

Screening hypochromism (sieve effect) in red blood cells: a quantitative analysis

K. Razi Naqvi*

Department of Physics, Norwegian University of Science and Technology, N-7491 Trondheim, Norway

[*razi.naqvi@ntnu.no](mailto:razi.naqvi@ntnu.no)

Abstract: Multiwavelength UV-visible spectroscopy, Kramers-Kronig analysis, and several other experimental and theoretical tools have been applied over the last several decades to fathom absorption and scattering of light by suspensions of micron-sized pigmented particles, including red blood cells, but a satisfactory quantitative analysis of the difference between the absorption spectra of suspension of intact and lysed red blood cells is still lacking. It is stressed that such a comparison is meaningful only if the pertinent spectra are free from, or have been corrected for, scattering losses, and it is shown that Duysens' theory can, whereas that of Vekshin cannot, account satisfactorily for the observed hypochromism of suspensions of red blood cells.

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References and links

1. M. N. Merzlyak and K. Razi Naqvi, "On recording the true absorption spectrum and the scattering spectrum of a turbid sample: application to cell suspensions of the cyanobacterium *Anabaena variabilis*," J. Photochem. Photobiol. B: Biol. **58**(2–3), 123–129 (2000).
2. T. Jávorfí, J. Erostryák, J. Gál, A. Buzády, L. Menczel, G. Garab, and K. Razi Naqvi, "Quantitative spectrophotometry using integrating cavities," J. Photochem. Photobiol. B: Biol. **82**(2), 127–131 (2006).
3. K. Razi Naqvi, M. N. Merzlyak, and T. B. Melø, "Absorption and scattering of light by suspensions of cells and subcellular particles: an analysis in terms of Kramers-Kronig relations," Photochem Photobiol Sci. **3**(1), 132–137 (2004).
4. A. Nonoyama, A. Garcia-Lopez, L. Garcia-Rubio, G. Lepare, and R. L. Potter, "Hypochromicity in red blood cells: an experimental and theoretical investigation," Biomedical Optics Express, **2**(8), 2126–2143 (2011).
5. L. N. M. Duysens, "The flattening of the absorption spectrum of suspensions, as compared to that of solutions," Biochim Biophys Acta. **19**(1), 1–12
6. K. Razi Naqvi, T. B. Melø, B. Bangar Raju, T. Jávorfí, and G. Garab, "Comparison of the absorption spectra of trimers and aggregates of chlorophyll *a/b* light-harvesting complex LHCII," Spectrochim. Acta Part A **53**(11), 267–269 (1999).
7. M. N. Berberan Santos, "Beer's law revisited," J. Chem. Educ. **67**(9), 757–759 (1990).
8. R. S. Daniels, "A random number model for Beers law—atom shadowing," J. Chem. Educ. **76**(1), 138–141 (1999).
9. J. Amesz, L. N. M. Duysens, and D. C. Brandt, "Methods for measuring and correcting the absorption spectrum of scattering suspensions," J. Theor. Biol. **1**(1), 59–74 (1961).
10. P. J. Halling, "Estimation of flattening coefficient for absorption and circular dichroism using simulation," Anal. Biochem. **387**(1), 76–81 (2009).
11. P. Latimer and C. A. H. Eubanks, "Absorption spectrophotometry of turbid suspensions: a method of correcting for large systematic distortions," Arch. Biochem. Biophys. **98**(2), 274–285 (1962).
12. R. A. MacRae, J. A. McClure, and P. Latimer, "Spectral transmission and scattering properties of red blood cells," J. Opt. Soc. Am. **51**(12), 1366–1372 (1961).

13. N. L. Vekshin, "Screening hypochromism of biological macromolecules and suspensions," *J. Photochem. Photobiol. B: Biol.* **3**(4), 625–630 (1989).
 14. N. L. Vekshin, "Screening hypochromism in molecular aggregates and biopolymers," *J. Biol. Phys.* **25**(4), 339–354 (1999).
 15. C. Bustamante and M. F. Maestre, "Statistical effects in the absorption and optical activity of particulate suspensions," *Proc. Natl. Acad. Sci. USA* **85**(22), 8482–8486 (1988).
 16. G. Papageorgiou, "Absorption of light by non-refractive spherical shells," *J. Theor. Biol.* **30**(2), 249–254 (1971).
 17. L. Fukshansky, "On the theory of light absorption in non-homogeneous objects," *J. Math. Biol.* **6**(2), 177–196 (1978).
 18. M. Das, E. Rabinowitch, L. Szalay, and G. Papageorgiou, "The "sieve effect" in *Chlorella* suspensions," *J. Phys. Chem.* **71**(11), 3543–3549 (1967).
 19. J. T. O. Kirk, *Light and Photosynthesis in Aquatic Ecosystems* (Cambridge University Press, Cambridge, 2010).
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1. Introduction

When an absorbing-and-scattering specimen is investigated by using a standard absorption spectrophotometer, a measurement of the attenuation $\lg(P_0/P)$ does not provide the true absorbance of the sample, and the measured attenuation depends on the acceptance angle of the instrument (or the sample-to-detector ratio); here P_0 denotes the output of the detector when it views the collimated incident beam leaving the reference cell and P is the output when the cell is filled by the sample under investigation. Reliable methods for finding the true absorbance, either by separating the contribution made by scattering [1] or by circumventing it altogether by placing the sample inside an integrating cavity [2], have been available for some time. A 2004 paper [3], written by the present author and his associates, suggested a practicable procedure for a quantitative analysis of the scattering spectrum by dividing it into two parts, one caused by selective scattering and the other by non-selective scattering; the approach, which relied on the assumption that absorption and selective scattering from a single pool of pigments satisfy the Kramers-Kronig relations and on a milder assumption concerning the wavelength dependence of non-selective scattering, was shown to account for the scattering and absorption spectra of a wide variety of suspensions, including those of red blood cells. An independent approach, utilizing similar ingredients, was presented some years later by Nonoyama and co-authors [4], who also addressed an issue not treated in the earlier work, namely the lower absorbance (or hypochromism) of a suspension of pigmented cells as compared to the absorbance recorded by disrupting the cells and allowing the pigments to disperse throughout the sample holder. They commented that "this perceived hypochromism can be accounted for by considering two important issues: the acceptance angle of the instrument and the combined scattering and absorption effect of light on the particles". The purpose of the present paper is to point out that hypochromism, properly so called, should be used when one compares two spectra neither of which suffers from scattering losses, and to show that Duysens' theory of screening hypochromism can be applied, in its original form [5] or in a trivially amended fashion [6], to understand the hypochromism of red blood cells.

2. Theoretical background

The problem before us, when stripped of all the distracting details specific to a particular system, may be stated as follows: Find the relation between A' and A , where A' is the true absorbance of a suspension of randomly distributed, identical clusters of subunits, and A is the absorbance recorded after disrupting the clusters, the sole purpose of disruption being that of turning the suspension of clusters at hand into a suspension of randomly distributed subunits. The reader should observe that a "subunit" (which will henceforth be called a particle) may be a single molecule or a macromolecule carrying one or more chromophores. Closely entwined with clustering, yet demanding a separate investigation, is the phenomenon of scattering. We

will assume that each cluster contains k subunits, and that scattering is either negligible or that an appropriate correction has been made to procure a scattering-free absorption spectrum of the suspension of clusters. It will be more convenient to work with the napierian absorbances $\mathcal{E} = \ln(10) \times A$ and $\mathcal{E}' = \ln(10) \times A'$, and no confusion is likely to arise if the shorter term absorbance is applied also to \mathcal{E} and \mathcal{E}' .

We consider two homogeneous samples of absorbing entities, each with a pathlength l ; in one sample, the absorbing entities are randomly dispersed particles at a concentration N , whereas the other sample has clusters, also randomly dispersed, whose concentration is $N' = N/k$. Using unprimed symbols for the former sample and primed symbols for the latter, we apply the BLB law to each sample by writing

$$\mathcal{E} = \sigma N l, \quad (1a)$$

and

$$\mathcal{E}' = \sigma' N' l = \sigma' (N/k) l. \quad (1b)$$

It is important to point out that, whatever the absorbing entity, the BLB law takes account of shadowing of an entity by another entity lying closer to the front of the absorption cuvette [7, 8].

Amesz, Duysens and Brandt [9] proposed the following expression for σ' , the absorption cross section of a cluster characterized by a physical cross section s (defined as the area of the projection of a cluster on a plane perpendicular to the monitoring beam):

$$\sigma' = s U_C = s(1 - T_C), \quad (2)$$

in which T_C is the transmittance of a cluster, so that $U_C = 1 - T_C$ equals the probability that a cluster will absorb an incident photon.

We now divide Eq. (1b) by Eq. (1a), and use Eq. (2) to get the relations

$$\frac{A'}{A} = \frac{\mathcal{E}'}{\mathcal{E}} = \frac{\sigma'}{\sigma k} = \frac{s(1 - T_C)}{\sigma k}, \quad (3)$$

the second of which will be rearranged, after introducing the symbol

$$Y = \sigma k / s, \quad (4)$$

as

$$\frac{\mathcal{E}'}{\mathcal{E}} = \frac{(1 - T_C)}{Y}. \quad (5)$$

All that remains for us now is to interpret the quantity $Y = \sigma k / s$.

In order to interpret Y , it is necessary to propose a concrete model for the shape of a cluster, which in turn makes it necessary to distinguish, by using an appropriate subscript, one model from another. If we assume that each cluster is a right circular cylinder of cross section s and length d , and assume further that each cluster is aligned so that its plane faces are perpendicular to the monitoring beam, a cluster can be treated as a minuscule absorption cell (containing k particles in a volume $v_c = sd$) and we can apply the BLB law to it, which gives the following expressions for the transmittance (T_c) and absorbance (\mathcal{E}_c):

$$T_c = e^{-\sigma(k/sd)d} = e^{-\sigma k / s} = e^{-Y}, \quad \mathcal{E}_c = \sigma k / s = Y, \quad (6)$$

and we note that the purpose of the lower-case subscript c is to remind the reader that the symbol carrying the subscript refers to a cylindrical cluster. Since the concentration of particles within a cylinder is $n_c = k/v_c$, we can introduce the symbol $\mathcal{E}_c = \sigma n_c d$, and thereby express the ratio $\mathcal{E}'_c / \mathcal{E} = A'_c / A$ as

$$\frac{\mathcal{E}'_c}{\mathcal{E}} = \frac{1 - T_c}{\mathcal{E}_c} = \frac{1 - e^{-\sigma n_c d}}{\sigma n_c d} \quad (7)$$

If, on the other hand, we assume that each cluster is a sphere of diameter d , cross section $s = \pi d^2/4$ and volume $v_s = \frac{2}{3}sd$, we can calculate its transmittance by using the relation

$$T_s = \frac{2[1 - (1 + \mathcal{E}_d)e^{-\mathcal{E}_d}]}{\mathcal{E}_d^2}, \quad (8)$$

where \mathcal{E}_d is the (napierian) absorbance measured along a diameter of the sphere (which could be determined, in principle, by using a sufficiently narrow beam passing through the centre of the sphere); that is to say, if n_s is the concentration of the particles inside the sphere and σ their absorption coefficient, $\mathcal{E}_d = \sigma n_s d$. To interpret Y , we multiply the numerator and denominator on the right-hand side of Eq. (4) by $\frac{2}{3}d$ and use the relation $n_s = k/v_s$ to arrive at the result shown below:

$$Y = \frac{2}{3}\sigma n_s d = \frac{2}{3}\mathcal{E}_d. \quad (9)$$

For spherical clusters, Eq. (5) and Eq. (8) lead us finally to the result

$$\frac{\mathcal{E}'_s}{\mathcal{E}} = \frac{3(1 - T_s)}{2\mathcal{E}_d}, \quad (10)$$

found first by Duysens [5], whose analysis has been considerably condensed in the derivation presented above.

For clusters of other shapes, it will be more convenient to use a numerical simulation, such as that developed by Halling [10] and found, in the case of spherical clusters, to be in agreement with the analytical approach discussed above.

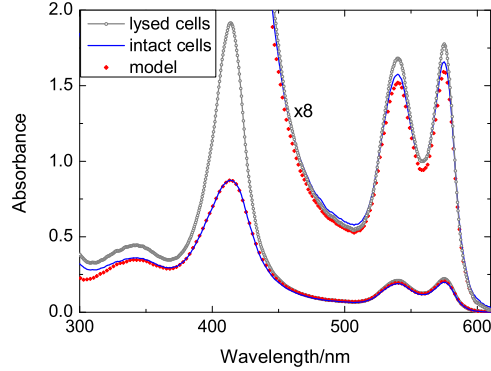


Fig. 1. Plots of $A(\lambda)$, $A'(\lambda)$ (representing absorption spectra of suspensions of lysed and intact human red blood cells, respectively), and of $A'_c(\lambda)$, a model spectrum calculated by applying Eq. (11). Corrections for scattering were applied by recording two spectra at different distances from the input port of an integrating sphere and analyzing the data in accordance with the method developed by Latimer and Eubanks [11]. For obtaining red blood cells, approximately 150 μl of freshly collected human peripheral blood was added to 5 ml of 0.6% NaCl solution containing 7 mM trisodium citrate as an anticoagulant; erythrocytes were sedimented at 1000 g, washed twice and resuspended either in 0.6% NaCl (intact cells) or in distilled water (lysed cells). For more experimental details, see [3].

3. Flattening in the spectrum of red blood cells

Once A becomes available, one can see whether a particular model succeeds in reproducing the absorption spectrum of a suspension of clusters. Though \mathcal{E}_c and \mathcal{E}_d are not known, each is

proportional to A , and the proportionality constant ($= m$, say) can be treated as an adjustable parameter in a non-linear least squares fitting procedure involving a comparison of the experimental spectrum A' and a particular model ($A'_c = \ln(10) \times \mathcal{E}'_c$ or $A'_s = \ln(10) \times \mathcal{E}'_s$). Figure 1 displays the absorption spectra of red blood cells suspended in 0.6% NaCl solution (intact cells) and in distilled water (lysed cells); a flattened spectrum A'_c calculated by using the relation [cf. Eq. (7)]

$$A'_c = A \frac{1 - \exp(-\mathcal{E}_c)}{\mathcal{E}_c} \quad (11)$$

and labeled as ‘model’, is also displayed in Figure 1. The fitting leads to the conclusion that $A_c = \ln(10) \times \mathcal{E}_c = 0.8$ at 414 nm. It is gratifying to note that an equally satisfactory fit was obtained by modeling each cluster as a sphere, and in this case $A_s = \ln(10) \times \mathcal{E}_s = 1.1$ at 414 nm, which is not far from the value $\frac{3}{2} \times 0.8$, expected on the basis of the foregoing analysis, and that these values are in fair agreement with the dimensions of a red blood cell and its decadic absorption coefficient at 414 nm, approximately $0.3/\mu\text{m}$ [12].

A different expression for the ration \mathcal{E}'/\mathcal{E} was proposed by Vekshin [13, 14]; though his model can account for the observed hypochromism of red blood cells, it is physically unreasonable because the best fit between the model and the experimental data leads to the conclusion that the number of heme subunits per cluster is smaller than ten [14].

A serious discrepancy between the experimental spectrum $A'(\lambda)$ and the predicted spectrum $A'_m(\lambda)$ (where $m = c, s, \dots$), would have implied that either the procedure used for finding the scattering-corrected absorption spectrum is at fault or that at least one of the basic assumptions underlying Duysens’ analysis is unrealistic. The absorbance of a cluster must always be lower than that of a solution containing the same number of subunits, and it will be noted in the region close to 500 nm, A' is slightly larger than A , but the difference is negligible for practical purposes. Figure 1 also reveals a noticeable difference between A' and A'_c at wavelengths shorter than 300 nm, but this is probably due to the contribution (in the latter spectrum) of absorption by the proteins. The central assumption in the Duysens approach is that the spectral properties of subunits do not change when they are inside a cluster, and this is most likely to hold good when the pigments are buried inside proteins, as in hemoglobin or in light-harvesting complexes [6], since pigment-pigment distances remain large even when neighboring proteins are tightly packed.

4. Concluding remarks

Since Duysens’ analysis has been criticized in the past [13, 15], a few clarifying remarks appear to be in order. Bustamante and Maestre [15] chose to treat a homogeneous suspension of clusters as an inhomogeneous assembly of subunits, where inhomogeneity is defined as the non-uniformity of the concentration of the absorbing entities across a plane perpendicular to the monitoring beam. When one replaces a homogeneous sample with an inhomogeneous sample, one can calculate an average transmittance, but one cannot speak of an absorbance, since the number of absorbing entities encountered by each ray in the monitoring beam is no longer a constant. As has been pointed out above, each of the two samples whose absorbances are compared here may be considered to be homogeneous, since the absorbing entities (subunits or clusters) are distributed uniformly throughout the cuvette in either case, and the BLB law is applicable to the subunits in one case and to the clusters in the other. Vekshin’s criticism of the papers of Duysens [5], Papageorgiou [16] and Fukshansky [17] has been marred by his unsympathetic reading, if not exactly by a wilful misreading, of these works, and he has failed to spot what a sympathetic and patient reader would sooner or later recognize as obvious typing errors or notational ambiguities. His concluding paragraph, which ends with the remark “Thus the ‘sieve effect’ is erroneous”, merely shows that he has not grasped the fact that what he calls

“screening hypochromism” is another name for the phenomenon named flattening by Duy-sens, and subsequently called “sieve effect” [18] or “package effect” [19]. The former term is infelicitous and misleading, for it carries the connotation that sieving is a consequence of clustering or packing. Even a sample in which chromophores (or subunits) are randomly dispersed may be said to act as a sieve if its transmission is not too low; all that clustering does is to make the sample a better (more transmissive) or leakier sieve.