

# Diffuse reflectance spectroscopy characterization of hemoglobin and intralipid solutions: *in vitro* measurements with continuous variation of absorption and scattering

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## 1 Introduction

Diffuse reflectance spectroscopy has been a useful tool for *in vivo* studies of tissues in both diagnostic and therapeutic applications.<sup>1-4</sup> The technique is based on the spectroscopic analysis of the light reflected from the sample under study by using a pair of optic fibers called optodes, which respectively inject and collect light from the medium. The aim is to determine the concentration of optically absorbing substances (chromophores) from the attenuation of the incident light, taking into account the scattering of the tissue.

Despite the important advances made in this field in recent years, leading for example to the design of tissue oxymeters for clinical use,<sup>4</sup> the determination of absorber concentrations from spectroscopic measurements continues to be an open

**Abstract.** Absorption and scattering processes in biological tissues are studied through reflectance spectroscopy in tissue-like phantoms. For this aim, an experimental setup is designed to independently control both processes in hemoglobin and intralipid solutions. From the analysis of the obtained spectra, a simple empirical power law equation is found that relates absorbance with scattering and absorption coefficients. This relationship includes three wavelength independent parameters, which can be determined geometry from *in vitro* measurements for each particular optical optode. The dependence of the optical path length on the absorption and scattering coefficients is also analyzed, and estimations of this parameter for physiological conditions are presented. This study is useful to better understand the scattering phenomena in biological tissue, and to obtain absolute concentration of absorber particles when a homogeneous medium can be assumed. © 2009 Society of Photo-Optical Instrumentation Engineers. [DOI: 10.1117/1.3149864]

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problem. Some active research lines in this matter are: the estimation of the optical path length,<sup>5</sup> the look-ahead distance or the area under study,<sup>6</sup> the comparison of different algorithms to measure oxygen saturation,<sup>1</sup> the determination of scattering and absorption coefficients,<sup>7,8</sup> or the simulation of light propagation in tissue using Monte Carlo methods.<sup>9,10</sup>

The basic theory of light propagation in tissues was formulated by Twersky.<sup>11-13</sup> This theory is quite complicated for practical use. Several methods are available in the literature to analyze light propagation in biological tissue, to cite some; the adding-doubling method,<sup>14</sup> the Kubelka-Munk theory,<sup>15</sup> and the already mentioned Monte Carlo simulation.<sup>9,10</sup>

Here we present an alternative empirical approach to quantitatively analyze the dependence of the measured absorbance on absorption and scattering processes. The method is based on using *in vitro* optical tissue-like phantoms: different con-

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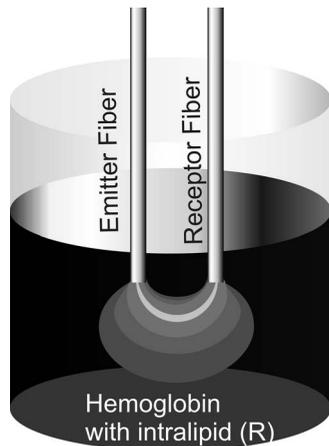


Fig. 1 Drawing of the experimental setup.

concentrations of absorbing and scattering substances are mixed, simulating the experimental conditions of biological tissues.<sup>1-10,16,17</sup> Hemoglobin has been widely used to simulate absorption, because it is the main absorber in the visible range in biological tissues.<sup>1-4</sup> Other artificial substances such as Nigrosin,<sup>7</sup> Trypan blue dye,<sup>16</sup> or epoxy resin<sup>18</sup> have also been used.

Scattering is usually simulated using intralipid or similar lipid suspensions.<sup>1-3,5-7,19-21</sup> These substances show strong forward scattering and very weak absorption at visible and NIR wavelengths, similar to biological tissues.<sup>19-21</sup> The main drawback is its bottle-to-bottle variation of the optical magnitudes. Other substances like amorphous silica spheres,<sup>18</sup> polystyrene micro-spheres,<sup>7,22</sup> or perfluorocarbon<sup>2</sup> have also been proposed. A review of *in vitro* optical tissue-like phantoms for optical spectroscopy has been recently published.<sup>23</sup>

We have carried out different sets of experiments where the hemoglobin or the intralipid progressively varied their concentration. The analysis of these results allows us to provide an empirical relation of the absorbance as a function of the scattering and absorption coefficients. Moreover, an expression for the optical path length related with these coefficients is also obtained.

## 2 Experimental

A drawing of the experimental setup is shown in Fig. 1. The incident light comes from a tungsten halogen lamp, and the captured light is sent to a spectrometer with a photodiode array detector. The parameters of the used optodes are: 100  $\mu\text{m}$  core, 130  $\mu\text{m}$  separation between centers, and 0.22 numerical aperture.

The samples were prepared by diluting different concentrations of hemoglobin and intralipid in distilled water. The former was extracted from the blood of a healthy human volunteer and the latter was supplied by Farmacia Ibérica. A magnetic stirrer was used to maintain homogeneity.

The experiments were carried out in two groups:

1. Constant scattering: absorption was continuously changed at different fixed intralipid concentrations (0.5, 1, and 2%), by progressively increasing the hemoglobin concentration from 0 to 200  $\mu\text{M}$ .

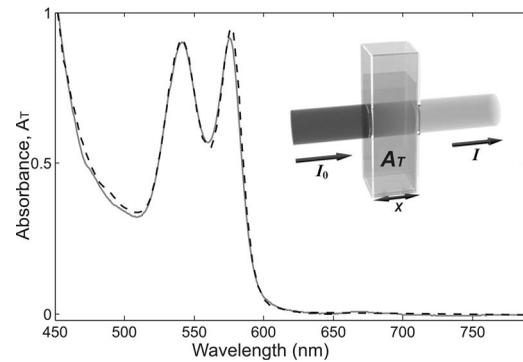


Fig. 2 Absorbance spectrum of diluted hemoglobin measured by transmittance (continuous) compared with the normalized spectrum reported by Prah1<sup>25</sup> (dashed) and setup used, top right.

2. Constant absorption: in this case, scattering was changed by increasing the intralipid concentration from 0 to 5% with different hemoglobin concentrations fixed at 40, 80, and 120  $\mu\text{M}$ .

The 0- to 120- $\mu\text{M}$  interval of hemoglobin concentration covers the usual range of interest in biological tissues,<sup>1,2,5,18,24</sup> while the 0 to 5% interval in the intralipid concentration, corresponding, in the visible range of wavelength, to a variation of the reduced scattering coefficient  $\mu'_s$  from 0 to 7  $\text{mm}^{-1}$ ,<sup>20-22</sup> is enough to simulate the scattering values reported in the literature for biological tissues.<sup>1,2,5,18,24</sup>

The first step was to measure the concentration of hemoglobin  $c$  without scattering using transmittance spectroscopy. According to the Beer Lambert law, the concentration  $c$  is given by

$$c = \frac{A_T}{\varepsilon \cdot x}, \quad (1)$$

where  $\varepsilon$  is the extinction molar coefficient,  $x$  is the optical path length (taken here as the width of the cuvette  $x = 10$  mm), and  $A_T$  is the absorbance measured by transmittance, as shown in Fig. 2. We found that, before dilution, the used hemoglobin had a concentration of 900  $\mu\text{M}$ .

Here the absorbance  $A_T$  corresponds to the measurement of the attenuation of the light intensity  $I$  after passing through the sample in relation with that incident on it  $I_0$

$$A_T = \log\left(\frac{I_0}{I}\right), \quad (2)$$

where  $\log$  is the base 10 logarithm. A similar relation is used in the following for reflectance spectroscopy.

Figure 2 shows the spectrum of the measured absorbance  $A_T$  and a diagram of the used setup. The two well-known peaks of oxyhemoglobin at 542 and 576 nm are clearly shown. It can be seen that our results are in good agreement with those given by Prah1,<sup>25</sup> with minor differences between both spectra due to the fact that our hemoglobin is slightly deoxygenated (94% OxyHb+6% DeoxyHb).

The absorption coefficient  $\mu_a$  can be calculated from the measured absorbance  $A_T$  as

$$\mu_a = \frac{1}{\log(e)} \frac{A_T}{x} \quad (3)$$

### 3 Variation of Reduced Absorbance with Hemoglobin Concentration

As already mentioned, in reflectance spectroscopy, the measured intensity depends on both scattering and absorption processes being difficult to analyze separately. So it is useful to introduce a magnitude that we call reduced absorbance  $A_S$ , defined as

$$A_S = \log\left(\frac{I_S}{I}\right), \quad (4)$$

where the incident radiation  $I_o$  is substituted by the intensity  $I_S$  measured at the receiver fiber when the concentration of absorbers is zero (only scattering is present).<sup>1-3</sup>

The reduced absorbance  $A_S$  corresponds to the difference between the absorbance for a certain concentration of absorbers  $A(\mu_a)$ , given by Eq. (2), and the absorbance  $A(0)$  measured if the absorbing particles are removed:

$$A_S = A(\mu_a) - A(0). \quad (5)$$

This approach is equivalent to the procedure proposed by Knoefel et al.,<sup>1,26</sup> based on the subtraction of the baseline extrapolated from the region of zero absorption, which is the usual way to take the reference for *in vivo* samples.

The use of the reduced absorbance  $A_S$ , instead of the absorbance  $A$ , has the advantage that it is not necessary to measure the input intensity  $I_o$ , which is hard to obtain in reflectance spectroscopy. It must also be noted that in this kind of experiment, the instrumental spectral response makes an additional contribution to the raw extinction spectrum as measured by the spectrometers. The process of referencing the measured extinction spectra to purely scattering intralipid phantoms also has the advantage that it removes that instrumental additional contribution.

In the first set of experiments, the reference intensity  $I_S$  was measured for intralipid concentrations of 0.5, 1, and 2%, without hemoglobin ( $\mu_a=0$ ). These reference spectra were used for the calculation of the reduced absorbance  $A_S$  when the hemoglobin was subsequently increased. The results for the particular fixed 0.5% intralipid concentration are plotted in Fig. 3. An increase of the reduced absorbance  $A_S$  with the hemoglobin concentration is observed for wavelengths under 650 nm, without appreciable changes over the 650- to 750-nm wavelength range.

We have selected the 576-nm peak to monitor the variation of the reduced absorbance  $A_S$  with the absorption coefficient  $\mu_a$  previously calculated through Eq. (3). The results are shown in Fig. 4 for two different intralipid concentrations of 0.5 and 2%. A linear behavior is observed in the log-log plot that corresponds to the following power law for  $A_S$ ,

$$A_S = K_a \cdot \mu_a^{C_a}, \quad (6)$$

where  $K_a$  and  $C_a$  are parameters obtained from the fitting.

We have analyzed the dependence of these  $K_a$  and  $C_a$  parameters with the intralipid concentration and hence with the

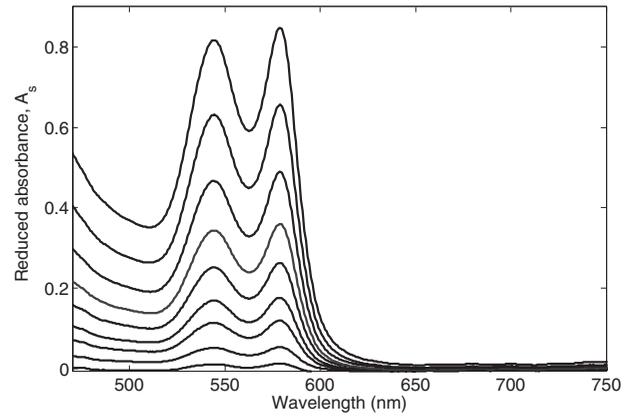


Fig. 3 Reduced absorbance spectra for 0.5% intralipid (fixed scattering) and different hemoglobin concentrations from 5 to 200  $\mu\text{M}$ .

reduced scattering coefficient. The results are shown in Fig. 5. The plot was made selecting the wavelengths of 460, 542, and 576 nm to obtain three different values of the reduced scattering coefficient for each intralipid concentration. The dependence of the reduced scattering coefficient on wavelength and intralipid concentration was taken from Staveren et al.<sup>20</sup>

Figure 5 shows that the exponent  $C_a$  does not appreciably change when the reduced scattering coefficient varies from 0.5 to 5  $\text{mm}^{-1}$ . This corresponds with the small variation of the slope in the log-log plot shown in Fig. 4. Assuming a mean value  $C_a=0.78$ , the dependence of the reduced absorbance on the absorption coefficient can be expressed as

$$A_S = K_a \cdot \mu_a^{0.78}. \quad (7)$$

It is important to realize that this result leads to a nonlinear relation between absorbance and absorber concentration, which significantly differs from the Beer Lambert law, which has frequently been used to calculate relative changes of chromophore concentrations in reflectance spectroscopy.

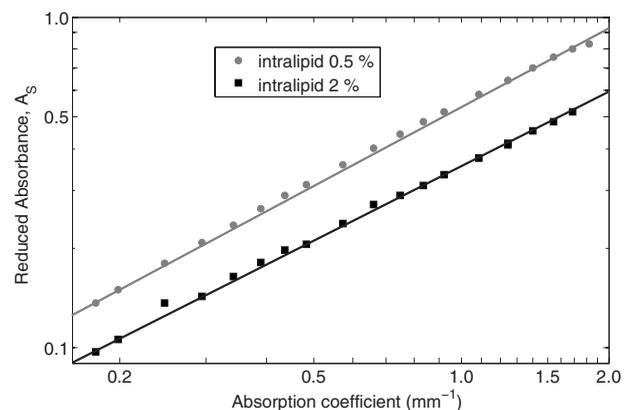
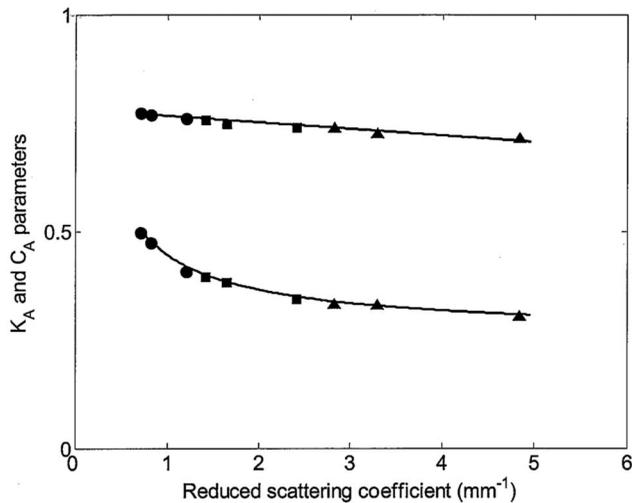


Fig. 4 Log-log plot of the *in vitro* measurements of the reduced absorbance at 576 nm versus the absorption coefficient, linear fits are also shown.

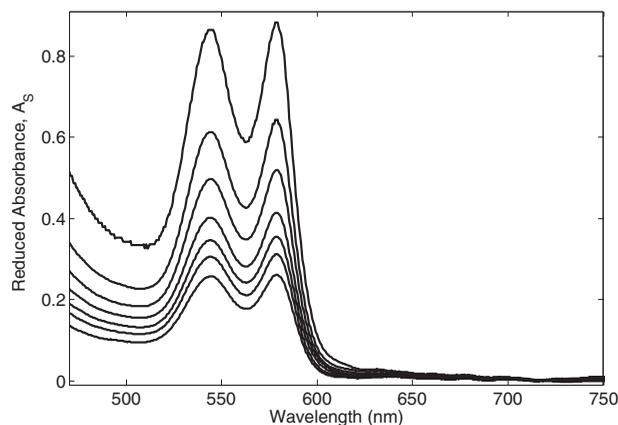


**Fig. 5** Variation of  $K_A$  and  $C_A$  parameters with reduced scattering coefficient. Circles, squares, and triangles represent respectively values for 0.5, 1, and 2% intralipid concentrations.

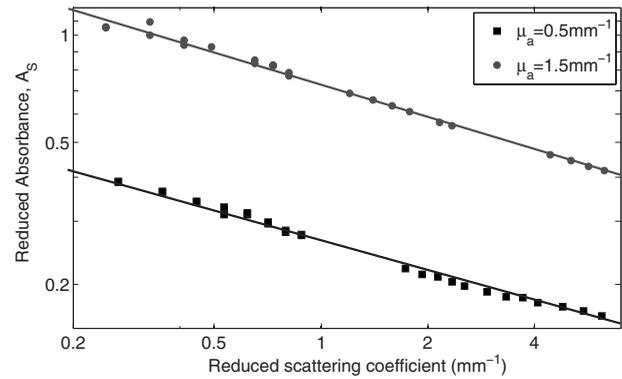
#### 4 Variation of Reduced Absorbance with Intralipid Concentration

In the second set of measurements, the intralipid concentration was continuously increased from 0.1 to 5% for different fixed hemoglobin concentrations (0, 40, 80, and 120  $\mu\text{M}$ ). In Fig. 6 it can be observed that the reduced absorbance  $A_S$  decreases with increasing intralipid concentration. This is due to the fact that the probability of catching photons at the receptor fiber grows with the concentration of scattering particles (as previously mentioned, scattering increases linearly with intralipid concentration). Notice that 0% of intralipid produces no reflectance, i.e.,  $I_S=0$ .

The variation of the reduced absorbance at 576 nm with the reduced scattering coefficient is plotted in Fig. 7 for two different values of the absorption coefficient. The log-log plot shows a linear behavior, so the relationship between the reduced absorbance and the reduced scattering coefficient can also be described by a power law:



**Fig. 6** Evolution of reduced absorbance for a fixed oxyhemoglobin concentration of 80  $\mu\text{M}$  and different intralipid concentrations from 0.1% (top) to 5% (bottom).



**Fig. 7** Log-log plot and linear fit of the *in vitro* reduced absorbance at 576 nm versus reduced scattering coefficient for two different hemoglobin concentrations.

$$A_s = K_s \cdot \mu_s'^{C_s}, \quad (8)$$

where  $K_s$  and  $C_s$  are parameters obtained from the fitting.

Figure 7 shows that the slope of the fits, corresponding to the parameter  $C_s$  in Eq. (8), is almost unchanged in the different sets of experiments, with  $C_s = -0.28$ , which means that this parameter does not change appreciably with the hemoglobin concentration. By the opposite, the y-intercept point  $K_s$  varies appreciably, with values of 0.34, 0.46, and 0.52 for hemoglobin concentrations of 40, 80, and 120  $\mu\text{M}$ , respectively.

It would be remarked from Fig. 7 that the reduced absorbance increases when the reduced scattering coefficient decreases, while it increases with the absorption coefficient, as shown in Fig. 4.

Combining the results from both sets of experiments [see Eqs. (6) and (8)], we reach the conclusion that for each optode geometric configuration, the reduced absorbance  $A_S$  can be written as

$$A_s = K \cdot \mu_a^{C_a} \cdot \mu_s'^{C_s}, \quad (9)$$

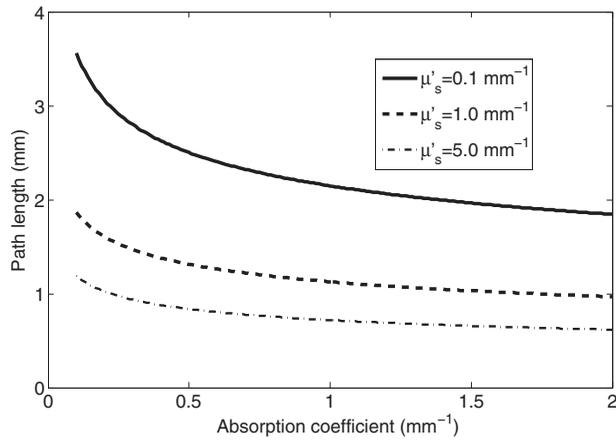
being, finally,  $K$ ,  $C_a$ , and  $C_s$  parameters that depend only on the used optode geometry. It would be remarked that these three parameters are not wavelength dependent. For our particular geometric configuration, Eq. (9) stays like

$$A_s = 0.49 \cdot \mu_a^{0.78} \cdot \mu_s'^{-0.28}. \quad (10)$$

We have taken different measurements varying the relative position of the fibers in the optode, as well as using a setup with 24 optodes for simultaneous multipoint monitoring. In all cases, the double power law, given by Eq. (9), resulted as valid, with different values for the  $K$ ,  $C_a$  and  $C_s$  parameters (data not shown here).

#### 5 Path Length Determination

The estimation of the path length is a key issue in diffuse reflectance spectroscopy, because it provides information about the extension of the sample under study. Moreover, this magnitude is useful because it relates absorbance and absorption coefficients in the modified Beer Lambert law.<sup>27,28</sup>



**Fig. 8** Dependence of the path length on the absorption coefficient for three different reduced scattering coefficients, using Eq. (12).

The path length can be evaluated from the reduced absorbance by,<sup>27</sup>

$$\bar{X} = \frac{A(\mu_a) - A(0)}{\log_{10}(e) \cdot \mu_a} = \frac{A_s}{\log_{10}(e) \cdot \mu_a}, \quad (11)$$

which in our case, using Eq. (9), leads to

$$\bar{X} = 2.30 \cdot K \cdot \mu_a^{C_a-1} \cdot \mu_s^{C_s}, \quad (12)$$

and finally, for our particular optode geometry, with the parameter values given in Eq. (10), stays like

$$\bar{X} = 1.13 \cdot \mu_a^{-0.22} \cdot \mu_s^{-0.28}. \quad (13)$$

Results obtained using Eq. (13) are depicted in Fig. 8 as a function of the absorption coefficient for three different reduced scattering coefficients, covering the range of values of biological interest. It is noteworthy that the path length decreases appreciably when absorption increases for a fixed reduced scattering coefficient. This can be understood by taking into account that photons following long paths, as shown in Fig. 1, have a greater probability of being absorbed than photons traveling shorter paths. In other words, the increase of the absorber concentration leads to the predominance of the short paths. As a result, the mean path covered by the photons decreases when absorption grows.

It would also be remarked that the results obtained for the path length are mainly from 1 to 3 mm, which is 1 order of magnitude larger than the distance between the centers of the optical fibers in the used optode (0.13 mm). This indicates that the area of the sample explored in our measurements is relatively deep.

Finally, it must be noted that the definition of the path length has been the subject of discussion and several attempts have been made to provide an adequate approach that allows us to use a modified version of the Beer Lambert law. A definition, alternative to Eq. (11), is<sup>29-31</sup>

$$\langle X \rangle = \left( \frac{\partial A}{\partial \mu_a} \right), \quad (14)$$

which is related to Eq. (11) as<sup>27</sup>

$$\bar{X} = \frac{1}{\mu_a} \int_0^{\mu_a} \langle X \rangle d\mu_a'. \quad (15)$$

The definition in Eq. (13) corresponds to the average of the paths followed by the photons when it is calculated by Monte Carlo simulation. A detailed comparison of measured and simulated path lengths, with clarification about the different path length definitions, will be presented in a forthcoming paper.

## 6 Conclusions

*In vitro* solutions of hemoglobin (absorber) and intralipid (scatterer) are systematically analyzed by means of reflectance diffuse spectroscopy, with the aim of obtaining a representative and simple relation between the absorbance and the coefficients of absorption and scattering. Results are in fairly good agreement with a power law for the dependence of the reduced absorbance on the coefficients of absorption and scattering. The proposed empirical relation includes three parameters that should be determined for each particular optode geometry, following the method described in this work. It is important to remark that these three parameters do not depend on the wavelength, which very much simplifies the application of this empirical relation.

From this empirical relation, an expression is derived for the path length, also as a function of the absorption and reduced scattering coefficients. Values for the path length were calculated from this empirical expression to estimate the variation of this parameter in biological tissues.

This equation where absorbance is related with scattering and absorption coefficients is the simplest and most straightforward relation among these magnitudes that, as far as we know, exists in the literature. This equation and the associated path length equation greatly clarify how these magnitudes are related. From our point of view, all this constitutes an important step toward the understanding of reflectance spectroscopy in turbid media like biological tissue.

Although our work was made using *in vitro* samples, the results reported can be applied to real tissue where nearly homogeneous optical conditions can be assumed, as it happens in many areas of the brain. These results represent a significant step in determining the absolute concentration of chromophores *in vivo* using reflectance diffuse spectroscopy.

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