HIGHER ORDER STRUCTURE COMPARISON OF A PROPOSED BIOSIMILAR AND THE INNOVATOR BIOTHERAPEUTIC TRASTUZUMAB USING CIRCULAR DICHROISM COUPLED WITH STATISTICAL ANALYSIS **

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We employ a novel circular dichroism (CD) technology coupled with statistical analysis to assess the higher order structure (HOS) similarity of commercially available trastuzumab and a proposed biosimilar. This technology shows good potential for enabling the comparison of similarities and differences in secondary and tertiary structure of proteins. Multiple CD spectra in far- and near-UV are obtained reproducibly from a CD spectrometer with fully integrated autosampler and flow cell to eliminate the errors typically associated with sample handling on manual CD spectrometers. The significance of similarities or differences was quantified statistically using three analytical methods (weighted spectral difference, correlation coefficient and area of overlap). HOS comparison of the secondary structure (far-UV CD spectra) suggested similarity between the innovator drug, trastuzumab (Herceptin®), and biosimilar products. However, the tertiary structure (near-UV CD spectra) suggested statistically significant differences. These differences may be due to changes in tertiary structure or to modifications and degradations during the process of production or storage. The results show that the automated circular dichroism technology coupled with statistical analysis is a robust tool for enabling the comparison of similarities and differences in secondary and tertiary structure of protein products.

Keywords: *comparability, biosimilar, trastuzumab, circular dichroism, statistical analysis.*

СРАВНЕНИЕ СТРУКТУРЫ ВЫСШЕГО ПОРЯДКА ПРЕДЛАГАЕМОГО БИОАНАЛОГА И ИННОВАЦИОННОГО БИОТЕРАПЕВТИЧЕСКОГО ПРЕПАРАТА ТРАСТУЗУМАБА С ИСПОЛЬЗОВАНИЕМ КРУГОВОГО ДИХРОИЗМА В СОЧЕТАНИИ СО СТАТИСТИЧЕСКИМ АНАЛИЗОМ

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Использована технология кругового дихроизма (КД) в сочетании со статистическим анализом для оценки сходства структур более высокого порядка коммерчески доступного трастузумаба и предлагаемого биоаналога. Эта технология демонстрирует хороший потенциал для обеспечения возможности сравнения сходств и различий во вторичной и третичной структуре белков. Множественные спектры КД в дальнем и ближнем УФ-диапазонах воспроизводятся с помощью КД-спектрометра с полностью интегрированным автосамплером и проточной ячейкой для устранения ошибок, обычно связанных с обработкой образцов на ручных КД-спектрометрах. Значимость сходств или различий оценивалась статистически с помощью трех аналитических методов (взвешенной спектральной разности, коэффициента корреляции и площади перекрытия). Сравнение вторичной структуры (спектры КД в дальнем УФ-диапазоне) показало наличие сходства между препаратом-новатором трастузумабом (герцептином®) и биоаналоговыми продуктами. Однако третичная структура (ближние УФ-спектры КД) свидетельствовала о статистически значимых различи-

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ях. Эти различия могли быть обусловлены изменениями третичной структуры или модификациями и деградациями в процессе производства или хранения.

Ключевые слова: *сопоставимость, биоаналог, трастузумаб, круговой дихроизм, статистический анализ.*

Introduction. A biosimilar is a biological therapeutic that is highly similar to the original product which aims to offer a cost-saving copy of an innovator biotherapeutic. Although there is still a debate around biosimilar develo*p*ment [1, 2], the approaching expiration of patent protection for original drugs arouses increasing interest in the biopharmaceutical industry. In 2015, the first biosimilar ZARXIO® was approved by the US Food and Drug Administration (FDA). Two further biosimilar products were authorized in 2016, while a monoclonal antibody (mAb) Inflectra® was the first mAb biosimilar approved within the European Union in 2013. Many regulatory agencies worldwide have already introduced guidelines to regulate the development of biosimilar products in their countries [3–5].

High similarity is the basis of biosimilar development and subsequent submission. The stepwise approach is recommended by FDA to demonstrate biocomparabilities, and the analytical comparability in structure is suggested to be preferentially established. Furthermore, the state-of-the-art technology is expected by FDA to be employed in the extensive structural analysis and physicochemical characterization for the proposed product and the reference product [4]. The FDA also recommends using statistical approaches to evaluate analytical similarity in the draft guidance [6] issued in September 2017. So, the use of novel analytical technology coupled with statistical analytical approaches for the assessment of analytical comparability between biosimilar candidate and originator product will be interesting and helpful.

For a protein product, including monoclonal antibodies, structural characterization includes primary structure (amino acid sequence), higher order structure, post-translational modifications, and other possible variations. In recent years, primary structure comparisons of a biosimilar and its originator have been performed satisfactorily by advances in analytical methods. However, the characterization and comparison of higher order structure (HOS) are more challenging due to HOS complexities and lack of suitable analytical methods. The HOS is one of the critical quality attributes (CQAs) for a protein product, and significant impact on efficacy and immunogenicity of a biotherapeutic can be caused by the slightest change in HOS. During a biosimilar development, the importance of detecting minor changes in HOS has been well recognized, and the HOS comparison of a proposed biosimilar and the innovator biotherapeutic was performed extensively [7–11].

Circular dichroism (CD) is an effective tool to analyze HOS and has been widely used in the physicochemical characterization of protein-based therapeutics [12–16]. Secondary structural elements absorb the circularly polarized light differently and thus generate characteristic CD spectra. Unfortunately, conventional CD measurement has two inherent shortcomings: errors associated with sample handling (variations in the CD spectra occur as a result of manual cuvette handling) and spectra must be compared visually, leading to an inherently subjective determination of comparability [17]. Using a fully integrated CD spectrometer equipped with autosampler and flow cell can ensure highly reproducible results and eliminate errors arising from manual cuvette handling. Converting spectral data into numerical data that can be subjected to robust statistical analysis provides objective, statistically validated HOS comparisons.

Herceptin® (Trastuzumab) is a monoclonal antibody that interferes with the HER2/neu receptor [18], and its main use is to treat certain breast cancers. It consists of a pair of heavy and light chains connected by disulfide bonds, and the molecular weight is approximately 150 kDa. Herceptin® is invented by Genentech Roche but in 2014, patent protection for Herceptin® expired in China. A Chinese biotherapeutics manufacturer has provided us a trastuzumab product which is planned to be developed as a Herceptin® biosimilar. In this paper, we will report the application of automated CD technology coupled with statistical analysis in the assessment of HOS comparability between the proposed biosimilar and Herceptin®.

Materials and methods. *Samples.* Herceptin® (trastuzumab, 440 mg/20 ml) is a product from Genentech Roche (Basel, Switzerland); four batches of trastuzumab biosimilar candidates (440 mg/20 mL each) were provided by a Chinese biotherapeutics manufacturer. The originator and follow-on products contain the same excipient composition: 4.65 mmol/L histidine, 54.4 mmol/L α,α-trehalose dehydrate, and 0.08 mmol/L polysorbate 20.

Peptide mapping analysis for the primary structure investigation. The amino acid sequence (primary structure) is the basis of high order structure. Before the HOS characterization, the peptide mapping analysis was conducted to investigate similarity for the primary structures. All the Herceptin® product and the pro-

posed biosimilar products were denatured with guanidine hydrochloride, followed by reduction with dithiothreitol and alkylation with iodoacetamide. Enzymatic digestion with trypsin (Roche Life Science, Indianapolis, IN, USA) was then performed for 18 h in 37°C. The digested peptide mixtures were separated on a Phenomenex Jupiter Proteo C18 column (250×4.6 mm, 5 μm, Phenomenex, Torrance, CA, USA) with UV detection at 214 nm.

Sample preparation for CD measurements. For far-UV measurements, samples were resuspended in water, each to a final concentration of 1.6 mg/mL; 90 mL aliquots were arranged in a 96 well plate. A reference aliquot of buffer (in this case water) was included for each sample aliquot. This approach results in each sample having an independent reference (necessary for robust statistical analysis) and provides evidence that the procedures chosen for cleaning the fixed position 0.1 mm pathlength flow cell were appropriate for the samples. For the near-UV, a 10 mm pathlength flow cell was used with a sample concentration of 0.8 mg/mL. The sample volume required for each independent measurement was 900 uL.

CD measurement. Absorbance and CD spectra of the supplied samples were recorded simultaneously using the conditions listed in Table 1. All the CD measurements including far-UV and near-UV were performed using the Chirascan Q100 circular dichroism spectrometer (Applied Photophysics Limited, U.K.) equipped with an autosampler system. Prepared samples were analyzed sequentially. Absorbance and far-UV CD spectra were collected simultaneously in the 190–260 nm range (1 nm step, 1 s integration time) with a bandwidth of 1 nm. For each sample loading, the monochromator was scanned five times with each scan taking less than 2 min. The analysis temperature was 20°C, and the microtiter plate was kept at 4°C. After each measurement the flow cell was washed and dried automatically using one of several selectable procedures. This process took approximately 6 min per wash/dry. The total time per microtiter plate well (analysis + cell wash/dry) was approx. 15 min. The near-UV CD spectra were measured in the 250–350 nm range.

TABLE 1. Experimental Conditions for Far-UV and Near-UV CD Measurements

Conditions	Far-UV	Near-UV
Sample concentration	1.6 mg/mL	0.8 mg/ml
Temperature in rack/measurement	4/20°C	4/20°C
Volume used in well/in measurement	100/39 μL	900/700 μL
Pathlength	0.1 mm	10 mm
Wavelength range/step	180 or 190–250/1 nm	250–350/0.5 nm
Bandwidth/time per point	0.5 nm/1 s	1 nm/1 s
Replicates (wells per sample)	5 or 7	5
Repeats (scans per well)	3	4

Spectral comparison and analysis of HOS biosimilarity. For CD and absorbance, data from each sample well was averaged, and the averaged data from the proceeding reference well subtracted. The spectral comparison and analysis for biosimilarity were performed using an HOS comparison software module, qBiC biocomparability Suite, applying a weighted spectral difference method [19], correlation coefficient [20], and area of overlap [21] to quantitatively compare spectral differences to establish the higher order structure comparability of the proposed biosimilar product to the reference product.

Biological activities analysis. The cell proliferation inhibition assay was performed using human breast cancel BT-474 cells, 1:2 folds of serial dilution of the samples were transferred into BT-474 cell culture plates and incubated at 37°C in a humidified 5% CO_2 incubator for 7 days. After incubation, each well was treated with CellTiter 96® aqueous one solution cell proliferation assay reagent (Promega), and the absorbance at 450 nm was read and recorded. Test results were expressed as the relative percentage of the EC50 from the dose-response curve of biosimilar candidates with respect to originator trastuzumab. The binding affinity to HER2 antigen was evaluated by surface plasmon resonance (SPR) using Biacore T200 (GE Healthcare). According to the manufacturer's recommendation, anti-human IgG-Fc antibodies were immobilized to a CM5 sensor chip surface via the amine coupling method. Each sample was diluted to 1 μg/mL, then injected at a flow rate of 15 μL/min for 40 s and captured by the anti-Fc antibody. The HER2 antigen was serially diluted and injected into the ligand-immobilized CM5 chip with an injection time of 100 s and a dissociation time of 800 s. The data was globally fitted using a 1:1 binding model. The dissociation equilibrium constant K_D value was evaluated using Biacore T200 software (v. 2.0).

Results and discussion. *Similarity of the primary structure.* Before the HOS comparison, it is necessary to perform the similarity inspection for the primary structure. The peptide profiles for innovator and the proposed biosimilar are visually indiscriminate, suggesting the comparability of the amino acid sequence (Fig. 1). Complementary characterization includes N terminal sequencing, intact protein mass analysis, deglycosylated protein mass analysis, amino acid composition analysis, isoelectric point determination, CE-SDS for HC and LC analysis, and glycan profiling. Results suggested no significant differences except for minor differences in the relative abundance of principal glycoforms. We conclude that the proposed biosimilar is similar to innovator product in terms of primary structure.

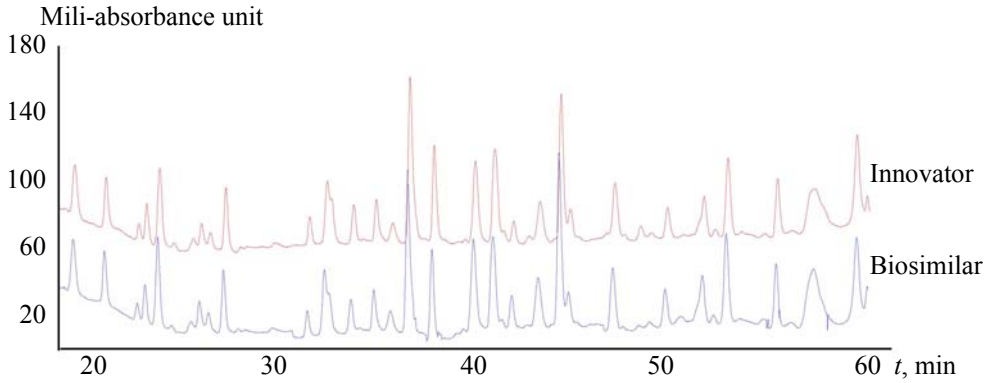

Fig. 1. A comparison of the trypsin digested peptide mapping for Herceptin ® and the proposed biosimilar suggests the similarity of amino acid sequence.

Repeatability for CD measurement. Measurement repeatability is vital to establish analytical comparability between similar proteins. Sources of experimental error generated by instrumentation, sample handling, and operator performance should be minimized as far as possible. For this reason, a Chirascan Q100 including autosampler and flow cell and the qBiC Biocomparability Suite were used in these experiments. An optics-based, multiwavelength calibration method eliminates errors arising from traditional single point chemical calibration and long-term instrument drift/variability. Automation eliminates errors arising from manual cuvette handling and increases the feasibility of taking multiple repeat measurements, thus further reducing error. Robust statistical methods then enable objective results to be obtained. Figure 2 shows representative overlay CD and absorbance spectra obtained from seven sample aliquots of the same protein with five repeated scans per sample. A total of 35 spectra appears to have the same overall spectral profile and show good measurement repeatability. Analysis with qBiC shows that differences between these spectra are not significant at the 2-sigma confidence.

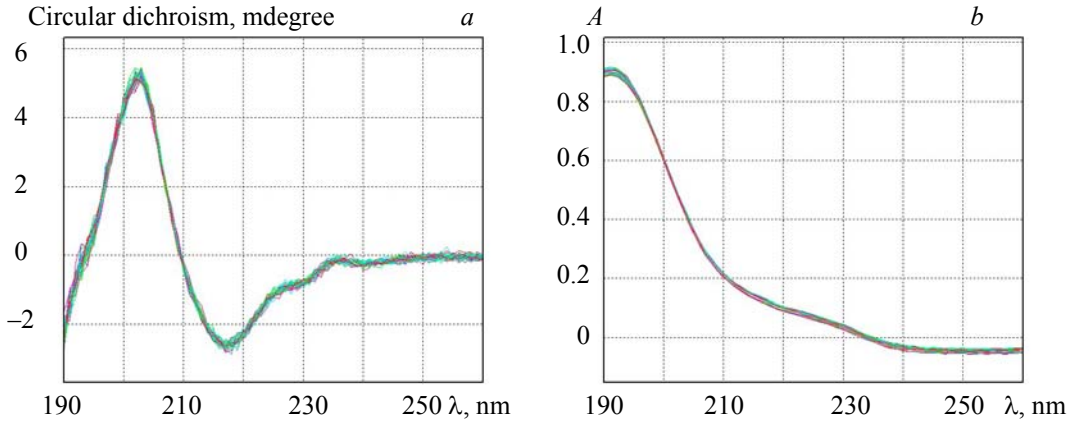

Fig. 2. Overlay CD (a) and absorbance (b) spectra obtained from seven sample aliquots of the same protein, five repeat scan per sample. No smoothing, averaging, or baseline correction.

Far-UV CD spectra of Herceptin® and biosimilar candidates. The autosampler system used in the CD measurements enables us to obtain highly reproducible results and prepare many replicates, which leads to increased signal-to-noise rate and robust statistical analysis. In our experiments, each sample (including innovator products and proposed biosimilar products) was loaded into seven separate wells, and five repeat CD scans were performed per sample loading. A total of 35 absorbance and 35 UV CD spectra was acquired for each innovator and proposed biosimilar sample. The data from each sample well was averaged, and then the average of the data from the proceeding reference well was subtracted. Figure 3 shows the CD spectra of these samples normalized to the average absorbance at 191 nm. These spectra show visual similarities, and the next step was a statistical analysis to ensure the comparison results are objective.

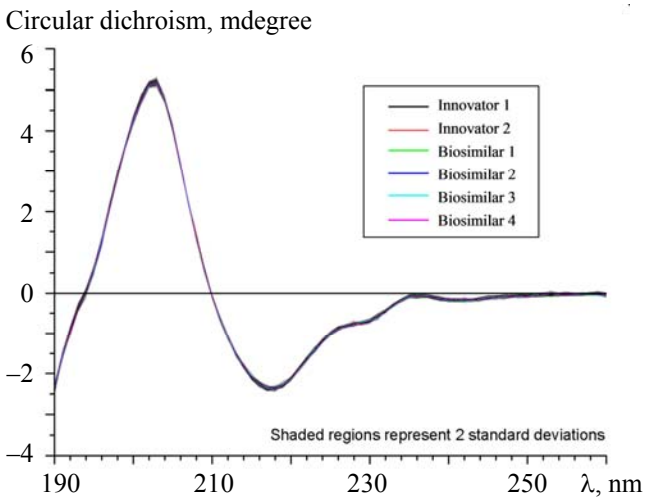

Fig. 3. Absorbance normalized absolute far-UV CD spectra for reference Herceptin® (trastuzumab) products and proposed biosimilars.

Quantitative comparisons of far-UV CD data. Difference spectra (Fig. 4) were calculated by subtracting the innovator product CD spectrum from those of the other samples. The shaded regions propagated represent two standard deviations, which provide a way of judging the extent of statistically significant differences between the two protein spectra. As can be seen, all spectrum lies completely within two standard deviations and show no statistically significant differences. Three independent mathematical approaches were used to calculate a series of similarity scores for spectra within the reference dataset and a second series of scores based on comparisons between reference and test datasets. A *t*-test was performed to assess whether the differences were significant, in this case, at the 2-sigma confidence interval, within the qBiC software.

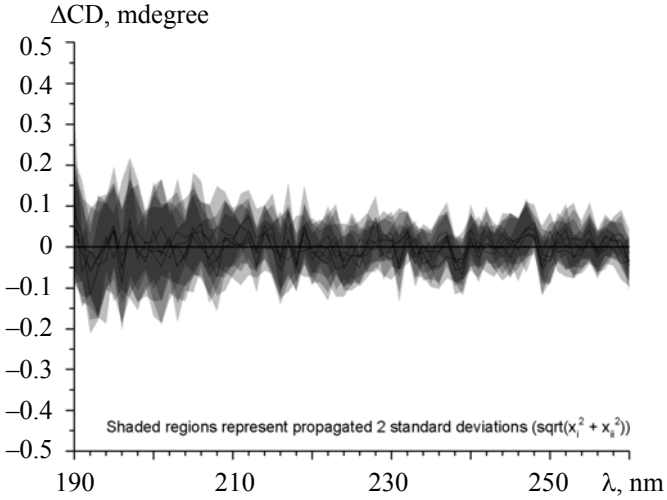

Fig. 4. Far-UV CD difference spectra for comparison of innovator products and proposed biosimilars.

TABLE 2. Similarities of Biosimilar Candidates to Herceptin® according to Far-UV CD Data Using three Independent Mathematical Approaches

Sample	Correlation coefficient		Weighted spectral difference		Area of overlap	
	Similarity	*p*-value (*t*-test)	Similarity	*p*-value (*t*-test)	Similarity	*p*-value (*t*-test)
Innovator	0.99987	0.522	0.031991	0.331	0.9904	0.365
Biosimilar 1	0.99987	0.241	0.029853	0.673	0.98949	0.163
Biosimilar 2	0.99984	0.463	0.037249	0.998	0.98986	0.427
Biosimilar 3	0.99988	0.158	0.028441	0.277	0.98993	0.0957
Biosimilar 4	0.99984	0.0865	0.038324	0.114	0.98859	0.0913

N o t e. Herceptin® (trastuzumab) is used as reference. $p > 0.05$ suggests the absence of significant differences between the datasets for the 2-sigma confidence interval.

Among the three spectra comparison approaches, the correlation coefficient and area of overlap methods are spectral similarity methods with defined ranges either from 0 to 1 or from –1 to 1, the higher values meaning higher similarity between spectra. While weighted spectral difference measures differences rather than similarities between spectra, the higher calculated values mean greater differences between spectra. As shown in Table 2, either the spectral similarity methods or the spectral difference method give similar reporting results. Furthermore, the p-values given by *t*-test are all greater than 0.05, meaning that there are no significant differences between the datasets. The conclusion from the comparison of the datasets is that far UV CD cannot find significant difference between these samples, demonstrating the high similarity between innovator herceptin® product and the proposed biosimilar products.

Near-UV CD spectra and data for Herceptin® and biosimilar candidates. Figure 5 shows near UV CD spectra for Herceptin® and biosimilar candidates; the spectra are averaged and baseline corrected with two standard deviation error bars. Correlation coefficient, weighted spectral difference, and area of overlap methods are used to calculate the spectral similarity (similarity score) for pairs of spectra. A *t*-test is performed to determine the probability (*p*-value) that the scores are part of the same population and that differences between them arise by chance alone – i.e., no significant change in structure. When working at the 2-sigma confidence interval, $p < 0.05$ indicate the presence of a significant difference between similarity scores and, by extension, the CD spectra. As seen in Table 3, comparison of the innovator control and biosimilar 1 and biosimilar 2 shows significant differences (all $p < 0.05$). Biosimilar 3 is similar to the original product according to results calculated by correlation coefficient methods, but results of the other two calculating methods show dissimilarities with the control. Biosimilar 4 is like biosimilar 3, two results show no significant differences, and the other one is significantly different. These results show the important differences between the spectral similarity calculating methods, and careful consideration is needed as to which one is the most appropriate.

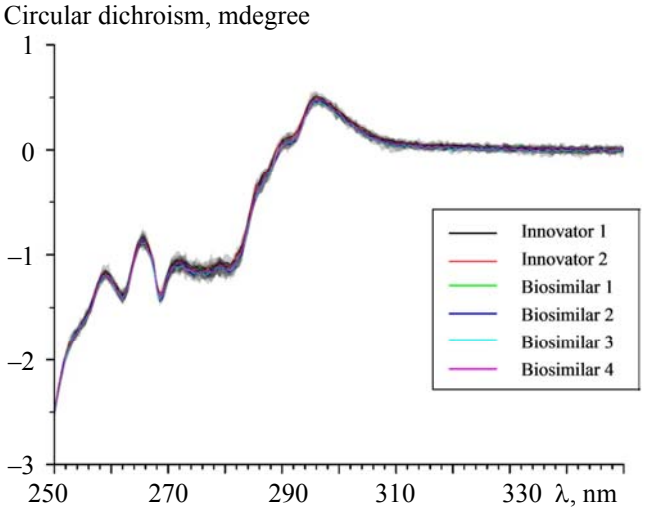

Fig. 5. Averaged and baseline corrected near-UV CD spectra of the innovator products and biosimilar products.

TABLE 3. Similarities of Biosimilar Candidates to Herceptin® Calculated by Near-UV CD Data Using Three Independent Mathematical Approaches

Sample	Correlation coefficient		Weighted spectral difference		Area of overlap	
	Similarity	*p*-value (*t*-test)	Similarity	*p*-value (*t*-test)	Similarity	*p*-value (*t*-test)
Innovator	0.99974	0.708	0.018354	0.114	0.98779	0.861
Biosimilar 1	0.99942	0.0196	0.037965	0.00669	0.97322	0.00159
Biosimilar 2	0.99938	0.0237	0.040491	0.00122	0.97422	0.00031
Biosimilar 3	0.99988	0.0633	0.042102	0.00605	0.96905	0.00528
Biosimilar 4	0.9995	0.186	0.033833	0.0984	0.97753	0.0235

N o t e. Herceptin® is used as reference. $p < 0.05$ indicates the presence of differences that are significant at the 2-sigma confidence interval.

According to previous research [22] by Marshall using the circular dichroism technique for HOS comparisons of monoclonal antibodies, it is possible that the above-mentioned differences in near-UV CD spectra between the innovator product and biosimilar candidate products are due to changes in the local environment of aromatic side chains (tertiary structure) of products caused by deamidation/isomerization, glycation, or oxidation during the process of production or storage, although the secondary structure contents are not changed according to the far-UV CD results. In another study on charge heterogeneities of Herceptin® and biosimilar candidates (as with this study), there existed differences between innovator and similar products in terms of acidic species (data not shown in this paper). The reasons for the formation of acidic species are possible modifications including sialylation, glycosylation, deamidation, cysteinylation, mismatched disulfide linkage, and so on [23]. We can assume that the reasons for the above-mentioned near-UV CD spectra differences and charge heterogeneities between innovator and similar products are similar. Further study is required for better characterization.

Other minor differences also were found when we performed the HOS comparisons using hydrogen/deuterium exchange (HDX) coupled with mass spectrometry (MS) analysis and nuclear magnetic resonance (NMR) spectral fingerprints (data not show in this paper). These results, in addition to the above-mentioned near-UV CD spectra differences, do not preclude the two products from being biosimilar.

Biological activities. To explore whether the above-mentioned HOS differences correlate with biological activities, we evaluated and compared the biological activities of Herceptin® and biosimilar candidates using a proliferation inhibiting bioactivity assay and a HER2 binding affinity assay. Surprisingly, biosimilar candidates exhibited highly similar antiproliferative activities and binding affinities compared with originator trastuzumab. The antiproliferative activities of candidate biosimilars were measured at 96.46–105.54% in comparison with the originator trastuzumab, while the SPR analysis showed that the binding affinity constant K_D to HER2 antigen was 1.02, 1.05, 0.85, 0.88, and 0.94 for four batch biosimilars and originator separately. The biological activity analysis indicated a minimal influence of observed HOS differences on antiproliferative activity and antibody-antigen interaction.

We compared the HOS of originator trastuzumab and its biosimilar candidates using the CD technology coupled with statistical analysis; meanwhile, we investigated the biological activities using bioanalytical methods. Some minor HOS differences were observed, and similarities were not determined, while the biological activities proved to be similar. This inconsistency did not mean that the CD measurement coupled with statistical analysis is incorrect; on the contrary, it can be confirmed from these results that this technology has high resolution and is powerful for protein HOS characterization and comparison.

Besides biosimilar development and regulatory approval, auto-CD measurement coupled with statistical analysis is a powerful analytical tool that can also be used in the assessment of comparability for a biological product before and after a manufacturing process change made by the same manufacturer according to ICH Q5E guidelines [24], and in the evaluation of batch-to-batch HOS heterogeneity for quality control during a biotherapeutic manufacturing processes.

Conclusions. An auto-CD technique coupled with rigorous statistical analysis was used to assess the degrees of similarity between comparator monoclonal antibody products in this paper. The circular dichroism spectral comparison method proved to be a sensitive technique for biosimilarity analysis and objective identification of very small differences in HOS of trastuzumab and its proposed biosimilar. After statistically significant differences are identified, the next step is to determine whether these differences are acceptable or

not. Other orthogonal methods for physicochemical characterization will, of course, be applied to support the totality of the evidence [25–30].

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