Release of Methylene Blue from dioctadecyldimethylammonium chloride vesicles

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The cationic dye Methylene Blue included in the internal aqueous microphase of dioctadecyldimethylammonium chloride (DODAC) vesicle shows the typical monomer–dimer equilibrium observed in homogenous aqueous solutions. Using a simple spectrophotometric method, values of 54 000 ± 1300 M⁻¹ cm⁻¹ and 3860 ± 130 M⁻¹ cm⁻¹ have been obtained for the extinction coefficient of Methylene Blue monomer at 680 nm (λmax) and the equilibrium constant (K), respectively, in homogeneous aqueous solution at 20 °C. At this wavelength dimer absorbance is negligible. Leakage of Methylene Blue from the inner aqueous pseudophase can be described in terms of Fick’s law. The dependence of the release rate on monomer concentration, the value of the permeability constant (1.09 × 10⁻⁹ cm s⁻¹) at 20.3 °C) and the dependence of the release rate on NaCl addition suggest that Methylene Blue diffuses across the lipidic bilayer of the vesicle as a monomer.

Introduction

Liposomes are considered to be potential delivering agents for a great variety of molecules including drugs, enzymes and dyes.1,2 The use of liposomes as carriers in biological systems allows the use of low dosages and hence can diminish harmful allergic and immunological side effects. These systems have been used for the treatment of afflicted tissues in cases of neoplastic and genetic deficiency diseases. However, the utilization of liposomes as target directed sensitizer carriers is hampered by their non-selective distribution and because the factors affecting dye entrapment and retention in liposomes or vesicles are not completely understood.

Methylene Blue is a hydrophilic cationic dye that is located mainly in the aqueous pseudophase of vesicle solutions. It has been extensively used in traditional medicinal applications. In the last few years, Methylene Blue has been studied as a model system for stacking interactions3 due to its oxidation–reduction properties4 as a photosensitizing dye in singlet oxygen reactions5 and for its properties as a photosensitizing dye in nucleic acid damage.6

Synthetic vesicles obtained from dioctadecyldimethylammonium chloride (DODAC) provide a suitable system to encapsulate Methylene Blue in the inner aqueous pseudophase, due to unfavourable electrostatic interactions arising from the interaction between the cationic dye and the positively charged bilayer surface. It can constitute an appropriate system to evaluate the factors that determine the capacity of the vesicle to retain dyes in their inner aqueous pseudophase.

In the present study we determine the rate of release of Methylene Blue from the internal compartment of DODAC vesicles and propose a simple model to account for this leakage. From the dependence of the release rate on temperature the apparent activation energy associated to the process is obtained.

Materials and methods

Methylene Blue (Aldrich) was used without further purification. Dioctadecyldimethylammonium chloride (DODAC) (Herga Ind. of Brazil) was purified by extraction with diethyl ether in a Soxhlet apparatus followed by at least six recrystallizations from acetone p.a.7 Unilamellar vesicles were prepared by controlled injection of a DODAC–chloroform solution (20 mM) in water (Omnisolv, HPLC quality) thermostatted at 75 ± 0.5 °C according to the procedure previously described.7 Final DODAC concentration was fixed by the amount of DODAC–chloroform solution injected. To obtain vesicle solutions with Methylene Blue included in the internal aqueous compartment, an appropriate quantity of Methylene Blue from an aqueous stock solution was added to the thermostatted water prior to the DODAC–chloroform solution injection. Subsequently, gel permeation chromatography was employed to remove Methylene Blue from the external aqueous pseudophase. Typically, 3 ml of freshly prepared Methylene Blue–vesicle solution were eluted through a 1 × 25 cm Sephadex G-25 (Sigma Chemical Co.) column. Water flow was controlled at 1 ml min⁻¹ with a Masterflex model 7521-55 peristaltic pump. In order to avoid the release of Methylene Blue from the internal aqueous compartment, the system was thermostatted to 5 ± 0.5 °C. Final analytical Methylene Blue concentrations were determined using UV–VIS spectrophotometry after obtaining of total release by heating the vesicle solution in a thermostatted water bath above 40 °C for at least 30 min. The Methylene Blue spectra obtained by following this procedure show the same monomer to dimer ratio as that observed in aqueous solution at the same analytical concentration. To prepare Methylene Blue–vesicle solutions 5 mM in NaCl, a constant salt concentration was maintained throughout the procedure.

A Unicam UV4 UV–VIS spectrophotometer equipped with a thermostatted cell holder was used to obtain the absorption spectra of the Methylene Blue–vesicle solutions. Short-path cuvettes were employed to obtain the spectra of highly absorbing solutions.

Results and discussion

Absorbance of organic dyes in aqueous solutions deviates from the Lambert–Beer law and Methylene Blue is no exception. This deviation is due to reversible aggregation to form dye oligomers. In the concentration range between 10⁻³–10⁻⁴ M,
Methylene Blue aggregation is limited to a rapid monomer–dimer equilibrium, with maximum absorbances in the visible spectra at 660 and 610 nm for monomer and dimer species, respectively.\(^1\,^2\)

Fig. 1 shows a comparison of the UV–VIS spectra of a vesicle solution with Methylene Blue included in the internal aqueous pseudophase of the microparticle (measured immediately after the solution was obtained from the gel permeation column) with the spectrum of the same solution at infinite time (once the system had reached equilibrium and no additional changes were observed in the spectrum). The spectrum at infinite time shows mainly the band corresponding to the monomer and the dimer absorption, respectively. The shape of this spectrum agrees with the spectrum observed for an aqueous solution containing the same analytical concentration of Methylene Blue. The spectrum obtained at zero time shows two maxima of similar absorbance at 660 and 610 nm, corresponding to the monomer and the dimer absorption, respectively. The appearance of this spectrum is explained in terms of the monomer–dimer equilibrium within the inner cavity of the vesicle, where the large extent of dimer contribution is a consequence of a local concentration effect. In order to apply a kinetic model to the release of Methylene Blue from the vesicle internal compartment, it is necessary to determine both monomer and dimer local concentrations in this cavity. Several methods have been used to measure the equilibrium constant, \(K\), corresponding to Methylene Blue monomer–dimer equilibrium in aqueous solution.\(^8\,^11\) However, these values have been obtained at different temperatures and the reported data for the temperature dependence of \(K\) differs by a factor of two.\(^8\,^11\)

In this study, a simple spectrophotometric method is employed to evaluate \(K\) and its temperature dependence.

If equilibrium in aqueous solution is expressed by eqn. (1):

\[
2M \rightleftharpoons D
\]  

the pseudo-equilibrium constant, \(K\), can be defined by eqn. (2):

\[
K = \frac{[D]}{[M]^2} = \frac{[M]_0 - [M]}{2[M]^2}
\]  

where \([M]_0\) and \([M]\) are the initial (analytical) and equilibrium Methylene Blue monomer concentrations, respectively. However, to relate eqn. (2) with absorbance at a given wavelength, concentrations and extinction coefficients of all species present in the system must be included and a multiparametric equation is obtained. In order to reduce the number of variable parameters, measurements were performed at a wavelength of 680 nm, at which dimer absorbance may be disregarded. This condition was verified by applying a convolution method to determine the contribution of both dimer and monomer to spectral data.

When measurements are performed at wavelengths equal to or greater than 680 nm, there are only two adjustable parameters, the monomer extinction coefficient, \(\varepsilon^M\), and the dimerization constant, \(K\), in the equation that relates absorbance with monomer concentration. Then, eqn. (2) can be solved for \([M]\) to give eqn. (3):

\[
[M] = \frac{1}{4K} (\sqrt{1 + 8K[M]_0} - 1)
\]  

Absorbance at 680 nm can then be written as eqn. (4).

\[
A_{680} = \frac{\varepsilon^M}{4K} (\sqrt{1 + 8K[M]_0} - 1)
\]  

Fig. 2 shows experimental data measured at 293 K, fitted according to eqn. (4). From this fit, values of 54 000 ± 1300 M\(^{-1}\) cm\(^{-1}\) and 3860 ± 130 M\(^{-1}\) were obtained for \(\varepsilon^M\) and \(K\), respectively. \(K\) at this temperature compares favourably with previously reported values.\(^8\)

Similar experiments to obtain \(K\) were performed in the temperature range between 6 and 30 °C. Plots of \(\ln K\) vs. 1/\(T\) fit eqn. (5), which accounts for the temperature dependence of the dimerization constant.

\[
\ln K = -(8.5 \pm 0.7) + (4900 \pm 200) T^{-1}
\]  

From eqn. (5) a value of 9.7 ± 0.4 kcal mol\(^{-1}\) is obtained for the dimerization enthalpy. This value ties intermediate values reported by Dunken et al.\(^7\) (\(\Delta H = -7.6\) kcal mol\(^{-1}\)) and Spencer\(^9\) (\(\Delta H = -13.9 \pm 0.8\) kcal mol\(^{-1}\)) using spectrophotometric and jump-temperature methods, respectively.

As mentioned above, a monomer–dimer equilibrium is clearly observable in freshly prepared DODAC vesicles with Methylene Blue included in the internal compartment (with local concentrations of Methylene Blue ranging from 0.47 to 0.62 mm). Additionally, time-dependent spectral changes are observed when the system evolves towards equilibrium. These spectral changes can be ascribed to Methylene Blue release from the internal aqueous pseudophase, thus decreasing the local concentration (inside the vesicle). Fig. 3 shows the time dependence of the system absorbance at 680 nm (the wavelength at which only monomer absorbance is significant) at
of Methylene Blue across the bilayer; (\(d\))

Various temperatures. As observed in these plots, absorbance at 680 increases with time as a consequence of Methylene Blue release from the internal aqueous pseudophase since in the external aqueous pseudophase, at low dye concentrations, the aggregation to form oligomers is negligible. Furthermore, as expected from the dependence of the membrane fluidity on temperature, the plots show that the rate of absorbance growth increases at higher temperatures and a nearly constant absorbance value is obtained at long times, when the system approaches equilibrium.

In this system, Methylene Blue release from the internal compartment of DODAC vesicles can be closely approximated by Fick’s law: [eqn. (6)], where \(J\) is the substrate flux, \((\delta C)/\delta t\), and \(D_m\) and \(D_o\) are the monomer and dimer diffusion coefficients in the lipidic bilayer. In our system, if the flow is assumed to be dominated by the monomer displacement, eqn. (6) can be expressed in terms of eqn. (7), where the derivative term is related to the flux of Methylene Blue across the bilayer; ([M], – [M]\(_d\)) corresponds to the flux going from the highest to the lowest concentration, \([M]\(_f\) is the total Methylene Blue concentration in the inner aqueous phase of the vesicle; \([M]\) is the monomeric Methylene Blue concentration in the inner aqueous pseudophase of the vesicle; \([M]\(_d\) is the monomeric Methylene Blue concentration in the outer aqueous pseudophase, which takes a value equal to zero at the initial time, and \(k_{rel}\) is a constant for the release process.

Fig. 4 shows typical plots obtained according to eqn. (7). Values of \(\delta [M]/\delta t\) were calculated from the slopes of a plot of \([M]\(_f\) vs. \(t\) at different \([M]\(_f\). Fig. 4 shows good linear correlations up to 50% of Methylene Blue release and appreciable deviations from linear behaviour when \(\delta [M]/\delta t\) reaches a nearly constant value at smaller ([M] – [M]\(_d\)) differences. These deviations can be explained considering that above this percentage of release practically equal concentrations of \(M_i\) and \(M_d\) are maintained by the dimer–monomer equilibrium in both inner and outer aqueous pseudophase. Additionally, it may be noted that the values of \(k_{rel}\) calculated from the slope of the curves in Fig. 5 are independent of the initial Methylene Blue concentration. This result implies that the permeant species across the bilayer is the monomer and supports the idea that dimer contribution to the release process can be disregarded.

Permeability coefficients for the diffusion of Methylene Blue across the lipidic bilayer in DODAC vesicles were calculated using eqn. (8) from the values of \(k_{rel}\) and including a correction factor, which corresponds to the ratio between \(v_i\) the volume of the internal aqueous pseudophase of the vesicle and \(a_i\) the inner surface of the bilayer.

Values of \(v_i\) and \(a_i\) were calculated assuming a spherical shape vesicle of internal radius \(R_i\). Changes in the vesicle shape and/or size due to Methylene Blue included in the internal aqueous pseudophase may be disregarded since no important changes in dispersion between 300 and 400 nm are observed upon comparing the absorption spectra of freshly prepared vesicle solutions not containing Methylene Blue with the spectra of vesicle solutions containing Methylene Blue included in the internal aqueous pseudophase, and with the spectra of vesicle solutions containing Methylene Blue after the dye had been released from

\[ P = k_{rel} \left( \frac{v_i}{a_i} \right) \]
the liposome. The value calculated for the permeability constant, $P$, at 20.3 °C is equal to $1.09 \times 10^{-6}$ cm s$^{-1}$. This value is larger than those reported by Marsh et al. for the permeation of the cationic spin label tempo-choline into dimyristoylphosphatidylcholine vesicles at transition phase temperature ($P = 2.4 \times 10^{-10}$ cm s$^{-1}$ at 24°C) and by Disalvo for the leakage of carboxy fluorescein in egg phosphatidylcholine liposomes. These differences can be ascribed to the different methods employed to obtain $P$ values and/or to the changes in membrane structure occurring when the membrane undergoes mechanical stress due to swelling or shrinking-induced leakage in these systems. Also, the $P$ value is several orders of magnitude larger than those values typically reported for the permeability of considerably smaller inorganic cations in unilamellar liposomes ($P = 10^{-11} \sim 10^{-14}$ cm s$^{-1}$).

The use of eqn. (7) is only valid for a process that involves independent fluxes. This means that substances penetrating and diffusing across the membrane do not interact with each other. In other words, the permeation of Methylene Blue through the DODAC bilayer vesicle corresponds to a process involving permeant-membrane interactions supporting the above postulate that Methylene Blue molecules diffusing across the membrane do not interact with each other.

Furthermore, experiments to determine salt effects on the release rate of Methylene Blue were performed at 20 °C. Fig. 6 shows data plotted according to eqn. (7) for Methylene Blue release from the internal compartment of vesicles prepared in 5 mM of NaCl in comparison with NaCl containing a membrane thickness of 50 Å, a diffusion coefficient ($D = \Phi 9$) equal to $5.5 \times 10^{-14}$ cm$^2$ s$^{-1}$ was determined. This value is similar to one reported for the permeation of tempo-choline in dimyristoylphosphatidylcholine vesicles at the phase transition temperature.

An apparent activation energy for the permeation process below the phase transition temperature of 16.0 ± 1.8 kcal mol$^{-1}$ was calculated from the slope of the plot of In $P$ vs. $1/T$ in Fig. 6. This value, which includes contributions from the passage across the interface and the diffusion within the bilayer to the activation energy, is greater than those reported for the permeation of small aliphatic amines in spherical lipid bilayers (ca. 10 kcal mol$^{-1}$). The large value determined for Methylene Blue permeation can be ascribed to two factors, the greater size of the Methylene Blue molecule and the repulsive interactions between the cationic dye and the positively charged bilayer surface.

Additionally, it can be noted in Fig. 6 that the value of $P$ increases appreciably at 35 °C, the temperature close to the gel-to-liquid crystalline phase transition for DODAC vesicles. Furthermore, the values of $P$ above the phase transition temperature are smaller than that obtained near the phase transition temperature. Several factors contribute to the origin of the high permeability at the phase transition temperature: the mismatch in molecular packing which occurs at the interfacial regions of the fluid and ordered phases that coexist during the transition, the formation of free volume holes, the area increasing and the thickness decreasing in the bilayer at this temperature. Above the phase transition temperature in the liquid crystalline state, lipid molecules still remain in a relatively ordered state.

Taking into account the tendency of Methylene Blue to form aggregates in aqueous solutions and to behave like a monomer in organic solvent, and the good fit between the experimental data and the model derived from Fick’s law, we can postulate that Methylene Blue from the inner aqueous pool of the vesicle diffuses across the vesicle interface and the lipidic bilayer as a monomer. Similar behaviour has been observed in bilayer aggregates formed from substituted diocadecyl 1-glutamate-derived anionic amphiphiles below the phase transition temperature. In this system Methylene Blue is bonded to the amphipathic head (as a counterion) and is incorporated into the crystalline hydrophobic region as a monomer.

Additionally, we can point out that the low leakage efficiency of Methylene Blue from DODAC vesicles at laboratory temperatures (15–20 °C) provides this system with remarkable characteristics with which to study sensitized photobleaching reactions of water soluble substrates, particularly for those systems in which the reaction between the substrate and the sensitizer (type I reaction) compete with the reaction between the substrate and singlet oxygen, $O_2^*(\Delta)$ (type II reaction). Studies related to this point will be published elsewhere.

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References