Refractive Index Effects on the Absorption Spectra of Macromolecules

L. H. Garcia-Rubio

Department of Chemical Engineering, College of Engineering, University of South Florida, Tampa, Florida 33620

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ABSTRACT: Qualitative and quantitative interpretations of comparative UV-vis spectroscopy of macromolecules are normally conducted on the basis of the Beer-Lambert law and on the basis of spectral shifts attributed to changes in the degree of ionization and/or to changes in molecular conformation. The theory of scattering of electromagnetic radiation indicates that in order to compare and interpret UV-vis spectra it is necessary to account for both molecular size effects and differences in the refractive index of the solvents used. In this paper, the differences in the intensity and the spectral shifts typically observed in comparative UV-vis spectroscopy of macromolecules are explained on the basis of the Mie theory. It is demonstrated that the refractive index of the solvent plays an important role in the estimation of the absorption coefficient of macromolecules and that a significant fraction of the spectral shifts normally attributed to changes in conformation can be readily explained in terms of changes in the refractive index of the solvent. As a result from this investigation it is shown that failure to consider the effects of the refractive index of the solvent can lead to serious errors in the interpretation of UV-vis spectroscopy and light scattering data.

Introduction

The primary purpose of this paper is to examine in detail the effect of changes in the refractive index on the absorption spectra of macromolecules in solution. This is achieved through the light scattering theory. The motivation for this study stems from the discrepancies found in literature-reported values of the absorption coefficients of macromolecules and on the difficulties encountered in the use of model molecules for the quantitative representation of chromophoric groups contained in macromolecules. The results from the investigation are particularly relevant to the quantitative characterization of macromolecules using spectroscopy and light scattering techniques and for the interpretation of the spectroscopy data from polymeric sensors.

Comparative absorption spectroscopy techniques are routinely used to investigate reaction kinetics and to determine the concentration and composition of macromolecules in chromatographic effluents. Comparative absorption spectroscopy techniques are also used for the quantitative characterization of proteins and for the establishment of cause-effect relationships between protein activity and purification conditions.[1-10] In these types of analyses, the spectra are recorded in optically transparent solvents at frequencies where at least some of the chromophores in the polymer absorb strongly. There are, however, three major difficulties for the quantitative interpretation of UV-vis spectroscopy data: the quantification of the scattered light from large macromolecules and from aggregates,[8,11,12] the selection of the appropriate model molecules that can represent the behavior of chromophoric groups within the macromolecules,[6,7] and the quantification of the spectral shifts observed as functions of the nature of the solvent (i.e., changes in solvent quality, pH, and denaturant concentration[1-5]). According to the Beer-Lambert law,[1] the decrease in the intensity of the incident light of any given wavelength, on passage through a homogeneous solution containing chromophores, is directly proportional to the concentration of chromophores (C) and to the length of the absorbing path (l).

$$\log (I_t/I_0)_\lambda = -\epsilon(\lambda)\, Cl \qquad (1)$$

The proportionality constant $\epsilon(\lambda)$ is generally interpreted as the molar absorption coefficient. The validity of the Beer-Lambert law hinges upon several assumptions: (i) the incident radiation is monochromatic; (ii) the decrease in the intensity is due only to absorption; (iii) absorption is limited to a volume of uniform cross section; and (iv) each absorbing center is independent of all others regardless of their kind and concentration. In many cases, the deviations from the Beer-Lambert law can be attributed to failures in one or more of the above assumptions. When the Beer-Lambert law is applied to macromolecules and colloids, assumptions ii and iv are of primary concern because of the finite size of the macromolecules and the close proximity of functional groups within the molecule. It has already been shown that the Beer-Lambert law is a limiting case of the Mie theory[11] and that the effect of the neighboring groups can be treated by considering the affected groups as independent chromophores with absorption patterns that depend on the nature of the neighboring groups.[6,7] In addition to assumptions ii and iv, there are two important solvent refractive index effects that are seldom considered in comparative spectroscopy: (i) The solvent refractive index affects the magnitude of the measured extinction coefficient; and (ii) the solvent refractive index affects the position of the absorption bands. These effects, predicted by the theory of electromagnetic radiation can, in many instances, explain the spectral shifts often attributed to changes in molecular conformation and/or molecular structure.[13,16] In this paper, the effects of the solvent refractive index are analyzed using the Mie theory. For this purpose, the analysis has been divided into three sections; the first one deals with the refractive index effects on the magnitude of the observed extinction coefficients; the second section analyzes the refractive index effects on the position of the absorption bands; the third section deals with the problems associated with the determination of the specific refractive index increments and the solvent effects in turbidity and light scattering measurements.

Experimental Methods

Narrow polystyrene standards were obtained from Scientific Polymer Products, Inc. Pituitary-derived porcine somatotropin was obtained from A. F. Parlow (UCLA, Los Angeles, CA). Spectral-grade tetrahydrofuran and dioxane are from Burdick

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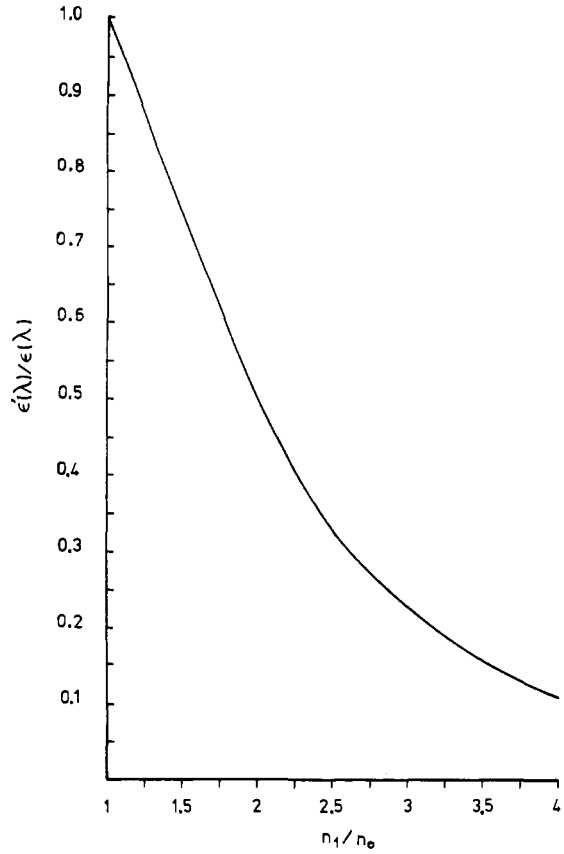

Figure 1. Ratio of the observed to the true absorption coefficient ($\epsilon'(\lambda)/\epsilon(\lambda)$) as a function of the refractive index ratio.

and Jackson Laboratories. Other chemicals were purchased from Sigma. The UV–vis spectra were recorded on a Perkin-Elmer 3840 photodiode array UV–vis spectrophotometer equipped with a thermoelectric cell holder and a temperature controller with temperature-programming capabilities. A 1-cm-path-length cell was used for all measurements. The extinction spectra of the macromolecules were obtained from measurements at 25 °C and several concentrations. Up to seven concentrations plus replicates were used in order to obtain good estimates of the optical constants as well as good estimates of the measurement errors. Special care was taken to ensure that the measurements were always within the linear range of the instrument. The spectroscopy data were stored in a Perkin-Elmer 7500 computer linked to a SUN 3/160 workstation for processing with the interpretation software developed in-house. The solvent refractive indices were measured with an Altex 156 refractive index detector calibrated with solutions of known refractive index.

Mie Theory

The Mie theory represents the ideal scattering behavior expected of suspensions of isotropic spherical particles and, although macromolecules are not in general spherical or isotropic, it has been shown that nonspherical globular particles can be evaluated as a distribution of spheres.[17,18] On this basis, the Mie theory has been selected as a reasonable starting point for the analysis.

As discussed previously,[11] in order to apply the Mie theory to the analysis of homogeneous solutions of macromolecules, it is necessary to deviate from the continuum representation of homogeneous solutions and consider that, relative to the size of the solvent molecules, macromolecules in solution form an optically heterogeneous system where the discrete entities are the macromolecules and the continuum is the solvent. In this context the terms macromolecules and particles will be used interchangeably. Under these conditions, if absorption and scattering are present, the logarithm of the ratio of transmitted to incident radiation is known as turbidity (τ). For dilute solutions, it has be shown that the turbidity is given by[19,21]

$$\hat{\tau}(\lambda) = Nl\int_0^\infty \frac{\pi}{4} D^2 Q(\alpha,m)\, f(D)\, dD \qquad (2)$$

or

$$\hat{\tau}(\lambda) = \left(\frac{3Cl}{2\rho}\right)\frac{\int_0^\infty D^2 Q(\alpha,m)\, f(D)\, dD}{\int_0^\infty D^3 f(D)\, dD} \qquad (3)$$

where α is the size parameter ($\alpha = \pi D/\lambda$); m represents the complex refractive index ratio given by

$$m = (n_1(\lambda) + ik_1(\lambda))/n_0(\lambda) = n(\lambda) + ik(\lambda) \qquad (4)$$

where n_1 and k_1 represent the real and the imaginary parts of the complex refractive index of the particles in free space, λ is the wavelength in the suspending medium (i.e., $\lambda = \lambda_0/n_0$), N is the number of particles per milliliter, C is the concentration in grams per milliliter, D is the particle diameter, $f(D)$ is the frequency distribution of particle sizes, ρ is the density of the particles, n_0 is the refractive index of the suspending medium, and $Q(\alpha,m)$ is Mie's overall extinction efficiency given by[19,21]

$$Q(\alpha,m) = (2/\alpha^2)\ \mathrm{Re}\left[\sum_{j=1}^{\infty}(2j+1)(a_j + b_j)/(2\pi n_0/\lambda)^2\right] \qquad (5)$$

a_j and b_j are known as the scattering coefficients, and they are given in terms of Bessel and Neuman functions.[20]

The Beer's law absorption coefficient ($\epsilon(\lambda)$) is related to Mie's absorption coefficient ($k(\lambda)$) through Bouger's law[19]

$$\epsilon(\lambda) = 4\pi k(\lambda)/\lambda = 4\pi k_1(\lambda)/\lambda_0 \qquad (6)$$

Refractive Index Effects on the Absorption Intensity

In order to visualize the effect of the solvent refractive index on the absorption spectra, it is convenient to consider a limiting case of the Mie theory. It has been shown that, in the limit of zero size, the turbidity equation (eq 3) reduces to a form of the Beer–Lambert law[11]

$$\tau(\lambda) = \left(\frac{Cl}{\rho}\right)\left(\frac{9n\epsilon(\lambda)}{(n^2+2)^2}\right) = \left(\frac{Cl}{\rho}\right)\epsilon'(\lambda) \qquad (7)$$

where $\epsilon'(\lambda)$ is the observed absorption coefficient. Notice that since $\epsilon(\lambda)$ is not a function of the solvent refractive index, the primary effect is given by the ratio $n = n_1/n_0$ in the term premultiplying $\epsilon(\lambda)$. It is clear from Figure 1 that, for the same turbidity, as the ratio of the refractive indices increases, the observed absorption coefficient will decrease.

Refractive Index Effects on the Position of the Absorption Bands

An important consequence of the solvent refractive index is the wavelength shift that occurs when the light enters the solution. The wavelength λ in eqs 2–7 corresponds to the wavelength in the medium where the light is interacting with the molecules or particles. Under normal operating conditions, the known wavelength of the source is used as the reference (i.e., the wavelength in vacuum). The relationship between the wavelength in free space and that of the solution is given by[19]

$$\lambda = \lambda_0/n_0 \qquad (8)$$

In general, n_0 is a function of both the wavelength and the

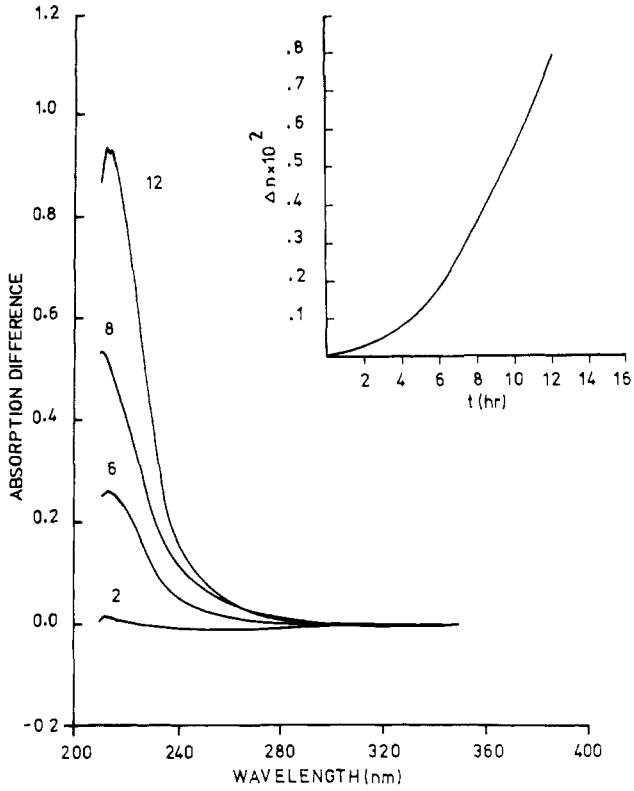

Figure 2. Differences in the absorption spectra and the refractive index (λ = 546 nm) of spectral-grade tetrahydrofuran as a function of time during a typical sample preparation sequence (the time is in hours).

absorption coefficient of the solvent.[21] However, in the region of high solvent transparency n_0 will be approximately constant and the measured spectra will appear to be displaced by a constant factor relative to the wavelength in free space. Such an effect does not represent any major problems by itself. The problems arise in comparative spectroscopy when two or more spectra in different solvents are being compared. For these cases, the spectra will appear to be shifted relative to the wavelength in free space and relative to each other. Therefore, in order to superimpose and compare spectra, it is necessary to refer them to the same wavelength (i.e., the same solvent). It can be readily shown that if two UV–vis spectra are to be compared, and the spectrum recorded in solvent 1 (n_{01}) is used as a reference, then the spectrum in solvent 2 (n_{02}) should be displaced by

$$\lambda_2 = \lambda_1(n_{01}/n_{02}) \tag{9}$$

The displacement indicated by eq 9 is generally ignored, and, as is shown below, it could be misleading in the interpretation of spectroscopy data.

There are three cases where the lack of consideration to the solvent refractive index could be misleading: (i) when the spectra to be compared were recorded in the same solvent but the solvent changes as a function of time; (ii) when the spectra to be considered have been recorded in solvents with different refractive indices but with similar solvating powers; (iii) when differences in the nature of the solvent lead to differences in the conformation and/or the composition (i.e., degree of ionization).

Case I. This problem arises as a consequence of the long dissolution times required by macromolecules and as a result of the sensitivity of some solvents, such as tetrahydrofuran (THF), to light and to the presence of oxygen. Figure 2 shows the variation of the UV–vis absorption spectra and the variation in the refractive index of spectral-grade THF during a sample preparation sequence. Notice from eq 9 that, for a 1-nm shift at 280 nm, less than a 0.4% change in the solvent refractive index is required (i.e., a change between 1.3000 and 1.2954). In UV–vis spectroscopy, a 1-nm difference is significant and it could give rise to considerable spectral differences. For example, Figure 3 shows the spectra of a narrow polystyrene standard (M_w = 13 000) measured from a freshly prepared THF solution and from the same solution after a period of 12 h. Figure 4 shows the comparison between the two spectra after the refractive index corrections indicated by eqs 7–9 have been applied.

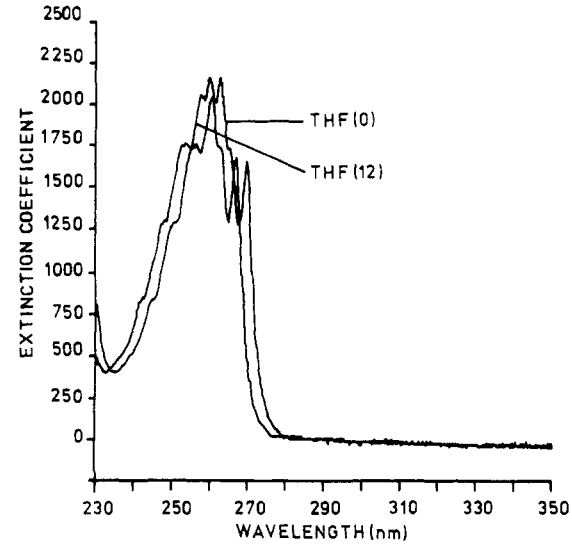

Figure 3. Comparison of the UV–vis absorption spectra of a narrow polystyrene standard (M_w = 13 000) from a freshly prepared solution and the same solution after 12 h.

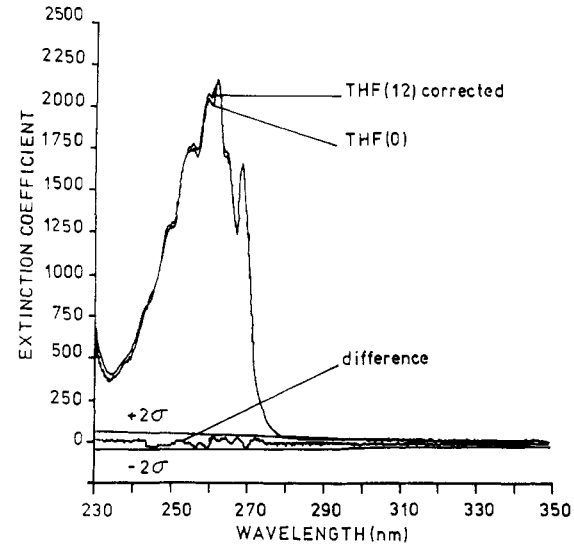

Figure 4. Comparison of the refractive index corrected UV–vis absorption spectra of a narrow polystyrene standard (M_w = 13 000) in THF.

Case II. This situation presents itself when the spectra have been recorded in transparent solvents with similar solvating powers and different refractive indices.[22] The fact that the solvents are transparent over the wavelength region of interest does not preclude the refractive index effects indicated through eqs 7 and 9. The expected wavelength shifts and the small changes in the intensity can be appreciated in Figure 5, where the spectra of a narrow polystyrene standard (M_w = 50 000) in THF and in dioxane are shown. If the literature values for the refractive index of polystyrene (n_1 = 1.5683 + 10.087 ×

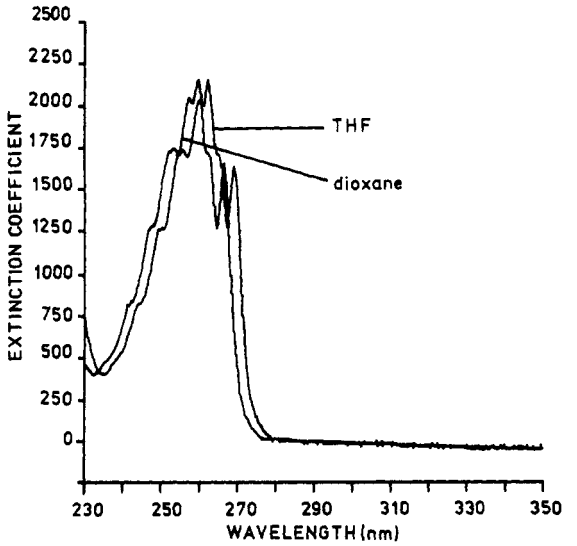

Figure 5. Comparison of the UV–vis absorption spectra of a narrow polystyrene standard (M_w = 50 000) in THF and in dioxane.

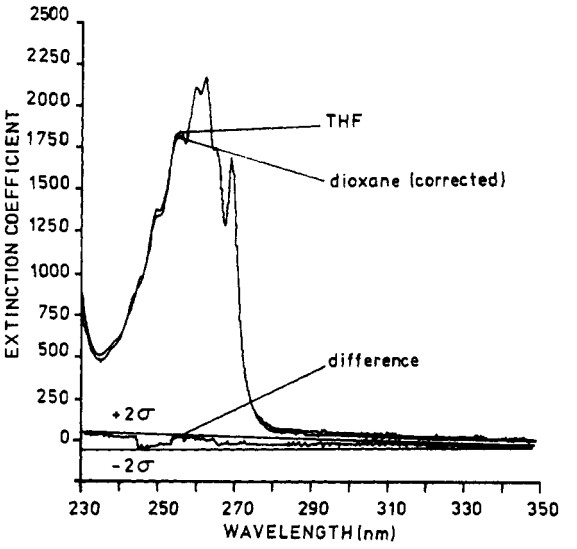

Figure 6. Comparison of the refractive index corrected UV–vis absorption spectra of a narrow polystyrene standard (M_w = 50 000) in THF and in dioxane.

$10^{-11}/\lambda_0^2$), tetrahydrofuran (n_0 = 1.3947 + 3.5562 × $10^{-11}/\lambda_0^2$), and dioxane ($n_0 = 1.4090 + 3.81767 \times 10^{-11}/\lambda_0^2$) are used,[23] the spectrum in dioxane can be readily corrected relative to the spectrum in THF (Figure 6). As expected for solvents with similar solvating powers, the differences from the corrected spectra are minimal.

Case III. In this case, the solvent is modified to induce changes in conformation.[1-5,12-16] Solvents are typically modified by changing the composition, the pH, and the ionic strength. Conformational changes may also be induced through temperature manipulation. In some cases, the solvent is further modified with the addition of a denaturant such as urea or guanidine/HCl.[1-5] This type of experimentation is extensively used in protein chemistry, and, for the comparison of spectra, it relies on the fact that the solvents remain fairly transparent in spite of relatively large changes in the salt concentration, pH, and denaturant concentration (typically, 3 < pH < 13 and guanidine/HCl between 0–6 M). Figure 7 shows the calculated effect of the solvent refractive index on the measured spectrum of porcine somatotropin in Tris-HCl buffer at pH = 8.25. The changes in the solvent refractive index were calculated as a function of the concentration

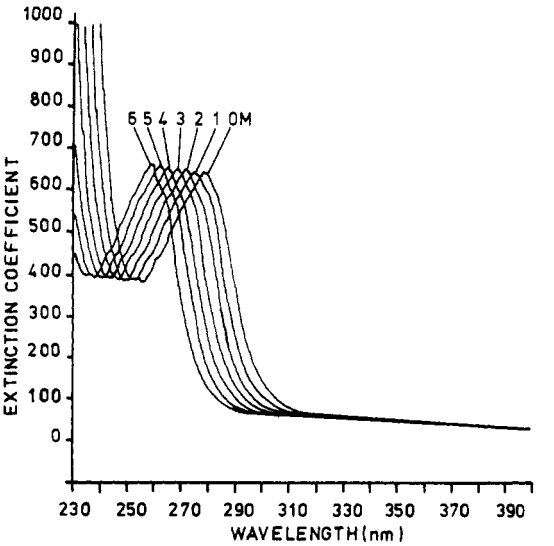

Figure 7. Calculated effect of the solvent refractive index on the measured spectrum of porcine somatotropin in a 0 M guanidine, 0.05 M Tris-HCl buffer at 25 °C and pH = 8.25. The changes in the solvent refractive index were calculated as a function of guanidine/HCl concentration (ref 23).

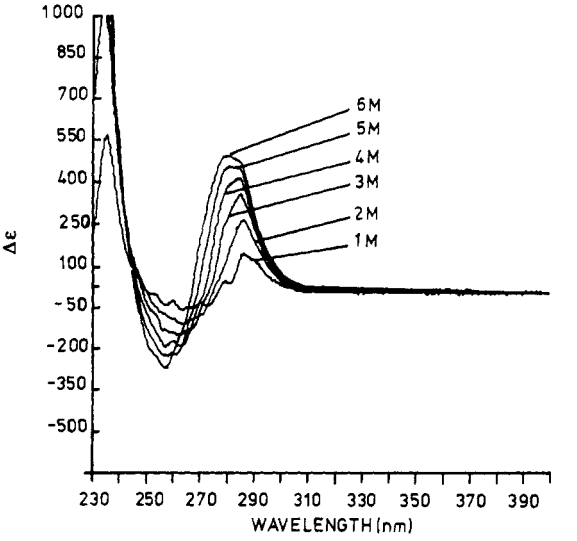

Figure 8. Difference spectra of porcine somatotropin in 0.05 M Tris-HCl buffer at 25 °C and pH = 8.25 as a function of the solvent refractive index (guanidine/HCl concentration). The differences were taken relative to 0 M guanidine (see Figure 7).

of guanidine/HCl in accordance with the data of Nozaki.[24] Notice that a significant blue shift has occurred as a function of the denaturant concentration. This blue shift is generally considered as indicative of conformational changes (i.e., protein denaturation).[1-5] It is clear, however, that at least a significant fraction of the wavelength shift observed can be attributed to changes in the solvent refractive index. The effect of the refractive index can be better appreciated in Figure 8, where the difference spectra relative to 0 M guanidine/HCl have been plotted. Notice that the difference spectra follow the typical patterns and displacements reported in actual denaturation experiments.[1-5,13-16]

Particle Size and Solvent Refractive Index on Light Scattering Measurements

It is evident from eqs 2–5 that the refractive index ratio (n_1/n_0) plays an important role in light scattering experiments[19-21] and in the corrections for scattered light in spectroscopy measurements.[11,12] The refractive index

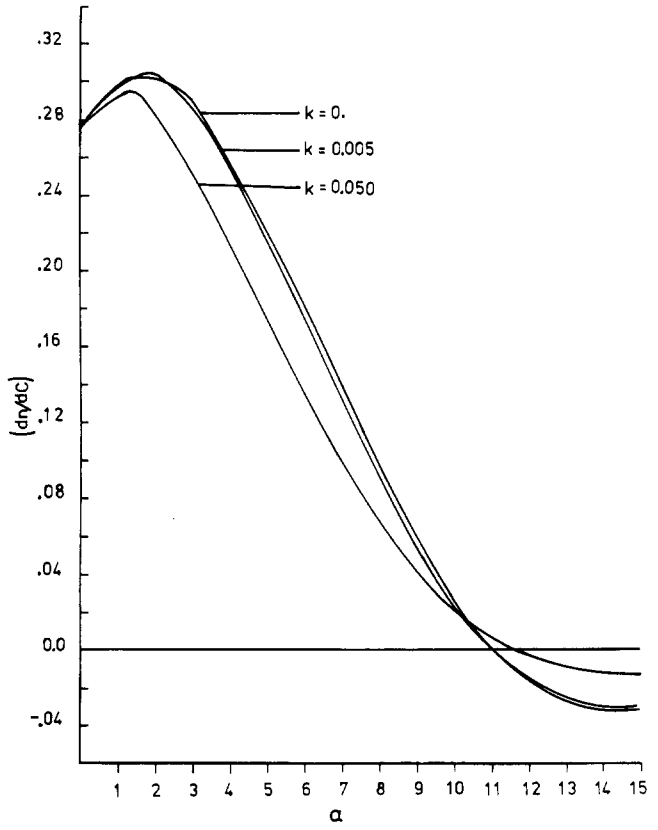

Figure 9. Effect of the molecular size and the absorption coefficient on the refractive index increments (n_0 = 1.45; n_1 = 1.74; ρ = 1).

of a polymer in solution is often obtained through measurements of the more convenient refractive index increments (dn/dc).[20,21] It has been shown theoretically and demonstrated experimentally that the refractive index increments depend on the molecular weight and therefore on the size of the polymer molecules.[25,28] On the basis of the Mie theory, Zimm and Dandliker[25] derived the following expression for the refractive index increment of a suspension of absorbing and scattering particles:

$$dn/dc = -(3/\rho\alpha^3)\ \mathrm{Im}\left[\sum_{j=1}^{\infty}(2j+1)(a_j+b_j)/2\right] \quad (10)$$

In the limit of zero size, eq 10 reduces to the well-known expression

$$dn/dc = (3n_0/2\rho)((n_1/n_0)^2-1)/((n_1/n_0)^2+2) \quad (11)$$

from which the specific refractive index increments are normally estimated.[23,27,28] The effects of the absorption coefficient (k) and the molecular size on dn/dc are shown in Figure 9. Notice that dn/dc is rather insensitive to the magnitude of the absorption coefficient for typical values of the absorption coefficients of macromolecules in the UV–vis region (i.e., $0.0 < k(\lambda) < 0.005$). On the other hand, changes in molecular or particle size have a marked effect on the refractive index increment values. Also, notice that for values of α typical of macromolecules in solution ($0 < \alpha < 1$), the magnitude of the changes in dn/dc is relatively small and it agrees well with the values reported in the literature.[27,28] It is clear, however, that for larger particles (i.e., macromolecular aggregates), eqs 3 and 10 should be solved simultaneously.

In light scattering studies of aggregation phenomena and protein denaturation processes, the refractive index of the solvent for different conditions is often approximated with the refractive index of the solvent free of denaturant. However, if the refractive index differences in the solvent are significant, this may not be a good approximation. The effect of the refractive differences on the estimation of particle diameters is demonstrated in Figure 10, where the variation of the observed to actual particle diameter in the Rayleigh regime is shown for refractive index differences typical of guanidine/HCl denaturation experiments (i.e., 0–6 M guanidine/HCl). Notice that for a solvent containing 6 M guanidine/HCl (Δn = 0.106 from ref 24) the particle diameter would be under estimated by approximately 100%! For particles in the Mie scattering regime (eqs 2 and 3), the refractive index effects can be expected to be even more pronounced.

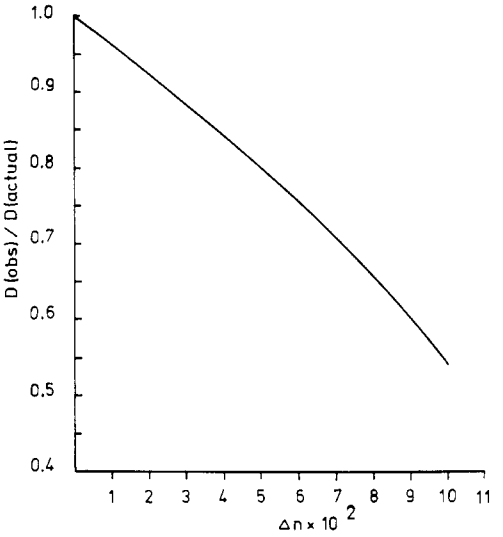

Figure 10. Ratio of the observed to the true particle diameter as a function of the refractive index difference between Tris-HCl buffer and the same buffer with varying concentrations of guanidine/HCl.

Applications to Macromolecular Characterization

The refractive index corrections described above allow for quantitative comparisons of UV–vis turbidity spectra on the same basis, making it possible to obtain good estimates of the scattering corrected absorption spectra which, in turn, can be interpreted in terms of composition and/or conformational changes. The refractive index corrections also have implications in the use of model molecules for the representation of chromophoric groups contained in macromolecules. By taking measurements of the refractive index of the solvent and the solution, it is possible to effectively deconvolute the spectra of complex macromolecules. This approach has been successfully used for the characterization of narrow polystyrene standards,[29] for the characterization of complex macromolecules in solution such as copolymers and proteins,[30] and for the quantitative interpretation of the signal from polymeric sensors.[31,32]

Summary and Conclusions

The effects of the solvent refractive index on the absorption spectra of macromolecules have been investigated using the Mie theory. It has been shown that the differences in intensity and the spectral shifts often observed in comparative UV–vis spectroscopy of macromolecules can be explained on the basis of differences in the refractive index of the solvents. A significant fraction of the spectral shifts normally attributed to changes in conformation may be interpreted in terms of differences in the refractive index of the solvent. It is also evident

that, for large molecules and/or particles, the particle size must be accounted for in the estimation of the refractive index increments. Clearly, the scattering and refractive index equations must be solved simultaneously in order to obtain good estimates of the molecular weights and/or the particle sizes. It is expected that other spectroscopy characterization techniques are subject to similar refractive index effects.

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