Biosimilar Profiling

Application Note NT-PR-010

Rapid and Precise Biosimilar Candidate Profiling by nanoDSF

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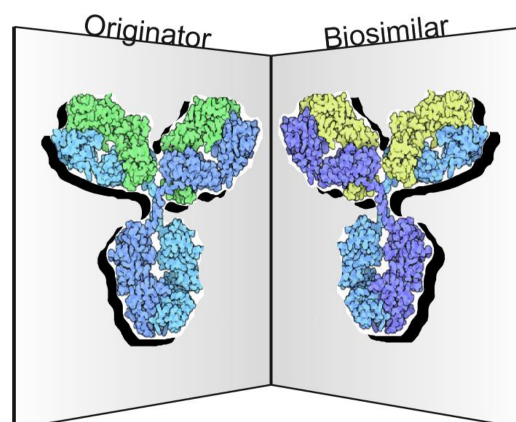
Abstract

The development of biosimilars requires extensive physicochemical characterization of biosimilar candidate molecules which should match the quality profile of the reference molecule (originator).

Here we use a novel method of thermal unfolding profiling to rapidly screen a variety of Fc-fusion protein biosimilar candidates. The best-in-class precision of the NanoTemper Technologies Prometheus NT.48 nanoDSF instrument allowed for the ranking of biosimilar candidates according to the comparability of their unfolding profiles to the reference molecule. The results were in excellent agreement with conventional screening methods, while dramatically reducing sample consumption and measurement times. Thus, nanoDSF (miniaturized differential scanning fluorimetry) is a new and powerful tool for rapid screening approaches in biosimilar development. It enables narrowing down the number of promising candidates in hours instead of days or weeks.

Introduction

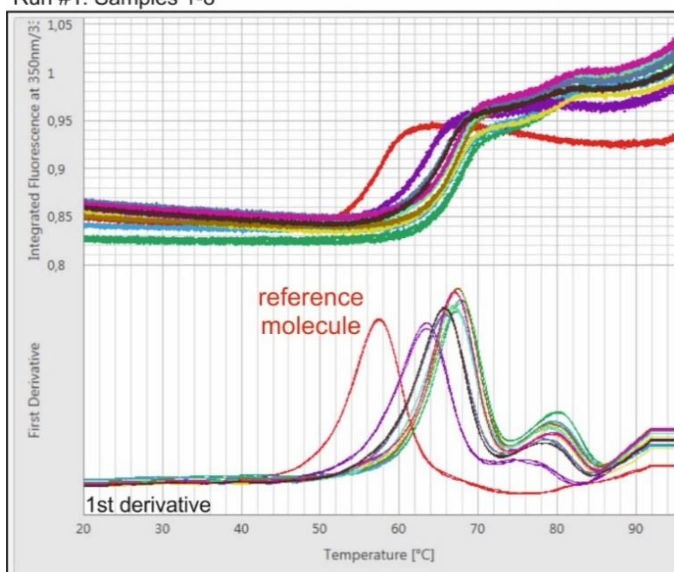
In contrast to the development of new biological entities (NBEs), where much time and resources are invested in clinical trials, the focus in the development of biosimilar medicinal products lies on a comprehensive physicochemical and biological protein characterization to ensure that the molecular fingerprint of the biosimilar matches that of the originator as closely as possible (van Aerts et al, 2014). Commonly, various analytical methods such as high-performance liquid chromatography, capillary electrophoresis, mass spectrometry, Raman spectroscopy, isoelectric focusing, and CD-spectroscopy are used to evaluate the degree of similarity between molecules (Berkowitz et al, 2012; Bui et al, 2015; Cai et al, 2011). While useful for in-depth characterization of biosimilar candidates, these methods suffer from either low throughput, high sample consumption, or laborious data analysis, and thus are not very well suited for rapid screening of a large number of biosimilar candidates.



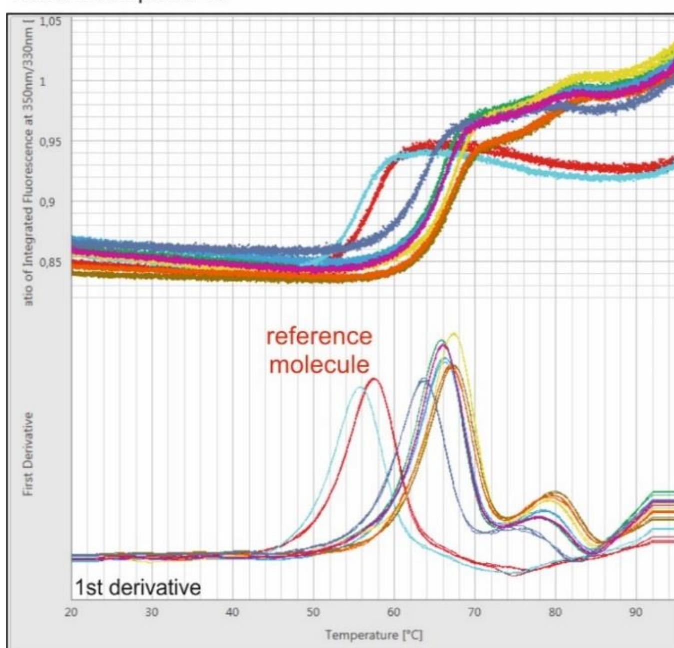
The 3D-structure of biosimilars is typically very complex since it is determined by the sum of a plethora of low-affinity intramolecular interactions.

A

Run #1: Samples 1-8



Run #1: Samples 9-16



B

Run #	Sample #	1st Tm	2nd Tm
1	Ref	57.5°C	n.a.
1	Ref	57.5°C	n.a.
1	Ref	57.6°C	n.a.
1	1	67.9°C	80.3°C
1	1	67.9°C	79.9°C
1	1	67.8°C	80.2°C
1	2	67.3°C	74.3°C
1	2	67.3°C	79.2°C
1	2	67.3°C	79.4°C
1	3	63.5°C	82.6°C
1	3	63.6°C	82.4°C
1	3	63.5°C	84.3°C
1	4	67.2°C	78.8°C
1	4	67.2°C	80.1°C
1	4	67.1°C	78.9°C
1	5	67.4°C	79.8°C
1	5	67.4°C	79.2°C
1	5	67.5°C	79.1°C
1	6a	67.0°C	79.0°C
1	6a	66.9°C	78.2°C
1	6a	67.0°C	78.7°C
1	6b	65.9°C	77.9°C
1	6b	65.9°C	77.8°C
1	6b	66.0°C	79.0°C
1	7	67.1°C	79.4°C
1	7	67.1°C	79.4°C
1	7	67.0°C	78.8°C
1	8	65.8°C	78.1°C
1	8	65.8°C	78.5°C
1	8	65.9°C	77.7°C
2	ref	57.5°C	n.a.
2	ref	57.4°C	n.a.
2	ref	57.5°C	n.a.
2	9	65.8°C	78.1°C
2	9	65.9°C	78.1°C
2	9	66.0°C	78.2°C
2	10	66.2°C	78.6°C
2	10	66.3°C	78.6°C
2	10	66.4°C	78.2°C
2	11	55.8°C	n.a.
2	11	55.7°C	n.a.
2	11	55.7°C	n.a.
2	12	67.4°C	78.9°C
2	12	67.4°C	78.9°C
2	12	67.3°C	78.6°C
2	13	67.4°C	79.7°C
2	13	67.3°C	79.6°C
2	13	67.3°C	79.6°C
2	14	67.0°C	79.5°C
2	14	67.0°C	79.3°C
2	14	67.1°C	78.1°C
2	15	63.7°C	75.3°C
2	15	63.7°C	74.3°C
2	15	63.7°C	76.0°C
2	16	66.0°C	78.6°C
2	16	66.1°C	78.3°C
2	16	66.0°C	78.2°C

Figure 1: Biosimilar profiling using nanoDSF. (A) Raw nanoDSF data of the thermal unfolding of the originator (red) and 17 biosimilar candidates. Measurements were carried out in triplicates. Changes in the F350/F330 fluorescence ratio are shown. (B) Comparison of T_m values of the samples. Note that the reference molecule and sample #11 do not show a 2nd unfolding transition in the F350/F330 ratio.

Importantly, since the single intramolecular interactions are weak, differences in the expression or purification process can introduce structural alterations of the biosimilar when compared to the originator. Controlled heating of the biosimilar gradually dissipates intramolecular interactions required for protein folding, resulting in multiple unfolding transition events. nanoDSF offers highly precise and reproducible detection of thermal unfolding patterns and therefore precisely recapitulates conformational stability and similarity between distinct unfolding events.

Here we show that the similarity in the unfolding profiles of biosimilar candidates serves as a direct measure for their conformational similarity to the originator. The nanoDSF thermal unfolding profiling approach allows for rapid screening of a large number of biosimilar variants in different stages of development in order to narrow down the number of candidates and to optimize the biosimilar development process.

Results

A CHO cell line expressing candidates biosimilar to a marketed Fc-fusion therapeutic protein was generated at UGA Biopharma. During early-stage biosimilar cell line development, sixteen different combinations of basal and feed media were screened in fed-batch mode.

The aim of media screening was to identify a baseline cell culture fed-batch process resulting in maximum comparability of biosimilar candidates to the reference molecule. Candidate biosimilars were purified from clarified cell culture fluid by capture chromatography using modified protein A. Biosimilar candidates, as well as the reference molecule, were then diluted to approximately equal concentrations in a sample buffer comparable to the formulation buffer of the reference product.

We measured the thermal unfolding of 16 candidates and the reference by nanoDSF. Measurements were performed in triplicates in 2 runs. The variance in unfolding transition temperatures (T_m values) and unfolding patterns between the different molecules was rather large: While the reference molecule showed a single T_m value at 57.5 °C in the F350/F330 fluorescence ratio, almost all candidates, except for sample #11, displayed two unfolding transitions (Figure 1A).

The T_m1-values differed by as much as 12 °C, ranging from 55.7 °C (sample #11) to 67.9 °C (sample #1) (Figure 1B). Interestingly, unfolding in the single-wavelength fluorescence showed two clear unfolding transitions for all samples, including the reference molecule and sample #11 (Figure 2). These differences in the unfolding signals are likely caused by differences in the secondary structures of the candidates. The fact that the reference molecule and sample #11 show a second transition only in the single wavelength and not in the F350/F330 ratio suggests that the responsible tryptophan(s) is/are already surface-exposed and thus do not undergo a red-shift.

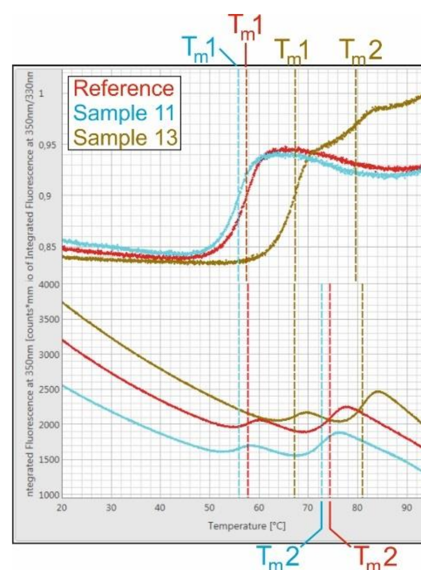
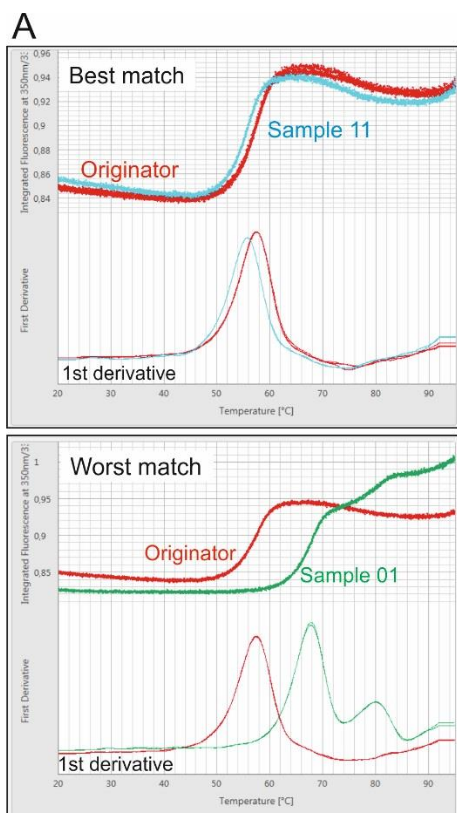


Figure 2: Comparison of triplicate thermal unfolding signals from the F350/F330 ratio and the single wavelength at 350 nm. While sample #13 displays two unfolding transitions in both signals, the reference and sample #11 display only one transition in the F350/F330 ratio.

Since the candidates not only differed in T_m, but even more in the entirety of their unfolding profiles, including unfolding amplitudes, unfolding transition slopes, and number of unfolding events, we quantified the overall similarity of their thermal unfolding patterns. For this, the first derivative data of the F350/F330 signal were analyzed by directly comparing the profile of the reference molecule to the unfolding profile of each candidate. The result is an “overlap index” which allows for ranking of

candidates according to their overall similarity to the reference molecule. A perfect match yields an overlap index value of 0. This analysis showed that sample #11 was the best match by a large margin (Figure 3A and B), while samples #1 and #5 had the lowest similarity scores.



B

Sample	1st derivative Overlap Index	rank
1	0.492	17
2	0.461	13
3	0.306	2
4	0.446	9
5	0.488	16
6a	0.461	14
6b	0.423	6
7	0.458	12
8	0.403	4
9	0.433	7
10	0.438	8
11	0.111	1
12	0.464	15
13	0.455	11
14	0.452	10
15	0.332	3
16	0.423	5

Figure 3: Quantitative similarity profiling of nanoDSF data. (A) Comparison of the unfolding signals and corresponding first derivative traces of the best match (sample #11) and the worst match (sample #01). (B) 1st derivative overlap indices and resulting ranking of all candidates in respect to the reference molecule.

In order to evaluate whether identification of biosimilar candidates correlates with complementary techniques, we compared the nanoDSF thermal profiling data to the data from HPLC methods used at UGA Biopharma to assess critical quality attributes of the Fc-fusion protein in question. Charge variants were profiled using a strong anion exchange column with a salt gradient. N-linked glycan profiling was done by HILIC with fluorescent glycan detection. Total sialic acid was quantified by fluorescent detection of released sialic acid. First, all biosimilar candidates were analyzed in charge variant profiling. Biosimilar candidates which showed dissimilar profiles (e.g. multiple peaks in place of a single peak) were not considered for further evaluation. For the remaining samples, similarity indexes were calculated using a proprietary UGA Biopharma algorithm, based on how close the match between the reference product and biosimilar candidate was (Table 1).

Table 1. Samples ranked according to the charge variant similarity index.

Rank	SampleID
1	09
1	11
2	03
3	15
4	07
5	05
6	06b

Next, biosimilar candidates were analyzed for their sialic acid content. Again, similarity to the reference product was used to calculate a similarity index (Table 2).

Table 2. Samples ranked according to the sialic acid content similarity index.

Rank	SampleID
1	11
2	09
3	03
4	06b
5	15
6	07
7	05

Finally, the N-linked glycan profiles were acquired for the samples and compared to the reference. Again, an appropriate algorithm of UGA Biopharma was used to rank the samples (Table 3).

Table 3. Samples ranked according to the N-linked glycan similarity index.

Rank	SampleID
1	15
2	11
3	09
4	06b
5	03
6	07
7	05

To compare the results obtained by a single method (nanoDSF thermal profiling) and more specialized analyses used in biosimilar development, we combined the ranks of charge variant profiling, sialic acid quantification and N-linked glycan profiles into a combined similarity rank (Table 4).

Table 4. Samples ranked according to the combined charge variants, sialic acid content and N-linked glycan similarity index.

Combined rank	SampleID
1	11
2	09
3	03
3	15
4	06b
5	07
6	05

Both approaches for biosimilar candidate selection, nanoDSF profiling and three combined HPLC methods identified sample #11 as the one showing the best match to the reference product (Figure 4). In addition, there was an overlap for 5 out of 7 samples with most similarity for the two ranking strategies. The comparison shows an excellent agreement between the similarity ranking obtained by combined charge variants, N-linked glycans and sialic acid content methods with the nanoDSF thermal unfolding profile ranking.

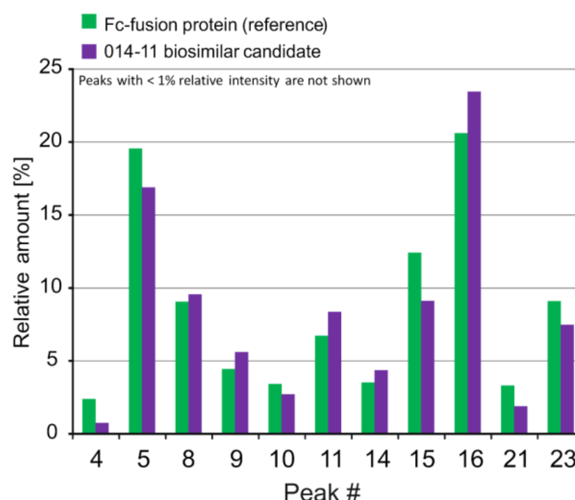


Figure 4: N-linked glycan analysis of the reference product and candidate 014-11.

Conclusions

This study shows that nanoDSF has the potential to revolutionize similarity screening processes in biosimilar profiling. The best-in-class precision, reproducibility and data quality of the Prometheus series delivers high-quality thermal unfolding profiles which show even smallest differences between biosimilar variants. Up to 48 samples, each with a volume of just 10 µl and less than 1 µg of protein, can be measured in parallel. nanoDSF is therefore orders of magnitude faster than the complimentary screening methods used here, which require ~ 1 week of lab work. Moreover, 150-times less protein is used for the analysis, rendering nanoDSF the perfect tool for large-scale screenings at early stages of biosimilar development.

The optional automation allows for unattended analysis of hundreds of samples per day, resulting in a yet unknown productivity. The flexibility, low consumable costs and maintenance-free operation of the system allows for its integration into virtually every step in the biosimilar development process. This enables scientists to perform quick and meaningful screenings with minimal time and material requirements to filter candidates with similar structural properties based on their unfolding patterns.

Methods

Thermal unfolding by nanoDSF. Samples 014-01 to 014-16 and the reference molecule were measured as triplicates in thermal unfolding experiments with a heating rate of 1 °C/min in standard treated capillaries. The reference was previously diluted in sample buffer to reach a final concentration of 2 mg/ml, matching the concentration range of the other samples. 10 µl of sample were required for one thermal unfolding profile.

For calculation of the overlap index, the first derivative data of the F350/F330 fluorescence ratio unfolding curves were used. Averages of the triplicate measurements were calculated and subtracted from the 1st derivative value of the reference molecule for each scan. Subsequently, absolute values were added to yield the 1st derivative overlap index for each sample.

Protein charge variant profiling. Protein charge variants were profiled using a strong anion exchange column with a salt gradient at pH 7 and detected. N-linked glycans were released from the protein by PNGase F digest and then labelled by 2-AB.

N-glycan profiling. N-linked glycan profiles were acquired by separation of labeled glycans on an amide column run in HILIC mode with fluorescent detection.

Sialic acid quantification. Sialic acids were released by acidic hydrolysis followed by DMB-labelling. Total sialic acid was then quantified by fluorescent detection of labelled sialic acid species separated on a reversed phase chromatography column following normalization with internal standards.

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Notes

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