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Triggered release of siRNA from poly(ethylene glycol)-protected, pH-dependent liposomes

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ABSTRACT

The ability of small interfering RNA (siRNA) to regulate gene expression has potential therapeutic applications, but its use is limited by inefficient delivery. Triggered release of adsorbed poly(ethylene glycol) (PEG)-b-polycation polymers from pH-dependent (PD) liposomes enables protection from immune recognition during circulation (pH 7.4) and subsequent intracellular delivery of siRNA within the endosome (pH ~5.5). Polycationic blocks, based on either poly[2-(dimethylamino)ethyl methacrylate] (31 or 62 DMA repeat units) or polylysine (21 K repeat units), act as anchors for a PEG (113 ethylene glycol repeat units) protective block. Incorporation of 1,2-dioleoyl-3-dimethylammonium-propane (DAP), a titratable lipid, increases the liposome's net cationic character within acidic environments, resulting in polymer desorption and membrane fusion. Liposomes encapsulating siRNA demonstrate green fluorescent protein (GFP) silencing in genetically-modified, GFP-expressing HeLa cells and glyceraldehyde-3-phosphate dehydrogenase (GAPD) knockdown in human umbilical vein endothelial cells (HUVEC). Bare and PD liposomes coated with PEG113-DMA31 exhibit a 0.16±0.2 and 0.32±0.3 fraction of GFP knockdown, respectively. In contrast, direct siRNA administration and Oligofectamine complexed siRNA reduce GFP expression by 0.06±0.02 and 0.14±0.02 fractions, respectively. Our in vitro data indicates that polymer desorption from PD liposomes enhances siRNA-mediated gene knockdown.

1. Introduction

The discovery of siRNA in mammalian cells [1] provides a new strategy in which protein expression can be regulated. siRNA is able to induce the destruction of specific mRNA sequences that may lead to disease or may regulate diseased cells. For example, siRNA-induced protein regulation has shown therapeutic benefits in breast cancer [2], colon cancer [3,4], dystonia [5] (a muscular disorder), and diabetes [6].

One of the limiting factors in the clinical use of siRNA technology is its delivery to target cells. Previously investigated avenues of delivery include direct intravenous injection of 'naked' or chemically-stabilized siRNA [7,8], insertion of siRNA into DNA plasmid vectors [4,6], transposon vectors (transgenic plasmids) [2,9], plasmid-infected viruses [5], virosomes (reconstituted viral envelopes) [10], lentiviral vectors [11,12], and liposomes [7,13]. The most successful of these in protecting siRNA and supporting in vivo transport are viral and liposomal delivery methods. Viral vectors have serious drawbacks such as immunogenicity and insertional mutations [10], whereas liposomes can be engineered to have a wide range of physical and biological characteristics [14].

Nucleic acid delivery can exhibit high transfection efficiency when complexed with lipid formulations that include cationic, fusogenic, and/or PEG-conjugated lipids [15–17]. Physical challenges for siRNA delivery, as compared to delivery of other nucleic acids, arise from these molecules being highly charged, relatively small, susceptible to nucleases, and the delivery of many siRNA molecules within one cell is required for therapeutic benefit. Efficiently loaded liposomes may protect siRNA from degradation and facilitate therapeutic administration.

Herein, we report a pH-dependent liposomal system for siRNA delivery that has the potential to passively target tumors or sites of inflammation. A PEG-b-polycation block copolymer is electrostatically bound to the liposome exterior for protection from immune system recognition. Endocytosis of the polymer-coated liposomes causes an

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increase in the cationic character of the liposome; this leads to desorption of the PEG-polycation chains since they are no longer electrostatically anchored to the liposome. The cationic liposome fuses with the anionic endosomal membrane, releasing the siRNA into the cell. Polymer-coated liposomes encapsulating siRNA protect from degradation and immune recognition, allow passive targeting, and provide a mechanism for endosomal release.

2. Materials and methods

2.1. Materials

1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (PC), 1,2-dioleoyl-3-dimethylammonium-propane (DAP), and 1,2-dioleoyl-*sn*-glycero-3-phosphoserine (PS) were obtained from Avanti Polar Lipids, Inc (Alabaster, AL). The poly(ethylene glycol)-b-poly[2-(dimethylamino) ethyl methacrylate] (PEG-DMA) polymers, were synthesized at the University of Sheffield (Sheffield, South Yorkshire, UK) as described by Deshpande et al. [18]. The block copolymers are designated as PEG "X"-DMA"Y", where *X* is the number of ethylene glycol repeat units and *Y* is the number of cationic DMA repeat units. The poly(ethylene glycol)-b-poly(lysine) (PEG122-K21, with 122 ethylene glycol repeat units and 21 K repeat units) sample was prepared at the University of California, Santa Barbara (Santa Barbara, CA) as described by Yu et al. [19]. GFP- and GAPD-interfering siRNA were purchased from Dharmacon RNA Technologies (Lafayette, CO). *N*-(7-Nitro-2,1,3-benzoxadiazol-4-yl)-1,2-dioleoyl-*sn*-phosphatidyethanolamine (NBD-PE), *N*-(lissamine rhodamine B sulfonyl)-1,2-dioleoyl-*sn*-phosphatidylethanolamine (Rh-PE), and Oligofectamine were acquired from Invitrogen (Carlsbad, CA). Quant-IT RiboGreen RNA reagent was procured from Molecular Probes (Eugene, OR). *N*-Tris(hydroxymethyl)methyl-2-aminoethane-sulfonic acid (TES) was purchased from Sigma-Aldrich (St. Louis, MO). Qiagen RNeasy mini kit and Triton X-100 were obtained from VWR International (Westchester, PA). Primers and reagents for quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) were purchased from Applied Biosystems (Foster City, CA).

2.2. Liposome preparation

PC (100 mol% PC), PD (PC:DAP, 9:1, mol:mol), and RET (PC/PS/NBD-PE/Rh-PE, 58:38:2:2, mol:mol:mol:mol) liposomes were prepared as described previously [20]. Stock solutions of 10 mM lipid were solubilized in chloroform. Lipids were mixed in a glass vial to a final concentration of 5 mM lipid. Evaporation of solvent results in the formation of bilayers. To this, 2 ml of an aqueous solution consisting of 2.5 μM siRNA in a TES Buffer (20 mM TES, 150 mM NaCl, pH 7.4) was added. The samples were vortexed, subjected to 7 freeze/thaw cycles in liquid nitrogen and a room temperature water bath, and then extruded. Samples were extruded through a Lipex extruder (Northern Lipids, Inc., Vancouver, British Columbia, Canada) 10 times through two 0.1 μm polycarbonate filters (Whatman, Florham Park, NJ) with nitrogen gas (200–250 psi).

A phosphate assay was performed to determine lipid composition as described previously [21]. Briefly, samples were ashed with 10% sulfuric acid at 200 °C for 1 h, followed by addition of 30% hydrogen peroxide and further heating at 200 °C for 40 min. After cooling, a color reagent (5% ammonium molybdate, 10% ascorbic acid) was added to the samples and incubated for 20 min. Absorption was measured on a SpectraMax Plus spectrophotometer (Molecular Devices, Sunnyvale, CA) at 820 nm, with appropriate backgrounds.

Samples were characterized for size and zeta potential on a ZetaPALS Zeta Potential Analyzer (Brookhaven Instruments, Corp., Holtsville, NY) in TES buffer (20 mM TES, 150 mM NaCl, pH 7.4). The prepared solutions were dialyzed (Float-A-Lyzer tubes, 1 ml, MWCO 25 kDa, Spectrum Laboratories, Los Angeles, CA) against TES Buffer (20 mM TES, 150 mM NaCl, pH 7.4), and sterile-filtered through a 0.22 μm polycarbonate membrane (Aerodisc Syringe Filters, Pall Life Sciences, Corp., East Hills, NY). For zeta potential measurements, a 1:20 dilution of liposomes into either a TES (20 mM TES, pH 7.4) or sodium citrate (20 mM Na Citrate, pH 5.5) buffer resulted in solutions with 7.5 mM NaCl.

2.3. Encapsulation efficiency

A Ribogreen assay (Molecular Probes, Eugene, OR) was performed to determine the encapsulation efficiency of siRNA within the liposome samples. A calibration curve was performed with appropriate backgrounds. Spectrofluorometry, (excitation 500 nm, emission 525 nm, Photon Technologies International, Birmingham, NJ, USA) was performed on diluted liposome samples. Equal concentrations of lipid were incubated with RiboGreen and measured for fluorescence, before and after administration of Triton X-100 [22]. Triton X-100 is a surfactant that lyses liposomes. Similar methods have been used previously [23]. The encapsulation efficiency is the encapsulated siRNA concentration divided by the initial siRNA concentration times 100.

2.4. Polymer adsorption

Liposomes were prepared at a concentration of 1.0 mM lipid in either 0.5 ml TES buffer (1 mM TES, pH 7.4) or 0.5 ml sodium citrate buffer (1 mM sodium citrate, pH 5.5). Polymer was added to achieve the desired concentration. All samples were vortexed and allowed to equilibrate overnight at room temperature with gentle shaking (American Rotator V, Miami, FL). The samples were added to Nanosep tubes and centrifuged at 1000 ×*g* for 10 min. The supernatant was then assayed for phosphate and polymer content.

The polymer concentration was quantified by an assay described by Baleux [24], wherein 25 μl of an iodine-potassium iodide solution (0.04 M I_2, 0.12 M KI) was added to 1 ml of a diluted supernatant sample. Samples were diluted to an optimal adsorption range ($0.1 < AU < 1.0$). After 5 min, the optical density (OD) of the solution was determined for $\lambda = 500$ nm at ambient temperature. Liposomes were retained by the Nanosep membrane and did not influence the Baleux assay. The variation in the calibration curve with different polymer architectures is due to the differences in hindrance to helix formation, which is the origin of the colored complex.

2.5. Membrane fusion assay

Fusion between liposomes containing both NBD-PE and Rh-PE and liposomes devoid of fluorescent lipid was measured by the decrease in resonance energy transfer resulting from probe dilution [25,26]. RET (PC/PS/NBD-PE/Rh-PE, 58:38:2:2, mol:mol:mol:mol) liposomes were prepared in TES buffer (20 mM TES, 150 mM NaCl, pH 7.4). PC and PD liposomes were incubated with polymer at the appropriate concentrations for 2 h. PC and PD liposomes with or without adsorbed polymer were incubated with RET liposomes at a 9:1 molar ratio (at a final 1 mM lipid) in a TES buffer (20 mM TES, 150 mM NaCl, pH 7.4). Fluorescence was monitored with excitation of 450 nm, emission of 535 nm, and an emission cut-off filter at 530 nm. After 15 min, the pH was adjusted to 5.5 with 1 mM HCl. Fluorescence (F) was normalized by subtracting the initial fluorescence (F_0) and dividing by the fluorescence achieved by maximal probe dilution, achieved by addition of Triton X-100 (F_{max}). The percent change in fluorescence is given by:

$$\text{Percent change in fluorescence} = \frac{F - F_0}{F_{max} - F_0} \times 100$$

2.6. Transfection

HeLa cells (CCL-2, ATCC, Rockville, MD, USA) and green fluorescent protein (GFP) modified HeLa cells (donated from P. Sharp and C.