Permeability Characteristics of Lipid Bilayers from Lipoic Acid Derived Phosphatidylcholines: Comparison of Monomeric, Cross-Linked and Non-Cross-Linked Polymerized Membranes†,[1]

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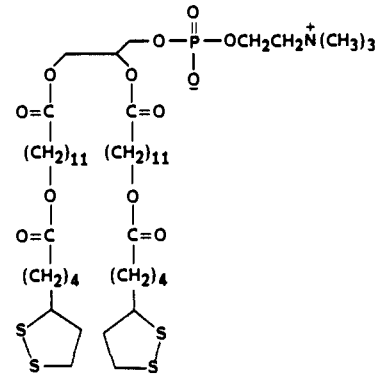
Abstract: Polymerized and monomeric forms of large unilamellar liposomes (ca. 1000-Å diameter) have been prepared from a cross-linkable phospholipid, 1,2-bis[12-(lipoyloxy)dodecanoyl]-*sn*-glycero-3-phosphocholine (**1**), a non-cross-linkable analogue, 1-palmitoyl-2-[12-(lipoyloxy)dodecanoyl]-*sn*-glycero-3-phosphocholine (**2**), and certain mixtures of **1** and **2**. On the basis of efflux measurements, membranes derived from monomeric **2** were found to be less permeable toward captured [^{14}C]sucrose than those prepared from monomeric **1**; increasing the mole percentage of **2** in mixed bilayers resulted in decreased permeability. While homopolymerization of liposomal **2** significantly increased bilayer permeability, analogous homopolymerization of **1** significantly decreased membrane permeability. Increasing the mole percentage of **1** in mixed polymerized liposomes resulted in a reduction in bilayer permeability. The trends that have been observed for the monomeric bilayers are interpreted in terms of the relative packing efficiency of **1** and **2**. A "polymer boundary" hypothesis is used to account for the permeability behavior of polymerized analogues.

Polymerized forms of liposomes (phospholipid bilayer vesicles) are receiving considerable attention as stable models for biological membranes and as carriers for drugs.[2-7] Although a large variety of polymerized liposomes have now been synthesized, only *one* specific system has been carefully examined with regard to its permeability characteristics; i.e., poly(butadiene)-based liposomes.[8] The fact that the transport properties of biomembranes play a central role in their biological function underscores the need to clearly define the permeation behavior of polymerized liposomes, if they are to be fully exploited as model membranes. Moreover, if polymerized liposomes can be designed with controllable permeability, they could be of considerable practical value as drug delivery devices having "tunable" release rates.[9]

One fundamental question regarding polymerized bilayer permeability remains unanswered: Does *cross-linking* (i.e., network formation within each vesicle) increase or decrease membrane permeability? Closely related to this question is the issue of whether one can modulate bilayer permeability by controlling the cross-link density of the membrane. In principle, a cross-linked, polymerized unilamellar liposome consists of *one* large spherical polymer molecule. A non-cross-linked analogue contains many individual polymer chains, the exact number of which depends on the average polymer size and the diameter of the liposome.[10-12] One might expect that a non-cross-linked polymerized liposome would be highly permeable due to "breaks" in the polymeric assembly. Such breaks would be analogous to "packing faults" which are believed to exist in conventional (monomeric) liposomes at the gel to liquid crystalline phase-transition temperature (T_m).[13] A cross-linked polymerized bilayer would be expected to contain a lower density of breaks, which could result in reduced permeability. Alternatively, one might imagine that a cross-linked bilayer might exhibit *higher* permeability due to poorer packing of rigid aliphatic chains. It is not obvious whether cross-linking should, in fact, increase or decrease the permeation properties of a polymerized bilayer. It is also not obvious whether polymerized bilayers should be more or less permeable relative to their monomeric counterparts. More experimental data are clearly needed before adequate theories can be developed.

We have previously reported the preparation of polymerized liposomes derived from 1,2-bis[12-(lipoyloxy)dodecanoyl]-*sn*-glycero-3-phosphocholine (**1**).[7] Based on its ease of synthesis, its ability to form highly stable liposomes under extremely mild conditions, and its potential biodegradability, we have concluded that **1** should become *the lipid of choice* for a large number of mechanistic and practical applications. In this paper, we report the permeability characteristics of polymerized and monomeric



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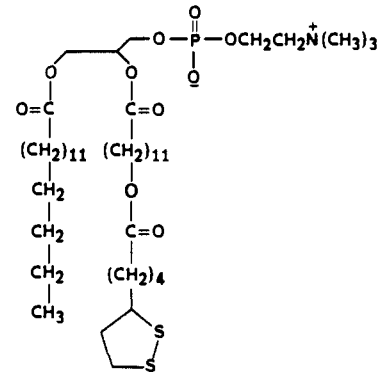

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† Dedicated to the memory of Professor Emil T. Kaiser.

(1) Supported by PHS Grant CA 42065 awarded by the National Cancer Institute.

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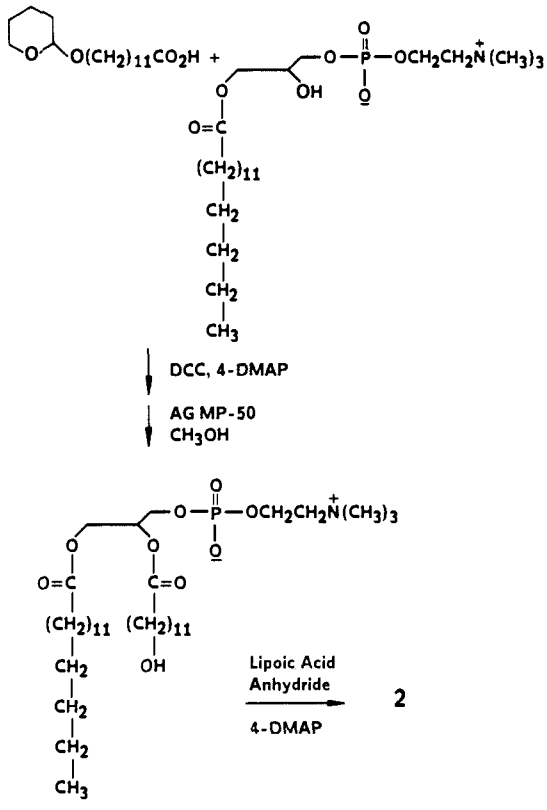
Scheme I



forms of liposomes derived from **1**, 1-palmitoyl-2-[12-(lipoyloxy)dodecanoyl]-*sn*-glycero-3-phosphocholine (**2**), and certain mixtures of **1** plus **2**. In principle, **1** should yield a highly cross-linked lipid bilayer; lipid **2** can only produce linear polymer chains. The fact that **1** and **2** have the same head-group structure and a similar lipophilic component suggests that they should form either completely miscible or nonideally miscible monolayers.[14] It further suggests that the degree of cross-linking and permeability may be controllable through a variation in the molar ratio of **1**/**2** employed. In this work we examine the influence of polymerization and cross-linking on the permeability properties of membranes derived from **1** and **2**.

Results

Lipid Synthesis. The synthesis of **1** has been previously described.[7] By analogous procedures, 1-palmitoyl-*sn*-glycero-3-phosphocholine was esterified with 12-(tetrahydropyranyloxy)-dodecanoic acid, deprotected, and subsequently esterified with lipoic acid anhydride to give **2** (Scheme I). Analysis of both **1** and **2** by 500-MHz ^{1}H NMR spectroscopy showed that the glycerol methine proton appeared exclusively at 5.24 ppm, consistent with the proposed 1,2-diacylphosphocholine structure.[15] This fact, together with the exclusive release of 12-(lipoyloxy)-dodecanoic acid upon treatment with phospholipase A_2, established the isomeric purity of **2**.[16]

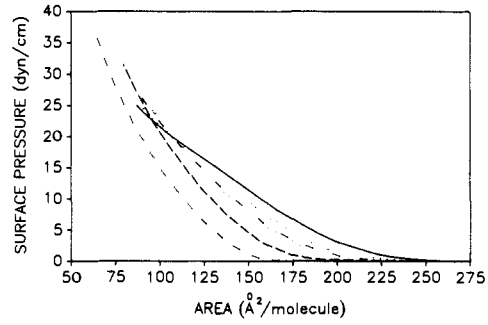


Figure 1. Surface pressure–area isotherms at 25 °C for **1** (—); **1** + **2** (80/20) (···); **1** + **2** (60/40) (-···-); **1** + **2** (20/80) (--); **2** (-·-).

Table I. Collapse Pressure as a Function of Lipid Composition

	collapse pressure, dyn/cm			collapse pressure, dyn/cm	
X_1	measd	calcd[a]	X_1	measd	calcd[a]
0.0	36.4 ± 0.5	36.4	0.8	25.8 ± 0.5	27.3
0.2	31.9 ± 0.5	34.1	1.0	25.0 ± 0.5	25.0
0.4	27.3 ± 0.5	29.6			

[a] Calculated by using eq 2.

Miscibility of 1 and 2. In order to determine the miscibility of **1** and **2**, we have measured surface pressure–area isotherms for various mixtures of the two lipids at the gas–water interface. For an *ideally miscible* or *completely immiscible* monolayer, the mean area per molecule, A_m, (at a specified surface pressure) is defined by the mole fraction of lipid employed (X_1) and by the partial molar areas of each lipid (A_1 and A_2, respectively), according to eq 1.[17] Thus, A_m is a linear function of X_1. Any

$$A_m = X_1A_1 + (1 - X_1)A_2 \quad (1)$$

deviation from linearity indicates nonideal miscibility, resulting from specific interaction between the lipids. By use of the phase rule of Defay and Crisp, it is also possible to distinguish between ideal miscibility and complete immiscibility.[18,19] In particular, for a completely immiscible two-component monolayer, the collapse points are equal to those of the pure components; for a completely miscible mixture, the collapse point is linearly related to the mole fraction of lipid employed (eq 2). Here, P_m equals

$$P_m = X_1P_1 + (1 - X_1)P_2 \quad (2)$$

the collapse pressure of the mixed monolayer, and P_1 and P_2 represent the collapse pressures for the pure lipids.

Figure 1 represents a composite of surface pressure–area isotherms recorded at 25 °C for mixtures of **1** and **2**, having X_1 = 0, 0.2, 0.4, 0.8, and 1.0, where X_1 represents the mole fraction of **1**. Based on the A_m values observed at 24 dyn/cm (Figure 2), and based on the variation in the collapse pressure for these mixed monolayers (Table I), it is evident that **1** and **2** are nonideally miscible.

Liposomes via Extrusion. All of the permeability studies reported in this paper were based on the use of large unilamellar vesicles (LUVs; 1000-Å mean external diameter), prepared by extrusion methods.[20,21] We have specifically avoided the use of sonicated liposomes for three reasons. First, sonicated dispersions normally consist of small unilamellar vesicles (SUVs) having mean external diameters of ca. 300 Å. Because lipid bilayers can *adsorb*

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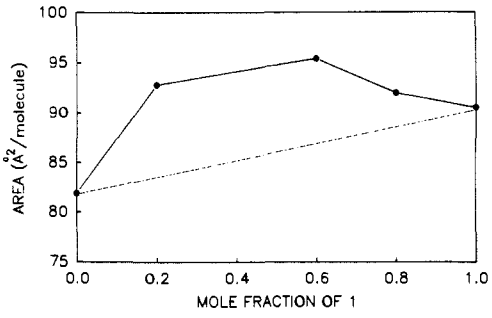

Figure 2. Plot of mean area per molecule, (estimated at 24 dyn/cm) as a function of the mole fraction of **1** in mixed monolayers of **1** plus **2**. A theoretical plot expected for a completely immiscible or ideally miscible monolayer (···) is included.

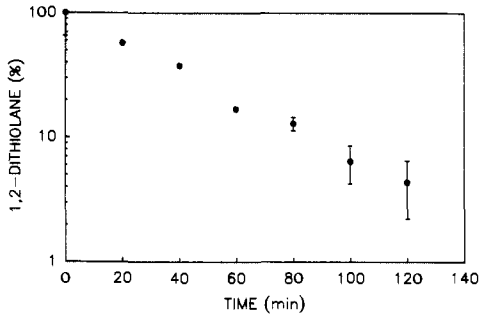

Figure 3. Semilogarithmic plot of percent of 1,2-dithiolane remaining as a function of time (23 °C).

as well as entrap permeants,[22,23] it is important to maximize the ratio of entrapped solute/adsorbed solute in order to maximize the accuracy of captured volumes and efflux rates; i.e., adsorbed permeants can increase apparent captured volumes and can contribute to apparent release rates via desorption processes. Because LUVs have a smaller total surface area per mole of lipid and a higher captured volume (as compared with SUVs), they exhibit a higher ratio of entrapped/adsorbed solute; they are, therefore, more attractive for permeation studies. Second, we wished to avoid any influence that membrane curvature might have on efflux rates. In particular, SUVs represent "strained" bilayers that are highly curved.[13,24] In principle, uneven lipid packing may not only affect the membrane's fluidity[25,26] and elasticity[27] but also its permeability characteristics. While a detailed study of how membrane curvature influences bilayer permeability would be of interest, in and of itself, this was not an intended goal of the present work. Third, **1** is unstable toward ultrasound; i.e., sonication induces polymerization. It was, therefore, not feasible to prepare sonicated monomeric dispersions of **1**.

By procedures similar to those described by Hope et al.,[21] lipid **1** was readily assembled into a multilamellar state by use of a borate buffer solution (pH 6.4). Sequential extrusion through 0.4-, 0.2-, and 0.1-μm polycarbonate filters afforded a liposome population having a mean external diameter of 1040 Å, with a range of 400–1700 Å (transmission electron microscopy); dynamic light scattering showed a mean diameter of 920 Å. Analyis of a 58 mM liposomal dispersion of **1** containing 2.0 mM Pr^{3+}, by ^{31}P NMR spectroscopy,[21] confirmed the unilamellar nature of these vesicles; i.e., the number of lipids in the outer leaflet of the bilayer, appearing at 17 ppm (relative to a triphenylphosphine reference), was equal to the number of lipids located in the inner leaflet (5 ppm). LUVs of **2** and mixtures of **1** plus **2**, were prepared by similar procedures.

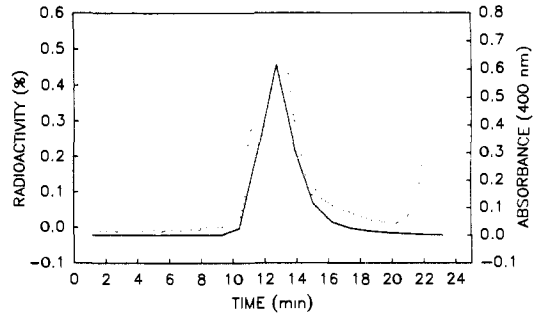

Figure 4. Gel filtration separation of [^{14}C]sucrose entrapped within polymerized liposomes of **1** from nonentrapped [^{14}C]sucrose, using a Sephadex G 50 column (···). Also shown is the apparent absorption at 400 nm due to light scattering of the liposomes (—).

Polymerized Liposome Formation. Upon raising the pH of a LUV dispersion of **1** to a value of 8.5, and after adding 10 mol % of dithiothreitol (DTT), a rapid ring-opening polymerization ensued which obeyed first-order kinetics over 4 half-lives (Figure 3). The extent of ring opening was monitored by following the disappearance of the 1,2-dithiolane moiety (333 nm).[7] At 23 °C, the observed first-order rate constant was 4.4×10^{-4} s^{-1}. Polymerization was essentially complete after 6 h, based on the disappearance of the 1,2-dithiolane chromophore (>95%), and the disappearance of starting monomer (only a trace amount of **1** could be detected by TLC). Within experimental error, there was no significant change in the mean external diameter of the dispersion upon polymerization (electron microscopy and dynamic light scattering). Lyophilization of the dispersion afforded a polymer that was completely insoluble in chloroform and chloroform/ethanol (1/1, v/v).

By using procedures similar to those described above, polymerized and monomeric liposomes were prepared from **2** and from mixtures of **1** and **2** having molar ratios (**1**/**2**) of 80/20, 60/40, and 20/80. In all cases, the extent of polymerization was estimated to be in excess of 90% (TLC). Polymers formed from lyophilized liposomes of pure **2** were soluble in chloroform/ethanol (1/1, v/v).

Captured Volume. Captured volumes were determined by dividing the fraction of the water-soluble, "impermeant" solute that was entrapped within the liposomal compartment by the lipid concentration in the dispersion. With [^{14}C]sucrose as the solute, LUVs of **1** showed a captured volume of 2.1 ± 0.1 L/mol. Figure 4 shows a typical gel filtration separation of entrapped from nonentrapped sucrose. This captured volume is in excellent agreement with that estimated for similar LUVs prepared from soya phosphatidylcholine via extrusion through 0.1-μm polycarbonate filters, using [^{14}C]inulin as the solute.[21]

While the above agreement is satisfying, one issue that warrants consideration is whether or not *polymerized* liposomes show the same counting efficiency as their monomeric counterparts. In particular, if polymerized liposomes are not disrupted by the scintillation cocktail, and if the radiolabel remains captured, then the observed counting efficiency will depend on the ability of the fluorophore to diffuse into the liposomal compartment. In a control experiment, a nonpolymerized LUV dispersion of **1** was prepared with entrapped [^{14}C]sucrose. After the dispersion was divided into two equal fractions, and after one was polymerized with DTT, nonentrapped radiolabel was removed from both by gel filtration. Within experimental error, the polymerized and nonpolymerized liposomes were recovered, quantitatively, in the void volume of a Sephadex G-50 column (phosphorus analysis), and both showed the same level of radioactivity. This fact, together with the fact that extended dialysis of nonpolymerized liposomes of **1** with borate buffer (pH 6.4) afforded a quantitative release (>95%) of sucrose into the dialysate (analyzed from the nonliposomal side of the dialysis membrane), establishes that the counting efficiencies for the radiolabel in the presence of polym-

(22) Sonicated vesicles prepared from dioctadecyldimethylammonium chloride, for example, have been found to adsorb almost twice the amount of [^{14}C]sucrose as that which is captured: Carmona-Ribeiro, A. M.; Chaimovich, H. *Biochim. Biophys. Acta* **1983**, *733*, 172.

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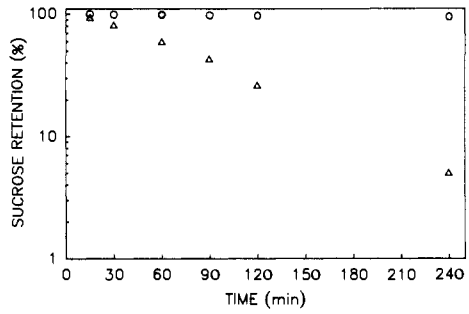

Figure 5. Semilogarithmic plot of percent of [^{14}C]sucrose retained within polymerized (○) and monomeric (△) liposomes derived from **1** (23 °C).

erized and nonpolymerized liposomes, and in the absence of phospholipid, are equivalent. Finally, when preformed polymerized and monomeric liposomes of **1** were incubated with [^{14}C]sucrose for 30 min and then subjected to gel filtration, "apparent" captured volumes were <0.02 L/mol. Under these conditions, adsorption of the sugar was negligible.

Membrane Permeability. The average velocity of transport of a solute across a bilayer is characterized by a permeation coefficient, P, which is expressed in units of centimeters per second. This parameter is a measure of the number of moles per second of solute permeating per unit of bilayer surface area per unit concentration difference of solute. For permeation coefficients that lie in the range of 10^{-9}–10^{-11} cm/s (typically found for small, uncharged solutes such as glucose diffusing across phosphatidylcholine bilayers), the transport kinetics may be conveniently monitored by measuring the efflux of the solute out of liposomes via dialysis.[8,28] Specifically, liposomes can be loaded with a radiolabeled form of the solute and then dialyzed against a buffer. If the label diffuses out of the aqueous liposomal compartment through the bilayer and into the external buffer at a rate that is much slower than it diffuses across the dialysis membrane, then the efflux kinetics is expected to conform to the first-order relationship shown in eq 3. Here A_1 = surface area of the bilayer,

$$\ln\left[\frac{N_1V_o}{V_1+V_o}-N_o\right]=\ln N_1\left[\frac{V_o}{V_1+V_o}\right]-k_1\left[\frac{V_o+V_1}{V_o}\right]t \tag{3}$$

V_1 = internal volume of the liposome, N_1 = counts in the liposome at $t = 0$, V_o = volume of dialysate, N_o = counts in the dialysate beyond $t = 0$, and k_1 = the first-order rate constant which is defined by eq 4.[8,29] If experimental conditions are chosen such

$$k_1 = (A_1/V_1)P \tag{4}$$

that $V_o \gg V_1$ (as in the present work), then eq 3 reduces to the simplified form shown in eq 5. Thus, the rate of disappearance

$$\ln\left[\frac{N_1-N_o}{N_1}\right]=-k_1t \tag{5}$$

of the radiolabel from within the liposomes should yield a first-order plot whose slope, k_1, depends on the size of the liposomes and the intrinsic permeability of the bilayer, according to eq 4.

In the present study, we have investigated the permeability of polymerized and monomeric liposomes prepared from **1** and **2** using radiolabeled glucose and sucrose as the permeants. Our reasons for choosing these specific sugar molecules were 3-fold. First, as mentioned above, each of these sugars was expected to diffuse out of phosphatidylcholine liposomes at a rate that would be convenient to monitor by dialysis methods. Second, their nonionic character and high water solubility should minimize adsorption by the liposomal membrane. Third, [^{3}H]glucose was used as the permeant in the only well-studied polymerized liposomal membrane reported to date, i.e., that prepared from a butadiene-based phosphatidylcholine.[8] It was, therefore, of interest to compare the permeation properties of our membranes with those of this previously investigated system.

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Table II. Estimated Permeability of Polymerized and Monomeric Liposomes toward [^{14}C]Sucrose at 23 °C[a]

lipid[b]	$10^{11}P$, cm/s[c]	lipid[b]	$10^{11}P$, cm/s[c]
poly(**1**)	16.8 ± 2.4[d]	poly(**1** + **2**), 60/40	2.34 ± 1.09
1	11.7 ± 1.1	(**1** + **2**), 20/80	4.60 ± 0.76
poly(**1**)	0.24 ± 0.13	poly(**1** + **2**), 20/80	5.63 ± 0.36[e]
(**1** + **2**), 80/20	9.54 ± 0.45	**2**	1.07 ± 0.58
poly(**1** + **2**), 80/20	0.70 ± 0.25	poly(**2**)	8.98 ± 0.13[e]
(**1** + **2**), 60/40	8.61 ± 0.39	egg PC[f]	0.31 ± 0.04

[a] Average of three independent experiments. In all cases, captured volumes ranged between ca. 1 and 2 L/mol; within experimental error, captured volumes were the same before and after polymerization. The first-order rate of diffusion of [^{14}C]sucrose across the dialysis membrane, in the absence of liposomes, was ca. 2.3 times greater than the fastest efflux rate measured. [b] Molar ratios of **1**/**2** are given as relative molar percentages. [c] Calculated by using a mean liposomal diameter of 1000 Å and a limiting area of 90 Å^2/molecule. In all cases, dynamic light scattering indicated mean particle diameters of ca. 1000 Å. [d] [^{3}H]glucose permeability. [e] Average of two experiments. [f] LUVs prepared from egg/yolk phosphatidylcholine.

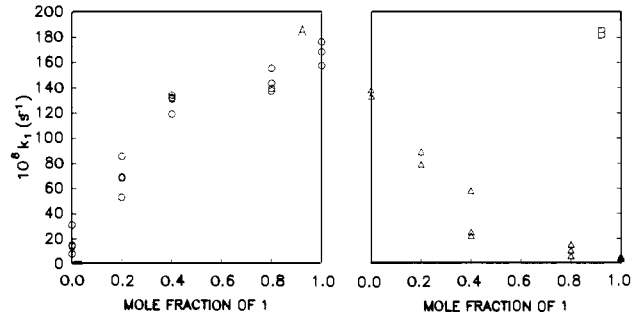

Figure 6. Efflux rates (10^6k_1) for [^{14}C]sucrose out of monomeric (A) and polymerized (B) liposomes as a function of mole percent of **1** (23 °C).

By use of procedures that are outlined in the Experimental Section, the release of [^{14}C]sucrose from nonpolymerized liposomes of **1** obeyed first-order kinetics for more than 4 half-lives (Figure 5). Polymerized liposomes of **1** exhibited substantially lower permeability; i.e., the first-order rate constant for release was decreased by a factor of ca. 50 (Table II). Attempted entrapment of [^{3}H]glucose in nonpolymerized liposomes of **1** led to very low and irreproducible captured volumes. Apparently, in this case, the membrane was too permeable toward the smaller sugar and substantial leakage occurred during gel filtration. In contrast, *polymerized* liposomes of **1** exhibited captured volumes of 1.61 ± 0.1 L/mol and first-order release over 4 half-lives for glucose. Because of the relatively high permeation rates found for glucose, principal comparisons that have been made in this work have been based on sucrose.

Experimental first-order rate constants for release of sucrose from monomeric and polymerized liposomes are presented in Figure 6. With the former, an increase in the mole percentage of **2** resulted in a decrease in membrane permeability; with the latter, the opposite was observed. Estimated values for the permeation constants, P, are reported in Table II.

Discussion

Before discussing the permeability characteristics of these lipid membranes, three structural issues warrant discussion. Specifically, the extent of polymerization, the degree of cross-linking, and the distribution of **1** and **2** within the bilayers must be considered. In this work, we have relied on two independent methods to judge the extent of polymerization. Thin-layer chromatography has shown, in all cases, that only trace amounts of starting monomer remain, under the polymerization conditions used. Further, the disappearance of the UV absorption at 333 nm (characteristic

of the five-membered ring cyclic disulfide)[7] provides additional support that extensive ring-opening polymerization has taken place; i.e., more than ca. 95% of the *total number* of five-membered ring cyclic disulfide groups have reacted.

In all instances where **1** has been used, an extensively cross-linked liposomal network may be formed. The specific cross-link density will be directly related to the percentage of **1** that has both disulfide rings reacting *intermolecularly* (with neighboring lipids in the same or adjacent monolayer). In addition, the ability of **1** to function as a cross-linking agent in mixed liposomes may or may not be a function of the molar ratio of **1**/**2** employed. At the present time, we have no experimental means that allows us to define the actual cross-link density in any of the systems investigated. Nonetheless, the insolubility of homopolymerized liposomes of **1**, together with the solubility of homopolymerized liposomes of **2**, infers that at least some degree of cross-linking is present in the former. We *assume* that an increase in molar composition of **1** results in an overall increase in the cross-link density of the membrane.

Monolayer studies carried out with **1** and **2** at the gas–water interface clearly show that these lipids are nonideally miscible. If we assume that they have similar miscibility in the lamellar phase, and that the reactivity of each of the disulfide rings in **1** and **2** is equivalent, then a nearly statistical distribution of the monomers should exist within copolymerized liposomal membranes. While each of the above assumptions remains to be proven either correct or incorrect, we believe that they are all reasonable and that this "picture" serves as a viable working model.

Monomeric membranes of **2** have been found to be less permeable toward [^{14}C]sucrose than those composed of **1**. The simplest interpretation of this result is that the replacement of the 12-(lipoyloxy)dodecanoyl group in the *sn*-1 position of **1** with a palmitoyl moiety significantly improves the lipid's packing efficiency and thus affords a tighter membrane. The higher compressability of **1**, relative to **2**, is consistent with this hypothesis (Figure 1). The decrease in the efflux rate that is observed, as the percentage of **2** is increased, clearly shows that "fine-tuning" of the permeability of these monomeric membranes is possible.

Homopolymerization of liposomes produced from **2** results in significantly *increased* permeability. In contrast, homopolymerization of liposomes derived from **1** results in significantly *decreased* permeability. A plausible explanation that would account for this difference is that "breaks" or "polymer boundaries" within the membrane serve as the primary avenue for release of sucrose.[30,31] Non-cross-linked liposomes of **2** are composed of many individual polymer molecules. They must, therefore, contain many polymer boundaries. If polymerized liposomes of **1** are highly cross-linked, the effective number of such boundaries should be greatly diminished. Similarly, the decrease in permeability that is observed as the mole percentage of **1** increases may also be explained by a decrease in the effective number of polymer boundaries within the bilayer.

Comparison of liposomes derived from **1** with those prepared from a cross-linkable butadiene-based phosphatidylcholine[8] reveals a strong similarity and also a striking difference. Both liposomes show a very substantial reduction in membrane permeability upon polymerization. In contrast to the poly(butadiene) liposomes, however, where first-order release was observed for only 20% (80% of the entrapped glucose appears to be released at a much slower rate, if released at all), polymerized liposomes of **1** exhibited a first-order release for more than 95% of the captured glucose.

Taken together, the results reported herein demonstrate the feasibility of modulating liposome permeability in the polymerized as well as in the monomeric state, by varying the percentage of cross-linkable and non-cross-linkable phospholipid employed. Studies that are now in progress are aimed at (1) elucidating the precise structure of polymerized liposomal membranes formed from **1** and **2**, (2) obtaining a more detailed understanding of how the structure of these polymerized membranes controls their permeability characteristics, and (3) examining the generality of these findings.

Experimental Section

General Methods. Unless stated otherwise, all reagents and chemicals were obtained from commercial sources and used without further purification. House-deionized water was purified by using a Millipore Milli-Q filtering system containing one carbon and two ion-exchange stages. *sn*-Glycero-3-phosphorylcholine was purchased as the cadmium chloride salt (Sigma, GPC·$CdCl_2$) and used directly. [^{14}C]Sucrose (400 mCi/mmol, 20% ethanol solution) and [^{3}H]glucose (18 Ci/mmol, 90% ethanol solution) were obtained from ICN Laboratories. DL-Thioctic acid (lipoic acid, Sigma), dihydropyran (Aldrich), and dicyclohexylcarbodiimide (Aldrich) were used as obtained. 12-Hydroxydodecanoic acid (Aldrich) and 4-(dimethylamino)pyridine (4-DMAP; Aldrich) were recrystallized once from toluene prior to use. Tetrahydrofuran was purified by distillation over sodium benzophenone ketyl. Vesicle dispersions were prepared in 10 mM borate buffer (pH 6.4) containing 140 mM NaCl and 2 mM NaN_3. Chloroform and methanol used for chromatography, and hexane used for monolayer studies, were HPLC-grade (Burdick & Jackson). Dichloromethane (Aldrich, Gold Label) was used as obtained. AG MP50 (a macroporous strongly acidic cation-exchange resin) was obtained from Bio-Rad Laboratories and was purified by extensive extraction (Soxhlet) with methanol prior to use. ^{1}H NMR, IR, and UV spectra were recorded on Bruker 500-MHz, Mattson Sirus 100, and Perkin-Elmer Lambda 5 spectrometers, respectively. Chemical shifts are reported relative to tetramethylsilane. ^{31}P NMR spectra were recorded on a JEOL FX 90Q instrument, using triphenylphosphine as an external standard. For separation of the outer from the inner lipids in the bilayer, $PrCl_3$ was used as the paramagnetic shift reagent.[33] Elemental analyses were performed by Robertson Laboratory, Inc. (Florham Park, NJ). Chromatographic separations were carried out by using precoated Merck 0.25-7-230 ASTM silica gel with the following eluting solvent mixtures: (A) $CHCl_3/CH_3OH$ (9/1, v/v); (B) $CHCl_3/CH_3OH/H_2O$ (4/5/1, v/v/v); (C) $CHCl_3/CH_3OH/H_2O$ (65/25/4, v/v/v). Detection on TLC plates was made by using iodine vapor, phosphomolybdic acid (10% in ethanol), or molybdenum blue. Vortex mixing was carried out by using a Thermolyne mixer. Specific procedures used for electron microscopy were similar to those previously described.[7] Electron micrographs were recorded by using a Philips 400 microscope. Freeze drying of vesicle dispersion was carried out by a Virtis freeze dryer. Liquid scintillation was performed with a Beckman instrument, Model LS 5801, using a liquid scintillation cocktail comprised of 70% 1,2,4-trimethylbenzene plus 30% surfactant (Beta Blend; ICN Laboratories). Phosphorus analysis was performed by using established procedures.[34] Dialysis tubing used for permeability studies was Spectrapor 7, MW cutoff 50 000. Equilibrium dialysis cells used for permeability measurements were purchased from Fisher Scientific. All vesicle extrusions were carried out with a Lipex Biomembrane apparatus (Vancouver, British Columbia). Polycarbonate filters (Nuclepore) were used as obtained.

1,2-Bis[(12-lipoyloxy)dodecanoyl]-*sn*-glycero-3-phosphocholine (1). The procedures used for the synthesis of **1** were similar to those previously described.[7] A scale-up of this synthesis, starting from 1.2 mmol of 1,2-bis[12-hydroxydodecanoyl]-*sn*-glycero-3-phosphocholine, afforded a 77% isolated yield of **1**: R_f 0.54 (solvent C); UV (EtOH) λ_{max} 331.4 nm (ϵ 275); ^{1}H NMR ($CDCl_3$) δ 1.3 (s, 32 H, $OCH_2(CH_2)_8$), 1.40–1.53 (m, 4 H, $SCHCH_2CH_2CH_2$), 1.53–1.76 (m, 12 H, $O{=}CCH_2CH_2$ + $SCHCH_2CH_2CH_2$), 1.92 (sext, 2 H, $SSCH_2CH_2$), 2.25–2.35 (m, 8 H, $O{=}CCH_2$), 2.48 (sext, 2 H, $SSCH_2CH_2$), 3.10–3.22 (m, 4 H, $SSCH_2$),

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(31) This polymer boundary hypothesis may also be used to account for some of the permeability properties of *non-cross-linked* vesicles that have recently been reported. In particular, polycondensation of amino acid amphiphiles to give vesicular oligopeptides resulted in an *increase* in membrane permeability toward both glucose and carboxyfluorescein.[32] In contrast, formation of linear polymers derived from ammonium-based lipids, having a high degree of polymerization (ca. 500 monomer units per average polymer chain) *reduced* the membrane's permeability toward glucose.[8a] Using our polymer boundary hypothesis, it is tempting to suggest that long linear polymer chains, which create relatively few polymer boundaries per vesicle bilayer, tend to decrease membrane permeability; also, short linear polymers, which produce many polymer boundaries, tend to increase vesicle permeability. Further studies are, however, clearly needed in order to define bilayer permeability as a function of polymer chain length in non-cross-linked polymerized vesicles.

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3.38 (s, 9 H, $^+N(CH_3)_3$), 3.58 (pent, 2 H, SSC*H*), 3.84 (br s, 2 H, CH_2N^+), 3.95 (m, 2 H, $CHCH_2OP$), 4.07 (t, 4 H, $O{=}COCH_2CH_2$), 4.13 (m, 1 H, $POCH_2CHCH_2$), 4.34 (br s, 2 H, $^+NCH_2CH_2$), 4.39 (d, 1 H, $POCH_2CHCH_2$), 5.2 (br s, 1 H, $POCH_2CH$). For storage purposes, this lipid should be dissolved in dichloromethane (0.02 M), filtered (0.2-μm FG Millipore filter), and kept at 10 °C in the dark.

1-Palmitoyl-2-[12-(lipoyloxy)dodecanoyl]-*sn*-glycero-3-phosphocholine (2). A mixture of 174.8 mg (0.582 mmol) of 12-(tetrahydropyranyloxy)dodecanoic acid,[7] 115.4 mg (0.233 mmol) of 1-palmitoyl-*sn*-glycero-3-phosphocholine, 32.7 mg (0.27 mmol) of 4-(dimethylamino)-pyridine, and 60.0 mg (0.291 mmol) of dicyclohexylcarbodiimide was suspended in 3 mL of dry dichloromethane (Gold Label; Aldrich), and stirred under nitrogen in the dark for 48 h. After removal of solvent under reduced pressure, the residue was dissolved in 1 mL of CH_3OH/H_2O (97/3, v/v) and stirred in the presence of 1 g of AG MP-50 (23 °C) to allow for complete deprotection of the hydroxyl groups (monitored by thin-layer chromatography). The resin was then removed by filtration and the solution concentrated under reduced pressure. The crude product, obtained after drying [12 h, 23 °C (0.05 mm)], was subjected to chromatographic purification by using a silica gel column (5 g) eluting sequentially with solvents A and C, to yield 62.9 mg (16%) of 1-palmitoyl-2-(12-hydroxydodecanoyl)-*sn*-glycero-3-phosphocholine: R_f 0.40 (solvent C); IR (KBr) ν_{OH} 3390, $\nu_{C=O}$ 1728, $\nu_{N(CH3)3}$ 967, 1055, 1090 cm^{-1}.

A solution of lipoic acid anhydride[7] (2.0 mL, 0.15 M) was added to 1-palmitoyl-2-(12-hydroxydodecanoyl)-*sn*-glycero-3-phosphocholine (63 mg, 0.09 mmol), followed by addition of 12 mg (0.1 mmol) of 4-(dimethylamino)pyridine. After the mixture was stirred for 6 h under nitrogen at room temperature, thin-layer chromatography (silica, solvent C) indicated complete conversion to **2**. The product mixture was filtered and concentrated under reduced pressure. The residue was dissolved in 5 mL of solvent B and passed through a 1.2 × 1.5 cm AG MP-50 cation-exchange column in order to remove 4-(dimethylamino)pyridine. The filtrate was concentrated under reduced pressure, dissolved in a minimum volume of ethanol, and then concentrated again. Chromatographic purification of the residue on a silica gel column (0.9 × 6 cm), eluting first with solvent A and then with solvent C (lipid **2** elutes as a single yellow band), afforded, after drying [10 h, 23 °C (0.05 mm)], 0.055 g (69%) of **2** as a yellow solid: R_f 0.48 (solvent C); UV (EtOH) λ_{max} 330.3 nm, (ε 144); IR (KBr) $\nu_{C=O}$ 1736, $\nu_{N(CH3)3}$ 968, 1065, 1095 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.88 (t, 3 H, CH_3CH_2), 1.30 (s, 40 H, $OCH_2(CH_2)_8$ + $O{=}CCH_2CH_2(CH_2)_{12}$), 1.40–1.53 (m, 2 H, $SCHCH_2CH_2CH_2$), 1.53–1.76 (m, 8 H, $O{=}CCH_2CH_2$ + $SCHCH_2CH_2CH_2$), 1.92 (sext, 1 H, $SSCH_2CH_2$), 2.25–2.35 (m, 6 H, $O{=}CCH_2$), 2.48 (sext, 1 H, $SSCH_2CH_2$), 3.10–3.22 (m, 2 H, $SSCH_2$), 3.38 (s, 9 H, $^+N(CH_3)_3$), 3.58 (pent, 1 H, SSC*H*), 3.84 (br s, 2 H, CH_2N^+), 3.95 (m, 2 H, $CHCH_2OP$), 4.07 (t, 2 H, $O{=}COCH_2CH_2$), 4.13 (m, 1 H, $POCH_2CHCH_2$), 4.34 (br s, 2 H, $^+NCH_2CH_2$), 4.39 (d, 1 H, $POCH_2CHCH_2$), 5.2 (br s, 1 H, $POCH_2CH$). Anal. Calcd for $C_{44}H_{88}O_{10}NPS_2$: C, 59.86; H, 9.52; N, 1.59; P, 3.51; S, 7.26. Found: C, 59.64; H, 9.81; N, 1.52; P, 3.42; S, 7.11. For storage purposes, this lipid should be dissolved in dichloromethane (0.02 M), filtered (0.2-μm FG Millipore filter), and kept at 10 °C in the dark.

The isomeric purity of **2** was established by reaction with phospholipase A_2.[35] A 0.3-mL dichloromethane solution containing 5 mg of **2** was stirred (under nitrogen) with 1 mg of phospholipase A_2, which was dissolved in 0.025 mL of 0.006 M $CaCl_2$ plus 0.025 mL of borate buffer (pH 8.5). The progress of the reaction was monitored by thin-layer chromatography [silica, $CHCl_3$/MeOH, (20/1, v/v)]. Only 12-lipoyloxydodecanoic acid (R_f 0.42) was released from **2** after 5 h; palmitic acid (R_f 0.55) was not detected.

Preparation of Liposomes via Extrusion. Typically, a thin film of **1** (12.0 mg, 0.012 mmol) was deposited onto the walls of a 13 × 100 mm test tube by concentrating 0.475 mL of a 0.025 M dichloromethane solution under a stream of nitrogen. The test tube was fitted with a No-Air stopper and repeatedly degassed, by evacuating the tube under reduced pressure (50 mm, 5 min) and replenishing the tube with nitrogen (10 times in succession). The lipid was dispersed in 1.2 mL of a borate buffer solution (10 mM borate, 140 mM NaCl, and 2 mM NaN_3, pH 6.4, purged with nitrogen for 2 min) with the aid of vortex mixing at room temperature. The test tube was subsequently plunged directly into liquid nitrogen and allowed to remain at this temperature for 2 min; it was then plunged immediately into a water bath (23 °C) and allowed to reach room temperature. This freeze–thaw procedure was repeated four times.[21] The resulting dispersion was sequentially extruded through two stacked polycarbonate filters of 0.4 μm (four extrusions), 0.2 μm (four extrusions), and 0.1 μm (six extrusions). The temperature of the 1.5-mL Lipex Biomembrane chamber that was used was maintained at 30 °C via an external water bath. Each pass through the double filter took less than 20 s, using a maximum pressure of 400 psi. A typical quantity of [^{14}C]sucrose that was included in the buffer, for entrapment and permeability studies, was 40 μCi.

Polymerization of Liposomes. Typically, the pH of a 0.5-mL extruded liposomal sample (6 mg, 0.0058 mmol) was raised to a value of 8.4 via the addition of 0.05 mL of a second borate buffer solution (10 mM borate, 140 mM NaCl, and 2 mM NaN_3, pH 10), and the dispersion was purged with nitrogen for 2 min. Polymerization was initiated by adding 58 μL of a 0.01 M aqueous solution of DTT to the dispersion. With a temperature of 23 °C, and the above experimental conditions, the extent of polymerization was normally complete within ca. 4 h, as judged by thin-layer chromatography. Polymerization rates that were observed with a pH <8.4 were significantly lower. When analysis was carried out by UV spectroscopy, a similar liposomal dispersion was prepared from 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine and used as a "blank" in the reference beam of the spectrometer. This was done in order to minimize overlapping absorption (turbidity) due to light scattering of the vesicles. Except for experiments in which the kinetics of the polymerization was monitored, dispersions were normally maintained at 20 °C for 6 h.

Gel Permeation Chromatography. A Sephadex G-50 column (1 × 43 cm) was prepared by first hydrating 3.0 g of dry gel with 30 mL of 10 mM borate buffer (140 mM NaCl and 2 mM NaN_3, pH 6.4 used for monomeric and 8.4 for polymerized liposomes), degassing the gel under low vacuum (water aspirator, 20 mm) for 1 h at room temperature, and allowing the swollen gel to equilibrate for a minimum of 6 h prior to use. The gel was added to the glass column in a single pouring, followed by passage of 200 mL of the same buffer. Typically, a 0.5-mL aliquot of a 9.5 mM liposomal dispersion of polymerized **1** containing entrapped and nonentrapped radiolabel was applied to the column and eluted with borate buffer (the pH was 8.5 for polymerized liposome samples and 6.4 for nonpolymerized analogues). The void volume of the column was determined in a prior elution by using blue dextran 2000; for all filtrations, more than 95% of **1** was recovered in the void volume (phosphorus analysis). For vesicles made from **2**, and mixtures of **1** plus **2**, recovery in the void volume generally ranged between 20 and 50%. The reason for this lower vesicle recovery is not presently clear. Forty fractions of 1.16 mL were collected, with the majority of the lipid eluting in fractions 11 and 12, as detected by turbidity (400 nm). All fractions were analyzed for radioactivity, using 100-μL aliquots.

In all cases, a well-defined separation between free and liposome-entrapped radiolabel was obtained. The ratio of entrapped sugar/free sugar was determined by dividing the counts per minute (cpm) in the void volume of the column by the total cpm added to the column. This ratio, when multiplied by 100, gives the percentage of sugar that is captured. In a control experiment, preformed polymerized liposomes of **1** (0.5 mL of a 9.7 mM dispersion in borate buffer, pH 8.5) were incubated with 20 μCi of sucrose for 0.5 h at room temperature. Subsequent gel filtration and analysis of the liposomal fraction indicated an apparent captured volume of <0.02 L/mol.

Scintillation Counting. Aliquots (20–100 μL) that were analyzed for radioactivity were placed in a 7-mL scintillation vial (the disposable micropipet that was used was rinsed with an equal volume of water, which was also added to the vial). The liquid scintillation cocktail (4 mL) was then added, and the vial was sealed with a screw-cap and shaken vigorously by hand for 30 s. All samples were counted for 10 min to give good statistics.

Dialysis Measurements. A 1-mL portion of combined fractions 11 and 12 of the gel filtration column (containing the majority of liposomes with encapsulated marker) was immediately added (within 2 min) to a previously prepared equilibrium cell. This cell consisted of two 1-mL chambers, separated by a Spectrapor 7 membrane (50 000 MW cutoff) which had been cut from a 23-mm-diameter dialysis tube. Prior to use, this tubing was washed by soaking in 150 mL of water for 24 h at 10 °C, with four exchanges of water. In a fifth exchange, the membrane was placed in a 10 mM borate buffer [140 mM NaCl and 2 mM NaN_3 (pH 8.5 for polymerized and pH 6.4 for nonpolymerized liposomes)]. The dialysis cell was assembled by (i) placing the membrane between the two 1-mL chambers, (ii) adding 1 mL of the same borate buffer solution to one empty chamber, and (iii) adding the liposome sample (1 mL) to the remaining chamber. The cell was immediately attached to a wrist-action shaker (Burrel) and agitated at the maximum rate at ambient temperatures (22–24 °C). NOTE: In order to ensure efficient agitation, a small air bubble should be visible on each side of the dialysis cell. Aliquots (40 μL) were periodically removed from both chambers, but only those samples that were removed from the chamber that were devoid of liposomes were analyzed for radioactivity. A maximum of 30% of the volume in each cell was removed. All permeability measurements for monomeric

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liposomes were carried out at pH 6.4 in order to maintain the monomeric state of the membrane (at higher pH values, a slow spontaneous polymerization occurs). Polymerized liposomes of **1** showed the same permeability toward sucrose at pH 6.4 as that found at pH 8.5.

Dynamic Light Scattering. Size distributions were determined with a Nicomp Model 200 laser particle sizer equipped with a 5-mW helium–neon laser at an excitation wavelength of 632.8 nm. This instrument employs digital autocorrelation to analyze the fluctuations in scattered light intensity generated by the diffusion of liposomes in solution. The measured diffusion coefficient is used to obtain the average hydrodynamic radius and, thus, the mean diameter. Samples were filtered by using a 0.45-μm HV4 Millipore filter prior to analysis.

Film Balance Experiments. Surface pressure–area isotherms were recorded by using a MGW Lauda film balance, maintained at 25 °C, and equipped with a computerized data acquisition station. All lipids were spread onto a pure water subphase (600 cm^2) that was purged with nitrogen, using a hexane/ethanol (4/1, v/v) solution. The concentrations of stock solutions of **1** and **2** that were used were 0.44 and 1.18 mg/mL, respectively. Monolayers were compressed under a nitrogen atmosphere up to the collapse point. The total quantity of lipid that was used in each experiment was 3.17×10^{-8} moles; the rate of compression was 60 cm^2/min.

Registry No. 1, 116405-86-8; **2**, 116405-87-9; 12-(tetrahydropyranyloxy)dodecanoic acid, 116405-88-0; 1-palmitoyl-*sn*-glycero-3-phosphocholine, 17364-16-8; 1-palmitoyl-2-(12-hydroxydodecanoyl)-*sn*-glycero-3-phosphocholine, 116405-89-1; lipoic acid anhydride, 91319-83-4.

Does a Stilbazolium Cation Adsorbing Poly(styrenesulfonate) Anion Form Micellelike Clusters?

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Abstract: Poly(styrenesulfonate) anion (PSS^{n-}) is shown to aggregate with a large number of stilbazolium cations (Stz^+) which undergo an electron-relay chain photoisomerization in the presence of $Ru(bpy)_3^{2+}$ sensitizer. The quantum yields for the cis-to-trans isomerization ($\Phi_{c\rightarrow t}$) were rather constant in the range of 50–110, irrespective of changing the solvent from water to acetone or of the degrees of polymerization of PSS^{n-} from 100 to 5000. Adsorption and aggregation numbers of benzyltrimethylammonium ion (BTA^+), an organic cationic substitute for Stz^+, on PSS^{n-} were determined by a methylanthracene quenching method. The results show that the thousands of Stz^+ ions bound to PSS^{n-} are divided into small aggregates of micellelike clusters and that their aggregation numbers are constant in the range of 80–90, irrespective of the degree of polymerization of PSS^{n-}. It was concluded that the electron-relay chain isomerization proceeds within one micellar cluster.

Interactions between polyions and organic counterions play an important role in a wide variety of biological and industrial systems.[1-4] The binding of organic materials to biopolymers or polyelectrolytes is a particularly interesting methodology to achieve artificial photosynthesis enabling a facile electron transfer between electron donors and acceptors.[5] We have reported an electron-relay chain reaction utilizing anionic micelles[6a] or colloidal silica gel surface for the $Ru(bpy)_3^{2+}$-sensitized cis-to-trans isomerization of stilbazolium cations (Stz^+).[6b] The resulting high quantum yields of 50–100 are suggestive of a possibility for developing a so-called photomultiplier, provided that the length of electron relay could be further increased by applying polyionic fields. Poly(styrenesulfonate) anion (PSS^{n-}), a polyelectrolyte, seems to be a good candidate for such fields with anionic sites available up to 5000. Here, we report our unexpected results that thousands of Stz^+ molecules bound to the anionic polymer PSS^{n-} are divided into micellelike clusters and that the electron relay is confined within one cluster.

Experimental Section

Materials. Tris(2,2′-bipyridine)ruthenium dichloride [$Ru(bpy)_3^{2+}$]-$(Cl^-)_2$[7] and *cis*-γ-styrylpyridine (*cis*-Stz)[8] were prepared according to the literature methods. Sodium poly(styrenesulfonates) [(PSS)Na's] were either of the standard sample grade from Pressure Chemical Co. or from Toyo Soda Manufacturing Co. β-Phenethylammonium chloride, tetramethylammonium bromide, and 9-methylanthracene of extra pure grade were used as received. Benzyltrimethylammonium chloride (BTAC) was prepared by the reaction of trimethylamine with benzyl chloride.

Preparation of *cis*-γ-Stilbazolium Poly(styrenesulfonate)[PSS^{n-}·(*cis*-$Stz^+)_n$]. A 0.05 M solution of *cis*-Stz in methanol (80 μL) was precisely neutralized by adding an equivalent quantity of 0.01 M poly(styrenesulfonic) acid (PSSH) in water and stored as a stock solution in the dark. The concentration of PSSH was based on the sulfonic acid group involved, and the neutralization point was checked potentiometrically. Here, the PSSH solution was obtained according to the following procedures: 50 mg of (PSS)Na in water (~1 mL) was passed through a column (17 mm i.d. × 200 mm length) packed with ca. 20 mL each of Amberlite IR and IRA and separated into fractions (ca. 10 mL). Fractions (no. 3–6) including PSSH were collected and diluted with ca. 50 mL of water to afford ca. 0.01 M PSSH solution. Its concentration was determined spectrophotometrically at 262 nm (ϵ 730) on a Shimadzu UV-265 spectrophotometer.

Photolysis of PSS^{n-}·(*cis*-$Stz^+)_n$ with $Ru(bpy)_3^{2+}$. An aqueous solution of 0.4 mM (PSS^{n-}/n)·*cis*-Stz^+ and 6.7 μM $Ru(bpy)_3^{2+}$ sensitizer was

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