Spectroscopy in heterogenous media and applications for bioprocess and environmental monitoring

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ABSTRACT. Diffuse reflectance measurements and photon migration studies with near infrared (NIR) diode lasers were employed to elucidate experimental methods for determining absorption and scattering coefficients and species concentrations in heterogenous media. Measurements were performed at a number of wavelengths utilizing several laser sources some of which were widely tunable. In order to establish the applicability of simple photon migration models derived from radiation transport theory and to check the experimental boundary conditions of our measurements, simple light scattering solutions (such as suspensions of titanium dioxide, latex particles, and solutions of milk powder) containing dyes (such as nile blue, isosulfan blue) were investigated. The results obtained from diffuse-reflectance studies at different source-detector distances were in accordance with predictions from simple photon diffusion theory. Applications of reflectance measurements for monitoring of cell growth during fermentation processes and for *in-situ* investigations of soils are presented.

1. INTRODUCTION

Accurate quantitative analysis of photophysical properties in heterogeneous media is an important task in many spectroscopic and analytical investigations of, e.g., particulate materials, biological matrices or environmental samples. Due to light scattering, determination of, e.g., absolute concentrations or molar absorption coefficients of absorbing species can in most cases not easily be achieved without extensive calibration, either because light scattering prevents simple Lambert-Beer's law analysis or the molar absorption coefficient under the conditions of the turbid media is not known with a sufficient certainty. For detailed interpretation of spectroscopic investigations of many environmental and biological samples, which often show absorption, multiple light scattering and luminescence, advanced models for the radiation transport in heterogeneous media are required. Appropriate theoretical models allow development of experimental procedures for the determination of spectroscopic parameters such as absorption coefficient (μ_a) and effective scattering coefficient (μ_s') [1]. Only on the basis of these parameters the accurate analysis of the light intensity absorbed, which is of particular interest in investigations of, e.g., photocatalysis, and characterization of light-scattering components (such as particles) is possible. In general, diffuse reflectance measurements are often preferable to transmission measurements because (i) the transmitted light intensity is often weak and (ii) for precise transmission measurements of multiply scattering media the theoretical and experimental effort is more demanding [2].

Recent improvements in room temperature diode laser technology for communications and data storage applications have given rise to a new class of optical near infrared (NIR) sensors with improved sensitivity. Compared to traditional lasers, diode lasers are generally compact, reliable, easy to operate, amenable to electronic high frequency modulation and temperature tuning, and are in most cases of low cost. Diode lasers recently enabled significant progress in ultrasensitive gas analysis in both research and industrial applications (e.g. [3] and references cited therein). High quality room temperature laser devices are now available in a number of discrete spectral windows between *ca.* 400 nm and 1900 nm and promise new perspectives for highly sensitive analytical *in-situ* techniques for investigations of heterogenous media.

Analysis of photon density waves launched by intensity-modulated lasers in light scattering media provides an elegant way for the determination of μ_a and μ_s' of multiply scattering media. The absorption coefficient can be used for qualitative and quantitative analysis of chemical constituents (which absorb in the range of the laser wavelengths). The effective scattering coefficient allows characterization of physical and morphological properties of the scattering components of the medium. Photon density waves for non-invasive optical analysis have found widespread applications in biomedical optics (see e.g. [4, 5] and references cited therein). The feasibility for investigations of colloid-polymer suspensions, biological cell debris and for particle sizing of suspensions has been in ivestigated in only few publications [6–8].

Monitoring of cell growth parameters (such as cell number density, size distribution, cell shape) is an important task in biotechnological fermentation processes. In practice, simple turbidity probes are employed, which usually require extensive calibration for,

e.g., determination of biomass, because they detect simple light attenuation and cannot distinguish between absorption and scattering [9]. Development of non-invasive sensor probes which can overcome this limitation is thus desireable. In the current work, the feasibility of diffuse-reflectance measurements at various distances between illumination and detection fiber optics for monitoring of cell growth of *S. cerevisiae* (yeast) is demonstrated. Soils can also be viewed as strongly scattering and absorbing media and are of outstanding analytical interest. Soils are porous media which can contain gaseous, liquid and solid phases. The solid phase can contain complex organic and inorganic material as humic substances and minerals like silicates, clays etc. A remarkable increase of interest in non-invasive analytical tools for monitoring pollutants and natural processes in soils is observed during the past decade (cf. [10, 11] and references cited therein). Techniques for investigations of processes like soil respiration and conversions of soil organic matter are of particular relevance because these processes are believed to play an important role in the global CO_2 balance with impact on the global atmosphere [12].

This contribution is organized as follows: (i) The experimental problem is defined and transmission measurements of multiply scattering solutions are discussed. (ii) In order to establish the applicability of simple photon migration models derived from radiation transport theory and to check the experimental boundary conditions of our measurements, simple light scattering solutions (such as suspensions of titanium dioxide, latex particles, milk powder solution) containing dyes (such as isosulfan blue, nile blue) are investigated. (iii) Results of photon migration studies to monitor cell growth in a bioreactor and *in-situ* investigations of sand, which served as a simple soil model, are discussed.

2. EXPERIMENTAL DETAILS

Absorption and total light attenuation measurements were carried out on a conventional Varian Cary 500UV/VIS-NIR absorption spectrometer. Fiber-optic diffuse-reflectance measurements and photon migration studies were performed with an intensity-modulated laser diode spectrometer (IMLDS). The IMLDS consisted of a home-built array of intensity-modulated diode lasers as light sources. Photodiodes, amplifier and a lock-in amplifier (EG&G 7260) or a network analyzer (HP 8712ET) set to the modulation frequency were employed for phase sensitive detection of scattered light intensity. Current laser diode wavelengths included $\lambda = 638, 678, 785, 1544$ nm (from standard Fabry-Perot laser diodes) and the wavelength regions *ca.* 1580–1690 and 1360–1420 nm (from tunable external cavity diode lasers, Sacher Lasertechnik, Marburg/Germany). Additionally, a Helium-Neon laser (633 nm) was employed. The laser diodes and the detectors were coupled to fiber-optics which allowed flexible illumination and detection geometries. For the photon migration studies, both the illumination and the detection fiber-optics were immersed in the medium in 0 degrees angle with respect to the axis perpendicular to the sample surface, the two fibers being separated by the distance r. Ultrafiltrated, deionized, non-degassed water and commercially available milk powder (Néstle) was used. TiO_2 (anatas) was obtained from Alfa Chemicals Co., nile blue from Lambda Physik GmbH, isosulfan blue from Aldrich. All chemicals were used without purification. PMMA latices were supplied by T. Ruhl and G. Hellmann, Deutsches Kunststoffinstitut, Darmstadt/Germany.

3. RESULTS AND DISCUSSION

In Figure 1, light attenuation (extinction)([1]) of nile blue sulfate, milk powder, and mixtures of them in aqueous solutions as measured with a standard absorption spectrometer are displayed. Absorption bands of nile blue at 637 nm (due to electronic transitions) and of water at 974 nm (due to second overtone of the O – H stretching vibrational transition) are clearly observable. By subtraction of the water spectrum from the spectrum of the dye solution, the absorption spectrum and thus μ_a for nile blue is obtainable. On the other hand, subtracting the water spectrum from the spectrum of the milk powder solution, yields the attenuation of milk powder. The attenuation of milk powder can in this spectral region attributed to mainly scattering. Only in the absence of multiple scattering effects, the attenuation coefficient μ_t, which is the sum of μ_s and μ_a, can be determined from this spectrum (see below). Furthermore, the absorption coefficient of an absorber in an emulsion like milk powder solution can be different from the absorption coefficient measured in clear solution. Inspection of the spectrum obtained for nile blue solution containing milk powder reveal that the band shape of the absorption peak is changed compared to the spectrum in clear solution (cf. Figure 1). In general, separate

([1]) The spectra are given terms of $\log(I_0/I)$, where I_0 and I are the light intensity without and with a sample in the spectrometer light path, respectively. $\log(I_0/I)$ is called attenuation and is for merely absorbing samples given by the linear absorption coefficient multiplied by the optical path length. Then attenuation equals absorbance. For samples, which show scattering and absorption, the attenuation per unit path length is given by the linear attenuation coefficient. Finally, the attenuation per unit path length for weakly scattering samples is given by the linear scattering coefficient. Please note that throughout this paper μ_a, μ_s, and μ_t denote the vaperian absorption, scattering, and attenuation coefficients, respectively. The linear coefficients μ_s and μ_a can be expressed by, e.g., the corresponding molar coefficients multiplied by the species concentration.

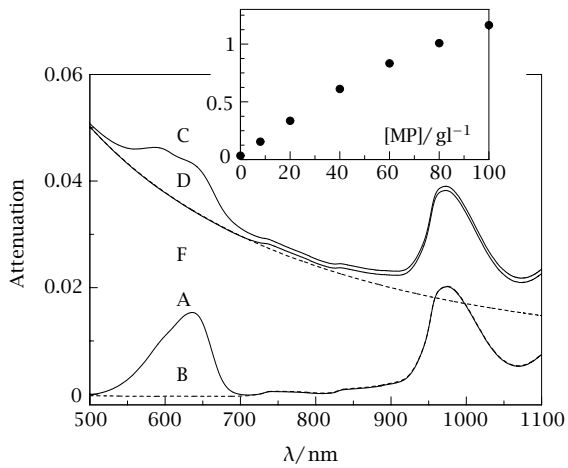

Figure 1. *Light attenuation of aqueous nile blue solution (A), pure water (B), aqueous nile blue solution containing milk powder (C) and of milk powder in aqueous solution (D) at room temperature as determined with transmission measurements over* 1 *mm optical path length. The absorbance of nile blue in water can be derived by subtracting B from A. The attenuation of milk powder in water is given by* $F = D - B$. *The nile blue concentration was* 5.8×10^{-6} *M for A and C, the milk powder concentration was* 0.474 gl^{-1} *for C and D. Inset: Attenuation of a milk powder solution in water at* 600 *nm vs. milk powder concentration over 0.16 mm optical path length.*

determination of absorption and scattering cofficients of multiple scattering media with simple transmission measurements on a standard spectrometer is not an easy task. The reasons for this can be devided in effects due to multiple scattering and in experimental effects due to characteristics of standard spectrometers: (i) In absence of luminescence, the light intensity detected after propagation of the incident light through a multiple scattering sample can be expressed as a sum of two portions. The first portion accounts for the attenuation of the incident beam by absorption and scattering in the medium (described by the linear attenuation coefficient μ_t). On the other hand, the detected intensity increases because a portion of scattered light from other directions can be scattered into the direction of detection. Furthermore, due to multiple scattering effects the optical path length in the sample can become longer than the cuvette length. (ii) The light beam in the sample compartiment of a standard spectrometer can usually not be described by an ideally collimated beam. For detection of scattered light, which can show complicate dependence on the angle between the detected and the incident beam, the measurement geometry is thus not very well defined. Furthermore, the geometrical characteristics of the incident and the detected beam can be different for spectrometers from different suppliers. These effects make accurate determination of μ_s (see below for descrimination of μ_s' and μ_s) and μ_a of multiple scattering media with standard spectrometers a difficult task. The limits of optical transmission measurements were previously studied in detail by Swanson *et al.* [13]. In the inset of Figure 1, the light attenuation of aqueous milk powder solution is plotted vs. the concentration. Due to multiple scattering effects and the reasons stated above, a non-linear dependence is observed and complicates accurate determination of the scattering coefficient. For spectroscopic investigations of clear dye solutions Lambert-Beer's law is applicable and can be employed for, e.g., determination of molar absorption coefficients or concentrations. Alternative approaches are required for solutions which show multiple light scattering.

In this study, results of photon migration studies are analyzed in terms of simplified photon diffusion theory [1]. In the framework of this theory, light transport through the solution is described by a transport equation derived from a particle diffusion approach of photon migration through random media. Solving the transport equation for appropriate boundary conditions yields to the following dependence of (intensity-modulated) light intensity on the separation distance between light source and detection point r [15]:

$$I_{AC} = \frac{\text{const}}{rD} \exp\left(-r\sqrt{3\mu_a\mu_s'}\right). \tag{1}$$

Equation (1) yields

$$\ln(I_{AC}r) = \ln\left(\frac{\text{const}}{D}\right) - r\sqrt{3\mu_s'\mu_a}, \tag{2}$$

I_{AC} denotes the AC light intensity which is measured as detector voltage at a 50 Ω resistor. $D = [3(\mu_a + \mu_s')]^{-1}$ is the optical diffusion coefficient with the absorption coefficient μ_a and the effective scattering coeffient μ_s'. Equation (1) is valid for $\mu_a \ll \mu_s'$, for moderate modulation frequencies and for infinite media. In this approximation, the slope m of a plot $\ln(I_{AC}r)$ vs. r is simply $\sqrt{3\mu_s'\mu_a}$. Thus m^2 is proportional to the absorption coefficient and to the effective scattering coefficient. In this work, I_{AC} measurements are persued which were performed at modulation frequencies in the kHz regime. However, the AC portion of the diffusely reflected light intensity provides further experimental information. The phase lag ϕ between the sinusoidally modulated laser intensity at r and r_0 that is given by [15]:

$$\phi = (r - r_0)[\mu_a(\mu_a + \mu_s')]^{1/2}\left(\frac{3}{2}\right)^{1/2} \times \left[\left(1 + \left(\frac{2\pi f}{\mu_a c}\right)^2\right)^{1/2} - 1\right]^{1/2}, \tag{3}$$

f denotes the modulation frequency. Detection of ϕ,

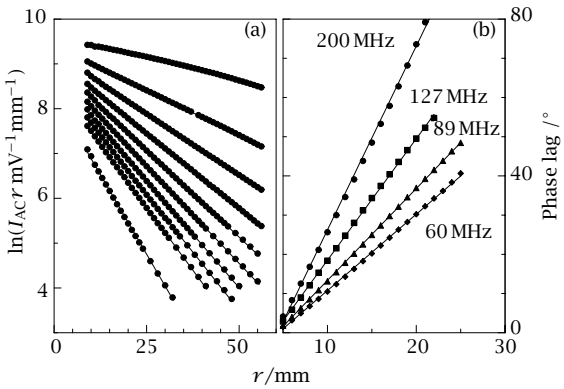

Figure 2. *(a) Photon migration studies of isosulfan blue solutions* ([ISB] $= 0 - 3 \times 10^{-6}\,M$) *in water containing* $5\,gl^{-1}$ *milk powder* (MP). *Logarithmic plot according to equation* (2) *of the light intensity vs. source/detector separation* r *measured at* 638 *nm. (b) Phase lag vs.* r *for* TiO_2 *suspension* ($4\,gl^{-1}$) *for various modulation frequencies* ($r_0 = 4$ *mm).*

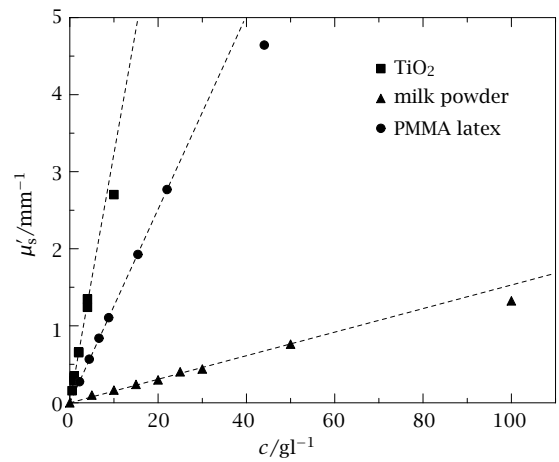

Figure 3. *Effective scattering coefficient* μ_s' *at* 638 *nm vs. concentration for various scatterer. The nominal particle diameter were* 32 *nm for* TiO_2, 0.1 μm *for PMMA latex beads, and* 0.2–10 μm *for milk powder.*

which is performed with modulation frequencies in the MHz regime, and I_{AC} allows determination of μ_a and μ_s', respectively. μ_a can be written as the sum of the absorption coefficients of all i components absorbing at the laser wavelength employed:

$$\mu_a = \sum_i \epsilon_i c_i. \tag{4}$$

ϵ_i and c_i denote the molar absorption coefficient and the concentration of the ith compound, respectively. The scattering coefficient for particulate material with scattering particles that are large compared to the wavelength can be expressed in the framework of Mie theory [6, 17]:

$$\mu_s' = \Phi_v \int_0^\infty \frac{3}{2x} Q_{scat}(x, n_r, \lambda) S f(x) \mathrm{d}x, \tag{5}$$

Φ_v denotes the volume fraction of scatterer. x is the particle size, $f(x)$ is the particle size distribution and $Q_{scat}(x, n_r, \lambda)$ is the angle-averaged scattering efficiency as function of x, the relative refractive index n_r, and the wavelength λ. S is the static structure factor and is a measure of local ordering of the particles due to particle-particle interactions. $Q_{scat}(x, n_r, \lambda)$ can be computed using appropriate scattering theory, for example Mie theory [17]. Thus, informations about species concentrations, scatter volume fraction, particle size and interactions among the particles are obtainable from diffuse-reflectance measurements for particulate media in the framework of photon diffusion and Mie theory. However, it is noted that due to the complexity of equation (5) accurate determination of some parameters can become a formidable problem.

Because the theoretical approach leading to equation (1) included a number of assumptions concerning, e.g., optical boundary conditions, we conducted investigations of simple model systems in order to established the applicability of equation (1). A typical model solution consisted of a scattering component (e.g. milk powder, titanium dioxide, PMMA latex beads), which exhibited negligible absorption in the spectral region of interest, and an absorbing dye (e.g. isosulfan blue, nile blue) in aqueous solution. These suspensions and emulsions allowed independent variation of absorption and scattering cofficients by simple variation of species concentration. Figure 2 shows the results of diffuse-reflectance measurements at different separation distances between the illumination and the detection fiber optics. The following conclusions can be drawn: (i) A plot of $\ln(I_{AC}r)$ vs. r exhibit good linearity in accordance with equation (2), (ii) the phase lag ϕ depends linearily on the separation distance r in agreement with equation (3) and increases with increasing modulation frequency and (iii) a plot of the square of the slope m^2 vs. absorber concentration is linear (not shown) in accordance to equation (2).

From phase lag measurements, μ_s' and μ_a can both be determined with equation (3). From intensity measurements alone, e.g., μ_s' can be determined by varying the absorber concentration and plotting m^2 vs. the absorber concentration. μ_s' can then be calculated from the slope if the molar absorption coefficient of the absorber is known. In Figure 3, μ_s' obtained for various scatterers is plotted vs. the scatterer concentration. Good linearity is observed for relatively small concentrations because of the proportionality of the volume fraction of the scatterer to the concentration. At higher scatterer concentrations, deviations from linearity are observed. This behaviour has been attributed to interactions between the scattering particles and can be a basis

for investigations of the structure factor S [6]. Furthermore, μ_s' is generally higher for scatterer with smaller nominal particle diameter x which is in agreement with expectations from equation (5). It is noted that since aggregation of the particles cannot be excluded, the actual diameters of the particles responsible for the observed scattering are not known.

One common way for additional characterization of the scatterer is determination of the anisotropy factor g which is the mean cosine of the scattering angle. g is related to μ_s' according to the following relation:

$$\mu_s' = (1 - g)\mu_s. \tag{6}$$

For very weakly absorbing scatterer, μ_s can be estimated from simple transmission measurements using a collimated light beam, low scatterer concentration, and short optical path length (to avoid multiple scattering). Under these conditions the total attenuation coefficient μ_t of scatterer solutions can be determined. Since the absorption coefficient μ_a of the scatterer is assumed to be negligible, μ_s can be estimated from μ_t:

$$\mu_t = \mu_a + \mu_s \approx \mu_s. \tag{7}$$

Finally, g can be calculated with equation (6) employing μ_s and μ_s' as obtained from the photon migration studies decribed above. $g = 0.69 \pm 0.02$ and 0.80 ± 0.02 was obtained at 633 nm for milk powder samples containing 1% fat and 17.5% fat, respectively. Previously, $g = 0.79 \pm 0.012$ was reported for whole milk powder as determined with experiments using a collimated laser beam [14]. Our result for g is very close to that value. We conclude that the equations (1) and (3) provide excellent basis for quantitative determination of absorption coefficient and effective scattering coefficients of turbid media and can be applied within the optical boundary conditions of our experiments.

The assumptions of the photon diffusion theory can also be applied to describe reflectance measurements of cell cultivations in the NIR spectral region between *ca.* 800 and 1100 nm. Diffuse-reflectance studies have been performed to monitor cell growth of *S. cerevisiae* (yeast) in suspensions and the results are shown in Figure 4. The plot of $\ln(I_{AC}r)$ vs. separation distance r exhibit good linearity in accordance with expectations from equation (1). As the cell cultivation grows, the number density of cells, μ_s' and thus m^2 increases. Furthermore, changes in cell size and form can be expected and can also influence μ_s'. Previous work in the literature underlines that the change of the absorption coefficient with increasing cell number is relatively small for yeast cells [16]. Thus the observed change in m^2 plotted in Figure 4 is mainly due to change in μ_s'. Further work is in progress in our laboratory to determine both μ_a and μ_s' as a function of cell number density for a number of microorganisms including algae, which contain considerable amounts of chlorophyll derivatives. Algae can thus be expected to show also significant changes of the absorption coefficient in the course of the cell growth.

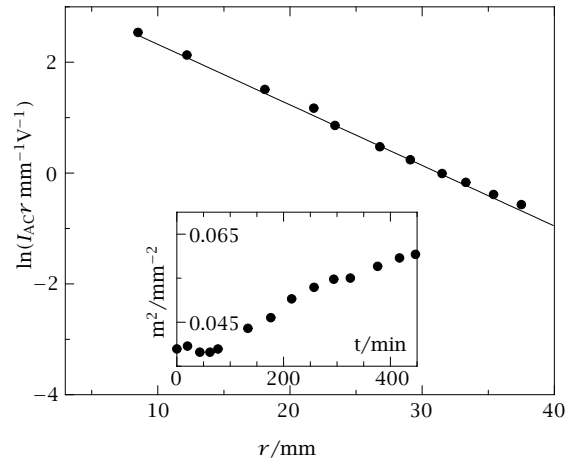

Figure 4. $\ln(I_{AC}r)$ *according to equation* (2) *vs. source/detector separation* r *for a cultivation of S. cerevisiae in malt beer measured at* 638 *nm. Inset: Square of the slope* m *vs. observation time.*

We have performed exploratory diffuse-reflectance studies of sand as a simple soil model utilizing several diode lasers. Our measurements aimed to determine the feasibility of diffuse-reflectance studies at various source/detector distances for characterization of soils. The plot of $\ln(I_{AC}r)$ vs. separation distance r is in accordance with expectations from equation (1) (Figure 5). Due to relatively high scattering of sand, the reflected intensity shows a remarkable decrease with increasing distance r. In the inset of Figure 5, m^2 is plotted for different wavelenghts. m^2 decreases with increasing wavelength by a factor of approximately 10

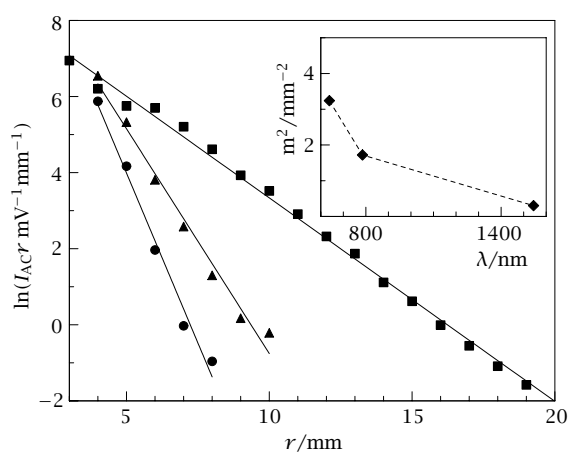

Figure 5. $\ln(I_{AC}r)$ *according to equation* (2) *vs. source/detector separation* r *for sand measured at* 1544, 785, *and* 638 *nm (top to bottom). Inset: Square of the slope* m *vs. laser wavelength.*

from 635 nm to 1544 nm. A decrease of μ_s' with increasing wavelength is generally expected in the framework of Mie theory [17]. However, wavelength dependence of μ_a has to be taken into account as well. Experimentally, investigation of diffuse-reflectance soils in the NIR spectral region is attractive for the following reasons: (i) Scattering is relatively weak at longer wavelengths leading to detectable $I_{AC}(r)$ functions. (ii) Many organic compounds in general and soil organic matter in particular exhibit destinct absorption features in this spectral region [18, 19]. (iii) Diode lasers are available for a number of wavelengths in the NIR and can be utilized for reflectance studies with outstanding signal to noise ratio. It can be concluded that diffuse-reflectance measurements at different separation distances between illumination and detection fiber optics provide a powerful tool also for investigations of soils. Further studies are now being conducted in our laboratory to achieve quantitative determination of μ_a and μ_s' for various soils at a number of wavelengths.

4. CONCLUSIONS

The current study demonstrates the enormous potential of intensity-modulated diode lasers for analytical and spectroscopic *in-situ* investigations of heterogenous media. It was shown that approaches derived from photon diffusion theory allow quantitative studies of multiply light scattering media with diffuse-reflectance measurements at various distances between illumination and detection fiber optics. Absorption and effective scattering coefficients can be determined under conditions for which the Lambert-Beer law is not applicable. In general, the absorption coefficient allows quantitative and qualitative analysis of chemical constituents. The effective scattering coefficient can be used to characterize physical and morphological properties of the medium. For particulate material, particle sizes, forms and interactions among the scattering particles can be investigated.

The media investigated in the current work include semiconductor and polymer suspensions, emulsions, a cultivation of biological cells, and sand. The bandwidth of possible applications of the applied diffuse-reflectance measurements range from investigations of photocatalytic reactions, dense colloidal solutions to on-line monitoring of cell growth in bioreactors and of environmental processes.

The technique used in this work employs compact diode laser sources which are reliable enough for implementation in sensor systems. Further progress can be expected from electronic components originally developed for telecommunication devices. These devices will also contribute significantly to the development of new sensor devices for biomedical, environmental and chemical tasks.

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