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Elastase activated liposomal delivery to nucleated cells

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Abstract

The specific activation of liposomes for delivery has been explored by enzyme mediated cleavage of a peptide substrate covalently conjugated to a fusogenic lipid. We have previously shown an elastase sensitive peptide conjugated to 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine (DOPE) could be activated by enzymatic cleavage, triggering liposome-liposome lipid mixing and fusion with erythrocyte ghosts (Pak et al., Biochim. Biophys. Acta, 1372 (1998) 13–27). Further optimization of this system has been aimed at obtaining substrate cleavage at or below physiological elastase levels and to demonstrate triggered delivery to living cells. Therefore a new peptide-lipid, MeO-suc-AAPV-DOPE (*N*-methoxy-succinyl-Ala-Ala-Pro-Val-DOPE), has been developed that exhibits greater sensitivity and selectivity for elastase cleavage and subsequent conversion to DOPE. This peptide-lipid was used with DODAP (dioleoyl dimethylammonium propane, a pH dependent cationic lipid) in a 1:1 mol ratio with the expectation that endocytosis would lead to a liposome with an overall positive charge if enzymatic cleavage had occurred. Elastase treated liposomes displayed pH dependent enhancement of binding, lipid mixing, and delivery of 10 000 MW dextrans, relative to untreated liposomes, when incubated with HL60 human leukemic cells. Heat denatured elastase did not activate DODAP/MeO-suc-AAPV-DOPE liposomes, indicating enzymatic activity of elastase is necessary. Liposomes bound to ECV304 endothelial cells at physiological pH could be activated by elastase to deliver an encapsulated fluorescent probe, calcein, into the cell cytoplasm. These results suggest enzyme substrate peptides linked to a fusogenic lipid may be used to elicit specific delivery from liposomes to cells. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Liposome; Fusion; Elastase; Peptide; Lipid; Cleavage

1. Introduction

A paradox of liposomal delivery vehicles is the requirement for liposomal membranes that are both stable and yet capable of undergoing fusion or destabilization at the desired site, so as to elicit delivery of

Abbreviations: DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine; MeO-suc-AAPV-DOPE, *N*-methoxy-succinyl alanyl alanyl prolyl valyl 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine; *N*-Ac-AA-DOPE, *N*-acetyl alanyl alanyl 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine; *N*-NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-phosphatidylethanolamine; *N*-Rho-PE, *N*-lissamine rhodamine B sulfonyl)-phosphatidylethanolamine; TMR-dextran, tetramethyl rhodamine dextran; DODAP, dioleoyl dimethylammonium propane; C12E8, octaethylene glycol monododecyl ether; HBSS, Hanks' balanced salt solution; TES, *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid; EDTA, ethylenediamine tetraacetic acid; RET, resonance energy transfer; RFU, relative fluorescence unit; TLC, thin layer chromatography; FDQ, fluorescence dequenching

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the contents of the liposome into the cell. We have previously described the use of enzymatic cleavage to trigger liposomal fusion and contents delivery [1]. Although other triggers have been utilized to induce liposomal fusion or membrane destabilization [2–7], activation by enzyme cleavage has several advantages over these methods.

Elevated enzyme activity is associated with numerous pathological conditions. Metastatic cancer cells display enhanced extracellular activity of several degradative enzymes, such as matrix metalloproteinases and urokinase-type plasminogen activator (for review see [8]). Elevated enzymatic activity facilitates the extravasation of these cells from the circulation and increases their invasive potential. Inflammatory conditions such as cystic fibrosis [9–12], rheumatoid arthritis [13,14], and emphysema [15,16] are accompanied by an increase in extracellular elastase activity due to release of elastase from phagocytic cells. Elevated elastase activity appears to be due, in part, to an imbalance in the elastase/anti-protease ratio [9,17,18]. Elastase has also been associated with tumor progression and development [19–21]. The ubiquitous yet specific nature of disease-associated enzymatic activity, its localization near or on the membranes of cells involved in tissue remodeling [22,23] and its association with several pathologies provide numerous opportunities for triggering specific liposomal delivery to desired targets using the activity of such enzymes. The triggering event would be expected to convert the liposome from a relatively inert state to a fusogenic state and may even trigger specific binding depending on the design.

The selectivity of liposomal activation can be modulated by the choice of an enzyme substrate conjugated to a fusogenic lipid so that enzymatic cleavage releases or unmasks fusogenic lipids. Thus liposomes may be designed for a selected site of activation and hence liposomal delivery could be targeted. We have chosen to use elastase mediated triggering of liposomal fusion as a model for the general principle of enzymatically activated delivery via liposomes.

We have previously described the activation of liposomal lipid mixing and fusion by enzyme triggering [1]. A peptide-lipid consisting of *N*-Ac-Ala-Ala-, an elastase sensitive peptide sequence, was conjugated to the headgroup of DOPE (1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine), a known fusogenic lipid. The fusogenic potential of this peptide-lipid, *N*-Ac-AA-DOPE, was limited until enzymatic cleavage of the peptide regenerated DOPE. Liposomes containing *N*-Ac-AA-DOPE could be triggered to lipid mix with target liposomes and fuse with RBC ghosts.

Although *N*-Ac-AA-DOPE was capable of demonstrating the utility of enzyme-triggered fusion, physiological relevance requires greater sensitivity to enzyme activation, demonstration of delivery to nucleated living cells and ultimately the ability to deliver in the physiological milieu which may include serum proteins. We chose to address the first two of these problems in this report.

First, it is necessary to demonstrate that the choice of an appropriate peptide can be used to optimize the cleavage and triggering for the desired target at physiological levels of the enzyme. Powers et al. [24] had shown that the peptide sequence MeO-suc-Ala-Ala-Pro-Val- was highly sensitive to elastase cleavage. Therefore we hypothesized that this sequence when conjugated to DOPE would create a triggerable peptide-lipid with greater sensitivity. In this study a new peptide-lipid, MeO-suc-AAPV-DOPE, was tested for elastase mediated DOPE generation. Included in this effort was the introduction of a lipid that would reduce the surface charge of the liposome compared to the previous version to enhance interaction of the positively charged enzyme, elastase, with the substrate. A second goal was to demonstrate delivery to nucleated cells, where at least some delivery may occur via an endosomal compartment. Liposomes were designed to take advantage of the low pH in that compartment in a manner that would also enhance elastase triggering. Here we report a liposomal system that could be shown for the first time, to be triggered by physiological levels of elastase to undergo lipid mixing with and aqueous contents delivery to nucleated cells.

2. Materials and methods

2.1. Reagents

Human leukocyte elastase was purchased from Calbiochem (San Diego, CA, USA). Lipids were purchased from Avanti Polar Lipids (Alabaster, AL,

USA) and were of 99% or greater purity. Calcein (>95% pure), tetramethyl rhodamine dextran (TMR-dextran), and streptavidin were from Molecular Probes (Eugene, OR, USA). Biotinylated-wheat germ agglutinin (WGA) was obtained from Pierce (Rockford, IL, USA). MeO-suc-Ala-Ala-Pro-Val-OH used for covalent linkage with DOPE was from Bachem Bioscience (King of Prussia, PA, USA). RPMI 1640, fetal bovine serum (FBS), and HBSS (Hanks' balanced salt solution) were purchased from Life Technologies (Gaithersburg, MD, USA). Medium 199 was purchased from BioWhittaker (Walkersville, MD, USA). 1,3-Dicyclohexylcarbodiimide (DCC), 4-dimethylaminopyridine (DMAP), *p*-nitrophenol and triethylamine were obtained from Sigma (St. Louis, MO, USA). TMD-8 ion exchange resin was purchased from Aldrich (Milwaukee, WI, USA). HPLC-grade solvents, tetrahydrofuran (THF), chloroform and methanol were purchased from Baxter (McGaw Park, IL, USA). All chemicals and solvents were used without further purification.

2.2. Cells

HL60 human leukemia and ECV304 human endothelial cells were obtained from ATCC (Rockville, MD, USA). HL60 cells were passaged as suspension cultures in RPMI 1640 supplemented with 10% heat inactivated FBS. Adherent ECV304 cells were grown in medium 199 supplemented with 10% heat inactivated FBS. Greater than 98% viability was observed during routine tissue culture.

2.3. Synthesis and characterization of N-methoxy-succinyl alanine alanine proline valine 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine (MeO-suc-AAPV-DOPE)

2.3.1. MeO-suc-AAPV-p-nitrophenyl ester

To a solution of MeO-suc-AAPV-H peptide (540 mg, 1.15 mmol) was added 142 mg (1.38 mmol) of *p*-nitrophenol, 175 mg (1.38 mmol) of DCC and a catalytic amount (a few crystals) of DMAP in 10 ml of dry chloroform. The reaction mixture was stirred overnight under nitrogen atmosphere at room temperature. At this point TLC (thin layer chromatography) analysis showed that the reaction had gone to completion. The precipitate, dicyclohexylurea, from the reaction mixture was filtered using a G-2 funnel and the filtrate concentrated under reduced pressure. The residual material was used in next step without purification.

2.3.2. MeO-suc-AAPV-1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine

To a solution of *p*-nitrophenyl ester of MeO-suc-AAPV-OH (600 mg, 1.01 mmol) was added 604 mg (0.81 mmol) of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine and 82 mg (113 ml, 0.81 mmol) of triethylamine in 20 ml of chloroform:tetrahydrofuran (1:4 v/v). The reaction mixture was stirred under nitrogen atmosphere at room temperature overnight. TLC analysis showed that the reaction had gone to the completion. The reaction mixture was concentrated under reduced pressure and passed through activated TMD-8 ion exchange resin in THF:H_2O (9:1 v/v). The phosphorus positive fractions were pooled and concentrated to get a residual product. The residual material was purified with silica gel column chromatography (the column was washed with 5% methanol in chloroform, then eluted with chloroform:methanol:ammonium hydroxide 65:25:4 v/v/v), giving 915 mg (95% yield on the basis of DOPE), which on lyophilization gave a white solid. The lipopeptide molecule tested positive with a molybdenum reagent and negative with a ninhydrin reagent. By TLC the lipopeptide gave a single spot and >99% purity. The lipopeptide was also characterized by NMR and FAB mass spec analysis. Some characteristic ^{1}H-NMR signals (300 MHz, $CDCl_3$) are shown here: d 0.87 (t, 3H, $J=7.15$ Hz), 1.27 (40H), 1.56 (4H), 2.0 (8H), 2.23 (t, 4H, $J=7.15$ Hz), 5.17 (1H), 5.32 (4H, $J=3.12$ Hz). The ^{31}P-NMR spectrum (121.5 MHz, $CDCl_3$) gave a single signal. FAB (M^+) calculated for $C_{62}H_{109}N_5O_{15}P$ was 1195.55, and masses of 1196.8 (MH^+) and 1234.9 (MK^+) were observed.

2.4. Liposome preparation

Large vesicles were prepared by aliquoting desired amounts of lipid from chloroform stocks into 13×100 mm pyrex tubes and drying under a nitrogen stream. After exposure to high vacuum 4 h overnight the lipid film was hydrated in 10 mM

TES (*N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid), 154 mM NaCl, 0.1 mM EDTA (ethylenediamine tetraacetic acid), pH 7.4. Unless specified otherwise, this buffer was used for all experiments. After vortexing, samples were freeze-thawed 8× and extruded under pressure 10× (Lipex, Vancouver, BC, Canada) through 0.1 μm polycarbonate filters (Nucleopore, Pleasanton, CA, USA). Liposomes were stored at 4°C until used.

Fluorescent dextran containing liposomes were prepared by hydrating the DODAP/MeO-suc-AAPV-DOPE lipid film with a 50 mg/ml solution of 10 000 MW TMR-dextran in TES/NaCl/EDTA buffer. Liposomes were then vortexed, freeze-thawed, and extruded through 0.1 μm filters as described above. To remove unencapsulated dextran the liposome solution was extensively dialyzed with the TES/NaCl/EDTA buffer using a Biodialyser (Sialomed, Columbia, MD, USA) fitted with 50 nm pore size filters. Calcein loaded liposomes were prepared by hydrating the lipid film in the presence of buffer containing 50 mM calcein. The calcein solution was pH and osmolarity adjusted prior to preparation of liposomes. Calcein loaded liposomes contained 0.75 mol% *N*-Rho-PE to monitor liposome binding. After preparation of large vesicles as described above, calcein loaded liposomes were transferred to a 10 000 MWCO Slide-A-Lyzer (Pierce, Rockford, IL, USA) and extensively dialyzed with TES/NaCl/EDTA buffer. The encapsulated volume of these liposomes was 0.8 l/mol of lipid. Sonicated vesicles were prepared by drying lipid in the same manner as described above but preparations were vortexed then water bath sonicated for >10 min at room temperature. Lipid concentration was monitored by phosphate assay [25]. The size of liposomes was verified by quasi-elastic light scattering using a Nicomp submicron particle sizer (Particle Sizing Systems, Santa Barbara, CA, USA). Freeze-thaw-extrusion vesicles and sonicated vesicles were 70–80 nm and 35–45 nm in diameter, respectively, as determined by number weighted Gaussian analysis.

2.5. Detection of MeO-suc-AAPV-DOPE cleavage

2.5.1. TLC detection of MeO-suc-AAPV-DOPE cleavage

100 nmol of MeO-suc-AAPV-DOPE sonicated vesicles were incubated with 0, 5, or 10 μg elastase in 0.4 ml TES/NaCl/EDTA buffer, pH 7.4, overnight at 37°C. Lipid was extracted by organic phase separation [26], dried under N_2 stream, and exposed to vacuum for 4 h. Samples were resuspended in chloroform and spotted onto TLC plates. TLC was run using chloroform:methanol:ammonium hydroxide (65:25:5), air dried, sprayed with molybdenate blue, and charred on a hot plate.

2.5.2. ^{31}P-NMR analysis

DODAP/MeO-suc-AAPV-DOPE (1:1 mol ratio) freeze-thaw-extrusion vesicles were prepared and treated with or without elastase (0–50 μg protein/1000 nmol lipid/4 ml) for 2 h in TES/NaCl/EDTA buffer, pH 7.4, at 37°C. Liposomes were transferred to 13×64 mm polyallomer centrifuge tubes (Beckman, Palo Alto, CA, USA), and pelleted by ultracentrifugation at 149 000×*g* for 1 h at 4°C with a L5-50E ultracentrifuge (Beckman, Palo Alto, CA, USA). The liposome pellet (approximately 90% of the total) was resuspended in 100 μl TES/NaCl/EDTA buffer, to which 400 μl of 10% deoxycholate, 100 mM EDTA, 20 mM HEPES, pH 7.4, buffer and 200 μl of deuterium oxide (Cambridge Isotope Laboratories, Woburn, MA, USA) were added. After transfer 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. 1 Hz line broadening was applied to all spectra. Peaks were identified by comparison with standards run under identical conditions.

2.6. Binding and lipid mixing of liposomes to HL60 cells

Lipid mixing was monitored by the *N*-NBD-PE/*N*-Rho-PE resonance energy transfer assay, as described [27]. Liposomes were prepared with 0.75 mol% *N*-NBD-PE and 0.75 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. DODAP/MeO-suc-AAPV-DOPE (1:1 mol:mol) lipo-

somes were incubated in TES/NaCl/EDTA buffer ± elastase (5 μg/100 nmol lipid, 250 μM lipid concentration) for 2 h at 37°C, pH 7.4. HL60 cells were washed with TES/NaCl/EDTA buffer and incubated with liposomes (1×10^6 cells, 10 nmol liposome) in 200 μl TES/NaCl/EDTA buffer. Samples were either at pH 7.4 or adjusted to pH 5 by the addition of dilute HCl. All samples were shaken in an Eppendorf Thermomixer (Brinkmann Instruments, Inc., Westbury, NY, USA), 700 rpm, for 30 min at 37°C. There was no reduction in cell viability following this procedure, as detected by trypan blue exclusion (unpublished data). Cells were then washed with TES/NaCl/EDTA buffer, pH 7.4, and transferred to Falcon 24 well plates (Becton Dickinson, Lincoln Park, NJ, USA). Fluorescence was monitored in a Cytofluor II multiwell fluorescence plate reader (Perseptive Biosystems, Framingham, MA, USA) with a quartz halogen lamp using 450 nm excitation/530 nm emission or 560 nm excitation/620 nm emission wavelengths for *N*-NBD-PE or *N*-Rho-PE fluorescence, respectively. Liposome binding was determined as the amount of *N*-Rho-PE fluorescence associated with washed cells relative to total fluorescence of liposomes added. This percentage was converted to number of liposomes bound by multiplying by the number of liposomes added (assuming all liposomes were 100 nm in diameter and 10^5 lipid molecules/0.1 μm diameter liposome. Therefore 6.02×10^{10} liposomes of 0.1 μm diameter were added per sample). The % fluorescence dequenching (FDQ) was calculated by the following formula:

$$[(F_t/F_{\text{max cells}})-(F_{\text{o alone}}/F_{\text{max alone}})]/$$
$$[1-(F_{\text{o alone}}/F_{\text{max alone}})]\times100$$

where F_t = *N*-NBD-PE fluorescence of liposomes incubated with cells at a given time, $F_{\text{o alone}}$ = initial *N*-NBD-PE fluorescence of liposomes only, $F_{\text{max cells}}$ and $F_{\text{max alone}}$ = maximal *N*-NBD-PE fluorescence of liposomes incubated either with cells or alone, respectively, as determined by addition of 0.5% C12E8 detergent. FDQ was assumed to result from all-or-none lipid mixing of liposomes with cells. Therefore % FDQ could be converted to number of liposomes mixed by simple multiplication of the total. This was done to take into account both the enhancement of binding and the lipid mixing after elastase activation.

2.7. DODAP/MeO-suc-AAPV-DOPE liposome-ECV304 binding, lipid mixing, and calcein delivery

Liposomes were bound to adherent ECV304 cells via a biotin-streptavidin linkage. To this end DODAP/MeO-suc-AAPV-DOPE (1:1 mol:mol) liposomes were prepared with 0.3 mol% *N*-biotinyl caproylamine-PE as well as fluorescent lipid probes or with encapsulated calcein. ECV304 cells were washed with HBSS buffer and then incubated sequentially at room temperature with biotin-WGA (20 μg/ml) and streptavidin (40 μg/ml) prepared in HBSS, 30 min/treatment. Cells were washed after each treatment. Liposomes were treated with or without elastase as described above. Certain aliquots of pretreated DODAP/MeO-suc-AAPV-DOPE (1:1 mol/mol) liposomes were freeze-thawed after dialysis and prior to the addition to cells to release the liposomal contents. Such freeze-thawed liposomes were exposed to a liquid nitrogen-37°C water bath for 5 cycles. The self-quenching of calcein was reduced by approximately 85% (maximal FDQ determined by detergent solubilization) after freeze-thawing, indicating the release of encapsulated calcein. In all cases, 50–100 nmol of liposomes were added to confluent ECV304 cell monolayers (1×10^5 cells/well of a 24 well plate) and incubated in HBSS for 30 min at room temperature to promote *N*-biotinyl cap-PE binding to streptavidin. Unbound liposomes were removed by repeated washes. After the final wash, fresh HBSS buffer was added to all wells and cells were incubated at 37°C for given times. Fluorescence was quantitated as described above.

2.8. Fluorescence microscopy of liposome-cell lipid mixing and aqueous contents delivery

DODAP/MeO-suc-AAPV-DOPE (1:1 mol/mol) liposomes were incubated for 2 h at 37°C without or with elastase (5 μg protein/100 nmol lipid). Liposomes containing the fluorescent lipid probes *N*-NBD-PE and *N*-Rho-PE were bound to HL60 in solution as described above. TMR-dextran loaded

DODAP/MeO-suc-AAPV-DOPE liposomes (40 nmol) were incubated with 1×10^5 HL60 cells in 200 μl TES/NaCl/EDTA buffer under pH 5, 37°C, conditions for 30 min to induce binding. Calcein loaded DODAP/MeO-suc-AAPV-DOPE liposomes were bound to ECV304 cells that had been plated on glass coverslips by the biotinylated-WGA-streptavidin linkage described above. In some experiments liposomes were freeze-thawed, as described above, prior to the addition to cells. Cells were washed with HBSS to remove unbound liposomes and observed with a Bio-Rad (Hercules, CA, USA) MRC-1000 laser scanning confocal imaging system equipped with an Olympus BX50 microscope (Olympus, Lake Success, NY, USA) using an apochromat 60× oil (N.A. 1.40) objective. Calcein fluorescence was excited with the 488 nm line of a krypton/argon laser and observed at 522 nm emission. *N*-Rho-PE and TMR-dextran fluorescence were observed with the 568 nm line and observed at 605 nm emission. Images were frame averaged and false color was applied. All images within a figure were obtained under identical conditions of confocal iris width, gain, and black level. Identical false color look up tables were also applied to images within a figure. Average fluorescence/cell μm^2 of all cells in an image was determined with the histogram feature of Bio-Rad CoMOS confocal imaging software.

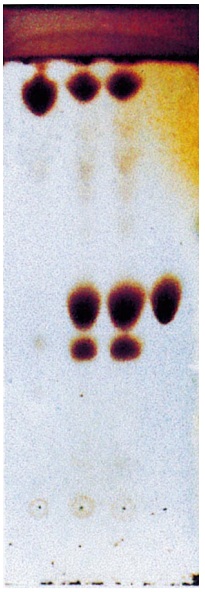

Fig. 1. Elastase mediated cleavage of MeO-suc-AAPV-DOPE as detected by TLC. 100 nmol of MeO-suc-AAPV-DOPE sonicated vesicles were incubated with 0, 5, or 10 μg elastase in 0.4 ml buffer. After overnight incubation at 37°C lipid was extracted and spotted onto TLC plates. Plates were run with a chloroform:methanol:ammonium hydroxide (65:25:5) solvent system and visualized by charring. Lane 1: MeO-suc-AAPV-DOPE only; lane 2: +5 μg elastase; lane 3: +10 μg elastase; lane 4: 20 μg DOPE.

3. Results

3.1. Cleavage of MeO-suc-AAPV-DOPE by elastase

Cleavage by elastase to yield DOPE is necessary for MeO-suc-AAPV-DOPE to act as a trigger for liposomal delivery. This was tested by incubating MeO-suc-AAPV-DOPE sonicated vesicles with or without elastase overnight at 37°C, after which lipid was isolated and analyzed by TLC. Incubation with either 5 or 10 μg elastase/100 nmol lipid resulted in the significant generation of DOPE (Fig. 1), although there also appeared to be a slower running phosphate positive component that may represent an intermediate peptide cleavage product. Only minimal cleavage of MeO-suc-AAPV-DOPE by proteinase K was observed at the same concentrations (unpublished data), indicating the tetrapeptide sequence is a more selective target than the previously studied *N*-Ac-AA-dipeptide [1].

The conversion of MeO-suc-AAPV-DOPE to DOPE was also quantitated by ^{31}P-NMR. Since subsequent experiments employed liposomes containing both DODAP and MeO-suc-AAPV-DOPE, vesicles with a 1:1 (mol:mol) ratio of these two components were prepared by the freeze-thaw-extrusion method (see Section 2) and incubated at 37°C for 2 h with increasing amounts of elastase (0–5 μg elastase/100 nmol lipid). ^{31}P-NMR analysis demonstrated an elastase concentration dependent cleavage of MeO-suc-AAPV-DOPE and appearance of DOPE (Fig. 2, solid line). A small shoulder that may represent an incomplete peptide cleavage product was also observed near the original peptide-lipid peak (unpublished data). Treatment with 5 μg elastase/100 nmol lipid yielded 20% DOPE. Longer incubation may have led to further digestion, though multiple lamellae and/or the surface charge of the liposome may limit the

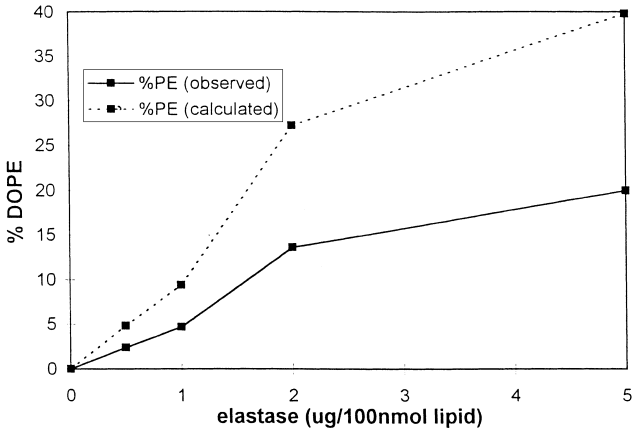

Fig. 2. Quantitation of elastase mediated cleavage of MeO-suc-AAPV-DOPE to DOPE by ^{31}P-NMR. DODAP/MeO-suc-AAPV-DOPE (1:1 mol/mol) freeze-thaw-extrusion vesicles were incubated with 0, 0.5, 1, 2, 5 μg elastase/100 nmol lipid. Samples were incubated for 2 h at 37°C, after which liposomes were pelleted by ultracentrifugation. Liposomes were solubilized and monitored by ^{31}P-NMR. Solid line: % of total MeO-suc-AAPV-DOPE converted to DOPE; dotted line: % of expected MeO-suc-AAPV-DOPE on outer monolayers converted to DOPE assuming unilamellar vesicles, i.e. result multiplied by 2.

ultimate amount digested (see Section 4). The maximum exposed peptide-lipid for intact liposomes would occur with unilamellar vesicles. Assuming only the outer leaflet peptide-lipid is available for digestion, the minimal percentage conversion of exposed MeO-suc-AAPV-DOPE to DOPE in the outer leaflet lipid was 40% in this situation (Fig. 2, dotted line). If the average number of lamellae were greater than one, the percentage conversion was even higher. In fact, the ratio of encapsulated volume to total lipid would appear to indicate an average of approximately 2.5 lamellae per vesicle for this preparation (see Section 2) which would indicate that 100% of available peptide-lipid had been cleaved under these conditions. The concentration of elastase required to produce this amount of cleavage (12.5 μg elastase/ml) is less than the amount of elastase activity found in the epithelial lining fluid from patients with cystic fibrosis [28]. The contents from human neutrophil granules were also able to cleave MeO-suc-AAPV-DOPE and generate DOPE (unpublished data). The number of neutrophils required to observe this effect was less than that observed in epithelial lining fluid from cystic fibrosis patients [28], indicating the amount of elastase required to cleave MeO-suc-AAPV-DOPE to DOPE is within concentrations that are physiologically or therapeutically relevant, with the caveat that the effect of serum proteins must still be established.

3.2. Optimum DODAP/MeO-suc-AAPV-DOPE composition for binding and lipid mixing with HL60 cells

MeO-suc-AAPV-DOPE containing liposomes were designed to deliver their contents after binding, endocytic internalization, and fusion with and/or disruption of the endosomal membrane. DODAP was chosen instead of the previously used DOTAP [1] because only 20% of the DODAP population is positively charged at pH 7.4 [29]. This allows a more

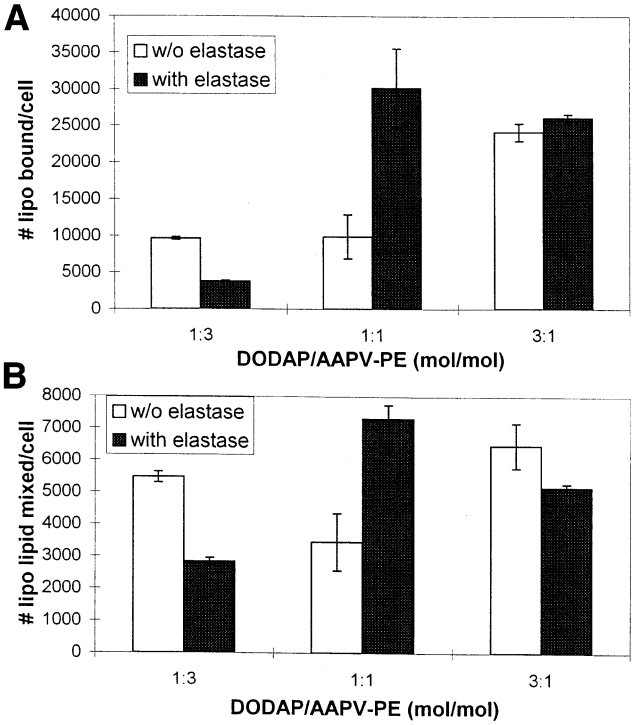

Fig. 3. Optimum DODAP/MeO-suc-AAPV-DOPE liposome composition for elastase activation of binding/lipid mixing with HL60 cells. DODAP/MeO-suc-AAPV-DOPE liposomes prepared at 1:3, 1:1, or 3:1 (mol/mol) ratios and labeled with 0.75 mol% *N*-NBD-PE and 0.75 mol% *N*-Rho-PE were incubated with or without elastase (5 μg elastase/100 nmol lipid) for 2 h at 37°C. 10 nmol of liposomes were then mixed with 1×10^6 HL60 cells and incubated for 30 min at 37°C, pH 5. After washing twice with 5× volume of TES/NaCl/EDTA buffer, pH 7.4, (A) binding and (B) lipid mixing was determined by monitoring *N*-Rho-PE and *N*-NBD-PE fluorescence, respectively.

complete peptide hydrolysis by elastase (unpublished data, see Section 4) so that we can model the more general case of cleavage on the liposome surface that is not affected by the high charge of the enzyme. For other enzymatic activators it may be possible to utilize a permanently positively charged lipid that would allow the liposome to become positively charged at physiological pH after peptide cleavage, so that extracellular binding would be triggered. By contrast, the tertiary amine of DODAP would be fully protonated at pH 5, suggesting liposomes containing DODAP would undergo stronger interaction with negatively charged cell membranes within the low pH environment of the endocytic compartment.

In order to determine the optimum combination of DODAP and MeO-suc-AAPV-DOPE for triggerable binding and lipid mixing with cells the two lipids were formulated into liposomes at different ratios. DODAP/MeO-suc-AAPV-DOPE liposomes prepared at 1:3, 1:1, and 3:1 mol ratios were pretreated with or without elastase and incubated with HL60 cells under low pH conditions to promote DODAP mediated binding to cells. Only DODAP/MeO-suc-AAPV-DOPE liposomes at a 1:1 mol ratio exhibited an elastase dependent increase in binding and lipid mixing with HL60 cells (Fig. 3), possibly as a result of increased positive charge after enzymatic cleavage. Apparently the amount of DODAP in 1:3 liposomes was insufficient to mediate binding to cells, even at pH 5 after elastase treatment. By contrast, DODAP/MeO-suc-AAPV-DOPE (3:1 mol ratio) liposomes were able to bind to cells with or without elastase treatment, reflecting the greater amount of DODAP in these liposomes. These liposomes were also able to lipid mix with cells even without elastase activation. The DODAP/MeO-suc-AAPV-DOPE 1:1 mol ratio liposomal formulation was chosen for all further studies in order to develop a delivery system that can be triggered by enzymatic cleavage.

3.3. *Elastase activated binding and lipid mixing of DODAP/MeO-suc-AAPV-DOPE liposomes with HL60 cells*

Since DODAP was included in liposomes with MeO-suc-AAPV-DOPE to enhance binding with cells under low pH conditions, we determined whether the pH dependence of DODAP mediated binding is within physiological levels. DODAP/MeO-suc-AAPV-DOPE (1:1) liposomes were pretreated with elastase and incubated with HL60 cells at different pH. Enhanced binding and lipid mixing of elastase pretreated liposomes with HL60 cells were observed when incubated at pH 4.6 or pH 5.1 (Fig. 4). Incubation at pH 5.8–7.4 did not yield any significant association of liposomes with cells. These results suggest that these liposomes are sensitive to elastase mediated activation of binding and lipid mixing when DODAP is maximally positively charged. The pH required to achieve this state is present under normal physiological conditions within the late endosome [30].

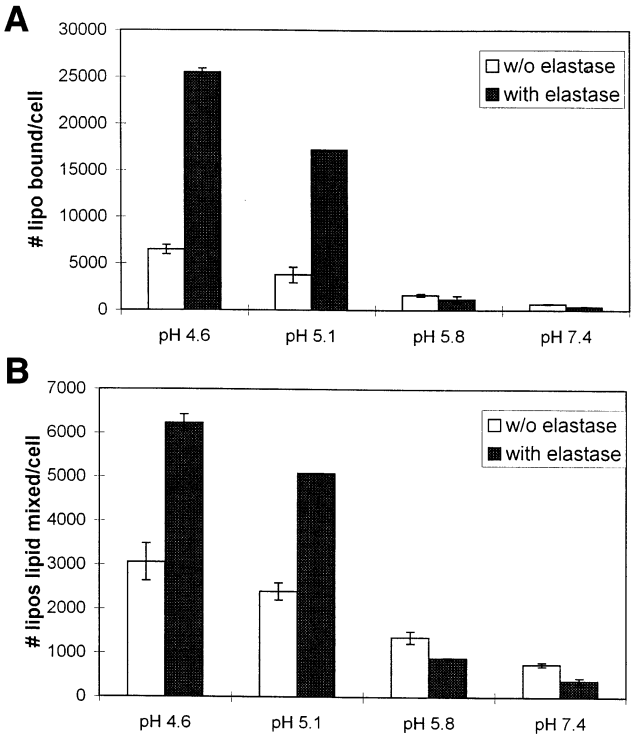

Fig. 4. pH dependence of DODAP/MeO-suc-AAPV-DOPE liposome binding/lipid mixing with HL60 cells. Fluorescent lipid probe labeled DODAP/MeO-suc-AAPV-DOPE liposomes were incubated with or without elastase (5 μg elastase/100 nmol lipid) for 2 h at 37°C. 10 nmol of liposomes were mixed with 1×10^6 HL60 cells in 200 μl TES/NaCl/EDTA buffer. Samples were incubated for 30 min at 37°C, at the given pH and washed. Binding (A) and lipid mixing (B) were determined by monitoring *N*-Rho-PE and *N*-NBD-PE fluorescence, respectively.

To confirm that the enzymatic activity of elastase

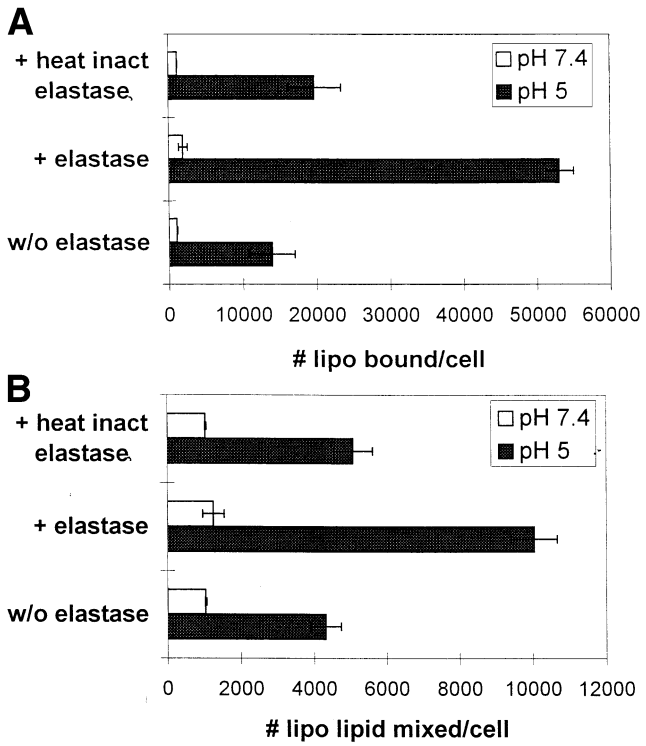

Fig. 5. Active elastase is required for triggering DODAP/MeO-suc-AAPV-DOPE liposome binding/lipid mixing with HL60 cells. DODAP/MeO-suc-AAPV-DOPE liposomes containing 0.75 mol% *N*-NBD-PE and 0.75 mol% *N*-Rho-PE were incubated alone, with elastase (5 μg elastase/100 nmol lipid), or with an equivalent amount of heat inactivated elastase (95°C, 1 h) for 2 h at 37°C. 10 nmol of pretreated liposomes were mixed with 1×10^6 HL60 cells in 200 μl TES/NaCl/EDTA buffer. Samples were incubated for 30 min, 37°C, at either pH 7.4 or pH 5 and washed. Binding (A) and lipid mixing (B) were determined by monitoring *N*-Rho-PE and *N*-NBD-PE fluorescence, respectively.

was responsible for triggering of binding and lipid mixing, DODAP/MeO-suc-AAPV-DOPE liposomes were pretreated with heat inactivated elastase. Heating elastase to 95°C for 1 h completely abrogated enzymatic cleavage of either a chromogenic substrate or MeO-suc-AAPV-DOPE (unpublished data). Pretreatment of DODAP/ MeO-suc-AAPV-DOPE liposomes with heat inactivated elastase did not enhance binding or lipid mixing with HL60 cells above background levels (Fig. 5). Only active elastase was capable of triggering the increased association. As expected, pH 5 conditions were required to observe this effect, since at pH 7.4 the binding of these liposomes to HL60 cells was greatly reduced. Confocal microscopy of liposomes with HL60 cells confirmed that elastase pretreatment was required for enhanced binding and lipid mixing. Bright fluorescent labeled cells indicate the *N*-Rho-PE fluorescent probe from the bound elastase activated liposomes had mixed into the cell plasma membrane (Fig. 6). In contrast, untreated liposomes displayed significantly less binding and lipid mixing (Fig. 6). Quantitation of fluorescence images revealed nearly 12 times as much fluorescence/cell area in HL60 cells that had been incubated with DODAP/MeO-suc-AAPV-DOPE liposomes treated with elastase, as compared to those cells incubated with untreated liposomes, although the distribution of this enhanced delivery may not be uniform across all the cells. Almost no fluorescence was associated with the cells at pH 7.4 (unpublished data).

3.4. Aqueous contents delivery from DODAP/MeO-suc-AAPV-DOPE liposomes to HL60 cells

To determine if the enhanced lipid mixing between elastase activated DODAP/MeO-suc-AAPV-DOPE liposomes and HL60 cells is truly indicative of fusion, it is necessary to monitor the delivery of an aqueous probe from the liposome to the cell cytoplasm. Therefore DODAP/MeO-suc-AAPV-DOPE liposomes were loaded with tetramethyl rhodamine labeled 10 000 MW dextran (TMR-dextran), treated with or without elastase, and incubated with HL60 cells under pH 5 conditions.

Only DODAP/MeO-suc-AAPV-DOPE liposomes that had been pretreated with elastase were capable of fusing with HL60 cells, as demonstrated by TMR-dextran labeling of the cytoplasm of these cells (Fig. 7). HL60 cells incubated with liposomes that had not been treated with elastase contained little or no cytoplasmic fluorescent dextran, indicating elastase cleavage was required to trigger the fusion of DODAP/MeO-suc-AAPV-DOPE liposomes with HL60 cells. TMR-dextran delivery to cells was not due to leakage of the fluorescent dextran out of the liposomes and subsequent uptake by HL60 cells. This possibility was investigated by inducing a TMR-dextran release from DODAP/MeO-suc-AAPV-DOPE liposomes (with or without elastase pretreatment) with repeated freeze-thaw cycles after the liposomes

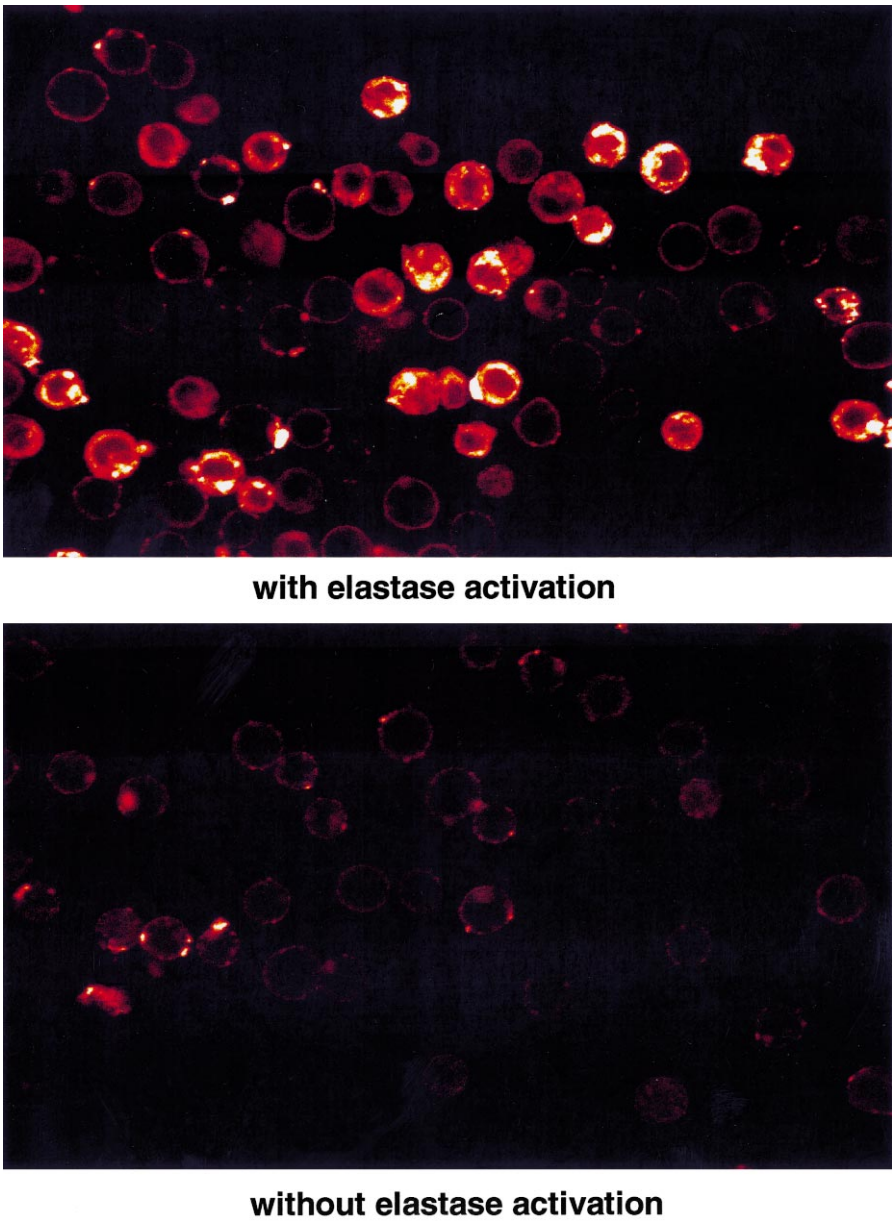

Fig. 6. Confocal microscopy of DODAP/MeO-suc-AAPV-DOPE liposome interaction with HL60 cells. DODAP/MeO-suc-AAPV-DOPE liposomes labeled with 0.75 mol% *N*-NBD-PE and 0.75 mol% *N*-Rho-PE were incubated alone or with elastase (5 μg elastase/100 nmol lipid) for 2 h at 37°C as described in Fig. 5. After washing, cells were observed for *N*-Rho-PE fluorescence. Color range of 0–255 pixel values (0, bottom; 255, top) is given in upper right corner of each image in Fig. 7.

were dialyzed and diluted into a buffer. Incubation of HL60 cells with this mixture of such freeze-thawed liposomes and free TMR-dextran did not result in HL60 cell labeling (Fig. 7). Only elastase activated, intact DODAP/Me-suc-AAPV-DOPE liposomes loaded with TMR-dextran could fuse and deliver dextran to cells.

3.5. Elastase activated binding, lipid mixing, and calcein delivery of DODAP/MeO-suc-AAPV-DOPE liposomes with adherent ECV304 endothelial cells

Pretreatment of DODAP/MeO-suc-AAPV-DOPE liposomes with elastase activated these liposomes to

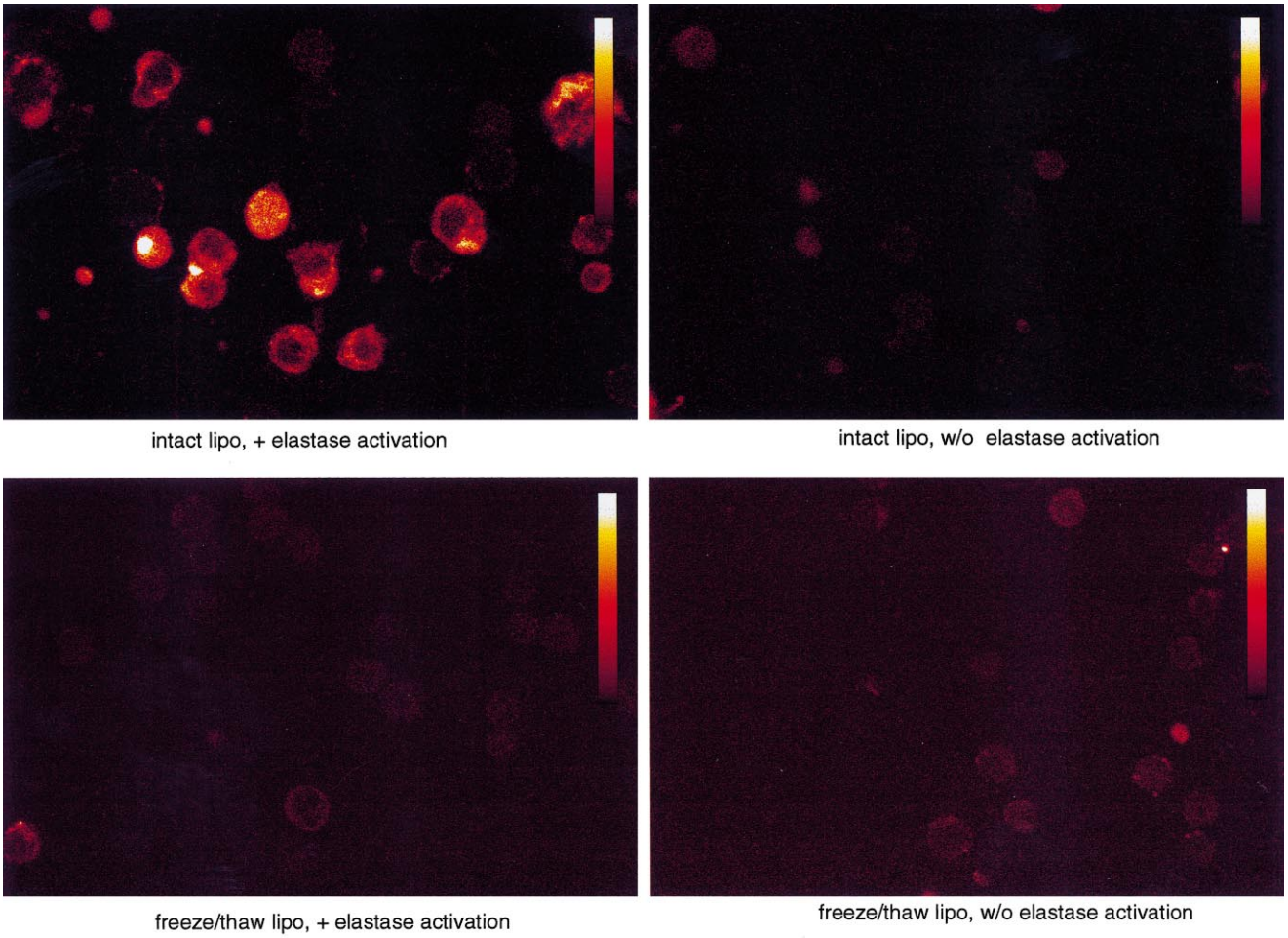

Fig. 7. Delivery of TMR-dextran from DODAP/MeO-suc-AAPV-DOPE liposomes to HL60 cells. DODAP/MeO-suc-AAPV-DOPE liposomes loaded with 10 000 MW TMR-dextran were incubated alone or with elastase (5 μg elastase/100 nmol lipid) for 2 h at 37°C. Liposomes were then added directly to cells or subjected to five freeze-thaw cycles to release encapsulated TMR-dextran. 40 nmol of intact or freeze-thawed liposomes were mixed with 2×10^5 HL60 cells and incubated for 30 min at 37°C, pH 5. After washing, cells were observed for TMR-dextran fluorescence by confocal microscopy. Color range of 0–255 pixel values (0, bottom; 255, top) is given in upper right corner of each image.

bind and fuse with HL60 cells when the pH was artificially lowered to mimic the endosomal environment (Fig. 7). We next tested whether a similar effect could be observed when elastase treated liposomes were internalized by endocytosis. In order to accomplish this it was necessary to bind these liposomes to cells under physiological conditions at pH 7.4. This was done via a biotin-streptavidin linkage. We turned to an adherent cell line, ECV304 endothelial cells, to circumvent complications arising from biotin-streptavidin mediated aggregation of the suspension HL60 cells. ECV304 cells were sequentially treated with biotinylated-WGA- streptavidin. DODAP/MeO-suc-AAPV-DOPE liposomes containing trace amounts of *N*-biotinyl cap-PE and pretreated with or without elastase were then added to the cells and incubated at pH 7.4. Elastase pretreated liposomes exhibited enhanced binding, as compared to untreated liposomes (Fig. 8), perhaps as a result of decreased negative surface charge or better accessibility of the biotinyl group. It appears that in this case the elastase activation augments the biotin-streptavidin mediated binding. The biotin-streptavidin linkage was required, as the absence of biotin-streptavidin treatment abrogated any binding of untreated or elastase pretreated liposomes at this pH

(unpublished data). Liposomes with or without elastase activation were localized within perinuclear endocytic vesicles (Fig. 10). Despite the enhanced binding of elastase pretreated liposomes and uptake into endocytic vesicles there was only a slight increase in lipid mixing of these liposomes with ECV304 cells (Fig. 8). This may be due to the nature of the assay. The fluorescence dequenching assay requires the diffusion of the *N*-NBD-PE and *N*-Rho-PE probe after lipid mixing. Diffusion of the fluorescent lipid probes after fusion with the endosomal membrane may be insufficient to completely diminish the resonance energy transfer. Therefore an aqueous contents delivery assay was employed.

DODAP/MeO-suc-AAPV-DOPE liposomes were loaded with self-quenched concentrations of calcein, a fluorescent aqueous probe. After extensive dialysis to remove unencapsulated calcein these liposomes were pretreated with or without elastase and bound to ECV304 cells by biotinylated WGA-streptavidin. Elastase pretreated liposomes displayed relatively rapid calcein dequenching that increased over the course of several hours (Fig. 9). Maximal dequenching of calcein appears to be achieved after 2 h at 37°C, which was consistent with the time course of

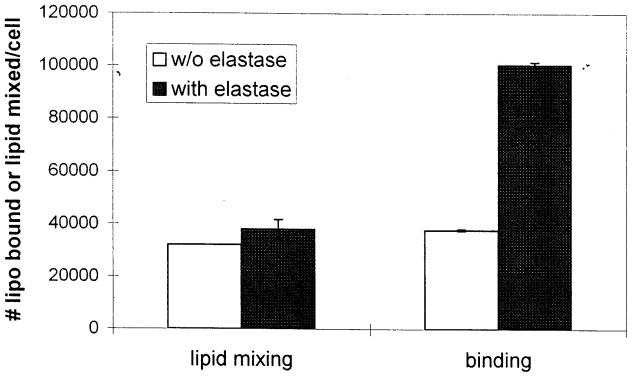

Fig. 8. Binding/lipid mixing of DODAP/MeO-suc-AAPV-DOPE liposomes with ECV304 cells at pH 7.4. DODAP/MeO-suc-AAPV-DOPE liposomes containing *N*-NBD-PE and *N*-Rho-PE were incubated alone or with elastase (5 μg elastase/100 nmol lipid) for 2 h at 37°C. 100 nmol of pretreated liposomes were added to ECV304 cells that had been pretreated with biotinylated WGA and streptavidin (see Section 2). After a 30 min incubation at room temperature the cell monolayer was washed to remove unbound liposomes and samples were incubated for additional 2 h at 37°C. Binding and lipid mixing were determined by monitoring *N*-Rho-PE and *N*-NBD-PE fluorescence, respectively.

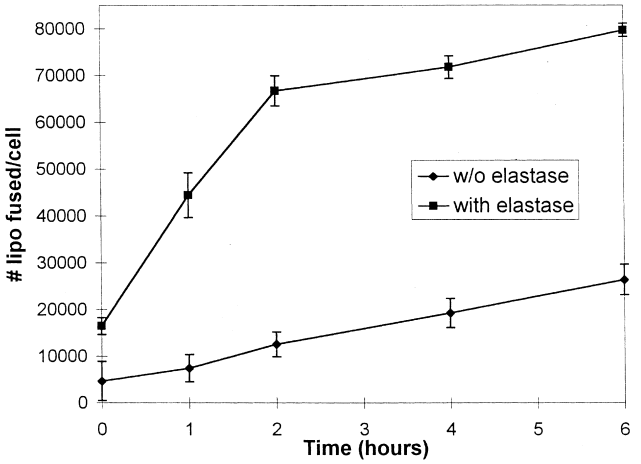

Fig. 9. Elastase triggered calcein delivery from DODAP/MeO-suc-AAPV-DOPE liposomes to ECV304 cells at pH 7.4. DODAP/MeO-suc-AAPV-DOPE liposomes loaded with self-quenched concentration of calcein and *N*-Rho-PE as a lipid marker were incubated with or without elastase (5 μg elastase/100 nmol lipid) for 2 h at 37°C. 100 nmol liposomes were added to ECV304 cells that had been pretreated with biotinylated WGA and streptavidin. After a 30 min incubation at room temperature the cell monolayer was washed to remove unbound liposomes and samples were incubated for 0, 1, 2, 4, or 6 h at 37°C. At the given times calcein delivery was determined by monitoring calcein fluorescence.

DODAP/MeO-suc-AAPV-DOPE liposome endocytosis (unpublished data) and cationic lipid:DNA complex uptake [31]. The apparent delivery of calcein was completely dependent upon liposome-cell interactions, as calcein loaded liposomes subjected to identical conditions of elastase pretreatment and pH 5 environment but without co-incubation with cells did not demonstrate any increase in calcein fluorescence dequenching (unpublished data). DODAP/MeO-suc-AAPV-DOPE liposomes that had not been treated with elastase exhibited much less initial calcein dequenching that slowly increased over time, albeit not to levels observed with the elastase activated liposomes (Fig. 9). Repeated freeze-thaw cycles of DODAP/MeO-suc-AAPV-DOPE liposomes results in almost complete release of encapsulated calcein. Incubation of this mixture of freeze-thawed liposomes and released free calcein with ECV304 cells resulted in only 2–3% of the calcein uptake observed with cells incubated with intact liposomes (unpublished data). This result indicates the fluorescence dequenching observed with elastase pretreated intact

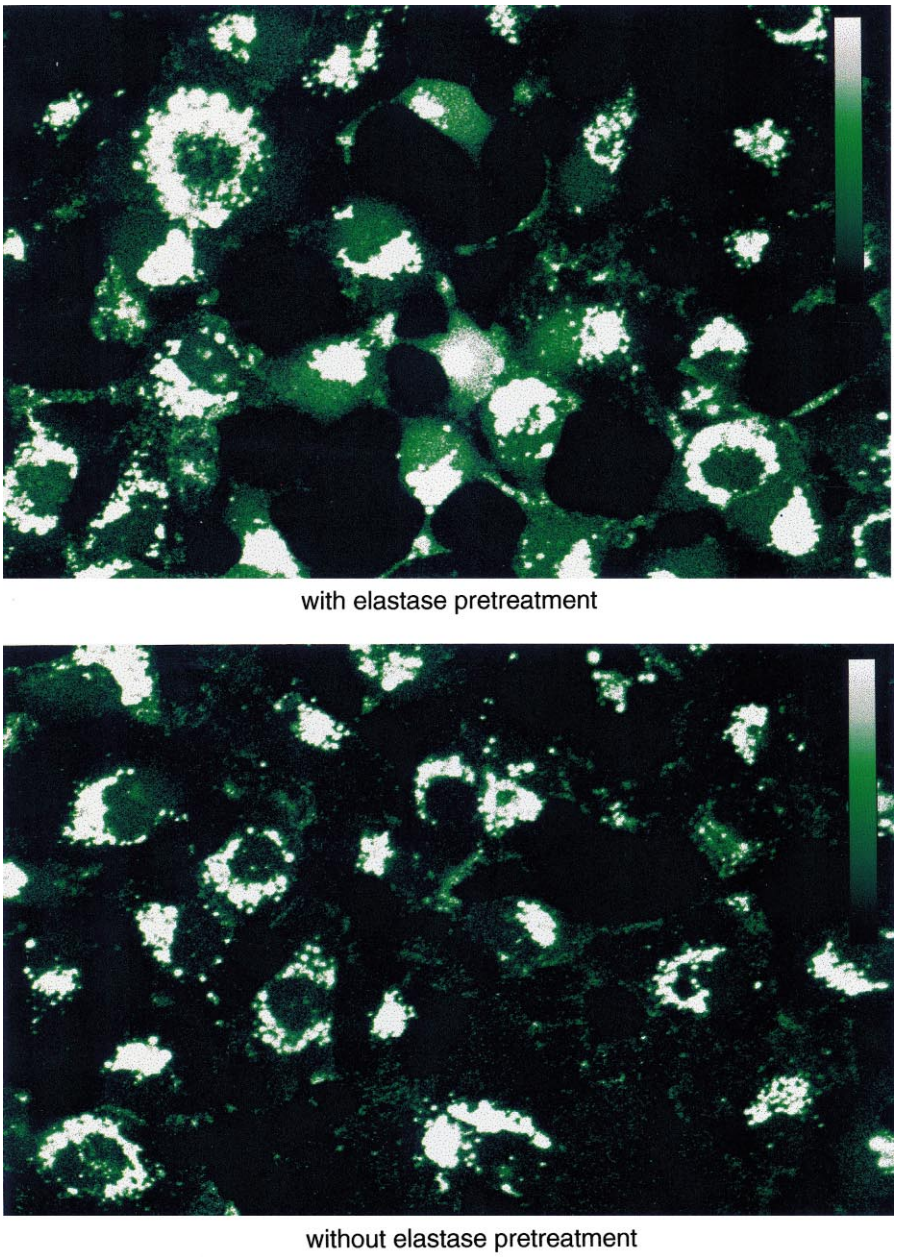

Fig. 10. Confocal microscopy of elastase activated calcein delivery from DODAP/MeO-suc-AAPV-DOPE liposomes to ECV304 cells at pH 7.4. DODAP/MeO-suc-AAPV-DOPE liposomes loaded with a quenched concentration of calcein were incubated alone or with elastase (5 μg elastase/100 nmol lipid) for 2 h at 37°C. 100 nmol of pretreated intact liposomes were added to ECV304 cells which had been pretreated with biotinylated WGA and streptavidin. After a 30 min incubation at room temperature the cell monolayer was washed to remove unbound liposomes and samples were incubated for an additional 4 h at 37°C. Cells were observed by confocal microscopy for calcein fluorescence. Color range of 0–255 pixel values (0, bottom; 255, top) is given in upper right corner of each image.

liposomes was due to delivery and not to non-specific uptake of free calcein.

Confocal microscopy of ECV304 cells incubated with elastase pretreated DODAP/MeO-suc-AAPV-DOPE liposomes contained bright fluorescent perinuclear localized vesicles that appear to be due to the uptake of the calcein loaded liposomes into endosomes. Importantly, these cells also had diffuse fluo-

rescence within the cells (Fig. 10) that is indicative of calcein delivery into the cytosol. The diffuse fluorescence was not due to non-specific uptake of calcein released from liposomes prior to endocytosis, as loaded liposomes subjected to freeze-thaw cycles did not result in fluorescently labeled cells (unpublished data). Unactivated liposomes were also endocytosed into vesicles near the nucleus. However, there was no diffuse fluorescence visible with these liposomes. Quantitation of the total fluorescence/cell area showed ECV304 cells incubated with elastase activated DODAP/MeO-suc-AAPV-DOPE liposomes had more than twice the amount of calcein fluorescence as those incubated with unactivated liposomes. Without elastase pretreatment there does not appear to be significant delivery of the encapsulated calcein into the cell cytoplasm.

4. Discussion

We previously demonstrated that liposomes containing the *N*-Ac-AA-DOPE peptide-lipid could be activated to lipid mix with target liposomes and fuse with RBC ghosts [1]. In this study, enzymatically triggered liposomes have been induced to mix lipids and fuse with adherent cells. The critical modifications required for increased fusogenicity are the creation of a new peptide-lipid that exhibits greater sensitivity and specificity for elastase cleavage, and the reformulation of liposomes with DODAP. This latter change decreases the initial positive charge and thereby enhances sensitivity to elastase mediated cleavage.

Creating a peptide-lipid with increased sensitivity and selectivity was accomplished by substituting *N*-Ac-AA with MeO-suc-AAPV, a highly sensitive substrate for elastase mediated cleavage [24]. Conjugation of this peptide to DOPE yielded a peptide-lipid with greatly enhanced susceptibility to elastase cleavage (Figs. 1 and 2), relative to *N*-Ac-AA-DOPE [1]. The increased sensitivity was accompanied by greater selectivity of cleavage. Proteinase K is a relatively non-specific protease that was able to cleave the ala-ala dipeptide from the *N*-Ac-AA-DOPE peptide-lipid [1]. In contrast, almost no DOPE was generated by incubation of proteinase K with MeO-suc-AAPV-DOPE (unpublished data). The improved selectivity of MeO-suc-AAPV-DOPE cleavage increases the likelihood that liposomes containing this peptide-lipid will be activated to fuse specifically by elastase.

The concentration of elastase used for most of the experiments in this study (12.5 μg elastase/ml = 0.42 μM) is threefold less than the amount of active elastase found, for example, in the epithelial lining fluid of children with cystic fibrosis [28] and is 15–20 times less than that found in adults with cystic fibrosis [9]. Our preliminary experiments have shown that the contents of granules isolated from human neutrophils were also capable of cleaving MeO-suc-AAPV-DOPE to DOPE (unpublished data). The minimum number of neutrophils required to provide crude neutrophil granule proteins capable of cleaving MeO-suc-AAPV-DOPE to DOPE was also less than the number of neutrophils reported in 20 μl of cystic fibrosis epithelial lining fluid [28]. These results suggest the amount of elastase required to activate DODAP/MeO-suc-AAPV-DOPE liposomes may not be a limiting factor for the use of these liposomes in vivo.

In our previous studies liposomes were formulated with the permanently cationic lipid, DOTAP [1]. Including a cationic lipid in the liposomal formulation is intended to promote closer interaction between liposomal and negatively charged cellular membranes after the initial binding. Preparation of liposomes with a cationic lipid and MeO-suc-AAPV-DOPE in equimolar amounts creates a charge balanced liposome that converts to an overall positive charge after enzyme cleavage. This is due to conversion of the negatively charged MeO-suc-AAPV-DOPE to the zwitterionic DOPE after elastase cleavage. Enhanced binding of elastase activated DODAP/Me-suc-AAPV-DOPE (1:1) liposomes to both HL60 and ECV304 cells was observed (Figs. 3–5 and 8), suggesting the charge conversion was capable of promoting binding. The increased binding was not due to bridging between liposomes and cells by the positively charged elastase, as heat inactivated elastase did not mediate the increase in binding (Fig. 5A).

DODAP was chosen as the cationic lipid in the current studies for several reasons. The structure of DODAP differs from DOTAP only in the presence of a tertiary amine instead of a quaternary amine. This structural difference translates to maximal pro-

tonation at pH 5 and 20% positive charge at pH 7.4 [29]. The pH dependence of DODAP/Me-suc-AAPV-DOPE liposomal binding/lipid mixing with HL60 cells (Fig. 4) supports the role of DODAP in liposomal binding and indicates these liposomes are capable of binding in the low pH environment of the endosome.

Because elastase is positively charged at pH 7.4, MeO-suc-AAPV-DOPE in liposomes containing 50% DOTAP was not cleaved (unpublished data), whereas the peptide-lipid was readily cleaved to DOPE in DODAP/MeO-suc-AAPV-DOPE liposomes (Fig. 2). Therefore, DODAP confers an advantage in enzymatic activity. The loss of negative charge on the liposomal surface as cleavage proceeds may partially explain the limited cleavage obtained after a 2 h incubation, i.e. elastase is less efficiently bound to the surface of liposomes that have been digested extensively.

Another advantage of DODAP is that a larger change in surface charge is ultimately possible without including so much positively charged lipid that liposome destabilization occurs or non-specific interaction with cells occurs before enzymatic cleavage or endocytosis. The ultimate change in net charge is from approximately 40% net negative surface charge to approximately 15% net positive surface charge under the conditions of our experiments. DODAP may also limit the non-specific association of these liposomes with negatively charged cell membranes until after endocytosis. In contrast, DOTAP is a potent binding agent at physiological pH [32]. Finally, the net negative charge of liposomes composed of DODAP and MeO-suc-AAPV-DOPE at physiological pH could limit liposomal aggregation and increase in vivo circulation [33].

Elastase treatment of DODAP/Me-suc-AAPV-DOPE liposomes triggered fusion between these liposomes and HL60 cells at low pH (Fig. 7). Interestingly, there was no uptake of fluorescent dextran within these cells when incubated with elastase treated, freeze-thawed liposomes and the accompanying free TMR-dextran. This suggests the fusion of DODAP/Me-suc-AAPV-DOPE liposomes with cells is a non-leaky event. If not, we would have expected to observe TMR-dextran within HL60 cells, due to entry of free dextran through the liposome-cell fusion contacts.

While elastase activated calcein delivery into live ECV304 cells was clearly demonstrated (Fig. 10), the mechanism of delivery is not yet certain. Possibilities include uptake of free calcein across the cellular plasma membrane prior to endocytosis, leakage of calcein from liposomes into the endosomal compartment followed by either transport across or disruption of the endosomal membrane, or fusion between liposomal and cellular membranes and delivery of encapsulated calcein into the cell cytoplasm.

The fluorescence dequenching of calcein encapsulated within DODAP/Me-suc-AAPV-DOPE liposomes remained unchanged after elastase treatment as well as at pH 5 (unpublished data), indicating these liposomes were stable under conditions mimicking the endosomal environment. However, contact with the endosomal membrane or exchange of cellular proteins into the liposome could alter the stability of the liposomes within endosomes. Notably, unactivated liposomes did not exhibit significant calcein delivery (Figs. 9 and 10) despite binding and uptake into cells. It appears the elastase activated liposomes are capable of further actions that permit calcein to reach the cytoplasm, consistent with the much larger calcein dequenching observed for elastase activated liposomes (Fig. 9). Transport of free calcein directly across the plasma membrane appears unlikely, as calcein released from freeze-thawed DODAP/Me-suc-AAPV-DOPE liposomes did not result in any calcein within the cell despite the release of nearly all of the encapsulated calcein (unpublished data). Non-specific diffusion of leaked calcein across the endosomal membrane remains a possible route of entry. However, it is expected that both elastase treated and untreated liposomes may exhibit this behavior. This was clearly not the case (Figs. 9 and 10). Although we are unable to distinguish between endosomal disruption and fusion as the primary entry mechanism, either is sufficient for the purpose of liposome mediated delivery.

The successful transfer of aqueous contents probes into adherent cells by elastase activation of DODAP/MeO-suc-AAPV-DOPE liposomes suggests that such systems may have great utility for both drug and gene delivery once appropriate modifications are made to maximize serum stability and minimize potential serum interference with binding and fusion. Coupling selective enzyme triggering with targeting

ligands may increase the likelihood of generating liposomes that are stable until localized to the site of interest. Efforts are also under way to encapsulate DNA within enzyme triggerable liposomes for selective transfection and gene expression.

References

[1] C.C. Pak, S. Ali, A.S. Janoff, P. Meers, Biochim. Biophys. Acta 1372 (1998) 13–27.
[2] V.C. Anderson, D.H. Thompson, Biochim. Biophys. Acta 1109 (1992) 33–42.
[3] D.E. Bennett, D.F. O'Brien, J. Am. Chem. Soc. 116 (1994) 7933–7934.
[4] J.L. Nieva, F.M. Goni, A. Alonso, Biochemistry 28 (1989) 7364–7367.
[5] A.S. Luk, E.W. Kaler, S.P. Lee, Biochemistry 32 (1993) 6967–6973.
[6] D. Kirpotin, K. Hong, N. Mullah, D. Papahadjopoulos, S. Zalipsky, FEBS Lett. 388 (1996) 115–118.
[7] L. Hu, R.J.Y. Ho, L. Huang, Biochem. Biophys. Res. Commun. 141 (1986) 973–978.
[8] L.A. Liotta, P.S. Steeg, W.G. Stetler-Stevenson, Cell 64 (1991) 327–336.
[9] N.G. McElvaney, R.C. Hubbard, P. Birrer, M.S. Chernick, D.B. Caplan, M.M. Frank, R.G. Crystal, Lancet 337 (1991) 392–394.
[10] D.D. Rees, J.D. Brain, Am. J. Physiol. 269 (1995) L195–L202.
[11] M. Berger, R.U. Soerensen, M.F. Tosi, D.G. Dearborn, G. Doring, J. Clin. Invest. 34 (1989) 1302–1313.
[12] S. Suter, U.B. Schaad, H. Tegner, K. Ohlsson, D. Desgrandchamps, F.A. Waldvogel, J. Infect. Dis. 153 (1986) 902–909.
[13] N. Al-Haik, D.A. Lewis, G. Struthers, Agents Actions 15 (1984) 436–442.
[14] P. Gysen, M. Malaise, S. Gaspar, P. Franchimont, Clin. Rheumatol. 4 (1985) 39–50.
[15] V.V. Damiano, A. Tsang, U. Kucich, W.R. Abrams, J. Rosenbloom, P. Kimbel, M. Fallahnejad, G. Weinbaum, J. Clin. Invest. 78 (1986) 482–493.
[16] G.L. Snider, D.E. Ciccolella, S.M. Morris, P.J. Stone, E.C. Lucey, Ann. NY Acad. Sci. 624 (1991) 45–59.
[17] E. Cavarra, P.A. Martorana, F. Gambelli, M. de Santi, P. van Even, G. Lungarella, Lab. Invest. 75 (1996) 273–280.
[18] G. Doring, Am. J. Respir. Crit. Care Med. 150 (1994) S114–S117.
[19] J.-I. Yamashita, M. Ogawa, S. Ikei, H. Omachi, S.-I. Yamashita, T. Saishoji, K. Nomura, H. Sato, Br. J. Cancer 69 (1994) 72–76.
[20] J. Yamashita, M. Ogawa, M. Abe, N. Hayashi, Y. Kurusu, K. Kawahara, T. Shirakusa, Chest 111 (1997) 885–890.
[21] B. Starcher, P. O'Neal, R.D. Granstein, S. Beissert, J. Invest. Dermatol. 107 (1996) 159–163.
[22] H. Sato, T. Takahisa, Y. Okada, J. Cao, A. Shinagawa, E. Yamamoto, M. Seiki, Nature 370 (1994) 61–65.
[23] C.A. Owen, M.A. Campbell, P.L. Sannes, S.S. Boukedes, E.J. Campbell, J. Cell Biol. 131 (1995) 775–789.
[24] J.C. Powers, B.F. Gupton, A.D. Harley, N. Nishino, R.J. Whitley, Biochim. Biophys. Acta 485 (1977) 156–166.
[25] G.R. Bartlett, J. Biol. Chem. 234 (1959) 466–468.
[26] E.G. Bligh, W.J. Dyer, Cancer J. Biochem. Physiol. 37 (1959) 911–917.
[27] D.K. Struck, D. Hoekstra, R.E. Pagano, Biochemistry 20 (1981) 4093–4099.
[28] R. Birrer, N.G. McElvaney, A. Rudeberg, C. Wirz Sommer, S. Liechti-Gallati, R. Kraemer, R. Hubbard, R.G. Crystal, Am. J. Respir. Crit. Care Med. 150 (1994) 207–213.
[29] A.L. Bailey, P.R. Cullis, Biochemistry 33 (1994) 12573–12580.
[30] M.C. Kielian, M. Marsh, A. Helenius, EMBO J. 5 (1986) 3103–3109.
[31] J. Zabner, A.J. Fasbender, T. Moninger, K.A. Poellinger, M.J. Welsh, J. Biol. Chem. 270 (1995) 18997–19007.
[32] L. Stamatatos, R. Leventis, M.J. Zuckerman, J.R. Silvius, Biochemistry 27 (1988) 3917–3925.
[33] P.L. Ahl, S.K. Bhatia, P. Meers, P. Roberts, R. Stevens, R. Dause, W.R. Perkins, A.S. Janoff, Biochim. Biophys. Acta 1329 (1997) 370–382.