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Biophysical Chemistry 104 (2003) 449–458

Biophysical Chemistry

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Interaction between DMPC liposomes and HM-PNIPAM polymer

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Received 24 October 2002; received in revised form 30 January 2003; accepted 31 January 2003

Abstract

The interactions of hydrophobically-modified poly-(*N*-isopropylacrylamides) (HM-PNIPAM) and dimyristoylphosphatidylcholine (DMPC) vesicles were investigated by the effect of the polymer on the binding of a fluorescent dye, oxonol VI, to DMPC vesicles, and on its diffusion across the membrane. On mixing with the vesicles, the dye exhibits an increase in fluorescence, which occurs in a two-stage process. The process was monitored by stopped-flow fluorescence spectrophotometry. According to the dependence of the reciprocal relaxation time on vesicle concentration, the rapid stage seems to be due to the second-order binding of the dye to the lipid membrane, a process that is almost diffusion-controlled, whereas the slow process is attributed to movement of the dye within the membrane phase. The polymer did not significantly affect the rate constant of the binding step, but it slowed down slightly the dissociation process of the dye from the membrane. However, the polymer affected the second stage, causing an increase in the reciprocal of its relaxation time, which suggests that the polymer makes the vesicle membrane more fluid.

Keywords: Dye binding; DMPC liposome; HM-PNIPAM polymer; Fluorescence

1. Introduction

Liposomes, i.e. lipid vesicles, can be used as carriers for the selective delivery of drugs to cells and tissues. Unmodified liposomes, however, are susceptible to aggregation or fusion as well as uptake and clearance by the immune system, which weaken the effectiveness of drug delivery within the body. Hydrophilic polymers are considered to be capable of improving the circulation times and stability of vesicles within tissues by forming a coating on the surface of the vesicles. Some polymers have been studied as potential lipid vesicle protectors, such as polyethylene glycol (PEG) [1,2], polysaccharides [3], polyoxazolines [4], and modified polyacrylamides [5]. Important properties of protective polymers such as PEG are their hydrophilicity, biocompatibility, molecular geometry, conformation, and high flexibility [1,6]. The use of PEG as a modifier for lipid vesicle surfaces has been shown to increase their circula-

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doi:10.1016/S0301-4622(03)00034-6

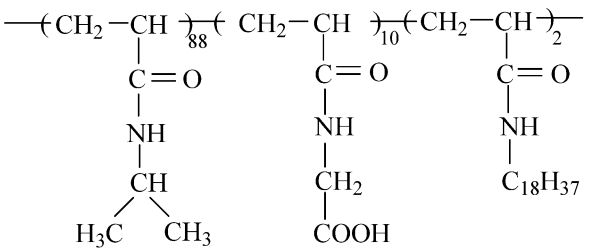

Fig. 1. Structure of HM-PNIPAM.

tion times up to 10-fold [7,8]. Hydrophobically modified poly-(*N*-isopropylacrylamides) (HM-PNIPAM) share several of these same characteristics, and thus have also been recognized as promising candidates for liposome protectors. Many researchers have studied the use of HM-PNIPAM derivatives as liposome coatings [9–15].

Oxonol VI has a pK_a near 4.2 [16] and is thus anionic at physiological pH values. The negative charge of the anionic dye is delocalized by resonance over the entire molecule, so that the dye remains membrane permeable. As it is extremely hydrophobic, it binds strongly to lipid membranes and changes in its absorbance and fluorescence spectra are observed upon binding to the membrane. At low dye/lipid concentration ratios, a fluorescence enhancement occurs, an effect probably due to the restricted motion of the dye molecule and to its protection from quenching agents once it is incorporated in the lipid phase. In previous work [17,18], we have used stopped-flow fluorescence spectrophotometry to explore the interaction of oxonol VI with lipid vesicles. It was found that on mixing with vesicles in the stopped-flow apparatus, two relaxation phases were observed, which are widely separated in terms of their time scales, the faster process occurring in the range of tens of milliseconds and the slower in the range of seconds.

In this work, a temperature- and pH-sensitive water-soluble polymer, hydrophobically modified poly(isopropyl acrylamide) (HM-PNIPAM) having the structure shown in Fig. 1 was used as a coating of dimyristoyl phosphatidylcholine (DMPC) liposomes. The polymer possesses a small fraction of carboxylic acid groups, so that it is soluble at 30 °C in solutions of physiological pH, the conditions used in this study [19–21].

In order to investigate the effect of the polymer on the vesicle structure, the fluorescent probe, oxonol VI (see Fig. 2) was introduced into the vesicle system. The stopped-flow technique was employed to observe the binding of dye molecules to the lipid and their diffusion process across the membrane by analyzing the patterns of change in fluorescence intensity. Using the stopped-flow technique with the aid of a fluorescent dye is a novel way for the investigation of the effect of polymers on liposomes, by analyzing the kinetics of the fluorescent dye's interaction with the membrane in the presence and absence of polymer. Photon correlation spectroscopy was used to determine the diameter of vesicles.

2. Experimental section

2.1. Materials

Dimyristoylphosphatidylcholine (DMPC) and oxonol VI were purchased from the Sigma Chemical Company (Castle Hill, NSW, Australia) and Molecular Probes (Eugene, OR, USA), respectively. The hydrophobically modified poly(*N*-isopropylacrylamide) was synthesized as previously reported [21].

2.2. Stopped-flow measurement

The interaction of oxonol VI dye molecules with the lipid membrane was investigated by means of stopped-flow fluorescence spectrophotometry using an SF-61 stopped-flow spectrofluorimeter from Hi-Tech Scientific (Salisbury, UK). Changes in fluorescence intensity were detected at right angles to the incident light beam, when

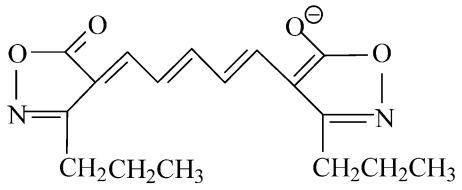

Fig. 2. Structure of oxonol-VI.

mixing a 120 nM dye solution with an equal volume of vesicle suspension at a temperature of 30 °C, a temperature which lies above the phase transition temperature of the lipid membrane (23 °C) and below the critical temperature of the polymer. The excitation wavelength was 577 nm and the fluorescence emission was observed at wavelengths ≥ 630 nm. Each individual kinetic trace consisted of 1024 data points. To improve the signal-to-noise ratio, typically between 6 and 20 experimental traces were averaged before the reciprocal relaxation time was evaluated.

2.3. Sample preparation

2.3.1. Lipid vesicles (LUV)

Unilamellar DMPC vesicles were prepared by the ethanol injection method [22]. Approximately 0.27 g of DMPC was dissolved in 1 ml of ethanol to form a solution, which was injected under agitation into 10 ml of buffer at 30 °C to form a vesicle suspension. The vesicle suspension thus formed was then transferred to dialysis tubing and dialyzed at 30 °C for 72 h against approximately 500 ml of the buffer, refreshing the buffer every 24 h. All buffer solutions used in this work consisted of 30 mM Tris, 150 mM NaCl, and 1 mM EDTA, with the pH value being adjusted to 7.2 with HCl. The vesicle size was measured by photon correlation spectrometry (PCS) using a wavelength of 514 nm with a photon correlation spectrometer from Malvern Instruments Ltd. (Malvern, UK). The concentration of the lipid in the vesicle suspension thus prepared was measured by the phospholipid B test from Wako (Osaka, Japan) with a Varian (French's Forest, Australia) DMS80 UV-Visible spectrophotometer.

2.3.2. Lipid–polymer mixtures

Polymer stock solutions (6.45 g l^{-1}) were prepared in the buffer. DMPC liposome suspensions and polymer solutions were mixed in prescribed proportions to obtain the desired final lipid/polymer ratios by weight, and allowed to equilibrate for 2 h at 30 °C before measurement. Unless otherwise specified, all the ratios between lipid and polymer in the work were 10/1 by weight.

3. Results

When dye oxonol VI was rapidly mixed with vesicles (both with and without polymer) in the stopped-flow apparatus, two relaxation processes were observed, which were characterized by an increase in the fluorescence intensity as shown in Fig. 3. The faster process occurred within tens of milliseconds and the slower one within the range of seconds.

When the reciprocal relaxation time of the faster stage was plotted as a function of the DMPC lipid concentration for systems both with (Fig. 4a) and without polymer (Fig. 4b). In both cases straight lines were obtained, indicating that the processes being observed are due to the binding of dye to the vesicle membrane.

According to Clarke and Apell [17], a vesicle can be considered as a supermolecule with a large number of binding sites for dye. The 'molecular weight' of a vesicle is then given by

$$M_v = N_A[4\pi/3\cdot(r_0^3 - r_i^3)]/\bar{\nu} \quad (1)$$

where r_0 and r_i denote the external and internal radii of the vesicle, respectively, N_A Avogadro's number, and $\bar{\nu}$ the partial specific volume of lipid in the vesicle membrane. It is known that the vesicle radii follow a Gaussian distribution, which causes an uncertainty in the value of M_v and the number of lipid molecules in a vesicle. The numbers given here are valid for an average vesicle with a mean radius. For large vesicles of dimyristoylphosphatidylcholine in the liquid crystalline phase $\bar{\nu}$ has been determined to be approximately 0.97 $cm^3\ g^{-1}$ [23]. According to PCS measurements the external radius, r_0, of the vesicles in the absence of polymer is 46 nm. Assuming a bilayer thickness of 3.5 nm [23], the internal radius, r_i, can be calculated to be 42.5 nm. Introducing these values into Eq. (1) gives $M_v = 5.35\times10^7$ g mol^{-1}. The molecular weight of dimyristoyl-phosphatidylcholine, M_1, is 677.95 g mol^{-1}. Thus, the average number of lipid molecules per vesicle, n, is given by [17]:

$$n = M_v/M_1 = 79\,000 \quad (2)$$

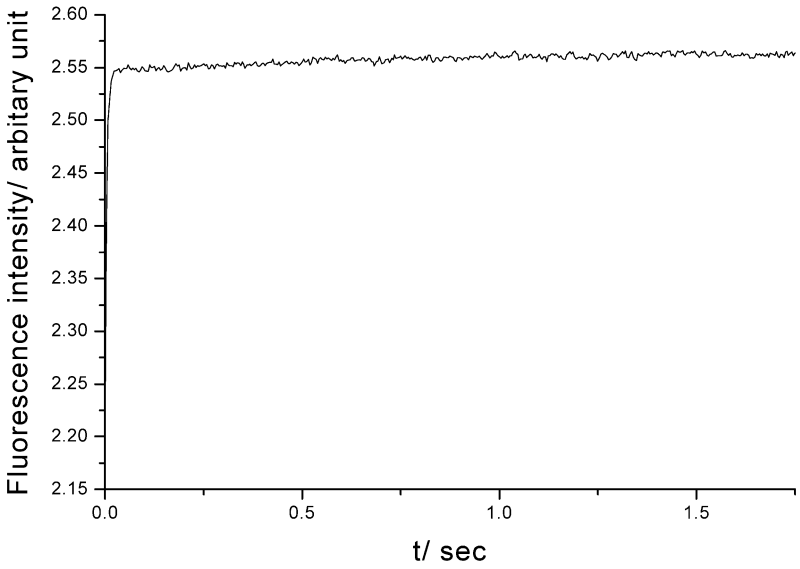

Fig. 3. Changing trace of fluorescence intensity in stopped-flow experiment for DMPC-polymer/oxonol VI system: [DMPC] = 135.6 μM; [oxonol VI] = 60 nM; and $T = 30$ °C.

This value was taken here as an approximation to convert the concentration of lipids into that of vesicles. The reciprocal relaxation times for both fast (Fig. 5a,b) and slow (Fig. 6a,b) stages were plotted vs. the DMPC vesicle concentrations for cases with and without polymer. From the slopes of the plots in Fig. 5a,b, the rate constants for the binding stage of the dye to vesicles can be estimated as $7.1(\pm 0.3)\times 10^{10}$ M^{-1} s^{-1} and $6.3(\pm 0.7)\times 10^{10}$ M^{-1} s^{-1}, respectively, indicating no significant difference between the values with and without polymer.

The rate constants for the binding of dye molecules to vesicles can be compared to the theoretical diffusion-controlled values of the rate constant, calculated from the Smoluchowski equation. Since the vesicles are much larger than dye molecules, they can be considered as remaining stationary during the course of the reaction and their diffusion coefficient may therefore be neglected. The radius of collision can also be approximated by the vesicle radius. Thus, the diffusion-controlled rate is given by

$$k'_{\mathrm{diff}} = 4\pi N_A D r \qquad (3)$$

where D denotes the dye diffusion coefficient and r the vesicle radius. The diffusion coefficient of oxonol VI is equal to 6.7×10^{-6} cm^2 s^{-1} [24]. Using these values and an average vesicle radius of 46 nm (according to PCS measurements), the theoretical diffusion-controlled rate constant k'_{diff} for the dye oxonol VI is calculated to be 2.32×10^{11} M^{-1} s^{-1}. The k'_{diff} is comparable to the experimentally determined values of the binding rate constants. This indicates that the dye-binding step is very close to being diffusion-controlled.

From the intercept of the reciprocal relaxation time at infinite dilution of the vesicles, the rate constant for dissociation of dye from the membrane can be determined. Values of $140(\pm 5)$ s^{-1} and $182(\pm 8)$ s^{-1} were found for the cases with and without polymer, respectively. The polymer therefore appears to slow down the dissociation of dye from the vesicles.

For the slow stage that occurs subsequent to the binding process, the reciprocal relaxation time is independent of vesicle concentration when polymer is absent and has a slight increasing tendency with increasing vesicle concentration when polymer is present (see Fig. 6b,a). The relative inde-

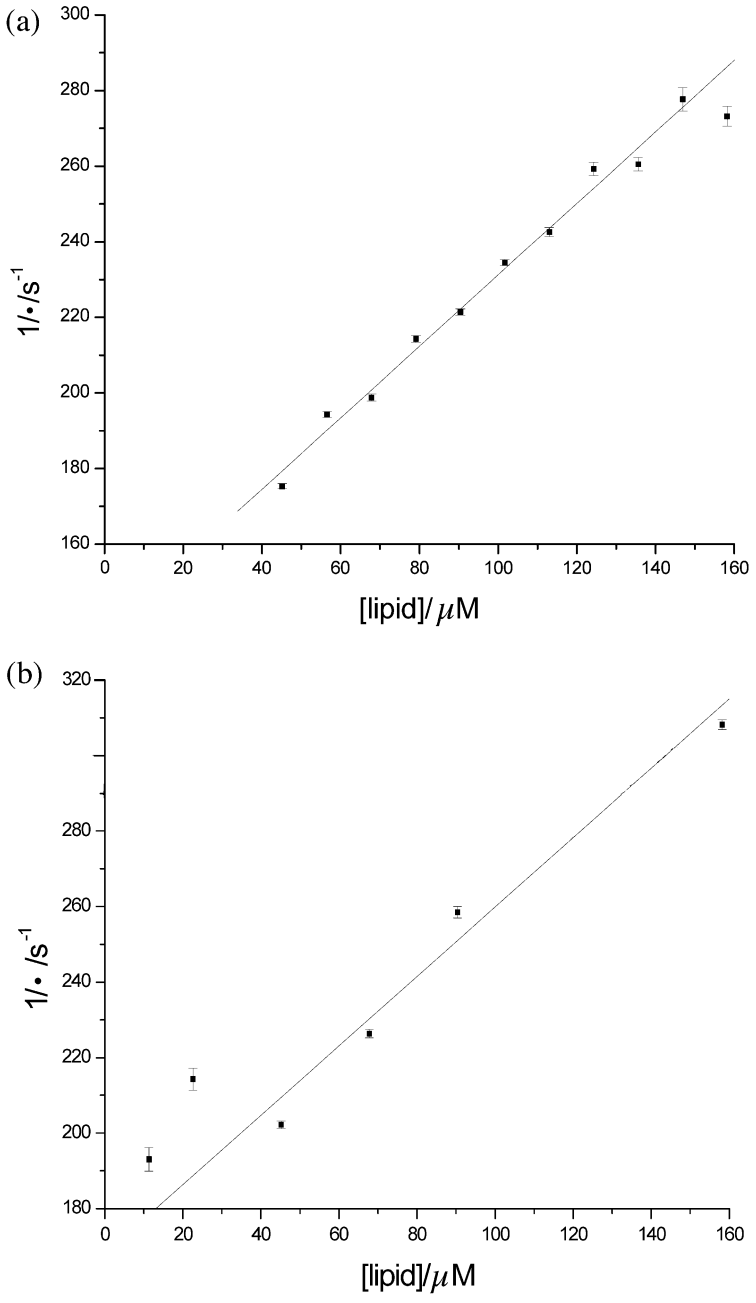

Fig. 4. (a) Dependence of the reciprocal of relaxation time on lipid concentrations in fast stage for DMPC-polymer/oxonal VI system; [oxonol VI] = 60 nM, T = 30 °C. (b) Dependence of the reciprocal of relaxation time on lipid concentrations in fast stage for DMPC vesicle/oxonal VI system; [oxonol VI] = 60 nM, T = 30 °C.

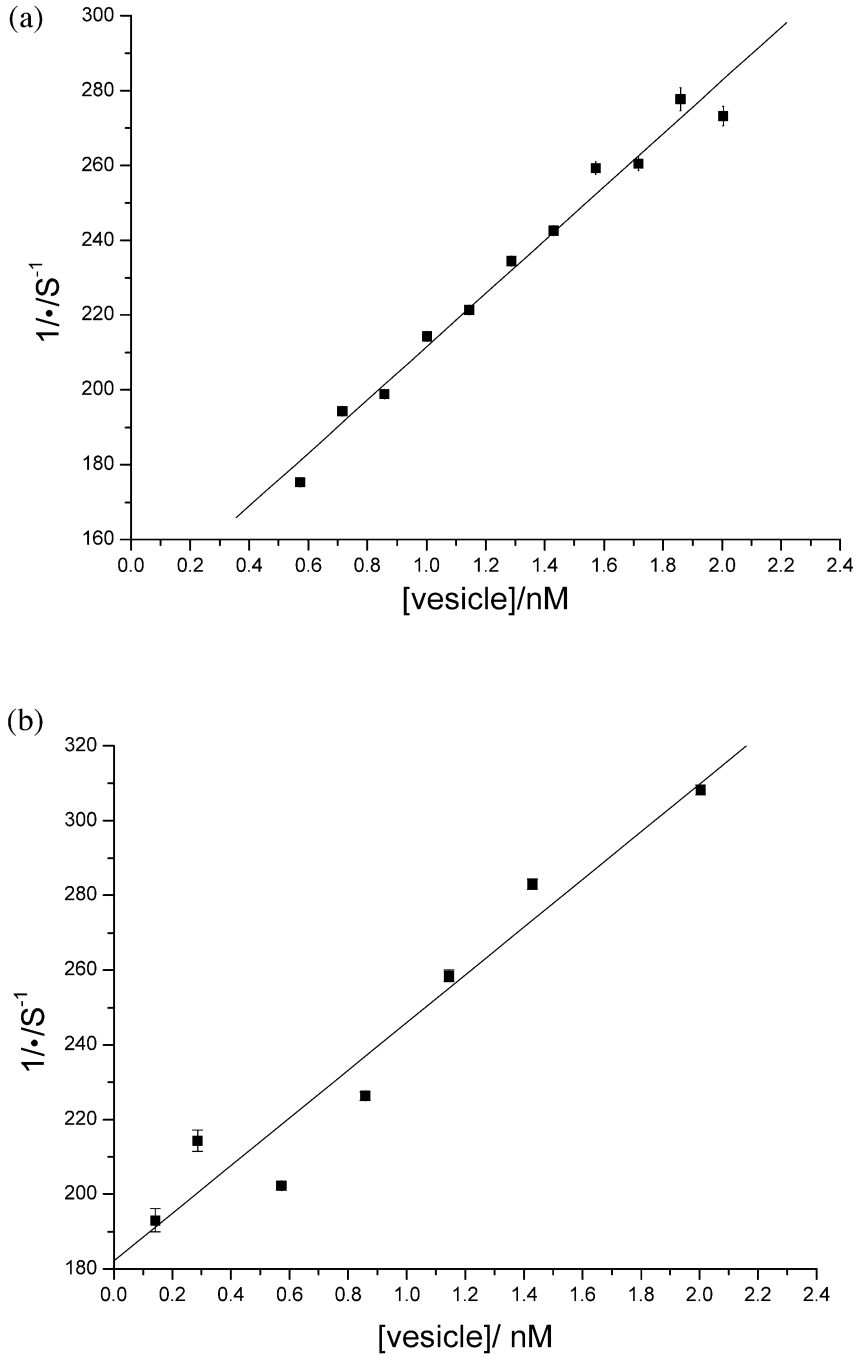

Fig. 5. (a) Dependence of the reciprocal of relaxation time on vesicle concentrations in fast stage for DMPC-polymer/oxonal VI system; [oxonol VI] = 60 nM, T = 30 °C. (b) Dependence of the reciprocal of relaxation time on vesicle concentrations in fast stage for DMPC vesicle/oxonal VI system; [oxonol VI] = 60 nM, T = 30 °C.

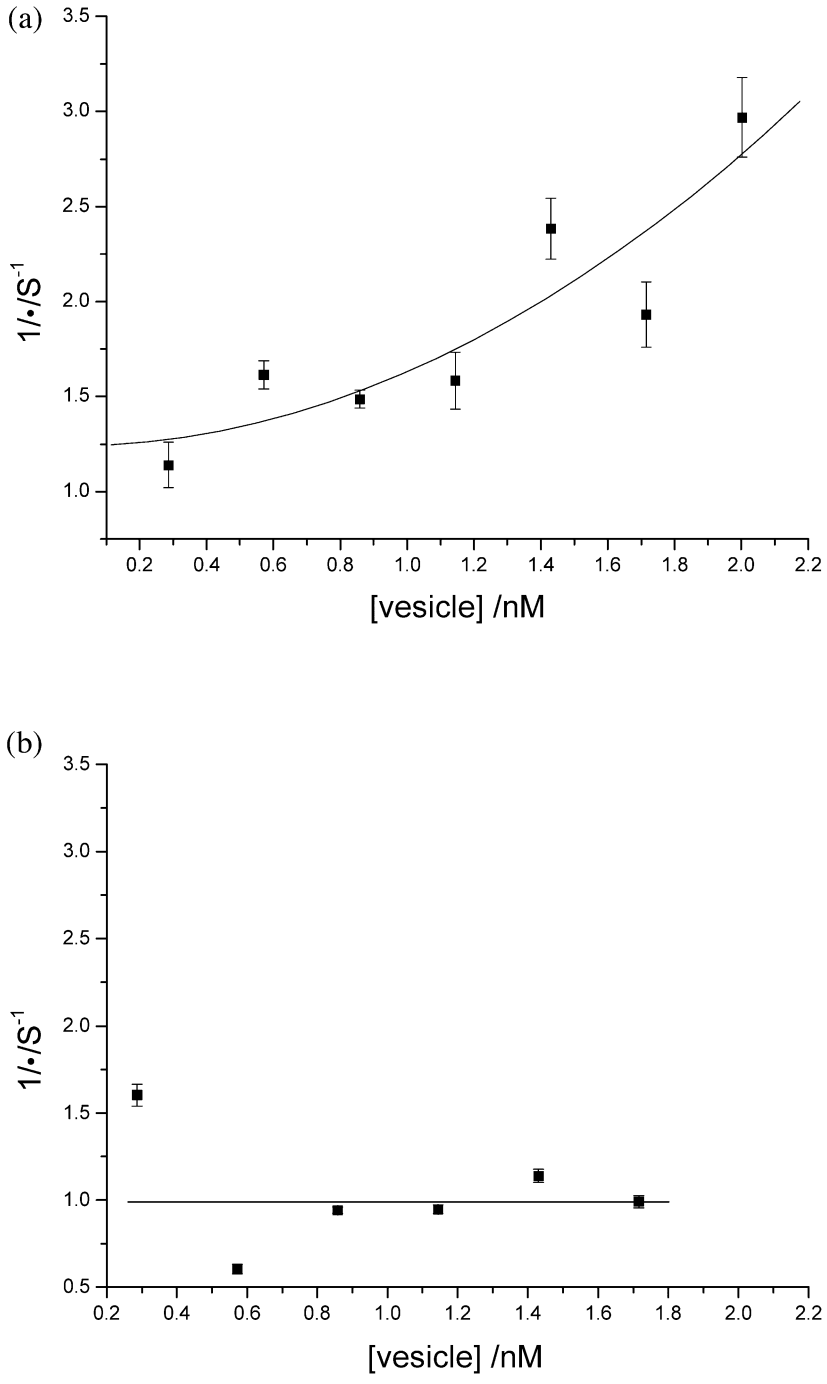

Fig. 6. (a) Dependence of the reciprocal of relaxation time on vesicle concentrations in slow stage for DMPC-polymer/oxonal VI system; [oxonol VI] = 60 nM, T = 30 °C. (b) Dependence of the reciprocal of relaxation time on vesicle concentrations in slow stage for DMPC vesicle/oxonal VI system; [oxonol VI] = 60 nM, T = 30 °C.

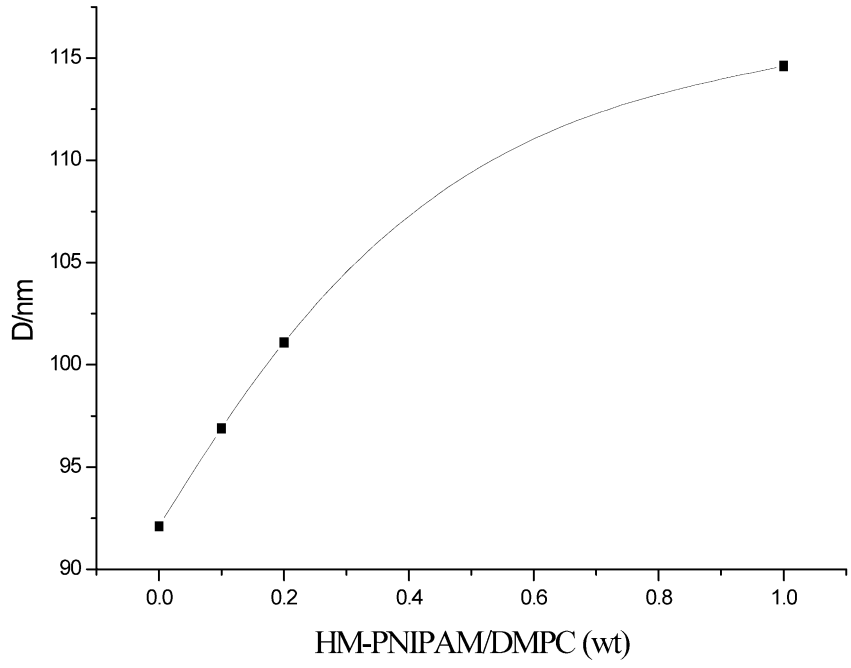

Fig. 7. Diameters of DMPC vesicle at different HM-PNIPAM/DMPC ratios by weight; [DMPC] = 0.326 mM.

pendence of the reciprocal relaxation time on the vesicle concentration for this phase implies a first order process, most likely dye diffusion within the membrane.

In order to further investigate the effect of polymer on the vesicle structure, the size of vesicles with different amounts of polymer were measured as shown in Fig. 7. It can be seen from Fig. 7 that the size of the DMPC vesicles tends to increase with increasing amount of polymer.

4. Discussion

The interaction between oxonol VI molecules and DMPC membrane exhibits a two-stage process both in the presence and absence of polymer. The presence of polymer has no significant effect on the fast process. The association rate constants for the cases with and without polymer are of the same order of magnitude as the theoretical diffusion-controlled constant, indicating that the binding of the dye to the lipid membrane is a diffusion-controlled process and that the polymer does not interfere with this process.

It can be seen that the polymer affected the second stage, causing an increase in the reciprocal of its relaxation time, which suggests that the polymer makes the vesicle membrane more fluid and does not hinder at all the movement of dye across the membrane. Such behavior is advantageous for the use of polymer-modified vesicles as drug carriers, since the polymer would be expected to increase the vesicles' circulation time without inhibiting drug release.

The changing tendency of the vesicle size with polymer would appear to contradict the results of previous work [12,13], in which it was found that HM-PNIPAM leads to the partial extraction of lipids from large vesicles and the formation of smaller polymer-coated vesicles. It is important to note, however, that the diameter of the vesicles used in the previous work was much larger (400 nm) than that of the vesicles used here (92 nm).

Since the vesicles used here are already relatively small, perhaps no further lipid extraction occurs and the increase in size can be explained by adsorption of polymer to the vesicle surface.

Acknowledgments

We are grateful to Professor Robert Gilbert for valuable discussions and for his support of this work. The Key Centre for Polymer Colloids is established and supported under the Australian Research Council's Research Centre Program. Also, the work was supported by the China Scholarship Council.

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