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Nanoengineering Artificial Lipid Envelopes Around Adenovirus by Self-Assembly

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ABSTRACT We have developed a novel, reproducible, and facile methodology for the construction of artificial lipid envelopes for adenoviruses (Ad) by self-assembly of lipid molecules around the viral capsid. No alteration of the viral genome or conjugation surface chemistry at the virus capsid was necessary, therefore difficulties in production and purification associated with generating most surface-modified viruses can be eliminated. Different lipid bilayer compositions produced artificially enveloped Ad with physicochemical and biological characteristics determined by the type of lipid used. Physicochemical characteristics such as vector size, degree of aggregation, stability, and surface charge of the artificially enveloped Ad were correlated to their biological (gene transfer) function. In monolayer cell cultures, binding to the coxsackie and adenovirus receptor (CAR) was blocked using a zwitterionic envelope, whereas enhanced binding to the cell membrane was achieved using a cationic envelope. Envelopment of Ad by both zwitterionic and cationic lipid bilayers led to almost complete ablation of gene expression in cell monolayers, due to blockage of virion endosomal escape. Alternatively, artificial Ad envelopes built from lipid bilayers at the fluid phase in physiological conditions led to enhanced penetration of the vectors inside a three-dimensional tumor spheroid cell culture model and delayed gene expression in the tumor spheroid compared to nonenveloped adenovirus. These results indicate that construction of artificial envelopes for nonenveloped viruses by lipid bilayer wrapping of the viral capsids constitutes a general strategy to rationally engineer viruses at the nanoscale with control over their biological properties.

KEYWORDS: nanomedicine · gene therapy · viral envelope · nonenveloped virus · liposome · nanobiotechnology · nanotechnology · nanoengineering · biomembrane

See the accompanying Perspective by Thompson on p 821.

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Received for review January 29, 2008 and accepted April 07, 2008.

Published online April 30, 2008.
10.1021/nn8000565 CCC: $40.75

Adenovirus (Ad) is a nonenveloped DNA virus commonly used for gene therapy applications.[1,2] Recombinant Ad vectors have been rendered replication deficient through deletion of essential viral genes, which are supplied in trans by a packaging cell line during vector production. Ad delivers a genetic payload of up to 35 kb[3] with high efficiency to a broad range of cell types that express the coxsackie and adenovirus receptor (CAR).[4] For adenovirus, determinants of tissue tropism are presumed to be the viral capsid proteins, hexon, penton base, and fiber.[5,6] The fiber contains the knob domain which interacts with CAR, its primary cellular receptor. The penton base protein contains the RGD sequence motif which is responsible for interaction with cell surface integrins, and this interaction is essential for cell internalization. Adenoviral vectors readily infect cell lines that are CAR sufficient, while it is difficult to infect those that are CAR deficient.[7] Although many attempts to alter adenoviral tropism have been made, the problem has been that while new targeting motifs can be added, the endogenous specificity to CAR and integrin usually remains.[8] *In vivo*, Ad primarily infects the liver[5] and the resulting hepatotoxicity is the major factor limiting the use of Ad vectors.[9]

As an alternative to viral gene delivery, synthetic vectors such as liposomes have been developed for use as gene delivery vehicles. Liposome-based systems do not suffer from problems such as native tropism or hepatotoxicity. In addition to use in gene therapy for nucleic acid delivery, liposomes are one of the most widely used drug delivery systems and have been investigated for delivery of chemotherapeutics,[10] vaccines,[11] and diagnostic imaging agents.[12] As with any nanoparticulate drug delivery system, the physicochemical characteristics of liposomes vary with their composition. Although liposomes lack any specific tropism, cell or tissue specificity can be added by conjugation or association with targeting moieties such as peptides or antibodies.[13,14] Despite the advantages and flexibility offered, liposome-DNA complexes, termed lipoplexes, have proven to be far less efficient at therapeutic gene transfer than Ad vectors.[15]

To address the limitations of viral and nonviral gene delivery vectors, we sought to engineer a modular, hybrid gene transfer vector system that could maintain the advantages offered by both its viral and nonviral components, while at the same time eliminate their respective shortcomings. We hypothesized that a nonenveloped

virus nanoparticle could act as a template on which artificial lipid bilayer envelopes (of varying compositions) could be built by self-assembly. The artificially enveloped virus would therefore feature novel physicochemical characteristics and biological functionality. Adenovirus (Ad) was used as the model nonenveloped virus onto which lipid bilayers were allowed to self-assemble.

Ad and preformed liposomes previously have been studied as reciprocal adjuvants of each other's gene transfer capabilities. Numerous groups have reported that adenovirus infectivity can be enhanced by interaction with cationic[16–25] or, more recently, zwitterionic preformed liposomes[26,27] by simple mixing prior to infection in a process commonly referred to as "lipoduction". These complexes are thought to be taken up by cells independent of virus-specific pathways, leading to enhanced gene expression when compared to Ad alone, particularly in CAR-deficient cell lines.[23] At high cationic liposome to Ad ratios, liposome encapsulation of Ad also has been observed;[28,29] however, such "mixing" protocols suffer from various limitations such as irreproducibility, restriction to cationic liposome formulations, and inherent colloidal instability, making *in vivo* applications or large scale production almost unfeasible.

In the present study, Ad was efficiently enveloped by self-assembly of lipid bilayers templated around the viral capsid. Through use of electron microscopy and dynamic light scattering, characteristics such as vector size, degree of aggregation, and stability were correlated to gene transfer efficiency. Enveloped particles showed significantly reduced gene transfer in cell monolayers, possibly due to failure to escape from the endosomal compartment. Confocal microscopy studies were conducted to monitor cell binding and intracellular trafficking in monolayer cell cultures. Binding to CAR was blocked using a zwitterionic envelope and enhanced binding to the cell membrane was achieved with a cationic envelope. Finally, penetration and gene expression inside a three-dimensional tumor spheroid cell culture model was studied. Use of an artificial viral envelope formulation containing phosphatidylethanolamine enhanced penetration into a three-dimensional tumor spheroid cell culture model and led to delayed gene expression in the tumor spheroid when compared to naked adenovirus. The results presented here provide a new paradigm for the modification of nonenveloped viral capsids and demonstrate the importance of understanding and manipulating adenoviral (used here as a model nonenveloped virus) physicochemical characteristics and how these modulate its biological activity.

RESULTS

Stability of Enveloped Adenoviruses by Dynamic Light Scattering. Initially two artificial lipid envelope compositions were selected: a cationic envelope, based on a positively charged phospholipid, 1,2-dioleoyloxypropyl-*N*,*N*,*N*-trimethylammonium chloride mixed at a 2:1 molar ratio with cholesterol (DOTAP/Chol), and a neutral (zwitterionic) envelope, that consisted of dimyristoyl phosphatidylcholine, also mixed at a 2:1 molar ratio with cholesterol (DMPC/Chol). Lipid bilayer envelope formation was carried out by a simple protocol in which an aqueous suspension of Ad nanoparticles was used to hydrate a lipid film, allowing the lipid molecules to self-assemble into bilayers on the virus surface (Figure 1a). Adenovirus (5×10^{10} pu/ml) in 1 mL of deionized water was used to hydrate either DMPC/Chol or DOTAP/Chol films to yield phospolipid concentrations ranging from 20 pM to 2 mM (with corresponding cholesterol concentrations ranging from 10 pM to 1 mM, respectively). We monitored the stability of the enveloped Ad prepared at the various lipid-Ad ratios by dynamic light scattering (DLS) to determine the mean hydrodynamic diameter of the hybrid vectors 3 h after preparation (to allow time for the bilayers to stabilize

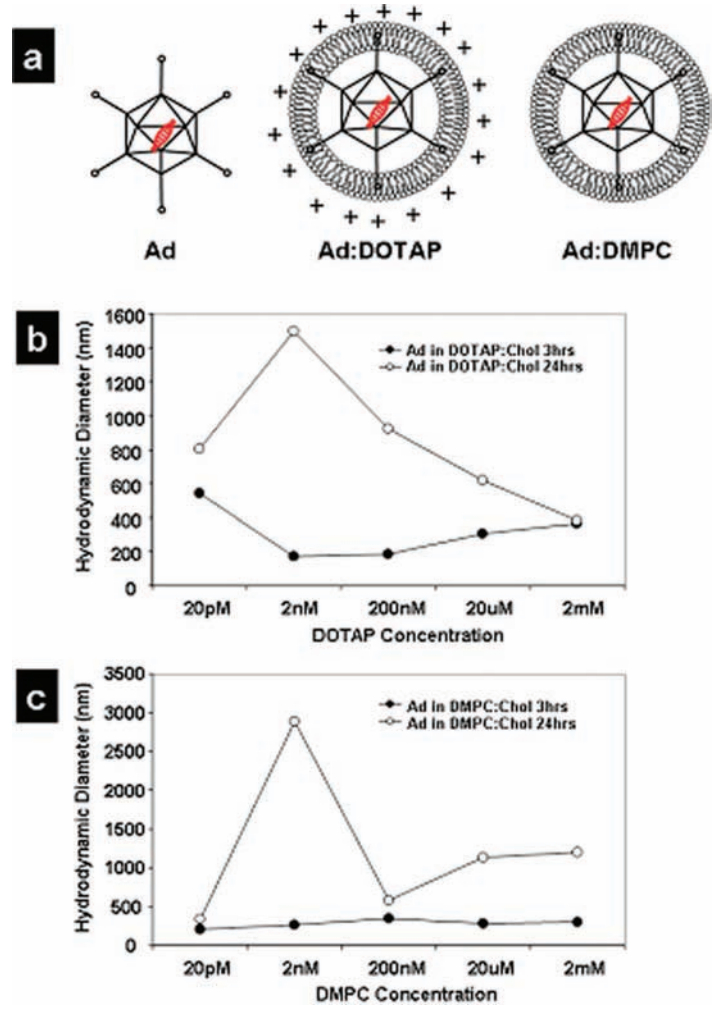

Figure 1. Assessment of enveloped Ad stability by dynamic light scattering (DLS): (a) Schematic of Ad and artificially enveloped Ad; (b) the effect of DOTAP/Chol or (c) DMPC/Chol lipid concentration on artificially enveloped Ad particle size containing 5×10^{10} pu/ml Ad. Hydrodynamic diameter was evaluated by DLS after 3 h at 23 °C or after storage overnight at 4 °C.

following bath sonication) and after overnight storage at 4 °C. The hydrodynamic diameter of nonenveloped Ad was measured (118 nm after 3 hours in deionized water), increasing slightly to 127 nm following storage overnight.

At the 3 h time point, the size of cationic-enveloped Ad particles varied from 363 nm at 2 mM DOTAP concentration, decreasing to 172 nm at 2 nM, then rising to 542 nm at 20 pM (Figure 1b). Following storage of these vectors overnight, evidence of aggregation leading to significant mean particle size increase was observed at all DOTAP concentrations below 2 mM, with the most severe aggregation occurring between 200 and 2 nM. The high stability against aggregation at 2 mM was thought to indicate that at this lipid concentration most Ad viruses were enveloped by a DOTAP/Chol bilayer. The aggregation observed over time in systems of lower than 2 mM lipid concentration was thought to be due to incomplete envelopment of the virus, leading to high colloidal instability due to attraction between the naked negatively charged Ad virions and the cationic liposomes formed, similar to previously described aggregation during mixing of preformed cationic liposomes with Ad reported by Qui *et al*. [25]

Ad enveloped with zwitterionic DMPC/Chol lipid bilayers exhibited very little variation in mean diameter with decreasing DMPC concentrations from 2 mM, 20 μM, to 200 nM at 3 h (Figure 1c). However, severe flocculation was observed following storage overnight at 4 °C, particularly for the 2 nM and 200 nM DMPC-enveloped Ad systems. At 200 nM DMPC, this led to precipitation of the aggregated particles, and only the smaller particles remained in suspension as indicated by the sharp decrease in the DLS curve. Some flocculation was observed at 20 μM and 2 mM DMPC as shown by the size increases obtained at the 24 h time point indicating that enveloping Ad with a zwitterionic lipid bilayer led to an unstable suspension. This type of behavior was expected in the case of incomplete envelopment of Ad by DMPC/Chol bilayers, since it has been previously described that Ad hexon proteins bind specifically to phosphatidylcholine (PC) (when mixed with preformed PC liposomes).[26,27] Generally, the sharp increases in mean diameter after 24 h observed by DLS at low concentrations of DOTAP/chol and DMPC/chol were thought to be caused by attractive interactions between naked (nonenveloped) Ad and formed liposomes as previously reported. This data also indicated the critical role of molar ratio in forming fully enveloped, stable Ad.

Determination of Enveloped Adenovirus Structure by Electron Microscopy. The structural characteristics of artificially enveloped viruses were elucidated by transmission electron microscopy (TEM). Our light scattering data indicated complete envelopment of the Ad particles occurring at 2 mM DOTAP/Chol, and this was confirmed by TEM (Figure 2b). Moreover, Ad virions appeared to be enveloped by multiple concentric DOTAP/Chol lipid bilayers and 98% of all Ad virions (estimated by TEM) were fully enveloped throughout all samples (see Supporting Information Figure 1). In contrast, envelopment of Ad using DMPC/Chol lipid bilayers appeared far less effective; the bilayers were more rigid than DOTAP/Chol bilayers, leading to incomplete envelope formation around the viral capsids in most cases (Figure 2c). However, zwitterionic bilayers, lipid bilayer

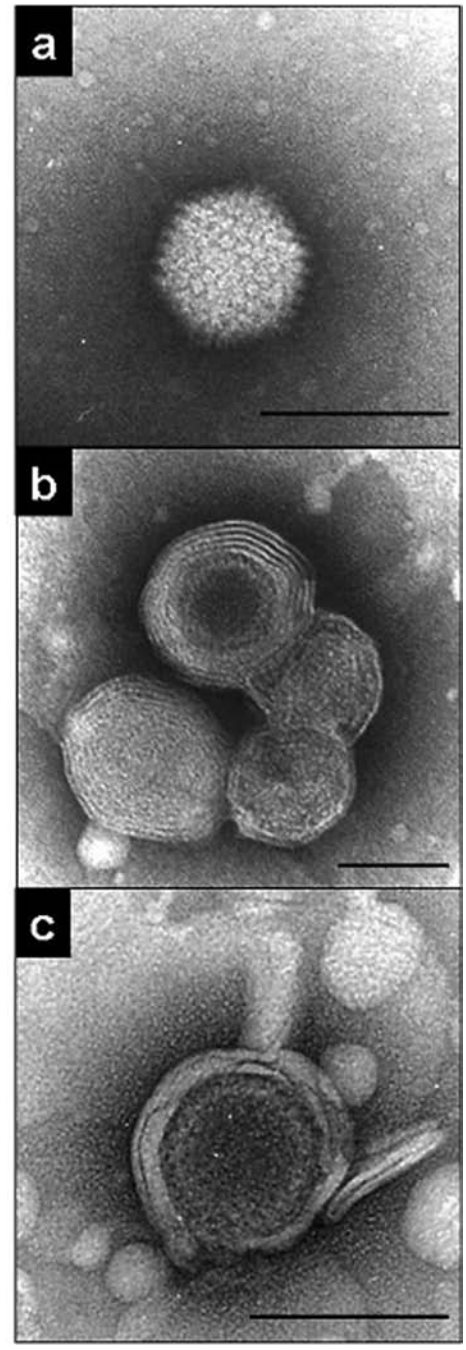

Figure 2. Structural analysis of artificially enveloped Ad by TEM: (a) 2×10^{11} pu/ml adenovirus alone; (b) 2×10^{11} pu/ml enveloped Ad with 2 mM:1 mM DOTAP/Chol; and (c) 2×10^{11} pu/ml enveloped Ad in 2 mM:1 mM DMPC/Chol. All samples were stained with 1% aqueous uranyl−acetate (scale bar is 100 nm).

fragments, or empty lipid vesicles were found associated with the majority of the Ad capsids for almost all of the virions present, indicating partial to complete envelopment.

Envelopes Inhibit *in Vitro* Gene Transfer in Cell Monolayers. To study the effect of the envelope on the biological capabilities of Ad, enveloped virus particles were evaluated for their *in vitro* gene transfer ability. Recombinant Ad encoding for the beta-galactosidase (β-gal) reporter gene was enveloped in cationic DOTAP/Chol or zwitterionic DMPC/Chol. The DLS and TEM data (Figures 1 and 2) indicated that a 2 mM phospholipid concentration produced fully enveloped viral capsids with the most uniform size and surface characteristics. The gene transfer efficiency of these vectors was compared to the unstable systems produced at 20 μM and 200 nM lipid concentrations. Moreover, in a control experiment, nonenveloped Ad was subjected to the same processing steps prior to use for gene transfer study to ensure that changes in activity were due to the envelope rather than any other part of the procedure.

The vectors were added to human lung epithelial carcinoma cells (A549), rich in the primary Ad receptor, CAR, at a dose of 500 particle units per cell. Only a minor loss in β-gal expression of the processed Ad was obtained when compared to unprocessed, freshly thawed Ad (Figure 3a). Use of cationic lipid (DOTAP/Chol) enveloped Ad 3 h after preparation at all DOTAP concentrations resulted in less than 10% of β-gal expression when compared to nonenveloped Ad (Figure 3b). However, if the vectors were stored overnight at 4 °C, high levels of gene expression were observed in the 200 nM and 20 μM DOTAP samples. At 2 mM DOTAP, β-gal expression remained at background levels even after storage. For the zwitterionic lipid bilayer enveloped Ad, we observed that envelopment with 2 mM or 20 μM DMPC led to poorly transfected A549 cells when used three hours after preparation, and that this effect was maintained following overnight storage at 4 °C (Figure 3c). It appears that the fragmented envelopes observed by TEM are sufficient to reduce the interaction between Ad and the cell surface, and that the association with the lipid bilayers was stable overnight.

We previously reported a strong affinity between Ad and cholesterol alone[31] and others have reported a specific interaction between phosphatidylcholine and the Ad capsid hexon protein.[27] The present results indicated that this interaction may be quite stable over time and responsible for the dramatic reduction in gene transfection. At the lowest phospholipid concentration studied, 200 nM DMPC, gene expression was reduced to background levels when the hybrid vector was used 3 h after preparation, but rose to nearly 30% that of nonenveloped Ad following overnight storage. The DLS data indicated aggregation of the suspension in this preparation, leading to the formation of a precipitate.

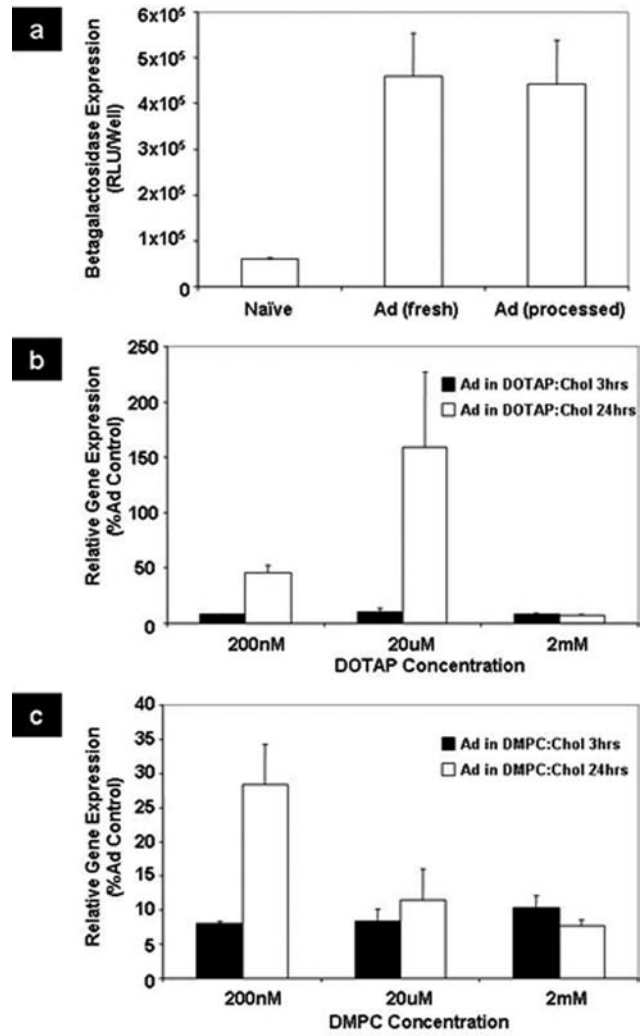

Figure 3. In vitro gene transfer efficiency of artificially enveloped Ad: (a) processed Ad (stirred, sonicated, and stored at 23 °C for 3 h in the absence of lipid), or freshly thawed Ad was added to A549 cells at a dose of 500 pu/cell. β-gal expression was assessed 24 h later; (b) 5×10^{10} pu/ml Ad was enveloped by DOTAP/Chol bilayers at various phospholipid concentrations. After 3 h at 23 °C or storage overnight at 4 °C, Ad in DOTAP/Chol was added to A549 cells at a dose of 500 pu/cell. β-gal expression was assessed 24 h later; (c) 5×10^{10} pu/ml Ad was enveloped by DMPC/Chol bilayers at various phospholipid concentrations. After 3 h at 23 °C or storage overnight at 4 °C, Ad in DMPC/Chol was added to A549 cells at a dose of 500 pu/cell. β-gal expression was assessed 24 h later. For panel a, data are represented as mean gene expression plus standard deviation. For panels b and c, data are expressed as relative gene expression compared to the processed Ad control plus standard deviation.

It is possible that these precipitates are able to transfect A549 cells in a manner similar to that of previous reports of Ad included in a calcium phosphate coprecipitate.[32] Overall, our results suggested that changes in transfectivity were due to the envelope lipid composition and the interaction between the artificial lipid bilayer envelopes and Ad rather than any step during the processing of the vector.

Artificial Envelopes Alter Cell Binding and Intracellular Trafficking of Ad. The most likely explanation for the lack of β-gal expression following transfection with DMPC/Chol enveloped Ad is due to reduced cell uptake. How-

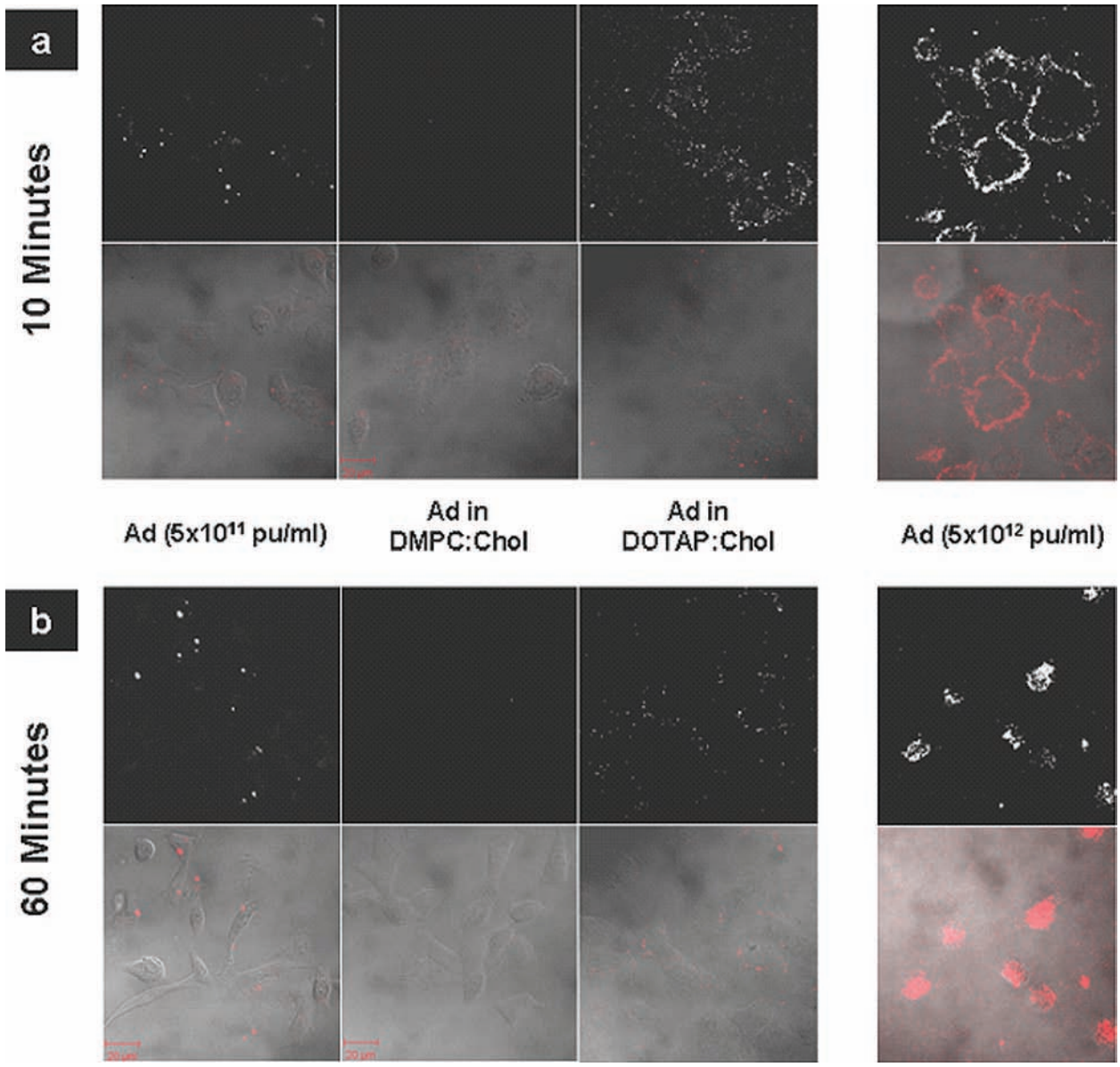

Figure 4. Binding and uptake of enveloped adenovirus (Ad) by confocal laser scanning microscopy: Ad was fluorescently labelled with the red dye Cy3 (Cy3-Ad). Five × 10^{10} pu/ml Ad was enveloped in 2 mM:1 mM DMPC/Chol or DOTAP/Chol bilayers, and added to A549 cells for (a) 10 min, then washed to remove any unbound vector, fixed, and mounted on slides; or (b) 10 min, then washed to remove any unbound vector, incubated for a further 60 min, fixed, and mounted on slides. The top panel in each pair of images is the red channel alone showing Cy3-Ad, while the bottom panel shows the pseudo-DIC image merge with Cy3 fluorescence. Panels to the far right show Cy3-Ad alone as control (using a 10× higher dose of Ad than the enveloped Ad).

ever, this does not explain the loss of transfectivity observed for DOTAP/Chol enveloped Ad as binding likely is enhanced compared to Ad alone. To investigate the mechanism responsible for the loss of gene expression observed in Figure 3, fluorescently labeled Ad was prepared by covalent conjugation of the red fluorophore cyanine 3 (Cy3), and Cy3-Ad was enveloped with 2 mM DOTAP/Chol or DMPC/Chol and then added to A549 cells. The enveloped vectors were allowed 10 min to bind to the cell surface, the excess washed away, and then given a further 60 min to translocate to the nucleus (Figure 4). We tested Cy3-Ad to ensure that our vector was functioning normally. Ad previously has been demonstrated to translocate to the nucleus within 30 min of binding to the plasma membrane,[33] and a similar result was achieved here (Figure 4a and 4b, far right panels). Studies using the enveloped virus were conducted at a 10-fold lower Ad dose to avoid any cationic liposome-induced toxicity. After a 10 min incubation, significantly more DOTAP/Chol enveloped Cy3-Ad vectors were associated with the cell surface than the control Cy3-Ad alone group, confirming that the cationic envelope did enhance the binding of the enveloped virus to the negatively charged cell membrane. After a 60 min chase, less virus was seen bound to the outside of the cell, but none was observed accumulating at or close to the nucleus indicating that the cationic

envelope disrupted normal Ad trafficking, likely causing the virus to be degraded in the lysosome following internalization. By comparison, the neutral DMPC/Chol enveloped Cy3-Ad bound poorly to the cell surface as seen by the absence of Cy-3 fluorescence on the cell periphery following the 10 min incubation or the 60 min chase. This indicated a disruption in the interaction between the enveloped Ad and its high affinity receptor, CAR, supporting the hypothesis that the envelope blocked binding of the Ad fiber to CAR. In the absence of a cationic surface charge to bind it to the cell membrane, very little vector associated with the cell surface.

Endosome Disrupting Agents Enhance Gene Transfer Efficiency of Enveloped Adenovirus. Because DOTAP/Chol enveloped Ad was able to bind to the plasma membrane and be taken up by cells but was unable to reach the nucleus, we hypothesized that an envelope designed to fuse with the endosomal membrane following uptake would help release the Ad and restore transfectivity. Therefore we designed a new lipid envelope which included the fusogenic lipid DOPE, which has been shown to be effective in enhancing lipoplex mediated gene delivery.[34,35] We then utilized an endosome disrupting agent, the null adenovirus (Ad.Null, a recombinant vector with no transgene in its expression cassette), to enhance the release of DOTAP/DOPE enveloped Ad.β-gal from the endosome. When used as an adjuvant to lipoplex mediated gene transfer, Ad.Null has been shown to greatly enhance plasmid DNA gene transfer through endosomolysis, allowing the DNA to escape degradation due to acidification of the endosomal compartment.[16] We hypothesized that Ad.Null virions would co-internalize with any enveloped Ad bound to the plasma membrane and that Ad.Null signaling from the cell surface could stimulate release of the DOTAP:DOPE enveloped Ad to the cytolsol through the previously described macropinosome and endosome activation.[46]

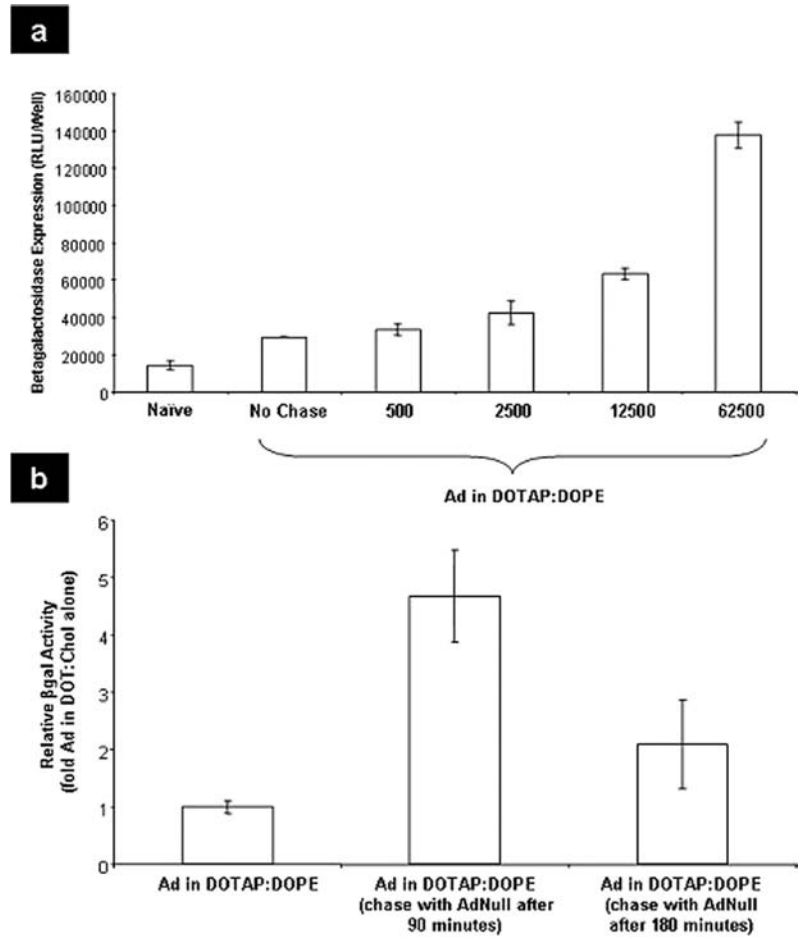

Figure 5. ***In vitro*** **gene transfer efficiency of artificially enveloped Ad following treatment with Ad.Null as an endosomolytic agent: (a) 5 × 10^{10} pu/ml Ad was enveloped in 1 mM:3 mM DOTAP/DOPE bilayer and incubated with A549 cells at a dose of 500 pu/cell for 90 min. The cells were then washed to remove any unbound vector, and Ad.Null at the indicated dose per cell was added for 30 min and then washed. Gene expression was analysed 24 h later and is expressed as relative light units (RLU) +/− standard deviation; (b) DOTAP/DOPE enveloped Ad was added to cells as in (a), then chased with 62500 pu/cell Ad.Null 90 or 180 min after the addition of the enveloped virus. Gene expression was analysed 24 h later and the data are expressed as relative gene transfer efficiency compared to the enveloped vector in the absence of the Ad.Null chase +/− standard deviation.**

To prevent the formation of a complex between Ad.Null and DOTAP/DOPE-enveloped Ad.β-gal, it was necessary first to incubate the cells with the enveloped vector for 90 min, then wash away any unbound vector and chase with Ad.Null, either immediately after the washing step, or 90 min later. In the absence of the Ad.Null chase, gene expression was minimal; however, following Ad.Null treatment, β-gal expression increased in a dose-dependent manner by increasing doses of Ad.Null (Figure 5a). The effect is reduced if the Ad.Null is added 180 min after the initial addition of the enveloped vector rather than immediately after washing (Figure 5b), likely due to degradation of the enveloped virus in the lysosome following uptake. This result offered further evidence that poor endosomal escape is the main cause of low gene transfer efficiency for the artificially enveloped Ad vectors.

Envelopes Enhance Interstitial Penetration and Delay Gene Expression in Tumor Spheroids. To study the gene transfer and expression capabilities of the DOTAP/DOPE bilayer-enveloped Ad in a more complex, closer to *in vivo* model cell culture environment, three-dimensional multicellular tumor spheroids of the BxPC-3 human pancreatic adenocarcinoma cell line were formed. We previously have shown that inclusion of the fusogenic lipid DOPE in combination with a cationic lipid of lower surface charge density such as DOTAP in lipid bilayer vesicles could lead to improvements in their degree of penetration within tumor spheroids.[36] Here we investi-

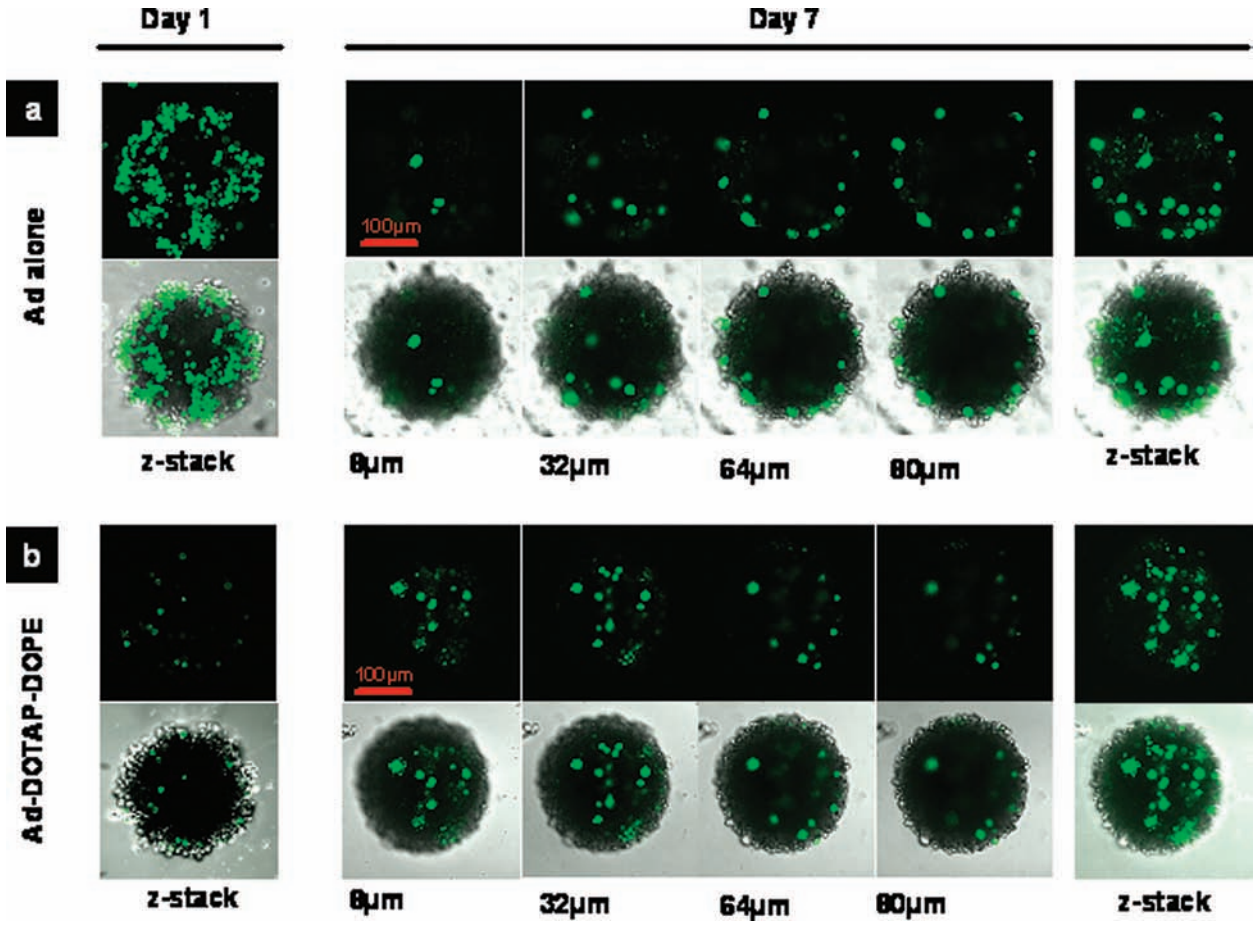

Figure 6. Distribution of GFP gene expression of artificially enveloped Ad in tumor spheroids: 2.5 × 10^{11} pu/ml Ad.GFP was encapsulated in 1 mM:3 mM DOTAP/DOPE liposomes and incubated with BxPC-3 spheroids at a dose of approximately 2.5 × 10^{9} pu/spheroid for 24 h. The spheroids (n = 100) were then washed and incubated in Ad-free media for 7 days, and then GFP expression was analyzed in BxPC-3 spheroids transfected with (a) non-enveloped Ad or (b) enveloped Ad . The reconstructed z-stack of half the spheroid is shown to the far left and right for days 1 and 7, respectively. Spheroids were optically sectioned by CLSM into 4 μm thick slices from the spheroid rim (first slice 8 μm thick) and towards the equatorial optical plane.

gated whether such enhanced penetration could lead to deeper tumor spheroid gene expression of DOTAP/DOPE enveloped Ad. The tumor spheroids were infected with both nonenveloped and artificially enveloped Ad encoding for the green fluorescent protein (Ad.GFP). The expression of green fluorescence protein (GFP) within the tumor mass was assessed optically by confocal laser scanning microscopy (CLSM) (Figure 6). One day after transfection, non-enveloped Ad efficiently transfected cells at the periphery of the tumor spheroid (Figure 6a, day 1 z-stack), though not within its interior. Little or no GFP expression was observed 1 day following transfection with DOTAP/DOPE-enveloped Ad vectors (Figure 6b, day 1 z-stack), similar to the results obtained in the cell monolayer transfection studies.

GFP expression at the spheroid periphery decreased 7 days following transfection with nonenveloped Ad (Figure 6a, day 7). However, artificially enveloped Ad transfected spheroids exhibited an increase in GFP expression, and GFP was observed within the spheroid volume rather than on the periphery (Figure 6b, day 7). This was attributed to the ability of DOTAP/DOPE-enveloped Ad vectors to penetrate deeper into the tumor spheroid and transfect many more cells at the inner layers of the tumor mass. This data indicated that DOTAP/DOPE-enveloped Ad vectors were capable of penetrating deeper into the interstitial tumor space and also transfect cells, despite the problematic endosomal release illustrated above. This suggested that enveloped Ad that localize into the tumor spheroid mass are capable of allowing Ad to escape from the lipid bilayer envelope in the tumor interstitium in order to infect cells, leading to the evidenced GFP expression. Moreover, it also suggests that in an *in vivo* scenario, enveloped Ad that accumulates in the tumor interstitium (*e.g.*, by extravasation) may be capable of gene delivery and expression deeper into the tumor mass, provided that enveloped Ad vectors are not drained out of the tumor volume. Further studies are needed to investigate the *in vivo* gene transfer capability and dynamics of these vectors by optimization of the artificial envelope characteristics.

DISCUSSION

Safety and efficacy in gene therapy depends upon selectively delivering the therapeutic gene to the correct tissue without inducing harmful side effects. As no current delivery system offers a solution, the aim and motivation for the work presented here was to develop a new system that could rapidly be adapted for clinical use. One of the most effective gene delivery vehicles is recombinant Ad, but its use is limited because of inherent tissue affinities leading to potentially toxic side-effects. Most of the negative aspects of Ad are related to interactions between the viral capsid and the host organism. If those interactions could be blocked by covering the virus in an inert material, it might be possible to reduce these undesired side-effects. Further, if the inert material could be shed upon uptake by the target cell, the virus would be able to function normally intracellularly, and deliver DNA to the nucleus with high efficiency. To test this hypothesis, we used the self-assembly of phospholipid bilayers to build artificial envelopes around the Ad capsid. We propose a conceptual shift in perspective whereby Ad enters the colloidal domain, is treated as a semisolid nanoparticle and the interaction of its surface with biologically relevant molecules determines its pharmacological profile. In this light, we demonstrate that envelopment of Ad with a lipid bilayer envelope of an appropriate composition dramatically changes its *in vitro* gene transfer profile and leads to a vector with enhanced tumor mass penetrating capabilities.

Moreover, a general methodology to nanoengineer artificial envelopes around nonenveloped viruses is presented. Complete envelopment of Ad within lipid bilayers creates a novel synthetic/viral hybrid vector platform with the possibility for a number of further modifications through engineering of the lipid envelope rather than altering the virus chemically or genetically. Ideally, this system offers tremendous versatility in terms of the possible physicochemical characteristics (*e.g.*, surface charge, elasticity) and targeting capabilities, while maintaining the very efficient gene transfer capacity of the enveloped viral component.

Fabrication of lipid bilayers onto solid or semisolid nanoparticles by self-assembly previously has been described, though not for any biomedical application.[37–39] The feasibility of delivering lipid bilayer-coated nanoparticles to cells also has been demonstrated.[40] When encapsulated in liposomes composed of phosphatidylcholine, dimyristoyl phosphatic acid and cholesterol, nanoparticles entered cells *via* endocytosis; whereas, by inclusion of Sendai virus envelope glycoproteins in the lipid bilayer, nanoparticles could be delivered directly to the cytosol *via* liposome fusion with the cell membrane. In previous studies more closely related to the present one, the encapsulation of intact viruses in liposomes has been attempted. Faller *et al*. encapsulated the enveloped Moloney murine leukemia virus (M-MuLV) in large, unilamellar liposomes demonstrating enhanced viral infection of resistant cells.[41] Mizuno *et al*. demonstrated that the nonenveloped adeno-associated virus (AAV) could be encapsulated in cationic liposomes leading to six times greater transfection efficiency of glioblastoma cells compared to AAV alone.[42] However, in the first case the process of encapsulation destroyed the M-MuLV structure, and the study by Mizuno *et al*. reporting liposome encapsulation of AAV lacked physicochemical characterization, making it very difficult to determine the resulting vector structures.

Recently, Hama *et al*. reported that liposome/DNA complexes compared to Ad are more efficiently uptaken by cells, have a similar efficiency of endosomal escape, and traffic to the nucleus at only a slightly slower rate than Ad, yet are still 1000 times less efficient at gene transfer.[15] This study concluded that this is a result of sharp differences in nuclear transcription efficiency of DNA packaged in Ad and not from differences in trafficking. This result emphasizes the importance of delivering intact Ad to cells in order to maximize gene transfer potential. While there have been reports of intact Ad encapsulated in liposomes by mixing Ad with prefomed liposomes,[28,29] such systems based upon the mixing of oppositely charged nanoparticles such as the negatively charged Ad with preformed cationic liposomes will be inherently unstable colloidally, making characterization, systematic manipulation, and clinical use unreliable and almost impossible to reproduce. The artificially enveloped virus vector system developed here allows for stability, reproducibility, and further modular design of the lipid envelope by keeping the virus intact.

In the present study, we demonstrated envelopment of adenovirus in both zwitterionic and cationic lipid bilayers by structural characterization using dynamic light scattering and electron microscopy, as well as biological function through confocal laser scanning microscopy and *in vitro* gene transfer assays. We optimized the stability of enveloped Ad vectors and found that when wrapped in a lipid bilayer, Ad was unable to transfect cells *in vitro* owing to low efficiency of endosomal escape. However, upon release from the endosome through the use of strong endosomolytic agents, this effect was reversible and transfectivity returned. Confocal microscopy studies indicated that binding to CAR-sufficient cells can be greatly reduced when neutral envelopes were used, and interactions between cells and enveloped Ad were dominated by the type of lipid used.

There are several possible mechanisms which could cause the loss of transfectivity. The first is that by covering the Ad surface with lipid, the fiber knob and penton base were completely retained within the bilayer and physically prevented from interaction with the CAR and integrins, leading to no cellular uptake of the vector. A

second possibility is that the enveloped Ad was prevented from several critical uncoating steps because of the high degree of lipid affinity for the hexon, preventing escape from the endosome and leading to acidic degradation. Normally Ad uptake is followed by rapid detachment of the adenovirus fiber during endocytosis, which has a dissociation rate with a half-time of 3 min, followed by a slower, pH dependent dissociation from the hexons, pentons, and other structural proteins and leads to escape of the Ad from the endosome into the cytosol[43] where it then translocates to the nucleus *via* interaction with cytoplasmic dynein.[44] From our data, it appears that both the interruption of CAR binding and disruption of intracellular trafficking are involved in the loss of Ad transfectivity. We hypothesized that inclusion of fusogenic lipids such as DOPE in the formulation could restore Ad transfectivity by allowing escape of the adenovirus into the cytosol. However, our results indicated that a strong endosomolytic agent was still necessary even when DOPE was incorporated into the envelope.

CONCLUSIONS

We have shown that engineering an artificial lipid bilayer envelope for adenovirus can lead to dramatic changes in the physicochemical and biological performance of these new gene transfer vectors. We also found that artificially enveloped Ad are unable to transfect cells *in vitro* due to poor endosomal escape. Moreover, CLSM studies indicated that CAR binding was greatly reduced, and interactions between cells and artificially enveloped Ad were dominated by the nature of the lipid composition of the artificial envelope. Further engineering of the lipid bilayer envelope with the inclusion of the fusogenic lipid DOPE led to deeper penetration and delayed gene expression inside avascular 3D tumor spheroids. In this way, the modular engineering capabilities of the new artificially enveloped viruses were exemplified. Ad vectors are widely used for cancer gene therapy, but success will depend on the ability to achieve high level tumor transduction through systemic vector administration. "Smart" vectors based upon an artificially enveloped adenoviral platform may finally help to overcome this hurdle. Nanoengineering of artificial envelopes for nonenveloped viruses is thought to offer a general new strategy to build hybrid structures between biological (virus) and synthetic (lipid) components, rationally designed for specific biomedical applications.

EXPERIMENTAL METHODS

Viral Vectors. Adenovirus (Ad) was purchased from the Baylor College of Medicine Vector Development Laboratory in Houston, Texas. For these studies, the vector Ad.βgal, encoding for the β-galactosidase reporter gene driven by the CMV promoter, Ad.GFP, encoding for the enhanced green fluorescent protein driven by the CMV promoter, or Ad.Null, with an empty expression cassette, were used. Stocks were stored at −80 °C in glycerol buffer at a concentration of 5×10^{12} particle units (pu)/ml until ready for use.

Envelopment of Adenovirus. DMPC (dimyristoyl phosphatidylcholine) and Chol (cholesterol) were purchased from Sigma, U.K. DOTAP ((1,2-dioleoyloxypropyl)-*N*,*N*,*N*-trimethylammonium chloride) and DOPE (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine) were purchased from Avanti Polar Lipids. DMPC/Chol, at a 2:1 molar ratio, DOTAP/Chol, at a 2:1 molar ratio, or DOTAP/DOPE, at a 1:3 molar ratio were dissolved in 4:1 chloroform/methanol in a 25 mL round-bottom flask. A lipid film was formed using a rotovaporator [BÜCHI, Switzerland]. After 1 h under a vacuum, the film was further dried for 15 min under a nitrogen gas stream. Adenovirus stock was thawed then diluted in 1 mL of distilled and deionized water (ddH2O) then added to the flask to hydrate the film. To ensure thorough hydration, a small magnetic stirring bar (10 mm) was added, and the hydrating films were stirred for 30 min at room temperature. The stirrer was removed, and the flask was then placed in an ultrasonic water bath [VWR model 300TH; VWR, U.K.] for 30 min at 30 °C.

Nanoparticle Sizing by Dynamic Light Scattering (DLS). All measurements were made using the Autosizer 4700 [Malvern Instruments, U.K.]; 90° scattering was assessed with the software set for automatic analysis and triplicate measurements were taken for each sample. Adenovirus at a concentration of 5×10^{10} pu/ml in ddH2O was added to DMPC/Chol or DOTAP/Chol lipid films to yield final phospholipid concentrations of 20 pM, 2 nM, 200 nM, 20 μM, or 2 mM. Vectors were allowed to sit at room temperature for three hours and were then vortexed for 15 s prior to measurement. A second measurement was taken of each sample after storage overnight at 4 °C. Samples were allowed to warm to room temperature and were vortexed for 15 s prior to measurement. Size analysis of adenovirus alone was conducted at an adenovirus concentration of 5×10^{10} pu/ml. Data are representative of two independent experiments.

Structural Analysis by Negative Stain Transmission Electron Microscopy (TEM). Digital images were taken using a CM120 BioTwin electron microscope [Philips/FEI]. One ml Adenovirus at a concentration of 2×10^{11} pu/ml in distilled water was added to DMPC/Chol or DOTAP/Chol lipid films to yield final phospholipid concentrations of 2 mM, and 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 5×10^{12} pu/ml in glycerol buffer.

***In Vitro* Gene Transfer Efficiency.** A549 cells [ATCC], a human lung adenocarcinoma cell line, were grown to confluency in 24-well tissue culture dishes [Corning B.V., The Netherlands] in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS and penicillin/streptomycin [all from Invitrogen, U.K.]. Ad.βgal, at a concentration of 5×10^{10} pu/ml, was added to DMPC/Chol or DOTAP/Chol lipid films to yield final phospholipid concentrations of 200 nM, 20 μM, or 2 mM as described above. The vectors were allowed to sit at room temperature for 3 h or overnight at 4 °C and then were diluted in serum-free DMEM to an adenovirus concentration of 1×10^{8} pu/ml. One ml of the diluted vector was added to triplicate wells of A549 cells, yielding a dose of approximately 500 Ad particles per cell. Each well of the 24-well plate was estimated to contain 2×10^{5}cells per well, which at a dose of 500 adenovirus particle units (pu) per cell yields a total adenovirus particle number of 1×10^{8} pu/well. As controls, an equivalent dose of Ad alone or media only was added to triplicate wells. Cells were incubated with the vector for 90 min, washed in PBS, and incubated overnight in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were harvested in 200 μl of Tropix Galactolight Plus lysis buffer [Applied Biosystems] and centrifuged at 13000*g* to removed any cell debris, and 10 μl of the supernatant was analyzed for β-galactosidase (β-gal) activity using the Tropix Galactolight Plus kit and a Berthold Lumat 9507 luminometer [Berthold Instruments, Germany]. Data are ex-

pressed as percent expression relative to the Ad alone control (100%) plus standard deviation of the mean.

In a separate control experiment, Ad was mock processed in the absence of lipids. Briefly, Ad (5×10^{10} pu/ml in ddH2O) was added to a 25 mL round-bottom flask from which 2 mL of 4:1 chloroform/methanol previously had been evaporated, stirred for 30 min, then sonicated in a water bath for 30 min at 30 °C, and allowed to sit at room temperature for 3 h. A549 cells were transfected as above and β-gal activity was compared to that of cells transfected with freshly thawed Ad, or DMEM alone. Data are expressed as relative light units per well plus standard deviation of the mean.

For endosomolysis experiments, A549 cells were transfected as before with Ad alone, or Ad enveloped in DMPC/Chol, DOTAP/Chol, and 1 mM:3 mM DOTAP/DOPE as described above. Immediately following washing of the enveloped Ad, or 90 min later, 1 mL of DMEM containing increasing concentrations of Ad.Null (1×10, 5×10^8, 2.5×10^9, 1.25×10^{10}, or 6.25×10^{10} pu/ml) equivalent to the dose per cell listed in Figure 5 was added, then washed away 30 min later and fresh, complete media was added. Cells were incubated overnight and analyzed for β-galactosidase expression as above.

Cell Uptake of Enveloped Adenonovirus by Confocal Laser Scanning Microscopy (CLSM). Ad was covalently conjugated to an *N*-hydroxysuccinimide (NHS)-ester of the cyanine 3 fluorophore [Amersham Bioscience, U.K.] as previously described yielding a fluorescently labeled vector (Cy3-Ad).[33] The fluorophore conjugated vector was stored at −20 °C in 30% glycerol stock [Sigma, U.K.]. Confocal microscopy was performed using a Zeiss LSM 510 Meta laser scanning confocal microscope, equipped with a 30 mW argon laser, a 1 mW, 543 nm HeNe laser, and a 5 mW, 633 nm HeNe laser and a Plan-Apochromat 63x/1.4 oil lens. Samples were analyzed using two channel confocal laser scanning microscopy to obtain a pseudo-DIC image combined with a Cy3 fluorescence image. Lipid enveloped Cy3-Ad was prepared as follows: AdCy3-Ad (5×10^{10} pu/ml in ddH2O) was added to a DMPC/Chol or DOTAP/Chol lipid film to yield a final phospholipid concentration of 2 mM as described above, then concentrated to 2×10^{12} pu/ml using a Vivaspin 4 concentrator with a 5 kD molecular weight cutoff [Vivascience, Germany]. The vector was diluted 1:1 in 2× binding buffer (2× minimum essential medium (MEM) [Invitrogen, U.K.], 2% bovine serum albumen (BSA) [Sigma, U.K.], and 20 mM HEPES [Invitrogen, U.K.]). A549 cells were plated on Laboratory-Tek 16-well chamber slides [Nalgene Nunc International] at a density of 3000 cells per well in DMEM supplemented with 10% FBS and penicillin/streptomycin [all from Invitrogen, U.K.]. Cells were washed three times in 1× binding buffer (1× MEM, 1% BSA, 10 mM HEPES), and 200 μl of enveloped Cy3-Ad, Cy3-Ad alone (5×10^{10} pu/ml), or liposomes alone were added to duplicate wells. Cells were incubated for 10 min at 37 °C, and unbound vector was removed with three washes in 1× binding buffer. To evaluate surface binding, half of the samples were further washed three times with PBS and fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. To evaluate cell uptake, the remaining samples were incubated for a further 60 min and washed and fixed as above. The chambers were removed from the slides, and cells were mounted in Citifluor AF1 antifade reagent [Citifluor, U.K.].

Gene Transfer of Multicellular Tumor Spheroids (MTS). BxPC-3 cells, a human pancreatic adenocarcinoma cell line, were grown to confluency in T-75 tissue culture flask [Corning B.V., The Netherlands] in Advanced RPMI-1640 supplemented with 10% FBS, 20 mM L-glutamine and 1% penicillin/streptomycin [all from Invitrogen, U.K.]. Multicellular spheroids were prepared using the liquid overlay technique of Yuhas *et al.*[45] Briefly, cells were seeded (1×10^4 cells/well) in 1% agar-coated nonadherent flat-bottomed 96-well plates [Fisher, U.K.] and incubated in a 5% humidified CO_2 atm at 37° for 3 days to allow for spheroid formation. The spheroids were collected from the 96-well plates by a multichannel pipet and transferred into 100 mm Petri dishes. Spheroids were then placed in a 15 mL centrifuge tube and were allowed to settle for 15 min. The cell culture medium was carefully removed by vacuum aspiration, and fresh, serum-free RPMI with 20 mM L-glutamine and 1% penicillin/streptomycin was added. Spheroids were subsequently used for enveloped virus gene transfer studies. AdCMV.GFP, at a concentration of 2.5×10^{11} pu/ml, was added to DOTAP/DOPE (1 mM:3 mM) lipid films to yield final phospholipid concentrations of 4 mM. The vectors were allowed to sit at room temperature for 3 h or overnight at 4 °C and then were diluted in serum-free advanced RPMI media supplemented with 20 mM L-glutamine and 1% penicillin/streptomycin to an adenovirus concentration of 2.5×10^{10} pu/ml. Ten ml of the diluted vector was added to 1% agar-coated 100 mm bacteriological dishes containing at least 100 spheroids, yielding a dose of approximately 2×10^9 Ad particles per spheroid. Each spheroid was estimated to contain 1×10^5 cells per well, which at a dose of 2.5×10^9 adenovirus particle units (pu) per spheroid yields 25000 virus per cell. As a control, an equivalent dose of Ad alone or media only was used. Cells were supplemented with 10% FBS after 3 h and incubated with the vector for 24 h, washed in PBS before being transferred to glass bottom 24-well plate, and viewed using CLSM using X20 using Cy2 singletrack (488 nm laser excitation source, a 505−530 nm output filter, and a Plan-Neofluar 20X lens). GFP expression was observed 1 and 7 days post-transfection.

Acknowledgment. The authors would like to thank The School of Pharmacy, University of London, for financially supporting this research, Mr. David McCarthy for assistance with the electron microscopy, and Dr. David Gathercole for his help with the confocal microscopy.

Supporting Information Available: Lower resolution (TEM) electron micrographs showing multiple DOTAP/Chol- and DMPC/Chol-enveloped adenoviruses. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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