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Triggerable liposomal fusion by enzyme cleavage of a novel peptide–lipid conjugate

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Abstract

A novel peptide–lipid sensitive to enzyme cleavage was designed to generate liposomes that could be triggered to fuse by enzymatic activation. Covalent linkage of dioleoyl phosphatidylethanolamine (DOPE) to an elastase substrate, *N*-acetyl-ala–ala-, resulted in a cleavable peptide–lipid (*N*-Ac-AA-DOPE) with no intrinsic fusogenic activity. Cleavage of *N*-Ac-AA-DOPE and concomitant conversion to the fusogenic lipid DOPE could be detected after treatment with human leukocyte elastase or proteinase K, two proteases with similar substrate specificities. A strategy to utilize this cleavage to trigger fusogenicity was tested by modeling the fusion of liposomes containing the expected product of complete cleavage. Based on these results liposomes were designed to contain *N*-Ac-AA-DOPE, DOTAP, and PE in the ratio of 15/15/70. These liposomes exhibited lipid mixing with acceptor liposomes after elastase or proteinase K protease treatment. Activation of fusion, as monitored by a lipid mixing assay, appeared to be dependent on protease activity, as (1) heat inactivated enzyme did not activate liposomal fusion, and (2) the time and concentration dependence of proteinase K mediated cleavage of *N*-Ac-AA-DOPE correlated with membrane mixing. Liposomes could also be formulated that exhibited lipid mixing and transfer of aqueous fluorescent probe with erythrocyte ghosts. These observations demonstrate fusogenic lipids conjugated to enzyme substrates serve as triggerable fusion systems that may be useful for gene and drug delivery.

Keywords: Liposome; Fusion; Elastase; Proteinase K; Enzyme; Triggering

1. Introduction

Specific delivery of encapsulated drugs or genes to cells by liposomes is dependent upon fusion between liposomal and cell membranes. Promotion of liposomal fusion is achieved by inclusion of lipids capable of undergoing the lamellar (L_α) to inverted hexagonal (H_{II}) phase transition, which is associated with enhanced fusogenicity [1,2]. Unfortunately, liposomes containing these lipids exhibit unregulated fusion. In the context of in vivo delivery, it is desirable

Abbreviations: DOPE (1,2-Dioleoyl-*sn*-glycero-3-phosphatidylethanolamine); *N*-Ac-AA-DOPE (*N*-acetyl alanine alanine 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine); PS (phosphatidylserine); *N*-NBD-PS (*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)PS); *N*-Rho-PE (*N*-lissamine rhodamine B sulfonyl)PE); DOTAP (dioleoyl trimethylammonium propane); LUV (large unilamellar vesicle); SUV (small unilamellar vesicle); C12E8 (octaethylene glycol monododecyl ether); TES (*N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid); EDTA (ethylenediamine tetraacetic acid); H_{II} (inverted hexagonal); RET (resonance energy transfer); RFU (relative fluorescence units)

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to formulate liposomes that are not fusogenic until activated by a specific trigger. In this way the liposomes and their contents retain stability and reduce non-specific fusion until present at the site of intended intervention. Liposomes have been previously developed that rely upon a variety of triggers to activate their fusogenic or membrane destabilization potential. These triggering signals include pH sensitive lipids [3–7], photoactivation [7,8], phospholipase C activity [9,10], chemical cleavage [11], and trypsin mediated cleavage [12].

Another approach to creating a triggerable fusogenic liposome is to rely upon a physiologically relevant signal that is associated with a pathological state. Specifically, the modification of DOPE by covalent conjugation of the headgroup to an enzyme substrate may mask its fusogenic potential until cleaved by the specific enzyme. DOPE is of interest because its tendency to form non-bilayer structures is associated with its ability to promote fusion [1]. Pure DOPE does not form stable membrane bilayers at physiological pH, instead organizing into H_{II} structures [13]. However, certain modifications of the PE headgroup generate phospholipids that are capable of forming stable lamellar membranes. For example, both mono-methylated PE [14,15] and negatively charged phosphatidylserine (PS) can be formulated into stable liposomes. This indicates the addition of a bulkier headgroup and/or charged group to PE inhibits the non-bilayer forming aspect of this phospholipid. Therefore, addition of an enzyme susceptible peptide to the headgroup of DOPE, resulting in increased headgroup size and conversion to a negatively charged lipid, may create a novel phospholipid capable of forming stable liposomal membranes.

Possible candidates for an enzyme substrate to attach to DOPE are numerous. Elastase, an enzyme released from the azurophil granules of activated neutrophils, is of particular interest because of its ubiquitous involvement in inflammatory and tumorigenic conditions. In addition, the ability of elastase to recognize a simple peptide sequence [16] simplifies the coupling of the substrate to DOPE, as well as potentially limiting the immunogenicity of the peptide–lipid.

In this report we describe the conjugation of the *N*-Ac-ala–ala peptide to the headgroup of DOPE, generating a peptide–lipid (*N*-Ac-AA-DOPE) that acts as the triggering component in a fusogenic liposome. Cleavage of *N*-Ac-AA-DOPE is in fact associated with activation of liposomal fusion. The design of such a liposome has immediate implications for delivery to inflammatory sites, as well as providing a rationale for expanding the repertoire of peptide–lipids for potential delivery to other pathological sites involving elevated enzyme concentrations.

2. Materials and methods

2.1. Reagents

Human leukocyte elastase was purchased from Calbiochem (San Diego, CA). Phenylmethylsulfonyl fluoride (PMSF), and trifluoroacetic acid (TFA) were purchased from Sigma Chemical (St. Louis, MO). Proteinase K was purchased from Sigma and Boehringer Mannheim (Indianapolis, IN). Lipids were purchased from Avanti Polar Lipids (Alabaster, AL) and were of 99% or greater purity. TX-red dextrans were from Molecular Probes (Eugene, OR). Triethylamine (Et_3N) was obtained from Fluka Chemical (Ronkonkoma, NY). Dry tetrahydrofuran (THF), Celite and 1,3-Dicyclohexylcarbodiimide (DCC) were from Aldrich Chemicals (Milwaukee, WI). Ac-ala–ala-OH used for covalent linkage with DOPE was from Bachem Bioscience (King of Prussia, PA). Packed red blood cells were purchased from Analytical Biological Systems (Wilmington, DE). Hexane and isopropanol were obtained from Burdick and Jackson (Muskegon, MI). Unless otherwise specified, buffer used was 10 mM TES, 154 mM NaCl, 0.1 mM EDTA, pH 7.4. All experiments conducted with human leukocyte elastase were performed using the same buffer adjusted to pH 8.8.

2.2. Synthesis and characterization of N-acetyl alanine alanine 1,2-Dioleoyl-sn-glycero-3-phosphatidylethanolamine (N-Ac-AA-DOPE)

N-Acetyl AA-OH (25 mg, 0.12 mmol), dissolved in 5 ml of dry THF, was stirred for 24 h at room temperature with DCC (25 mg, 0.12 mmol), DOPE (50 mg, 0.06 mmol) and excess of Et_3N (100 ml, 0.7 mmol). The white precipitate of dicyclohexyl urea was filtered through a Celite bed and the filtrate was

concentrated. The product was purified by preparative TLC using $CHCl_3$:MeOH:H_2O (60:15:2) to yield 23 mg as a white flaky powder. The product was characterized by TLC and ^1H NMR (300 MHz, $CDCl_3$). Purity was 90% or greater. The resulting peptide–lipid (*N*-Ac-AA-DOPE) resolved as a single spot by TLC (Fig. 2) and generated a single peak by reverse-phase HPLC (unpublished data). A predominant single peak was also observed by ^{31}P-NMR analysis, although this technique detected a small amount ($<10\%$) of an apparent impurity that was present in all batches (unpublished data).

2.3. Liposome preparation

Large unilamellar vesicles (LUVs) were prepared by aliquoting desired amounts of lipid into 16×100 mm pyrex tubes from chloroform stocks and drying under a nitrogen stream. After exposure to high vacuum 4 h overnight, the lipid film was hydrated in TES/NaCl/EDTA buffer. After vortexing, samples were freeze–thawed $8\times$ and extruded under pressure $10\times$ (Lipex, Vancouver, BC, Canada) through 0.1 μm polycarbonate filters (Nucleopore, Pleasanton, CA). Liposomes were stored at 4°C until used. Dextran loaded liposomes were prepared by hydrating the lipid film in the presence of buffer containing 10 kDa TX-red dextran (50 mg/ml). Liposomes were then prepared as described above. Small unilamellar vesicles (SUVs) were prepared by drying lipid in the same manner as described above but preparations were water bath sonicated after vortexing for >10 min at room temperature. Lipid concentration was monitored by phosphate assay [17]. The size of liposomes was verified by quasi-elastic light scattering using a Nicomp Submicron Particle Sizer (Particle Sizing Systems, Santa Barbara, CA). LUVs and SUVs were 70–80 nm and 35–45 nm in diameter, respectively.

2.4. RBC ghost preparation

Ghosts were prepared as described previously [18]. Briefly, washed RBC were hypotonically lysed at 4°C by addition of 10 mM Tris, 2 mM $MgCl_2$, 0.1 mM EGTA, 0.1% BSA, pH 7.4. After 2 min, isotonicity was restored by addition of 1.22 M NaCl, 30 mM KCl, 0.15 M Na_2HPO_4, 50 mM KH_2PO_4, 1 mM $MgCl_2$, pH 7.4. Cells were incubated at 37°C for 40 min, then washed $3\times$ with the appropriate buffer for the planned experiments.

2.5. Detection of N-Ac-AA-DOPE cleavage

2.5.1. TLC detection of N-Ac-AA-DOPE cleavage

About 100–200 nmol of *N*-Ac-AA-DOPE SUVs were incubated with 1 mg enzyme in 0.1 ml overnight at 37°C. Lipid was extracted by organic phase separation [19] twice. Collected lipid was dried under N_2 stream and exposed to vacuum for 4 h overnight. Samples were resuspended in chloroform and spotted onto TLC plates. TLC was run using chloroform/methanol/water (65:25:4), air dried, sprayed with molybdenate blue, and charred on a hot plate.

2.5.2. HPLC detection of N-Ac-AA-DOPE cleavage

Liposomes composed of DOTAP/*N*-Ac-AA-DOPE (1:1) or DOTAP/*N*-Ac-AA-DOPE/PE (15/15/70 mol%) were incubated with enzyme under given conditions. Lipid was extracted by the Bligh Dyer procedure [19] twice. Collected lipid was dried under N_2 stream and exposed to vacuum for 4 h overnight. Samples were resuspended in 100% ethanol and injected in 30 μl aliquots into Spherisorb silica columns (150×4.6 mm, 0.3 μm, Keystone Scientific). HPLC was performed using a hexane:isopropanol:water:TFA mobile phase. Hexane and TFA were held constant at 37% and 0.2%, respectively. The *N*-Ac-AA-DOPE peak was detected using a gradient of 59–55% isopropanol:4–8% water. Flow rate was 1.5 ml/min, column temperature was set at 45°C, and peaks were detected by a UV detector set at 205 nm. Lipid peaks were quantitated in comparison to standard curves generated by injecting 5–200 nmol of DOTAP or *N*-Ac-AA-DOPE and monitoring 205 nm signal. Percent cleavage was calculated by normalizing peaks to DOTAP (which is not cleaved by enzyme treatment, unpublished observation), then determining the decrease in *N*-Ac-AA-DOPE peak size relative to starting amounts.

2.5.3. ^{31}P-NMR analysis

N-Ac-AA-DOPE LUVs were prepared and treated with or without proteinase K (1.5 mg protein/100 nmol lipid) overnight at 37°C. Samples were mixed

with buffer (10% deoxycholate, 100 mM EDTA, 20 mM Hepes) and deuterium oxide (Cambridge Isotope Laboratories, Woburn, MA) (1:4:2) and transferred to 5 mm NMR tubes. Samples were monitored at room temperature in a Bruker AC300 spectrometer operating at 121.5 MHz, with 110 μs 90° radio frequency pulse for proton decoupling and set to 2 s interpulse delay to avoid signal saturation. Sweep width was set at 50 kHz. A 1 Hz line broadening was applied to all spectra.

2.6. Lipid mixing assay

Lipid mixing was monitored by the *N*-NBD-PE/*N*-Rho-PE resonance energy transfer assay, as described [20]. Liposomes were prepared with 1 mol% *N*-NBD-PE and 1 mol% *N*-Rho-PE, which results in quenching of the *N*-NBD-PE fluorescence signal. Membrane fusion results in probe diffusion and relief from self-quenching, which is monitored as an increase in *N*-NBD-PE fluorescence. Liposome–liposome lipid mixing was initiated by addition of 10 nmol of fluorescently labeled liposomes to 90 nmol unlabeled liposomes in microcentrifuge tubes containing 1 ml of TES/NaCl/EDTA buffer with 1.5 mM Ca^{++}/1.5 mM Mg^{++}. For fusion with cells 1×10^8 RBC ghosts were substituted for unlabeled liposomes. All samples were shaken in an Eppendorf Thermomixer (Brinkmann Instruments, Westbury, NY), 700 rpm/min, during the 37°C incubation for 30 min. *N*-NBD-PE fluorescence was monitored in a T-format PTI Alphascan spectrafluorometer (Princeton, NJ) with a xenon short arc lamp using 450 nm excitation/530 nm emission wavelengths and 5 nm slitwidths. Band pass of 450 nm and 500 nm cutoff filters were utilized for excitation and emission light paths, respectively, to reduce stray light. Maximal fluorescence dequenching was determined by addition of 0.1% C12E8 detergent.

2.7. Protease activation of fusion

Liposomes were incubated with protease at a 1 mg protease/100 nmol lipid/0.1 ml buffer ratio, unless otherwise stated. This concentration of proteinase K was found to have comparable activity, within an order of magnitude, with that of elastase in rheumatoid arthritis synovial fluid [[21]; unpublished data]. Mixtures were incubated at 37°C in microcentrifuge tubes with constant shaking in an Eppendorf Thermomixer, 700 rpm/min. Treated liposomes were then assayed for *N*-Ac-AA-DOPE cleavage by HPLC, as described above. For fusion experiments liposomes containing fluorescent membrane probes were treated with protease and then the concentrations of liposomes were determined by monitoring direct *N*-Rho-PE fluorescence (550ex/590em) and comparing with a known amount of stock liposomes. Aliquots of these fluorescently labeled protease treated liposomes were incubated with unlabeled target liposomes or cells and lipid mixing was determined as described above.

2.8. Fluorescence microscopy of dextran delivery from liposomes to RBC ghosts

DOTAP/*N*-Ac-AA-DOPE/PE (20/10/70 mol%) liposomes loaded with 10 kDa TX-red dextran were incubated overnight at 37°C with proteinase K. Some 10 nmol aliquots were incubated with 1×10^8 RBC ghosts in 1 ml buffer for 30 min at 37°C. Cells were washed with buffer 3× to remove unbound liposomes. The cell pellet was resuspended in 0.1 ml buffer and observed under an Olympus BH-2 fluorescence microscope (Olympus, Lake Success, NY) using an apochromat 40× oil (N.A. 1.00) objective. TX-red fluorescence was excited by a xenon lamp transmitted through a green excitation cube (580 nm dichroic mirror, 545 nm excitation filter). Non-fluorescent images were observed with transmitted light Nomarski differential interference contrast microscopy.

3. Results

3.1. N-Ac-AA-DOPE cleavage by elastase

We postulated that covalent conjugation of an enzyme substrate to the headgroup of DOPE would create a novel peptide–lipid with limited fusogenicity that could be reactivated to fuse upon removal of the substrate by enzymatic cleavage (Fig. 1). The elastase substrate, *N*-Ac-ala–ala, was covalently conjugated to DOPE via an amide linkage and subsequent purification was performed as described in Section 2. In

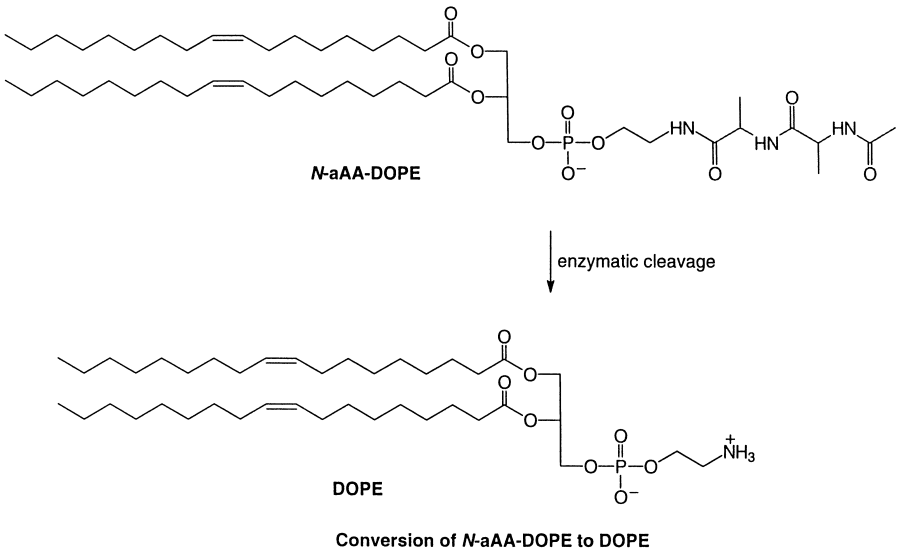


Fig. 1. Structure of *N*-Ac-AA-DOPE and postulated scheme of conversion to DOPE by enzymatic cleavage.

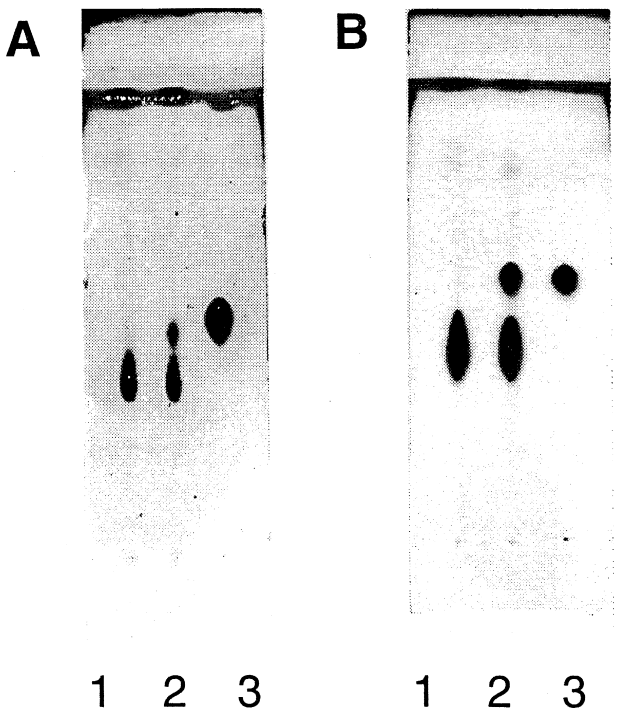


Fig. 2. TLC determination of protease mediated cleavage of *N*-Ac-AA-DOPE. *N*-Ac-AA-DOPE SUVs were incubated with (A) elastase or (B) proteinase K (1 mg enzyme/100 nmol lipid/0.1 ml buffer) overnight at 37°C. Lipid was collected and separated by TLC. Lipid spots were developed as described in Section 2. Lane 1, *N*-Ac-AA-DOPE without enzyme; lane 2, *N*-Ac-AA-DOPE with enzyme treatment; lane 3, DOPE from stock solution.

contrast to pure DOPE, stable liposomes composed solely of *N*-Ac-AA-DOPE could be prepared at room temperature, pH 7.4, indicating the addition of the ala–ala dipeptide to the headgroup of DOPE either elevated the H_{II} transition temperature or rendered the lipid incapable of converting to a non-bilayer structure. When cleavage of *N*-Ac-AA-DOPE was monitored by TLC, treatment of *N*-Ac-AA-DOPE liposomes with elastase generated a product corresponding to DOPE, whereas untreated *N*-Ac-AA-DOPE showed no change (Fig. 2A). Therefore, elastase recognized *N*-Ac-AA-DOPE and cleaved the dipeptide to yield DOPE.

3.2. Modeling of elastase mediated cleavage of N-Ac-AA-DOPE by proteinase K

Extensive experimentation with human leukocyte elastase was precluded because of cost considerations. Therefore, several proteases were tested to determine whether an enzyme with similar substrate specificity could be used as a model for elastase mediated cleavage of *N*-Ac-AA-DOPE. Proteinase K is a serine protease that, similar to elastase, can cleave at peptide bonds C-terminal to aliphatic residues. Upon incubation of *N*-Ac-AA-DOPE liposomes with proteinase K, the peptide–lipid was cleaved and DOPE was generated (Fig. 2B). The

conversion of *N*-Ac-AA-DOPE to DOPE was also monitored by ^{31}P-NMR analysis. *N*-Ac-AA-DOPE liposomes treated with proteinase K (1.5 mg protease/100 nmol lipid) resulted in the appearance of a peak 0.3 ppm upfield from *N*-Ac-AA-DOPE, corresponding to pure DOPE (unpublished data).

Elastase and proteinase K mediated cleavage of *N*-Ac-AA-DOPE was quantitated using liposomes composed of *N*-Ac-AA-DOPE and DOTAP, a positively charged lipid. DOTAP was included to provide a counterbalancing positive charge (see Section 3.3), and was used as a standard by which different samples could be normalized and compared. After treatment with elastase or proteinase K the reduction in the amount of *N*-Ac-AA-DOPE was monitored by HPLC. Both enzymes cleaved *N*-Ac-AA-DOPE to a similar extent (Fig. 3). To verify that the cleavage of *N*-Ac-AA-DOPE was due to proteinase K enzymatic activity, liposomes were treated with heat inactivated proteinase K. Proteinase K was inactivated by heating at 95°C for 1 h, after which the enzyme was incapable of cleaving the chromogenic substrate *N*-Ac-AAA-pNA (unpublished data). Treatment of

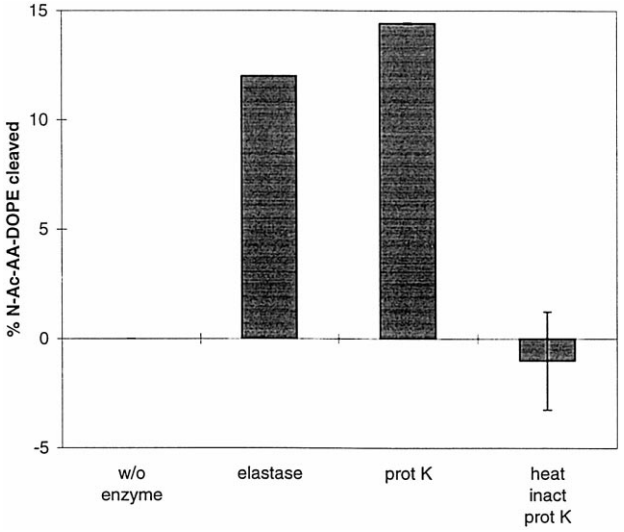


Fig. 3. Proteinase K mediated cleavage of *N*-Ac-AA-DOPE. DOTAP/*N*-Ac-AA-DOPE (1:1) SUVs were incubated with or without elastase, proteinase K, or heat inactivated proteinase K (95°C, 1 h) at a 1 mg protease/100 nmol/0.1 ml buffer lipid concentration overnight at 37°C. Lipid was collected and analyzed by HPLC. The *N*-Ac-AA-DOPE peak was quantitated and the amount of cleavage was calculated as a percentage of the starting lipid.

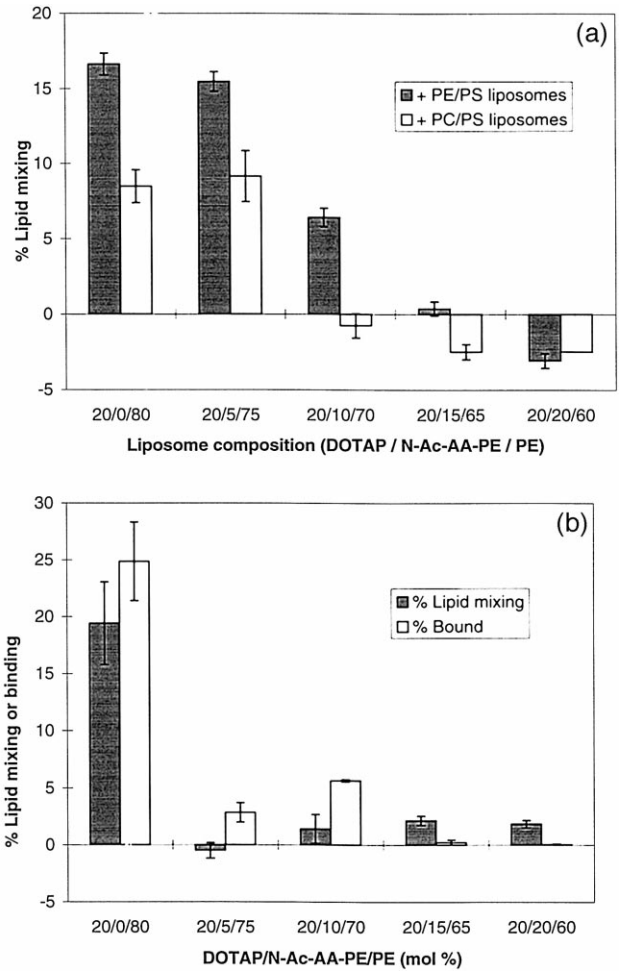


Fig. 4. Determination of optimal liposomal composition. Liposomes were prepared in given molar ratios of DOTAP, *N*-Ac-AA-DOPE, PE. A total of 1 mol% *N*-NBD-PE and *N*-Rho-PE fluorescent probes were included in all preparations. Liposomes were mixed with (A) unlabeled PE/PS or PC/PS (80/20 mol%; 1:10 effector:acceptor ratio; 60 μM total lipid) or (B) 2×10^8 RBC ghosts at 37°C for 1 h. Lipid mixing was calculated as the percentage of *N*-NBD-PE FDQ relative to maximal FDQ, as determined by detergent addition. Binding of liposomes to RBC ghosts was quantitated after washing cells with buffer by calculating the amount of *N*-Rho-PE fluorescence associated with the cell pellet relative to the total input fluorescence.

DOTAP/*N*-Ac-AA-DOPE liposomes with heat inactivated proteinase K did not result in any cleavage of *N*-Ac-AA-DOPE (Fig. 3), indicating the requirement for active proteinase K. Since proteinase K has been shown to share substrate specificity with elastase [22,23] and is considerably less costly than human

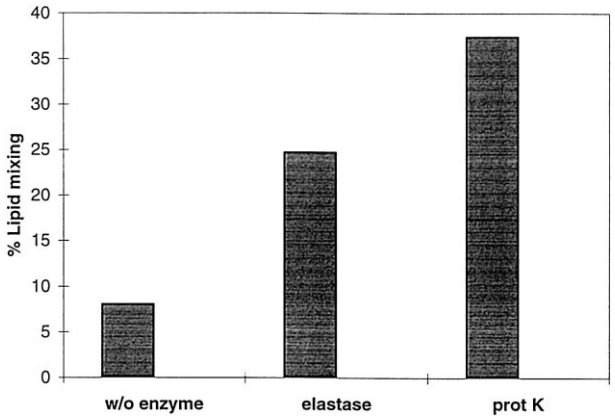


Fig. 5. Elastase and proteinase K mediated activation of liposomal lipid mixing. DOTAP/*N*-Ac-AA-DOPE/PE (15/15/70 mol%) liposomes containing fluorescent membrane probes were pretreated with human leukocyte elastase or proteinase K (1 mg protein/100 nmol lipid/0.1 ml buffer) overnight at 37°C. Some 10 nmol aliquots were incubated with unlabeled PE/PS acceptor liposomes (80/20 mol%; 1:10 effector:acceptor ratio) for 60 min at 37°C. Lipid mixing was determined by monitoring *N*-NBD-PE FDQ.

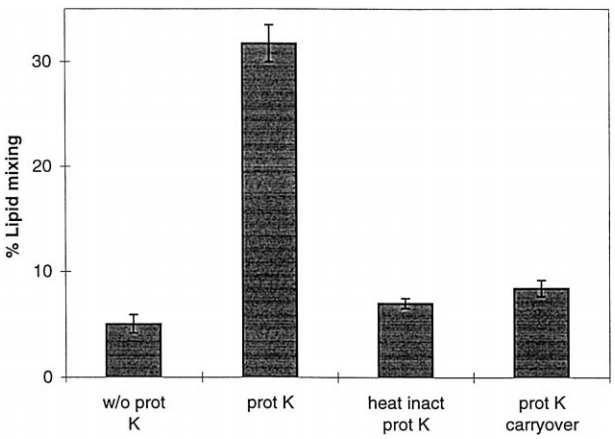


Fig. 6. Requirement for active proteinase K for DOTAP/*N*-Ac-AA-DOPE/PE liposome lipid mixing activation with PS/PE liposomes. DOTAP/*N*-Ac-AA-DOPE/PE (15/15/70 mol%) liposomes (100 nmol) containing fluorescent membrane probes were pretreated with or without 1 mg of proteinase K or heat inactivated proteinase K (1 h, 95°C) overnight at 37°C in 0.1 ml buffer. Aliquots of 10 nmol were incubated with unlabeled PE/PS acceptor liposomes (80/20 mol%; 1:10 effector:acceptor ratio), after which lipid mixing was determined. Prot K carryover = effect of residual proteinase K carried over to incubation mixture with PE/PS liposomes was monitored by incubating untreated DOTAP/*N*-Ac-AA-DOPE/PE (15/15/70 mol%) liposomes with PE/PS liposomes in presence of freshly added proteinase K equivalent to the expected transferred amount (see text).

leukocyte elastase, the majority of subsequent experiments were conducted with proteinase K.

3.3. Design of fusion-triggerable liposomes containing N-Ac-AA-DOPE

Liposomes containing *N*-Ac-AA-DOPE were designed with the following goals in mind: (1) formulate liposomes with the maximum amount of PE so as to maximize the fusogenic potential without actually

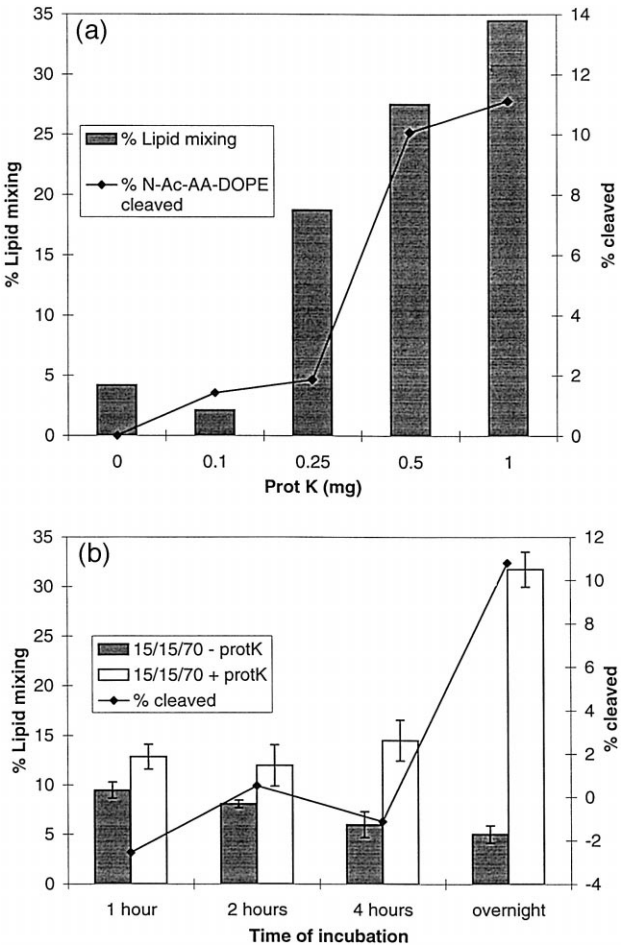


Fig. 7. Concentration and time dependence of proteinase K activity. Activation of lipid mixing: DOTAP/*N*-Ac-AA-DOPE/PE (15/15/70 mol%) liposomes (100 nmol) containing fluorescent membrane probes were incubated in 0.1 ml buffer at 37°C either (A) overnight with given amounts of proteinase K or (B) with 1 mg proteinase K for given times. Aliquots of 10 nmol were incubated with unlabeled PE/PS acceptor liposomes (80/20 mol%; 1:10 effector:acceptor ratio), after which lipid mixing was determined. *N*-Ac-AA-DOPE cleavage: unlabeled DOTAP/*N*-Ac-AA-DOPE (1:1 mol ratio) liposomes were treated identically as for fusion activation, after which lipid was extracted and analyzed by HPLC.

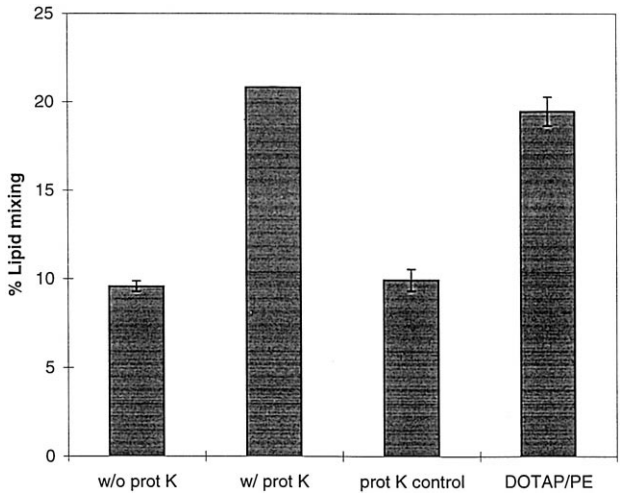


Fig. 8. Activation of DOTAP/*N*-Ac-AA-DOPE/PE liposomes by proteinase K for lipid mixing with RBC ghosts. DOTAP/*N*-Ac-AA-DOPE/PE (20/10/70 mol%) liposomes (100 nmol) containing fluorescent membrane probes were incubated overnight at 37°C with or without 1 mg of proteinase K in 0.1 ml buffer. Aliquots of 10 nmol of DOTAP/*N*-Ac-AA-DOPE/PE liposomes as well as DOTAP/PE (20/80 mol%) liposomes were incubated with 1×10^8 RBC ghosts in buffer containing 0.5 mM PMSF for 30 min at 37°C, after which lipid mixing was determined. Effect of transferred proteinase K on lipid mixing was monitored by incubating untreated liposomes with RBC ghosts in the presence of an equivalent amount of proteinase K (prot K control).

allowing fusion in the absence of the activating trigger, and (2) counterbalance the negatively charged *N*-Ac-AA-DOPE with a positively charged lipid so that upon conversion of the negatively charged *N*-Ac-AA-DOPE to zwitterionic DOPE, the liposome may acquire an overall positive charge. This latter characteristic is expected to enhance the binding of activated liposomes to cells, which tend to have an overall negative charge. The threshold of fusogenicity was determined by preparing liposomes with increasing amounts of PE transesterified from egg PC. This PE was preferred over DOPE because of its higher H_{II} transition temperature ($\sim$37°C vs. 10°C, respectively), which aids in the preparation of stable liposomes yet does not inhibit fusion. DOTAP was chosen as the positively charged lipid.

Lipid mixing assays were performed for DOTAP/*N*-Ac-AA-DOPE/PE liposomes containing the fluorescent membrane probes *N*-NBD-PE and *N*-Rho-PE and inversely varying amounts of *N*-Ac-AA-DOPE and PE. These liposomes were monitored for enhanced fusogenic potential by observing lipid mixing with either unlabeled target liposomes or for lipid mixing and binding with RBC ghosts. It became readily apparent that the threshold for fusogenicity depends upon the target in question. Liposomes composed of DOTAP and PE exhibited lipid mixing with both target liposomes and RBC ghosts, as has been shown previously [24]. Inclusion of 10 mol% *N*-Ac-AA-DOPE with a corresponding decrease in PE to 70 mol% generated liposomes that were still capable of lipid mixing with PE/PS liposomes but not PC/PS liposomes (Fig. 4A). The requirements for membrane mixing with RBC ghosts appeared to be more stringent, with inclusion of 5 mol% *N*-Ac-AA-DOPE inhibiting both the lipid mixing and the binding significantly (Fig. 4B). Defining the different threshold of fusogenicity for different targets creates a gradient of sensitivity for fusion that can be used to determine optimum conditions for activating *N*-Ac-AA-DOPE containing liposomes to fuse. As PE/PS liposomes appeared to be the most sensitive target,

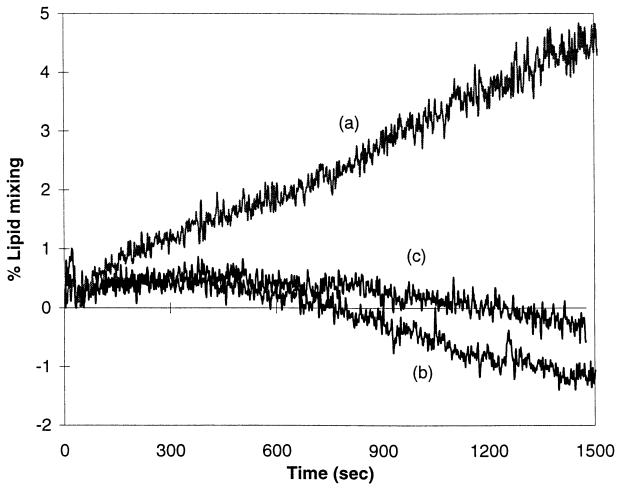
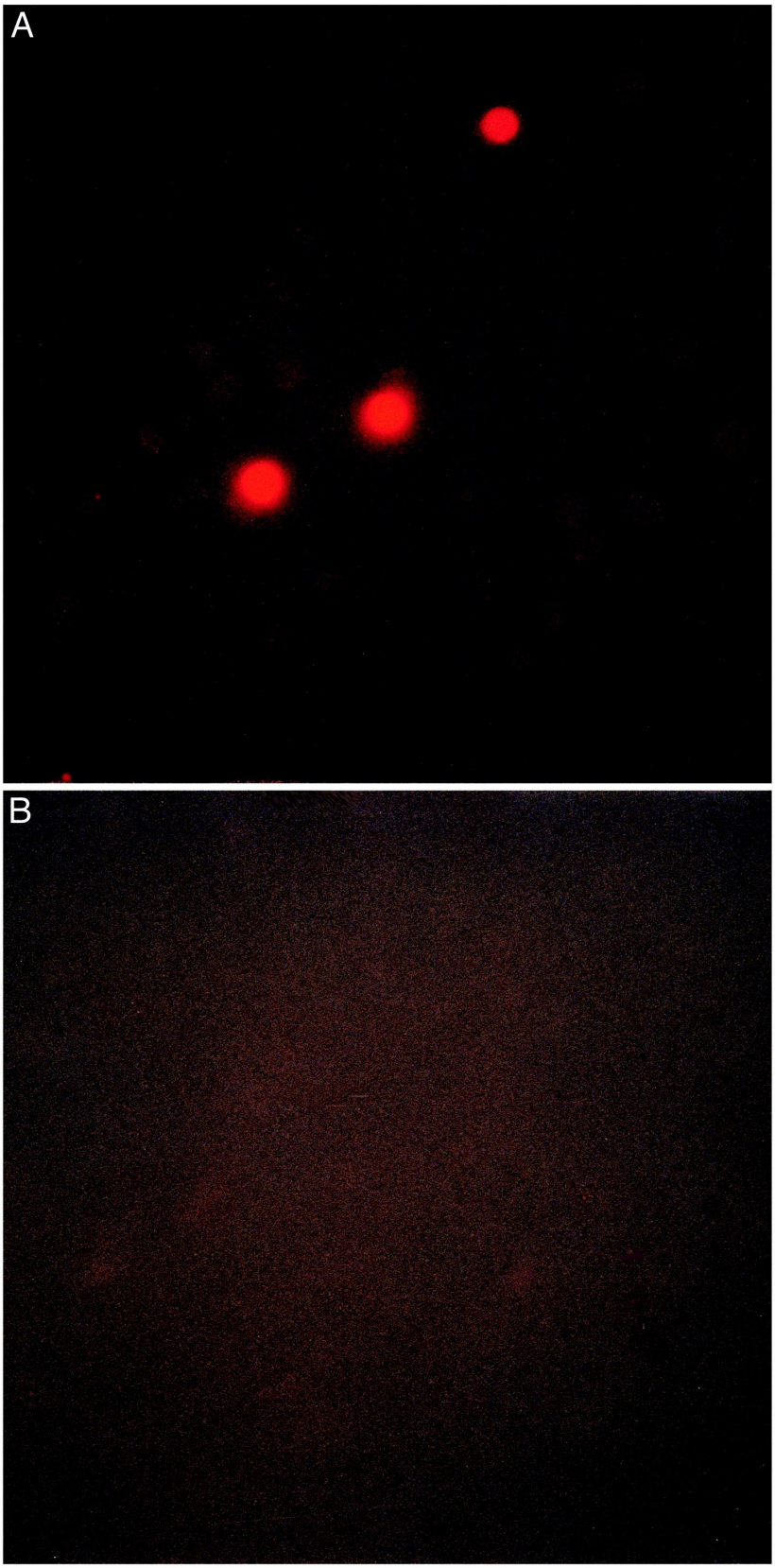


Fig. 9. DOTAP/*N*-Ac-AA-DOPE/PE liposome with RBC ghosts: continuous kinetics of lipid mixing. Aliquots of 10 nmol of DOTAP/*N*-Ac-AA-DOPE/PE (20/10/70 mol%) liposomes incubated (a) with or (b) without proteinase K overnight at 37°C were added to a cuvette containing 2 ml buffer with 0.5 mM PMSF under continuous stirring and 37°C conditions. *N*-NBD-PE fluorescence recording was initiated and 1×10^8 RBC ghosts were added at 30 s. (c) Effect of carryover proteinase K on lipid mixing was monitored by incubating untreated liposomes with RBC ghosts in presence of equivalent amount of proteinase K.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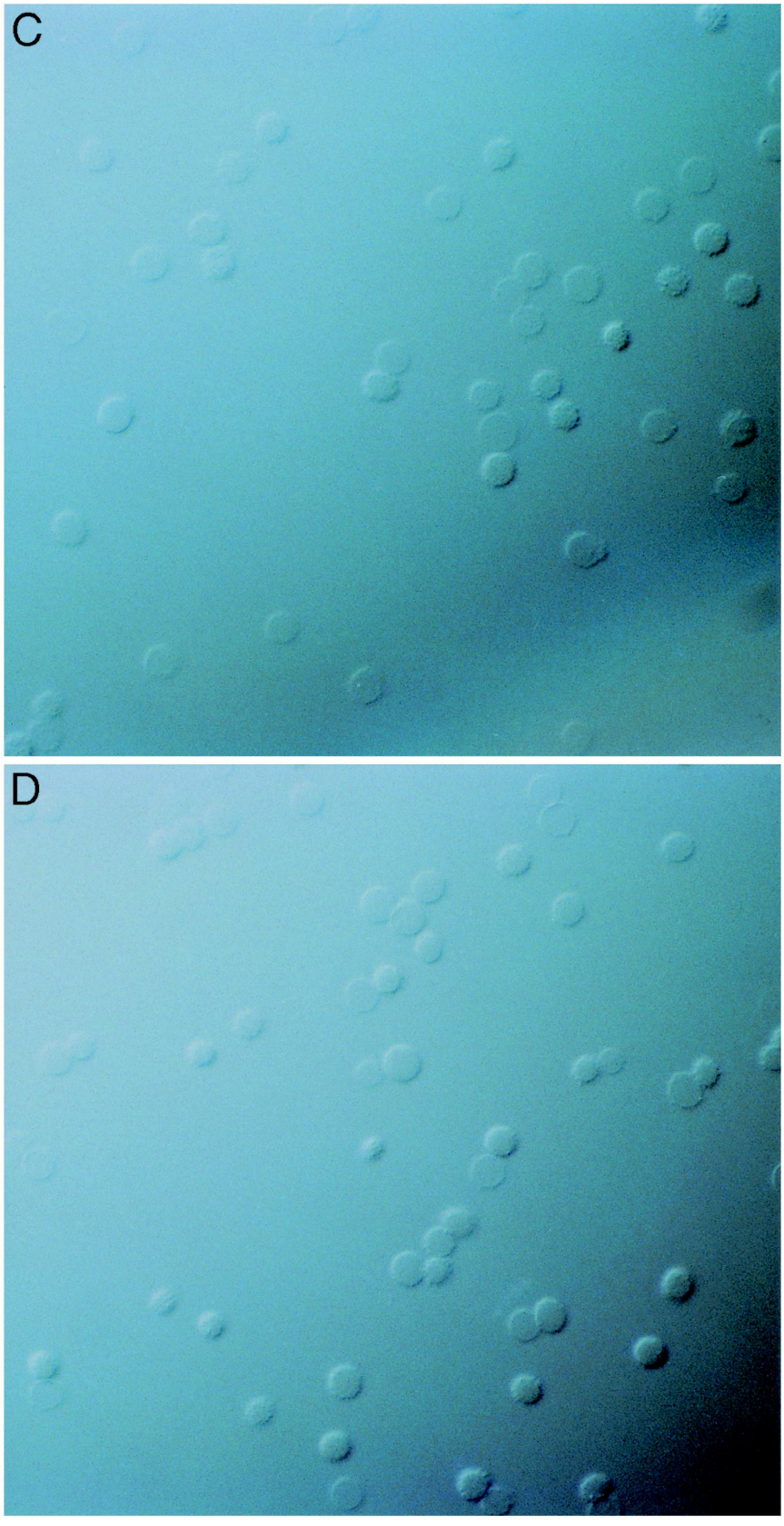
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D

we focused on a composition of DOTAP/*N*-Ac-AA-DOPE/PE liposomes that could be activated to fuse. The threshold of PE content appeared to be between 65–70 mol%. In order to create a liposome that is not initially highly positively charged, DOTAP and *N*-Ac-AA-DOPE were added in equivalent amounts to yield liposomes composed of DOTAP/*N*-Ac-AA-DOPE/PE in a 15/15/70 mol ratio.

3.4. Activation of liposome–liposome lipid mixing by enzyme cleavage

Since elastase and proteinase K were capable of cleaving *N*-Ac-AA-DOPE to DOPE (Figs. 2 and 3), both enzymes were tested for their ability to activate DOTAP/*N*-Ac-AA-DOPE/PE (15/15/70 mol%) liposomes to undergo lipid mixing. These liposomes were treated overnight at 37°C with elastase, or proteinase K, or without either enzyme, after which liposomes were incubated with PE/PS liposomes and lipid mixing monitored by relief of *N*-NBD-PE fluorescence quenching. Treatment by either enzyme resulted in a greater extent of lipid mixing over that of untreated liposomes (Fig. 5). This result, coupled with the shared substrate specificity of proteinase K and elastase, suggests proteinase K activation serves as a suitable substitute for elastase to characterize the fusion activation of *N*-Ac-AA-DOPE containing liposomes.

A causal relationship between cleavage of the *N*-Ac-AA-DOPE peptide–lipid and fusion activation of DOTAP/*N*-Ac-AA-DOPE/PE liposomes was studied using heat inactive proteinase K. DOTAP/*N*-Ac-AA-DOPE/PE (15/15/70 mol%) liposomes containing the fluorescent membrane probes *N*-NBD-PE and *N*-Rho-PE were incubated overnight at 37°C with active or heat inactivated proteinase K, after which an aliquot of the liposomes was incubated with unlabeled PS/PE acceptor liposomes to monitor the extent of lipid mixing. Treatment of DOTAP/*N*-Ac-AA-DOPE/PE liposomes with active proteinase K resulted in $\sim$30% fluorescence dequenching, a six-fold increase in lipid mixing over the untreated liposomes (Fig. 6). However, treatment with an identical amount of the heat inactivated enzyme did not activate liposomes to mix lipids (Fig. 6). Therefore, enzymatic activity is essential for the liposomes to become fusogenic, indicating *N*-Ac-AA-DOPE cleavage is crucial for triggering the fusogenic potential.

Because the experimental protocol involved transferring a small portion of active proteinase K (10 μg protease/100 nmol lipid; 100-fold dilution) along with the pretreated liposomes to the subsequent liposome–liposome incubation, it was possible that this amount of enzyme mediated the observed lipid mixing by non-specific protein effects. This possibility was tested by adding the expected carryover amount of active proteinase K to the untreated DOTAP/*N*-Ac-AA-DOPE/PE + PE/PS incubation mixture. Lipid mixing of this sample was the same as that of liposomes incubated without proteinase K (Fig. 6). Furthermore, continuous fusion kinetics showed an immediate increase in fluorescence dequenching upon mixing of proteinase K treated DOTAP/*N*-Ac-AA-DOPE/PE liposomes with PS/PE liposomes (unpublished data). The enzymatically triggered threshold would presumably not be reached immediately, suggesting non-specific enzymatic cleavage of target liposomes was not responsible for fusion. The mere presence of protein cannot be responsible for fluorescence dequenching, as heat inactivated proteinase K did not mediate a similar response. Conversely, proteinase K did not physically prevent fluorescence dequenching, as exogenous proteinase K added to mixtures of the fusogenic DOTAP/PE liposomes with target liposomes did not inhibit lipid mixing (unpublished data). Taken together, these results indicate that only pretreatment with enzymatically active proteinase K triggers fusion of *N*-Ac-AA-DOPE containing liposomes.

The reliance of activation upon enzyme cleavage of *N*-Ac-AA-DOPE was further assessed by examining the concentration and time dependencies of both

Fig. 10. Dextran loaded DOTAP/*N*-Ac-AA-DOPE/PE liposome fusion with RBC ghosts. DOTAP/*N*-Ac-AA-DOPE/PE (20/10/70 mol%) liposomes were loaded with 10 kDa TX-red conjugated dextrans. Liposomes were incubated with proteinase K overnight at 37°C. Aliquots of 40 nmol of dextran loaded liposomes (A, C) or unloaded liposomes + free dextran (B, D) were incubated with 1×10^8 RBC ghosts in 1 ml buffer for 30 min at 37°C, after which the cells were washed and observed by fluorescence microscopy (A, B) or Nomarski differential interference contrast (C, D).

events. DOTAP/*N*-Ac-AA-DOPE/PE liposomes were either incubated with 0, 0.1, 0.25, 0.5, and 1 mg proteinase K/100 nmol lipid overnight, or with 1 mg proteinase K/100 nmol lipid for 1, 2, 4 h or overnight. These liposomes were monitored for *N*-Ac-AA-DOPE cleavage by HPLC or for lipid mixing with acceptor liposomes by *N*-NBD-PE fluorescence dequenching. A similar concentration dependence was evident for both *N*-Ac-AA-DOPE cleavage and liposomal lipid mixing (Fig. 7A). Treatment with 0.5 or 1 mg proteinase K yielded apparently maximal cleavage and fusion activity. Only background levels of both activities were observed when 0 or 0.1 mg of the enzyme were used. The kinetics of proteinase K mediated cleavage and fusion activation were also correlated, with overnight incubation giving the highest amount of cleavage and lipid mixing (Fig. 7B). These results further support the contention that the activation of fusogenicity of DOTAP/*N*-Ac-AA-DOPE/PE liposomes is due to enzymatic cleavage of *N*-Ac-AA-DOPE.

3.5. Activation of DOTAP / N-Ac-AA-DOPE / PE fusion with RBC ghosts

Since DOTAP/*N*-Ac-AA-DOPE/PE liposomes could be activated to lipid mix with target liposomes after enzymatic cleavage, we determined if the activated fusogenicity of *N*-Ac-AA-DOPE containing liposomes with cells could also be observed. As lipid mixing with RBC ghosts (Fig. 4B) appeared to exhibit a different threshold than liposomes (Fig. 4A), we prepared DOTAP/*N*-Ac-AA-DOPE/PE liposomes at a 20/10/70 mol ratio. The overall positive charge of these liposomes improves the binding to cells, relative to the 15/15/70 composition, but the positive charge is not high enough to induce liposome fusion with cells in the absence of an activation trigger (Fig. 4B, unpublished data). After an overnight, 37°C, incubation of these liposomes with proteinase K, lipid mixing with RBC ghosts was observed in the presence of the protease inhibitor PMSF (Fig. 8). The activity of residual proteinase K transferred from the initial incubation was negligible (Fig. 8). Specific activation of DOTAP/*N*-Ac-AA-DOPE/PE (20/10/70 mol%) lipid mixing with RBC ghosts was also observed under continuous kinetics conditions. Only liposomes pretreated with proteinase K were capable of apparently fusing with RBC ghosts while untreated liposomes did not (Fig. 9). The addition of active proteinase K to untreated liposomes also did not induce fluorescence dequenching (Fig. 9, curve c), indicating the observed increase for proteinase K treated DOTAP/*N*-Ac-AA-DOPE/PE liposomes was due to specific activation of lipid mixing, and presumably, fusion.

To determine if the lipid mixing observed after proteinase K activation was due to true fusion of liposomes with cells and not potential artifacts of the lipid mixing assay, such as membrane probe exchange or hemifusion between outer leaflet membranes, DOTAP/*N*-Ac-AA-DOPE/PE (20/10/70 mol%) liposomes were loaded with 10,000 MW fluorescent aqueous probe TX-red dextran. Dextrans were used because small MW probes (e.g., Tb^{3+}, 159 MW) rapidly leaked from the liposomes (unpublished observations). Liposomes were then treated with proteinase K and incubated with RBC ghosts. After washing the cells extensively to remove unbound liposomes, the RBC ghosts were observed under fluorescence microscopy. Bright diffuse fluorescence could be observed in a portion of the cells (Fig. 10), indicating complete fusion occurred between liposomes and certain cells with subsequent transfer of the fluorescent aqueous probe. Differences in fluorescence levels may be due to differences in the number of liposomes fusing with a single cell. The observed fluorescence does not appear to be due to non-specific uptake of dextran out of leaky liposomes, as incubation of RBC ghosts with unlabeled liposomes and free TX-red dextran did not result in observable aqueous probe labeling (Fig. 10). Thus, DOTAP/*N*-Ac-AA-DOPE/PE liposomes can be activated by enzymatic cleavage of the peptide–lipid to fuse with cells and deliver their aqueous contents.

4. Discussion

Liposomes have been activated to fuse or release their internal contents by a variety of triggering signals. pH sensitive lipids have been included in liposomal formulations such that exposure to an acidic environment mimicking the endosome destabilizes the liposomal membrane and in some cases leads to fusion [3–7]. Photoactivation has been used to either

generate membrane destabilizing surfactants [7] or to polymerize photosensitive lipids such that enriched DOPE domains could form and promote fusion [8]. Phospholipase C activation [9,10], chemical cleavage [11], and trypsin mediated cleavage [12] have also been used with specific liposomal formulations to generate fusogenic liposomes. These approaches have their unique advantages and disadvantages. Activation of fusion by light has the advantage that the triggering signal can be tightly controlled but in turn is limited to sites where the activating signal can be directed. Low pH sensitive liposomes are effective in releasing their aqueous contents under acidic conditions [4] and have been shown to deliver their encapsulated contents to cells [5,25]. Unfortunately, non-endocytic cells are excluded as delivery targets and fusion are not actively physiologically targeted. Furthermore, it may be advantageous to avoid the endosomes for low pH sensitive molecules. Phospholipase C triggered fusion depends on an enzyme that is not selectively found at pathological sites. Chemical and trypsin dependent cleavage are not feasible for in vivo applications.

Recent reports of protease mediated delivery systems indicate the appeal of this approach. Peng et al. [26] showed that expression in retrovirus of epidermal growth factor (EGF) linked to a viral envelope glycoprotein via a matrix metalloproteinase (MMP) cleavage signal enhanced the ability of the retrovirus to infect cells expressing MMP and EGF receptor. However, this vector presumably still suffers from the problems associated with retrovirus delivery, such as immunogenicity and potential active virus production. Another application of protease activation is the modification of α-hemolysin, a self-assembling, pore-forming bacterial protein. α-Hemolysin has been remodeled so that the pore-forming ability can be activated by a number of triggers, including cathepsin B, a protease secreted by certain tumor cells [27]. Development of a delivery system based on modified α-hemolysin has not been described as of yet. These reports demonstrate the potential of an enzyme triggered system.

Covalent conjugation of the ala–ala dipeptide to DOPE generates a novel peptide–lipid that differs from DOPE in two significant ways. First, the overall shape of the phospholipid may be converted from that of an inverted cone to a cylindrical form, which apparently affects the intrinsic curvature of the phospholipid such that its fusogenic potential is reduced and its ability to form stable membrane bilayers is enhanced. Thus, the finding that liposomes composed solely of *N*-Ac-AA-DOPE can be prepared at neutral pH is not unexpected. Second, the overall charge of the phospholipid changes from net neutral to negative (Fig. 1). Both the size and charge effects are reversed upon removal of the *N*-Ac-ala–ala headgroup by protease treatment, resulting in enhanced fusogenicity and loss of negative charge.

We took advantage of these properties to design liposomes that would be quiescent until activated to fuse by enzymatic cleavage. Liposomes with this property could prove to be superior to liposomes that are inherently fusogenic. The charge reversibility of *N*-Ac-AA-DOPE containing liposomes is an attractive feature because it permits the preparation of negatively charged liposomes, which has been shown to decrease liposomal aggregation and increase in vivo circulation for certain liposomal formulations [28]. Further refinements of the liposomal composition may involve substitution of a specific targeting component for the positively charged lipid. This would permit retention of the negative charge and triggerable fusogenicity while selectively delivering the liposome and its contents. We also foresee the inclusion of components that facilitate in vivo circulation or alternate fusogenic lipids to promote fusion under different conditions. This modular approach to liposome design would permit us to prepare delivery vehicles that are best suited for a given environment.

Several potential mechanisms by which cleavage of *N*-Ac-AA-DOPE mediates liposomal fusion may be postulated. Previously described triggerable systems appear to proceed by one of two mechanisms, depending upon the composition of the liposomes. One mechanism relies upon temporarily stabilizing PE with a labile component, then releasing the inherent fusogenicity of the liposome by modification of the latter component. Another method to trigger fusion or liposomal contents release is the generation in situ of membrane destabilizing components. Fusion of *N*-Ac-AA-DOPE containing liposomes can conceivably proceed by a combination of the two methods. The conversion of *N*-Ac-AA-DOPE to DOPE by active proteinase K generates additional DOPE that may drive the liposomes over the threshold of nega-

tive curvature stress required for fusion [29,30]. At the same time, the decrease in negatively charged *N*-Ac-AA-DOPE may reduce the charge repulsion between liposomal and cell membranes. Furthermore, an additional mechanism of action for membrane destabilization and subsequent fusion may derive from generation of a transmembrane asymmetry in PE content. If the localized increase of DOPE is confined to the outer leaflet, it may enhance the propensity for negative curvature on the outer monolayer without affecting the inner monolayer. Several experiments [31,32] suggest that fusion can be optimized by induction of negative curvature stress in the outer monolayer, but the inner monolayer may require lipids inducing positive curvature stress. Fusion may, in fact, be inhibited by induction of negative curvature stress on the inner monolayer [33].

Improvements in the design of the enzyme substrate may enhance the efficiency of enzyme activated liposomal fusion. One approach may be to substitute a more reactive enzyme substrate for *N*-Ac-ala–ala. This dipeptide was chosen for conjugation with DOPE because of its greater reactivity with elastase [34], relative to a single alanine linked to a chromogenic substrate [16], as well as to minimize immunogenic potential. However, other peptide sequences such as ala–ala–pro–val have a higher turnover rate for cleavage [34]. Therefore, conjugation of this peptide or similar peptide sequences to DOPE may enhance the proteolytic cleavage. It was also shown that increased solubility of synthetic substrates was related to enhanced cleavage by human leukocyte elastase [34]. Thus, the efficiency of peptide–lipid cleavage may also be improved by substituting the *N*-acetyl group of *N*-Ac-ala–ala with alternatives with greater hydrophilicity.

The variety of proteolytic enzymes present within the inflammatory environment may provide another method to enhance the cleavage and fusion activation of peptide–lipid liposomes. Activated neutrophils release numerous enzymes in addition to elastase, such as cathepsin G and the matrix metalloproteinases gelatinase [35] and collagenase [36]. A similar substrate specificity between cathepsin G and elastase [34] and among MMPs [37,38] suggests that some peptide–lipids containing a substrate for elastase may also be cleaved by other neutrophil derived enzymes, thereby enhancing the activation of fusion.

The ubiquitous nature of elastase at inflammatory sites hints at the potential applications of an elastase susceptible peptide–lipid. Elastase and the neutrophils from which it is produced have been implicated as contributing factors to rheumatoid arthritis [21,39], acute respiratory distress syndrome [40], emphysema [41], and cystic fibrosis [42,43]. Elevated levels of elastase have also been associated with breast [44] and skin cancer [45]. Liposomes composed of *N*-Ac-AA-DOPE could be suitable delivery vehicles for therapeutic agents to these sites. Furthermore, other disease states may be addressed by substitution of the elastase substrate of the peptide–lipid with substrates for other enzymes. For example, metastases of tumor cells may be targeted because of their correlation with elevated matrix metalloproteinase levels [46]. This study demonstrates that the general concept of enzyme cleavage triggered fusion is feasible and holds promise as a means of directed delivery.

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