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Synthesis and Self-organizing Properties of Iminodiacetic Phospholipids and Their Role in Noncolvant Enzyme Immobilization on Organized Assemblies

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Diacetylenic phospholipids with metal chelating iminodiacetic acid (IDA) headgroup, [R-O-CH_2-CH(OR)-CH-OP(OH)(O)-$(OCH_2CH_2)_n$-$N(CH_2COOH)_2$, where R = palmitoyl or tricosa-10,12-diynoyl and n = 1-3], have been synthesized to explore their technical utility. The lipids homogeneity was confirmed by ^1H and ^{13}C NMR spectrometry. The microstructure formation properties of the lipids were studied by Langmuir film balance technique and transmission electron microscopy (TEM). Synergistic influence of copper ions, ionic strength and pH of the dispersion medium, and length of the ethyleneoxy spacer in the headgroup caused the formation of ribbons and tubules. Increasingly, racemic diacetylenic phospholipids upon binding with copper produced ribbons and tubules when di- and tri-ethylenoxy spacer was inserted to separate phosphate and IDA moieties. In fully formed tubules, helical striations of uniform handedness were observed. Mixture of metal chelating lipids with diacetylenic phosphatidylcholine produced vesicles. To these vesicles and enzyme bovine carbonic anhydrase II (EC 4.2.1.1) was non-covalently linked with the aid of copper ion. An affinity between copper-IDA complex and histidines available on enzyme surface facilitated this binding without affecting enzyme activity. Polymerized vesicles showed improved stability and sustained activity of enzymes.

14. SUBJECT TERMS

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Synthesis and Self-organizing Properties of Iminodiacetic Phospholipids and Their Role in Noncovalent Enzyme Immobilization on Organized Assemblies

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Abstract

Molecular self-assembling behavior of diacetylenic phospholipids has been studied to explore their technical utility. Diacetylenic phospholipids equipped with metal chelating iminodiacetic acid (IDA) headgroup of the following general formula (R-O-CH_2-CH(OR)-CH-OP(OH)(O)-$(OCH_2CH_2)_n$-N$(CH_2COOH)_2$, where R = palmitoyl or tricosa-10,12-diynoyl and n = 1-3 have been synthesized. The lipids were characterized by ^1H and ^{13}C NMR spectrometry. The microstructure formation properties of these lipids were characterized by transmission electron microscopy (TEM) and the monolayer properties were examined at the air-water interface. The formation of ribbons and tubules from lipid bilayers of diacetylenic IDA lipids was dependent on the synergistic influence of copper ions, ionic strength and pH of the dispersion medium, and length of the ethyleneoxy headgroup spacer. The copper ion-bound racemic phospholipids produce ribbons made from lipid bilayers which attained a hollow cylindrical shape when distance between phosphate group and IDA moiety was changed from O-CH_2-CH_2- to -(O-CH_2-CH_2-)$_3$. In fully formed tubules, helical striations were observed without any preference for handedness. Upon mixing the metal chelating lipids with diacetylenic phosphatidylcholine lipids, vesicles were produced. Copper ions were bound to the metal chelating headgroups and the vesicles were then utilized to bind the enzyme bovine carbonic anhydrase II (EC 4.2.1.1) to the outer vesicle membrane. This process utilized the affinity of copper-IDA groups towards histidines present on the surface of carbonic anhydrase. The catalytic activity of surface-bound protein molecule on polymerized and non-polymerized vesicles was measured. Only the polymerized vesicles showed improved stability and sustained activity of enzymes. These studies demonstrate that metal ions bound to lipid headgroup may be used for non-covalent immobilization of enzymes and proteins, as well as to influence the morphology of the lipid micro-structures by affecting, a) headgroup to acyl chain ratio, b) charges on the polar headgroup, and c) geometrical shape of the molecules.

Introduction

The fabrication of molecular assemblies is driven by both a desire to understand the relationship between molecular geometry and microscopic shape, and to explore the utility of microstructures in various applications.[1,9] Our aim is to synthesize new molecular materials to gain a better understanding of the self-assembling processes, to create novel microscopic structures of defined

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morphologies efficiently and cost-effectively, and to explore the technical utility of the resultant structures.

A survey of recent literature reveals the existence of three major strategies that are currently being followed to construct supramolecular assemblies: Host-guest chemistry involving hydrogen bonding or metal ions[1,9-11]; self assembly of molecules into aggregates of macroscopic structures depending on the molecular shape[12]; and molecular self-organization of amphiphiles including diacetylenic lipids.[7,13,14] It is the latter approach that interests us due to the simplicity in achieving a macroscopic structure through molecular design of the amphiphiles[11,15] which can then be utilized in a variety of technological applications.[7,16]

Self-assembling of diacetylenic phospholipids is known to be influenced by the diacetylenic functionality in the acyl chains.[7] Thus diacetylenic lipids are unique in that in addition to the formation of closed bilayer vesicles formed by other lipids, they also form helices and hollow, cylindrical structures (tubules).[7,17] Morphologically, tubules consist of tightly wrapped and overlapped helices.[18-20] Changes in the headgroup region of amphiphiles allow control of the resultant morphology[14] and enable tailoring of the chemistry at membrane interface.[21,22]

Our interest in how phospholipid headgroups and dicetylenic acyl chains synergistically influence the formation of self-organized structures prompted us to design diacetylenic phospholipids with metal chelating headgroups to facilitate metal ion- assisted supramolecular formation. The self-assembling properties of pure enantiomers of negatively charged lipids has been extensively explored.[14] The constant diameter of tubules was altered by modulating headgroup geometry and orientation through metal ion binding and by changing the ionic strength and pH of the dispersion medium.

One goal of this study was to examine the influence of metal ions bound to the metal iminodiacetic acid (IDA) headgroups present in lipid microstructure assemblies. A second goal was to take advantage of Cu-iminodiacetate affinity towards histidines to utilize polymerizable vesicles in noncovalent protein immobilization. The bivalent metal ion - IDA complex is known to have a high binding affinity towards histidine containing compounds.[23-24] This strategy may allow any protein or enzyme containing surface available histidines to be effectively immobilized on the surface of microstructures.[25] To achieve the aforementioned goals, metal chelating phospholipids with palmitoyl and diacetylenic acyl chains, and ethylenoxy spacers of varying length between the phosphate and IDA functionality in headgroup region have been synthesized (Figure 1). All lipids used in this study were racemic mixtures.

Materials and Methods

General. Chloroform, methanol, ether, and acetone were obtained from Burdick and Jackson. All other chemicals and solvents were purchased from Aldrich Chemical Company. Bovine carbonic anhydrase II (EC 4.2.1.1)and p-nitrophenyl acetate were obtained from Sigma Chemical Company (St. Louis, MO). An 80 μg/mL enzyme stock solution was prepared in 100 mM Tris -HCl (pH 8.5) buffer and stored refrigerated. Triple distilled water was used in the lipid characterization studies. Polymerizable diacetylenic lipid 1,2-bis(tricosa-10,12-diynoyl)-sn-glycero-3-phosphocholine was synthesized.[26] For the purpose of clarity in the discussion, the phospholipids reported in this paper

have been abbreviated as follows: dipalmitoyl phosphoiminodiacetic acid lipids as DPPIDA (n=1, 2 or 3) and diacetylenic phospholipids as $DC_{8,9}PIDA$ (n=1, 2 or 3) where n represents the number of ethyleneoxy units in the spacer between the phosphate and the iminodiacetic acid group. The term phospho-IDA is used for all the phospholipids containing iminodiacetic acid headgroup. The designation of various protons and carbon chemical shifts of lipids and their intermediates is made according to the letters depicted in Figure 2. The glycerol carbons (and the attached protons) are labelled as a, b and c. The ether headgroup carbons are sequentially labelled d, e, f, g, h and i. Thus the labels for the ether headgroup carbons would be d, e for n=1, d, e, f, g for n=2 and d, e, f, g, h and i for n=3. The iminodiacetic acid carbons are designated as k and l while the ketal carbon is j. ^{13}C NMR chemical shifts for compounds **1-6** are compiled in Tables 1-4.

Purity of all the compounds was monitored by thin layer chromatography on silica gel (E.M. Merck) using chloroform : methanol : water (65:25:4, Solvent A) and chloroform : methanol : water : acetic acid (65:25:4:6, Solvent B). For column chromatography and TLC analysis, silica gel 60, 70-230 mesh and silica gel 60 F254 (both E. Merck) were used. For flash chromatography 230-400 mesh silica gel 60 (Merck) was used. Cationic impurities were removed by using Biorad resin AG 50W-X8, 200-4000 mesh.The lipids on TLC plates were visualized by a combination of three techniques; UV, iodine, and molybdenum blue spray. Synthetic intermediates and lipids were characterized by IR (Perkin-Elmer 1800 FT-IR or a Mattson Instruments Inc. Galaxy RS/1 research series FT-IR), and NMR (Bruker MSL-360 250 MHz) spectrometer. Ultrasound agitation was carried out on a NEY 300 ultrasonic bath. Enzyme assays were performed using a Durrum stopped-flow spectrophotometer equipped with an A/D converter and the OLIS stopped-flow operating system from Online Instrument Systems Inc. (Jefferson, GA). Force-area isotherms were recorded on a thermostated Wilhelmy plate film balance (NIMA) with an automatic data collection unit. A Zeiss EM-10 microscope was used for transmission electron microscopy (TEM).

Preparation of Buffer Solution. The pH of the dispersion medium was maintained using acetate buffer (pH 5.6) and Tris-HCl buffers (pH 7.0, 8.0, 9.0). Ionic strength (I) was maintained by adding an appropriate volume of NaCl solution. Copper chloride concentration in the buffers was adjusted by adding aliquots from a 50 mM aqueous copper chloride solution.

Monolayer Studies. Lipids were dissolved in $CHCl_3$ and monolayers were spread with a Hamilton syringe on subphase of water containing $CuCl_2$ ($[CuCl_2]$ = 0 to 10 mM, 23 °C) and compressed 15 min. after spreading. Monolayers were compressed at a rate of 10-12 A^2/molecule/min up to their collapse pressure and were reproducible to 1 A^2/molecule.

Microstructure Preparation and Microscopy. A thin film of lipid was hydrated in either acetate or Tris-HCl buffer containing $CuCl_2$ at 80 °C for 1 h. The lipid concentration in each sample was 2 mg/mL and the lipid to Cu^{2+} ratio was one. Hydrated lipids were dispersed with the aid of Branson sonifier (model 450, outfitted with a cuphorn attachment) until the solution became translucent. The sonicated solution was cooled to room temperature (2 °C/min). Dispersions which were to be thermally cycled were further cooled to 4 °C and kept at that temperature overnight. Then, the lipid samples were heated above the phase transition temperature and allowed to cool to room temperature.[27] Air-dried samples of the microstructures on carbon coated copper grids were

examined unstained by TEM.

Preparation of Enzyme Immobilized Vesicles. Polymerizable lipid 1,2-bis(tricosa-10,12-diynoic)-sn-glycero-3-phosphocholine ($DC_{8,9}PC$) was separately mixed with nonpolymerizable metal chelating lipid **1a** (0.8 mg) and polymerizable metal chelating lipid **2a** in 90:10 (mole/mole) ratio using chloroform as solvent. The solvent was removed to form a thin lipid film on the walls of glass tubes. The vacuum dried (4 h.) film was dispersed in 2 mL 0.05M Tris-HCl (pH 8.5) by incubating at 55°C for 2 h followed by sonication at 50°C for 12 m. in a bath sonicator. The vesicle dispersions were then treated with 0.01mM $CuCl_2$ in the Tris buffer. The unbound copper salt was removed by gel filtration on a sephadex G 75-125 column. Copper bound vesicles were then divided into two equal volumes. One portion was polymerized at 4 °C by irradiating with 254 nm light for 10 minutes. Both, polymerized and unpolymerized samples were incubated with enzyme bovine carbonic anhydrase II (EC 4.2.1.1) at room temperature for 3-4 h, and gel filtered to remove unbound enzyme from enzyme bound to vesicles.

General Procedure for Enzyme Assay. One syringe of the stopped-flow spectrophotometer was loaded with substrate (5mM p-nitrophenyl acetate in 30% acetone/water), and the other syringe was loaded with the preparation to be tested in 100mM Tris (pH 8.5). Data was collected at the rate of 200 points in 120 seconds. Absorbance was monitored at 402 nm with a slit width set to 1.0 nm. Data was analyzed using the "DATAFIT" module of the stopped-flow operating system. All data analyzed were fit with a linear regression.

Synthesis of Phospholipids and their Intermediates.

General Procedure for the Synthesis of 3: In a two-necked round bottomed flask equipped with a pressure equalized dropping funnel, a magnetic stir bar and nitrogen inlet and outlet adapters, the appropriate amount of phosphorous oxychloride was added. The entire set-up was placed in an ice-bath while maintaining a gentle flow of nitrogen. Phosphorus oxychloride was in 10 mol % excess to that of bromo or chloroalkanol. Ω-Haloalkanol was slowly added to the phosphorous oxychloride. After the addition was complete, the ice bath was removed and the reaction mixture was stirred overnight at room temperature with nitrogen bubbling through solution to remove the HCl formed during the reaction. The $POCl_3$ was removed under reduced pressure and the residue, if possible, was vacuum distilled and stored under nitrogen.

2-Bromoethyl dichlorophosphate (3a): Following the general procedure, reaction between bromoethanol (54.0 g 0.40 mol) and phosphorus oxychloride (85g, 0.55 mol) afforded 68 g (65% yield) **3a** as a colorless liquid after vacuum distillation. ^{1}H NMR ($CDCl_3$) δ ppm 3.59 (td, J=6.1 Hz, CH_2(e), 2H), 4.54 (t, CH_2(d),1H), 4.58 (t, CH_2(d),1H). ^{13}CNMR ($CDCl_3$) δ ppm 69.9 (CH_2(d)), 27.4 (CH_2(e)). IR (neat); 3032, 871, 1301, (P=O), 1070, 1017 cm^{-1}.

2-(2-Chloroethoxy)ethyldichlorophosphate (3b): Reaction between 2-(2-chloroethoxy)ethanol (34 g, 0.27 mol) and $POCl_3$ (52 g, 0.34 mol) gave 53 g (80%) **3b** as colorless liquid. ^{1}H NMR ($CDCl_3$) δ ppm 3.59 (td, J=6.1 Hz, CH_2(d), 2H), 3.74 (td, J = 6.1, CH_2 (e),2H), 3.79 (m, CH_2(f),2H), 4.38 (m,

CH_2(g),2H). ^{13}CNMR ($CDCl_3$) δ ppm 70.4 (d, $^2J_{P-C}$=9.3,CH2(d)), 68.7 (d, $2J_{P-C}$ =8.8, CH_2(e)), 71.3 (CH_2(f)), 42.4 (CH (g)). IR (neat) 2964, 2872, 1296 (P=O), 1136, 1043 cm^{-1}.

2-(2-(2-Chloroethoxy)ethoxy)ethyldichlorophosphate (3c): 2-(2-(2-chloroethoxy)- ethoxy)ethanol 50 g (0.3 mol) was reacted with 57.6 g (0.37 mol) phosphorus oxychloride to yield 81 g (94%) **3c**. ^{1}H NMR ($CDCl_3$) δ ppm: 3.57 (td, J=6.1 Hz, CH_2(d), 2H), 3.63 (overlapping multiplets, CH_2(f,g) 4H), 3.70 (dt, J= 6.1 Hz, CH_2(e), 2H), 3.77 (m, CH_2(h), 2H), 4.38 (m, CH_2(i), 2H). ^{13}CNMR ($CDCl_3$) δ ppm 69.3 (d, $^2J_{P-C}$=8.9,CH_2(d)), 71.0, 70.7, and 70.5 CH_2(e-h)), 42.6 (CH_2 i)). IR (neat) 2958, 2873,1452, 1354, 1297, 1141, 1013, 827 cm^{-1}.

General Procedure for the Synthesis of 4: An equimolar quantity of base (triethyl amine or pyridine) was added to an ice-cooled chloroform solution of **3** placed in a reaction flask equipped with magnetic stirrer, nitrogen inlet and outlet, and a pressure equalized addition funnel. An equimolar amount of solketal in chloroform was then slowly added. The course of the reaction was monitored by proton NMR. After about 1 h, the reaction was quenched by addition of aqueous saturated sodium bicarbonate solution. The chloroform layer was separated and then washed with water and dried over sodium sulfate. Removal of solvent under reduced pressure provided the product as a viscous colorless oil.

1,2-Isopropylidene-3-bromoethylglycerophosphate, sodium salt (4a): Following the general procedure, solketal (6.60 g, 50 mmol) was reacted with **3a** (12.5 g, 51.6 mmol) dissolved in 30 mL chloroform in the presence of triethylamine (7.2 mL, 51.6 mmol) to provide 11.0 g (70%) of **4a** as viscous liquid. ^{1}H NMR ($CDCl_3$) δ ppm, 1.12 and 1.20 (s, 6H), 3.35 (t, 2H), 3.60 (m, 2H), 3.86(m, 4H), 4.1 (quintet, 1H). IR (neat) 3425, 2978, 2891, 1451, 1371, 1257, 1217, 1150, 1030 cm^{-1}.

1,2-Isopropylidene-3-(2-(2-chloroethyloxyethylphosphate)-sn-glycerol (4b): Solketal (8.76 g, 66.2 mmol), dichlorophosphidate **3b** (16.8 g, 69.8 mmol) and triethylamine (9.7 ml, 69.8 mmol) 18 g (90%) of **4b** as a viscous liquid. ^{1}H NMR ($CDCl_3$) δ 1.26 and 1.33 (s, 6H), 3.55 (td, J=6,1 Hz, 2H), 3.68 (overlapping td and m, J=6, 1 Hz, 6H), 3.98 (m, 2H), 4.09(m, 2H), 4.27 (quintet, 1H) IR (neat) 3486, 2991 2885, 1634, 1454, 1367, 1254, 1214, 1134, 1035 cm^{-1}.

1,2-Isopropylidene-3-(2-(2-(2-chloroethyl)ethoxy)ethylphosphate)-sn-glycerol (4c): Solketal (8.64 ml, 9.0 g, 68 mmol), dichlorophosphidate **3c** (20 g, 69.5 mmol) and pyridine (5.7 ml, 5.5 g, 70 mmol) yielded 17 g (68%) of **4c** as a viscous liquid. ^{1}H NMR ($CDCl_3$) δ 1.17 and 1.24 (s, 6H), 3.48 (overlapping multiplets, 9H), 3.66 (m, 2H), 3.89(m, 3H), 4.03 (m, 2H), 4.15 (quintet, 1H). IR (neat) 3509, 2987, 2888, 1450, 1372, 1298 (P=O), 1217, 1129, 1042, 838, 754 cm^{-1}.

General Procedure for the Synthesis of 5: The reaction was carried out by stirring equimolar amounts of sodium salt of **4** with iminodiacetic acid dissolved in 7N aqueous solution of potassium hydroxide for three days at room temperature. At this time the reaction mixture was treated with methylene chloride to remove unreacted starting material **4**. The reaction mixture was freeze dried and the product was treated with methanol. Cooling the methanol solution to ice temperature helped remove inorganic salts from the solution. Removal of methanol yielded pure **5** as waxy solid.

1,2-Isopropylidene-3-(2-(2-(2-dipotassiumminodiacetate)ethoxy) ethylphosphate)-sn-glycerol (5a): Glycerophosphidate **4a** (8.25 g, 25.8 mmol), imminodiacetic acid (3.45 g, 26.2 mmol) and KOH (ca.7 M, 11 ml, 77 mmol) produced 8 g (67%) of **5a** as a waxy solid. ^{1}H NMR (D_2O) δ 1.43 and 1.47 (s, 6H), 3.55(t, 2H), 3.84(s, 4H), 3.62-4.0 (m, 4H, 4.14 (t, 2H), 4.42 (quintet, 1H). IR (neat) 3425, 2982, 2832, 1615, 1400, 1241, 1057, 910 cm^{-1}.

1,2-Isopropylidene-3-(2-(2-(2-dipotassiumiminodiacetate)ethoxy) ethylphosphate)-sn-glycerol (5b): Glycerophosphidate **4b** (7.1g, 22.3 mmol), imminodiacetic acid (2.81g, 21.1 mmol) and KOH (ca.7M, 10ml) was stirred for 3d. The solution was then extracted with dichloromethane to remove unreacted starting material. The water was then evaporated and the residue dissolved in methanol. The precipitate was then removed by repeated concentration and filtration of the methanol solution. Evaporation of methanol gave 7.0 (82%) of **5b** as a waxy solid. ^{1}H NMR (D_2O) δ 1.38 and 1.45 (s, 6H), 2.80(s, 2H), 3.30(s, 4H), 3.59-4.13 (m, 8H), 4.13 (t, 2H), 4.42 (quintet, 1H). IR (neat) 3400, 2995, 2898, 2818, 1600, 1464, 1404, 1331, 1224, 1064, 954, 827 cm^{-1}.

1,2-Isopropylidene-3-(2-(2-(2-dipotassiumiminodiacetate)ethoxy)ethyl phosphate)-sn-glycerol (5c): Glycerophosphidate **4c** (8.44 g, 23.2 mmol), imminodiacetic acid gave 2.38 g (17%) of **5c** as a waxy solid. ^{1}H NMR (D_2O) δ 1.38 and 1.45 (s, 6H), 2.87 (s, 2H), 3.27(s, 4H), 3.62-4.18 (m, 12H) 4.14 (t, 2H), 4.42 (m, 1H). IR (neat) 3331, 2939, 2822, 1628, 1401, 1331, 1244, 1115, 950, 835 cm^{-1}.

Deprotection of Ketal 5: All three compounds (**5a-c**) were deprotected by stirring aqueous solution of **5** with excess 3N HCl (>6 mol equivalent) for 6 hours at room temperature. The reaction was monitored by TLC using methanol:10% aq. ammonium acetate (2:1) solvent system and considered complete when only one spot at R_f 0.75 was observed. Also, the deprotected diol had a R_f of 0.48 in methanol. Water was removed and the resulting residue was dissolved in methanol to remove inorganic salts produced during the reaction. Final purification was achieved by column chromatography on a silica gel column by employing methanol as solvent. The waxy material was checked for purity by IR, NMR and elemental analysis for C and H. Deprotected **6** was dissolved in methanol in a known concentration and stored at ca. -15°C until further use.

Rac-glycero-3-phospho-N-(2-ethyl)-iminodiacetic acid (6a): Deprotection of **5a** (1.57 g, 3.37 mmol) with 18 mL 3N aq. HCl provided **6a** quantitatively. ^{1}H NMR spectra in D_2O showed the absence of methyl protons. IR spectra showed signals at 1750 (COOH), 1241 (P=O), and 1045 (P-O-C) cm-1 that confirmed the formation of **6a**.

Rac-glycero-3-phospho-N-(2-(2-ethoxy)ethyl)-iminodiacetic acid (6b): Deprotection of 5b (1.97 g, 3.80 mmol) with 12 mL 3N aq. HCl provided **6b** quantitatively. ^{1}H NMR spectra in D_2O showed the absence of methyl protons. IR spectra showed signals at 1751 (COOH), 1234 (P=O), and 1017 (P-O-C) cm-1 that confirm the formation of **6b**.

Rac-glycero-3-phospho-N-(2-(2-(2-ethoxy)ethoxy)ethyl)-iminodiacetic acid (6c):

Deprotection of **5c** (2.3 g, 4.1 mmol) with 12 mL 3N aq. HCl provided **6c** quantitatively. ^{1}H NMR spectra in D_2O showed the absence of methyl protons present in the protecting group. IR spectra showed signals for COOH, P=O, and 1017 P-O-C cm^{-1} that confirmed the formation of **6c**.

General Procedure for the Synthesis of PhosphoIDA Lipids Containing 1,2 Dipalmitoyl and 1,2-bis(tricosa-10,12-diynoyl) Acyl chains 1 and 2: Details of the acylation of **6a** have been described elsewhere.[26] In general, methanol solution of **6** was transferred to a single necked, round bottomed flask of appropriate size . Methanol solution was evaporated under a stream of nitrogen followed by evacuation under high vacuum for 6-8 hours. To the resulting anhydrous **6** were sequentially added a solution of palmitic or tricosa-10,12-diynoic anhydride in chloroform (freshly distilled over phosphorus pentoxide), and 4-dimethylaminopyridine in chloroform and the contents were mixed by ultrasound agitation for 2 hours and then by magnetic stirring overnight. Care was taken to protect the reaction mixture from direct laboratory light. The course of the reaction was monitored by TLC using chloroform: methanol: water (65:25:4) as developing solvent. The lipid spots were revealed by iodine and molybdenum blue spray. Most of the time the reaction was found to be complete after overnight stirring. After removal of the chloroform, the residue was dissolved in the chloroform:methanol (1:1) solution and passed through a column of Biorad resin AG50WXD, 200-400 mesh) to remove DMAP and cationic impurities. Lipid mixture thus obtained was chromatographed by flash chromatography on a 230-400 mesh Merck grade silica gel column using chloroform, 5% methanol/chloroform, and then 10% methanol/chloroform. The phosphoIDA lipids were revealed at Rf 0.45-0.50 in chloroform: methanol: water (65:25:4) solvent system.

Synthesis of 1,2-Dipalmitoyl-rac-glycero-3-phospho-N-(2-ethyl)-iminodiacetic acid (1a): Following the general procedure 300 mg (0.9 mmol) **6a** was reacted with 1.36 g (2.75 mmol) palmitic anhydride in 10 mL chloroform in the presence of 50 mg (3.68 mmol) DMAP to give 300 mg (41% yield) of the lipid after column chromatography. ^{1}H NMR ($CDCl_3$) δ ppm: 0.87(t, 6H), 1.25 (br m, 48H), 1.6 (m, 4H), 2.35 (t, 4H), 3.7-4.5 (m, 12H), 5.25 (m, 1H). IR (film) 3559, 3052, 918, 2938, 1734, 1461, 1421, 1260, 736 cm^{-1}.

Synthesis of 1,2-Dipalmitoyl-rac-glycero-3-phospho-N-(2-(2-ethoxy)ethyl)-iminodiacetic acid (1b) : Following the general procedure 220 mg (0.58 mmol) **6b** was reacted with 880 mg (1.77 mmol) palmitic anhydride in 10 mL chloroform in the presence of 220 mg (1.8 mmol) DMAP to give 290 mg (58% yield) of the lipid 1b after column chromatography. ^{1}H NMR ($CDCl_3$) δppm: 0.87(t, 6H), 1.25 (br m, 48H), 1.60 (m, 4H), 2.35 (t, 4H), 3.7-4.5 (m, 16H), 5.24 (m, 1H). IR (film) 3326, 2918, 2854, 1734, 1554, 1461, 1260, 1220, 1100, 1030 cm^{-1}.

Synthesis of 1,2-Dipalmitoyl-rac-glycero-3-phospho-N-(2-(2-(2-ethoxy)-ethoxy ethyl)-iminodiacetic acid (1c): Following the general procedure 370 mg (0.88 mmol) 6c was reacted with 1.3 g (2.62 mmol) palmitic anhydride in 10 mL chloroform in the presence of 400 mg (3.27 mmol) DMAP to give 140 mg (18% yield) of the lipid 1c after column chromatography. ^{1}H NMR ($CDCl_3$) δ ppm: 0.86 (t,6H), 1.25 (br m, 48H), 1.60 (m, 4H), 2.29 (t, 4H), 3.55-4.6 (m, 16H), 5.22 (m, 1H). IR (film) 3326, 2918, 2854, 1734, 1554, 1461, 1260, 1220, 1100, 1030 cm^{-1}.

Synthesis of 1,2-bis(tricosa-10,12-diynoyl)-rac-glycero-3-phospho-N-(2-ethyl)-iminodiacetic acid

(2a): Acylation of 320 mg (0.97 mmol) 6a with tricosa-10,12-diynoic anhydride (1.35 g, 2.0 mmol) in the presence of 350 mg (2.89 mmol) DMAP provided 270 mg (28%) of lipid **2a**. ^{1}H NMR ($CDCl_3$) δ ppm: 0.86(t, 6H), 1.28 (br m, 56 H), 1.46 (m, 4H), 2.23 (t, 12H), 3.35-4.19 (m, 12H), 5.10 (m, 1H). IR (film) 3250, 2925, 2845, 2264, 2164, 1741, 1628, 1574, 1461, 1264, 1177, 1066, 1011 cm^{-1}.

Synthesis of 1,2-bis (tricosa-10,12-diynoyl)-rac-glycero-3-phospho-N-(2-(2-ethoxy)ethyl)-iminodiacetic acid (2b): Acylation of 260 mg (0.69 mmol) **6b** with tricosa-10,12-diynoic anhydride (1.39 g, 2.06 mmol) in the presence of 250 mg (2.06 mmol) DMAP provided 290 mg (41%) of lipid **2b**. ^{1}H NMR ($CDCl_3$) δ ppm: 0.83(t, 6H), 1.22 (br m, 56 h), 1.44 (m, 4H), 2.19 (t, 12H), 3.35-4.50 (m, 16H), 5.10 (m, 1H). IR (film) 3394, 3055, 2925, 2855, 2305, 2160, 1735, 1652, 1456, 1265, 1066 and 976 cm^{-1}.

Synthesis of 1,2-bis(tricosa-10,12-diynoyl)-rac-glycero-3-phospho-N-(2-(2-(2-ethoxy)-ethoxy)ethyl)-iminodiacetic acid (2c): Acylation of 200 mg (0.47 mmol) **6c** with tricosa-10,12-diynoic anhydride (1.10 g, 1.63 mmol) in the presence of 40 mg (2.78 mmol) DMAP provided 107 mg (21%) of lipid **2c**. ^{1}H NMR ($CDCl_3$) δ ppm: 0.87(t, 6H), 1.25 (br m, 56 h), 1.50 (m, 4H), 2.23 (m, 12H), 3.15-4.75 (m, 20 H), 5.11 (m, 1H). IR (film) 3547, 2928, 2855, 2251, 2162, 1737, 1618, 1465, 1265, 1168, 1106,1066,976 cm^{-1}.

Results and Discussion

Synthesis of phospholipids. All six phospholipids were synthesized following the synthetic route illustrated in Scheme 1. Four approaches (outlined in Scheme 2) were considered and attempted for the synthesis of phosphoIDA lipids 1 and 2. Acylation of the 1,2-hydroxyl groups of glycerol backbone after linking the headgroup phosphoIDA to the glycerol 3 position provides a problem free route without any possibility of side reaction (Path A and the process reported in this work). Carboxylic acid groups of IDA moiety were not protected because anhydride/dimethylaminopyridine (DMAP) will acylate the hydroxyl groups of glycerol backbone.[28] Additionally, protection of acid functionality is not practical in the current synthetic scheme because the conditions that are required for deprotection may cause hydrolysis of acyl chains. Recently, in a synthesis of an IDA headgroup lipid, the carboxylic groups were protected but the lipid acyl chains were linked to glycerol backbone via an ether group.[29]

Linking IDA to 1,2-diacyl glycerophosphate ester (path B) was a possible synthetic alternative. This route suffered from problems of solubility of IDA in organic solvent and the use of extreme basic conditions (pH >10) to link IDA with the phospholipid headgroup. Hydrolysis of acyl chains occurred under these conditions. Our attempts to search for milder reaction conditions to couple these reactants were not successful. Pathway C, which has been reported for the synthesis of thiol lipids, involves reaction of phosphatidic acid with an appropriate alcohol in the presence of triisopropyl phenylsulfonyl chloride.[30] This would have been an efficient synthetic procedure since phosphatidic acid may be quantitatively obtained from corresponding phosphatidylcholine by

phospholipase D reaction[31], if hydroxyethyl substituted IDA would have been soluble in organic solvents. Path D is similar to path C in many respects except that dichlorophosphate moiety in glycero 3 position is capable of directly reacting with an alcohol group.[32] This classical process is good for reacting organic soluble functionalities. However, insolubility of substituted IDA makes the reaction biphasic, which left the reagents unreacted. Therefore this path was unacceptable as a synthetic route.

Haloalkanol upon reaction with phosphorousoxytrichloride yielded the dichlorophosphate **3a-c**. In each case proton NMR revealed the characteristic phosphorous coupling of $^{2}J_{P\text{-}H}$ of 1Hz for the CH_2(d) protons. The CH_2(e) protons of **3a** appeared as two separate protons with the characteristic first order coupling of 7Hz. Their separate appearance is most likely a conformational effect, with one proton being proximal to the phosphorous and one distal. We did not observe any $^{3}J_{P\text{-}H}$ coupling to the CH_2(e) protons. The carbon NMR of **3a** showed(d) and CH_2(e) carbons at 69.9 and 27.4 ppm, respectively. In **3b** assignments of the CH_2 protons of d and g were made by proton decoupling experiments. Irradiation of the 4.4 ppm protons attached to the chlorine caused the multiplet at 3.8 ppm to collapse indicating that they were directly coupled. In addition to coupling with their neighbors, both CH_2 protons at d and g couple to each other causing an appearance of a multiplet rather than a simple triplet. The carbon NMR of **3b** showed CH_2 (d, e) carbons at 7.4 ppm and 68.6 ppm with a phosphorous coupling of 9.3 and 8.8 Hz. In **3c**, CH_2 f and g protons appear as a broad multiplet at 3.6 ppm. The carbon assignments are completely consistent with those seen for **3b** with the CH_2(d) carbon at 70.5 ppm and the other carbons from 70.7 to 71 ppm. The compounds **3a-c** are colorless viscous oils and are moisture sensitive decomposing to give dark colored liquids with the evolution of HCl. They can be repurified by distillation.

Reaction of solketal with **3a-c** produced **4a-c** as colorless viscous oils. These have limited stability to air and moisture and thus have finite shelf-life. Decomposition is most likely via acid catalyzed cleavage of the ketal moiety. The proton nmr showed considerable overlapping of the headgroup and glycerol backbone protons. The chemical shifts and splitting of the CH(b) proton, and the chemical shift of the carbon remain virtually unchanged in proceeding from **3a-c** to **4a-c**. Only a slight upfield shift from 4.3 to 4.2 in the proton and negligible change in the carbon was observed. This suggests that once the phosphorous is appended, negligible conformational changes occur in the glycerol backbone. The solketal derivatives **4a-c** were then linked with the iminodiacetic acid in aqueous basic media to produce **5a-c**. In all three cases yields were moderate to high. The long reaction times are readily understandable considering the low solubility of the haloethylenoxyphosphates **4a-c** in water and the insolubility of the sodium or potassium salt of iminodiacetic acid in organic solvents. Attempts to expedite the reaction rate by substituting iodine for the chlorine or bromine were unsuccessful. In addition to the product, a myriad of side products, most likely arising via decomposition of the iodo precursor during the basic iminodiacetic reaction, were obtained. An accurate and meaningful comparison of the chemical shifts of these compounds with their precursors cannot be made since they were acquired in D_2O due to their insolubility in chloroform. The ^{13}C NMR chemical shifts of **4** and **5** are compiled in Tables 1 and 2.

Hydrolysis of **5a-c** in aqueous HCl produced the lipid precursors **6a-c** in quantitative yields. These colorless to off-white waxy materials were stored as methanol solutions in known concentration in the freezer at ca. -15 °C. The transformation of the ether to an alcohol caused considerable alteration of the glycerol carbon and proton resonances. The only conspicuous CH(b)

proton resonance shifts from 4.40-4.42 ppm in **5a-c** to 4.2-4.3 ppm in **6a-c**, consistent with fact that carbon is changing from an ether to an alcohol. The CH_2 (k) protons of the IDA moiety also shifted to 4.12-4.22 ppm . Similarly, the CH(b) carbon shifts from 77.3-77.4 in **5a-c** to 72.9-73.0 in **6a-c** which is consistent with the fact that CH(b) has changed from an ether appended to an alcohol appended carbon (Table 3). The other carbons could not be unambiguously assigned. The reaction of compounds **6a-c** with the appropriate anhydride in the presence of DMAP in chloroform produced the lipids **1a-c** and **2a-c** in modest 30-40% yields. The lipids **1a-c** are white solids while **2a-c** are off-white waxy solid.

The proton NMR of the lipids reveal the acyl tail group resonances quite clearly. However, the headgroup and the glycerol backbone are severely broadened or are not observed. It is possible that water occluded in the lipid may cause broadening of peaks. A similar observation has recently been reported for headgroup modified lipids containing hydroxyl or ethereal moieties.[33] The prominent signal in these compounds is due to CH(b) proton appearing as a broad peak at 5.20-5.5 ppm. With the notion that the protons and carbons of the glycerol backbone would overlap with those of the head group, we prepared some glycerol phosphate derivatives and analyzed them along with solketal to assign the proton and carbon resonances (scheme-3). Reaction of solketal **i** with $POCl_3$ gave the dichlorophosphidate **ii** in quantitative yields. Hydrolysis of **ii** produced **iii**. Compound **iii** was made in order to compile values to compare with the values of lipids and lipid precursors that contained phosphate esters. Distinctive dddd patterns seen for the glycerol protons CH_2(a) and CH_2(c) in **i** appeared transformed to a complex overlapped pattern in **ii** and **iii**. The CH(b) proton appeared quite distinct as multiplet at 4.2 ppm in both **ii** and **iii**. DEPT showed the CH_2(b) carbon at 73.7 ppm, CH_2(a) at 67.7 ppm and CH_2(c) carbon at 65.8 ppm with $^2J_{PC}$ and $^3J_{P\text{-}C}$ coupling of 4.8 and 7.6 Hz for **ii** and 5.8 and 7.9 Hz for **iii**. In **ii** and **iii**, the chemical shifts of CH proton shifted downfield from 3.5 to 4.2 and an upfield shift was observed from 76.1 to 73.8 for carbon.

Table 4 compiles carbon-13 chemical shifts for compounds **1** and **2**. In the ^{13}C NMR, the lipids display the acyl tail group region in the 14-34 ppm range, and the headgroup and the backbone areas in the 50-80 ppm range. The CH of the lipids appears at ca. 70-71 ppm. This is completely consistent with the fact that in the precursors, this carbon is ether bound and occurs at ca. 73 ppm. Thus it is not unusual to expect the ester carbon to appear downfield as the chemical shifts vary in the order alcohol<ether<ester for a given carbon. The ester and the acid carbonyl occur at 173 and 180 ppm, respectively. The quaternary carbons of the diacetylenic moieties occur at 65 and 77 ppm. The resonances at 77 ppm belonging to the outer relatively deshielded pair while those at 65 ppm for the comparatively shielded inner pair. The typical Rf on TLC of **1a-c** and **2a-c** is 0.45 to 0.5 in chloroform:methanol:water (65:25:4) using molybdenum blue spray reagent. These lipids are quite stable when stored under inert atmosphere in cold (ca.-15%C). Any decomposition can be routinely checked by TLC and NMR. On TLC, spots of the palmitic or the diacetylenic acid and lyso compound appear at Rf 0.9 and 0.2, respectively. In proton nmr, the decomposition is evidenced by an upfield shift of the methine proton with respect to the lipid, which appeared as broad multiplet due to lyso compound. The lipids also decompose on extended contact with silica gel. Thus, flash chromatography was employed to purify lipids. Efforts to alleviate product loss by doing chromatography without removing amine by ion-exchange were unsuccessful. A considerable amount of the dimethylamino pyridine (DMAP) co-eluted with the product. Attempts to stabilize

the lipid using 1% acetic acid in methanol were futile. The entire mode of the lipid decomposition is unclear but acid catalyzed cleavage of the ester linked to the CH (b) carbon occurs leading to the formation of lyso compound and free fatty acid.

Langmuir Film Properties. The surface pressure-area isotherms for the six lipids are shown in Figure 3 and the limiting areas and collapse pressures are presented in Table 5. In general, the monolayers are liquid expanded and the monolayers of the dipalmitoyl phosphoIDA lipids are more stable and have smaller molecular areas than the diacetylenic phosphoIDA lipids. As seen in Table 5, monolayers on water were more stable than those on subphases containing Cu^{2+}. This is particularly so for dipalmitoyl lipids **1b** and **1c**. This would suggest that on water the phosphoIDA headgroups are able to form a hydrogen bonded network which imparts additional stability to the monolayer. Since the pKa's of the phosphate, carboxylates, and amine of b-aminoethylphosphonic acid-N,N-diacetic acid (a compound similar to the phosphoIDA headgroup of the lipids) are 2.00, 2.45, 6.54 and 10.46, respectively, it would appear that the hydrogen bonded network would arise primarily from the amine and carboxylates of the IDA group.[34] Also, the phosphonate and copper-IDA groups of β-aminoethylphosphonic acid-N,N-diacetic acid interact.[34] This suggests that any intermolecular hydrogen-bonded network between the phosphoIDA lipids in the monolayer would be broken up when Cu^{2+} was available to bind to the IDA group. In addition, the stability of the lipids with n = 3 (**1c** and **2c**) on all subphases was greater than that for the corresponding lipids with shorter spacer groups suggesting that the intermolecular interactions between the headgroups of the n = 3 lipids are significantly different than those of the other lipids. For both the diplamitoyl lipids (**1a-c**) and the diacetylenic lipids (**2a-c**), the most interesting changes in monolayer behavior are observed when n is increased from 1 to 2 and 3. For the dipalmitoyl lipids **1a-c**, there is a small decrease in limiting area at some [Cu^{2+}] as n is increased from 1 to 2 and a larger decrease at all [Cu^{2+}] as n is increased further to 3. For the diacetylenic lipids **2a-c**, the limiting areas on all Cu^{2+} subphases decrease significantly when the length of the spacer group is increased from one to two etheleneoxy units. A smaller decrease is observed at some [Cu^{2+}] when n is increased from 2 to 3. For both sets of lipids, the effects of varying the [Cu^{2+}] are not as great for the lipids where n = 2 or 3 (**1b,c** and **2b,c**) as they are for the n = 1 lipids (**1a** and **2a**). The influence of the spacer length on monolayer behavior suggests that some intramolecular interaction between the phosphate and IDA groups when n = 1 exists which changes as n is increased.

The initial rise in limiting area for **1a** as Cu^{2+} is introduced ([Cu^{2+}]=0.01 mM) may be do to the creation of repulsive interactions or packing mismatches between the headgroups. Possible explanations for these adverse interactions include but are not limited to binding of the copper ion to the IDA group but not the phosphate group and/or participation of the phosphate group along with the IDA group in the binding of the copper ion. As the [Cu^{2+}] increases, copper ion would bind to the phosphate and thereby either neutralizing the negative charge of the phosphate or breaking up the interaction between the phosphate and the IDA-copper ion complex. Interestingly, the effect of initially added copper ion decreases as n is increased for the dipalmitoyl lipids but increases for the diacetylenic lipids as n is increased.

There is a large difference in limiting areas between the dipalmitoyl lipids and the corresponding diacetylenic lipids. This is in contrast to the small difference between the limiting areas of dipalmitoyl phosphatidyl choline (DPPC) (54 A^2/molecule) and 1,2-bis(tricosa-10,12-

diynoyl)-sn-glycero-3-phosphocholine ($DC_{8,9}PC$) (50 A^2/molecule).[35] Indeed, the limiting area of DPPC is similar to that of the dipalmitoyl phosphoIDA lipids. Since the corresponding headgroups of the two sets of lipids are identical, the differences in monolayer behavior between the dipalmitoyl and diacetylenic lipids must be due to the differences in the acyl chains of the lipids. A comparison of the monolayers of DPPC and $DC_{8,9}PC$ reveals that the dipalmitoyl acyl chains pack in a more fluid state than the diacetylenic acyl chains.[35,36] The diacetylenic group is known to produce a kink in the acyl chain which does not exist in the saturated acyl chains of the dipalmitoyl lipids[37,38] and the diacetylenic acyl chains are known to be more ordered than the hexadecanoyl acyl chains.[35,39] Also, the monolayer behavior of the diacetylenic phosphoIDA lipids contrasts with that of $DC_{8,9}PC$ which forms a condensed film when compressed indicating that the phosphoIDA headgroups require more freedom of movement to orient in a condensed packing as the monolayer is compressed than the phosphocholine headgroup. It may be that the combination of the kinks and the rigidity in the diacetylenic acyl chains may not allow the phosphoIDA headgroups the freedom of movement required to achieve the more condensed packing obtained with the dipalmitoyl lipids which has more fluid acyl chains.

Microstructure Formation. Diacetylenic phosphoIDA lipids were examined for their microstructure formation properties by dispersing them in buffers of varying pH (5.6, 7.0, 8.0 and 9.0) at varying ionic strength (I) in the presence of copper ions. In all cases, the bulk [Cu^{2+}]:[phosphoIDA] was one. Table 6 summarizes the results obtained with lipids **2a-c**. The n = 1 and n = 2 lipids (**2a** and **b**) were initially screened for microstructure formation properties at ionic strength (I) 0.2 and 0.4. The lipids did not disperse in the pH 5.6 buffer. At ionic strength 0.2, no well defined structures were obtained at any pH. This result is quite different to that reported earlier for negatively charged diacetylenic phospholipids which formed well defined tubules at pH 5.6 (I 0.18).[14] However, at I 0.4 some identifiable structures were observed both at pH 7.0 and 8.0. Since, the most promising results in terms of chiral microstructure formation were observed at pH 8.0 (I 0.4), the dispersions of all 3 lipids at pH 8.0 (I 0.4, 0.6, 0.8) were thermally cycled in order to optimize the amount of microstructures formed.[27] Figures 4 and 5 show the transmission electron micrographs of microstructures formed from lipids **2a-c** at pH 8.0 (I 0.4). Note that lipid **2c** produced tubule structures with visible helical striation on the surface suggesting helices as the structural precursors (Fig. 5).

The importance of headgroup interactions in metal ion assisted formation of helices and tubules from racemic phospholipid mixtures is demonstrated by the results of this study. While tubule structures are formed from lipid **2c**, only ribbons and are formed from **2a** and **2b**. These results reveal that a separation of some length between the phosphate and IDA groups is required for tubule formation. As mentioned previously, interactions between phosphonate and copper-IDA groups in compounds containing both functionalities has been shown to occur. It is possible that as the length of the spacer group increases, intramolecular interactions between the phosphate and the copper-IDA groups decrease or are eliminated thereby allowing the headgroup freedom of movement to reorient and the acyl chains to pack in an alignment conducive to tubule formation. Also, tubule formation by **2c** is observed under select conditions of pH (8.0) and I (0.4) further suggesting that the morphological transformations are sensitive to the molecular geometry and orientation of the lipids in the bilayer.

Helices and ribbons have reported as precursor in the formation of tubules, for example, helical structures have been observed in alcohol/water solvent systems from diacetylenic phosphocholines,[19,20] non-diacetylenic single and double chain amphiphiles via hydrogen bonded networks between the headgroups,[40-44] and from non-amphiphiles due to influence of metal ion addition to oligobipyridine.[45] Featureless microstructures with flat surfaces were formed from the meso component of chiral tartrate amphiphiles but left and right handed helices were formed from racemic solutions of the chiral amphiphiles.[44] Formation of right or left handed helices has been reported from a racemic 1,2-bis(tricosa-10,12-diynoic)-sn-glycero-3-phosphocholine, $DC_{8,9}PC$, when the structures were made by an alcohol-water precipitation method.[19] The reason for the formation of the helices in that case was the lateral phase separation of R and S lipids. Recently, experimental evidence has been provided through circular dichroism (CD) studies that both the diacetylene in acyl chains and a chiral center in the glycerol backbone are needed to produce left or right handed helical structures.[18]

Enzyme immobilization on Vesicles. Vesicles were made by mixing polymerizable lipid 1,2 bis (tricosa-10,12-diynoyl)-sn-glycero-3-phosphocholine ($DC_{8,9}PC$) with nonpolymerizable 1,2 dipalmitoyl-sn-glycero-3-phospho-(N,N-bis-carboxymethyl)-2-aminoethanol (**1a**, DPPIDA) or polymerizable 1,2 bis (tricosa-10,12-diynoyl)-sn-glycero-3-phospho-(N,N-bis-carboxymethyl)-2-aminoethanol (**2a**, $DC_{8,9}PIDA$). The first step involves the demonstration of the proof of principle that enzyme can be immobilized on the surface of vesicles and can maintain its catalytic activity. Randomly distributed IDA-phospholipids in lipid membranes offer such an opportunity for binding with proteins containing multiple histidine sites. The metal chelating IDA lipids were passed through cation exchange resin before the preparation of vesicles. Saturated DPPIDA lipid **1a** showed a chain melting transition peak at 55.1 °C in DSC thermogram. Lipid $DC_{8,9}PC$ has chain melting transition at 43.1 °C and **2a** should have melting transition lower to that of **1a**. Using these transition numbers as guides, we prepared vesicles at 55 °C which is above their chain melting transition temperatures of the mixed system.

The two sets of vesicles were prepared by mixing lipid $DC_{8,9}PC$ with **1a**, and $DC_{8,9}PC$ with **2a**. Sonication for about 10 minutes provided dispersion with constant turbidity at 400 nm. Each set of vesicles showed an increased turbidity upon mixing with copper chloride solution, which diminished upon vortex mixing. This effect could be due to an abrupt change in ionic strength of the medium as well as to electrostatic attractions between the copper bound vesicles. The copper bound vesicles came in the void volume during gel-filtration while the free copper eluted later. The fractions with free copper salt were visible due to their intense blue color. The copper bound vesicles were divided into two parts, one of which was polymerized. Polymerization produced an orange to reddish color dispersion. Polymerization was confirmed by TLC analysis. Most of the lipid was found at the origin of the TLC plate(solvent A). The average size (determined by TEM) of Cu-bound vesicles before polymerization was found to be 1300 A. We did not observe any changes in vesicle size during polymerization.

The mixing behavior of $DC_{8,9}PIDA$(**2a**) and $DC_{8,9}PC$ at the air-water interface has been reported.[46] The plot of molecular area of the lipid mixtures versus mole fraction of $DC_{8,9}PIDA$ demonstrated that mixtures containing 50% or less $DC_{8,9}PIDA$ exhibited more ideal mixing behavior than the other mixtures. Lipid miscibility is required for the extent of lateral motion of the lipids

needed to facilitate binding of more than one histidine group of a protein at the surface of lipid assemblies. For our experiments, a mixture of $DC_{8,9}$PIDA and $DC_{8,9}$PC containing 10 mole % IDA lipid was used in vesicle preparation. The enzyme carbonic anhydrase was bound to the surface of both polymerized and non-polymerized vesicles. Due to their affinity towards Cu^{2+}-iminodiacetate, the surface available histidine moieties of the protein were utilized for immobilization.[23] The vesicle/protein mixtures were eluted on a sephadex column. The fractions were monitored at 400 nm for the presence of vesicles which eluted in the void volume. Carbonic anhydrase was retained on the column longer than the vesicles eluting after 1 to 2 column

To examine the stability and activity of the vesicle-bound enzyme, three sets of experiments were performed employing; a) unpolymerized vesicles containing diacetylenic PC and PIDA lipids, b) polymerized vesicles containing diacetylenic PC and PIDA lipids , and c) polymerized vesicles containing non-polymerizable dipalmitoyl PIDA and diacetylenic PC lipids. The results are summarized in Figure 6. Maximum activity was observed for enzyme-bound polymerized vesicles containing diacetylenic phosphocholine and metal chelating lipids. The graph depicted in figure 6A illustrates that the estimated activity is equivalent to an enzyme concentration between 1.6-3.2 μg/mL carbonic anhydrase. Enzyme-bound polymerized vesicles containing non-polymerizable **1a** and diacetylenic PC had an enzyme activity which was equivalent to enzyme concentration between 0.4-0.8 μg/ml (figure 6B). No enzyme activity was observed for the enzyme-bound unpolymerized vesicles (figure 6C). Detailed studies on the kinetics of enzyme binding with non-polymerizable metal-chelated lipid inserted in polymer constrained environment of polymerized vesicles may provide an insight about the role of polymerization on enzyme immobilization.

Summary and Conclusions

Synthesis of phospholipids containing both saturated and diacetylenic chains and IDA headgroup has been accomplished. Chemical shifts of phospholipids and their intermediates have been assigned through extensive proton and carbon NMR experiments. Based on monolayer and TEM analysis, the spacer length has shown to exert influence on the headgroup geometry and microstructure morphology. Depending on spacer length, bilayer sheets, ribbons, or tubules were formed. Formation of tubules with helical striations from diacetylenic phosphoIDA **2c** in the presence of metal ions demonstrates that relatively inexpensive racemic lipids may produce structures similar to that of expensive enantiomers. The relative activity of immobilized carbonic anhydrase on unpolymerized and fully polymerized vesicles demonstrates that cross-linking of the bilayers is a necessary step for the noncovalent immobilization of enzymes on vesicle surfaces. The results demonstrate that this method of noncovalent attachment of proteins to surfactant membranes provides an attractive alternative for immobilization of macromolecules to surfaces.

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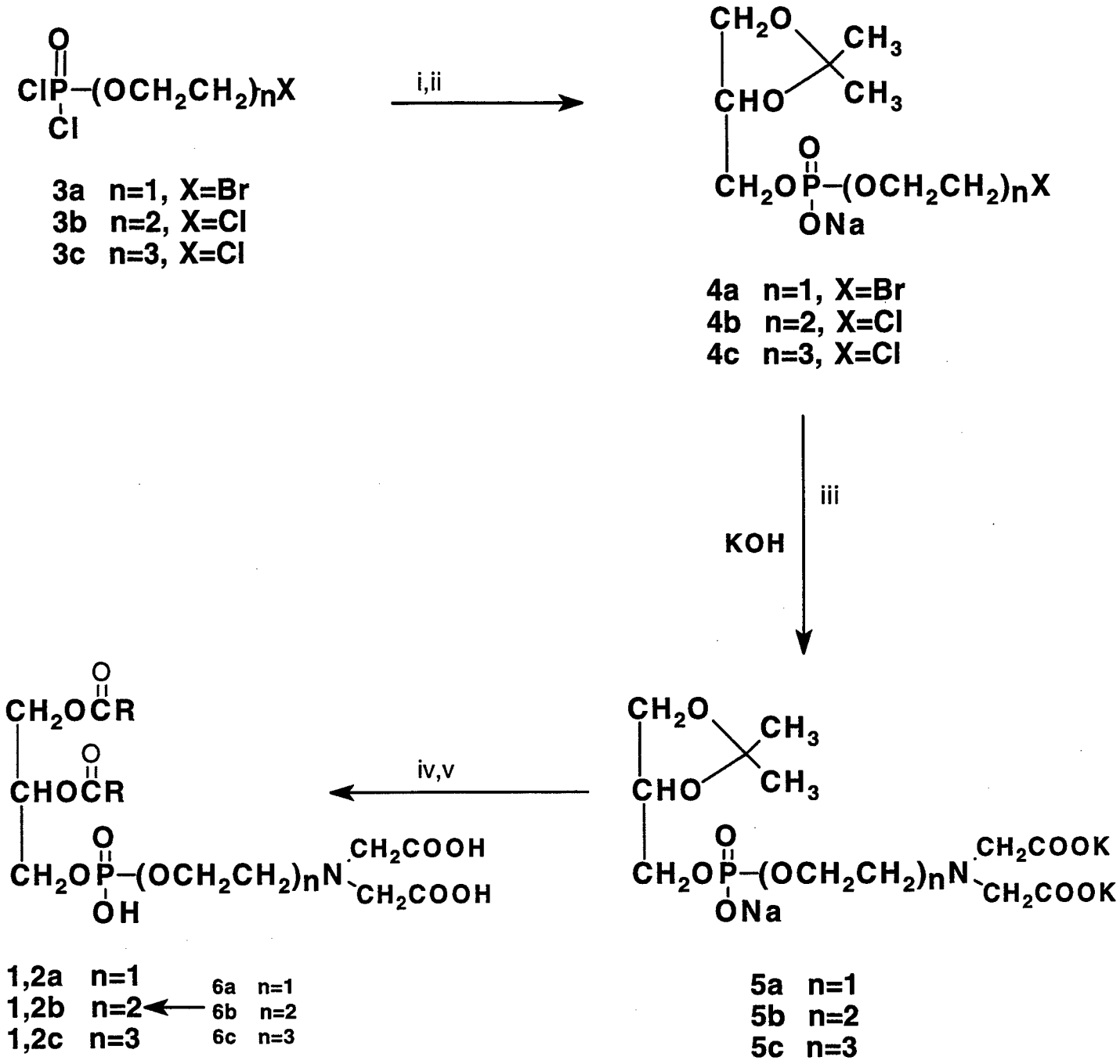
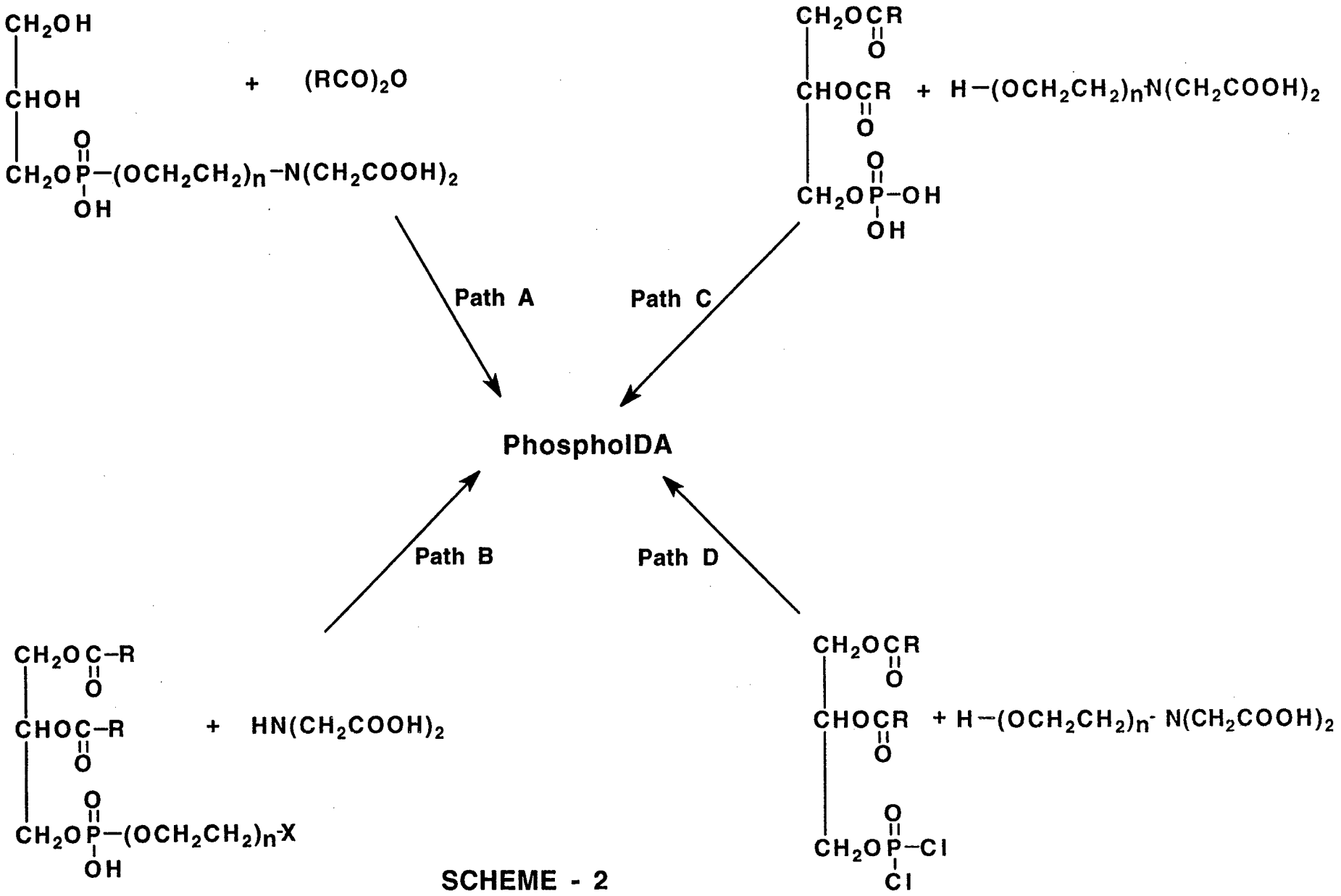


i) solketal, base, O°C; ii) Na_2CO_3; iii) IDA/7M KOH, 3d,r.t; iv) 3N HCl, 6hr, r.t; iv) DMAP, $R(CO)_2O$

SCHEME-1

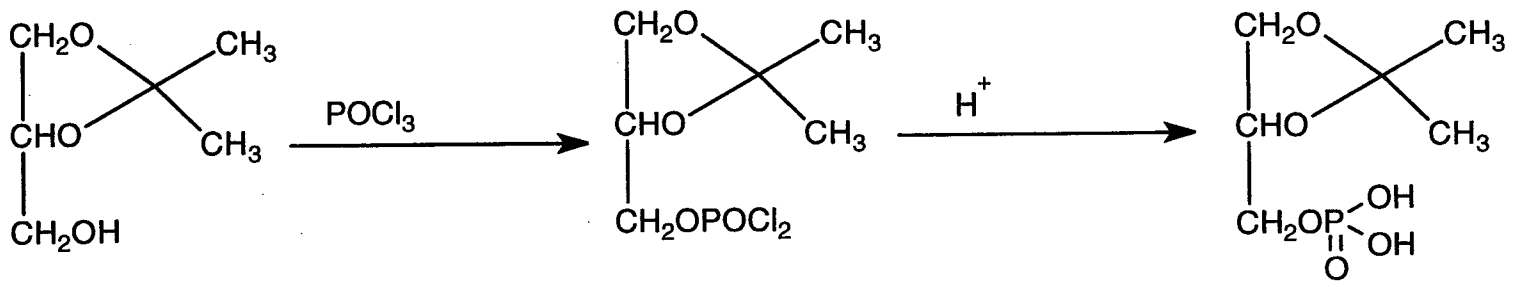
SYNTHESIS OF PHOSPHOLIPIDS

Possible routes:



SCHEME - 2

SCHEME -3



(i) (ii) (iii)

Table -1 Carbon-13 Chemical Shifts (δ_{ppm}) of 4a-c

4a	4b	4c
24.7, 26.2 (CH_3)	*25.0, 26.5* (CH_3)	*24.8, 26.9* (CH_3)
67.4 (d) CH_2^a, $^2J_{P-C}$=5.8Hz	*67.5* (d) CH_2^a, $^2J_{P-C}$=5.9Hz	*67.1* (d) CH_2^a, $^2J_{P-C}$=5.6Hz
73.8 (d) Ch^b, $^3J_{P-C}$=7.7 Hz	*73.8* (d) Ch^b, $^3J_{P-C}$=8.0 Hz	*73.5* (d) Ch^b, $^3J_{P-C}$=8.1 Hz
65.3 CH_2^c	*65.8* CH_2^c	*65.6* CH_2^c
66.4 (d) CH_2^d, $^2J_{P-C}$=4.9Hz	*69.7* (d) CH_2^d, $^2J_{P-C}$=6.5Hz	*69.5* (d) CH_2^d, $^2J_{P-C}$=6.6Hz
29.1 (d) CH_2^e, $^3J_{P-C}$=7.6Hz	*66.5* (d) CH_2^e, $^3J_{P-C}$=5.9Hz	*66.5* (d) CH_2^e, $^3J_{P-C}$=5.9Hz
	71.0 CH_2^f	*70.11,70.14,70.9* $CH_2^{f,g,h}$
	42.4 CH_2^g	*42.4* CH_2^I
109.3 CH_2^j	*109.7* CH_2^j	*109.3* CH_2^j

TABLE - 2 Carbon 13 Chemical Shifts (δ_{ppm}) for 5a-c

5a	5b	5c
26.9, 28.3 (CH_3)	*26.9, 28.3* (CH_3)	*27.1, 28.5* (CH_3)
68.5 (d) CH_2^a, $^2J_{P-C}$=5.0 Hz	*68.3* (d) CH_2^a, $^2J_{P-C}$=5.2Hz	*68.4* (d) CH_2^a, $^2J_{P-C}$=5.1Hz
77.3 (d) CHb, $^3J_{P-C}$=8.5 Hz	*77.4* (d) CHb, $^3J_{P-C}$=8.4 Hz	*77.4* (d) CHb, $^3J_{P-C}$=8.1 Hz
67.8 CH_2^c	*67.8* CH_2^c	*67.9* CH_2^c
62.3 (d) CH_2^d, $^2J_{P-C}$=4.2Hz	*72.9* (d) CH_2^d, $^2J_{P-C}$=6.8Hz	*72.8* (d) CH_2^d, $^2J_{P-C}$=7.5Hz
57.7 (d) CH_2^e, $^3J_{P-C}$=7.7Hz	*67.3* (d) CH_2^e, $^3J_{P-C}$=5.4Hz	*67.5* (d) CH_2^e, $^3J_{P-C}$=5.0Hz
	68.0 CH_2^f	*70.0,72.0,72.2* $CH_2^{f,g,h}$
	57.6 CH_2^g	*56.9* CH_2^I
112.9 CH_2^j	*112.8* CH_2^j	*112.8* CH_2^j
59.7 CH_2^k	*60.7* CH_2^k	*61.8* CH_2^k
172.8 CH_2^l	*180.4* CH_2^l	*180.4* CH_2^l

TABLE-3 Carbon 13 Chemical Shifts (δ_{ppm}) for 6a-c

6a	6b	6c
63.3 (d) CH_2^a, $^2J_{P-C}$=5.0 Hz	*62.9* (d) CH_2^a, $^2J_{P-C}$=5.0Hz	*73.0* (d) CH^b, $^3J_{P-C}$=7.9 Hz
72.9 (d) CHb, $^3J_{P-C}$=7.2Hz	*73.0* (d) CH^b, $^3J_{P-C}$=7.2 Hz	
64.3 CH_2^c ,69.7 CH_2^d,57.5 CH_2^e	*69.5, 68.0,67.6, 64.5* CH_2^{c-g}	*72.4, 72.3, 71.9 69.5, 67.4, 64.5, 62.9* $CH_2^{a,c-I}$
58.3 CH_2^k	*58.6* CH_2^k	*58.6* CH_2^k
170.3 CH_2^l	*170.5* CH_2^l	*170.7* CH_2^l

TABLE-4 Carbon 13 Chemical Shifts (δ_{ppm}) for 1a-c and 2a-c

1a	1b	1c	2a	2b	2c
76.9 CH^b	*77.1* CH^b	*77.2* CH^b	*77.3* CH^b	*77.2* CH^b	*77.5* CH^b
173.3 $C=O^m$	*173.6* $C=O^m$	*173.5* $C=O^m$	*173.5* $C=O^m$	*173.0* $C=O^m$	*173.1* $C=O^m$
179.8 $C=O^l$	*179.9* $C=O^l$	*179.1* $C=O^l$	*179.7* $C=O^l$	*179.8* $C=O^l$	*179.9* $C=O^l$
			65.2 $C^{acetylene}$	*65.2* $C^{acetylene}$	*65.3* $C^{acetylene}$
			77.4 $C^{acetylene}$	*77.2* $C^{acetylene}$	*77.3*
$C^{acetylene}$					
52.8, 55.1, 55.4,	*52.8, 56.1,*	*53.2, 55.1, 62.3,*	*52.6, 55.2, 55.4,*	53.2, 53.5, 55.4,	53.2, 53.5,
62.4, 63.1, 68.6,	*62.0,63.1,*	*63.1, 64.2, 69.8,*	*60.5, 70.6*	*62.6, 63.1, 68.6,*	*62.6, 63.1,*
70.3	*71.1*	*71.7*		*70.2*	*70.2*
Headgroup	Headgroup	Headgroup	Headgroup	Headgroup	Headgroup
3.9, 22.4, 24.6, 22.6,	*14.1, 22.7, 24.7,*	*13.9, 19.0, 22.5,*	*14.0, 19.1, 22.6,*	*13.9, 19.0, 22.5,*	*14.1, 19.2,*
29.2, 31.7, 33.8 28.8,	*24.8, 29.3, 29.4,*	*24.7, 28.3, 28.8,*	*24.7, 28.3, 28.8,*	*24.7, 28.3, 28.8,*	*24.8, 28.4,*
	29.5, 29.7, 31.9,	*28.9, 29.2, 29.4,*	*28.9, 29.0, 29.4,*	*28.9, 29.2, 31.8*	*28.9, 29.1,*
29.4,	*33.7*	*31.8*	*31.8*		*29.5*
Acyl chain	Acyl chain	Acyl chain	Acyl chain	Acyl chain	Acyl chain

TABLE-5

Monolayer Properties of PhosphoIDA Lipids

Lipids	Limiting Area(A^2/mol)					Collapse Pressure(mN/m)				
	water	*mM* $[CuCl_2]$				*water*	*mM* [CuCl2]			
		0.01	0.1	1.0	10.0		0.01	0.1	1.0	10.0
1a	56	101	73	75	85	22	19	24	27	24
1b	57	70	81	75	75	41	24	20	23	22
1c	60	58	67	60	58	51	35	29	39	37
2a	160	145	133	103	126	20	12	19	12	17
2b	93	102	99	87	87	21	12	11	11	10
2c	89	118	89	89	76	28	19	26	19	27

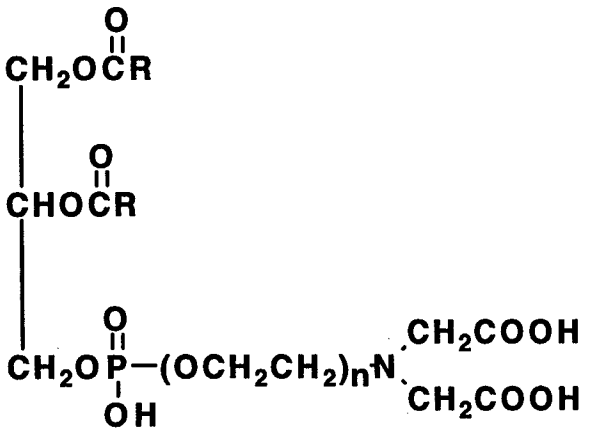
TABLE-6

Microstructures from diacetylenic lipids at various pH and ionic strength

pH	IS	2a (n=1)	2b(n=2)	2c(n=3)
7.0				
	0.2	**a**	**a**	**b**
	0.4	**vesicles (width 0.8-2.8 um)**	**ribbons**	**b**
8.0				
	0.2	**b**	**ribbons**	**a**
	0.4	**ribbons**	**ribbons**	**tubules(1.6um dia.)**
	0.6	**ribbons**	**flat ribbons (0.8-2.8 dia)**	**crystallites**
	0.8	**crystallites**	**ribbons**	**cyrstallites**
9.0				
	0.2	**crystallite**	**crystallite**	**a**
	0.4	**crystallites**	**crystallites**	**a**

a. Not attempted; b. no identifiable micro structure observed

List of Figures



1 a-c R = $-(CH_2)_{14}CH_3$

2 a-c R = $-(CH_2)_8-C\equiv C-C\equiv C-(CH_2)_9CH_3$

a- n=1; b- n=2; c- n=3

FIGURE-1

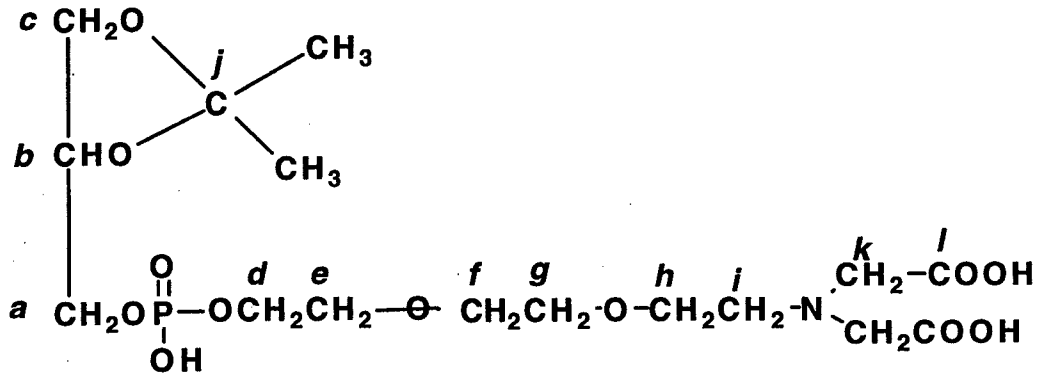
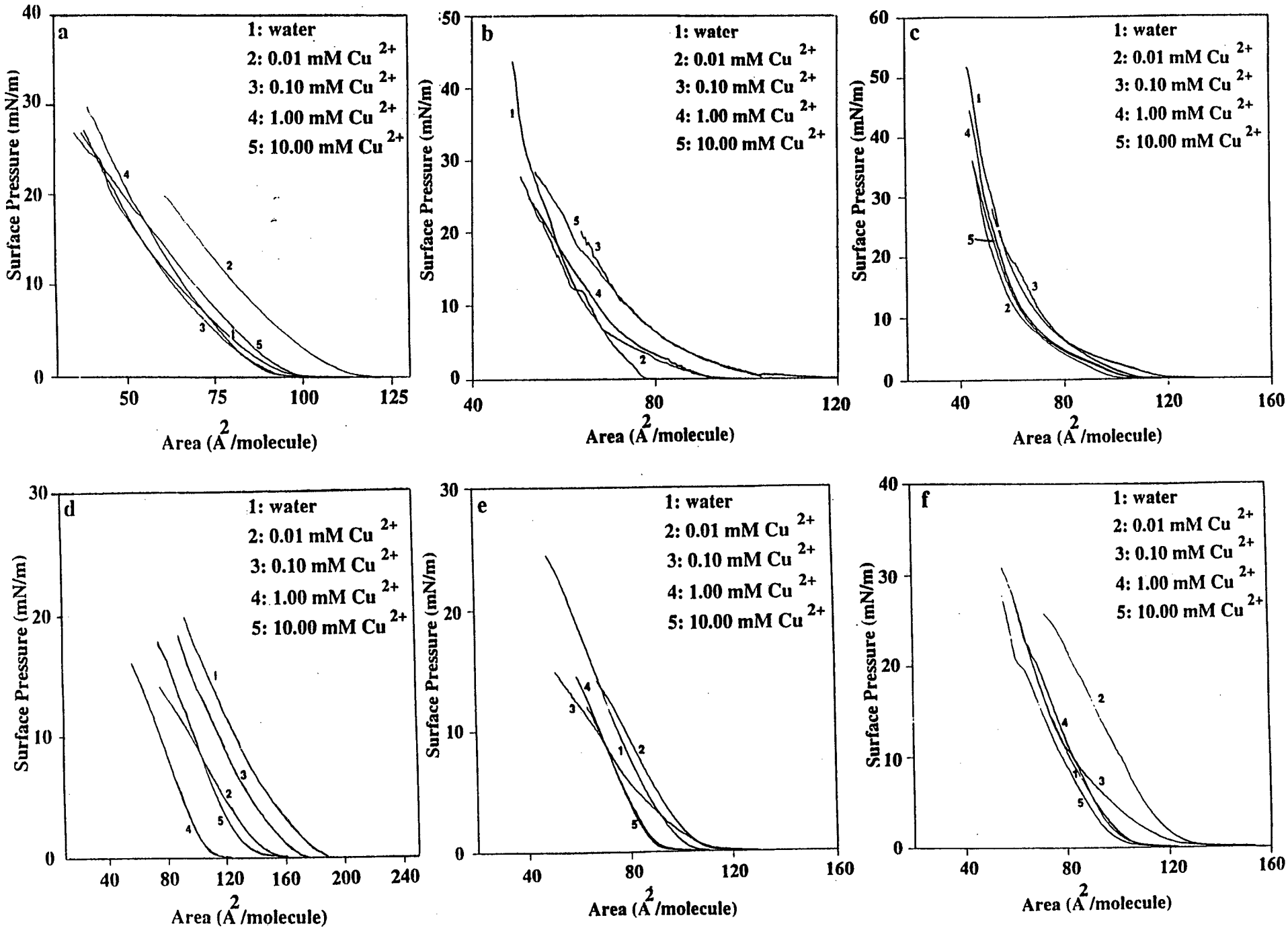

c CH2O
j
CH3
C
b CHO
CH3
k
l
O
d e
f g
h j
CH2–COOH
a CH2O P–OCH2CH2–O CH2CH2–O–CH2CH2–N
CH2COOH
OH

FIGURE- 2

FIGURE 3


a
1: water
2: 0.01 mM Cu 2+
3: 0.10 mM Cu 2+
4: 1.00 mM Cu 2+
5: 10.00 mM Cu 2+
Surface Pressure (mN/m)
Area (Å2/molecule)
b
1: water
2: 0.01 mM Cu 2+
3: 0.10 mM Cu 2+
4: 1.00 mM Cu 2+
5: 10.00 mM Cu 2+
Surface Pressure (mN/m)
Area (Å2/molecule)
c
1: water
2: 0.01 mM Cu 2+
3: 0.10 mM Cu 2+
4: 1.00 mM Cu 2+
5: 10.00 mM Cu 2+
Surface Pressure (mN/m)
Area (Å2/molecule)
d
1: water
2: 0.01 mM Cu 2+
3: 0.10 mM Cu 2+
4: 1.00 mM Cu 2+
5: 10.00 mM Cu 2+
Surface Pressure (mN/m)
Area (Å2/molecule)
e
1: water
2: 0.01 mM Cu 2+
3: 0.10 mM Cu 2+
4: 1.00 mM Cu 2+
5: 10.00 mM Cu 2+
Surface Pressure (mN/m)
Area (Å2/molecule)
f
1: water
2: 0.01 mM Cu 2+
3: 0.10 mM Cu 2+
4: 1.00 mM Cu 2+
5: 10.00 mM Cu 2+
Surface Pressure (mN/m)
Area (Å2/molecule)

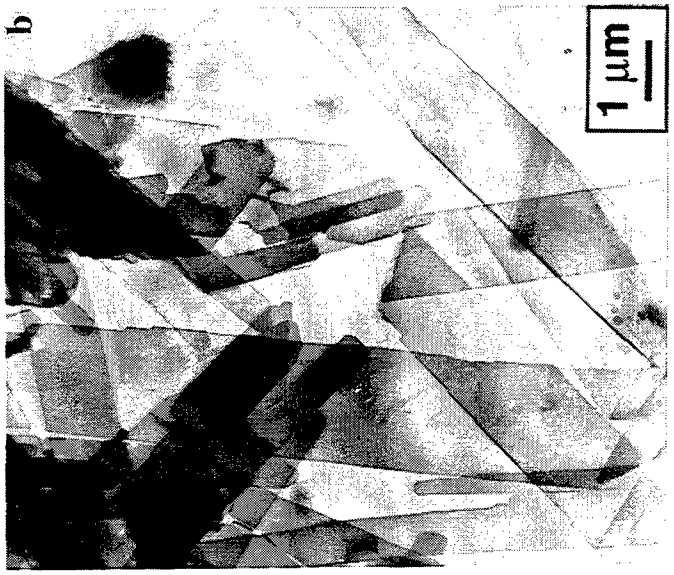
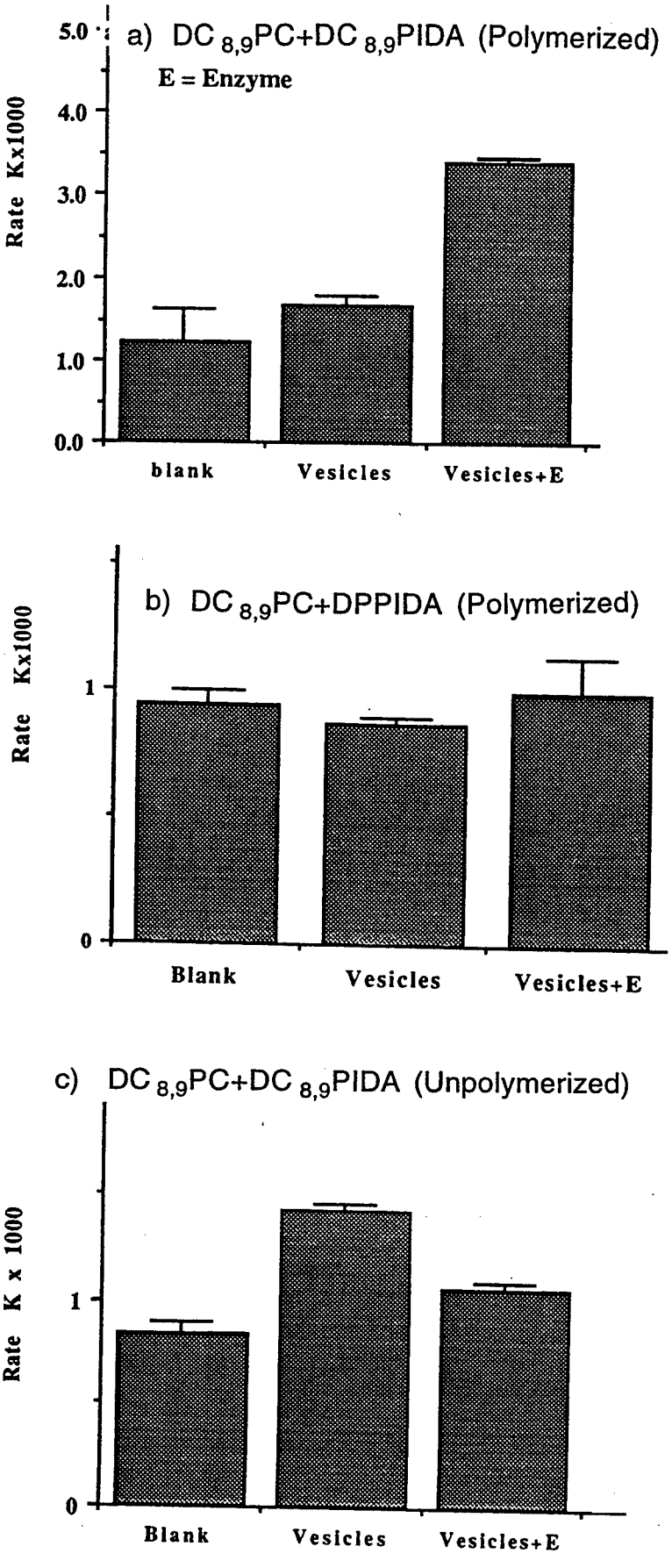


FIGURE 4

FIGURE 5

FIGURE 6


a) DC 8,9PC+DC 8,9PIDA (Polymerized)
E = Enzyme
Rate Kx1000
5.0
4.0
3.0
2.0
1.0
0.0
blank
Vesicles
Vesicles+E
b) DC 8,9PC+DPPIDA (Polymerized)
Rate Kx1000
1
0
Blank
Vesicles
Vesicles+E
c) DC 8,9PC+DC 8,9PIDA (Unpolymerized)
Rate K x 1000
1
0
Blank
Vesicles
Vesicles+E