

Detection of particulate contaminants and protein aggregates in protein solution using multiwavelength transmission spectroscopy (MWT)

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ABSTRACT

Recent publications have utilized multiwavelength transmission spectroscopy (MWT) to quantify particle sizes, particle mixtures and the effects of coating in protein systems. In this work, the limits of detection achieved using MWT spectroscopy are tested using NIST (National Institute of Standards and Technology) traceable polystyrene and glass standard particles as surrogates for contaminants or aggregates in solutions of bovine serum albumin. A range of concentration thresholds have been determined by leveraging the wavelength-dependent properties of the complex refractive indices of each material. These results indicate that MWT spectroscopy is a sensitive and effective means for the measurement of particulates (inherent as well as foreign) in protein systems.

KEYWORDS: spectroscopy, absorbance, particles, proteins, aggregates

INTRODUCTION

The presence of particulates in protein products is a challenge to quality control and production yield [1]. Further, protein aggregates and particles may result in loss of protein activity [2] and are considered to be a risk to immunogenicity [3] even though the link between aggregation and clinical immunogenicity has not been clearly

established [4]. Further, the amount and type of aggregates that trigger immune reactions remain unknown [5]. One of the factors limiting the studies of the immunogenicity of proteinaceous particulates has been the challenges in quantifying proteinaceous particulates [2]. None of the currently available commercial particle counting systems or methods is capable of the analysis of the entire spectrum of particle sizes, ranging from nanometers to millimeters. Consequently, particle analysis results obtained from different instruments have to be pieced together and reconciled to provide a composite view of the entire size range that may be present [6, 7]. However, because different techniques are based on a number of varying measurement principles, comparison of the data obtained from different analytical methods may be inappropriate and misleading [8].

The utilization of current techniques for particle quantification is further hindered by limitations regarding robustness, sensitivity and type of particles that can be quantified accurately. These limitations also apply to the industry standards such as light obscuration and microscopy for quantification of visible and subvisible particles that are $>10\ \mu\text{m}$ [8]. All the currently available methods and instruments for particle analysis have certain limitations in their application for routine monitoring of particulates of $<10\ \mu\text{m}$ size [6, 7, 8, 9]. The variability of the methods for measuring particles $<10\ \mu\text{m}$ and the difficulties with differentiation between proteinaceous and

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extrinsic particles are the main arguments against setting specific limits for these particles in protein products [2, 4]. Although the identification of particles is not required by the Pharmacopeias, it has been recognized as essential for an understanding of protein stability, response to harsh conditions, structural integrity, and compatibility with package materials, etc. [6, 8]. However, only a few techniques (Raman, IR, methods involving fluorescent dyes, and flow imaging microscopy methods) are able to distinguish between proteinaceous and non-proteinaceous particles [4, 6] and these methods are further limited by sample preparation and handling issues [4, 7]. Overall, it has been acknowledged that current particle-counting techniques are reaching the limits of their capabilities to analyze protein samples [1, 8]. There is a clear need for analytical methods capable of simultaneous measurements of orthogonal attributes (size distribution, count, composition, structure, etc.) for particulates over the entire size range from oligomers to subvisible particles [1, 2, 4, 6, 7, 9, 10].

In this paper, MWT UV-Vis-NIR spectroscopy is systematically evaluated for the quantification of low concentrations of particles in protein solutions using bovine serum albumin (BSA) which is a well characterized protein, and a set of NIST traceable polystyrene and glass particle size standards having certified particle diameters from 30 nm to 50 μ m. The particle standards are used as surrogates for both proteinaceous and contaminating particles in protein products to quantitatively modify the optical properties of protein solutions and, thus, to simulate the spectroscopic effect of the presence of particles on the MWT spectra of the protein. The experimental results demonstrate detectable and quantifiable changes in the optical density spectra of BSA induced by particle standards that are consistent with the expectations set by light scattering theory. It is further demonstrated that the composition of the protein/particle mixtures can be identified and that the relative concentrations of the particle populations present in the sample can be quantified. Because of the considerable dynamic range of MWT UV-Vis-NIR spectroscopy for particle analysis and its real-time measurement capabilities, it can be effectively used for the characterization of particle content in protein products.

MATERIALS AND METHODS

Materials

BSA was obtained from Sigma-Aldrich (St. Louis, USA) and NIST traceable polystyrene standards with nominal sizes of 30, 50, 100, 500, 1300, 10000, and 50000 nm were purchased from Thermo Scientific (Freemont, USA). Glass standard beads with nominal sizes 470 and 1580 nm were also purchased from Sigma-Aldrich (St. Louis, USA). Table 1 lists the properties of particles relevant to this study.

Preparation of BSA-PS/GS samples

A series of measurements were taken to explore the detection limits for a range of particle sizes in varying concentrations of BSA acting as a background. Three different preparations of each particle size and type were prepared ($1:10^3$, $1:10^4$, and $1:10^5$) and added to 2 ml of stock BSA solution ($0.4\text{--}1.5\text{ mg ml}^{-1}$) in a 1 cm pathlength quartz cuvette. Baseline MWT measurements of the BSA (2 ml) were taken before the addition of the particles. The cuvette was gently inverted 5 to 6 times before each measurement. The number of measurements, BSA concentration range, and particle dilutions explored for each particle type are given in Table 2.

Spectroscopy measurements

UV-Vis-NIR MWT spectra from all protein/particle suspensions were recorded using a diode array spectrometer (Agilent 8453, Santa Clara, CA). All measurements were conducted at room temperature using a 1 cm pathlength cuvette and 1 s integration time.

Theoretical analysis

The quantitative analysis of the collected MWT spectra in terms of the composition of the mixtures of BSA and particle standards was performed with the interpretation model from Smith *et al.* [11]. The optical density $\tau(\lambda_o)$ at a given wavelength λ_o for M populations was written as:

$$\tau(\lambda_o) = N_p \ell \left(\frac{\pi}{4} \right) \sum_{i=1}^M x_i \int_0^{\infty} Q_{ext,i}(m_i(\lambda_o), D) D^2 f_i(D) dD \quad (1)$$

Table 1. Manufacturer estimates of particle diameter (D) and concentration (C) of NIST traceable polystyrene and glass standards.

D (nominal) [nm]	D (certified) [nm]	CV% (PSD)	C [g/mL]
30	33 ± 1.4		0.00924
50	46 ± 2.0	15.7	0.01187
100	97 ± 3.0	4.6	0.01053
500	491 ± 4.0	1.3	NA
1300	1361 ± 24	1.5	NA
10000	*NA	NA	NA
50000	NA	NA	NA
470**	NA	NA	NA
1580**	NA	NA	NA

*not available

**glass

Table 2. Summary of the nominal diameter (D), nominal concentration (C) of NIST traceable PS/GS particles together with the information on the number of PSA/particle experiments, BSA concentration, particle dilution, and the particle weight fraction range on the BSA/particle mixtures.

Diameter in nm	Number of measurements	Initial BSA concentrations (mg/ml)	Resulting particle weight fraction range (%)
30	20	0.4-1.0	0.005-1.1
50	15	0.8-1.0	0.006-1.4
100	25	0.4-1.5	0.0003-1.2
500	20	0.4-1.5	0.0003-1.2
1,300	25	0.4-1.5	0.0003-1.1
10,000	10	1.5	0.0007-0.008
50,000	10	1.5	0.0005-0.06
470	10	0.8-1.5	0.007-0.014
1,580	10	0.8-1.5	0.001-0.08

where N_p is the total number of particles per unit volume, ℓ is the pathlength, x_i ($i = 1 \rightarrow \mathbf{M}$) is the number fraction corresponding to each population, D represents the i^{th} particle diameter, $f(D)$ is the normalized particle size distribution, and Q_{ext} corresponds to the Mie extinction efficiency of i^{th} particle population. In this analysis four populations have been identified (i.e., $\mathbf{M} = 4$). Statistically and on the basis of the principle of parsimony, the

solution with the minimum number of populations that adequately describe the data is the most likely and in this case, $\mathbf{M} = 4$. This is the basis of Claro's algorithm. Therefore, although mathematical uniqueness cannot be ensured, statistically we ensure the most likely solution given the data. These four populations include three BSA populations (monomer, oligomer, and aggregate) and a population of the appropriate particle standard. The extinction

efficiency of each particle population is a function of the particle size and the optical properties of the particles and suspending medium through the complex refractive index, $m(\lambda_o)$:

$$m(\lambda_o) = \frac{n(\lambda_o) + \kappa(\lambda_o)}{n_o(\lambda_o)} \quad (2)$$

where $n(\lambda_o)$ and $\kappa(\lambda_o)$ represent the real and imaginary components of the complex refractive index of the particles and $n_o(\lambda_o)$ represents the real refractive index of the suspending medium. There is an important variable defined in the process of deriving working equations from the theory of electromagnetic radiation for particle analysis [2, 12, 13]. This quantity, known as the scattering vector (q), represents the vector difference between the incident beam and scattered beam in the θ direction and it can be expressed as:

$$q = \frac{4\pi}{\lambda} \sin\left(\frac{\theta}{2}\right) \quad (3)$$

Equations 1 and 3 contain two key concepts for the comparison of MWT measurements with other spectroscopy techniques such as angularly resolved static light scattering and DLS which do not routinely use multiwavelength spectral measurements. These concepts are:

If Eq. 1 is normalized by dividing by the number density of particles times the pathlength (i.e., $N_p \ell$) or by any other suitable normalization scheme, the effect of the particle concentration is eliminated across the wavelength measurement range. It becomes apparent from the normalized turbidity spectra that the spectral features are only a function of the optical properties (i.e., Eq. 2) and the particle size distribution. The resulting particle size distribution is equivalent to volume-based particle size distributions obtained from DLS. Furthermore, if the particle system is monodisperse, then the spectral features depend only on the optical properties and the particle size. Therefore, as long as there are spectral differences among particle sizes, MWT spectroscopy should be able to resolve them.

The scattering vector q obtained from Eq. 3 is typically observed at a finite set of angles (θ) and at fixed wavelengths (λ). As transmission measurements are the difference among the incident, scattered

and absorbed light, the transmission measurement includes the integral of the scattering vector over all scattering angles at each and every one of the wavelengths observed. Therefore it is implicit in Eq. 1 that all angular information is contained therein. In other words, angularly resolved scattering and MWT spectroscopy should yield equivalent results provided that absorption is either accounted for or zero. In practical terms, as detectors have a finite aperture, the light accepted by the transmission detector is in fact forward scattered light and must also be accounted for.

An additional feature of MWT spectroscopy reflected in Eq. 1 is that the effect of the absorption properties of the particles (i.e., the imaginary part of the complex refractive index, $\kappa(\lambda_o)$ in Eq. 2) becomes explicit and further accentuates the spectral differences that are due to the size of the particles. This has been shown theoretically through a moment expansion of Eq. 1, and has been experimentally demonstrated that the absorption properties of the particles tend to dominate the spectral features as the particle size decreases. Conversely, as the particle size increases, spectral features arising from scattering and diffraction effects tend to dominate the observed spectra. Thus absorption properties provide an additional element of discrimination for particle size distribution analysis.

The wavelength dependent refractive index of BSA was taken from the data reported by Andersen and Painter [14], Arwin [15] and Fu *et al.* [16]. The wavelength-dependent refractive index of polystyrene was from the work of Inagaki *et al.* [17] and the more recent reports from Velazco-Roa *et al.* [12] and the references cited therein. The refractive index of water $n_o(\lambda_o)$ as a function of wavelength was calculated from the correlation reported by Thormählen [18]. Small changes in the wavelength dependent refractive index induced by the state of aggregation are not significantly meaningful in terms of the particle analysis sought.

To solve the optical density as expressed in Eqs. 1-3 in terms of the composition, size and concentration of the particles in the samples, the regularized solution approach reported by Elicabe [13, 19] was used.

RESULTS

The real parts of the wavelength-dependent relative refractive indices of polystyrene, glass, and BSA are compared in Figure 1. Here, we chose to compare only the real parts of the refractive indices since these play the dominant role in defining the spectral features of particles larger than 100 nm. For the effect of the imaginary components on smaller particles spectra, see Serebrennikova *et al.* [20].

Figure 2 shows the particle size distributions obtained from the interpretation of MWT spectra of the glass particle standards having nominal diameters of 470 nm and 1.58 μm together with the comparison between the corresponding measured and calculated spectra. The excellent agreement between the measured and calculated spectra, and the good agreement between the MWT spectroscopy-derived mean particle sizes and the certified values can be readily appreciated (Table 3).

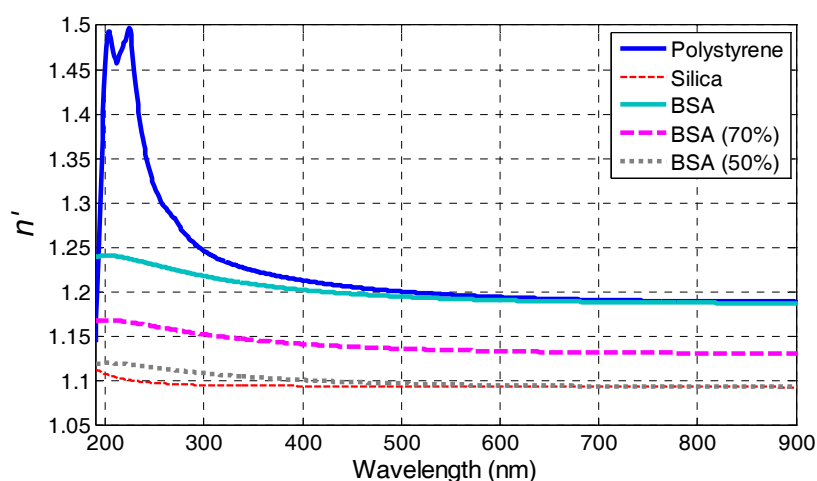


Figure 1. Comparison of the real parts of the relative refractive indices of polystyrene, glass, and BSA.

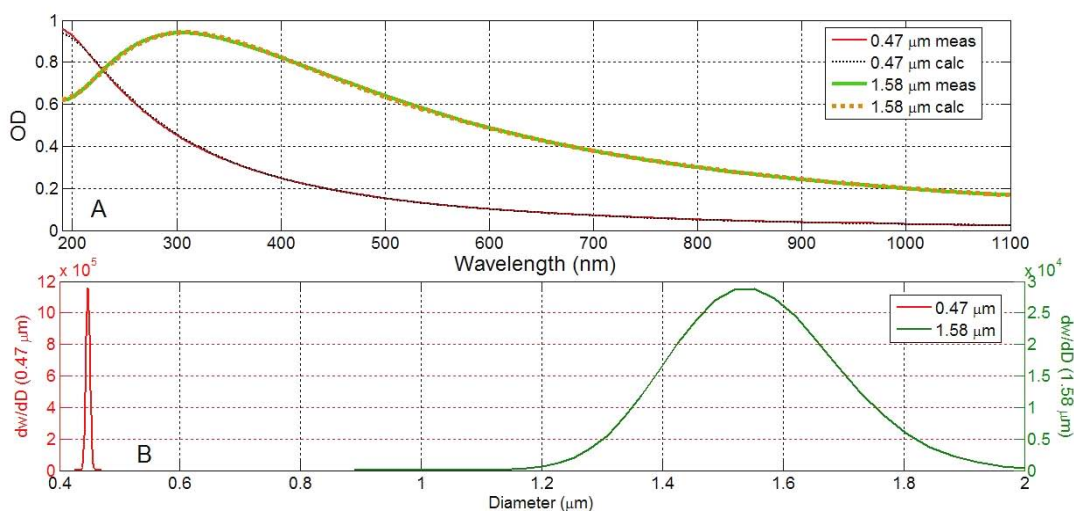


Figure 2. Quantitative analysis of glass particle size standards with nominal sizes 0.47 and 1.58 μm . Top panel shows the comparison between measured and calculated spectra as a demonstration of model adequacy and goodness of fit, and bottom panel shows the weigh-based particle size distributions obtained from the solution of Eq. 1 (See also Table 3).

Table 3. Comparison of manufacturer estimates of size and concentration compared to those obtained from MWT spectra.

Manufacturer				MWT		
D (nom) (μm)	D (cert) (μm)	CV% (PSD)	C [g/mL]	D (μm)	CV% (PSD)	C [g/mL]
0.47	0.49 ± 0.02	4.1	$9.9\text{E-}05$	0.45 ± 0.003	1.7	$9.0\text{E-}05$
1.58	1.57 ± 0.02	2.5	$1.9\text{E-}04$	1.56 ± 0.14	5.7	$1.33\text{E-}04$

Selected particle size standards are compared in Figure 3A. The distinct spectral features of particles due to their size can be clearly appreciated. Further, it is worthwhile noting the distinct spectral features of 0.47 nm glass and 0.5 nm polystyrene standards. These standards are close enough in size to have similarities in their spectra. Yet, the spectral features are very distinct due to different refractive indices of the materials. Since both the size and composition affect the MWT spectra of particles, the parameters pertaining to these particle characteristics can be extracted from the measured MWT spectra.

Measured MWT spectra of mixtures of BSA and particle standards are shown in Figure 3B. Using Eq. 1, each measured spectrum was approximated as a sum of the weighted fractions of the spectral contributions from the components of the mixture. The weights of the spectral contributions of the components correspond to the relative weights of these components in the mixtures. This linearity holds for dilute suspensions, i.e. single scattering

regime, and negligible interactions between the mixture components. The measured spectra of BSA/particle mixtures were resolved in terms of four components: monomer, oligomer, and aggregate forms of BSA and particles. Although the contribution of the aggregated BSA species to the spectra of the mixtures was small, it was necessary to account for it given how low the fractions of particles in the mixtures were.

The measured MWT spectra of the mixtures of BSA and particle standards were quantitatively analyzed to determine the concentrations of particle standards in the mixtures. These values were compared to the nominal concentration values calculated on the basis of the experimental protocol. The results are graphically summarized in Figure 4.

In order to quantitatively evaluate the agreement between the MWT based values for the weight fractions of particles in the protein/particle mixtures, relative error (RE) was calculated as follows:

$$RE (\%) = \frac{|(MWT \text{ weight fraction}) - (nominal \text{ weight fraction})|}{nominal \text{ weight fraction}} * 100$$

The lower limits of detection for MWT analysis are presented using a 10% maximum relative error as an upper boundary or threshold for accurate particle enumeration, in a protein solution. These values are summarized in Table 4.

DISCUSSION

In order to evaluate a technique for the detection of aggregates and particulates in protein products it is advantageous to use well characterized particle standards as proxy for protein aggregates and particulates because of stability and conformation issues that occur when dealing with

real aggregated macromolecules. One of the main arguments against the use of particle standards, especially polystyrene, is the difference in the refractive indices between particles and protein. Nevertheless, polystyrene and glass particle standards appear to be suitable and have been used for this purpose [11, 20].

Figure 1 shows that the relative refractive index of pure BSA is close to that of polystyrene at wavelengths above 400 nm. This means that polystyrene standards can approximate the spectral behavior of compact protein particulates effectively. When protein forms amorphous aggregates that incorporate solvent

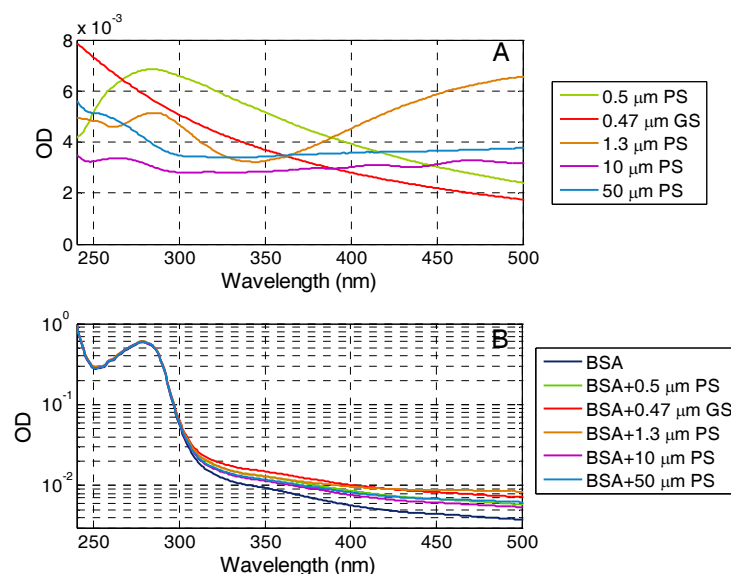


Figure 3. Panel A: Measured MWT spectra of polystyrene (PS) and glass (GS) particle standards of various nominal diameters. Panel B: Measured MWT spectra of BSA and BSA mixtures with polystyrene (PS) and glass (GS) particle standards from Panel A.

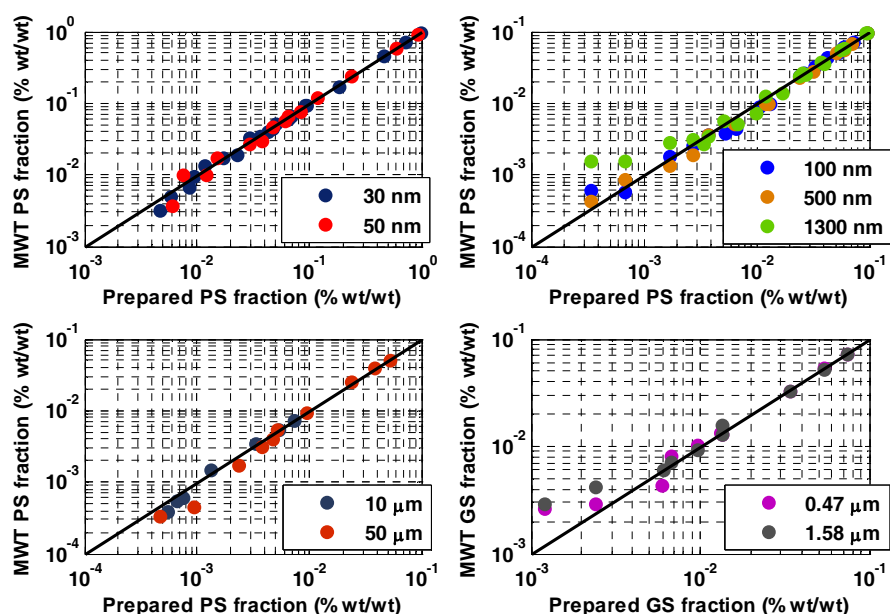


Figure 4. Comparison between the weight fraction values of polystyrene (PS) and glass (GS) particle standards in BSA/particle mixtures obtained using MWT spectroscopy and the nominal weight fractions calculated on the basis of the experimental protocol.

into their structure, there is a decrease in its relative refractive index. The relative refractive index of glass is about 1.1 and can approximate well the spectral behavior of highly amorphous protein

(only 50% protein by weight) particulates of 100 nm in size. Therefore, it is reasonable to evaluate the aggregate/particle detection with MWT spectroscopy using mixtures of protein and polystyrene or glass

Table 4. Summary of the detection thresholds expressed as weight fraction (% wt/wt) and number density (# ml⁻¹) of polystyrene and glass particles in 1 mg ml⁻¹ BSA for 10% relative errors (RE).

Nominal diameter (nm)	Detection threshold (<10% RE)	
	% wt/wt	# ml ⁻¹
30	0.04	3 x 10 ¹⁰
50	0.03	5 x 10 ⁹
100	0.02	4 x 10 ⁸
500	0.02	3 x 10 ⁶
1300	0.02	1 x 10 ⁵
10000	0.005	80
50000	0.01	1-2
470*	0.01	8 x 10 ⁵
1580*	0.01	2 x 10 ⁴

*glass

particle standards. The spectral differences observed as functions of the size and concentration of the particle standards could be representative of those of protein particles. Further, they can be representative for possible contaminants in protein products such as silicon, glass fragments, or metal particles.

Smith *et al.* [11] demonstrated accurate retrieval of the mean particle sizes, particle size distributions and concentrations from MWT spectra using NIST polystyrene standards of a large particle size range. Here we demonstrate that the same accuracy is achieved for the glass standards having substantially different refractive index from that of polystyrene. At very low concentrations, the effect of the spectral features of the particles on the MWT spectra of protein/particle mixtures is still evident (Figure 3B). While the strong absorption features of BSA mask the particle features in the UV region of the spectrum, they can be clearly seen in the visible-NIR region where BSA absorption is negligible. The differences in the sizes and composition of particles lead to different effects on the MWT spectra in the mixtures of BSA and particles (Figure 3B). Therefore, when the entire multiwavelength spectrum is analyzed, the information on the size, composition, and

concentration of the particles in a protein sample can be extracted. In this study we were able to experimentally demonstrate that MWT measurements contain quantitative information on the size distribution of particle suspensions that spans at least three orders of magnitude in size from 30 to 50,000 nm. Further, it can be seen that this method can meet or exceed the detection limits reported today for many existing as well as emerging technologies [5].

CONCLUSIONS

In this study mixtures of BSA and NIST traceable polystyrene and glass particle size standards were used to experimentally evaluate concentration thresholds for particle detection in protein using MWT spectroscopy. It was demonstrated that the particle type, size, and concentration can be resolved from a single MWT measurement using a suitable interpretation algorithm and the wavelength-dependent optical properties of the materials. The values of particle concentrations in BSA/PS mixtures obtained using MWT spectroscopy are in excellent agreement with the corresponding nominal values. These results are facilitated by the multiwavelength aspect of the measurement and analysis. The difference in the refractive indices of materials is far less critical compared to techniques based on single wavelength measurements. This multiwavelength capability is not available from commercially available particle characterization instrumentation and, therefore, a multitude of techniques would have otherwise been required to obtain this breadth of information.

The advantages of the MWT spectroscopy are many-fold: high reproducibility of the measurement, accuracy, simplicity and rapidity of sample analysis. Therefore, economic and reliable instrumentation built upon a MWT spectroscopy foundation can be used to provide valuable and accurate data on sample concentration and sample composition. This single optical technology can be used in the plant and in the laboratory, for batch and continuous measurements (i.e., liquid chromatography), thus preempting issues arising from having to reconcile data obtained with instrumentation using different measurement principles.

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