Gene Therapy (2010), 1–6

www.nature.com/gt

ORIGINAL ARTICLE

Surface immobilization of hexa-histidine-tagged adeno-associated viral vectors for localized gene delivery

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Adeno-associated viral (AAV) vectors, which are undergoing broad exploration in clinical trials, have significant promise for therapeutic gene delivery because of their safety and delivery efficiency. Gene delivery technologies capable of mediating localized gene expression may further enhance the potential of AAV in a variety of therapeutic applications by reducing spread outside a target region, which may thereby reduce off-target side effects. We have genetically engineered an AAV variant capable of binding to surfaces with high affinity through a hexa-histidine metal-binding interaction. This immobilized AAV vector system mediates high-efficiency delivery to cells that contact the surface and thus may have promise for localized gene delivery, which may aid numerous applications of AAV delivery to gene therapy.
Gene Therapy advance online publication, 27 May 2010; doi:10.1038/gt.2010.81

Keywords: *AAV; localized gene delivery; substrate-mediated gene delivery; hexa-histidine*

Introduction

Adeno-associated virus (AAV) is a nonpathogenic parvovirus that depends on the presence of a helper virus, such as adenovirus, to replicate. It has a single-stranded 4.7-kb genome containing two open reading frames, *rep* and *cap*, which encode proteins that mediate replication of the viral genome and form the viral capsid, respectively. Recombinant AAV vectors, in which a transgene is inserted in place of the viral genome, have the capacity to deliver genes to both dividing and nondividing cells in numerous tissues, such as muscle,[1] brain[2] and retina.[3] Furthermore, AAV has enjoyed recent clinical success, which includes clinical trials that used AAV2-mediated gene delivery to the retina for treatment of Leber's congenital amaurosis and resulted in significant improvements in sight for numerous patients.[4,5] Despite these numerous advantages, a number of challenges remain in engineering AAV gene delivery systems, such as achieving targeted and/or localized gene delivery to specific cells and tissues.

Gene delivery typically involves the direct injection of a vector in solution; however, this mode of administration is accompanied by its local or systemic spread away from the injection site.[6] Such spreading can reduce vector levels at the target site, lead to possible side effects in off-target regions and potentially enhance immune responses against the vector. As an alternative, immobilizing gene delivery vectors onto material surfaces followed by implantation into target regions, that is, substrate-mediated gene delivery, is a strategy that can potentially yield effective and localized gene expression while preventing systemic vector spread.[7,8] Specifically, substrate-mediated delivery places the vectors into sustained contact with target cells to facilitate subsequent cellular internalization, and in some systems it has been shown to overcome mass transfer barriers or limitations to gene transfer.[9] In addition, substrate-mediated delivery has the potential to reduce the vector quantities required for high-level gene expression, and in the cases of adenoviral vectors and nonviral vehicles, the use of lower doses in substrate-mediated delivery has been shown to result in reduced cellular toxicity, which is typically caused by the initial burst of vector on direct injection *in vivo*.[10–12] Because of these advantages, a variety of substrate-mediated gene delivery systems have been used, primarily involving nonviral vectors and biomaterials.[12,13] Although viral vectors can have significantly higher gene transfer efficiencies than nonviral vectors, few studies have investigated viral vector delivery from substrates, presumably because of the lack of moieties on the viral surfaces that can specifically interact with biomaterials compared with engineered nonviral vectors.[8,14–16] However, developing substrate-mediated delivery approaches for viral vectors may yield systems that combine cell contact-mediated delivery with high efficiency. Owing to an increasing

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Received 30 November 2009; revised 6 April 2010; accepted 6 April 2010

understanding of its capsid, AAV vectors have the potential to be readily modified to interact with a substrate, thereby mediating 'localized' AAV delivery to cells that come into contact with the substrate.

Results

We have developed a strategy to specifically immobilize AAV vectors directly onto a substrate with which cells subsequently interact, thus concentrating the virus for direct contact with cells. Specifically, we had previously inserted a hexa-histidine (6xHis) onto a physically exposed loop of the AAV2 (that is, at amino acid 587 position) (Figure 1a) and AAV8 capsids, which enabled an efficient, single-step viral purification through immobilized metal affinity chromatography.[17] The resulting tagged virus was able to mediate high-efficiency gene delivery *in vivo* and elicited a macrophage and T-cell immune response equivalent to that of a phosphate-buffer control injection.[17] In this study, the same 6xHis tag was harnessed to immobilize AAV onto a material surface that presents nickel ions chelated by biotin-nitrilotriacetic acid (biotin-NTA) moieties bound to a streptavidin-coated surface (Figure 1b). Once the AAV vectors were bound to the substrate, various cell types—including HEK293T, Chinese hamster ovary (CHO), HeLa and B16F10 cells—were plated on these surfaces, and the subsequent gene delivery was analyzed and compared with gene expression achieved by the direct addition (that is, bolus delivery) of AAV vector (with either wild-type (wt) AAV2 or 6xHis tag capsids) to the cell-culture medium.

To modulate the interactions between histidine residues and multiple Ni-NTA groups, chimeric 6xHis AAV vectors were generated by mixing AAV packaging plasmids encoding the 6xHis mutant (pXX2 His6) or the wt capsid 2 (pXX2Not) at various mass ratios (that is, 100, 75, 50, 25 and 0% (w/w) pXX2 His6). In the case of 100% pXX2 His6 plasmid, all 60 viral protein subunits would display histidine residues on the capsid,[17] and as the proportion of pXX2 His6 is reduced, the number of 6xHis residues exposed on the viral shells is anticipated to decrease (Figure 2a).[18] This reduced presence of histidine tags on the virus likely decreases the extent of multivalent interaction between a single vector and Ni-NTA, thereby reducing the overall affinity of the virus for the surface. We anticipate that the capsids should contain sufficient levels of histidine to become effectively immobilized to the surfaces; however, overly strong interactions may inhibit subsequent vector release from the surface and thereby reduce gene delivery. Therefore, vector with low or intermediate histidine levels should both adsorb effectively and subsequently

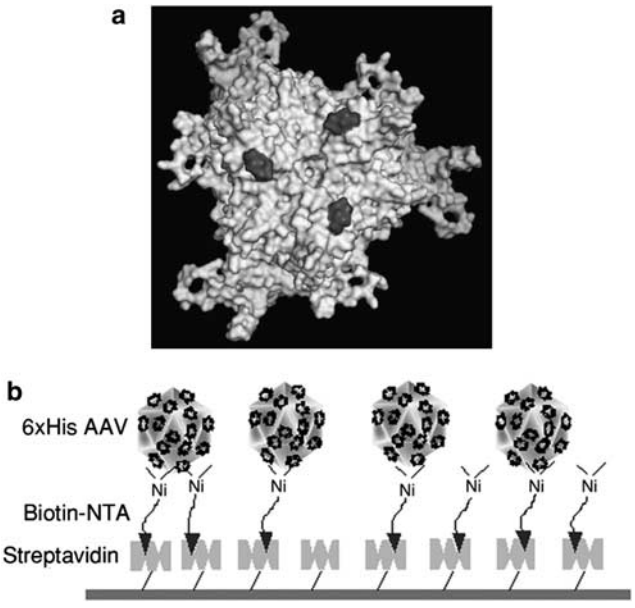

Figure 1 Schematic illustration of 6xHis AAV vectors and immobilization of the vectors onto the surface. (**a**) A view of the trimer at the three-fold axis of symmetry in the capsid (Rasmol). The heparan sulfate proteoglycan-binding site, where the 6xHis insertion occurs, is shaded in dark gray. (**b**) Binding of 6xHis AAV vectors on Ni-NTA-biotin conjugated on the streptavidin surface.

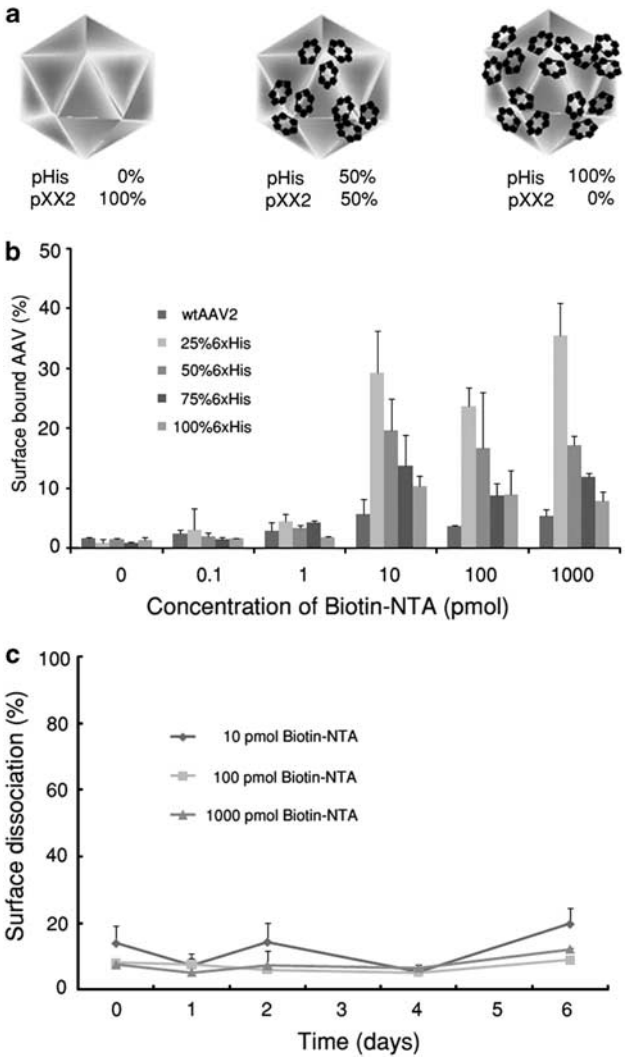

Figure 2 (**a**) Schematic illustration of 6xHis AAV vectors formed by varying the mass ratio of pHis to pXX2. (**b**) Surface-bound quantity of 6xHis-tagged AAV as a function of both the concentration of biotin-NTA on the surface and the fraction of histidine residues on the viral surface. (**c**) The amount of 25% 6xHis AAV vectors dissociated from the surface containing no cells.

be released in close proximity to adjacent cell surfaces, where viral binding to cellular receptors may even aid vector desorption, to mediate gene delivery.

We first analyzed the extent of AAV immobilization to surfaces as a function of histidine content. Interestingly, low levels of 6xHis in the vector resulted in effective AAV immobilization, and the amount of bound virus decreased with increasing histidine. That is, maximal surface binding occurred for vectors packaged with 25% of 6xHis and 75% wt AAV2 capsid helper plasmids (that is, 25% 6xHis AAV vectors, Figure 2b). The reduced binding with higher levels of 6xHis may be due to free capsid proteins not incorporated into viral particles competing with assembled capsids for binding to the surface. In addition to modulating the capsid, changing the concentration of biotin-NTA on the streptavidin substrate was a major factor that modulated the amount of immobilized 6xHis AAV vector (Figure 2b). Viral binding increased substantially as the level of biotin-NTA was elevated up to 10 pmol, but higher concentrations of biotin-NTA (that is, 100 and 1000 pmol) did not further enhance AAV binding. This binding saturation at 10 pmol was observed for all 6xHis AAV formulations. The increased viral binding with higher biotin-NTA levels may be due to a progressive increase in the number of sterically accessible NTA groups for the virus to bind.

The capacity for localized gene delivery to cells that come into contact with a vector-loaded substrate vector requires that the surfaces not prematurely release the vector. To assess desorption in the absence of cells, we incubated the AAV-laden substrates with cell-culture medium. Approximately 6–14% of bound vector initially dissociated from the surface, but for the subsequent 6 days no additional vector desorbed under any conditions (Figure 2c). To investigate the extent to which cells internalize virus introduced by direct addition vs substrate-mediated delivery, cell-internalized AAV vector was quantified as a function of time and the concentration of biotin-NTA after plating HEK293 cells on the virus using a previously published approach.[19] Approximately 15–20% of the surface-bound 6xHis AAV vector was associated with cells by 2 days, and the level progressively increased through day 6 (Figure 3a). In contrast, the majority of wt AAV2 vector and 25% 6xHis AAV vector directly added to the medium was internalized into cells within 2 days of exposure to HEK293T (Figure 3a). The difference in cellular internalization between wt AAV2 and 25% 6xHis AAV on direct addition may represent differences in the affinities of each vector for cell surface receptors, presumably heparan sulfate proteoglycans,[20] as our previous study indicated that insertion of 6xHis into amino acid 587 resulted in a slightly reduced affinity for a heparin column compared with wt AAV2.[17] Regardless, the cellular internalization results for the immobilized vector importantly indicate that cell contact with the bound vector can lead to localized vector uptake.

To analyze substrate-mediated gene delivery, 293 cells were incubated on surfaces bound with AAV at a number of 6xHis formulations, and after 2 days luciferase gene expression was assayed. The 25% 6xHis formulation, which yielded optimal vector binding to the surface (Figure 2a), resulted in the highest substrate-mediated gene expression. This result indicates that the binding

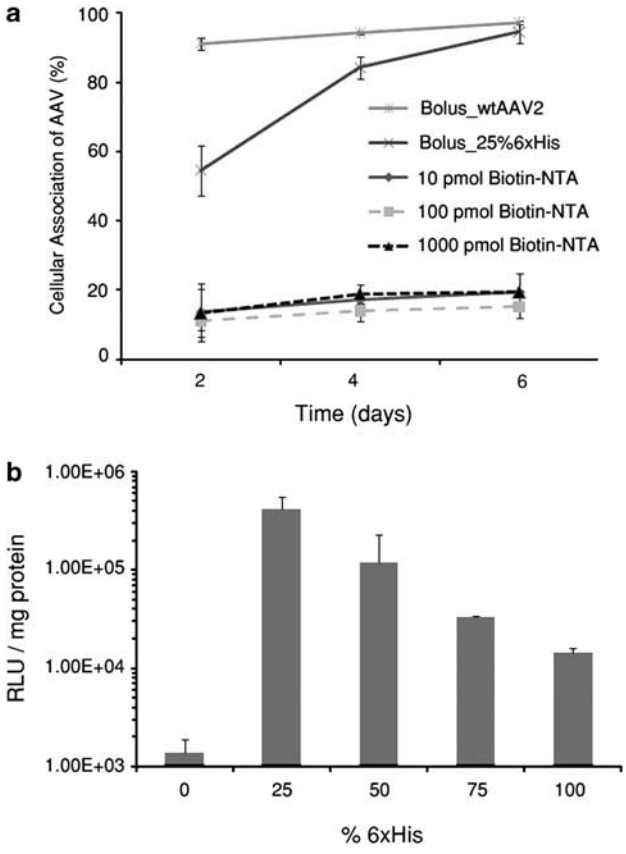

Figure 3 (**a**) Quantification of vector internalization into cells after Ni-NTA surface-mediated delivery or 25% 6xHis AAV or bolus delivery of vectors. (**b**) HEK293T cell infection and luciferase gene expression by substrate-bound AAV vectors, which were formulated with different ratios of pHis6/pXX2.

capacities of the 6xHis AAV vector are likely an important determinant of gene delivery efficiency (Figure 3b).

We next analyzed whether surface-immobilized AAV could mediate transduction of several additional cell types, including CHO, HeLa and B16F10 cells. HEK293T and HeLa cells, which are highly permissive to AAV2, were used as positive controls, and CHO cells and B16F10 human melanoma cells were chosen to assess delivery to cells known to be nonpermissive to AAV2. Infection with AAV carrying a luciferase reporter gene varied as a function of both the levels of histidine residues in the vector and biotin-NTA on the surface, demonstrating the potential for modulating localized gene delivery through engineering and tuning the virus–substrate interactions. Luciferase gene expression following bolus vs substrate-mediated delivery of 25% 6xHis AAV and wt AAV2 was examined at days 2, 4 and 6. It can be noted that, to enable a comparison with bolus delivery, the level of AAV directly added to the medium was fixed at 1×10^7 viral genomes, equal to the levels of vector immobilized to the substrate before addition of cells based on viral binding results (Figure 2).

The onset of gene expression following bolus infection was more rapid and could be detected by 2 days; however, luciferase expression mediated by substrate-mediated delivery reached that of bolus delivery over time (Figure 4). Substrate-mediated delivery to 293 cells

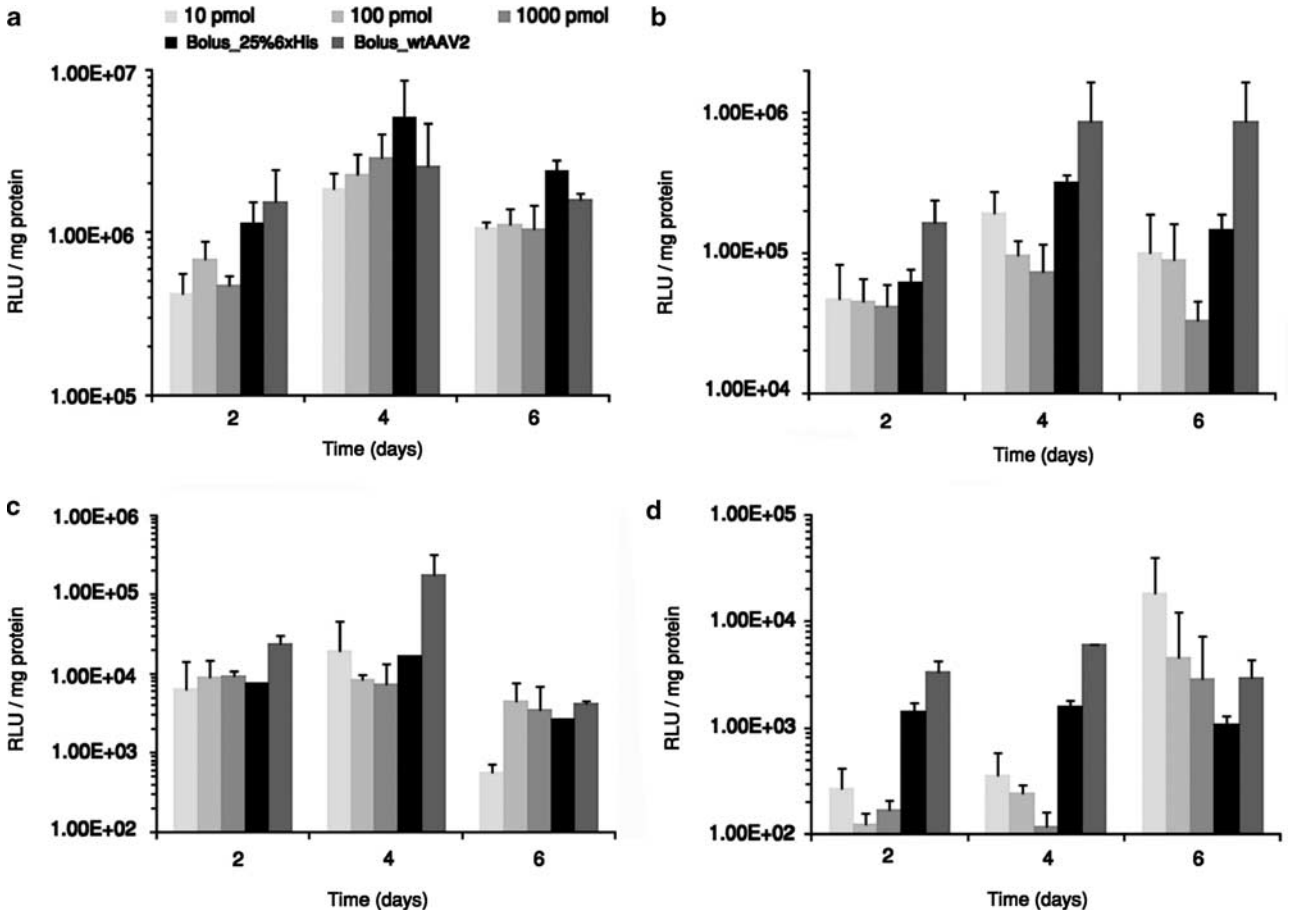

Figure 4 Luciferase expression following substrate-mediated and bolus gene delivery. Luciferase expression for various cell types—including (**a**) HEK293T, (**b**) HeLa, (**c**) CHO and (**d**) B16F10 cell lines—were investigated.

demonstrated similar or slightly improved gene transfer capabilities as compared with direct addition method. One interesting and important aspect of this substrate-mediated delivery is that comparable gene expression could be obtained even with significantly reduced AAV quantities of internalized vector as compared with bolus delivery (~70% less in Figure 3a), indicating that substrate-mediated delivery may somehow alter intracellular processing of the vector. In addition to 293 cells, substrate-mediated delivery to HeLa and CHO cells was comparable or slightly reduced compared with bolus delivery. Finally, delivery to B16F10 cells, a human melanoma cell line reported to be nonpermissive to AAV transduction,[21] was equivalent or slightly higher for substrate-mediated delivery vs bolus addition.

Discussion

We have developed a surface-mediated AAV vector delivery approach that, by releasing vector in close proximity to a cell surface, offers the potential for localized transduction. Furthermore, because the immobilization concentrates the virus into direct contact with the cell, the system may ultimately increase cellular uptake, thereby achieve similar gene expression with reduced viral dosages and/or potentially enhance delivery efficiency. In particular, we inserted a 6xHis onto a physically exposed loop of the AAV capsid protein (amino acid 587 position). This tag mediated vector binding to the surface when exposed to surfaces presenting nickel ions chelated by NTA. The levels of viral immobilization varied with the levels of histidine on the viral surface and biotin on the substrate, such that an optimal balance between the two led to high levels of viral adsorption and subsequent release to mediate gene delivery to multiple cell types. As a result, this system yielded comparable gene expression with significantly reduced internalized viral quantities compared with bolus or direct addition to medium.

In future, we anticipate that the level and kinetics of gene expression can be tuned by controlling binding capacities (for example, bound quantity, strength of binding and so on), indicating that 'smart' gene delivery devices can be developed for controlled release of vector. Moreover, as the substrate can be potentially 'upgraded' to three-dimensional scaffolds, this system may have future application in tissue engineering and regenerative medicine efforts. The AAV vector can be incorporated into such a material or device as the final step, such that complex scaffold fabrication processes would not affect the activity of surface-bound AAV. The development of systems with the capacity for local, efficient gene transfer therefore represents an additional gene delivery mode with the potential for application to a number of disease therapies.

Materials and methods

Cell culture

HEK293T and B16F10 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Mediatech, Herndon, VA, USA), HeLa in Iscove's modified Dulbecco's medium (Mediatech), and CHO in DMEM/F-12 (1:1) (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS) (Invitrogen) and 1% penicillin and streptomycin (Invitrogen) at 37 °C and 5% CO_2. All cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). For viral packaging, AAV293 cells (Stratagene, La Jolla, CA, USA) were grown in DMEM with 10% FBS and 1% penicillin and streptomycin at 37 °C and 5% CO_2.

Production and purification of 6xHis AAV vectors

Construction of 6xHis-coding plasmids ($pHis_6$) and pXX2 are described in a previous study,[17] and histidine residues were inserted at 587 amino acid sequence, corresponding to physically exposed loop domain: -LQRGNLGHHHHHHSRQA- (wt AAV2: ^{583}LQRGNRQA-). 6xHis AAVs were packaged by varying the total mass ratios of 6xHist-coding plasmids (pHis6) to pXX2. Recombinant AAV encoding luciferase was produced by methods described in a study.[22] Briefly, viral vectors were packaged using calcium phosphate transient transfection of pAAV CMV luc, pHelper, pXX2Not and pXX2 His_6, and were harvested as previously described.[23] The levels of 6xHis-presenting capsid proteins within a preparation were changed by varying the relative amounts of pXX2 His_6 and pXX2Not: 100% (7 μg pXX2 His_6:0 μg pXX2Not), 75% (5.25:1.75), 50% (3.5:3.5), 25% (1.75:5.25) and 0% (0:7). Subsequently, 1 volume of cell lysate was mixed with 0.5 volume of binding buffer (10 mM Tris (pH 8), 300 mM NaCl and 20 mM imidazole) and 500 μl of 50% Ni-NTA agarose beads (Qiagen, Valencia, CA, USA). This mixture was agitated gently overnight at 4 °C and then loaded onto a plastic column (Kontes, Vineland, NJ, USA). The loaded mixture was washed twice with 5 ml of wash buffer (10 mM Tris (pH 8), 50 mM imidazole), and viral vectors bound to the Ni-NTA beads were eluted with 2–3 ml of elution buffer (10 mM Tris (pH 8), 500 mM imidazole). The eluted virus was then concentrated using Microcon spin columns (Millipore, Billerica, MA, USA) according to the manufacturer's instructions and buffer exchanged into phosphate-buffered saline (PBS)/0.01% Tween.

Immobilization of 6xHis AAV vectors

6xHis-tagged AAV vectors were immobilized onto the surface by varying the concentration of biotin-NTA and the quantity of histidine residues on the viral surfaces. Before immobilizing the 6xHis AAV vectors, the streptavidin-coated polystyrene surfaces (Roche, Pleasanton, CA, USA) were prewashed with PBS/0.01% Tween according to the manufacturer's instructions, and a mixture (100 μl) of nickel chloride (100 mM) with biotin-NTA (0, 0.1, 1, 10, 100 and 1000 pmol) was agitated gently on the surface at room temperature for 4 h. The surface was then washed three times with PBS/0.01% Tween to remove unbound Ni-NTA-biotin. Finally, purified viral vector (10^7 genomic particles) was added onto the surface and agitated gently at 4 °C overnight. The surface was rinsed three times with PBS/0.01% Tween to remove unbound AAV, and each cell type (5000 cells per well, 200 μl) was then seeded onto the surface.

Quantification of surface-bound and dissociated AAV vectors

For quantification of the surface-bound 6xHis AAV, viral genomic titers were determined by quantitative PCR. Surface-bound AAV vectors were incubated with cell-culture media without cell seeding. At each time point, cell-culture medium was collected, and the surface was rinsed twice with PBS, which was also collected for quantification. Subsequently, viral vectors in the collected were harvested and quantified using quantitative PCR. For quantification of the surface-dissociated 6xHis AAV, immobilized 25% 6xHis AAV vectors were incubated with cell-culture media without cell seeding for each time point (4 h, 1, 2, 4, 6 days). As with quantifying surface-bound AAV, both cell-culture medium and rinsed buffers were collected at each time point, and viral vectors were harvested and quantified using quantitative PCR.

Quantification of vector internalization

Viral vectors associated with cells were quantified to examine each delivery mechanism (bolus vs substrate-mediated delivery). Note that percentages for each delivery mechanism were calculated from the initial quantities that were added to the medium (bolus) and to the surface for binding (substrate). After a 2- , 4- or 8-day incubation at 37 °C, the media were removed, and the surface was rinsed twice with PBS. Cells were subsequently trypsinized and collected by centrifugation.[19] To prevent the immediate association of surface-bound AAV vectors on the detached cells during the trypsinization, the cultures were incubated and blocked with heparin (30 μg ml^{-1}) at 37 °C for 30 min before trypsinization. Vectors in the collected buffers were harvested and quantified using quantitative PCR to estimate the amount of cell-internalized AAV vectors.

Transduction assay

At several time points (2, 4 and 6 days), luciferase levels were measured using a luminometer (Turner Biosystems, Sunnyvale, CA, USA), which was set for a 2-s delay with signal integration for 10 s, using the luciferase assay system (Promega, Madison, WI, USA), with levels normalized to the total amount of protein, which was measured using a BCA Protein Assay Kit (Pierce, Rockford, IL, USA). For bolus delivery, 10^7 genomic particles of wt AAV2 or 25% 6xHis AAV were directly added to the cell-culture medium (5000 cells per well).

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

This work was supported by NIH R01HL081527. In addition, JJ was supported by a grant from the California Institute for Regenerative Medicine (Training Grant

Number T1-00007). The contents of this publication are solely the responsibility of the authors and do not necessarily represent the official views of CIRM or any other agency of the State of California.

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