

DIMERS OF MEROCYANINE 540 II. IN EGG LECITHIN LIPOSOMES

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The electronic absorption spectroscopy was applied to study the occurrence and equilibrium of different species of a fluorescent probe - merocyanine 540 (M-540) in suspensions of egg lecithin (EL) liposomes. The analysis of the absorption spectra enabled to differentiate three coloured species of M-540 dye with different optical properties: i/ M-540 monomers bound to EL liposomes, ii/ M-540 dimers bound to liposomes, iii/ dye molecules in water - unbound to liposomes. In case the ratio of molar concentrations $[EL] : [M-540] > 73$ we may consider nearly all M-540 molecules in suspension to be bound to liposomes, and thus we may neglect the contribution to absorption from the dye molecules in water, unbound to liposomes. When the molar concentrations fulfils the condition $73 < [EL] : [M-540] < 43000$ then the distribution of M-540 monomers and dimers bound to liposomes was quantitatively determined and there the monomer $\rightleftharpoons$ dimer equilibrium constant $K = (14.8 \pm 0.4) \mu\text{mol/l}$ was calculated.

Introduction

Merocyanine 540 is a lipophilic fluorescent dye, earlier widely used to measure membrane potential changes in nerves, muscle, submitochondrial particles and phospholipid membranes /1,2,6,11/. Later on M-540 was applied to studies of structural modifications in membranes /3,4,5/ and the degree of lipid packing of bilayers /13/. SIEBER et al. /7/ studied selective killing of leucemic cells by M-540 - mediated photosensitization. In the above mentioned works optical response of M-540 dye is often ascribed to dimerization of M-540 dye followed by change of physical state of membrane: ROSS et al. /6/ first suggested that M-540 shows a potential-dependent equilibrium between dye monomers and dye dimers of differing optical properties. WAGGONER and GRINVALD /11/ published calculated absorption spectra of egg phosphatidylcholine vesicle bound M-540 monomers and dimers. DRAGSTEN and WEBB /2/ studied electrical field perturbations on M-540 fluorescence polarization in hemispherical bilayer membranes and found dye reorientation within the membrane coupled with

dye dimerization. VERKMAN and FROSCH /9/ published temperature-jump studies of M-540 relaxation kinetics in lipid bilayer membranes based on the existence of its two forms - monomers and dimers.

In the present work there are reexamined WAGGONER's and GRIN-VALD's experiments /11/ of titration of EL liposomes with M-540 probe but for a much wider range of M-540 concentrations than stated in the paper /11/. The aim of our work is to determine by absorption spectroscopy the monomer-dimer distribution and the monomer-dimer equilibrium constant of M-540 dye in liposomes. It was also necessary to determine the dye concentration range when all the dye in suspension can be considered to be bound to liposomes.

Materials and methods

Merocyanine 540 (molecular weight 569.68) was obtained from Eastman Kodak, Co., and was used without further purification. Egg lecithin (molecular weight cca 775) was prepared at the Charkow State University (USSR). Egg lecithin was employed in the preparation of the "alcohol" liposomes according to the standard method of VLADIMIROV and DOBRETISOV /10/. Fresh M-540 solutions were prepared daily. The M-540 concentrations were in the range $(0.06-176) \times 10^{-6}$ mol/l. Bidistilled water of $\text{pH}=6.2^{\pm 0.2}$ was used. All experiments were carried out at the temperature of $22^{\pm 2}^{\circ}\text{C}$. The visible absorption spectra were recorded by spectrophotometer SPECORD M-40 (Zeiss Jena, GDR).

Results and discussion

FIG. 1 illustrates the visible absorption spectra of M-540 in EL liposomes for M-540 concentrations ranging within the extent of $(0.06-176) \times 10^{-6}$ mol/l and constant EL concentration (2.58×10^{-3} mol/l). There are three absorption maxima on the stated spectra: $\lambda_1=571$ nm, $\lambda_2=535$ nm, and $\lambda_3=506$ nm. Our data of λ_1 corresponds well to the data in /4,5,9,11/. The data on λ_2 can be found in /9,11/ which correspond with our results of measurements. The peak in λ_3 is registered only in reference /9/ and it is in agreement with our data. The absorption peak at λ_1 pertains prevaillingly to M-540 monomers bound to EL liposomes and peak at λ_2 corresponds prevaillingly to M-540 dimers bound to liposomes. Peak at λ_3 can be registered on absorption spec-

tra only when M-540 concentration are larger than 88×10^{-6} mol/l which corresponds to a molar ratio of $[EL]:[M-540] < 29$. The comparison of M-540 spectra in lecithin liposomes with M-540 spectra in water shows that peak at λ_3 results from M-540 molecules in water, i. e. from M-540 molecules unbound to EL.

The spectral characteristics of M-540 dye in H_2O were presented in the preceding article /8/, thus we wish to concentrate in this paper to dye components bound to liposomes.

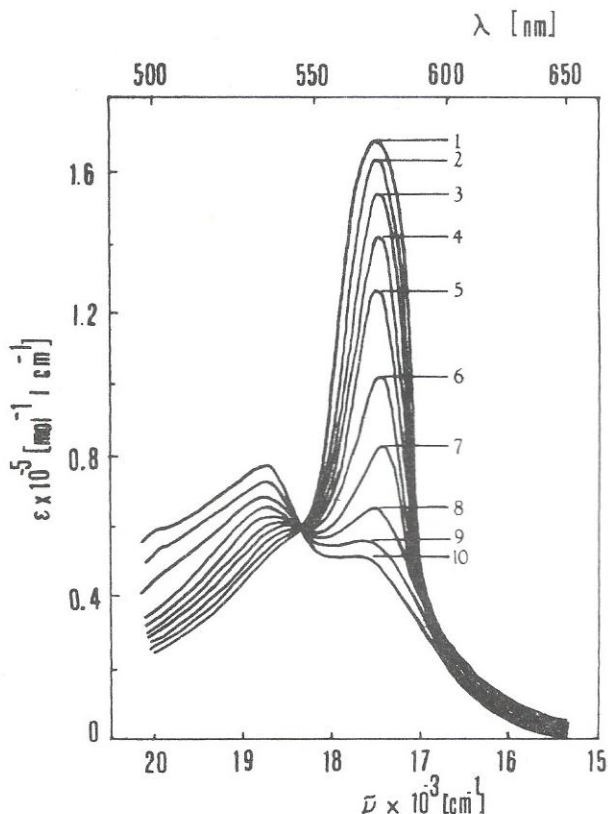


FIG. 1 Visible absorption spectra of M-540 in suspension of EL liposomes. Suspension contained constant EL concentration (2.58 mmol/l) and varying M-540 concentration: 1/ $0.06 \text{ } \mu\text{mol/l}$; 2/ $0.35 \text{ } \mu\text{mol/l}$; 3/ $0.88 \text{ } \mu\text{mol/l}$; 4/ $1.76 \text{ } \mu\text{mol/l}$; 5/ $3.51 \text{ } \mu\text{mol/l}$; 6/ $8.78 \text{ } \mu\text{mol/l}$; 7/ $17.56 \text{ } \mu\text{mol/l}$; 8/ $35.11 \text{ } \mu\text{mol/l}$; 9/ $87.78 \text{ } \mu\text{mol/l}$; 10/ $175.55 \text{ } \mu\text{mol/l}$

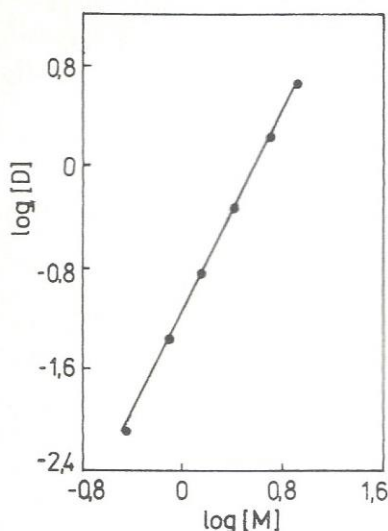


FIG. 2 Plot of $\log [D]$ vs. $\log [M]$ for M-540 in EL liposomes. The concentrations are taken in $\mu\text{mol/l}$. The line is least square fitted with the correlation coefficient 0.999 and its equation is: $\log [D] = 1.976 \log [M] - 1.168$

When the whole amount of M-540 dye is bound to lecithin, the following equation for molar concentration is valid:

$$[M-540] = [M] + 2 [D], \quad (1)$$

where $[M-540]$ is total dye concentration, $[M]$ and $[D]$ are concentrations of monomers and dimers, respectively. The values of $[M]$ and $[D]$ can be calculated using the method of WEST and PEARCE /12/ from the data of optical densities OD in case we know the absorption coefficients of monomers ϵ_M and dimers ϵ_D :

$$OD = \epsilon_M [M] + \epsilon_D [D] \quad (2)$$

We have used the values of $\epsilon_M = 170000 \text{ mol}^{-1} \text{ l cm}^{-1}$ and $\epsilon_D = 6000 \text{ mol}^{-1} \text{ l cm}^{-1}$ determined by WAGGONER /11/ at 571 nm. The concentrations of $[M]$ and $[D]$ calculated in such a way are stated in TAB. 1 together with the value of monomer $\rightleftharpoons$ dimer equilibrium constant K , defined by the following equation:

TAB. 1 Titration of EL (2.58×10^{-3} mol/l) liposomes with M-540. [M-540] - total molar concentration of the dye; [M] and [D] - the computed concentration of M-540 monomers and dimers, respectively; OD⁵⁷¹ - the measured optical density at the monomeric maximum 571 nm, K - the computed monomer $\rightleftharpoons$ dimer equilibrium constant

N ^o	[M-540] 10 ⁶ mol/l	[EL]: [M-540]	OD ⁵⁷¹ cm ⁻¹	[M] 10 ⁶ mol/l	[D] 10 ⁶ mol/l	K 10 ⁶ mol/l
1	0.06	43000	0.010	0.060	0.000	-
2	0.35	7371	0.057	0.334	0.008	14.03
3	0.88	2932	0.135	0.794	0.043	14.66
4	1.76	1466	0.250	1.468	0.146	14.76
5	3.51	735	0.444	2.612	0.449	15.19
6	8.78	294	0.885	5.205	1.787	15.16
7	17.56	147	1.419	8.346	4.607	15.12
8	35.11	73	2.295	-	-	-
9	87.78	29	6.232	-	-	-
10	175.55	15	13.166	-	-	-

$$\text{Av. } K = 14.820 \times 10^{-6} \text{ mol/l}$$

$$SD = \pm 0.407 \times 10^{-6} \text{ mol/l}$$

$$K = \frac{[M]^2}{[D]} \quad (3)$$

In case the [M] and [D] values fulfil the mass law for monomer dimer equilibrium, according to equation (3) the dependence log [D] vs. log [M] should be linear with a slope of two. Using our data we have plotted the stated dependence (FIG. 2). The equation of the straight line with the slope of two is fulfilled only by the data for M-540 concentration within the interval $(0.06-35) \times 10^{-6}$ mol/l, i. e. molar ratio [EL] : [M-540] within the 43000-73 interval.

For concentration of [M-540] $> 35 \times 10^{-6}$ mol/l, i. e. [EL] : [M-540] < 73 , [M] and [D] values no longer conform to the mass law for monomer $\rightleftharpoons$ dimer equilibrium. Also M-540 molecules in water contribute to values of absorbances in case of these concentrations. At M-540 concentrations $(0.06-35) \times 10^{-6}$ mol/l the values

of $K=14.8 \times 10^{-6}$ mol/l are constant within a standard deviation of 0.4×10^{-6} mol/l.

Results of our measurements proved that for the employing of M-540 probe for the study of "alcohol" liposomes, it is necessary to use M-540 concentrations fulfilling the condition $[EL] : [M-540] > 73$. Further increase of dye concentration does not lead to increase of probe bounding to lecithin but the M-540 molecules already remain in water and the resulting spectrum is the superposition of M-540 spectrum in liposomes and M-540 spectrum in water. This work did not ascertain any limiting factors for the use of low M-540 concentrations in egg lecithin liposomes.

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