PHYSICOCHEMICAL PROPERTIES OF THERMOSENSITIVE LIPOSOMES ENCAPSULATING FLUORESCEIN DIPHOSPHATE

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Abstract. Fluorescein diphosphate (FDP) is a fluorogenic alkaline phosphatase substrate that has recently been isolated in pure form and made commercially available. FDP is colorless and nonfluorescent. Sequential alkaline phosphatase-mediated hydrolysis of the two phosphate substituents yields weakly fluorescent fluorescein monophosphate followed by strongly fluorescent fluorescein (excitation/emission ~ 490/514 nm). The long-term motivation behind this work was to develop methods in which chemical reactions can be initiated and followed in complex liposomes synthesized using combinatorial schemes so that rates and mechanisms of chemical reactions can be studied in environments that approximate true cellular environments. In this work negatively charged fluorescein diphosphate was encapsulated in thermosensitive liposomes composed of Dipalmitoylphosphatidylcholine (DPPC), Hydrogenated Soy phosphatidylcholine (HSPC), Cholesterol (Chol) at a molar ratio of (1:1:1). The release of FDP in response to 42 ºC heating was followed in a medium containing 10% serum and alkaline phosphatase enzyme. More that 60% of the encapsulated FDP was released in the outer medium in response to heating, once released, It emits strong fluorescent light at a wavelength of 514 nm indicating complete hydrolysis to FDP, yielding fluorescein monophosphate which emits fluorescent light upon excitation at a wavelength of 490 nm. The phase transition temperature of the liposomes was followed using a differentiated fluorescence scan. Right angle light scattering intensity at 436 nm per unit lipid concentration was measured. The optical parameters were explained by the Rayleigh – Gans – Debye theory in which the liposomes were modeled as homogenous spheres with mean refractive indices determined by the volume fractions of the lipids in vesicles.

Key words: Liposomes, fluorescein diphosphate, light scattering, cell models, fluorescence.

INTRODUCTION

Liposomes consisting of single phospholipid membranes (unilamellar vesicles) are excellent model systems for studying the dynamics and structural features of many cellular processes, including viral infection, endocytosis, exocytosis, cell fusion, and transport phenomena. In addition to having importance for basic research in biological disciplines, liposomes are used as vehicles for drug application [13], for gene transfer in medical therapy and genetic engineering [12], and as microcapsules for proteolytic enzymes in the food industry [14]. Vesicles also open many exciting possibilities for chemical reactions in small confined

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volumes, 10^{-12} to 10^{-21} liters. As examples, ion-channels, and photosynthesis machinery have been reconstituted in liposomes thereby having the capacity to serve as chemical drug-screening, and light-responsive/harvesting devices [2]. Liposomes have also been used to reconstruct simple cell models or properties of such models that are believed to be important in understanding the origin of life and evolution of primitive life forms [17].

Many of the elegant studies performed with liposomes are bulk experiments. Thus, they do not offer the capability of altering or designing the properties of a single selected liposome. Liposomes are composed of materials similar to those in cells and are cell-sized microscopic objects that are difficult to manipulate individually [19].

The size distribution of liposomes is commonly determined by dynamic light scattering and electron microscopy. These techniques are, however, time-consuming and therefore not appropriate for systems in which the particle size changes rapidly. Good examples are the aggregation, fusion and micellization of liposomes induced by peptides and other agents. In these cases, right angle light scattering [15] has often been continuously monitored using conventional spectrofluorimetry as a measure of vesicle size. However, the interpretation of the results is not straight forward, even if liposomes are assumed to be ideal monodispersed spheres. First, light scattering is known to be complicated oscillating functions dependent on both the particle size and wavelength of incident light. Second, liposomes are not optically homogenous particles.

As summarized by Kerker [11], Rayleigh scattering is a theory to explain light scattering by particles much smaller than the wavelength of incident light. Many studies have been performed to clarify the relationships among vesicle size, light wavelength and optical properties. The total volume of liposomes was found to be reciprocally proportional to turbidity at 450 nm [15]. In an earlier study, Chong and Colbow [8] calculated the specific 90º light scattering intensity as a function of vesicle size and aqueous radius in the framework of the Rayleigh – Gans – Debye (RGD) theory.

In this report, the development of miniaturized methods to mimic and manipulate cells and cell nanoenvironments, as well as methods to initiate chemical reactions in ultrasmall biological and biomimetic systems are treated. The release of FDP in response to 42 ºC heating was followed in a medium containing 10% serum and alkaline phosphatase enzyme. Right angle light scattering intensity at 436 nm per unit lipid concentration was measured, and treated in the light of RGD theory for light scattering from homogenous spheres. Phase transition temperature of the liposome system was measured using a modified fluorescence method.

THEORY

The intensity of light scattered by a sphere is dependent on the ratio of particle radius, R, to the wavelength of incident beam in the medium, λ. The size parameter, α, is defined by:

$$\alpha = \frac{2\pi R}{\lambda} \tag{1}$$

$$\lambda = \frac{\lambda_0}{n_W} \tag{2}$$

The wavelength of the incident beam in vacuum and the refractive index of the medium (water) are denoted by λ_0 and n_W, respectively. In this study, we investigated light scattering by liposomes at 436 nm where the refractive indices of both water (1.340) and lipid (n_L = 1.497) are known [8].

Liposomes are treated as optically homogenous spheres with the average refractive index, n_{av}, calculated according to [16]

$$\frac{n_{av}^2 - 1}{n_{av}^2 + 2} = f\,\frac{n_L^2 - 1}{n_L^2 + 2} + (1 - f)\,\frac{n_W^2 - 1}{n_W^2 + 2} \tag{3}$$

The volume fraction of lipid in the vesicles, f, can be estimated as follows:

$$f = \frac{3v_L}{RXA_L} \tag{4}$$

where v_L (= 1.3 nm^3) is the volume occupied by a lipid molecule [18]. A_L (= 0.68 nm^2) denotes the area per lipid [15]. The value of X can be given by:

$$X = \frac{4\pi R^2}{N_T A_L} \tag{5}$$

where N_T is the total number of lipid molecules per vesicle. The form factor for optically homogenous sphere is given by [11]:

$$P(\theta) = \left[\frac{3(\sin u - u\cos u)}{u^3}\right]^2 \tag{6}$$

$$u = 2\alpha \sin(\frac{\theta}{2}) \tag{7}$$

The lipid concentration is denoted by L (M^{-1}).

MATERIALS AND METHODS

MATERIALS

Hydrogenated Soy Phosphatidylcholine (HSPC) of > 98% purity was obtained from Nattermann phospholipid, Koln, Germany. Diplamitoyl - phosphatidylcholine (DPPC) of > 98% purity was purchased from Avanti Polar Lipids (Alabaster, Alabama). Cholesterol (Chol) of > 99% purity was obtained from Calbiochem (La Jolla, California). Fluoresecin diphosphate (FDP), high purity from Molecular Probes Inc. (Eugene, Oregon). Alkaline phosphatase enzyme (shrimp origin) from Organon Teknika Co. (Durham, North Carolina). Bovine serum from the cell culture facility of the Egyptian National Institute for Vaccine (Egypt, Cairo).

METHODS

Preparation of liposomes and FDP loading

Lipid mixture, composed of DPPC, HSPC, and Chol at a molar ratio of (1:1:1) were dissolved in Chloroform and placed in a round-bottomed flask. The solvent was removed in a rotary evaporator. After drying under vacuum, the residual lipid film was hydrated with Tris buffer containing fluorescein diphosphate (FDP). Unentrapped FDP was removed by gel filtration on Sephadex G-75. The concentration of FDP was adjusted to be 0.1 μmole FDP/μmole phospholipid. The suspension was subjected to five freeze – thaw cycles to produce multilamellar vesicles (MLV). The freeze thaw procedure reduces the lamellarity of the vesicles [6]. Small unilamellar vesicles (SUV) were obtained by sonicating the MLV for 30 min to clarity on ice under an atmosphere of Nitrogen. The lipid concentration was determined in triplicate by phosphorus analysis [4].

Release Kinetics of Fluorescein diphosphate from liposomes

In order to measure the release behavior of FDP from thermosensitive liposomes in response to heat, 0.13 μmole from liposomes encapsulating FDP was added to 20 μl buffer (dephosphorylation buffer adjusted to pH 8.5) containing 30 μl bovine serum and alkaline phosphatase. The whole mixture was incubated in a small glass vial at 37 ºC and 5 μl was taken each 15 minutes and diluted to 2 ml with HEPES buffer. The fluorescence intensity was measured at an excitation wavelength of 498 nm and 514 nm emission wavelength. Tritton X-100 was added at the end of the time course and the percentage of FDP release was estimated. The same experiment was repeated at 42ºC and the percentage of FDP release was estimated at 42ºC. For each sample collected after 15 minutes, the temperature was adjusted to 37 ºC again in order to stop the release of FDP from liposomes at the intact period of time. Since the liposome composition is thermosensitive and would not release any of the FDP contents unless heated to the desired phase transition

temperature, we believe that our kinetics curve at 42 ºC reflect the true kinetics behavior for the release rate from liposomes.

Phase transition temperature measurement for liposomes

In order to measure the phase transition temperature of the liposome sample, liposomes encapsulating calcein, were prepared following the same procedure described above. However, instead of hydrating liposomes with FDP, we used 20 mM solution of calcein adjusted to pH 7.4 and 290 osmolarity. The release of calcein from liposomes was determined by fluorimetry using 490 nm as an excitation wavelength and 520 nm as an emission wavelength. The release of calcein was monitored as a function of temperature with a heating rate of 0.5ºC/min. Differentiated fluorescence intensity was plotted as a function of temperature, where the phase transition temperature of the liposome sample can be estimated.

Right angle light scattering

The right angle light scattering intensities at various wavelengths were determined on the spectrofluorometer by simultaneously scanning the excitation and emission wavelengths (synchronous spectra). The scattered light intensity at 436 nm was measured on a Schimadzu RF – 1501 Spectrofluorimeter. During the measurement, the temperature of the sample was maintained at 25 ºC.

RESULTS

Figure 1 shows the effect of temperature on the fluorescence spectrum of FDP encapsulated in thermosensitive liposomes, before and after adding Tritton X-100 detergent to liposomes. The upper graph shows the effect at 37 ºC. Middle graph, shows the same effect at 42ºC, while lower graph shows the effect at 42ºC after 30 minutes incubation time.

Figure 2 shows the release of FDP from thermosensitive liposomes at 37ºC and 42ºC in a medium containing 10% serum and alkaline phosphatase enzyme. It is clear from the figure that 60% of the encapsulated FDP was released from liposomes upon heating to 42ºC. The plateau of the release curve starting after 30 minutes indicates full consumption of the hydrolyzing phosphatase enzyme giving a typical enzyme kinetics curve.

Figure 3 shows the phase transition temperature of the liposome sample as measured by a differentiated fluorescence thermal technique. It is clear from the figure that the phase transition temperature is ~ 42 ºC.

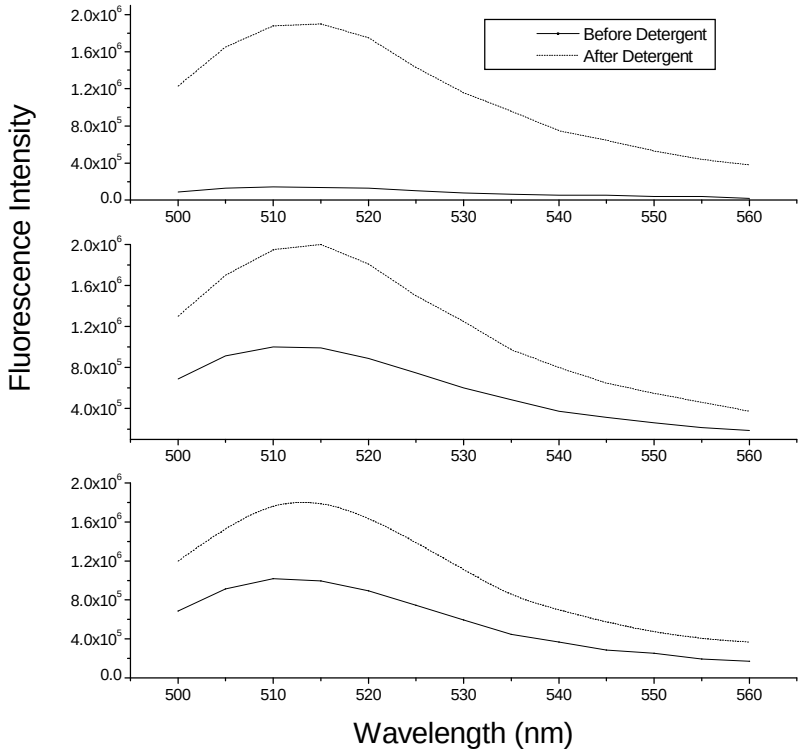

Fig. 1. The effect of temperature on the fluorescence spectrum of FDP encapsulated in thermosensitive liposomes. The upper graph shows the effect at 37 ºC. Middle graph, shows the same effect at 42 ºC, while lower graph shows the effect at 42 ºC after 30 minutes incubation time.

The right angle light scattering intensity $I(90^o)$, at 436 nm is plotted as a function of lipid concentration in Figure 4. The intensity was proportional to lipid concentration, indicating the absence of any effects due to multiple scattering. The theoretical relative $I(90^o)/L$ values were calculated based on the homogenous model described in the theoretical section and shown in Figure 5. The average size of the liposome sample was estimated and was found to be near 100 nm.

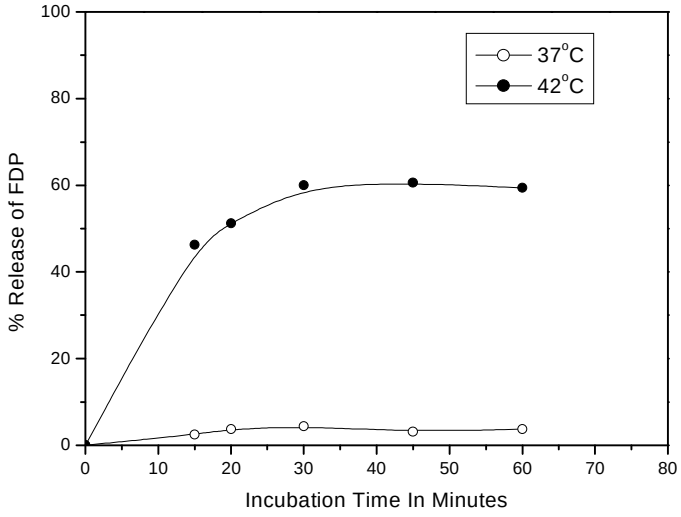

Fig. 2. The release of FDP from thermosensitive liposomes at 37ºC and 42ºC in a medium containing 10% serum and alkaline phosphatase enzyme.

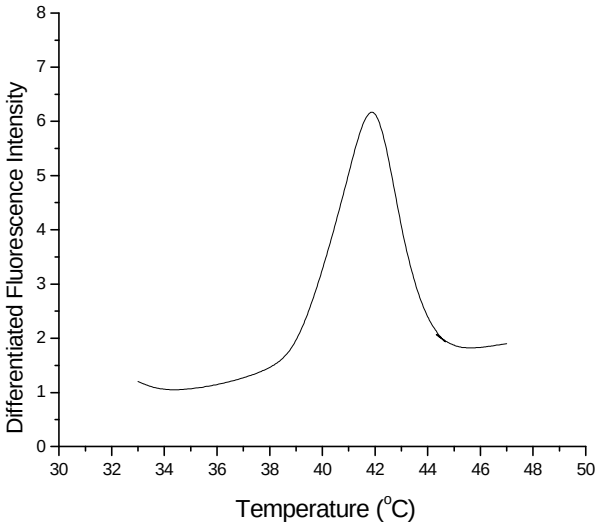

Fig. 3. The phase transition temperature of liposomes composed of DPPC/HSPC/Chol at a molar ratio of (1:1:1).

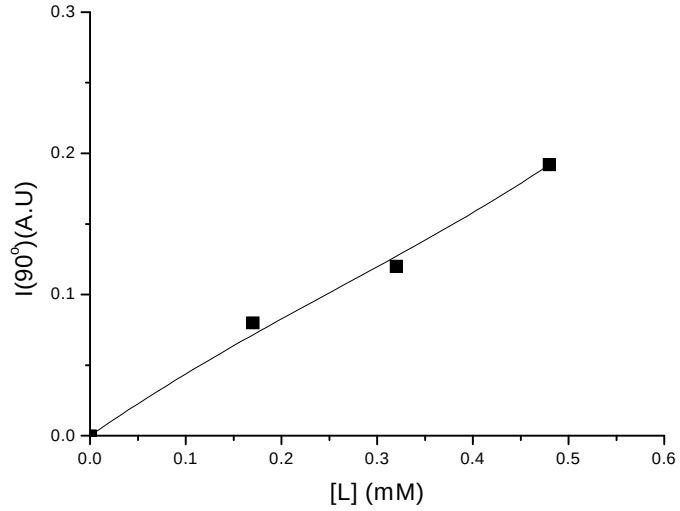

Fig. 4. Dependence of right angle light scattering intensity on lipid concentration at 436 nm.

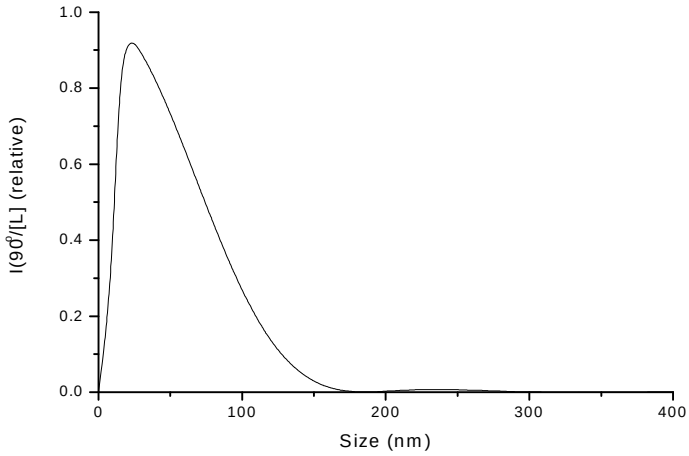

Fig. 5. Simulation of Optical properties. Specific light scattering at 436 nm simulated by homogenous sphere model.

DISCUSSION

Living systems usually carry out biochemical transformations within cellular compartments defined by a phospholipid bilayer boundary. At such small dimensions [zeptoliters (10–21 liters) to femtoliters (10–15 liters)], the surface-to-volume ratio is very high and the contained molecules experience collisions with the phospholipid surface at high frequencies. A hard-sphere approximation and a simulation of Brownian motion indicate that, in a 170 nm diameter vesicle, a single enzyme and a single substrate collide at a frequency of 300 kHz, which might be compared with the substrate-wall collisional frequency of 200 MHz. Thus, the biochemical reactivity of the contained molecules can be dominated by surface interactions, and such interactions can profoundly influence enzyme kinetics [7]. A tool to study confined chemical reactions under biologically relevant conditions would offer valuable insights into *in vivo* reaction conditions.

Various approaches exist for carrying out chemical reactions in aqueous solutions at small dimensions [3]. Most open volume methods involve micromachining techniques [5, 10] by which nanoliter to femtoliter wells can be created in silicon-based substrates. For self-enclosed volume elements, microdroplets in an immiscible solvent have been used. However, these techniques do not create the biologically relevant nanoenvironment that can be achieved in lipid vesicles [9].

Using a recently developed rotaevaporative technique, we prepared unilamellar vesicles between 100 nm and 200nm in diameter from a wide range of different phospholipids [6]. The vesicles can encapsulate one or more reagent molecules of choice. Because of the small volume element in a vesicle, the initial number of substrate molecules is limited and typically becomes largely depleted during the time required to prepare the vesicle. One way to overcome this drawback is to use the lipid bilayer as a partition between the reactants. The reaction can then be initiated by heating the liposomal preparation to their phase transition temperature.

In this work we succeeded to perform an enzymatically chemical reaction using a nanoliters liposome reservoirs. The reaction was initiated by heating the liposomes to a temperature very close to their phase transition and within a hyperthermia range (42 ºC). The designed nanoliter reservoirs were characterized using a 90º light scattering technique. The data were analyzed in the light of Rayleigh – Gans – Debye (RGD) approximation assuming that the vesicles are optically homogenous spheres with average refractive indices determined by the volume fraction of lipid in the vesicle. Using the simulation model of the RGD theory, we found that the fitting quality can be improved if the size distribution of liposomes was taken into consideration. However, its precise determination is difficult [1]. For example, the size distribution of a given sample by the dynamic light scattering histogram method differs between measurements. Size measurement by electron microscopy is time consuming and requires collection of huge numbers

of vesicles. In contrast, the cumulant radii used here were reproducibly measurable and gave rough estimates of optical parameters.

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