Vol. 203, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 30, 1994 Pages 296-305

Formation and Properties of a Network Gel Formed from Mixtures of Diacetylenic and Short-Chain Phosphocholine Lipids

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Received June 27, 1994

Summary: A polymerizable gel superaggreagate has been formed from low concentrations (6 mM) of phospholipid mixtures of polymerizable 1,2-*bis*(tricosa-10,12-diynoyl)-*sn*-glycero-3-phosphocholine and 1,2-*bis*(dinonoyl)-*sn*-glycero-3-phosphocholine. Transmission electron microscopy (TEM) revealed that the superaggregate structure consisted of a network of twisted, braided fibers and that the pore size of the gel ranged from 0.1 to 1.0 μm. TEM of gel plated with Ni revealed that the width of the fibers was 280 A. Optical microscopy demonstrated that the onset of the gel phase occurred at mole fraction 0.43 DNPC in the absence of salt and 0.36 in the presence of 0.25 M NaCl. Polymerization did not affect the morphology of the gel but did increase its temperature stability.

Studies on pure lipid systems have led to a better understanding of the relationships between lipid structural features and lipid membrane properties. Many of these structure/property correlations can carryover into systems of mixed lipids, but other novel membrane properties may also arise as a result of lipid mixing. Mixed-lipid systems consisting of polymerizable and nonpolymerizable lipids have previously been studied to examine the effect of mixing on polymerization, membrane integrity, and other physical chemical properties (1, 2). The polymerization moiety in lipids, usually methacrylates or dienes, was located either in the beginning or at the terminus of the hydrocarbon chains. The non-polymerizable lipids can be long-chain zwitterionic lipids, such as dipalmitoyl phosphatidylcholine (DPPC), or dipalmitoylphosphatidylethanolamine (DPPE), a nonbilayer forming lipid.

Polymerization in the middle of the acyl chains generally requires precise alignment, as in the case of the diacetylenes. However, it has been shown that addition of the short-chain 1,2-*bis*(dinonoyl)-*sn*-glycero-3-phosphocholine (DNPC), as "spacer" lipids mixed with 1,2-*bis*(tricosa-10,12-diynoyl)-*sn*-glycero-3-phosphocholine ($DC_{8,9}PC$) significantly enhances the efficiency of polymerization of the diacetylenic lipids (3,4). This mixture of lipids, furthermore, can incorporate significant amount of membrane protein even after extensive polymerization (5). The addition of DNPC changes the permeability of the membrane. Solute leakage across the

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membrane increases with DNPC content, but DNPC stabilizes polymerized liposomes to detergent lysis (6). We report the formation of a new state of superaggregation for mixtures of $DC_{8,9}PC$ with DNPC. The observed network-gel-like aggregation is a unique behavior for lecithin/water systems and also represents the first known case of a polymerizable gel-network.

Materials and Methods

Materials: 1,2-*Bis*(tricosa-10,12-diynoyl)-*sn*-glycero-3-phosphocholine was synthesized according to literature procedures (7). DNPC was purchased from Avanti Polar Lipids (Birmingham, AL). Chromatographically pure carboxyfluorescein (CF) was obtained from Calbiochem (La Jolla, CA).

Lipid Preparation: $DC_{8,9}PC$ and DNPC were mixed in $CHCl_3$, dried under Ar, and stored in vacuum overnight. The lipids were dispersed in either distilled water or buffer above 55 °C. Polymerization was achieved using UV radiation (254 nm) at 18 °C (15 min) and polymerization monitored by phosphate analysis of the thin layer chromatographed gel according to literature procedures (8).

Encapsulation Assay: $DC_{8,9}PC$ PC and DNPC at 1:1 were dried as above. One mL of 50 mM CF was added to 6 umol of lipid mixture and the dispersion allowed to sit at room temperature until a gel-like flocculent is observed on the bottom of the test tube. Exogenous CF was washed off by centrifuging for 3 min. at 2,000 rpm (IEC Centra-8R centrifuge), pulling off the supernatant, re-infusing with 130 mM NaCl and 20 mM Tris (pH 8.0). The procedure was repeated six times. Samples for fluorimetric analysis were prepared with 25 µL of the gel diluted with 3 mL of buffer. Excitation wavelength was 492 nm and emission measured at 520 nm (SLM 8000, Urbana IL).

Microscopy: The structures formed from the aqueous dispersions were observed using a Nikon model diathote inverted optical microscope equipped with a temperature controlled stage. The gel structures were heated at rates of 2 to 5 °C/min for thermal stability studies. Transmission electron microscopy (TEM) of microstructures was accomplished with a Zeiss EM-10 microscope. Samples were pipetted onto carbon coated copper grids, air dried and observed directly. All unpolymerized samples were stained with phosphotungstic acid while polymerized samples were examined unstained. Scanning electron microscopy of the Nicoated gel was performed on an ISI-DS130 scanning electron microscope. The gel was electrolessly plated with Ni according to literature procedures (9).

Calorimetry: Calorimetric measurements were taken with a Perkin-Elmer DSC 7. Samples were sealed in stainless steel pans. Heating scans were done at 10 °C/min.

Results

The morphology and aggregate properties of mixtures of $DC_{8,9}PC$ and DNPC change greatly as a function of lipid ratio and salt concentration. Tubular growth can be initiated from pure $DC_{8,9}PC$ within a narrow range about T_m (43 °C)(10). When DNPC is present in a mixture with $DC_{8,9}PC$ at mole fraction of 0.50, cylindrical structures are formed that slowly (within a period of 2 to 24 hours) transform into a gel-like network of entangled, "braided" fibers (Figure 1a,b). DSC measurements (10 °C/min) of the gel showed a broad endotherm exists between 35 - 48 °C indicating a co-existence region between two phases within that temperature range.(data not shown). The fibers are themselves composed of helically wrapped threads. In order to determine the diameter of the threads, the gel was electrolessly plated with Ni. The metallized

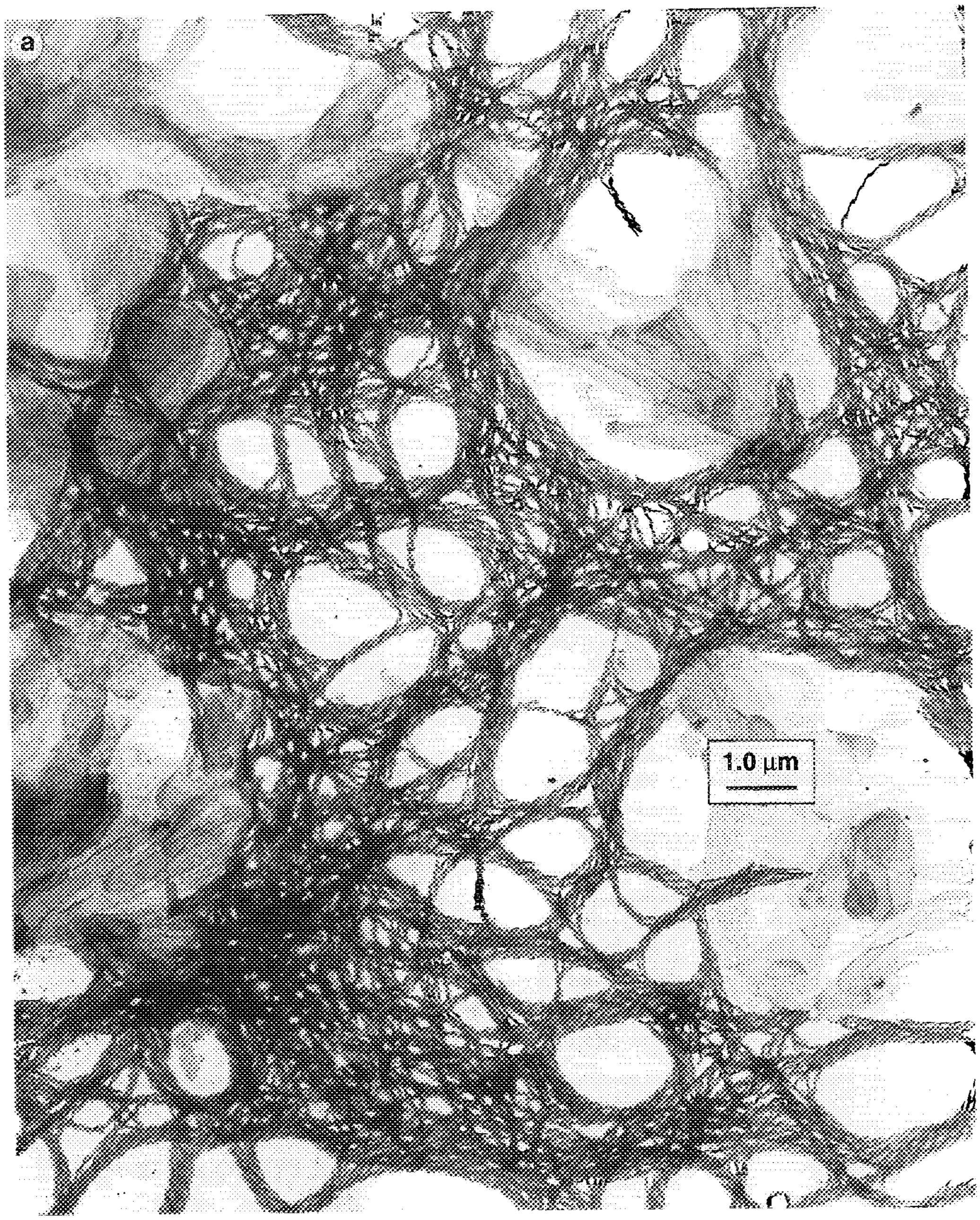

Fig. 1. Transmission electron micrographs of gel superaggregate.

gel was then examined by SEM and TEM (200 kV) and the thread diameter was found to be 280 A (Figure 2a,b).

In order to determine the phase boundaries of this gel, the change in phase behavior as the concentration of DNPC and ionic strength varied was monitored by optical microscopy. In

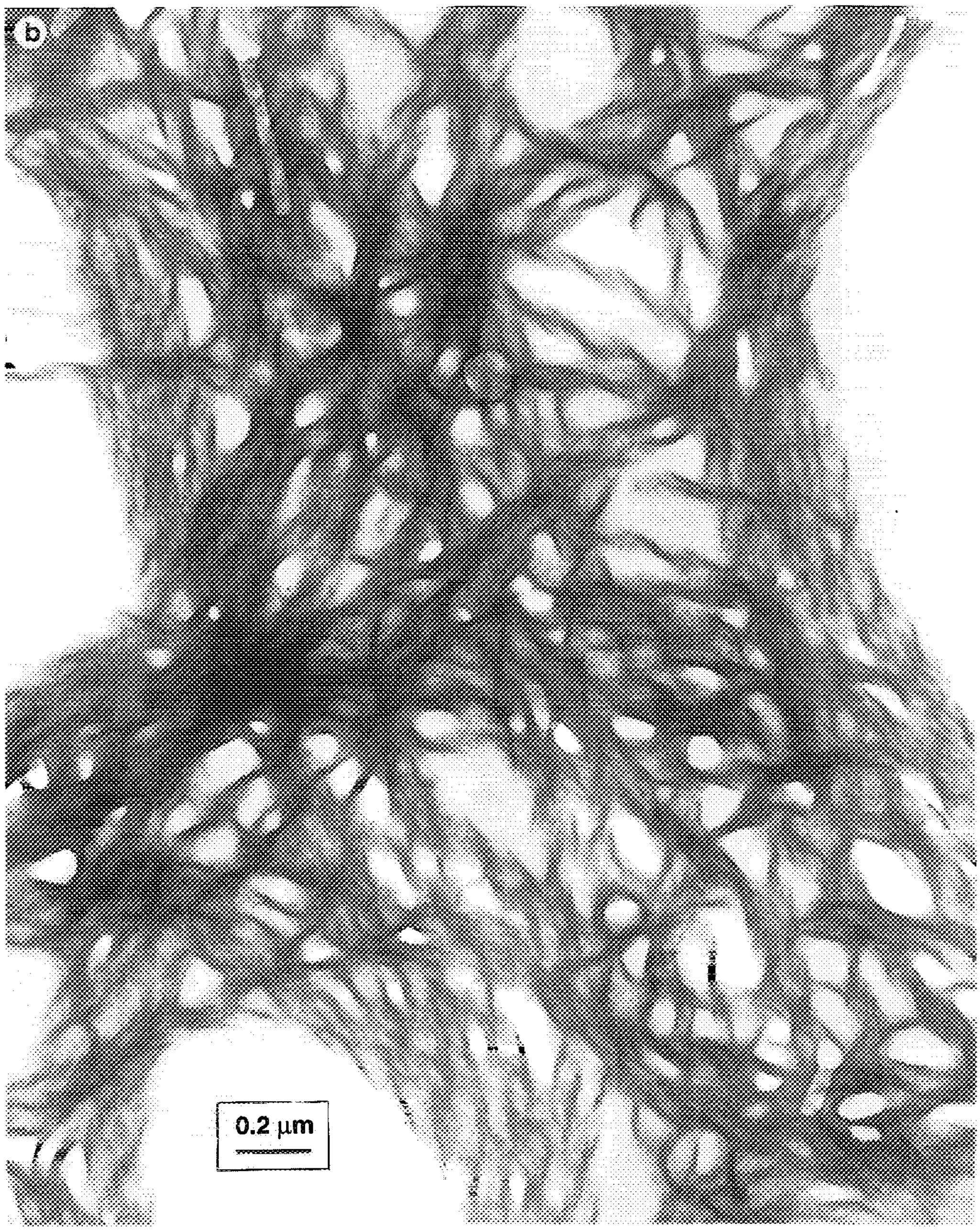

Fig. 1. - Continued

the absence of salt, a sharp change in morphology from vesicles to a superaggregate structure was observed as the molar percentage of DNPC increased from 0.41 to 0.43 (Figure 3). As seen in figure 3b, the structure of the gel superaggregate was comprised of large clusters which had aggregated to form domains with distinct boundaries. The DNPC mole fraction region in which this superggregate was observed extended from 0.43 to 0.50. In the DNPC mole fraction region

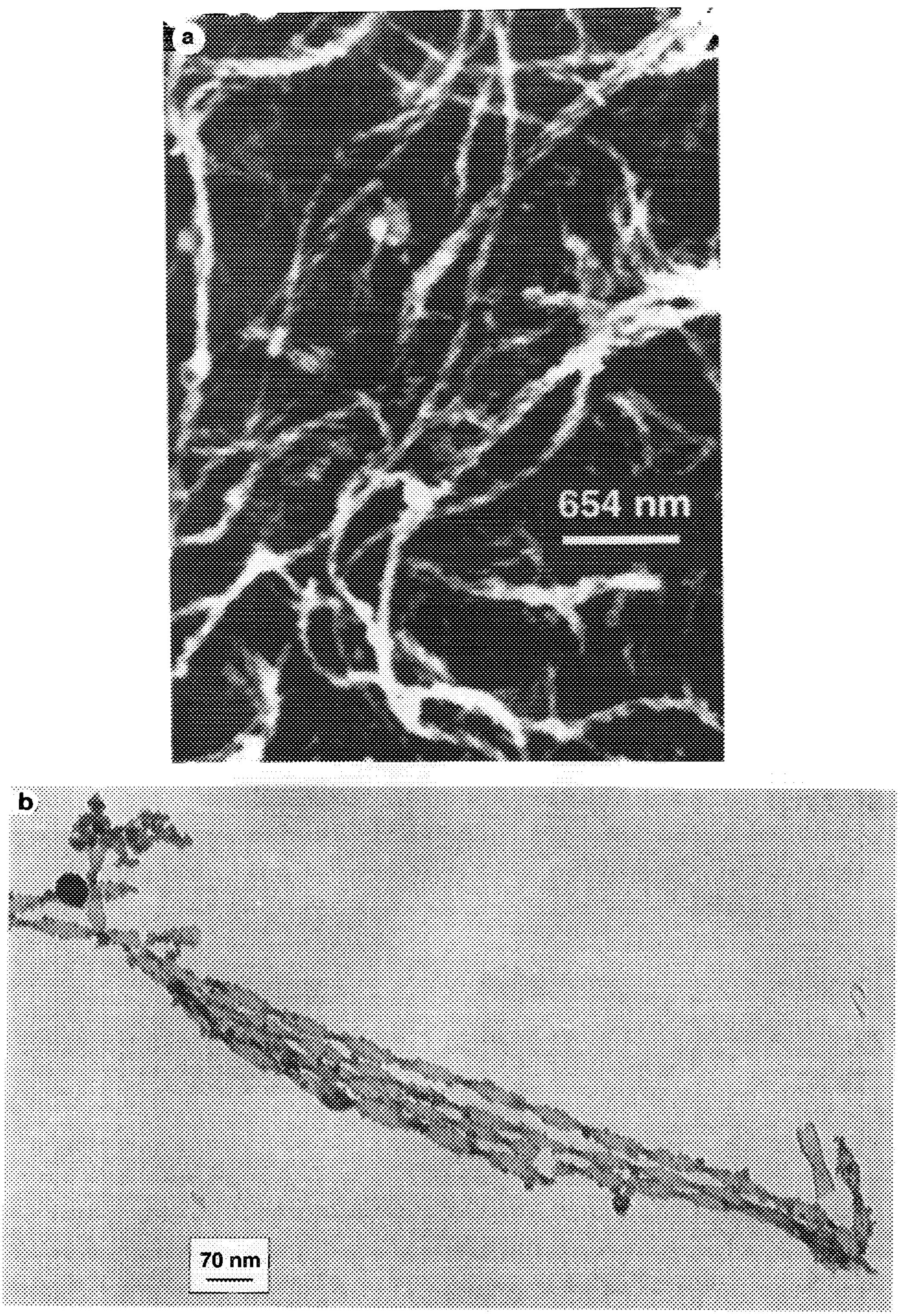

Fig. 2. a)Scanning electron micrograph and b) transmission electron micrograph of Ni plated gel superaggregate.

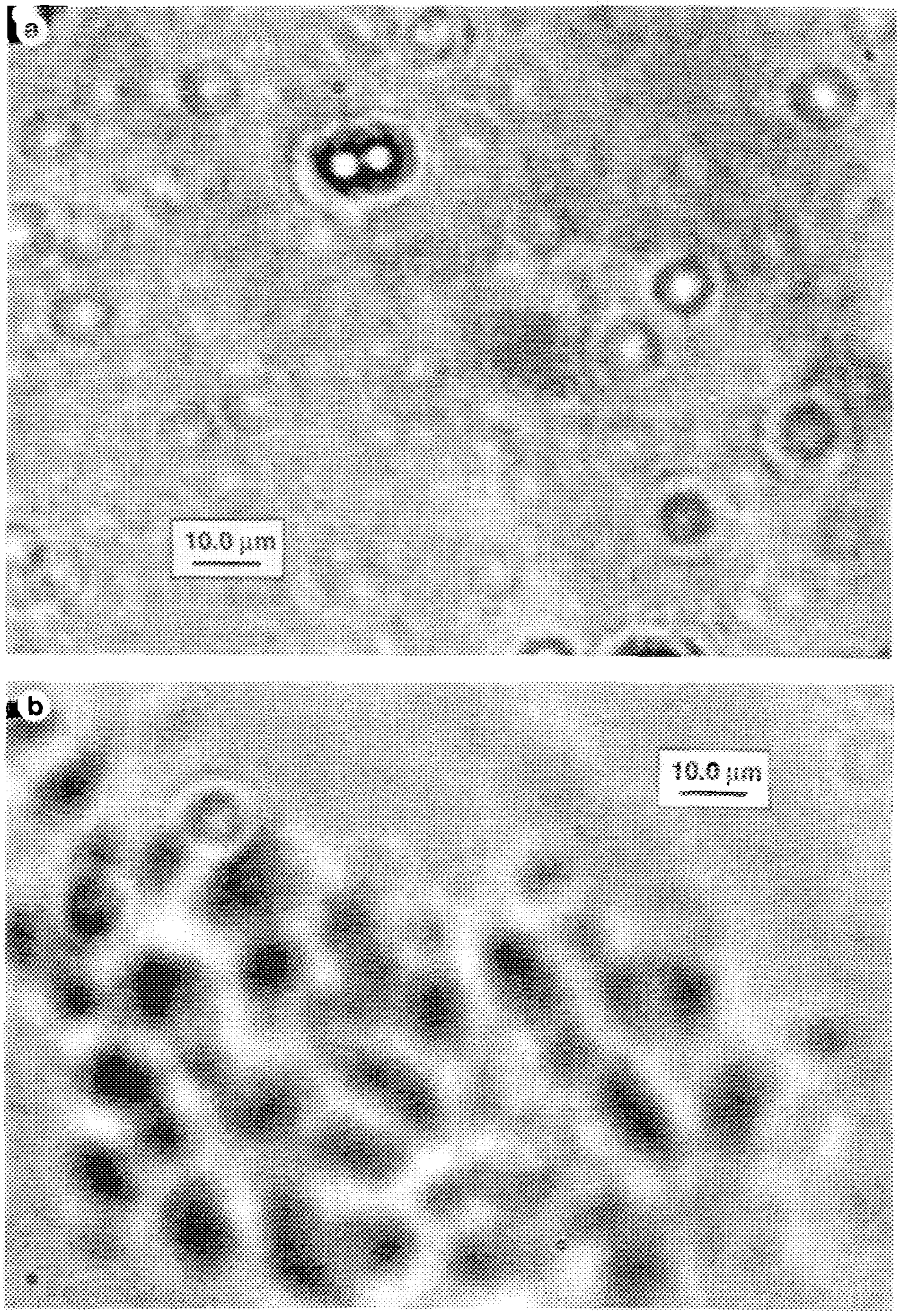

Fig. 3. Optical micrographs of mixtures of DC$_{8,9}$PC and DNPC at a) 0.41 mole fraction DNPC and b) 0.43 mole fraction DNPC.

0.51 to 0.59, the superaggregate was no longer comprised of a continuous array of domains but rather consisted of irregularly shaped structures which had agglomerated into separate clusters. The size of both the structures and the clusters progressively decreases as the mole fraction of

DNPC increases from 0.59 to 0.67. Also, the morphology of the structures became more spherical as the mole fraction of DNPC increased.

In the presence of 0.25 M NaCl, the types of structures observed by optical microscopy were similar to those observed in the absence of salt. However, the DNPC mole fraction boundaries between the various structures was altered. At 0.25 M NaCl, gel-formation took place within 1 hour instead of proceeding slowly through the large cylindrical structure intermediates. The onset of gel-superaggregate comprised of individually aggregated domains began at DNPC mole fraction of 0.36 as opposed to 0.43 in the absence of NaCl. The superaggreagate structure was observed as the mole fraction of DNPC was increased to 0.50 and then clusters of irregularly shaped spheres were observed in the DNPC mole fraction region of 0.51 to 0.59. As the DNPC mole fraction was further increased from 0.59 to 0.67, the size of the clusters of structures decreased but the size of the structures did not. In addition, the morphology of the structures did not change.

Both optical microscopy and TEM demonstrated that polymerization did not disrupt braided structure of the gel (Figure 4). Exposure of the gel to UV radiation (254 nm) for 15 min at 18 °C produced a change in color of the gel from white to red. Phosphate analysis of the thin layer chromatographed gel formed from a 1:1 mixture of the phospholipids revealed that, both in the presence and absence of salt, 33 % of the diacetylenic phospholipid monomer had reacted. The effect on the thermal stability of the gel can be seen from the results of optical microscopy studies of the effect of temperature on the gel structure. At 43 °C (the T_m of $DC_{8,9}PC$), the domain structure of the unpolymerized gel began to break apart and by 50 °C the gel structure was completely disrupted. This behavior was consistent with the existence of the broad endotherm observed by DSC in this temperature range. In contrast, the polymerized superaggregate separated into large clusters when the gel was heated to 50 °C. No further disruption of superaggregate structure was observed when the temperature was maintained at 50 °C for 6.5 min. Upon heating the gel to 70 °C, the larger clusters broke up into smaller clusters.

Fluorescence measurements of washed gels (both polymerized and unpolymerized) showed no release of CF in the presence of excess triton, implying that neither the unpolymerized nor the polymerized networks are capable of retaining aqueous solutes.

Discussion

While $DC_{8,9}PC$ is known to form elongated tubules, the tubules are relatively stiff and do not exhibit any tendency to form a gel. We report the discovery here that when $DC_{8,9}PC$ is mixed with DNPC close to 1:1 ratios, the lipids form twisted, flexible, braided fibers that entangle to form a polymerizable, viscous gel with pores on the order of 0.1 to 1.0 um. This state is annealed only slowly in distilled water, but appears much more rapidly at higher ionic

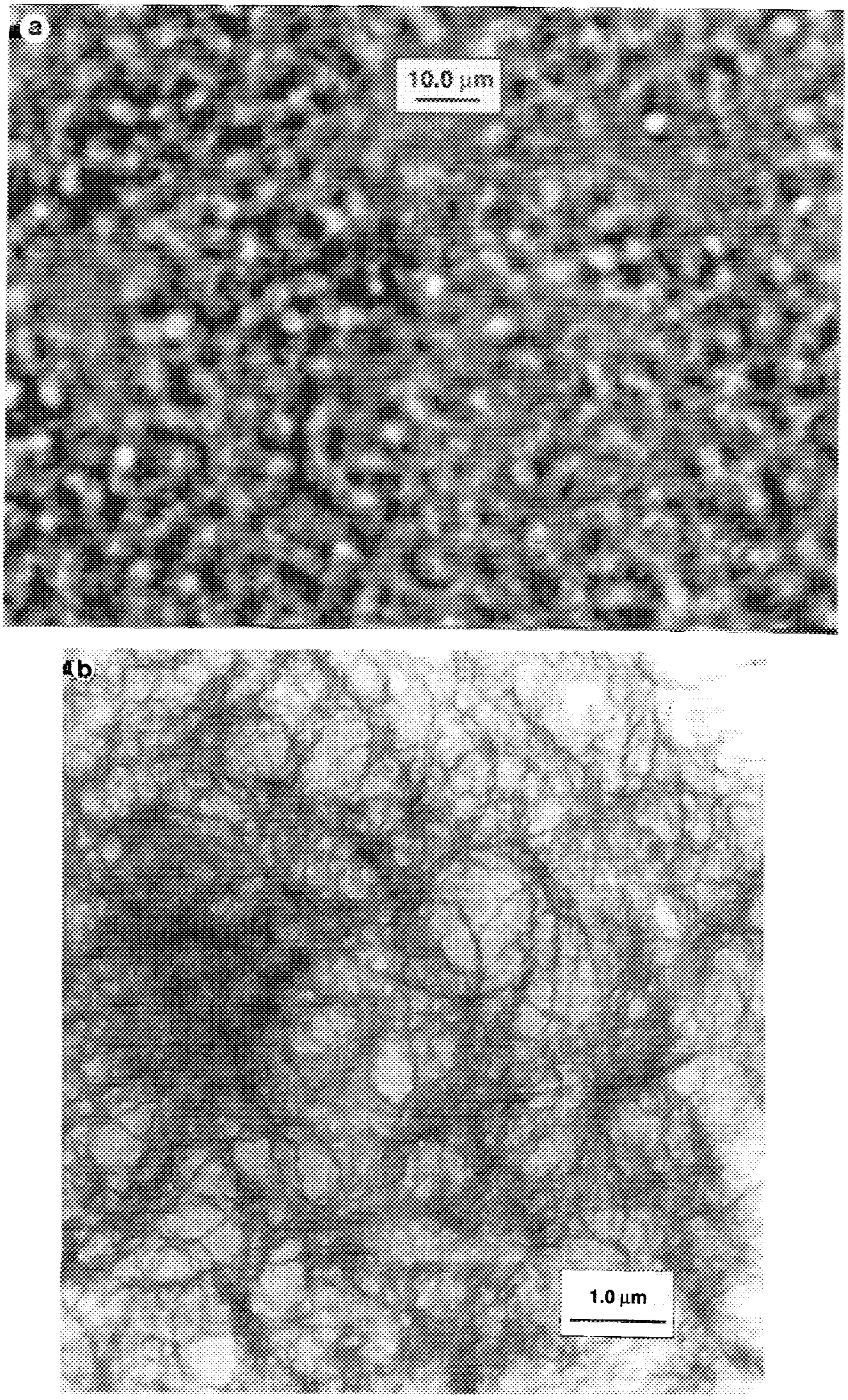

Fig. 4. a)Optical micrograph and b) transmission electron micrograph of polymerized 1:1 mixture of DC$_{8,9}$PC and DNPC.

strength. The long braids can further be polymerized to preserve the entangled state, but the gel does not appear to have a capacity to encapsulate aqueous solute.

The existence of bulk gel-like phases for amphiphiles are of interest both for verifying theories of entangled-network phases (11,12) and for potential biomedical applications (13,14). However, previously reported systems were either composed of ionic amphiphiles at high concentrations and/or high mineral salts , or involved mixtures of amphiphiles with organic additives. There has been one report on the aggregation of N-octylaldomamides, a class of non-ionic amphiphilic molecules, forming gels in aqueous solutions with as little as 0.4 % (w/v) (15). However, systems where flexible, elongated structures occur in aqueous solutions at very low concentrations have not been reported until now. In addition to the ability of creating gels at low concentrations, the long entangled fibers of $DC_{8,9}PC$ and DNPC have the further advantage of polymerizability. That is, the gel structure, once formed, can be locked in place through polymerization of the diacetylenic bonds. Once polymerized, the external solution conditions may be changed as needed without destroying the gel structure. In terms of pharmaceutical applications, this system could potentially serve as a porous support material with the added advantage of being biocompatible with tissues. In addition, the low concentrations of lipids that can result in formation of a gel state may also make this system a potentially valuable model to test the power law dependencies of rheological properties on concentration for polymer solutions in the dilute regime.

Acknowledgments

The authors would like to thank Dr. B. Ratna for access to her optical microscope. This research was supported by the Office of Naval Research through an NRL Core program on Synthetic Membranes.

References

1. Okahata, Y. (1986) Acc. Chem. Res. 19: 57-63.
2. Singh, A. and J. M. Schnur (1993) In Phospholipids Handbook. (G. Cevc, Ed.), pp. 233-291, Marcel Dekker, Inc., New York.
3. Rhodes, D. G. and Singh, A. (1991) *Chem. Phys. Lipids*, *59*, 215-224.
4. Singh, A. and Gaber, B. P. (1988) In *Applied Bioactive Polymeric Materials* (Gebelein, Carraher Jr and Foster Eds), pp. 239-249, Plenum Press, New York.
5. Ahl, P. L., Price, R, Smuda, J., Gaber, B. P. and Singh A. (1990) *Biochim. Biophys. Acta*, *1028*, 141-153.
6. Markowitz, M. A,, Chang, E. and Singh, A. (1994) In *Diagnostic Biosensor Polymers* (A. Usmani and N. Akmal), pp. 264-274, American Chemical Society, Washington, D.C.
7. Singh, A. (1990) *J. Lipid Res.*, *31*, 1422-1425.
8. Regen, S. L., Kirzensztejn, P. and Singh, A. (1983) *J. Am. Chem. Soc.*, *105*, 335-338.

9. Price, R, and Patchan, M. (1991) *J. Microencapsulation*, *8*, 301-306.
10. Yager, P. and Schoen, P. (1984) *Mol. Cryst. Liq. Cryst.*, *106*, 371-381.
11. Makhloufi, R., E. Hirsch, S. J. Candau, W. Biana-Limbele, and R. Zana (1989) *J. Phys. Chem.*, *93*, 8095-8101.
12. Porte, G. (1983) *J. Phys. Chem.*, *87*, 3541-3550.
13. Capitani, D., Segre, A. L., Sarapani, R., Giustini, M., Scartazzini, R. and Luisi, P. L. (1991) *Langmuir*, *7*, 250-253.
14. Scartazzini, R. and Luisi, P. L. (1988) *J. Phys. Chem.*, *92*, 829-833.
15. Fuhrhop, J.-H., Schnieder, P., Boekema, E. and Helfrich, W. (1988) *J. Am. Chem. Soc.*, *110*, 2861-2867.