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Efficient In Vitro Gene Delivery by Hybrid Biopolymer/Virus Nanobiovectors

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Abstract

Recombinant retroviruses provide highly efficient gene delivery and the potential for sustained gene expression, but suffer from significant disadvantages including low titer, expensive production, poor stability and limited flexibility for modification of tropism. In contrast, polymer-based vectors are more robust and allow cell- and tissue-specific delivery via conjugation of ligands, but are comparatively inefficient. The design of hybrid gene delivery agents comprising both virally derived and synthetic materials (nanobiovectors) represents a promising approach to development of safe and efficient gene therapy vectors. Non-infectious murine leukemia virus-like particles (M-VLP) were electrostatically complexed with chitosan (χ) to replace the function of the viral envelope protein. At optimal fabrication conditions and compositions, ranging from 6–9 μg chitosan/10^9 M-VLP at 10×10^9 M-VLP/ml to 40 μg chitosan /10^9 M-VLP at 2.5×10^9 M-VLP/ml, χ/M-VLP were ~300–350 nm diameter and exhibited efficient transfection similar to amphotropic MLV vectors. In addition, these nanobiovectors were non-cytotoxic and provided sustained transgene expression for at least three weeks in vitro. This combination of biocompatible synthetic agents with inactive viral particles to form a highly efficient hybrid vector is a significant extension in the development of novel gene delivery platforms.

Keywords

Chitosan; Gene therapy; Gene delivery; Retrovirus; Hybrid vectors

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INTRODUCTION

Human gene therapy relies upon the introduction of exogenous genetic material to diseased cells or tissue for the replacement of damaged and mutated genes, introduction of new genes, or supplementation of deficient gene expression [1]. Thus, gene therapy has the potential to treat inherited diseases including cystic fibrosis [2, 3], muscular dystrophy [3–5] and severe combined immunodeficiency (SCID) [3], as well as acquired diseases such as cancers [6], cardiovascular diseases and viral infections [7, 8]. This technology has yet to deliver on its promise, however, due in large part to the lack of safe and efficient means of delivering therapeutic genes [9].

Delivery of nucleic acids to mammalian cells can be achieved via recombinant viruses or synthetic materials including cationic lipids and polymers [10]. Recombinant retroviruses, in particular, are common vectors in the laboratory and in clinical trials, and are capable of integrating their genetic cargo with the target cell genome with remarkable efficiency. However, the lack of cell-specific targeting, immunogenicity, risk of insertional mutagenesis followed by oncogene activation, as well as poor stability and expensive and low-titer production of retroviruses have been critical limitations [11]. In order to move retroviral vectors toward clinical application, safety has been enhanced by introduction of features such as chromatin insulators, conditional and cell type-specific gene expression, targeted transduction, and site-specific integration [12].

In the last two decades, numerous synthetic materials have been developed as alternatives for delivering genetic material. Two prominent classes of these synthetic materials are polycations, such as poly-L-lysine (PLL) [13], polyethylenimine (PEI) [14] and polyamidoamine (PAMAM) [15], and various cationic lipids [16]. These materials electrostatically bind and condense nucleic acids forming nanometer-scale particles [10, 17]. Such synthetic vectors typically offer safety, flexibility, functionality for attachment of cell-specific targeting ligands, and more facile manufacturing, but are comparatively inefficient and typically provide short duration of gene expression [18].

Developments in nanobiotechnology provide the means to generate hybrid vectors, comprising viral and synthetic components, for efficient and safe gene delivery. For example, murine leukemia virus-like particles (M-VLP) are similar in composition, size and morphology to murine leukemia virus (MLV) but lack the envelope protein (Env), which is an important cause of many of the limitations of retroviral vectors including their instability, which leads to relatively low titers and difficult purification, and difficulty in cell-specific targeting [19–21]. Env is responsible for binding receptors on target cells and initiating infection, making M-VLP non-infectious [22]. Gene delivery is possible, however, if a synthetic agent associated with M-VLP provides entry of the viral particle into the cell and release of the viral cargo into the cytosol. Once the M-VLP are internalized, native viral mechanisms lead to transport of viral genes to the cell nucleus and highly efficient transgene expression.

We have reported transfection employing nanobiovectors comprising M-VLP with the aide of cationic polymers [21, 23] and liposomes [24]. Here we have expanded upon the polymer

parameter space using a more biocompatible polymer, chitosan (χ), to form nanobiovectors with improved physicochemical properties and reduced cytotoxicity. This paper demonstrates assembly of the chitosan/virus-like particle (χ/M-VLP) nanobiovectors, highly efficient gene delivery and sustained transgene expression in a human cell line in vitro.

MATERIALS AND METHODS

Cell lines

Human embryonic kidney cells, HEK293, were purchased from the American Type Culture Collection. The MLV producer cell line, GP293Luc, expressing the MLV viral *gag-pro-pol* genes and a viral packaging sequence encoding neomycin resistance and luciferase reporter genes was purchased from Clontech. Both cell lines were grown in DMEM supplemented with 10% FBS (Gemini Bio-Products) and cultured at 37 °C in 5% CO_2. Dulbecco's Modified Eagle's Medium (DMEM) and phosphate-buffered saline (PBS) were produced by the Cell Culture Media Facility, School of Chemical Sciences, University of Illinois.

M-VLP production and quantification

M-VLP were produced in GP293Luc cells seeded at 2×10^6 cells in a 10 cm dish. The cells were cultured for four days before the M-VLP containing supernatant was collected and filtered through a 0.45 μm surfactant-free cellulose acetate syringe filter. M-VLP supernatant was either used immediately or stored at 4 °C for short-term storage (<1 month) or −80 °C for long term storage.

The concentration of M-VLPs in the supernatant was measured using quantitative reverse-transcriptase PCR [23]. RNA standards were obtained from the Clontech q-PCR Retroviral Quantification Kit and stored at −80 °C before further use. Viral RNA was extracted using the QIAGEN Viral RNA Extraction kit and stored in a 60 μl eluate at −80 °C before further use. Standards and viral RNA samples were prepared for reverse transcription using Taqman reverse transcription reagents (Applied Biosystems, Carlsbad, CA). Twenty μl samples were mixed in 200 μl PCR tubes with 250 nM sequence-specific primers. Thermal cycling was carried out on a Peltier Thermocycler (PTC-100, MJ Research).

Real-time PCR of the cDNA standards and samples was carried out in triplicate in 10 μl/well samples on a 384-well plate in a Taqman 7900 Real-Time PCR Machine (Applied Biosystems) and analyzed using SDS software (Applied Biosystems). The final reaction mixture ratio of the components was 5:1:1:3 (2× SYBRGreen real-time PCR reagent:forward primer:reverse primer:cDNA volume). The final concentration of the sample RNA was calculated using the calibration curve obtained via the cDNA standards. Each viral particle has two RNA copies, which enabled us to calculate the total number of M-VLPs in a given volume of supernatant. Two RNA extracts were collected for each M-VLP sample and quantified using three dilutions of each cDNA sample.

Assembly of polymer/M-VLP hybrid vectors

PEI (750 kDa, Sigma-Aldrich, 1 mg/ml in ultrapure water), PLL (150–300 kDa, Sigma-Aldrich, 1 mg/ml in ultrapure water) or chitosan (190–310 kDa, Sigma-Aldrich, 1 mg/ml

dissolved overnight at 55 °C in 0.6 % acetic acid and filtered through a 0.22 μm surfactant-free cellulose acetate syringe filter) was added drop-wise to the required volume of M-VLP supernatant while vortexing to achieve the desired polymer:M-VLP ratio. The hybrid vectors were then incubated at 4 °C for 4 h.

Transfections

HEK293 cells were seeded 18–24 h prior to transfection at 4×10^5 cells/well in 12-well plates. Growth media containing serum was replaced with serum-free DMEM prior to drop-wise addition of vectors and replaced again with normal growth media 4 h post-transfection. For serum studies, the transfection media contained 0–50% fetal bovine serum. For uptake inhibition studies, growth media was replaced with serum-free DMEM along with predetermined concentrations of drugs 1 h prior to addition of the hybrid vectors.

Luciferase expression assay

Luciferase expression was quantified 48 h post-transfection using the Promega luciferase assay system following the manufacturer's protocol. Luciferase activity was measured in relative light units (RLU) using a Lumat LB 9507 luminometer (Berthold, GmbH, Germany). Lysate protein concentration was then determined by BCA assay (Thermo Scientific) to standardize expression values.

Cellular uptake

Twenty ml of M-VLP supernatant was centrifuged at 60,000g for 2 h at 4 °C and the pellet was resuspended in 50 mM HEPES with 145 mM NaCl to obtain a M-VLP density of 10^{10} M-VLPs/ml. 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt (DiD) (Invitrogen, 1 mM in DMSO, Ex = 644 nm, Em = 665 nm) was added to the resuspended M-VLP to give a final concentration of 2 μM DiD. The mixture was incubated for 1 h at room temperature. Labeled M-VLPs were separated from the free dye through a PD-10 gel filtration column (GE Healthcare). HEK293 cells were seeded in 12-well plates at 4×10^5 cells/well 18–24 h prior to transfection. Hybrid vectors composed of polymer and DiD-labeled M-VLP were assembled as described above and used immediately for transfection. The target cells were washed with PBS containing 0.001% SDS 2 h post-transfection to remove surface-bound, uninternalized vectors followed by a PBS wash. Cells were then trypsinized followed by neutralization with 50 μl of FBS, collected, and analyzed by flow cytometry using a Becton Dickinson LSR II Flow Cytometer with a 633 nm laser.

Negative stain transmission electron microscopy

M-VLP and χ/M-VLP vectors were fixed by adding 1 ml of Karnovsky's fixative to 3 ml of sample. The sample was then centrifuged at 60,000g for 2 h at 4 °C. The pellet was resuspended in a small volume of 5% glucose and diluted with the fixative. A 40 μl drop of the sample was placed on a parafilm sheet, and a copper grid was placed face down on the sample drop and incubated for 30 minutes. Excess sample was wicked off the grid using filter paper. The grid was then placed grid-face down on a drop of 7% uranyl acetate stain

for 1 min. Excess stain was again wicked off using filter paper and the grid was dried for 15 min before viewing in a Hitachi H600 Electron Microscope.

Size and zeta potential measurements

Vectors were prepared as described above and diluted 10-fold in 5% glucose. The sizes of the resulting complexes and the constituents were measured using dynamic light scattering with a Brookhaven 90Plus Particle Size Analyzer (Brookhaven Instruments). Light scattering was measured 10 times at 10-second intervals for each sample.

Cytotoxicity

The toxicity of polymers and χ/M-VLP was determined using the Cell Titer-Blue™ Cell Viability Assay (Promega). Target cells were seeded in a 96-well plate at 5×10^4 cells/well 18–24 h prior to addition of polymer or χ/M-VLP. The polymers (0 – 300 μg/ml) or χ/M-VLP were added to the cells in serum-free medium and left for 4 h before the medium was replaced. Fifteen μl of CellTiter-Blue™ reagent was added to each well 24 h post-addition of polymers or χ/M-VLP. The cells were incubated for 4 h at 37 °C before adding 100 μl per well of stop reagent following which fluorescence (λ = 570 nm, 650 nm) was measured in a SpectraMax 340PC 96-well plate reader.

Confocal fluorescence microscopy

HEK-293 cells were grown on glass coverslips and transfected with DiD-labeled χ/M-VLP (pH 3 chitosan) as described above. After 8 h, the cells were fixed with 4% formalin, permeabilized in 0.1% triton X-100 for 5 min on ice, and blocked with buffer A (1% w/v bovine serum albumin in PBS) for 30 min. The cells were labeled with primary antibody (LAMP1 (Abcam), 1:700 dilution; caveolin and clathrin (Santa Cruz Biotechnology), 1:1000 dilution) at 4 °C overnight, followed by Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody (2 μg/mL) in buffer A at 22 °C for 1 h at room temperature. After being washed three times with PBS and one time with distilled water, the coverslips were mounted in ProLong Gold Antifade reagent with DAPI (Life Technologies) and imaged on a Zeiss Axiovert 200M confocal microscope equipped with an Apotome illuminator at 100× magnification.

Stable transgene expression assay

Hybrid vectors were assembled as described above. HEK293 cells were seeded 18–24 h prior to transfection at 8×10^5 cells/well in 6-well plates. Growth media containing serum was replaced with serum-free DMEM prior to drop-wise addition of vectors and replaced again with normal growth media 4 h post-transfection. After 48 h, the cells in three wells were lysed and quantified for luciferase expression as described above. The cells in the other three wells were split into another 6-well plate at a 1:8 split ratio. This process was repeated every 3–4 days depending on the confluency of the cells for up to at least three weeks.

Statistical Analysis

All statistical analyses mentioned in this study were performed using the Student’s t-test.

RESULTS

Assembly and physicochemical characterization of χ/M-VLP nanobiovectors

Chitosan/M-VLP complexes were assembled in aqueous suspension through electrostatic interaction of varying amounts of the polymer with the M-VLP lipid membrane. The sizes of the resulting χ/M-VLP nanobiovectors were measured via dynamic light scattering (Table 1). M-VLP alone exhibited diameters of approximately 95 ± 3.5 nm, which is typical of retroviruses. The sizes of the nanobiovectors were >1000 nm. For measurement of vector zeta potentials (Figure S1), nanobiovectors were formed in 5% glucose solution. (DMEM contains a high concentration of salts and resulted in noisy measurements and extensive blackening of electrodes.) M-VLP exhibited a zeta potential of −6.8 ± 1.2 mV. Addition of chitosan at 2 μg/10^9 M-VLP resulted in positively charged nanobiovectors (8.5 ± 0.5 mV) and zeta potential continuously increased with further increase in χ:M-VLP ratio. In contrast to nanobiovectors formed in DMEM, χ/M-VLP formed in 5% glucose were smaller in size (Figure S1).

We also visualized the morphology of nanobiovectors via TEM (Figure 1). The structure of M-VLP (Figure 1A) is similar to that of the enveloped retrovirus [25] allowing them to be readily identified in the complexes. χ/M-VLP complexes appeared as aggregates of multiple M-VLP held together by a darkly stained material, presumably corresponding to a domain of chitosan. Such morphology seems to be a common trait of polymer/M-VLP nanobiovectors and was observed in PEI/M-VLP and PLL/M-VLP [21]. The size of the vectors ranged from 1200–1400 nm, consistent with the sizes obtained from DLS.

χ/M-VLP-mediated transfection

As with non-viral vectors, the polymer:M-VLP ratio is an important factor controlling transfection efficiency. Chitosan (in 0.6% w/v acetic acid, pH 5), PEI or PLL was added to M-VLP suspension in different ratios of polymer/10^9 M-VLPs to form nanobiovectors, which were then used to transfect HEK293 cells (Figure 2A). Chitosan provided higher transgene expression compared to PEI and PLL at all polymer:M-VLP ratios. At the optimal transfection stoichiometries, 6–9 μg chitosan/10^9 M-VLP, 3–5 μg PEI/10^9 M-VLP and 8 μg PLL/10^9 M-VLP, χ/M-VLP mediated a three-fold improvement over PEI/M-VLP ($p < 0.002$) and a 13-fold improvement over PLL/M-VLP ($p < 0.0005$).

We also characterized cellular internalization of fluorescently labeled nanobiovectors assembled at various polymer:M-VLP ratios (Figure 2B). Uptake for all three types of nanobiovector was greatest at the lowest polymer:M-VLP ratio (2 μg/10^9 M-VLP) and decreased with increasing polymer content. A notable difference between χ/M-VLP compared to PEI/M-VLP and PLL/M-VLP was the correlation between uptake and transfection efficiency. Transfection efficiency of χ/M-VLP decreased ten-fold between 8 μg/10^9 M-VLP and 12 μg/10^9 M-VLP (Figure 2A), but the vector internalization remained fairly constant. In contrast, transfection efficiency of PEI/M-VLP and PLL/M-VLP remained relatively constant at polymer:M-VLP ratios of 4–12 μg polymer/10^9 M-VLP, but cellular uptake decreased 2- to 4-fold. Additionally, PEI/M-VLP exhibited greater uptake than either χ/M-VLP or PLL/M-VLP at low polymer:M-VLP ratios, which is not surprising

given the higher cationic charge density of PEI. Transfection efficiency of PEI/M-VLP, however, was intermediate between the other two nanobiovectors.

Previously, we demonstrated that the density of the M-VLP suspension plays an important role in determining the optimal stoichiometry for PEI/M-VLP vectors [23]. We found a similar trend for χ/M-VLP (Figure S3). Various amounts of chitosan were complexed with M-VLP suspension at three different densities. The optimal χ:M-VLP ratios were 8, 17 and 40 μg/10^9 M-VLP at M-VLP densities of 10 × 10^9 M-VLP/ml, 5 × 10^9 M-VLP/ml and 2.5 × 10^9 M-VLP/ ml, respectively. Furthermore, the transfection activity of vectors formed with M-VLP at 10 × 10^9 M-VLP/mL was approximately double that for the nanobiovectors formed with less dense suspensions.

An important advantage of recombinant retroviral vectors over other vectors such as adenoviruses or non-viral vectors is their ability to provide stable transgene expression. Murine leukemia virus mediates stable transgene expression due to integration of viral genes with host DNA. M-VLP contain the viral reverse transcriptase and integrase and are expected to be capable of genomic integration. HEK293 cells were transfected with a conventional amphotropic pseudo-typed murine leukemia virus (MLV-A) or χ/M-VLP and cultured as normal for 21 days. Luciferase activity was maintained at constant levels during this three-week period, despite sub-culturing the cells 10 times (Figure 2C). Upon transfection of HEK293 with non-viral PEI/pDNA polyplexes, no luciferase was detected after 5–7 days (not shown).

Cytotoxicity

Chitosan is biodegradable and hence potentially more desirable as a component of gene delivery vectors compared to PEI or PLL [26, 27]. We investigated the cytotoxicity of chitosan, PEI and PLL using an assay of cellular metabolic activity in HEK293 cells in the presence of the polymers (Figure 3A). Chitosan exhibited negligible cytotoxicity even at very high concentrations (up to 300 μg/ml). In contrast, PEI and PLL exhibited notably deleterious effects above 20 μg/ml. The polymer concentration at which metabolic activity was reduced to 50% of the control was between 40–50 μg/ml for PEI and 80–90 μg/ml for PLL. In addition, χ/M-VLP vectors exhibited negligible cytotoxicity at all χ/M-VLP ratios (Figure 3B).

These results suggest that transfections with χ/M-VLP vectors may be further improved by increasing nanobiovector dose, which is not feasible with PEI/M-VLP and PLL/M-VLP vectors due to the toxicity of these polymers. We tested this hypothesis by transfecting HEK293 cells with χ/M-VLP vectors at doses of 1–4 × 10^9 M-VLP/well (Figure 3C). In order to maintain the same χ:M-VLP ratio, chitosan amounts were increased as well. At optimal vector composition (7 μg/10^9 M-VLP), transgene expression increased nearly proportionally to M-VLP dose. At sub-optimal composition of 4 μg/10^9 M-VLP, transfection increased 10-fold, while at 10 and 13 μg/10^9 M-VLP transfection decreased with increasing dose.

Alternative assembly of χ/M-VLP nanobiovectors

The initial χ/M-VLP nanobiovectors described above provide remarkably efficient gene delivery, low cytotoxicity and sustained transgene expression. However, the complexes were large (>1000 nm diameter; Table 1). In order to improve vector morphology and reduce sizes, polymer-M-VLP interactions were tuned by varying complexation parameters such as the chitosan solution pH and chitosan concentration. Remarkably, the transfection efficiency improved 60- and 120-fold when nanobiovectors were formed with chitosan solutions at pH 4 and 3, respectively, compared to pH 5 (Figure 4). Formation of complexes with pH 3 chitosan resulted in transfection comparable to intact MLV-A. Decreasing chitosan solution pH also led to decreases in the polymer requirements for optimal transfections (4 μg/10^9 M-VLPs at pH 3 and 6 μg/10^9 M-VLPs at pH 4, compared to 7–8 μg/10^9 M-VLPs at pH 5). χ/M-VLP assembled using pH 6 chitosan failed to transfect HEK293 cells. Importantly, the average size of the nanobiovectors decreased from >1000 nm to ~300–350 nm with the use of lower pH chitosan solutions (Table 1 and Figure 1C). Finally, χ/M-VLP assembled with pH 3 chitosan exhibited no cytotoxicity (Figure 3B).

Investigation of χ/M-VLP transfection mechanisms

Understanding the mechanisms of nanobiovector delivery is important for design of new vectors. In particular, the pathway of cellular uptake has been shown to be critical for gene delivery by both non-viral vectors [28, 29] and recombinant retrovirus [30]. Thus, nanobiovector efficiency was investigated in the presence of pharmacological inhibitors of caveolin-dependent endocytosis and endocytic vesicle acidification (Figure 5A). Genistein prevents the production of caveolae through inhibition of tyrosine phosphorylation [31]. Bafilomycin A1 (BafA1) inhibits ATPases preventing endosome acidification [32]. MLV-A infection is known to proceed via caveolae [33], which is validated by the reduction in infection by MLV-A in HEK293 cells in the presence of genistein. Genistein also inhibited χ/M-VLP transfection. Additionally, BafA1 inhibited transfections by χ/M-VLP but did not affect MLV-A infection. This suggests that χ/M-VLP is capable of gene delivery upon internalization via an acidified pathway such as clathrin-mediated endocytosis or macropinocytosis, as well as caveolin-dependent endocytosis.

To further investigate the effect of cellular uptake and endocytic localization on hybrid vector delivery, HEK293 cells were transfected with fluorescently labeled MLV-A or χ/M-VLP, caveolae, clathrin-coated vesicles and lysosomes were immunolabeled, and the cells were observed by confocal fluorescence microscopy 8 h post-transfection (Figure 5B). Although MLV-A infection is known to proceed via caveolae, MLV-A colocalized with caveolin, clathrin and lysosomes, suggesting that internalization occurs through both caveolin- and clathrin-dependent pathways. MLV-A in clathrin-coated vesicles and lysosomes are not involved in infection and are presumably degraded. Similarly, χ/M-VLP were observed in caveolae, clathrincoated vesicles and lysosomes. In contrast to MLV-A, inhibition of transgene expression by both genistein and BafA1 suggests that χ/M-VLP in all three compartments may lead to effective transfection.

DISCUSSION

Delivery systems for human gene therapy should be safe (i.e., low toxicity, non-pathogenic, non-immunogenic), provide highly efficient gene delivery, and exhibit appropriate cell or tissue specificity, for example through passive or active targeting and/or via cell-specific transcriptional regulation. Neither recombinant viruses nor non-viral vectors currently provide all of these characteristics. A top-down approach, combining viral and synthetic components, is being developed to produce hybrid nanobiovectors that exhibit advantages of both components. In particular, the synthetic components may provide biocompatibility, immunoprotection and cell-specific targeting, while the viral component provides efficient intracellular processing including the potential for transfection of non-dividing cells and sustained gene expression [34, 35]. Facilitated by the convergence of nanotechnology, colloidal self-assembly and retrovirology, this approach is capable of producing highly efficient nanobiovectors.

χ/M-VLP formed with pH 5 chitosan solution comprised aggregates of multiple M-VLP >1000 nm in diameter (Figure 1B). Such large structures may be formed because of the low negative surface charge on M-VLP. Other studies have reported smaller polymer/virus vectors through complexation of polycations with dilute solutions of viral particles (< 10×10^6 viral particles/ml) [36], and significant transfection efficiency was achieved despite the low viral particle numbers used. However, here inactive M-VLP, rather than complete retroviruses, were complexed with polymers.

Uptake of χ/M-VLP did not correlate directly with the transfection efficiency of these complexes (Figure 2A and B), suggesting that trafficking within the cell largely dictates the overall efficiency of these vectors. Also, transgene expression increased more than three-fold after 48 h compared to 24 h (Figure S2). Normal growth of HEK293 cells, given a cell doubling time of ~24 hours, should lead to at most two-fold increase in total protein content. This further suggests that relatively slow intracellular processing, occurring during the intervening 24 h period, is an important barrier for χ/M-VLP. We observed a similar trend with PEI/M-VLP wherein transgene expression increased 10-fold between 24 and 48 h [20].

The difference in efficiency of transfection and uptake with the choice of polymer (PEI, PLL or chitosan) suggests that the properties of the synthetic component are crucial for optimal delivery. Thus, chitosan may provide more efficient escape from endocytic vesicles or release of M-VLP in the cytosol compared to the other polymers. It has been reported that the choice of the envelope protein affects the pathway of the retrovirus towards the nucleus [30]. It is possible that the choice of polymer might also control the trafficking pathway for the M-VLP. Higher uptake (Figure 2B) but poorer transfection (Figure 2A) of PEI/M-VLP compared to χ/M-VLP suggests that the pathway of choice for χ/M-VLP is better suited for the nanobiovectors compared to PEI/M-VLP. The mechanism by which chitosan effects more efficient delivery is the subject of ongoing research.

The effects of chitosan solution pH on vector morphology and transfection are somewhat surprising as there was no significant change in the pH of the nanobiovector suspension after complexation when using chitosan solution at different pH values, with the resulting pH

between 7 and 7.5 at all vector stoichiometries. Deprotonation of chitosan upon entering the buffered M-VLP suspension is expected to be fast compared to the rate of chitosan/M-VLP interaction. Thus, the protonation state of chitosan, when a polymer chain encounters a M-VLP particle, should be the same regardless of the chitosan solution pH. We suggest, however, that slower changes in chitosan conformation and/or chitosan-chitosan interactions may contribute to the difference in nanobiovector size and gene delivery activity.

In summary, we have explored the combination of chitosan and M-VLP to form efficient hybrid gene delivery vectors. This study marks an important step towards the development of novel gene delivery vectors that alleviate the disadvantages of using either viral or non-viral vectors. χ/M-VLP are more effective compared to earlier hybrid vector designs [21, 23] with activity similar to intact recombinant retrovirus, and are non-cytotoxic, facilitating extension to clinical application. Importantly, controlling the complexation conditions reduced χ/M-VLP size. Nevertheless, cellular uptake of these vectors is size dependent and, given their relatively large sizes, remains a significant challenge to this class of hybrid vectors. In order to move these vectors toward in vivo applications, future studies will explore further reduction of complex size, attachment of targeting ligands and PEGylation, as well as studying mechanisms of intracellular navigation. Together with recent and ongoing advances in retroviral vector safety, particularly reduction of insertional mutagenesis, such hybrid nanobiovectors may provide the means to further advance retroviral vectors for clinical application.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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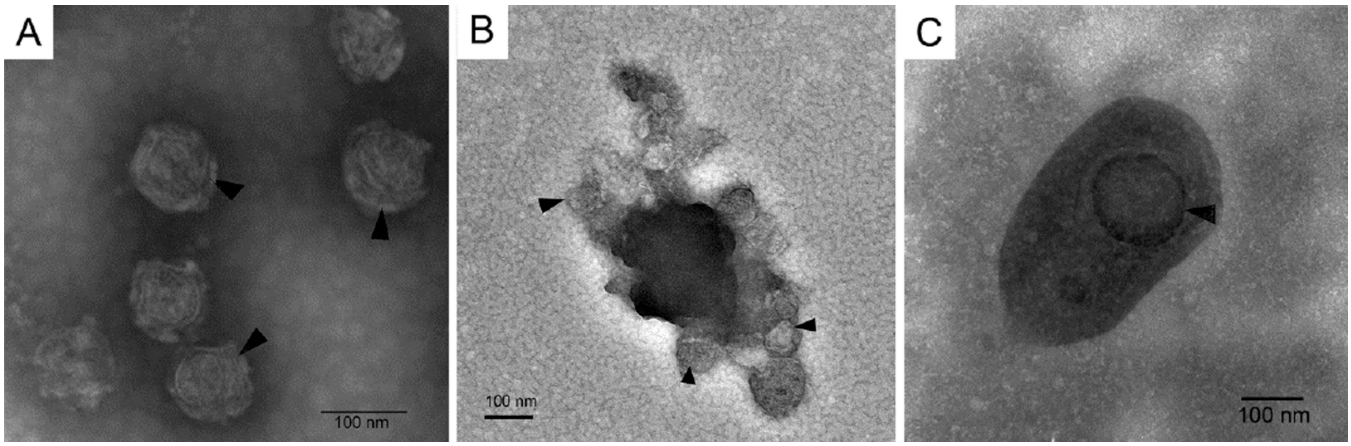

Figure 1.

Negative-stain transmission electron micrographs of (A) M-VLP alone (scale bar = 100 nm) and χ/M-VLP formed at a stoichiometry of 10 μg chitosan/10^9 M-VLPs using 1 mg/ml chitosan in 0.6% acetic acid at (B) pH 5 (scale bar = 200 nm) and (C) at pH 3 (scale bar = 100 nm).

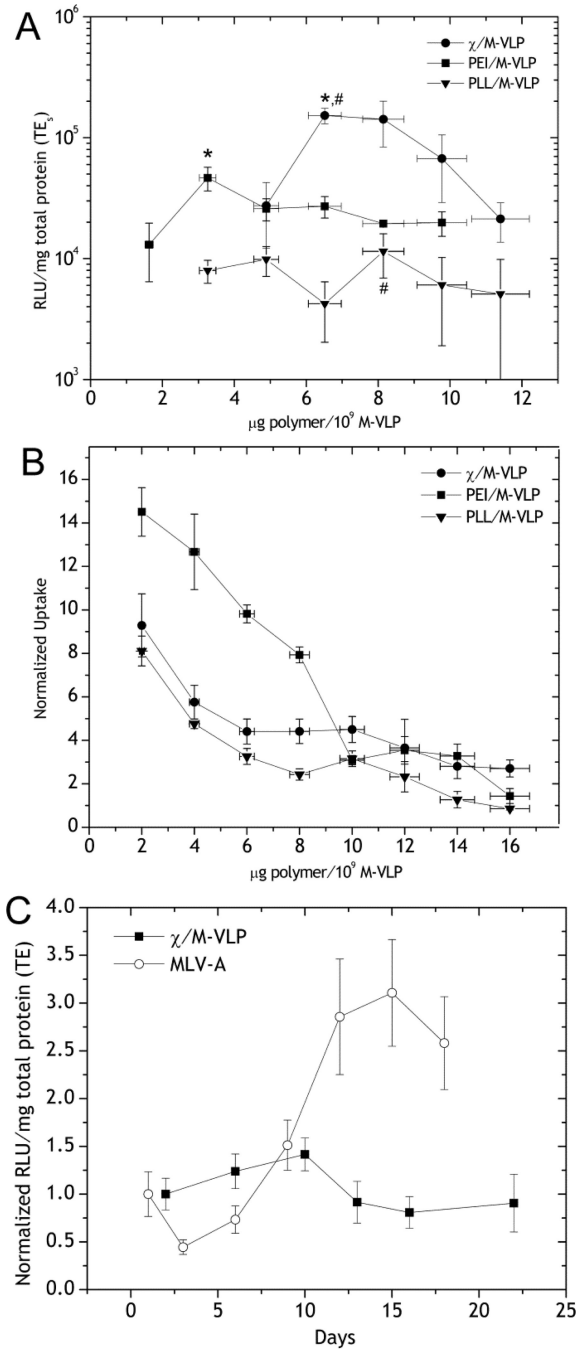

Figure 2.

(A) Transfection efficiency and (B) cellular uptake in HEK293 cells of χ/M-VLP, PEI/M-VLP and PLL/M-VLP with 1×10^9 M-VLP/well of a 12-well tissue culture plate using M-VLP density of 10×10^9 M-VLPs/ml. Normalized Uptake = (mean fluorescence of treated sample - mean fluorescence of untreated samples)/mean fluorescence of untreated samples. Error bars indicate standard deviation (n=3). (C) Expression of luciferase reporter gene in HEK293 cells by χ/M-VLP in comparison to MLV-A over three weeks. Cells were trypsinized, split and re-seeded ~10 times during this period. (*, $p < 0.002$; #, $p < 0.0005$).

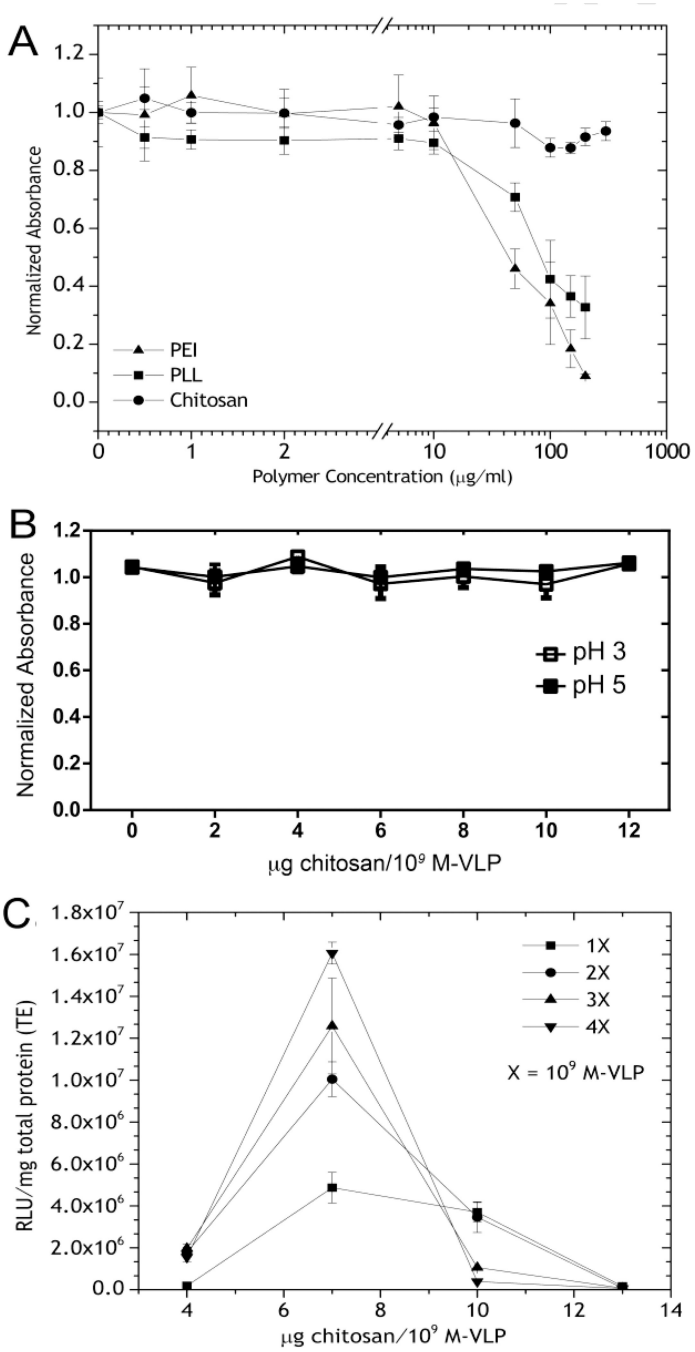

Figure 3.

(A) Cytotoxicity of chitosan, PEI and PLL, and (B) χ/M-VLP on HEK293 cells. Metabolic activity measured after 4 h in the presence of polymer or vector was normalized to control cells with no polymer. (C) Transfection of HEK293 cells at vector doses of 1–4 × 10^9 M-VLP/well in a 12-well plate. (n = 3, error bars represent standard deviation).

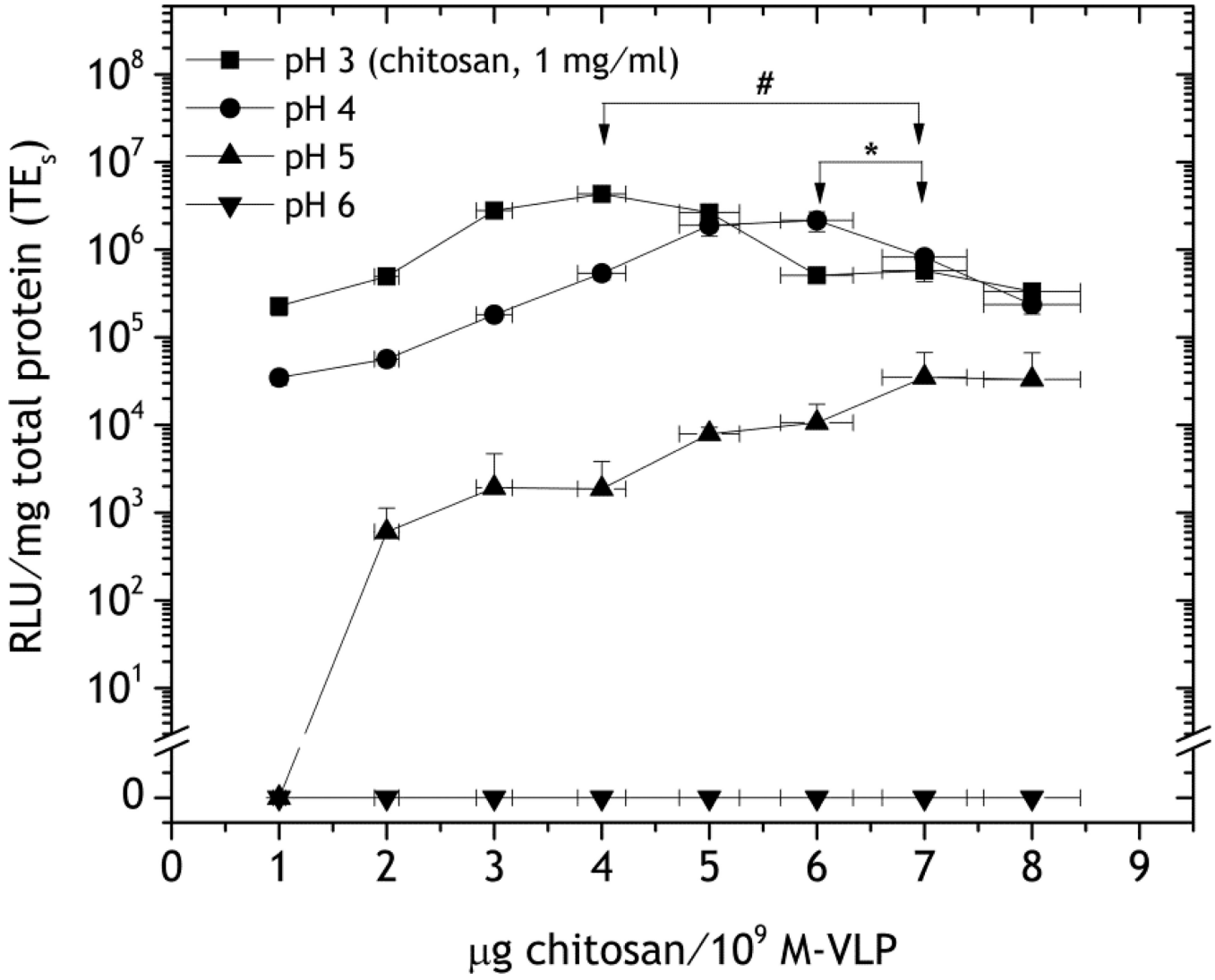

Figure 4.

Transfection of HEK293 cells with χ/M-VLP formed with chitosan solutions at pH 3, 4, 5 and 6. (#, $p<0.001$; *, $p<0.003$; n = 3, error bars represent standard deviation).

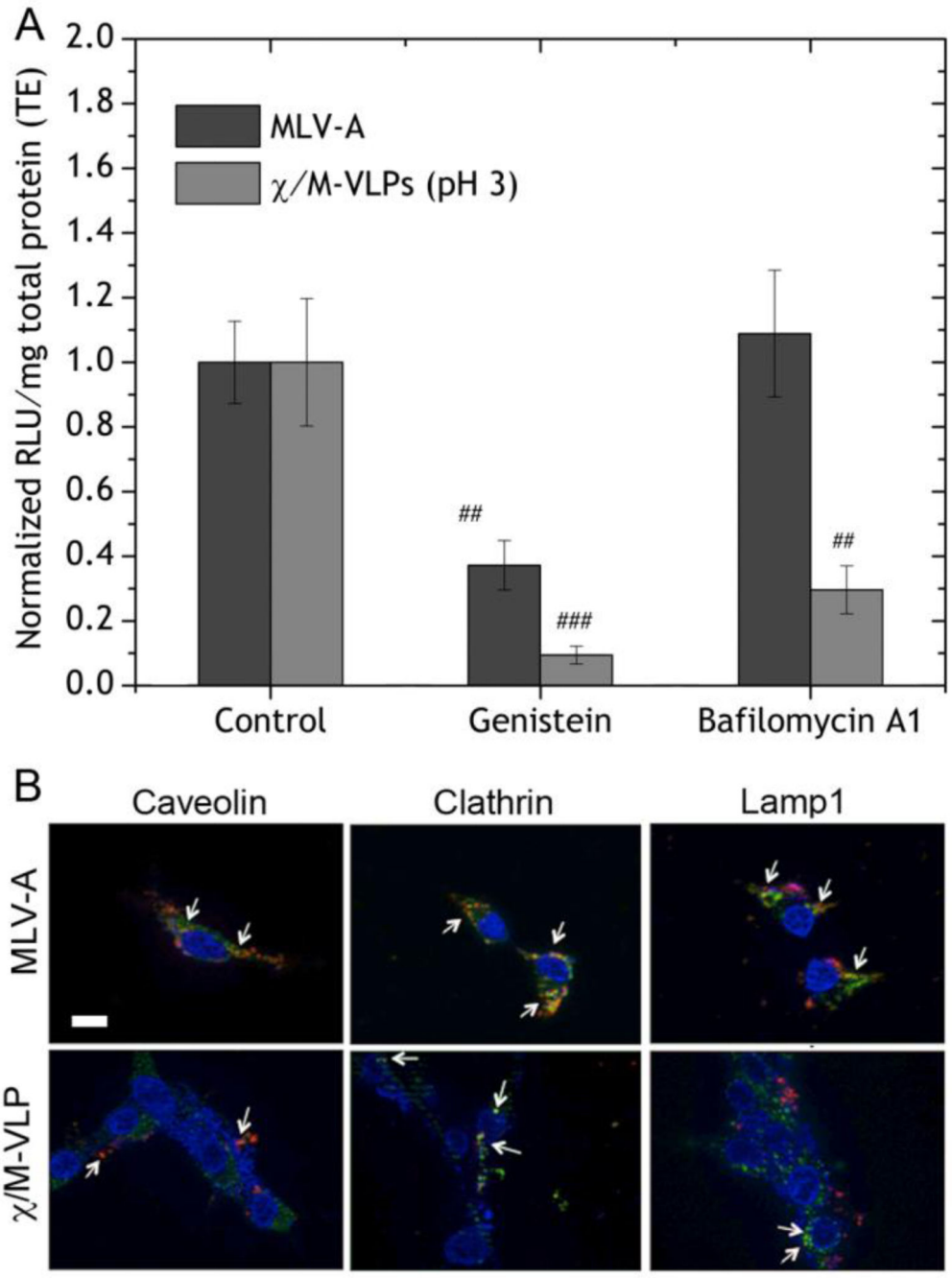

Figure 5.

(A) Transfection of HEK293 cells with χ/M-VLP formed with pH 3 chitosan solutions at 5 μg/10^9 M-VLPs in the presence of 20 μg/mL genistein and 5 nM BafA1. Luciferase expression (RLU) per mass total protein was normalized to that in the absence of drug. (##, $p < 0.02$; ###, $p < 0.002$; $n = 3$, error bars represent standard deviation). (B) Confocal fluorescence micrographs of HEK293 cells 8 h post-transfection with DiD-labeled χ/M-VLP (red) formed with pH 3 chitosan solutions at 5 μg/10^9 M-VLPs and immunostained with

antibodies against caveolin, clathrin or Lamp1 (green). White arrows indicate colocalization (yellow) of M-VLP and antibody. Scale bar = 10 μm.

Table 1

Hydrodynamic diameter measured via dynamic light scattering of χ/M-VLP. Errors are standard deviations (n=10).

Vector stoichiometry (μg/10^9 M-VLPs)	Diameter (nm)	
	pH 5	pH 3
4	-	311 ± 9
5	1033 ± 84	342 ± 24
7	-	593 ± 32
10	1010 ± 171	330 ± 7