# Use of the Gamma-Ray Perturbed Angular Correlation (PAC) Technique for Monitoring Liposomal Phospholipid Bilayer Integrity

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A membrane labeling method based on the principle of gamma-ray perturbed angular correction (PAC) was developed to monitor the structural integrity of liposomal membranes. The reporter group was $^{111}$In(III) complexed with the lipophilic diethylenetriaminepentaacetic acid (DTPA) derivative of dipalmitoylphosphatidylethanolamine (DPPE) embedded in the phospholipid bilayers of small unilamellar liposomes. Using this method, complete chemical digestion of the constituent phospholipids in these DTPA-conjugated liposomes by phospholipase $A_2$ or phospholipase C in the presence of $Ca^{2+}$ was found not to be followed by an immediate disruption of the liposomal membrane. Compared with other methods, the method developed permits the continuous noninvasive monitoring of the microenvironment of the lipid bilayer at the molecular level. It may potentially be applicable to evaluate liposomal fusion, screen for penetration enhancers under development for enhancement in mucosal drug penetration, and monitor liposomal degradation within the living animal.

KEY WORDS: gamma-ray perturbed angular correlation; liposomes; lipophilic probe; indium.

## INTRODUCTION

The effectiveness of liposomes as drug carriers depends partly on their stability to phospholipases in the serum and various tissues (1). Hydrolysis by phospholipase $A_2$, C, or D requires phosphatidylcholine as the substrate, which is the most widely used phospholipid in liposomes. Because dipalmitoylphosphatidylcholine (DPPC)[4] easily forms small unilamellar vesicles (SUVs) under mild conditions, DPPC/SUV was used as a model system in the present study to evaluate phospholipase-mediated hydrolysis.

Methods for monitoring liposomal permeability include the liposome swelling assay (2), dialysis method (3), two-compartment growth inhibition assay (4), fluorescence-quenching assay (5), double-radioisotope method (6), and methods based on nuclear magnetic resonance (1,7) and electron spin resonance (8). All of the above methods suffer from a number of disadvantages, ranging from lengthy, cumbersome separation processes (2) to light-scattering problems (5), unsynchronized release of markers (6), and difficulty with interpreting spectra quantitatively (9). These disadvantages can be overcome by the perturbed angular correlation (PAC) technique. First, since PAC is a radioisotopic method, it is not subject to problems due to heat, oxygen, light, solvent, or optical turbidity. Second, its inherent high sensitivity allows the detection of subtle changes in the bilayer membrane. Third, its nondestructive nature allows continuous monitoring of integrity of the bilayer membrane (10).

Originally used by nuclear physicists to study excited nuclear states (11), the PAC technique was later applied to study conformational changes of macromolecules (12), estimate the rotational correlation times of bovine serum albumin, and measure the antibody–ligand binding constant (13). The principle of the technique is that $^{111}$In emits two gamma-rays in the decomposition cascade, the angular correlation between the direction of propagation of which is affected only by the rotational (or tumbling) motion of the $^{111}$In nucleus, and not by relative translational motion. If the size of the molecule to which the $^{111}$In is bound is changed, the rate of rotation of the $^{111}$In in solution, hence the angular correlation of the two gamma-rays in the cascade will also change. The extent of perturbation of the angular correlation is an experimentally measurable parameter, called the time-integrated perturbation factor $G\langle 22\rangle$.

The objective of the present study was to develop a membrane labeling technique using a hydrophobic membrane-bound probe that chelates $^{111}$In(III)—dipalmitoylphosphatidylethanolamine–diethylenetriaminepentaacetic acid (DPPE-DTPA)—for monitoring liposomal membrane integrity during digestion by phospholipases. By virtue of having the same physicochemical characteristics as the two fatty acid chains of DPPC, the dipalmitoyl chain of the probe is readily incorporated into the liposomal membrane itself. The binding constant between $^{111}$In(III) and the DPPE-DTPA probe is similar to that between $^{111}$In(III) and DTPA, so that $^{111}$In(III) is not expected to dissociate from the chelate to any significant extent. The reasonable size and length of the DPPE-DTPA polar head group exposed on the surface of the liposomal membrane provide a rigid environment for the tight binding of $^{111}$In(III), so that the angular correlation time reflects liposomal membrane rotation rather than independent flexibility of the chelating moiety. Thus, when the liposome is intact and DPPE-DTPA is tightly anchored in the bilayer, $^{111}$In(III) bound to DTPA-conjugated liposomes has a slow tumbling rate with a low $G\langle 22\rangle$ reading. After phospholipase digestion, the liposome loses its structural integrity; DPPE-DTPA is loosely incorporated or even released in free form; and $^{111}$In(III) will have a fast tumbling rate, giving rise to a high $G\langle 22\rangle$ reading. The percentage of liposomal degradation can then be calculated from the following equation:

$$G\langle 22\rangle_P = X * G\langle 22\rangle_D + (1 - X) * G\langle 22\rangle_I \qquad (1)$$

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[4] Abbreviations used: DCCI, 1,3-dicyclohexylcarbodiimide; DPPC, dipalmitoylphosphatidylcholine; DPPE, dipalmitoylphosphatidylethanolamine; DTPA, diethylenetriaminepentaacetic acid; 8-HOQ, 8-hydroxyquinoline; LPC, lysophosphatidylcholine; NTA, nitrilotriacetic acid; PAC, perturbed angular correlation; $PLA_2$, phospholipase $A_2$; PLC, phospholipase C; SUV, small unilamellar vesicle.

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where the $G\langle 22\rangle$ with subscripts I, D, and P represent the values of intact (I), fully degraded (D), and partially degraded (P) liposomes, and $X$ is the percentage of the partial degradation at any time after the onset of degradation.

## MATERIALS AND METHODS

### Materials

L-α-Dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylethanolamine (DPPE) were purchased from Avanti (Pelham, AL). Phospholipase $A_2$ (from bee venom), phospholipase C (Type I; from *Clostridium perfringens*), palmitic acid, and lysophosphatidylcholine (LPC) were purchased from Sigma (St. Louis, MO). Diethylenetriaminepentaacetic acid (DTPA), nitrilotriacetic acid (NTA), and 1,3-dicyclohexylcarbodiimide (DCCI) were obtained from Aldrich (Milwaukee, WI). All other chemicals and solvents were reagent grade.

Indium chloride was purchased from NEN Dupont (Boston, MA). Sephadex G-50 and Sepharose 2B and 4B were purchased from Pharmacia (Piscataway, NJ). AG1-X8 ion-exchange resin (chloride form) was obtained from Bio-Rad (Richmond, CA). The analytical TLC plate (silica gel HLF; 0.25-mm thickness) was purchased from Analtech (Newark, DE), and the preparative TLC plate (silica G-200) was obtained from Alltech (Deerfield, IL). Phospray was obtained from Supelco Inc. (Bellefonte, PA).

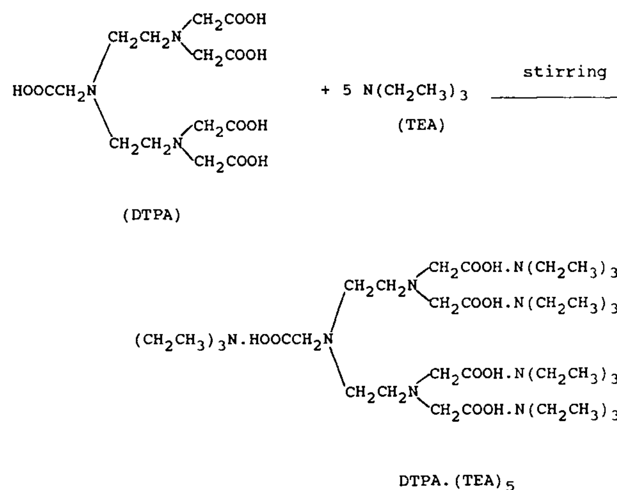
### Purification of $^{111}$In

To enhance the radiolabeling efficiency of liposomal DTPA, it is imperative that $InCl_3$ be free of heavy metal ion contaminants. Purification was achieved by eluting indium off a plastic column packed with AG 1-X8 resin with a step gradient of 18 ml (1 bed vol) of 2 $N$ HCl and 40 mL of 0.1 $N$ HCl. The fractions containing indium were collected in plastic disposable minibeakers, dried overnight under heat lamps, and then stored behind lead bricks.

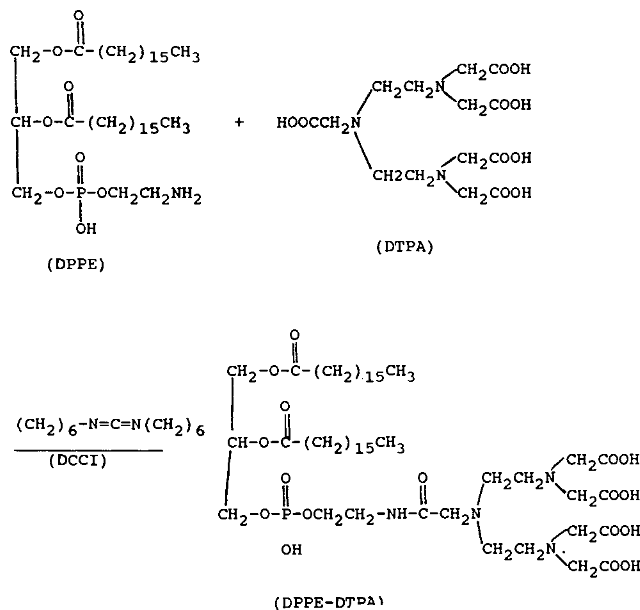
### Synthesis and Purification of DPPE-DTPA

DPPE-DTPA was synthesized in two steps, as shown in Scheme I. In the first step, 1 m$M$ DTPA (393 mg) was suspended in 20 mL of deionized water and solubilized by slowly adding 506 mg (5 m$M$) of triethylamine (TEA) under stirring. The solution was frozen with dry ice and lyophilized overnight to obtain a gel-like DTPA-TEA complex. In the second step, 1 m$M$ DTPA-TEA complex was dissolved in 6 mL of $CHCl_3$ and combined with 1 m$M$ (207 mg) DCCI dissolved in 1 mL of $CHCl_3$. To this mixture was added dropwise 100 μ$M$ (69.2 mg) of DPPE suspended in 6 mL of $CHCl_3$ after a brief reflux. After 4 hr of stirring at room temperature, the dicyclohexylurea precipitate was removed by filtration and washed with a small quantity of $CHCl_3$. The combined filtrate and wash were dried by flash evaporation. Thin-layer chromatographic analysis of the reaction mixture on silica gel, using $CHCl_3$:$CH_3OH$:$H_2O$ (65:25:4) as the mobile phase, demonstrated complete conversion of DPPE to DPPE-DTPA. This was indicated by the disappearance of the purple color characteristic of the free amino group after treatment of the plate with ninhydrin spray. The phosphate-

Step 1. Synthesis of DTPA(TEA)$_5$ Complex




Step 2. Synthesis of DPPE-DTPA




Scheme I. Synthesis of dipalmitoylphosphatidylethanolamine–diethylenetriaminepentaacetic acid (DPPE-DTPA).

positive spot indicated by phospray migrated from an $R_f$ value of 0.4 (DPPE) to one of 0.2 (DPPE-DTPA product).

DPPE-DTPA was purified by dialysis and preparative TLC to remove hydrophilic and hydrophobic impurities, respectively. The residue from flash evaporation was resuspended in Millipore-grade water, transferred to a dialysis bag with a MW cutoff of about 12,000–14,000, and dialyzed twice against 1 L of 1 $M$ acetic acid and twice against 1 L of Millipore-grade water over a 48-hr period. The product was recovered by lyophilization and further purified by preparative TLC on silica gel (HLF; 2-mm thickness, 20 × 20 cm) using $CHCl_3$:$CH_3OH$:$H_2O$ (65:25:4) as the mobile phase. The band containing DPPE-DTPA, as revealed by phospray, was scraped from the plates and eluted with $CHCl_3$:$CH_3OH$

(2:1). After removing the solvent by flash evaporation, the compound was suspended in Millipore-grade water and lyophilized to yield a white powder. The overall yield was about 85%.

### Preparation of DPPC:DPPE-DTPA Liposomes

Twenty milligrams of DPPC and 1 mg of DPPE-DTPA were dissolved in 0.6 mL of $CHCl_3$:$CH_3OH$ (2:1) and dried under nitrogen to obtain a thin film of lipid. Liposomes were formed by bath sonication (Ultrasonic Cleaner, Model 0112SP1T, Laboratory Supplies Company Inc., Hicksville, NY) for 15 min at 52°C in the presence of 1 mL of 10 m*M* Tris–HCl isotonic buffer (pH 7.4), followed by annealing for 30 min at 52°C for binding and enzymatic digestion studies.

### Phospholipase-Mediated Digestion of DTPA-Conjugated Liposomes

The binding of $^{111}$In(III) to DTPA-conjugated liposomes and the subsequent phospholipase-mediated digestion were monitored by the PAC technique. The custom-built PAC instrument was calibrated before each use by determining the $G\langle 22\rangle$ reading with freshly purified $InCl_3$, In citrate ($pK_a$ = 6.2), and In serum (transferrin or other iron-binding proteins in the plasma; $pK_a$ = 3.1). To demonstrate that the $G\langle 22\rangle$ value responded to changes in the microenvironment where $^{111}$In was situated, the $G\langle 22\rangle$ reading was measured before and after the addition of 8-hydroxyquinoline (8-HOQ) and DPPC/SUV with entrapped Tris buffer or 1 m*M* free NTA inside.

Approximately 0.2 mL of liposomes in a 13 × 100-mm glass tube was placed in a 37°C water jacket positioned at the geometric center of the four detectors. Thereafter, either phospholipase $A_2$ (0.497 or 48.9 μg/mg DPPC) or phospholipase C (2 U/mg DPPC) in the presence of $CaCl_2$ was added. The percentage of degradation at different times was calculated according to Eq. (1), using the $G\langle 22\rangle$ value of 1% Triton X-100-treated In liposomes as 100% degradation and the initial In liposome $G\langle 22\rangle$ value of 0.25 before enzymatic treatment as 0% degradation.

The phospholipase $A_2$ ($PLA_2$) degradation products of DPPC/DPPE-DTPA liposomes were identified by TLC on silica gel using palmitic acid and lysophosphatidylcholine as standards. Approximately 10 μL of the liposome-phospholipase reaction mixture was applied to the silica gel plate and developed by the $CHCl_3$:MeOH:$H_2O$ (65:25:4) solvent system. The position of the phosphate positive spot was identified by phospray.

The size changes of DPPC/DPPE-DTPA liposomes before and after $PLA_2$ treatment were monitored by gel chromatography on Sepharose 2B (or 4B) and Sephadex G-50 columns, which were preequilibrated with Tris–HCl buffer. The preparation was eluted from the column with Tris–HCl buffer, and fractions were collected for gamma-counting or lipid assay. The lipid assay was performed according to Charles and Stewart (14).

## RESULTS

### $G\langle 22\rangle$ Values of Indium Situated in Different Environments

The $G\langle 22\rangle$ values of $^{111}$In(III) in various microenvironments varied greatly (Table I). 8-HOQ, an amphiphilic chelate molecule capable of forming a stable complex with indium, functioned as a mobile carrier to transport $^{111}$In(III) across liposomal lipid bilayers. The partitioning of 8-HOQ-In into the liposomal bilayers of DPPC/SUV resulted in the reduction of the $G\langle 22\rangle$ value from 0.54 to 0.30 (Fig. 1, top). In the presence of a stronger water-soluble NTA chelate in the internal aqueous compartment of the liposome, the 8-HOQ-In complex traveled farther across the membrane to complex with NTA, resulting in a rise in the $G\langle 22\rangle$ to 0.51 (Fig. 1, bottom). As expected, when free NTA was added to the 8-HOQ-In liposomes, the $G\langle 22\rangle$ reading was increased to 0.69 (Fig. 1, top).

### Binding of $^{111}$In(III) to DPPC:DPPE-DTPA Liposomes

Upon adding DPPC/DPPE-DTPA SUV to an In-citrate complex, there was an immediate drop in the $G\langle 22\rangle$ value from 0.58 to 0.25 (Fig. 2), reflecting the large difference in mass, hence rotational rate, between citrate and DTPA-conjugated liposomes. No change in $G\langle 22\rangle$ value occurred with control DPPC/DPPE liposomes even after 20 hr of incubation at 37°C (data not shown). The inability to remove the bound $^{111}$In(III) from DTPA liposomes by free DTPA suggests tight binding of $^{111}$In(III) to the liposomes. The $G\langle 22\rangle$ value increased instantly as the liposomes were destroyed by the addition of 1% Triton X-100. The above observations were consistent with the gel filtration results (data not shown). For DTPA-conjugated liposomes, all the $^{111}$In(III) radioactivity stayed with the liposomes and eluted in the void volume; for the control DPPC/DPPE liposomes, $^{111}$In(III) stayed with the citrate molecule and the radioactivity eluted in the low molecular weight fraction. The same peak position from the lipid assay and the radioactivity measurement in a Sepharose 4B column (Fig. 3) indicates the coexistence of the DPPC and the probe in the liposomal

Table I. $G\langle 22\rangle$ Values of Various $^{111}$In-Bound Molecules and Intact and Degraded DTPA-Conjugated Liposomes

| Microenvironment | $G\langle 22\rangle$ ± SD ($n$ = 3) |
|---|---|
| 1. $^{111}$In(III) in 3 m*M* HCl solution | 0.88 ± 0.010 |
| 2. 1.6 m*M* In–citrate in 10 m*M* Tris–HCl buffer (pH 7.4) | 0.58 ± 0.002 |
| 3. 8-Hydroxyquinoline–$^{111}$In(III) complex in sodium acetate (pH 5.4) | 0.54 ± 0.010 |
| 4. 8-Hydroxyquinoline–$^{111}$In(III) complex in DPPC/SUV | 0.30 ± 0.002 |
| 5. $^{111}$In(III)–NTA complex in Tris–HCl buffer aqueous compartment of DPPC/SUV | 0.51 ± 0.006 |
| 6. $^{111}$In(III)–NTA complex in Tris–HCl buffer | 0.69 ± 0.013 |
| 7. In–$Cl_3$, In–citrate, or In–NTA plus rabbit serum | 0.20 ± 0.013 |
| 8. $^{111}$In(III) bound to intact DPPC/DPPE-DTPA SUVs | 0.25 ± 0.005 |
| 9. Phospholipase C-digested DPPC/DPPE-DTPA SUVs | 0.50 ± 0.004 |
| 10. Phospholipase $A_2$ digested DPPC/DPPE-DTPA SUVs | 0.49 ± 0.005 |

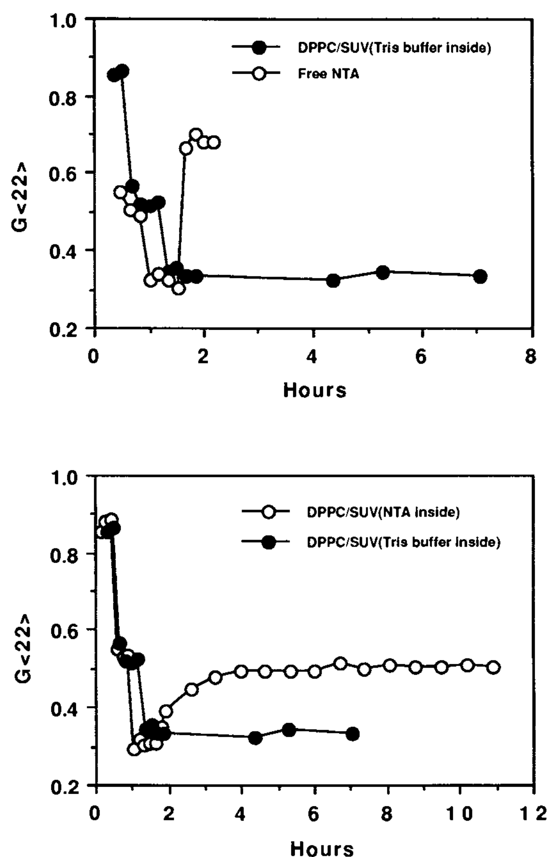



Fig. 1. Partitioning of 8-hydroxyquinoline (8-HOQ)–In into a DPPC/SUV membrane with 1 m*M* free NTA on the outside (top) and with 1 m*M* NTA or Tris buffer entrapped inside (bottom).

membrane. This peak position is consistent with small unilamellar vesicles, in the size range of 40–100 nm, as measured by the light-scattering method on a PCS 100 system (Malvern Instruments, California Institute of Technology, Pasadena).

### Degradation of DPPC:DPPE-DTPA Liposomes by Phospholipases

After DPPC:DPPE-DTPA liposomes were treated with different concentrations of phospholipase $A_2$ and phospholipase C, the $G\langle 22\rangle$ value changed significantly (Fig. 4). The slowly increased $G\langle 22\rangle$ value after enzymatic treatment indicated liposomal membrane transition from a tight to a loose packing arrangement and, finally, to a micellar or free molecular state. The $G\langle 22\rangle$ value was unaffected by the addition of enzymes that had been heated at 100°C for 30 min (Fig. 4).

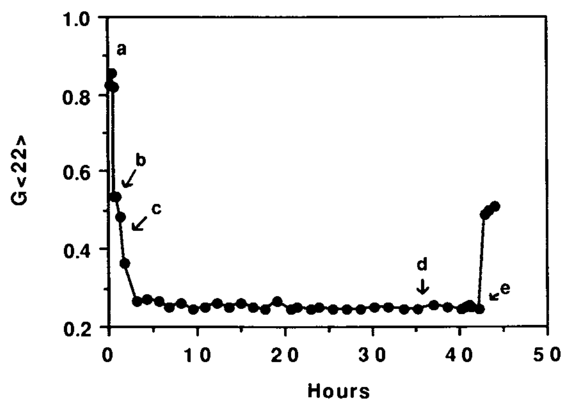



Fig. 2. Binding of $^{111}In(III)$ to DPPC/DPPE-DTPA liposomes under various conditions. (a) $G\langle 22\rangle$ of $InCl_3$ solution; (b) after addition of In–citrate; (c) after addition of DPPC-DPPE/DTPA SUVs; (d) after addition of free DTPA; (e) after addition of 1% Trition X-100.

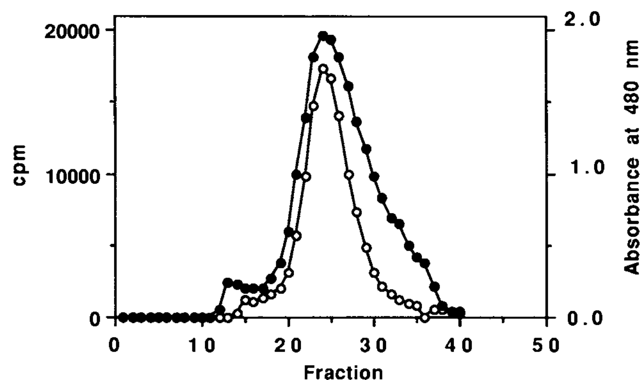



Fig. 3. Chromatographic separation of $^{111}In(III)$-bound DPPC/DPPE-DTPA liposomes on a Sepharose 4B column. (○) Lipid assay; (●) radioisotopic assay.

TLC data indicated that DPPC ($R_f$, 0.45) was completely hydrolyzed to palmitic acid ($R_f$, 0.9) and LPC ($R_f$, 0.2) after a 30-min treatment with 0.50 and 48.9 μg $PLA_2$/mg DPPC. The angular correlation measurement, on the other hand, showed that the liposomes did not lose their integrity until having been treated for 15 hr with 48.9 μg $PLA_2$/mg DPPC and that they retained 50% of their integrity after treatment with 0.50 μg $PLA_2$/mg DPPC for as long as 30 hr.

## DISCUSSION

### Conjugation and Labeling Studies on the Hydrophobic Probe DPPE-DTPA

A simple, rapid, and reproducible procedure has been developed to bind $^{111}In(III)$ tightly to a DTPA group anchored on the DPPC/DPPE liposomal membrane. This pro-

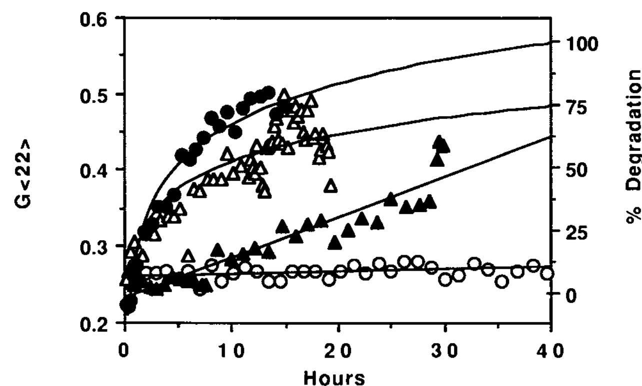



Fig. 4. Changes in $G\langle 22\rangle$ of $^{111}In(III)$-bound liposomes after phospholipase $A_2$ treatment. (△) Native $PLA_2$ (48.9 μg $PLA_2$/mg DPPC); (▲) native $PLA_2$ (0.50 μg $PLA_2$/mg DPPC); (●) native PLC (2 U/mg DPPC); (○) $PLA_2$ (48.9 μg/mg DPPC) pretreated by heat inactivation at 100°C for 30 min.

cedure is a viable alternative to the direct binding procedure whereby $^{111}$In(III) is bound to preformed DPPC/DPPE SUVs through the amino group on the liposomal surface—a difficult and lengthy procedure (15). This is a serious limitation in light of the short half-life of $^{111}$In (2.73 days).

There are two ways to prepare DTPA-conjugated liposomes: (i) by conjugating DTPA to preformed amino groups in DPPC-DPPE liposomes using the carbodiimide method and (ii) by first preparing the DPPE-DTPA phospholipid derivative and then mixing it with DPPC to form liposomes. The former method is not preferred since the lower pH (pH 3) required may compromise the stability of liposomes (11) and since the reaction yield may be low due to steric hindrance by the liposomes.

There is no reported method for making DTPA-conjugated phospholipids. There are, however, two reported methods for making DTPA-coupled albumin that may be adapted for phospholipids: the DTPA carboxycarbonic mixed anhydride method (16,17) and the cyclic DTPA anhydride method (18,19). Both methods, however, suffer from the drawback of going through a moisture sensitive DTPA anhydride. To overcome this disadvantage, a two-step nonaqueous carbodiimide method was developed in the present study to conjugate DTPA to DPPE (Scheme I). Instead of making DTPA anhydride in the first step, a lipophilic DTPA · $(TEA)_5$ complex was made, which was then codissolved and reacted with DPPE in $CHCl_3$. The ratio of DPPE:DTPA$(TEA)_5$:DCCI, sequence of addition, and time to complete addition were carefully chosen to avoid the formation of cross-linked, high molecular weight products (possibly DPPE$_n$-DTPA; $n = 1$–5), as indicated by the appearance of additional phosphate-positive spots with $R_f$ values between 0.2 (DPPE-DTPA) and 0.4 (DPPE). Only a very small amount (3% molar ratio) of the DTPA probe is required for labeling the liposomal membrane to satisfy the amount of activity required by the PAC technique, namely, approximately $10^{11}$ molecules of $^{111}$In. For a sample containing approximately $10^9$ liposomes, a probe/liposome ratio of 100 will be adequate.

### Phospholipase-Mediated Degradation of DPPC/DPPE-DTPA Liposomes

The hydrolysis of phosphatidylcholine vesicles catalyzed by phospholipase has been studied extensively (20,21). Hydrolysis follows Michaelis–Menten kinetics (22–24). Consistent with literature results (21,24), a fast initial phase followed by a considerably slower second phase was observed in the hydrolysis catalyzed by $PLA_2$ and PLC (Fig. 4). In sharp contrast to the results from the TLC method (which measures fatty acid and LPC release as well as disappearance of DPPC), the results from PAC (which measures angular correlation) showed a 15- to 30-hr delay of degradation. Thus, liposomes do not necessarily lose their structural integrity even though the constituent phospholipid molecules have been chemically hydrolyzed. Since the addition of 1% Triton X-100 to $^{111}$In(III) DTPA-conjugated liposomes caused an instantaneous increase in the $G\langle 22\rangle$ value, the long lag time observed here cannot be attributed to the unlikely possibilities of low sensitivity of the PAC method or failure of the $G\langle 22\rangle$ reading to respond to loss in liposomal structural integrity. The PAC method can therefore reveal information about the true physical characteristics of the membrane, whereas the chemical method cannot.

At low $PLA_2$ concentrations (0.50 μg $PLA_2$/mg DPPC), fusion of the liposomes induced by the degradation product, LPC, possibly occurred. The increased size of the liposomes reduced the tumbling rate of the bound $^{111}$In(III), resulting in a lower $G\langle 22\rangle$ reading compared to that at a higher PLA concentration (Fig. 4). At a higher $PLA_2$ concentration, most of the DPPC molecules in the outer leaflet of the membrane were probably hydrolyzed, producing a high LPC concentration. Under this condition, LPC may behave as a detergent, dissolving the DPPC liposomes and causing a steady increase in the $G\langle 22\rangle$ value (Fig. 4). Based on the average standard deviation of 0.02 in the $G\langle 22\rangle$ measurement, only 8% of the vesicles need to be degraded to cause a detectable change in the $G\langle 22\rangle$ value.

In conclusion, the PAC technique using the lipophilic probe DPPE-DTPA can detect subtle structural changes in the liposomal membrane. Direct information about molecular motion in bilayer membranes renders this method suitable for studying the mechanism of phospholipid (or lipoprotein) exchange and liposomal fusion and screening for penetration enhancers under development for enhancement in mucosal drug penetration. In addition, the gentle, nondestructive nature of this method may be applicable to the direct monitoring of the extent and rate of change in liposomal integrity in living cells.

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