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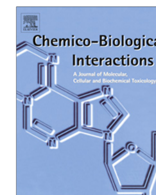

Quaternary ammonium salt containing soybean oil: An efficient nanosize gene delivery carrier for halophile green microalgal transformation

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ABSTRACT

Dunaliella salina, a halophile green microalga, is considered a robust photobioreactor and a remarkable cost beneficial system for the production of therapeutic recombinant proteins. In this study, with low overall cost, a proper cationic lipid was synthesized from renewable soybean oil as an efficient gene delivery carrier for *D. salina* cells to create appropriate protein-producing transformed cell lines. To obtain an effective carrier, quaternary ammonium salt containing soybean oil (QASSO) was synthesized through the ring opening reaction of the epoxy groups of epoxidized soybean oil with diethylamine. QASSO was characterized using nuclear magnetic resonance and Fourier-transform infrared instruments. QASSO was used to prepare nanolipoplex construct using plasmid DNA molecules containing green fluorescent protein (GFP) as reporter gene. These nanolipoplexes (QASSO-pGFP, N/P = 3) and QASSO had diameter of 63.62 and 110.63 nm, and zeta potential of –68.89 and 48.25 mV at pH 7.0, respectively. Results indicated the GFP gene expression and cytoplasmic accumulation of GFP protein in the transformants after incubation under desirable conditions for 48 h and 1 week. The transformation efficiency was quantitatively assayed by flow cytometry, which yielded transformations of 58.87% and 48.34% for QASSO and 38.32% and a negligible percentage for Polyfect® after 48 h and 1 week incubation, respectively.
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1. Introduction

Dunaliella salina, a unicellular eukaryotic green microalga, is one of the most suitable bioreactors because of its unique property to produce therapeutic proteins [1,2]. This photobioreactor has both animal and plant properties, such as the absence of a rigid cell wall and presence of chloroplasts, respectively [3,4]. In addition, rapid and autotrophical growth using inexpensive sources and halotolerance to different concentrations of NaCl provide the lowest cost and highest yield for bioreactor systems, which are important economic points [5,6]. By contrast, to solve the insoluble therapeutic recombinant proteins produced in non-halotolerant systems, cultivation under osmotic stress or addition of compatible solutes (e.g., glycine betaine) is commonly applied to protect solved proteins in peripheral spaces of cells. The measured second osmotic virial coefficients of several proteins indicate that at low-salt concentration conditions, protein–protein interactions follow either attractive or repulsive mechanisms. However, at high salt concentrations, the interaction behavior depends on the type of salt. In NaCl, protein interactions generally show little salt dependency up to a very high salt concentration, which results in most proteins to have a flat dimensionless second virial coefficient profile with increasing NaCl concentration. This phenomenon explains the high solubility of most proteins in highly concentrated NaCl solution [7]. Given the protein interaction behavior in high NaCl concentrations and the potential of *D. salina* as a robust photobioreactor with its growth condition (2–5 M NaCl), the establishment of a highly efficient transformation procedure for this halophile green microalga presents a valuable strategy to generate cost beneficial trans-

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formed cell lines that typically produce recombinant therapeutic proteins.

The most important challenge for gene transformation in microalgae is the development of methods that facilitate the fast delivery of target genes into cells with high transformation yield. Thus, proper gene expression and desirable protein yield in transformants would be achieved. Thus, several methods have been developed for microalgal transformation. These methods include particle bombardment [8,9], electroporation [10,11], glass bead methods [12], silicon carbide whiskers [13], *Agrobacterium tumefaciens*-mediated method [14–16], and cell wall-deficient strains [17]. The usual biologically mediated methods for gene transformation cannot be used for *D. salina* because of the high NaCl concentration and high ionic strength of its culture media. However, some of the aforementioned methods, in particular, particle bombardment, electroporation, and glass bead methods, have been used for gene transformation in *D. Salina*. These methods suffer from some critical limitations such as the need for expensive laboratory equipment, low repeatability, low cell viability in transformants, and low transformation yield.

Nanotechnology offers remarkable advantages in enhancing the efficacy of non-viral gene delivery system, particularly for gene delivery into cells without a rigid cell wall [18]. With the progressive advances in nanotechnology, gold nanoparticles, alone or in combination with cationic carriers, have been used for non-viral gene delivery. Easy preparation procedures, possibility of surface chemical modification for further targeting, and considerable biocompatibility are the main merits of these gold materials [19–24]. However, these gold nanoparticles cannot be used for *D. salina* transformation because of their precipitation in culture media with high NaCl concentrations. Effective polymeric nanoparticles (polyethyleneimine and polyamidoamine dendrimers) [25–27], lipids (cationic liposomes) [28–31], and carbohydrates (chitosan) [32–34] have been extensively used for in vitro/in vivo gene delivery in animal and plant cells. However, to the best of our knowledge, these nanosystems have never been used for halophile green microalgal transformation. These kinds of carriers also possess some deficiencies, specifically cytotoxicity. Several methods that reduce cytotoxicity and improve buffering capacity via proton sponge effect, which results in osmotic swelling, membrane disruption, and eventually DNA release, are known to blend and conjugate the cytotoxic cationic materials with low cytotoxic biodegradable and biocompatible compounds [35]. Moreover, to improve water solubility and gene transfection efficiency, these carriers are often modified using varying degrees of quaternization [36–38]. Previous studies have investigated the quaternized cationic lipid tetraesters dimyristoyl bis (N,N-trimethylammonioglycyl) and dioleoyl bis (N,N-trimethylammonioglycyl) assessments in terms of gene delivery and cytotoxicity [39]. In these studies, the quaternary amines were presumed to enhance the rate of hydrolysis to yield a nontoxic dilipid with efficient transfection and reduced cytotoxicity [39]. Large-scale systems based on cationic lipids have also been reported for gene delivery applications. These lipids include cationic lipophosphoramide with diunsaturated lipid chains [40], pyridinium cationic lipids [41–43], multivalent cationic lipids [44], glutamide-containing cationic lipids [45], symmetric 1,3-dialkoylamidopropane-based cationic surfactants containing single primary and tertiary amine polar head groups [46,47], N,N-diacyl-1,2-diaminopropyl-3-carbamoyl-(dimethylaminoethane) amphiphilic derivatives [48], cationic glycolipids [49], and long chain alkyl acyl carnitine esters [50]. Additionally, the derivatives from Vernonia oil (extracted from *Vernonia galamensis* oilseeds), such as bolaamphiphiles, have been synthesized to construct nanovesicles as efficient gene delivery carriers for plant or animal cell transformation [51–53]. The present study is aimed to synthesize quaternary ammonium salt containing soybean oil (QASSO) as an efficient gene delivery carrier with high biocompatibility and low cytotoxicity as well as a cost beneficial carrier because of soybean oil abundance. Additionally, given the benefit of *D. salina* as a robust photobioreactor in the production of recombinant proteins (i.e., therapeutic proteins), the synthesis of a suitable gene delivery carrier with efficient transformation potency at *D. salina* cell culture condition (with 2–5 M NaCl) is advantageous to achieve recombinant protein-producing transformant lines.

2. Experimental section

2.1. Materials

Epoxidized soybean oil (ESBO) (PATSTAB 901) with average molecular weight of ca. 1000 and epoxy content of 3.32 mmol epoxy kg^{-1} was purchased from PATCHEM (Sharjah, UAE) and used as received. Diethylamine (DEA, >99%) and methyl iodide (MeI) were obtained from Merck, Germany and used without further purification. Zinc chloride ($ZnCl_2$) from Merck was dried and ground to fine powder prior to use. Magnesium sulfate ($MgSO_4 \cdot 7H_2O$), sodium bicarbonate ($NaHCO_3$), NaCl, ethyl acetate, and tetrahydrofurane (THF) all from Merck were used as received. Phosphate-buffered saline (PBS) was prepared via dissolving NaCl (5.85 g), KH_2PO_4 (0.6 g), and Na_2HPO_4 (6.4 g) (all from Merck) in distilled water, and the volume was adjusted to 1 L. The pH was then adjusted to 7.0 by HCl or NaOH solutions (0.2 M). PolyFect® was purchased from QiaGen, and all other materials used in this research were obtained from Merck or Sigma.

2.2. Synthesis of QASSO

This compound was prepared according to the procedure described in Ref. [54]. In the first step, tertiary amine-functional soybean oil (TASO) was synthesized through the ring opening reaction of epoxy groups of ESBO with DEA. Briefly, ESBO (20 g, 66.4 mmol epoxy groups), DEA (8.64 g, 118.2 mmol), and anhydrous $ZnCl_2$ (2.21 g, 16.2 mmol) was placed in a 250 mL round-bottomed flask equipped with a condenser, oil bath, and magnetic stirrer. The reaction mixture was heated to 80 °C and stirred for 4 h. Then, the flask was cooled down to room temperature. The unreacted DEA was removed under vacuum at 60 °C. The residue was dissolved in ethyl acetate (50 mL) and washed thrice with saturated $NaHCO_3$ solution (50 mL) to remove the residual $ZnCl_2$ catalyst, followed by repeated washing with distilled water (50 mL) and saturated NaCl solution (50 mL). The dried organic layer (yellow–brown oil of TASO at 85% yield) was obtained after evaporation of solvent at 60 °C under vacuum.

In the second step, TASO was alkylated by MeI. TASO (20 g, ~20 mmol tertiary amine) and MeI (4.6 g, 32.4 mmol) were placed in a 100 mL round-bottomed flask (38 °C for 20 h). The excess MeI was removed at 60 °C under vacuum. The prepared QASSO was dehydrated via heating in a vacuum oven at 60 °C.

2.3. Characterization of modified soybean oils

Proton nuclear magnetic resonance (^{1}HNMR) spectra were recorded on a 400 MHz Bruker instrument (Model Avance 400, Germany) at room temperature using $CDCl_3$ as solvent. Fourier-transform infrared spectra from 4000 to 400 cm^{-1} were obtained using a Bruker Instrument (Model Aquinox 5S, Germany) under air at a resolution of 0.5 cm^{-1} and signal averaged over eight scans.

2.4. Preparation of complexes for transformation

The green fluorescent protein (GFP) expression plasmid (pGreen, 35S cassettes, pGFP) was used to prepare lipid-DNA

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