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FULL LENGTH ARTICLE

A simple protocol for preparation of a liposomal vesicle with encapsulated plasmid DNA that mediate high accumulation and reporter gene activity in tumor tissue

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ABSTRACT

The systemic delivery of gene therapeutics by non-viral methods has proven difficult. Transfection systems that are performing well *in vitro* have been reported to have disadvantageous properties such as rapid clearance and short circulation time often resulting in poor transfection efficiency when applied *in vivo*. Large unilaminary vesicles (LUV) with encapsulated nucleic acids designated stabilized-plasmid-lipo-particle (SPLP) have showed promising results in terms of systemic stability and accumulation in tumor tissue due to the enhanced permeability and retention effect (EPR). We have developed a simple protocol for the research-scale preparation of SPLPs from commercially available reagents with high amounts of encapsulated plasmid DNA. The SPLPs show properties of promising accumulation in tumor tissue in comparison to other organs when intravenously injected into xenograft tumor-bearing nude mice. Although transcriptionally targeted suicide gene therapy was not achieved, the SPLPs were capable of mediating reporter gene transfection in subcutaneous flank tumors originating from human small cell lung cancer.

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1. Introduction

In the field of gene therapy, efficient gene delivery *in vivo* based on non-viral methods remains a major challenge, with an overwhelming variety of polymeric and liposomal compounds being tested [1]. A major obstacle has been the fact that extremely efficient methods involving cationic liposomes for gene delivery to cells *in vitro*, perform very poorly when tested in animals [2]. Although a regime of transfection-potent lipoplexes has been established *in vitro* [3], *in vivo* applications require different physical–chemical properties and only limited information about these have been described.

The development of liposomal carriers with enhanced systemic stability has mainly been advanced by the liposomal formulation of chemotherapeutics, i.e. doxorubicin into DOXIL® that is FDA-approved for use against several cancers [4]. Here a great advantage of therapeutic efficiency over the naked drugs has been accomplished [5]. A great accumulation in disease area, i.e. tumor tissue due to the so-called enhanced permeability and retention effect (EPR) is a hallmark of these liposomal formulations [6] where the property of long circulation is accomplished by a 5–10% PEG polymers screen on the liposomal surface.

Furthermore, efficient encapsulation of plasmid DNA in liposomes can be achieved using an ethanol-mediated condensation procedure [7,8], and this was established in our laboratory [9]. The technology of stabilized plasmid lipo-particles (SPLPs) has progressed in recent years [10] and we decided to investigate these methods for laboratory scale studies of a gene therapy strategy in mice using conventional lipid reagents, hence we included a tritium-labeled lipid in the formulation enabling evaluation of systemic circulation and biodistribution of SPLPs [11]. A robust laboratory-scale protocol allows for researchers to perform experiments investigating the biological properties of SPLPs and the interaction with the biological milieu in order to characterize the barriers to successful gene delivery.

Aiming at gene therapy of small cell lung carcinoma (SCLC) [12] we have recently showed high and specific effect of a suicide gene therapy system [13]. At the time of diagnosis SCLC often appears disseminated to various extra-thoracic organs [14], and therefore a systemic distribution of the therapeutic agent is

Abbreviations: SPLP, Stabilized plasmid–lipid particle; PEG, Polyethylene glycol; SCLC, Small cell lung carcinoma; EPR, Enhanced permeability and retention effect; PDI, Polydispersity index; SCD, Super cytosine deaminase

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demanded. Hence in the current study we have exploited the potential of transcriptionally targeted suicide gene therapy using SPLPs as a delivery vehicle for systemic treatment of a mouse model of SCLC.

2. Materials and methods

2.1. Materials

All chemicals, e.g. synthetic cholesterol were purchased from Sigma-Aldrich Inc. (Brøndby, Denmark) unless otherwise stated. DDAB: Dimethyl-dioctadecyl-ammonium bromide, DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine) and DSPE-PEG2000 (1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA) [1,2-3H]-Cholesteryl Hexadecyl Ether (^{3}H-CHE) was purchased from Perkin Elmer (Skovlunde, Denmark). High quality plasmids; pCMV-LUC (sequence available upon request) and pEGFP-N1 (Clontech, Mountain View, CA, USA) preparations were made with the Endo-free Giga kit from Qiagen GmbH (Hilden, Germany) according to the manufacturer's instructions. Glassware used for lipid work was washed and rinsed in mQ water, baked at 180 °C for 6 h and de-dusted by pressurized air prior to use. Lipid solutions in chloroform were handled with gastight glass syringes (Hamilton, VWR, Herlev, Denmark) reserved for this purpose. Syringes were rinsed with chloroform and 20% EtOH in water only.

The H1299 and NCI-H69 cell lines (obtained from ATCC, Boras, Sweden) were cultivated in RPMI medium supplemented with pen-strep and 10% fetal calf serum (Invitrogen Inc., Taastrup, Denmark). Six-week old male NMRI mice were from Taconic Europe (Lille Skensved, Denmark) and housed at Department of Experimental Medicine, University of Copenhagen. All animal experiments were performed according to ethical guidelines and under valid license from the Danish Animal Experimentation Board.

2.2. Methods

2.2.1. Preparation of SPLPs: Cholesterol/DSPC/DDAB/DSPE-PEG2000 liposomes encapsulating plasmid DNA

Chloroform solutions of lipids (10–20 mg/ml) were mixed in a 12×75 mm^2 glass tube (Thermo Fischer Scientific, Slangerup, Denmark) at the following composition (20 μmol total lipid, mole percent): Cholesterol 55%, DSPC 20%, DDAB 15% and DSPE-PEG2000 10%. In experiments where a radioactive label was used ^{3}H-CHE (50 μCi, 50 Ci/mmol in toluene) was added. The solvent was evaporated under vortexing and under a thin nitrogen gas stream allowing a thin, fairly even lipid film to form on approximately 6 cm of the glass surface. High vacuum was applied overnight to ensure complete solvent evaporation. A Tris–HCl buffer (300 μl, 50 mM, pH 7.0) was used to hydrate the lipids and allow for vesicle formation. The tube was rotated and lipids allowed to hydrate overnight at room temperature. The next day the liposome preparation was placed in a metal basket and sonicated for 2 min using a Bransonic water bath (MT-1510, 42 kHz, 80 W, setting "sonics", Branson Ultrasonics, Danbury, CT, USA). Plasmid DNA (Endo-free GIGA prep, 200 μg, 5.7 μg/μl in Tris-buffer) was added to the tube and after collecting the material at the bottom of the tube by a brief spin exactly one volume of 80% ethanol in Tris-buffer (50 mM, pH 7.0) was added dropwise and with mixing during one minute.

The tube was closed and subjected to five cycles of freeze-thaw between dry ice/EtOH and 37 °C water bath with 2–3 min in each step. Liposomes were downsized using 11 passes in a hand-held, small-scale extruder (Avestin Europe GmbH, Mannheim, Germany) with polycarbonate nucleopore filters (400 nm, 200 nm and 100 nm, Whatman, Frisenette, Knebel, Denmark). For each step a small volume of buffer to wash the extruder ensure a complete liposome recovery. The entire SPLP volume (typically 1 ml) after the extrusion process was transferred to a dialysis cassette (PIERCE, Thermo, 10 kDa MWCO) and dialyzed against 0.5 l HEPES buffer (pH 7.4) overnight at room temperature with one buffer exchange.

MALDI-TOF mass spectrometry analysis of liposome preparation made at 20 μmol scale confirmed the lipids in the composition and did not reveal any degradation (data not shown).

2.2.2. Characterization of SPLP

Plasmid encapsulation and ability to migrate in an electrical field was investigated using agarose gel electrophoresis [9,15,16]. Samples of SPLP from different stages of the encapsulation procedure were loaded on a standard 1% agarose–Tris–Borate–EDTA gel containing 2 μg/ml ethidium bromide. After completion the gel was photographed under UV light. Subsequently, the concentration of plasmid DNA in liposome was determined using a variation of the PicoGreen assay (Invitrogen) as described by Jeffs et al. [7]. A typical dose for intravenous injection contained 20 μg DNA and 4 μmol lipid in 200 μl HEPES buffer.

A Zetasizer Nano ZS (Malvern Instruments Inc., Malvern, UK) was used for characterizing the particle size by dynamic light scattering. Preparations of liposomes were diluted to approximately 1 mM total lipid and placed in a clear disposable zeta cell (Malvern). Size was determined using 4 cycles of 3 min. at standard settings for vesicles and with "general purpose" parameter settings. The quality of size measurements given as the volume-weighted mean diameter were analyzed by evaluating polydispersity index (PDI), scattering correlation and cumulants fit. Subsequently, samples were analyzed for zeta potential of particles using standard settings with three repeated measurements of 20 zeta runs and assessing the quality of measurements by evaluation of the phase plot.

2.2.3. Gene expression analysis in vitro

Adherent H1299 were plated the day prior to the experiment in 6-well plates, 300,000 cells per well. NCI-H69 cells growing in suspension were single-cell resuspended on the day of the experiment and counted in a hemocytometer using Trypan Blue (0.4%) staining to discriminate from dead cells before placing 2×10^6 cells in 6 well plates. Forty microlitres (2–4 μg/0.8 μmol) of plasmid DNA/liposome preparation was added to cells in full growth medium and incubated for 2 days at 37 °C before analysis of reporter activity. Here, cultured cells were washed with phosphate-buffered saline (PBS) and lysed in 100 μl passive lysis buffer (Promega Inc., Madison, WI, USA) for 10 min. After centrifugation for 1 min, the supernatant was analyzed for luciferase activity (20 μl, Luciferase kit, Promega) using a luminometer (Lumat LB9507, Berthold, Bad Wildbad, Germany) and total protein concentration (20 μl, 10 times diluted, BCA kit, Pierce/Thermo, Rockford, IL, USA) using an OpsysMR microplate reader (Dynex Technologies GmbH, Berlin, Germany). Using a purified, recombinant firefly luciferase (Promega) for standardization, luciferase activity was expressed as picogram luciferase enzyme per milligram of total protein (pg luc/mg protein).

2.2.4. SCLC tumor model establishment and evaluation of growth

The SCLC xenograft model was established as previously described [13,17]. Briefly, 5×10^6 NCI-H69 cells per flank were injected subcutaneously into 6–8-week old male nude NMRI mice. Tumors from injected mice (termed passage 0) were used for serial transplantation of mice that entered experimental

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