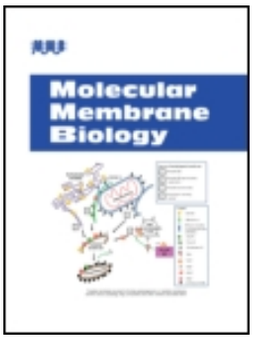

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Alkylated derivatives of poly(ethylacrylic acid) can be inserted into preformed liposomes and trigger pH-dependent intracellular delivery of liposomal contents

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Summary

Poly(ethylacrylic acid) (PEAA) is a pH-sensitive polymer that undergoes a transition from a hydrophilic to a hydrophobic form as the pH is lowered from neutral to acidic values. In this work we show that pH sensitive liposomes capable of intracellular delivery can be constructed by inserting a lipid derivative of PEAA into preformed large unilamellar vesicles (LUV) using a simple one step incubation procedure. The lipid derivatives of PEAA were synthesized by reacting a small proportion (3%) of the carboxylic groups of PEAA with C_{10} alkylamines to produce C_{10}-PEAA. Incubation of C_{10}-PEAA with preformed LUV resulted in the association of up to 8% by weight of derivatized polymer with the LUV without inducing aggregation. The resulting C_{10}-PEAA-LUV exhibited pH-dependent fusion and leakage of LUV contents on reduction of the external pH below pH 6.0 as demonstrated by lipid mixing and release of calcein encapsulated in the LUV. In addition, C_{10}-PEAA-LUV exhibited pH dependent intracellular delivery properties following uptake into COS-7 cells with appreciable delivery to the cell cytoplasm as evidenced by the appearance of diffuse intracellular calcein fluorescence. It is demonstrated that the cytoplasmic delivery of calcein by C_{10}-PEAA-LUV could be inhibited by agents (bafilomycin or chloroquine) that inhibit acidification of endosomal compartments, indicating that this intracellular delivery resulted from the pH-dependent destabilization of LUV and endosomal membranes by the PEAA component of the C_{10}-PEAA-LUV. It is concluded that C_{10}-PEAA-LUV represents a promising intracellular delivery system for *in vitro* and *in vivo* applications.

Keywords: Liposomes, intracellular delivery, pH-sensitive polymers, poly(ethylacrylic acid), pH-dependent membrane fusion.

Abbreviations: PEAA, poly(2-ethylacrylic acid); LUV, large unilamellar vesicles; PEAA-LUV, LUV coated with alkylated PEAA; ^{3}H-CHE, [^{3}H] cholesteryl hexadecyl ether; DTNB, 5,5′-dithiobis (2-nitrobenzoic acid); AET, 2-aminoethanethiol; EDC, 1, Ethyl-3 (3-dimethylaminopropyl) carbodiimide; 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-1-1,3-benzoxadiazol-4-yl), RET, resonance energy transfer; HBS, 5 mM HEPES in 150 mM NaCl pH 7.5.

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Introduction

Liposomal drug delivery systems have demonstrated clinical utility for improving the efficacy of certain antifungal and anticancer drugs [1–13]. This clinical utility relies in part on the ability of small, long-circulating liposomes to preferentially accumulate in sites of infection, inflammation and tumours following intravenous administration [14–17]. More sophisticated liposomes that are targeted to specific cells using antibodies or other ligands are also being developed [14,18–22]. However, the general utility of liposomes would be significantly improved by an ability to deliver their contents to the interior of target cells. For example, the therapeutic potential of antisense oligonucleotides for the down-regulation of target genes or plasmid DNA for gene therapy applications cannot be realized without a transport vehicle that delivers these macromolecules to the interior of target cells. Presently available liposomal systems are accumulated into cells by endocytosis leading to sequestration in lysosomes and consequent degradation of biologically active cargoes.

Considerable efforts have been made to develop liposomes that are stable under normal physiological conditions but which release their contents in response to environmental changes such as changes in pH or temperature [17,23–39]. Particular attention has been given to the development of liposomal systems that would release their contents under the acidic environments encountered during endocytosis. Such pH sensitive liposomes can be constructed by incorporating pH-sensitive lipids [23–25,35–39] or pH-sensitive polymers [26–34] into the liposomal membrane. In the test tube these systems can usually be demonstrated to release their contents in response to pH changes, however few can achieve pH-dependent delivery of their entrapped cargo to the cytoplasm of cultured cells [24,28].

Previous work in this laboratory has led to the development of liposomes grafted to the pH sensitive polymer poly(ethylacrylic acid) (PEAA), a polymer that becomes membrane lytic at pH values below 6.3 [29]. These pH sensitive liposomes were made by conjugating thiolated PEAA to maleimide-lipid in the outer monolayer of large unilamellar vesicles (LUV). The resulting PEAA-LUV exhibited contents leakage and fusion at pH values below 6, however the coupling of thiolated PEAA to the surface of

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LUV is an irreproducible process that leads to variability in the amount of coupled polymer [29,30].

In the present study a simple one step process was developed to coat preformed LUV with PEAA. This process utilized modified polymers containing a low density of alkyl chains that spontaneously associate with LUV on incubation. It is shown that PEAA-LUV prepared by this method exhibit proton induced membrane destabilization and fusion. It is also demonstrated that PEAA-LUV deliver their contents into the cytoplasm of COS cells in a pH-dependent manner.

Results

C_{10}-PEAA can be post-inserted into preformed LUV without causing aggregation

As previously shown, poly(ethylene glycol) (PEG) polymers attached to a lipid anchor can be post-inserted into preformed LUV employing a simple incubation procedure [40]. In order to determine whether a similar procedure could be employed to attach PEAA to preformed LUV, alkylated derivatives of PEAA containing C_{10}, C_{14} and C_{18} alkyl chains were first synthesized as indicated in Methods. This procedure resulted in modification of 3% of the PEAA carboxyl groups. These alkylated PEAA were then incubated with preformed EPC/Chol (55/45; mol/mol) LUV at a 10% and 20% w/w derivatized PEAA to lipid ratio and the excess alkylated PEAA removed by chromatography employing Sepharose CL-6B column chromatography as illustrated in Figures 1 and 2. The percent insertion of alkylated PEAA into the LUV following chromatography is summarized in Table 1. All three alkyl chains (C_{10}, C_{14} and C_{18}) were sufficient to anchor alkylated PEAA in LUV at levels corresponding to 8% (w/w). The alkylated PEAA that was associated with the LUV appeared to be irreversibly incorporated for all three species of alkyl anchors as subsequent chromatography on a second Sepharose CL-6B column did not result in removal of any additional PEAA. The C_{10} variety of alkylated PEAA (C_{10}-PEAA) was chosen for further investigations of LUV containing derivatized PEAA.

The next series of experiments investigated the effect of the extent of alkylation and the initial ratio of C_{10}-PEAA to LUV on the amount of polymer associated with the LUV. PEAA polymers where 3, 5 and 7% of the carboxyl functions were modified with C_{10} alkyl chains were incubated with LUV at different polymer-to-lipid ratios and the polymer association determined. The results are summarized in Table 2. In the absence of alkylation, only trace amounts of PEAA adsorbed to the LUV. The amount of polymer associated with the LUV increased as the percentage of alkylation increased and also as the initial C_{10}-PEAA concentration increased. A C_{10} anchor density corresponding to modification of 3% of PEAA carboxyl functions did not result in vesicle aggregation either during or after the post-insertion process. Incubation of LUV with C_{10}-PEAA containing 5% alkyl chains resulted in some degree of aggregation as detected by a 24 to 84% increase in vesicle size. More extensive aggregation with an apparent vesicle size of >500 nm was observed for the C_{10}-PEAA in which 7% of the carboxyl functions were modified. The system that had the highest incorporation of C_{10}-PEAA that did not result in appreciable aggregation was the C_{10}-PEAA-LUV system achieved employing C_{10}-PEAA with 3% carboxyl group modification incubated with LUV at a

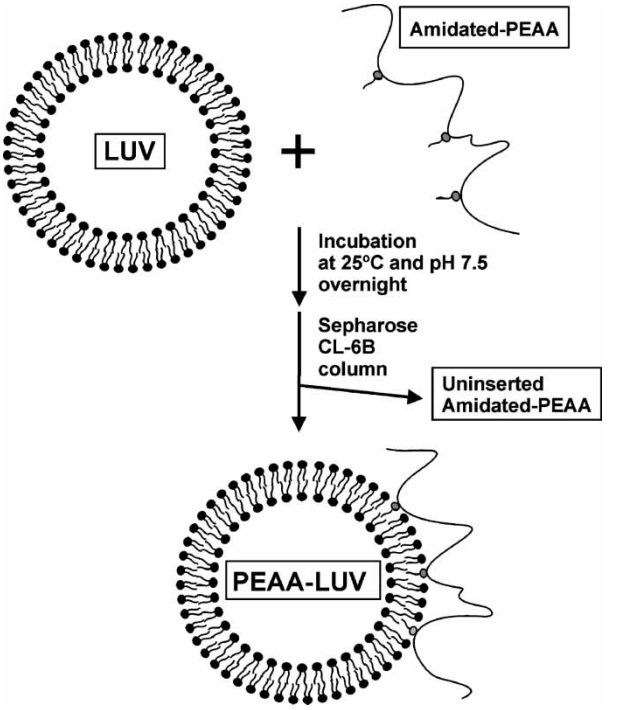


Figure 1. Scheme for the formation of polymer coated liposomes. Alkylated PEAA is added to large unilamellar liposomes resulting in spontaneous insertion of the hydrophobic alkyl chain into the outer monolayer of the lipid vesicles. The alkylated PEAA was formed by randomly modifying 3% of the carboxyl groups in PEAA with alkylamine.

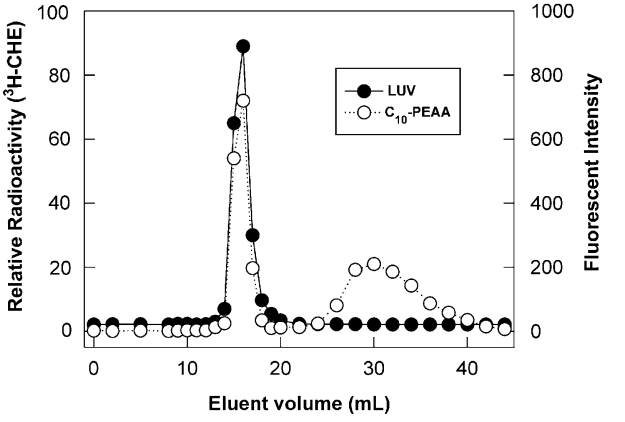


Figure 2. Chromatography profile of polymer coated liposomes. C_{10}-PEAA was added to LUV composed of EPC/Chol (55:45; mol/mol) at a w/w ratio of 20:80 and incubated overnight at room temperature as outlined in Material and Methods. Excess C_{10}-PEAA was removed by chromatography on a Sepharose CL-6B column equilibrated with HBS buffer. Fractions were analyzed for lipids (^{3}H-CHE) and polymer (pyrene fluorescence, excitation at 345 nm and emission at 379 nm) and plotted as a function of eluted volume.

Table 1. Polymer insertion into preformed lipid vesicles as a function of the length of the alkyl chain used to modify the PEAA.

Anchor chain length	Initial polymer-to-lipid ratio[a] (w/w)	% Insertion	% Polymer association[b]
C_{10}	10:90	40	4
	20:80	40	8
C_{14}	10:90	48	4.8
	20:80	38	7.6
C_{18}	10:90	48	4.8
	20:80	39	7.8

[a]Alkylated PEAA (3% of carboxyl groups modified with alkyl chains) was incubated with lipid vesicles (EPC/Chol; 55:45 mol:mol) overnight at room temperature at polymer to lipid ratio as indicated and excess free alkylated PEAA removed by Sepharose CL-6B column chromatography.
[b]Polymer association is expressed in as % of polymer weight of total vesicle weight.

20:80 w/w ratio. This system contained approximately 8% by weight of C_{10}-PEAA and was used for all further experiments.

C_{10}-PEAA-LUV exhibit pH-dependent leakage and fusion

The pH-dependent leakage properties of C_{10}-PEAA-LUV was examined employing systems where the component LUV contained calcein at self-quenching concentrations. These systems were incubated at different pH values and calcein release determined as indicated in Materials & Methods. A typical profile of calcein leakage as a function of pH is shown in Figure 3A. No leakage of calcein was observed for C_{10}-PEAA-LUV at pH 7.5, however at pH values below 7 progressive release of calcein was observed. No significant calcein release was observed for LUV that did not contain the C_{10}-PEAA over the pH range 7.5 to 4.0. The addition of Triton X-100 elicited the complete release of calcein. As shown in Figure 3B, the kinetics of calcein release from C_{10}-PEAA-LUV upon acidification was rapid; significant calcein release was observed as early as 2 min after acidification and over 80% leakage was achieved within 10 min after acidification to pH 5.

Table 2. The effect of the alkyl chain density on the insertion of C_{10}-PEAA into LUV.

Anchor density[a] (%)	Initial polymer concentration[b] (weight %)	Final polymer association[c] (weight %)	Vesicle size (nm)
	12.5	0.5	122
0	20	0.6	122
	33	0.8	122
	12.5	3.7	122
3	20	8.2	110
	33	12	122
	12.5	11	153
5	20	17	185
	33	23	220
	12.5	ND[d]	>500
7	20	ND	>500
	33	ND	>500

[a]Percent carboxyl groups modified with alkyl chains.
[b]LUV (EPC/Chol; 55:45 mol:mol) were incubated with C_{10}-PEAA (weight % as indicated) overnight at room temperature and free C_{10}-PEAA was removed by Sepharose CL-6B column chromatography.
[c]Polymer association is expressed in as% of polymer weight of total vesicle weight.
[d]Not determined.

The pH dependent leakage of calcein from C_{10}-PEAA-LUV was accompanied by inter-vesicular fusion and/or lipid mixing. This was characterized using the resonance energy transfer assay (RET assay) employed to detect lipid mixing and/or fusion events occurring between closely opposed membranes. In this study we used Rh-PE and NBD-PE as fluorescent RET probes as described in Materials & Methods. C_{10}-PEAA-LUV containing Rh- and NBD-PE were mixed with unlabeled C_{10}-PEAA-LUV and the NBD fluorescence was monitored following acidification. As shown in Figure 4A, little or no intervesicular fusion of C_{10}-PEAA-LUV was observed at neutral pH; however, significant fusion occurred when the pH was lowered past 6.5. No fusion was observed for the LUV in the absence of C_{10}-PEAA over the pH range 7.5 to 4.0. As shown in Figure 4B, pH-induced fusion on acidification of C_{10}-PEAA-LUV to pH 5.0 was very rapid and reached equilibrium in as little as 5 min.

C_{10}-PEAA-LUV exhibit pH dependent intracellular delivery of LUV contents following endocytosis

During endocytosis, the endosome containing accumulated material is acidified to pH values as low as 5.0 [41]. The pH sensitive leakage and fusion properties of C_{10}-PEAA-LUV suggest that these systems could exhibit pH-dependent intracellular delivery of LUV contents following uptake into cells. This ability was examined by fluorescence and phase contrast microscopy following uptake of calcein-containing C_{10}-PEAA LUV into COS-7 cells. As shown in Figure 5, incubation of the cells with calcein-containing C_{10}-PEAA-LUV resulted in the observation of bright punctuate and diffuse intracellular fluorescence as early as 5 min after the start of the incubation. Further, the intensity of intracellular fluorescence increased as the amount of C_{10}-PEAA polymer associated with the LUV increased.

Although the preceding results are consistent with pH-dependent intracellular delivery of LUV contents by the C_{10}-PEAA-LUV system, they could also be interpreted as arising from some non-pH dependent leakage of the C_{10}-PEAA-LUV following uptake. The pH-dependent nature of intracellular release from C_{10}-PEAA-LUV was confirmed by pre-treating the COS-7 cells with 100 nM bafilomycin A1, an inhibitor of vacuolar-type H(+)-ATPases or 0.1 mM chloroquine, a lysosomotropic amine. As shown in Figure 5 for chloroquine, the intracellular calcein fluorescence intensity following incubation with C_{10}-PEAA-LUV was significantly reduced by such pretreatments. The inhibitory effects of both reagents were maintained for up to 45 min after addition of the liposomes. In order to confirm that the bafilomycin-induced inhibition of fluorescence was not caused by an inhibition of liposome endocytosis in the bafilomycin-treated cells, COS-7 cells were incubated in 100 nM bafilomycin or control buffer before the addition of the rhodamine labeled

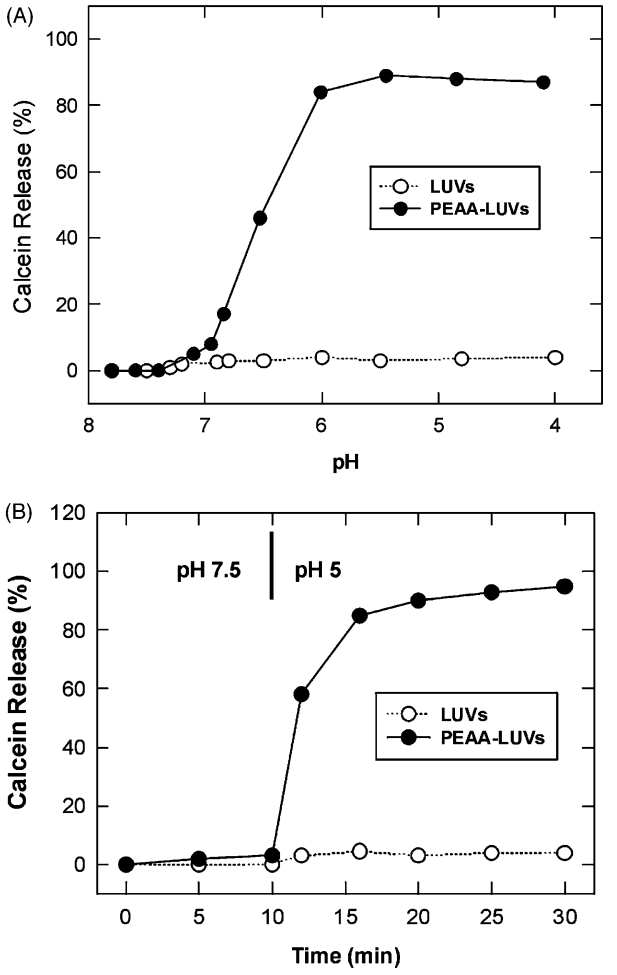


Figure 3. pH dependence and kinetics of pH-induced calcein release from LUV coated with C_{10}-PEAA. C_{10}-PEAA (3% alkyl modification) was inserted into LUV composed of EPC/Chol (55:45 mol/mol) containing calcein (100 mM) as in Figure 2. The pH dependence is demonstrated in panel A: the pH was adjusted to the desired pH and following a 10-min incubation, the pH was increased to 6.5–7.5 and the calcein fluorescence intensity measured at 530 nm with excitation at 495 nm. In panel B, the kinetics of calcein release are shown. In these studies the pH of the LUV solution was kept at 7.5 for 10 min and then the pH was adjusted to 5. At the times indicated an aliquot was removed, the pH was re-adjusted to pH 6.5–7.5 and calcein fluorescence was measured (the temperature was maintained at 25°C). The percent calcein release from C_{10}-PEAA-LUV (●) and LUV incubated with PEAA lacking alkyl chains (○) is plotted as a function of pH in A and incubation time in B.

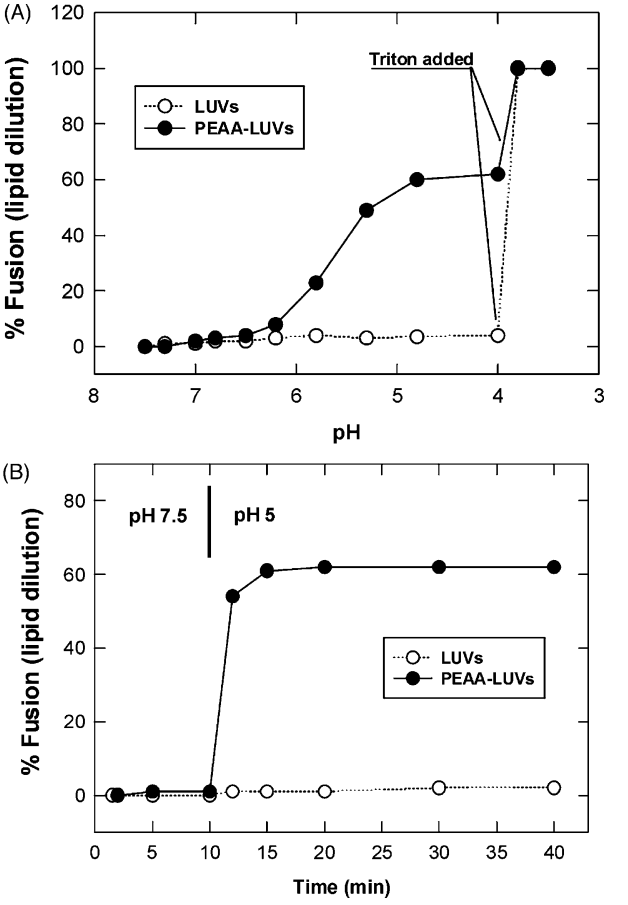


Figure 4. pH dependence and kinetics of proton-induced fusion/lipid mixing of C_{10}-PEAA-LUV with target LUV. C_{10}-PEAA (3% alkyl modification) was inserted into LUV composed of EPC/Chol/NBD-PE/Rh-PE) (54:45:0.5:0.5 mol/mol/mol/mol) containing calcein (100 mM) as in Figure 2. The polymer-coated vesicles (100 μg/ml) were mixed with target LUV (EPC/Chol; 55:45 mol/mol) (400 μg/ml) at 25°C and the pH adjusted as indicated. The pH dependence of fusion/lipid mixing is shown in panel A: the NBD-PE fluorescence intensity was determined after a 10 min incubation at 530 nm with excitation at 465 nm. In panel B, the kinetics of lipid mixing/fusion were determined by incubation at pH 7.5 for 10 min, and then monitoring NDB fluorescence following a reduction in pH to 5. The percent of lipid mixing was calculated for C_{10}-PEAA-LUV (●) and LUV incubated with PEAA lacking alkyl chains (○) as outlined in Materials and Methods and are plotted as a function of pH and time in A and B respectively.

C_{10}-PEAA-LUV for 15 min at 37°C. After extensive washes, lysis of the cells and fluorimetry of the cell lysates revealed that there was no significant difference between the cell-associated fluorescence in the control or bafilomycin-treated cell populations ($p > 0.2$) (data not shown).

Discussion

This work describes a straightforward method for producing pH sensitive liposomes that exhibit an ability to deliver their contents to the cytoplasm of cells in a pH dependent manner following uptake. There are three aspects of these results that are of particular interest, concerning the post-insertion process and the nature of the particle produced, the mechanism whereby these particles deliver material to the cytoplasm of cells and the potential utility of these systems as intracellular delivery vehicles. We discuss these points in turn.

The ability to insert material into the outer monolayer of preformed liposomes (post-insertion) is a useful procedure that has been applied to insertion of lipids conjugated to poly(ethylene glycol) (PEG) [32,40] as well as to proteins conjugated to PEG-lipids [42]. The benefits of this procedure are that reproducible amounts of well-defined material can be attached to the liposome in a manner that does not result in

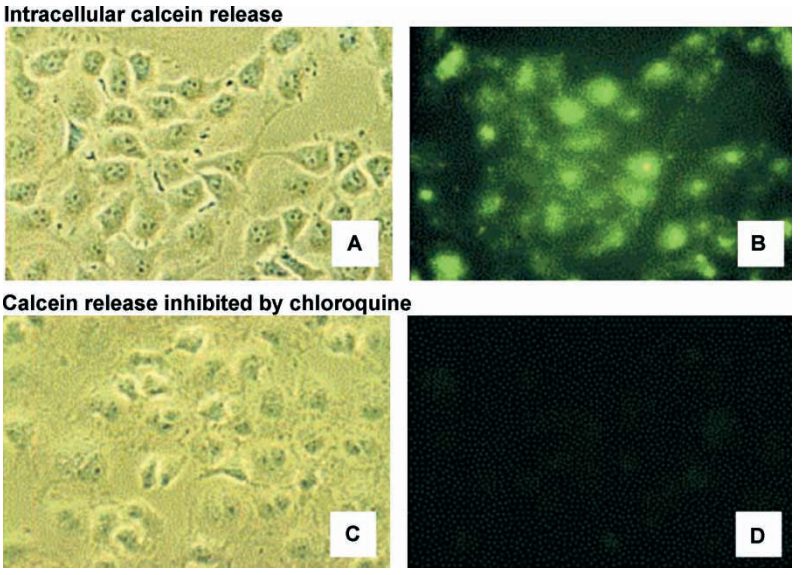


Figure 5. Intracellular release of calcein from C_{10}-PEAA-LUV is inhibited when cells are treated with chloroquine. The COS-7 cells shown in panels C and D were pre-incubated with 100 μM chloroquine 30 min before addition of C_{10}-PEAA-LUV. No chloroquine was added to cells in panels A and B. Cells were incubated with C_{10}-PEAA-LUV loaded with calcein and labeled with Rh-PE for 15 min. Panels A and C were taken by phase contrast and panels B and D by confocal fluorescence microscopy. This Figure is reproduced in colour in *Molecular Membrane Biology* online.

appreciable leakage of contents. As detailed here, the amidation of 3% of the carboxyl functions of PEAA results in a molecule that is soluble in aqueous media at neutral pH but which associates irreversibly with LUV following incubation at room temperature. Given that the average molecular weight of the PEAA employed is 20,000, the amidation procedure results in the attachment of approximately 6 alkyl chains to each polymer molecule. The observation (Table 1) that approximately 40% of the amidated polymer is associated with LUV at both initial polymer to lipid ratios of 0.1 and 0.2 is not currently understood, but may reflect a sub-set of the modified polymer that is more avidly retained by the LUV during the procedure to remove free alkyl-PEAA. There is clearly a balance between the alkyl chain density and the degree to which the modified PEAA associates with LUV. As shown in Table 2, approximately twice as much of the modified PEAA containing 5% C_{10} associates with LUV as does the PEAA containing 3% modification. Finally, there is a definite limit to the number of acyl chains that can be incorporated, since modification of 7% of the carboxyl functions of the PEAA results in a molecule that induces aggregation at all of the polymer to lipid ratios examined. This presumably arises due to the increased ''stickiness'' of highly modified PEAA leading to cross-linking between LUV in addition to association with individual vesicles. The PEAA-LUV system containing 8% by weight of PEAA with 3% modification represents a good balance between achieving sufficient amidation to lead to incorporation by post-insertion and excess modification leading to aggregation. It may be noted that even 5% modification leads to some aggregation during the post-insertion process as evidenced by increased vesicle size (Table 2).

The C_{10}-PEAA-LUV system has remarkable pH-dependent leakage and intracellular delivery capabilities that can be directly attributed to the properties of the PEAA component. Essentially immediate and complete release of LUV contents is observed on reducing the external pH from pH 7.5 to pH 5.0, behaviour that is not observed for LUV that do not contain C_{10}-PEAA. Also, there is a correlated lipid mixing and/or fusion of the PEAA containing liposomes as the pH is reduced to 6.5 and below (Figure 4A). The membrane fusion/lipid mixing occurs at a lower pH than leakage of liposomal contents (calcein leakage below pH 7) which may be attributed to a larger percentage of polymer required to induce membrane instability as compared to the amount required to initiate leakage of a small molecule. Similarly, C_{10}-PEAA-LUV exhibit intracellular release of LUV contents as evidenced by the appearance of intracellular fluorescence following incubation with COS-7 cells. The fact that the part of this intracellular fluorescence is diffused throughout the cell rather than remaining in punctate form indicates that at least a portion of released calcein has not only escaped the LUV but also the endosome; consistent with an ability of the PEAA associated with the C_{10}-PEAA-LUV to destabilize not only the LUV membrane but also the surrounding endosomal membrane. Finally, the intracellular delivery properties of the C_{10}-PEAA-LUV system are dependent on acidification of the endosomal compartment following uptake into the cell, as evidenced by the ability of agents that inhibit endosomal acidification processes to inhibit intracellular release of calcein. The mechanism whereby PEAA is able to destabilize membranes at lower pH values has been attributed to an increased hydrophobicity of PEAA polymers following protonation of associated carboxylate ions at low pH. This protonation of carboxylate ions induces a coil-to-globule transition followed by partitioning into available membranes, leading to membrane disruption [43].

The potential utility of C_{10}-PEAA-LUV for intracellular delivery applications should be evaluated in the context of other intracellular delivery systems. As noted previously, intracellular delivery has been an area of considerable interest for over 20 years. However, no generally applicable pH-sensitive intracellular delivery system has yet been devised even for *in vitro* applications. This is despite intensive efforts employing pH-sensitive lipids, peptides and polymers. With regard to pH-sensitive lipids, the large bulk of studies have focused on mixtures of dioleoylphosphatidylethanolamine (DOPE) stabilized into a bilayer organization by weakly acidic lipids such as oleic acid [44,45] or cholesteryl hemisuccinate (CHEMS) [46]. Such systems are inherently leaky, are not applicable to delivery of lipophilic weak bases (which includes many drugs) and are unstable in biological media. PEG lipids can stabilize DOPE-containing systems in serum [47] but result in significantly reduced pH sensitivities [46]. Enhanced intracellular delivery has also been observed with pH-sensitive detergents that become micellar once protonated [48–50] and pH-labile lipid derivatives such as plasmalogens that hydrolyze at low pH resulting in increased liposome permeability [51]. However, there is a tendency for lysolipids to partition out of the membrane, reducing the degree of membrane destabilization possible [51] and the processes depending on pH-sensitive lipid lability tend to be relatively slow, leading to inefficient intracellular delivery properties.

pH sensitive systems based on pH-sensitive peptides such as the N-terminus of hemaglutinin (INF peptides from influenza) [52,53], GALA peptides [33,54–57] and the N-terminus of human adenovirus fibre protein [52,58–60] have demonstrated reasonable intracellular delivery properties but suffer from tedious preparation procedures to couple the peptides to the liposomes and are immunogenic [61–63], limiting *in vivo* applications.

Intracellular delivery systems based on pH sensitive polymers have clear advantages over systems based on pH sensitive lipids and peptides in that such polymers can exhibit rapid membrane destabilization at well-defined pH values, are stable, are relatively straightforward to manufacture [27,31,43,64–66] and display pH sensitivity in the presence of serum [67]. Further, polyanionic polymers with optimized pH ranges for activity at an early or late endosomal stage have recently been synthesized [68]. A feature limiting the general applicability of pH-sensitive polymers to construct pH-sensitive liposomes concerns the tedious, often irreproducible coupling procedures required for attachment to the liposome surface. The demonstration here that hydrophobic derivatives of PEAA can be post-inserted in a rapid, simple manner into preformed LUV containing the agent to be delivered and that these systems exhibit pH-sensitive intracellular delivery properties would appear to remove such difficulties. Further studies will be required, however, to evaluate the influence of biological media on intracellular delivery capabilities as well as the exchangeability of the C_{10}-PEAA moiety from the LUV surface.

It is likely that the C_{10}-PEAA LUV system, in common with other negatively charged liposomal systems [69] will be rapidly cleared from the circulation by the mononuclear phagocytic system following intravenous administration, thus limiting the ability of these systems to access disease sites such as tumour sites. In this context the most useful applications could lie in intracellular delivery to the fixed and free macrophages of the MPS to upregulate or downregulate specific immune responses, and these avenues are currently under investigation. Alternatively, acyl-linked PEAA conjugated liposomal systems may also have utility in direct tissue injection therapies [70] and intratumoral injection of liposomes carrying immunomodulatory genes which have already been shown to evoke a powerful destructive response to tumor cells [71].

In summary, this study has demonstrated that alkylated derivatives of the pH-sensitive polymer PEAA can be post-inserted into preformed LUV, leading to pH-sensitive liposomes that are capable of intracellular delivery of the liposomal contents. It is anticipated that these well-defined and readily constructed systems will be of general utility for *in vitro* intracellular delivery applications and may have significant applications for intracellular delivery to cells of the immune system *in vivo*.

Experimental procedures

Materials

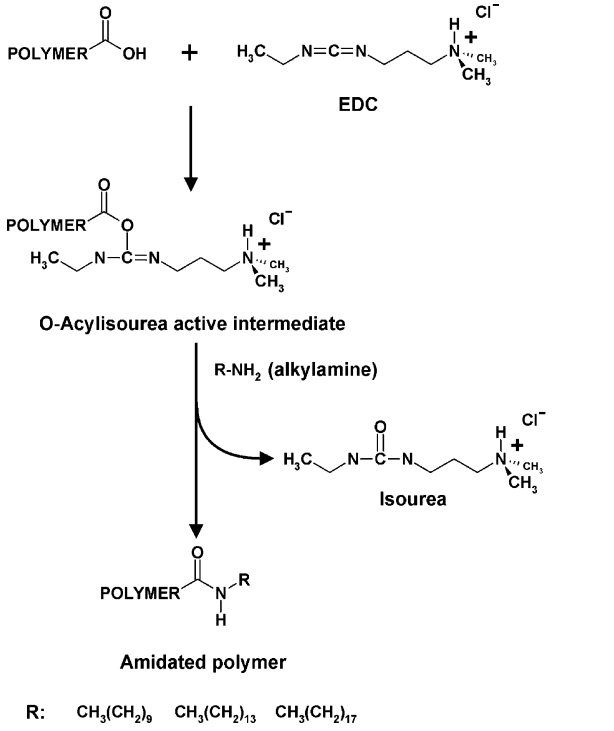
All reagents were of the highest purity available commercially and were used without further purification. Pyrene labeled poly (2-ethylacrylic acid) (*Py*-PEAA, Xn = 200, MW = 20 000) was synthesized as described previously [30]. Egg phosphatidylcholine (EPC) was obtained from Northern Lipids (Vancouver, Canada). [^{3}H] cholesterylhexadecyl ether (^{3}H-CHE) was obtained from DuPont (NEN Research Products, Boston, MA, USA). Cholesterol, calcein and N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC) were obtained 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-1-1,3-benzoxadiazol-4-yl) (NBD-PE) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL, USA).

Synthesis of amidated PEAA

The outline for the synthesis of the lipid derivatives of PEAA is shown in Figure 6. 100 mg (1 mmol unit, unit MW = 100) of PEAA and 0.03 mmol of 1-alkylamine (RNH_2, R = $C_{10}H_{21}$, $C_{14}H_{29}$, $C_{18}H_{37}$) were dissolved in water (pH 7.0). A solution of EDC (20 mg/ml) was slowly added to the reaction mixture until all the amine had reacted. The reaction was monitored by thin-layer chromatography ($CHCl_3$: MeOH: triethylamine = 8:2:0.2; amine was visualized employing ninhydrin). The resulting derivative was precipitated by adjusting the pH of the solution to pH 2–3. The supernatant was removed and the pellet was re-dissolved in a 2M NaOH solution, stirred for 0.5 h and the pH re-adjusted to pH 2–3. The suspension was centrifuged and the resulting pellet was washed 3–5 times in water and lyophilized. A typical yield was 60–85%.

Preparation of PEAA-containing liposomes

Large unilamellar vesicles (LUV) were prepared by extrusion as described by Hope *et al*. [72]. Appropriate amounts of lipid (EPC/Chol, 55:45 mol/mol) with trace amounts of ^{3}H-CHE (1.33 μCi/μmol) in chloroform, were dried under a stream of nitrogen gas to form a homogeneous lipid film. Any remaining trace solvent was then removed under vacuum overnight. The lipid film was hydrated in HBS buffer (5 mM HEPES, 150 mM NaCl, 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 at 55°C through two-stacked 100 nm



Polyelectrolyte:

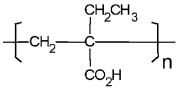
Polymer name	Chemical structure
PEAA	

Figure 6. Reaction scheme to attach alkyl chains randomly to carboxyl groups in PEAA polymers forming amidated-PEAA.

polycarbonate filters (Nuclepore) employing an extrusion device (Northern Lipids, Inc., Vancouver, BC, Canada). Non-entrapped free calcein was removed by chromatography using a 1.1 × 20 cm Sepharose CL-6B column (Sigma Chemical Co., St. Louis, MO, USA) equilibrated with HBS buffer.

C_{10}, C_{14} or C_{18}-PEAA-LUV were prepared by incubation of the preformed LUV with the C_{10}, C_{14} or C_{18}-PEAA solution (pH 7.4) (Figure 1) overnight at room temperature at a derivatized PEAA to LUV lipid ratio of 20:80 (w/w), unless indicated otherwise. Excess free polymer was separated from LUV-associated polymer by chromatography on a Sepharose CL-6B column (1.5 × 20 cm) and the fractions were assayed for lipid and polymer content.

Determination of liposome size

The mean diameter of the liposomes was determined by quasi-elastic light scattering (QELS) using a Nicomp 370 submicron particle sizer (Santa Barbara, CA, USA).

Calcein release

In order to determine the effect of pH on the permeability of PEAA-LUV, fluorescent aqueous calcein was encapsulated in LUV at self-quenching concentrations (100 mM). These LUV were then associated with derivatized PEAA as indicated above. Solutions of PEAA-LUV containing calcein were acidified to the desired pH with 1% HCl. After a 10 min equilibration an aliquot was withdrawn and the pH was adjusted to 6.5–7.5 to minimize the effect of pH on calcein fluorescence. The fluorescence intensity of the aliquot was measured using an Aminco Bowman® Series 2 luminescence spectrofluorometer (SLM-AMINICO, Urbana, IL, USA) at 530 nm (slit width 4 nm) under steady-state excitation at 495 nm (slit width 4 nm).

To determine the kinetics of calcein release, the initial pH of 7.5 was maintained for a period of 10 min before acidifying the solution to pH 5. At different time points an aliquot was withdrawn, the pH was adjusted to 6.5–7.5, and the fluorescence intensity of the solution was measured. The maximum fluorescence intensity (F_{max}), representing complete release of encapsulated calcein, was determined following solubilization of the vesicles with Triton X-100 (10% of lipid concentration). The percentage of calcein released was calculated using the following equation:

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

where F_t is the fluorescence intensity at time t, or at a given pH; F_o is the initial fluorescence intensity.

Fusion assay

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*. [73]. 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 PEAA-LUV (acceptor). These mixed vesicles were maintained at their initial pH of 7.5 for 10–15 min and then acidified to pH 5. At different time points, an aliquot was withdrawn and the fluorescence intensity of NBD-PE was determined using a spectrofluorometer at 530 nm under steady-state excitation at 465 nm. The fusion of labeled LUV (donor) with probe-free LUV (acceptor) results in probe dilution and an increased distance between the NBD-PE and Rh-PE, thereby decreasing RET efficiency. The maximum 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:

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

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

Cell Culture

COS-7 cells were cultured in DMEM supplemented with 10% FBS at 5% CO_2 and 37°C. The cells were maintained in monolayer culture and studied at subconfluence.

Fluorescence microscopy

The uptake of PEAA-LUV by COS-7 cells and the subsequent intracellular release of calcein fluorescence were studied. COS-7 cells grown in 24 well tissue plates were washed 3 times with PBS containing 0.4 mM calcium and 0.42 mM magnesium (PBS-CM). Cells were then incubated for various time periods (5, 15, 30 and 60 min) with liposomes (1.0 μmol lipid/ml medium) that were labeled with Rh-PE in the bilayer and which contained encapsulated calcein. Another group of cells was pre-incubated with either 100 nM bafilomycin or 100 μM chloroquine for 30 min before addition of the PEAA liposomes. The fluorescence distribution of the calcein and rhodamine was visualized by confocal fluorescence microscopy.

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