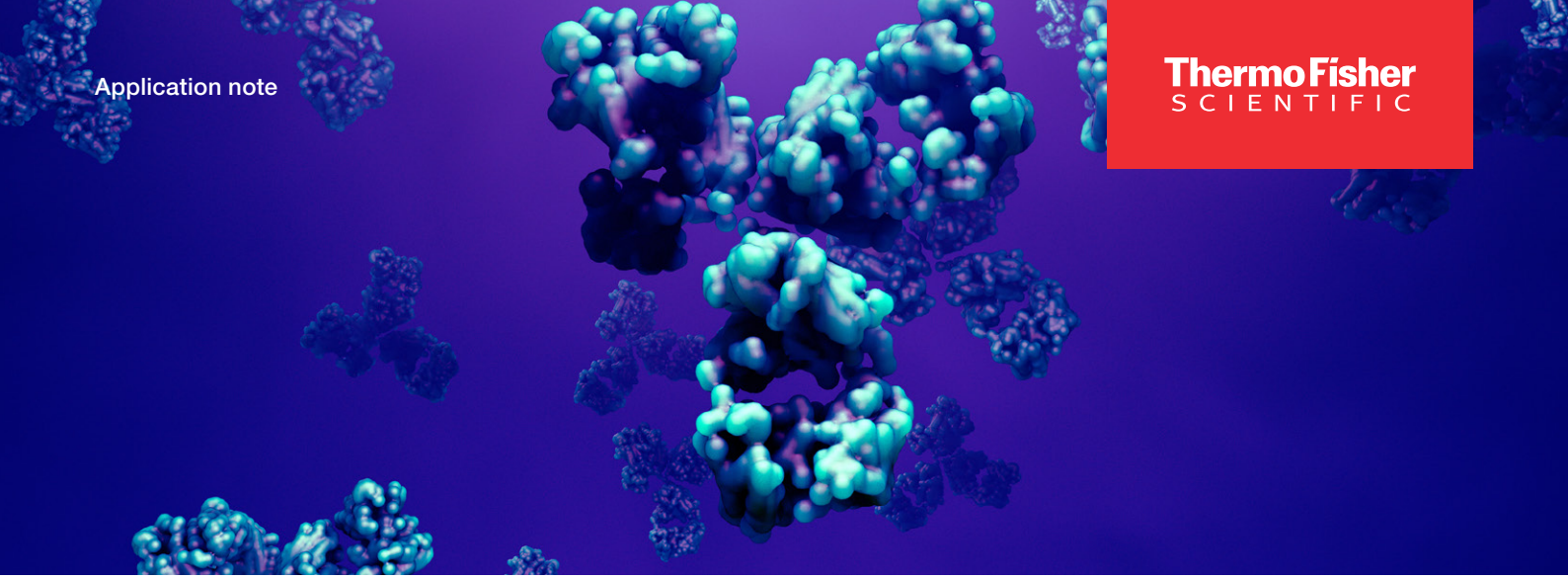

Application note

Protein aggregation identified through UV-Visible absorption spectroscopy

Introduction

Misfolded or denatured proteins can associate in solution,[1] forming insoluble aggregates (Figure 1). This process is often irreversible, effectively removing useful proteins from solution and making the detection of aggregates critical for further downstream use of protein solutions. This is particularly important when studying unstable or abnormal proteins, which are more likely to form aggregates.[2,3]

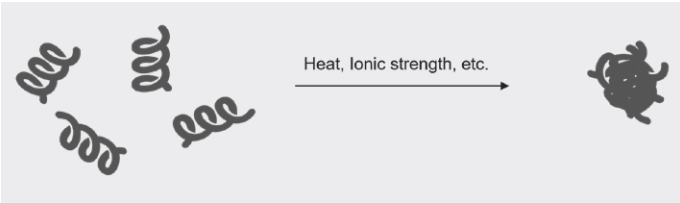

Figure 1. Visualization of protein aggregation induced by heat or changes in ionic strength.

The formation of protein aggregates in the body has also been linked to several diseases, including Alzheimer's and Parkinson's disease.[1,4,5] In the pharmaceutical industry, protein therapeutics, such as insulin,[6] have been developed to effectively treat a variety of diseases but have been difficult to synthesize.[7] The presence of aggregates in these products can lead to lower product yields and can reduce the efficacy of the final therapeutic.[5, 8] For example, protein therapeutics that undergo aggregation have been linked to lowered immune responses and, in some cases, can even induce allergic reactions.[8]

In the food industry, protein composition can have a large impact on the palatability of the final product. Protein aggregates can significantly change a food's organoleptic properties (e.g., taste, smell, etc.), as well as the digestibility of the material.[5]

Size-exclusion chromatography has previously been used to identify the presence of aggregates in a sample.[9] This characterization method is time-consuming, however, and sample retrieval can be difficult. An alternative method for the detection of protein aggregates uses UV-visible (UV-Vis) absorption spectroscopy, a technique that measures a sample's light absorption. Aggregates in solution are known to scatter incoming light, resulting in an apparent absorption artifact across the entire spectrum.[5, 10] This scattering artifact does not represent the true absorption of the sample and instead indicates that the solution contains aggregates large enough to scatter the incoming light.

In this application note, UV-Vis absorption spectroscopy was used to identify the presence of protein aggregates in aqueous bovine gamma globulin (BGG) samples. Aggregation was induced in these samples using heat or the addition of NaCl. An integrating sphere was further used to measure the scatter-free spectra of the samples. Scatter-correction methods were used to determine the concentration of free, non-aggregated BGG in solution.

Experimental

Absorption spectra were collected using a Thermo Scientific™ Evolution™ One Plus UV-Vis Spectrophotometer. Samples were held in a 10 mm quartz cuvette, and measurements were collected between 220 and 400 nm. A stock 1.1 mg/mL BGG solution was made by diluting standard Thermo Scientific Pierce™ BGG Standard (2.0 mg/mL, Lot Number MH162604) with phosphate buffer (PBS, 1×) to achieve the appropriate concentration. A 5.3 M NaCl solution in phosphate buffer was made by dissolving 1.5 g NaCl (Fisher Scientific) in 6.0 mL of phosphate buffer. BGG samples were prepared as described in Table 1.

	BGG sample		Volume of 1.1 mg/mL BGG (mL)	Volume of PBS (mL)	Volume of 5.3 M NaCl (mL)
	Temperature (°C)	NaCl concentration (M)			
1	25.0	0.00	1.0	1.0	0.0
2	25.0	2.65	1.0	0.0	1.0
3	75.0 (60 min incubation)	0.00	1.0	1.0	0.0
4	75.0 (30 min incubation)	0.00	1.0	1.0	0.0

Table 1. BGG solution preparation.

BGG samples were heated using a single-cell Peltier accessory at 75°C for 30 or 60 minutes. Sample measurements were collected using a Thermo Scientific™ Evolution™ ISA-220 Integrating Sphere Accessory in transmission geometry. The collected data was reported using the Kubelka-Munk transformation. An 8° wedge was used for optimized light collection. After integrating sphere measurements were completed, Sample 4 (Table 1) was filtered using a syringe filter. The absorption spectrum of the filtrate was then measured using the Evolution One Plus Spectrophotometer, without the Evolution ISA-220 Accessory.

Results

The absorption spectrum of BGG (not aggregated), depicted in Figure 2a (blue curve), is in agreement with literature values.[11] Upon addition of NaCl, the entire spectrum appears to have a higher absorbance, an artifact resulting from the presence of larger particulates. Increased ionic strength of a protein solution (due to high salt concentration) has been shown to induce protein aggregation;[4] this scattering signal can therefore be attributed to the presence of small BGG aggregates. Scattering is observed regardless of the visual (clear, non-turbid) appearance of the solution (Figure 2c). This indicates that, while it is difficult to confirm through visual observation alone, aggregate scattering can be measured using UV-Vis absorption, and the technique can be used as a test for protein aggregation.

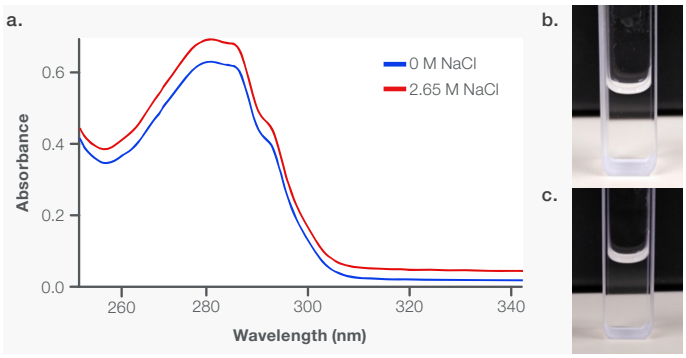

Figure 2. Absorption spectra of 0.55 mg/mL BGG in PBS with (red) and without (blue) 2.65 M NaCL. Images of a solution of BGG with (b) and without (c) 2.65 M NaCl.

Scattering appears as a raised baseline at longer wavelengths but also influences the apparent absorption across the entire spectrum and is highly dependent on the wavelength of the incident light. This influence can be estimated using the following equation:

$$A_{scatter} = \log (I_0/I_{no\ scatter}) + A_{offset} = \log (I_0/I_0\text{-}(f/\lambda^4))) + A_{offset} \quad (1)$$

In the equation above, $A_{scatter}$ is the scattering artifact/apparent absorption due to scattering, I_0 is the intensity of the light before it interacts with the sample, $I_{no\ scatter}$ is the intensity of the light that reaches the detector (not scattered by the solution), f is an arbitrary scaling factor, λ is wavelength in nanometers, and A_{offset} is an offset. This equation uses Beer's law,

$$A = \log (I_0/I) \quad (2)$$

and the relationship between the wavelength of light and the intensity of the scattered light, which is defined by the Rayleigh equation,[12]

$$I_{scatter} \propto (1/\lambda^4) \quad (3)$$

to determine an estimated intensity of the scattered light ($I_{scatter}$). Assuming I_0 is 1 and the intensity of the scattered light is less than 1, Equation 2 includes only two parameters that must be fit to determine the scattering contribution. The relationship between scattering intensity and wavelength indicates that there is a larger effect in the UV region (Figure 3a), where there are prominent absorption features for proteins. This effect must therefore be carefully corrected.

Figure 3b shows the data corrected using two different methods. The first, referred to as "baseline correction," involves taking the average of the absorption reported in the spectral region in which the sample should not absorb. The calculated average is then subtracted from each point in the spectrum, as described by:

$$A_{corrected,\lambda} = A_{measured,\lambda} - A_{average,(330\text{-}350\ nm))} \quad (4)$$

In this equation, $A_{measured\lambda}$ is the absorption spectrum collected, $A_{average,\ (330–350\ nm)}$ is the average of the absorption measured between 330 and 350 nm, and $A_{corrected\lambda}$ is the corrected absorption spectrum. The resulting spectrum is shown in Figure 3b (green curve); the maximum absorption from the band is still higher than that of the untreated BGG sample. This does not match the expected result, as formation of aggregates should remove free BGG from solution, leading to a lower concentration and lower absorbance in the region of interest. Consequently, the "baseline correction" does not properly account for the scattering artifact present in the collected spectrum.

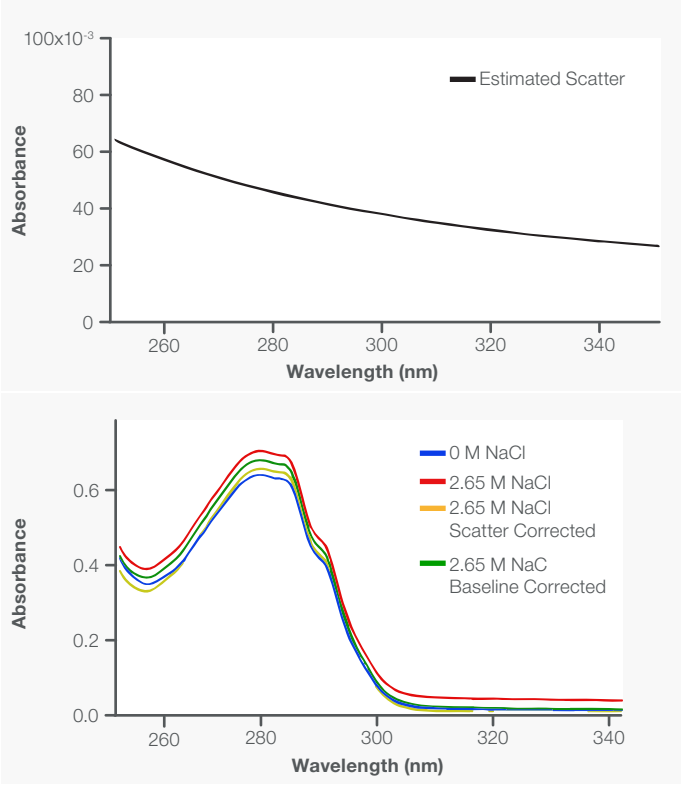

Figure 3. a) Estimated scattering calculated using Equation 1. b) Absorption spectra of BGG with and without NaCL. Baseline-corrected data is shown in green, calculated using Equation 4. Scatter-corrected data is shown in yellow, calculated using Equation 5.

The second method, called "scatter corrected", fits the long wavelength baseline to Equation 1, where f and A_0 are fit such that the resulting function matches the long wavelength signal well. The scattering function described in Figure 3a was fit using f = 6.1 x 10^8 and A_0 = 0.006. The resulting scatter function was then subtracted from the absorption spectrum, as shown in the following equation,

$$A_{(corrected,\lambda)} = A_{measured,\lambda} - A_{scatter,\lambda} \quad (5)$$

where $A_{scatter\lambda}$ is the calculated scatter estimate. This correction results in the yellow spectrum in Figure 3b. Unlike the baseline corrected spectrum (green curve, Figure 3b), the maximum absorption of the scatter-corrected spectrum is below the absorption maximum of the spectrum for untreated BGG, as expected.

The concentration of free, non-aggregated BGG in the sample was found to be 0.54 mg/mL using Beer's law:

$$A = c l \varepsilon \quad (6)$$

In the equation above, A is the measured absorbance, c is the concentration, l is the path length (1 cm), and ε is the extinction coefficient of the protein. Therefore, the concentration of proteins that contribute to aggregation in this sample is 0.01 mg/mL.

For samples with a relatively low scatter contribution, the mathematical scatter-correction method works well. However, for samples that are visibly cloudy/turbid, this correction is not ideal, as only a small portion of the light is allowed to interact with the detector. To study a sample that is turbid, a 0.55 mg/mL BGG sample was held at 75°C for 60 minutes using a single-cell Peltier accessory for the Evolution One Plus Spectrophotometer, producing a cloudy solution (Figure 4b). The resulting absorption spectrum is depicted in Figure 4a. The scattering artifact present indicates that ~30% of the light is transmitted through the sample at 310 nm, where BGG begins to absorb, and even less is transmitted at shorter wavelengths. This suggests there is a high concentration of aggregates present in this heated sample.

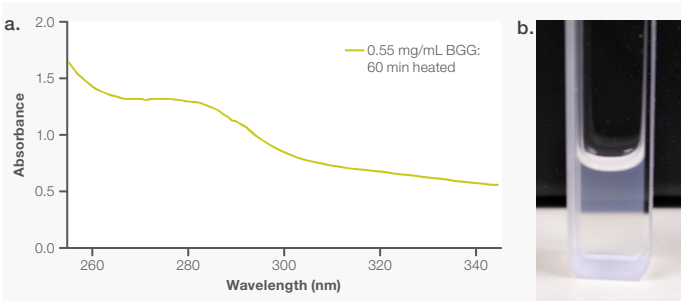

Figure 4. Absorption spectrum of 0.55 mg/mL BGG following a 60-minute incubation at 75°C.

As mentioned previously, the small amount of light reaching the detector makes it difficult to mathematically correct for scattering. Instead, an integrating sphere can be used—this accessory allows for the collection of scattered light diffusely reflected off the inner walls of the sphere. As the diffuse light reflects many times, it can be uniformly collected, removing the scattering artifact. To correct for the scatter shown in Figure 4a, a spectrum for the aggregated BGG sample (Table 1, Sample 3) was collected using an Evolution ISA-220 Accessory. Through the instrument software, the signal was reported using Kubelka-Munk units, F(R), which is proportional to both the absorption coefficient, k, and scattering coefficient, s, of the material:

$$F(R)=k/s \quad (7)$$

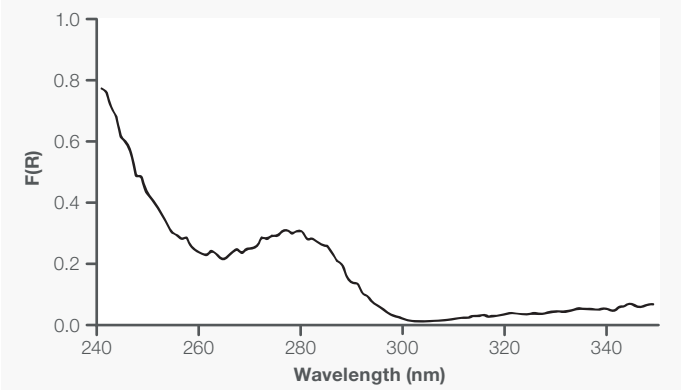

Figure 5. Kubelka-Munk spectrum of 0.55 mg/mL BGG after a 60-minute incubation at 75˚C.

Figure 5 demonstrates the Kubelka-Munk spectrum of the BGG solution shown in Figure 4; the scattering signal is largely removed from the spectrum.

F(R) is not equivalent to absorbance, indicating Beer's law cannot be used to determine concentration from the collected results. However, as F(R) is proportional to the absorption coefficient, it is also proportional to the absorbance, A, and the concentration, c, of the free proteins in solution:

$$F(R) \propto A \propto c. \quad (8)$$

To determine the concentration of aggregated and non-aggregated proteins in solution using the Kubelka-Munk formula, the fully non-aggregated sample (control) was measured using the integrating sphere. The resulting Kubelka-Munk spectrum collected is shown in Figure 6a (gray curve). A second BGG sample heated to 75˚C for 30 minutes (Table 1, Sample 4), which also resulted in a large scattering artifact, was analyzed using the Evolution ISA-220 Accessory as well.

If the collected F(R) of the sample at a given wavelength is assumed to be equivalent to the concentration of the proteins in solution multiplied by some constant, b, that is shared between all BGG samples, then we can construct a series of equations:

$$F_{control}(R) = c_{control} b \quad (9)$$

$$F_{sample}(R) = c_{sample} b \quad (10)$$

$$c_{sample} = c_{control} * F_{sample}(R)/F_{control}(R) \quad (11)$$

The equations above can be used to relate the concentration of non-aggregated BGG in the sample that was incubated at 75˚C (c_{sample}) to the concentration of the non-aggregated BGG control ($c_{control}$), the Kubelka-Munk signal of the sample ($F_{sample}(R)$), and the control ($F_{control}(R)$). For more complex samples, constructing a standard curve with multiple control samples of differing concentration would be a more effective analysis tool.

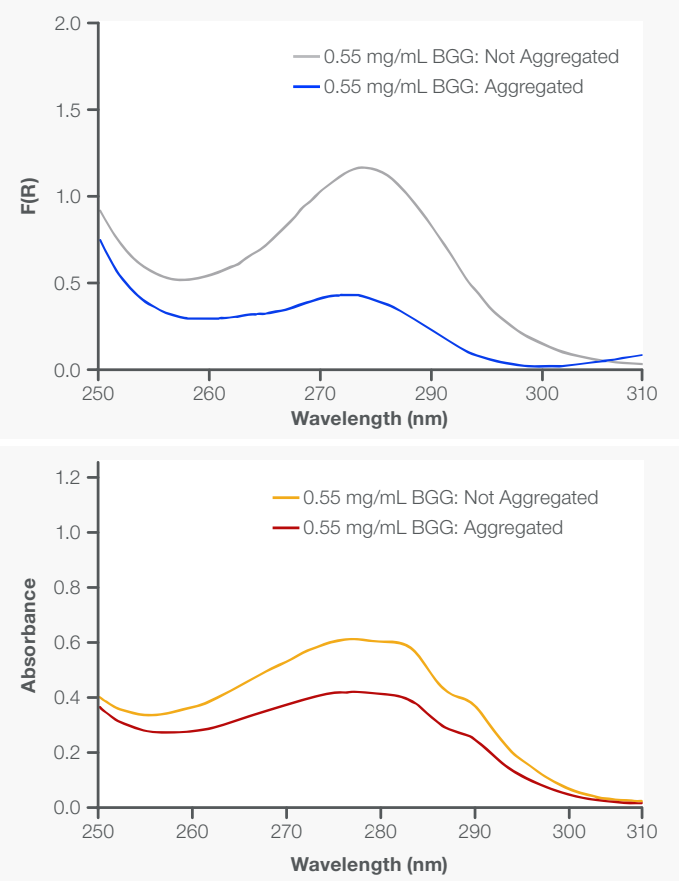

Figure 6. a) Kubelka-Munk spectra of 0.55 mg/mL BGG after a 30-minute incubation at 75˚C (blue) and 0.55 mg/mL non-aggregated BGG (gray). b) Absorption spectra of filtered 0.55 mg/mL BGG after a 30-minute incubation at 75˚C (brown) and 0.55 mg/mL non-aggregated BGG (orange). The incubated BGG sample was filtered using a Millipore Millex-GV PVDFA filter.

Using Equation 11, the concentration of non-aggregated materials in the BGG sample was found to be 0.20 mg/mL, implying 0.35 mg/mL of BGG contributed to the formation of aggregates in this sample. To verify this equation, the BGG sample containing aggregates was filtered using a syringe filter and the absorption spectrum of the filtrate was collected using a traditional cell holder. Using Beer's law, the concentration of the BGG filtrate was found to be 0.20 mg/mL, matching the calculated concentration determined using the integrating sphere. This further implies that BGG aggregates in solution do not absorb an appreciable amount of light in the spectral region of interest for this sample.

Conclusion

Protein aggregates in solution can quickly be detected using the Evolution One Plus UV-Visible Spectrophotometer. For samples with a low concentration of aggregate present, the resulting scattering artifact can be corrected by estimating the scattering contribution and subtracting that estimate from the measured spectrum. For highly scattering solutions, the Evolution ISA-220 Integrating Sphere Accessory works well in removing the scattering artifact from the spectrum. The concentration of free proteins in solution can then be solved for the corresponding spectrum of a known standard or a series of known standards.

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