

PROTON-INDUCED PERMEABILITY AND FUSION OF LARGE UNILAMELLAR VESICLES BY COVALENTLY CONJUGATED POLY(2-ETHYLACRYLIC ACID)

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ABSTRACT

Proton sensitive large unilamellar vesicles (LUV) were constructed by immobilization of the pH sensitive synthetic polymer poly(2-ethylacrylic acid) onto the outer monolayer. Thiolated poly(2-ethylacrylic acid) (PEAA-SH) was covalently conjugated to the surface of LUVs composed of egg phosphatidylcholine (EPC) and cholesterol (Chol) through the thiol-reactive maleimide lipid MPB-DSPE (N-(4-(p-maleimidophenyl)butyryl)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine). The resulting PEAA- LUVs were shown to be stable at neutral pH (pH 7.0 to 8.0). Under acidic conditions, however, protonation of PEAA resulted in interaction with both the membrane it was linked to and the membrane of target vesicles, causing membrane destabilization and release of vesicle contents. Moreover, conjugated PEAA is shown to mediate fusion with target membranes in a pH dependent manner. PEAA-mediated permeabilization and vesicle-vesicle fusion occurred only when the polymer was covalently linked to the LUV surface. Proton dependent fusion of PEAA-LUVs was also observed with erythrocyte ghosts. This pH-dependent release

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of vesicle contents and fusion of PEAA-LUVs occurred below pH 6.8, which is well within the pH range expected to be encountered inside the endosomes in the endocytic pathway, indicating the potential of PEAA-LUVs as a drug carrier system for intracellular drug delivery.

Key Words: Liposome; Intracellular delivery; Carrier system; Membrane fusion; Polyelectrolyte

INTRODUCTION

Liposomes have demonstrated considerable promise as drug delivery systems in both animal studies and human clinical trials (1-4). Enhanced therapeutic properties have been reported for a variety of drug classes including antineoplastics, antibiotics and antifungal agents. These benefits are believed to reflect liposome mediated improvements in drug pharmacokinetics and biodistribution. In particular, small, long circulating liposomes have been shown to accumulate at tumor sites, sites of infection and inflammation, and can therefore allow preferential drug delivery to the site of action (5,6).

The primary mechanism of liposome uptake by these target cells is via endocytosis followed by sequestration of the vesicles into acidic intracellular vacuoles (7). The therapeutic potential is consequently dependent on the efficient drug release from liposomes in the endosomal compartment and release into the cytoplasm. pH-sensitive liposomes are designed to trigger the release of encapsulated drugs when they are exposed to a lower pH-environment. This approach takes advantage of the considerably lower pH in the endosomes (pH 4.5-6.5) compared to pH 7.4 in the blood (8). In addition, sites of primary tumors, metastases, inflammation and infection have reduced local pH environments (9-12). The major advantage of utilizing pH-sensitive liposomes is that they remain stable at physiological pH, and only release their contents when an acidified environment is encountered such as in endosomes following internalization through the endocytic pathway.

Most of the pH-sensitive liposomes studied earlier have utilized the strong tendency of unsaturated phosphatidylethanolamine (PE) to form non-bilayer structures (13,14). The unsaturated PE adopts a bilayer structure only in combination with bilayer-forming lipids. To obtain pH-sensitive liposomes, acidic lipids such as oleic acid, palmitoylhomocysteine, cholesterylhemisuccinate, N-succinyldioleoylphosphatidylethanolamine and dipalmitoylsuccinylglycerol, have been employed in order to form stable liposomes with PE (15-21). These lipids form bilayer structures when charged at neutral pH, however, when exposed to an acidic environment, these pH-sensitive lipids become protonated and lose their stabilizing potential. This results in bilayer destabilization, fusion and in the release of entrapped contents. However, pH-sensitive liposomes can have serious stability problems under various conditions (22,23).

Fusion proteins of viruses, such as hemagglutinin (HA), mediate viral cell entry and fusion (24-27). The viral proteins catalyze fusion between the viral envelope and endosomal membrane at acidic pH. Based on information obtained from viral proteins, a number of fusion peptides (amphiphilic peptides) have been synthesized and shown to be fusion-active in a pH-dependent manner (28-30). pH-sensitive liposomes known as virosomes have been developed with reconstituted hemagglutinin, the membrane fusion protein (31,32). These vesicles show pH-dependent fusion with target membranes. However, the immunogenicity of viral proteins and peptides limits their application as systemic delivery systems.

Recently, synthetic polymers have been described that exhibit a pH-dependent effect on the lipid bilayer. Such polymers have potential for construction of pH-sensitive liposomes with utility in drug delivery systems. These systems have gained attention due to their non-immunogenic nature, straightforward large-scale synthesis and their potential for structural modifications. Poly(2-ethylacrylic acid) (PEAA) (33,34) and succinylated poly(glycidol) (35) have been reported to mediate the H⁺-induced release of liposomal contents from small unilamellar vesicles (SUV) under acidic conditions.

In the present study PEAA was covalently coupled to large unilamellar vesicles (LUV) and the potential of the polymer to induce pH dependent membrane destabilization and membrane fusion was evaluated. The data demonstrate that the thiolated PEAA can be conjugated to the surface of non-fusogenic maleimide-containing-LUVs. The resulting PEAA-LUVs exhibit H⁺-induced membrane destabilization with release of calcein from both LUVs PEAA is linked to and from target vesicles. Most importantly, PEAA linked to LUVs can induce pH-dependent fusion with target membranes.

MATERIALS AND METHODS

Materials

All chemical reagents were of the highest purity commercially available and were used without further purification. The polymer PEAA (poly(2-ethylacrylic acid); MW =20000) was fluorescently labeled with a trace amount of pyrene (0.1 mol% pyrene to permit quantification of PEAA and was obtained from Dr. D. A. Tirrell(34). EPC and MPB-DSPE were purchased from Northern Lipids (Vancouver, BC, Canada). [3H] cholesteryl hexadecyl ether (3H-CHE) was obtained from Du Pont (NEN Research Product). Cholesterol, dithiothreitol (DTT), Ellman's reagent [5,5'-dithiobis(2-nitrobenzoic acid)] (DTNB), calcein, 2-aminoethanethiol (AET), 4-pyrrolidinopyridine and 1,3-dicyclohexylcarbodiimide (DCC) were purchased from Aldrich Chemical Corp. 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N- (lissamine Rhodamine B sulfonyl) (Rh-PE) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-1,3-benzoxadiazol-4-yl) (NBD-PE) were obtained from Avanti Polar-Lipids, Inc. (Alabaster, AL, USA).

Thiolation of PEAA

PEAA (225 mg, 2.25 mmol repeating unit), 2-aminoethanethiol (48 mg, 0.62 mmol) and 4-pyrrolidinopyridine (10 mg, 0.07 mmol) were dissolved in 30 ml dimethylformide (DMF). DCC (510 mg, 2.5 mmol) was added to the reaction mixture while stirring. After 24 h at room temperature, 0.5 ml water was added to hydrolyze excess DCC, the precipitated dicyclohexyl urea (DCU) was removed by centrifugation (2800 rpm, 10 min) in a Beckman GS-6 Centrifuge (Beckman Instruments, Fullerton, CA, USA) and the supernatant was filtered. The filtrate was concentrated to approximately 1 ml and the crude product in DMF was diluted with 3 ml water, acidified to pH 2 and triturated with diethyl ether (3 x 40 ml). The solid product was dissolved in methanol (5 ml), precipitated with ether and dried under vacuum (70% yield, 162 mg).

The number of thiol groups introduced into PEAA was determined using Ellman's reagent. Briefly, PEAA-SH was first treated with DTT (20 mM) in HBS (20 mM HEPES, 100 mM NaCl, pH 7.5) for 30 min. The reduced PEAA-SH was purified on a 1.1 x 20 cm Sephadex G-25 column (SIGMA,) equilibrated with HBS. The polymer fractions were collected and quantified by pyrene fluorescence intensity (Excitation at 345 nm and emission at 379 nm). Aliquots (50 μ l) of each fraction were treated with equal volumes of DTNB solution and diluted with phosphate buffer at pH 8 to 1 ml. The thiol content was calculated based on the absorbance of the solution measured at 412 nm with a Beckman DU-7 spectrophotometer (Beckman Instruments, Fullerton, CA, USA) and the extinction coefficient of $\epsilon = 1.36 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$. The degree of modification was 2.6 mol%.

Preparation of Large Unilamellar Vesicles (LUVs)

LUVs were prepared by extrusion as described previously (36). Appropriate amounts of lipid mixtures with MPB-PE (EPC/Chol/MPB-DSPE; 53 : 45 : 2 mol/mol/mol) or without MPB-PE (EPC/Chol; 55:45 mol/mol) in chloroform containing trace amounts of $^3\text{H-CHE}$ (1.33 $\mu\text{Ci}/4 \mu\text{mol}$), were dried under a stream of nitrogen gas to form a homogeneous lipid film. Trace amount of solvent was then removed under a vacuum overnight. The lipid film was hydrated in HBS buffer (pH 7.5) with or without calcein (100 mM) by vortex mixing. The resulting multilamellar vesicles (MLVs) were freeze/thawed (liquid nitrogen / 55°C) 5 times and extruded 10 times through two stacked 100 nm polycarbonate filters (Nuclepore, Pleasanton, CA) employing an extrusion device (Lipex Biomembranes, Inc., Vancouver, BC, Canada) at 55°C. Non-entrapped free calcein was removed by chromatography using a 1.1 X 20 cm Sepharose CL-6B column (Sigma Chemical Co., St. Louis, MO, USA) equilibrated with HBS buffer.

Coupling of PEAA-SH to LUV

Freshly purified PEAA-SH was incubated with LUVs (EPC/Chol/MPB-DSPE; 53 : 45 : 2 mol/mol/mol) with or without entrapped calcein at a ratio of 2.5 :

1 (w/w, lipid-to-polymer) in HBS (pH 7.5) for 16 h at room temperature. Unreacted excess PEAA-SH was removed on a 1.1 X 20 cm Sepharose CL-6B column. To determine non-covalent adsorption of polymer to LUVs thiolated PEAA was incubated with LUVs lacking MPB-PE and non-thiolated PEAA with LUVs containing MPB-PE. Following Sepharose CL-6B chromatography the amount of adsorbed polymer was detected measuring pyrene fluorescence.

Determination of Liposome Size

Liposome size was determined by quasi-elastic light scattering (QELS) using a Nicomp 370 submicron particle sizer (Santa Barbara, CA, USA).

Calcein Release Assay

The fluorescent calcein was encapsulated as an aqueous permeability marker in LUVs at self-quenching concentrations as described above. Subsequently, the solutions of polymer-conjugated LUVs (calcein) and related control samples were acidified to the desired pH. Calcein release was determined by measuring the fluorescence intensity using an Aminco Bowman@ Series 2 luminescence spectrofluorometer (SLM-AMINCO, Urbana, IL) at 530 nm (slit width 4 nm) under steady-state excitation at 495 nm (slit width 4 nm). To minimize the pH effect on calcein fluorescence, the liposome solutions was adjusted to pH 6.5-7.5 prior to the fluorescence measurements.

For the pH-dependent calcein release from the PEAA-coupled LUV (calcein), the sample solution was adjusted with 1% HCl to the desired pH. After equilibration for 10 minutes an aliquot was withdrawn, the pH adjusted to 6.5-7.5 and the fluorescence intensity measured. To determine the kinetics of calcein release, the initial pH of 7.5 was maintained for a period of 10 minutes, prior to acidification to pH 5. At the different time points an aliquot was withdrawn and the fluorescent intensity measured following adjustment of the pH to 6.5-7.5. The maximal fluorescence intensity (F_{\max}), representing the complete release of encapsulated calcein, was determined after solubilization of the vesicles with Triton X-100 (10% of lipid concentration). The percentage of calcein released was calculated using the following equation:

$$\% \text{ Release} = \frac{F_t - F_0}{F_{\max} - F_0} \times 100$$

F_t is the fluorescence intensity at time t, or at a given pH and F_0 represents the initial fluorescence intensity.

Fusion Assays

Liposome fusion was monitored by a fluorescence assay based on the resonance energy transfer (RET) between the lipid headgroup-labeled probes, NBD-PE and Rh-PE, as described by Struck *et al.* (37). In a typical experiment, the

donor vesicles contained both probes (0.5 mol% each of NBD-PE and Rh-PE) and the polymer was conjugated to their surfaces. The Donor vesicles were mixed in different ratios with probe-free LUVs (Acceptor). These mixed vesicles were maintained at their initial pH of 7.5 for 10–15 minutes and then acidified to pH 5. At different time points, an aliquot was withdrawn, the pH was adjusted back to 6.5–7.5 and the fluorescence intensity of NBD-PE was determined at 530 nm with excitation at 495 nm. The fusion of labeled LUVs (Donor) with probe-free LUVs (Acceptor) results in probe dilution and an increased distance between the NBD-PE and Rh-PE, thereby decreasing RET efficiency. The maximal fluorescence intensity (F_{\max}) in each sample was determined following the solubilization of vesicles with Triton X-100 (10% of lipid concentrations) to reach an infinite dilution of the probe. The percentage of fusion (or lipid dilution) was calculated using the following equation:

$$\% \text{ Fusion} = \frac{F_t - F_0}{F_{\max} - F_0} \times 100$$

where F_t is the fluorescence intensity at each time point and F_0 the initial fluorescence intensity.

RESULTS

Coupling of PEAA-SH to MPB-LUVs

The strategy for coupling the synthetic polyelectrolyte poly(2-ethylacrylic acid) (PEAA; MW 20,000) to the surface of calcein loaded large unilamellar lipid vesicles (LUV) is outlined in Figure 1. Prior to the coupling the thiolated poly(2-ethylacrylic acid) (PEAA-SH containing 2.6 mol% thiol groups) was treated with an excess of DTT to reduce any disulfide bond and DTT was then removed by gel filtration chromatography (Sephadex G-25). The PEAA lacking thiol modification used for control experiments was also subjected to the same DTT treatment. The amount of PEAA coupled to the surface of LUVs was calculated based on the fluorescence of the polymer bound pyrene measured in the LUV fractions following removal of excess free PEAA by chromatography on a Sepharose CL-6B column.

When PEAA-SH was coupled to the surface of calcein loaded LUVs composed of EPC and MPB-DSPE (98 : 2 mol/mol) calcein was released rapidly during this procedure even at neutral pH. To overcome this leakage problem, cholesterol was incorporated into the LUVs. With LUVs composed of EPC/Chol/MPB-DSPE (53 : 45 : 2 mol/mol/mol) only minor calcein release was detected during the coupling procedure and also during subsequent incubation of the PEAA-LUVs at pH 7.5. Typically 40–50 μg polymer per mg of lipid was coupled to the surface of the LUVs (Table 1). The calcein entrapped in the LUV had no significant effect on the coupling reaction. Based on the pyrene fluorescence a significant amount of polymer did also adsorb nonspecifically to the LUVs and could not be removed

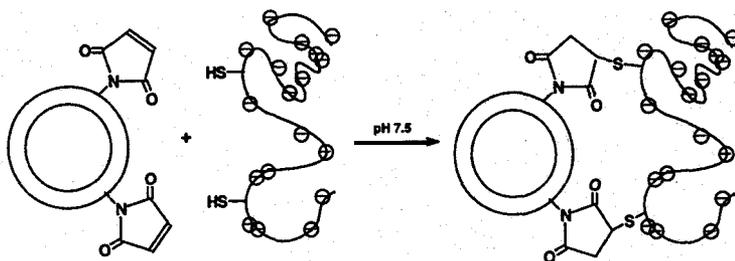
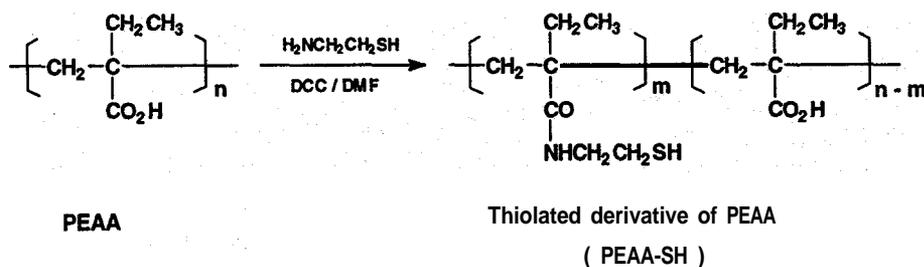


Figure 1. Synthetic scheme for preparing thiolated derivative of PEAA (PEAA-SH) and subsequent coupling of PEAA-SH to maleimide containing large unilamellar vesicles.

even by repeat chromatography. Therefore, in all the following experiments LUVs with adsorbed polymer were included as a control. The net amount of polymer covalently conjugated to LUVs was calculated by subtracting the amount of non-specific adsorbed PEAA-SH from the total amount of polymer associated with vesicles.

The mean diameters of LUVs and LUVs with coupled PEAA determined

Table I. Conjugation of PEAA-SH to MPB-LUVa

MPB-LUVsb	POLYMER CONJUGATED LUVsc
	($\mu\text{g}/\text{mg}$, polymer/lipid)
LUVS	42 ± 6
LUVs(calcein)	50 ± 4
LUVs(NBD/Rh)	36 ± 6

aLipid compositions for LUVs and LUVs (calcein) were EPC/Chol/MPB-PE (53 : 45 : 2, mol/mol); for LUVs(NBD/Rh) were EPC/Chol/MPB-PE/NBD-PE/Rh-PE(52 : 45 : 2 : 0.5 : 0.5: mol%).

bReactions were carried out at 2.5: 1 lipid-to-polymer ratio overnight.

cAmount of PEAA conjugated with was corrected by subtracting the amount of non-specifically adsorbed polymer.

by QELS were 128 ± 30 nm and 134 ± 34 nm respectively. The slight size increase after the coupling reaction is likely due to the flexible, conjugated polymer on the liposomes rather than inter-vesicular crosslinking. Other laboratories observed similar increases in vesicle size following insertion of polymers into the lipid membrane (38,39).

Proton-Induced Release of LUV Contents

Calcein was encapsulated in the LUV at selfquenching concentrations as a marker. LUVs with coupled PEAA (PEAA-LUVs) were stable at pH 7.5, however, upon acidification to pH 6 and lower, calcein fluorescence increased indicating release of the entrapped calcein to over 80% (Figure 2). In the control sample with non-covalently adsorbed polymer only a minor fluorescence increase was detected upon acidification. Addition of Triton X-100 released the entrapped calcein completely in both samples. The release of calcein upon acidification from PEAA-LUVs occurred very rapidly within less than 10 minutes (Figure 3). In contrast, in the different control samples where PEAA was only adsorbed to the LUVs surface only limited leakage was observed over the 20 minutes time course following acidification. The extend and kinetics of the proton-induced calcein release was not affected by the lipid concentration since the calcein release pattern remained unchanged following addition of empty LUVs. However, an increase in the acid-induced calcein release of approximately 20% was observed when

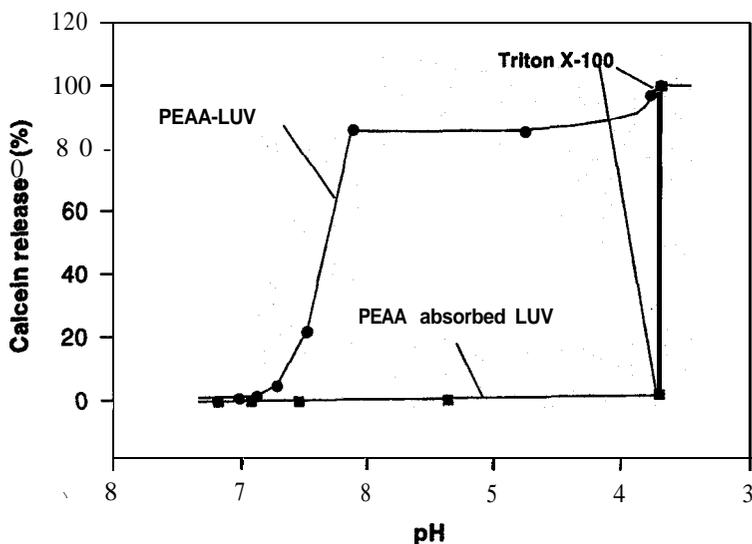


Figure 2. pH-dependent calcein release from LUVs with surface coupled PEAA. PEAA-SH was coupled to LUVs composed of EPC/Chol/MPB-PE(53 : 45 : 2; mol/mol/mol) with encapsulated calcein. The pH was adjusted to the desired pH and after 10 minute incubation the pH was increased to 6.5-7.5 and calcein fluorescence measured at 530 nm with excitation at 495 nm. Percent calcein release from PEAA-LUVs (•) and LUVs with adsorbed polymer (■) is plotted as a function of pH.

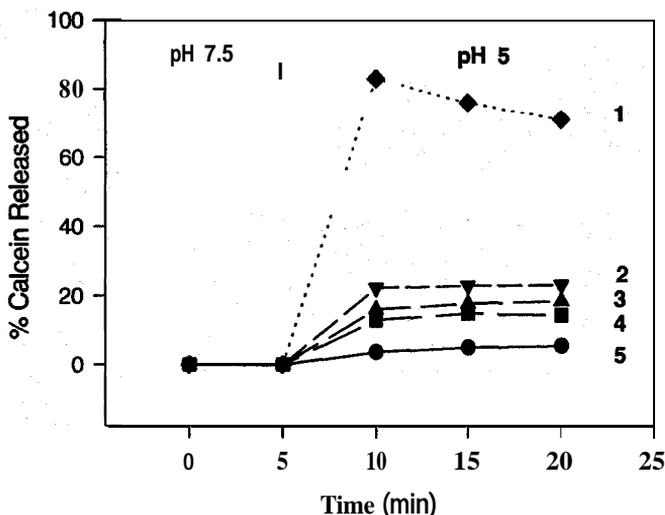


Figure 3. H⁺-induced calcein release from LUVs as a function of time. LUVs with surface conjugated PEAA (as in Figure 2) and various control-LW preparations with entrapped calcein were incubated at pH 5 for times indicated. Following incubation the pH was adjusted to pH 6.5 – 7.5 and the calcein fluorescence determined as in Figure 2. Calcein release is plotted as a function of incubation time for (1) PEEA-LUVs (◆); (2) LUVs (EPC:Chol; 55:45 mol/mol) lacking MPB-PE with thiolated polymer non-specifically adsorbed (7); (3) LUVs with MPB-PE (EPC:Chol:MPB-DSPE; 53:45:2 mol/mol/mol) and non-specifically adsorbed non-thiolated PEAA (A); (4) LUVs with MPB-PE and non-specifically adsorbed non-thiolated PEAA (■) and (5) LUVs without any polymer (○).

empty LUVs with coupled PEAA were added (data not shown). This suggests that the conjugated polymer can destabilize not only the liposome bilayer it is linked to but also the membranes of neighboring liposomes. The ability to destabilize the membrane of targeted LUVs was confirmed by the results of pH-dependent calcein release from target vesicles. In this experiment equal amounts of PEEA-LUVs without entrapped calcein and target-LWs with entrapped calcein but lacking PEAA were mixed together. When the pH of this mixture was adjusted to pH 5, over 30% of calcein was released (Figure 4). In the control sample, where PEAA was only adsorbed to the LUVs, no calcein release from the target-LUVs was observed upon acidification.

Proton-Induced Fusion of PEAA-LUVs

The fusion of PEAA-LUVs was studied using the resonance energy transfer assay (BET assay) with Rh-PE and NBD-PE as fluorescent probes. An increase in NBD-PE fluorescence as consequence of probe dilution indicates lipid mixing due to the vesicle-vesicle fusion. A low percentage of the fluorescent-labeled lipids Rh-PE and NBD-PE, with either a more hydrophobic or a negative charge in their head group incorporated in the bilayer, had no significant effect on polymer

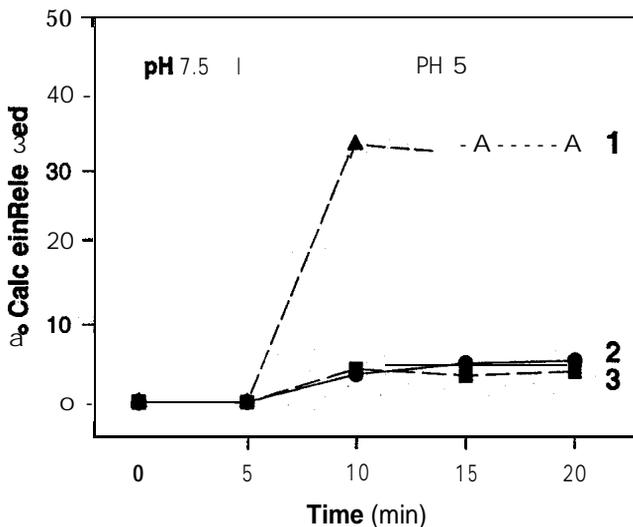


Figure 4. Release of calcein from target vesicles. Target vesicles (100 $\mu\text{g}/\text{ml}$ lipid) composed of EPC:Chol (55:45 mol/mol) with entrapped calcein were incubated with PEAA-LUVs (100 $\mu\text{g}/\text{ml}$ lipid) prepared as in Figure 2 but without entrapped calcein. Following incubation at pH 5 for times indicated an aliquote was removed, the pH adjusted to pH 6.5 – 7.5 and calcein fluorescence determined. The percentage of calcein release is plotted as a function of incubation time for (1) PEAA-LUVs with calcein loaded target vesicles (A); (2) LUVs lacking MPB-PE with non-specifically adsorbed thiolated polymer together with calcein loaded target LUVs (\bullet) and (3) target LUVs only (m).

coupling (Table 1). PEAA-LUVs containing Rh- and NBD-PE (donor) were mixed with unlabeled LUVs (acceptor) and the NBD-fluorescence was monitored following acidification. At neutral pH no fluorescence change was observed, while, upon acidification to pH 5 the fluorescence increased rapidly indicating lipid mixing / fusion (Figure 5). In control samples with PEAA adsorbed to LUVs only minor fluorescence changes were observed following acidification of the medium.

The rate of H^+ -induced fusion of PEAA-LUVs was very fast. As shown in Figure 5, the NBD-fluorescence increased within 5 minute following acidification equivalent to almost 80% lipid mixing / fusion. On the other hand, only 8% lipid mixing was detected for polymer adsorbed-LUVs over the same time period following acidification. These fluorescence changes were similar to the one observed with donor vesicles without adsorbed PEAA (Figure 5). The extent of lipid mixing / fusion was dependent on the ratio of donor PEAA-LUVs to acceptor vesicles in the mixture. When the concentration of donor vesicles was kept constant, the degree of fusion increased with increasing concentration of probe-free acceptor vesicles (Figure 6). In this lipid-mixing assay the degree of probe dilution, the distance between NBD-PE and Rh-PE respectively, is a measure for the extent of fusion. Therefore, a higher concentration of probe-free acceptor vesicles is expected to lead to more rounds of fusion between the donor and acceptor,

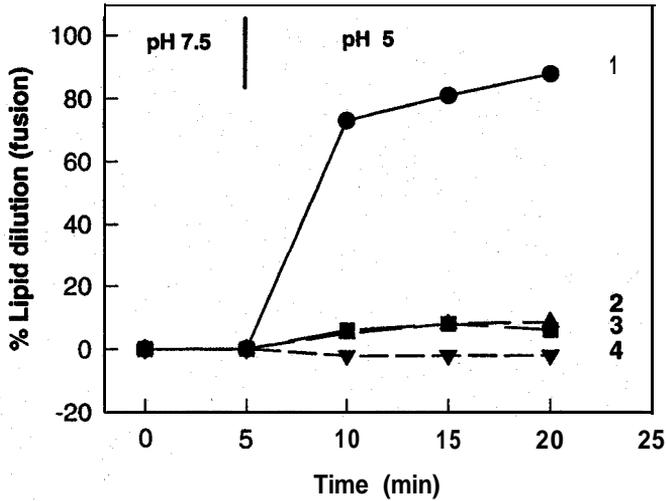


Figure 5. H⁺-induced lipid mixing / fusion of PEAA-LUVs with target LUVs. LUVs containing 0.5% each NBD and Rh-PE with conjugated PEAA (100 μg/ml lipid) were mixed with target (acceptor) vesicle (400 μg/ml lipid) composed of EPC/Chol (55 : 45 mol/mol). After 5 minutes incubation at pH 7.5 the pH was lowered to pH 5 and aliquots removed at times indicated. Following adjustment of the pH to pH 6.5-7.5 the calcein fluorescence was determined at 530 nm using excitation at 495 nm. Percent of lipid mixing was calculated as outlined in Method and Material and plotted as a function of incubation time for (1) PEAA-LUVs (○); (2) LUVs (EPC:Chol:MPB-DSPE : NBD-PE : Rh-PE; 52 : 45 : 2 : 0.5 : 0.5 mol%) with adsorbed non-thiolated polymer (▲); (3) LUVs without adsorbed polymer (■) and (4) PEAA-LUVs only without target vesicles (▼).

resulting in higher probe dilution as was observed in Figure 6. No detectable fluorescence change was observed in absence of probe-free acceptor vesicles as expected, since no probe dilution can occur. However, in this case a significant increase in the vesicle size occurred following acidification (Table 2). The size changes of the vesicles, summarized in Table 2 for different donor / acceptor ratios, is an additional indication for actual fusion. There was a significant increase in vesicle size with the isolated PEAA-LUVs itself and in the presence of acceptor vesicles. In the absence of polymer or with polymer absorbed to vesicles, acidification did not result in a change of the vesicle size.

Fusion with Erythrocyte Ghosts

The ability of PEAA-LUVs to fuse with a biological membrane was studied using erythrocyte ghosts. PEAA-LUVs containing Rh- and NBD-PE were mixed with erythrocyte ghosts to give a molar ratio of 1: 20. Fluorescence change was monitored following acidification to pH 5.0. Extensive probe dilution / fusion was observed with PEAA-LUVs (Figure 7). The extent of fusion of LUVs with only adsorbed PEAA with erythrocyte ghosts was much smaller than with PEAA coupled to the LUVs, with 30% compared to 60-80%. Fusion occurred rapidly within the first 10 minutes after acidification.

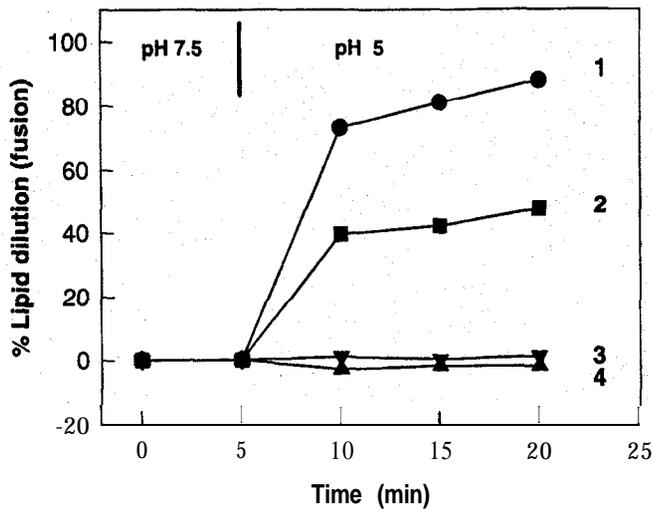


Figure 6. PEAA-LUV fusion is dependent on the concentration of target vesicles. PEAA-LUVs with 0.5% NBD-PE and 0.5% Rh-PE (100 $\mu\text{g/ml}$ lipid) were incubated with LUVs composed of EPC and Chol (55:45 mol/mol) at different concentrations. The extent of fusion was determined by measuring the NBD fluorescence at times indicated for PEAA-LUV to target LUV ratio (w/w) of 1:4 (○); 1:1 (■) and 1:0 (◐). For the 1:0 ratio 200 $\mu\text{g/ml}$ lipid were used. As control for the effect of adsorbed polymer 100 $\mu\text{g/ml}$ LUVs (NBD/Rh) with adsorbed non-thiolated polymer were incubated with 400 $\mu\text{g/ml}$ target LUVs (A).

Table 2. Changes of vesicle size following H⁺-induced fusion of PEAA-LUVs and polymer adsorbed LUVs (donor) with different amount of target vesicles (acceptor).

DONOR LUVs-a	DONOR/ACCEPTOR RATIO-b	FUSION-c	VESICLE SIZE (nm)
without polymer	1:4	0	128 \pm 30
absorbed PEAA	1:4	5	134 \pm 28
Conjugated PEAA	1:4	89	449 ^d
Conjugated PEAA	1:1	44	2619 ^d
Conjugated PEAA	1:0	NA-e	4203 ^d

a-Lipid composition for donor LUVs were EPC/Chol/MPE-PE/NBD-PE/Rh-PE (52 : 45 : 2 : 0.5 : 0.5 : 2, mol%) for PEAA-LUVs and EPC/CHOL/NBD-PE/Rh-PE)52 : 45 : 2:0.5 :0.5: mol%) with non-specifically adsorbed PEAA, or without polymer.

b-Lipid composition for acceptor LUVs were EPC/Chol (55:45 mol/mol)

c -Determined by lipids-mixing assay

d-Heterogeneous particle size, only the peak with highest percentage was reported.

e-There was no fluorescence change since fusion between PEAA-LUVs does not result in Rh/NBD probe dilution.

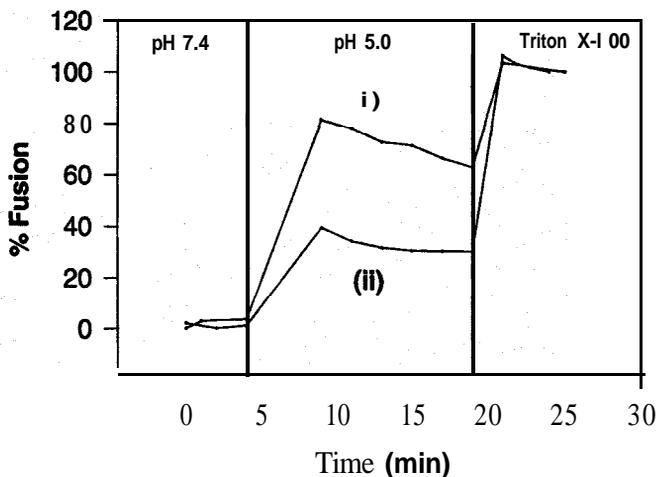


Figure 7. Fusion of PEAA-LUVs with erythrocyte ghosts. PEAA-LUVs containing 0.5 mol% of each NBD-PE and Rh-PE prepared as in Figure 5 were mixed with human erythrocyte ghosts at a molar ratio of 1:20 at pH 7.4. Following acidification to pH 5.0 the fluorescence change was monitored at 530 nm with excitation at 495 nm. (i) represents PEAA-LUVs and (ii) PEAA non-specifically absorbed to LUVs.

DISCUSSION

This study demonstrates that the hydrophobic polyelectrolyte, PEAA, when coupled to the surface of large unilamellar vesicles (LUVs) can destabilize the lipid bilayer it is linked to and also bilayers of target membranes in a pH dependent manner causing leakage of vesicle contents. Furthermore, it is shown that the surface linked PEAA can promote fusion of the LUV with target vesicles and biological membranes at lower pH. The capacity of PEAA to cause membrane destabilization and fusion indicate the potential of PEAA as an active component in a drug carrier system to facilitate intracellular drug release.

Previously it was shown that at lower pH PEAA could disrupt the lipid bilayer of small unilamellar vesicles (SUVs) composed of EPC resulting in release of entrapped calcein (34). However, SUV with PEAA linked to their surface exhibit only limited stability at pH 7.4 and complete leakage of vesicle content occurred within 24 h when stored at 4°C. In addition, in our hands significant leakage of SUV contents can occur during the PEAA coupling process (data not shown). SUVs in general are less stable than LUVs of the same lipid composition due to their higher curvature and less ordered state of the lipid molecules in the bilayer compared to LUV. In any case, SUVs are not really useful as drug carrier systems for systemic applications because of their low loading capacity due to a limited internal volume and their unfavorable pharmacokinetics, being cleared rapidly from the blood through the RES.

The lipid composition of EPC and cholesterol (5545 mol%) chosen to form the LUVs permitted the coupling of PEAA to LUV surface without loss of vesicle

contents. Coupling to preloaded LUVs without significant loss of contents is important in order to employ active loading methods for drugs into LUVs and to reduce excess loss of costly drugs. In contrast to the PEAA-SUVs the PEAA-LUVs described here, were stable at pH 7.4 at 4°C and no significant calcein leakage was observed over a three-week period. The PEAA linked to LUVs at a density as low as 5 % w/w (polymer/lipid) is shown to disrupt the LUV bilayer causing rapid release of vesicle content in a pH dependent manner. Furthermore, it is demonstrated that PEAA could also rupture the lipid bilayer of target membranes (Figure 4). The observed calcein release induced in target LUVs by vesicles with conjugated polymer of approximately 30% compared to 80% for PEAA-LUVs indicates that penetration of the polymer into the membrane bilayer it is coupled to is energetically more favorable than insertion into the bilayer of target vesicles.

Interaction of the of hydrophobic polyelectrolyte PEAA with lipid membranes at lower pH is anticipated as the result of the protonation of carboxylic groups leading to an increase in the hydrophobicity of the polymer. These hydrophobic segments are unstable in an aqueous environment and are therefore expected to penetrate into the hydrophobic interior of a lipid bilayer through Van der Waals forces and, at high polymer concentration, leading to solubilization / micellization of lipid membrane as shown by Tirrell and coworkers (40,41). At lower concentrations with PEAA linked to the surface of a lipid bilayer the polymer probably causes defects in the lipid packing and disruption of the hydration layer upon acidification leading to release of vesicle contents. Apparently some polymer-lipid interaction exists even at pH 7.4 based on the leakage observed with PEAA-SUVs but it is not disruptive for the LUV bilayer.

The pH dependent rapid and extended lipid mixing observed with polymer conjugated-LUVs and target LUVs (Figures 5 and 6) in conjunction with the detected increase in vesicle size (Table 2) are suggesting that PEAA actually triggers a complete fusion event and not just semifusion in conjunction with aggregation. The final size was dependent on the concentration of target vesicles and decreased with increasing concentration of target vesicles (Table 2). The role of PEAA in triggering membrane fusion is further supported by the observation that the fusion is dependent on the polymer concentration. For an inter-membrane adhesion polymer one would expect an increase in vesicle aggregation with increasing polymer concentration but a decrease in vesicle fusion due to steric constraints caused by the polymer. However, the degree of fusion of PEAA-LUVs with the target vesicles increased with increasing polymer concentration (data not shown) suggesting that PEAA plays both an adhesion and fusion role.

The capacity of the conjugated PEAA on the liposomal surface to promote fusion seems to be related to two common characteristics of many membrane-associated proteins, to confer specificity to the initial inter-membrane interaction and to trigger the fusion reaction. PEAA plays an intricate role as an adhesion agent and as trigger and modulator of the actual vesicle-vesicle fusion event. The driving force for the vesicle-vesicle contact with protonized (neutral) PEAA is

Van der Waals attraction. This is different from the electrostatic interactions of the positively charged fusion peptides and fusion proteins such as melittin (42), cytochrome c (43), clathrin (44), cardiotoxin (45), and polylysine (43) with negatively charged liposomes. Insertion of the hydrophobic segments of PEAA into the membranes of neighboring liposomes is leading to close vesicle-vesicle contact, facilitates local dehydration at the contact site and causes defects in the packing of the membrane lipids and eventually fusion.

The nature of the pyrene labeled fraction that strongly adsorbed to the LUVs was not characterized further but could consist of either small molecular weight polymer or a contaminant from polymer synthesis containing a relatively high percentage of pyrene. Thiolation of the polymer or the presence of MPB on the liposomal surface did not affect the amount of polymer adsorption. However, the adsorbed polymer did not cause any significant pH-induced membrane destabilization or fusion. Therefore, we conclude that the pH-induced activities observed are indeed specific to the conjugated polymer.

Lipid vesicles have been shown to be taken up by cells via endocytosis (7). The pH required to trigger content release and fusion of PEAA-LUVs is well within the range of the pH in maturing endosomes. Therefore, these PEAA-coated LUVs present a potential drug carrier system with an intracellular drug release mechanism, triggering drug release from LUVs and facilitating escape from the endosomal compartment into the cytoplasm by fusion and membrane disruption. There are five beneficial features that distinguish the PEAA based pH-sensitive vesicles presented here from previously reported pH-sensitive liposomes (22,24,31,34,35). 1) The internal volume to carry drugs of large unilamellar vesicles is much larger than in SUV and LUV exhibit extended circulation lifetime while SUV are cleared rapidly from the blood. Moreover, SUV are less stable and tend to fuse with any cell membrane independent of pH because of the lipid asymmetry in the bilayer. 2) The lipid composition employed of egg phosphatidylcholine and cholesterol (55/45, mol/mol) results in vesicles that are known for their stability and drug retention during circulation in the blood following iv application and also during storage. This sets PEAA-LUVs apart from pH-sensitive liposomes composed of fusogenic PE and pH-sensitive lipids. 3). The range of pH triggering fusion and content release from PEAA-LUVs is well within the range of the pH encountered in maturing endosomes. Therefore, PEAA-LUVs taken up by endocytosis are expected to efficiently destabilize the endosomal membrane and release their contents into the cytoplasm. 4) PEAA is expected to be non-immunogenic and should permit repeated injections of PEAA-LUVs. 5) The most important advantage of PEAA-LUVs is that the pH-induced permeability and release rate of the vesicles can be modulated by varying the amount of polymer conjugated onto the liposomes, modification of the polymer's structure (46), or its molecular size (47). These characteristics demonstrate the potential of the PEAA-LUVs as drug carriers with efficient intracellular drug delivery triggered by acidic pH. In particular, the capacity to fuse with target membranes provides a release mechanism particularly for larger and highly charged membrane

impermeable drugs such as proteins and poly-oligonucleotides (antisense, plasmid).

In conclusion, polymer conjugated-LUVs were formed by coupling a modified PEAA to pre-formed MPB-LUVs. The resulted polymer conjugated-LUVs are stable under physiological conditions, but they efficiently released their liposomal contents upon acidification and most importantly have the capacity to fuse with target membranes. The extensive calcein release and fusion of PEAA-LUVs observed suggest that PEAA is an efficient fusogen that can be employed to form pH-sensitive fusogenic vesicles.

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ABBREVIATIONS

PE, phosphatidylethanolamine; PEAA, poly(2-ethylacrylic acid); SUV, small unilamellar vesicle; LUV, large unilamellar vesicle; PEAA-SUV, PEAA covalently conjugated to SUVs; PEAA-LUVs, PEAA covalently conjugated to LUVs; PEAA-SH, thiolated poly(2-ethylacrylic acid); EPC, egg phosphatidylcholine; Chol, cholesterol; MPB-DSPE, N-(4-(p-maleimidophenyl)butyryl)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine, sodium salt; 3H-CHE, [3H] cholesteryl hexadecyl ether; DTT, dithiothreitol; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); DCC, 1,3-dicyclohexylcarbodiimide; Rh-PE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine Rhodamine B sulfonyl); NBD-PE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-1,3-benzoxadiazol-4-yl); RET, resonance energy transfer; AET, 2-aminoethanethiol;

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