement system



- Fully automated measurement of up to 192 samples.
- Pre-registered flush method for protein or DNA/ RNA samples can be selected to eliminate sample carry-over.

FT/IR-4X FTIR spectrometer with ATR PRO 4X single-reflection ATR



- High S/N and resolution with small body size.
- One drop (approx. 10 μ L) ATR measurement

NRS-4500 Raman spectrometer



- Microanalysis
- Chemical imaging

JWQHOS-531 qHOS



- Statistical HOS similarity test program developed by JASCO.
- High-sensitive detection of spectral difference by noise-weighted Euclidean distance.
- Support for spectra obtained by multiple instruments.

Measurement Conditions

<Far-UV/CD measurement>

Optical path length: 0.2 mm
Bandwidth: 1.0 nm

Auto washing: On
Scanning speed: 20 nm/min

<Near-UV/CD measurement>

Optical path length: 0.5 mm
Bandwidth: 1.0 nm

Auto washing: On
Scanning speed: 50 nm/min

<IR measurement>

Detector: TGS
ATR crystal: Diamond

Resolution: 4 cm⁻¹
Accumulation: 128

<Raman measurement>

Laser: 532 nm
Grating: 900 gr./mm

Exposure: 45 sec.
Number of scans: 32

Analysis conditions

Distance calculation method:
Euclidean distance
Weighting: Noise

Statistical testing: Welch's *t*-test*
Significance level: 0.05

*Welch's *t*-test tests for significant differences in spectral distances, taking into account the variances in both standard and sample spectral distances. Data for multiple standard and unknown samples are needed. This method is used for comparing the HOS of innovators and biosimilars, the HOS for antibody drugs before and after manufacturing process changes and for lot inspections.

Results

First, to confirm that this system can discriminate between the tertiary structures of antibody drugs with different targets and formulation conditions, we determined the similarity of the tertiary structures of MabThera®, the innovator of Rituximab, and Herceptin®, the innovator of Trastuzumab (Fig. 2). The shapes of the near-UV/CD spectra of MabThera® and Herceptin® differ significantly (Fig. 2a). The Cotton effect at around 260, 280 and 290 nm reflects the intermolecular interactions and environment of aromatic amino acids, such as phenylalanine, tyrosine and tryptophan, respectively, indicating that the tertiary structures of these antibody drugs are different. MabThera® and Herceptin® have similar amino acid sequences and the difference is only in the Fv region, but the near UV/CD spectra can sensitively detect the difference in their tertiary structures.

Similarly, the average distances between MabThera® and Herceptin® calculated from the CD spectra show a large difference (Fig. 2b). The *p*-value obtained from the *t*-test is below the significance level of 0.05, indicating that Herceptin® has a different tertiary structure to MabThera®. These results show that the system can clearly distinguish differences in the tertiary structure of different antibody drugs.

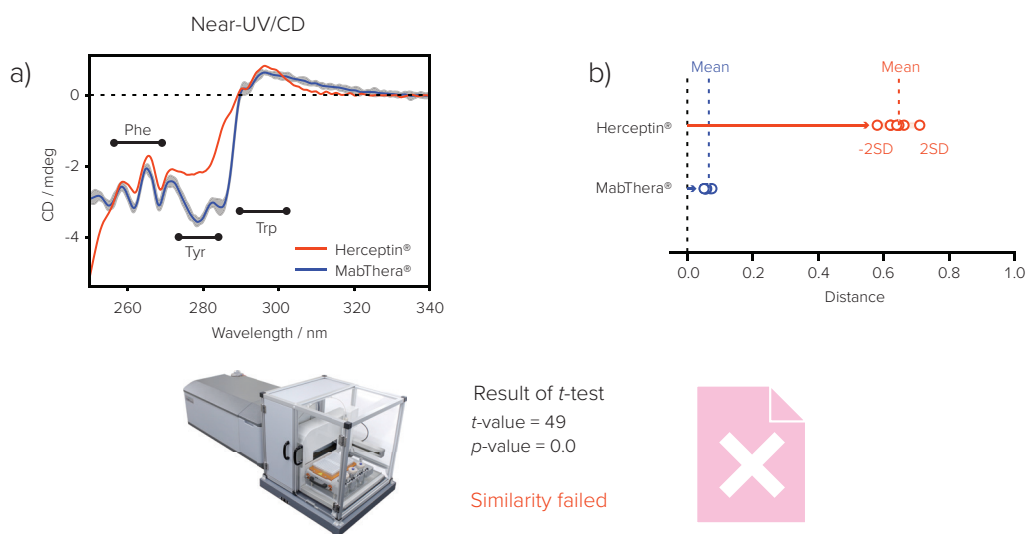


Fig. 2 Similarity assessment of the tertiary structure of MabThera® and Herceptin®, antibody drugs with different targeting and formulation conditions.

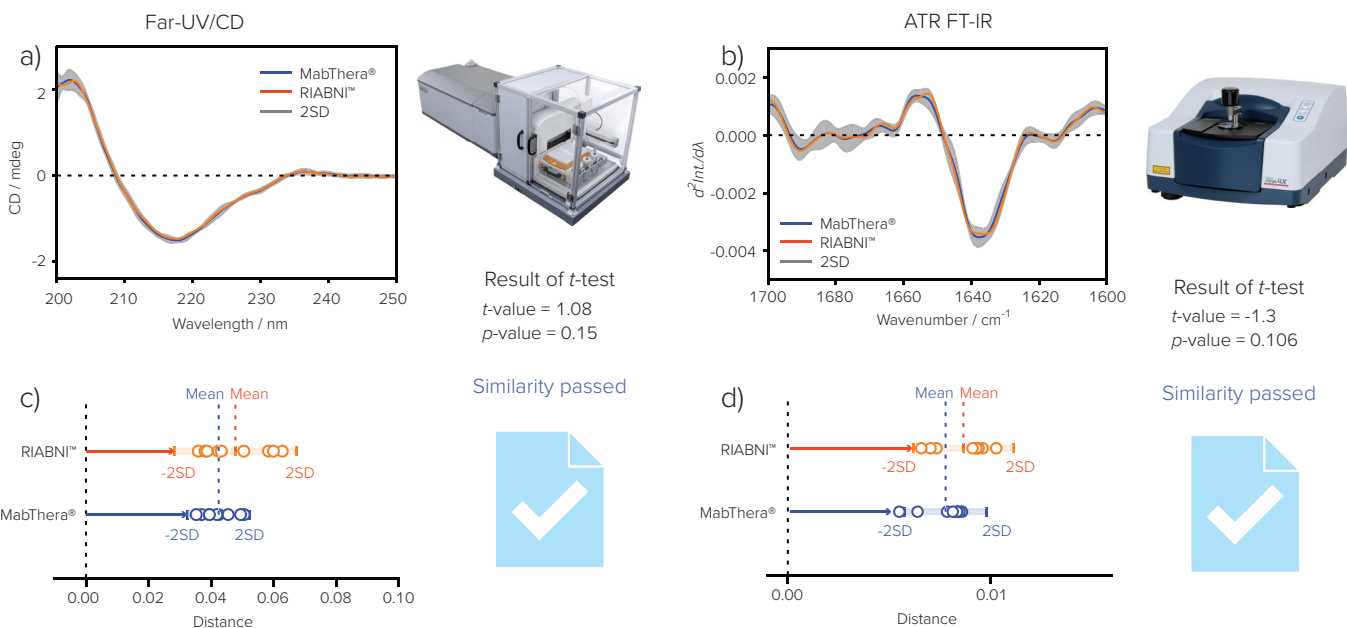


Fig. 3 Orthogonal similarity assessment for the secondary structure of MabThera® and RIABNI™

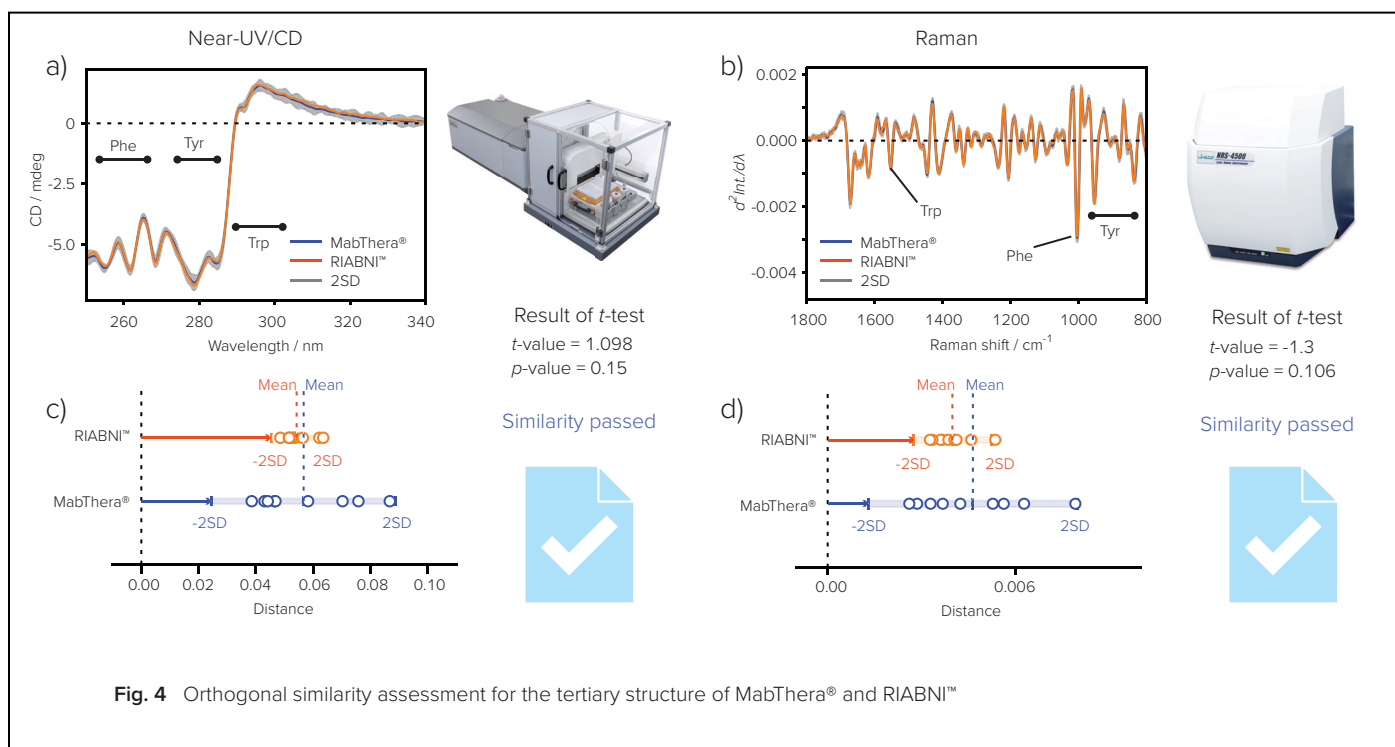


Fig. 3 shows the results of orthogonal similarity assessment of the secondary structure. Both MabThera® and RIABNI™ Far-UV/CD spectra show a negative Cotton effect at 217 nm, which is characteristic of β -sheets (Fig. 3a). IR spectra around 1700 to 1600 cm^{-1} are derived from amide I, and the spectral shape is known to change reflecting the secondary structure (Fig. 3b). The secondary differential spectrum of amide I shows a negative peak around 1640 cm^{-1} , which is characteristic of β -sheets, indicating that the structure is rich in β -sheets, as indicated by the CD spectrum. Furthermore, both the CD and IR spectra of MabThera® and RIABNI™ show good agreement in spectral shape.

The distributions of the distances between MabThera® and RIABNI™ are close to each other (Figs. 3c and 3d). The p -value is larger than the significance level of 0.05.

Fig. 4 shows the results of orthogonal similarity assessment of the tertiary structure. Although the near-UV/CD spectra of MabThera® and Herceptin® show significant differences despite most of the amino acid sequences being identical, the near-UV/CD spectra of MabThera® and its biosimilar, RIABNI™, show very good agreement (Fig. 4a).

The same is true for the Raman spectra: the Raman second derivative spectra at 1800 to 700 cm^{-1} including tryptophan (around 1550 cm^{-1}), phenylalanine (around 1000 cm^{-1}) and tyrosine (around 850 cm^{-1}) are identical for MabThera® and RIABNI™. The entire spectrum below 1600 cm^{-1} is in agreement with those for MabThera® and RIABNI™ (Fig. 4b).

The distribution of the distances between MabThera® and RIABNI™ are close to each other (Figs. 4c and 4d). The p -value is larger than the significance level of 0.05.

The similarity of the biosimilar and innovator HOS was confirmed in the secondary and tertiary structures.

Table 1. Raman shift and assignments to tertiary structure for protein

Raman shift	Assignment
1555 cm^{-1}	Tryptophan
1000 cm^{-1}	Phenyl alanine
850 cm^{-1}	Tyrosine
750 cm^{-1}	Tryptophan

Conclusions

By combining the latest HTCD Plus, FT/IR-4X and NRS-4500 with qHOS, we were able to comprehensively assess the HOS similarity of biosimilars against innovators in an orthogonal manner. This system can be used not only to evaluate the similarity of biosimilars, but also to easily and accurately determine the changes in the HOS of antibody drugs caused by post-translational modifications and external stimuli.

Additional Information

- Application: Orthogonal HOS Similarity Assessment of Biosimilar Using Multi Spectroscopic Technique and Statistical Calculation.
<https://www.jasco-global.com/solutions/orthogonal-hos-similarity-assessment-of-biosimilar-using-multi-spectroscopic-technique-and-statistical-calculation/>
- Product information: CD spectrophotometer.
<https://www.jasco-global.com/product-spectroscopy/circular-dichroism/>
- Product information: FTIR spectrometer.
<https://www.jasco-global.com/product-spectroscopy/ftir-spectrometer/>
- Product information: Raman imaging microscope.
<https://www.jasco-global.com/product-spectroscopy/raman/>

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- 3) Department of Biotechnology, Government of India: “*Guidelines on similar biologics: Regulatory requirements for marketing authorization in India*” (2012)
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About the Author

Taiji Oyama is a spectroscopy application scientist at JASCO Corporation (Tokyo, Japan) specializing in CD, IR, Raman, UV-vis, and fluorescence spectroscopy.

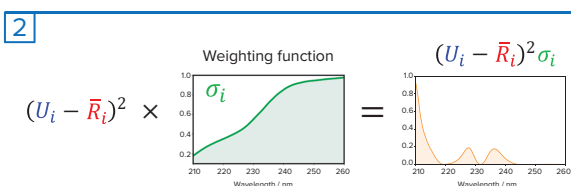
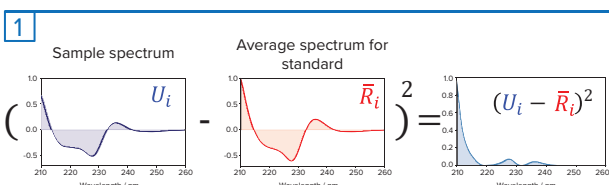
Spectral Similarity Test - qHOS Program

The qHOS program converts the spectral difference into a parameter called the "Euclidean distance". By weighting the Euclidean distance to account for noise and spectral variation, differences between spectra can be detected with high sensitivity ("weighted Euclidean distance"). The qHOS program allows statistical significance testing to determine whether the differences between spectra are due to noise, sample preparation errors or are in fact chemically meaningful. Three types of statistical tests can be selected according to the purpose: Student's *t*-test, Welch's *t*-test and Equivalence test (TOST).

Features of each testing method

Assay	Features
Student's <i>t</i> -test	This method tests for significant differences in spectral distances, taking into account the variance in standard sample spectral distances. Data for multiple standard samples and a single unknown sample are used. This test is used for quality assessment of manufactured antibody drugs.
Welch's <i>t</i> -test	This method tests for significant differences in spectral distances, taking into account the variances in both standard and sample spectral distances. Data for multiple standard and unknown samples are used. This test is used for comparing the HOS of innovators and biosimilars, the HOS for antibody drugs before and after manufacturing process changes, and for lot inspections.
Equivalence test (TOST)	This method tests for significant differences in spectral distances, taking into account the variances in both standard and sample spectral distances. In this method, a range (equivalence margin) is set in which the distance between the spectrum of each standard sample and the average spectrum of standard samples, and the distance between the spectrum of each unknown sample and the average spectrum of unknown samples, are equivalent. Data for multiple standard and unknown samples are used. This test is based on the guidelines for similarity tests published by the FDA.

Calculation of distance between standard and sample spectra



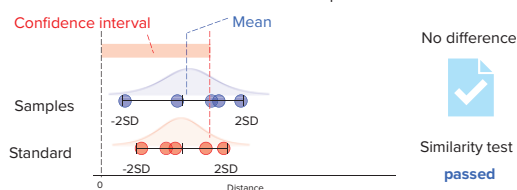
3

Weighted Euclidean distance:

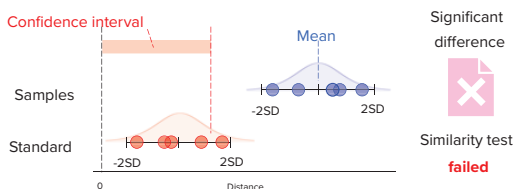
$$E = \sqrt{\frac{1}{n} \sum_{i=1}^{\lambda_n} (U_i - \bar{R}_i)^2 \sigma_i}$$

Evaluate difference in distance between standard and sample

There is a small difference in the distance between the standard and the sample



There is a large difference in the distance between the standard and the sample

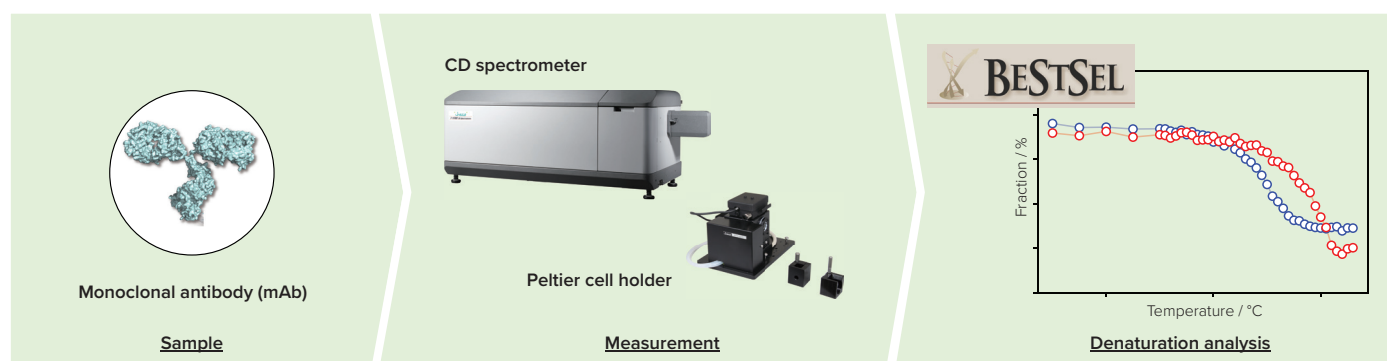


Confidence interval = $Mean_{std} + t_{\alpha}(v) \sqrt{\frac{SD_{sam}^2}{m} + \frac{SD_{std}^2}{n}}$

$t_{\alpha}(v)$: t-value at significance level α
 m : Number of sample
 n : Number of standard

Procedure for spectral similarity assessment.

Thermal Denaturation Analysis of Monoclonal Antibodies by CD Spectroscopy



Graphical abstract

Introduction

Antibodies, which are proteins, form their structure and exert their activity through a complex series of non-covalent bonds, and may lose their activity due to various external stimuli. Therefore, the evaluation of structural stability is extremely important in the development and formulation of candidate antibodies. Thermodynamic stability of antibodies is most frequently evaluated by applying heat stress, and Differential Scanning Calorimetry (DSC) is used to directly measure the calorimetric changes that accompany the transition from the natural state to the denatured state.¹⁾ On the other hand, DSC does not provide detailed information on conformational changes induced by heat. CD spectroscopy is an easy and rapid method for obtaining information on the secondary structure and other higher-order structures of proteins in solution and can be used to evaluate the structure of proteins under perturbations, such as heat. Therefore, CD spectroscopy is widely used as the method that can estimate the specific conformational changes of proteins induced by heat.²⁾

Recently, Andras et al. developed the BeStSel algorithm, which can accurately estimate the proportion of secondary structure in a protein based on the shape of its CD spectrum. BeStSel has the following features: 1) high accuracy in analyzing β -sheet littered proteins such as antibodies, 2) 8 types of secondary structure categories and 3) high accuracy in classifying CATH.^{3,4)} Therefore, we have employed BeStSel to trace the structural changes of antibodies induced by thermal stress in detail using temperature dependent CD spectroscopy.

Keywords

Circular Dichroism (CD) spectroscopy, secondary structure prediction, BeStSel, Herceptin®, trastuzumab, IgG, monoclonal antibody (mAb), therapeutic antibody, biopharmaceutical, secondary structure, denaturation, heat

Experimental

Sample

- Herceptin®(trastuzumab, Roche): powder dissolved in ultrapure water to 0.025 mg/mL
- IgG, from human serum (Sigma Aldrich): powder dissolved in ultrapure water to 0.025 mg/mL

Measurement and analysis system

J-1500 CD spectrometer



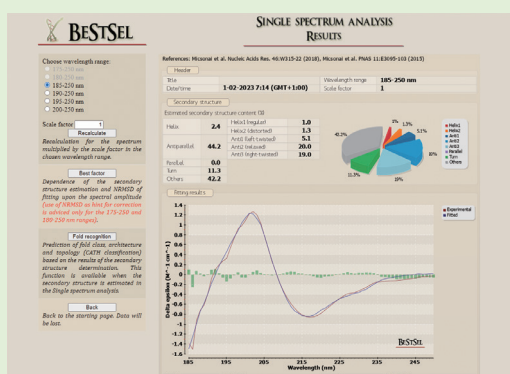
- Accurate and efficient normalization of protein CD spectrum realized by simultaneous CD and Abs measurements.

PTC-510 single-position Peltier cell holder



- Temperature range of 0 to 100 °C with a measurement probe that can be placed inside or adjacent to the cell and a magnetic stirrer to eliminate thermal gradients.

BeStSel



- Secondary structure prediction method developed by Dr. József Kardos at Eötvös Loránd University.
- Highly accurate estimation of β -sheet content by dividing β -sheets into four classes.

Measurement Conditions

Bandwidth: 1.0 nm
D.I.T.: 2 sec.
Data pitch: 0.1 nm
Scanning speed: 50 nm/min.

Accumulation: 4
Optical path length: 0.01 mm
Sample volume: 1.8 μ L
Temperature range: 30 to 95 °C

Results

The thermal denaturation spectra of Herceptin® and human IgG are shown (Figs. 1a and 1b). Both antibodies showed a spectral shape with a minimum around 217 nm, which is characteristic of the β -sheet at 30 °C, but the peak disappeared as the temperature increased.

In order to analyze these structural changes in detail, secondary structure prediction was performed on these temperature change data using BeStSel to determine the change in the ratio of each secondary structure with temperature. The secondary structure ratio of others, β -strands and random coils at room temperature was consistent with that for human IgG (PDB ID 1IGT) obtained from the X-ray crystal structure (data not shown). The ratio of others increased while the ratio of β -strand decreased with heating for both Herceptin® and human IgG (Figs. 1c and 1d). The T_m values for Herceptin® and human IgG estimated from these curves were consistent with previously reported values.^{5,6)}

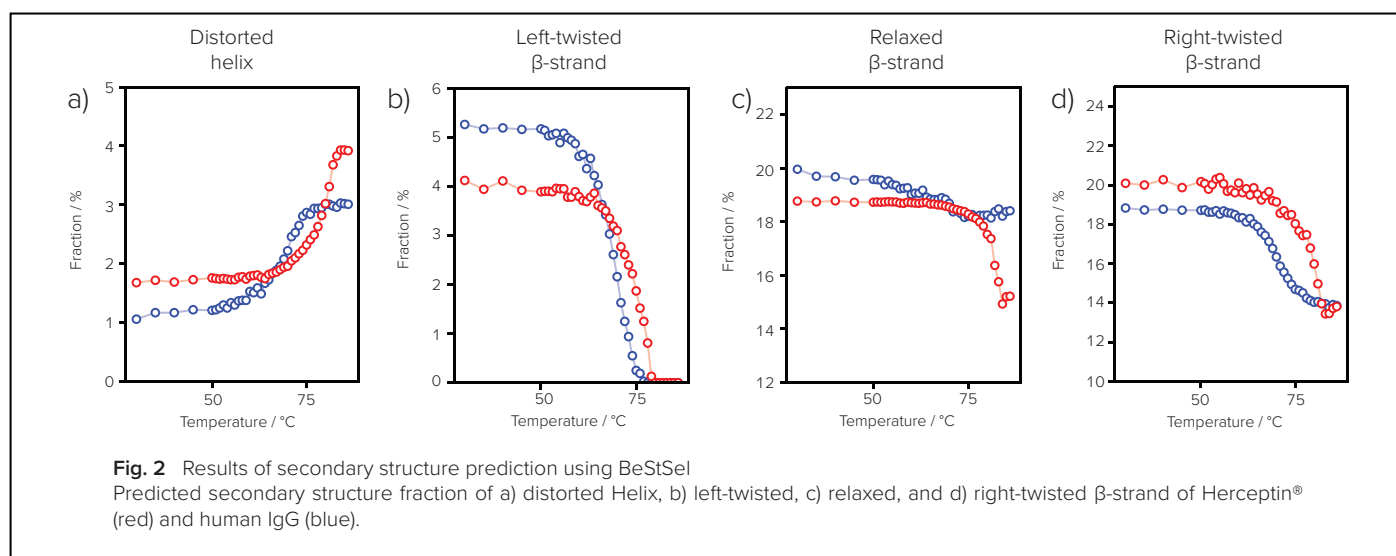
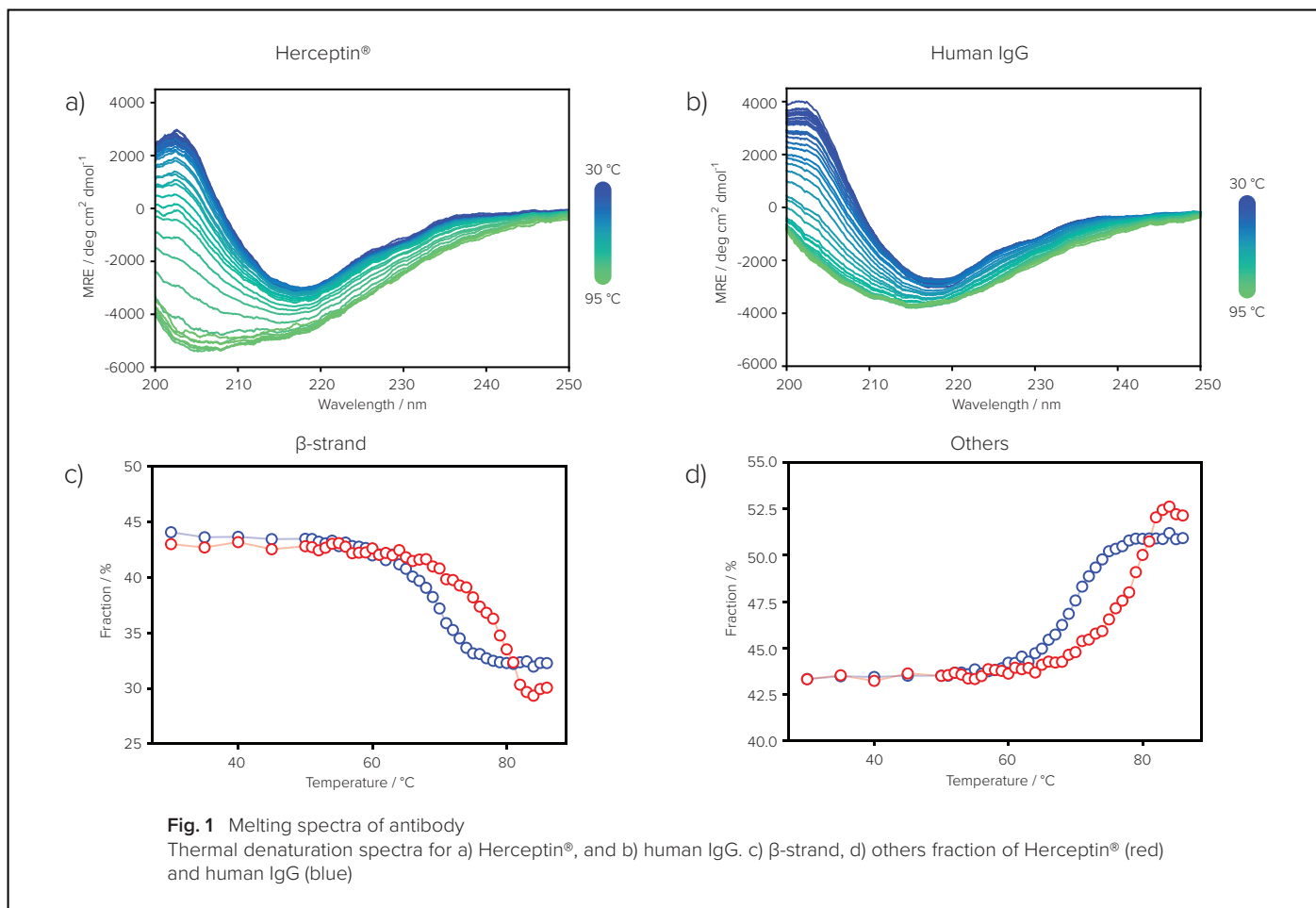


Fig. 2 shows the changes in the secondary structure ratios of the helix and the β -strand as a function of temperature. A large difference was observed between Herceptin® and human IgG. This result indicates that the combination of temperature variation spectroscopy and BeStSel can provide detailed information on the structure of antibodies.

Conclusions

By combining BeStSel with temperature change measurements by CD spectroscopy, we were able to precisely trace the thermally-induced conformational changes of antibodies.

Additional Information

- Product information: CD spectrophotometer.
<https://www.jasco-global.com/product-spectroscopy/circular-dichroism/>

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About the Author

Taiji Oyama is a spectroscopy application scientist at JASCO Corporation (Tokyo, Japan) specializing in CD, IR, Raman, UV-vis, and fluorescence spectroscopy.

Thermal Denaturation Analysis of Monoclonal Antibodies by IR Spectroscopy



Graphical abstract

Introduction

In recent years, therapeutic antibodies have been actively developed, manufactured and marketed. Antibody drugs are often used as therapeutic agents to inhibit the proliferation of malignant tumor cells or the activity of immune cells and are highly anticipated for diseases for which complete treatment is not yet available. Active ingredients of therapeutic antibodies are proteins, which have the potential to undergo aggregation when exposed to various external stimuli. Thus, efforts have been made to minimize aggregation in R&D and manufacturing processes of antibody drugs.¹⁾

Infrared (IR) spectroscopy is widely used to analyze the secondary structure of proteins in trace amounts and in a short time. It is used to analyze the ratio of the secondary structure because the shape of the amide I peak varies depending on the structure of the main chain of the protein (i.e. the secondary structure). In addition, it has recently been shown that Fourier-transform infrared spectrometer (FTIR) can detect intermolecular β -sheet formation associated with protein aggregation and is used as a tool for detecting aggregates and elucidating the mechanism of aggregation.^{2,3)} In this study, we report on the ATR-FTIR detection of aggregation induced by sample heating of MabThera®, a commercially available antibody drug.

Keywords

Fourier-transform infrared (FTIR) spectroscopy, Attenuated Total Reflectance (ATR), MabThera®, rituximab, therapeutic antibody, biopharmaceutical, secondary structure, aggregation, denaturation, heat, intermolecular β -sheet

Experimental

Sample

- MabThera® (rituximab, Roche) 100 mg/mL
- Additives: sodium citrate dihydrate 7.4 mg/mL, sodium chloride 9.0 mg/mL, sodium hydroxide 9.0 mg/mL, polysorbate 80 0.7 mg/mL

FT/IR-4X FTIR spectrometer with ATR PRO 4X single-reflection ATR



- High S/N and resolution with small body size.
- Protein solution measurement with one drop of sample (approx. 10 μL) with ATR PRO 4X.
- Easy measurement of suspensions and powder samples that contain aggregates as well as solutions

Measurement Conditions

Resolution: 4 cm^{-1}
Sample volume: 10 μL

Accumulation: 100 (2 min.)
 N_2 purge: Yes

Results

Fig. 1 shows IR spectra measured after MabThera[®] was heated at various temperatures for 10 min., and then returned to room temperature. All spectra are subtracted from the buffer spectrum. Fig. 1a shows that the spectral shapes for amide I and amide II, which reflect the higher-order structure (HOS) of the protein, change as the heating temperature increases. Fig. 1b shows the second-derivative spectra at each temperature. Fig. 1c shows the change in the intensity of the 1640 and 1620 cm^{-1} peaks as a function of temperature. As the heating temperature increases, the intensity of the 1640 cm^{-1} peak derived from intramolecular β -sheets becomes weaker, while the intensity of the 1620 cm^{-1} peak derived from intermolecular β -sheets becomes stronger. This behavior is consistent with previous reports showing the formation of intermolecular β -sheets of antibodies by aggregation.

Conclusions

It was found that FTIR combined with ATR can be used to measure aqueous antibody solutions containing aggregates, and easily detect structural changes in trace amounts. This system has proven to be a useful tool for the evaluation of antibody drug aggregates and the elucidation of their molecular mechanisms in the screening and formulation of antibody candidates.

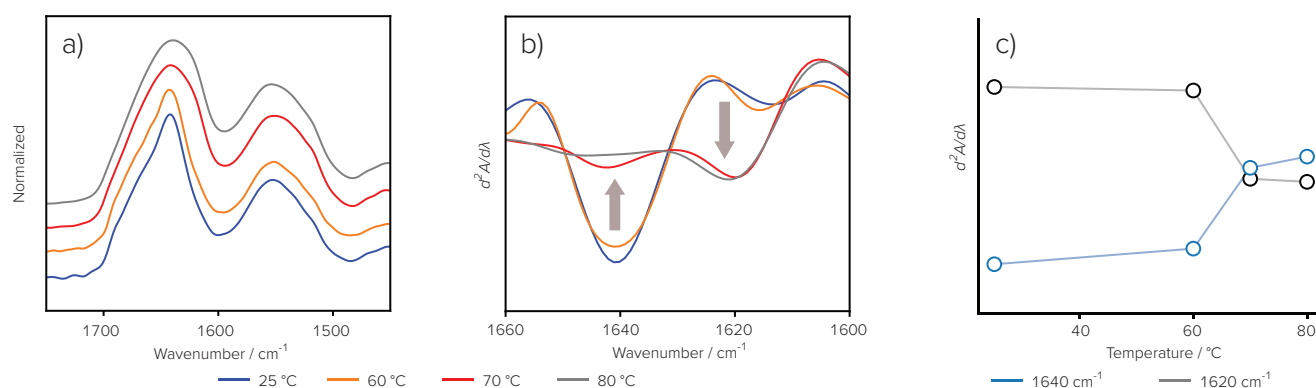


Fig. 1 Thermal denaturation analysis of MabThera[®] using FTIR.
a) Normalized IR absorbance spectra, b) second-derivative spectra, and c) transition of inter- and intramolecular β -sheets.

Additional Information

- Product information: FTIR spectrometer.
<https://www.jasco-global.com/product-spectroscopy/ftir-spectrometer/>

References

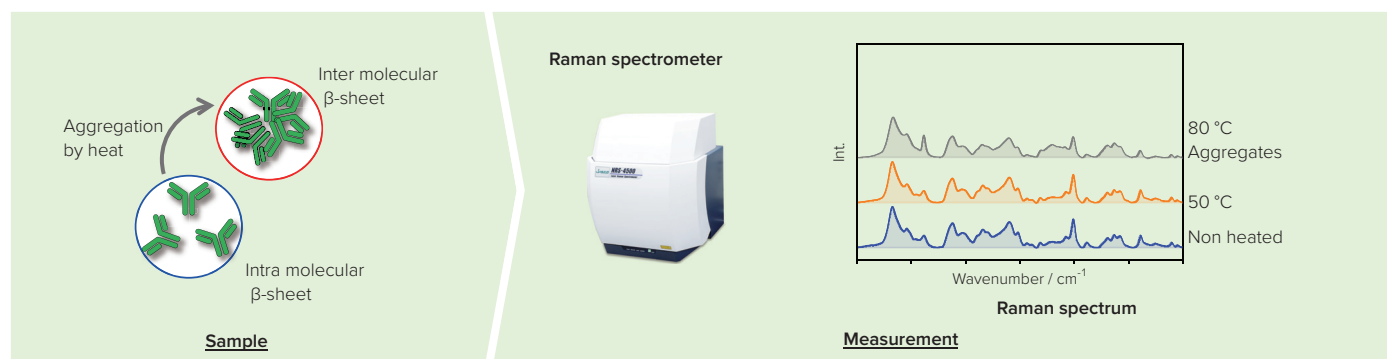
- 1) Y. Yunyu and L. Zang: *Am. Pharm. Rev.*, **21** (2), (2018). <<https://www.americanpharmaceuticalreview.com/Featured-Articles/348833-Factors-Influencing-Biotherapeutic-Monoclonal-Antibody-Aggregation/>>, (accessed 2022. 5. 25).
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About the Author

Taiji Oyama is a spectroscopy application scientist at JASCO Corporation (Tokyo, Japan) specializing in CD, IR, Raman, UV-vis, and fluorescence spectroscopy.



Thermal Denaturation Analysis of Monoclonal Antibodies by Raman Spectroscopy



Graphical abstract

Introduction

Antibody drugs are currently employed to inhibit the proliferation of malignant tumor cells or the activity of immune cells, and are prime research candidates for treatment of conditions which currently have no medical routes to treatment. Antibody therapeutics have several factors involving environment, storage condition and manufacturing that can spur detrimental behavior, such as aggregation when exposed to various external stimuli. To develop and provide conditions which prevent the aggregation of antibody drugs, there is a large amount of R&D being conducted to identify when, how and why aggregation occurs during the manufacturing processes.^{1–3)}

Raman spectroscopy can analyze the secondary and tertiary structures of proteins in solid or aqueous solution nondestructively and without pretreatment. Because of its wide applicability from microscopic measurement to process monitoring using fibers, Raman spectroscopy has attracted much attention as a method to evaluate physical and scientific properties and to monitor the quality of therapeutic antibodies.⁴⁾

Here we report a Raman spectroscopic evaluation of tertiary structural changes in MabThera®, a commercially available antibody drug, due to aggregation.

Keywords

Raman spectroscopy, MabThera®, rituximab, therapeutic antibody, biopharmaceutical, higher-order structure (HOS), secondary structure, tertiary structure, aggregation, denaturation, heat

Table 1. Various Raman measurement system

Micro Raman	Fiber Raman
 NRS-5500/7500 NRS-4500	 RMP-500
• Microanalysis • Chemical imaging • High sensitivity	• In-situ measurement • Process monitoring • High flexibility

Experimental

Sample

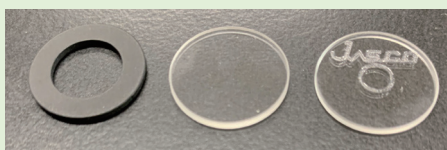
- MabThera® (rituximab, Roche) 100 mg/mL
- Additives: Sodium citrate dihydrate 7.4 mg/mL, sodium chloride 9.0 mg/mL, sodium hydroxide 9.0 mg/mL, polysorbate 80 0.7 mg/mL

NRS-4500 Raman spectrometer



- Desktop micro Raman
- High sensitivity
- Multi laser excitation
- Quick Raman imaging
- Full automated and Class 1 laser safety

MSD-462 Micro sampling disc*



- Sample volume: 10 μ L
- Optical path length: 1 mm

* Accessory for J-1500 CD spectrometer

Measurement Conditions

Laser: 532 nm
Exposure: 70 sec.
Slit: Φ 100

Accumulation: 20
Grating: 900 gr./mm
Objective: LMPFLN 50x

Results

Fig. 1 shows Raman spectra of MabThera® without heating, MabThera® heated at 50 °C for 10 min. and the aggregates generated by heating at 80 °C for 10 min. Except for the spectrum of the aggregates, the spectra of the buffer were subtracted. In each spectrum, peaks characteristic of the secondary and tertiary structures of the protein were clearly observed, as shown in Table 2.

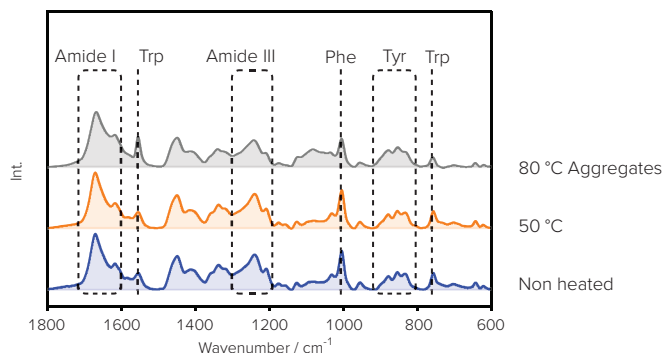


Fig. 1 Raman spectra of MabThera® and its aggregates
Blue: Non heated, Orange: Heated at 50 °C for 10 min, Gray: aggregates generated by heating at 80 °C for 10 min. Each spectrum was normalized by the amide I band.

Table 2. Raman shift and assignments.⁵⁾

Raman shift	Assignment
1650 cm^{-1}	Amide I
1555 cm^{-1}	Tryptophan
1300 cm^{-1}	Amide III
1000 cm^{-1}	Phenylalanine
850 cm^{-1}	Tyrosine
750 cm^{-1}	Tryptophan

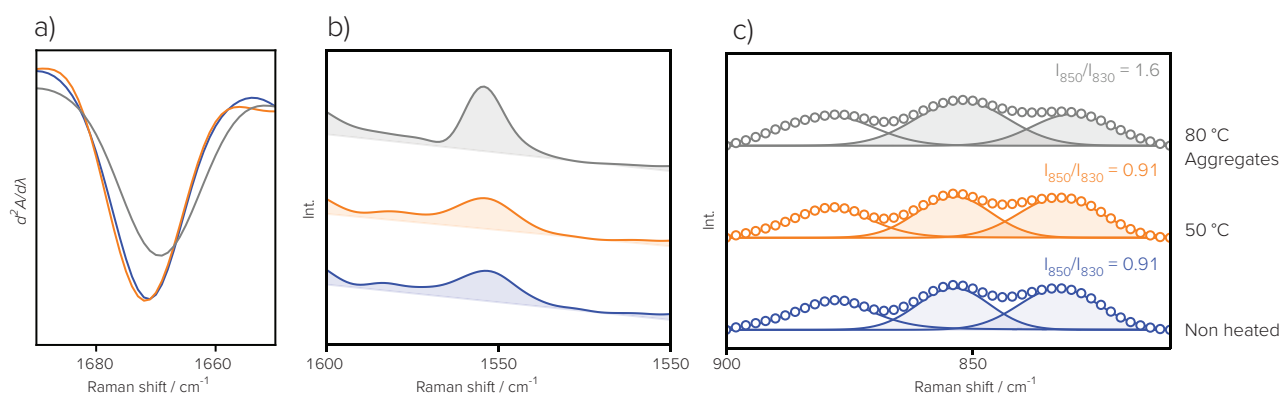


Fig. 2 Enlarged view of a) second-derivative Raman spectra and b,c) Raman spectra
Blue: Non heated, Orange: Heated at 50 °C for 10 min, Gray: aggregates generated by heating at 80 °C for 10 min. Each spectrum was normalized by the amide I band.

The second-derivative bands for amide I are consistent for the unheated and 50 °C heated samples, but a peak shift is observed in the aggregates, indicating that the secondary structure has changed (Fig. 2a). The band at 1550 cm^{-1} derived from tryptophan also shows a change in the band shape of the aggregates compared to the unheated and 50 °C heated samples, indicating a change in the tertiary structure (Fig. 2b). Similar changes are observed for the triplet bands derived from tyrosine (Fig. 2c). Curve fitting was applied to these bands, and the intensity ratio of I_{850}/I_{830} increased in the aggregates, indicating that the phenolic -OH group of tyrosine changed from a donor to an acceptor during aggregation process.⁵⁾

Conclusions

Raman spectroscopy has proven to be a useful tool for easily evaluating the secondary and tertiary structures of therapeutic antibodies in aqueous solution, aggregates and other forms. JASCO offers a variety of Raman spectrometers, including microscopic and fiber type systems, to meet the needs of various applications, from development to quality monitoring of antibody drugs.

Additional Information

- Product information: Raman spectrometer.
<https://www.jasco-global.com/product-spectroscopy/raman/>

References

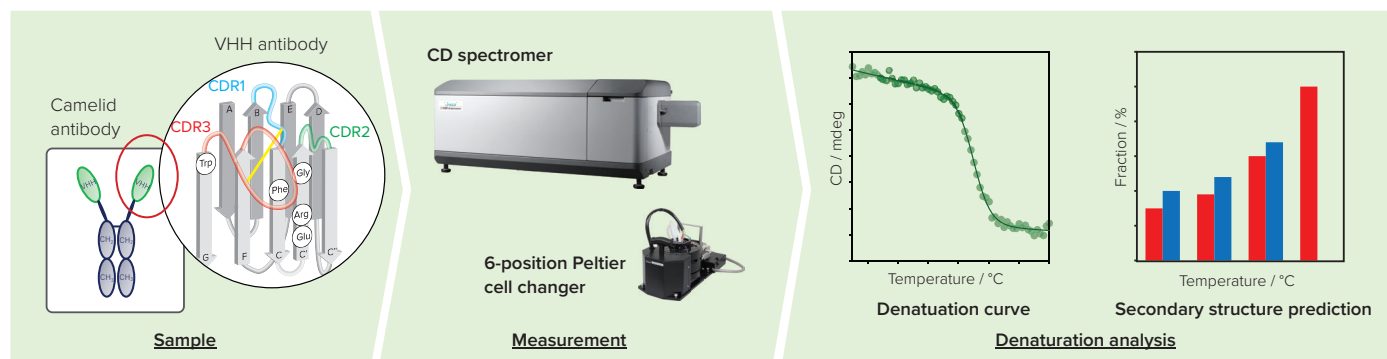
- 1) Y. Yunyu and L. Zang: *Am. Pharm. Rev.*, **21** (2), (2018). <<https://www.americanpharmaceuticalreview.com/Featured-Articles/348833-Factors-Influencing-Biotherapeutic-Monoclonal-Antibody-Aggregation/>>, (accessed 2022. 5. 25).
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About the Author

Taiji Oyama is a spectroscopy application scientist at JASCO Corporation (Tokyo, Japan) specializing in CD, IR, Raman, UV-vis, and fluorescence spectroscopy.



Heat-Induced Denaturation and Refolding Study of VHH Antibody



Graphical abstract

Introduction

The coronavirus S glycoproteins sit on the surface of the virus particle and facilitate receptor binding and membrane fusion, which leads to entry of the virus into host cells (Fig. 1).¹⁾ Therefore, the S proteins are the main target for therapeutic drug design by inhibiting binding.

VHH antibodies are small, single-domain antibodies corresponding to the variable region of a heavy chain camelid antibody (see “Sample” in the graphical abstract above). They are highly stable with respect to heat, have high affinity and binding specificity, a low molecular weight which increases mass production efficiency and can be easily modified.²⁾ Because of these features, VHH antibodies have been pursued as therapeutic modalities.²⁾ Since the structure of antibodies may change and cause a loss in their activity depending on the formulation conditions and storage environment, it is necessary to evaluate their stability in order to ensure quality when they are applied to therapeutic uses. In particular, thermal denaturation is highly reversible depending on the protein, increasing the need to determine the denaturation temperature and refolding ability in the stability evaluation of antibody drugs.

Here, we evaluate the thermal stability and refolding ability of three different types of VHH antibodies using a CD spectrometer. The secondary structure contents were estimated using the BeStSel^{3,4)} program to further correlate the denaturation temperatures with structural changes to the VHH antibody framework.

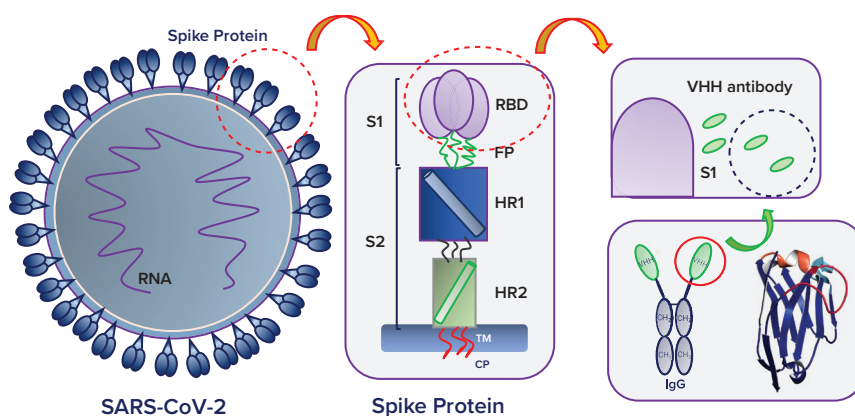


Fig. 1 Schematics of the spike protein and VHH antibody structures.

Keywords

Circular Dichroism (CD) spectroscopy, secondary structure prediction, BeStSel, VHH antibody, therapeutic antibody, biopharmaceutical, secondary structure, denaturation, refolding, heat, SARS-CoV-2

Experimental

Sample

Denaturation study

- Protein: anti-SARS-Cov-2 VHH antibody (three types with different sequences)
No. 1 (0.4 mg/mL), No. 2 (0.28 mg/mL), No. 3 (0.2 mg/mL)
- Buffer: 20 mM PBS

Refolding study

- Protein: anti-SARS-Cov-2 VHH antibody (three types with different sequences)
No. 1 (0.1 mg/mL), No. 2 (0.1 mg/mL), No. 3 (0.1 mg/mL)
- Buffer: 20 mM PBS

Measurement and analysis system

J-1500 CD spectrometer

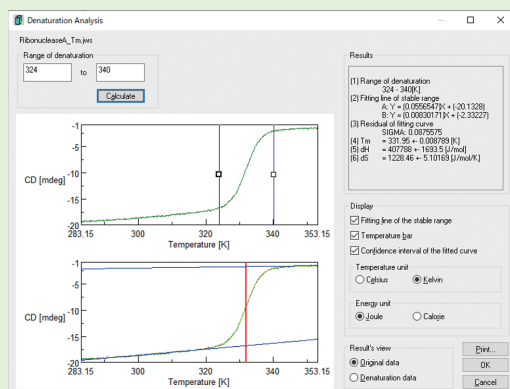


- Accurate and efficient normalization of protein CD spectrum obtained by simultaneous CD and Abs measurements.

MPTC-513 automatic 6-position Peltier cell changer

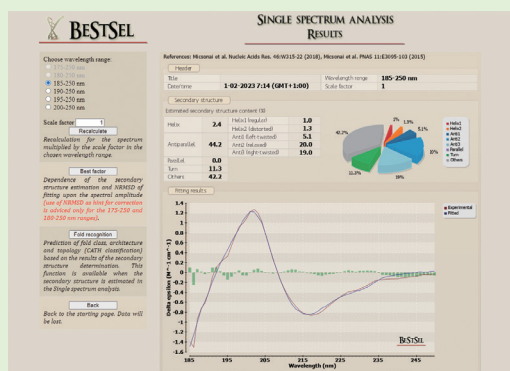
- Automated measurements of 6 samples while maintaining or ramping their temperature.
- Temperature range of 0 to 100 °C.

JWTDA-519 denatured protein analysis program



- T_m calculation based on temperature vs. CD sigmoid curve.

BeStSel



- Secondary structure prediction method developed by Dr. József Kardos at Eötvös Loránd University.
- Highly accurate estimation of β -sheet content by dividing β -sheets into four classes.

Measurement Conditions

Denaturation study

The denaturation curve for the VHH antibody was obtained by heating the sample from 25 to 90 °C while measuring the CD value at 217 nm to track the amount of β -sheet structure. The onset temperature (T_o), denaturation temperature (T_m) and end temperature (T_e) were calculated from the obtained denaturation curves.

Wavelength:	217 nm	Bandwidth:	1 nm
D.I.T.:	2 sec.	Temp. interval:	1 °C
Temp. ramp rate:	1 °C/min.	Temp. range:	25 to 90 °C

Refolding study

The sample was heated from 25 °C to the end temperature (T_e) obtained from the denaturation curves, and then cooled back to 25 °C. CD spectra were measured at the start temperature, onset (T_o), denaturation temperature (T_m), and end temperature (T_e). Secondary structure analysis was performed on the obtained CD spectra using BeStSel and the refolding ability was calculated from the amount of change in the β -sheet structure at 25 °C before and after heating.

Bandwidth:	1 nm	Scanning speed:	50 nm/min.
D.I.T.:	4 sec.	Accumulation:	9
Data pitch:	0.1 nm	Optical path length:	1 mm

Results

Denaturation study

Fig. 2 shows the denaturation curve based on the CD value at 217 nm, which reflects changes in the β -sheet structure. Table 1 shows the onset (T_o), denaturation temperature (T_m) and end temperature (T_e) obtained from the denaturation curve. Comparing the denaturation temperatures, the thermal stability of the samples decreases in the following order: No. 3, 1 and 2.

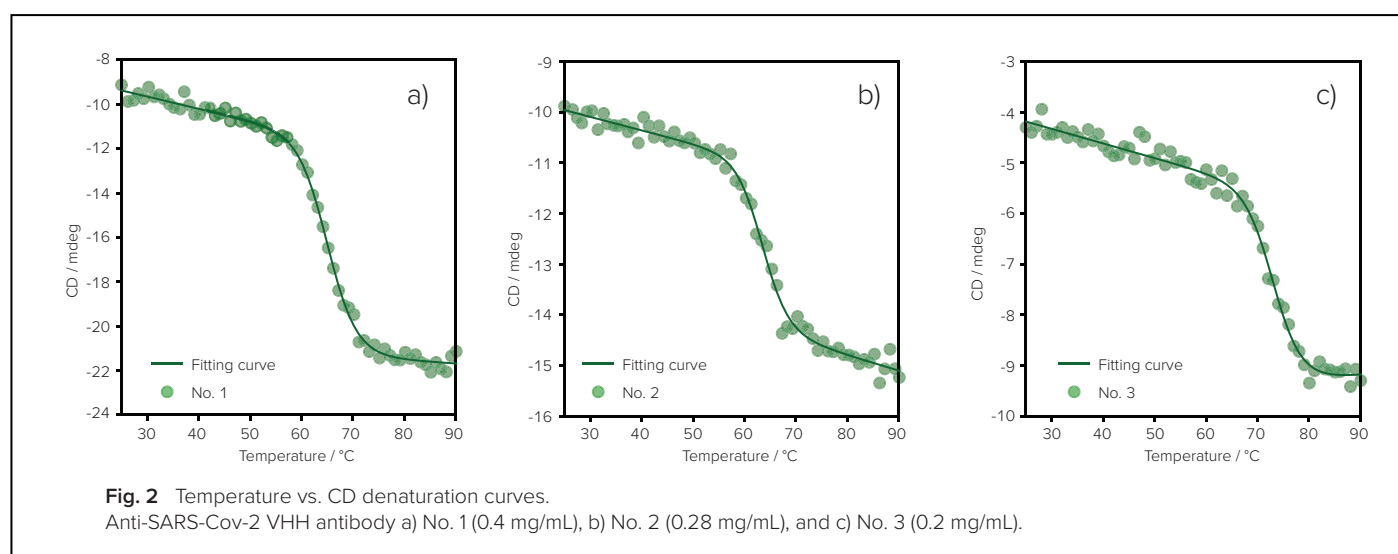


Table 1. Onset temperature (T_o), denaturation temperature (T_m), and end temperature (T_e).

Sample	T_o / °C	T_m / °C	T_e / °C
No. 1	57	65	75
No. 2	57	63	75
No. 3	65	73	80

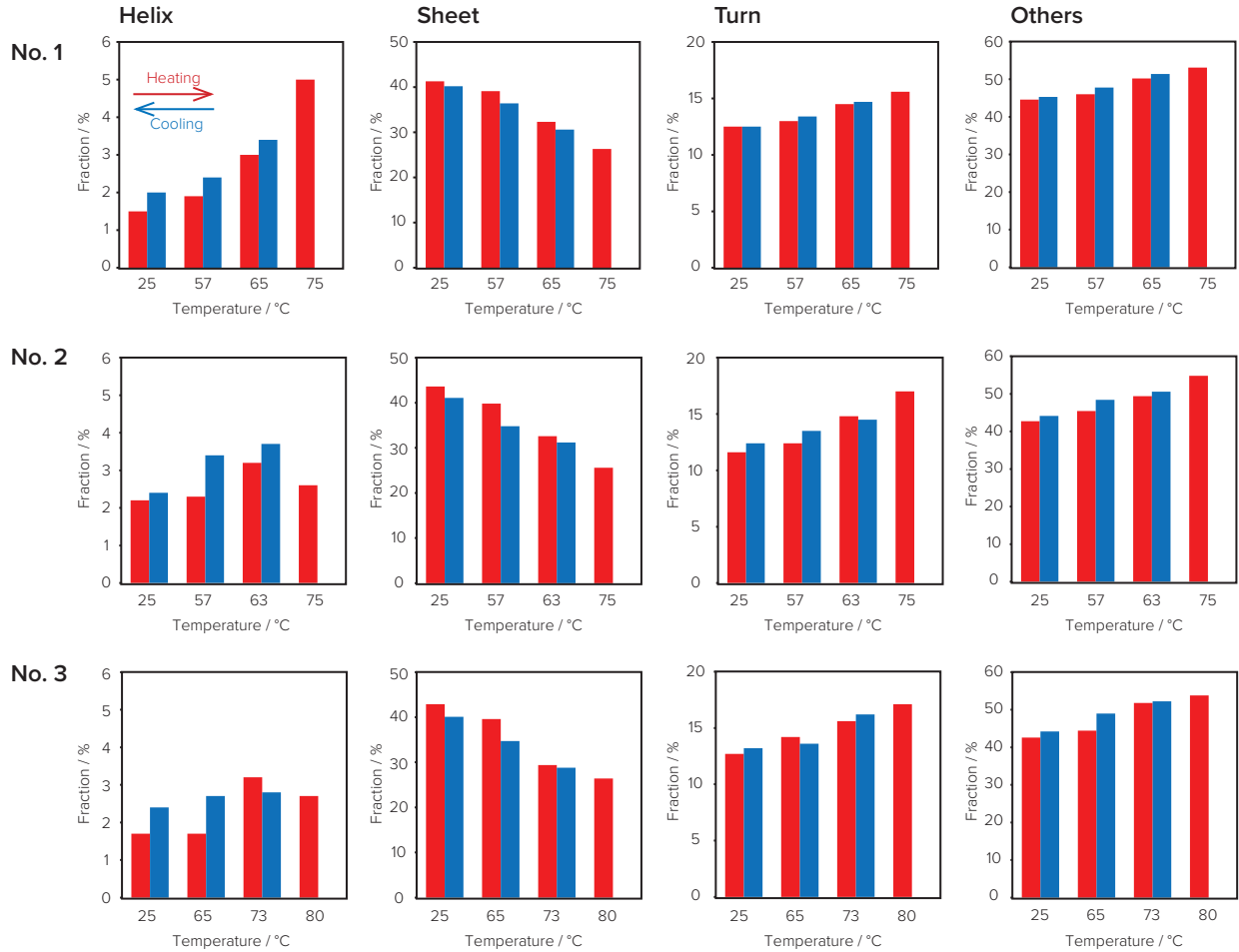


Fig. 3 Changes in estimated secondary structure content as a function of temperature. Red bars show changes in heating process, while blue bars show changes in cooling process.

Refolding study

In order to reduce the effect of aggregation, the sample was heated to the end temperature (T_e) determined from the previously obtained denaturation curves and cooled immediately after the sample became denatured. Fig. 3 shows the secondary structure analysis result of the obtained CD spectra using BeStSel.

Conclusions

For all samples, when the temperature increases, the β -sheet content decreases, and the proportion of turn and other structures increases. In addition, the proportion of α -helix structures increases with increasing temperature for sample No. 1 only. The thermal reversibility study indicates that the β -sheet, turn, and other structures in all three samples are reversible, as well as the α -helix structure of No. 1. While the framework of VHH antibodies is considered to be conserved, the structure of the Complementarity Determining Regions (CDR) that bind to the antigens is variable. The framework is primarily composed of β -sheet structures, and any changes in structure can potentially affect the structure of the CDR. Focusing on the percentage of the β -sheet secondary structure, the effective refolding before and after heating was calculated for samples No. 1, 2 and 3 and were 97.3 %, 95.0 % and 93.5 %, respectively. Therefore the ability of the samples to refold after heating and cooling assumes the following order, No. 1, 2 and 3, further supporting the melting temperature data shown in Table 1.

Additional Information

- Product information: CD spectrophotometer.
<https://www.jasco-global.com/product-spectroscopy/circular-dichroism/>
- BeStSel web server:
<https://bestsel.elte.hu/index.php>

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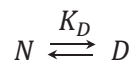
Satoko Suzuki is a spectroscopy application scientist at JASCO Corporation (Tokyo, Japan) specializing in CD, CPL, UV-vis, and fluorescence spectroscopy.

* VHH antibody was kindly provided by RePHAGEN <https://rephagen.com/>



Denatured Protein Analysis Program

This program evaluates a single protein substance in solution that is in equilibrium at a certain temperature, and enables calculation of the thermodynamic parameters (T_m , ΔH and ΔS) of its protein from the data with variable temperatures.

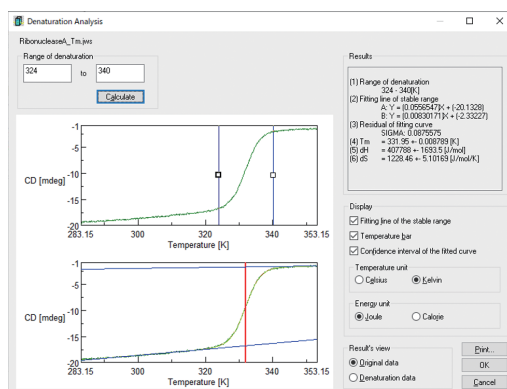


$$\ln K_D = -\frac{\Delta H}{R} \cdot \frac{1}{T} + \frac{\Delta S}{R}$$

K_D is the ratio of $[N]$ and $[D]$, and is calculated by using the experimental data, which is the measured CD values θ_i as a function of temperature T_i . This program approximates θ_i by the following equation, and calculates the K_D .

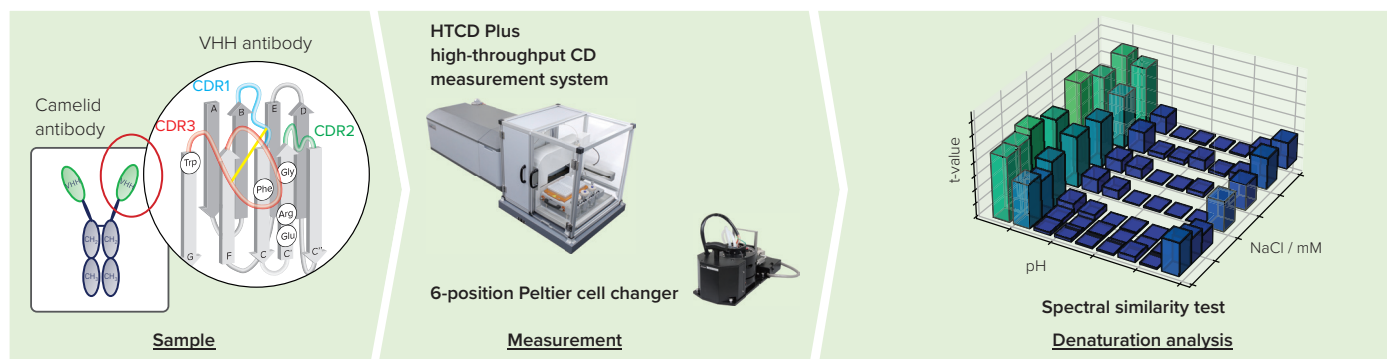
$$\tilde{\theta}(T) = \frac{\theta_N(T) + K_D(T)\theta_N(T)}{1 + K_D(T)}$$

$$\theta_n(T) = \sum_{i=1}^N a_{i,n} T^{N-i} \quad K_D(T) = \sum_{i=1}^N A_{i,D} T^{N-i} \quad N = 2$$



JWTDA-519 denatured protein analysis program

pH and Salt-Induced Denaturation Study of VHH Antibody using CD Spectroscopy



Graphical abstract

Introduction

Since their first approval in 1986, therapeutic antibodies have dramatically expanded their market over the past decades.¹⁾ Antibodies are known to lose their native structure due to changes in the surrounding environment, such as temperature, pH and salt concentration, ultimately resulting in the loss of their function. Therefore, the key to achieving a robust life cycle of therapeutic antibodies is to understand the effects of formulation and storage conditions on their structure.²⁾

Circular Dichroism (CD) spectroscopy is a classic technique for monitoring the structural stability of biological macromolecules, such as proteins and nucleic acids. It is especially well-known for providing fast secondary structure prediction of proteins under homogeneous conditions.

In this study, the CD spectral difference between denatured VHH and native-state VHH was statistically analyzed using the qHOS program. To assess the effects of pH and salt concentration, the VHH was measured under 63 different solvent conditions using an HTCD Plus, high-throughput CD measurement system, that provides fully automated sample loading to flow washing. The denaturation temperature (T_m) for VHH under the different solvent conditions was obtained from the CD denaturation curves. The spectral difference and the T_m showed a high correlation, suggesting that pre-screening of the pH and salt concentration can be performed before time-consuming thermal screening.

Keywords

Circular Dichroism (CD) spectroscopy, similarity assessment, VHH antibody, therapeutic antibody, biopharmaceutical, denaturation, pH, salt concentration, heat, high-throughput screening, SARS-CoV-2

Experimental

Sample

- Protein: anti-SARS-Cov-2 VHH antibody 0.5 mg/mL
- Buffer*: 50 mM citric acid for pH 3, 4, 5, and 6, 50 mM phosphoric acid for pH 7 and 8, and 50 mM CHAPS for pH 9, 10, and 11.

*NaCl concentration varied as follows: 10, 100, 200, 400, 600, 800, and 1000 mM.

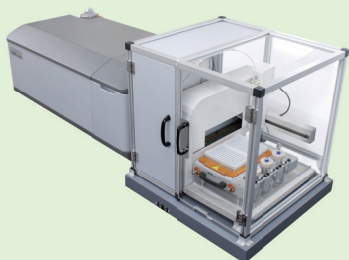
Measurement and analysis system

J-1500 CD spectrometer



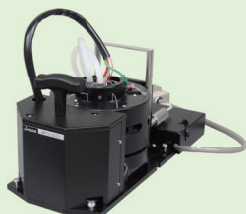
- Accurate and efficient normalization of protein CD spectrum obtained by simultaneous CD and Abs measurements.

HTCD Plus high-throughput CD measurement system



- Fully automated measurement of up to 192 samples.
- Pre-registered flush method for protein or DNA/RNA samples can be selected to eliminate sample carry-over.

MPTC-513 automatic 6-position Peltier cell changer



- Automated measurements of 6 samples while maintaining or ramping their temperature.

JWQHOS-531 qHOS



- Statistical HOS similarity test program developed by JASCO.
- High-sensitive detection of spectral difference by noise-weighted Euclidean distance.
- Support for spectra obtained by multiple instruments.

Measurement Conditions

pH and salt induced denaturation

Bandwidth: 1 nm
D.I.T.: 2 sec.
Data pitch: 0.1 nm

Scanning speed: 100 nm/min.
Accumulation: 4
Optical path length: 0.2 mm

Thermal denaturation

Bandwidth: 1 nm
D.I.T.: 2 sec.
Temp. interval: 1 °C

Temp. ramp rate: 1 °C/min.
Optical path length: 1 mm
Wavelength: 236 nm

Spectral Distance Test

- Reference: 10 samples of 0.5 mg/mL anti-SARS-Cov-2 VHH antibody at pH 7 and 200 mM NaCl were measured as a representative native state to create a reference set.
- Subjects: 0.5 mg/mL VHH was prepared at different pHs using the buffers described in the “Sample” section.

See page 36 for more details of the spectral similarity test using the qHOS program.

Results

pH- and salt-induced denaturation

CD spectra of VHH for 200 mM NaCl and different pH values are shown in Fig. 1a. The CD spectra for pH 3 and pH 4 display a negative peak at 217 nm, reflecting the β -sheet structure, and a peak at 227 nm, which is considered to be due to β -sheet interactions. These peaks deviate from the native VHH CD spectrum for pH 7.

CD spectra of VHH were measured while changing the NaCl concentration from 10 to 1000 mM and the pH from 3 to 11. The CD spectrum changes from the native state (200 mM NaCl and pH 7) were quantified using the qHOS program. Fig. 1b shows the relationship among the VHH antibody pH, the NaCl concentration and the t -value. The t -value is small in the pH range from 6 to 9, and the structure is not significantly different from that of the reference sample at pH 7 and an NaCl concentration of 200 mM. On the other hand, at pH 5 and lower, or pH 10 and higher, the t -value is large, indicating that the structure has changed. It can also be seen that the structure changes as the NaCl concentration increases.

Thermal denaturation

Using the 6-position Peltier cell changer, which automatically rotates 6 cells while changing the temperature, the denaturation temperature (T_m) was obtained for each combination of pH and NaCl concentration. T_m was calculated from the thermal denaturation curves obtained by monitoring the CD signal at 236 nm. Fig. 2 shows the relationship between the t -value shown in Fig. 1a and the T_m for each solvent condition.

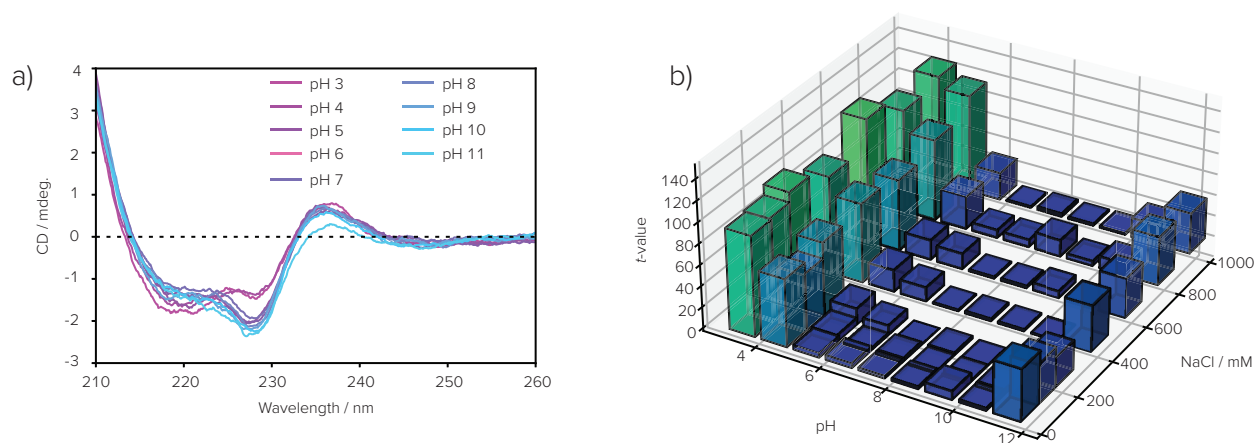


Fig. 1 Screening of pH and NaCl concentration.

a) CD spectra of VHH antibody for 200 mM NaCl and different pH. b) relationship between pH and NaCl concentration of VHH antibodies and t -value.

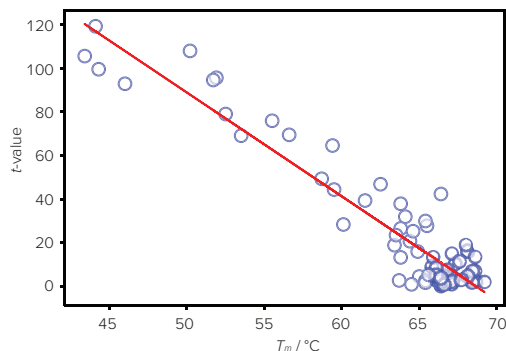


Fig. 2 Relation between denaturation temperature and t-value.

Conclusions

The combination of the HTCD Plus, an automated CD measurement system and the qHOS program is a breakthrough approach to statistically analyzing the effects of solvent conditions. This approach is especially convenient for understanding the robust conditions for preparation, synthesis and storage of biopharmaceuticals.

In the thermal denaturation study, there was a high correlation between the t -value and the T_m . This implies that the spectral difference test is effective for primary screening of a large number of buffers before conducting a time-consuming thermal stability evaluation.

Additional Information

- Application: Structure Evaluation of G-quadruplex aptamers Using High-Throughput CD Measurement System and Principal Component Analysis.
<https://www.jasco-global.com/solutions/structure-evaluation-of-g-quadruplex-aptamers-using-high-throughput-cd-measurement-system-and-principal-component-analysis/>
- News: JASCO releases new CD accessory HTCD Plus.
<https://www.jasco-global.com/news/jasco-releases-new-cd-accessory-htcd-plus/>
- Product information: CD spectrophotometer.
<https://www.jasco-global.com/product-spectroscopy/circular-dichroism/>

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About the Author

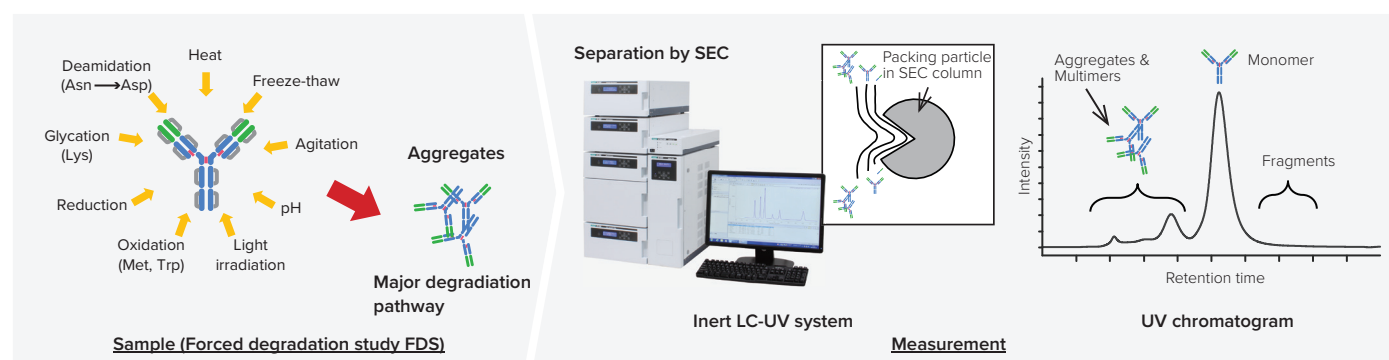
Satoko Suzuki is a spectroscopy application scientist at JASCO Corporation (Tokyo, Japan) specializing in CD, CPL, UV-vis, and fluorescence spectroscopy.

* VHH antibody was kindly provided by RePHAGEN <https://rephagen.com/>



* This application was developed under the guidance of Professor Kohei Tsumoto of the University of Tokyo.

Monitoring of Aggregates and Degradation Products by SEC in FDS of Antibody Drugs



Graphical abstract

Introduction

Since the first monoclonal antibody (mAb) was approved by the U.S. Food and Drug Administration (FDA) in 1986, the market for therapeutic proteins has expanded rapidly, and antibody drugs have become predominant treatment modalities for various diseases over the past 25 years.¹⁾ The stability of a drug molecule is very important because it affects the safety and efficacy of the drug. In products with higher-order structures (HOS), such as antibody drugs, the maintenance of molecular structure and biological activity depends on non-covalent bonds, as well as covalent bonds of the molecule and is affected by various environmental factors.²⁾

In the guidelines of the FDA and International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH), the need for stability test data is described to understand how the quality of drug substances and drug products changes over time in different environments^{3, 4)}. Therefore, a Forced Degradation Study (FDS) to purposely deteriorate and degrade the drugs is conducted at each stage from research and development through marketing approval of antibody drugs. FDS is an effective method for clarifying the degradation pathways and structures of degradation products of drug substances and drug products and guaranteeing stability. Also, it is useful for the development of manufacturing processes and selection of storage conditions and containers.^{5, 6)} Common test conditions for FDS include heating, freezing and thawing, agitation, pH, light irradiation, oxidation, reduction and glycation.^{7, 1)} Since one of the degradation pathways that occurs under many of these conditions is aggregates that are deeply related to immunogenicity, Size Exclusion Chromatography (SEC) that can successfully separate and detect monomer and aggregates, including multimers, is used as an analytical method.^{5, 7)}

In this article, we performed various forced degradation tests on MabThera® (rituximab), which is a therapeutic drug for non-Hodgkin's lymphoma, and measured untreated and forcibly degraded MabThera® by SEC in order to compare the changes in the amount of monomer and aggregates, including multimers and degradation products.

Keywords

High Performance Liquid Chromatography (HPLC), Size Exclusion Chromatography (SEC), TSKgel G3000SW_{XL}, UV detector, Forced Degradation Study (FDS), MabThera®, rituximab, therapeutic antibody, biopharmaceutical, aggregation, degradation

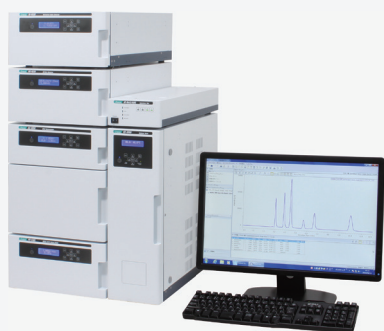
Experimental

Sample

- MabThera® (rituximab, Roche) 100 mg/10 mL
Additives: sodium citrate dihydrate 7.4 mg/mL, sodium chloride 9.0 mg/mL, sodium hydroxide 9.0 mg/mL, polysorbate 80 0.7 mg/mL

Measurement and analysis system

LC-4000 HPLC system



<System configuration>

Pump: PU-4080i
Autosampler: AS-4050i (with TC-4000-1)
Column oven: CO-4060
UV detector: UV-4075 (with inert flow cell unit)
Chromatography data system:
ChromNAV Ver. 2
(with GPC calculation program)

Measurement Conditions

Column: TSKgel G3000SW_{XL}
(7.8 mmI.D. x 300 mmL, 5 μm)
Flow rate: 0.8 mL/min
Wavelength: 220 nm
Standard: Protein standard mix
15-600kDa (Merck)
30 mg/mL in water

Eluent: 0.2 M sodium phosphate
buffer (pH 6.7)
Column Temp.: 25 °C
Injection volume: 10 μL

Results

Fig. 1 shows UV chromatograms of a blank (ultrapure water) and a protein standard sample. The column used in this experiment has an exclusion limit with globular proteins of 800 kDa and a measurement range of approximately 10 to 500 kDa. As shown in Fig. 2, a molecular weight calibration curve was created from the retention times and molecular weights of the four components, except for *p*-aminobenzoic acid, that eluted after the permeation limit (around 13.8 min). Based on this calibration curve, the molecular weight of each sample was calculated.

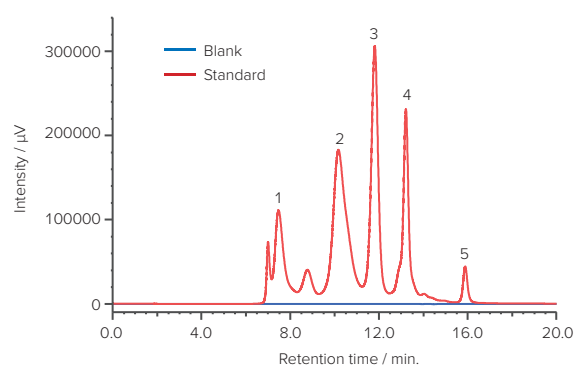


Fig. 1 UV chromatograms of a blank and a protein standard
1: Thyroglobulin (670 kDa), 2: γ-globulins (150 kDa), 3: Ovalbumin (44.3 kDa), 4: Ribonuclease A (13.7 kDa), 5: *p*-amino-benzoic acid (0.137 kDa)

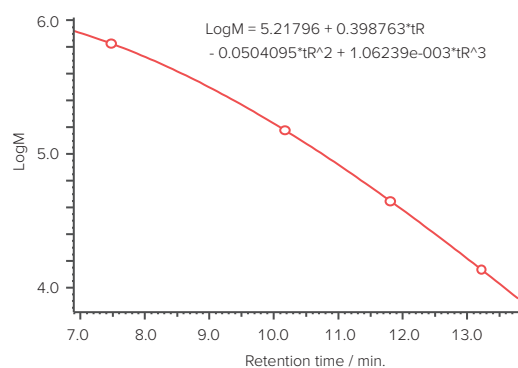


Fig. 2 Molecular weight calibration curve

Fig. 3 shows the UV chromatogram of MabThera® without degradation treatment. Figs. 4 to 7 show UV chromatograms of MabThera® that had been oxidized, glycosylated, deamidated and heated, respectively. In addition, Figs. 8 to 10 show the UV chromatograms of light-irradiated MabThera® with the irradiation time changed in three steps of 1, 2 and 5 minutes. The peaks around 14 minutes in Figs. 3 to 10 are the peaks that eluted after the permeation limit of the column. Although detailed calculation results of molecular weight are not shown here, the monomer peaks were identified from each peaks top molecular weight in Figs. 3 to 10.

In Figs 3 to 10, the peaks of each chromatograms were categorized into monomer, High Molecular Weight Species (HMWS), including aggregates that elute earlier than monomer, and Low Molecular Weight Species (LMWS), including degradation products that elute later than monomer. Table 1 shows the comparison results of each category ratio (area %) to the total peak area. From these results, the ratio of HMWS of MabThera® that had been oxidized, glycosylated, deamidated, heated and light-irradiated (1 minute) increased by about 0.2–0.6% and that of LMWS increased by about 0.1–0.4% as compared to untreated MabThera®. Regarding light irradiation, it was found that the ratios of the monomer gradually decreased and those of the HMWS and LMWS increased significantly as the irradiation time increased.

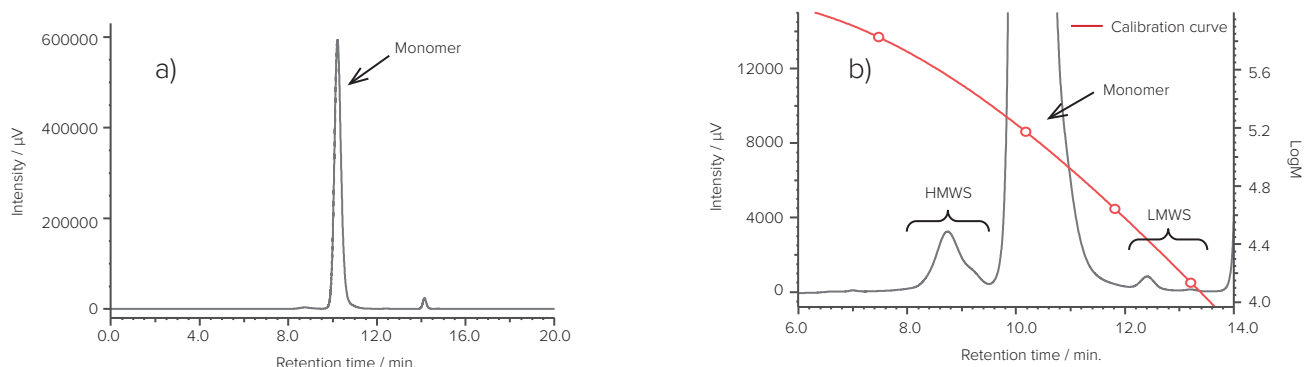


Fig. 3 UV chromatogram of untreated MabThera®

a) Full scale and b) enlarged view near the baseline at 6–14 min. (shown with molecular weight calibration curve)

Pretreatment: 100 mg/10 mL of MabThera® was diluted with ultrapure water to 1 mg/mL.

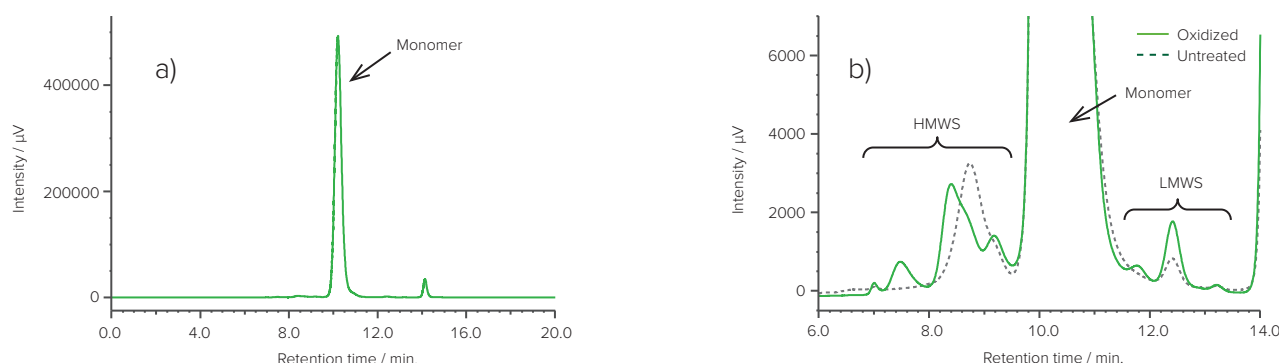


Fig. 4 UV chromatogram of oxidized MabThera®

a) Full scale and b) enlarged view near the baseline at 6–14 min. (shown with chromatogram of untreated MabThera®)

Pretreatment: One μL of 30% hydrogen peroxide solution was added (final concentration: 0.3% hydrogen peroxide) to 100 μL of MabThera® 100 mg/10 mL solution, and the solution was left at room temperature for 3 hours. Then, the solution was dialyzed against 10 mM sodium phosphate buffer (6 mM disodium hydrogen phosphate-4 mM potassium dihydrogen phosphate, pH 7.0) using a dialysis cellulose tube (Visking tubing, manufactured by Serva), and finally diluted 10 times with ultrapure water.

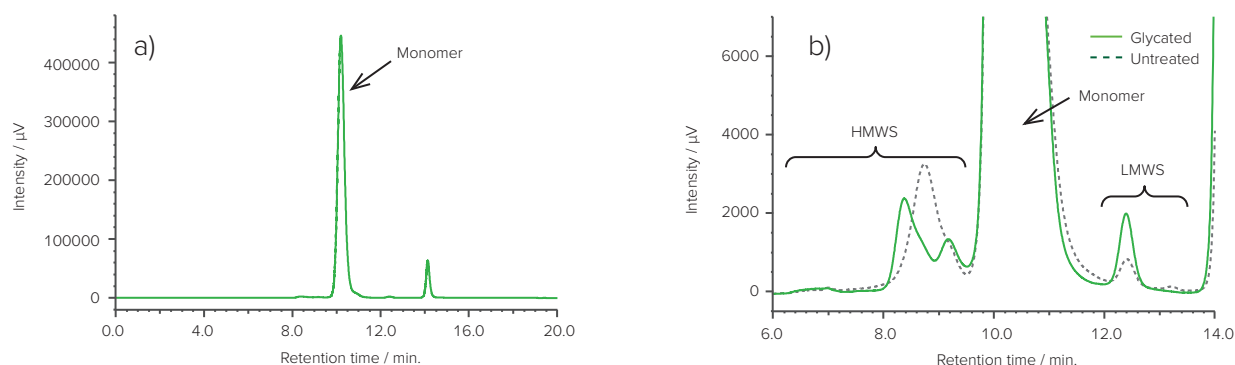


Fig. 5 UV chromatogram of glycated MabThera®

a) Full scale and b) enlarged view near the baseline at 6–14 min. (shown with chromatogram of untreated MabThera®)

Pretreatment: Fifty-four mg of glucose was added to 100 μ L of MabThera® 100 mg/10 mL solution and dissolved (final concentration: 2.5 M glucose), and then left in a constant-temperature oven (SCF-Sro) at 40 °C for 1 week. Then, the solution was dialyzed against 10 mM sodium phosphate buffer (6 mM disodium hydrogen phosphate-4 mM potassium dihydrogen phosphate, pH 7.0) using a dialysis cellulose tube (Visking tubing, manufactured by Serva), and finally diluted 10 times with ultrapure water.

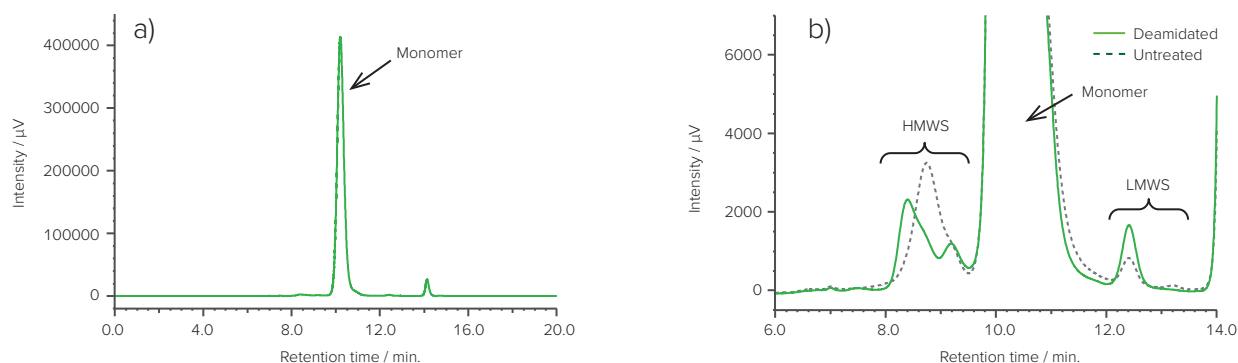


Fig. 6 UV chromatogram of deamidated MabThera®

a) Full scale and b) enlarged view near the baseline at 6–14 min. (shown with chromatogram of untreated MabThera®)

Pretreatment: 100 μ L of MabThera® 100 mg/10 mL solution was dialyzed against 10 mM sodium phosphate buffer (6 mM disodium hydrogen phosphate-4 mM potassium dihydrogen phosphate, pH was adjusted to 8.5 by 5 M sodium hydroxide solution) using a dialysis cellulose tube (Visking tubing, manufactured by Serva), and then left in a constant-temperature oven (SCF-Sro) at 40 °C for 1 week. Then, the solution was dialyzed against 10 mM sodium phosphate buffer (6 mM disodium hydrogen phosphate-4 mM potassium dihydrogen phosphate, pH 7.0) again, and finally diluted 10 times with ultrapure water.

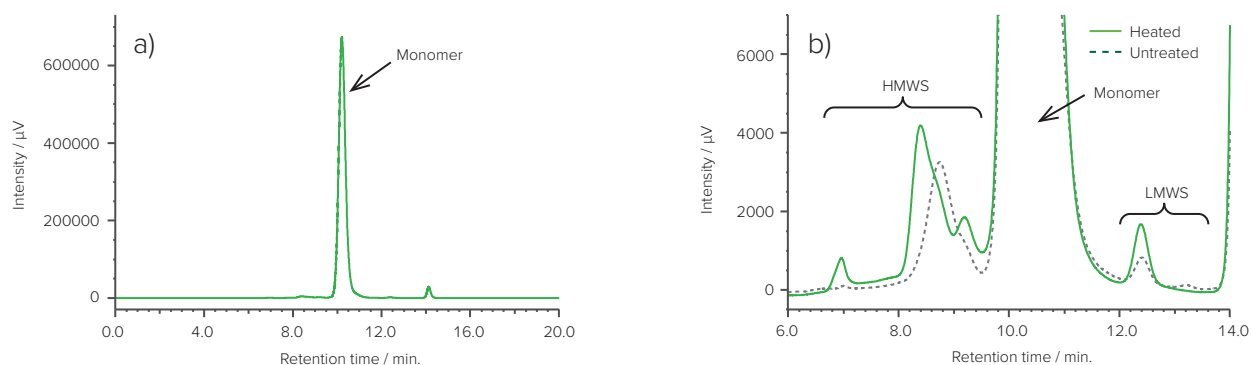


Fig. 7 UV chromatogram of heated MabThera®

a) Full scale and b) enlarged view near the baseline at 6–14 min. (shown with chromatogram of untreated MabThera®)

Pretreatment: 100 μ L of MabThera® 100 mg/10 mL solution was left in a constant-temperature oven (SCF-Sro) at 40 °C for 8 days and then diluted 10 times with ultrapure water.

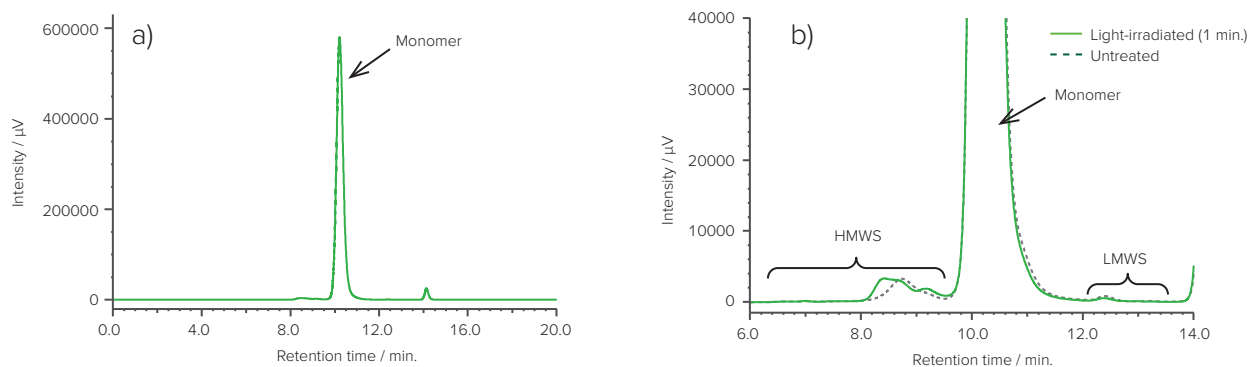


Fig. 8 UV chromatogram of light-irradiated MabThera® (irradiation time: 1 min)
a) Full scale and b) enlarged view near the baseline at 6–14 min. (shown with chromatogram of untreated MabThera®)
Pretreatment: 100 μL of MabThera® 100 mg/10 mL solution was irradiated with light (energy: 16.3 mW, center wavelength: 247 nm) for 1 minute using a light irradiation system (MM-3, manufactured by Bunkoukeiki).

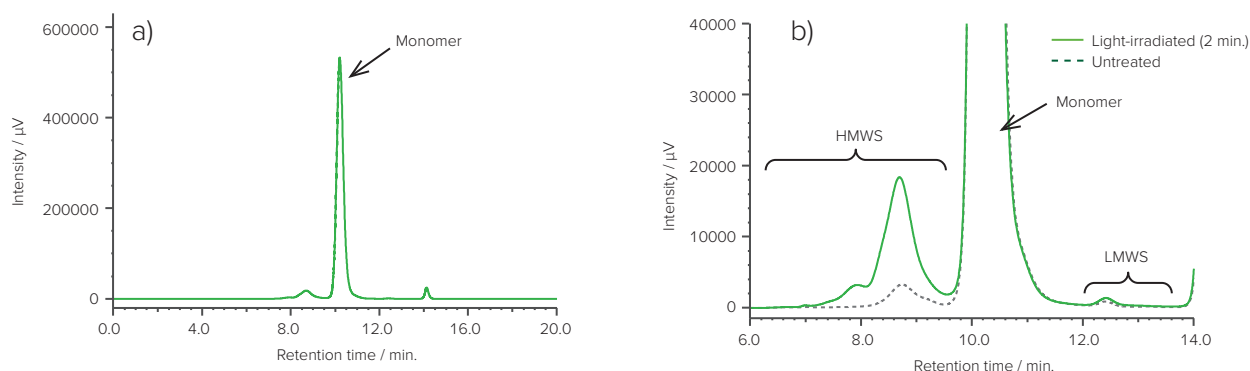


Fig. 9 UV chromatogram of light-irradiated MabThera® (irradiation time: 2 min)
a) Full scale and b) enlarged view near the baseline at 6–14 min. (shown with chromatogram of untreated MabThera®)
Pretreatment: 100 μL of MabThera® 100 mg/10 mL solution was irradiated with light (energy: 16.3 mW, center wavelength: 247 nm) for 2 min. using a light irradiation system (MM-3, manufactured by Bunkoukeiki).

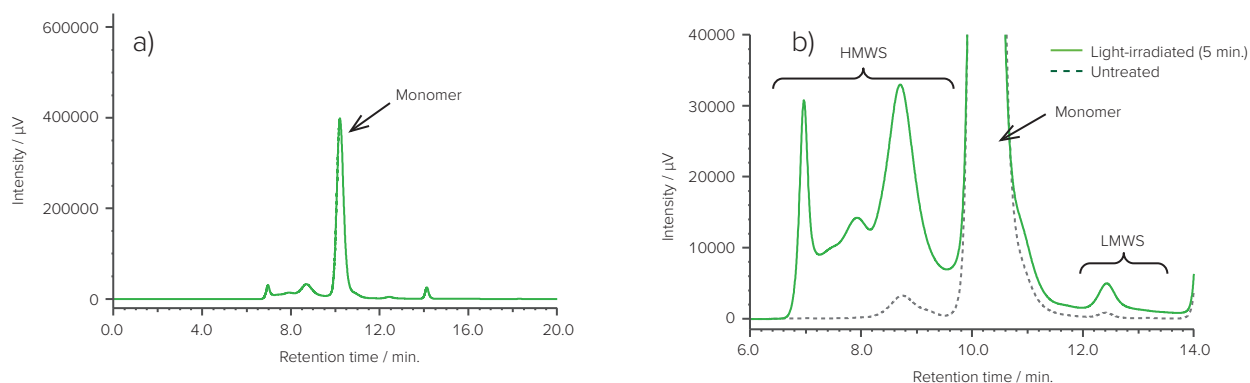


Fig. 10 UV chromatogram of light-irradiated MabThera® (irradiation time: 5 min)
a) Full scale and b) enlarged view near the baseline at 6–14 min. (shown with chromatogram of untreated MabThera®)
Pretreatment: 100 μL of MabThera® 100 mg/10 mL solution was irradiated with light (energy: 16.3 mW, center wavelength: 247 nm) for 5 min. using a light irradiation system (MM-3, manufactured by Bunkoukeiki).

Table 1. Comparison of ratios of HMWS, monomers, and LMWS in untreated and forcibly degraded MabThera®

MW classification Type of treatments	HMWS	Monomer	LMWS
Untreated	1.0 %	98.8 %	0.2 %
Oxidized	1.5 %	97.9 %	0.6 %
Glycated	1.2 %	98.3 %	0.5 %
Deamidated	1.3 %	98.2 %	0.5 %
Heated	1.6 %	98.1 %	0.3 %
Light-irritated (1 min)	1.5 %	98.3 %	0.2 %
Light-irritated (2 min)	6.7 %	93.0 %	0.3 %
Light-irritated (5 min)	21.0 %	74.4 %	4.6 %

Conclusions

In this experiment, it was confirmed that aggregates and degradation products in antibody drugs generated under various FDS test conditions can be well separated from monomers and detected by using SEC, and SEC is a powerful method for monitoring aggregates in the FDS.

Additional Information...

- Application: Analysis of Aggregates in Antibodies by SEC.
<https://www.jasco-global.com/solutions/analysis-of-aggregates-in-antibodies-by-sec/>
- Product information: HPLC.
<https://www.jasco-global.com/product-chromatography/integrated-hplc-system/>

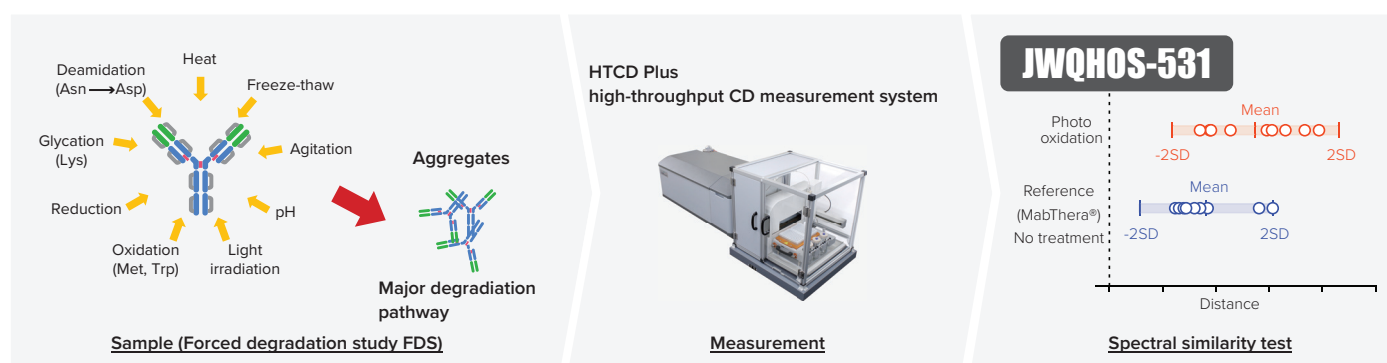
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Statistical Similarity Assessment of Antibody Drugs in FDS by Circular Dichroism (CD) and qHOS



Graphical abstract

Introduction

In recent years, therapeutic antibodies have been actively developed, manufactured and marketed. Antibody drugs are widely used as therapeutic agents to inhibit the proliferation of malignant tumor cells and the activity of immune cells and are also highly anticipated for diseases for which complete treatment is not yet available.

Antibodies generally exhibit high affinity and highly specific activity due to their higher-order structure (HOS) driven by covalent and non-covalent bond formation. In the production of therapeutic antibodies, there are several processes, such as cell cultivation, purification and virus removal, which may lead to various stimuli, modifications and degradations that may significantly impair the function of the drugs. Therefore, Forced Degradation Study (FDS) is frequently conducted at each stage from the selection of candidate antibodies to post-approval.^{1,2)} ICH Q5C provides guidelines for stability testing of biologics and describes the importance of testing under accelerated and severe conditions.³⁾ The main stimuli for forced degradation testing include heating, freeze-thawing, agitation, pH, light exposure, oxidation/reduction and glycation. Rapid detection of changes in physicochemical properties of antibodies due to these stimuli is critical for R&D and quality control of antibody drugs.

Circular Dichroism (CD) spectroscopy is a simple method to obtain information on the HOS of antibodies and to identify minute changes in HOS to modification or degradation as described above. Therefore, it is recognized as a method to evaluate HOS in ICH Q6B and is widely used as one of the measurement methods to control antibody quality.⁴⁾ With the implementation of these guidelines, there is a growing demand for a method that can objectively and sensitively evaluate CD spectra and identify significant differences between them. The qHOS program, developed to meet these demands, is capable of detecting small-differences in spectra with high sensitivity based on statistical tests.

Here, we report the results of statistical determination of higher-order conformational changes for rituximab caused by forced degradation testing using the HTCD Plus, high-throughput CD measurement system, in combination with the qHOS program.

Keywords

Circular Dichroism (CD) spectroscopy, similarity assessment, Forced Degradation Study (FDS), MabThera®, RIABNI™, rituximab, therapeutic antibody, biopharmaceutical, aggregation, degradation, high-throughput screening

Experimental

Sample

MabThera® (rituximab innovator, Roche) and RIABNI™ (rituximab biosimilar, AMGEN) 100 mg/10mL
Additives: sodium citrate dihydrate 7.4 mg/mL, sodium chloride 9.0 mg/mL, sodium hydroxide 9.0 mg/mL, polysorbate 80 0.7 mg/mL

Preparation

- Photo-oxidation
10 mg/mL MabThera® solution was irradiated with 16.3 mW of light energy and a central wavelength of 247 nm for 1 minute at room temperature using a light irradiation system. (MM-3, manufactured by Bunkoukeiki) before measurement.
- Reduction
Tris (2-carboxyethyl) phosphine was added to a 10 mg/mL MabThera® solution to a final concentration of 0.001% and left at room temperature for 30 minutes before measurement.

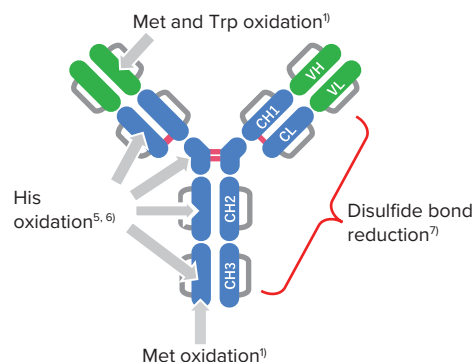
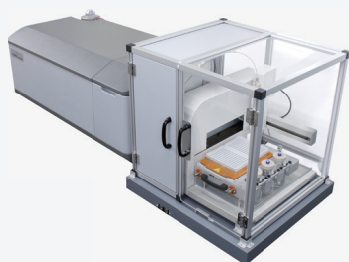


Fig. 1 Types of post-translational modifications and affected domain of antibodies by oxidation and reduction

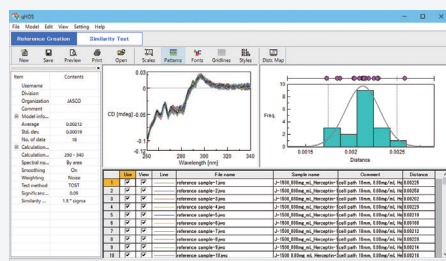
Measurement and analysis system

HTCD Plus high-throughput CD measurement system



- Fully automated measurement of up to 192 samples.
- Pre-registered flush method for protein or DNA/ RNA samples can be selected to eliminate sample carry-over.

JWQHOS-531 qHOS



- Statistical HOS similarity test program developed by JASCO.
- High-sensitive detection of spectral difference by noise-weighted Euclidean distance.
- Support for spectra obtained by multiple instruments.

Measurement Conditions

Bandwidth: 1 nm
D.I.T.: 4 sec.
Accumulation: 4

Scanning speed: 20 nm/min.
Data pitch: 0.1 nm
Optical path length: 0.5 mm

Analysis conditions

Distance calculation method:	Smoothing:	Savitzky-Golay
Euclidean distance	Weighting:	Noise
Number of samples: 9	Statistical testing:	Welch's <i>t</i> -test
Concentration correction: Absorbance	Significance level:	0.05
(far-UV: Abs_{200})		
(near-UV: $0.5 Abs_{280}$)		

See page 36 for more details of the spectral similarity test using the qHOS program.

Results

Through the near-UV CD spectra the tertiary structure of RIABNI™ was determined to be identical to that of MabThera® (Fig. 2). The near-UV CD spectrum of the biosimilar RIABNI™ was in excellent agreement with that of the innovator MabThera®, and the distances calculated from the spectra were also small. The *t*-test results showed that the *p*-values were above the significance level of 0.05, objectively confirming that RIABNI™ has the same tertiary structure as MabThera®.

The spectra and identity test results for MabThera® after photo-oxidation and reduction treatments are shown in Fig. 3. The spectra after the photo-oxidation and reduction treatments do not visually appear to be significantly different from the spectrum of the untreated MabThera®, which was used as the reference for each. However, when the spectra are converted to distances from each other, the distributions of distances between the reference and post-stimulation treatments are far apart.

Furthermore, a Welch's *t*-test based on these distances statistically determined that the stimulus-treated data are significantly different from the untreated data. These demonstrate that the treatments have caused significant structural and spectral changes, which would otherwise go unnoticed if visually inspected.

Conclusions

Using HTCD Plus and the qHOS program, we were able to determine, based on statistical tests, that while the tertiary structures of MabThera® and RIABNI™, antibody drugs with the same amino acid sequence and formulation conditions, are identical, there are significant differences in tertiary structure when MabThera® is exposed to stimuli, such as photo-oxidation and reduction. Based on statistical tests, we found that the tertiary structures of MabThera® and RIABNI™ are identical. Thus, by combining HTCD Plus and qHOS, we were able to objectively evaluate significant differences that could not be determined visually, and this system proved to be useful in the evaluation of HOS in FDS.

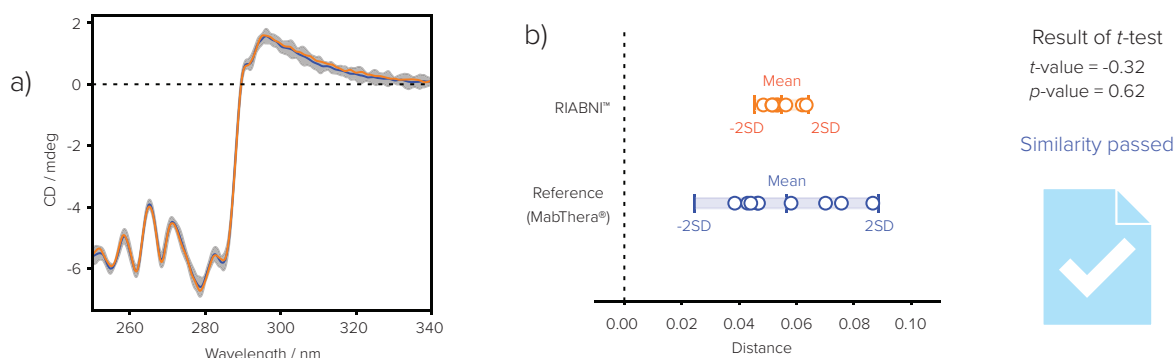


Fig. 2 Results of spectral similarity test for MabThera® (red) and RIABNI™ (blue).

a) and b): mean spectra of MabThera® (red), RIABNI™ (blue), standard deviation for MabThera® (gray).

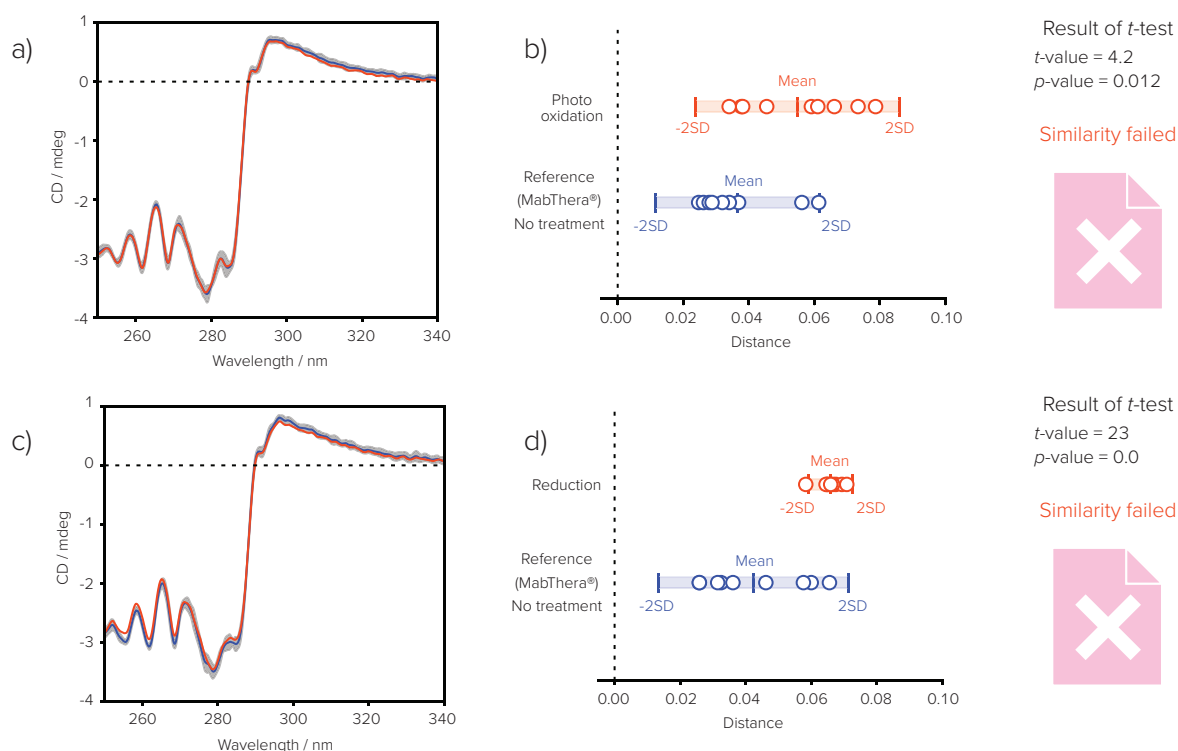


Fig. 3 Results of spectral similarity test for non-treated (red) and stimulated (blue) MabThera®. a) and c): mean spectra of MabThera (red), stimulated sample (blue), standard deviation for MabThera (gray). b) and d): distances and test results for MabThera (red) and stimulated sample (blue).

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