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Accelerated gene transfer through a polysorbitol-based transporter mechanism

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ABSTRACT

Here we report an accelerated gene transfer through a polysorbitol-based osmotically active transporter (PSOAT) that shows several surprising results through interesting mechanisms. The nano-sized and well-complexed PSOAT/DNA particles are less toxic, stable at serum and show no aggregation after lyophilization due to their polysorbitol backbone. The transfection is remarkably accelerated both *in vitro* and *in vivo*, presumably due to a transporter mechanism of PSOAT in spite of possibility of reduction of transfection by many hydroxyl groups in the transporter. PSOAT possesses a transporter mechanism owing to its polysorbitol backbone, which enhances cellular uptake by exerting polysorbitol transporter activity, thus accelerates gene transfer to cells because transfection ability of PSOAT is drastically reduced in the presence of a cyclooxygenase (COX)-2-specific inhibitor, which we have reported as an inhibitor of the transporter to cells. Moreover, the gene expression is found to be enhanced by hyperosmotic activity and buffering capacity due to polysorbitol and polyethylenimine backbone of PSOAT, respectively. The polysorbitol in PSOAT having polyvalency showed more efficiency in accelerating gene transfer capability than monovalent sorbitol. The above interesting mechanisms display PSOAT as a remarkably potential system to deliver therapeutic (small interfering RNA) and diagnostic agents for effective treatment of cancer.

1. Introduction

In the nascent years of gene therapy research, gene delivery vehicles with greater efficacy have been documented with regular frequency. However, among the investigated reports, the rate of significant and clinically successful advancements has been almost asymptotic, even though the field has matured over the past couple of decades and the rate of published articles has grown exponentially [1]. The role of gene therapy is application oriented to either attenuation or overriding of the malfunctioning gene. It can also be implemented for prevention of disease through vaccination using an antigenic component encoding a gene for a specific pathogen [1]. In reality, the primary barrier to successful gene therapy remains the lack of a safe and effective gene delivery strategy. Until today, the majority of gene therapy clinical trials have used modified viruses as gene delivery vectors, which, while effective for transport of DNA to cells, potentially suffer from immunogenicity, severe toxicity, and production problems [2,3]. Therefore, non-viral polymeric systems for gene delivery have been extensively studied due to their well defined chemistries, diversity, and tunable physicochemical properties, especially cationic polymers, which offer several advantages, including effective condensation of anionic DNA, stability of polyplexes, and reduction of immunogenicity, as well as toxicity and ability to resolve vector size limitations. However, despite these exciting advantages, existing polymeric gene carriers are far less efficient, compared with viral vectors, owing to their low level of gene transporting capability [3]. Therefore, the hunting of that 'magic bullet' for effective gene

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transportation to cells remains the most outstanding challenge to the scientific community.

Recently, production of molecular transporters has aroused tremendous attention due to their ability to overcome biological barriers, including the cellular plasma membrane, the blood-brain barrier (BBB), and the nuclear and mitochondrial membranes [4–6]. In this context, peptide-based molecular transporters, such as cell penetrating peptides and related ones, have been extensively studied in recent years for their potential use in protein, nucleic acid, and gene delivery [7–9]. However, they are mostly vulnerable to various endogenous proteases in the body, thereby limiting their bioavailability. Thus, for successful gene delivery, an effective transporter system, which is currently lacking, is a priority demand. For effective gene delivery, the transporters should have several key features, including biocompatibility, biodegradability, charge/receptor mediated uptake, tissue specificity, endosomal escape, nuclear tropism, and vector unpackaging, all of which contribute to the canon of requirements. Sorbitol-based molecular transporters have recently been documented in two previous reports. First, Maiti et al. described a guanidine-containing sorbitol-based molecular transporter [5]. Later, Higashi et al. investigated the potential applications of these transporters for DNA and small interfering RNA (siRNA) delivery, where they documented a lipidated sorbitol-based molecular transporter containing guanidine moieties [6]. Through these transporters, on one hand, Maiti et al. found a very interesting and enhanced cellular uptake, as well as high intracellular localization properties, and, on the other hand, Higashi et al. found enhanced transfection activity using pDNA and increased silencing effect using siRNA. However, the mechanism behind the above accelerated cellular uptake by these transporters was not investigated. In the present study, we have developed a polysorbitol-based osmotically active transporter (PSOAT) based on sorbitol dimethacrylate (SDM) and a low molecular weight linear polyethylenimine (LMW LPEI), which is entirely different from the aforementioned studies because our transporter is based on polysorbitol without guanidine residues. We hypothesized that polysorbitol having polyvalency will accelerate cellular uptake more than sorbitol as a monovalency by its improved transporter mechanism; thus, increase transfection activity of the transporter in combination with LPEI. Since our transporter system is based on polysorbitol, it can be expected that they would exert more accelerated gene transport capability compared to the previously reported transporters based on sorbitol itself [5,6] owing to the polysorbitol capacity.

Sorbitol (D-glucitol), an organic osmolyte, is widely produced in plants, particularly in those of the *Rosaceae* family, including apples, cherries, pears, and others. It is produced commercially by reduction of D-glucose or D-glucono-1, 4-lactone and is used extensively in the food industry due to its complete water solubility and lack of any perceptible toxicity [5]. Our transporter system possesses polysorbitol backbone which contains many hydroxyl groups. It will provide several beneficial properties because reduced cytotoxicity and stable gene transfer efficiency of gene carriers by inclusion of hydroxyl groups in polycations have been reported [10], although the hydroxyl groups of polymers such as poly (3-amino-2-hydroxypropyl methacrylate) (PAHPMA) [11] and poly(ethylene glycol) (PEG) [12] reduced gene transfection efficiency *in vitro*. Hence, it is interesting to see the transfection capability of our PSOAT system. We hypothesized that it will provide accelerated transfection efficiency by its transporter mechanism even though the PSOAT possesses many hydroxyl groups than PAHPMA and PEG.

Polyethylenimine (PEI), a 'gold standard' polycation, has been used extensively for its high DNA complexation ability, and, most importantly, its ability to exert 'proton sponge effects' for endosomal escape of polyplexes. Simultaneously, this highly cationic polymer exhibits high cytotoxicity, depending on the molecular weight and type of polycation. In several previous reports, LMW LPEI was found to be significantly less cytotoxic, compared with its high molecular weight counterparts, and more tolerable than branched PEI as well [13,14]. Until today, PEI has been exclusively focused by most of the experts in the field of polymer-mediated gene delivery technology [12–21]. However, our novelty in this study is the introduction of a transporter concept mainly based on polysorbitol which was hypothesized to accelerate transfection efficiency of the transporter by increasing cellular uptake through an improved transporter mechanism. Because it is worth mentioning that the difference in gene expression depends mainly on cellular uptake rather than endosomal escape as hypothesized earlier [6].

Here we demonstrate a class of gene transporter, the PSOAT, in order to provide accelerated transfection efficiency with low cytotoxicity and also show the insight mechanisms of their high transfection. Nevertheless, the physicochemical characterizations of PSOAT, including chemical composition by ^{1}H nuclear magnetic resonance (NMR) spectroscopy, molecular weight by gel permeation chromatography (GPC), gel retardation assay, protection and release assay of DNA, particle size, and zeta potential, were precisely examined. We have checked particle size and transfection efficiency of the polyplexes in the presence of various serum concentrations, which could be a beneficial first step toward their use in future clinical trials. Most important, we have explored and studied transfection efficiency in various ways and the high transfection of PSOAT was potentially investigated by various kinds of mechanisms, including one indigenous strategy, which can be helpful for future study in this research area. The potentiality of this gene transporter system was also explored *in vivo*.

2. Materials and methods

2.1. Materials

Linear PEI (Mn: 423 Da), branched PEI (Mn: 25 kDa), dimethyl sulfoxide (DMSO), 3-(4, 5-dimethyl thioazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) reagent, bafilomycin A1, and D-sorbitol were purchased from Sigma (St. Louis, Mo, USA). Sorbitol dimethacrylate (SDM) was purchased from Monomer-Polymer & Dajac Labs, Inc. (Trevose, PA 19053, USA) and was used as received. Luciferase reporter of 1000 assay for *in vitro* transfection study and pGL3-control vector with SV-40 promoter, and enhancer encoding firefly (*Photonus pyralis*) luciferase were obtained from Promega (Madison, WI, USA). The pEGFP-N_2, which has an early promoter of cytomegalovirus (CMV) and an enhanced green fluorescent protein (EGFP) gene, were obtained from Clontech (Palo Alto, CA, USA). A competent *Escherichia coli* strain, JM109, was used for amplification of the plasmids and purified with a plasmid DNA purification kit (DNA-spin™ iNtRoN Biotechnology, Inc.). The concentration of the purified DNA was determined at 260 nm of UV absorbance. Deoxyribonucleic acid (DNA) sodium salt from salmon testes (Sigma) was used for the particle size and zeta potential measurement. All other chemicals used in this study were of analytical reagent grade.

2.2. Synthesis of polysorbitol-based osmotically active transporter (PSOAT)

The PSOAT was successfully prepared based on SDM and LPEI by simple Michael addition reaction with a slight modification. In brief, LPEI (Mn: 423 Da) and SDM (Mn: 318.32) were dissolved separately in DMSO. SDM solution was then added drop by drop to LPEI solution while gently stirring at a mole ratio of 1:1 (Supplementary Table S1). The reaction mixture was kept at 80 °C and maintained for 24 h with continuous stirring. Following completion of the reaction, the reaction mixture was dialyzed using a Spectra/Por® membrane (MWCO: 1000 Da; Spectrum Medical Industries, Inc., Los Angeles, CA, USA) for 24 h at 4 °C against distilled water. Finally, the synthesized polymer was lyophilized and stored at −70 °C for later use.

2.3. Characterization of PSOAT

The PSOAT was characterized by ^{1}H nuclear magnetic resonance (^{1}H NMR, Avance™ 600, Bruker, Germany) spectroscopy to confirm the synthesis and the composition of the synthesized PSOAT. The actual molecular weight of the polymer was measured using a gel permeation chromatography column coupled with