

Selective Precipitation of Antibody with Ligand-Modified Phospholipids: Effect of Lipid Chain Length

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The selective precipitation from aqueous solutions of goat polyclonal anti-biotin antibody (pABA) by complexation with ligand-modified phospholipids (LMPs) is described. In this study, the effect of varying the acyl chain length of the LMP from six to 18 carbon atoms on the rate and yield of precipitation is detailed. As the acyl chain length increases, the hydrophobic driving force for interaction of ligand-bound antibody molecules also increases, resulting in a larger yield of precipitated antibody. The rate of selective precipitation, however, is observed to pass through a sharp maximum at an acyl chain length of 10–12 carbon atoms. In the range of target antibody and LMP concentrations studied (1–10 μM), the maximum rates of precipitation are observed for those LMPs in sufficiently low concentrations in aqueous solution to be below their critical micelle concentration (CMC). The longer chain length LMPs (12–18 carbon atoms at concentrations of 5–10 μM) gave considerably slower rates of precipitation and were all observed to be micellar solutions. The yield of target antibody as a percentage of antibody precipitated was not observed to pass through a maximum, rather all LMPs with acyl chain lengths longer than 12 carbon atoms were observed to give the maximum yield. Thus the optimal structure of an LMP for precipitation of a target antibody corresponds to the maximum chain length (10 carbon atoms) at a concentration level (5–10 μM) which still falls below its CMC. The kinetics of precipitation, as monitored by measuring turbidity, are well modelled by a theory which combines the Mie theory of light scattering with the Smoluchowski theory for the kinetics of precipitation. The maximum rate constants corresponding to Smoluchowski kinetics for precipitating pABA were approximately 25 000–30 000 $\text{M}^{-1} \text{s}^{-1}$, while the maximum yields were 65–70%. The molecular picture which emerges is one in which the optimal rate is obtained by maximizing hydrophobic driving force for interaction of LMP acyl chains while still maintaining a submicellar state of aggregation.

Introduction

A new method has been described recently (Guzman et al., 1990; Powers et al., 1992, 1994) for the affinity precipitation of multivalent proteins employing a ligand-modified phospholipid, or LMP, solubilized in aqueous solutions of a nonionic surfactant [octakis(ethylene glycol) mono-*n*-dodecyl ether or C_{12}E_8]. This method can be used to selectively precipitate a target protein from a fairly complex mixture in one step and, as such, has implications for selective protein purification, immunoassay, biosensing, and other applications as discussed in our first several papers on the subject. The phospholipid-surfactant solution used to effect the precipitation can be added to a mixture containing the target protein, thus lowering the surfactant concentration below its critical micelle concentration (CMC). Once the target protein binds to the specific ligand moieties of the phospholipid, precipitation is effected by hydrophobic interactions between the nonpolar alkyl chains of the protein-bound LMP. The precipitation is observed to proceed on a time scale of a few minutes, resulting in yields of precipitated protein in excess of 30–40%, provided the detergent concentration during precipitation is below the CMC. A schematic depiction of the process on a molecular level is shown in Figure 1. The precipitation proceeds more slowly above the CMC of the surfactant; at detergent concentrations well in excess of the CMC ($3\text{--}4 \times \text{CMC}$), no precipitation is observed. Apparently, the hydrophobic solubilization of LMP acyl chains in detergent micelles

inhibits the inter-LMP hydrophobic interactions required for precipitation.

In our first reports (Guzman et al., 1990; Powers et al., 1992), this technique was illustrated using ditetradecanoylphosphatidylethanol-amidobiotin (DTDPE-B) as the LMP (also known as dimyristoylphosphatidylethanol-amidobiotin, DMPE-B) and avidin as the multivalent target protein. The binding constant between avidin and its small molecule ligand biotin is exceedingly strong, and the binding pocket on avidin is deep within the molecule (9 Å below the effective molecular surface). Subsequently, we described (Powers et al., 1994) the precipitation of both polyclonal goat anti-biotin antibody (pABA) and mouse monoclonal anti-biotin antibody (mABA). The purification of specific antibodies from mixtures of IgGs by this affinity precipitation technique represents a greater selectivity challenge than the purification of avidin because the specific antibody (pABA or mABA) is purified from virtually identical antibody molecules which differ only in the binding site region. In addition, antibodies have considerably lower association binding constants ($10^6\text{--}10^9 \text{M}^{-1}$) than does biotin for avidin (10^{15}M^{-1}). Antibodies are also considerably larger (155 versus 64 kDa for avidin) and have only two binding sites per molecule as compared to four binding sites with avidin. In this report, we describe the effects of varying the hydrophobic acyl chains of the LMP on the kinetics and yield of this selective precipitation. We also describe the effect of removing entirely the nonionic surfactant C_{12}E_8 from the precipitating solution. It is possible to solubilize the LMPs in aqueous buffer solution without nonionic detergent C_{12}E_8 . This is due to the resulting charge on

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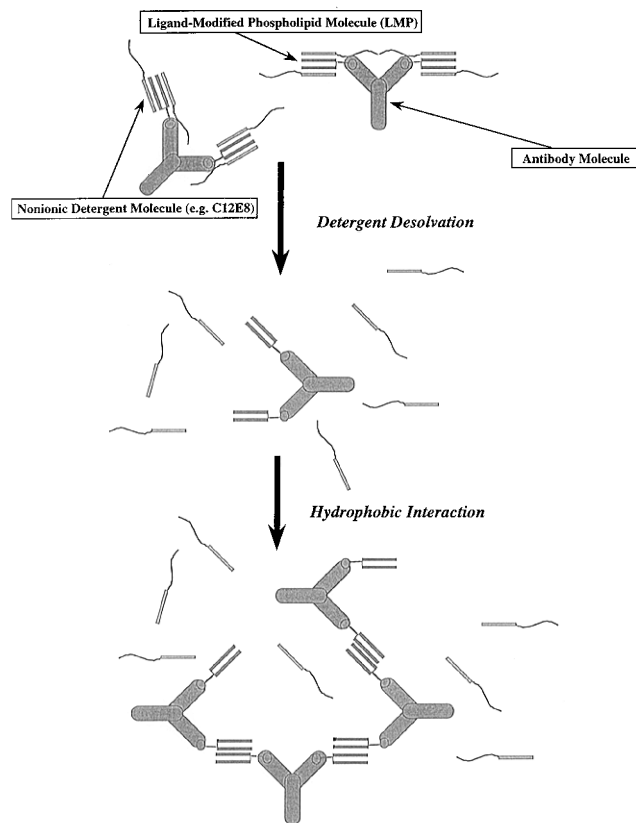


Figure 1. Molecular mechanism of precipitation with detergent showing desolvation and hydrophobic interaction steps.

the LMPs upon derivatization of the zwitterionic phosphatidylethanolamine which converts the protonatable primary amine to a neutral amide group and confers a net negative charge on the LMP. The range of acyl chains used is from C-6 to C-18, and this variation is observed to have a profound impact on kinetics and yield. A molecular interpretation of this effect of acyl chain on the kinetics and yield of selective precipitation is developed and tested by studying a particular LMP (didecanoylphosphatidylethanol–amidobiotin or DDPE) at LMP concentrations above and below the CMC of the LMP.

Materials and Methods

Materials. Dihexanoyl-*L*- α -phosphatidylethanolamine (DHPE), dioctanoyl-*L*- α -phosphatidylethanolamine (DOPE), didecanoyl-*L*- α -phosphatidylethanolamine (DDPE), didodecanoyl-*L*- α -phosphatidylethanolamine (DDDPE), ditetradecanoyl-*L*- α -phosphatidylethanolamine (DTDPE), dihexadecanoyl-*L*- α -phosphatidylethanolamine (DHDPE), and dioctadecanoyl-*L*- α -phosphatidylethanolamine (DODPE) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). 4'-Hydroxyazobenzyl-2-carboxylic acid (HABA), avidin, phosphate buffered saline (PBS), molybdenum blue reagent, and 4-(dimethylamino)cinnamaldehyde reagent were obtained from Sigma Chemical Co. (St. Louis, MO). Octakis(ethylene glycol) mono-*n*-dodecyl ether ($C_{12}E_8$) was purchased from Nikko Chemicals (Tokyo, Japan) and biotinyl-*N*-hydroxysuccinimide ester (biotin-NHS) from Pierce (Rockford, IL). Sodium hydroxide pellets (NaOH) and all solvents (HPLC grade) were obtained from Fisher Scientific Co. (Raleigh, NC). All chemicals were of the highest purity available and were used without further purification. Deionized water was obtained by passage through a Barnstead Nanopure System (Newton, MA). Whatman high-performance thin-layer chromatography (HPTLC) silica gel plates were

Table 1. Summary of Ligand-Modified Phospholipids Synthesized and Their Nomenclature

$$\begin{array}{c} \text{O} \\ \parallel \\ \text{CH}_3-(\text{CH}_2)_{n-2}-\text{C}-\text{O}-\text{CH}_2 \\ \text{O} \\ \parallel \\ \text{CH}_3-(\text{CH}_2)_{n-2}-\text{C}-\text{O}-\text{CH} \\ | \\ \text{H}_2\text{C}-\text{O}-\text{P}-\text{O}-(\text{CH}_2)_4-\text{NH}-\text{C}-\text{O}-(\text{CH}_2)_4-\text{S} \\ \parallel \\ \text{O} \end{array}$$

no. of C atoms in acyl chain	short name	IUPAC name
6	di-C6	DHPE-biotin (dihexanoyl)
8	di-C8	DOPE-biotin (dioctanoyl)
10	di-C10	DDPE-biotin (didecanoyl)
12	di-C12	DDDPE-biotin (didodecanoyl)
14	di-C14	DTDPE-biotin (ditetradecanoyl)
16	di-C16	DHDPE-biotin (dihexadecanoyl)
18	di-C18	DODPE-biotin (dioctadecanoyl)

obtained from American Scientific Products (Charlotte, NC). Buffer used for all solution preparations and dilutions was PBS with a 0.01 M phosphate buffer concentration (pH 7.4).

Protein Solutions. Goat polyclonal anti-biotin antibody (pABA) as obtained from Sigma was a lyophilized powder. The powder was reconstituted in PBS and filtered through a 0.2 μm Acrodisc syringe filter (Gelman, Ann Arbor, MI). The concentration of this antibody stock solution was determined by absorbance at 280 nm. All the ABA stock solutions were stored at 4 °C. pABA was shown to be essentially pure antibody by SDS-PAGE analysis.

Synthesis of Biotinylated Phospholipids. Biotin was covalently bound to each of the 1,2-diacyl-*sn*-glycerol-3-phosphatidylethanolamines studied by following the protocol described by Bayer et al. (1979) as modified by Powers et al. (1992, 1994). Biotin-NHS was mixed with the phospholipids to obtain biotinylated phospholipids having the general structure shown in Table 1. Seven different ligand-modified phospholipids were synthesized in this manner varying solely in the length of their respective acyl tail groups. Table 1 contains a listing of the biotinylated phospholipids, the number of carbons in their acyl chain groups, and their chemical abbreviations. The purity of the products was determined using thin-layer chromatography as described by Powers et al. (1994). The products yielded single spots that migrated differently than standards of NHS-biotin and unmodified phospholipid. Impurities from cleaved NHS were detected using ultraviolet light. Each product was used only after confirmation that the impurities described above were absent from the analyzed sample.

Solubilization of Phospholipids in Surfactant Solutions. Predetermined aliquots (0.1–2 mL) of biotinylated phospholipid stock solutions in chloroform were measured into 3 mL reaction vials. The chloroform was evaporated off using a nitrogen stream, and a volume of 10 mM $C_{12}E_8$ in PBS was added to obtain a 2.5 mM biotinylated lipid stock in surfactant solution. The amount of surfactant solution added was varied to yield mole ratios of $C_{12}E_8$ to LMP of 0, 4, or 7. The solution was vortexed vigorously and was stirred for several minutes. These mixtures were then heated in a warm water bath (60 °C) for 4 min or until they reached their cloud point. The solutions were then allowed to cool to room temperature at which time they were diluted with PBS to create 0.5 mM working solutions. This generated LMP- $C_{12}E_8$ solutions which were indefinitely stable. LMP solutions with no added $C_{12}E_8$ were prepared by heating the solutions to 70–80 °C, stirring for 30–60 min,

and then cooling. This was repeated until all of the ligand-modified phospholipid had solubilized.

Titration To Determine the Ligand-Modified Phospholipid Concentration. Before the test solutions were used, it was necessary to identify the biotinylated phospholipid concentration. This was done by using the titration method described in Powers et al. (1992, 1994) where standardized avidin solutions were titrated with hydrolyzed aliquots of sample solution. A standard titration was performed by adding aliquots of 0.5 mM biotin solution in buffer to a similar standardized avidin solution. The absorbance was monitored at 500 nm for each titration using a Shimadzu Model UV-160 spectrophotometer with a temperature-controlled sample cell maintained at 25 °C. The slope determined for the absorbance plot of the analyzed sample was divided by the slope obtained from the absorbance plot of the biotin standard. This calculation yields the concentration of ligand-modified phospholipid in the hydrolyzed solution. This method was used to determine the ligand-modified phospholipid concentration for all working and stock solutions employed for this experiment.

Purification and Concentration Determination of Anti-biotin Antibody (ABA). pABA, a polyclonal goat IgG, was initially dissolved in PBS buffer at concentrations near 2 mg/mL. The ABA solution was centrifuged at 6000 rpm for about 10 min and was filtered with a 0.2 mL Acrodisc filter into a clean vial. All anti-biotin antibody used for this study was purified in this manner. The concentration of ABA was measured using a Shimadzu Model UV-265 spectrophotometer. The antibody sample was diluted with PBS buffer, and the sample absorbance was zeroed against a reference cell of buffer at 340 nm. The absorbance of the sample cell was then measured at 320 and 280 nm. By subtracting the absorbance measurement observed at 320 nm, caused by light scattering from the measurement taken at 280 nm for protein absorbance, a relative number can be obtained that when multiplied by a predetermined factor will yield the antibody concentration in a sample. Such procedures were used for all protein concentration determinations.

Kinetic Measurements of pABA Aggregation. Aggregation of pABA with biotinylated phospholipid was followed by monitoring absorbance values at 340 nm in a model UV-265 spectrophotometer (Shimadzu, Kyoto, Japan) equipped with a temperature-controlled sample compartment maintained at 25 °C. For test aggregations a masked semimicrocuvette was used as a reaction vessel to contain a total volume of 1.0 mL. For larger scale aggregations, a regular cuvette was employed and the total reaction volume was 3.0 mL. Appropriate aliquots of pABA stock solutions (in 0.01 M PBS) were placed in the sample cuvette, and the spectrophotometer was zeroed electronically with only PBS in the reference cuvette. At time zero, biotinylated phospholipid solution was added to the sample cuvette and absorbance values were recorded until a decrease was observed. The decrease in absorbance indicated that the aggregate particles had begun to settle, giving a corresponding decrease in the turbidity of the solution. Stock solutions were prepared and aggregations carried out in PBS buffer.

Yield of Affinity Precipitation. Yields of pABA aggregations were obtained as a function of LMP chain length in the presence and absence of nonionic detergent C₁₂E₈. The C₁₂E₈ concentration was maintained at 72.8 μM for all of these experiments, which is just below the CMC of the surfactant. Experiments were also performed in which there was no added C₁₂E₈. Data were obtained by monitoring the aggregations at 340 nm until absorbance did not increase further. The mixture was

transferred from the cuvette to a 1.5 mL polypropylene centrifuge tube and centrifuged, supernatant was removed, and the pellet was washed once with 1.0 mL of PBS. Samples were centrifuged at room temperature for 3–5 min at 11000g in a Sorvall MC 12V microcentrifuge. After centrifugation, the wash supernatant was removed and the pellet dissolved in 1.0 mL of PBS containing 10 mM C₁₂E₈. To estimate the amount of pABA present at each stage, absorbance measurements (280 nm) were made on the clear supernatant, the wash supernatant, and the solution obtained by resolubilizing the pellet. The extinction coefficient of pABA was taken to be 1.4 (absorbance units mL)/(mg cm).

Surface Tension Measurements for Determination of Critical Micelle Concentrations. The surface tensions of aqueous solutions of LMPs were measured by the Du Nuoy ring method as described previously (Powers et al., 1992). Maximum forces at detachment were corrected for the volume of pendant fluid as described in Harkins and Jordan (1930). Accuracies were estimated at ±0.1–0.3 dyn/cm.

Results and Discussion

Effect of LMP Acyl Chain Length and Nonionic Detergent on pABA Precipitation. In the first set of experiments performed, the effects of varying LMP acyl chain length on rate and yield of precipitation were probed. In a typical precipitation experiment, the LMP of interest was dissolved in a C₁₂E₈ aqueous solution at detergent concentrations above the CMC (75 μM). The ratio of C₁₂E₈ detergent to LMP was approximately 10:1 in all cases. An aliquot of this solution was then combined with an aliquot of pABA solution to yield a mixture containing 0.15 mg/mL pABA (1 μM), 7 μM LMP, and 72.9 μM C₁₂E₈ (a concentration just below the CMC) in 0.01 M phosphate-buffered saline. The mixture was prepared in a spectrophotometric cuvette and the turbidity monitored at 340 nm immediately after mixing as described in the Materials and Methods. This experiment was performed several times with each LMP, and the results are shown in Figure 2. The apparent turbidity is followed in time with each of the seven LMPs (Table 1). A characteristic Smoluchowski rate constant β, as described in Powers et al. (1992, 1994), can be obtained from the time dependence of the turbidity. The rate constant β has units of volume/(mol time) and equals the particle–particle intrinsic rate constant divided by the sum of the two interacting particle diameters [see Powers et al. (1992) and Drake (1972)]. A natural dimensionless time *t** is given by

$$t^* = \beta N_0 t \quad (1)$$

where *N*₀ is a characteristic number concentration of the precipitating species. As indicated in Figure 1, the species which precipitate are antibody molecules with LMPs bound to them. We therefore set *N*₀ equal to the concentration of precipitable antibody, calculated by assuming that LMP in solution binds to antibody with a binding equilibrium constant equal to 1 μM⁻¹. Most of the experiments were performed at a pABA concentration of 0.95 μM (0.15 mg/mL) and an LMP concentration of 6.65 μM (mole ratio of LMP:pABA of 7). Under these conditions, *N*₀ is 0.82 μM. In those few experiments in which the LMP concentration was varied, such as the DDDPE-B experiments described in Figure 10, *N*₀ is calculated from the equilibrium expression:

$$N_0 = K_A [\text{pABA}] [\text{LMP}] \quad (2)$$

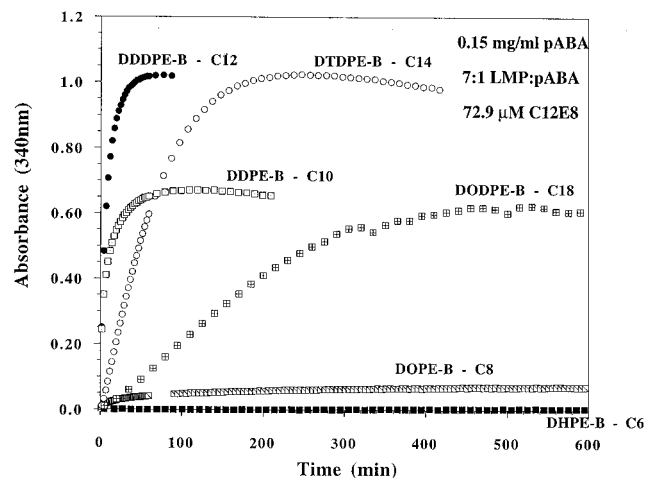


Figure 2. Effect of varying LMP acyl chain length on kinetics of affinity precipitation.

Experimentally, β is estimated by measuring the characteristic time, $t_{1/2}$, required for the LMP-pABA mixture to achieve one-half of the long-time turbidity and setting

$$\beta = \frac{t_{1/2}^*}{N_0 t_{1/2}} \quad (3)$$

In eq 3, $t_{1/2}^*$ is the theoretical dimensionless time required to obtain one-half the maximum theoretical turbidity and is equal to 3.1 (Powers et al., 1992, 1994). This assumes that the void fraction ϵ in the precipitating antibody-LMP aggregates is equal to 0.9. With a different void fraction, a different value of $t_{1/2}^*$ would result. This model assumes that binding between the ligand on the LMP and the binding site on the antibody occurs rapidly relative to the hydrophobic binding, an assumption which is well borne out by experiment. One would thus expect three things to potentially dictate the magnitude of the rate constant β : (1) the diffusivities of the aggregating species, (2) the hydrophobic driving force, or "sticking efficiencies", for interactions between phospholipid acyl chains, and/or (3) the rate of micelle or aggregate breakup. As is evident from the data in Figure 2, this rate constant increases with increasing LMP acyl chain length, achieves a maximum at an acyl chain length of 12 (DLPE-B), and then decreases with a further increase in chain length. This behavior was somewhat unexpected since precipitation ratios should be directly proportional to hydrophobic driving forces which increase with increasing acyl chain length.

It was also observed that reducing the $C_{12}E_8$ detergent concentration increased the rate of precipitation. As mentioned in the Introduction, it was observed that the detergent concentration could be eliminated altogether and LMP alone could be solubilized in PBS and used to precipitate pABA. A typical turbidity profile of pABA precipitation in the presence and absence of $C_{12}E_8$ is shown in Figure 3 in which DDPE-B (acyl chain length of 10) is contacted with pABA in a mole ratio of 7:1 in the presence and absence of $72.9 \mu\text{M } C_{12}E_8$. Removing the nonionic detergent not only speeds the rate of precipitation (increasing the Smoluchowski rate constant β from 22 to $27 \text{ mM}^{-1} \text{ s}^{-1}$) but also increases the yield of precipitation. The yield was quantified, as described in the Materials and Methods, by centrifuging the precipitated pABA and measuring the UV absorbance at 280 nm of the residual supernatant. In this particular case, the yield increased from about 30% to 43% (see Figure 7 for a comparison of the other LMPs). It is not surprising that removing the detergent speeds the rate of precipita-

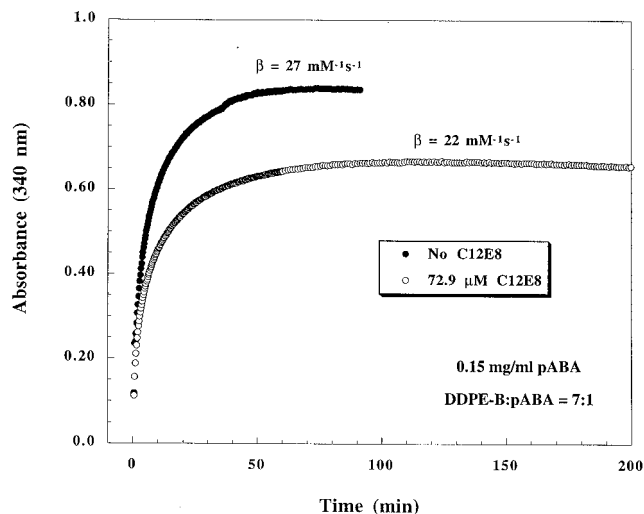


Figure 3. Effect of removing $C_{12}E_8$ on rate and yield of precipitation: DDPE-B (di-C10).

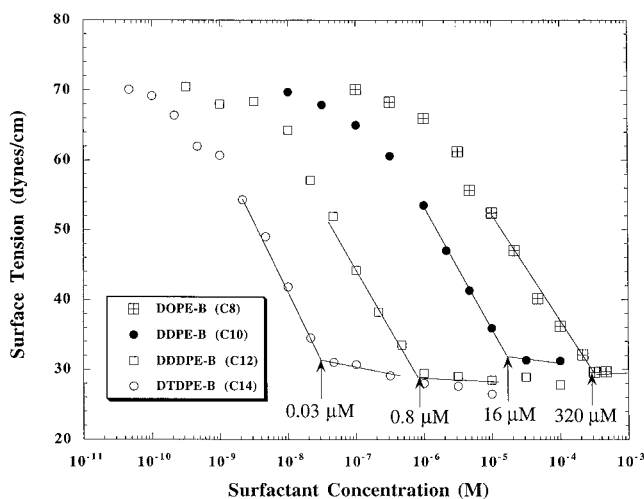


Figure 4. Surface tension of aqueous solutions of LMPs of increasing acyl carbon chain length as functions of increasing LMP concentration. The value of apparent CMCs of each LMP are indicated on the plot.

tion as the LMP tail groups must be desolvated from the detergent before pABA-LMP precipitation can occur (Figure 1). The modest effect observed with DDPE-B ($\approx 25\%$ increase in β with removal of $C_{12}E_8$) indicates that this detergent solvation of LMP tail groups is not particularly strong.

Measurement of CMCs of LMPs and Comparison with Phosphatidylcholine CMCs. The aggregation state of the LMPs in the absence of nonionic detergent $C_{12}E_8$ was determined by measuring the CMC of each modified phospholipid in aqueous 0.01 M phosphate buffer. This was accomplished by measuring the surface tension by DuNuoy ring tensiometry as a function of increasing LMP concentration. The results are shown for four of the LMPs in Figure 4. As expected, increasing the acyl chain length of the LMP leads to a decrease in the CMC. The incremental effect of increasing the acyl chain length can be determined by plotting the logarithm of the CMC of the LMPs versus acyl chain length (Figure 5). Also shown in Figure 5 is the dependence of the CMCs of dialkanoylphosphatidylcholines (PCs) with acyl chain length. It is clear from a comparison of the CMCs of these two types of double-tailed phospholipids that the CMCs of the LMPs are about 60% higher than those of the PCs. This is likely due to the charge conferred on the LMPs by the functionalization of the PE group as

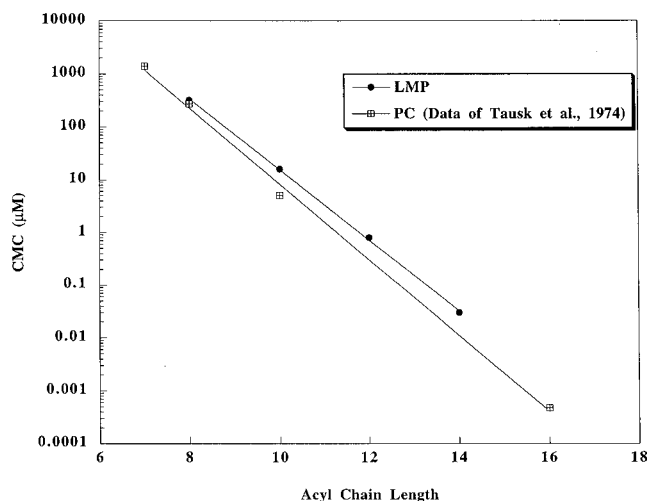


Figure 5. Dependence of CMC of LMPs and PCs on acyl chain length.

compared to the zwitterionic PCs. It is also clear that the incremental changes in CMC per CH_2 group given by the slope of the CMC lines in Figure 5 are approximately the same for both types of lipids. The slope of this plot multiplied by the universal gas constant R and the absolute temperature T yields the chemical potential increment per added carbon unit to the acyl chain length of the LMPs for transferring an acyl chain from water into the interior of the micelle.

$$RT \left(\frac{\partial \ln[\text{CMC}]}{\partial n_c} \right)_T = \mu_{\text{mic}}^\circ - \mu_w^\circ \quad (4)$$

This increment is approximately 910 cal/mol for the LMPs and 960 cal/mol for the PCs (Tanford, 1980; Chapter 7). This is to be compared with approximately 700–750 cal/mol obtained for a wide variety of single-alkyl-chain surfactants (Tanford 1980). Clearly, one would expect a larger transfer chemical potential for symmetric double-chain phospholipids as compared to single-chain surfactants. The fact that the transfer energy is not twice that for single-chain simply suggests that either (1) the acyl chains of the LMPs in monomeric form are not completely solvated by water (there is some lateral interaction of tails on the same molecule) and/or (2) the micelles formed from LMPs allow more penetration of water into the core of the micelles than micelles formed from single-chain surfactants. The difference in the transfer chemical potential for LMPs versus PCs is likely due to the charge and bulkiness of the head groups of the LMPs relative to the PCs. This probably leads to less effective packing of tails in the micelle with LMPs and a lower transfer chemical potential.

Removal of Nonionic Detergent: Effect on Kinetics and Yield. The precipitation rate data in Figure 2 were all fit to the Mie–Smoluchowski theory described in Powers et al. (1992, 1994) to obtain characteristic rate constants β as a function of LMP chain length. These rate constants, obtained with and without added C_{12}E_8 , are shown in Figure 6. All experiments were performed at a pABA concentration of $1 \mu\text{M}$ and an LMP concentration of $7 \mu\text{M}$. With acyl chain lengths less than and equal to 10 carbon atoms, the LMP concentration of $7 \mu\text{M}$ falls below the CMC. Thus, in these experiments, the LMP is present in solution as either monomers or perhaps dimers or small submicellar oligomers. With acyl chain lengths greater than or equal to 12, the LMP concentration of $7 \mu\text{M}$ falls above the CMC and the majority of the LMP in solution is organized into micelles. A clear demarcation exists between the rate constant dependence

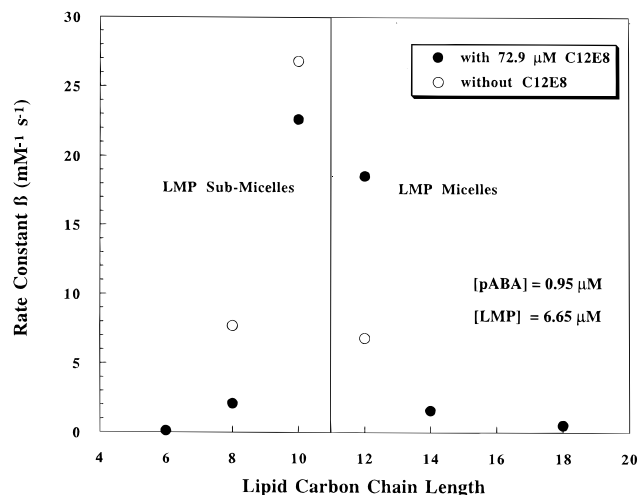


Figure 6. Smoluchowski rate constant β as a function of lipid chain length in presence and absence of nonionic detergent C_{12}E_8 .

on acyl chain length when the state of aggregation is submicellar (monomers or small oligomers) as compared to when there are micelles present. In those examples in which the LMP is in the form of monomers and small oligomers, the rate constant increases dramatically with increasing acyl chain length. This is expected due to the increasing hydrophobic interactions between acyl chains with increasing chain length. Presumably, the diffusing species in solution need only collide with each other and an interaction between tail groups can lead to a growing aggregate. Apparently, hydrophobic interactions control the rate by controlling the effectiveness (i.e., the sticking efficiency) of tail group interactions. With longer acyl chain lengths in which the LMP is organized into micelles at $7 \mu\text{M}$, increasing the chain length decreases the rate of precipitation. One might suspect that this is due to the rate at which micelles can diffuse in solution relative to the rate at which monomers or oligomers can diffuse. Alternatively, this decrease in rate constant with increasing acyl chain length may be attributed to the ease (or lack thereof) with which the micelles dissociate into monomers or small oligomers for binding to the pABA followed by cross-linking and precipitation. It is clear that micelles must dissociate into small oligomers because the relative inventory of LMP to antibody is not sufficiently high (7:1) to allow for micelles to coprecipitate with antibody. A very interesting trend is observed in the presence and absence of nonionic detergent C_{12}E_8 . Adding C_{12}E_8 to LMP solutions below their CMC decreases the rate of precipitation. Again, this is consistent with both a reduced diffusion rate of LMP oligomers when solvated by detergent as well as a reduced sticking efficiency. However, when C_{12}E_8 is added to LMP solutions above their CMC, the rate of precipitation jumps considerably. This is likely due to the breakup of micelles by detergent into smaller aggregates which diffuse much faster, or this may also be due to the enhancement of the rate of micelle breakup by the solvating effect of the nonionic detergent. Thus, to maximize the rate of precipitation, it is desirable to use the longest chain length LMP whose CMC still falls above the concentration used in the affinity precipitation.

In addition to rates of precipitation, the yields of precipitated pABA were also measured as described in the Materials and Methods. These data, with and without C_{12}E_8 detergent, are shown in Figure 7. In contrast to the rate constants, the yield of precipitated antibody is observed to increase monotonically with increasing acyl chain length. It achieves a plateau value

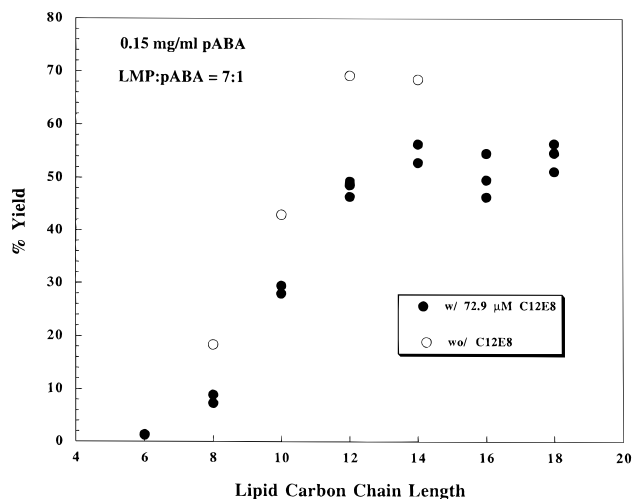


Figure 7. Yield of precipitation as a function of lipid chain length in the presence and absence of nonionic detergent C₁₂E₈.

of approximately 60% with acyl chains of 14 carbon atoms and longer when the precipitation is performed in the presence of 72.9 μM C₁₂E₈. With experiments performed in the absence of nonionic detergent, the yield is observed to rise to 70% at acyl chain lengths of 12 and 14 carbon atoms. Thus, removing the detergent enhances the hydrophobic interactions between LMP chains and increases the yield.

Molecular Interpretation of Rate Data. The dependence of the yield of affinity precipitation on LMP acyl chain length suggests that the magnitude of the hydrophobic interactions between tail groups dictates the yield of antibody precipitated up to a plateau value of about 55–60% corresponding to di-C14 LMP in the presence of C₁₂E₈. Removing the C₁₂E₈ increases the yield to about 70%, indicating again that yield is controlled by strength of hydrophobic interactions. Apparently, the aggregation and precipitation occurs over a sufficiently long time scale that local thermodynamic equilibrium can be achieved in the interactions among ligands, antibody binding sites, and hydrophobic tail groups. The rate of aggregation and precipitation, however, is dominated by either (1) diffusion of aggregating species, (2) the “sticking efficiencies” of LMP-bound antibodies, and/or (3) the rate of micelle or aggregate breakup. It is presumed that the effectiveness of a collision between two LMP-bound antibody oligomers in leading to a growing aggregate is primarily determined by the ability of the hydrophobic acyl chains on differing pABA oligomers to contact each other and interact sufficiently strongly to lead to a long-lived molecular cluster. Three molecular factors, therefore, should potentially dictate the rate of aggregation: (1) the diffusivity of LMP and LMP bound to pABA to other antibodies, (2) the intrinsic hydrophobic interaction energy between LMP acyl chains (determined solely by acyl chain length), and (3) availability of LMP acyl chains to interact with those acyl chains on a different LMP bound to an adjacent antibody molecule. This availability is determined primarily by the state of aggregation of the LMP (submicellar or micellar) and the presence or absence of solvating detergent. The diffusivity of LMP monomers and small oligomers (dimers, trimers) relative to micelles suggests that the rate of precipitation of pABA in the presence of submicelles should be more rapid (higher β) than with micelles, all other contributions being the same. This is borne out in Figure 6 by comparing the rate constant for di-C10 with no added C₁₂E₈ to that for di-C12 with no added C₁₂E₈, the latter being micellar. This distinction is depicted in a schematic fashion in Figures 8 and 9, in which the precipitation of

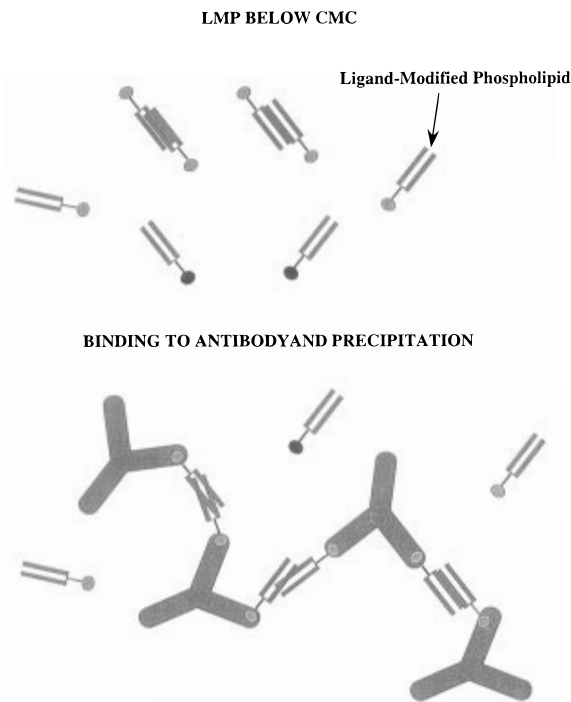


Figure 8. Molecular schematic depiction of precipitation with LMP monomers or small oligomers.

pABA by either submicellar oligomers (Figure 8) or micellar aggregates (Figure 9) is shown. The intrinsic hydrophobic interaction energy between acyl chains suggests that the sticking efficiency should increase with increasing acyl chain. This is confirmed by the increasing rate constant with increasing acyl chain for submicellar LMP in Figure 6. The effects of detergent on the availability of LMP for forming cross-linking interactions are seen in comparing the rate constant data of Figure 6 with and without added C₁₂E₈. With submicellar di-C10 LMP, the addition of C₁₂E₈ decreases the rate constant. This is due to the disruption of hydrophobic interactions between LMP acyl chains by the presence of C₁₂E₈ detergent. With micellar di-C12 and di-C14 LMP, the addition of C₁₂E₈ actually *increases* the rate constant! This is due to the “dissolution” of pure LMP micelles by C₁₂E₈ leading to submicellar mixed aggregates of LMP and detergent which can diffuse faster to the appropriate binding and interaction sites. To further explore and clarify the role of LMP aggregation state on rate constant, experiments were performed with a single LMP (di-C12 or DDDPE-B) in which the concentrations of the LMP and pABA were varied (keeping the ratio fixed at 7:1) above and below the CMC of the LMP (0.8 μM). These results are described in the next section.

Variation of DDDPE-B Concentration above and below the CMC: Effect on Rate Constant. The dependence of the Smoluchowski rate constant β on DDDPE-B concentration (in the absence of any added C₁₂E₈ detergent) is shown in Figure 10. The ratio of LMP to pABA was maintained constant at 7:1, and the LMP concentration was varied from 0.5 to 30 μM , the CMC being 0.8 μM (indicated on the figure). At LMP concentrations exceeding 7 μM , the rate constant β was approximately 6 $\text{mM}^{-1} \text{s}^{-1}$. At these concentrations, more than 90% of the LMP is in micellar form and the diffusivity and monomer lifetime in micelles dominates the rate of aggregation. As LMP concentration is lowered to just above the CMC, the rate constant increases to about 12 $\text{mM}^{-1} \text{s}^{-1}$, while below the CMC, the rate constant jumps to 27 $\text{mM}^{-1} \text{s}^{-1}$. Again, these observations confirm the molecular interpretation of our rate

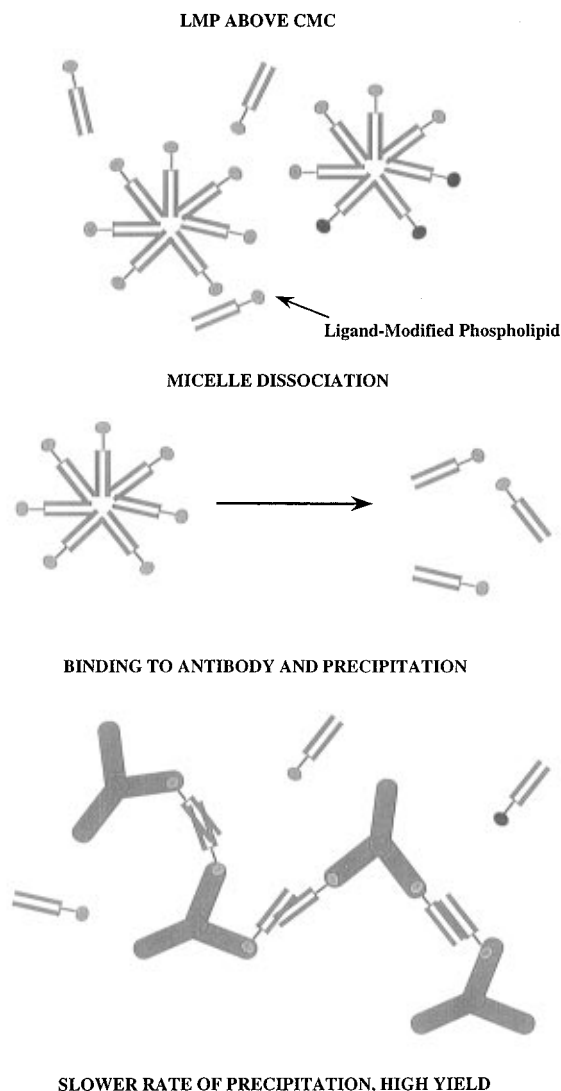


Figure 9. Molecular schematic depiction of precipitation of antibody with LMP micellar aggregates.

data that diffusivity, hydrophobic interactions, and state of aggregation dictate the rate and effectiveness of collisions leading to binding and precipitation of LMP-pABA aggregates.

Conclusions

The affinity-binding-mediated precipitation of polyclonal anti-biotin antibody (pABA) by LMPs of varying acyl chain length has been studied. The LMPs varied in acyl chain length from C6 to C18, corresponding to a state of aggregation at $7 \mu\text{M}$ from submicellar to micellar. Both the rate of affinity precipitation, as gauged by the apparent Smoluchowski rate constant β , and the yield of precipitated antibody were quantified. The yield increased monotonically with increasing acyl chain length and achieved a maximum plateau value of about 70% with LMP acyl chains longer than 12 carbon atoms. The rate constant was observed to pass through a maximum with increasing acyl chain length, achieving a maximum value of approximately $25\text{--}30 \text{ mM}^{-1} \text{ s}^{-1}$ with d-C10 and di-C12 LMPs below their CMC. This was interpreted by assuming that the rate is controlled by the frequency of collisions among LMP-bound pABA (dictated by diffusivities and states of aggregation) and by the effectiveness of collisions in resulting in hydrophobic interactions among LMP acyl chains on adjacent pABA oligomers.

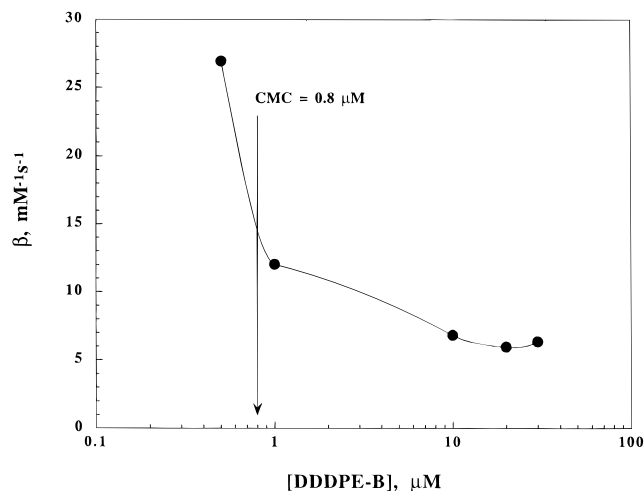


Figure 10. Effect of DDDPE-B concentration on Smoluchowski rate constant β .

This effectiveness is in turn dictated by intrinsic hydrophobic interactions among tail groups and by the state of aggregation and availability of acyl chains to interact hydrophobically. The molecular model was capable of rationalizing all of the observed trends in the rate constant data.

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