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Complexes of non-cationic liposomes and histone H1 mediate efficient transfection of DNA without encapsulation

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Abstract

Transfection competent complexes were assembled using a three component system. The constituents of the basic system were plasmid DNA, cationic DNA binding protein (NLS-H1) and anionic liposomes (dioleoyl phosphatidylethanolamine (DOPE) or phosphatidylserine (PS)). In contrast to cationic liposome/DNA binary complexes, all of the DNA in these ternary complexes was sensitive to DNase I degradation and ethidium bromide intercalation. Transmission electron microscopy revealed that these ternary complexes formed unique structures in which the DNA was located either on the outside of individual liposomes or bridging two or more liposomes. This provides evidence that plasmid DNA encapsulation is not essential for transfection competency.

Keywords: Gene therapy; Gene delivery; Liposome

1. Introduction

For effective gene therapy to become a reality the development of new vectors are needed. One approach involves the use of liposomes for gene delivery. Liposomes are attractive for a variety of reasons including, their ease of use, low level of toxicity and high fusogenic potential. Many early studies used neutral or anionic liposomes to deliver DNA to cells. From a structural standpoint it was generally thought that the DNA must reside in the aqueous interior of the liposome to be transfectable. While complete encapsulation of DNA within liposomes can be accomplished by several methodologies [1–10], low entrapment efficiency remains a significant problem.

The caveats encountered using uncharged or anionic liposomes have been largely alleviated by the use of cationic liposomes such as Lipofectin [11]. From transfection experiments it quickly became evident that electrostatic interactions between DNA and cationic liposomes were sufficient for functional complex formation. From structural studies it is now clear that a large percentage of plasmid DNA in cationic lipid/pDNA complexes is sequestered inside the complex in such a way that it is inaccessible to ethidium bromide and DNase I [11–13]. Thus, it appeared that complete coating of plasmid DNA by cationic liposomes might be an important parameter for transfectability regardless of the charge of the liposome.

In this study novel transfection competent complexes were formed by using a component system that included cationic DNA binding protein (NLS-H1), fusogenic liposomes (DOPE) and supercoiled plasmid DNA. In these ternary complexes the cationic protein compacts the DNA as well as facilitates binding to the anionic (at pH 8.5) DOPE liposomes. Efficient transfection of marker genes by these complexes provided evidence for a functional ternary complex interaction. Interestingly, structural analysis of these transfection competent complexes suggested that in contrast to previously described pre-formed complexes (i.e., Lipofectin/pDNA) the plasmid DNA in these ternary complexes was located on the outside of the liposomes.

2. Materials and methods

2.1. Proteins and lipids

The DNA binding protein NLS-H1 consisting of the nuclear localizing sequence of SV40 T antigen and human

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histone H1 was prepared as described by Fritz et al. [18]. Dioleoyl phosphatidylethanolamine (DOPE) and phosphatidylserine (PS) were purchased from Avanti Polar Lipids (Birmingham, AL). Lipofectin (DOTMA/DOPE) was purchased from Gibco-BRL (Grand Island, NY).

2.2. Liposome preparation and complex formation

2 mg of lipids (DOPE, PS) in chloroform were transferred to 1.5 ml microfuge tubes and vacuum dried overnight to remove traces of solvent. 1 ml of sterile 30 mM Tris-HCl (pH 8.5) was added and tubes were sonicated in a base sonicator (Branson 2200) until emulsion was clear. Three different liposome/plasmid DNA complexes were formed, two using anionic liposomes (PS) and/or DOPE at pH 8.5 and one using the cationic liposome Lipofectin (DOTMA/DOPE). Ternary complexes were formed by first mixing DOPE liposomes and NLS-H1 in 30 mM Tris-HCl pH 8.5 (10 min room temperature) at a 9:1 wt/wt ratio. Plasmid DNA was then added at a protein: DNA weight ratio of 1.5:1 which yields a 1.2 to 1 positive charge ratio. The final weight ratio was 1 part pDNA, 1.5 parts NLS-H1 and 12 parts DOPE. This overall ratio yielded a slightly positively charged complex as determined by charge calculation. Ternary complexes prepared using negatively charged liposomes (PS/DOPE) were formed similarly except the Tris-HCl pH 8.5 was replaced with Opti-MEM (Gibco-BRL). Binary complexes consisting of cationic lipid/pDNA were prepared according manufacturers recommendations. Specifically, Lipofectin (DOTMA/DOPE) was complexed with pDNA at a 3:1 wt/wt ratio. By charge calculation this ratio yields positively charged complexes. After all components were added complexes were incubated for an 10 additional minutes at room temperature prior to transfection or DNase I treatment.

2.3. DNase assays

Following complex formation, buffer was adjusted to 100 mM Tris-HCl pH 8.5, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and DNase I was added. Complexes were incubated for 30 min at 37°C. EDTA was added to 20 μM final concentration to stop DNase I digestion. For transfections, DNase I treated complexes were added directly to wells as described below. For agarose gel analysis, complexes were extracted with phenol/chloroform to remove protein and lipid components prior to loading. This effectively removed the excess positive charge and allowed the remaining DNA to migrate into the 1% agarose gel.

2.4. Transfections

NIH 3T3 cells (mouse fibroblast) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. For gene expression studies the luciferase encoding plasmid pBS.RSVLux was used [14]. All plasmid DNA was purified by alkaline lysis and two CsCl gradients as previously described [15]. At transfection, cells were washed once in Opti-MEM followed by addition of 2 ml of Opti-MEM to each 35 mm well. Pre-formed complexes (3 μg pDNA/well in a 150 μl volume) were then added to each well and dishes were placed at 37°C in 5% CO_2. After a 1–4 h incubation, complexes were removed and 2 ml of fresh growth medium was added. Cells were harvested after 40–48 h as previously described and assayed for luciferase expression [16,17]. Total cellular protein levels were determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA).

2.5. Recombinant proteins

NLS-H1 is a bacterially expressed fusion protein consisting of the nuclear localizing sequence (NLS) of SV40 T antigen fused to the C-terminal portion (amino acids 99–193) of human histone H1 [18].

2.6. Sucrose gradients

Pre-formed ternary complexes were analyzed by density centrifugation through a sucrose gradient. Complexes were prepared as described for transfection studies. Fluorescent DNA was prepared by mixing pDNA with 5 μM TOTO (Molecular Probes, Eugene, OR). This results in a labeling efficiency of approximately one TOTO molecule per 40 base pairs. TOTO has a binding constant for DNA in the range of 10^{10}–10^{12} M^{-1} [19]. Fluorescent liposomes were prepared by mixing DOPE (2 mg/ml) and rhodamine-DPPE 10 μg/ml (Molecular Probes). A 10 ml linear 5–20% sucrose gradient was poured and pre-formed complexes were carefully layered on top. Centrifugation was performed in a SW-41T rotor at 35 000 rpm for 17 h. After centrifugation, 0.6 ml fractions were collected from the bottom. After evacuation the centrifuge tubes were washed with Tris pH 8.5 and sonicated (fraction #0) to remove pelleted material from the tube. The rhodamine (λex-572 nm and λem-590 nm) and TOTO (λex-509 nm and λem-533 nm) fluorescence were determined using a spectrofluorimeter (Hitachi).

2.7. Electron microscopy

Ternary complexes were formed as described for transfections. Colloidal gold labeled plasmid DNA was prepared as previously described [20] and transmission electron microscopy was performed [21]. Briefly, 300 mesh copper grids were coated with pioloform (Ted Pella Instruments) and treated with 0.1 mg/ml bacitracin solution. After bacitracin removal and drying, one drop of liposome (complex) suspension was placed on the grid for 3 min. Adsorbed complexes were then negatively stained with

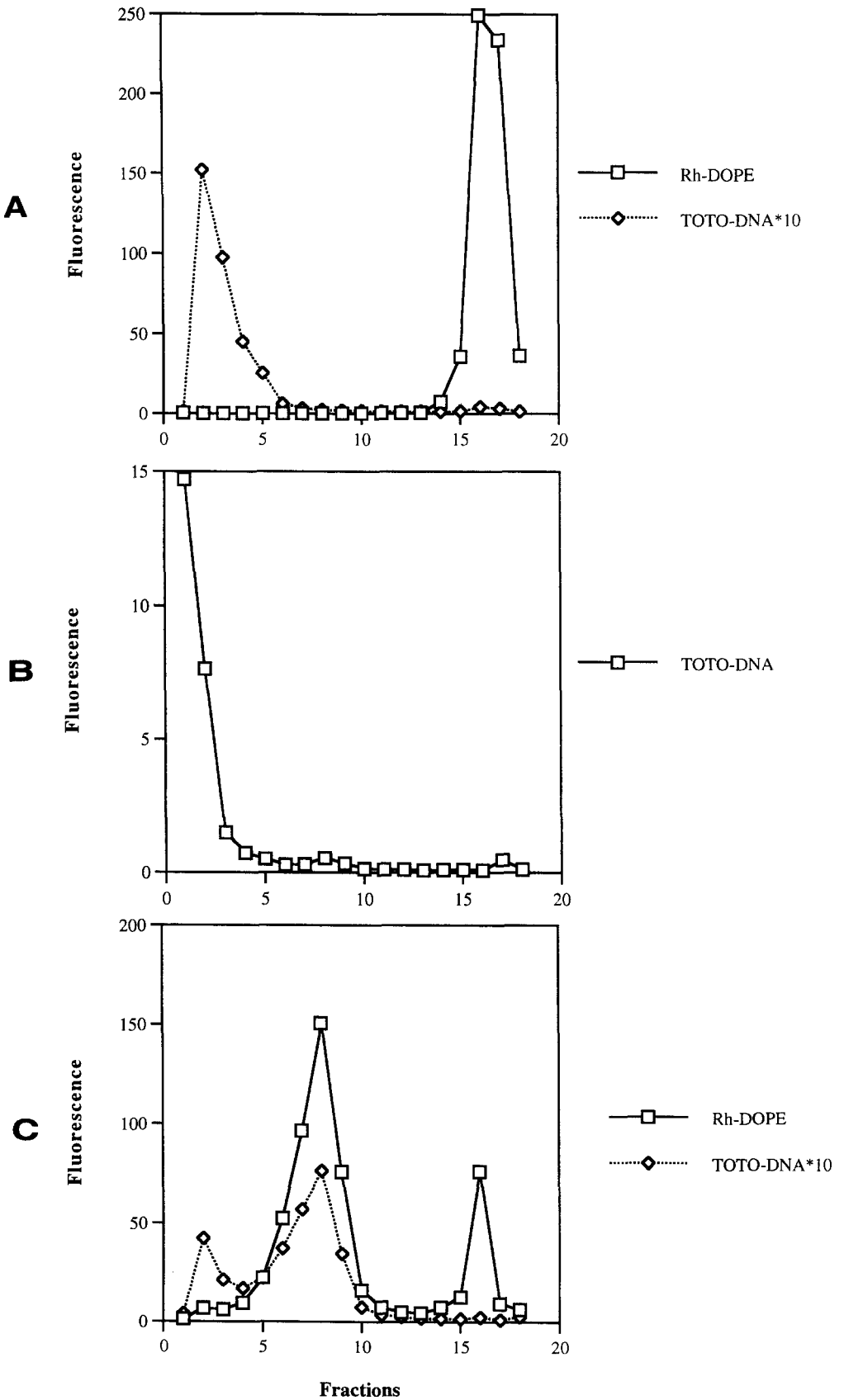

Fig. 1. Migration of fluorescently-labeled DOPE liposomes (0.5% rhodamine-DPPE + DOPE) and plasmid DNA (TOTO-pDNA) before and after complex formation using density gradient centrifugation (5–20% sucrose). (A) Rh-DOPE (120 μg) + TOTO-pDNA (10 μg); (B) NLS-H1 (15 μg) + pDNA (10 μg); (C) NLS-H1 (15 μg) + TOTO-pDNA (10 μg) + Rh-DOPE (120 μg).

one drop of a 1% aqueous uranyl acetate solution for 3 min. After removal of the negative stain the grids were air dried and analyzed in the transmission electron microscope JEOL JEM 100-CX with 80 kV accelerating voltage.

2.8. Fluorescence microscopy

Fluorescent DOPE liposomes were formed by mixing DOPE with rhodamine-DPPE (Molecular Probes Eugene, OR) prior to sonication. Final concentration of rhodamine-DPPE in DOPE liposomes was 0.5%. 3T3 cells were grown on sterilized, untreated glass coverslips. Following an 80 min incubation with the complexes in Opti-MEM (Gibco-BRL), the cover slips were removed from the 35 mm dishes and washed 2 × in PBS and then fixed in a freshly prepared 4% paraformaldehyde/PBS solution for 20 min. After fixation, cells were washed twice in PBS, counterstained with a 1 μg/ml Hoechst 33258 solution for 5 min, and washed two more times in PBS. Cover slips were mounted onto glass slides using Gel-Mount (Biomeda) and analyzed on a Leitz Orthoplan microscope equipped with a Ploemopak fluorescence vertical illuminator.

3. Results

3.1. Plasmid DNA, NLS-H1 and DOPE liposomes associate to form ternary complexes at pH 8.5

To make transfection competent complexes using three different molecules we needed to determine if there was a stable association of all the components. Dioleoyl phosphatidylethanolamine liposomes were incubated with an excess of the cationic DNA binding protein NLS-H1 in 30 mM Tris-HCl pH 8.5. The alkaline pH of 8.5 stabilizes the DOPE liposomes in the lamellar phase and gives them a net negative charge allowing them to interact electrostatically with the positively charged NLS-H1 protein. This interaction results in positively charged DOPE/protein complexes which are then mixed with plasmid DNA. To confirm that all three components (pDNA, NLS-H1 and DOPE) were present in the final complexes, density centrifugation of the complexes was performed through a 5–20% linear sucrose gradient. Using both rhodamine-labeled DOPE liposomes and TOTO-labeled plasmid DNA as markers we found that the location of the TOTO-pDNA in the gradient varied dramatically depending on which components were present in the mixture. When the plasmid DNA and DOPE liposomes were mixed together at pH 8.5 (without NLS-H1) we found no interaction as evidenced by their separated elution profile (Fig. 1A). The location of the TOTO-DNA and DOPE liposomes in the sucrose gradient was similar if they were mixed together or run separately (data not shown). Conversely, when the labeled plasmid DNA was mixed with either NLS-H1 alone or NLS-H1 + DOPE liposomes there was a dramatic shift in mobility. With protein (NLS-H1) alone the complexes decreased in density but became insoluble so that they pelleted during the gradient centrifugation (Fig. 1B). Only when all three components were present did the majority of pDNA and DOPE liposomes co-migrate at the same position (Fig. 1C). NLS-H1 + DOPE resulted in binary complexes with a density greater than DOPE liposomes alone but less than ternary complexes (data not shown).

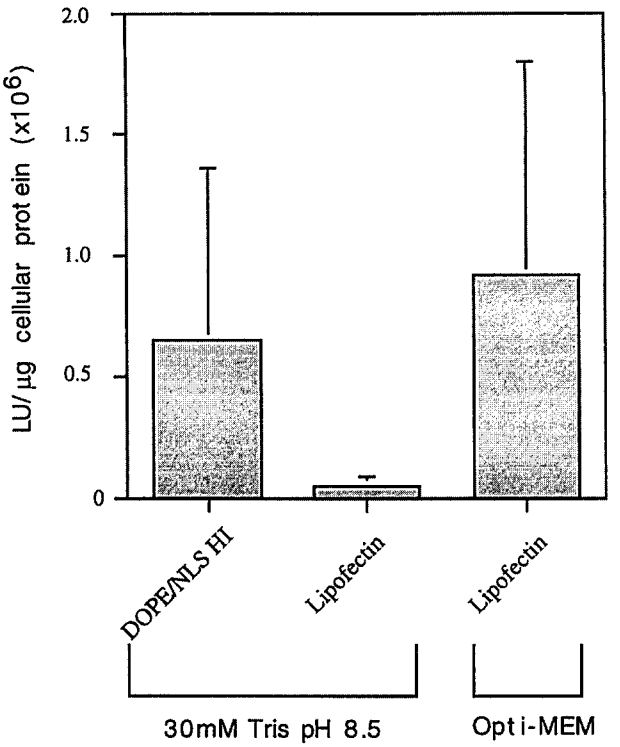

Fig. 2. Comparison of transfection efficiencies of Lipofectin/pDNA binary complexes and DOPE/NLS-H1/pDNA ternary complexes in NIH 3T3 cells. The pH dependent ternary complexes were prepared in Tris-HCl (pH 8.5) and compared with Lipofectin/pDNA binary complexes prepared in both the same buffer and manufacturer recommended buffer (Opti-MEM - Gibco/BRL). Lipofectin/pDNA in Opti-MEM ($n = 38$), Lipofectin/pDNA in Tris pH 8.5 ($n = 4$), DOPE/NLS-H1/pDNA in Tris pH 8.5 ($n = 14$). Error bars indicate standard deviation.

3.2. Plasmid DNA / NLS-H1 / DOPE ternary complexes can efficiently transfect NIH3T3 cells

NIH3T3 cells were exposed to ternary complexes consisting of pBS.RSVlux plasmid DNA encoding the firefly luciferase cDNA, NLS-H1 and DOPE liposomes. Luciferase values from cells transfected with the ternary complexes averaged higher than those transfected with the cationic lipid Lipofectin when done in similar buffers (Fig. 2).

Transfections were also performed using ternary complexes in which the DOPE liposomes contained 5% phosphatidylserine (PS). The addition of 5% PS to DOPE

created fusogenic liposomes that were negatively charged (anionic) at neutral pH. Ternary complexes containing these anionic liposomes (PS-DOPE/pDNA/NLS-H1) were as transfection competent as when DOPE alone was the lipid (data not shown). The cell lines Cos and HepG2 were also efficiently transfected using DOPE/pDNA/NLS-H1 ternary complexes (data not shown).

3.3. Plasmid DNA in DOPE/NLS-H1/pDNA complexes is not encapsulated

To determine if the plasmid DNA in a ternary complex is sequestered within a DOPE liposome or associated with the outside of the vesicle, we incubated the complexes with either DNase I or ethidium bromide. If in fact the pDNA is encapsulated within a lipid bilayer it will not be accessible to either of these agents. Following incubation of DOPE liposome/NLS-H1/pDNA ternary complexes (or PS-DOPE ternary complexes) with DNase I (Fig. 3A) no supercoiled pDNA remains intact, indicating that all pDNA is accessible to the enzyme. Conversely, the cationic liposomes DOTMA/DOPE (Lipofectin) interact with plasmid DNA in such a way as to provide protection for approximately 50% of the supercoiled pDNA band (Fig. 3A). The pDNA contained in ternary complexes was also accessible to ethidium bromide intercalation (data not shown).

Further studies were done to determine whether or not the protected pDNA within the cationic lipid/DNA complexes was the primary mediator of transfection. 3T3 cells were exposed to cationic lipid/pDNA binary complexes pre-treated with a large excess of DNase I. The 50% reduction in luciferase expression was consistent with the completely protected supercoiled pDNA (i.e., encapsulated) accounting for exogenous gene expression (Fig. 3B). Alternatively, DNase I pretreatment of DOPE/NLS-H1/pDNA transfection complexes completely abolished exogenous gene expression (Fig. 3B). Taken together these results argue that while pDNA/NLS-H1/DOPE ternary complexes and cationic lipid/pDNA complexes are both transfection competent they are structurally very different.

3.4. Plasmid DNA is located on the outside of (or between) individual liposomes after ternary complex formation

Transmission electron microscopy (TEM) was performed on ternary complexes consisting of plasmid DNA (labeled with 5 nm colloidal gold particles) [20], NLS-H1 and DOPE liposomes. This revealed that the gold labeled pDNA in these ternary complexes was located on the outside of individual liposomes or was bridging two or more independent liposomes (Fig. 4). The location of the labeled pDNA appears similar whether it is found associated with large or small DOPE liposomes (compare Fig. 4A and B). During complex formation we often found large aggregates which appeared to be dependent on the

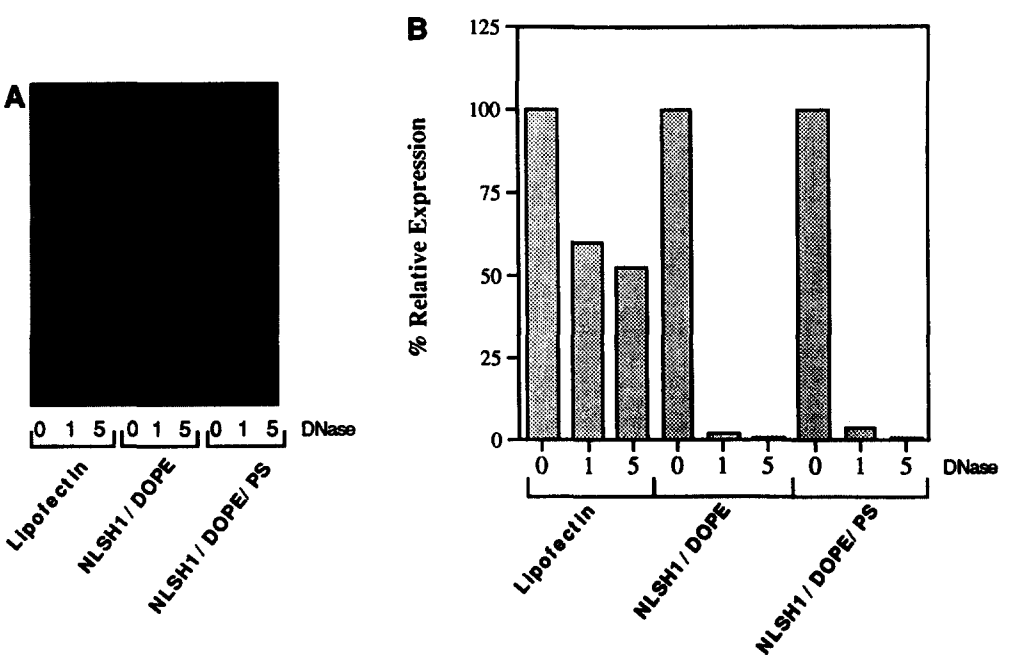

Fig. 3. DNase I analysis of pDNA following complex formation. Binary complexes were formed using 1 μg plasmid DNA and cationic liposomes (3 μg Lipofectin). Ternary complexes were formed using plasmid DNA, NLS-H1 and either pH sensitive liposomes (DOPE) or anionic liposomes (DOPE 95%/phosphatidylserine 5%). Following complex formation, DNase I was added in the indicated amounts and complexes were incubated at 37°C for 30 min. (A) 1% agarose gel electrophoresis of DNase I digested complexes. Numbers below the lanes represent microliters of DNase I added (2.7 units/μl). (B) Relative luciferase activity following transfection of DNase I treated and untreated complexes into NIH 3T3 cells.

amount of cationic protein present during complex formation (data not shown). Even after aggregate formation it appeared that the pDNA remained on the outside of the liposomes (Fig. 4C). When DOPE liposomes and colloidal gold labeled pDNA were incubated together (minus NLS-H1) no labeled liposomes were observed (data not shown).

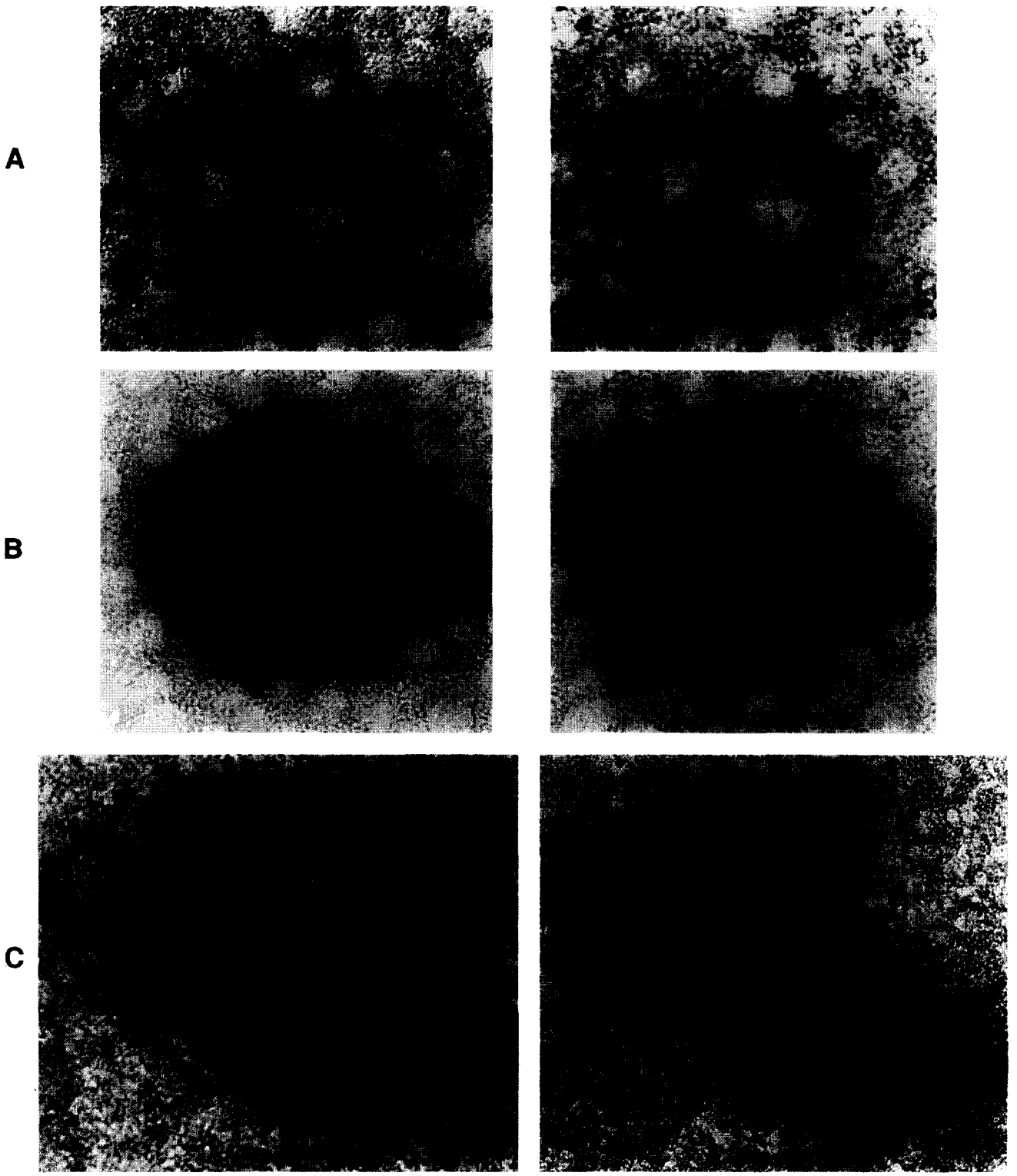

Fig. 4. Stereographic transmission electron micrographs (TEM) of DOPE/pDNA/NLS-H1 ternary complexes (12:1:1.5 wt/wt/wt ratio) using colloidal gold (5 nm) labeled plasmid DNA. All observed complexes were from a single complex formation using sonication prepared DOPE liposomes. (A) ternary complexes with 20–40 nm DOPE liposomes; (B) ternary complexes with 100–150 nm DOPE liposomes; (C) ternary complex aggregates. Final magnification is 500 000 ×.

3.5. Ternary complex formation enhances the fusogenicity of DOPE liposomes

Fluorescently labeled DOPE (Rh-DOPE) liposomes were used to monitor uptake of individual DOPE liposomes (control) or liposome complexes by NIH3T3 cells. Interestingly, neither DOPE liposomes alone nor NLS-H1/DOPE binary complexes were able to fuse with the cells (data not shown). Although NLS-H1 and DOPE form complexes at pH 8.5 they appear as large aggregates on EM (data not shown). Only when ternary complexes were formed and incubated with the cells did we see a high level of fluorescently labeled lipid in the cytoplasm (Fig. 5). Based on rhodamine fluorescence (yellow cytoplasm) nearly 100% of the 3T3 cells took up the liposomes when incubated with the ternary complex.

4. Discussion

While liposomes have proven to be efficient mediators of transfection there are still limitations associated with the current liposome delivery vehicles. Specifically, there remains a wide range of transfectabilities with regard to different cell lines or cell types in vitro. Additionally, and perhaps more importantly transfection of cells in vivo, with few exceptions, [22] has been refractory to efficient liposome mediated DNA delivery [23]. Therefore, there is a need for new types of transfection competent assemblies.

In this study we used three components to build a basic transfection system. The constituents of the basic system consist of plasmid DNA, cationic DNA binding protein (NLS-H1) and fusogenic lipid (DOPE). The cationic protein serves a dual role whereby it functions as a DNA compacting agent and as a bridge to initiate and maintain

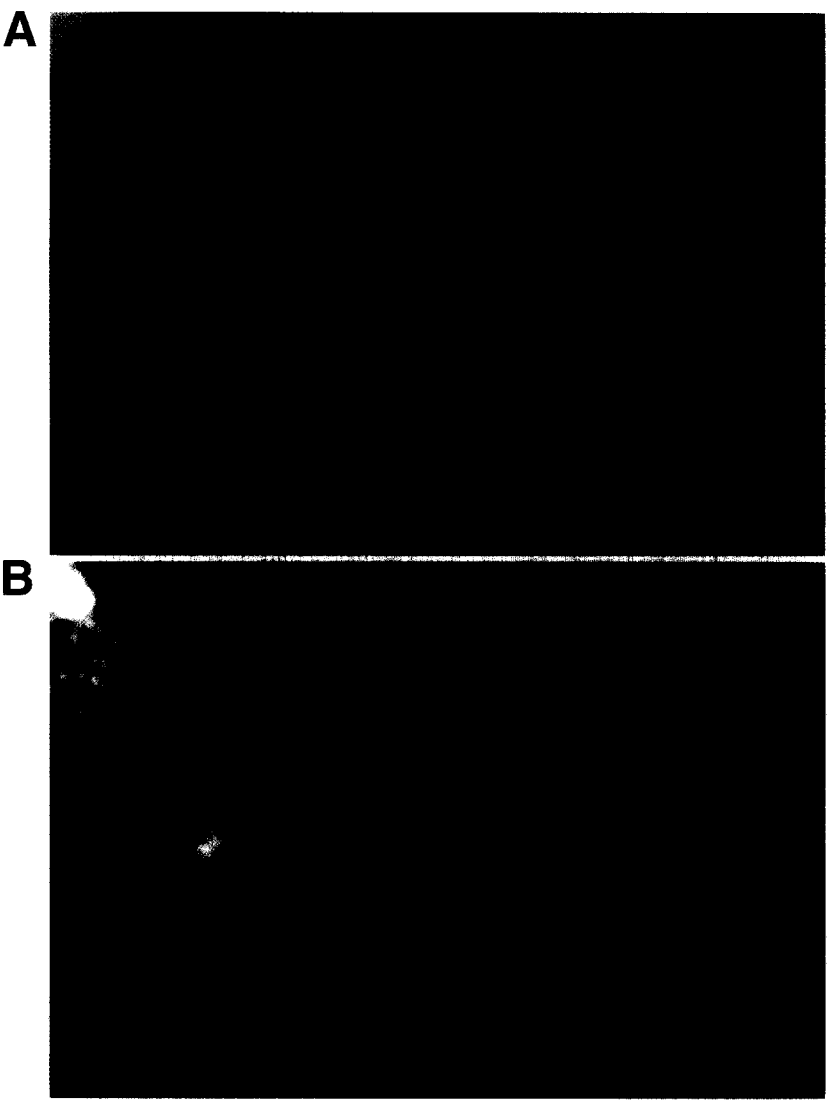

Fig. 5. NIH 3T3 cells following incubation with ternary complexes containing rhodamine-labeled DOPE/pDNA/NLS-H1. Complexes were prepared as previously described and incubated with the cells for 80 min at 37°C. The paraformaldehyde fixed cells were counterstained with Hoechst 33258 to show nuclear location. (A) NIH 3T3 cells after incubation with ternary complexes. (B) Hoechst staining of NIH 3T3.

stable complex association between the liposomes and the DNA. DNA binding/compacting agents have previously been shown to be effective at reducing the size of both linear and plasmid DNA [12,24] consequently, allowing them to be packaged into DNA/liposome complexes more efficiently. We have found NLS-H1 to be superior to a variety of other DNA binding agents including histones 2a, 2b, 3, 4, protamine, poly-L-lysine, lysozyme, cytochrome *c*, nuclear localizing peptides and gramicidin S [18,25] (data not shown). The criterion for assessing superiority was by measuring transfectability as assayed by reporter gene expression (luciferase). At present it is unclear what NLS-H1 confers on the transfection complex that allows for higher expression as compared to other DNA binding proteins. Fritz et al. [18] have shown that it is likely not the simple presence of the SV40 T antigen nuclear localizing signal in this construct since calf thymus histone H1 functions similarly. In all transfections we have performed to date, NLS-H1 or histone H1 has functioned to increase transfectability regardless of the cell type. Based on these results it is reasonable to believe that the benefits that this protein confers on transfectability might be applicable to transfections in a wide variety of cell types both in vitro and in vivo.

An early postulate of liposome-mediated transfection had been the idea that DNA had to be encapsulated in order for it to be transferred into and expressed within cells [9,26]. In the context of anionic or neutral liposomes, encapsulation is the primary means by which DNA can be tightly associated with the liposomes. However, complexes of cationic liposomes and DNA can form a variety of species. The encapsulation of DNA by cationic liposomes has been suggested by fluorescence-quenching studies which indicated that DNA induced fusion of cationic liposomes and also by electron microscopic studies which revealed tubular structures that presumably represented DNA enveloped by a lipid bilayer [12,27]. Protection of DNA by cationic liposomes from interaction with DNase or ethidium bromide is also consistent with its encapsulation. On the other hand, electron microscopic studies also revealed that many of the cationic liposomes form aggregates in the presence of DNA [27,28]. Shielding from DNase or ethidium bromide interactions could result from the DNA being 'sandwiched' between bilayers of the cationic liposomes. Thus, it remains unclear whether transfection by cationic liposomes is mediated by either encapsulation or aggregation of DNA.

The current study used histone H1 protein to associate DNA with anionic liposomes. In contrast to the studies of cationic liposomes, all of the pDNA responsible for the expression in the ternary complexes was not protected. These results provide unequivocal evidence that pDNA protection is not essential for transfection competency. The EM results also indicate that pDNA is not encapsulated. While it is possible that H1 could have increased the leakiness of the liposomes for small molecules such as ethidium bromide, it is highly unlikely that the DNase I ($\sim$ 31 000 daltons) could have accessed DNA within even leaky liposomes.

The fact that DNA located on the outside of a liposome complex is still able to efficiently transfect a cell raises a series of mechanistic questions. From a simplistic standpoint, it is easy to visualize that once DNA is encapsulated within the aqueous environment of a liposome, a fusion event with either the plasma membrane or an internal endosome would allow the aqueous contents to be extruded into the cytoplasm. On the other hand, if the DNA is associated with the outside of a liposome it is unclear how it would access the cytoplasm even after a liposome mediated fusion event. Experiments are currently being conducted to better understand this process.

The initial goal of this work was to design a component system for liposome mediated DNA delivery. The benefit of a component system lies in its inherent flexibility with regard to assembling a wide variety of complexes by changing only one component. Up until now, only cationic liposomes could be used to package DNA into complexes quickly and efficiently. We now have evidence that pDNA/NLS-H1 can form transfection competent complexes with anionic (Fig. 3), and cationic [18] liposomes. Thus a wide range of very different liposomes can be incorporated into these ternary complexes and tested for transfection capabilities both in vitro and in vivo.

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