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Short communication

Pyrocatechol violet as a marker to characterize liposomal membrane permeability using the chelation and the first-order derivative spectrophotometry

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Abstract

Pyrocatechol violet (PV), a chelating agent for cupric ions was used to characterize liposomal membrane permeability. After cupric ions were added to PV liposomes, free PV turned into its chelate (PV-Cu), and encapsulated PV kept stable since liposomal membranes prevented metal ions from permeating. After the light scattering background of liposomes and the absorbance of PV were eliminated by the first-order derivative spectrophotometric method, PV-Cu i.e. free PV in liposome suspensions could be determined without separation. The released PV from liposomes could also be determined. Because PV release is relevant to liposomal membrane permeability, PV becomes a marker to characterize the membrane permeability. This new method was simple, rapid, sensitive, and was used to measure the temperature-dependent liposomal membrane permeability in this paper. Dipalmitoylphosphatidylcholine (DPPC) and soybean lecithin liposomes showed the peaks of release at 40 °C and 39 °C, respectively.

Keywords: Pyrocatechol violet; Chelation; Derivative spectrophotometry; Liposomes; Membrane permeability

1. Introduction

Liposomes (lipid vesicles) have been studied extensively as artificial biomembranes and drug delivery systems, and in biochemistry [1,2]. Liposomal membrane can function as control of the entrance and efflux of substances. Membrane permeability is very important because it determines chemicals' transfer, drug-loaded efficacy, in vitro/in vivo stability of liposomes, and etc. [3,4]. The release of encapsulated chemicals from lipid vesicles directly indicates membrane permeability. Generally, the released chemicals can be determined after being separating from liposomes by filtration, centrifugation or dialysis [5]. However the complicated and time-consuming processes of separation may promote unexpected release of chemicals and lead to errors. Some methods such as the self-quenching fluorescent methods were developed without separation steps [6]. But, the fluorescent agents such as carboxyfluorescein or calcein must be at high concentration (up to 100 mM), which may bring some inconvenience.

Derivative spectrophotometric methods are convenient to directly determine one ingredient in the mixture of chelating agents and their metal chelates after the absorption interference of another ingredient being eliminated [7]. The methods can also eliminate the interferences of some background signals such as the light scattering of liposomes [8]. A new method was developed to measure the release of encapsulated chemicals from lipid vesicles without separation procedures based on the above principles in the paper. When metal

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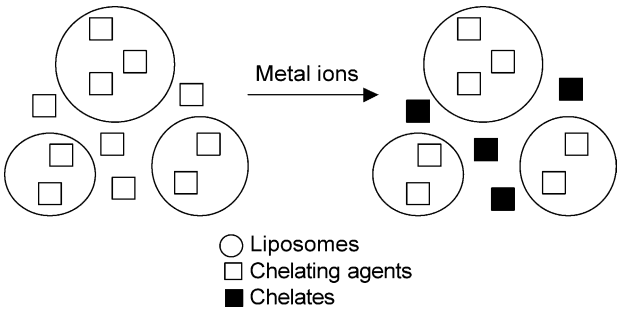

Fig. 1. Schematic illustration of the principle of the new method for determining the chelates in liposome suspensions directly.

ions are added to liposome suspensions containing chelating agents, only the chelating agents in the external aqueous medium turn into the chelates while those in the internal space of vesicles do not change since liposomal membranes prevent metal ions from permeating (Fig. 1). So, the chelates that represent the free chelating agents can be directly determined in the mixture after the interferences of the absorbance of chelating agents and the light scattering of liposomes are eliminated by the derivative spectrophotometric method.

2. Experimental

2.1. Materials

Pyrocatechol violet (PV) and dipalmitoylphosphatidylcholine (DPPC) were purchased from Acros Organics. Soybean lecithin was supplied by Shanghai First Fat Factory (China). All the other reagents were in analytical grade. Double distilled water and PV aqueous stock solution at 4 mM (pH 6.0) were used. PV-Cu chelates were prepared by adding an extra concentrated cupric sulfate solution (50 mM) to PV solution in the molar ratio of 20 or more.

2.2. Liposome preparation

Liposomes, in reverse-phase-evaporation vesicles (REVs), were prepared according to the method of Szoka and Papahadjopoulos [9] and 4 mM PV solution was encapsulated. DPPC (54.2 mg) or soybean lecithin (50.4 mg) was added to a 100 mL round-bottom flask, and 12 mL of chloroform/isopropyl ether (1:1, v/v) was used to dissolve the phospholipids. Four milliliters of water or PV solution was added to the organic solution, and then the mixture was sonicated briefly (10 min) in a bath-type sonicator (Bath Sonicator CX-300, Beijing Second Medical Instrument Co.) until the mixture became a homogeneous emulsion that did not separate for at least 30 min. The mixture was then placed on a rotary evaporator and the organic solvent was removed under reduced pressure (with a water aspirator) at 45 °C. When the solvent was removed, the mixture first formed a viscous gel and subsequently became an aqueous suspension. The aqueous suspension was evaporated for an additional 30 min at 50 °C to remove traces of the organic solvent. All liposomes were extruded through 450 nm polycarbonate filters. The gel filtration chromatographic column of Sephadex G-50 (Amersham Biosciences) was used to separate free PV from liposomes in the control experiments.

2.3. Spectrometric properties of samples

PV solution (30 μM) and PV-Cu solution (30 μM) were prepared in 50 mM sodium acetate buffer (pH 6.0). Blank liposomes containing 12 mg mL^{-1} phospholipid, were added to the above solutions to obtain the dispersions containing about 0.1 mg mL^{-1} phospholipid. Cupric sulfate solution (50 mM) was added to the buffer-diluted PV liposomes (containing about 0.1 mg mL^{-1} phospholipid) to chelate free PV. The molar ratio of additional cupric sulfate to total PV in the mixture was 20 to get the complete chelation. All of the samples above were scanned on a Shimadzu UV-2501PC double-beam spectrophotometer in the range of 400–800 nm at room temperature. The first-order derivative data of spectra were calculate by UVPC v3.91 Personal Spectroscope Software of Shimadzu with $\Delta\lambda = 10$ nm and scaling factor = 1.0.

2.4. Measurement of the release of encapsulated chemicals

The standard curves of PV ethanol solutions with the absorption maxima at 435.5 nm and PV-Cu buffer solutions according to the first-order derivative spectral data at 657.0 nm were prepared. PV liposomes containing about 4 mM PV, were diluted about eighty folds with buffer, followed by the addition of cupric solution (50 mM) and mixing to chelate free PV. The dispersion was diluted 100 folds with buffer at last and scanned. Then PV-Cu i.e. free PV in liposomes was determined. Total PV was determined after absolute ethanol dissolved PV liposomes from the same sample. Highly concentrated ethanol can ensure encapsulated chemicals to release completely [10,11]. Encapsulated PV was calculated after free PV was subtracted from total PV. Therefore, PV release was equal to the decrease of encapsulated PV or the increase of free PV. The classical gel chromatographic method was used to separate free PV from liposomes as control.

The temperature-dependent membrane permeability of liposomes was characterized by the release of encapsulated PV. The measurement details are described as follows. Sodium acetate buffer solutions (5 mL) were introduced into the dry tubes, sealed, and incubated in bath at designed temperatures for 5 min. PV liposomes (50 μL) were added to the tubes, mixed, and incubated for 10 min. The mixture was cooled to room temperature, immediately followed by the addition of 80 μL of cupric solution (50 mM), then mixed thoroughly, and scanned without delay. Free or encapsulated PV at each temperature point was determined.

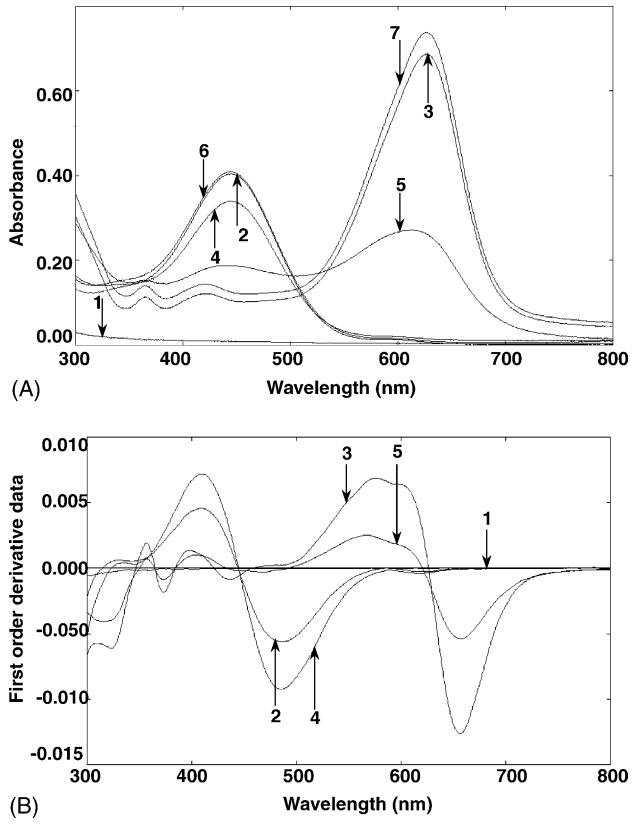

Fig. 2. The absorption spectra (A) and the first-order derivation (B) of the measurement system in sodium acetate buffer (pH 6.0). (1) The diluted blank liposome suspension; (2) PV solution; (3) PV-Cu solution; (4) PV liposomes; (5) the mixture of PV liposomes and cupric sulfate solution; (6) the mixture of PV solution and blank liposomes; (7) the mixture of PV-Cu solution and blank liposomes.

3. Results and discussion

3.1. Selection of measurement system

PV-Cu solution, the mixture of PV-Cu solution and blank liposomes, and the mixture of PV liposomes and cupric solution showed an absorption peak in the range of 600–700 nm, whereas PV solution and the diluted blank liposome suspension showed low and plane absorption curves in the same field (Fig. 2A). No interactions between PV or PV-Cu and liposomes were found. Upon the first-order derivation of these zero-order spectra, the interferences of PV absorption and liposomal light scattering were eliminated, and PV-Cu could be determined at 657.0 nm (Fig. 2B). Total PV could be determined by the zero-order spectrophotometry at 435.5 nm after liposomal lipid and PV were dissolved by absolute ethanol. The concentration of phospholipids in the detected samples should be below 0.5 mg mL^{-1}, above which the strength of liposomal light scattering increased significantly and the measurement errors were not negligible.

Because liposomal membranes can mostly prevent polar chemicals or ions from permeating, the key of this new method is that, as soon as the chemicals release from lipid vesicles, the released chemicals change to other forms with some physicochemical properties different from original chemicals. Chelating reaction happens quickly and completely. But this is not enough. The ideal measurement system should have the following characteristics: (1) the chemicals are freely soluble in water and soluble in alcohol; (2) the chelation takes place at neutral pH range with a high stability constant; (3) the chemical to be determined has a high molar absorbance coefficient and a long maximum absorption wavelength; (4) the significant spectral differences occur between the determined chemicals and the interfering components; (5) the chemicals keep stable on measurement; (6) no interactions occur between the chemicals and the lipid vesicles. PV is a sensitive metallochromic indicator with the triphenylmethane structure for determining copper, and high solubility in both water and ethanol. The instability constant of PV-Cu is 1.818×10^{-4} [12]. All the physicochemical properties of PV including its spectral characteristic make it as a good marker in this new method.

Calcium-loaded liposomes were prepared recently [13]. The addition of chelating agent to metal ions-loaded liposomes seems to become another measurement system. And, more measurement systems other than the chelation may be developed based on the above rules.

3.2. Chemicals release from liposomes depending on temperature

Total PV in liposomes was determined according to the standard curve of PV ethanol solutions obeying Beer's law in the range of 5–80 μM. PV-Cu i.e. free PV in the external medium of liposomes was determined according to the standard curve of PV-Cu buffer solutions in the same concentration range. Encapsulation fraction (E) was the ratio of encapsulated PV to total PV. E of DPPC liposomes was $22.3 \pm 0.3\%$ (mean ± S.D., $n = 3$) with the new method, and $18.2 \pm 0.2\%$ (mean ± S.D., $n = 3$) with the gel chromatographic method. In the case of soybean lecithin liposomes, E was $43.4 \pm 0.6\%$ ($n = 3$) and $41.2 \pm 1.0\%$ ($n = 3$) correspondingly. It could be concluded that the dilution and the long duration in the gel chromatographic method promoted a little release of PV from lipid vesicles.

Release of encapsulated PV can be given as:

$$R(\%) = \frac{([\mathrm{PV}]_f - [\mathrm{PV}]_{f0})}{([\mathrm{PV}]_t E)} \times 100 \quad (1)$$

in which, R is release percentage, the subscripts of [PV] such as f, $f0$ and t, represent free PV after releasing, initial free PV before releasing and total PV, respectively. In the Eq. (1), $[\mathrm{PV}]_t$ was equal to $[\mathrm{PV}]_{t0}/\beta$, where $[\mathrm{PV}]_{t0}$ is the concentration of total PV in the original liposomes, and β is the dilution folds. β was generally 100 in the paper. All parameters in the Eq. (1) could be got according to the above analytical steps, and the temperature-dependent PV release curves of DPPC and soybean lecithin liposomes were then obtained (Fig. 3). The maximum release temperature of DPPC

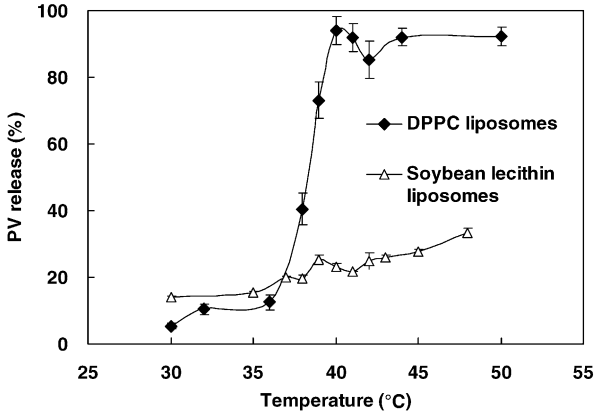

Fig. 3. The temperature-dependent release curves of encapsulated PV from dipalmitoylphosphatidylcholine (DDPC) liposomes and soybean lecithin liposomes. The data represented the mean ± S.D. ($n = 3$).

liposomes was 40 °C, which was in agreement with the literature [14]. Soybean lecithin liposomes had an insignificant peak of PV release at 39 °C, which could be attributed to that the natural phospholipids was composed of many kinds of phospholipid molecules with different phase transition temperatures (T_c) [15]. In fact, the membrane permeability was mainly determined by liposomal T_c. The liposomes prepared by the different phospholipids have various T_c, and generally the longer or the higher saturation the lipid chain is, the higher T_c [15]. The measurement of encapsulated molecules release from liposomes is a direct method to characterize liposomal membrane permeability.

PV is a good marker to characterize the temperature-dependent liposomal membrane permeability. Other factors disturbing membrane permeability could also be investigated by this method. In addition, if PV was used along with other hydrophilic compounds, PV could become an indicator for these compounds permeating membranes.

4. Conclusion

The permeability of liposomal membranes depends on temperature or surroundings. The increase of liposomal membrane permeability may lead to the release of encapsulated compounds. Separation of the released molecules from liposomes may bring measurement errors and inconvenience. By using PV as a marker to indicate encapsulated molecules releasing, it not only makes the determination of PV release more convenient but also facilitates the understanding of the influences of surroundings factors such as temperature. And, the new method had been applied to detect the temperature dependence of liposomal membrane permeability in the paper. It is simple, rapid, sensitive, and proper to determine small volume samples.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jpba.2004.10.019.

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