FEBS Letters 352 (1994) 37–40

FEBS 14525

Absorption flattening in the optical spectra of liposome-entrapped substances

Pernilla Wittung[a], Johan Kajanus[a], Mikael Kubista[b], Bo G. Malmström[b,*]

[a]*Department of Physical Chemistry, Chalmers University of Technology, S-412 96 Göteborg, Sweden*
[b]*Department of Biochemistry and Biophysics, Göteborg University, Medicinaregatan 9C, S-413 90 Göteborg, Sweden*

Received 8 August 1994

Abstract Measuring optical spectra of chromophore molecules entrapped in liposomes can lead to considerable distortion because of absorption flattening. This phenomenon is analyzed theoretically, and it is shown that the deviation from the Beer–Lambert law becomes larger as the molar absorptivity of the chromophore increases and as the number of entrapped molecules becomes larger. The theoretical equations are tested experimentally with intermediate-sized phospholipid vesicles containing entrapped cytochrome *c*. It is shown that considerable absorption flattening is observed with vesicles containing about 50 chromophore molecules. The equation given can be used to correct the spectrum.

Key words: Absorption flattening; Optical spectrum; Liposome; Cytochrome *c*

1. Introduction

It is well known that in recording optical spectra of suspensions, deviations from the Beer–Lambert law are observed because of the phenomenon of absorption flattening [1], but this fact is often ignored by biochemists studying colored substances entrapped in liposomes. The phenomenon is easy to understand on the basis of simple considerations, as illustrated in Fig. 1. Here we visualize three situations in which the same number of absorbing particles are dissolved in the same total volume, but distributed in different ways. The normal situation is a homogeneous distribution (top), but consider instead that all solute molecules are occupying only half the sample volume, the remaining half being pure solvent. If the volume elements are located after each other along the light path (middle), then the absorption will be the same as for a homogeneous solution, since the decrease in the length of the light path is exactly compensated for by an increase in concentration. This is not the case, however, if the volume elements are parallel to the incident light (bottom). In this situation the absorption will always be smaller than for a homogeneous sample, because even if the concentration is infinite, 50% of the incident light will still reach the detector. This corresponds to the situation when working with suspensions, including liposomes, rather than with homogeneous solutions.

Strictly speaking, all non-crystalline samples are heterogeneous because of entropy effects. For ordinary solutions containing a very large number of independently moving chromophores, the relative concentration fluctuations are extremely small, and the Beer–Lambert law will be obeyed. In solutions of large macromolecules, aggregates or microparticles, each containing a large number of chromophores and thus having a high optical absorption, the number of freely moving particles may, however, be considerably smaller, and the local concentration fluctuations may become important. We analyse this situation theoretically in this communication and show that deviations from Beer–Lambert behavior can be appreciable if the molar absorptivity of the substance under study is considerable and the number of chromphore molecules entrapped in each particle is large. We also show experimentally that large deviations may be found when many strongly colored molecules are entrapped in intermediate-sized unilamellar vesicles of phospholipids.

2. Materials and methods

2.1. Chemicals

Soybeen phospholipids (containing ca. 15% L-α-phosphatidyl choline) was obtained from Sigma (type II-S; Sigma P 5638). Horse heart cytochrome *c* was also from Sigma (C 7752). All other chemicals were of analytical grade.

2.2. Preparation of liposomes

Liposomes were prepared by an extrusion method [2]. A solution of cytochrome *c* (2, 4 or 8 μM) in a 5 mM Na-phosphate buffer, pH 7.2, was mixed with phospholipids (10 mg/ml). The suspension was vortexed for a few minutes and then passed 21 times through a 100 nm polycarbonate filter in a commercial, small-volume (0.5 ml) extrusion apparatus (LiposoFast–Basic). The liposomes were then separated from free cytochrome *c* by gel-filtration on a Sephadex G-100 column. The optical spectrum was recorded, and then the entrapped cytochrome *c* was released by the addition of 5 vol% of a solution of Triton X-100 (20%), whereafter the spectrum was again recorded.

2.3. Spectroscopic methods

Optical spectra were recorded at room temperature (22°C) in a 1-cm cell in a Cary 2300 spectrophotometer in the wavelength region 400–640 nm. The size of the liposomes was determined by quasi-elastic light scattering (Malvern Instruments) with a helium–neon laser as light source.

3. Theory

According to the Beer-Lambert law the intensity of the incident light decreases exponentially on its way through the sample in proportion to the average concentration of the solute molecules:

$$I_{B-L} = I_0\, 10^{-\varepsilon_p C_p l} \quad (1)$$

where ε_p is the molar absorptivity of the particles, C_p is their molar concentration, and l is the cell length. This equation gives the well-known expression for sample absorption:

*Corresponding author. Fax: (46) (31) 773 3910.

SSDI 0014-5793(94)00912-0

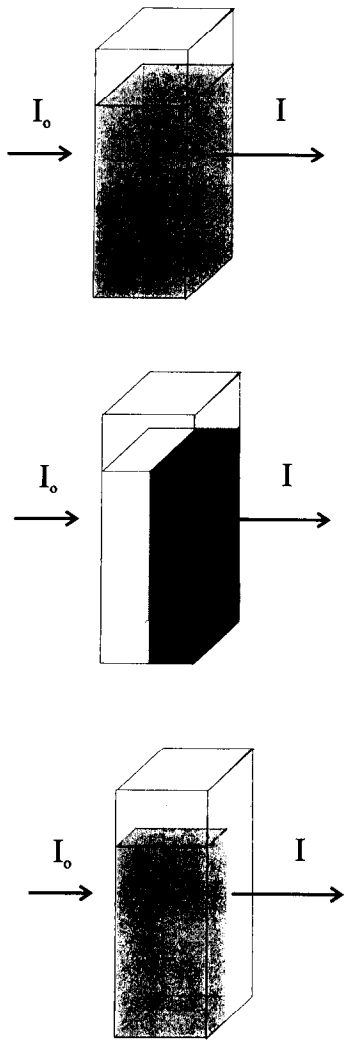

Fig. 1. Three possible distributions of solute in relation to the direction of the light path. I_0 is the intensity of the incident light and I that of the transmitted light. For further explanations, see the text.

$$A_{B\text{-}L} = \log\left(\frac{I_0}{I_{B\text{-}L}}\right) = \varepsilon_p C_p l \tag{2}$$

However, since real samples are not homogeneous, some of the incident light will pass through regions of more concentrated solution and some through less concentrated solution. To account for these concentration fluctuations in calculating sample absorption, we think of the sample cell as being composed of a very large number of independent small cells, or channels (N_{ch}), with the same length as the sample cell and a cross-section corresponding to the size of the absorbing particles, through each of which a fraction, I_0/N_{ch}, of the total incident light passes. Representing the total number of particles in the sample cell by N_p, we can calculate the number distribution of the particles in the channels. The probability of finding n particles in a channel is given by the binomial expression:

$$p(n) = \binom{N_p}{n}\left(\frac{1}{N_{ch}}\right)^n\left(\frac{N_{ch}-1}{N_{ch}}\right)^{N_p-n}, \quad \binom{N_p}{n} = \frac{N_p!}{n!(N_p-n)!} \tag{3}$$

According to Moivres-Laplace theorem, the binomial expression can be approximated with the normal distribution:

$$p(n) \approx \frac{1}{\sigma\sqrt{2\pi}} e^{-(n-\mu)^2/2\sigma^2} \tag{4}$$

with mean $\mu = N_p/N_{ch}$ and standard deviation $\sigma = (N_p/N_{ch}(1 - 1/N_{ch}))^{0.5} \cong (N_p/N_{ch})^{0.5}$. For $C_p < 2C_{ch}$, this approximation is, however, not valid, and eq. 1 was used with Sterling's approximation to calculate factorials:

$$\ln(N!) = 0.5\ln(2\pi N) + N\ln(N) + 1/(12N) - N \tag{5}$$

The concentration of particles in a channel containing n particles is

$$C_n = \left(\frac{n}{N_p}\right) N_{ch} C_p \tag{6}$$

and the light intensity emerging from that channel is

$$I_n = \left(\frac{I_0}{N_{ch}}\right) 10^{-C_n \varepsilon_p l} \tag{7}$$

The average intensity emerging from a single channel is then obtained

$$\langle I_{ch}\rangle = \sum_{n=0}^{N_p} p(n) I_n \tag{8}$$

which gives the total intensity registered by the detector:

$$I = N_{ch}\langle I_{ch}\rangle \tag{9}$$

Combining eqs. 3–9 and using the definition of absorption we obtain:

$$A = \sum_{n=0}^{N_p}\left(\binom{N_p}{n}\left(\frac{1}{N_{ch}}\right)^n\left(\frac{N_{ch}-1}{N_{ch}}\right)^{N_p-n} 10^{\left(-n\left(\frac{N_{ch}}{N_p}\right)\varepsilon_p C_p l\right)}\right) \tag{10}$$

$$\approx \frac{1}{\sigma\sqrt{2\pi}} \sum_{n=0}^{N_p} e^{-(n-\mu)^2/2\sigma^2} 10^{\left(-n\left(\frac{N_{ch}}{N_p}\right)\varepsilon_p C_p l\right)} \tag{11}$$

From eq. 11 it is possible to understand why the Beer–Lambert law fails for very dilute solutions and becomes progressively more valid as the concentration increases. The standard deviation, σ, is a measure of the fluctuations in concentration, and increases with increasing concentration (recall $\sigma \approx (N_p/N_{ch})^{0.5}$). However, owing to the second term ($10^{-const \times C_p}$), the relative fluctuation in concentration, $\sigma/\mu \approx (N_p/N_{ch})^{-0.5}$, which decreases with increasing concentration, is important. For example, with 16 particles per channel $2\sigma = 8$, only 50% of the channels will have a number of particles in the range $\mu \pm \sigma$ (8–24), whereas with $N_p/N_{ch} = 400$, 90% will be in the range $\mu \pm \sigma$ (380–420). At sufficiently high concentrations the relative fluctuations are negligible, so that n in the second term can be replaced by the mean concentration (N_p/N_{ch}), and eq. 11 becomes the Beer–Lambert law.

With eq. 11 we can calculate how much the measured absorbance deviates from Beer–Lambert behavior depending on the particular molecular parameters of the system under study. Calculated curves showing how the aborbance varies with the molar absorptivity and the number of entrapped molecules with vesicles 100 nm in outer diameter are shown in Fig. 2. The straight line is obtained under conditions when the Beer–Lambert holds, whereas curved lines indicate deviations, which increase with increasing absorbances (hence the term absorption flattening). With just a few chromophore molecules per vesicle,

the Beer–Lambert law still applies up to a molar absorptivity as high as 10^5 $M^{-1} \cdot cm^{-1}$, but as the number of entrapped molecules increases, significant deviation is found even for relatively modest absorptivities.

4. Results and discussion

The phenomenon of absorption flatterning has been analyzed earlier by Duysens [1], who used his equations to correct a measured absorption spectrum of a *Chlorella* suspension and showed that the corrected spectrum was very close to that of a methanol extract of the cells. We have also performed an experimental test of the validity of the theoretical results in Fig. 2. For this purpose we chose cytochrome *c* entrapped in phopholipid vesicles of intermediate size (ca. 100 nm). Cytochrome *c* was selected because its spectrum is not changed by any electrostatic or other interaction with the phopholipids, and it has molar absorptivities ranging from essentially zero to 1.1×10^5 $M^{-1} \cdot cm^{-1}$ in the spectral range studied.

To compare our experimental results with the theoretical curves in Fig. 2, we would have to vary the molar absorptivity and the number of the entrapped molecules. Rather than varying the absorptivity by working with different entrapped substances we chose to record the spectrum of cytochrome *c* from 400 to 640 nm, but instead of arranging the data points along a wavelength axis we used increasing observed absorbances for the homogeneous solution as our abscissa and the corresponding measured absorbances with entrapped cytochrome *c* as our ordinate; this approach has been used earlier for experimental correction of the inner-filter effect in fluorescence spectra [3]. The number of entrapped molecules was varied by the use of different cytochrome *c* concentrations, in the range 2–8 μM, in the liposome preparation.

Some of our experimental results are given in Fig. 3; in constructing this, the measured absorbances have been normalized. The heavy line with a slope of 1 is the measured absorbances of the Triton-treated (homogeneous) samples plotted against themselves, whereas the other two curves contain the vesicle data plotted against the corresponding curve for the homogeneous samples; data obtained with 4 μM cytochrome *c* fell between the curves for 2 and 8 μM concentration. The noise in the curves is a result of the same absorbance value being found at more than one wavelength. The results agree qualitatively with the calculated curves and show that substantial absorption flattening occurs even at rather modest concentrations of a highly colored substance.

Our measured vesicle size was 128 ± 32 nm, which allowed us to calculate the number of entrapped molecules and to make a more exact comparison with Fig. 2. Unilamellar vesicles of this size made from 10 mg of phospholipids should have a total inner volume of 50 μl in 1 ml of suspension, and the number of vesicles in the same volume should be about 8×10^{11}, as read from a nomogram in [4]. The number of cytochrome *c* molecules entrapped in each vesicle should then be 60 and 240, respectively, for the two concentrations shown (Fig. 3). The measured absorbances indicate, however, that the actual numbers of chromophores associated with each vesicle are about twice as large. This may be due to some electrostatic binding of the positively charged protein to the negative phospholipid surface. Thus, the agreement between the experimental results and the theoretical curves in Fig. 2 is remarkably good.

In summary, experimental spectra measured with suspensions of vesicles with entrapped chromophores can be considerably distorted by absorption flattening and must consequently be corrected. This can readily be done with the theoretical equation given here, provided that the vesicle size and the

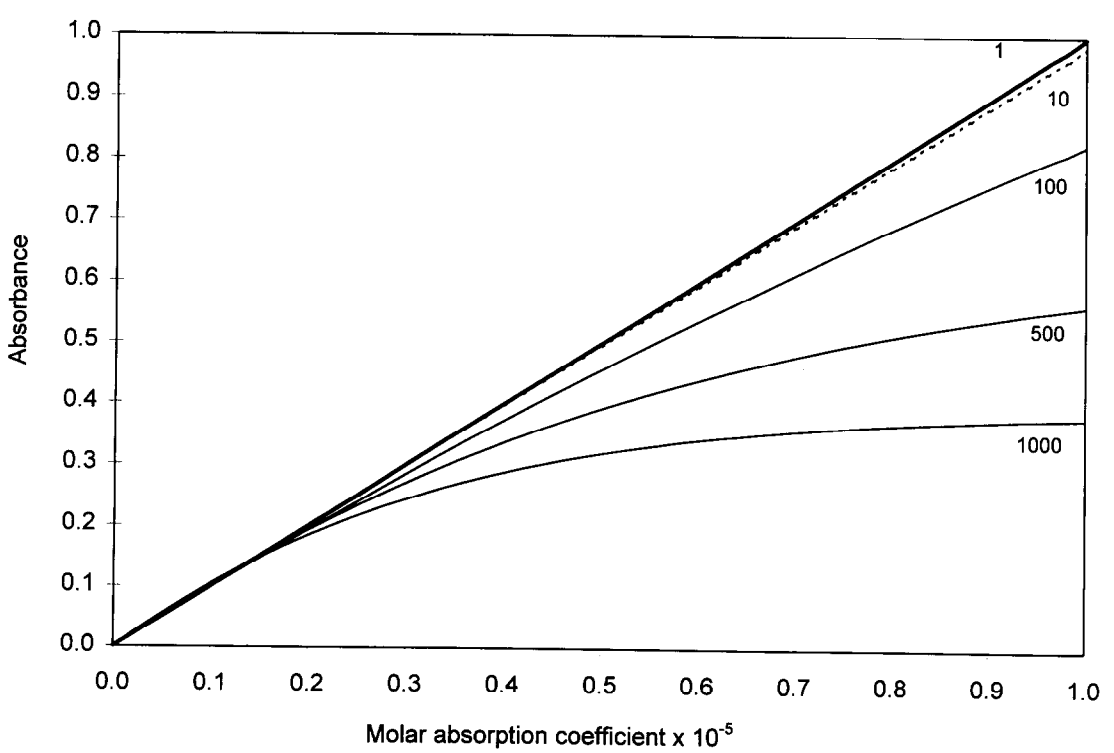

Fig. 2. Curves calculated from eq. 11 showing how the measured absorbance varies with the molar absorptivity and the number of molecules entrapped (the figures given at the different lines) in vesicles with an outer diameter of 100 nm.

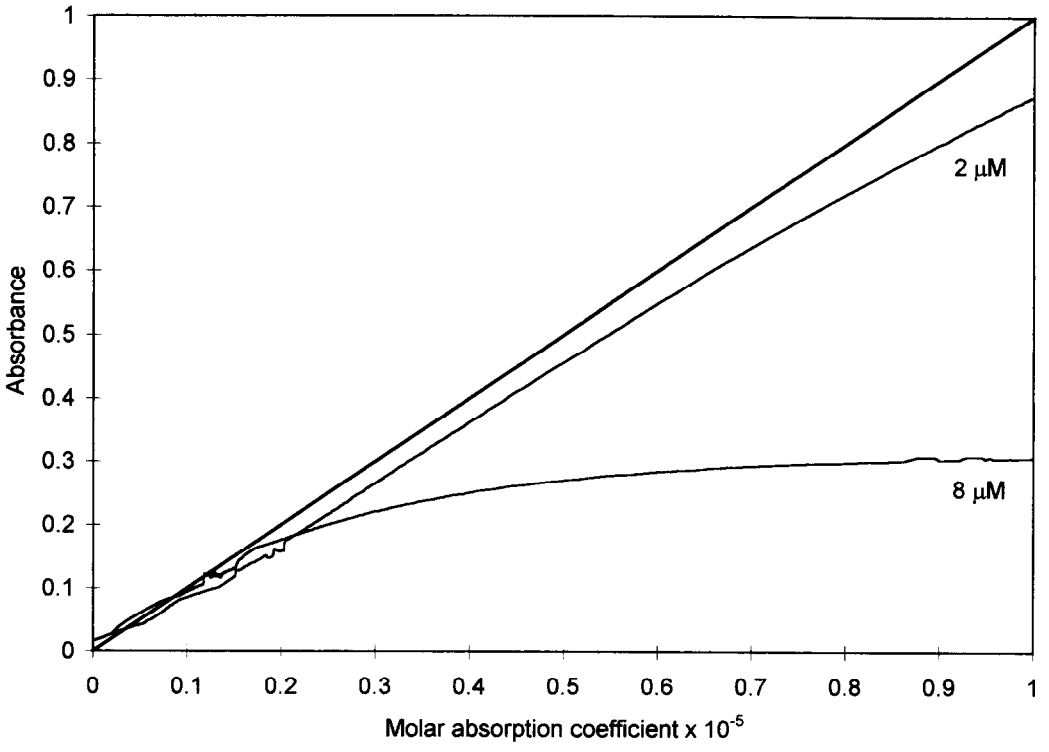

Fig. 3. Experimental curves relating the measured absorbance of cytochrome *c* entrapped in phospholipid vesicles to the absorbance after the release of the entrapped molecules. The concentrations of cytochrome *c* used were 2 and 8 μM. The curves have been normalized.

number of entrapped chromophore molecules have been determined.

Acknowledgements: This study has been supported by the Swedish Natural Science Research Council, The Royal Society of Arts and Sciences in Gothenburg, and Wilhelm and Martina Lundgrens Vetenskapsfond. We are very grateful to Dr. Bruno Källebring for assistance in preparing Fig. 3 from our original data, and to Dr. Örjan Hansson for helpful discussions. Prof. Sture Nordholm has given advice on the derivation of our theoretical equation.

References

[1] Duysens, L.M.N. (1956) Biochim. Biophys. Acta 19, 1–12.
[2] MacDonald, R.C., MacDonald, R.I., Menco, B.P.M., Takeshita, K., Subbarao, N.K. and Hu, L.-r. (1991) Biochim. Biophys. Acta 1061, 297–303.
[3] Kubista, M., Sjöback, R., Eriksson, S. and Albinsson, B. (1994) Analyst 119, 417–419.
[4] New, R.R.C. (1990) in: Liposomes: A Practical Approach (New, R.R.C., ed.) p. 25, IRL Press, Oxford.