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Surface modification of adenovirus by zwitterionic (DMPC:Chol) liposomes can up- or down-regulate adenoviral gene transfer efficiency *in vitro*

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The interaction of adenovirus with liposomes formulated from dimyristoyl phosphatidylcholine (DMPC) and cholesterol (Chol) was characterized. Turbidity and particle sizing studies do not indicate the formation of large adenovirus-liposome aggregates leading to large-scale flocculation of nanoparticles. However, a high degree of association between liposomes and adenovirus (Ad) can be identified by electron microscopy (TEM) analysis. Most strikingly, gene transfer experiments demonstrate that adenovirus infectivity can be positively and negatively modulated by Ad surface modification achieved by mixing Ad with different concentrations of DMPC:Chol liposomes. These results indicate that it may be possible to engineer viral nanoparticles and alter adenoviral tropism through interaction with zwitterionic phosphtidylcholine and cholesterol containing liposomes.

Key words: Adenovirus – Liposome – Gene therapy – Nanoparticles - Surface engineering – Nanotechnology - Dimyristoyl phosphatidylcholine – Cholesterol – Tropism – Targeting.

Adenoviridae are DNA viruses comprised of a 36 kb linear, double stranded DNA genome and core proteins surrounded by capsid proteins. There are at least 49 human adenovirus (Ad) serotypes, categorized into six subgroups based upon DNA homology. The subgroup C viruses, types 2 and 5, are most highly characterized, and are the basis for gene transfer vectors currently in use [1]. The vector, once administered, binds to high affinity receptors on the target cell surface, translocates to the nucleus, and inserts the genetic material it carries to the nucleus. This material, including the transgene carried in its expression cassette, remains separate from the target cell chromosomes, yet begins to express the transgene, allowing a functional gene product to be produced [2].

For adenovirus, determinants of tissue tropism are presumed to be the viral capsid proteins, hexon, penton base and fiber. The fiber contains the knob domain which interacts with the primary cellular receptor, Coxsackie/Adenovirus Receptor (CAR). The penton base protein contains the RGD sequence motif which is responsible for interaction with cell surface integrins, and this interaction is essential for internalization. Adenoviral vectors easily infect cell lines that are CAR sufficient and those that are CAR deficient are difficult to infect. While some attempts to alter adenoviral tropism have been made, the problem of such methods in the past has been that while specificity can be added, the endogenous specificity to CAR and integrin is difficult to overcome [3-5].

By comparison, non-viral vectors, particularly liposome based vectors, are easily produced and are able to deliver DNA to a variety of cells and tissues both *in vitro* and *in vivo*. Due to the electrostatic nature of the liposome:DNA interaction with the plasma membrane, non-viral vectors, are capable of delivering genes efficiently to a much broader range of cell types, however, compared to their viral counterparts, with far less efficiency *in vivo* [6, 7]. Like their viral counterparts, intravenously injected liposome-based vectors are generally found in the liver, where most are cleared from the circulation by macrophages. Moreover, liposomes are easily modified in the laboratory by changing their chemical make-up, thereby altering the physicochemical characteristics of the vectors, and can be tailored to specific needs. They have little or no toxicity, and though cleared from the blood stream by components of the immune system, they induce no long-lasting immunity. Additionally, as their pharmacological profile is determined entirely by their physicochemical properties, alteration of any of their components to modify liposome-based vector tropism, does not suffer from the problem of overcoming endogenous affinity for a specific receptor as in the case of adenoviruses [8, 9].

Numerous groups have reported that adenovirus infectivity can be upregulated by interaction with null (DNA-free) cationic liposomes by simple mixing prior to infection in a process commonly referred to as lipoduction [10-12]. It is believed that the virus forms a complex with the positively charged liposomes, which then associate with the negatively charged cell membrane. Similarly, the interaction of null (transgene-free) adenoviruses with liposome-DNA complexes has been shown to increase non-viral gene expression, in what is called adenofection [13, 14]. All such studies aimed at enhancing either viral or non-viral gene expression by assisting the transgene-carrying vector through interaction with components capable of more efficient electrostatic binding (lipoduction) or endocytosis (adenofection).

More recently, Balakireva *et al*. reported that adenovirus infectivity, following interaction with liposomes made from a neutral phospholipid, dipalmitoyl phosphatidylcholine (DPPC), is upregulated in the human lung adenocarcinoma cell line A549 [15]. In this study, it is believed that liposomes interact specifically with the hexon proteins on the Ad capsid, forming some sort of complex with the viral particles. These complexes are thought to translocate through the target cell membrane independently of virus-specific pathways, leading to enhanced gene expression when compared to Ad alone, particularly in CAR deficient cell lines.

The effect of liposome physicochemical characteristics on the interaction with viral nanoparticles (such as adenoviruses) will determine the gene transfer capabilities of the complex vectors. Factors such as complex size, degree of aggregation, stability in solution, and charge, all play a role in vector targeting [16-18]. In order to fully understand the nature of the interaction of adenovirus with liposomes and how that influences transfection efficiency, it is necessary to study these properties.

It is our aim to engineer novel synthetic-viral nanoparticles through surface modification of recombinant adenovirus vectors with lipids using basic self-assembly principles. In this study, we

set out to characterize the interaction of adenovirus with liposomes formulated from the neutral phospholipids dimyristoyl phosphatidylcholine (DMPC) and cholesterol. Interestingly, our turbidity studies (UV/vis spectrophotometry) do not indicate the formation of large adenovirus-liposome aggregates leading to large-scale flocculation of nanoparticles. However, we can identify a high degree of association between liposomes and adenovirus by electron microscopy (TEM) analysis. Most strikingly, we are able to both up- and down-regulate adenovirus mediated gene transfer in A549 cells simply by altering the adenovirus to liposome ratio. These results indicate that it may be possible to engineer the viral nanoparticles and alter adenoviral tropism through interaction with zwitterionic phosphtidylcholine containing liposomes.

I. MATERIALS AND METHODS

1. Liposomes

DMPC (dimyristoyl phosphatidylcholine), DOTAP ((1,2-dioleoyloxypropyl)-N,N,N-trimethylammonium chloride) and Chol (cholesterol) all were purchased from Sigma, UK. DMPC:Chol , at a 2:1 molar ratio, or DOTAP:Chol, at a 1:1 molar ratio, liposomes were prepared by the lipid film method. Briefly, lipids were dissolved in 4:1 chloroform:methanol in a 50-ml round bottom flask. A lipid film was formed using a rotovaporator. After 1 h under a vacuum, the film was futher dried for 5 min under a nitrogen stream. One millilitre of PBS or distilled and deionized water was then added to the flask to hydrate the film. To insure thorough hydration, a small magnetic stirrer was added and the hydrating films were stirred for 30 min. The stirrer was removed and the film was then added to a sonicating water bath for 30 min. Liposomes were allowed to sit at room temperature for at least 30 min before use.

2. Viral vectors

Adenovirus (Ad) was purchased from the Baylor College of Medicine Vector Devolopment Lab in Houston, Texas, USA. For these studies, the vector Ad.βgal, encoding for the betagalactosidase gene driven by the CMV promoter was used. Stocks were stored in glycerol buffer at a concentration of 5 x 10^{12} particle units (pu)/ml.

3. Preparation of adenovirus-liposome complexes

Adenovirus stock was thawed then diluted in distilled and deionized water (ddH2O) or PBS and added to an equal volume of liposomes suspended in ddH2O or PBS and mixed by rapid pipetting in a 1.5-ml polypropylene microcentrifuge tube (Eppendorf, UK). The complex was allowed to stabilize at room temperature for 30 min prior to analysis. For turbity studies, complexes were formed in PBS at a final adenovirus concentration of 5 x 10^{10} pu/ml and 2 mM DMPC or DOTAP concentration by mixing 500 μl of adenovirus (1 x 10^{11} pu/ml) with 500 μl of either DMPC:Chol (4 mM) or DOTAP:Chol (4 mM) liposomes. For photon correlation spectroscopy experiments, complexes were formed in PBS at a final adenovirus concentration of 2 x 10^{11} pu/ml and 2 mM DMPC concentration by mixing 250 μl of adenovirus (4 x 10^{11} pu/ml) with 250 μl of DMPC:Chol liposomes (4 mM). Because PBS can cause precipitates to form in uranyl acetate solutions, complexes for transmission electron microscopy were formed in ddH2O at a final adenovirus concentration of 2 x 10^{11} pu/ml and 2 mM DMPC concentration by mixing 125 μl of adenovirus (4 x 10^{11} pu/ml) with 125 μl of DMPC:Chol liposomes (2 mM). For the assessment of gene transfer efficiency of adenovirus mixed with liposomes, three separate complexes were formed in ddH20 at a final adenovirus concentration of 2 x 10^{11} pu/ml and 2, 1 or 0.5 mM DMPC concentration by mixing 40 μl of adenovirus (4 x 10^{11} pu/ml) with 40 μl of liposomes (4, 2 or 1 mM, respectively).

4. Turbidity assay (UV/vis spectrophotometry)

Measurements: all measurements were made in PBS, pH 7.4 using a DU 650 spectrophotometer (Beckman Instruments, UK). DMPC:Chol, DOTAP:Chol, and adenovirus-liposome complexes were prepared as described. Absorbance of adenovirus alone was measured at an adenovirus concentration of 5 x 10^{10} pu/ml in 1 ml of PBS.

5. Mean nanoparticle sizing assay (photon correlation spectrometry)

All measurements were made using the Zetasizer 3000 (Malvern Instruments, UK). Ninety degree scattering was assessed and the software was set for automatic analysis. DMPC:Chol and adenovirus-liposome complexes were prepared as described. Size analysis of adenovirus alone was conducted at an adenovirus concentration of 2 x 10^{11} pu/ml.

6. Electron microscopy (negative stain transmission electron microscopy)

Images were made using a CM120 BioTwin electron microscope (Philips/FEI, USA). DMPC:Chol and adenovirus-liposome complexes were prepared as described, then dried onto a gold grid and stained with 1% aqueous uranyl acetate. Images of adenovirus alone were taken using adenovirus at a concentration of 5 x 10^{12} pu/ml in glycerol buffer.

7. Gene transfer studies

For all studies, A549 cells (ATCC, USA), a human lung adenocarcinoma cell line, were grown to confluency in 24 well tissue culture dishes (Corning BV, The Netherlands). Each well of the 24 well plate was estimated to contain 2 x 10^5 cells per well, which at a dose of 500 adenovirus particle units (pu) per cell yields a total adenovirus particle number of 2.5 x 10^8 pu/50 μl or 5 x 10^9 pu/ml. DMPC:Chol and adenovirus-liposome complexes were prepared as described, then were diluted to an adenovirus concentration of 5 x 10^9 pu/ml in 50 μl of serum-free DMEM and added to the A549 cells, yielding a dose of approximately 500 Ad particles per cell. All experiments were done in triplicate wells. Cells were incubated with the virus for 90 min, washed, then incubated in complete media for 48 h. Cells were harvested in 200 μl of lysis buffer and 10 μl of the lysate was analysed for β-gal activity using the Tropix Galactolight Plus kit (Applied Biosystems, USA) and a Berthold Lumat 9507 luminometer (Berthold Instruments, Germany).

II. RESULTS

In order to test the hypothesis that viral nanoparticles can be engineered with different gene delivery capabilities depending on their interaction with non-ionic liposomes, characterisation of the physicochemical properties of complex vectors formed by interacting adenovirus and DMPC:Chol liposomes was carried out. As a measure of liposome aggregation, absorbance of 400 nm light was assessed using a UV/vis spectrophotometer. Typically, changes in turbidity of a liposome suspension can be monitored by measuring absorbance at 400 nm. In parallel, it may be possible to monitor the resulting aggregation of adenovirus. Bondoc and Fitzpatrick have reported that by measuring the ratio of the absorbance of an adenovirus preparation at 320 nm to that of 260 nm, it is possible to detect the formation of Ad aggregates [19]. They indicate that this ratio should be approximately 0.23-0.27 in non-aggregated samples of Ad and will increase as aggregates form. Unfortunately, at the concentration of Ad and liposomes used for this study, Ad measurements were overwhelmed by the presence of liposomes in the sample, and the data proved inconclusive (data not show). However, this may be of use in future experiments with higher Ad and lower liposome concentrations.

Liposomes formulated at a 2:1 DMPC:Chol molar ratio were maintained at 25ºC and 2 mM DMPC concentration in PBS. As a control, we also measured the interaction of adenovirus with cationic DOTAP:Chol liposomes, formed at a 2 mM DOTAP concentration.

Ad, at a concentration of 1 x 10^{11} pu/ml in 500 μl of PBS, pH 7.4 was mixed with 500 μl of the liposomes and the mixture was allowed to stabilize for 30 min, then absorbance at 400 nm was assessed. Representative data can be seen in *Figure 1*. There is no increase in the 400 nm absorbance spectrum of the DMPC:Chol liposomes after they have been mixed with Ad, while, as expected, the adenovirus-DOTAP:Chol mixture exhibits higher turbidity relative to the liposomes alone. The observation that no increase in turbidity occurs in the adenovirus-DMPC:Chol sample indicates the absence of extensive aggregation occurring following interaction between the DMPC:chol liposomes and the adenovirus. This could be expected as, in the absence of strong, long-distance electrostatic forces to draw virus and liposomes together, any molecular interaction between the virus and the lipids would likely require close proximity. However, this spectrophotometic assay is not sensitive enough to obtain interactions between virus particles and liposomes that do not lead to extensive flocculation, and therefore considerable increase in turbidity. Moreover, the liposome concentration in our experiments is overwhelmingly responsible for the observed turbidity, masking any molecular interactions that do not lead to colloidal aggregation. Nanoscale associations between the viruses and liposomes had to be investigated using different experimental techniques.

Greater insight into the ensuing interactions between adenoviruses and liposomes was obtained by studying the distribution of mean particle sizes using photon correlation spectroscopy. For these experiments, we increased the final adenovirus concentration to 2 x 10^{11} pu/ml as the instrument being used, the Malvern Zetasizer 3000, was unable to accurately measure lower concentrations of adenovirus. The liposome concentration remained constant at 2 mM DMPC. Equal volumes of liposomes and adenovirus were mixed and the complex was allowed to stabilize. We observed a small increase in the mean hydrodynamic diameter of the adenovirus-DMPC:Chol mixture compared to liposomes alone (*Figure 2*). From this data a simple averaging effect between the liposomes and the highly monodisperse adenovirus is occurring. Association of adenovirus with liposomes does not lead to appreciable increases in light scattering. In this way, the PCS data correlate with our observations from the turbidity study (*Figure 1*) indicating that extensive flocculation between adenovirus particles and DMPC:Chol liposomes leading to increases in light scattering is not occurring.

Next, we looked at the structural characteristics of the interacting nanoparticles by studying transmission electron micrographs of the Ad-DMPC:Chol mixtures. Our previous data indicated that no extensive virus-liposome aggregation occurred, nor any considerably large aggregate structures were formed. These observations were confirmed by electron microscopy (*Figure 3*). Adenovirus can be seen closely associated with liposomes ranging in size from less than 50 nm to greater than 300 nm in diameter. Moreover, viruses appear to associate with single or multiple liposomes by specific points of contact; however, no appreciable aggregation leading to structures in the hundreds or thousands of nanometers was found throughout the samples. These images strongly support the hypothesis that phosphatidylcholine containing liposomes have a affinity for the surface proteins of adenovirus [15], and therefore can be used to modify the adenovirus surface.

Finally, we looked at the transfection efficiency of adenovirus mixed with various concentrations of DMPC:Chol (*Figure 4*). A549 cells were selected as they retain many of the characteristics of type II alveolar epithelium, one of the major targets for pulmonary gene therapy. We observed that when adenovirus was mixed with liposomes at a 0.5 mM DMPC concentration, we were able to enhance expression by almost 25% over adenovirus alone. Surprisingly, as the DMPC concentration was increased during the mixing stage, thereby increasing the lipid to adenovirus ratio, Ad transfection efficiency is markedly reduced, falling to less than one fourth of the activity of Ad alone. The increase at low DMPC concentration followed by a decrease at higher DMPC concentrations was striking, especially since we were unable to detect the formation of aggregates at similar virus and liposome concentration. As A549 cells express high levels of CAR, the high affinity adenovirus receptor, it is surprising that transfection efficiency was decreased. This observation certainly bears further investigation as it indicates that surface modification of adenovirus by DMPC:Chol liposomes is able to both positively and negatively modify adenovirus transfection efficiency, possibly through alteration of cellular binding, intracellular trafficking or both.

III. DISCUSSION

The present work demonstrates that adenovirus infectivity can be positively and negatively modulated by Ad surface modification achieved by mixing Ad with different concentrations of DMPC:Chol liposomes. Further, this modulation does not appear to be due to the formation of large adenovirus-liposome aggregates, but by some other mechanism.

It has been demonstrated that other phosphatidylcholine (PC) containing liposome formulations such as DPPC liposomes form complexes with adenovirus [15]. As the degree of fluidity of the li-

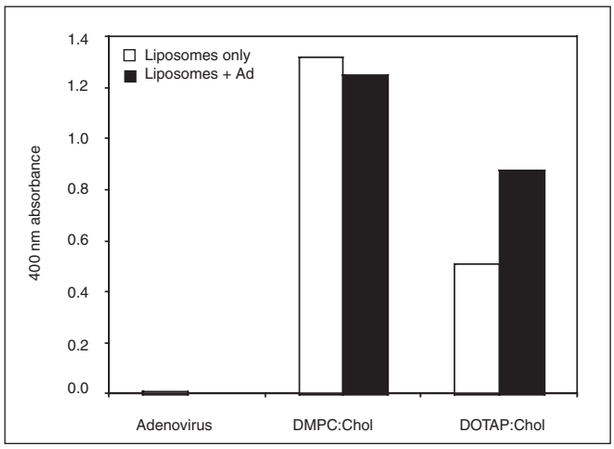

Figure 1 - Assessment of liposome flocculation following mixing with adenovirus by spectrophotometric absorbance of 400 nm wavelength light. DMPC:Chol or DOTAP:Chol liposomes were prepared at a 2 mM DMPC or DOTAP concentration in PBS, pH 7.4. Liposomes were mixed with an equal volume of PBS containing 1 x 10^{11} pu/ml of adenovirus.

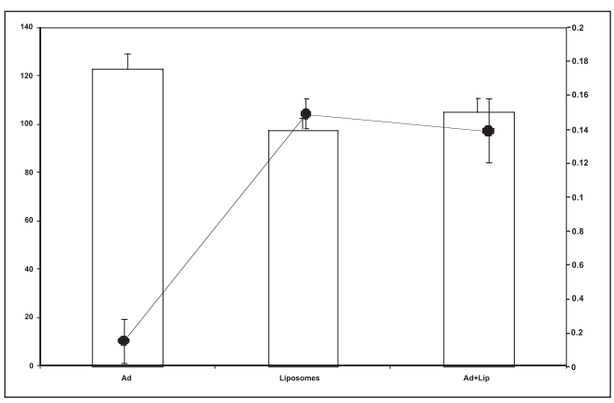

Figure 2 - Measurement of hydrodynamic diameter (bars and left hand axis) and polydispersity (line and right hand axis) of virus-liposome mixtures by PCS at 90 degree deflection; DMPC:Chol liposomes at a 4 mM DMPC concentration, were mixed with an equal volume of water containing 4 x 10^{11} Ad pu/ml. Complexes were allowed to form for 30 min prior to measurement. Data is expressed as the mean ± SD of triplicate samples.

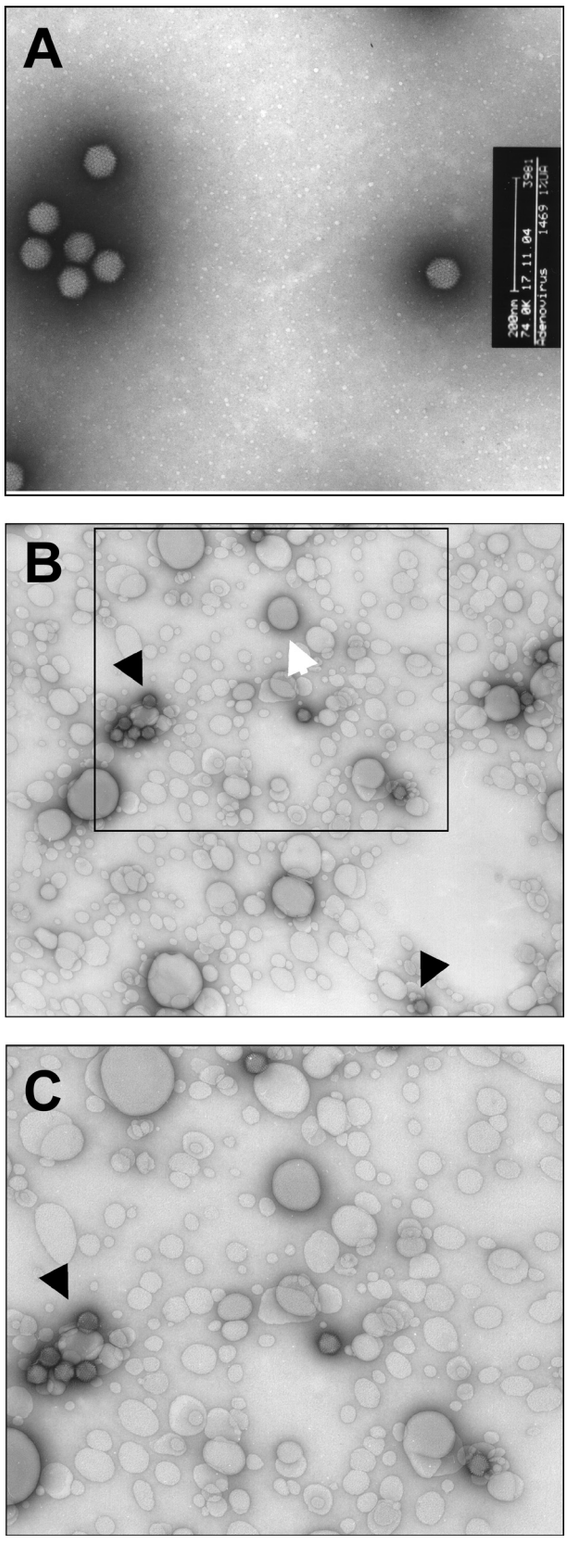

Figure 3 - TEM analysis of virus-liposome mixtures. DMPC:Chol liposomes at a 4 mM DMPC concentration were mixed with an equal volume of water containing 4 x 10^{11} Ad pu/ml. Complexes were allowed to form for 30 min then placed onto gold grids and stained with uranyl acetate. Black arrows indicate adenovirus and white arrows indicate liposomes. A. Adenovirus alone. B. Adenovirus mixed with DMPC:Chol liposomes. C. Higher resolution image of inset in B. Size bar represents 200 nm in A, and 500 nm in B and C.

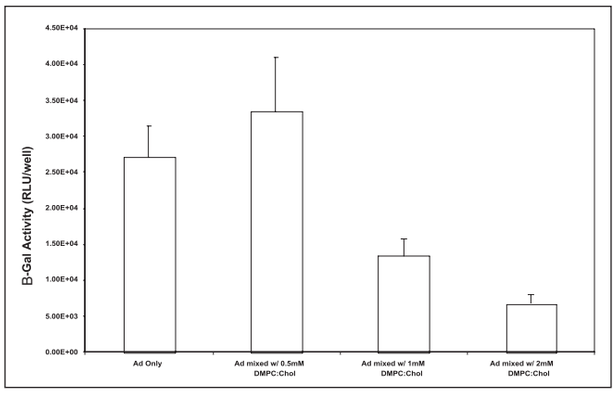

Figure 4 - Assessment of gene transfer efficiency of adenovirus mixed with liposomes. Ad.βgal at a concentration of 4 x 10^{11} pu/ml in water was mixed with DMPC:Chol liposomes to a final DMPC concentration of 0.5, 1, or 2 mM in water, and then diluted in serum-free DMEM and added to A549 cells, yielding 500 Ad particles per cell. After 48 h, cells were lysed and β-gal expression was assessed using a luminometer.

posome bilayer may effect this interaction, we have elected to study the interaction of adenovirus with DMPC due the fact that it has a far lower gel to liquid crystal phase transition temperature (T_M) than DPPC, 23°C vs. 41°C for DPPC. While it is possible to use even shorter chain phosphatidylcholines with lower T_M, these phospholipids have more limited miscibility with longer chain phospholipids, and therefore offer limited options for future formulation modifications through incorporation of other lipids into the bilayer. Additionally, earlier work had demonstrated that free cholesterol can enhance adenovirus transfection efficiency, and that the interaction of adenovirus and cholesterol leads to the formation of adenovirus aggregates [20]. By including cholesterol in the phospholipid bilayer, we believed that there would be even greater virus-liposome association. Close examination of both the spectrophotometric absorbance data and the PCS data indicate that large viral-DMPC:Chol aggregates do not form in suspension under the concentrations studied. This was further supported by TEM analysis which indicated that although there appears to be a high degree of association between adenovirus and DMPC:Chol liposomes, the resulting structures tend to be small and well isolated from each other.

There is a great body of work detailing the ability of cationic liposomes to alter intrinsic properties of adenovirus including cellular affinity *in vitro* and *in vivo*, as well as reduction of immunogenicity. Lee *et al*. demonstrated that Ad complexed to DOTAP:DOPE, DOTAP:DOPE:PEG, or lipofectamine at a cationic lipid to adenovirus ratio of approximately 0.1 to 1 ug lipid to 10^7 adenovirus particles produced increased transfection over Ad alone in CAR sufficient and deficient cells [21]. The mechanism of uptake was further defined to be CAR independent [12]. Yotnda *et al*. demonstrated that DOTAP:Chol liposomes complexed with adenovirus type 2 are protected from neutralization by the immune system *in vivo* [22]. Additionally, the interaction of cationic liposomes has been shown to alter adenovirus biodistribution *in vivo*, enhancing targeting to tumor and pulmonary tissue [23, 24].

Although it is clear that cationic liposomes can be very effective at altering virus behavior, the actual mechanism of the virus-liposome interaction has not been studied. Much of the previous work has not looked directly at the interaction of Ad and liposomes in suspension, but instead has been focused on the biological effects (gene transfer efficiency) of such interactions. As far as structural structural characterisation of the complex vectors is concerned, electron microscopy or fractionation studies have been performed to demonstrate complex formation; however such techniques may introduce additional

experimental variables during sample handling, such as drying or centrifugation, which may alter the virus-liposome interaction. By using photometric techniques in addition to microscopy, we are able to directly examine what is occurring in solution and to compare our predictions with the microscopic images.

Our work focuses on Ad mixed with zwitterionic liposomes and shows some parallels to that of Balakireva *et al*. [15]. However, while Balakireva *et al*. present excellent data regarding the interaction of adenovirus proteins with phosphatidylcholine-based liposomes, they do not investigate the structures resulting from the interaction of adenovirus with zwitterionic liposomes in supension. In addition, they demonstrate that DPPC, but not egg yolk derived phosphatidylcholine (EYPC), is able to upregulate adenovirus mediated gene transfer. This still leaves in question whether this upregulation is specific only to DPPC liposomes, or a more general phenomenon. Our gene transfer studies show the greatest degree of upregulation of gene expression at 0.5 mM DMPC concentrations, within the same range as the upper end of the data presented in the previous study, where optimal transfection was seen at 0.625 mM DPPC concentration. Investigation of transfection efficiency of adenovirus–liposome vectors at higher DMPC concentrations (2 mM) shows that gene transfer activity is down regulated.

An indication of a possible mechanism for this inhibition is presented by Balakireva's group, as they mention that at DPPC concentrations higher than 1.25 mM they are able to precipitate Ad, along with DPPC liposomes, by low speed centrifugation. They also demonstrate that DPPC has a specific interaction with the adenovirus hexon protein, most probably due to the charge on the phosphatidylcholine head group. While this is certainly possible, our data indicate that electrostatic forces, which can act over long distances and often lead to the formation of aggregates, do not appear to dominate the Ad-liposome interaction. Instead, the liposomes may, in conjunction with the activity of weak electrostatic forces, interact with specific hydrophobic domains on the adenovirus hexon. Indeed, it has been demonstrated that adenovirus hexon proteins contain several hydrophobic and hydrophilic domains, and that the balance of the accumulated surface charge in these regions correlates with the tissue tropism of various adenovirus serotypes [25]. Differences in the degree of exposure of these hydrophobic domains caused by surface modification of adenovirus by various concentrations of phosphatidylcholine and cholesterol containing liposomes could lead to the observed alteration of adenoviral gene delivery.

While the mechanism of how DMPC:Chol is able to modulate Ad transfection efficiency remains unclear, the fact that DMPC:Chol liposomes can both upregulate or inhibit adenovirus mediated gene transfer has the potential to have great impact on *in vivo* gene therapy. By studying, or altering, the lipid profiles of different individuals or tissues, it may be possible to improve viral targeting, and thus increase the therapeutic index of the vector. Indeed, Fechner et al have demonstrated that adenovirus biodistribution *in vivo* does not correlate with CAR and intregrin expression [26]. It is possible that this is due to interactions of Ad with lipids within the blood stream. However, this hypothesis needs further investigation.

It is our objective to engineer new, complex gene delivery systems, and to analyse the ability of these novel vectors to target different types of tissue by modifying the chemical composition of various components of synthetic and hybrid (synthetic-viral) vectors. In order to be successful, vectors need to be developed not only for their therapeutic capabilities, but also for the ability to apply standardized, routine production procedures for their large-scale manufacture to ensure a high degree of homogeneity and reproducibility, as well as stable formulation. One advantage of using DMPC:Chol formulations is that, unlike cationic liposomes, mixtures with adenovirus do not appear to aggregate, making them far easier to store and characterize, nor do DMPC:Chol liposomes attract serum proteins when injected intravenously; yet, they have the ability to both positively and negatively modulate Ad transfection. Because of this, we believe that our observations, with further research, may lead to the development of a novel gene delivery platform.

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