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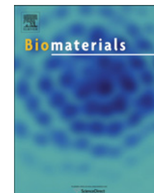

Intracellular trafficking and gene expression of pH-sensitive, artificially enveloped adenoviruses *in vitro* and *in vivo*

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ABSTRACT

Recombinant adenovirus (Ad) has shown great promise in gene therapy. Artificial envelopment of adenovirus within lipid bilayers has previously been shown to decrease the immunogenicity and hepatic affinity of naked Ad *in vivo*. Unfortunately, this also resulted in a significant reduction of gene expression, which we attributed to poor endosomal release of the Ad from its artificial lipid envelope. In this work, we explored the artificial envelopment of Ad within pH-sensitive DOPE:CHEMS bilayers and characterized this vector by TEM, AFM, dot blot, dynamic light scattering and zeta potential measurements. The artificially enveloped viral vectors exhibited good stability at physiological pH but immediately collapsed and released naked Ad virions at pH 5.5. Intracellular trafficking using confocal laser scanning microscopy (CLSM) revealed that Cy3-labelled Ad enveloped in DOPE:CHEMS bilayers exhibited the characteristic Ad distribution within the cytoplasm that led to virion accumulation around the nuclear membrane, indicating endosomal release of Ad. We obtained equivalent levels of gene expression as those of naked Ad in a series of CAR-positive (CAR+) and CAR-negative (CAR-) cell lines. This suggested that the mechanism of infection for the artificially enveloped Ad remained dependent on the presence of CAR receptors. Finally, the pH-sensitive enveloped Ad were injected intratumorally in human cervical carcinoma xenograft-bearing nude mice, also illustrating their capacity for efficient *in vivo* marker gene expression. This study is a step forward toward the engineering of functional, artificially enveloped adenovirus vectors for gene transfer applications.

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1. Introduction

Gene therapy vectors can be divided into two categories, viral and non-viral, with viral vectors being the most efficient gene transfer and expression agents. Recombinant adenovirus (Ad) allows for the delivery of a genetic payload up to 35 kb [1] and has been shown to hold great promise for gene therapy purposes [2]. However, it suffers from poor pharmacokinetics and extensive preclinical and clinical studies have indicated immunogenic responses and overwhelming virus accumulation in the liver resulting in severe hepatoxicity [3,4]. Despite significant promise, these issues currently inhibit widespread clinical use of adenoviral vectors for genetic medicine.

One approach to alter the native tropism of Ad includes chemical conjugation of hydrophilic polymers, among which polyethylene glycol (PEG)-based polymers or copolymers are the most popular due to the reduced protein surface opsonisation offered by PEG [5]. However, as the majority of these modifications are through random covalent modifications of the viral capsid they tend to result in structural and biological irreproducibility. Site-specific modifications have also been achieved through genetic engineering of the virions, but are cumbersome and do not ameliorate the immunological risks associated with Ad administrations. Moreover, both strategies have been shown to significantly decrease viral activity and gene expression *in vitro* [6].

Liposomes constitute the most clinically established type of nanoparticle today, with an extensively investigated pharmacological profile for various therapeutic and diagnostic applications [7]. They have received considerable interest as non-viral gene therapy vectors [8] and drug delivery systems [9] as they allow for the incorporation and delivery of both hydrophobic (within the lipid bilayer) and hydrophilic (within the aqueous compartment) molecules. In addition, it is possible to further engineer liposomes with cell- or tissue-specific signals by conjugation with targeting moieties such as peptides, antibodies or small molecules [7].

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We have recently proposed the construction of a hybrid liposome-adenovirus vector system by building lipid bilayer artificial envelopes around adenovirus particles [10]. Our previous investigations have shown that artificial envelopment of Ad can alleviate one of the main handicaps of adenoviral gene transfer by dramatically reducing gene expression in the liver responsible for hepatic toxicity. Ad could be enveloped by a variety of different lipid bilayers (zwitterionic, cationic, polymer-coated), however, envelopment resulted in significant reduction of gene expression *in vitro* due to poor release from the endosomal compartment following endocytosis [10]. More recently, we have also shown that it is possible to achieve delivery of enveloped Ad to solid tumours through the systemic circulation by use of PEGylated lipid envelopes that resulted in increased blood circulation, decreased immunogenic response and liver uptake compared to naked Ad [11]. These results indicated that the molecular characteristics of the artificial envelope will have a critical role on the resulting biological activity of the hybrid vector.

Acid triggering of drug delivery systems takes advantage of the decrease in the pH during endosomal localisation (reaching values less than 5.5) [12] and has previously been shown to increase the efficacy of drug delivery systems when compared to pH-stable systems. [9,13,14] Liposomes are well known to undergo endocytosis and the use of pH-sensitive liposomes has been extensively explored in the design of vectors for drug and gene delivery [15]. One of the most facile pH-sensitive liposome systems is based on the construction of lipid bilayers that consist of the fusogenic lipid dioleoyl-phosphatidylethalonamine (DOPE) with protonable amphiphiles such as cholesteryl hemisuccinate (CHEMS) [16]. The mechanism behind the pH-dependent destabilization of this bilayer system is that within an acidic environment, head group protonation of CHEMS occurs that causes DOPE molecules to revert from a bilayer (lamellar) to an inverted hexagonal II phase (non-lamellar). The use of pH-sensitive DOPE:CHEMS liposomes in drug delivery has been well studied, however they have met limited success as non-viral gene transfer vectors, since complexation with anionic nucleic acids (pDNA or siRNA) is generally required [17].

In this study we have investigated the possibility of increasing the gene transfer efficacy of artificially enveloped Ad by building pH-sensitive lipid bilayer envelopes around the virions made of DOPE:CHEMS. We have characterized this pH-sensitive, artificially enveloped vector by dynamic light scattering (DLS), atomic force microscopy (AFM), transmission electron microscopy (TEM), and surface charge (zeta potential) measurements. The efficiency of *in vitro* gene expression was further studied using various receptor CAR efficient (CAR+) and deficient (CAR-) cell lines. The intracellular trafficking of fluorescently-labelled (Cy3) Ad capsids was determined by confocal laser scanning microscopy. Finally, *in vivo* gene expression of these new vectors was evaluated following intratumoural administration into human cervical tumor (C33a; CAR+) xenografts.

2. Materials and methods

2.1. Viral vectors

Adenovirus (Ad) was purchased from the Baylor College of Medicine Vector Development Laboratory (Texas, USA). For these studies, the vector Ad β-gal, encoding for the β-galactosidase reporter gene driven by the CMV promoter was 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.

2.2. Envelopment of adenovirus

CHEMS (cholesteryl hemisuccinate) was purchased from Sigma–Aldrich (UK). DOTAP (1,2-dioleoyloxypropyl)-*N*,*N*,*N*-trimethylammonium chloride) and DOPE (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine) were purchased from Avanti Polar Lipids (USA). DOTAP/Chol, at a 2:1 M ratio, or DOPE/CHEMS, at a 3:2 M ratio were dissolved in 4:1chloroform/methanol in a 25 mL round-bottom flask. A lipid film was formed using a rotovaporator (BÜCHI, Switzerland). After 1 h under vaccum, the lipid film was flushed under nitrogen gas stream to remove any organic solvent residue. Adenovirus stock was thawed and diluted at a concentration of 10^{10} pu/mL in 1 mL of HEPES buffer (pH 7.4) which was used hydrate the lipid film. The flask was then placed in an ultrasonic water bath (VWR, UK) for 15 min at room temperature, after which the encapsulated viruses were allowed to anneal for 3 hr at room temperature.

2.3. Dynamic light scattering (DLS)

All measurements were made using the Zetasizer Nano ZS (Malvern Instruments, UK). Adenovirus at a concentration of 1×10^{10} pu/ml in HEPES buffer was added to DOPE:CHEMS films to yield final lipid concentrations of 2–10 mM. The pH sensitivity of DOPE:CHEMS envelopes was determined through incubation of the samples at pH 5 for a few minutes prior to size measurement. The average of threemeasurements was used and the results expressed as mean diameter (nm) ± S.D.

2.4. Transmission electron microscopy (TEM)

50 μl of a solution with an Ad concentration corresponding to 2×10^{11} Ad particles/ml was added onto a gold grid, and excessive material was removed using a filtered paper. These were stained with 1% aqueous uranyl acetate. Samples were allowed to dry in air and then imaged using a CM120 BioTwin electron microscope (Philips/FEI).

2.5. Atomic force microscopy (AFM)

20 μl of a Ad solution in water containing 10^{10} particles/ml was deposited on the surface of freshly cleaved mica (Agar Scientific, Essex, UK). The samples were allowed to adsorb for 30 s. Unbound vesicles were removed by washing with filtered dH_2O. Samples were then dried under a nitrogen stream. Imaging was carried out in Tapping Mode using a Multimode AFM, E-type scanner, Nanoscope IV controller, Nanoscope 5.31r1 control software (all from Veeco, Cambridge, UK), and a silicon tapping tip (NSG01, NTI-Europe, Apeldoorn, The Netherlands) of 10 nm curvature radius, mounted on a tapping mode silicon cantilever with a typical resonant frequency of 150 kHz and a force constant of 5.5 N/m, to image 5 × 5 μm square areas of the mica surface with a resolution of 512 × 512 pixels and a scan rate of 1 Hz. All AFM images were performed in air.

2.6. Dot blot

Ad (5×10^{10} pu/ml), enveloped Ad (5×10^{10} pu/ml) in DOPE:CHEMS (10 mM, 5 mM, 1 mM) and empty liposomes were spotted onto a Hybond ECL nitrocellulose membrane (GE Healthcare, UK). After blocking in 1.5%BSA-TBS overnight, the blots were incubated with mouse anti-hexon antibody (ab8251, Abcam plc, UK) at 1:1000 dilution. Membranes were washed with either TBS alone or TBS-0.1% Tween-20. HRP-linked anti-mouse IgG (Sigma, UK) at 1:1000 dilution was used as secondary antibody. The specific spots were detected with the ECL detection system (Pierce, USA).

2.7. 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 β-gal, at a concentration of 1×10^{10} pu/ml, was added to DOPE/CHEMS lipid films yielding final phospholipid concentrations of 3.33 mM 6.66 mM and 10 mM. The DOTAP:Chol liposomes were formed at a DOTAP concentration of 2 mM as described before. The vectors were allowed to sit at room temperature for 3 h and 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. 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 for 22 more hours. The β-galactosidase expression was examined using the TROPIX β-gal assay (Applied Biosciences, Warrington, UK) using a Lumat LB 9507 tube luminometer (Berthold Technologies, Bad Wildbad, Germany).

2.8. Intracellular trafficking by confocal laser scanning microscopy (CLSM)

Ad was covalently conjugated to an N-hydroxysuccinimide (NHS)-ester of the cyanine 3 fluorophore (Amersham Bioscience, UK.) as previously described yielding a fluorescently labeled vector (Cy3-Ad) [18]. The fluorophore conjugated vector was stored at - 20 °C in 20% glycerol stock (Sigma, UK). Confocal microscopy was performed using a Zeiss LSM510 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 three channel confocal laser scanning microscopy to obtain a pseudo-DIC image combined with a Cy3 and TOPRO-3 fluorescence image. Lipid enveloped Ad-Cy3 was prepared as follows: Ad-Cy3 (1×10^{12} pu/ml in HEPES buffer 7.4) was added to a DOPE/CHEMS or DOTAP/Chol lipid film to yield a final lipid concentration of 10 mM as described

Table 1
Size and surface charge characteristics of pH-sensitive enveloped Ad. The mean average diameter (nm), polydispersity index and surface charge of naked Ad, empty DOPE:CHEMS liposomes and pH-sensitive enveloped Ad by dynamic light scattering.

Vector Type	Mean Diameter (nm) ± S.D[a]	Polydispersity Index ± S.D[a]	Surface Charge (mV) ± S.D[a]
Adenovirus	115.1 ± 0.777	0.0587 ± 0.0123	−13.13 ± 0.723
DOPE:CHEMS	129.1 ± 0.0577	0.154 ± 0.00793	−69.733 ± 0.896
Ad-DOPE:CHEMS	107.7 ± 1.31	0.271 ± 0.0275	−63.3 ± 3.7

[a] Mean ± standard deviation; $n = 3$.

above. A549 cells and NIH 3T3 cells were plated on Laboratory-Tek 16-well chamber slides (Nalgene Nunc International, USA) at a density of 3000 cells per well in DMEM supplemented with 10% FBS and penicillin/streptomycin (all from Invitrogen, UK). The cells were incubated with 1×10^{10} pu/ml of enveloped (both DOPE:CHEMS and DOTAP:Chol formulations) Cy3-Ad, Cy3-Ad alone for 1 h and 3 h at 37 °C, after which the cells were washed three times using 1 X PBS and fixed with 4% paraformaldehyde in PBS for 15 min at room temperature after which the nuclei were stained using TOPRO-3 (Molecular Probes, USA). The chambers were removed from the slides, and cells were mounted in Citifluor AF1 antifade reagent (Citifluor, UK), after which confocal microscopy was performed using a Zeiss Axiovert LSM510 confocal with a 63 × oil immersion objective (Carl Zeiss Inc.).

2.9. In vivo gene expression in (human cervical carcionoma) xenograft-bearing nude mice

All animal experiments were performed in compliance with the UK Home Office Code of Practice for the Housing and Care of Animals used in Scientific Procedures. 6 week-old female CD-1 nude mice (Charles River Laboratories, UK) were caged in groups of 5 with free access to water. A temperature of 19–22 °C was maintained, with a relative humidity of 45–65%, and a 12-h light/dark cycle. Mice were inoculated subcutaneously with 1×10^7 C33a human cervical carcinoma cells in a volume of 150 μl PBS, into the right flank, using 26G needles. The tumor volume was estimated by measuring three orthogonal diameters (a, b, and c) with calipers; the volume was calculated as $(a \times b \times c) \times 0.5$ mm^3. Intratumoral injections were performed when tumor reached 100 mm^3. For intratumoral administration, mice were anaesthetized using isofluorane, and injected with 50 μl of naked or enveloped Ad at a final Ad dose of 1.25×10^9 pu. β-gal gene expression in the tumors was assessed after 24hrs of injection and expressed as RLU/mg proteins ($n = 3$–4). Statistical analysis: Data were analyzed using two samples, single sided, student's *t-test*. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Construction, structure characterisation, and pH sensitivity of DOPE:CHEMS enveloped Ad

We have previously engineered and characterized artificially enveloped Ad within cationic, zwitterionic and PEGylated liposomes [10,11]. In the present work, we attempted the construction of artificial pH-sensitive envelopes (DOPE:CHEMS) around the adenoviral capsid and studied their physicochemical properties. The Ad envelopment protocol followed was as previously described [10]. Briefly, DOPE:CHEMS lipids (3:2 M ratio) were dissolved in a chloroform:methanol (4:1 v/v) mixture and the organic solvent was evaporated under vacuum. The resulting lipid films were hydrated with a solution of 1×10^{10} pu/ml of Ad in HEPES buffer to achieve different lipid concentrations and lipid:Ad ratios. The liposome dispersions were bath-sonicated for 15 min and left to anneal for 3hr prior to analysis. The Ad enveloped in DOPE:CHEMS bilayers were found to be 107.7 (+/− 1.31) nm in size with a surface charge of −63.3 (+/− 3.7) mV, similar to empty liposomes (Table 1). The structure of artificially enveloped Ad was further elucidated using atomic force microscopy (AFM) and transmission electron microscopy (TEM). Fig. 1 represents AFM images of the pH-sensitive enveloped Ad. Cross-section analysis of naked Ad showed a height image of 65 nm on mica surface (Fig. 1, top right), while empty DOPE:CHEMS (2 mM:1.33 mM) liposomes were only 10 nm high as imaging was done in vacuum (Fig. 1, middle right). On the other hand, artificially enveloped Ad imaging showed the Ad particle surrounded by a lipid bilayer (Fig. 1, bottom left), in agreement with the cross-section analysis results (Fig. 1, bottom right). AFM was preferred for structural analysis of the vectors compared to TEM since imaging could be performed at neutral pH with no additional staining. TEM images also indicated Ad envelopment within DOPE:CHEMS lipid bilayer (Figure S1A). However, due to the use of acidic uranyl acetate stain, the DOPE:CHEMS envelopes collapsed rapidly (even after 10 min), similar to empty DOPE:CHEMS liposomes.

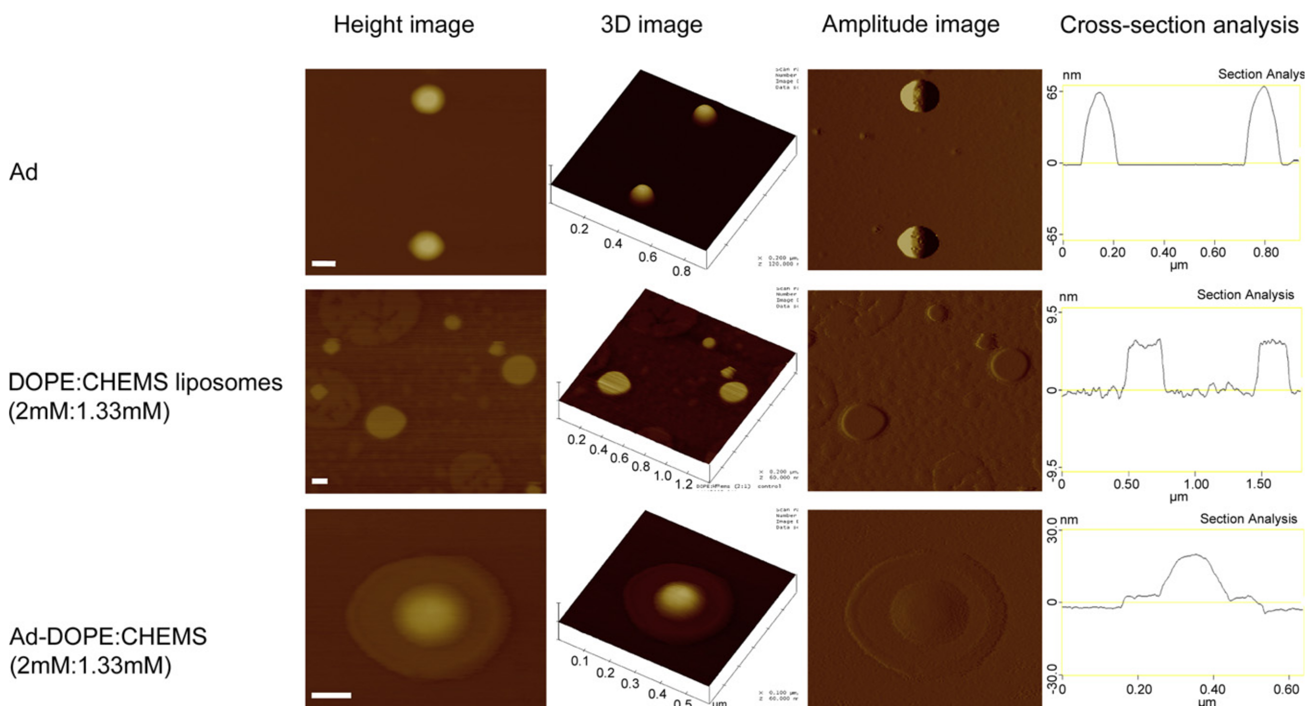

Fig. 1. *Structural elucidation of artificially enveloped Ad in pH-sensitive (DOPE:CHEMS) bilayers*. AFM images: Height image, 3D image, amplitude image, and cross-section analysis (left to right) of 1×10^{10} pu/ml naked Ad; DOPE:CHEMS empty liposomes; and enveloped Ad in DOPE:CHEMS. Scale bars are 100 nm.

In addition to the physicochemical information above, we further attempted to quantitatively determine the envelopment efficiency (% virions) of Ad within the lipid bilayers. In order to do that, we studied the effect of lipid concentration and lipid:Ad ratios on envelopment efficiency using the dot blot technique. Anti-hexon antibody (hAb) was used to probe the capsid hexon protein of naked Ad (Fig. 2A). The hAb bound specifically the capsid proteins (Fig 2A, Left) and no signals were detected following interaction with DOPE:CHEMS liposomes (Fig. 2A, right). Increasing the lipid concentration during Ad envelopment dramatically reduced availability and recognition of the Ad hexon (Fig. 2A, top). At 10 mM total lipid the envelopment efficiency obtained was in the order of 90% virions (Fig. 2B). In addition, incubating the enveloped Ad with 0.1% Tween solubilized the liposome bilayers at all lipid:Ad ratios tested and revealed Ad, regaining recognition by the anti-hexon Ab.

We then compared the stability of the enveloped Ad and the empty DOPE:CHEMS liposomes at pH 7.4 and 5.5 using DLS.

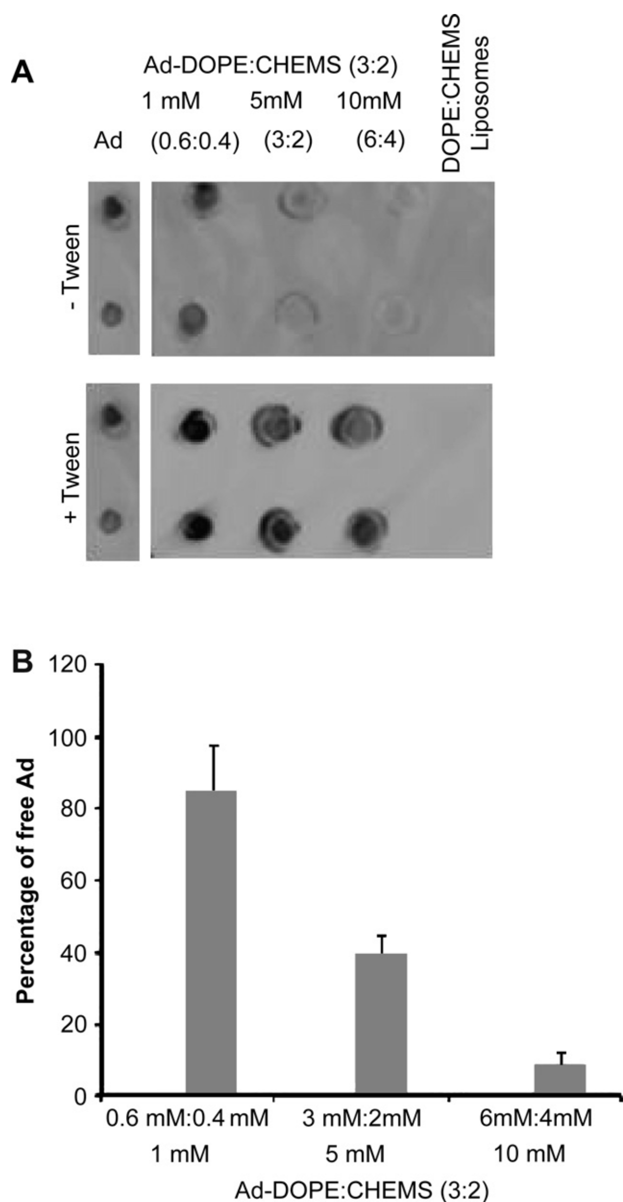

Fig. 2. *Envelopment efficiency of Ad in pH-sensitive DOPE:CHEMS bilayers.* (A) Dot blot of naked Ad and Ad enveloped in 1, 5 and 10 mM total lipid (left to right) of DOPE:CHEMS (3:2) in the absence (top) and presence (bottom) of 0.1% Tween. (B) Percentage of free Ad after envelopment in 1, 5 and 10 mM total DOPE:CHEMS bilayers.

DOPE:CHEMS liposomes and enveloped Ad at 2 mM DOPE:1.33 mM CHEMS (low lipid:Ad ratio) exhibited hydrodynamic diameters of 102 nm and 106 nm, respectively, at pH 7.4 (Figure S1B, black curves). The hydrodynamic diameter showed little or no change over 24 hr (data not shown) indicating good stability of the system at this pH. However, when empty liposomes and enveloped Ad were incubated at pH 5.5 we observed a rapid increase in the turbidity of the sample, indicating collapse of the liposome system. This collapse was confirmed by an increase in hydrodynamic diameter from approximately 100 nm to over 1000 nm (Figure S1B, striped curves). It seemed that irrespective of the lipid concentration and lipid:virus ratios, pH-sensitive artificially enveloped Ad systems reverted to the H_{II} phase of DOPE at pH 5.5, due to protonation of cholesteryl hemisuccinate (Figure S1C) [15]. Destabilization of pH-sensitive vector systems is indeed required at this pH range to achieve appropriate biological activity. This data indicated that we could modify the lipid enveloped Ad particles resulting in a pH-responsive vector system [13]. In addition, it provided further proof that envelope properties will have the most significant impact on the structural and physicochemical characteristics of the artificially enveloped Ad.

3.2. *In vitro gene expression of pH-sensitive, artificially enveloped Ad in CAR + cells*

The biological behaviour of pH-sensitive enveloped Ad was studied *in vitro* using reporter (beta-galactosidase; β-gal) gene transfer experiments. We investigated the effect of the artificial lipid envelopes on Ad gene expression by comparing Ad transfection after envelopment in pH-sensitive (DOPE:CHEMS) and pH-stable (DOTAP:Chol) lipid bilayers. Although the latter have the advantage of cationic surface charge and should exhibit more avid cellular binding, they suffer from inability to escape the endosomal compartment [10]. Recombinant Ad.β-gal was enveloped in DOPE:CHEMS and DOTAP:Chol liposomes at different lipid concentrations and lipid:Ad ratios. Human lung epithelial carcinoma cells (A549), rich in the primary Ad receptor, CAR, were incubated for 90 min with 10^8 Ad pu/well and β-gal expression was assessed after 24 hr of incubation. From the data obtained (Fig. 3A) the envelopment of Ad within pH-sensitive lipid bilayers is shown to have a significant advantage over cationic (non-pH-sensitive) bilayers. We observed that β-gal expression for the pH-sensitive enveloped Ad was at a similar level to that of naked Ad. Interestingly, we found that increasing the concentration of cationic lipids from 2 mM to 4 mM, significantly reduced the Ad gene expression due to higher Ad envelopment efficiency with no release from the endosome following endocytosis (Fig. 3A, black bars). The opposite trend was observed with pH-sensitive lipids (Fig. 3B), in this case Ad gene expression increased at the highest lipid to Ad ratio (envelopment within 6 mM DOPE:4 mM CHEMS).

Correlation of the dot blot data (Fig. 2B) to β-gal expression for pH-sensitive enveloped Ad viruses allowed us to conclude that increased Ad envelopment efficiency led to increased gene expression. To exclude the effect of lipid dose on Ad gene expression in cells, Ad was also enveloped at low lipid:Ad ratios and then mixed with empty DOPE:CHEMS liposomes before incubation with cells, to achieve a final lipid concentration equal to Ad enveloped in 10 mM. Interestingly, incubating cells with higher lipid concentrations did not seem to improve transfection, since the β-gal expression (Figure S3, white bars) was similar to the enveloped Ad prior to liposome mixing (Figure S3, black bars). This finding highlights the importance of the structural characteristics and lipid:Ad ratio for the design of vectors to maintain Ad transfectivity. All further experiments in this study were conducted with Ad

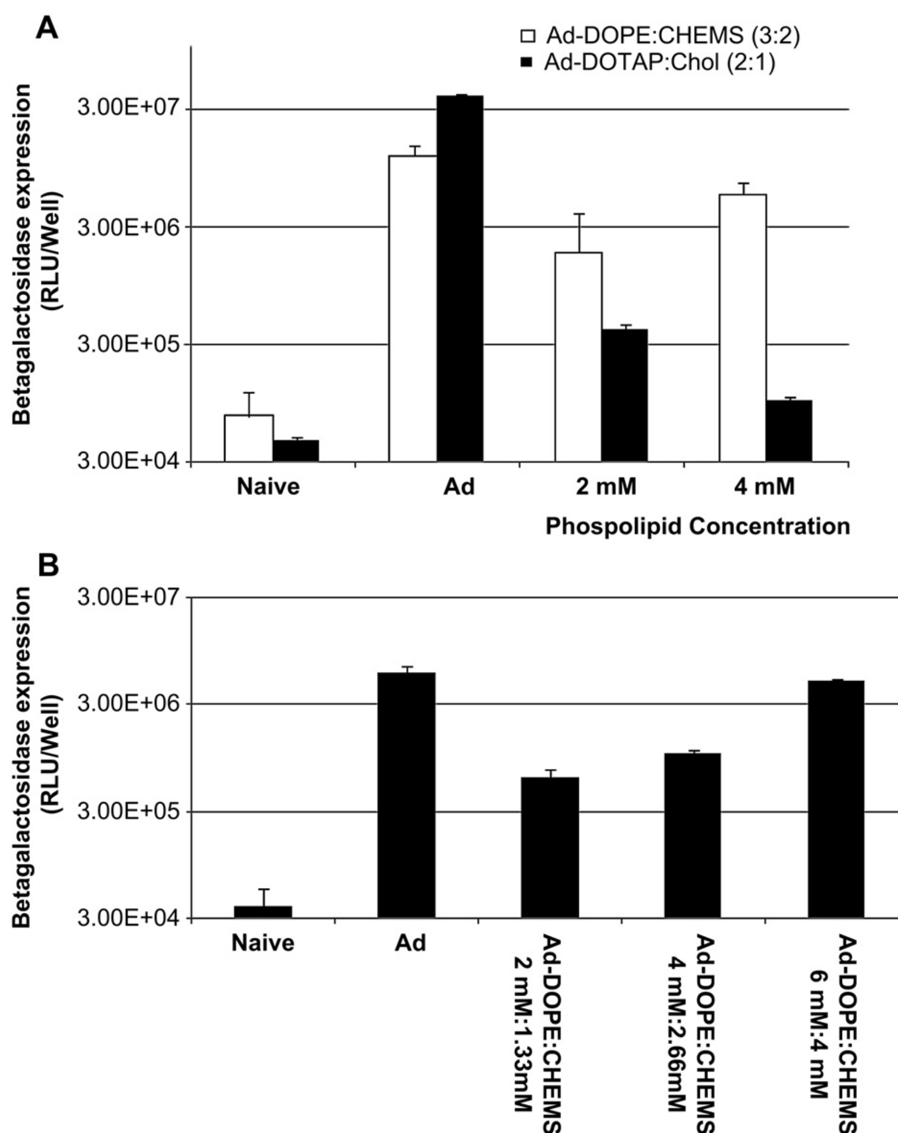

Fig. 3. *In vitro gene transfer efficiency of pH-sensitive enveloped Ad*. (A) β-gal expression of artificially enveloped Ad in pH-sensitive (DOPE:CHEMS) and pH-stable (DOTAP:Chol) at different Ad:lipid ratios (concentrations indicate that of DOPE or DOTAP used only). (B) β-gal expression of Ad enveloped into different DOPE:CHEMS lipid bilayer concentrations. 1×10^{10} virion pu were always enveloped and diluted to 1×10^{8} pu/well. A549 cells were incubated for 90 min with the vectors and analyzed 24 hr after incubation.

enveloped in 10 mM total lipid (6 mM DOPE : 4 mM CHEMS) to maintain the highest gene transfection efficacy.

3.3. Gene expression of pH-sensitive enveloped Ad in CAR+ and CAR- cells

Cellular uptake of Ad is determined by the presence of CAR receptors, leading to poor levels of gene expression in cell lines that do not express high amounts of this protein. We therefore investigated the β-gal expression of enveloped Ad in pH-sensitive (DOPE:CHEMS) compared to pH-stable (DOTAP:Chol) lipid bilayers in five cell lines with different levels of CAR expression. A549 and C33a were CAR-positive (CAR+) and B16F10, NIH 3T3 and CT26 were CAR-negative (CAR-) [19,20,21]. The results in Fig. 4 show (as expected) lower levels of gene expression for the naked Ad in the CAR- compared to CAR + cells. Interestingly, high transfection capacity was maintained in both CAR + cell lines (A549, C33a) for Ad enveloped within pH-sensitive lipid bilayers, while gene expression was decreased in all cell lines when cationic, pH-stable lipids were used to envelope the virus. When CAR- cell lines (B16F10, NIH 3T3, CT26) were incubated with pH-sensitive enveloped Ad, low levels of gene expression were obtained compared to naked Ad. Overall, these data indicated that the high levels of CAR receptors were still necessary for efficient gene expression of the pH-sensitive artificially enveloped vectors.

3.4. Intracellular trafficking of pH-sensitive artificially enveloped Cy3-Ad using CLSM

Fluorescently (Cy3) labelled Ad was prepared by incubation of Ad with the NHS-activated form of Cy3, dialyzed to remove unreacted dye molecules[18] and imaged by CLSM (Figure S2A). The Cy3-Ad construct was further characterized and compared to unmodified Ad in terms of mean size and gene expression activity. The hydrodynamic diameter and β-gal gene expression of Cy3-Ad were similar to the unmodified Ad (Figure S2B–D) which indicated that fluorescent labelling of the Ad did not significantly alter Ad characteristics.

To study the cellular uptake and trafficking of pH-sensitive enveloped Ad *in vitro*, 1×10^{12} p/ml of Cy3-Ad were enveloped with

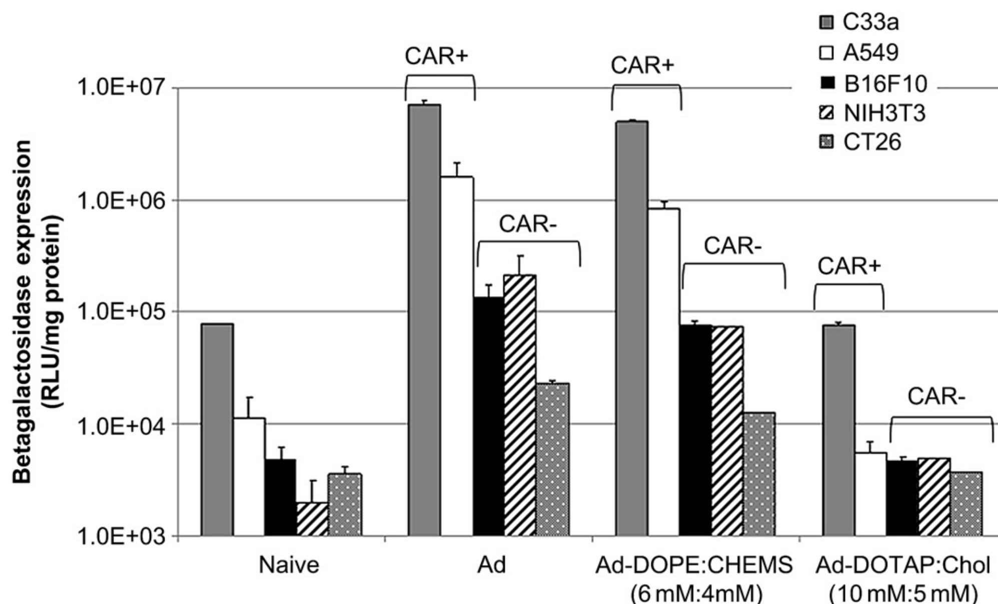

Fig. 4. *In vitro* gene transfer efficiency of artificially enveloped Ad in CAR+ (A549, C33a) and CAR- (B16F10, NIH 3T3, CT26) cell lines. 1×10^{10} Ad was enveloped in DOPE:CHEMS and DOTAP:Chol bilayers and diluted to 1×10^{8} pu/well. Cells were incubated for 3hr with the vectors and analyzed 24 hr after incubation.

DOTAP:Chol (2 mM:1 mM) and DOPE:CHEMS (6 mM:4 mM) lipid bilayers. Human lung epithelial carcinoma cells (A549; CAR+) and fibroblasts (NIH 3T3; CAR-) were incubated for 3 hr in serum free media with naked and enveloped Cy3-Ad (Fig. 5). The cells were fixed with paraformaldehyde and the nuclei were counterstained with TO-PRO3. Fig. 5 shows the intracellular trafficking of naked Cy3-Ad or Cy3-Ad enveloped in pH-stable (DOTAP:Chol) and pH-sensitive (DOPE:CHEMS) envelopes. In the CAR + cell line (A549) Cy3-Ad efficiently trafficked to the nucleus and the punctuate fluorescence detected in the cytoplasm following incubation with enveloped Ad in DOTAP:Chol agreed with our previous reports [10], confirming that these cationic-enveloped Ad were undergoing endocytotic internalisation, however suffered from lack of endosomal release. On the other hand, intracellular uptake and trafficking of Cy3-Ad enveloped within pH-sensitive lipid bilayers (DOPE:CHEMS) showed a completely different trafficking pattern. They distributed throughout the cell volume at early time points that indicated endosomal release of the enveloped Ad and exhibited the characteristic perinuclear localisation of naked virions after a few minutes.

In CAR- cells (NIH 3T3), Ad intracellular trafficking was dramatically reduced for both naked Ad (Fig. 5B, left) and DOPE:CHEMS enveloped Ad (Fig. 5B, right) that emphasised the need for the presence of CAR receptors in order to achieve efficient trafficking to the nucleus. The release of enveloped Ad from pH-sensitive bilayers compared to non-pH sensitive ones and the differences between CAR+ and CAR- cell lines obtained from CLSM were in good agreement with the gene transfection data (Fig 4).

3.5. Gene expression of pH-sensitive enveloped Ad in vivo following intratumoral administration

Lastly, the gene expression and cell trafficking studies *in vitro* led us to investigate how pH-sensitive enveloped Ad would interact with tumor cells in living animals. CAR + human cervical carcinoma (C33a) xenografts were implanted subcutaneously and grown in CD-1 nude mice. Naked Ad and Ad enveloped in pH-sensitive (DOPE:CHEMS) and pH-stable (DOTAP:Chol) envelopes were injected intratumorally into fully grown C33a tumor xenografts at a final Ad dose of 1.25×10^{9} particle units per animal (pu/animal). β-gal gene expression in the tumors was assessed 24 hr post administration. Fig. 6 represents the *in vivo* β-gal gene expression data from analysis of the excised C33a tumors. Similar to our *in vitro* results, the pH-sensitive enveloped Ad showed high levels of gene expression in the tumor, comparable to naked Ad ($p > 0.05$), and contrary to pH-stable enveloped Ad which significantly reduced the Ad transfection efficacy ($p < 0.01$). Therefore, the pH-sensitive release of the virions capable of gene expression from the artificial envelopes was also validated *in vivo*.

4. Discussion

Efficient gene therapy requires protection of the genetic material from degradation and efficient release from the delivery vector at the tissue and cells of interest [22]. Adenovirus has been an excellent candidate for gene delivery because its viral genome is protected by the capsid and is delivered very efficiently in CAR + cells, to achieve high levels of gene expression [23]. However, upon systemic administration, Ad is rapidly removed from the blood stream and captured in the liver. This leads to severe hepatotoxicity and induces immunogenicity in patients upon repeated injections and at therapeutically-relevant doses [3,4].

The Ad capsid has been previously modified genetically or synthetically (with hydrophilic polymers) to prolong blood circulation and reduce its immunogenicity [24]. Our group has described an alternative approach to circumvent some of the Ad drawbacks by artificial envelopment of Ad within self-assembled lipid bilayers [10]. In these studies, we showed that the viral particles could be enveloped with cationic, zwitterionic and PEGylated lipid bilayer envelopes that allowed great design flexibility in terms of the resulting physicochemical [10] and pharmacokinetic properties [11]. However, these envelopes dramatically reduced the transfection capability of Ad *in vitro*, presumably due to their failure to escape the endosomal compartments following endocytosis [10].

Cationic liposomes are one of the most extensively studied non-viral gene delivery systems, as their positive surface charge allows complexation with nucleic acids, such as plasmid DNA, oligonucleotides and siRNA into various forms and types of lipoplexes. In

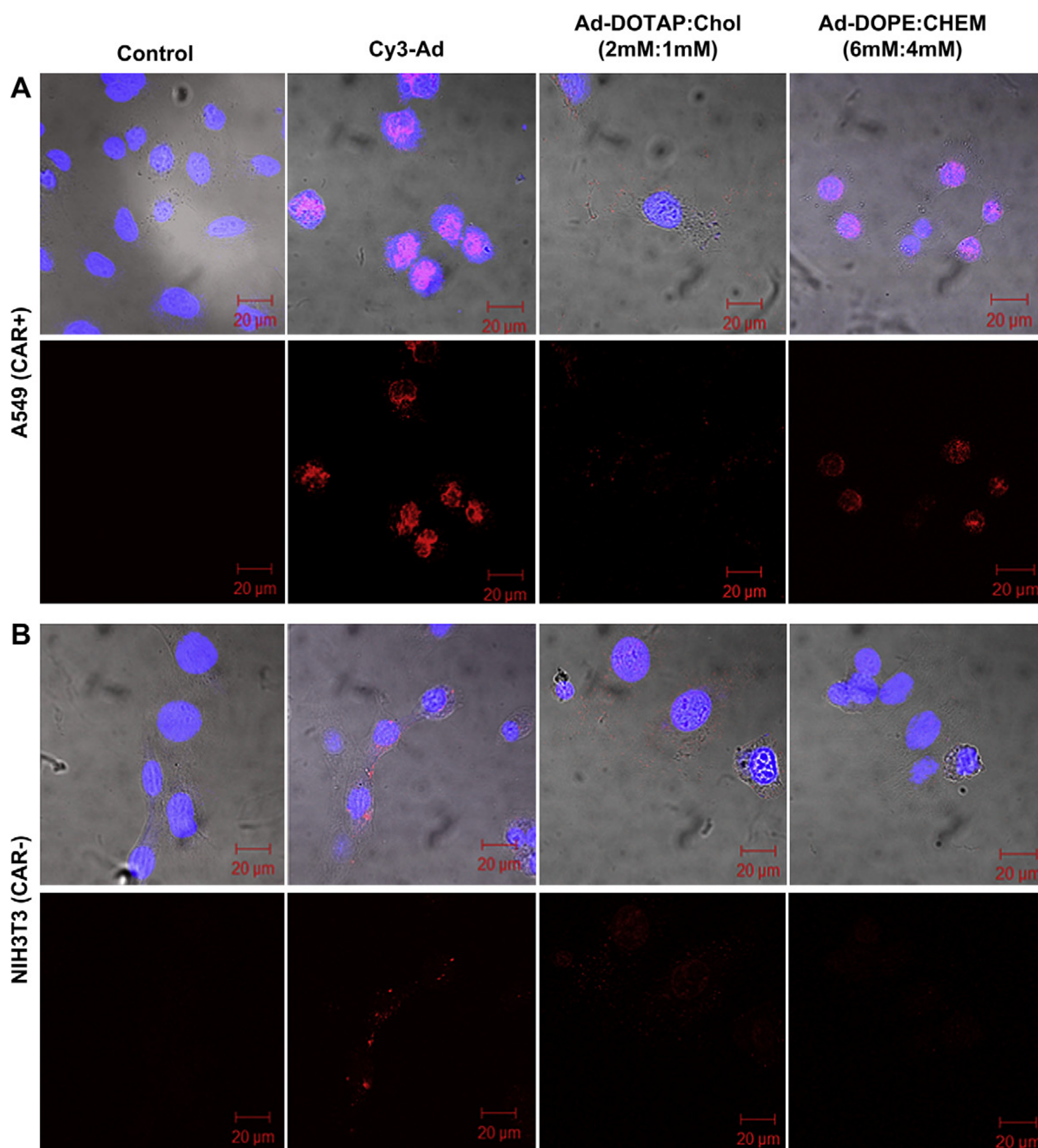

Fig. 5. *Intracellular trafficking of artificially enveloped Cy3-Ad in CAR + and CAR- cell lines.* Confocal and light transmitted DIC images of: (A) A549 (CAR+) and (B) NIH3T3 (CAR-) cells incubated with DMEM, Cy3-Ad, or Cy3-Ad enveloped in DOTAP:Chol and DOPE:CHEMS (left to right) for 3hr. Cells were washed, fixed and the nuclei were counterstained with TO-PRO-3.

addition, their net positive charge offers improved cellular binding due to electrostatic affinity for the anionic plasma membrane. Unfortunately the therapeutic index of lipoplexes is often poor due to inadequate endosomal release [13,25]. A change in pH is involved in many physiological processes, such as during endosome maturation, and a number of viruses contain pH-responsive, amphiphilic peptides on their surface that are known to play an important role in the destabilization of the endosomal membrane and the release of the viral genome into the cytoplasm of the infected cell [26]. Various groups have successfully used this physiological change in pH to trigger more efficient gene and drug intracellular delivery [13]. In addition to triggered release, pH-sensitive liposome systems have been shown to reduce cytotoxicity *in vitro* and *in vivo* in comparison to pH-stable analogues [25,27]. DOPE:CHEMS is one such pH-sensitive lipid combination that can also form stable anionic vesicles. Liposomes consisting of DOPE:CHEMS have been shown to become rapidly unstable below pH 5.5 due to protonation of the amphiphile CHEMS resulting in successful release of the encapsulated material from the endosomal compartment. [16] In order to overcome the problem of endosomal virion release when Ad was artificially enveloped, we engineered pH-sensitive envelopes around the Ad capsid that could successfully escape the endosomal compartment (Fig. 3) and maintain levels of exogenous gene expression equivalent to naked Ad.

The data obtained in this study indicates that the optimum ratio of lipid-to-virus for complete Ad envelopment is dependent on the nature and composition of the lipid envelope used. Previously, we reported that zwitterionic and cationic lipid bilayers required as low as 2 mM lipid concentration to achieve high levels of Ad envelopment [10]. Here, using an anionic (DOPE:CHEMS) envelope,

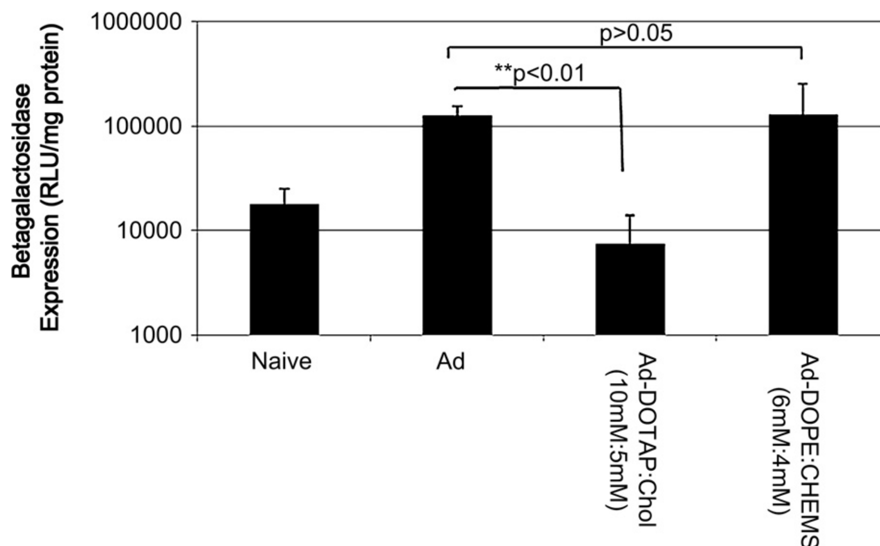

Fig. 6. *Gene expression of pH-sensitive artificially enveloped Ad in C33a tumor xenografts.* C33a tumors were injected (i.t.) with PBS, 1.25×10^9 pu naked Ad and Ad enveloped in DOTAP:Chol and DOPE:CHEMS bilayers. β-gal gene expression in the excised tumors was assessed 24 hr post-injection and results were expressed as RLU/mg protein. Statistical analysis: Data were analyzed using two samples, single sided, student's *t*-test. Differences were considered significant at $p < 0.05$.

both the transfection and dot blot data indicated that a higher lipid concentration of DOPE:CHEMS (6 mM:4 mM) was required to offer full envelopment of 10^{10}pu Ad (Fig. 2) and maintain effective levels of gene expression *in vitro* (Fig. 3B) and *in vivo* (Fig. 6). Besides the lipid envelope surface charge, bilayer fluidity will also determine the efficiency of Ad envelopment. Singh et al. showed that a relatively 'rigid' zwitterionic bilayer could lead to incomplete Ad envelopment by TEM [10]. These previous studies also illustrated that cationic envelopes resulted in high cellular uptake due to interaction with the cell membrane [10], PEGylated lipid envelopes could prolong blood circulation times compared to naked Ad[11], while the present study showed that pH-sensitive envelopes can fully restore gene transfer capacity to naked Ad levels (Fig. 3B).

pH-sensitive enveloped Ad showed similar levels of gene expression as those of naked Ad in all cell lines (Fig. 4) and no broadening of gene expression tropism could be achieved in CAR-cells. Intracellular trafficking studies showed that both Ad and pH-sensitive enveloped Ad successfully escaped the endosomes and trafficked to the perinuclear region within 3 hr of interaction with cells (Fig. 5). Overall, the data suggested that in order to trigger efficient Ad trafficking to the nucleus, the presence of CAR receptors was essential even if the Ad virions were released from the collapsed lipid envelopes within the endosomal compartment. Further work is therefore warranted to fully understand the details of cellular uptake, endosomal release and cytoplasmic trafficking mechanism of different artificially enveloped Ad and other types of virions. Lastly, *in vivo* gene expression was shown in CAR + human tumor xenografts to levels equivalent to naked Ad for artificially enveloped virions within pH-sensitive bilayers only and not pH-stable bilayers (Fig. 6). This offers encouragement for the further *in vivo* development of such vector systems. Successful translation of these pH-sensitive artificially enveloped Ad vectors in therapeutic preclinical studies will greatly depend on control of their pharmacokinetic and tissue distribution profile, as well as the immunological responses they will elicit.

5. Conclusion

We have successfully engineered pH-sensitive enveloped Ad and demonstrated that viral envelopment within pH-sensitive artificial envelopes can allow escape of virions from the endosomal compartment after endocytosis and almost complete restoration of gene expression efficacy *in vitro* and *in vivo*. We envision that such pH-sensitive artificially enveloped viral vectors can offer a viable alternative for gene delivery with the capacity to maintain high levels of gene expression in the absence of any severe innate immunogenicity and hepatotoxicity.

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Appendix. Supplementary data

Supplementary data associated with this article can be found in online version at doi:10.1016/j.biomaterials.2010.12.043.

Appendix

Figures with essential color discrimination. Fig. 1 and 5 in this article are difficult to interpret in black and white. The full color images can be found in the online version, at doi:10.1016/j.biomaterials.2010.12.043.

References

[1] Bergelson JM, Cunningham JA, Droguett G, Kurt-Jones EA, Krithivas A, Hong JS, et al. Isolation of a common receptor for coxsackie B viruses and adenoviruses 2 and 5. Science 1997;275(5304):1320–3.
[2] Rein DT, Breidenbach M, Curiel DT. Current developments in adenovirus-based cancer gene therapy. Future Oncol 2006;2(1):137–43.
[3] Campos SK, Barry MA. Comparison of adenovirus fiber, protein IX, and hexon capsomeres as scaffolds for vector purification and cell targeting. Virology 2006;349(2):453–62.
[4] Einfeld DA, Schroeder R, Roelvink PW, Lizonova A, King CR, Kovesdi I, et al. Reducing the native tropism of adenovirus vectors requires removal of both CAR and integrin interactions. J Virol 2001;75(23):11284–91.
[5] Allen TM, Hansen C, Martin F, Redemann C, Yau-Young A. Liposomes containing synthetic lipid derivatives of polyethylene glycol show prolonged circulation half-lives in vivo. Biochim Biophys Acta. Biomembr 1991;1066 (1):29–36.
[6] Croyle MA, Chirmule N, Zhang Y, Wilson JM. "Stealth" adenoviruses blunt cell-mediated and humoral immune responses against the virus and allow for

significant gene expression upon readministration in the lung. J Virol 2001;75 (10):4792–801.
[7] Torchilin VP. Recent advances with liposomes as pharmaceutical carriers. Nat Rev Drug Discov 2005;4(2):145–60.
[8] Kostarelos K, Miller AD. Synthetic, self-assembly ABCD nanoparticles; a structural paradigm for viable synthetic non-viral vectors. Chem Soc Rev 2005;34 (11):970–94.
[9] Gerasimov OV, Boomer JA, Qualls MM, Thompson DH. Cytosolic drug delivery using pH- and light-sensitive liposomes. Adv Drug Deliv Rev 1999;38(3): 317–38.
[10] Singh R, Al-Jamal KT, Lacerda L, Kostarelos K. Nanoengineering artificial lipid envelopes around adenovirus by self-assembly. ACS Nano 2008;2(5):1040–50.
[11] Singh R, Tian B, Kostarelos K. Artificial envelopment of nonenveloped viruses: enhancing adenovirus tumor targeting in vivo. FASEB J 2008;22(9): 3389–402.
[12] Watson P, Jones AT, Stephens DJ. Intracellular trafficking pathways and drug delivery: fluorescence imaging of living and fixed cells. Adv Drug Deliv Rev 2005;57(1):43–61.
[13] Guo X, Szoka Jr FC. Chemical approaches to triggerable lipid vesicles for drug and gene delivery. Acc Chem Res 2003;36(5):335–41.
[14] Duncan R. The dawning era of polymer therapeutics. Nat Rev Drug Discov 2003;2(5):347–60.
[15] Drummond DC, Zignani M, Leroux J. Current status of pH-sensitive liposomes in drug delivery. Prog Lipid Res 2000;39(5):409–60.
[16] Ellens H, Bentz J, Szoka FC. pH-induced destabilization of phosphatidylethanolamine-containing liposomes: role of bilayer contact. Biochemistry 1984;23 (7):1532–8.
[17] Rosa M, Penacho N, Simoes S, Lima MC, Lindman B, Miguel MG. DNA precondensation with an amino acid-based cationic amphiphile. A viable approach for liposome-based gene delivery. Mol Membr Biol 2008;25(1): 23–34.
[18] Leopold PL, Ferris B, Grinberg I, Worgall S, Hackett NR, Crystal RG. Fluorescent virions: dynamic tracking of the pathway of adenoviral gene transfer vectors in living cells. Hum Gene Ther 1998;9(3):367–78.
[19] McDonald D, Stockwin L, Matzow T, Zajdel MEB, Blair GE. Coxsackie and adenovirus receptor (CAR)-dependent and major histocompatibility complex (MHC) class I-independent uptake of recombinant adenoviruses into human tumour cells. Gene Ther 1999 Sep;6(9):1512–9.
[20] Takayama K, Reynolds PN, Short JJ, Kawakami Y, Adachi Y, Glasgow JN, et al. A mosaic adenovirus possessing serotype Ad5 and serotype Ad3 knobs exhibits expanded tropism. Virology 2003;309(2):282–93.
[21] Han SY, Lee YJ, Jung HL, Lee SW, Lim SJ, Hong SH, et al. Gene transfer using liposome-complexed adenovirus seems to overcome limitations due to coxsackievirus and adenovirus receptor-deficiency of cancer cells, both in vitro and in vivo. Exp Mol Med 2008;40(4):427–34.
[22] Parra-Guillen ZP, Gonzalez-Aseguinolaza G, Berraondo P, Troconiz IF. Gene therapy: a pharmacokinetic/pharmacodynamic modelling overview. Pharm Res 2010;27(8):1487–97.
[23] Hidaka C, Milano E, Leopold PL, Bergelson JM, Hackett NR, Finberg RW, et al. CAR-dependent and CAR-independent pathways of adenovirus vector-mediated gene transfer and expression in human fibroblasts. J Clin Invest 1999;103(4):579–87.
[24] Weaver EA, Barry MA. Effects of shielding adenoviral vectors with polyethylene glycol on vector-specific and vaccine-mediated immune responses. Hum Gene Ther 2008;19(12):1369–82.
[25] Boomer JA, Thompson DH, Sullivan SM. Formation of plasmid-based transfection complexes with an acid-labile cationic lipid: characterization of in vitro and in vivo gene transfer. Pharm Res 2002;19(9):1292–301.
[26] Carrasco L. Entry of animal viruses and macromolecules into cells. Febs Letters 1994;350(2–3):151–4. 22.
[27] Aissaoui A, Martin B, Kan E, Oudrhiri N, Hauchecorne M, Vigneron JP, et al. Novel cationic lipids incorporating an acid-sensitive acylhydrazone linker: Synthesis and transfection properties. J Med Chem 2004;47(21):5210–23.