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Novel cationic vesicle platform derived from vernonia oil for efficient delivery of DNA through plant cuticle membranes

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Abstract

Novel cationic amphiphilic compounds were prepared from vernonia oil, a natural epoxidized triglyceride, and studied with respect to vesicle formation, encapsulation of biomaterials such as DNA, and their physical stability and transport through isolated plant cuticle membranes. The amphiphiles studied were a single-headed compound III (a quaternary ammonium head group with two alkyl chains) and a triple-headed compound IV, which is essentially three molecules of compound III bound together through a glycerol moiety. Vesicles of the two amphiphiles, prepared by sonication in water and solutions of uranyl acetate or the herbicide 2,4-D (2,4-dichloropenoxy acetic acid), were examined by TEM, SEM, AFM, and confocal laser systems and had a spherical shape which encapsulated the solutes with diameters between 40 and 110 nm. Vesicles from amphiphile IV could be made large enough to encapsulate a condensed 5.2 kb DNA plasmid (pJD328). Vesicles of amphiphile IV were also shown to pass intact across isolated plant cuticle membranes and the rate of delivery of encapsulated radio-labeled 2,4-D through isolated plant cuticle membranes obtained with these vesicles was clearly greater in comparison to liposomes prepared from dipalmitopyl phosphatidylcholine (DPPC) and the control, nonencapsulated 2,4-D. Vesicles from amphiphiles III and IV were found to be more stable than those of liposomes from DPPC. The data indicate the potential of vesicles prepared from the novel amphiphile IV to be a relatively efficient nano-scale delivery system to transport DNA and other bioactive agents through plant biological barriers. This scientific approach may open the way for further development of efficient *in vivo* plant transformation systems.

Keywords: Cuticle membrane; Dipalmitopyl phosphatidylcholine; Epoxy fatty acid; Nano-vesicles; Uranyl acetate; Vernonia oil

1. Introduction

There is growing interest in the preparation of lipid vesicles for encapsulating biological substances such as drugs, pesticides, organic and inorganic materials, lipids, genes, and proteins for pharmaceutical, medical, cosmetic, agricultural, and chemical applications, e.g., gene transfection therapy, pesticides, aroma, mineral or vitamin encapsulation (Ollivon et al., 2000).

Controlled delivery of active agents, as well as transfection with DNA is, in general, the limiting factor in the development of the plant biotechnology industry. Methods of nucleic acid delivery can be classified into two main groups: the indirect method, via *Agrobacterium* and the direct methods that are specifically used in plant species where *Agrobacterium*-mediated transformation is not possible. Direct methods include, but are not limited to, microprojectile bombardment, electroporation, electrophoresis, and carbide fiber-mediated and liposome-mediated transformation (Rakoczy-Trojanowska, 2002). Most plant transformations are *Agrobacterium*-mediated, while among direct methods, microprojectile bombardment is most widely used. These direct methods habitually require the use of protoplasts, which offer many possibilities but are also associated with major disadvantages compared with organized tissues,

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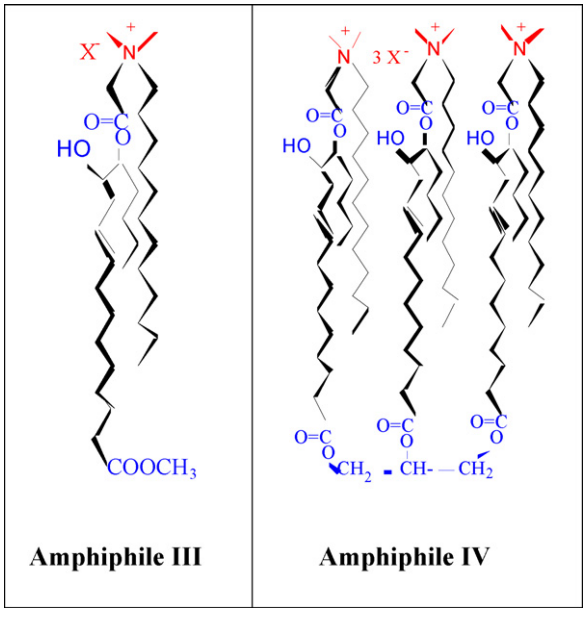
such as laborious isolation procedures, which may induce stress and recalcitrance to systems in culture, problems in plant regeneration, and low transient expression of transgenes (Rakoczy-Trojanowska, 2002; Davey et al., 2005).

The ideal vehicle for these compounds should be stable *in vitro* and *in vivo*, be efficient as a delivery platform, protect the biomaterials from degradation *e.g.*, from nucleases (Davey et al., 2005), deliver the bioactive agents to their target, be non-toxic and non-immunogenic, and be available in large quantities (Deshmukh and Huang, 1997). In addition, the vehicle should be efficient in penetrating structured plant tissues and work in a wide range of species. Liposomes (spherical phospholipid vesicles) can encapsulate water-soluble and lipid-soluble molecules in their aqueous and lipid phases, respectively, and have been used since the 1970s (Gregoriadis and Ryman, 1972) to deliver a variety of pharmacologically active agents to specific sites in the body where pharmacological intervention is needed (Gregoriadis et al., 1998). In plants, the use of liposomes for transformations was reported for wheat (Patnaik and Khurana, 2001), but is in general extremely limited. Most commercial liposomes are a combination of phospholipids, cholesterol, diverse lipids, and sometimes various polymers. One of the disadvantages of liposomes from natural phospholipids is their limited stability in biological environments, from which they are rapidly cleared (Hans-Hening et al., 1980; Po-Shun et al., 1981).

In this communication we describe the further characterization of novel nano-vesicles recently synthesized (Grinberg et al., 2002; Grinberg et al., 2005) from derivatives of vernonia oil, a natural triglyceride of vernolic acid (*cis*-12,13-epoxy, *cis*-9-octadeconic acid).

Amphiphile III (Scheme 1) is synthesized from the methyl ester of vernolic acid as the starting material, to form a single-headed amphiphile with two alkyl chains and a single quaternary ammonium head group. Amphiphile III forms bilayer vesicles and has been partially characterized with respect to structure–function characteristics of vesicle formation and DNA transfection (Grinberg et al., 2005). Amphiphile IV is essentially three molecules of amphiphile III bound together through a glycerol moiety. The differences in their intrinsic curvature was used to explain why amphiphile III could easily form encapsulating vesicles while compound IV formed micelle-like vesicles. Amphiphile IV was reported to form encapsulating vesicles when cholesterol was used. Amphiphile IV alone, however, was shown in this earlier study to be more efficient as a DNA complexant and transfecting agent.



Scheme 1. Chemical structure of the amphiphilic compounds III (based on methyl vernolate) and IV (based on trivernolin).

In this paper, vesicles made from amphiphiles III and IV are further characterized with respect to size, shape, self-aggregation/fusion, and encapsulation of DNA and the herbicide 2,4-D. Emphasis is placed on amphiphile IV vesicle encapsulation of condensed DNA, stability, and the transport rate of radio-labeled encapsulated biomarker (2,4-D) through a model system of isolated plant cuticle membranes.

2. Materials and methods

2.1. Chemicals

Vernonia oil was purchased from Vertech Inc. (Falls Church, VA, USA). Amphiphiles III and IV were synthesized following the procedure reported in Grinberg et al. (2005). L-α-Phosphatidylcholine from dried egg yolk was purchased from Sigma (St. Louis, MO, USA). For differential encapsulated DNA labeling study, FITC-ULSIS Alexa Fluor® 488 Nucleic Acid Labeling Kit (Molecular Probes, Leiden, The Netherlands), rhodamine B (tetramethylrhodamine-5-2′-deoxy-uridine-5′-triphosphate) for microscopy was purchased from Merck (Darmstadt, Germany), and fluroescein from BDH (Poole, England). Uranyl acetate (UA) for microscopy was purchased from BDH (Poole, England). 2,4-Dichlorophenoxy acetic acid (2,4-D), 2,4-D[^{14}C] (specific activity 19.2 mCi/mmol) and protamine were purchased from Sigma (St. Louis, MO).

All the chemicals employed were analytical grade. Double-distilled water was used in all experiments.

2.2. Vesicle preparation

Two main methods were used to produce the vesicles–(1) *Injection method*: 100–200 μl of the amphiphile dissolved in methanol (in a range of 0.008–0.016 g in 10 ml methanol) was injected into 2–4 ml aqueous solution containing the compounds to be encapsulated. The mixture was sonicated in a 60 °C bath sonicator (Delta Ultrasonic Cleaner, D80, 43 kHz, 80 W) for short (1, 2, 5, 10 min) or long (60 min) periods depending on the desired vesicle size and uniformity (Barenholtz et al., 1979). (2) *Reverse phase evaporation*: Using the same amphiphiles as in (1) above, methanol (250–1000 μl) was added to a 50 ml round bottom flask, and the solvent removed under reduced pressure by a rotary evaporator (BÜCHI) to form a thin oily film on the flask bottom. An aqueous solution containing the compounds intended to be encapsulated (1–4 ml) was added to the flask