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The effect of artificial lipid envelopment of Adenovirus 5 (Ad5) on liver de-targeting and hepatotoxicity

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ABSTRACT

Human Adenovirus type 5 (Ad5) has been extensively explored in clinical gene therapy, but its immunogenicity dramatically affects the kinetics and toxicity profile of the vector. We previously designed a variety of artificial lipid bilayer envelopes around the viral capsid to develop safer hybrid vectors. Here, we studied the interaction of enveloped Ad in cationic (DOTAP:Chol) or anionic (DOPE:CHEMS) lipid bilayers with different blood components. When Ad was enveloped by cationic lipids, significantly high levels of viral uptake in HepG2 cultured cells were achieved, independent of blood coagulation factors present. *In vitro* experiments also showed that artificial envelopment of Ad completely altered the affinity towards both human and murine red blood cells. After intravenous administration in BALB/c mice, real-time PCR and transgene expression studies indicated that cationic lipid envelopes significantly reduced hepatocyte transduction significantly increasing virus lung accumulation compared to DOPE:CHEMS enveloped or naked Ad. ALT/AST serum levels and liver histology showed that envelopment also improved hepatotoxicity profiles compared to naked Ad. This study suggests that artificial envelopes for Ad significantly alter the interactions with blood components and can divert viral particles from their natural liver tropism resulting in reduced hepatotoxicity.

1. Introduction

Many studies have attempted to reveal the potential benefits and limitations of adenoviral (Ad)-mediated gene transfer preclinically and clinically [1–3]. The halted University of Pennsylvania clinical trial in 1999 has had a lasting impact on the whole field, highlighting the serious concerns regarding the safety of Ad gene therapeutics [4]. Numerous studies have since tried to understand the immune mechanisms and reactions in response to systemic administration of Ad vectors [5–7].

The interaction of Ad with various hematological components is critical, often responsible for the extensive accumulation of Ad in the liver leading to hepatocyte transduction [8]. Kupffer cells (KCs) are hepatic macrophages and constitute one of the first lines of host defense against blood-borne pathogens. Liver KCs are considered a major limiting factor for systemic Ad delivery as they rapidly sequester and destroy virions [9]. KCs have been found to be responsible for the elimination of more than 90% of Ad after intravenous administration. Alemany et al. showed that the half-life of Ad5 in mice was 2–5 min, with more than 99% of Ad cleared from the blood in 1 h and most of the viral genomes found in the liver, predominantly in KCs [10–12]. Ad uptake by KC results in the release of inflammatory cytokines such as IL-6 or IL-12 through NF-KB activation [13]. Ad virions that escape KCs are taken up by hepatocytes where non-specific replication results in hepatotoxicity. Adenovirus-related gene or transgene overexpressing hepatocytes are attracted and hepatolysed by T- and B-cells that results in severe toxicity [14].

The natural tropism of Ad to hepatocytes is mediated mainly by blood coagulation factors [8,15–17]. Coagulation factor IX and complement binding protein-4 were shown to bridge Ad through the fiber knob to the receptors in the liver. Blood factor X, a vitamin K-dependent coagulation factor, was later found to be the major player in CAR-independent uptake by hepatocytes. When mice were pre-treated with warfarin to deplete vitamin K-dependent coagulation factors, liver infection was reduced by several orders of magnitude. In 2008, both Waddington et al. and Kalyuzhniy et al. showed that there are hypervariable regions on hexons that provide binding sites for FX Gla domains [8,15], that results in hepatocyte binding and transduction. These findings provided

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critical understanding for the interaction of Ad with host tissue and have valuable implications for the development of safe and efficient cell- and tissue-type specific Ad vectors for gene therapy applications.

The present work proposes engineering artificial lipid envelopes around the virion capsid to improve the inherent hepatotoxicity of Ad5 vectors. Previously, we showed the effect of different lipid envelopes on the resulting biological activity of the artificially enveloped Ad vectors. Ad can be successfully enveloped by both cationic and zwitterionic lipid bilayers, however a significant reduction in gene expression was observed due to poor release of the enveloped virions from the endosomal compartment [18,19]. When a fusogenic lipid dioleoylphosphoethanolamine (DOPE) was incorporated into the envelope, enhanced penetration of the vectors in 3D-tumor spheroids and delayed gene expression was observed [18]. More recently, we have shown that the efficacy of lipid bilayer-enveloped Ad can be further improved by engineering pH-sensitive artificial envelopes [20]. These studies showed that Ad can be enveloped in DOPE:CHEMS (cholesteryl hemisuccinate) lipid bilayers and lead to restoration of the biological activity of the virus by efficient endosomal escape and nuclear translocation.

In this work we studied the *in vitro* and *in vivo* behavior of Ad enveloped in cationic non-pH-sensitive (DOTAP:Chol) and anionic pH-sensitive (DOPE:CHEMS) lipid bilayers in comparison to naked Ad. This study focuses on the interactions between enveloped Ad, blood factors and hepatocytes in order to evaluate the fate and hepatotoxicity of enveloped Ad following systemic administration.

2. Materials and methods

2.1. Materials

Recombinant Adenovirus type 5 vectors (Ad.β-Gal and Ad.luc) were purchased from the Baylor College of Medicine, Vector Development Laboratory in Texas, USA. Ad.β-Gal encodes β-Galactosidase and Ad.luc carries luciferase reporter gene under the control of cytomegalovirus (CMV) promoter. Viral stocks were obtained at a concentration of 5×10^{12} particles/ml (5×10^{12} p/ml), which were equivalent to 1.2×10^{11} plaque-forming units (pfu)/ml, in 20 mM HEPES, 150 mM NaCl and 10% glycerol at pH 7.8 and stored at −80 °C until ready for use. Human hepatocarcinoma HepG2 cells (ATCC, USA) were maintained in Minimum Essential Medium (MEM), supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin, 50 μg/ml streptomycin, 1% L-glutamine and 1% non-essential amino acids at 37 °C in 5% CO_2. DOTAP ((1,2-dioleoyloxypropyl)-N,N,N-trimethylammonium chloride) and DOPE (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine) lipids were purchased from Avanti Polar Lipids (Alabama, USA) .Cholesterol (Chol), cholesteryl hemisuccinate (CHEMS), Hank's balanced salt solution (HBSS), bovine serum albumin (BSA) and HRP-labeled anti-mouse IgG were obtained from Sigma, UK. BCA Protein assay kit, Gibco Liver Digest Medium, 1-Step Ultra TMB-ELISA substrate and ECL detection system were purchased from Fisher, UK. Mouse anti-hexon antibody (ab8251) was from Abcam plc., UK. Hybond ECL nitrocellulose membrane was purchased from GE Healthcare, UK. Tropix Galacto-light Plus lysis buffer and Galacto-light Plus System were obtained from Applied Biosystems, UK. Human RBC lysis buffer was from Roche, Germany and EDTA-treated microtainers were from BD, UK. Primers for the Ad fiber (5′TGGCTGTTAAAGGCAGTTTGG and 3′GCACTCCATTTTCGTCAAATCTT), as previously described [21], were custom synthesized by Invitrogen, UK. The Quantifect SYBR Green Kit, DNA Mini Kit and DNeasy Blood and Tissue Kit were from Qiagen, UK. All human blood factors were purchased from Haematologic Technologies (Vermont, USA).

2.2. Artificial envelopment of Ad in lipid bilayers

DOTAP:Chol (2 mM:1 mM) and DOPE:CHEMS (6 mM:4 mM) were dissolved in 4:1 chloroform:methanol with a total volume of 2 ml in a 25 ml round-bottom flask. Lipid films were prepared using a rotovaporator (Buchi, Switzerland) at 42 °C with a speed of 120 rotations/min. After 45 min under a vacuum, the film was further dried for 5 min under a nitrogen gas stream. Lipid films were hydrated with 1 ml of 1×10^{10} p/ml or 5×10^{10} p/ml Ad in 20 mM HEPES, 150 mM NaCl pH 8.0 buffer. Enveloped Ad was sonicated in a cuphorn sonicator (Sonics, Switzerland) for 10 cycles of 4 min (15 s pulse on and 5 s pulse off) at 20% amplitude. The temperature of the water bath during sonication was maintained at room temperature by changing the water between the sonication cycles. Samples were incubated at 4 °C for 2 h to ensure annealing.

2.3. Characterization of enveloped Ad vectors

The size and zeta potential analysis of naked and enveloped Ad vectors were performed by Dynamic Light Scattering (DLS). The measurements were conducted in Zetasizer Nano ZS (Malvern Instruments, UK). Size was measured in 1 ml cuvettes and samples were diluted with 20 mM HEPES, 150 mM NaCl buffer at pH 8.0. Zeta potential was measured in disposable zetasizer cuvettes, and samples were diluted in distilled water. Three measurements for sizing and five measurements for zeta potential were taken. To determine envelopment efficiencies, 5×10^{10} p/ml Ad.β-Gal or enveloped Ad.β-Gal in DOPE:CHEMS or DOTAP:Chol were spotted onto a Hybond ECL nitrocellulose membrane. The blots were blocked with 1.5% BSA in TBS and then incubated with mouse anti-hexon antibody at 1:1000 dilution. Membranes were washed with either TBS alone or TBS-0.1% Tween-20 between different incubation steps. HRP-linked anti-mouse IgG at 1:1000 dilution was used as secondary antibody. The specific spots were detected using an ECL detection system.

2.4. In vitro gene transfer with enveloped Ad

HepG2 cells were seeded onto 24-well plates and incubated at 37 °C, 5% CO_2, overnight. Ad stock was thawed then diluted to desired concentrations with 20 mM HEPES, 150 mM NaCl pH 8.0 buffer. Cells were washed with PBS and pre-incubated with blood factor IX (4 μg/ml), factor X (8 μg/ml), factor XI (3 μg/ml) in serum-free MEM media. After 45 min, 10^8 p/ml of naked Ad.β-Gal or enveloped Ad.β-Gal in DOTAP:Chol or DOPE:CHEMS were added to the wells for 3 h. After 3 h of incubation with viral vectors, the transfection medium was removed and cells were washed with PBS. Fresh complete medium was added and plates were incubated overnight at 37 °C. Gene expression was analyzed with β-Galactosidase assay, normalized by BCA assay at 24 h.

2.5. In vivo gene transfer with enveloped Ad

All experiments were performed with prior approval from the UK Home Office. Female BALB/c mice, 6 weeks old, (4 mice/group) were purchased from Harlan, UK. Mice were allowed one week to acclimatize prior to use. Enveloped Ad.β-Gal or Ad.luc samples were prepared. In brief, DOPE:CHEMS (6 mM:4 mM) or DOTAP:Chol (2 mM:1 mM) lipid films were hydrated with 5×10^{10} p/ml Ad and enveloped viral particles were sonicated as described previously. Mice were warmed in a 37 °C heating chamber, anesthetized with isofluorane and administered from the intravenous route with 200 μl of Ad and enveloped Ad samples. Experiments involving the inactivation of vitamin K-dependent blood coagulation factors, mice were injected subcutaneously with 5 mg/kg warfarin (W) in peanut oil 3 and 1 days before Ad administration [16,22]. To study the natural liver tropism, 24 h after virus injections, mice livers were perfused as previously described with some modifications [23,24]. In brief, livers were first perfused with Ca^{2+} and Mg^{2+} free HBSS and then with liver digest medium at 37 °C. After digestion, liver was washed with 1.5% BSA in HBSS and cell suspension was passed through a 100 μm cell strainer at 4 °C. Cells were centrifuged at 50 *g* for 5 min to separate parenchymal cells (PC including hepatocytes) which were in pellet and non-parenchymal cells (NPC including Kupffer cells and epithelial cells) which stayed in supernatant. PC were resuspended in 1.5% BSA in HBSS and centrifuged twice at 50 *g* for 5 min. NPC suspension was centrifuged twice at 50 *g* for 5 min and finally at 450 *g* for 2 min. After perfusion, spleen and lung were collected and homogenized with Ultra-Turrax Tissue Homogenizer (Jencons-PLS, UK). Following liver perfusion and isolation of PC/NPC cells, DNeasy Blood and Tissue Kit were used to isolate DNA. For tissue-distribution studies, luciferase activity at 24 h was imaged by using IVIS lumina (Caliper life science, USA). Mice were anesthetized with isofluorane and administered intraperitoneally with D-Luciferin (Caliper life science, USA) at 15 mg/kg (200ul of 15 mg/ml in PBS). 10 min post luciferin injection, mice were images and ROI analysis on the abdominal area was performed using Live imaging software 3.2®. Lungs, livers, spleens and kidneys were dissected and ROI analysis was performed.

2.6. Isolation of red blood cells and their interactions with enveloped Ad vectors

Human blood in EDTA-vacutainers was obtained from Sera-Lab, UK. To obtain mouse RBC, mice were anesthetized with isofluorane and blood was collected from inferior vena cava into EDTA-treated microtainers. To isolate RBC, whole blood was centrifuged at 2000 *g* for 5 min. RBC population was suspended in PBS and washed in 4 times in a 10× excess volume of PBS. Then cells were suspended in PBS at physiological concentrations, 5×10^9 cells/ml. Mouse RBC concentration was adjusted to 10^{10} cells/ml in PBS. Ad or enveloped Ad vectors (2×10^8 p/ml for human blood experiments and 10^{10} p/ml for mouse blood experiments) were incubated with RBC at 37 °C for 30 min. After incubation, unbound viral particles were separated from the cellular fraction by centrifugation at 1000 *g* for 5 min. Number of viral particles bound to cells were analyzed by qPCR.

2.7. β-Gal reporter gene assay

Transfected cells or homogenized organs were washed twice with PBS and lysed with Tropix Galacto-light Plus lysis buffer. Then lysates were centrifuged for 10 min

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at 13,000 rpm β-Gal activity in each cell or tissue lysates were assessed with Galacto-light Plus System using a Lumat LB 9507 tube luminometer (Berthold Technologies, Bad Wildbad, Germany). Total protein concentration was assessed with the BCA protein assay kit. Relative light unit/mg protein was calculated in each treatment group.

2.8. Quantification of Ad copies by real-time PCR

Real-time PCR was performed with the DNA samples of transfected cells or organs. DNA was isolated either by DNA Mini Kit for cell culture experiments or by DNeasy Blood and Tissue Kit for *in vivo* studies. Primers for the Ad fiber (5′TGGCTGTTAAAGGCAGTTTGG and 3′GCACTCCATTTTCGTCAAATCTT) were used at 200 nM concentration. The Quantifect SYBR Green Kit was used for PCR reaction mixture. Samples were run on CFX-96 Real Time System (Bio-Rad, UK) with the following protocol: 95 °C, 15 min, 1 cycle; denaturating: 95 °C, 30 s, annealing: 60 °C, 1 min, − repeated for 40 cycles. Standard curve was obtained with 4 different Ad concentrations: 10^{10}, 10^{9}, 10^{8}, 10^{7}, 10^{6} p/ml.

2.9. Evaluation of hepatotoxicity profile

Mice were injected with 1×10^{11} p of naked Ad.β-Gal or enveloped Ad in DOTAP:Chol or DOPE:CHEMS lipid bilayers or empty liposomes. Blood and livers were collected after 5 days. Serum levels of ALT and AST were determined by Diagnostic Laboratories in the Royal Veterinary College, UK. After fixing in para-formaldehyde and paraffin embedding, liver sections were stained with H&E by Diagnostic Laboratories. Random images were captured by light microscopy (×4 and ×20) for different treatment groups.

2.10. Assessment of NAb levels

Blood was taken from mice injected with Ad (5×10^{10} p/mouse) and enveloped Ad in DOTAP:Chol or DOPE:CHEMS lipid bilayers after 28 days. Sera were isolated from each treatment group and analyzed with ELISA and A549 transfection studies. The presence of Ad specific antibodies was determined by ELISA. 96-well plates were coated with 100 μl of Ad at a concentration of 5×10^{9} p/ml overnight. After washing with PBS − 0.01% Tween-20, blocking was performed with 3% BSA in PBS for 2 h. Serum samples (100 μl) at a dilution of 1:1000 were added to the wells for 2 h and followed by the incubation with HRP-labeled anti-mouse IgG at a 1:1000 dilution. After washing with PBS, 1-Step Ultra TMB-ELISA was added to the wells and the absorbance values of each sample were presented. To assess the NAb titers, sera obtained from each treatment group (1:100 dilution) was incubated with Ad.β-Gal (1×10^{9} p/ml) at 37 °C for 30 min and added to the wells pre-seeded with A549 cells. Data was expressed as the percentage of the β-Gal expression in cells treated with Ad in the presence of serum obtained from naive group.

2.11. Statistical analysis

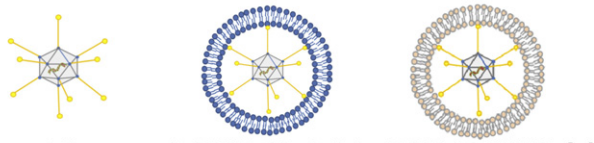
In vitro experiments were performed in triplicates on at least three independent occasions. *In vivo* experiments were performed with at least four animals per group. Statistical analysis was performed by analysis of variance and Tukey's pairwise comparison using SPSS software, version 16.0.

3. Results

3.1. Characterization of artificially enveloped Ad5

Artificially enveloped Ad was prepared using lipid film hydration method followed by sonication. We have previously engineered and fully characterized enveloped Ad in different artificial lipid bilayers [18–20]. However, in this study, we focused on two different envelopes: cationic non-pH-sensitive DOTAP:Chol and

a



Physiochemical Characterization	Ad	DOTAP:Chol-Ad	DOPE:CHEMS-Ad
Size (nm)	115.1 +/- 0.8	128.5 +/- 1.6	144.6 +/- 0.8
Polydispersity index	0.058 +/- 0.012	0.169 +/- 0.011	0.223 +/- 0.017
Zeta Potential (mV)	-13.1 +/- 0.7	+62.8 +/- 3.5	-63.1 +/- 3.4

b

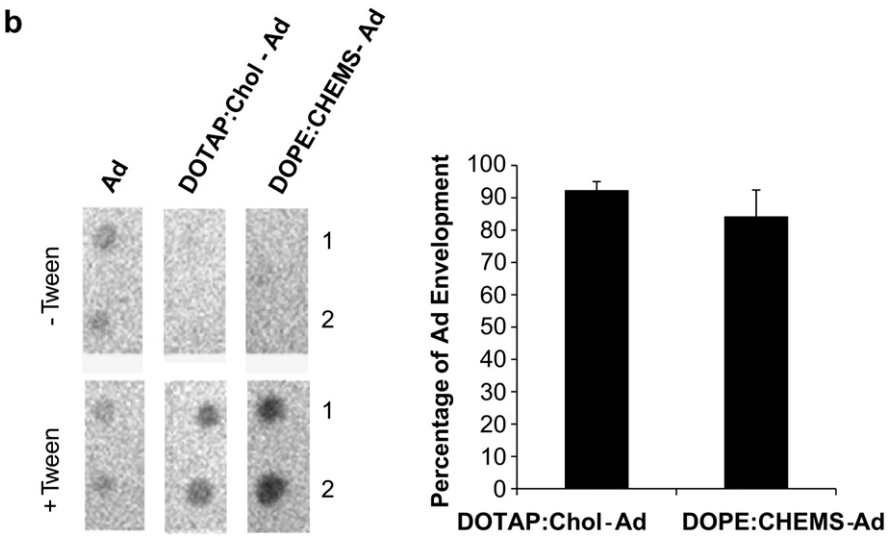


Fig. 1. Schematic representation and characterization of enveloped Ad vectors. The naked Ad and enveloped Ad in cationic non-pH-sensitive DOTAP:Chol, anionic pH-sensitive DOPE:CHEMS lipid bilayers are the vectors examined in this study. (a) The mean average diameter (nm), polydispersity index and surface charge (mV) for each vector were obtained by DLS. (b) Dot blot of naked Ad and enveloped Ad in DOTAP:Chol (3 mM) or DOPE:CHEMS (10 mM) in the absence (top) and presence (bottom) of 0.1% Tween-20. After analysis by GeneSnap software, percentage of Ad envelopment was plotted.

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anionic pH-sensitive DOPE:CHEMS. Dynamic Light Scattering (DLS) measurements showed a slight increase in the hydrodynamic diameter where Ad size increased from 115.1 nm to 128.5 nm and 145.6 nm following envelopment in DOTAP:Chol and DOPE:CHEMS envelopes, respectively (Fig. 1a). Lipid envelopment also altered the Ad surface charge (−13.1 mV), where DOTAP:Chol bilayers resulted in a positive surface charge (+62.8 mV) and DOPE:CHEMS resulted in negatively charged (−63.1 mV) viral particles (Fig. 1a), in accordance with the characteristics of the lipid molecules used to form the bilayers.

In order to determine the efficiency of envelopment, naked and enveloped Ad samples were spotted onto a nitrocellulose membrane to determine the percentage of envelopment. In agreement with previously published results, when Ad particles were in lipid envelopes, antibodies could not recognize hexon regions [20]. However, when membranes were washed with Tween-20, a non-ionic detergent used to solubilize the bilayer and releases the Ad, hexon signals appeared after chemiluminescent development. Fig. 1b shows that DOTAP:Chol at 3 mM and DOPE:-CHEMS at 10 mM lipid concentration successfully achieved >80% envelopment efficiency.

3.2. The influence of blood factors on viral transfection efficiency in vitro

Most of the toxicity after systemic administration of Ad is seen in the liver, particularly hepatocytes. Blood coagulation factors play a key role in liver transduction [8,15]. It has been shown that blood factor X (FX) is mainly mediating Ad binding to hepatocytes, whereas blood factor IX (FXI) determines liver tropism to a lesser extent and blood factor XI (FXI) does not play a significant role. To study the interaction between artificially enveloped Ad and blood factors, human hepatocarcinoma HepG2 cells were incubated with Ad.β-Gal in the presence or absence of FX, FXI, and FIX. After 24 h, qPCR was performed to determine the number of particles taken up by cells (Fig. 2a) and the β-Gal assay was performed to analyze the levels of gene expression (Fig. 2b). Cells pre-incubated with FX, followed by Ad infection, increased viral uptake and gene expression by approximately 3-fold ($p < 0.01$), while pre-incubation with FIX increased viral uptake and expression by about 2-fold. FXI was used as a negative control and did not influence the levels of gene expression, as expected. When Ad was enveloped in cationic liposomes (DOTAP:Chol) the levels of viral uptake were significantly

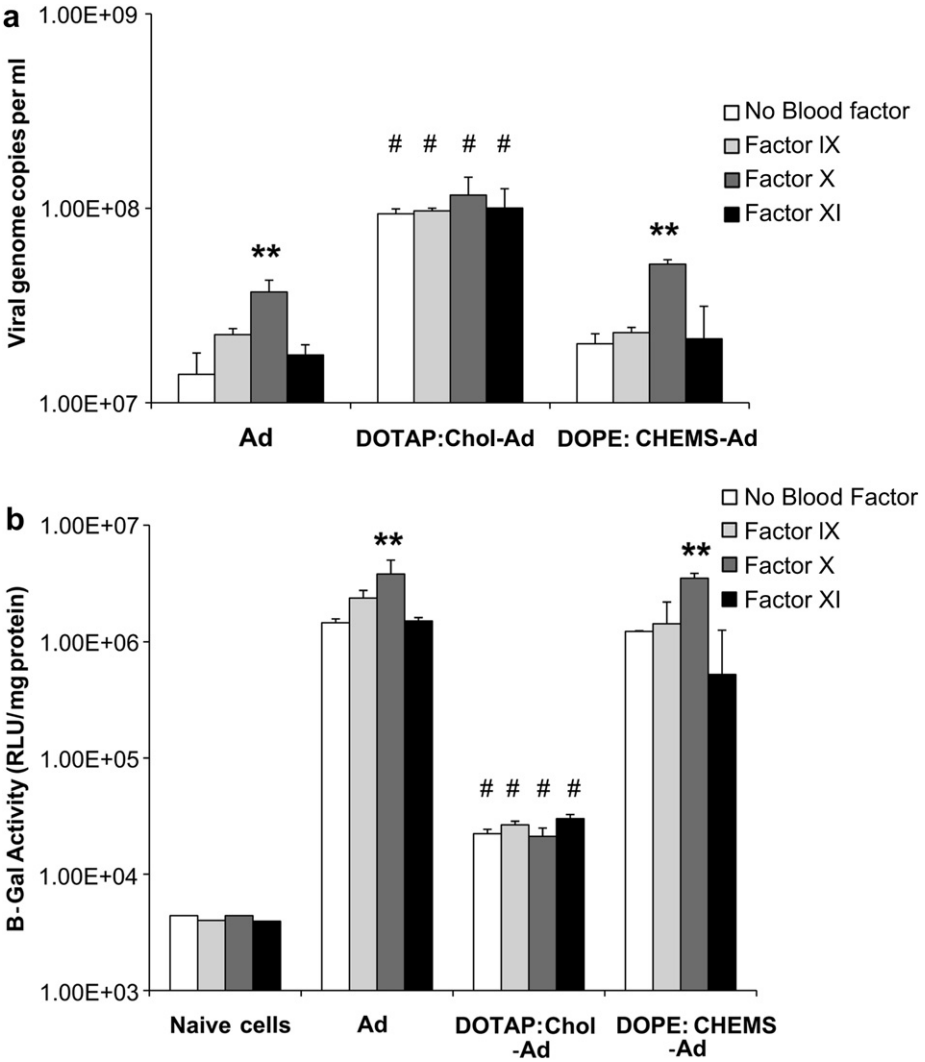

Fig. 2. The effect of blood factors on Adenovirus transfection *in vitro*. HepG2 cells were pre-incubated with or without blood factor-X, -IX and -XI for 45 min. Later, Ad.β-Gal (10^8 p/ml) and enveloped Ad.β-Gal (10^8 p/ml) in DOPE:CHEMS or DOTAP:Chol were added onto the wells. After 24 h, cells were lysed and (a) total genomic DNA was isolated to perform qPCR; and (b) β-Gal assay was performed to assess RLU/mg protein in each sample. $^{**}p < 0.01$ versus no blood factor group, $\#p < 0.01$ versus Ad.

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higher than Ad treated cells ($p < 0.01$), but no increase in gene expression was obtained independent of blood factors which agrees with the low gene expression previously observed with this envelope [18]. However when anionic pH-sensitive (DOPE:CHEMS) lipids were used to envelop Ad, a pattern similar to that of Ad alone was observed. FX significantly enhanced the DOPE:CHEMS enveloped Ad uptake and gene expression compared to FXI, FIX or no blood factor.

3.3. Interaction with red blood cells ex vivo

The interaction between blood cells and Ad can dramatically affect virus biodistribution and toxicity *in vivo* [25]. In order to study this further, we incubated human RBC with naked Ad and artificially enveloped Ad. After 30 min, unbound viral particles were washed away and the number of viral particles bound to cells was quantified by qPCR. Fig. 3a shows that Ad bind to human RBCs due to the high expression of CAR on their surfaces [26]. Following envelopment the affinity towards RBC was significantly higher with DOTAP:Chol envelope compared to Ad alone ($p < 0.01$). DOPE:-CHEMS envelopment also increased the interaction between human RBC ($p < 0.05$). To gain further information on the type of interaction between enveloped Ad and RBC, we also used mouse RBC, which does not express any CAR receptors, for comparison. Fig. 3b shows that the affinity of the enveloped vectors towards the murine RBC obtained were similar to that with the human cells.

3.4. Liver sequestration of artificially enveloped Ad5: hepatocyte versus Kupffer cell uptake

We then examined the cellular uptake and distribution between hepatocytes and Kupffer cells of the enveloped viral vectors *in vivo*. Ad and artificially enveloped Ad were intravenously administered in BALB/c mice and liver cells (parenchymal and non-parenchymal cells) were isolated via liver perfusion and differential centrifugation. Parenchymal cell (PC) populations mainly consist of hepatocytes, and non-parenchymal cells (NPC) consist of Kupffer cells and epithelial cells. Before quantification of viral particles in the different cell extracts, the percentage of hepatocytes and KCs in PC and NPC populations was determined respectively. We analyzed the extracted fractions with flow cytometry by staining using KC- and hepatocyte-specific markers. Anti-F4/80 antibody and anti-CD68 antibody were used for KC identification whereas hepatocytes were detected with anti-albumin antibody (Fig. S1). The extracted NPC cell population was found to be 100% viable, whereas we obtained 79.30% viability for the PC fraction. The NPC cell population was also F4/80 and CD68 positive. In the PC population, intracellular albumin staining indicated that 66% of the cells were hepatocytes.

Following liver perfusion, the number of viral genome copies in the hepatocyte (PC) and KC (NPC) fractions was compared by qPCR (Fig. 4a). Naked Ad.β-Gal showed the highest accumulation with nearly 5×10^7 particles were detected in hepatocytes and 5×10^8 particles in KC. When Ad was enveloped by DOTAP:Chol lipid bilayers, a significantly lower number of viral particles (3.4×10^5 particles) was detected in hepatocytes (compared to the naked Ad group), however similar number of viral copies were found in KC. The envelopment of Ad in anionic pH-sensitive (DOPE:CHEMS) led to a decrease in the number of viral particles in hepatocytes ($p < 0.01$), however not as low as with the cationic envelopes ($p < 0.001$). KCs sequestered a similar number of viral particles after injection of DOPE:CHEMS enveloped Ad compared to all other

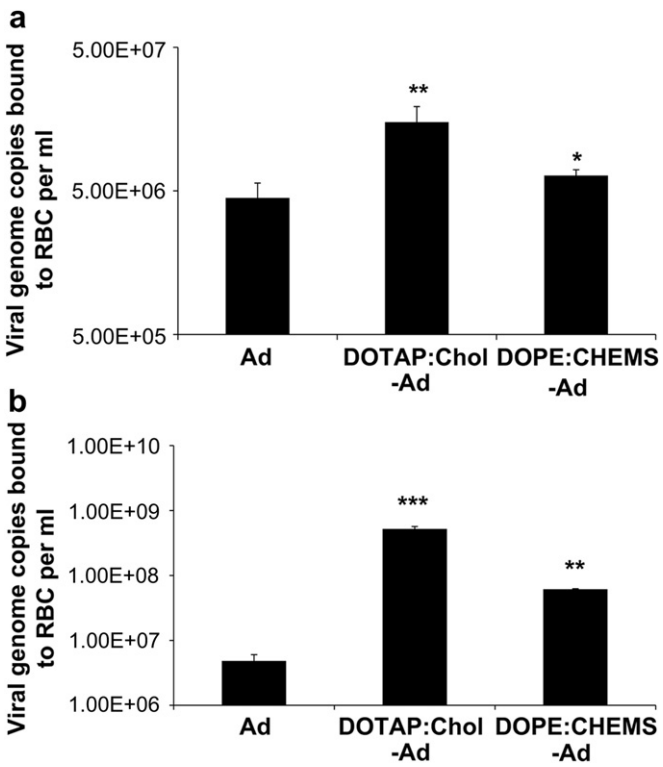

Fig. 3. The interaction between blood cells and enveloped Ad. Ad.β-Gal (2×10^8 p/ml for human blood experiments and 10^{10} p/ml for mouse blood experiments) or enveloped Ad.β-Gal in DOTAP:Chol or DOPE:CHEMS lipid envelopes were incubated with (a) human RBC (5×10^9 cell/ml) or (b) mouse RBC (10^{10} cells/ml) at 37 °C for 30 min. After incubation, unbound viral particles were separated from the cellular fraction by centrifugation at 1000 *g* for 5 min. Number of viral particles bound to cells were analyzed by qPCR. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus Ad.

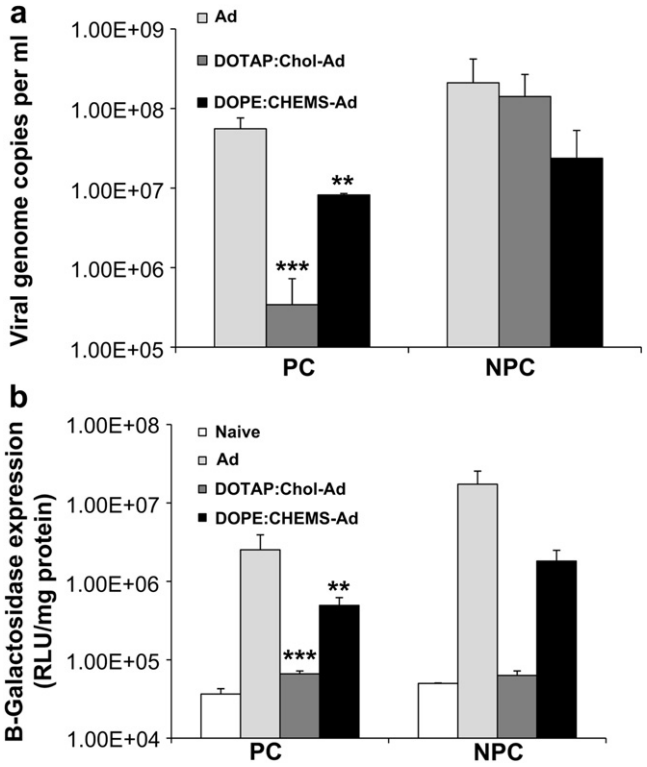

Fig. 4. The effect of artificial envelopment on Ad translocation in mouse liver. BALB/c mice were injected with either Ad.β-Gal (10^{10} p in 200 μl) or enveloped Ad.β-Gal (10^{10} p in 200 μl) in DOTAP:Chol or DOPE:CHEMS lipid envelopes. 24 h later, livers were perfused and parenchymal cells (PC) and non-parenchymal cells (NPC) were isolated. (a) After DNA isolation, real-time PCR was performed to determine the number viral copies in each cell population. (b) PC and NPC populations were lysed and gene expression was assessed by β-Gal assay. $^{**}p < 0.01$, $^{***}p < 0.001$ versus Ad.

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treatment groups. The β-Gal expression data obtained were in accord with the qPCR results. In the case of the non-pH-sensitive (DOTAP:Chol) enveloped Ad, no gene expression was observed in both PC and NPC fractions as expected (Fig. 4b), while gene expression was reinstated with DOPE:CHEMS in both hepatocytes and KCs, albeit at lower levels in comparison to naked Ad.

3.5. Blood circulation in mice after systemic administration

In order to test the effect of artificial envelopment on the blood circulation profile of viral particles, BALB/c mice were tail-vein injected with naked or enveloped Ad vectors. Blood was collected at different time points (5, 10 and 30 min) and the number of viral genomes in blood was determined via qPCR to show that Ad and DOTAP:Chol enveloped Ad were rapidly cleared from blood circulation within 5 min. Even though DOPE:CHEMS enveloped Ad also showed rapid clearance from blood immediately after injection, the number of viral genome copies in blood was significantly higher than naked or DOTAP:Chol enveloped Ad at all time points (Fig. 5).

3.6. Tissue-distribution of artificially enveloped Ad5

Next, we also evaluated the tissue-distribution of artificially enveloped Ad5 in different organs following systemic administration. 24 h post injection, the number of viral genomes in each tissue was quantified by qPCR (Fig. 6a) and luciferase activity in whole body (Fig. 6b) and dissected organs (Fig. 6c and Fig. S2) were analyzed by the IVIS Lumina imaging system. Naked Ad treated mice showed high viral uptake (Fig. 6a) and luciferase activity in the liver and spleen (Fig. 6b and c), whereas depletion of blood factors using warfarin decreased gene expression (Fig. 6b and c) but not viral uptake in the liver (Fig. 6a), as previously reported [27]. Envelopment of Ad in DOTAP:Chol lipid bilayers significantly decreased liver and spleen uptake, while significantly increasing lung accumulation of the vectors (Fig. 6a), however no gene expression was observed in any of the organs. DOPE:CHEMS enveloped Ad showed significantly lower viral uptake in liver (Fig. 6a) compared to naked Ad or Ad + W (with warfarin) treated groups. The qPCR results were also confirmed by IVIS imaging, since DOPE:CHEMS envelopment resulted in significantly lower Ad gene expression in the liver and spleen (Fig. 6b and c) compared to Ad or Ad + W treated animals.

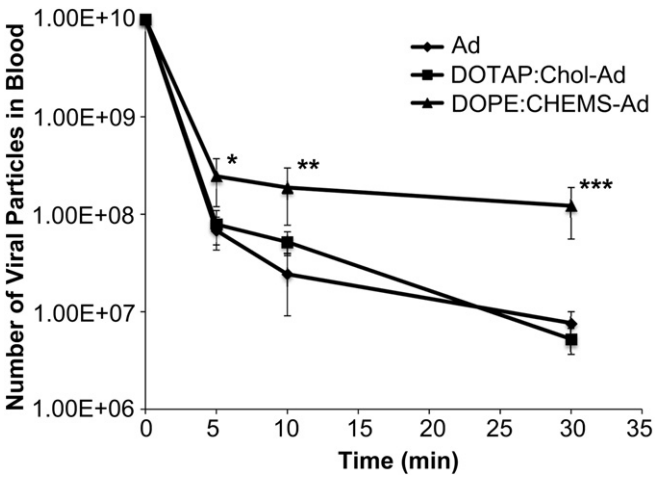

Fig. 5. Blood circulation profile of enveloped Ad vectors. BALB/c mice were injected with either Ad.luc (10^{10} p in 200 μl) or enveloped Ad.luc (10^{10} p in 200 μl) in DOTAP:Chol or DOPE:CHEMS lipid bilayers. Blood was collected 5, 10 and 30 min after systemic administration. Number of viral particles in blood was quantified after DNA isolation via qPCR. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus Ad and DOTAP:Chol-Ad.

3.7. Hepatotoxicity profiles of artificially enveloped Ad

The increase in alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels constitutes established markers of hepatic damage and pathophysiology. Ad hepatotoxicity is known to increase ALT and AST levels following systemic administration [28]. To evaluate such hepatotoxic effects of our enveloped Ad, mice were injected intravenously with naked or enveloped Ad in DOTAP:Chol or DOPE:CHEMS and after 5 days blood was collected and serum analyzed for ALT and AST enzyme levels. Administration of naked Ad resulted in up to 300–400 units/ml in serum for both enzymes, that was between 8 and 10 times higher compared to naive groups. Artificial envelopment of Ad5 in DOTAP:Chol or DOPE:CHEMS lipid bilayers greatly rescued hepatocytes and reduced the levels of both liver enzymes to 50 units/ml and 100 units/ml, respectively (Fig. 7). No increase in the liver enzymes was observed in animals injected with the empty liposomes. To support the serum analysis data, formalin-fixed and paraffin embedded liver sections were stained with H&E. Fig. 7b shows that Ad administration resulted in extensive hepatic damage and infiltration of leukocytes (as indicated by the black arrows). In agreement with liver enzyme results, mice administered with DOTAP:Chol enveloped Ad showed liver histology similar to naive or empty liposome treated groups. Ad enveloped in DOPE:CHEMS resulted in reduced hepatotoxicity with few signs of tissue damage and leukocyte infiltration compared to naked Ad (Fig. 7b). This data indicated that artificial Ad envelopment significantly improved hepatotoxicity by diverting viral particles away from liver.

3.8. Neutralizing antibody production following systemic administration of artificially enveloped Ad

All previous results evaluated the tissue-distribution and short term hepatotoxicity of the enveloped Ad. Gene expression and qPCR of the viral genome could not be performed at longer time points due to transient protein expression and the clearance of viral genomes from the liver [29]. For these reasons, a long-term humoral immunity study was designed by measuring the neutralizing antibody formation *in vivo*. For this experiment, mice were injected intravenously with naked Ad or enveloped Ad in DOTAP:Chol or DOPE:CHEMS and 28 days after Ad administration serum was collected and the levels of anti-Ad antibodies were determined by ELISA. Lipid bilayer envelopment of Ad significantly decreased the levels of antibodies formed against viral proteins compared to mice injected with naked Ad (Fig. 8a). Moreover, NAb titers in serum were determined by transfecting A549 cells with naked Ad in the presence of sera obtained from mice injected with the different vectors. Serum from naked Ad-injected mice showed up to 90% inhibition of *in vitro* gene expression, whereas artificial envelopment could maintain levels of gene expression up to 60% (Fig. 8b).

4. Discussion

Ad vectors have been shown to be effective in various gene therapy approaches, however toxicity-related issues are still the main obstacles limiting their clinical translation [30–32]. Natural liver tropism of Ad5 vectors in particular is often viewed as a critical impediment, and much effort has been invested in ways to re-direct these vectors from the liver [27,31,33,34]. Artificial envelopment of Ad by self-assembled lipid bilayers is a novel strategy we have proposed in an attempt to achieve both better pharmacokinetic control of non-enveloped viral vectors away from the liver and at the same time benefit from shielding of the viral capsids to alleviate toxicological responses.

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Taking into consideration the critical role of host blood cells and plasma proteins following systemic administration of Ad, we studied the interaction of artificially enveloped Ad in cationic, non-pH-sensitive (DOTAP:Chol) and anionic, pH-sensitive (DOPE:-CHEMS) lipid bilayers with several different blood components. Blood factors, especially blood factor X, play a critical role in increasing the affinity of Ad for the liver [8,15]. When Ad was enveloped with cationic lipids, very high levels of viral uptake in HepG2 cells was achieved, independent of any blood factor (Fig. 2a) which can be due to electrostatic interactions between the cationic envelope and negatively charged cell membrane. However, when an anionic lipid envelope was used, the levels of cellular uptake and gene transfection of those liver cells were similar to naked Ad vectors. Such data indicated the importance of negative surface charge in the interactions between viral vectors and blood factor proteins. Zwaal et al. previously reported that blood coagulation factors interacted with anionic lipids as Ca^{2+} could bridge the Gla domains of the proteins with the phospholipid headgroups of the anionic lipids [35].

The interaction between gene therapy vectors and red blood cells is also important. Blood cell binding of Ad5 vectors has been shown to dramatically alter their blood circulation and tissue-distribution, usually leading to reduce infectivity [26,36]. Artificial envelopment of Ad5 resulted in significantly higher binding to human RBC compared to naked virus (Fig. 3). The strongest interaction was seen with DOTAP:Chol enveloped Ad, possibly due to the positive surface charge of this envelope. The interaction with mouse RBC was similar to that with human cells for the enveloped Ad, even though mouse RBCs have been previously described to be both CAR and CR1 negative and show reduced affinity to naked Ad [26]. Overall, it can be concluded that artificial envelopment can lead to stronger electrostatic interactions with blood cells that may result in altered pharmacokinetic behavior after systemic administration.

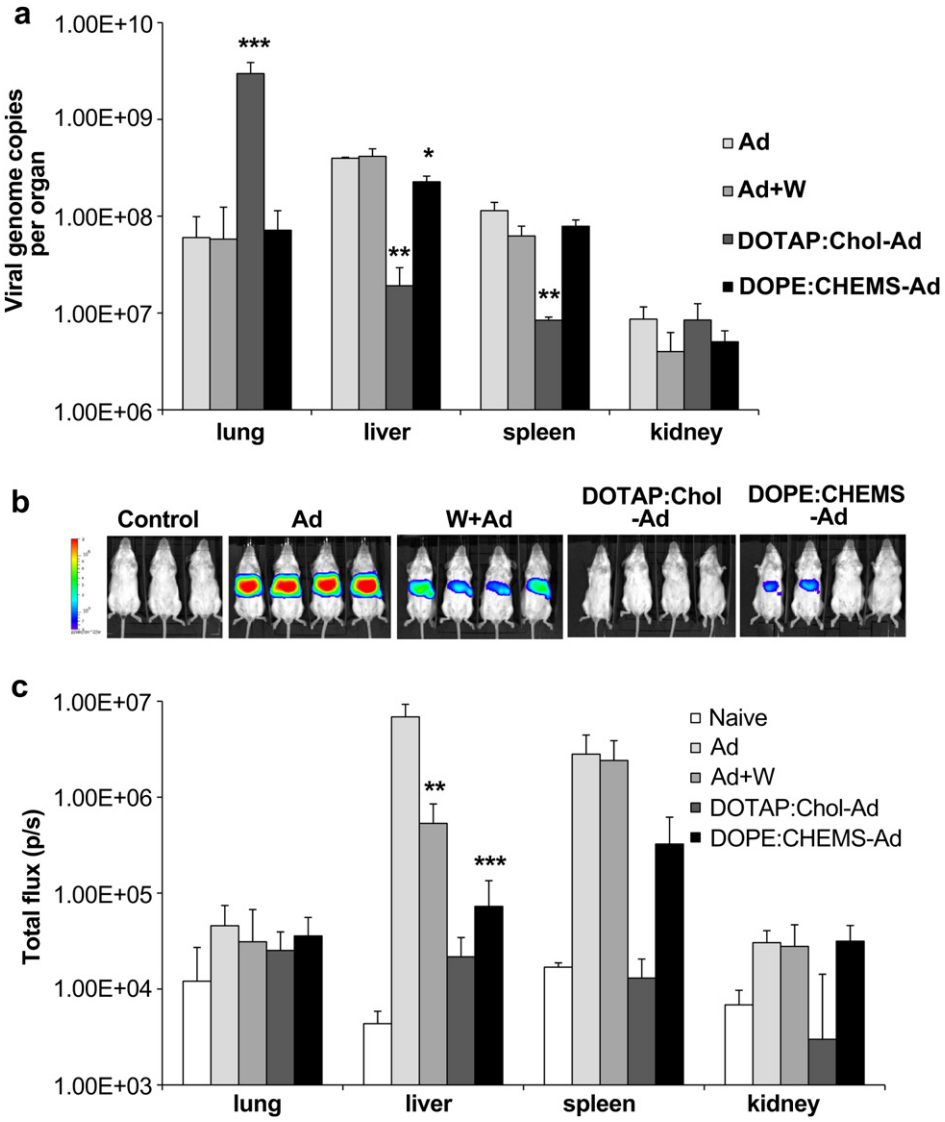

Fig. 6. The effect of artificial envelopment on Ad tissue-distribution. BALB/c mice were injected with either Ad.luc (10^{10} p in 200 μl) or enveloped Ad.luc (10^{10} p in 200 μl) in DOTAP:Chol or DOPE:CHEMS lipid bilayers. To inactivate vitamin K-dependent blood coagulation factors, mice were injected subcutaneously with 5 mg/kg warfarin (W) in peanut oil 3 and 1 days before Ad administration. (a) Levels of viral uptake in different organs were quantified after DNA isolation 24 h after virus administration; (b) Luciferase expression in whole-body imaged with the IVIS camera after i.p. luciferin injection; (c) The total flux (photons/s) from the dissected organs (lung, liver, spleen and kidney) of each treatment group were quantified. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus Ad.

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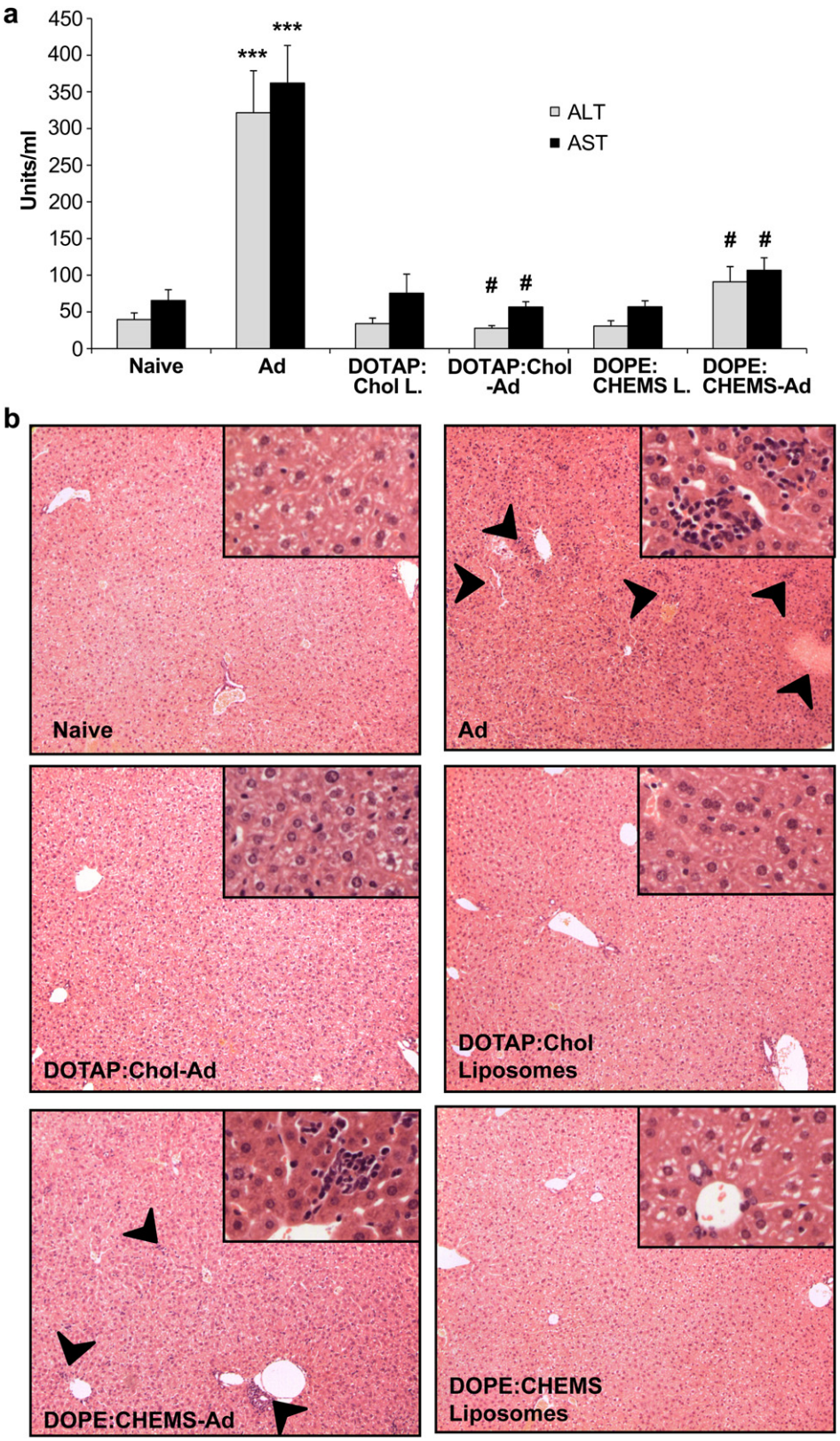

Fig. 7. Hepatotoxicity profiles following systemic administration of enveloped Ad. BALB/c mice were injected with either Ad.β-Gal (10^{11} p in 200 μl) or enveloped Ad.β-Gal (10^{11} p in 200 μl) in DOTAP:Chol or DOPE:CHEMS lipid bilayers. (a) Blood was collected at day 5 for the analysis of ALT and AST in serum. (b) Livers were harvested and stained with H&E. Sections were imaged under light microscopy (×4 and ×20 objectives) and arrows indicate damaged areas. $^{***}p < 0.001$ versus naive and $\#p < 0.001$ versus Ad.

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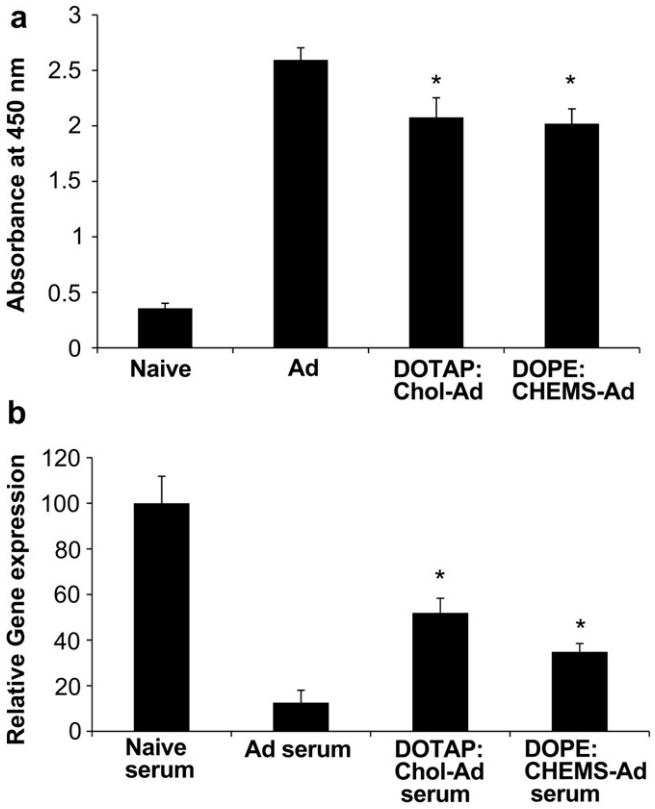

Fig. 8. The effect of artificial envelopment on the production of neutralizing antibodies against Ad5. BALB/c mice were injected with either 5×10^{10} p of Ad.β-Gal or enveloped Ad.β-Gal in DOTAP:Chol or DOPE:CHEMS lipid bilayers and blood was collected after 28 days. (a) Serum was analyzed for the presence of Ad specific antibodies by ELISA. The absorbance values of each sample were presented. (b) To assess the neutralizing antibody titers, serum at 1:100 dilution was incubated with Ad.β-Gal (1×10^9 p/ml) at 37 °C for 30 min and added to the wells pre-seeded with A549 cells. Data was expressed as the percentage of the β-Gal expression in cells treated with Ad in the presence of serum obtained from the naive group. $^*p < 0.05$ versus Ad.

We then hypothesized that the observed change in the affinity towards blood components would alter natural liver tropism, blood circulation and tissue-distribution for the case of the artificially enveloped Ad vectors [25]. For this reason, blood, liver, spleen, kidney and lung samples were analyzed for viral uptake and reporter gene activity. The most striking difference was obtained in the lung, where artificially enveloped Ad in DOTAP:Chol showed dramatically higher viral genome accumulation (Fig. 6a). Such higher lung uptake can be expected for positively charged particles based on previous experience from intravenous injections of cationic liposomes that rapidly lead to aggregation with the negatively charged serum proteins and entrapment in the pulmonary capillary bed (the diameters of these capillaries are relatively small and the blood flow is slower) [37]. Different blood and tissue-distribution profiles were obtained for artificially enveloped viruses using the negatively charged DOPE:CHEMS bilayers. Whole-body imaging of *Luc* expression at 24 h after intravenous administration showed an overall decrease in liver transduction, similar to that observed for naked Ad injected in warfarin-treated mice (Fig. 6). Therefore artificial envelopment can be proposed as a general strategy to inactivate the "redundant and synergistic" mechanisms [27] mediating the sequestration of blood-borne Ad viruses into the liver, and dependent on the physicochemical characteristics of the envelopes used, to re-direct or passively target specific tissues.

Artificial envelopment of Ad masks hexon, fiber and the rest of the viral capsid proteins that all contribute to liver accumulation in various ways, therefore we further investigated the mechanism of artificially enveloped Ad5 sequestration in the liver. Our qPCR and β-Gal assay results showed that naked Ad was uptaken almost equally by KCs and hepatocytes. When the virus was artificially enveloped in the cationic DOTAP:Chol bilayers, a dramatic decrease in the number of viral particles uptaken by hepatocytes was observed (Fig. 4a), while KCs captured similar number of these vectors compared to naked Ad5. The pH-sensitive, anionic artificial envelope DOPE:CHEMS also led to a significantly lower number of virions in hepatocytes and even less in KCs. This data indicated a surface charge-dependent mechanism that determined the liver cell type mainly responsible to capture the artificially enveloped vectors: cationic enveloped Ad escaping almost entirely from hepatocyte uptake and anionic envelopment leading to significant reduction in both hepatocyte and KC capture. Both types of envelopes led to significant reduction in hepatocyte sequestration and transgene expression that has been previously considered responsible for some of the most serious toxicological responses from Ad5 administrations [17]. More importantly, the reduced accumulation and gene expression in hepatocytes was considered responsible for the significant improvement in hepatotoxicity observed after tail-vein injection of the artificially enveloped Ad5 vectors (Fig. 7). Furthermore shielding the Ad capsid proteins led to a lower level of neutralizing antibody formation which lasted up to 28 days post Ad injection (Fig. 8).

Artificial envelopment of Ad5 was therefore shown able to re-direct viruses away from hepatocytes. Others have previously reported that overall lower liver transduction *in vivo* by genetic and chemical modification of Ad vectors can lead to improved toxicity [38]. Various different strategies have been described that can lead to reduced liver transduction. Doronin et al. demonstrated that chemical modification with 20-kDa PEG reduced liver gene expression by 19- to 90-fold followed by a decrease in hepatotoxicity [39]. Also when the Ad5 fiber shaft was exchanged for Ad3, liver transduction was suppressed by 5-fold compared to wild-type Ad vectors [40]. Moreover, following i.v. injection of Ad with shortened fiber shafts resulted in a 10-fold reduction of intracellular viral DNA and transgene expression in the liver [41]. Another study showed that natural liver tropism can be decreased by 700-fold by developing Ad vectors with neither CAR nor integrin binding [42]. In all of these studies as Ad binding properties were modified to decrease liver transduction, gene transfer efficacy also became deficient. In this context, artificial envelopment of Ad with DOPE:CHEMS bilayers could offer a promising alternative for gene therapy or vaccine development, since it demonstrated a significantly reduced hepatotoxicity with concomitant retention of its gene delivery efficiency *in vivo* as previously reported [20].

Finally, humoral immunity studies further elucidated the behavior of artificially enveloped Ad vectors *in vivo*. It is well established that NAb production has an enormous impact in determining the efficacy of Ad vectors *in vivo* [32,36]. In this study, we showed that artificial envelopment can significantly decrease NAb production *in vivo* (Fig. 8). It is well-known that hexon regions are the most available sites for antibody binding and cationic liposomes or polymers (such as polyethylene glycol) can cover these regions and evade the adaptive immune system in the presence of neutralizing antibodies [43–46]. Thus artificial lipid envelopes would similarly shield the virus capsid and may allow increased blood circulation as well as the possibility of repetitive administration protocols of the enveloped Ad vectors in patients with pre-existing immunity.

5. Conclusion

We have previously reported the effect of different lipid envelopes on the resulting biological activity of the artificially enveloped

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Ad vectors *in vitro*. In this study, we propose that artificial envelopment of Ad5 can improve the inherent hepatotoxicity of Ad5 vectors *in vivo*. Our results showed that engineering artificial lipid envelopes around the Ad5 capsid dramatically alter the virus tissue-distribution, the interactions leading to liver sequestration and can provide an improved hepatotoxic profile by maintaining the capacity for efficient gene transfer and expression.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2012.10.053.

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