Oligonucleotide Delivery with Serum into HeLa Cells Using Polycation Liposomes

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ABSTRACT

Antisense oligonucleotides (ON) are promising novel therapeutic agents against viral infections and cancer. Polycations have gained increasing attention as nonviral gene delivery vectors in the past decades. Significant progress has been made in understanding complex formation between polycations and nucleic acids, entry of the complex into the cells, and subsequent entry into the nucleus. There is an efficient, systemic transgene expression in many cell lines (in vitro) by using anionic liposomes (AL), composed of equimolar amounts of 1, 2-dilauroyl-sn-glycero-3-phosphoethanolamine (DLPE), dioleoyl phosphatidyl serine (DOPS) and cholesterol (CHOL). These liposomes, dispersed in 10% serum-containing growth medium, efficiently delivered ON precondensed by protamine sulfate (PS) into HeLa cells. AL-liposomes complexed with PS/ON thus represent an efficient method for delivering encapsulated drugs and/or genes in the presence of serum, and they represent a true advance in the field of vector development.

INTRODUCTION

Antisense oligonucleotides (ON) have several properties that make them attractive as therapeutic agents. Design of therapeutic antisense agents can be made more rotationally compared with most traditional drugs ie, they can be designed on the basis of target ribonucleic acid (RNA) sequences and their secondary structures. Despite these advantages, the design and use of antisense ON for therapeutic use are still faced with several obstacles. One major obstacle is their inefficient cellular uptake and poor accessibility to target sites.[1] The molecular weight (5 to 10 kDa) of the ON drugs and their negative charges prevent their passive diffusion across the cell membranes to their sites of action in the cytoplasm (antisense, ribozymes) or in the nucleus (antigene, aptamers).[2,3] Nonviral gene delivery systems based on polycation/plasmid deoxyribonucleic acid (DNA) complexes are quickly gaining recognition as alternatives to viral gene vectors because of their potential in avoiding immunogenicity and toxicity problems inherent in viral systems. Gene delivery using protamine sulfate (PS) involves condensation of ON into compact particles, uptake into the cells, release from the endosomal compartment into the cytoplasm, and uptake of the ON into the nucleus.

Liposomes and lipid-based drug delivery systems have been used for the

delivery of relatively large DNA and RNA-based drugs, including plasmids, antisense ON ,and ribozymes.[4] Polylysine-condensed DNA entrapped into folate-targeted anionic liposomes have been successfully used for tumor cell-specific gene transfer.[5]

An anionic liposome was used recently for the encapsulation of condensed plasmid DNA. The efficiency and safety of the in vitro use of these liposomes have been shown in many cell lines.[6,7] These liposomes have special fusogenic properties, allowing them to transport encapsulated or associated drugs into cells.

PS can condense plasmid DNA efficiently for delivery into several different types of cells in vitro by several different types of cationic liposomes. Its primary role is that of a condensation agent, although it also possesses several amino acid sequences resembling that of a nuclear localization signal (NLS).[8]

PS is a US Pharmacopeia compound that is approved by the US Food and Drug Administration and is used clinically as an antidote to heparin-induced anticoagulation. PS has also been complexed with insulin (also known as NPH) and can serve as a long-acting delivery system, which is administered to patients on a daily basis. PS is nonantigenic due to the lack of aromatic amino acids and lack of a rigid structure. Therefore, issues of toxicity and immunogenicity are minimal. For these reasons, it was hypothesized that PS may be safer for condensation, as well as the delivery, of plasmid DNA to the nucleus.[8,9] The physicochemical properties, such as particle size and surface charges of the liposome DNA complex, may be important factors to obtain a higher transfection efficiency of the liposomal vectors. This study was intended to characterize precondensed ON/liposome complexes in terms of zeta (ξ) potential and particle size and to see whether these physicochemical properties have any influence on their disposition characteristics and hepatic uptake process. The ability of PS to condense ON in order to enhance the transfection efficiency of ON after their complexation with the anionic liposomes in HeLa cell lines was also studied. Furthermore, the condensing agent must also possess the ability to protect ON from enzymatic degradation, be compatible with the nonviral delivery system, and resist displacement of the polycation from the ON due to a biologically relevant protein (albumin, fibrinogen, serum, etc) until it has reached its target.

The presence of serum often reduces the transfection efficiency of liposomal vectors.[10-13] This may be due to the premature release of DNA from the complexes or its degradation by the nucleases. So, in the presence of serum, anionic liposomes effectively transport precondensed ON into the cell nuclei of HeLa cells in the present work.

MATERIALS AND METHODS

Materials

1, 2-dilauroyl-sn-glycero-3-phosphoethanolamine (DLPE) and dioleoyl phosphatidyl serine (DOPS) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). Cholesterol (CHOL) was obtained from Calbiochem (La Jolla, CA). Protamine sulfate (PS) was obtained from Eli Lilly (Indianapolis, IN). Fetal calf serum (FCS), L-glutamine (200 nM solution), and penicillin 5000 units/streptomycin 5000 mg were obtained from Bio Whittaker Europe, Verviers, Belgium.

A 5′ FAM oligonucleotide is obtained from Eurogentec EGT Group, 4102 Seraing- Belgium. It is a labeled 68-mer of sequence (5′-TGT-CAA-GCA-GAT-CGT-GGG-GGA-CCC-CTT-TTG-GGG-TCCCCC- ACG-ATC-TCC-TTG-ACA-GCG-CGT-TTT-CGC-GC-3′). Toto-3 and propidium iodide (PI) were

purchased from Molecular Probes (Eugene, OR). All other reagents were of analytical reagent grade.

Preparation of Liposomes

Anionic liposomes (AL) were composed of equimolar amounts of DLPE, DOPS, and CHOL.[7] In short, the appropriate phospholipid composition was mixed in an organic solvent in a 50-mL round flask. The organic solvent was evaporated to dryness by a mini rotary evaporator. The resulting lipid suspension was extruded through 100-nm polycarbonate membranes (Nucleopore GmbH, Germany), using a commercially available extruder (Liposofast, Avestin Inc, Canada). Size measurement was performed by laser light scattering, and the size was in the range of 100 nm.

Zeta Potential

In de-ionized water, the pure liposomes and their complexes were dispersed with different PS/ON charge ratios (+/-) and then the corresponding zeta-potential (ξ) was measured by using a Zetasizer 3000 HS, (Malvern Instruments GmbH, Herrenberg, Germany). The lipid concentration was 40 µg lipid/2.5 µg ON.

Electron Microscopy

The liposomes and their complexes with PS/ON were also characterized by using a negative stain electron microscope. On a copper grid, the appropriate concentration from each sample was added. Then 1 drop of 20% uranyl acetate was added. After 2 minutes at room temperature, the excess solution was removed with a filter paper and then examined under the electron microscope.

ON Transfection Experiment

In this study, ON was condensed with PS and coated with a negatively charged liposomal formulation.[7] Cellular distribution of the ON was investigated in HeLa cells following the kinetics of this process using confocal laser scanning microscopy. In general, liposomes were in the range of 100 nm in diameter. ON and liposomes were appropriately diluted in 10 mM tris-buffered saline (pH 7.8). PS solution was added to ON solution to achieve the desired charge ratio (+/-). Diluted liposomes were added to the PS/ON complexes to form AL/PS/ON complexes. The final complex had a size of approximately 150 nm, was diluted with the appropriate cell cultured medium containing 10% FCS, and added to HeLa cells. PS/ON complex experiments were prepared in the same way.

HeLa cells were grown on glass coverslips in 6 well plates (105-106 cells per well) in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FCS, 1% glutamine, and 1% penicillin/streptomycin solutions. The transfection system and cells were incubated for 6 hours at 37°C in 5% carbon dioxide. The cells were washed away by rinsing 3 times with cold phosphate buffer saline (PBS). Cells were fixed with formaldehyde. Cells nuclei were stained with PI stains and were examined with a laser scanning microscope. Images were converted to TIF-format with Scanware 5.1, (Leica, Germany). Integration analysis of images was employed with Scion Image Release Beta 3b (Scion Corporation, Frederick, MD).

RESULTS AND DISCUSSION

The physicochemical properties, such as particle size and surface charges, of the liposome-ON complexes may be important factors needed in order to obtain a higher transfection efficiency of the liposomal vectors. Physicochemical properties play an important role in the interaction of lipid vesicles with biological membranes. Although gene transfection of plasmid and/or ON complexed with anionic liposomes through polycations is documented,[6,7,9] little attention

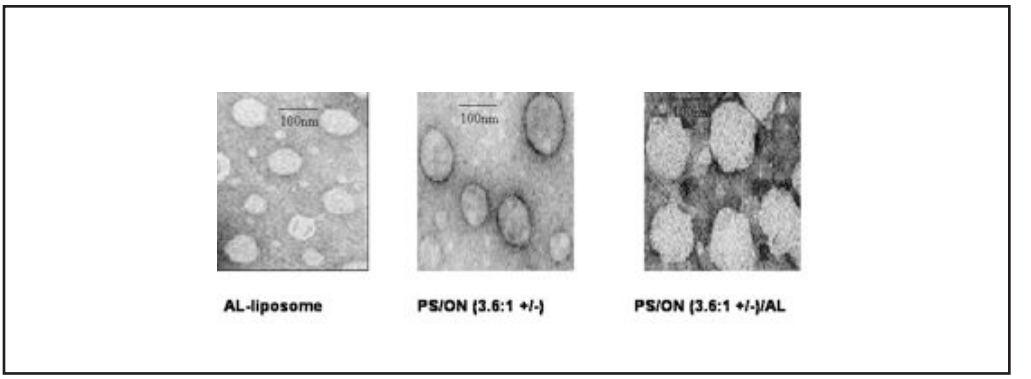

Figure 1. Negative stain electron micrographs of anionic liposomes (AL) and their complexes with PS/ON.

seems to be paid to understanding their physicochemical characteristics and cellular uptake mechanisms.

Transmission electron microscopy (TEM)

The ability of polycations to increase the transfection activity of cationic lipid DNA complexes is well documented.[9,14,15] This is believed to occur because of electrostatic interactions between the polycation and the ON, resulting in a charge neutralization of the complex and the formation of a condensed structure. This condensed structure, due to its diminished size, may be more readily endocytosed by the cell, resulting in the increased levels of transgene expression. Cationic polymers, such as polylysine, histone, and protamine, are known to complex and condense ON from an extended conformation to highly compact structures of about 30 to 100 nm in diameter.[16] Negative stain electron microscopy is a useful method for addressing questions concerning size distribution of the liposome, and it is a reliable technique that is simple to perform and requires only limited specialized equipment. In addition, negative staining can also provide information on whether liposomes produced in a particular manner are multi- or unilamellar.[17] Electron micrographs of AL-liposomes and their complexes with PS/ON (3.6:1 +/-) are shown in Figure 1. The majority of the particles in the pictures appeared spherical, small (≤100 nm in diameter). One possible mechanism by which the complex may have formed is illustrated in Lee and Huang.[5] In this model, ON is first condensed into a cationically charged complex with PS. The cationic complex is then entrapped into anionic liposomes by spontaneous charge interaction.

The data indicated the condensation ability of ON by PS. There are several possibilities that may explain the potentiation effect of the condensing agent on the transfection activity of liposomes. First, on the basis of the current endocytosis model, there is a size limitation for particles to be taken up efficiently by cells.[18] Direct size measurement of the complexes showed that the condensing agent significantly reduced the size of the complex formed over a wide range of liposome/ON ratios. Electron microscopic evidence was obtained that the complexes were smaller than or close to 100 nm in diameter. These small complexes should be more favorable to enter the cells via an endocytosis pathway than the larger ones.

NLS generally is a cluster of positively charged amino acid sequences or 2 clusters of positive amino acid sequences separated by a spacer.[19] Since

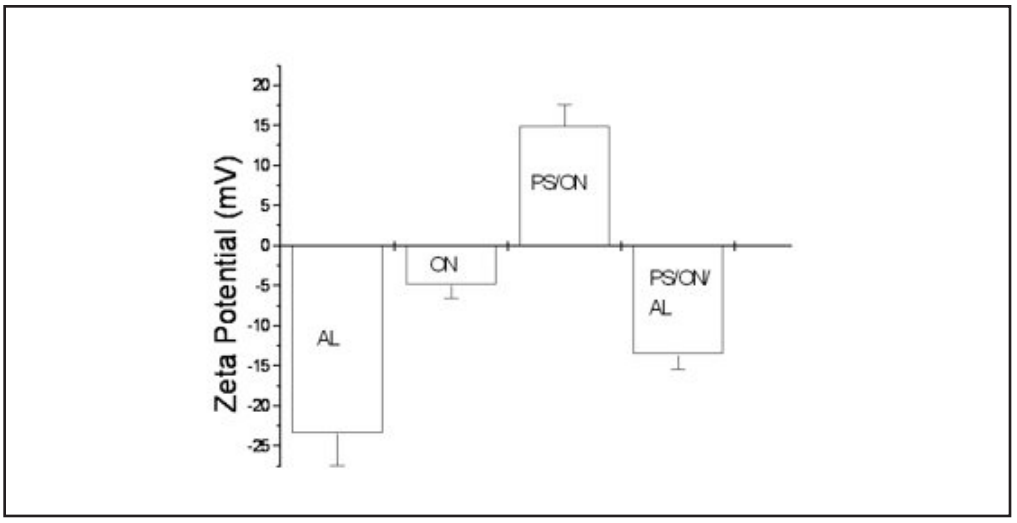

Figure 2. Zeta potential measurements of different formulations; (n=5).

protamine contains an NLS, there is a possibility that its molecules might be able to facilitate the active nuclear uptake of the complex into the nucleus. Finally, once the complex enters the nucleus, ON may have to dissociate from the polycations in order to be read by the host transcription machinery. PS, which is biodegradable and structurally more similar to other nuclear proteins than cationic liposomes, might be more readily dissociated from ON. Thus, polycations may play several roles such as reducing the complex size, providing an excess of cationic charges and therefore enhancing the cellular uptake by the endocytosis pathway, protecting ON from damage during this process, and possibly helping the release and relocalization of the delivered ON.[20]

Zeta Potential Measurements

Transfection complex formation is based on the interaction of the positively charged polycations with the negatively charged phosphate groups of the nucleic acid. The size and surface charge density of transfection complexes can be related to the transfection efficiency of a reagent. Values of the zeta potential of liposomes indirectly reflect vesicle surface net charge and therefore can be used to evaluate the extent of the interaction of the liposomal surface anionic charges with the precondensed ON. On this basis, the zeta potential of AL-liposomes was investigated before and after complexing with PS/ON complexes.

Results in Figure 2 show the negative zeta potential of the naked ON (-4.9 ± 1.6) and the AL-liposomes (-8.16 ± 1.7 mV), n=5. It was positive (14.96 ± 2.7) for PS/ON (3.6: 1 +/-). It became negative after adding liposomes, PS/ON was (-13.6 ± 1.8) for AL/PS/ON complex. The zeta potential values were negative in the case of AL/PS/ON.

ON Transfection

Success of human gene therapy depends on the development of delivery vehicles or vectors that can selectively deliver therapeutic genes to target cells with efficiency and safety. A lack of sufficient cellular and nuclear uptake of naked ON has been reported by several investigators,[9,22] presumably due to their high negative charge. In general, the nucleotide delivery by cationic lipids is substantially decreased in vitro if serum-

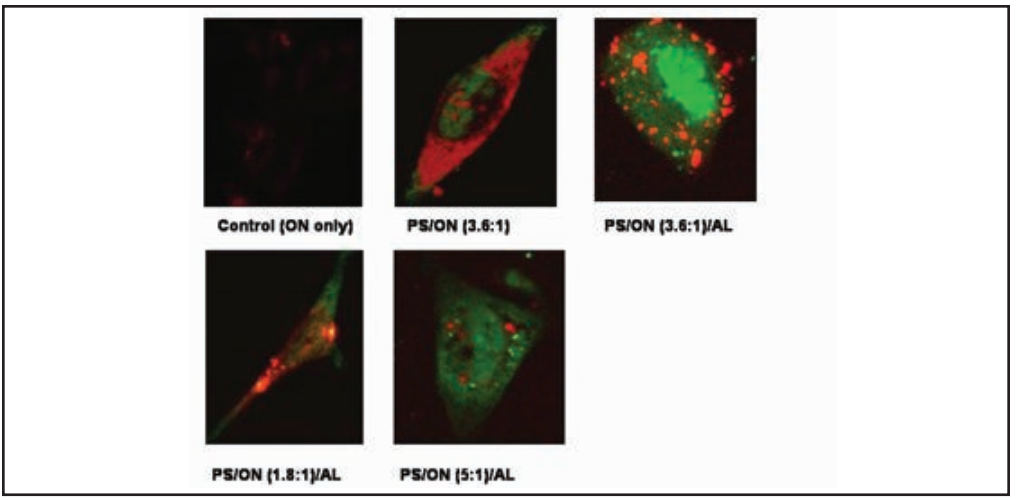

Figure 3. Confocal laser scanning microscopy of HeLa cells incubated with different complexes in culture medium supplemented with 10% fetal calf serum (FCS), at 37°C and 5% CO_2 after 6 hours.

containing medium is used.[23,24] Serum contains multitude of macromolecules and nucleases. The effect of serum is most substantial when the complexes are positively charged and subject to binding with anionic macromolecules of serum.[24] In contrast, negatively charged liposomes (like AL-liposomes) also interact with the biological environment in a nonspecific manner, but a liposomal gene delivery system with a net negative surface potential should exhibit less nonspecific tissue uptake and a better overall biocompatibility than cationic carrier systems.[6, 21]

Figure 3 shows the mean densities, which were calculated by the integration analysis of the images. In this case, HeLa cells transfected with AL/PS/ON and PS/ON complexes were (210 ± 14) and (169.24 ± 4.3), respectively. These results indicated that AL-liposomes effectively transported the PS/ON complex into the nucleus in the presence of serum more than PS/ON complexes alone.

Because of its positive charge, PS can help to fold ON and increase the transfection efficiency in the liposome-mediated gene transfer system.[8] PS proved to effectively enhance the transport of ON into the nuclei of HeLa cells. The primary role of PS is that of a condensation agent, although it also possesses several amino acid sequences that resemble that of an NLS.[8] With cationic charge in excess, ON is presumably protected from enzymatic degradation in the cytoplasm, and therefore sufficient ON entered the nucleus.

PS is an effective adjuvant for in vitro cationic lipid-mediated gene transfer. Due to its excellent safety profile, its use in vivo as a systemic transfection vector should be rigorously evaluated.

Figure 4 shows that the transfection of HeLa cells with PS/ON (3.6:1 +/-)/AL complex was more than that of PS/ON (1.8:1 +/-)/AL and PS/ON (5:1 +/-)/AL complexes under the same experimental conditions. The mean densities were (160 ± 62) and (133 ± 45) in PS/ON (1.8:1 +/-)/AL and PS/ON (5:1 +/-)/AL complexes, respectively. The results showed that PS/ON (3.6:1)/AL is the optimal amount for in vitro transfection, because the addition of more protamine did not increase the ON transfer efficiency. It is possible that, because of its

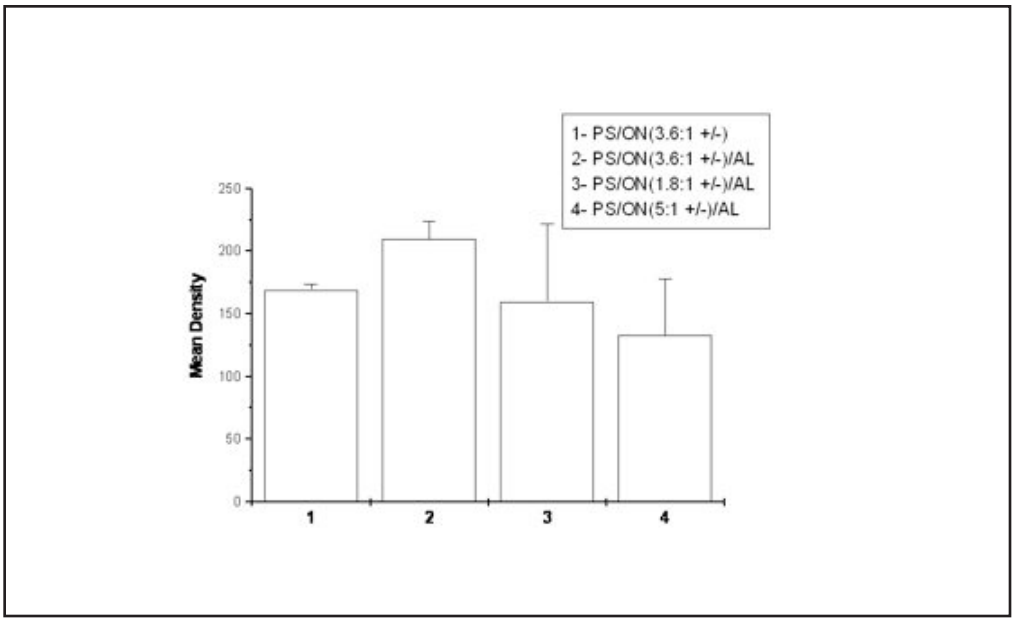

Figure 4. The mean densities (calculated by the integration analysis of the images in Figure 3) for the different formulations.

high affinity for ON, protamine hinders binding of transcription factors to ON and thereby inhibit expression when high levels are used for transfection.[25] At PS/ON (1.8:1 +/-) charge ratio, ON was not condensed totally by this amount of PS.

Successful transfection with anionic liposomes and PS-condensed plasmid was demonstrated in HepG2 cells in the presence of serum.[9,26] Anionic liposomes associated with PS-condensed ON, and this complex was able to be taken up by cells and to deliver ON to the nucleus. PS/ON complexes were released from the liposomal formulation, mainly as an extra nuclear enzymatic degradation of the liposomal phospholipids.[9]

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