

Biphasic protonation of hydrophilic cargo agents in unilamellar phospholipid vesicles: implications about cargo location

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Abstract

The commonly used cargo agents for liposome entrapment, chromate and 5(6)-carboxyfluorescein (CF), have been sequestered in small unilamellar vesicles composed of dipalmitoylphosphatidylcholine through preparations involving either sonication or extrusion methods. Once loaded, these water-soluble chromophoric cargo agents have been exposed to small quantities of externally applied acid solution, which decreases the pH from neutral to approx. 6. By monitoring photometrically the time profile of the protonation of the sequestered chromophores, it is evident that the uptake of protons by each cargo agent is biphasic. An immediate spectral change is followed by further change over 10–40 min, where the extent of protonation occurring in each time frame is approximately equal. The vesicles themselves are unaffected by the induced pH change. The leakages of both chromate and CF from loaded sonicated vesicles were monitored at both 25°C and 45°C. Overall, the leakage processes exhibited a deceleration over time. The biphasic protonation and decelerating leakage phenomena are together interpreted in terms of a mechanism of cargo loading involving an intercalation of the water-soluble agent along with water into the vesicle bilayer, rather than involving internal capture of the cargo inside the vesicles, or through electrostatic interactions with the bilayer surfaces. In addition, the measured extents of cargo loading are more consistent with calculated estimates of loading through bilayer intercalation than with those for internal capture.

Keywords: Vesicle; Phospholipid; Chromate; Carboxyfluorescein; Protonation; Leakage

1. Introduction

The incorporation of a broad range of hydrophilic cargo agents into various types of liposomes has been well-characterized [1]. The definitive determination that a hydrophilic protein cargo agent is located within the aqueous compartments of the liposomes is based on three criteria [2]: (a) There must be a clear separation of free from sequestered cargo by size-exclusion gel chromatography; (b) There must be no hydrophobic or charge interaction between the outermost bilayer and the cargo, as demonstrated by the absence of co-elution of the cargo with preformed liposomes after the cargo is added to a suspension of unloaded liposomes; (c) Disruption of gel-filtered liposomes by detergents must induce a shift in the gel-

filtration pattern of the cargo agent from a position coincident with the liposomes to one coincident with the free cargo. With minimal reservation [3], fulfillment of these criteria has since been considered sufficient evidence to conclude that liposome sequestration of the cargo has occurred and that the cargo is isolated from the external environment.

It is reasonable that liposome sequestration of large protein cargo that fulfills the three criteria above is equated in the literature with localization within the internal aqueous region of the vesicles. However, it has become general practice in liposome systems to apply these criteria to all hydrophilic cargo agents, both large and small. Specifically, simple co-elution of hydrophilic cargo agents with gel-filtered liposomes has become essentially the only criterion for concluding that the cargo is enclosed within the interior of the vesicles [3–5]. This generalization extends further to the associated proposal that hydrophobic cargo molecules are embedded inside the lipid bilayer, and

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that amphiphilic molecules may intercalate within each layer of the vesicle membrane. In addition, charged cargo molecules are considered capable of binding to the exterior and interior surfaces of the liposome [4]. Peptides which initially interact with liposome surfaces, have had their polarity adjusted by pH variation that consequently allows them to penetrate into the membrane bilayer [6].

The simplicity of these general correlations of cargo location with cargo solubility character extends to situations where, for example, not only is the chromatographic co-elution criterion taken as the only basis for locating the cargo inside the vesicles, but also, the intravesicular concentration of the cargo is assumed to be the same as that in the original preparation solution [7]. However, recent fluorescence studies have shown that hydrophilic dyes as cargo agents exhibit multiple environments in their association with liposomes, including a membrane-bound component [8,9].

The systematic examination of correlations of cargo entrapment efficiency with the molecular structure of the cargo, and with the liposome composition and morphology, is distinctly lacking in the literature. While protein cargoes have received some attention in this regard [10], where few consistent correlations were evident, other classes of cargo have not been examined. Before meaningful correlations can be attempted, a better understanding of the basic principles of cargo sequestration needs to be developed.

In this study, the protonation and leakage of small hydrophilic cargo agents associated with unilamellar phospholipid vesicles have been examined quantitatively. The pH-gradient imposed across the bilayer of the loaded vesicles is substantially greater in this study than for previous investigations of proton-permeability or pH-induced permeability shifts [11–13]; the larger pH-gradient is intended to model the effect of the gastric environment on vesicles taken orally for nutrient delivery. One previous study has examined cargo release over a pH range of 5–8, but without a gradient imposed [14], while another has focussed on proton diffusion both at the membrane surface, and between the surface and the bulk solution [15]. A pH-gradient method has been developed to prepare small unilamellar vesicles (SUV) composed of charged phosphatidic acid [16]. In addition, increasing the pH external to the vesicles has been found to stabilize phosphatidic acid SUV to the leakage of carboxyfluorescein cargo, while conversely, decreasing the external pH destabilizes these SUV [17].

The results obtained here, predominantly with chromate and carboxyfluorescein as cargoes, suggest that a significant proportion of the loaded cargo is accessible to the external environment, and is physically separated from the remainder of the cargo. The simplistic concept of internal encapsulation of these cargoes, which are both commonly used as standard marker agents for vesicle loading and stability [18,19], is thus called into question.

2. Materials and methods

2.1. Materials

The phospholipids dipalmitoylphosphatidylcholine (DPPC), dimyristoylphosphatidylcholine (DMPC), diarachidoylphosphatidylcholine (DAPC), and phosphatidic acid (PA) were all purchased as >98% pure reagents from Sigma Chemical (St. Louis, MO), and were used without further purification. The caffeine, theophylline, glucose, serotonin, procaine, bovine serum albumin (BSA), and Sepharose-4B were also purchased from Sigma, as was the hexokinase assay for glucose (Glucose [HK] 50). 5(6)-carboxyfluorescein (CF) was purchased from Eastman Chemical, and potassium chromate from Fisher Chemical. All reagents used to prepare the phosphate-buffered saline solution (PBS) were reagent grade, and made up in Milli-Q deionized water (Millipore, Milford, MA) to a phosphate concentration of 0.010 M, and 0.15 M in NaCl at pH 7.4.

2.2. Preparation of vesicles

Liposomes were prepared by two methods:

The first involved sonication, and is based on the methods of Barenholz et al. [20] and Wong et al. [21]. The phospholipid (PL) was firstly dissolved in 3:1 chloroform/methanol (20 μ l/mg PL); the solvent was then removed by vacuum at 55°C for 20 min with vigorous intermittent vortexing. To the dried PL was added PBS solution containing 0.10 M of the desired cargo agent (75 μ l/mg PL), followed by incubation at 55°C for DPPC, 80°C for DAPC, and 38°C for DMPC, for 5 min with vigorous intermittent vortexing. The suspension was then sonicated with a Branson Sonifier 350 with a micro-tip horn at the temperatures stated above, with 1 min blasts and 0.5 min breaks until two blasts after optical clarity was obtained. After sonication, the suspension was incubated at the specified elevated temperature for 45 min, then centrifuged at 13 000 rpm (10 000 \times g) at 15–20°C for 30 min. The top 4/5 of the supernatant was removed and stored at 20°C usually for 1 day before use. The post-sonication incubation and storage procedures allow the small unilamellar vesicles to stabilize into larger fused unilamellar analogs [22]. These stabilized vesicles maintain opalescence, and are referenced in this work as sonicated vesicles (SV).

The second method used to prepare vesicles involved extrusion of PL suspensions through 100 nm polycarbonate filters at the specified elevated temperatures using a LiposoFast apparatus (Avestin, Ottawa, Canada), based on the method of Nayar et al. [23]. The preparation involved 21 passages through the filter. The PL suspension was initially prepared just as for the sonication procedure, as was the procedure used for the centrifugation and storage steps. No post-extrusion incubation at elevated temperature was performed. These vesicles are referenced here as extruded vesicles (EV).

Just before use, aliquots of the vesicle preparations were passed through a Sepharose 4B-200 size exclusion gel chromatography column to remove the external cargo solution. Protonation and leakage studies of these loaded vesicles were initiated immediately after collection of the eluted vesicle fraction from the gel column. Extensive elution with PBS was conducted before re-using the column; eluants were checked photometrically for the complete removal of residuals. The protonation study of chromate-loaded DPPC SV was unaffected by using a BioGel A-5m size-exclusion column instead of the Sepharose analog.

2.3. Analysis of absorbance profiles

Once loaded, the absorbance profiles of vesicle preparations in the UV-visible region are composites of both the chromophoric cargo absorbance and the vesicle turbidity contributions; the high-purity saturated PL used have no chromophore in the accessible UV-visible region. In order to separate these contributions, the UV-visible spectrum in PBS of each vesicle-free cargo was firstly measured. A linear artificial baseline was then drawn beneath a major peak in the cargo spectrum; 371 nm for chromate, 492 nm for CF, 272 nm for caffeine and theophylline, 275 nm for serotonin, and 290 nm for procaine. This baseline, which spanned across the absorbance minima on each side of the absorbance peak, enables a peak-amplitude value to be determined from it to the top of the absorbance peak. The peak-absorbance value, representing the absorbance from the actual (zero-absorbance) baseline up to the peak maximum, was also measured, enabling the ratio of the peak-absorbance to the peak-amplitude to be determined. Analogous ratios of measured off-peak absorbance values to the peak-amplitude are also determinable in the cargo spectrum. Also, the molar absorbance of the cargo agent is readily determinable from the peak-absorbance value, using Beer's Law.

Having correlated the absorbances of each cargo spectrum with its respective peak-amplitude value, the composite spectrum of a loaded vesicle sample was deconvoluted as follows: An artificial baseline was drawn beneath the chosen absorbance peak in the manner described above. The peak-amplitude was measured. By removing the known proportion of the peak-amplitude corresponding to the peak-absorbance of the cargo from the top of the peak in the composite spectrum, the remaining absorbance corresponds to the vesicle turbidity. The reliability of this separation of the absorbance contributions is bolstered by the featureless character of the vesicle scatter profile, which uniformly sweeps upward with decreasing wavelength.

The concentration of cargo associated with loaded vesicles can be readily determined by Beer's Law from the estimated cargo absorbance contribution and the known molar absorbance value. The concentration of DPPC SV

was determined indirectly by a previously described technique [24], based on rigorous quantitative analyses of the UV-scatter profiles of this vesicle type. Specifically, parallel experiments were conducted involving spectroturbidimetric and quasi-elastic laser scattering measurements of DPPC SV, along with standard quantitative phosphorus analyses of the vesicle samples. A correlation was made between measurements of vesicle turbidity at 250 and 350 nm and the concentration and mean diameter of the vesicles in the sample; mean diameters of 75–95 nm were routinely obtained. In essence, this technique relies on the fact that only two factors affect the measured turbidities of a given vesicle type; viz, the number of vesicles and their size. For loaded vesicle samples, estimates of vesicle turbidities at 250 and 350 nm were made by removing the contribution of the chromophoric cargo from the composite spectrum, as described above. This methodology represents a significant advancement of the photometric quantitation of cargo and phospholipid concentrations over that reported earlier [25].

With both the estimation of the PL concentration in the vesicle samples, and the known amount of PL initially used in the vesicle preparation, the extents of PL conversion into SV were also determined; vesicle yields of approx. 65% were obtained.

2.4. Calculations of theoretical entrapments

The entrapment of hydrophilic cargo in PL vesicles was estimated for two mechanisms of sequestration.

First, assuming the cargo is enclosed inside the aqueous internal volume of the vesicles [18]:

Consider 85 nm vesicle diameter,

then the surface area for a monolayer = $2.3 \cdot 10^4 \text{ nm}^2$

then the surface area for the bilayer = $4.5 \cdot 10^4 \text{ nm}^2$.

Assuming 0.50 nm^2 surface area/PL molecule [26]

then the number of PL molecules/vesicle bilayer = $9.0 \cdot 10^4$.

For 10 mM PL vesicles in 1 L,

then the number of PL molecules/L = $6.0 \cdot 10^{21}$

and the number of vesicles/L = $6.7 \cdot 10^{16}$.

For 85 nm vesicles,

volume of one vesicle = $3.2 \cdot 10^{-19} \text{ L}$

then the total volume within vesicle sample = $2.1 \cdot 10^{-2} \text{ L}$.

For 0.10 M cargo in prep solution,

then the amount of cargo/L of vesicle vol = $2.1 \cdot 10^{-3} \text{ mol/L}$.

Then, LF% of cargo (vide infra) = 21%.

Second, assuming the cargo is intercalated only into each side of the PL bilayer along with the bulk water:

For 10 mM PL vesicles in 1 L,

then the number of PL molecules/l = $6.0 \cdot 10^{21}$.

If there are 8 water molecules/PL in the bilayer [27], the number of water molecules/L within the bilayer

= $4.8 \cdot 10^{22}$,

then amount water/L within the bilayer = $8.0 \cdot 10^{-2}$ mol.

With 0.10 M cargo and 55 M water in the prep solution, then (cargo) associated with the vesicles = $1.4 \cdot 10^{-4}$ M.

Then, LF% of cargo = 1.4%.

2.5. Instrumentation

The leakage of CF from loaded DPPC SV was examined fluorimetrically (19) on a SPEX Fluorolog-2 Model F212 double spectrometer. As incubation at 45°C proceeded, 100 μ l aliquots of the loaded liposomes were removed and immediately diluted to 1000 μ l with PBS at 25°C to arrest the leakage. The fluorescence emission intensity was then immediately measured at 515 nm with 492 nm excitation.

All photometric analyses were conducted on a Gilford Response Spectrophotometer, using 10 mm semimicro quartz cuvettes and PBS solution as reference.

Differential scanning calorimetry (DSC) was performed on a MicroCal MC-2 high-sensitivity solution calorimeter, using PBS solution as reference.

3. Results

In Fig. 1 is shown the UV-visible absorption spectrum of a 700 μ l sample of 200 μ M potassium chromate in PBS at pH 7.4 in the wavelength range 300–420 nm, immediately after additions of 150 μ l each of 1.0 M NaCl and 1.0 M HCl; it is readily evident through the dramatic decrease in the peak absorbance at 371 nm that CrO_4^{2-} is protonated to HCrO_4^- by the acid addition. Less dramatic decreases in 371 nm absorbance (ΔA) were induced by smaller additions of HCl solution, as shown in Fig. 2; initial values of A_{371} are corrected for the small dilution effect of the acid addition (acid dilution). Substantial,

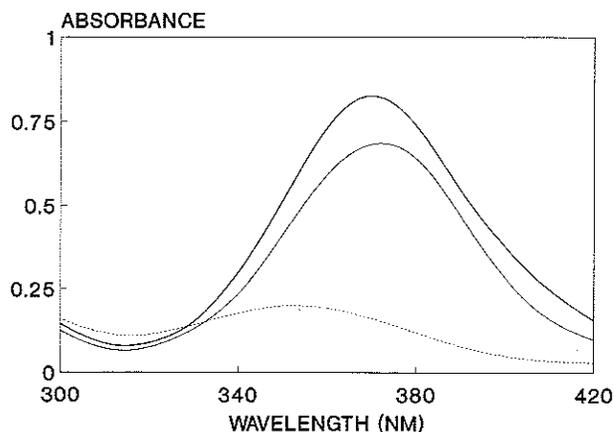


Fig. 1. Thick line, UV-visible absorption of 200 μ M potassium chromate in PBS at pH 7.4 (700 μ l); thin line, after addition of 150 μ l of 1.0 M NaCl; dotted line, after addition of 150 μ l of 1.0 M HCl.

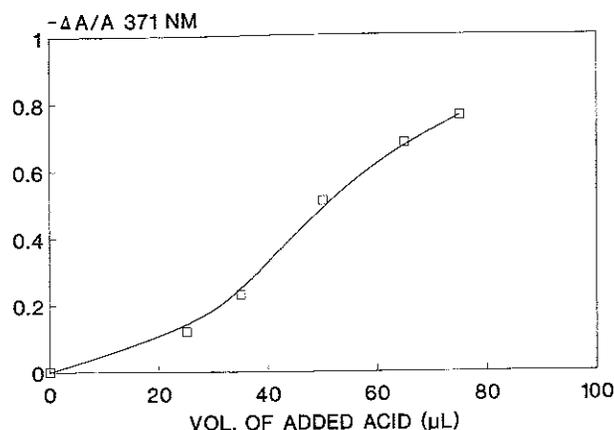


Fig. 2. Profile of $\Delta A/A_{371}$ against the volume of 0.10 M HCl added to 700 μ l of a 100 μ M potassium chromate solution in PBS at pH 7.4.

reproducible ΔA values are obtained for 50 μ l additions of 0.10 N HCl (pH 1.14) to 700 μ l samples of 60 μ M chromate in PBS initially at pH 7.4. The final pH after such an acid addition is approx. 5.8, which is close to the pK_a of hydrogen chromate of 6.5 [28]. Values of $\Delta A/A_{371}$ induced by these additions of 0.10 N HCl, measured 15–20 s after the addition, were independent of the original chromate concentration in the observed range of 25 μ M to 300 μ M.

Addition of 50 μ l of 0.10 N HCl to 700 μ l of unloaded DPPC SV in PBS at pH 7.4, where $[\text{DPPC}] = 0.5$ mM, leads to a marginal 4% immediate decrease in the vesicle turbidity at 371 nm. (Such an addition of 50 μ l of 0.10 M HCl to a 700 μ l sample of PBS at pH 7.4 is considered hereafter as a 'standard acid addition'). Furthermore, a standard acid addition to PBS leads to the same final pH regardless of the presence of up to 2.0 mM DPPC SV. Once corrected for the acid dilution, the turbidity profile of the vesicles in the 250–500 nm range is essentially unaffected by the standard acid addition. Consequently, the DPPC SV are considered to be unaffected by the acid addition.

Standard acid additions were analyzed within 20 s for two types of solution each containing 40 μ M chromate and 0.5 mM DPPC SV at pH 7.4, identical except for the following; in one case the chromate was loaded into the vesicles, along with subsequent gel filtration, and in the other case, the chromate was simply added externally to a preparation of unloaded vesicles. The values of $\Delta A/A_{371}$ were determined after corrections for the acid dilution and the deconvolution of the total absorbance at 371 nm into contributions of the chromate absorbance, used in the ratio, and the background vesicle turbidity. The chromate absorbance ratio obtained for the loaded vesicles was reproducibly 40–50% of that obtained for the unloaded vesicles. In addition, the $\Delta A/A_{371}$ value for the unloaded chromate system was the same as that for the vesicle-free chromate system, as in Fig. 2. Therefore, under otherwise identical conditions, the loaded chromate exhibits partial accessibil-

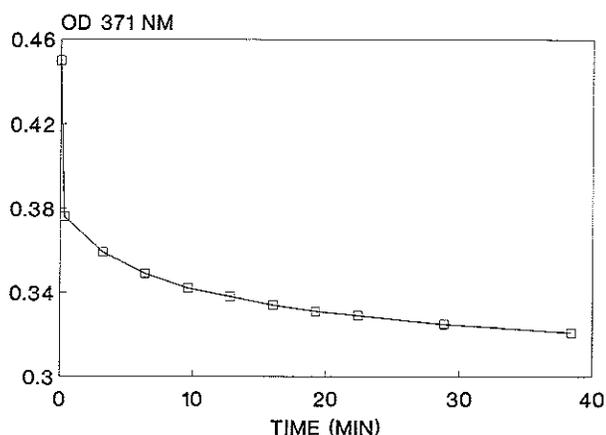


Fig. 3. Profile of the absorbance at 371 nm as a function of time after addition of 50 μ l of 0.10 M HCl to 700 μ l of 80 μ M potassium chromate loaded in DPPC SV at pH 7.4.

ity to external protonation, compared with the unloaded chromate, which, as expected, is completely accessible. The same results were obtained with an analogous HNO₃ solution, and the effect was immediately reversible by a subsequent equimolar addition of NaOH solution. To assess the role of vesicle morphology on the protonation of loaded chromate, DPPC extruded vesicles were examined for the standard acid addition at 25°C. Again, 40–50% of the absorbance change, compared with unloaded chromate, occurred immediately after the acid addition.

Fig. 3 shows the absorbance profile over time after standard addition to a DPPC SV preparation with 80 μ M of loaded chromate in PBS at 371 nm. The fast protonation within 15–20 s is followed by a much slower absorbance change indicative of protonation extending over several hours; overall, the protonation is clearly biphasic. The rate of the secondary slow process depends on both the vesicle morphology (SV and EV) and the duration that the vesicle preparation is stored in its parent solution at 20°C before gel filtration and the standard acid addition. Fig. 3 shows the acid addition to a SV sample stored 1 day at 20°C, which represents the fastest of the observed secondary protonations. The kinetics of both protonation steps have not been fully examined, and require further detailed study.

When the standard addition of acid is applied to chromate-loaded vesicles at 50°C, above the T_c of DPPC at 41°C [29], the protonation monitored by $\Delta A/A_{371}$ is the same as that observed for free chromate at 25°C. That is, there occurs complete protonation of the chromate immediately after the acid addition; the biphasic (fast-then-slow) protonation at 25°C becomes all fast at 50°C.

CF has been widely used as a fluorescent marker for loading and leakage studies of cargo associated with phospholipid vesicles. Inasmuch as CF has a pK_a of 6.2 [14], it was examined as an alternative cargo for protonation studies. The standard acid addition to a 5 μ M CF in PBS solution at pH 7.4 produced a dramatic decrease in the measured absorbance at its peak maximum at 492 nm.

DPPC SV loaded with CF were prepared, and examined after gel filtration by the standard acid addition. Again, the biphasic behavior was observed, with 50–60% of the protonation occurring in the immediate 20 s time-frame; the slow protonation occurred over several hours.

Inasmuch as the loaded chromate and CF appear to be partially accessible to the external acid, the possibility was explored that these cargo agents are associated through binding on the inner and outer surfaces of the vesicle bilayer, rather than through simple internal entrapment. This possibility has been proposed for ferricyanide as cargo in sonicated egg phosphatidylcholine vesicles [30]. Because both chromate and CF are negatively charged at neutral pH, the possibility of a favorable electrostatic interaction with the positive choline moiety on each bilayer surface was examined further.

The extent of loading chromate in the sonicated PL vesicles is expressed here in terms of a simple 'loading factor' (LF%), where

$$\text{LF}\% = [\text{cargo}]/[\text{PL}] \times 100\%.$$

The molar concentrations in the ratio were each determined photometrically (vide supra). In PBS solution at pH 7.4, the LF% of chromate and CF in DPPC SV are in the range 7–9%. When the ionic strength of the PBS used in the vesicle preparation was increased substantially by the inclusion of 0.5 M sodium sulfate, the LF% of chromate was unaffected. When 10% PA was included into the bilayer during vesicle preparation, thereby introducing negative charge into the bilayer surfaces, the LF% of chromate increased to 11%. Loading factors for positively charged (at neutral pH) cargo agents in DPPC SV were determined photometrically for procaine (2%) and serotonin (7%). (The pH of these solutions was pre-adjusted to pH 7.4 by prior additions of 1 M HCl before vesicle preparation). Using the standard hexokinase assay, the LF% of non-chromophoric uncharged glucose was determined to be 4% in DPPC SV. (Triton X-100 was used in this assay to lyse the vesicles and release the loaded glucose). To assess the extent of loading a large hydrophilic cargo, DPPC SV were prepared in a PBS solution containing 50 mg/ml of BSA; no absorption at 278 nm attributable to sequestered BSA was evident in the gel-filtered vesicles. In the case of chromate-loaded DPPC SV, DSC analysis was performed to assess whether the cargo sequestration affected the endotherm profile in the temperature range 20–60°C relative to the cargo-free analog; the profile was unaffected by the loading of chromate.

Further insights into the location of the acid-accessible chromate were sought from studies of the leakage of this cargo from the vesicles. Freshly prepared chromate-loaded DPPC SV were gel-filtered then stored at 20°C. Initially, and over time, portions of the stored sample were removed, again gel-filtered to remove any released cargo, and examined photometrically. Leakage of chromate from these vesicles at 20°C was monitored for several weeks, as

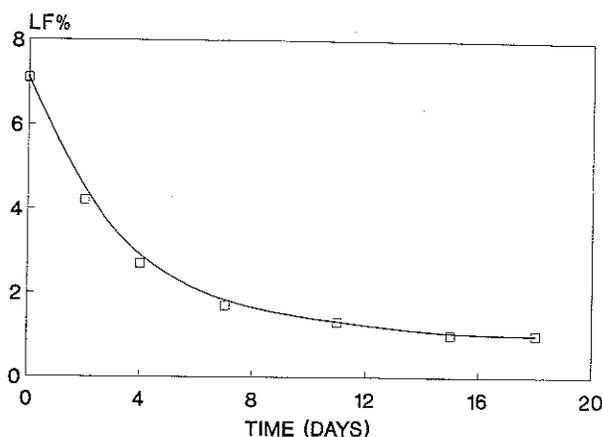


Fig. 4. Leakage of chromate from DPPC SV at 25°C monitored by diminished LF% of chromate over time.

shown in Fig. 4. When incubated at 45°C, however, the leakage of chromate was markedly accelerated as shown in Fig. 5, plot a, where release occurred over a duration of several hours, rather than the days taken at 20°C. After each time period at 45°C, the chromate remaining with the vesicles was examined by the standard acid addition at 25°C for the biphasic protonation. Fig. 5, plot b, shows a profile of the extent of the fast protonation, as a percentage of the total acid-induced conversion of the chromate, against the duration that the vesicles were at 45°C. Although less chromate remains associated with the vesicles as time passes at 45°C, increasingly more of this remaining chromate becomes accessible to the external acid.

The leakage of CF from loaded DPPC SV was also examined, but in this case by the standard fluorimetric method [19]. Fig. 6 shows a plot of the fluorescence emission intensity of CF at 515 nm measured at 25°C in diluted solution after incubation at 45°C for the designated

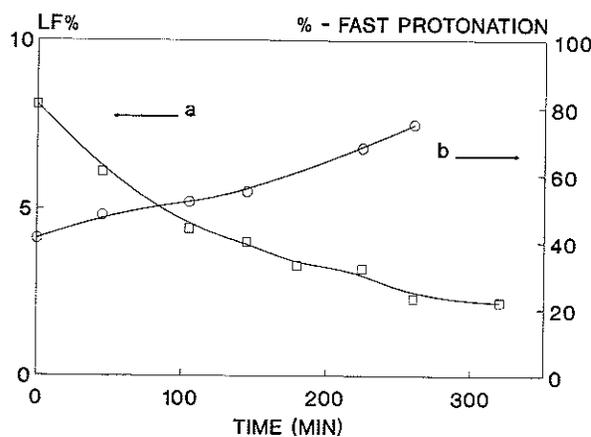


Fig. 5. (a) Leakage of chromate from DPPC SV at 45°C, monitored photometrically at 371 nm by diminished LF% of chromate over time. (b) Profile of the percentage of fast protonation of the residual chromate loaded in the DPPC SV (approx. the total acid-induced conversion of the loaded chromate) in PBS at pH 7.4 at 25°C, against the time the vesicles are kept at 45°C.

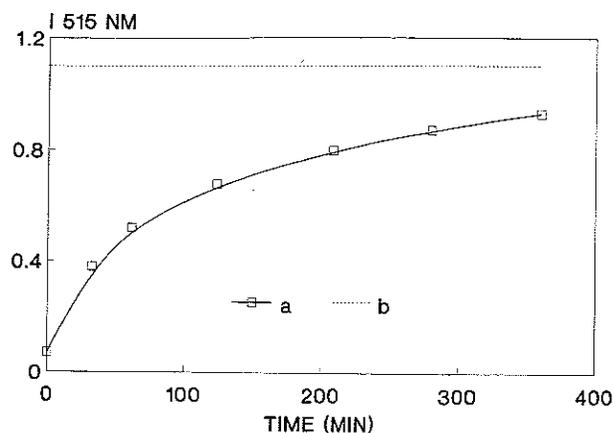


Fig. 6. (a) Profile of the fluorescence emission intensity at 515 nm of CF in loaded DPPC SV measured at 25°C in PBS diluted (1:10) solution against the time of incubation at 45°C. (b) Emission intensity obtained after 10 μ l of 50 mM Triton X-100 is added.

time duration; here, increased fluorescence correlates with CF release from the self-quenching environment of the loaded vesicles.

The photometric analysis of chromate leakage was applied to longer-chain C-20 DAPC vesicles having a T_c value of 68°C. No leakage of the loaded chromate was observed during 24 h at 45°C, over which time the standard acid addition at 25°C indicated that approx. 45% of the protonation was fast; completion of the protonation at 25°C took several days. An attempt to examine the analogous leakage from short-chain C-14 DMPC vesicles led to a notable result; the preparation of DMPC vesicles at 38°C, by both the sonication and extrusion techniques, did not load any detectable quantity of chromate from the original 0.10 M solution. This result was quite reproducible.

Inasmuch as acid accessibility can monitor the progressive release of loaded chromate, the possibility of monitoring the influx of external chromate into unloaded DPPC SV was explored. To an unloaded vesicle sample was added external potassium chromate to a concentration in the PBS of 0.10 M. This solution was then monitored photometrically over time of storage at 20°C for sequestration of the chromate by the vesicles after gel filtration. After 15 days at 20°C, associated chromate was marginally detectable at a LF% < 1%. An analogous study was conducted at a temperature of 45°C. Fig. 7, plot a, shows the LF% of associated chromate as a function of the incubation time at 45°C. After each time period, the associated chromate was examined by the standard acid addition at 25°C for the distribution of fast and slow protonation. Fig. 7, plot b, shows a profile of the percentage of fast protonation of the sequestered chromate against the duration at 45°C, analogous to Fig. 5b. Clearly, as more of the chromate becomes associated with the vesicles, a greater percentage of this chromate becomes inaccessible to the externally applied acid.

To assess the possibility of cargo association with the

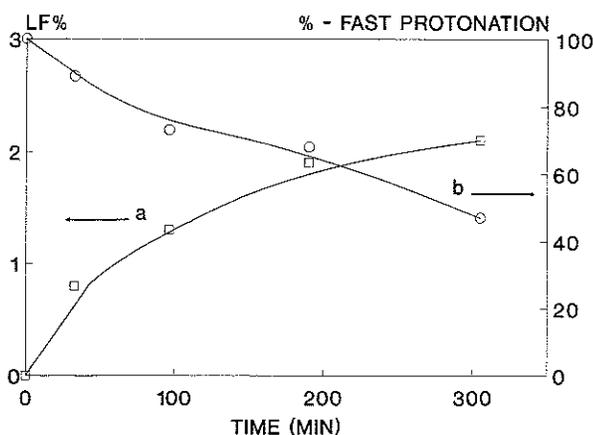


Fig. 7. (a) Penetration of external chromate (0.10 M) into DPPC SV at 45°C, monitored photometrically at 371 nm by increasing LF% of chromate over time. (b) Profile of the percentage of fast protonation of the chromate sequestered by the DPPC SV (approx. the total acid-induced conversion of the loaded chromate) in PBS at pH 7.4 at 25°C, against the time of incubation at 45°C.

hydrophobic portion of the phospholipid bilayers, the solubility of potassium chromate was determined at 25°C in both chloroform and oleic acid as representative non-polar solvents. The solubility, assessed by vortexing approx. 5 mg in approx. 2 ml of each solvent, was analyzed photometrically against the pure solvent. No solubility of chromate in either solvent was detectable.

To probe further the location of sequestered hydrophilic cargo in PL vesicles, a cargo whose absorption spectrum depends on the polarity of the solvent medium was examined; methylene blue (MB) is reported to have this spectral characteristic [31]. Combinations of MB individually with water, chloroform, oleic acid, and carbon tetrachloride were prepared. The peak maxima of MB in water and chloroform were similar at approx. 660 nm, although the peak molar absorbance in water was much higher than in chloroform. MB showed no detectable solubility in oleic acid and carbon tetrachloride. A PBS solution 0.10 M in MB was used in preparing DPPC SV. The visible absorption spectrum of the resulting gel-filtered vesicles showed the MB spectral overlay on the vesicle turbidity, indicating that a limited loading (LF% < 1%, using the molar absorbance determined in water) of MB had occurred. The peak wavelength of the loaded MB was the same as that of vesicle-free MB in PBS solution.

4. Discussion

The protonation of chromate is clearly altered by its association with the phospholipid vesicles. When chromate has been loaded into the vesicles by either of the literature methods used here, the protonation occurs in two greatly different time-frames, leading ultimately to the same over-

all extent of protonation as for free chromate. The biphasic behavior occurs for additions of acid, independent of its identity, and is readily reversible by the subsequent addition of strong base. The vesicles themselves are unaffected by the acid addition, as would be expected for the phosphatidic head-group of the lipid having a pKa < 3 [15]. The protonation becomes entirely monophasic in the fast time-frame when the bilayer phospholipids gain mobility above the gel to liquid-crystalline transition temperature. Furthermore, the protonation behavior cannot be correlated with an acid-induced change in the vesicle morphology; DSC analysis of DPPC SV shows no change in the phase transition peak at 41°C due to the standard acid addition. This observed insensitivity to acid is consistent with a report using ESR methodology that egg phosphatidylcholine liposomes are morphologically stable throughout the 2–11 pH range [32], unlike unsaturated ethanolamine analogs that form a hexagonal phase at low pH [33].

The biphasic protonation is not unique to sonicated vesicles, but is also evident for extruded vesicles. The literature characterizing these vesicle types clearly establishes that they are unilamellar [21,23], so the step-wise protonation cannot then be dismissed as an effect of vesicle multilamellarity. Chromate cargo is not unique in exhibiting the biphasic protonation. Structurally dissimilar CF shows the same behavior, with a quantitatively similar distribution of the fast and slow protonation, approximating one-half each fast and slow. It is notable that the time-frame for proton permeability across the vesicle membrane observed in the slow phase of Fig. 3 is comparable to that observed for an earlier fluorescence study of proton permeability across liposome membranes [12].

The possibility of electrostatic interaction of chromate at the membrane surfaces is not supported by the data. Both an increase in the ionic strength of the medium, and the inclusion of like charge into the bilayer, should diminish the association of an electrostatically bound load, which is not observed. Furthermore, neutral and positively charged cargoes would not be expected to load the vesicles by such a mechanism, yet experimentally they do load. Also, if chromate were electrostatically bound to the vesicle surfaces, the cargo associated on the outer surface of longer-chain DAPC SV would be expected eventually to break free at 45°C, leaving that on the inner surface locked inside. That is, the externally bound chromate should be freely capable of release into the bulk medium. However, no leakage at all was observed over 24 h.

The inverse processes of leakage/penetration of chromate from/into, respectively, the vesicles indicate that the cargo gradually makes a transition to/from a location that is accessible to the external acid probe. From Fig. 5a, it is further evident that the maximum load of chromate that penetrates into association with the vesicles approaches a limit of approx. 10 μ M, which translates to an upper LF% limit of approx. 3%. Clearly, the loading of the vesicles by external penetration is not limited by a surface saturation

of the membrane phospholipids, ostensibly at the choline moieties.

In our earlier study [24], we developed a method of estimating the mean diameter of our DPPC SV preparations using quantitative characteristics of the vesicle turbidity profile in the UV region. Specifically, the ratio of the measured turbidities at 250 and 350 nm was correlated with vesicle size. In the current study, the turbidity ratio of the unloaded and chromate-loaded SV was consistently in the range 3.5–3.7, corresponding to mean vesicle diameters of 80–90 nm. Using the approach of Deamer and Uster [18], the LF% of water-soluble cargo in 85 nm idealized spherical vesicles was estimated for the preparation conditions used in this study; viz, approx. 10 mM vesicled DPPC (based on approx. 65% yield of vesicles by the sonication method), 0.10 M cargo agent in PBS, and assuming approx. 0.50 nm² surface area/PL molecule in the bilayer [26]. As outlined in Section 2, a LF% of approx. 20% is obtained, a value dramatically higher than that observed for the hydrophilic cargoes used, which is highest for chromate at 8%, and lowest for caffeine and theophylline at 0%. Although cargo entrapment in excess of that expected from such an analysis of the trapped aqueous volume has been interpreted in terms of cargo binding with the lipid bilayer [3], measured entrapments of small cargoes less than expected have not received attention. Low interior aqueous concentrations of cargo have been found in multilamellar vesicles, but this effect is attributed to be a result of the sequential hydration of the original dry lipid, and the differential permeability of water and the cargo across the series of bilayers [34].

Both the variation of the LF% with the identity of the water-soluble cargo, and the substantially lower observed LF% values compared with the theoretical estimate, together suggest that the actual loading mechanism is not readily attributable to simple encapsulation or entrapment of the cargo solution within the internal aqueous volume of the vesicles. This conclusion is supported by the inability of BSA to load into the DPPC SV. The constancy of trapping efficiencies observed for a variety of small cargo types has been previously used to justify proposing an internalization of the cargo inside unilamellar phospholipid vesicles [35]. The lack of correlation of the LF% with the charge characteristics of the cargoes at pH 7 is also not consistent with an electrostatic interaction of the cargo with the bilayer surfaces.

If the cargo is not loaded entirely within the vesicles, nor on the vesicle surfaces, the possibility of some cargo inclusion within the bilayer itself must be considered. Although such a loading mechanism is well-accepted for hydrophobic cargo agents [4], its consideration for hydrophilic cargo as the dominant means of cargo sequestration is most unusual. This possibility appears to be further diminished by the results that chromate exhibits no detectable solubility in oleic acid or chloroform, neither with, nor without, the co-presence of aqueous PBS solution,

where these solvents are presumed to share the non-polarity characteristics of the hydrocarbon chains of the bilayer phospholipids.

By eliminating the logical alternatives, it is apparent that the association of chromate with the vesicles must inherently involve the hydrophobic membrane. However, the chromate is insoluble in non-polar solvent analogs. This apparent paradox suggests that the membrane assembly itself may be instrumental in cargo loading. Specifically, there are several lines of evidence suggesting that a significant amount of water exists within the bilayer. NMR studies have shown that at least the first 7 carbons in micelles composed of 16-carbon detergent molecules are in an aqueous environment, beyond which there exists a non-polar core [27]. The exceptionally high permeability of protons and hydroxyls compared to other ions across egg phosphatidylcholine vesicle membranes (above the gel to liquid crystalline transition temperature) has been rationalized on the basis of hydrogen-bonded water channels within the vesicle bilayer [11]. The ordered membrane evidently imposes an order on the associated water molecules that facilitates the channel for proton-transfer across it. Furthermore, it is reported in a hydrodynamic study of sonicated egg phosphatidylcholine vesicles that the total bound water, including surface associations, approaches 25 mol/mol PL [36].

The concept of water association within the bilayer phospholipid can be developed further. In Section 2, the LF% is estimated for cargo that is incorporated along with the associated water into the vesicle bilayer under the conditions used in this study, viz, approx. 10 mM vesicled DPPC, and 0.10 M cargo agent. It is assumed in this estimation that each of the first eight carbon atoms of each phospholipid has a water molecule in association with it when it assembles into the bilayer structure. Furthermore, it is assumed that the co-inclusion of cargo is purely random, based on the relative concentrations of the cargo (0.10 M) and water (55 M) in the bulk medium. An LF% of approx. 2% is estimated, which is at the low end of the range of the observed LF% values for the cargoes that loaded, and an order of magnitude less than the value estimated by assuming that the cargo is simply captured internally.

Based solely on these estimated loading factors, it appears uncertain whether the observed LF% of chromate and CF of approx. 7% represents an enhanced loading by the intercalation mechanism, or a diminished loading by internal entrapment. However, the external accessibility of a portion of these cargoes, indicated by their biphasic protonation behavior, tends to favor the enhanced intercalation option. The observed variability of LF% with cargo identity, extending between 0–8%, may then indicate a selectivity of inclusion/exclusion of the cargo along with the bulk medium into the inner and outer layers of the membrane. The influences of the intermolecular interactions among the cargo, the water, the head-group, and the

hydrocarbon chain may dictate this variability. Such influences are not wholly electrostatic, as rationalized earlier, but may be of sufficient magnitude to enhance or diminish the purely random basis for the cargo intercalation, as proposed above. A minor interaction of CF with vesicle membrane lipid has been proposed through time-resolved fluorescence studies [8].

It is notable that the activity coefficients of both sodium and potassium chromate are approx. 0.47 at 0.10 molal [37], indicating that substantial ion-pairing occurs among these ions in the solution medium used in this study. Significant ion-pairing of the chromate could thereby diminish the electrostatic barrier for its intercalation into the hydrocarbon region of the bilayer, beyond the phosphate moiety in the outer polar head-group of the phospholipids. Also, because of the structured molecular packing of the PL molecules in the bilayer assembly [26], it can be expected that the negative charge character of the phosphate moieties is significantly diminished by their network of zwitterionic interactions with adjacent choline moieties.

The observed biphasic protonation of loaded chromate below the T_c of the phospholipid supports the concept of limited penetration of water into the bilayer. After fast protonation of the accessible intercalated cargo in the outer layer, the central hydrophobic core presents a barrier to further protonation of the cargo located in the inside layer. The observation that the stoichiometry of the biphasic protonation for both chromate and CF proceeds approximately one-half fast and slow is further consistent with the suggestion that these cargoes have intercalated into both halves of the vesicle bilayer. This proposition is bolstered by the observation that the secondary slow protonation step of chromate loaded in C-20 DAPC vesicles proceeds about an order of magnitude slower than for the C-16 DPPC analog.

Above the T_c value, the greatly enhanced mobility of the hydrocarbon chains may effectively eliminate the barrier to proton transfer. The barrier evidently still remains substantial above T_c for the permeation of chromate from one side of the bilayer to the other in either direction, whether tested by leakage or penetration. However, the greater membrane permeability of H^+ relative to other ions is well established [12]. It is further notable that the observed upper-limit LF% value of chromate (approx. 3%) obtained by external penetration above T_c into empty DPPC SV (in Fig. 7a) is comparable to that estimated for chromate loading by the cargo intercalation mechanism (approx. 2%), and is clearly inconsistent with loading into the interior of the vesicles (approx. 20%). That this upper-limit value is about half of the observed LF% of chromate-loaded DPPC SV is consistent with an intercalation of cargo only into the outside of the bilayer.

The leakage profiles of chromate from DPPC SV at 20°C and 45°C in Figs. 4 and 5a can be interpreted further. At both temperatures, which are below and above T_c , respectively, it is evident that the leakage rate of chromate

decelerates over time. Specifically, the half-life for chromate release increases as the leakage progresses. This feature is also evident in the results of previously-studied leakages of chromate [25], [3H]glucose [38], CF [39], and [^{14}C]sorbitol [35] from unilamellar phospholipid vesicles. If the initial leakage of cargo is only from the internal aqueous volume of loaded vesicles, it should be impeded by the diffusional passage of the hydrophilic cargo across the hydrophobic core of the lipid bilayer before emerging into the exterior bulk medium. Although the kinetics of cargo leakage are probably not strictly Fickian [40], if it is assumed that there is no cargo initially within the membrane nor external to it after gel-filtration, at least an initial time lag would be expected as the cargo traverses the bilayer [41]. This lag feature would translate as a slower initial leakage of cargo, and, an accelerating rate of cargo leakage would then be observed. If on the other hand, the cargo is loaded initially as a distribution within each side of the bilayer, the initial leakage of outer cargo would proceed more rapidly than that in the inner side, analogous to a surface evaporation phenomenon [42], and be consistent with the observed decelerating leakage phenomenon. Although chromate leakage is rationalized here in terms of slow cargo migration through the bilayer, it is notable that for the leakage rates observed, the possible role of transbilayer diffusion (flip-flop) of the phospholipids themselves cannot be ruled out [43]. The results for CF in Fig. 6 confirm that the faster initial release of cargo is not unique to chromate-loaded DPPC SV, nor is it uniquely correlated with the specific methodology used to monitor it.

Although the weight of evidence presented here signals a need for reassessing the location of hydrophilic cargo in PL vesicles beyond that based on simple interior encapsulation, the alternative proposition of membrane intercalation of the cargo is not without its problems. Specifically, this proposition offers no clear explanation of why caffeine and theophylline do not load into DPPC vesicles, nor why chromate does not load into DMPC SV under the standard preparation conditions used. These results inherently require a process of reverse-osmosis that excludes solute sequestration during the energy-intensive conditions of vesicle preparation. In addition, an intercalation of cargo along with water into the bilayer would be expected to diminish with increasing size of the cargo molecules, but, while larger CF (MW = 377) does load, smaller caffeine (MW = 194) does not. The inability of BSA to load, however, shows a consistency with the cargo-intercalation mechanism. In the case of fluorescent CF, the observed self-quenching of the sequestered cargo requires that the original environment in the bulk of the preparation solution be maintained after the intercalation of the water and CF into the bilayer. Such a situation requires that the assembled saturated PL molecules are transparent to the self-quenching interactions among the intercalated CF molecules. Also, contrary to expectations [31], the sequestration of MB in DPPC SV did not provide insight into the

polarity of the location of this cargo; in our hands, MB was insoluble in non-polar solvents, and exhibited no absorption peak-shift when loaded into the vesicles.

The conventional understanding of the locations of cargo agents in PL vesicles is that hydrophilic agents are captured inside the internal volume, while hydrophobic agents are associated within the membrane bilayer [4]. This examination of both the protonation and leakage of loaded chromate, long used as a standard probe for determinations of encapsulation efficiency and captured volumes for hydrophilic cargo agents [18], casts doubt on the simplicity of the conventional assumptions about cargo location. Because a vesicle population emerges from a size-exclusion chromatography column with a hydrophilic cargo associated, does not, per se, establish that the cargo is loaded inside the vesicles, as most current practice assumes. While the location of hydrophilic cargo agents in unilamellar PL vesicles is not conclusive from this study, it is clearly evident that simplistic interpretations are inadequate, and that further studies of loaded liposomes are required to resolve this issue.

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