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Characterization of bevacizumab by dynamic light scattering while maintaining its native structure

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Bevacizumab, is a humanized monoclonal antibody and patents on Avastin® (Bevacizumab, Roche) will expire in the US in 2019 and in Europe in 2022. Therefore, bevacizumab is a popular target for biosimilar developers. One of the most common problems in the formulation of antibody drugs is protein aggregation. Dynamic light scattering (DLS) is a well-established method for the determination of hydrodynamic dimensions, aggregates, and aggregation points of proteins. In contradistinction to other techniques that require diluted samples or specific conditions, proteins and aggregates can maintain their native structure during DLS measurements. In recent studies, bevacizumab was characterized by DLS using diluted samples. In this study, we aimed at investigating the hydrodynamic dimensions, aggregates, and aggregation onset of bevacizumab (Altuzan®, Turkey, Roche) by DLS, while maintaining its native structure. The intensity, volume, and number-based particle size distribution profiles of the test samples were evaluated and the aggregation onset of the formulation was successfully determined against increasing temperature. It is shown that the preservation of the native structure of commercial formulations in DLS measurements provides an opportunity to the characterization of commercial products and development of biosimilars.

1. Introduction

The market for antibodies and Fc fusion proteins is increasing day-by-day. Up to now, 74 antibodies and Fc fusion proteins have been approved by at least one major authority (Czajkowsky et al. 2012; Strohl et al. 2018). One of them, bevacizumab, is a humanized monoclonal antibody, which can inhibit the angiogenic activity of the vascular endothelial growth factor (VEGF) *via* extracellular binding. Bevacizumab is indicated for the treatment of metastatic colorectal cancer, recurrent or metastatic non-squamous non-small cell lung cancer, recurrent glioblastoma, metastatic renal cell carcinoma, persistent, recurrent, or metastatic cervical cancer, and recurrent epithelial ovarian, fallopian tube, or primary peritoneal cancer. Patents on Avastin® (Bevacizumab, Roche) will expire in the US and Europe in July 2019 and January 2022, respectively; the 2016 sales of Avastin were ~5.95 billion Euros, which makes it a popular target for biosimilar developers. Currently, there are nearly 15 bevacizumab biosimilars in development (Genentech 2016; Gabi Online 2014; Monk et al. 2017).

One of the most important problems in the production and formulation of monoclonal antibodies is protein aggregation (Vazquez-Rey and Lang 2011). It is well known that the determination of aggregates is of great importance in therapeutic protein formulations as they induce immunological responses, impairing the potency of protein-based drugs and leading to stability problems (Vazquez-Rey and Lang 2011; Zolls et al. 2012; Pathak et al. 2013). Previously, various techniques have been described to determine the hydrodynamic radii of proteins, aggregates, and aggregation onset of protein formulations (Ye 2006; Demeule et al. 2007; Mahler et al. 2009; Philo 2009). However, the development and optimization of these methods are fraught with considerable challenges (Teraoka 2004; Philo 2009). For instance, to characterize proteins and their aggregates in pharmaceutical products, size exclusion-high performance liquid chromatography (SE-HPLC) is often used (Khodabandehloo and Chen 2017); despite the many advantages of SE-HPLC, misleading results were reported in previous studies. These studies demonstrated that the aggregate levels and sizes could not be measured accurately in the drug product container (Carpenter et al. 2010). When a sample is injected onto the SE-HPLC column, it is diluted by the mobile phase, which is compositionally different or has a relatively higher ionic strength compared to the product formulation (Carpenter et al. 2010; Khodabandehloo and Chen 2017). Further, sample dilution might be necessary before injection to obtain the required signal strength. As a result of these factors and others that may not be obvious, aggregates can be dissociated or formed (Carpenter et al. 2010; Zolls et al. 2012; Khodabandehloo and Chen 2017). Additionally, pH or excipient alteration in the medium is important for hydrodynamic radius determination and for the balance of repulsive and attractive intermolecular interactions between protein molecules in the solution (Kim et al. 2014; Quigley and Williams 2015).

Dynamic light scattering (DLS) is a well-established method for the determination of hydrodynamic dimensions, aggregates, and aggregation points of proteins (Khodabandehloo and Chen 2017). In contradistinction to other techniques that require diluted samples or specific conditions, the native structures of proteins and aggregates can be maintained during DLS measurements (Nobbmann et al. 2007; Khodabandehloo and Chen 2017). DLS is based on the scattering of light from particles or macromolecules (Khodabandehloo and Chen 2017). The incident laser beam light scatters in all directions when confronted by macromolecules. The intensity of the scattered light is determined by a detector (Stetefeld et al. 2016) and used to compute the intensity, volume, and number-based particle size distribution (Mahl et al. 2011). In spite of DLS being considered a popular technique for protein analysis, it faces several disadvantages and limitations (Khodabandehloo and Chen 2017). For instance, the intensity of the scattered light is proportional to the particle size raised to the sixth power; this

situation causes a tendency to produce artifacts when aggregates or larger particles are present in the samples (Mahl et al. 2011; Khodabandehloo and Chen 2017). DLS data can be represented in the form of size, number, and volume-based distributions, but the ideal distribution type is not clear for protein analysis (Mahl et al. 2011). Finally, sample concentration is critical for reproducible analyses (Stetefeld et al. 2016).

In recent studies, bevacizumab was characterized by DLS using diluted samples (Li et al. 2011; Paul et al. 2012; Wen et al. 2013; Signorello et al. 2014; Khalili et al. 2015). In this investigation, we aimed to analyze the hydrodynamic diameter, aggregates, and aggregation point of bevacizumab [Altuzan® (Avastin's commercial name in Turkey), Roche], while maintaining its native structure in commercial formulations. To achieve this objective, DLS measurements were performed using Altuzan® without any dilution. The intensity, volume, and number-based particle size distribution profiles were measured and the aggregation onset of the formulations were determined against increasing temperature to develop improved analysis tools for the characterization and formulation development of antibodies and their biosimilars.

2. Investigations, results, and discussion

DLS is a well-established, fast, and sensitive technique for measuring particle size and particle-size distribution. Thanks to these features, this technique has attracted a lot of attention from the pharmaceutical industry. DLS is based on the scattering of light from particles and their inherent Brownian motion. In previous studies, it was reported that the DLS technique has many limitations (Khodabandehloo and Chen 2017). For instance, a low

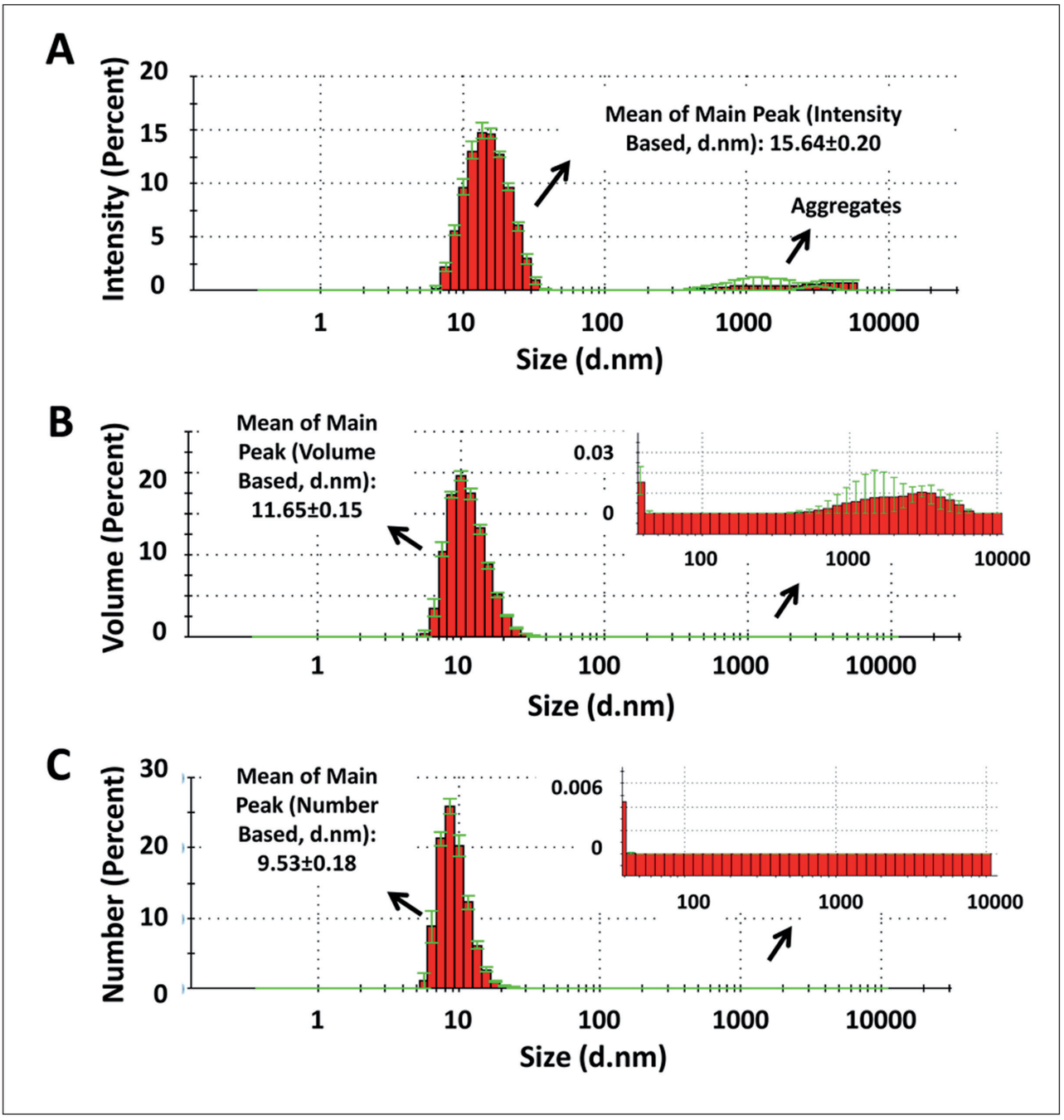

Fig. 1: DLS distribution analysis of Altuzan®. A) Intensity-based distribution. B) Volume-based distribution. C) Number-based distribution. DLS analysis was performed with Altuzan® without any dilution. Quality reports of all the measurements were checked to ensure that the obtained data met the quality criteria. The graphs represent the average results of six measurements.

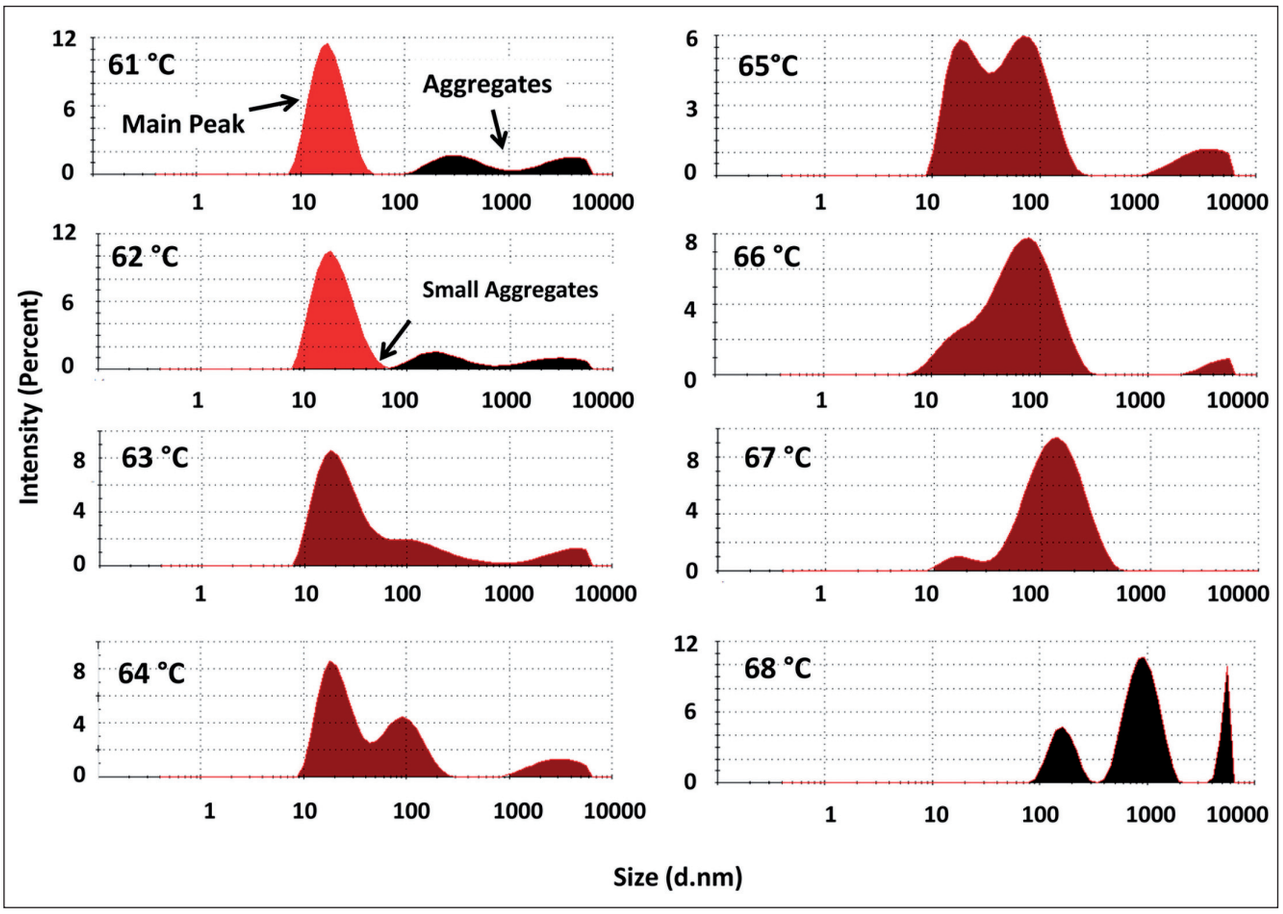

Fig. 2: Intensity-based aggregation onset analysis of Altuzan®. Measurements were performed in triplicate and the figure represents one of these measurements.

sample concentration could lead to a low signal-to-noise ratio. On the other hand, a high concentration can cause multiple scattering and viscosity alteration (Khodabandehloo and Chen 2017). Therefore, in previous studies, DLS analysis of bevacizumab was performed by diluting the original formulations; further, some of these solutions were filtered (Li et al. 2011; Paul et al. 2012; Wen et al. 2013; Signorello et al. 2014; Khalili et al. 2015). It is clear that when samples are diluted or filtered, the determined levels and sizes of aggregates do not accurately reflect the actual values as they might undergo dissociation or formation during dilution (Zolls et al. 2012; Khodabandehloo and Chen 2017).

In this study, the hydrodynamic diameter, aggregates, and aggregation point of bevacizumab [Altuzan®, Roche] were measured, while maintaining its native structure. To accomplish this task, the hydrodynamic diameter of bevacizumab (25 mg/mL in Altuzan®) was measured and computed as intensity, volume, and number-based particle size distributions (Fig. 1 A, B, and C, respectively). Quality reports of all the measurements were checked to ensure that the obtained data met the quality criteria. The means of the main peaks were found to be 15.64±0.20 nm, 11.65±0.15 nm, and 9.53±0.18 nm in the intensity, volume, and number-based distributions, respectively. According to previous studies, the hydrodynamic radius of bevacizumab varies from 3.5 to 6.5 nm (Li et al. 2011; Paul et al. 2012; Wen et al. 2013; Signorello et al. 2014; Khalili et al. 2015; Hirvonen et al. 2016). A detailed analysis of a recent study showed that the radius of bevacizumab is approximately 4.58±0.01 nm and this measurement was corrected with the calculations based on molecular weight and other experimental measurements (Hirvonen et al. 2016). Upon comparing our results with this analysis, the number-based distribution was found to be the optimum tool for determining hydrodynamic radius. This result might be explained in terms of the effect of oligomers on intensity-based distribution analysis. Intensity-based distributions indicated that the hydrodynamic diameter of the mean peak increased owing to oligomeric presence; however, such oligomeric effects are minimized in number-based analysis. On the other hand, it was hypothesized that a high sample concentration could cause multiple scattering and alteration in viscosity (Khodabandehloo and Chen 2017). In our study, when Altuzan® was diluted with its formulation medium (1 mg/mL), the hydrodynamic radius at the mean peak was found to be 6.5 nm, which is similar to the DLS results reported in the literature (data not shown). However, the obtained hydrodynamic radius is still different compared to the results obtained by time-resolved phosphorescence anisotropy (Hirvonen et al. 2016). Additionally, it is clear that aggregates were formed after dilution. This aggregation might have been masked in other DLS studies, owing to the additional filtration step. When the diluted and non-diluted measurements were compared, it could be observed that the hydrodynamic radius of bevacizumab decreased slightly after dilution. This may be caused by multiple scattering; it has been reported that multiple scattering leads to an artificially low measured particle size at high concentrations. Meanwhile, changes in the viscosity can also lead to changes in the hydrodynamic radius. Lastly, dissociated or formed oligomers could also contribute to this alteration. During the DLS measurement of Altuzan® using non-diluted samples, the main peaks exhibited a uniform size distribution and the presence of aggregates (>1 μm) was observed (Fig. 1A). The aggregates were present in higher quantities according to the intensity-based distribution than in the volume and number-based distributions. In volume-based measurements, the presence of aggregates was not observed in the main plot, but they could be observed when the volume (percent) was in the range of 0%–0.03% (Fig. 1B). On the other hand, no aggregates could be observed in the number-based results (Fig. 1C). In our measurements, it was found that intensity percent analysis is more suitable for aggregation or oligomeric

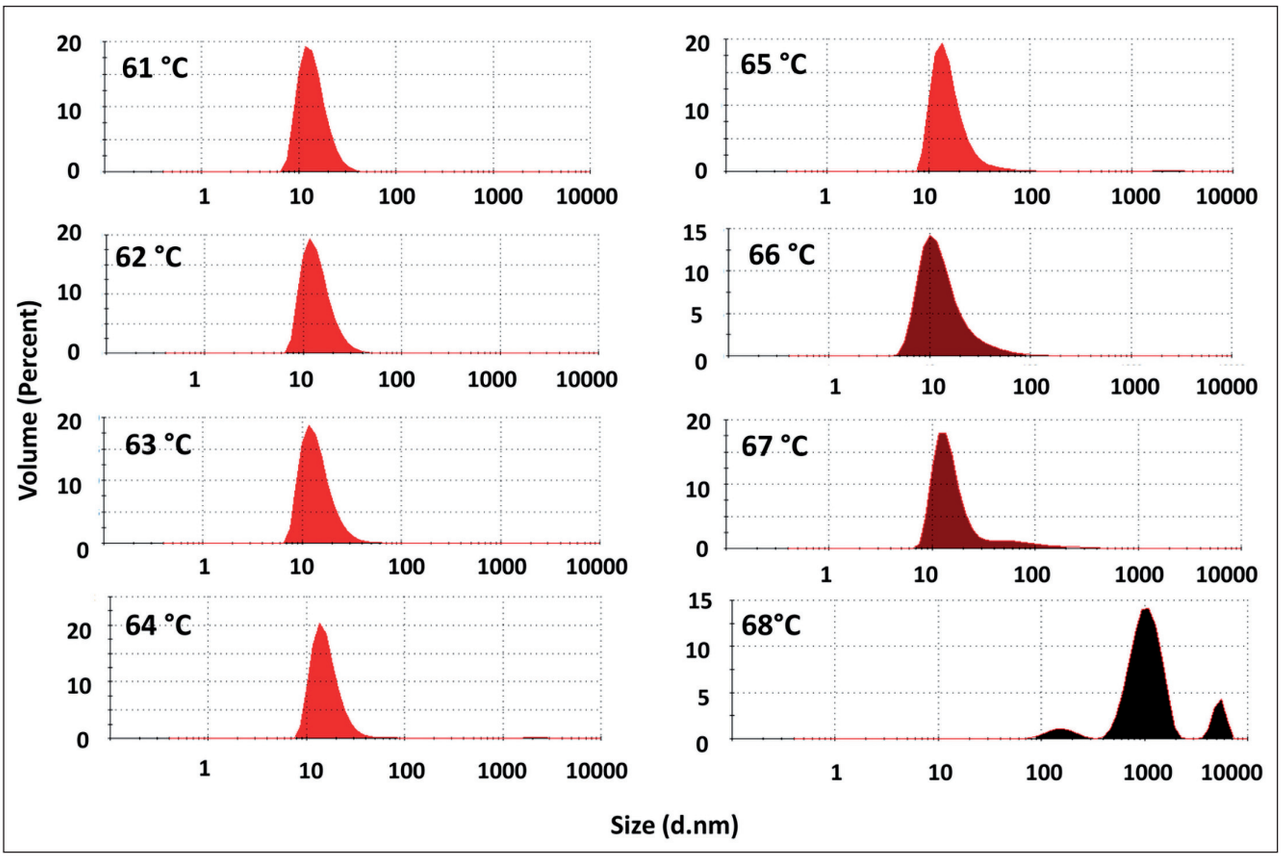

Fig. 3: Volume-based aggregation onset analysis of Altuzan®. Measurements were performed in triplicate and the figure represents one of these measurements.

analysis, while number-based analysis was more suited for the determination of hydrodynamic radius (Hirvonen et al. 2016). This situation could be explained as follows. The intensity of the scattered light is proportional to the sixth power of particle radius and number-based analysis can normalize this factor.

The onset of aggregation (T_{onset}) of proteins in a formulation is important for the development of protein-based drugs and in determining the temperature limit for preparing protein-containing drug delivery systems. Additionally, aggregates do not always grow to large sizes and can remain as soluble oligomers and multimers (Roberts 2014a, b). It can be said that high temperatures usually cause protein unfolding and are directly proportional to the amount of aggregates (Tsai et al. 1998). In this context, DLS provides an opportunity to analyze small-sized aggregates and the T_{onset} of proteins. The conformational stability of a protein can be analyzed by determining its melting temperature ($T_{melting}$), which is generally related to the observed aggregation rate (Chaudhuri et al. 2014). Usually, differential scanning calorimetry (DSC) is used to evaluate the effect of temperature on proteins. However, special and expensive DSC systems, such as nano/micro DSC, are required for temperature measurements. On the other hand, DLS is a cheaper, faster, and more sensitive method to evaluate the effect of temperature on proteins. To develop fast, applicable, and reliable $T_{melting}$ or T_{onset} determination methods, firstly, the temperature was increased from 25 °C to 80 °C at 5 °C intervals. It was found that the aggregation of bevacizumab increased between 60 °C and 70 °C. Based on these preliminary measurements, tests were conducted in the range of 60–70 °C at 1 °C intervals. It is known that viscosity and refractive index change with temperature. Measurement of these values against for all temperature is not applicable approach. To overcome this problem, the same measurements were edited at different viscosity and refractive index values (Altuzan's medium and water) with Zetasizer Software v7.11 and the obtained results were compared. This preliminary experiment demonstrated that only a small shift (2 nm) occurred. Later, water was selected as the dispersant for aggregation onset analysis to allow automatic alteration by Zetasizer Software v7.11. The main peak of bevacizumab could be clearly observed in the measurements at 61 °C and 62 °C but increased aggregate formation could also be observed (Fig. 2). These aggregates were formed upon heating and incubating at 60 °C (equilibration time: 180 s). After exposure at 63 °C, the aggregate peaks and main peak of bevacizumab began to merge and appeared as a non-uniform peak up to 67 °C. At 68 °C, the main peak of bevacizumab disappeared (Fig. 2). However, when the same aggregation point results were interpreted by volume-based analysis, it could be observed that the antibody structure was intact up to 65 °C and denaturation began at 66–67 °C, while complete aggregation occurred at 68 °C (Fig. 3). When the particle sizes at the same temperatures were examined in the number-based analysis results, they were found to be approximately the same as those obtained using volume-based analysis (data not shown). The aggregates seen in the intensity-based distribution at similar temperatures (for e.g., 61 °C and 62 °C) were not seen in the volume-based measurements. In the intensity-based distribution, aggregation could be determined more easily than in the volume and number-based distributions because the intensity of the scattered light is calculated to be proportional to the 6^{th} power of the particle size. The thermal ramp in Fig. 4A shows that T_{onset} for bevacizumab was ~63 °C (according to the Z-average size). Bevacizumab starts to degrade between 62 °C and 64 °C. As the temperature rises, the polydispersity index (PDI) width began to change at 64 °C and increased dramatically at 66 °C (Fig. 4B). In the graph showing the relationship between the temperature and derived count rate (kcps, derived count rate = measured count rate/attenuation factor), it is observed that the derived count rate increases abnormally when the temperature reaches 66 °C (Fig. 4 C). The thermal aggregation point of bevacizumab obtained from DLS is in agreement with melting point obtained using the

Micro-DSC technique, as reported by Signorello et al. (2014) The denaturation temperature measured by DSC was determined to be 69.94±0.07 °C. It can be seen from the DSC thermogram of bevacizumab (as determined from the graph using ImageJ software) that the antibody begins to denature at 63 °C. When these results are compared, it can be inferred that DLS is an optimum, fast, practical, and reliable method to determine T_{onset}. However, before the T_m is reached, the intensity of the peak corresponding to aggregates is higher than that of the bevacizumab peak, which disappeared at 68 °C. These results could be explained in terms of the sixth power of the aggregates' diameter, which is higher than the sixth power of bevacizumab diameter at 68 °C. Moreover, sample dilution with phosphate-buffered saline (PBS) in DSC analysis also has an effect on T_m (Signorello et al. 2014).

In conclusion, intensity-based distribution was found to be the optimum way to determine and analyze small-sized soluble aggregates. On the other hand, number-based distribution is an optimum tool for determining the hydrodynamic diameter of non-diluted samples. The aggregation onset of formulations was successfully determined using DLS but their melting point could not be determined by DLS, as the peak intensity of the aggregates is higher than that of bevacizumab before the melting point is reached. Collectively, our findings suggest that DLS provides an opportunity for the characterization of proteins, following aggregates, and determining aggregation onset, while maintaining their native structure.

3. Experimental

3.1. Materials

Sodium phosphate monobasic monohydrate and sodium phosphate dibasic (anhydrous) were obtained from J.T. Baker, Holland; α,α-trehalose dihydrate was purchased from Sigma Aldrich, USA and polysorbate 20 was purchased from Merck KGaA, Germany. Type 1 water with a resistivity of 18.2 MΩ cm was obtained using a Milli-Q® Reference Water Purification System (Millipore, USA). Bevacizumab was obtained from the commercially available product Altuzan® (Avastin's commercial name in Turkey); 100 mg in 4 mL, formulated in 51 mmol/L sodium phosphate, pH 6.2, 60 mg/mL trehalose dihydrate, and 0.4 mg/mL polysorbate 20.

3.2. Characterization of bevacizumab

The hydrodynamic diameter and aggregation point of bevacizumab were measured with a Malvern Zetasizer Nano ZS instrument (Malvern Instruments Ltd., UK). The settings for these measurements are listed in Tables 1 and 2. Briefly, for DLS analysis, Altuzan® was directly measured without any dilution. The sample (45 µL) was initially placed in a measurement cell [quartz micro cuvette, ZEN2112 (Hellma Analytics, Germany)]. Before and after measurements, the cells were washed with medium of Altuzan® to minimize aggregation risk during sample addition. To this end, a solution containing 51 mmol/L sodium phosphate, 60 mg/mL trehalose dihydrate, and 0.4 mg/mL polysorbate 20 was prepared and filtered using a 0.22 µm filter; the pH of the solution was 6.2. Further, the viscosity and refractive index of the medium were measured using an Ubbelohde viscometer and a refractometer, respectively. Quality reports of all the measurements were checked to ensure that the obtained data met the quality criteria. To evaluate the effect of dilution on the hydrodynamic diameter of bevacizumab and aggregates, Altuzan® was diluted to 1 mg/mL with its formulation medium and measured as described above.

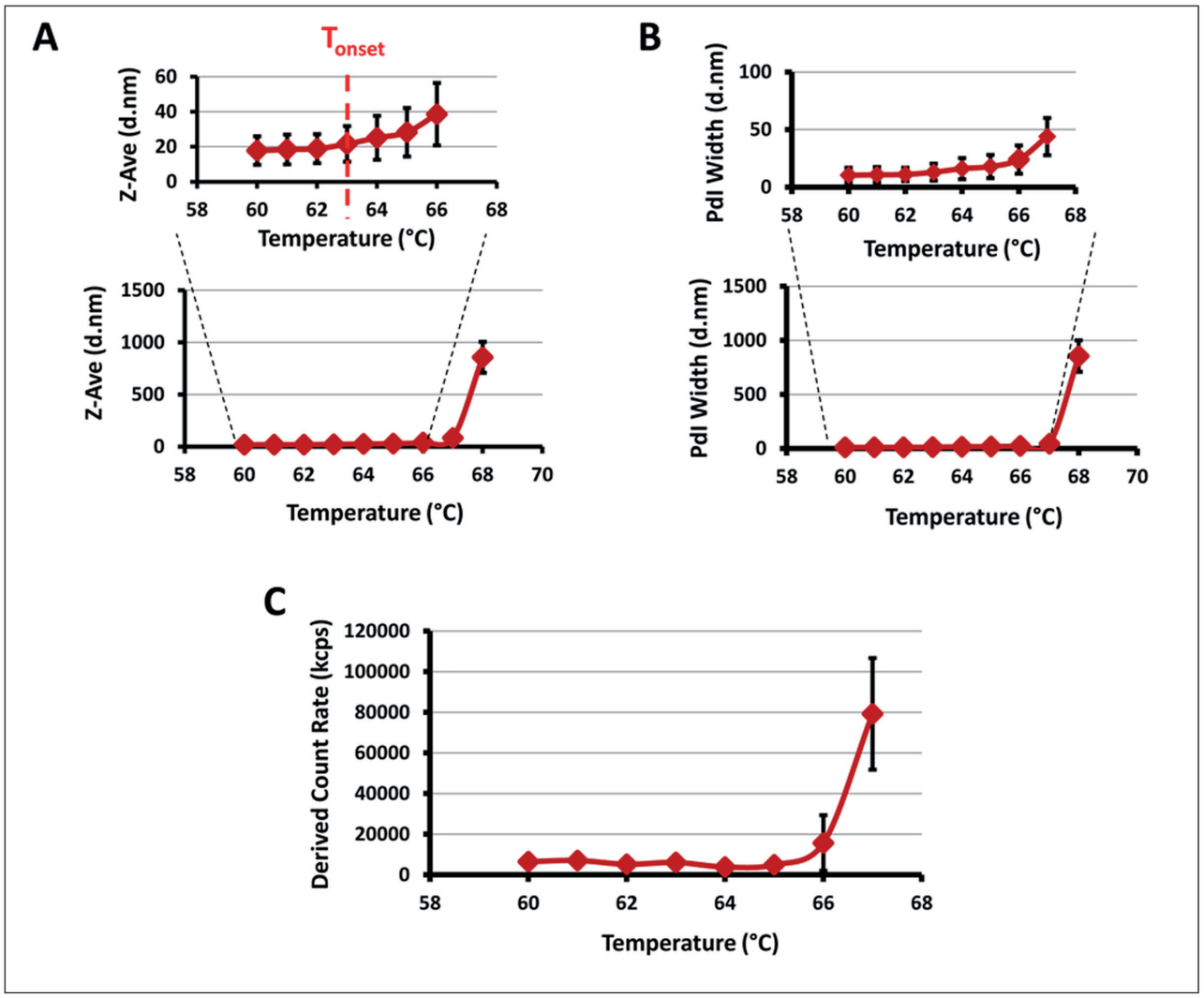

Fig. 4: T_{onset} analysis of AltuzanA) Z-Ave alteration, B) PDI width, and C) derived count rate alteration against increasing temperature. The measurements were performed in triplicate.

3.3. Determination of aggregation onset in formulations

For aggregation onset analysis, firstly the temperature was increased from 25 to 80 °C at 5 °C intervals. Based on these preliminary measurements, analysis was conducted between 60 °C and 70 °C at 1 °C intervals. It is known that viscosity and refractive index change with temperature. Measurement of these values against for all temperature is not applicable approach. To overcome this problem, the same measurements were edited at different viscosity and refractive index values (Altuzan's medium and water) with Zetasizer Software v7.11 and the obtained results were compared. This preliminary experiment demonstrated that only a small shift (2 nm) occurred. Later, water was selected as the dispersant for aggregation onset analysis to allow automatic alteration by Zetasizer Software v7.11. The aggregation onset of the formulation was evaluated against increasing temperature considering intensity, volume, and number-based particle size distribution profiles. The Z-average particle size (Z-Ave), PDI width, and derivative of the count rate were selected as the parameters to determine the aggregation onset.

Table 1: Measurement settings for hydrodynamic diameter analysis

Sample Name	Bevacizumab 25 mg/mL
Cell	ZEN2112 (Hellma Analytics, Germany)
Sample Volume	45 µL
Material	Protein
Dispersant	51 mmol/L sodium phosphate, pH 6.2, 60 mg/mL trehalose dihydrate, and 0.4 mg/mL polysorbate 20 Viscosity: 1.0178 mPa s, refractive index: 1.342
Measurement Temperature	25 °C
Measurement Angle	173°
Number of Runs	11
Run Duration	10 s
Number of Measurements	3
Positioning Method	Seek for optimum position
Automatic Attenuation	Yes

Table 2: Measurement settings of aggregation point analysis

Sample Name	Bevacizumab 25 mg/mL
Cell	ZEN2112 (Hellma Analytics, Germany)
Sample Volume	45 µL
Material	Protein
Dispersant	Water for automatic alteration of viscosity and refractive index against temperature
Start Temperature	60 °C
End Temperature	70 °C
Temperature Interval	1 °C
Equilibration Time	180 s
Measurement Duration	Automatic
Number of Measurements	1
Positioning Method	Seek for optimum position
Automatic Attenuation	Yes

Conflicts of interest: None declared.

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