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Optimizing Nanoparticle Design for Gene Therapy: Protection of Oligonucleotides from Degradation Without Impeding Release of Cargo

Oksana Fihurka, Juan Sanchez-Ramos*, Vasyl Sava

Department of Neurology College of Medicine, University of South Florida, Tampa, FL, USA

***Corresponding author:** Juan Sanchez-Ramos, Department of Neurology College of Medicine, University of South Florida, 13220 Laurel Drive Rm 4105, Tampa, FL 33612, USA. Tel: +18139745841; Email: jsramos@health.usf.edu

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Abstract

Gene therapy delivery systems that rely on synthetic nanocarriers can be optimized by assays of nucleic acid protection and kinetic studies of nucleic acid release. These empirical measurements ensure nanoparticle stability and predict potential *in vivo* efficacy. Quantitative methods for assessment of the capacity of nanoparticles to protect oligonucleotide cargo and to measure the rate of release of the cargo were developed and tested based on six commercial cationic matrices. *in vitro* study of drug release kinetics provides predictable release rates under a variety of conditions which can be adapted to appropriate physiological factors that affect release *in vivo*. In brief, *in vitro* DNA release and DNase I degradation assays described here will be useful for optimization of nanocarrier-mediated gene therapy administration by various routes.

Keywords: Gene Therapy; Nanoparticles; Protection of Nucleic Acid Cargo; Release of Nucleic Acids from Nanoparticles

Introduction

Gene therapy can profoundly impact disease progression by modulating gene expression, either by suppressing deleterious genes or increasing expression of beneficial genes. Gene expression can be silenced with Anti-Sense Oligonucleotides (ASO) or small interfering RNA (siRNA). Delivery of genes that upregulate biosynthesis of neurotransmitters or neurotrophic factors provides another avenue for modifying disease progression. The efficacy of foreign genetic material delivery is limited by the fact that oligonucleotides are inefficiently transported into most cells and nuclei, and these molecules are unstable in the cytosol due to degradation by nucleases [1]. Prerequisites for gene therapy include a) optimization of the delivery system, b) demonstration of the capacity of the oligonucleotide to enter the host cell, c) resistance to degradation and d) production of specific biological effects such as decreasing or increasing gene expression [2]. Packaging of nucleic acid payloads into nanoparticles is one of the most promising approaches for protection and optimizing the drug-like properties of oligonucleotides, such as bioavailability and cell targeting.

The composition and design of nanoparticles utilized for gene delivery plays a crucial role in determining the drug half-life time and biological effect. Nanoparticle matrices composed of polycations are the subject of interest, due to their versatility and proven gene delivery efficiency [3,4]. These structures are often used as the matrix for gene delivery systems because they have the ability to electrostatically bind oligonucleotides and compact it into nanostructures [5]. The resulting nanoparticles have colloidal dimensions, can protect genetic material from degradation, and facilitate cellular entry. Generally, these nanoparticles regulate gene expression via a mechanism which includes nanoparticle adhesion to the cell surface followed by cellular uptake, entry into the intracellular space and final release of the oligonucleotide [6].

Based on the above-mentioned considerations of nanoparticle properties, protection against enzymatic degradation and cargo release rate are crucial features for evaluation of the efficacy of drug delivery systems, despite a good safety profile [7]. Currently, the correlation between these factors and nanoparticles efficacy remains unclarified. We developed methods for quantitative evaluation of the capacity of nanocarriers to protect oligonucleotides from nuclease degradation with minimal interference with cargo release. Based on these methods six polycations were tested and their properties as oligonucleotide nanocarriers were studied.

Materials and Methods

Low molecular weight chitosan (mol. wt. 60,000-120,000 Da), chitosan oligosaccharide lactate (chitosan lactate Mn 4,000-6,000

Da), silica nanoparticles dispersion in water (<30 nm DLS), poly-L-ornithine hydrobromide (PLO mol wt 30,000-70,000 Da) and hexadimethrine bromide (polybrene Mn 1122 Da) were purchased from Sigma-Aldrich (Inc. St. Louis, MO). Polyethylenimine hydrobromide (PEI-MAX mol wt 40,000 Da) was purchased from Polysciences (Inc. Warrington, PA). In this work, oligonucleotide duplexed DNA (5`-TATATCAGTAAAGAGATTAA-3`, 5`-TTAATCTCTTTACTG-3`) model of hsiRNA directed against the huntingtin gene (htt) [8] was used for nanoparticles formulation.

Polycation/DNA nanoparticles were prepared via ionotropic gelation. Nanoparticles were spontaneously fabricated by mixing equal volumes of the polycation and DNA solutions under vigorous stirring at room temperature. The interaction, polycation/DNA molar and N/P ratios of full condensation of DNA by each nanocarrier were confirmed by gel retardation assay (Table 1). The hydrodynamic particle diameters of the nanoparticles, size distribution and surface charge (zeta potential) were measured at 25 C using Malvern Zetasizer Nano ZS90 (Westborough, MA) (Figure 1).

Ratio	Chitosan	Chitosan lactate	PEI-MAX	Polybrene	Poly-L-ornithine	Silica
molar	3	5	4.5	1.6	1.1	20
N/P	3.5	2	15	1	2.5	-

Table 1: Polycation/DNA molar and N/P ratios of six nanoparticles formulations.

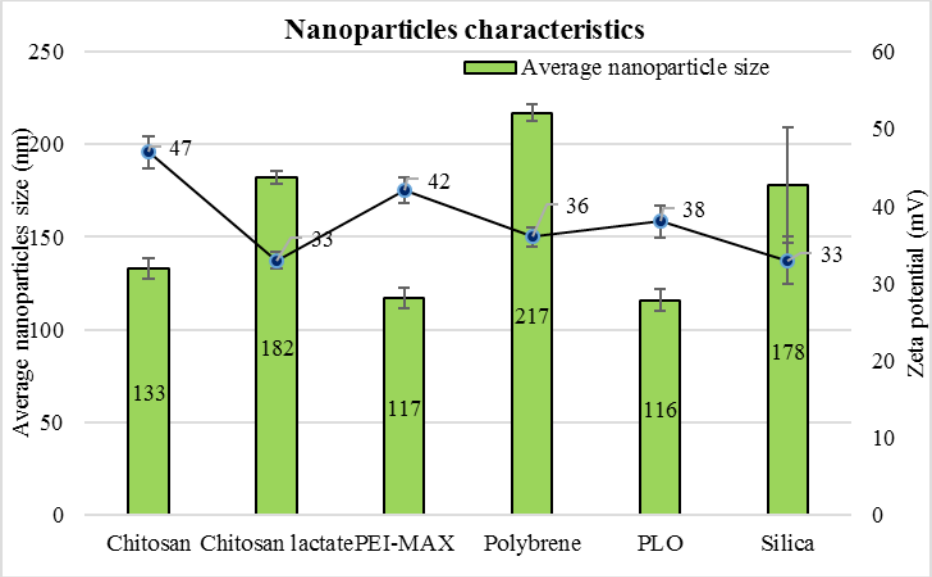

Figure 1: Average particle size and zeta potential of DNA containing nanoparticles with different polycations.

DNase I Degradation Assay

Naked DNA and six different DNA incorporated formulations (DNA amount - 13.5 µg) were placed in appropriate tubes. Then 2 µL of Ethidium Bromide (EtBr) solution (concentration - 1 µg/µL) were added to each tube. All samples were incubated with 13 µL of DNase I enzyme solution, approximately 1 U per 1 µg DNA. DNase I was maintained in Hank's Balanced Salt Solution *(HBSS)* with Ca^{2+} and Mg^{2+} enzyme activators (pH 7.2-7.4). Upon adding DNase, I, the kinetic measurements were conducted per 1 hour at 37°C with shaking. The digested DNA was quantified by determining the fluorescence of DNA and EtBr complex (excitation - 520 nm, emission - 605 nm), using a microplate reader (Spectra Max Gemini XPS). After each 10-minutes, fluorescence of each sample was recorded, and the relative fluorescence was calculated as follows: $F_r = (F_s\text{-}F)/(F_o\text{-}F)$, where F_r is the relative fluorescence, F_s is the fluorescence of the sample recorded at different time point, F_o is the fluorescence of appropriate nanoparticles solution with EtBr in the absence of DNase I enzyme, and F is the fluorescence of the matrix and EtBr solution. The amount of DNA was calculated based of the change of relative fluorescence as the result of the enzymatic digestion. The results were expressed as mean ± SD (n = 3).

DNA Release Assay of Nanoparticles in PBS

DNA and six different DNA containing formulations (400 µL) containing 54 µg of DNA were placed in appropriate tubes. Then 10 µL of ethidium bromide (EtBr) solution (concentration - 1

μg/μL) were added to each sample. Periodic samples were subject to centrifugation for 30 min at 10000 rpm, then the supernatant was removed and analyzed for the release of DNA. Then the 100 μL of the supernatant were placed into well (DNA amount 13.5 μg) and fluorescence of supernatants was measured. Samples were kept for different time points (24, 48, 72, 96 and 120 hours) under moderate heating at 37°C, the PBS was replaced at each time point. The amount of released DNA was calculated from the free DNA content in the supernatants, which was determined applying calibration curve DNA amount vs fluorescence response. Kinetics of DNA release from nanoparticles represented as the relative fluorescence value of DNA and EtBr complex (excitation - 520 nm, emission - 605 nm), using a microplate reader (SpectraMax Gemini XPS). All experiments were repeated three times. The results were expressed as mean ± SD (n = 3).

Results and Discussion

The ability of the carriers to protect cargo against enzymatic cleavage was studied using deoxyribonuclease I (DNase I) as an enzyme model. The protection assays showed that unprotected DNA was completely degraded by DNase I enzyme within 20 minutes, whereas DNA packaged in nanoparticles was stable for some time, depending on the nanoparticle matrix composition (Figure 2). Clearly, formation of compact nanoparticles not only condenses DNA but also protects DNA by sterically blocking access of nucleolytic enzymes [9].

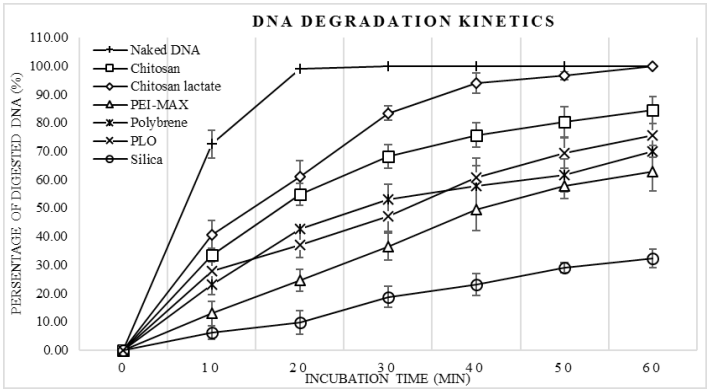

Figure 2: DNase I degradation assay. Naked DNA and six different DNA incorporated formulations were reacted with DNase I enzyme. The amount of undigested DNA was calculated based on the change of relative fluorescence as the result of the enzymatic digestion. The results were expressed as mean ± SD (n = 3).

Silica-based nanocarriers efficiently protected the DNA from degradation for all time periods. PEI-MAX, poly-L-ornithine, polybrene, and chitosan matrices exhibited approximately the same protective capacity as these. Chitosan lactate appears to be a loosely binding nanocarrier as it could not protect the DNA in DNase I degradation assay. Compared to all the other nanocarriers tested, the most tightly binding to nucleic acid cargo and the most protective against DNase I was the silica-based nanoparticle. A high degree of protection against DNA degradation is provided by strong binding between the polymer and DNA, with tight wrapping of cargo impeding the release of DNA. Another factor responsible for protection is localization of the cargo inside the particle which blocks enzymatic access to the nucleic acid. In contrast, when the DNA or oligonucleotide is located on the surface of the nanoparticle, it is easily exposed to rapid enzymatic cleavage. It is important to note that the concentration of DNase I in animal or human biofluids is much lower than used in this study. Even the administration of the nanoparticles formulated with chitosan oligosaccharide lactate may provide good protection under physiological conditions [10,11]. A critical step in gene or oligonucleotide delivery consists in efficient release of the cargo, because that is only way an oligonucleotide (e.g. anti-sense oligonucleotide) is able to interact with mRNA to lower specific gene expression. Drug release profiles are considered critical for determining the nanoparticle therapeutic efficacy *in vivo* [12,13]. It has been reported that more easily dissociated oligonucleotide-polymer nanoparticles also mediated higher transgene expression levels *in vitro* and *in vivo [14]*. Therefore, *in vitro* release studies are considered to be one of the key standards to evaluate and optimize the efficiency of delivery systems.

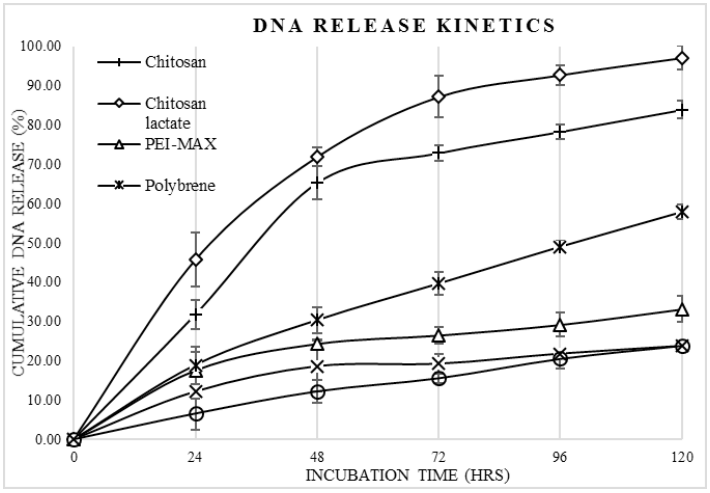

Figure 3: DNA release assay of nanoparticles in PBS. The kinetics of DNA release from six nanoparticles formulations represented as the relative fluorescence value of DNA and EtBr complex (excitation - 520 nm, emission - 605 nm), using a microplate reader (SpectraMax Gemini XPS). All experiments were repeated three times. The results were expressed as mean ± SD (n = 3).

DNA release kinetics of nanoparticles at pH 7.4 was conducted in a PBS buffer, as the model of some biological fluids: blood, cerebrospinal fluid, intracellular fluid, etc. The cumulative percentages of DNA released from nanoparticles based on different matrices as a function of time are shown in (Figure 3). The pH of the environment influences the stability of chitosan and chitosan

lactate nanoparticles profoundly, because amine groups of chitosan at neutral and basic conditions are less protonated and its chains become too rigid to exhibit noticeable electrostatic interaction activity [15]. Weakening of the electrostatic interactions with the negatively charged DNA initiated high and rapid, burst-like release from nanoparticles within the first two days of incubation, then DNA was constantly released up to more than 80%. The rapid release may be due to the outer DNA while the slowest release is due to DNA incorporated within the matrix. The achieved kinetic profile shows the existence of two different release mechanisms. The first one, occurring within 48 hours, is likely due to the release of DNA from the nanoparticle surface, while the second phase occurs later as DNA is constantly released from the particle core.

The polybrene-based matrix nanocarriers exhibit a constantly slow release of the cargo. At the end of the experiment it released more than 50 % of DNA. Compared to others, two polycations PEI-MAX and PLO released DNA very slowly, and the final released amount within 48 hours was close to 20%. The release rate of these nanoparticles was less than 5% per day over the final days. These nanoparticle matrices form small and compact particles due to the flexibility of linear chains (Figure 1). All these facts are indicators of very strong interactions between carrier and cargo. Nanoparticles with silica matrix demonstrated the lowest release rate and the highest protective capacity compared to all the tested matrices which characterize them as very stable.

From these data, long-term releasing matrices can be characterized as excellent carriers and protectors for systemic delivery or local application designed for slow and sustained constant release of cargo. With this slow release formulation, other properties of the delivery system must be carefully evaluated for a good safety profile [16]. Otherwise, polycationic carriers which show very high stability at basic conditions would have much higher stability at the pH<7 because of protonation of amino groups. As a result, depot effects may take place or the cargo might be not completely released.

Fast oligonucleotide release by matrices in our model indicates that these kinds of carriers might not effectively deliver the drug to the brain through such biological fluids as blood or cerebrospinal fluid. However, this formulation could be used for the non-invasive transmucosal route, as the pH level of mucous membrane secret is kept slightly acidic [17], thereby stabilizing the nanocarriers during migration. As soon as nanoparticles penetrate the cell and get into the intracellular fluid, due to their good unpackaging ability at the increased pH, genetic material will be able to regulate gene expression. Burst-like releasing matrices will require regular and frequent administration, which can be implemented by a nose-to-brain delivery route as reported previously [18].

Conclusion

In conclusion, nanoparticle stability is necessary for oligonucleotide protection, although, unpackaging is necessary for the delivered oligonucleotide to be able to interact with intracellular mRNA. The stability of nanoparticles is beneficial for the protection of nucleic acid cargo in transit to cellular and intracellular targets, but it limits the release of cargo that can result in low regulation of gene expression. Some optimal balance of oligonucleotide protection and release must be pursued, as an ideal carrier would provide total protection of incorporated DNA or other therapeutic nucleic acids from degradation prior to releasing it efficiently within the target cell. The studies of oligonucleotide enzymatic degradation and release kinetics are valuable as they provide information about nanoparticle stability and can be used for prediction of gene delivery systems biological effect *in vivo* from *in vitro* characterization studies.

Acknowledgements

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