Polymer-stabilized phospholipid vesicles with a controllable, pH-dependent disassembly mechanism

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Experimental

Materials

2-Hydroxyethylmethacrylate (HEMA), 2,2-dimethoxypropane (DMOP), anhydrous benzene, and p-toluene sulfonic acid (pTSA) were purchased from Aldrich and used as received. 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) was purchased from Avanti Polar Lipids (Alabaster, AL) and used as received. Butyl methacrylate (BMA) and ethylene glycol dimethacrylate (EGDMA) were obtained from Aldrich and the inhibitor was removed using a neutral alumina column prior to use. Irgacure 907 was purchased from Ciba Specialty Chemicals (Tarrytown, NY) and used as received. A poly(BMA) 14 kD standard was obtained from Polymer Source, Inc. and used as received (Dorval (Montreal), Quebec H9P 2X8, Canada). ^{1}H and ^{13}C NMR were conducted on a Bruker 500 MHz instrument.

Synthesis of 2,2-Dimethacroyloxy-1-ethoxypropane (DMOEP)

All glassware was baked in a 150 ºC oven for 24 hrs prior to use. The synthesis was performed in a 250 ml round bottom flask using a Dean-Stark trap and water-cooled reflux condenser plugged at one end. Prior to synthesis, 16 mL of dry molecular sieves were added to the Dean-Stark trap, and the apparatus was evacuated and backfilled with Ar three times. HEMA (20.0 mL, 165 mmol), DMOP (10.1 mL, 82.1 mmol), anhydrous benzene (100 mL), and pTSA (52.5 mg, 0.276 mmol) were added to the flask, and the entire apparatus flushed with Ar. The reaction was heated and refluxed for 20 hours at 95 ºC. Benzene was then removed by distillation and drained using the valve on the Dean-Stark trap. The product was purified by column chromatography on silica using a mobile

phase of 85:14:1 hexane:ethyl acetate:TEA. The fractions containing pure product were pooled, the solvent removed by rotary evaporation, and dried overnight under vacuum yielding 18.6 g (75.4 % yield). The product was stored at -80 ºC, and passed through neutral alumina prior to use. *^{1}H NMR (500 MHz, $CDCl_3$)* δ 1.396 (6H, s, $C(C\boldsymbol{H}_3)_2$); 1.954 (6H, s, $OCOCC\boldsymbol{H}_3CH_2$); 3.711 (4H, t, J = 5 Hz, $C(CH_3)_2(OC\boldsymbol{H}_2CH_2O)_2$); 4.280 (4H, t, J = 5 Hz, $C(CH_3)_2(OCH_2C\boldsymbol{H}_2O)_2$); 5.568 (2H, bs, $OCOCCH_3C\boldsymbol{H}_2$ – syn to methyl); 6.110 (2H, d, J = 6 Hz, $OCOCCH_3C\boldsymbol{H}_2$ – anti to methyl). *^{13}C NMR (125 MHz, $CDCl_3$)* δ 18.287 ($OCOC\boldsymbol{C}H_3CH_2$), 24.756 ($C(\boldsymbol{C}H_3)_2$), 58.845 ($C(CH_3)_2(O\boldsymbol{C}H_2CH_2O)_2$), 63.940 ($C(CH_3)_2(OCH_2\boldsymbol{C}H_2O)_2$), 100.109 ($\boldsymbol{C}(CH_3)_2$), 125.491 ($OCOCCH_3\boldsymbol{C}H_2$), 136.143 ($OCO\boldsymbol{C}CH_3CH_2$), 167.208 ($O\boldsymbol{C}OCCH_3CH_2$). *FT-ICR MS.* $M+Na^+ = C_{15}H_{24}O_6Na$, m/z = 323.1475 Da observed, m/z = 323.1471 calculated, error = 1 ppm.

Preparation of DOPC Vesicles

Unilamellar vesicles were prepared by placing 10 mg of DOPC in chloroform in a 0.5 dram glass vial, removing the solvent with a stream of Ar, and drying the lipid under vacuum for four hours. The lipid was rehydrated with 1.00 mL buffer consisting of 10 mM phosphate or 10 mM citrate (pH 7.4 in both cases), 137 mM NaCl, and 2.7 mM KCl by vortexing and shaking in a warm water bath (42 ºC). Lipid solution was freeze-thawed ten times using a dry ice/isopropanol bath (-79 ºC) and a warm water bath (42 ºC). The resulting multilamellar vesicles were extruded 21 times through two Nucleopore polycarbonate membranes with a mean pore diameter of 200 nm.

Polymer Stabilization of DOPC Vesicles

A 30.0 μL aliquot of 10 mg/mL Irgacure 907 in MeOH was placed in a 2 dram glass vial, the MeOH evaporated under a stream of Ar, and dried under vacuum for 10 min. One mL of 200 nm DOPC vesicles (10 mg total lipid) was added to the dried initiator and stirred in the dark for 30 min. BMA (2.40 μL) and EGDMA (2.00 μL) were added to the vesicle suspension and stirred overnight after which the suspension was diluted to 2 mg/mL DOPC, placed in a quartz cuvet, and purged with Ar for 10 min. The cuvet was sealed with a Teflon stopper and polymerized with UV irradiation (100 W mercury lamp, Newport model 6281 with Newport 69907 power supply operated at 100 W) for 30 min with a water IR filter and a UV bandpass filter (Edmund U-330). This procedure was also used to make acid degradable vesicles with the following modifications: 24.7 μL 10 mg/mL Irgacure 907, 1.99 μL BMA, and 2.42 μL DMOEP.

TEM of Stabilized Vesicles

Small mica squares coated with a 5 nm thick carbon layer were dipped into the desired solution of vesicles, and dropped into a saturated solution of uranyl acetate. The delaminated carbon film was retrieved using a 300 mesh TEM grid, and filter paper was used to wick away the excess liquid. The samples were analyzed using a JEM-100CX-II (JOEL) electron microscope operated at 80 kV.

Dynamic Light Scattering

An aliquot containing 0.04 mg lipid was diluted to 0.800 mL with matching buffer, and analyzed using a BI8000 autocorrelator (Brookhaven Instrument Corp.,

Holtsville, NY) with the scattering angle held constant at 90º. Cumulant analysis was applied to determine particle sizes of micellar assemblies. The samples were spiked with aliquots of 50 mM Triton X-100 producing the desired surfactant to lipid mole ratio, triturated, and reanalyzed.

GC-MS Analysis of Volatile Byproducts

A 1.60 mL aliquot of polymer-stabilized vesicle (10 mg/mL lipid) suspension was placed in a clean 20-mL headspace vial, and the pH was adjusted using HCl. The vials were sealed and stored in the dark for two weeks. The byproducts were analyzed by immersing an 85 μm Carboxen SPME fiber (Supelco) into the sample for 10 min without stirring, and injecting the adsorbed material into a HP 5890 GC with a HP 5971 Mass Selective Detector. The column was held at 50 ºC for four minutes, and the temperature was then ramped linearly to 250 ºC at 15 ºC/min.

Separation of the Polymer Scaffold from Lipid Bilayers

The aqueous solution of polymer-stabilized vesicles was lyophilized using a FreeZone freeze dry system (Labconco), and the white powder collected from lyophilization was dispersed in 4 mL of THF. The lipids dissolved in THF while the polymers precipitated. The polymer precipitates were then collected by centrifugation followed by methanol washing five times. Finally, the isolated polymers were collected and dried in a lyophilizer.

Acid-catalyzed Degradation of Isolated Cross-linked Polymers

The cross-linked polymers were dispersed in THF, hydrochloric acid (2 M) was added into the THF solution dropwise, and the pH of the solution was monitored with pH paper. Once an apparent pH of 2.0 was reached, the addition of hydrochloric acid was stopped. After incubating the polymer dispersion in acidified THF overnight, the solution was neutralized by adding 2 M KOH and then lyophilized. The dried polymer was collected and washed with water five times to reduce the salt content, then dried and dissolved in THF for further analysis. The polymer obtained from vesicles stored at pH 7.0 was suspended in THF spiked with a volume of water equivalent to the volume of 2M HCl added for acidified THF solutions.

Results and Discussion

Transmission Electron Microscopy

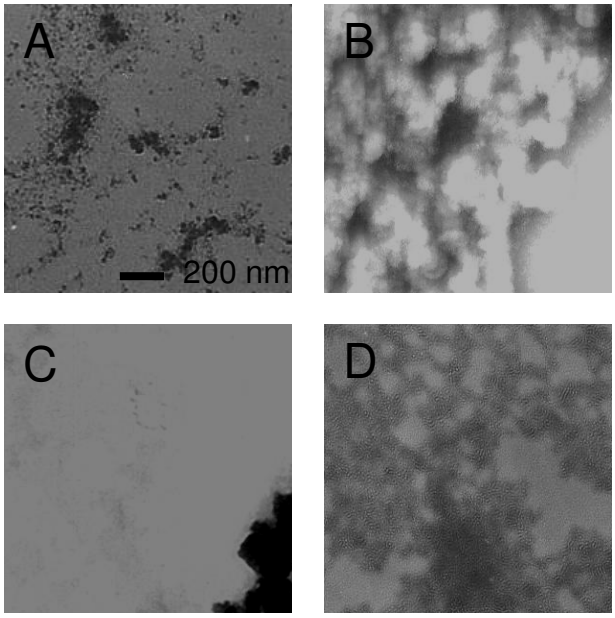

Figure S-1: TEM images of DOPC/BMA/DMOEP vesicles stored at pH 2.0 in the absence (A) and presence (B) of 10:1 mole ratio Triton X-100:DOPC, and DOPC vesicles stored at pH 7.0 in the absence (C) and presence (D) of 10:1 Triton X-100.

Polymer stabilized vesicles stored at pH 2.0, as well as DOPC vesicles stored at pH 7.0 were unable to withstand the drying conditions used to prepare the samples for TEM analysis, suggesting that pH 2.0 sufficiently degrades the acetal cross-linked polymer network to render DOPC/BMA/DMOEP polymer-stabilized vesicles indistinguishable from vesicles composed of only DOPC.

GC-MS Analysis of Polymer Degradation

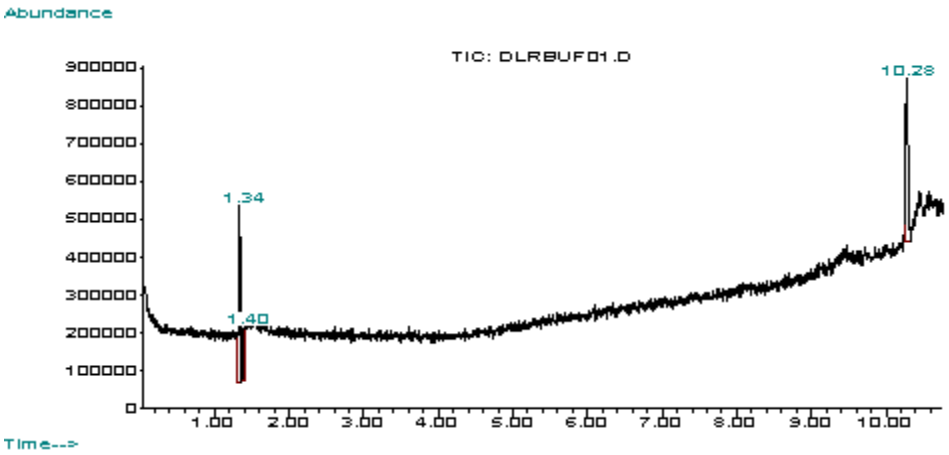

Figure S-2a: GC-MS Total Ion Current (TIC) chromatogram of 10 mM citrate pH 7.4, 140 mM NaCl.

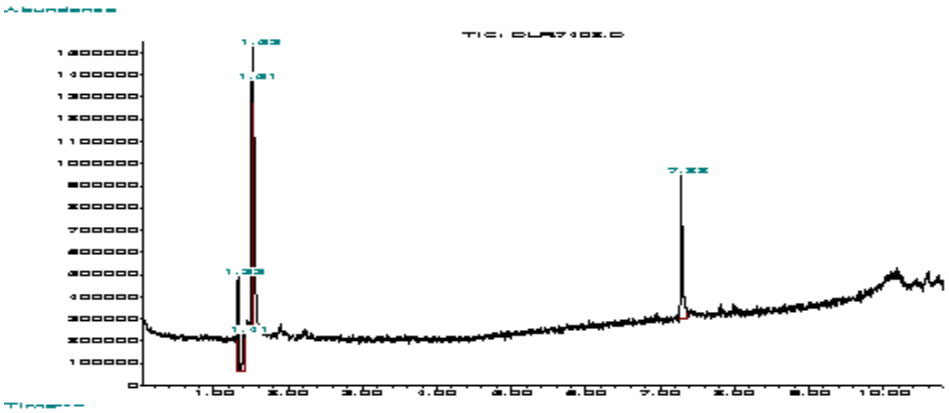

Figure S-2b: GC-MS TIC chromatogram of DOPC/BMA/DMOEP vesicles stored in 10 mM citrate pH 7.4, 140 mM NaCl

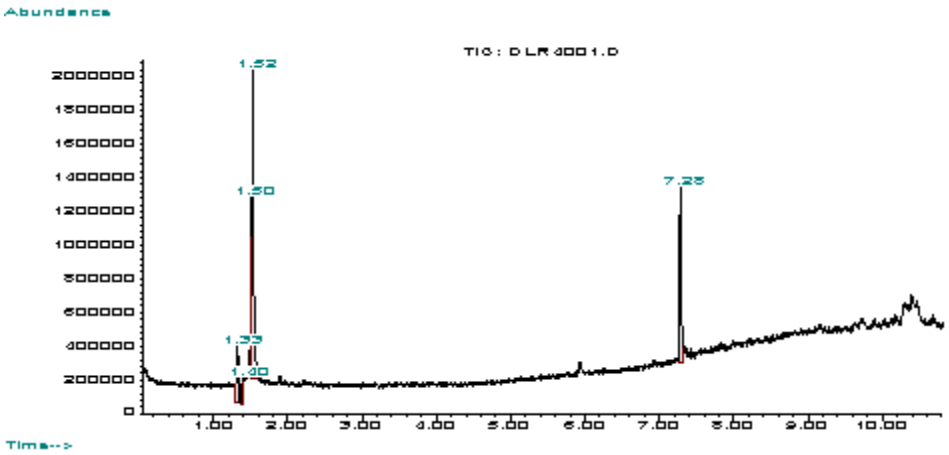

Figure S-3: GC-MS TIC chromatogram of DOPC/BMA/DMOEP vesicles stored in 10 mM citrate pH 4.0, 140 mM NaCl.

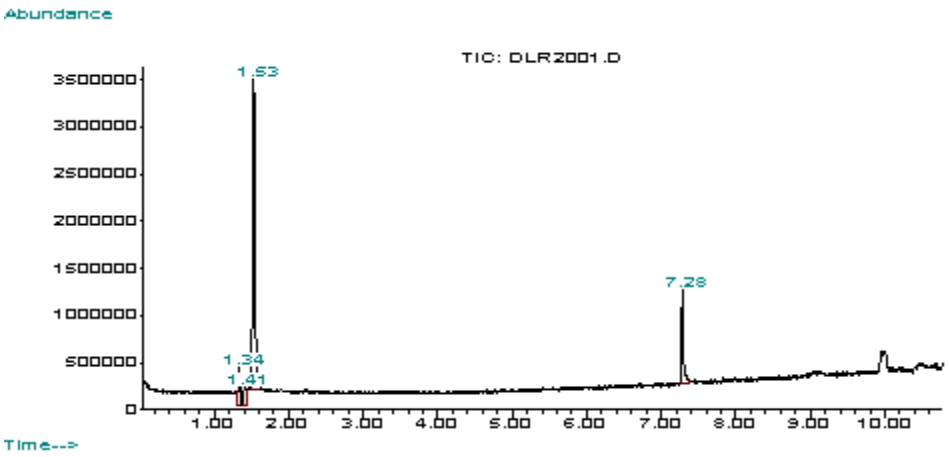

Figure S-4: GC-MS TIC chromatogram of DOPC/BMA/DMOEP vesicles stored in 10 mM citrate pH 2.0, 140 mM NaCl

Table S-1: Identification and peak areas for GC-MS analysis of DOPC/BMA/DMOEP stabilized vesicles

Peak Time (min)	Peak ID	pH 7.4 Area	pH 4.0 Area	pH 2.0 Area
1.53	Acetone	3264066	4038100	6931981
7.28	HEMA	1127394	1496456	1622637
	Ratio	2.9	2.7	4.3

All chromatograms contained only two peaks, neither of which was present in the blank sample. The first, at 1.53 min, was easily identified as acetone, and the second, at 7.28 min, was determined to be HEMA. These two compounds are products from the hydrolysis of DMOEP, and both increase in concentration in a pH-dependent manner. The ratio of acetone to HEMA is very similar when the vesicles are stored at pH 7.4 and 4.0 suggesting that the acetone is not generated from the degradation of the polymer network, but from the DMOEP that either does not partition into the bilayer, or is not incorporated into the polymer network and subsequently diffuses back into solution. The second is the more likely as both samples contained the same amount of DMOEP, and the lower pH should degrade the DMOEP in solution more readily. At pH 2, not only is there an increase the amount of acetone in solution, the ratio of acetone to HEMA increases as well. This implies that the polymer network is being degraded releasing more acetone, but not HEMA since it is incorporated into the polymer network. These results support the hypothesis that these vesicles are stable at pH 7.4, are only partially degraded at pH 4.0, and are fully degraded at pH 2.0.

Visual Observations of Polymer Solutions

Figure S-5: Poly(BMA) dissolved in THF.

A sample of poly(BMA) (14 kDa) was added to THF, sonicated, and observed to completely dissolve. This is in keeping with the general trend that lower molecular weight polymers are more soluble, and serves as a control for the observation that the polymer extracted from vesicles is more soluble as the acetal cross-links are degraded.