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H. H. Hub[a]; B. Hupfer[a]; H. Koch[a]; H. Ringsdorf[a]
[a] Institut fÜr Organische Chemie Johannes Gutenberg-UniversitÜt, Mainz, West-Germany

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Polymerization of Lipid and Lysolipid Like Diacetylenes in Monolayers and Liposomes

H.H. HUB, B. HUPFER, H. KOCH, and H. RINGSDORF

Institut für Organische Chemie
Johannes Gutenberg-Universität
D-6500 Mainz, West-Germany

ABSTRACT

Since conventional model membranes (Black Lipid Membranes (BLM), liposomes) lack in long time stability, a number of polymerizable lysophospholipid and phospholipid like monomers were synthesized, where the diacetylene moiety was the polymerizable unit. The hydrophilic head groups were simple ammonium, ether, or amine structures as well as phosphorus containing groups also occurring in nature. A first characterization of the monomers was achieved by surface pressure-area diagrams at the gas-water interface. Additionally, these monolayers could be polymerized via UV irradiation yielding stable,totally rigid polymer films. The mechanism of the polyreaction was followed with a new device permitting the recording of monolayer UV spectra. Electron microscopy showed that sonication of aqueous suspensions of phospholipid and lysophospholipid analogs yielded spherical vesicles, which could be polymerized by UV light under complete retention of their structure.

INTRODUCTION

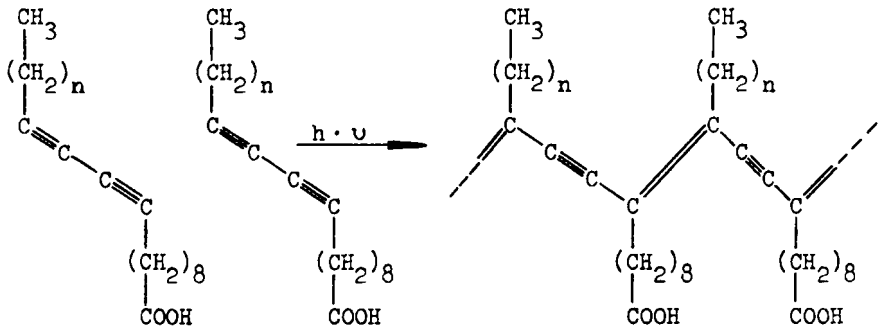
One of the most exciting phenomena in cell and molecular biology is the cell-cell interaction. If mice lymphocytes come in

contact with tumor cells, the result can be an interaction of the antigens of the cancer cell with the sensitized lymphocyte, which finally results in a destruction of the tumor cell [1]. This kind of interaction is an interesting problem to mimic. The simplest way to do this is to use phospholipid liposomes as cell models. Unfortunately, these vesicles have a very low stability, and in the collision of a tumor cell and a liposome the latter would be the less stable partner and would be destroyed or endocytosed. Therefore, our attention turns toward more stable cell models. These models can be obtained by connecting via polymerization the molecules forming a spherical liposome [6,7,8].

As initial model compounds we used simple diacetylene carbonic acids with different alkyl chain lengths (I). Since a monolayer at the gas-water interface can be viewed as one half of a biological membrane, we spread these monomers on a Langmuir trough and polymerized the resulting oriented monolayers via UV irradiation yielding very rigid, red colored polymer films (II) of a thickness of approximately 2,5 nm [2]. The same kind of topochemical diacetylene polymerization had formerly been observed in the solid state [3] and in multilayers [4] leading to the same kind of conjugated red colored polymers.

Our monolayer polymer films were also found to form bilayers if deposited onto a porous substrate such as an electron microscope grid. These bilayers can span holes as large as 0,5 mm in diameter, are stable in air and water for months,and are very good, stable cell membrane models.

Simple carbonic acids as (I) are not at all perfect analogs to naturally occurring phospholipids, and thus we synthesized



(I) (II)

compounds containing head groups also occurring in natural membranes such as the polymerizable phosphatidic acid analog (III), the cephalin analog (IV), and the lecithin analog (V) [5]. The lactone (XI) was the end product of a reaction, which was expected to lead to betain (XIV) [8]. Since natural phospholipids contain two alkyl chains per molecule, as initial models we prepared polymerizable compounds with two alkyl chains and simple ether (VI, VIII), amine (VII), ammonium (IX) [6,7], and betain (X) [8] head groups. Additionally, the head groups of the lysophospholipid analogs (III - V) were used to prepare the first real phospholipid analogs polymerizable in oriented systems (XII, XIII) [5].

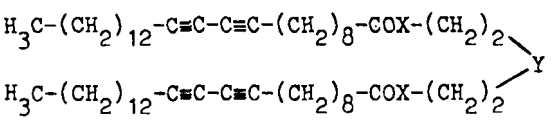
$H_3C-(CH_2)_{12}-C\equiv C-C\equiv C-(CH_2)_9-OR$

(III): R = PO_3H_2

(IV): R = $P(O^{\ominus})-O-(CH_2)_2-\overset{\oplus}{N}H_3$

(V): R = $P(O^{\ominus})-O-(CH_2)_2-\overset{\oplus}{N}(CH_3)_3$

$H_3C-(CH_2)_{12}-C\equiv C-C\equiv C-(CH_2)_8-CO-O-(CH_2)_2-N$ (XI)



(VI) : X = Y = O

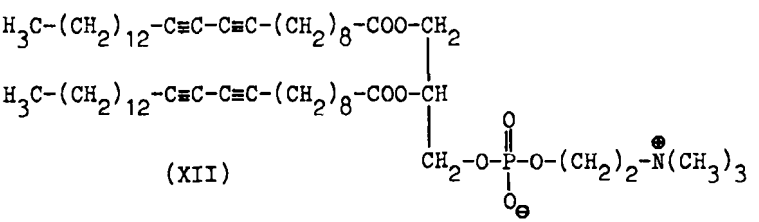
(VII) : X = O; Y = NCH_3

(VIII): X = NH; Y = O

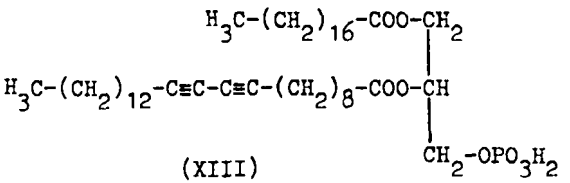
(IX) : X = O; Y = $\overset{\oplus}{N}(CH_3)_2\ Br^{\ominus}$

(X) : X = O; Y = $N\text{-}(CH_2)_2\text{-}SO_3H$

(XIV) : X = O; Y = $N\text{-}CH_2\text{-}COOH$



(XII)



(XIII)

The present work describes a) the spreading behavior of these monomers at the gas-water interface, b) their polymerization behavior in monolayers, and c) particularly their polymerization in liposomes, i.e. in defined spherical vesicles under retention of the orientation of the monomers.

EXPERIMENTAL

The preparation of the acids and alcohols of the type (I) has already been described [9]. For our experiments we used the acid (F: 68-69°C) and alcohol (F: 59-60°C) with n=12, which can be obtained from 1-Iodopentadecyne-1 and 10-Undecynoic acid and 10-Undecyne-1-ol, respectively as starting compounds. The phosphoric acid ester (III),(F: 79-81°C), was synthesized by the reaction of the corresponding alcohol (see above) with excess $POCl_3$ in dry CCl_4 and hydrolysis of the crude reaction product with H_2O [5,10]. In a similar manner, the cephalin (IV) and lecithin (V) analogs were prepared. β-Bromoethyl-phosphoric-acid-dichloride [10] and β-Phthalimidoethyl-phosphoric-acid-dichloride [11] were the phosphorylating reagents. The phthalic acid group was split off with Hydrazine [11] yielding (IV), (F: >230°C); the Bromo compound was quaternized with Trimethylamine in $CHCl_3/CH_3CN$ and bromine ions were removed with Ag_2CO_3 in $CHCl_3/CH_3OH/H_2O$ [13] yielding (V), (F: >250°C). Acylation of Diethyleneglycol, N-Methyldiethanolamine, Bis(2-aminoethyl)ether Hydrochloride, and N,N-Bis-(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES), respectively with the chloride of (I), n=12 (from (I) and Oxalylchloride) at 0°C in dry $CHCl_3$ in the presence of pyridine lead to the formation of (VI), (F: 59,5-60,5°C [6]);(VII), (F: 47,5-48,5°C [6]); (VIII), (F: 100-101°C [6]); and (X), (F: 109°C [8]). (IX), (F: 93,5-94,5°C [7]), was obtained by quaternization of (VII) with CH_3Br in dry acetone at 0°C. Conventional reactions of phospholipid chemistry [11,12] were used for the synthesis of (XII), (F: soft. 58°C, melted 190°C, cp. ref. 12 [5]). (XIII), (F: 37-38°C [5]) was prepared via the iodohydrine method with silver di-t-butyl phosphate as phosphorylating agent [14]. N,N-Bis(2-hydroxyethyl)glycine (BICIN) did not react with the chloride of (I), n=12 under the formation of the expected (XIV), but after

one alcoholysis step ring formation occurred and the lactone (XI) (F: 45°C [8]) was obtained.

Monolayers were spread from chloroform solutions all having concentrations of about 1 mg/ml. The films were spread on a LAUDA film balance, where surface pressure and area were automatically recorded. Polymerization was carried out via UV irradiation (254 nm) with an energy of 5 mW/cm^2 at the water surface under nitrogen [2]. UV absorbance in the monolayer was measured with a new device described recently [15].

Liposomes were prepared by sonication of aqueous suspensions (1 mg/ml) of the monomers in water under nitrogen at 50°C (BRANSON sonifier, model B 15). Polymerization of liposomes was achieved by irradiation of these solutions with multichromatic light (mercury high pressure lamp) at 18°C with a water filter between beam source and sample. In between, the samples were removed from the lamp and the optical density was measured (BECKMAN model 25 UV spectrometer).

Scanning electron micrographs were obtained with a ISI-40 scanning electron microscope operated at 15 kV at a magnification of 15,000.

SPREADING BEHAVIOR OF THE MONOMERS

The surface pressure-area diagrams of the lysophospholipid analogs (III, IV, V, XI) exhibit significant differences due to the different head groups. The phosphatidic acid analog (III) at 20°C shows a single condensed phase with a collapse point of 0,25 nm^2/molecule, whereas the cephalin analog (IV) exhibits both a condensed and an expanded film. In the diagram of compound (V) in the entire investigated temperature region (2°C to 50°C) only a liquid expanded phase occurs. This is due to the great volume of the trimethylammonium group, which prevents a dense packing of the alkyl chains in the monolayer. The lactone (XI) at 20°C also exhibits only a liquid expanded phase (Fig. 1).

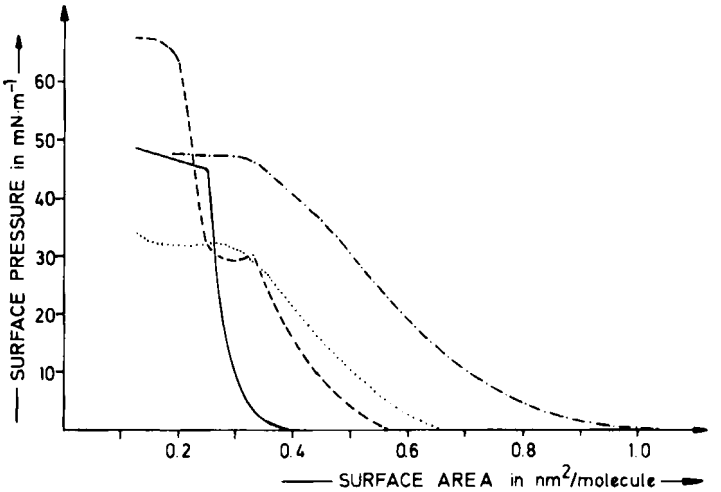


FIG. 1. Surface pressure-area diagrams of lysophospholipid analogs (III, IV, V, XI) at 20°C. (III): (————); (IV): (– – – –); (V): (—·—·—·—); (XI): (·····).

In contrast to the lysophospholipid analogs the lipid analogs (VI - X) show collapse areas greater than 0,40 nm^2/molecule as to be expected from the two long chains per molecule (Fig. 2). Monomers (VI) and (VII) at 20°C exhibit collapse areas of 0,42 and 0,43 nm^2/molecule, respectively without real sharp collapse points, whereas (VIII - X) show collapse areas of 0,48, 0,51, and 0,48 nm^2/molecule with sharp collapse points similar to monomer (III). The increase in the collapse areas for (VIII) and (IX) may be explained by hydrogen bonding between the molecules of (XIII) and a steric and charge repulsion for (IX) and (X).

MONOLAYER POLYMERIZATION

The UV initiated polymerization of diacetylenes is a topochemical reaction and does not take place in solution [3]. In the monolayer the polyreaction only occurs, if the temperature of the subphase is below the phase transition temperature i.e. in the

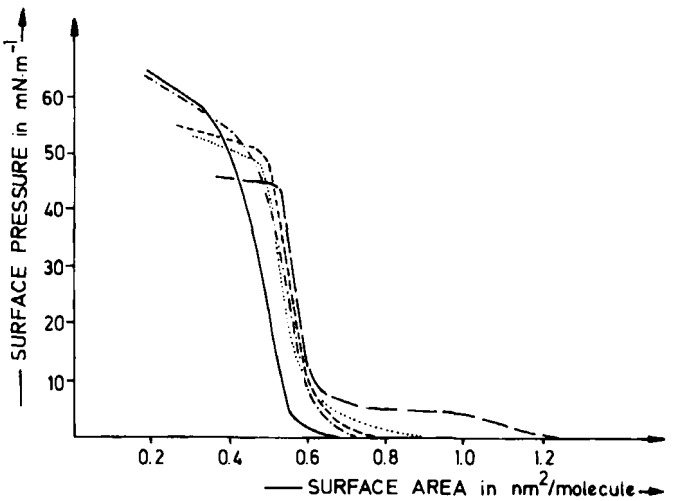


FIG. 2. Surface pressure-area diagrams of lipid analogs (VI - X) at 20°C. (VI): (—·—·—·—); (VII): (————); (VIII): (- - - - - - -); (IX): (—— ——); (X): (·····).

condensed phase, which again points to a topochemical effect. The polymerization under constant surface pressure can qualitatively be followed by the change of surface area [2]. However, an unchanged area after some time does not necessarily mean a complete conversion to the polymer. This can be proved with our new device [15] that permits the recording of monolayer UV spectra during the polymerization process.

As a result of the conjugated nature of the polymer backbone (II) the final polymerized monolayers of all the monomers have a high extinction coefficient in the visible spectrum and can be seen as a reddish tint on the water surface. Because the initial monomers are colorless the degree of absorbance of the monolayer is a criterion for the rate of conversion. With our apparatus the monolayer UV spectra of compounds (VII - IX) were recorded during the polymerization. Fig. 3 summarizes the absorbance curves of (VIII) at various irradiation times.

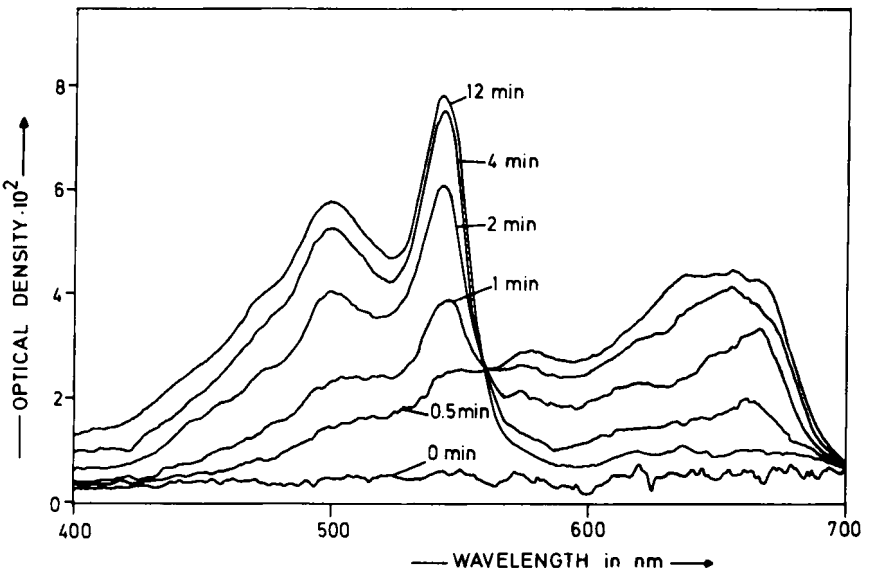


Fig. 3. UV/VIS Multiplot of absorbance of a monolayer of (VIII) versus polymerization time. Constant surface pressure: 10 $mN \cdot m^{-1}$; 20°C, N_2 atmosphere.

It becomes obvious that the blue species of the polymer (λ_{max}: 660 nm) can only be observed in the time interval from 0 up to 2 min. After that time the conversion to the red polymer takes place. The absorption spectrum of the final red polymer monolayer film (λ_{max}: 500, 540 nm) is identical with the spectra of polymer monolayers [6]. The color change blue→red during polymerization is also observed in the solid state and in multilayers and is probably due to a phase change from a monomer-rich to a polymer-rich solid solution. The blue color most likely results from the polymer dissolved in a monomer matrix. There is probably stress of some sort on the polymer backbone, causing a different electronic structure and therefore a different absorbance. Once enough polymer is formed, a transition from a monomer rich to a polymer rich phase occurs.

The reaction rates of these bifunctional monomers are higher than those of the acids (I), and also the maximum absorbances are slightly different from those of the monofunctional compounds. By further UV irradiation both show a decrease of the maximum absorbance, which could eventually be due to a degradation of the polymer backbone by radicals.

POLYMER LIPOSOMES

The investigations of Kunitake [16], Deguchi [19], and Fendler [20] have shown that a great variety of amphiphiles with two long chains are able to form a membrane like bilayer assembly. According to these investigations sonication of aqueous suspensions of the polymerizable phospholipid analogs (VII, IX, X, and XIII) results in the formation of clear colorless solutions of monomer liposomes. Electron microscopy proves the formation of bi- and multilayered vesicles of a defined spherical shape and of different diameters (range: 100 nm up to several μm). The diameter and number of bilayers strongly depend on sonication time and intensity. After 30 min of sonication only bilayer liposomes of a diameter of about 100 nm are formed. Further sonication does not alter the size.

We could also confirm the results of other investigators [17,18], who reported the formation of vesicles from a number of single chain amphiphiles. Aqueous suspensions of the acid (I), n=12, the corresponding alcohol, and the lactone (XI) did form liposomes upon sonication with ultrasound. Additionally, (XI) was found to form black lipid membranes [22], if a decane solution of this lactone is deposited on a small aperture of a teflon foil in an apparatus similar to that described by Mueller and Rudin [21]. Investigations concerning the polymerization behavior of these BLM are in progress.

UV irradiation of the monomer liposome solutions results in the formation of polymer liposomes indicated by a color change from colorless via blue to red. The polymerization rate can be followed by the change of optical density. Approximately the same kind of absorption spectra as for the polymer monolayers (Fig. 3) is obtained. Fig. 4 shows the UV/VIS spectrum of a liposome solution of (XI) after 45 min of UV irradiation showing that only traces of the blue polymer (λ_{max}: 606, 656 nm) are still present.

Electron microscopy shows that the shape of the liposomes remains unchanged during the polymerization. Only the diameters of the spheres are smaller than those of the monomer liposomes. As far as we know, this is the first example for a transformation of a defined monomeric liposome into a defined polymeric liposome

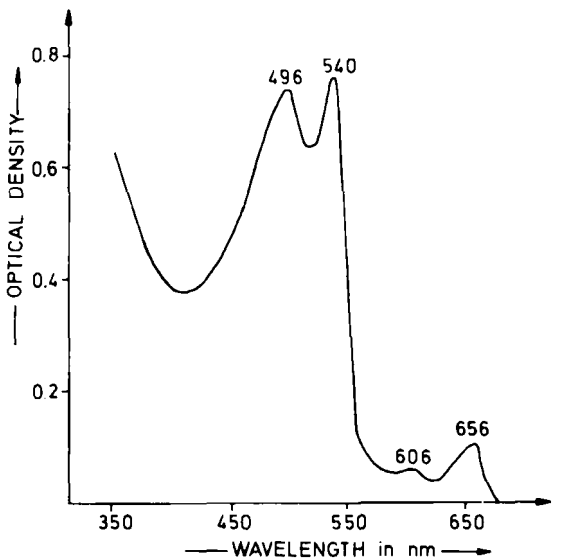


FIG. 4. UV/VIS Spectrum of red polymer liposomes of (XI) after 45 min of UV irradiation. Conc. of (XI): 1 mg/ml.

under complete retention of the orientation and shape of the vesicle.

The polymeric character of our liposomes is also established by their enhanced stability. In contrast to their monomeric analogs aqueous solutions of the polymer liposomes are stable for months. Moreover, they cannot be destroyed by organic solvents. The dilution of an aqueous polymer liposome solution with 50% of ethanol, for instance, does not result in a precipitation. The liposomes retain their structure, what could be shown by electron microscopy. Precipitation of the vesicles can be achieved by the addition of salts, but again it has to be pointed out that in contrast to low molecular weight liposomes the layer structure of our vesicles is not destroyed.

The possibility of obtaining scanning electron micrographs of the polymer vesicles (Fig. 5) is an additional proof of the enhanced stability. Contrary to the monomer vesicles, which are destroyed during the preparation of the samples. The polymerization of diacetylene monomers in liposomes points to a topochemical effect even in the spherical double layer orientation of these monomers.

In the meantime, additionally phospholipid analogs containing butadiene and vinyl moieties have been prepared [23,24]. Their syntheses and polymerization behavior will be described elsewhere.

CONCLUSIONS

A variety of phospholipid analogs containing polymerizable diacetylene groups were polymerized in monolayers at the gas-water interface as well as in liposomes yielding either extremely rigid planar polymer monolayers or very stable polymer vesicles. Application possibilities of these stable synthetic model

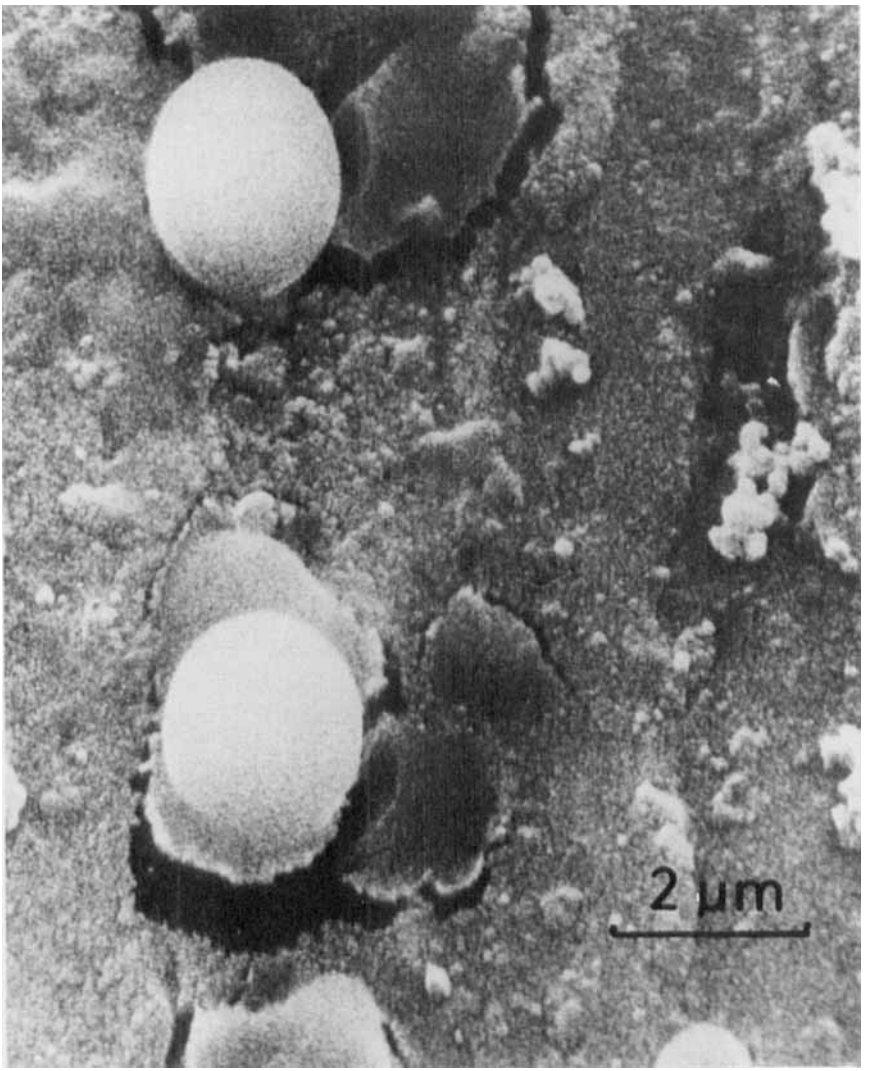


FIG. 5. Scanning electron micrograph of red polymer liposomes of (IX). Magnification: 15,000.

membranes are, for instance, their use as drug carriers or as models for the cell-cell interaction. Investigations on leakage and ruptures in the shell of the polymer vesicles are in progress as are attempts to cosonicate natural and polymerizable lipids with the presence of proteins to obtain totally stable cell models after polymerization.

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