Improved Cytoplasmic Delivery to Plant Protoplasts via pH-Sensitive Liposomes

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ABSTRACT

We demonstrated that the liposomes composed of dioleoylphosphatidylethanolamine/cholesterol/oleic acid (4:4:2) dramatically release their contents at a pH of less than five equal to 6.0 and are capable of delivering their contents into the cytoplasm of higher plant protoplasts. This is shown by using a soluble fluorescent dye, calcein, as a liposome-entrapped marker. We found that calcein fluorescence was equally distributed in the cytoplasm of wild carrot protoplasts after the incubation of protoplasts with liposomes in the presence of polyethylene glycol 6000. At 0.45 micro molar phospholipid per 6 x 10^5 protoplast, for example, the percentage of protoplasts which took up liposomes was 89%, which was much higher than that achieved by conventional pH-insensitive liposomes. In this study, liposomes were prepared by a detergent dialysis method which avoided sonication and organic solvents. Thus macromolecules such as proteins and nucleic acids could be entrapped in the liposomes and delivered to the cytoplasm of the protoplasts.

Liposomes have been used as a vehicle to deliver biologically active molecules into animal cells (24, 29); however, the use of liposomes with higher plant cells is still being developed. Recently, liposomes have been used to deliver RNA or DNA to plant protoplasts. These studies used either the Ca-EDTA chelation method or the modified reverse-phase evaporation method to produce liposomes (7, 8). The frequencies of transformation with liposome-mediated nucleic acid transfer were fairly low (10^-8 ~ 10^-3) when liposomes were prepared by both Ca-EDTA chelation and REV^2 method; however, compared with the calcium phosphate coprecipitation method for transformation, liposome entrapment is capable of keeping the nucleic acid from the nuclease attack.

The mechanisms of interaction between protoplasts and liposomes have been investigated. Although it is still not clear, fusion with plasmalemma (19, 20) and endocytosis of liposomes by protoplasts have both been demonstrated. It is the demonstration of the endocytosis which has prompted us to look into the possible use of pH-sensitive liposomes for an improved liposome-mediated delivery in higher plant protoplasts.

Recently, pH-sensitive liposomes have been developed in several laboratories including ours (4, 5). We demonstrated that the pH-sensitive liposomes composed of PE and palmitoylhomocysteine or OA become unstable and fusion active at mildly acidic pH (5.0-6.0) (4) and can deliver the entrapped contents to the cytoplasm of mouse L-929 cells after being endocytosed by the cells (3). It was proposed that these liposomes encounter the acidic pH in endosomes and fuse with the endosomal membrane, thus releasing the entrapped contents into the cytoplasm (15). The present study is designed to test the cytoplasmic delivery potential of the pH-sensitive liposomes for the higher plant protoplasts. We have used a water soluble fluorescent dye, calcein, as a commoner marker for observing cytoplasmic delivery. We have also compared several other pH-insensitive, conventional liposome compositions.

MATERIALS AND METHODS

Materials. Dioleoylphosphatidylethanolamine (DOPE), dioleoylphosphatidylcholine (DOPC) and brain phosphatidylserine (PB) were purchased from Avanti (Birmingham, Al). Oleic acid, cholesterol, dicetyl-P, calcein, lysophosphatidylcholine, stearylamine (SA), and n-octylglucoside were obtained from Sigma. SM-2 beads were purchased from Bio-Rad Laboratories. Cellulysin and macerase were obtained from Calbiochem, sorbitol from Sigma.

Liposome Preparation. Large unilamellar vesicles (LUV) were prepared according to Philippot et al. (27) with modifications. Lipid films of various composition were formed under a nitrogen stream and suspended in Hepes buffer containing 1 mM EDTA and 183.3 mM d-glucose. Hexadecyl-[-H]cholesterol ether was included in the lipid mixture to monitor the lipid (28). The lipid suspension was sonicated with a bath sonicator (Laboratory Supplies, Hicksville, NY) and the pH was adjusted to 8. After the small unilamellar vesicles (SUV) were formed by sonication, octylglucoside and calcein were added and the lipid suspension was transferred to a dialysis bag. Octylglcose was chosen because it has a high critical micellar concentration, which facilitates rapid removal from the mixed micelle complex (13). The molar ratio of octylglcose to lipid used in this study was 10. The optimal condition for the production of vesicles occurred when the detergent/lipid ratio was above 10:1 (23). The concentration of calcein was 23.5 mM in the mixture of a final volume of 0.34 ml. After the mixture was dialyzed against 100 ml of Tris buffer (pH 8.0) containing 1 mM EDTA, 183.3 mM d-glucose, 33.5 mM calcein and 1 g washed SM-2 beads (14) for 24 h, the liposomes were extruded through a polycarbonate filter of 0.2 μm pore diameter (Nuclepore Corp.) to form vesicles of a uniform size distribution. Subsequently, the liposomes were separated from free calcein using a column of autoclaved Sepharose CL-2B.

pH Sensitivity of Liposomes. To monitor the pH sensitivity of liposomes, 23.5 mM calcein was entrapped in the liposomes. Fluorescence of calcein at this concentration is approximately 26% quenched due to frequent collision. Fluorescence is signifi-
currently enhanced when calcein is released from the liposomes (1). Measurements were carried out in phosphate buffer (pH 8.0) with continuous stirring. Fluorescence was measured by Perkin-
Elmer LS5 spectrofluorometer with $\lambda_{ex}$ = 490 nm and $\lambda_{em}$ = 520 nm. After addition of the liposomes to the cuvette, the pH of the suspension was titrated to the desired value by adding HCl and maintained for 5 min. The pH of the suspension was then returned to the original value by adding an equivalent amount of NaOH. Deoxylate (0.15%) was then added to completely release calcein from the liposomes.

The percent leakage of liposomes was calculated from the dequenching of calcein according to the following equation:

$$\% \text{ Leakage} = \frac{F_I - F_0}{F_I - F_0} \times 100$$  \hspace{1cm} (1)$$

where $F_I$ stands for the fluorescence intensity of liposomes in phosphate buffer (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH$_2$PO$_4$, 1.1 mM Na$_2$HPO$_4$, and 183.3 mM D-glucose, pH 8.0) $F_I$ stands for the fluorescence intensity of the liposome suspension after acid treatment. $F_0$ stands for the fluorescence intensity of liposome suspension after adding deoxycholate. The compositions of liposomes, used in this study are shown in Table I.

**Maintenance of Carrot Cultures.** Cell cultures of Daucus carota (wild carrot) were obtained from Dr. D. K. Douglas and maintained as suspension cultures in WCM-1 medium (30). Cell suspensions were subcultured by adding 5 ml of 14 d-old suspension to 20 ml of fresh WCM-1.

**Isolation of Protoplasts.** Preliminary experiments indicated that best protoplast yield and viability was obtained with cell suspensions in early log phase, 4 d after subculture. Ten ml of a cell suspension from 4 d old cultures was added to a sterile 15 ml test tube and centrifuged at 100g for 5 min to pellet the cells. The medium was removed and cells resuspended in digestion solution (2.5% Cellulysin, 0.5% Macerase, 0.5 m sorbitol in Chapeau’s salts plus Chapeau’s minor elements (2)). The pH of the solution was brought to 5.8 and the solution was filter sterilized. The mixture was placed in a 15 ml Petri dish, sealed with parafilm and placed on a rotary shaker at 40 rpm at room temperature in the dark for 15 to 17 h. Following digestion, cells were pelleted and washed three times with Kao’s K8P medium (16). Cells were resuspended in 10 ml of K8P medium and the number of protoplasts/ml were determined with a hemacytometer. Cells were resuspended in the phosphate buffer immediately before incubation with liposomes.

**Cell Incubations.** Calcein-entrapped liposomes at a lipid concentration of 0.6 ml were incubated with $8 \times 10^4$ ml of protoplasts at room temperature for 3.5 h. The above ratio of lipid to protoplasm was chosen to achieve minimal structural damage of protoplasts. Then PEG-6000 was added into the incubation medium to a final concentration of 11% as described (21). An additional incubation of 1.5 h at room temperature was carried out.

Following the second 1.5 h incubation, the protoplasts were washed 3 times with phosphate buffer and then observed under a Leitz Orthoplan epiluminescence microscope equipped with an Orthomat-W camera. Photographs were taken with both phase contrast and fluorescent microscopy. All of the fluorescent pictures were taken using bandpass filter BP 470 to 490 with 2 min of film exposure time.

**Protoplast Viability.** Following incubation with liposomes, cells were cut with K8P medium to 3 x 10$^5$ cells/ml and cultured in sterile 48-well tissue culture clusters (Costar) with 0.2 ml of protoplasts per well. Protoplasts were cultured in the dark at 25°C for 7 d. After 1 week of culture, cells were stained with Calcofluor white (25) and observed with a fluorescence microscope equipped with DANS filter (BP 355–420 nm). Cell divisions and cell wall formation can be easily seen with this technique. Preliminary studies determined that the number of cells showing cell wall regeneration and one or more division plates are not increased after 7 d. The percent of dividing cells was recorded for each experiment.

**Incubation with Chloroquine and Methylamine.** Chloroquine and methylamine are weak bases which can penetrate membranes of cells or organelles and raise the pH of the acidic organelles (12). To scrutinize the mechanism of the cytoplasmic delivery of calcein by a pH-sensitive liposome, protoplasts were preincubated with methylamine or chloroquine for 30 min before liposomes were added.

### RESULTS

**pH Sensitivity of Liposomes.** The pH sensitivities of liposomes of different composition as shown in Figure 1 were determined by monitoring the release of entrapped calcein. All liposomes except those composed of DOPC or DOPC/stearylamine showed acid sensitivity, but DOPE/chol/OA liposomes showed the highest sensitivity in the seven compositions tested. The entrapped calcein was released dramatically when the pH of solution was dropped to 6.0 or below. The pH for half-maximal dye release lies at about 6.0. The maximum dye release was about 85% of the total entrapped calcein. When oleic acid was replaced with PS, the release of entrapped calcein decreased significantly. Furthermore, it also required more acidic pH for release. The same phenomena took place when the DOPE was replaced by DOPC. DOPC/dicetyl-P/lysoPC and DOPC/SA liposomes have been developed to deliver the exogenous DNA to the cytoplasm/nucleus of plant protoplasts (18, 22). However, the pH sensitivity of DOPC/dicetyl-P/lysoPC is much less than that of DOPE/chol/OA liposomes. The maximum dye release was only 20% of the entrapped calcein. It is about one-fourth of the maximum dye release from the DOPE/chol/OA liposomes. There was no evidence of entrapped calcein being released from the DOPC/SA and DOPC liposomes at pH between 7.0 and 5.0. Although the calcein release of DOPE/OA liposomes was slightly higher than that of DOPE/chol/OA at pH 5.5, the maximum release of calcein from the DOPE/OA liposomes was only about 30% of that from the DOPE/chol/OA liposomes. It is clear that DOPE and OA are both essential to the maximal pH sensitivity of liposome.

**Cell Incubation.** We have examined the dependence of the

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**Table 1. Effect of Liposome Composition on the Uptake of Liposome by Protoplasts, the Dye Release Distribution and Viability of Protoplast**

<table>
<thead>
<tr>
<th>Liposome Composition</th>
<th>Protoplast Taken Up Dye</th>
<th>Dye Distribution</th>
<th>Protoplasts Divided</th>
</tr>
</thead>
<tbody>
<tr>
<td>molar ratio</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DOPE/chol/OA</td>
<td>89 ± 4</td>
<td>Internal and homogeneous</td>
<td>79 ± 2</td>
</tr>
<tr>
<td>(4:4:2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DOPE/chol/PS</td>
<td>29 ± 4</td>
<td>Surface aggregate</td>
<td>72 ± 1</td>
</tr>
<tr>
<td>(4:2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DOPC/chol/OA</td>
<td>84 ± 5</td>
<td>Surface aggregate</td>
<td>78 ± 8</td>
</tr>
<tr>
<td>(4:2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DOPC/OA (8:2)</td>
<td>81 ± 5</td>
<td>Surface aggregate</td>
<td>73 ± 1</td>
</tr>
<tr>
<td>DOPC</td>
<td>77 ± 6</td>
<td>Surface aggregate</td>
<td>75 ± 4</td>
</tr>
<tr>
<td>DOPC/SA (10 =</td>
<td>63 ± 2</td>
<td>Surface aggregate</td>
<td>82 ± 2</td>
</tr>
<tr>
<td>1. w/w)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DOPC/dicetylP/lysoPC (8:2:0.4)</td>
<td>59 ± 4</td>
<td>Surface aggregate</td>
<td>71 ± 2</td>
</tr>
</tbody>
</table>

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The above phenomena and results were published in Plant Physiol. Vol. 82, 1986.
cytoplasmic delivery of calcein on the lipid composition of the liposomes. After protoplasts were incubated with liposomes, the percentage of protoplasts which took up fluorescence was counted. There was no fluorescence in the protoplasts which had not been treated with calcein-entrapped liposomes (photograph not shown). For DOPE/chol/OA liposomes, 89% of protoplasts took up liposomes and calcein was released from liposomes into the protoplast cytoplasm. The dye distribution was homogeneous within the protoplasts (Fig. 2). In some cells the fluorescence in the vacuole was brighter than that out of the vacuole (Fig. 2, arrow). In contrast, there was no dye release into the protoplasts when the DOPE and OA were replaced by DOPC and PS, respectively, although liposomes aggregated on the surface showing localized bright fluorescence instead of homogenous distribution inside the protoplast (Fig. 3). It is worth noting that partial internal release of calcein took place when the protoplasts were incubated with DOPC/dicetyl-P/lysoPC liposomes (Fig. 4). However, the degree of internal release in this case was not nearly as high as that for DOPE/chol/OA liposomes. Between 77 and 84% of protoplasts incubated with other DOPE-containing liposomes took up liposomes; however, the calcein was not released from the liposomes. Figures 5 to 7 show that liposomes aggregated on the surface of those protoplasts preincubated with DOPC/OA, DOPC or DOPC/SA liposomes. The results of photographs are summarized in Table I.

The use of PEG in the incubation protocol was essential for a high level of liposome uptake by protoplasts. In the absence of PEG only about 20% of protoplasts showed fluorescence after incubation with DOPE/chol/OA liposomes; however, the dye distribution in the protoplasts was still internal and homogeneous.

The relationship between the presence of cell wall on protoplasts and liposome uptake was examined by staining the protoplasts with 0.2% Calcofluor white. Results showed that the liposome uptake by protoplasts was not correlated with the existence of undigested cell wall; 58% of the protoplasts (counted from 124 protoplasts) which had taken up liposomes had no obvious cell wall remaining (photographs not shown). This is consistent with the results by Fukunaga et al. (10) who studied the infection of protoplasts by liposome-entrapped TMV-RNA and found that the efficiency of infection was not correlated with the existence of undigested cell wall.

Viability of Liposome-Infused Protoplasts. The viability of protoplasts after incubation with different liposomes are shown in Table I. The viability of liposome incubated protoplasts was between 71 and 82%. The viability of protoplasts incubated with the DOPC/SA liposomes was the highest while that with DOPC/dicetyl-P/lysoPC was the lowest. There was no obvious difference

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**Fig. 1.** pH sensitivities of liposomes suspended in glucose-containing phosphate buffer as mentioned in methods. The leakage percentage was calculated according to equation 1 and indicated the degree of calcein release from liposomes at indicated pH values. DOPE/chol/OA liposomes (O--O); DOPC/dicetylphosphate/lysoPC (Δ--Δ); DOPE/chol/OA (■--■); DOPE/chol/PS (V--V); DOPC/OA (○--○); and DOPC (□--□) and DOPC/stearylamine (▽--▽).

**Fig. 2.** Fluorescence labeling of protoplasts by calcein containing pH-sensitive liposomes. Carrot protoplasts were incubated with calcein-containing liposomes composed of DOPE/chol/OA (4:4:2). A-E are phase contrast micrographs and a-e are corresponding fluorescence micrographs. Bar is 10 μm. Even distribution of fluorescence in the cytoplasm of protoplasts is evident. Arrow indicates a vacuole of the protoplast.

**Fig. 3.** Fluorescence labeling of protoplasts by calcein-containing liposomes composed of DOPC/chol/OA (4:4:2) (Aa and Bb) and DOPE/chol/PS (4:4:2) (Cc). A-C are phase contrast micrographs and a-c are fluorescence micrographs. Bar is 10 μm. Surface aggregation of fluorescence is seen.
was partially released inside the protoplasts. A–C are phase contrast micrographs and a–c are fluorescence micrographs. Bar is 10 μm.

Fig. 5. Fluorescence labeling of protoplasts by calcein-containing liposomes composed of DOPC/OA (8:2). Surface aggregation of fluorescence is seen. A–C are phase contrast micrographs and a–c are fluorescence micrographs. Bar is 10 μm.

between the viability of protoplasts incubated with DOPE/chol/OA liposomes and that with DOPC/chol/OA liposomes. The viability of untreated cells is approximately 78%. Thus, the liposome treatment is mild and nontoxic.

Incubation with Methylamine and Chloroquine. Preincubation of protoplasts with 30 mM of methylamine or 100 μM chloroquine showed that the calcein-entrapped liposomes aggregated on the surface of protoplast and some were endocytosed by the protoplast but the dye was not released. Figure 8 shows punctate fluorescence on the surface and inside the protoplasts which had been preincubated with methylamine or chloroquine. Compared with cells shown in Figure 2 which had not been preincubated with chloroquine or methylamine only homogeneous cytoplasmic distribution of fluorescence was seen.

Fig. 6. Fluorescence labeling of protoplasts by DOPC liposomes. The calcein-entrapped liposomes aggregated on the surface or inside of protoplasts without dye release. A–C are phase contrast micrographs and a–c are fluorescence micrographs. Bar is 10 μm.

with chloroquine or methylamine only homogeneous cytoplasmic distribution of fluorescence was seen.

DISCUSSION

pH-sensitive liposome systems have recently been developed in this (3) and other laboratories (5) to deliver efficiently liposomal contents to tissue cultured mammalian cells. In this study we have examined the delivery potential of the pH-sensitive liposomes for higher plant protoplasts. In other previous studies, liposomes have been used to deliver DNA and RNA into the protoplasts of higher plants (11, 19, 22). Fukunaga et al. (11) have prepared liposomes by Ca-EDTA chelation and used phosphatidylserine with or without chol. In Lurquin’s and Fraley’s studies, liposomes were prepared by REV method (29) and composed of SA with phosphatidylcholine or PS and chol. Those liposomes were either positively or negatively charged. Although 80% of protoplasts were infected by liposome-mediated TMV-RNA in Fukunaga’s study, the entrapment efficiency of liposomes produced by Ca-EDTA chelation method was quite low for large size DNA. The efficiency of entrapment was improved with the REV method. Particularly, the modified REV method performed at low salt condition promoted the efficiency up to 65% of aqueous phase (29). Nevertheless, the efficiency of transformation of protoplasts which were transformed by liposome-entrapped nucleic acids was still low (10^{-4}) (7). The low efficiency of transformation may be caused by the defective nucleic acids which were damaged during entrapment or by the incomplete release of the nucleic acids from liposomes. Liposomes used in our study were produced with a mild detergent-dialysis method in which the use of sonication and organic solvent was avoided. Our results showed that the delivery efficiency was high with the use of the pH-sensitive liposomes in the presence of PEG. Lipo-
Figure 7. Fluorescence labeling of protoplasts by DOPC/stearylamine (10:1, w/w) liposomes. Surface aggregation of fluorescence is seen. A and B are phase contrast micrographs and a and b are fluorescence micrographs. Bar is 10μm.

Fig. 8. Effects of methylimine and chloroquine on calcein distribution. The protoplasts were preincubated with 30 mM methylimine (A and a) or 100 μM chloroquine (B and b, C and c) at room temperature for 30 min before the addition of DOPC/chol/OA liposomes. Punctate fluorescence on the surface and inside the protoplasts is evident. A–C are phase contrast micrographs and a–c are fluorescence micrographs. Bar is 10 μm.

Some liposomes composed of DOPE/chol/OA (4:4:2) showed the highest sensitivity to acid (Fig. 1), and most efficiently delivered calcein into the cytoplasm of protoplasts (Fig. 2 and Table 1). Other liposome compositions, including the DOPC/dicetyl-P/lysoPC used by Matthews et al. (22), DOPC/SA used by Lurquin and Sheehy (19), showed lower or no sensitivities to acid and delivered calcein poorly into the cytoplasm of the protoplasts. Thus, the high delivery efficiency seems to correlate with the acid sensitivity of the liposomes.

The mechanisms of the interaction between liposomes and protoplasts have been studied. Using EM, Lurquin and Sheehy (19) suggested that nucleic acid transfer occur via fusion between liposome and the plasma membrane (19). In contrast, Fukunaga et al. (11) indicated that liposomes enter plant protoplast via endocytosis in the presence of PEG and the nucleic acids were released from liposomes when the endocytosed liposomes fused with the endosomal membrane. Fukunaga also suggested that the role of PEG be to increase the adhesion of liposomes to the protoplasts rather than to induce the fusion between them. Others suggested that PEG causes the dehydration between membranes which induced the fusion events (6, 20).

Since PEG is required in the cytoplasmic delivery of calcein by pH-sensitive liposomes, one possible mechanism of delivery of calcein was that the fusion took place between liposomes and the plasma membrane of the protoplast, which would have resulted in an even distribution of fluorescence in the cytoplasm. However, some of the protoplasts shown in Figure 2 showed stronger fluorescence in the vacuole which is the lysosome equivalent of higher plant cells (26). The vacuolar pH of protoplasts has been measured to be around 5.5 (17). Additionally, the incubation of protoplasts with methylimine or chloroquine showed that the cytoplasmic release of fluorescent dye was blocked although the internalization of liposomes still took place (Fig. 8). These observations support the probable mechanism that the fluorescent dye is delivered into the cytoplasm of protoplasts via endocytosis of liposomes. The liposomes may be internalized by protoplasts and then fused with the endosome or vacuole membrane, resulting in the release of the fluorescent dye into the cytoplasm. The fusion between liposomes and endosome/vacuole membrane may be caused by the destabilization of liposomes when the liposomes encounter the acidic environment in these organelles. Duzgunes et al. (5) suggested that the OA was protonated at low pH. Thus this mechanism is similar to what we have proposed for animal cells (3, 15).

The present observation is likely to find its application in the delivery of nucleic acids into protoplasts. Since the method of liposome preparation is mild and relatively rapid, entrapment of structurally intact DNA or RNA should be a relatively easy task. Philippot et al. (27) have shown that up to 45–50% of RNA can be trapped in the liposomes prepared with a method similar to ours. Furthermore the liposomal delivery is not limited to nucleic acids.

Transfer of exogenous DNA into plant protoplasts has recently been carried out by electroporation (9). Although the transformation frequency achieved by this method is very high, the pores in the plasma membrane created by the electric field for DNA penetration are likely to cause the loss of intracellular macromolecules and metabolites. Although the cells can eventually recover from the initial damage, it is not clear if the method is suitable for the delivery of molecules with which the immediate, short-term effect is to be investigated. In contrast, results in this paper showed that a small molecule such as calcein was retained
in the protoplasts after delivery by the pH-sensitive liposomes. It is clear that our method is mild.

We have shown that small metabolites (J Connor, L Huang, unpublished data) and enzymes (D Collins, L Huang, unpublished data) can be efficiently delivered to the cytoplasm of animal cells using the pH-sensitive liposomes. Therefore it is likely that one can find a broad usage of this new type of liposomes.

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