PLASMID CONDENSATION INDUCED BY CATIONIC COMPOUNDS: HYDROPHILIC POLYLYSINE AND AMPHIPHILIC CATIONIC LIPID

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Abstract: The construction of an efficient carrier for genetic material is a major research objective that needs to be achieved before gene therapy can become a viable pharmacological approach. Artificial aggregates containing nucleic acids are one of the options for the systemic delivery of genetic information. The diversity of functions the aggregate is expected to fulfill necessitates its complex architecture. In order to obtain a complex supramolecular aggregate, formed from elements that are themselves complex molecules, appropriate procedures based on the detailed understanding of processes at the molecular level are required. In this study, we investigated how the various properties of cationic compounds affect nucleic acid condensation. The combination of two condensing agents, differing in their affinity towards water, when mixed with plasmids, resulted in aggregates which are resistant to enzymatic digestion and which form particles with well-defined size distributions. Such uniform and well-defined complexes may subsequently be further modified in order to obtain a fully functional genetic material carrier.

Key Words: DNA Condensation, Polylysine, Cationic Lipid

INTRODUCTION

Gene therapy is widely considered to be an attractive pharmacological strategy to cure both hereditary and non-hereditary diseases including cancer. Cell transfection is routinely achieved by applying viral vectors. However, problems with scaling up virus production and safety concerns have spurred intense
research aimed at developing a synthetic carrier for the nucleic acids. So far, this approach has had limited success, mainly due to low efficiency of transfection and poor control over formulation homogeneity. The major difficulty in developing such a synthetic carrier for genetic material lies in the design of a suitable supramolecular aggregate and the establishment of production protocols that would ensure the desired aggregate topology, sample homogeneity and high reproducibility. Applying genes for therapeutic purposes requires additional efforts, necessitated by the molecular properties of the nucleic acids themselves. Nucleic acids are highly charged linear polymers the dimensions of which highly exceed those of the cells intended for treatment. In addition, they are highly exposed to enzymatic hydrolysis in all body and cellular compartments. Nucleic acid properties, including size and effective charge can be altered by association with selected compounds. It has been shown that when aggregated with polycations, nucleic acids condense [1]. Numerous cationic amphiphiles have been synthesized and tested as potential components in aggregates containing DNA [2-5]. Despite more than a decade of lasting efforts, there has been very limited success in achieving the desired level of transfection efficiency. The major obstacle in developing an efficient synthetic vehicle for genetic material is in building the structure of the aggregate itself. In order to produce the particulate form of a nucleic acid, suitable production procedures need to be developed. The complexity of such a process is likely to depend on the desired aggregate composition and structure. There is numerous experimental data regarding the physicochemistry of nucleic acids, but surprisingly very little of it is utilized in synthetic vector formation protocols [6-8]. It is well documented that the structure of aggregates containing nucleic acids depends on sample history and the precise control of formation conditions. Consequently, each step in the production of a synthetic vector should be characterized in complete detail. Relatively few research efforts exist that aim at the full characterization of the intra- and intermolecular interactions between the compounds that will form such aggregates. The current state of knowledge regarding these problems is fragmentary and incomplete. For example, it has been established that polycationic compounds are needed to achieve nucleic acid condensation, but an understanding of their role in the resulting aggregate’s interaction with the cell membrane is still far from complete [9, 10]. Major difficulties arise from the complexity of events involved in such processes. In order to control and potentially utilize the processes occurring when an aggregate interacts with biological structures, the complexes of a nucleic acid and cationic compounds need to be well controlled and thoroughly characterized. Most protocols used to prepare the transfection reagents do not take the process of aggregate formation into account. It is very unlikely that the desired structure, which should contain molecules similar to plasmid in size, will form spontaneously in a one-step procedure. The complexity of the aggregate formation process has been recognized and appropriate efforts have been made by several groups. They tried to correlate desired aggregate topology with appropriate formation protocols.
As a result, a two-stage process was proposed. The procedure consists of the association of an amphiphilic cationic compound with a nucleic acid and the subsequent addition of compounds forming the aggregate outer shell. It relies on the differences between the intermediate forms of the aggregate in terms of their affinity towards water. The resulting aggregate topology, i.e. a nucleic acid core covered with a lipid layer, allows the processes of nucleic acid core formation and its subsequent coverage with a lipid surface to be independently manipulated [13]. In this paper, we present studies that aim to determine how condensing agent hydrophobicity affects nucleic acid core formation. The “core” will become a starting point for the subsequent steps in final aggregate formulation.

MATERIALS AND METHODS

AluI was obtained from MBI Fermentas (Germany). Ethidium bromide (EtBr) and poly-L-lysine solution (mol. wt. 70,000-150,000) (PLL) was purchased from Sigma-Aldrich (USA), and dioleoyl-3-trimethylammonium-propane (DOTAP) from Avanti Polar Lipids (USA). The pHßAPr-1-neo [10000 bp, [15]] and pUC19 [2686 bp, Gibco BRL (USA)] plasmids were purified according to the method described elsewhere [16], with slight modifications during the final purification stage [17]. Plasmid concentrations were determined spectroscopically by measuring absorbance at 260 nm.

The aggregate was formed by adding an appropriate amount of PLL and/or DOTAP, the latter from a concentrated chloroform stock solution, to the aqueous solution of DNA in 6 mM Tris-HCl buffer pH 7.4. The amount of chloroform added to the sample never exceeded 0.5 % v/v, therefore the effect of the organic solvent can safely be ignored. After obtaining the desired weight ratio, water was added in order to obtain a final volume of 600 µl.

For fluorescence measurements, 100 µl of the sample was mixed with 2880 µl of 6 mM Tris-HCl buffer pH 7.4. After aggregate formation, 20 µl of EtBr solution (100 µg/ml water) was added, and fluorescence measured at the excitation and emission wavelengths – 518 and 605 nm, respectively. Fluorescence measurements were carried out on an LS 50 Perkin Elmer Spectrometer (UK).

Aggregate sizes were determined using a ZETASIZER 5000, Malvern Instruments (UK) at 25°C. Samples used for size determination were prepared as follows: 160 µl of aqueous DNA solution (100 µg/ml) and 100 µl of 6 mM TrisHCl buffer, pH 7.4, were mixed with the appropriate amount of PLL and/or DOTAP. Finally, water was added to a final volume of 600 µl.

RESULTS AND DISCUSSION

The aggregate formation procedure proposed by Stuart et al. [13], initially designed for oligonucleotides, was later adapted for large nucleic acid molecules [11]. It consists of two well-defined steps: the condensation of the nucleic acid,
and aggregate surface formation. In order to obtain the desired final particulate topology, the result of the first step should be a nucleic acid condensate with well-defined physicochemical parameters. The quality of this intermediate structure will determine the final aggregate’s form. Nucleic acid condensation is achieved by mixing plasmids with polycationic compounds. The mixing procedure depends on the nature of the condensing agent; when hydrophilic, the condensation is carried out in the aqueous phase, while amphiphilic cations require more sophisticated approaches. Cationic lipids are routinely added to nucleic acid in the form of small liposomes or via combined multiphase systems of water and organic solvents [18, 13]. Electrostatic interactions between negatively charged DNA phosphate groups and the cationic residues of the condensing agent cause changes in spatial plasmid dimensions by four to five orders of magnitude [1]. The cationic lipids are routinely used as condensing agents for cell transfections in vitro as well as in vivo. However, there are a number of difficulties with their formulation. First, it is difficult to obtain a homogenous sample, and thus the resulting lipid-DNA aggregates have ill-defined properties. Second, cationic lipids are known to have toxic effects on cells [10]. It is therefore desirable to reduce their quantity to the minimum necessary. We have previously shown that the amount of DOTAP in a lipid formulation can be reduced to about 20 mol% of the used compounds [19]. Another strategy is to substitute the cationic lipid with other condensing agents that are less toxic. The difficulty with this approach is that the aggregate properties will then depend on the nature of the substitute as well as its quantity. In order to address this problem, in this study, two different cationic compounds were selected as condensing agents: hydrophilic PLL and amphiphilic cationic lipid (DOTAP). Their condensation with plasmid DNA was analyzed individually and as a mixture. The resulting aggregates were characterized with respect to their: (i) size distribution; (ii) accessibility to enzymatic digestion; and (iii) fluorescence labeling by ethidium bromide. The latter two tests are considered to be a measure of the extent of condensation [6]. Two plasmids of different sizes, pUC19 2686 bp and pHβAPr-1-neo 10000 bp, were used in the preliminary study to confirm that the condensation process is only weakly dependent on plasmid size, as was shown for lipopolyamine-DNA complexes [20].

Incubation of both plasmids with PLL (Fig. 1) as well as DOTAP (data not shown) gave similar results when the condensing effect was measured by the reduction in ethidium bromide fluorescence. For this reason, only plasmid pHβAPr-1-neo was used in further studies.

Fig. 2A shows that the accessibility of DNA binding sites for ethidium bromide decreases as the ratio of PLL/DNA in the aggregates increased, suggesting that the complexes formed from both plasmids and PLL are compact. However, when plasmids were condensed with DOTAP, there was a very limited reduction in ethidium bromide adsorption, indicating a loose aggregate structure (Fig. 3A).
Fig. 1. Complex formation of plasmid DNA representing different sizes (pUC19 2686 bp ○, pHβAPr-1-neo 10 000 bp ■) with PLL at the indicated ratios (w/w) of PLL/DNA. The increasing amounts of PLL were added to constant amounts of plasmid DNA in the presence of ethidium bromide (100 µg/ml water).

Additional information indicating that the PLL/DNA complexes were tightly packed came from experiments where aggregates were incubated with AluI. As shown in Fig. 2B, by contrast to DNA alone, no visible fragmentation of plasmid was seen for PLL/DNA complexes, regardless of the PLL:DNA ratio. On the other hand, treatment of DOTAP/DNA aggregates with restriction enzyme revealed that at the ratio of 1:1 (w/w), there was detectable DNA fragmentation. However, when the ratio of DNA to cationic lipid increased (lower amounts of cationic lipid in relation to DNA), enzymatic hydrolysis was substantially reduced (Fig. 3B). This observation contradicts a great deal of data available in the literature [2, 6, 21]. This discrepancy may result from changes in the aggregate preparation protocol. When cationic lipid is added from stock solution, its interaction with charged DNA phosphate groups will differ from that when it is added as preformed vesicles. In our preparation, an excess of lipid may result in vesicle formation and the adsorption of DNA onto their surface. Such a process gives an explanation for the dependence of DNA hydrolysis on the amount of lipid added.

When the sizes of aggregates were measured, the best distribution was obtained when the PLL/DNA ratio was 0.7:1 (w/w) (Fig. 2C). A single population of aggregates with a maximum around 0.5 µm was observed. When this ratio varies in either direction, the size distribution is less homogenous. The measurement of DOTAP/DNA aggregate size distribution shows that when the DOTAP:DNA ratio was higher than 1, a broad range of aggregate sizes was observed with a mean value of about 0.5-1 µm (Fig. 3C). When the lipid:DNA ratio was 0.7:1, a single aggregate population with a well-defined size distribution was present.
(the average size was about 0.5 μm). This result indicates that the excess of cationic lipid gives rise to an aggregate population with a large, broad size distribution.

Fig. 2. Properties of PLL/DNA complexes. A. Differences in ethidium bromide fluorescence intensity after complex formation of PLL/DNA (plasmid pHβAPr-1-neo) at the indicated ratios (w/w) of PLL/DNA. R is the ratio of fluorescence for the sample with the indicated ratios (w/w) of PLL/DNA and that with plasmid DNA alone. The background fluorescence level was subtracted from both values. B. Protection of plasmid pHβAPr-1-neo DNA complexes by PLL to AluI. PLL/DNA complexes were subjected to electrophoresis in 1% agarose at different PLL/DNA ratios. Lane 1 – DNA + AluI; lane 2 – PLL/DNA (1:1) + AluI; lane 3 – PLL/DNA (0.7:1) + AluI; lane 4 – PLL/DNA (0.5:1) + AluI; lane 5 – DNA; lane 6 – PLL/DNA (1:1); lane 7 – PLL/DNA (0.7:1); lane 8 – (0.5:1). C Distribution of PLL/DNA aggregate sizes at indicated ratios (w/w) of PLL/DNA.
Fig. 3. Properties of DOTAP/DNA complexes. A. Differences in ethidium bromide fluorescence intensity after complex formation of DOTAP/DNA (plasmid pHβAPr-1-neo) at the indicated ratios (w/w) of DOTAP/DNA. R is the ratio of fluorescence for the sample with the indicated ratios (w/w) of DOTAP/DNA and that with plasmid alone. The background fluorescence level was subtracted from both values. B. Protection of plasmid pHβAPr-1-neo DNA complexes by DOTAP to AluI. DOTAP/DNA complexes were subjected to electrophoresis in 1% agarose gel at different DOTAP/DNA ratios (w/w). Lane 1 – DNA; lane 2 – DOTAP/DNA (1:1); lane 3 - DOTAP/DNA (0.7:1); lane 4 - DOTAP/DNA (0.5:1); lane 5 – DNA + AluI; lane 6 – DOTAP/DNA (1:1) + AluI; lane 7 – DOTAP/DNA (0.7:1) + AluI; lane 8 – DOTAP/DNA (0.5:1) + AluI; lane 9 – DOTAP/DNA (2:1); lane 10 – DOTAP/DNA (2:1) + AluI. C. Distribution of DOTAP/DNA aggregate sizes at indicated ratios (w/w) of DOTAP/DNA.

Taking DNA accessibility to enzymatic digestion into account, it can be stated that an optimal fraction of amphiphilic compound exists that ensures a tight packing of the complex. However, condensation induced by cationic lipid does not protect DNA from ethidium bromide fluorescent labeling. It has been shown previously that the association of cationic lipids with nucleic acids results in the formation of aggregates the topologies of which depend on the formation conditions and the composition of the condensing mixtures [6, 21].
Fig. 4. Properties of DOTAP/PLL/DNA complexes. A. Differences in ethidium bromide fluorescence intensity after complex formation of DOTAP/PLL/DNA (plasmid pHβAPr-1-neo) at indicated ratios (w/w/w) of DOTAP/PLL/DNA. R is the ratio of fluorescence for the sample with indicated ratios (w/w/w) of DOTAP/PLL/DNA and that with plasmid DNA alone. The background level of fluorescence was subtracted from both values. B. Protection of plasmid pHβAPr-1-neo DNA complexes by DOTAP/PLL to AluI. DOTAP/PLL/DNA complexes were subjected to electrophoresis in 1% agarose gel at different DOTAP/PLL/DNA ratios (w/w/w). Lane 1 – DNA; lane 2 – DNA + AluI; lane 3 – DOTAP/PLL/DNA (1:0.7:1); lane 4 – DOTAP/PLL/DNA (1:0.7:1) + AluI; lane 5 – DOTAP/PLL/DNA (0.7:0.7:1); lane 6 – DOTAP/PLL/DNA (0.7:0.7:1) + AluI; lane 7 – DOTAP/PLL/DNA (0.5:0.7:1); lane 8 – DOTAP/PLL/DNA (0.5:0.7:1) + AluI; lane 9 – DOTAP/PLL/DNA (1:0.5:1); lane 10 – DOTAP/PLL/DNA (1:0.5:1) + AluI; lane 11 – DOTAP/PLL/DNA (0.7:0.5:1); lane 12 – DOTAP/PLL/DNA (0.7:0.5:1) + AluI; lane 13 – DOTAP/PLL/DNA (0.5:0.5:1); lane 14 – DOTAP/PLL/DNA (0.5:0.5:1) + AluI. C.
Distribution of DOTAP/PLL/DNA aggregate sizes at indicated ratios (w/w/w) of DOTAP/PLL/DNA.

The general strategy of obtaining an aggregate suitable for subsequent modifications, i.e. covering the DNA condensate with a lipid bilayer, requires the DNA to be simultaneously compact and hydrophobic. Results presented thusfar show that PLL condenses DNA very efficiently, but the resulting structure is hydrophilic, making the next step of the procedure, outer lipid layer formation, difficult or even impossible. DOTAP, on the other hand, ensures aggregate hydrophobicity, but the packing is somewhat looser as judged by the enzymatic fragmentation and ethidium bromide labeling. An optimal aggregate structure would be that of tightly packed DNA with a hydrophobic outer shell formed of the hydrocarbon chains of cationic lipids [11]. Therefore, a combination of PLL and DOTAP may possibly result in such a structure. In this case, DNA was condensed in two steps. First, the plasmids were mixed with an appropriate amount of PLL, and then DOTAP was added.

Fig. 4A shows selected examples of condensates treated with ethidium bromide. Fluorescent labeling reached levels intermediate to that of each compound alone. Exposure to enzyme digestion shows a lack of DNA fragmentation (Fig. 4B). Aggregate size distribution was very homogenous, with an average aggregate size of about 0.4 μm at an DOTAP:PLL:DNA ratio of 0.7:0.7:1 (w/w/w), (Fig. 4C).

In summary, we have shown that the combination of two condensing agents, differing in their affinity towards water, when mixed with plasmids resulted in aggregates which are resistant to enzymatic digestion and form particles with well-defined size distributions. These aggregates represent the intermediate stage of the final structure during the process of genetic material carrier formation. Such uniform and well-defined complexes may subsequently be further modified in order to obtain the fully functional aggregate with physicochemical parameters that can be modified at will.

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REFERENCES


