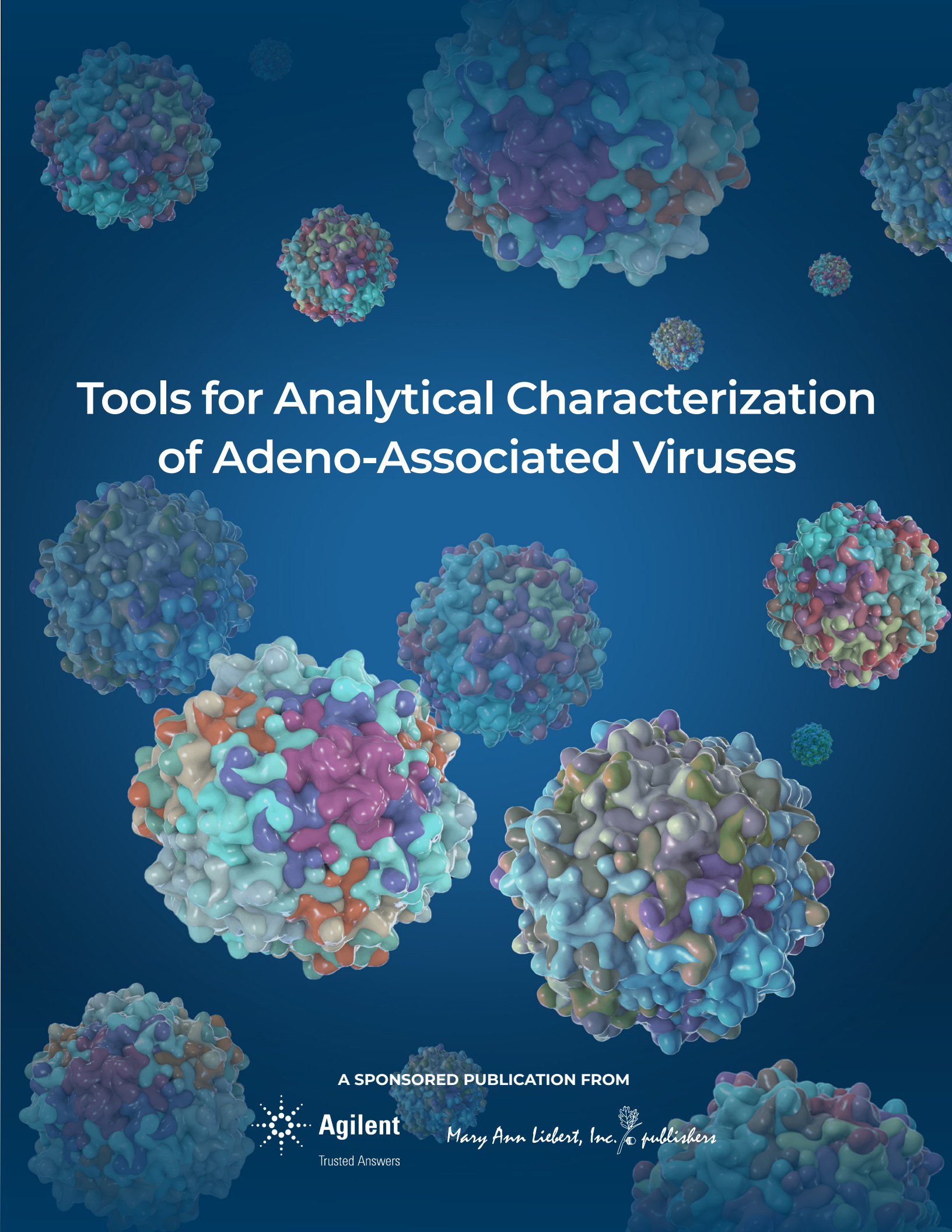


Tools for Analytical Characterization of Adeno-Associated Viruses

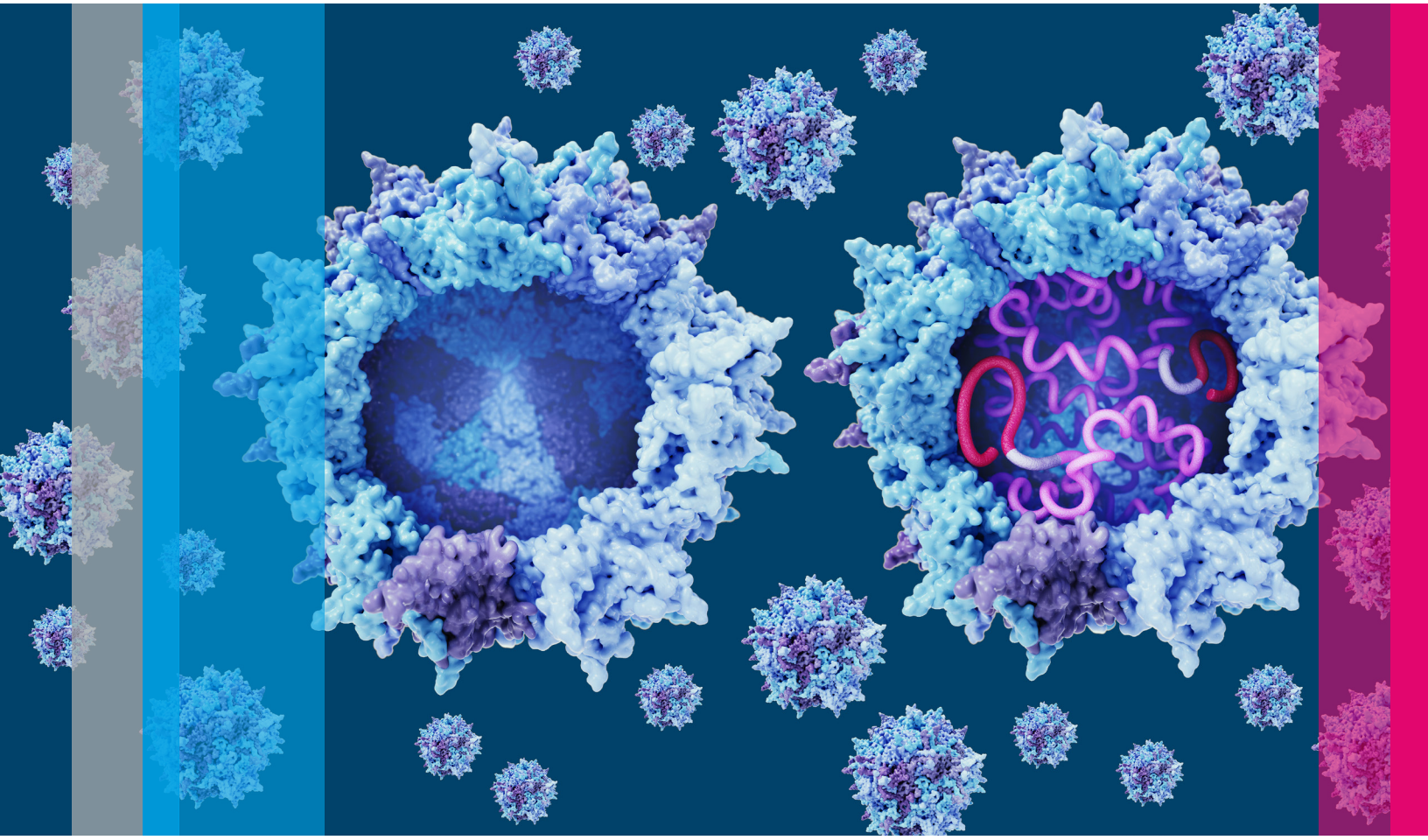
A SPONSORED PUBLICATION FROM

Agilent
Trusted Answers

Mary Ann Liebert, Inc. publishers

Agilent

Trusted Answers



Be Agilent Sure in Your CQA Monitoring

Measure what matters

Understanding the attributes of a viral vector, and the processes used to manufacture it, is critical to ensuring safety, efficacy, and desired pharmacokinetics.

Agilent AdvanceBio HPLC columns deliver results you can count on when analyzing complex biotherapeutic molecules. They can help you confidently monitor critical quality attributes (CQAs).

www.agilent.com/chem/advancebio-aav

Agilent offers BioHPLC columns for the analysis of:

- Cell culture media, including amino acids
- Intact analysis of VP1, VP2, and VP3
- Aggregates and fragments
- Peptide mapping of capsid proteins
- Empty/full payload analysis
- Oligonucleotide analysis

Contents

4 Analysis of Full/Empty Capsid Ratios in Adeno-Associated Virus 1 and 6 Serotypes

9 Capsid Engineering of Adenovirus Vectors: Overcoming Early Vector-Host Interactions for Therapy

26 LC/MS of Intact Adeno-Associated Virus (AAV) Capsid Proteins for Product Identity

36 Chemical Modification of the Capsid for Redirecting and Improving the Efficacy of Adeno-Associated Virus Vectors

44 Resolution of Adeno-Associated Viral Vector Aggregates and Fragments with Agilent Bio SEC-5

Analysis of Full/Empty Capsid Ratios in Adeno-Associated Virus 1 and 6 Serotypes Using Biocompatible Liquid Chromatography

Excellent linearity and reproducibility using the Agilent 1290 Infinity II Bio LC fitted with Agilent Bio SAX strong anion exchange columns

Author | Brian Liau, Agilent Technologies, Inc.

Abstract

Ion-exchange chromatography is an invaluable tool both for the purification of adeno-associated viruses (AAV) and analysis of their quality attributes. This study demonstrates the determination of the full/empty capsid ratio in AAV-1 and AAV-6 samples using the Agilent 1290 Infinity II Bio LC fitted with Agilent Bio SAX strong anion exchange columns. The method showed excellent linearity and reproducibility, establishing the 1290 Infinity II Bio LC as an advanced liquid chromatography system with industry-leading performance.

Introduction

During production of recombinant AAVs, a significant proportion of viral capsids contain no genetic payload and are therefore called “empty” capsids.[1] Empty capsids have been shown to lower the gene transduction efficacy of AAV preparations[2], and are therefore considered by regulatory authorities to be a process-related impurity that must be characterized.[3]

In this study, the Agilent 1290 Infinity II Bio LC fitted with Agilent Bio SAX column was used to determine the full/empty capsid ratio in purified AAV-1 and AAV-6 samples. The 1290 Infinity II Bio LC has a biocompatible flow path. When paired with Agilent nonporous poly(styrene divinylbenzene) (PS-DVB) Bio SAX strong anion exchange PEEK columns, the LC ensures the integrity of biomolecules by minimizing unwanted surface interactions, making it ideal for large molecule applications.

Experimental

AAV reference standards were bought from Vigene Biosciences as full- and empty-enriched samples. All chemicals were bought from Sigma-Aldrich, unless otherwise stated.

Instrumentation

Two configurations of the 1290 Infinity II Bio LC were used in this study (Figure 1). The LC was equipped with either a quaternary Flexible Pump, or binary High-Speed Pump. The Flexible Pump accommodates four solvent lines at once, reducing the number of different mobile phases that

must be prepared during method development. The High-Speed Pump combines the highest efficiency mixing with the lowest delay volume providing excellent levels of sensitivity and reproducibility. Both configurations are resistant to corrosion from high salt concentrations and can withstand high backpressures of up to 1300 bar. An in-line Agilent 1260 Infinity II Fluorescence Detector was used to achieve the high sensitivity required for analysis of the relatively dilute AAV samples.

Figure 1. Agilent 1290 Infinity II Bio LC.

Table 1. Composition of mobile phases.

	Flexible Pump	High-Speed Pump
Type Of Pump	Quaternary	Binary
Mobile Phase Composition	A: De-ionized water B: 1 M tetramethylammonium chloride C: 0.3 M HCl D: 0.2 M bis-tris propane A to D contain 2 mM $MgCl_2$	A: 70 mM bis-tris propane, pH 9.0 B: 70 mM bis-tris propane + 1 M tetramethylammonium chloride, pH 9.0 A and B contain 2 mM $MgCl_2$

Table 2. Flexible Pump gradient conditions.

Time	%A	%B	%C	%D	Flow Rate (mL/min)
0	36.2	15	13.8	35	0.1
40	15.8	35	14.2	35	0.1
40.1	0.6	50	14.4	35	0.3
43	0.6	50	14.4	35	0.3

Table 3. High-Speed Pump gradient conditions.

Time	%A	%B	Flow Rate (mL/min)
0	85	15	0.1
25	72.5	27.5	0.1
25.1	0	100	0.3
28	0	100	0.3

Table 4. Agilent OpenLab CDS 2.3 software integration settings.

Parameter	Value
Slope Sensitivity	0.02
Peak Width	2
Area Reject	0.05
Height Reject	0.05
Shoulders Mode	Tangential
Tangent Skim Mode	Straight
Tail Peak/Skim Height Ratio	0.05
Front Peak/Skim Height Ratio	0.05
Skim Valley Ratio	20
Baseline Correction Mode	Classical
Peak-to-Valley Ratio	500
Blank Subtraction	Yes

Chromatography

An Agilent Bio SAX NP5 column in PEEK hardware (2.1 × 50 mm, 5 µm, part number 5190-2472) was used to separate full and empty AAV capsids. Salt gradient elution was performed using the mobile phase and gradient conditions shown in Tables 1 to 3.

For the Flexible Pump method, Agilent Buffer Advisor Software was used to determine the percentages of each mobile phase component required to attain 70 mM *bis-tris* propane, pH 9.0, during the analysis. The AAV-1 and AAV-6 standards were diluted in 50 mM Tris-HCl, pH 7.4, before analysis, either individually as empty- or full-enriched, or as mixtures. Data acquisition and analysis was controlled by Agilent OpenLab CDS software using the integration settings shown in Table 4.

Results and discussion

The relative response factors of AAV-1 and AAV-6 were first estimated by injecting identical numbers of capsids from either full- or empty-enriched samples. Full capsids were observed to be brighter than empty capsids with an apparent response ratio relative to empty capsids of approximately 1.2 for both serotypes. This value is in good agreement with the published response ratio of 1.3 for AAV-6.2.[4]

As shown in Figure 2, separations of full and empty capsids in the mixed samples were achieved with acceptable resolutions (R = 1.1 for AAV-1, and R = 1.3 for AAV-6) using the Flexible Pump. Sample recovery was found to be negatively correlated with flow rate (data not shown), which was consistent with observations reported in the literature.[5] Therefore, the flow rate for this method was maintained at a low value of 0.1 mL/min during the analytical gradient.

The method was transferred to a 1290 Infinity II Bio LC equipped with a binary High-Speed Pump. Admixtures of full and empty capsids at different ratios were injected repeatedly over three days to assess the accuracy, linearity, and reproducibility of the assay. As shown in the overlaid chromatograms in Figure 3, the retention times and peak shapes of full and empty capsid peaks were reproducible over a wide range of capsid ratios.

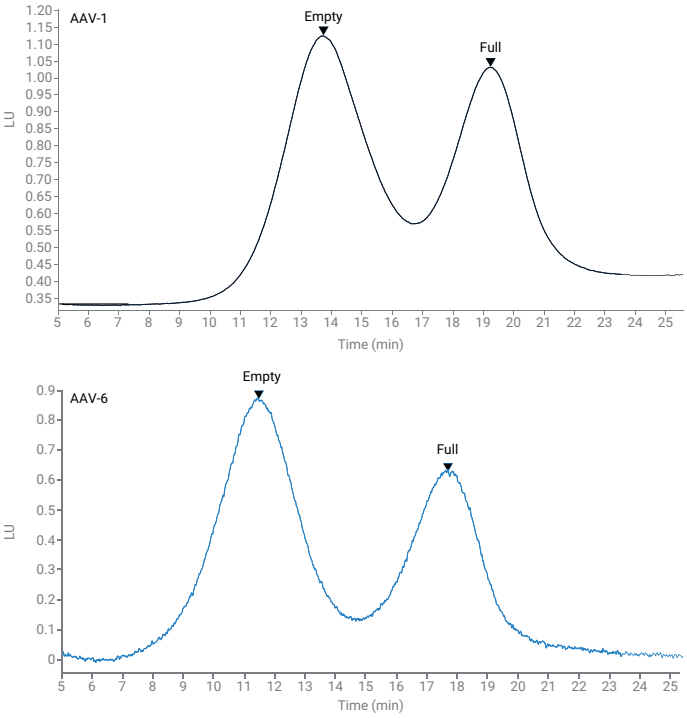


Figure 2. Separation of mixtures of full and empty capsids using the Agilent quaternary Flexible Pump.

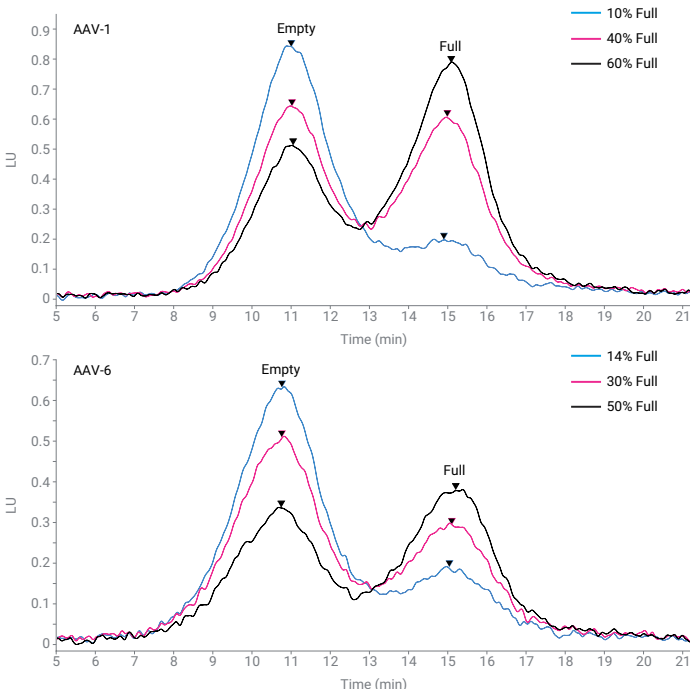


Figure 3. Separation of AAV-1 and AAV-6 samples with different full/empty capsid ratios using the Agilent binary High-Speed Pump.

As previously demonstrated[6], relative peak heights were found to provide more accurate and robust estimates of the full/empty capsid ratio compared to relative peak areas. This finding was because the peaks possessed some degree of tailing so were not fully resolved. Figure 4 illustrates the differences in linearity and accuracy for both AAV-1 and AAV-6 using either relative peak heights or peak areas as estimators. Linear trendlines for relative peak heights were closer to the origin and had higher R^2 values

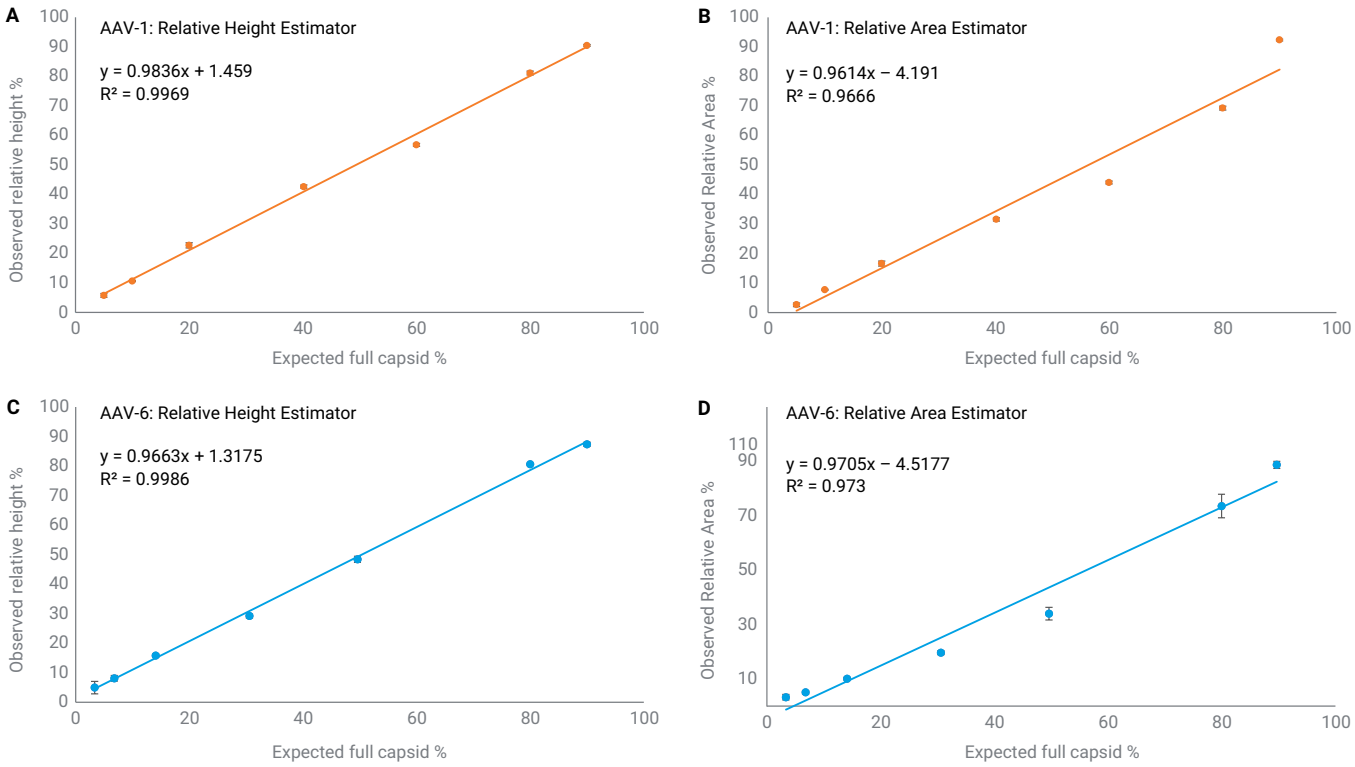


Figure 4. Comparison of relative peak heights and relative peak areas as estimators of full/empty capsid ratio. Relative peak height (A and C) provides superior accuracy and linearity compared to relative peak area (B and D).

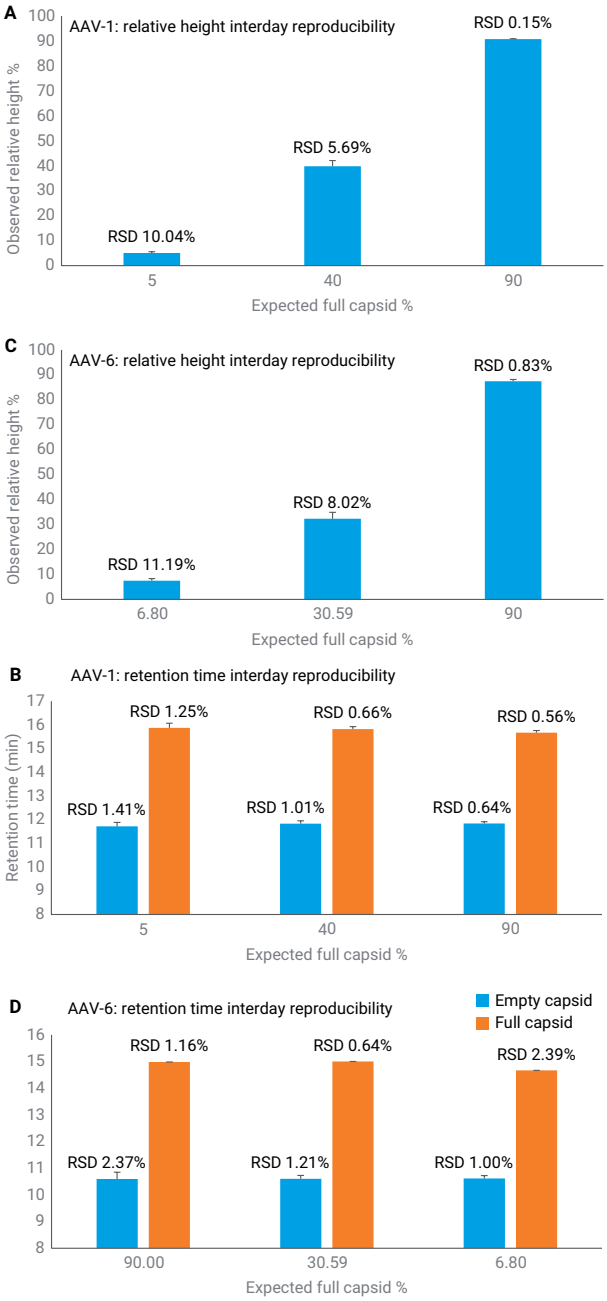


Figure 5. Interday reproducibility of relative height estimators and peak retention times.

Figure 5 shows the method's interday reproducibility. The relative standard deviations (RSD) of full/empty capsid ratios were ≤10.1% for AAV-1 and ≤11.2% for AAV-6 for samples containing between ~5 to 90% full capsids. The results show that the method has a broad linear range. The lower limit of quantitation for both serotypes were ≤5% full capsids, assuming an acceptable RSD of under 15%. The retention times for both full and empty capsid peaks were also highly reproducible, with RSDs of ≤2.4% for both serotypes for all tested samples.

Conclusion

This study shows that accurate and reproducible full/empty capsid ratios can be measured for AAV-1 and AAV-6 serotypes using the Agilent 1290 Infinity II Bio LC fitted with Agilent Bio SAX strong anion exchange columns.

References

1. Clark, K. R. *et al*. Highly Purified Recombinant Adeno-Associated Virus Vectors are Biologically Active and Free of Detectable Helper and Wild-Type Viruses. *Hum. Gene Ther*. **1999**, *10*, 1031–1039.
2. Gao, K. *et al*. Empty Virions in AAV8 Vector Preparations Reduce Transduction Efficiency and May Cause Total Viral Particle-Dose-Limiting Side Effects. *Mol. Ther. - Methods Clin.* Dev. **2014,** 1, 9.
3. Chemistry, Manufacturing, and Control (CMC) Information for Human Gene Therapy Investigational New Drug Applications (INDs) - Guidance for Industry. US *Food and Drug Administration* **2020**.
4. Wang, C. *et al*. Developing an Anion Exchange Chromatography Assay for Determining Empty and Full Capsid Contents in AAV6.2. M*ol. Ther. - Methods Clin. Dev*. **2019**, *15*, 257–263.
5. Trilisky, E. I.; Lenhoff, A. M. Flow-Dependent Entrapment of Large Bioparticles in Porous Process Media. *Biotechnol. Bioeng*. **2009**, *104*, 127–133.
6. McCoy, R. W. *et al*. Results of a Cooperative Study Comparing the Precision of Peak Height and Area Measurements in Liquid Chromatography*. *J. Chromatogr. Sci*. **1984**, 22, 425–431.

Capsid Engineering of Adenovirus Vectors: Overcoming Early Vector–Host Interactions for Therapy

Claudia Hagedorn and Florian Kreppel

Chair of Biochemistry and Molecular Medicine, Center for Biomedical Education and Research (ZBAF), Witten/Herdecke University, Witten, Germany.

Adenovirus-based vectors comprise the most frequently used vector type in clinical studies to date. Both intense lab research and insights from the clinical trials reveal the importance of a comprehensive understanding of vector–host interactions. Especially for systemic intravenous adenovirus vector delivery, it is paramount to develop safe and efficacious vectors. Very early vector–host interactions that take place in blood long before the first cell is being transduced are phenomena triggered by the surface, shape, and size of the adenovirus vector particles. Not surprisingly, a multitude of different technologies ranging from genetics to chemistry has been developed to alter the adenovirus vector surface. In this review, we discuss the most important technologies and evaluate them for their suitability to overcome hurdles imposed by early vector–host interactions.

Keywords: adenovirus, vector, capsid modification, oncolysis, genetic vaccination

Introduction

ADENOVIRUS (Ad)-BASED VECTORS are currently the most frequently used vectors for gene transfer in gene therapeutic clinical trials. They offer a number of advantageous features: Ad vectors can be produced to high titers, are able to efficiently transduce both dividing and quiescent cells, are amenable to genetic modifications, and have a large cargo capacity. During the last decade, cumulating comprehension of the complex molecular basis of diseases fostered the rational design of Ad-based vectors for therapeutic delivery. While an Ad can be delivered locally (*e.g.*, for genetic vaccination), systemic delivery through the bloodstream faces several hurdles that need to be overcome. The first and most complex hurdle is the induction of strong innate immune responses that occur probably due to interactions of Ad vectors with antigen-presenting cells such as macrophages and dendritic cells, resulting in the release of proinflammatory factors such as interleukin-6 and tumor necrosis factor-α (reviewed by Hendricx *et al.*[1]). A second hurdle is the widespread anti-Ad immunity and interactions with plasma proteins other than antibodies. Preexisting antibodies,[2,3] either naturally occurring or from previous treatments, can prevent the vector from reaching its target cell, but also blood factors like coagulation factors FIX, FX, and complement protein C4BP were shown to interact with Ad capsid proteins, thereby redirecting the virus from bloodstream into the liver.[4] The next hurdle is the endothelial layer. The vector must exit the bloodstream when reaching the target tissue or organ and get past the endothelial

**Correspondence: Dr. Florian Kreppel, Chair of Biochemistry and Molecular Medicine, Center for Biomedical Education and Research, Witten/Herdecke University, Stockumer Strasse 10, D-58453 Witten, Germany. E-mail: florian.kreppel@uni-wh.de*

Reprinted with permission from: Human Gene Therapy, Vol. 28, No. 10, 2017; p. 820-832; DOI: http://doi.org/10.1089/hum.2017.139

layer to transduce target cells. In most cases the vector is unable to move either through or across the epithelium, making further vector modifications necessary. Studies in rabbits indicated that size of the respective sinusoidal fenestrae is a major obstacle for efficient transduction.[5] Modifications that alter particle size or pharmacological treatment affecting sinusoidal fenestrae sizes may represent strategies for organ-specific transduction. This appears to be a janus-faced problem in vector retargeting: Modifications of the capsid that are required for tissue- or cell-specific targeting (*e.g.*, peptide coupling or PEGylation) may increase particle sizes, which in turn might then have consequences on vector's ability to get past the endothelial layer. However, hurdles following intravenous (i.v.) delivery are basically consequences of unwanted interactions between the capsid surface and plasma proteins (*e.g.*, FX binding) or antibodies (antibody neutralization). Therefore, to prevent these unwanted interactions and enable beneficial and specific interactions, modifications of the viral capsid are necessary. This review describes the complex vector–host interactions and gives an overview of capsid modification strategies that were applied to enable safe and specific Ad vector targeting. Moreover, we will discuss specific requirements for capsid modification strategies in the light of systemic delivery in cancer therapy (oncolysis) and vaccination.

Adenovirus biology and capsid structure

Adenoviruses, members of the family Adenoviridae, are nonenveloped DNA viruses. So far more than 70 types of human-infectious adenoviruses have been identified. Based on genome structure, hemaglutination properties, and sequencing results, these types are divided into seven species (human adenovirus A–G),[6,7] with Ad species B, C, and D comprising the types being most commonly used in gene therapy. The human Ad is a 38-kb double-stranded DNA virus encapsulated by an icosahedral nucleocapsid.[8] This capsid

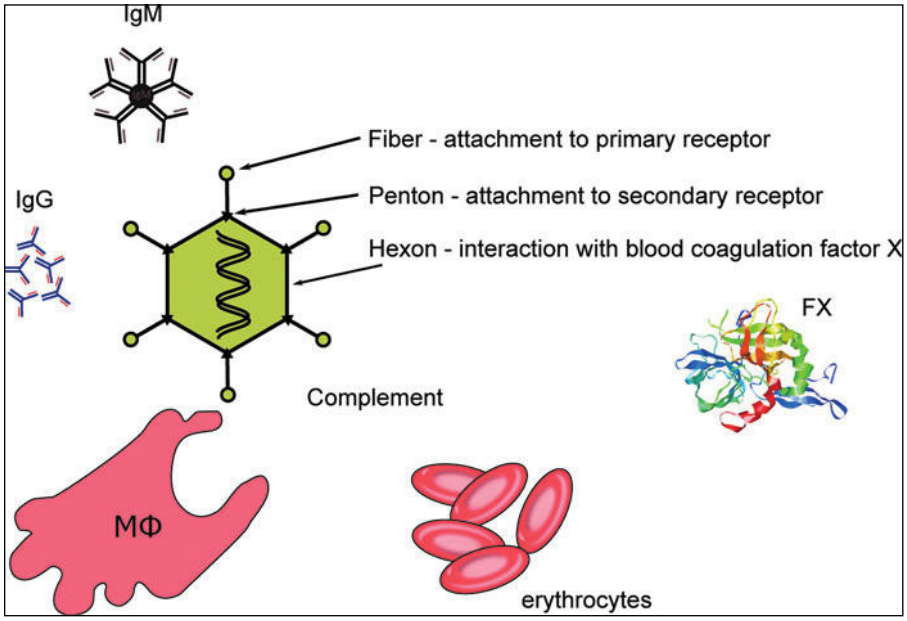


Figure 1. Adenovirus capsids and barriers for delivery. Fiber (36 copies per particle), Penton (60 copies per particle), and Hexon (720 copies per particle) are the most important capsid proteins involved in adenovirus vector sequestration and mistargeting by various barriers. FX, blood coagulation factor X; IgG, immunoglobulin G; IgM, immunoglobulin M; MΦ, macrophages. (Not drawn to scale.)

consists of nine proteins of which hexon, penton base, and fiber are referred to as major capsid proteins (Fig. 1). With 720 monomers, hexon is not only the largest, but also the most abundant capsid protein, forming 20 capsid facets each consisting of 12 hexon homotrimers.[9,10] Homopentamers of penton base protein are located on each icosahedral edge (vertex) forming part of the penton and representing the base for the vertex's spike that consists of trimers of glycolysated fiber protein.[10,11] Ad entry is composed of two major steps. Step one involves attachment of the fiber knob to its primary receptor. For Ad types from species A, C, E, and F, this primary receptor is the coxsackie and adenovirus receptor (CAR), while Ad types from species B and D were shown to interact with receptors distinct from CAR. This interaction brings virion and cell surface in spatial proximity to each other facilitating the interaction of cellular integrins with the Arg-Gly-Asp (RGD) tripeptide in penton base. In a second step, this interaction induces cellular responses that change the cytoskeleton, internalize the virion via clathrin-coated vesicles, and transport to the endosome.[12] In the endosome, the Ad virion partially disassembles and is released to the cytoplasm, where it ultimately travels to the nucleus for viral replication.

Successful application of Ad vectors as transfer vehicles requires efficient transduction of the respective targets cells without affecting non target cells. The utilization of tissue-specific promoters that are active in target cells only (transcriptional targeting, reviewed by Sadeghi and Hitt[13]), represents one approach. While potential toxic side effects of transgene expression can be reduced or even eliminated, this approach is still prone to side effects that may result from mislocalization of vector particles. However, irrespective of replication competence and infectivity, viral capsid proteins themselves have toxic side effects provoking an innate immune response with systemic release of cytokine interleukin-6.[14] Using transcriptional targeting as a single strategy, the efficient transduction of the target cell or tissue is not sufficiently addressed. One major obstacle is that the native tropism of the virus rarely meets the therapeutic need. Hence, efficient transduction of the correct cells often requires ablation of the native tropism and/or modification of the vector to infect cells that are naturally not a target (transductional targeting). Based on the knowledge about native Ad entry mechanisms, non-CAR-expressing cells (*e.g.*, many cancer cells) are predicted to be refractory to Ad transduction. In this scenario, high-CAR-expressing non target cells are efficiently transduced while low-CAR-expressing target cells would show poor transduction. However, *in vivo* the biodistribution appeared to be independent of CAR expression profiles.[15] Upon intravenous Ad delivery in the mouse the majority of transduction occurred in the liver, followed by spleen, heart, lung, and kidney; this transduction profile, however, does not correlate with CAR expression levels.[16] Likewise, ablating CAR and integrin binding in Ad capsids had only a small effect on virus uptake in the liver.[17,18] A major role of Ad fiber in virion interactions with blood coagulation factors was reported. Modification of Ad5 fiber preventing its interaction with FIX and C4BP resulted in a distinct decrease in liver transduction.[4] In other studies, a specific interaction between FX and hexon hypervariable regions (HVR) 3, 5, and 7 was defined. This interaction could be inhibited by either pharmacological factors or mutated forms of hexon.[19,20]

Capsid modifications

As mentioned above, most hurdles that are currently faced in Ad vector development arise from interactions with viral capsid proteins upon intravenous delivery, and numerous vector–host interactions influencing Ad tropism have been described in the past years. These new insights pave the way for promising modifications of the Ad capsid that are summarized in Table 1. In the following we will focus on genetic and chemical modifications of the Ad capsid. Figure 2 provides a schematic overview of the different technologies.

Chimeric capsids and directed evolution

Modifying vector tropism via genetic alteration of the Ad capsid represents the most direct approach. While the native entry biology of the commonly used Ad5 is based on a fiber–CAR interaction, primary attachment of other Ad species is CAR-independent. Accordingly, several attempts to modify Ad tropism focused on generation of chimeric Ad vectors. In this approach, parts of or the complete fiber are genetically substituted with its counterpart of other species resulting in altered tropisms, for example, enhanced transduction of malignant glioma.[21,22] The new types are preferentially generated from species B and D. Species B viruses have been shown to interact with receptors such as CD46,[23] CD80, and CD86,[24] or desmoglein 2 (types Ad3, 7, 11, 14),[25] while viruses of species D interact with CD46 and the glycoprotein component α(2-3)-linked sialic acid.[26] Interestingly, chimeric Ad vectors with fibers from species B Ad7 and Ad35 displayed an altered intracellular trafficking pathway.[27,28] These chimera resided longer in the endosome. While some virions eventually travelled to the perinuclear lysosome, others were recycled back to the cell surface [28]. *In vivo*, a chimeric, CAR and integrin-ablated Ad5 derived from Ad35 or Ad40 showed decreased liver transduction.[29,30] Additionally, chimeric Ad vectors were not only constructed for transductional targeting purposes, but also to evade anti-Ad5 immunity.

The high immunogenicity and high titers of Ad5-neutralizing antibodies in the human population indeed represents an important hurdle that needs to be overcome. Since the neutralizing antibodies are primarily directed against the HVRs of hexon, replacing HVRs of Ad5 with those of a less prevalent type (*e.g.*, Ad48)[31] was one successful strategy. Replacing all seven HVRs of Ad5 with their counterparts from Ad48 evaded the majority of preexisting Ad5 immunity *in vivo*.[32] Although some studies postulate that the majority of anti-Ad5 antibodies is directed against HVR1 and HVR5,[33,34] partial HVR-chimeric Ad5 vectors with only a subset of HVR substituted only insufficiently evaded preexisting Ad5-specific immunity in mice.[35] These data indicate that swapping all HVRs of Ad5 is necessary to evade neutralising antibodies. Such a vector has been evaluated recently as a human immunodeficiency virus (HIV) vaccine vector in phase 1 clinical trial.[36] However, Ad5:Ad48 chimeric vectors intravenously injected in mice triggered a robust inflammatory response,[37] with serum cytokine and chemokine responses elicited by Ad5:Ad48 being higher than those elicited by Ad5.[38]

In an approach termed "directed evolution," Kuhn *et al.* (2008) passaged a pool of seven Ad types (from species B–F) on cancer cell lines to invite recombination.[39] Subsequently, potent Ad variants or types showing early signs of cytopathic effects were selected. In this process a novel chimeric Ad3/Ad11p

Table 1. Overview of possible adenovirus capsid modification strategies

Modification strategy	Advantages	Disadvantages	Suitable to overcome
Chimeric vectors	• Exploiting tropism of other Ad types or species for retargeting • Evade pre-existing immunity toward Ad5	• Limited knowledge of Ad biology receptor usage • Induction of chimera-specific immunity	- AntiAd5 and FX mediated neutralization - limitations in targeting
Directed evolution	• Assay-dependent simultaneous addressing of multiple barriers	• Time-consuming approach • Risk of hazardous recombinants	• Limitations in targeting
Insertion of peptides and point mutations	• Expansion of Ad tropism	• Native tropism is not necessarily inhibited • Limitations in size of inserted Peptides • Limited availability of targeting peptides • Noncovalent attachment of targeting molecules	• Limitations in targeting
Single-component adapters Chemical modification	• Easy production and homogeneous populations • Covalent attachment of targeting molecules • Reduced antigenicity and immunogenicity • Efficient detargeting	• Large moieties required leading to impairment of virus bioactivity and bioreversibility • No site-specific attachment, shielding of genetically inserted targeting molecules	• Antibody neutralization • FX-mediated clearance • Macrophage and Kupffer cell scavenging
Geneti-chemical	• Site-specific shielding • No effect on additional targeting molecules • Evasion of antibody neutralization • Efficient detargeting	• Can require bioreversibility for optimal bioactivity	• Antibody neutralization • FX-mediated clearance • Macrophage and Kupffer cell scavenging

The table summarizes advantages and disadvantages of different capsid modification strategies, outlining specific obstacles that can be overcome. Ad, adenovirus.

oncolytic virus, ColoAd1, was generated. ColoAd1 displayed enhanced potency (9- to 100-fold over Ad5) on various colon tumor cell lines and in an intravenous liver tumor model. Relative to the parent Ad11p, ColoAd1 displayed a chimeric region in E2B and deletions in E3 and E4.[39] Interestingly, recombination did not occur in late genes encoding for viral capsid proteins. Since the seroprevalence of Ad11p is rather low,[40] ColoAd1 represents an attractive approach to systemically treat colon cancer and is currently undergoing a series of early phase clinical trials.

Capsid modifications with genetically inserted peptides or protein domains

A different approach uses short peptides inserted in the HI-loop, an exposed loop connecting β-sheets H and I, or C-terminus of fiber knob domain for Ad vector targeting. In principle, the C-terminus is the ideal location for peptide insertion. Linking an integrin-binding RGD-motif or heparin sulphate binding motif (pK7) in the C-terminus resulted in increased transduction of fibroblast and smooth muscle cells expressing alpha(v)integrin and cells lacking high levels of CAR expression, respectively.[41] However, since the interaction of fiber with CAR is not inhibited by insertions of short peptides, Ad vectors with expanded tropism are created. When combining both motifs in Ad fiber, RGD motif in HI-loop and pK7-motif in C-terminus, increased transduction efficiencies compared with nontargeted and singly modified Ad vectors in both CAR-positive and CAR-negative cells was shown. Moreover, incorporation of both motifs in Ad fiber knob resulted in a loss of its natural CAR-affinity.[42] In further studies it was shown that fiber knob can accept up to 89 amino acids without a loss or impairment of function. Specifically, the HI-loop was shown to accept an RGD-containing sequence of penton base of 83 amino acids in length,[43] while the C-terminus was fused to a 89 amino acid linker and biotin acceptor peptide (BAP),[44] demonstrating the applicability of adapter strategies at fiber knob. Besides

tropism-expanding peptides, cell-specific peptides were also incorporated: A vascular-endothelial cell targeting peptide was shown to enhance Ad infectivity for a variety of cancer cell lines.[45] Insertion of an Affibody, an antibody mimetic peptide, resulted in HER2/neu and Taq-polymerase-specific targeting.[46] However, besides fiber also other Ad capsid proteins gained attention due to their ability to display peptides. Protein IX, a polypeptide known to stabilize the capsid, is capable of displaying large polypeptides and proteins like EGFP with marginal effects on virion thermostability and bioactivity,[47] demonstrating that large proteins can also be linked to Ad capsid proteins. Another site for peptide insertion is the HVR5 surface loop of hexon. However, this site has been shown to accept only small insertions like RGD or His6,[48–50] with the exception of a 71 amino acid BAP.[51] In a combinatory approach, Hesse *et al.* (2007) demonstrated that insertion of

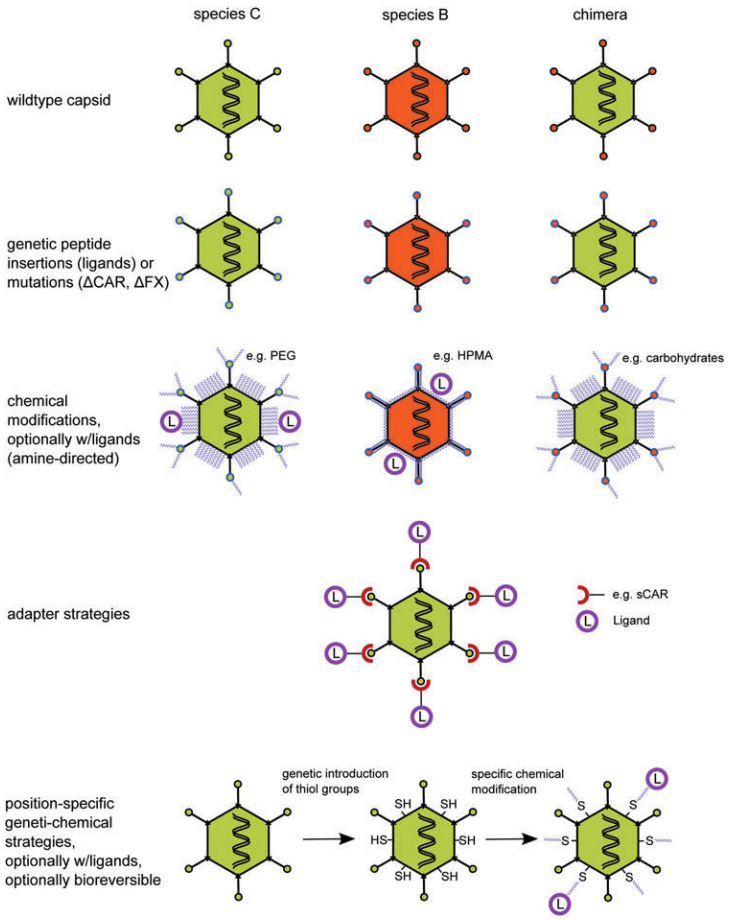


Figure 2. Schematic overview of capsid modifications technologies to improve delivery. To improve delivery by adenoviral vectors, multiple capsid modifications technologies exist. Chimeric adenovirus (Ad) particles can be generated by swapping entire capsid proteins or domains (top panel, *wildtype capsid*). Small genetic modifications can be employed to introduce peptidic ligands and to ablate coxsackie and adenovirus receptor (CAR)-binding or blood coagulation factor X (FX) binding. Such modifications have also been performed for types other than Ad5 and chimeric vectors (second panel, *genetic peptide insertions*). Amine-directed *chemical modifications* can be used to shield the vector capsids by synthetic polymers like polyethylene glycol (PEG) or poly-N-hydroxypropylamid (HPMA). In addition, ligands (L) can be coupled to the polymer shield. Chemical modifications are feasible with types other than Ad5 and chimeric vectors (third panel, chemical modifications). In contrast with chemical approaches, adapter strategies rely on noncovalent attachment of ligands via bifunctional adapters. Shown is sCAR, the soluble extracellular domain of the Ad receptor CAR that can be fused to ligands (fourth panel, *adapter strategies*). Geneti-chemical modifications combine small genetic modifications with position-specific, thiol-directed chemical modifications. The advantage is a precise shield instead of a dense cloaking. This way the infectivity of the particles can easily be maintained and ligands can be attached at advantageous positions (lower panel, *position-specific genti-chemical strategies*).

a 9 amino acid RGD peptide into one of the three loops of Ad41 short fiber knob (EG, HI, and IJ) was feasible without loss of fiber trimerization.[52] These successfully modified Ad41s fibers were then used to generate chimeric Ad5 vectors that exhibited increased transduction efficiencies in several cell lines.[52] However, despite some success incorporation of peptides is limited by a small number of motifs and therefore not suitable for specific targeting for all tissues. Moreover, there is no guarantee that any given peptide will be tolerated. Peptide insertion, although only 5–15 amino acids in length, may interfere with folding and/or multimerization of the modified capsid protein preventing vector rescue. It is also conceivable that peptides, although tolerated, lose their specificity in the structural context of the modified protein domain.

Adapter strategies

Another strategy for transductional targeting is based on the expression of single-component fusion proteins that serve as adapters. Fusing a single-chain antibody (scFv) against fiber knob to a scFv directed against epidermal growth factor (EGF) increased Ad gene transfer to EGF receptor–expressing cells.[53] Likewise, based on a truncated and soluble portion of CAR (sCAR) fused to a target molecule, a novel adapter system was developed. The first sCAR adapter, fused to EGF, transduced several EGF-overexpressing cancer cell lines with higher efficiency when compared with its untargeted counterpart.[54] Here the construction of trimerized sCAR fused to targeting ligands was shown to further improve the interaction of sCAR with fiber knob.[55] Other strategies fused sCAR to a scFv against polySia, a homopolymer that is frequently expressed on tumors of neuroendocrine origin, thus enabling effective targeting of oncolytic Ad to polySia expressing tumors.[56] The observation that some proteins like Tat (transactivator of transcription) of hHIV[57] or VP22 of herpes simplex virus[58] are taken up by mammalian cells via receptor-independent pathways led to the identification of protein transduction domains (PTD). PTDs are 10–30 amino acids in length and have been reported to deliver therapeutic proteins,[59] antisense oligonucleotides,[60]and plasmid DNA[61] into mammalian cells. Fusing PTDs to sCAR strongly increased affinity to and infection of CAR-negative nonpermissive cells. Moreover, when co-infected with conditionally replicating Ad vectors, uptake rate and lysis of permissive tumor cells was also increased.[62] Although adapter-based strategies provided vast evidence for retargeting of native Ad tropism, the preparations of these adapter molecules can be highly variable. The noncovalent nature of the adapter molecule–Ad capsid binding may limit its use for *in vivo* purposes and clinical translation as these interactions may be partially disrupted upon delivery. Further, endogenous CAR receptors or naturally occurring antibodies can compete for adapter binding thus circumventing vector targeting.

Chemical and genti-chemical capsid modifications

While the above described modification strategies are limited due to the noncovalent binding-nature and/or size of inserted or attached molecules, a modification approach originally used to modify therapeutic proteins has been adapted for Ad vectors. Polyethylene glycol (PEG) has been shown to reduce antigenicity and immunogenicity of therapeutic protein compounds.[63,64] Therefore, the covalent coupling of polymers to Ad capsid proteins opened new perspectives for transductional targeting and shielding

strategies. These chemical modifications are performed after production and purification of the virus avoiding specific production cells. Covering Ad vector particles with polymers like PEG and poly-N-(2-hydroxypropyl) methacrylamid (pHPMA) shields the vectors from undesired vector–host-interactions. Due to the hydrophilic nature of covalently attached polymers, vector particles in solution are surrounded by a stable water shell that reduces amongst others enzymatic degradation and immune cell recognition. Commonly, polymer shielding targets ε-amine groups from lysine side groups that are randomly distributed on the capsid surface. PEGylation has been performed for the first time in 1999. In their work, O'Riordan *et al*. demonstrated that PEGylated Ad vectors evaded neutralization by purified anti-hexon antibodies *in vitro* and in pre-immunized mice *in vivo*.[65] Besides vector shielding, polymers can also be used to covalently attach ligands for targeting. Using polymer-incorporated ligands like FGF resulted in 6-fold higher transduction of skeletal muscle in mice.[66] While nonreactive ends of monovalent PEG molecules protrude from the capsid surface, multivalent pHPMA molecules are linked to a multiplicity of reactive sites coupling the polymer to the particle surface. Polymer shielding using pHPMA led to promising results in terms of evasion from neutralizing antibodies,[67] binding to blood components,[68] and prolonged blood circulation *in vivo*.[69] Further, when analyzing transgene expression in the liver after systemic delivery of pHPMA-decorated Ad vectors, an up to 10,000-fold decrease in transgene expression for pHPMAylated vectors was demonstrated. These data indicate that polymer modifications can be used for detargeting from the liver.[69] Virus particles with pHPMA coupled to the capsid surface via degradable disulfide bonds showed higher infectivity when compared with virions with covalently coupled pHPMA,[68] a phenomenon probably due to its multivalent nature. However, the beneficial effects of polymer shielding required large moieties which impaired virus bioactivity.[68] Further, amine-directed shielding occurs randomly throughout the whole vector surface and does not allow for shielding of specific capsomers. Hence, polymer-modified vector particles show high heterogeneity even within one preparation. Further, extensive capsid modifications are needed to achieve above described effects and in combination with genetic insertion of targeting ligands the subsequent shielding also would modify these. To overcome these limitations, Kreppel *et al*.[70] introduced a geneti-chemical concept for vector re- and detargeting. Cysteines were genetically introduced in the virus capsid at solvent-exposed positions like fiber HI-loop,[70] protein IX,[71] and hexon.[72,73] Although not naturally occurring, cysteine-bearing Ad vectors can be produced at high titers in normal producer cells. Importantly, insertion of cysteines in not only certain capsomers, but also in different positions within one capsomer, allows for highly specific modifications with thiol group-reactive moieties. This geneti-chemical approach has been shown to overcome numerous obstacles in Ad vector design. The combination of amine-based PEGylation for detargeting and thiol-based coupling of transferrin to the fiber knob HI-loop has been proven to successfully retarget modified Ad vectors to CAR-deficient cells.[70] Since hexon is involved in most undesired interactions (neutralizing antibodies, blood coagulation factor FX), thiol-based modification strategies were also applied to hexon. Coupling small PEG moieties to HVR5 of hexon prevented Ad vector particles to transduce SKOV-3 cells in the presence of FX, whereas large PEG moieties increased

hepatocyte transduction.[72,74] Ad vector particles carrying mutations in the fiber knob to inhibit CAR binding and in HVR7 inhibiting binding of FX, and bearing inserted cysteines in HVR1 for position-specific PEGylation, were shown to evade antibody- and complement-mediated neutralization as well as scavenger receptor-mediated uptake, without loss of infectivity. Interestingly, despite lack of the natural FX shield, PEGylation again improved transduction of hepatocytes as a function of PEG size [73]. However, it was shown that covalent shielding does have an impact on intracellular trafficking processes. Prill *et al*. compared irreversible versus bioresponsive shields based on pHPMA and demonstrated that neither the mode of shielding nor co-polymer charge had an impact on cell entry, but rather affected particle trafficking to the nucleus. Employing a bioresponsive shield with positively charged pHPMA co-polymers allowed for particle trafficking to the nucleus maintaining the high transduction efficiencies of Ad vectors *in vitro* and *in vivo*.[75]

In summary, these data indicate that, even under the assumption all vector–host interactions were known and considered, excessive capsid modifications are necessary to overcome all hurdles associated with systemic vector delivery. The individual modification strategies possess their specific limitations. While genetic strategies are limited in the number of positions that can be modified without impairing production titers, the insufficient characterization of other human Ad types is limiting for the development of novel chimeric Ad viruses. The chemical approach at least overcomes limitations due to coupled molecule sizes and attachment stability; nevertheless, excessive polymer shielding impairs viral bioactivity and requires bioreversibility for efficient disassembly in the cytoplasm. Therefore, for successful utilization of Ad vectors in gene therapeutic approaches a rational and application-specific combination of genetic and chemical modification strategies is inevitably.

Virotherapy for cancer treatment

For systemic delivery of therapeutic Ad vectors, riddance of unwanted vector–host interactions is mandatory. This can be deemed an enhanced understanding of detargeting. At the same time, efficient targeting approaches are required. However, only the combination of highly specific targeting technologies and the addressing of all unwanted vector–host interactions at once will lead to the successful construction of safe and efficient therapeutic Ad vectors.

Oncolytic cancer therapy is a strategy that uses engineered viruses to treat malignancies. Oncolytic viruses (OVs) are modified such as they specifically infect cancer cells and replicate in these cells only. Malignant cells may be eliminated directly by the viral infection and tumor cell lysis, releasing additional virus particles to infect neighboring cells. Also, therapeutic viruses activate and support the immune system to recognize and attack tumor cells and unmask clandestine tumor antigens. A broad range of OV has been delivered by intratumoral injections with a perceptible success in treating reachable solid tumors.[76] However, for the treatment of advanced or metastatic malignancies, or inaccessible tumor entities such as pancreatic cancer, a systemic delivery of OVs is favorable, allowing the simultaneous treatment of primary tumor and disseminated metastases. Nevertheless, the systemic delivery of virus particles is complex and faces specific hurdles

that will be discussed in the context of cancer virotherapy in the following.

The systemic delivery of OV by i.v. injection requires careful considerations regarding tumor targeting. Cancer cell–specific promoters (*e.g.*, hTERT) and surface markers that are selectively expressed on tumor cells, such as EGF receptor, HER2/neu, and folate receptor can be used for transductional targeting and conditional replication. For example, genetic insertion of fiber displaying a single-domain antibody directed against human carcinoembryonic antigen (hCEA) in combination with the cancer cell-specific CXC4 promoter resulted in a retargeted and oncolytic conditionally replicating Ad vector (CRAd) showing increased infection of hCEA positive cells.[77] Likewise, an oncolytic CRAd expressing the human CD40 ligand induced activation of dendritic cells leading to an induction of a Th1 response and increased tumor-specific T-cells.[78] However, cancer cells exhibit high rates of mutation and chromosomal rearrangement, giving the tumor a high plasticity to modulate gene expression that allows for adaption to any kind of cytotoxic stress. This also results in a in a high intratumoral heterogeneity: different parts of the tumor may express different genes and therefore present different tumor cell-specific receptors. This limits the utilization of genetic modifications of the Ad capsid. Incorporation of peptides or the generation chimeric viruses may result in partial targeting of only a subset of tumor cells. Another challenging aspect is the presence of a dense stroma composed of different kinds of cells (*e.g.,* fibroblasts, macrophages, endothelial cells), blood vessels, and extracellular matrix. Diffusion of OV is blocked by this stroma and tumor cells that are sparsely embedded within evade antitumor therapeutics. Since Ad viruses are highly immunogenic, a collateral induction of innate and adaptive immune responses against tumor cells has been observed[79] and is currently explored as an approach in oncolytic virotherapy (reviewed in Woller *et al*.[80]). However, the efficient delivery of OV to the tumor by the blood stream represents another hurdle. As outlined above, in nonphysiological conditions when injected in the blood stream, Ad vector particles transduce hepatocytes. Interactions with soluble factors and cellular receptors result in fast clearance with a half-life of minutes. Ablating CAR and integrin binding did not affect liver tropism,[18] whereas in combination with fiber shaft exchange a reduced gene transfer into hepatocytes was observed.[29,30] However, compared to conventional Ad5 chimeric Ad vectors were shown to trigger robust immune responses.[38] Further, the presentation of chimeric capsid proteins may eventually trigger the generation of adapted neutralizing antibodies, impeding repeated administration of therapeutic Ad vectors.

A key role in hepatocyte transduction can be drawn to capsid protein hexon. Binding of blood coagulation factor FX to Ad5 hexon HVRs bridges the virus to heparan sulphate proteoglycans on the surface of hepatocytes. The pharmacological or genetical inhibition of FX-hexon interaction resulted in reduced transduction rates of the liver.[19,20] In mice, macrophages and Kupffer cells are the main cells removing Ad vectors from the blood using scavenger receptor A (SR-A). Combining ablation of FX-binding to hexon with ablation of SR-A binding or binding to other scavenger receptors such as SREC-I[81] will be interesting next steps, as a combined pharmacological inhibition of these factors resulted in tumor targeting.[82] However, since neutralizing anti-Ad antibodies are

also mainly directed to hexon, particle "coating" by FX protects the virus from host immunoglobulin M and complement-mediated neutralization. In humans, neutralizing antibodies will have a major impact on vector half-life. If tumor targeting using Ad vectors is challenging in Ad-naïve patients, it is even more challenging in patients that have experience Ad vector treatment before or have preexisting neutralizing antibodies. In this scenario, shielding virus particles with complex polymers represents a promising approach. Shielding of Ad6 with low seroprvalence with PEG blunted liver damage and cytokine production after systemic delivery but may also reduce its oncolytic efficacy,[83] The position-specific oupling of PEG using a geneti-chemical approach[70] enables for both particle shielding and genetic capsid modifications for transductional targeting. As demonstrated recently, the combination of genetically ablated CAR and FX binding with PEGylation of HVR1 prevented complement and antibody neutralization as well as particle sequestration via scavenger receptor-mediated mechanisms,[73] Due to polymer attachment, Ad vector particles are slightly increased in size, which may have an interesting side effect on liver transduction. The size of the sinusoidal fenestrae in humans without liver pathology (107±1.5nm)[84] allows unmodified Ad particles (80–100nm) to translocate. Studies in rabbits suggest that the sinusoidal fenestrae size may function as a natural barrier for Ad vectors when particle sizes are increased.[5]

Being the most abundant blood cell type, binding of Ad5 to human erythrocytes is of high relevance. Human erythrocytes were shown to sequester circulating Ad5 via CAR and complement receptor 1–mediated binding.[85] Due to the lack of integrins on human erythrocytes surface, CAR-mediated binding is reversible and does not prevent extravasation and organ transduction [86]. However, complement receptor 1–mediated binding of Ad5 to erythrocytes is targeted for rapid clearance, presumably involving the classic complement pathway.[85]

Despite the encouraging results of oncolytic Ad5-viruses being currently tested in clinical trials (reviewed in Uusi-Kerttula *et al*.[87]), rapid elimination of the therapeutic vector from the bloodstream upon systemic delivery severely limits their use to either Ad5-naïve patients and/or intra-tumoral or *ex vivo* applications. Noteworthy, the ablation of the ability of Ad5 particles to bind FX detargeted the particles from hepatocytes,[20] but at the same time rendered them highly susceptible for neutralization by natural antibodies and complement.[88] This example demonstrates that the vector biology in an organism is much more complex than simple on/off switches. However, since Ad5 is the best-characterized type so far, and given the high immune-prevalence for Ad5 type, further development and combination of the above discussed approaches is absolutely essential for the design of safe and efficient therapeutic vectors. In this context, the genti-chemical approach might offer advantages. The comparably minor modifications typically maintain the potency of the virus and can be applied to both well-characterized Ad types such as Ad5 but also to largely unknown types. Being a rational approach, geneti-chemical capsid modifications are solely based on the knowledge of hurdles that need to be overcome. A wide choice of ligands like antibodies, peptides, aptamers, affilins, lipids, and carbohydrates can be coupled by stable maleimide and bismaleimide groups or in a bioreversible manner forming *e.g.*, disulphide bridges, yielding in defined

particles. However, a vector for successful systemic delivery through the blood stream will very likely be generated by a combination of existing technologies.

Vaccination

Traditionally, vaccination against viral pathogens is based on attenuated or inactivated viruses or virus subunits. Progress in molecular virology and immunology has led to the generation of vectored vaccines, expressing an antigen of choice. Due to their aptitude to induce strong innate and adaptive immune responses, to efficiently transduce many cell types including professional antigen presenting cells (APC) and the potential adjuvant function of hexon, Ad vectors have been extensively studied as vaccination vectors.

Capsid modifications for targeting and shielding

A successful vaccination strategy requires the induction of a cytokine profile orchestrating the maturation of B- and cytotoxic T-cells, resulting in a prolonged and boostable immune response. Upon vector-receptor interaction, Ad5 was shown to trigger rapid release of IL-6, Il-12, and TNFα [89] thus activating the differentiation of immature dendritic cells to professional APC. Induction of $CD8^+$ cells is primarily stimulated by *de novo* synthesized peptides, while $CD4^+$ cells are favorably activated by peptides derived from phagocytosis and lysosomal cleavage. Naturally, Ad virions cause lytic infections resulting in short antigen presentation time of individually infected cells, thereby potentially favoring a $CD4^+$ immune response. Most Ad vectors currently used for vaccination are replication defective or single cycle,[90] resulting in prolonged antigen presentation time for both transgene and capsid proteins. Additionally, the generation of chimeric Ad5 vectors carrying fiber from Ad30 and Ad35 resulted in increased transduction of dendritic cells without affecting transgene-specific B- or T-cell responses,[91] Another study demonstrated that besides improved tropism for dendritic cells, an Ad5/ Ad35 chimeric vector also enhanced induction of $CD8^+$ cells *in vitro* and *in vivo*.[92] Incorporation of an RGD peptide in Ad5 fiber knob also increased the efficiency in transducing dendritic cells and was shown to induce higher transgene-specific $CD4^+$- and $CD8^+$-specific immune responses.[93] Further, due to the promiscuous nature of Ad vectors, leakage of vector particles from injection sites such as muscle or skin, will contribute to systemic transduction of nonlymphoid tissue, particularly the liver. It was demonstrated that vector dissemination from an intramuscular injection site to the liver resulted in early priming of transgene-specific $CD8^+$ cells. However, hepatocyte-specific silencing mediated by miRNAs significantly enhanced early priming of transgene-specific $CD8^+$ cells, allowing up to 100-fold reduction of vector dose.[94] However, in pre-exposed humans, hexon-specific $CD4^+$ and $CD8^+$ T-cell responses have been demonstrated.[95] Binding of neutralizing Ad-specific antibodies prevents cell transduction and thus transgene (epitope) expression and presentation. Recent efforts to circumvent antibody neutralization focus on Ad types exhibiting low serum prevalence in human population. Promising candidates such as human Ad6, as well as chimpanzee (Ch) Ad types ChAd3 and ChAd63 were studied for their use as vectored vaccines for hepatitis C virus, malaria, and HIV.[96–98] Another strategy to overcome Ab neutralization may be the utilization of Ad dodecahedrons. In the 1960s, the spontaneous formation of symmetric particles that were significantly smaller than Ad virions was observed in transduced

cells.[99,100] In the late 1990s the spontaneous formation of these dodecahedral particles was observed upon expression of either Ad3 penton base alone or combined expression of penton base and fiber.[101,102] Since Ad dodecahedrons contain fiber and integrin-binding RGD motif in penton base they can efficiently enter cells, whereas the lack of hexon greatly reduced the probability of being neutralized by anti-Ad antibodies. Another strategy to overcome antibody-mediated neutralization is chemical shielding of vector particles, as described above. Shielding of Ad vectors with PEG resulted in efficient detargeting upon systemic and intramuscular delivery. Despite being coated, PEGylated Ad vectors were still capable to induce a potent transgene-specific immune response while evading neutralization by preexisting antibodies.[103]

Capsid modifications for antigen presentation

An alternative approach to vector-encoded epitope expression is to incorporate the antigen in the viral capsid, either by genetic fusion or using adapter molecules. This strategy has firstly been used in 1994. An eight-amino-acid sequence of the VP1 capsid protein of poliovirus type 3 was genetically incorporated into two regions of Ad2 hexon. Antiserum derived from one of these vectors was shown to specifically recognize the VP1 capsid protein.[48] In similar studies, the immunodominant 14 -amino-acid peptide Epi8 of *pseudomonas* was incorporated in Ad5 HVR5. Mice vaccinated with this vector showed an increased antibody response consisting of anti-*pseudomonas* IgG1 and IgG2s subtypes and a remarkable number of pre-immunized mice survived when challenged with *pseudomonas*.[104] In a rather exceptional approach, a third-generation cocaine hapten termed GNE was conjugated to the capsid of a disrupted Ad5 vector. Vaccination with this vector evoked anti-cocaine antibodies in rats and prevented cocaine-induced hyperactivity and addiction related behavior.[105] In nonhuman primates, repetitive delivery maintained high levels of anti-cocaine titers that were sufficient to prevent cocaine from binding to dopamine transporter.[106] However, the presentation of target epitopes, either vector-encoded or capsid-incorporated, competes with numerous viral proteins for major histocompatibility complex (MHC) molecules and thus the release of stimulatory cytokines, which are needed for activation and proliferation of T- and B-cells. This might have negative consequences for the desired immune response. Therefore, one obstacle is to keep the balance between Ad- and transgene-specific immune responses; exploiting the adjuvant function of Ad capsid proteins while inducing a strong transgene-specific immune response. This might be achieved with a partial, position-specific shielding of hexon to preserve its adjuvant function but decrease the probability of neutralization and unwanted immune responses at the same time.

Besides being less prone to antibody neutralization, Ad dodecahedrons also proved to be suitable to present antigens on their surface. The prolin-containing motifs xPPxY in penton base were exploited to couple protein cargo to Ad dodecahedral particles by fusing WW domains to target proteins. Using this technique full-length proteins up to 150kDa in size can be efficiently coupled to dodecahedra and transported into cells (reviewed by Kron and Kreppel[107]). Ad3 dodecahedra coupled with the influenza matrix protein M1 entered myeloid dendritic cells with an efficiency of up to 90% and exhibited an immunostimulatory effect.[108] The potential of Ad dodecahedra

to induce humoral and cellular immune responses *in vivo* was further investigated with the model antigen ovalbumin. Upon subcutaneous delivery of ovalbumin-loaded dodecahedra in mice, ovalbumin-specific T-cells were detected in splenocytes. Moreover, these ovalbumin-loaded dodecahedra could induce ovalbumin-specific antibody response and titers could be boosted upon repeated delivery.[109] In summary, these studies impressively demonstrate the high potential of Ad dodecahedra in vaccination strategies.

Despite the advances in understanding the molecular basis of diseases, molecular virology, and viral immunology, little is known about the mechanisms of Ad APC transduction *in vivo* and the number of APC that must be transduced to induce a potent immune response.[91,110,111] Therefore, the rational design of Ad-based vaccines requires a thorough knowledge of the infectious agent the vaccination vector is developed for. However, although additional obstacles lie ahead the application of Ad vectors for vaccination looks promising.

Acknowledgements

This work was partly funded by the Center for Biomedical Education and Research (ZBAF) of the Witten/Herdecke University (UW/H).

Author disclosure

No competing financial interests exist.

References

1. Hendrickx R, Stichling N, Koelen J, et al. Innate immunity to adenovirus. Hum Gene Ther 2014;25:265–284.
2. Sumida SM, Truitt DM, Lemckert AAC, et al. Neutralizing antibodies to adenovirus serotype 5 vaccine vectors are directed primarily against the adenovirus hexon protein. J Immunol 2005;174:7179–7185.
3. Zhi Y, Figueredo J, Kobinger GP, et al. Efficacy of severe acute respiratory syndrome vaccine based on a nonhuman primate adenovirus in the presence of immunity against human adenovirus. Hum Gene Ther 2006;17:500–506.
4. Shayakhmetov DM, Gaggar A, Ni S, et al. Adenovirus binding to blood factors results in liver cell infection and hepatotoxicity. J Virol 2005;79:7478–7491.
5. Lievens J, Snoeys J, Vekemans K, et al. The size of sinusoidal fenestrae is a critical determinant of hepatocyte transduction after adenoviral gene transfer. Gene Ther 2004;11:1523–1531.
6. Benko M, Harrach B. Molecular evolution of adenoviruses. Curr Top Microbiol Immunol 2003;272:3–35.
7. Davison AJ, Benko M, Harrach B. Genetic content and evolution of adenoviruses. J Gen Virol 2003;84:2895–2908.
8. Rowe WP, Huebner RJ, Gilmore LK, et al. Isolation of a cytopathogenic agent from human adenoids undergoing spontaneous degeneration in tissue culture. Proc Soc Exp Biol Med 1953;84:570–573.
9. van Oostrum J, Burnett RM. Molecular composition of the adenovirus type 2 virion. J Virol 1985;56:439–448.
10. Stewart PL, Fuller SD, Burnett RM. Difference imaging of adenovirus: bridging the resolution gap between X-ray crystallography and electron microscopy. EMBO J 1993;12:2589–2599.
11. Stewart PL, Burnett RM, Cyrklaff M, et al. Image reconstruction reveals the complex molecular organization of adenovirus. Cell 1991;67:145–154.
12. Meier O, Boucke K, Hammer SV, et al. Adenovirus triggers macropinocytosis and endosomal leakage together with its clathrin-mediated uptake. J Cell Biol 2002;158:1119–1131.
13. Sadeghi H, Hitt MM. Transcriptionally targeted adenovirus vectors. Curr Gene Ther 2005;5:411– 427.
14. Schnell MA, Zhang Y, Tazelaar J, et al. Activation of innate immunity in nonhuman primates following intraportal administration of adenoviral vectors. Mol Ther 2001;3:708–722.
15. Fechner H, Haack A, Wang H, et al. Expression of coxsackie adenovirus receptor and alphav-integrin does not correlate with adenovector targeting *in vivo* indicating anatomical vector barriers. Gene Ther 1999;6:1520–1535.
16. Wood M, Perrotte P, Onishi E, et al. Biodistribution of an adenoviral vector carrying the luciferase reporter gene following intravesical or intravenous administration to a mouse. Cancer Gene Ther 1999;6:367–372.
17. Alemany R, Curiel DT. CAR-binding ablation does not change biodistribution and toxicity of adenoviral vectors. Gene Ther 2001;8:1347–1353.
18. Martin K, Brie A, Saulnier P, et al. Simultaneous CAR- and alpha V integrin-binding ablation fails to reduce Ad5 liver tropism. Mol Ther 2003;8:485–494.
19. Kalyuzhniy O, Di Paolo NC, Silvestry M, et al. Adenovirus serotype 5 hexon is critical for virus infection of hepatocytes *in vivo*. Proc Natl Acad Sci U S A 2008;105:5483–5488.
20. Waddington SN, McVey JH, Bhella D, et al. Adenovirus serotype 5 hexon mediates liver gene transfer. Cell 2008;132:397–409.
21. Staba MJ, Wickham TJ, Kovesdi I, et al. Modifications

of the fiber in adenovirus vectors increase tropism for malignant glioma models. Cancer Gene Ther 2000;7:13–19.

22. DiPaolo N, Ni S, Gaggar A, et al. Evaluation of adenovirus vectors containing serotype 35 fibers for vaccination. Mol Ther 2006;13:756–765.
23. Gaggar A, Shayakhmetov DM, Lieber A. CD46 is a cellular receptor for group B adenoviruses. Nat Med 2003;9:1408–1412.
24. Short JJ, Pereboev AV, Kawakami Y, et al. Adenovirus serotype 3 utilizes CD80 (B7.1) and CD86 (B7.2) as cellular attachment receptors. Virology 2004;322:349–359.
25. Wang H, Li Z-Y, Liu Y, et al. Desmoglein 2 is a receptor for adenovirus serotypes 3, 7, 11 and 14. Nat Med 2011;17:96–104.
26. Arnberg N, Edlund K, Kidd AH, et al. Adenovirus type 37 uses sialic acid as a cellular receptor. J Virol 2000;74:42–48.
27. Miyazawa N, Leopold PL, Hackett NR, et al. Fiber swap between adenovirus subgroups B and C alters intracellular trafficking of adenovirus gene transfer vectors. J Virol 1999;73:6056–6065.
28. Shayakhmetov DM, Li Z-Y, Ternovoi V, et al. The interaction between the fiber knob domain and the cellular attachment receptor determines the intracellular trafficking route of adenoviruses. J Virol 2003;77:3712–3723.
29. Koizumi N, Mizuguchi H, Sakurai F, et al. Reduction of natural adenovirus tropism to mouse liver by fiber-shaft exchange in combination with both CAR- and alphav integrin-binding ablation. J Virol 2003;77:13062–13072.
30. Nakamura T, Sato K, Hamada H. Reduction of natural adenovirus tropism to the liver by both ablation of fiber-coxsackievirus and adenovirus receptor interaction and use of replaceable short fiber. J Virol 2003;77:2512–2521.
31. Barouch DH, Pau MG, Custers JHHV, et al. Immunogenicity of recombinant adenovirus serotype 35 vaccine in the presence of preexisting anti-Ad5 immunity. J Immunol 2004;172:6290–6297.
32. Roberts DM, Nanda A, Havenga MJE, et al. Hexon-chimaeric adenovirus serotype 5 vectors circumvent preexisting anti-vector immunity. Nature 2006;441:239–243.
33. Abe S, Okuda K, Ura T, et al. Adenovirus type 5 with modified hexons induces robust transgene-specific immune responses in mice with preexisting immunity against adenovirus type 5. J Gene Med 2009;11:570–579.
34. Shiratsuchi T, Rai U, Krause A, et al. Replacing adenoviral vector HVR1 with a malaria B cell epitope improves immunogenicity and circumvents preexisting immunity to adenovirus in mice. J Clin Invest 2010;120:3688–3701.
35. Bradley RR, Maxfield LF, Lynch DM, et al. Adenovirus serotype 5-specific neutralizing antibodies target multiple hexon hypervariable regions. J Virol 2012;86:1267–1272.
36. Baden LR, Walsh SR, Seaman MS, et al. First-in-human evaluation of a hexon chimeric adenovirus vector expressing HIV-1 Env (IPCAVD 002). J Infect Dis 2014;210:1052–1061.
37. Coughlan L, Bradshaw AC, Parker AL, et al. Ad5:Ad48 hexon hypervariable region substitutions lead to toxicity and increased inflammatory responses following intravenous delivery. Mol Ther 2012;20:2268–2281.
38. Teigler JE, Penaloza-MacMaster P, Obeng R, et al. Hexon hypervariable region-modified adenovirus type 5 (Ad5) vectors display reduced hepatotoxicity but induce T lymphocyte phenotypes similar to Ad5 vectors. Clin Vaccine Immunol 2014;21:1137–1144.
39. Kuhn I, Harden P, Bauzon M, et al. Directed evolution generates a novel oncolytic virus for the treatment of colon cancer. PLoS One 2008;3:e2409.
40. Holterman L, Vogels R, van der Vlugt R, et al. Novel replication-incompetent vector derived from adenovirus type 11 (Ad11) for vaccination and gene therapy: low seroprevalence and non-cross-reactivity with Ad5. J Virol 2004;78:13207–13215.
41. Wickham TJ, Tzeng E, Shears LL, et al. Increased *in vitro* and *in vivo* gene transfer by adenovirus vectors containing chimeric fiber proteins. J Virol 1997;71:8221–8229.
42. Wu H, Seki T, Dmitriev I, et al. Double modification of adenovirus fiber with RGD and polylysine motifs improves coxsackievirus-adenovirus receptor-independent gene transfer efficiency. Hum Gene Ther 2002;13:1647–1653.
43. Belousova N, Krendelchtchikova V, Curiel DT, et al. Modulation of adenovirus vector tropism via incorporation of polypeptide ligands into the fiber protein. J Virol 2002;76:8621–8631.
44. Parrott MB, Adams KE, Mercier GT, et al. Metabolically biotinylated adenovirus for cell targeting, ligand screening, and vector purification. Mol Ther 2003;8:688–700.
45. Nicklin SA, Dishart KL, Buening H, et al. Transductional and transcriptional targeting of cancer cells using genetically engineered viral vectors. Cancer Lett 2003;201:165–173.
46. Myhre S, Henning P, Friedman M, et al. Re-targeted adenovirus vectors with dual specificity; binding specificities conferred by two different Affibody molecules in the fiber. Gene Ther 2009;16:252–261.
47. Le LP, Everts M, Dmitriev IP, et al. Fluorescently labeled adenovirus with pIX-EGFP for vector detection. Mol Imaging 2004;3:105–116.
48. Crompton J, Toogood CI, Wallis N, et al. Expression of a foreign epitope on the surface of the adenovirus hexon. J Gen Virol 1994;75 (Pt 1):133–139.
49. Vigne E, Dedieu J-F, Brie A, et al. Genetic manipulations of adenovirus type 5 fiber resulting in liver tropism attenuation. Gene Ther 2003;10:153–162.
50. Wu H, Han T, Belousova N, et al. Identification of sites in adenovirus hexon for foreign peptide incorporation. J Virol 2005;79:3382–3390.
51. Campos SK, Parrott MB, Barry MA. Avidin-based targeting and purification of a protein IX-modified, metabolically biotinylated adenoviral vector. Mol Ther 2004;9:942–954.

52. Hesse A, Kosmides D, Kontermann RE, et al. Tropism modification of adenovirus vectors by peptide ligand insertion into various positions of the adenovirus serotype 41 short-fiber knob domain. J Virol 2007;81:2688–2699.

53. Haisma HJ, Grill J, Curiel DT, et al. Targeting of adenoviral vectors through a bispecific single-chain antibody. Cancer Gene Ther 2000;7:901–904.

54. Dmitriev I, Kashentseva E, Rogers BE, et al. Ectodomain of coxsackievirus and adenovirus receptor genetically fused to epidermal growth factor mediates adenovirus targeting to epidermal growth factor receptor-positive cells. J Virol 2000;74:6875–6884.

55. Kim J, Smith T, Idamakanti N, et al. Targeting adenoviral vectors by using the extracellular domain of the coxsackie-adenovirus receptor: improved potency via trimerization. J Virol 2002;76:1892–1903.

56. Kloos A, Woller N, Gürlevik E, et al. PolySia-specific retargeting of oncolytic viruses triggers tumor-specific immune responses and facilitates therapy of disseminated lung cancer. Cancer Immunol Res 2015;3:751–763.

57. Frankel AD, Pabo CO. Cellular uptake of the tat protein from human immunodeficiency virus. Cell 1988;55:1189–1193.

58. Elliott G, O'Hare P. Intercellular trafficking and protein delivery by a herpesvirus structural protein. Cell 1997;88:223–233.

59. Phelan A, Elliott G, O'Hare P. Intercellular delivery of functional p53 by the herpesvirus protein VP22. Nat Biotechnol 1998;16:440–443.

60. Normand N, van Leeuwen H, O'Hare P. Particle formation by a conserved domain of the herpes simplex virus protein VP22 facilitating protein and nucleic acid delivery. J Biol Chem 2001;276:15042–15050.

61. Eguchi A, Akuta T, Okuyama H, et al. Protein transduction domain of HIV-1 Tat protein promotes efficient delivery of DNA into mammalian cells. J Biol Chem 2001;276:26204–26210.

62. Kühnel F, Schulte B, Wirth T, et al. Protein transduction domains fused to virus receptors improve cellular virus uptake and enhance oncolysis by tumor-specific replicating vectors. J Virol 2004;78:13743–13754.

63. Delgado C, Francis GE, Fisher D. The uses and properties of PEG-linked proteins. Crit Rev Ther Drug Carrier Syst 1992;9:249–304.

64. Parveen S, Sahoo SK. Nanomedicine: clinical applications of polyethylene glycol conjugated proteins and drugs. Clin Pharmacokinet 2006;45:965–988.

65. O'Riordan CR, Lachapelle A, Delgado C, et al. PEGylation of adenovirus with retention of infectivity and protection from neutralizing antibody *in vitro* and *in vivo*. Hum Gene Ther 1999;10: 1349–1358.

66. Menezes KM, Mok HS, Barry MA. Increased transduction of skeletal muscle cells by fibroblast growth factor-modified adenoviral vectors. Hum Gene Ther 2006;17:314–320.

67. Fisher KD, Stallwood Y, Green NK, et al. Polymer-coated adenovirus permits efficient retargeting and evades neutralising antibodies. Gene Ther 2001;8:341–348.

68. Subr V, Kostka L, Selby-Milic T, et al. Coating of adenovirus type 5 with polymers containing quaternary amines prevents binding to blood components. J Control Release 2009;135:152–158.

69. Green NK, Herbert CW, Hale SJ, et al. Extended plasma circulation time and decreased toxicity of polymer-coated adenovirus. Gene Ther 2004;11:1256–1263.

70. Kreppel F, Gackowski J, Schmidt E, et al. Combined genetic and chemical capsid modifications enable flexible and efficient de- and retargeting of adenovirus vectors. Mol Ther 2005;12:107–117.

71. Corjon S, Wortmann A, Engler T, et al. Targeting of adenovirus vectors to the LRP receptor family with the high-affinity ligand RAP via combined genetic and chemical modification of the pIX capsomere. Mol Ther 2008;16:1813–1824.

72. Prill J-M, Espenlaub S, Samen U, et al. Modifications of adenovirus hexon allow for either hepatocyte detargeting or targeting with potential evasion from Kupffer cells. Mol Ther 2011;19:83–92.

73. Krutzke L, Prill JM, Engler T, et al. Substitution of blood coagulation factor X-binding to Ad5 by position-specific PEGylation: preventing vector clearance and preserving infectivity. J Control Release 2016;235:379–392.

74. Khare R, Reddy VS, Nemerow GR, et al. Identification of adenovirus serotype 5 hexon regions that interact with scavenger receptors. J Virol 2012;86:2293–2301.

75. Prill J-M, Subr V, Pasquarelli N, et al. Traceless bioresponsive shielding of adenovirus hexon with HPMA copolymers maintains transduction capacity *in vitro* and *in vivo*. PLoS One 2014;9:e82716.

76. Khuri FR, Nemunaitis J, Ganly I, et al. a controlled trial of intratumoral ONYX-015, a selectively-replicating adenovirus, in combination with cisplatin and 5-fluorouracil in patients with recurrent head and neck cancer. Nat Med 2000;6:879–885.

77. van Erp EA, Kaliberova LN, Kaliberov SA, et al. Retargeted oncolytic adenovirus displaying a single variable domain of camelid heavy-chain-only antibody in a fiber protein. Mol Ther Oncolytics 2015;2:15001.

78. Zafar S, Parviainen S, Siurala M, et al. Intravenously usable fully serotype 3 oncolytic adenovirus coding for CD40L as an enabler of dendritic cell therapy. Oncoimmunology 2017;6:e1265717.

79. Sobol PT, Boudreau JE, Stephenson K, et al. Adaptive antiviral immunity is a determinant of the therapeutic success of oncolytic virotherapy. Mol Ther 2011;19:335–344.

80. Woller N, Gürlevik E, Ureche C-I, et al. Oncolytic viruses as anticancer vaccines. Front Oncol 2014;4:188.

81. Piccolo P, Annunziata P, Mithbaokar P, et al. SR-A and SREC-I binding peptides increase HDAd-mediated liver transduction. Gene Ther 2014;21:950–957.

82. Koski A, Rajecki M, Guse K, et al. Systemic adenoviral gene delivery to orthotopic murine breast tumors with ablation of coagulation factors, thrombocytes and Kupffer cells. J Gene Med 2009;11:966–977.

83. Nguyen TV, Heller GJ, Barry ME, et al. Evaluation of polymer shielding for adenovirus serotype 6 (Ad6) for

systemic virotherapy against human prostate cancers. Mol Ther Oncolytics 2016;3.

84. Wisse E, Jacobs F, Topal B, et al. The size of endothelial fenestrae in human liver sinusoids: implications for hepatocyte-directed gene transfer. Gene Ther 2008;15:1193–1199.

85. Carlisle RC, Di Y, Cerny AM, et al. Human erythrocytes bind and inactivate type 5 adenovirus by presenting Coxsackie virus-adenovirus receptor and complement receptor 1. Blood 2009;113:1909–1918.

86. Rojas LA, Moreno R, Calderón H, et al. Adenovirus coxsackie adenovirus receptor-mediated binding to human erythrocytes does not preclude systemic transduction. Cancer Gene Ther 2016;23:411–414.

87. Uusi-Kerttula H, Hulin-Curtis S, Davies J, et al. Oncolytic Adenovirus: strategies and insights for vector design and immuno-oncolytic applications. Viruses 2015;7:6009–6042.

88. Xu Z, Qiu Q, Tian J, et al. Coagulation factor X shields adenovirus type 5 from attack by natural antibodies and complement. Nat Med 2013;19:452–457.

89. Rea D, Schagen FH, Hoeben RC, et al. Adenoviruses activate human dendritic cells without polarization toward a T-helper type 1-inducing subset. J Virol 1999;73:10245–10253.

90. Crosby CM, Matchett WE, Anguiano-Zarate SS, et al. Replicating single-cycle adenovirus vectors generate amplified influenza vaccine responses. J Virol 2017;91.

91. 91. Mercier S, Verhaagh S, Goudsmit J, et al. Adenovirus fibre exchange alters cell tropism *in vitro* but not transgene-specific T CD8+ immune responses *in vivo*. J Gen Virol 2004;85:1227–1236.

92. Rea D, Havenga MJ, van Den Assem M, et al. Highly efficient transduction of human monocyte-derived dendritic cells with subgroup B fiber-modified adenovirus vectors enhances transgene-encoded antigen presentation to cytotoxic T cells. J Immunol 2001;166:5236–5244.

93. Worgall S, Busch A, Rivara M, et al. Modification to the capsid of the adenovirus vector that enhances dendritic cell infection and transgene-specific cellular immune responses. J Virol 2004;78:2572–2580.

94. Kron MW, Espenlaub S, Engler T, et al. miRNA-mediated silencing in hepatocytes can increase adaptive immune responses to adenovirus vector-delivered transgenic antigens. Mol Ther 2011;19:1547–1557.

95. Leen AM, Christin A, Khalil M, et al. Identification of hexon-specific CD4 and CD8 T-cell epitopes for vaccine and immunotherapy. J Virol 2008;82:546–554.

96. Barnes E, Folgori A, Capone S, et al. Novel adenovirus-based vaccines induce broad and sustained T cell responses to HCV in man. Sci Transl Med 2012;4:115ra1.

97. Biswas S, Choudhary P, Elias SC, et al. Assessment of humoral immune responses to blood-stage malaria antigens following ChAd63-MVA immunization, controlled human malaria infection and natural exposure. PLoS One 2014;9:e107903.

98. Herath S, Le Heron A, Colloca S, et al. Analysis of T cell responses to chimpanzee adenovirus vectors encoding HIV gag–pol–nef antigen. Vaccine 2015;33:7283–7289.

99. Norrby E. The relationship between the soluble antigens and the virion of adenovirus type 3. I. Morphological characteristics. Virology 1966;28:236–248.

100. Norrby E. The relationship between the soluble antigens and the virion of adenovirus type 3. II. Identification and characterization of an incomplete hemagglutinin. Virology 1966;30:608–617.

101. Schoehn G, Fender P, Chroboczek J, et al. Adenovirus 3 penton dodecahedron exhibits structural changes of the base on fibre binding. EMBO J 1996;15:6841–6846.

102. Fender P, Ruigrok RW, Gout E, et al. Adenovirus dodecahedron, a new vector for human gene transfer. Nat Biotechnol 1997;15:52–56.

103. Wortmann A, Vöhringer S, Engler T, et al. Fully detargeted polyethylene glycol-coated adenovirus vectors are potent genetic vaccines and escape from preexisting anti-adenovirus antibodies. Mol Ther 2008;16:154–162.

104. Worgall S, Krause A, Rivara M, et al. Protection against *P. aeruginosa* with an adenovirus vector containing an OprF epitope in the capsid. J Clin Invest 2005;115:1281–1289.

105. 105. Wee S, Hicks MJ, De BP, et al. Novel cocaine vaccine linked to a disrupted adenovirus gene transfer vector blocks cocaine psychostimulant and reinforcing effects. Neuropsychopharmacology 2012;37:1083–1091.

106. Maoz A, Hicks MJ, Vallabhjosula S, et al. Adenovirus capsid-based anti-cocaine vaccine prevents cocaine from binding to the nonhuman primate CNS dopamine transporter. Neuropsychopharmacology 2013;38:2170–2178.

107. Kron MW, Kreppel F. Adenovirus vectors and subviral particles for protein and peptide delivery. Curr Gene Ther 2012;12:362–373.

108. Naskalska A, Szolajska E, Chaperot L, et al. Influenza recombinant vaccine: matrix protein M1 on the platform of the adenovirus dodecahedron. Vaccine 2009;27:7385–7393.

109. Villegas-Mendez A, Garin MI, Pineda-Molina E, et al. *In vivo* delivery of antigens by adenovirus dodecahedron induces cellular and humoral immune responses to elicit antitumor immunity. Mol Ther 2010;18:1046–1053.

110. Suleman M, Galea S, Gavard F, et al. Antigen encoded by vaccine vectors derived from human adenovirus serotype 5 is preferentially presented to CD8+ T lymphocytes by the CD8a+ dendritic cell subset. Vaccine 2011;29:5892–5903.

111. Cheng C, Gall JGD, Kong W, et al. Mechanism of ad5 vaccine immunity and toxicity: fiber shaft targeting of dendritic cells. PLoS Pathog 2007;3:e25.

Received for publication July 31, 2017;
accepted after revision August 30, 2017.

Published online: August 30, 2017.

LC/MS of Intact Adeno-Associated Virus Capsid Proteins for Rapid Confirmation of Product Identity

Author | Brian Liau, Agilent Technologies, Inc.

Introduction

Adeno-associated viruses (AAVs) are a promising new class of biotherapeutic capable of treating a host of rare and intractable genetic diseases such as spinal muscular atrophy and inherited retinal degeneration.[1] AAVs are large molecular complexes consisting of a protein capsid and encapsulated DNA, with each requiring dedicated analytical techniques to ensure the quality and safety of the overall product. AAV capsids comprise three different capsid proteins, commonly denoted VP1–3, in approximately a 1:1:10 stoichiometric ratio with a total of 60 copies of protein per capsid.[2]

The U.S. Food and Drug Administration recommends viral capsids and encapsulated DNA of all AAV therapeutics to be unambiguously identified before release, especially in facilities where multiple serotypes or engineered variants are produced.[3] To date, antibody-based methods such as ELISA and immunoblotting have been the most common techniques for viral capsid analysis. These methods suffer from several drawbacks: in addition to being cumbersome and error-prone, antibody-based methods require highly specific antibodies to be generated for each type of AAV being analyzed. This is challenging because AAV capsids from different products may have a high degree of homology – for example, AAV serotypes 1 and 6 differ by only six amino acids (99% homology), making them difficult to distinguish by antibody binding.[4]

In principle, LC/MS offers the ideal combination of speed and specificity for viral capsid protein analysis because direct measurement of protein masses obviates the need to generate antibodies for each type of AAV. However, previous efforts have suffered from relatively poor chromatographic separation of virus capsid proteins.[5] This is problematic because coelution may compromise signal intensity and mass accuracy, in addition to precluding accurate quantitation of capsid stoichiometry, which is an important determinant of AAV infectivity.[6]

This application note demonstrates an optimized LC/FLD-MS method for the rapid analysis of AAV capsid proteins to confirm the primary sequences of seven different serotypes. This method has simple sample preparation requirements and is robust to high salt conditions as well as the common nonionic detergent additive Pluronic F-68.

Experimental

AAV Samples

AAV serotypes 2, 7, 9, 7m8, DJ, rh10, and Anc80 were purchased from the Vector Core @ GIS (A*STAR, Singapore). AAV samples were expressed in HEK-293 cells by triple transfection and purified by analytical ultracentrifugation. Samples were provided at a concentration of ~1.2 × 10^{10} viral genomes/µL in phosphate-buffered saline containing 0.001% Pluronic F-68 and used as received without buffer exchange.

Sample preparation

A denaturation buffer consisting of 12 M guanidine HCl + 40 mM DTT in 200 mM ammonium bicarbonate (pH ~8.0) was freshly prepared for each analysis. Denaturation buffer was added to AAV samples in a 1:3 ratio to achieve a final guanidine HCl concentration of 3 M. Samples were then heated to 70 °C for 15 minutes to ensure complete denaturation. 1.5 × 10^{11} viral genomes were injected per analysis.

LC/FLD-MS for intact capsid protein analysis

Samples were analyzed using the following instrumentation:

- Agilent 1290 Infinity II LC system including:
  - Agilent 1290 Infinity II High Speed Pump (G7120A)
  - Agilent 1290 Infinity II Multisampler (G7167B)
  - Agilent 1290 Infinity II Multicolumn Thermostat (G7116B)
  - Agilent 1260 Infinity Fluorescence Detector (G1321B) with 8 µL flow cell
- Agilent 6545XT AdvanceBio LC/Q-TOF

For separation, Agilent ZORBAX RRHD 300Å StableBond C18 (SB-C18) and SB-Diphenyl columns with dimensions 2.1 × 100 mm, 1.8 µm (part numbers 858750-902 and 858750-944) were used. A ZORBAX RRHD 300Å SB-C3 column (part number 858750-909) was also used for development, but not the optimized method.

A high eluotropic strength mobile phase consisting of 0.1% TFA + 0.1% FA in DI water (mp A) and 90% isopropyl alcohol + 9.8% water + 0.1% TFA + 0.1% FA (mp B) was chosen. In combination with high temperature, similar mobile phase conditions have achieved superior resolving power on ZORBAX StableBond columns for monoclonal antibody analysis.[7]

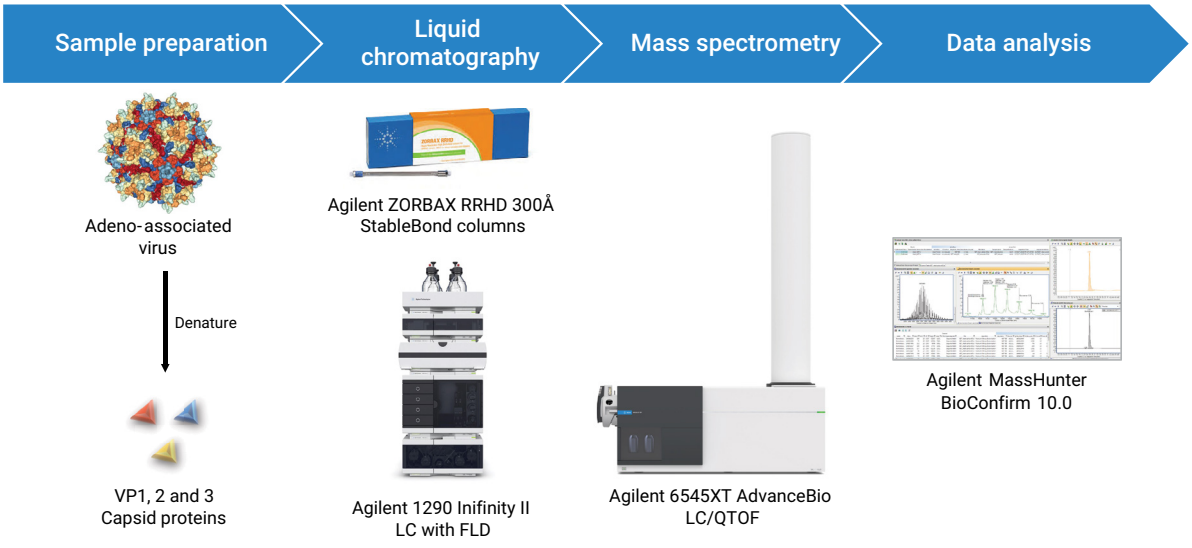


Figure 1. Equipment and consumables used in this application note.

Table 1. Intact capsid protein chromatographic parameters.

Agilent 1290 Infinity II LC System						
Condition	Testing	Optimized	Testing	Optimized	Testing	Optimized
Column	Agilent ZORBAX RRHD 300Å StableBond C18, 2.1 × 100 mm, 1.8 µm (p/n 858750-902)		Agilent ZORBAX RRHD 300Å StableBond Diphenyl, 2.1 × 100 mm, 1.8 µm (p/n 858750-944)		Agilent ZORBAX RRHD 300Å StableBond C3, 2.1 × 100 mm, 1.8 µm (p/n 858750-909)	
Solvent A	0.1 % FA+ 0.1% TFA in DI water	0.1 % FA+ 0.1% TFA in DI water	0.1 % FA+ 0.1% TFA in DI water	0.1 % FA+ 0.1% TFA in DI water	0.1 % FA+ 0.1% TFA in DI water	–
Solvent B	80% IPA + 10% ACN + 9.8% DI water + 0.1% FA + 0.1% TFA	90% IPA + 9.8% DI water + 0.1%FA + 0.1%TFA	80% IPA + 10% ACN + 9.8% DI water + 0.1% FA + 0.1% TFA	90% IPA + 9.8% DI water + 0.1%FA + 0.1% TFA	80% IPA + 10% ACN + 9.8% DI water + 0.1% FA + 0.1% TFA	–
Gradient	0 to 2 min, 20% B 42 min, 35% B 42.5 min, 80% B 45 min, 80% B	0 to 5 min, 28% B 23 min, 32.5% B 23.5 min, 80% B 26 min, 80% B	0 to 2 min, 28% B 42 min, 43% B 42.5 min, 80% B 45 min, 80% B	0 to 5 min, 33% B 21 min, 37% B 21.5 min, 80% B 23 min, 80% B	0 to 2 min, 20% B 42 min, 35% B 42.5 min, 80% B 45 min, 80% B	–
Column Temperature	75 °C	80 °C	75 ˚C	80 °C	75 ˚C	–
Flow Rate	0.4 mL/min					
Sample Quantity	1.5 × 10^{11} Viral genomes/injection					

Table 2. Intact capsid protein mass spectrometry parameters.

Agilent 6545XT AdvanceBio LC/Q-TOF System	
Source	Agilent Dual Jet Stream
Gas Temperature	350 ˚C
Gas Flow	12 L/min
Nebulizer	35 psig
Sheath Gas Temperature	350 ˚C
Sheath Gas Flow	11 L/min
Vcap	4 kV
Nozzle Voltage	2 kV
Fragmentor	180 V
Skimmer	65 V
Mass Range	900 to 3,200 *m/z*
Acquisition Rate	1 spectrum/sec
Reference Mass	922.0098
Acquisition Mode	Positive, 3,200 *m/z* mass range, Extended dynamic range (2 Ghz)

Table 3. Intact capsid protein deconvolution settings.

Agilent MassHunter BioConfirm B10.0 Settings	
Defined Chromatograms	FLD
Integrate When Extracted	Yes
Adjust Delay Time	0.1 min (MS Detector)
Deconvolution	Maximum entropy
Deconvolution *m/z* Range	1,000 to 3,200
Deconvolution Mass Range	55 to 85 kDa
Mass Step	0.5 Da
Deconvolution Subtract Baseline	7.0
Match Tolerance	32 ppm

For fluorescence detection, an 8 µL flow cell with single excitation (280 nm)/multiple emission (315, 330, 345, and 360 nm) mode was used as the emission spectra of aromatic residues may vary depending on number and the degree of solvent exposure. Emission at 360 nm was found to be most intense and was therefore used for all analyses. All data analysis was carried out in Agilent BioConfirm 10.0. Tables 1, 2, and 3 show the instrument and software settings.

LC/MS peptide mapping analysis

A 10 µL amount of sample containing 6.0 × 10^{11} viral genomes of AAV 2 (~3.6 µg of protein) was denatured and reduced in 30 µL of 150 mM Tris-HCl (pH 8) containing 760 mg/mL guanidine HCl + 3.8 mg/mL TCEP for 60 minutes at 60 °C. After cooling to room temperature, alkylation was performed by adding 10 µL of 133 mM iodoacetamide and incubating at room temperature in darkness for 30 minutes. The sample was then diluted with 210 µL of Tris-HCl, after which 0.2 µg of Promega's Sequencing Grade Modified Trypsin (part number V5117) was added. Digestion was carried out for 2 hours at 37 °C, after which a further 0.2 µg of trypsin was added before digesting overnight at 37 °C. The following day, the reaction was stopped by adding 30 µL of 10% formic acid, followed by C-18 cleanup using an Agilent AssayMap Bravo system (G5542A). Peptide mapping was performed on a 1290 Infinity

Table 4. Peptide mapping chromatographic parameters.

Agilent 1290 Infinity II LC System	
Solvent A	0.1% FA in DI water
Solvent B	0.1% FA in Acetonitrile
Gradient	0 to 15 min, 5 to 40% B 15 to 18 min, 40 to 90% B 18 to 20 min, 90% B
Column Temperature	60 °C
Flow Rate	0.4 mL/min
Injection Volume	20 µL

II LC with 6545XT AdvanceBio LC/Q-TOF. An AdvanceBio Peptide Mapping column (part number 653750-902) with dimensions 2.1 × 150 mm, 2.7 µm was used for separation. Tables 4 and 5 show the instrument settings.

Results and discussion

LC/MS method development

AAV-2 was used for method development as it is the most well-studied serotype. The sequences of capsid proteins VP1–3 are shown in Figure 2. All capsid proteins are transcribed from the same DNA sequence in the Cap gene, with mRNA splicing and leaky ribosomal scanning resulting in three proteins with a high degree of homology.[6] Specifically, the VP3 sequence is common to all three proteins, with VP1 and VP2 differing only in their N-terminal sequences.

Table 5. Peptide mapping mass spectrometry parameters.

Agilent 6545XT AdvanceBio LC/Q-TOF System	
Source	Agilent Dual Jet Stream
Gas Temperature	290 °C
Gas Flow	13 L/min
Nebulizer	35 psig
Sheath Gas Temperature	275 °C
Sheath Gas Flow	12 L/min
Vcap	4 kV
Nozzle Voltage	2 kV
Fragmentor	175 V
Skimmer	65 V
Mass Range	100 to 1,700 *m/z*
Acquisition Rate	5 spectra / sec
Auto MS/MS Range	50 to 1,700 *m/z*
Min. MS/MS Acquisition Rate	3 spectra / sec

Isolation Width	Medium (~4 *m/z*)
Precursors/Cycle	Top 10
Collision Energy	3.6*(*m/z*)/100 – 4.8
Threshold for MS/MS	On; 3 repeat then exclude for 0.2 minutes
Precursor Abundance-Based Scan Speed	Yes
Target	25,000
Use MS/MS Accumulation Time Limit	Yes
Purity	100% stringency, 30% cutoff
Isotope model	Peptides
Sort precursors	By abundance only; +2, +3, >+3
Reference mass	922.0098
Acquisition mode	Positive, 3,200 *m/z* mass range

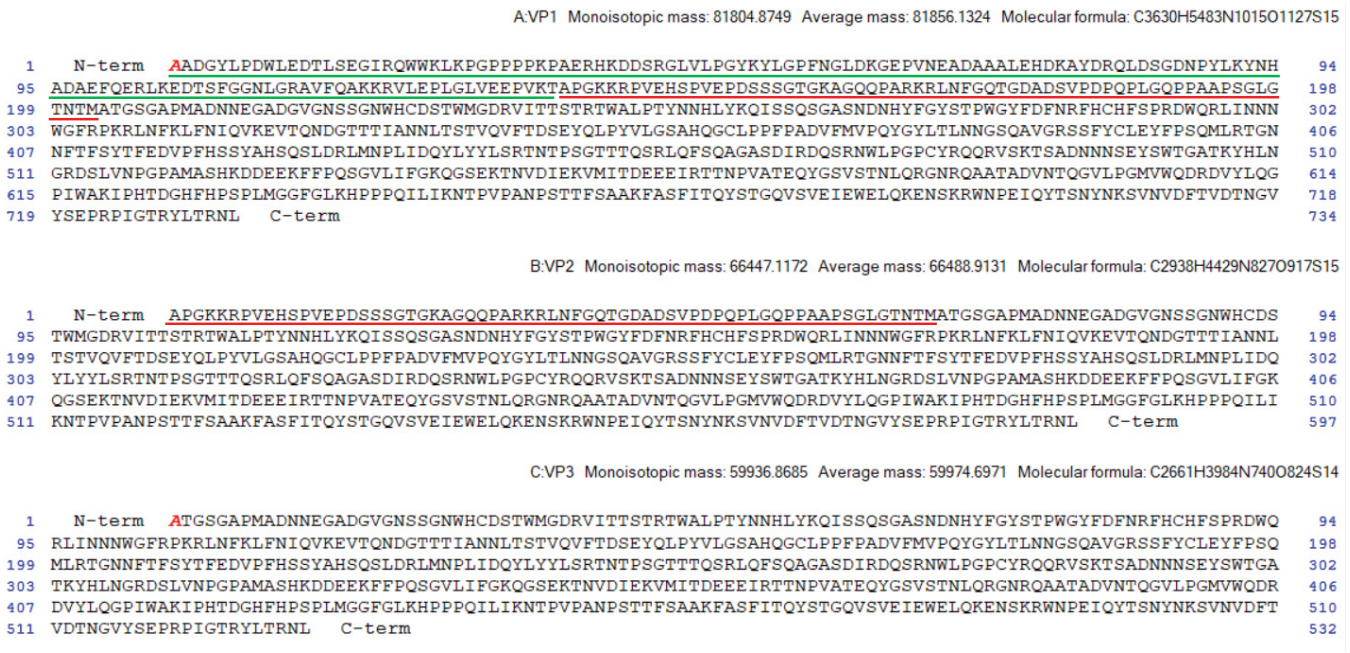


Figure 2. Primary amino acid sequence of AAV-2 capsid proteins. Shown in Agilent MassHunter Sequence Manager software, VP1–3 differ only in their N-terminal sequences: each contains the VP3 sequence, with an additional N-terminal sequence underlined in red (VP2) or red and green (VP1). The monoisotopic mass, average mass, and molecular formula are shown above for each protein.

As published elsewhere,[5] these post-translational modifications were anticipated: (i) removal of the initiator methionine on VP1 and VP3, and (ii) acetylation of the amino acid immediately following the removed methionine. Acetylated amino acids were indicated in Agilent MassHunter Sequence Manager software as red, italicized letters, and the theoretical average masses of VP1–3 were 81,856.13, 66,488.91, and 59,974.70 Da respectively (Figure 2).

Denatured AAV-2 samples were separated on ZORBAX RRHD 300Å SB-C3, SB-Diphenyl and SB-C18 columns (Figures 3A to 3C) using the Testing gradients shown in Table 1. VP1–3 separated as two peaks on SB-C3 and SB-Diphenyl columns, with VP1 and VP3 coeluting as a single peak. Only the SB-C18 column possessed the selectivity necessary to separate each capsid protein.

Deconvoluted mass spectra of the VP1 + VP3 peak (Figures 3D to 3E) showed a far more intense VP3 signal at 59,975.52 Da compared to VP1, reflecting its order of magnitude greater abundance. The coelution of VP3 may constitute a matrix effect, potentially interfering with detection and mass accuracy of VP1 through ion suppression.[8] In contrast, the superior separation of VP1 on the SB-C18 column (Figure 3F) greatly reduced interference by VP3, permitting reliable and accurate mass spectrometry analysis of VP1.

As shown in Figure 4, AAV-2 samples were then separated on a SB-C18 column using the optimized gradient shown in Table 1, improving peak resolution and shape and shortening analysis time. Note that the slight background undulation in the total ion current was likely caused by residual Pluronic F-68, which did not interfere significantly with the analysis.

Importantly, the observed deconvoluted mass of VP1 was 81,885.72 Da (Figure 4E), which was +29.6 Da greater than the theoretical value of 81,856.13 Da. In contrast, the observed masses of VP2 and VP3 were in good agreement with their theoretical values. Therefore it was hypothesized that a single amino acid substitution had occurred in the N-terminal region of VP1 (sequence underlined in green, Figure 2).

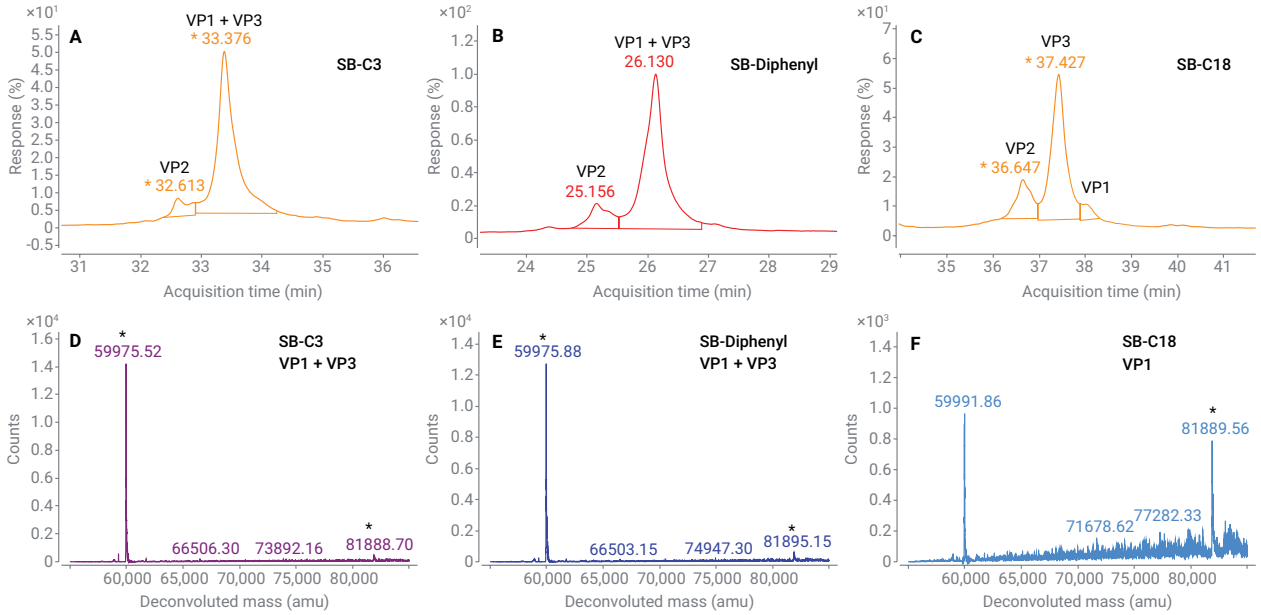


Figure 3. Method development with AAV-2. Fluorescence chromatograms depicting separation using (A) Agilent ZORBAX RRHD 300Å SB-C3, (B) SB-Diphenyl, and (C) SB-C18 columns. Note the superior chromatographic resolution of SB-C18. (D to F) Deconvoluted mass spectra of chromatographic peaks showing the coelution of VP1 and VP3.

Confirmation of single amino acid substitution in AAV-2

A trypitc digestion and peptide mapping of AAV-2 was performed to test this hypothesis. Identification was made of a triply charged 1,102.1864 m/z peptide whose MS/MS spectrum confirmed an alanine & threonine substitution at amino acid 77 of the VP1 sequence, which was indeed located in the N-terminal region (Figure 5).

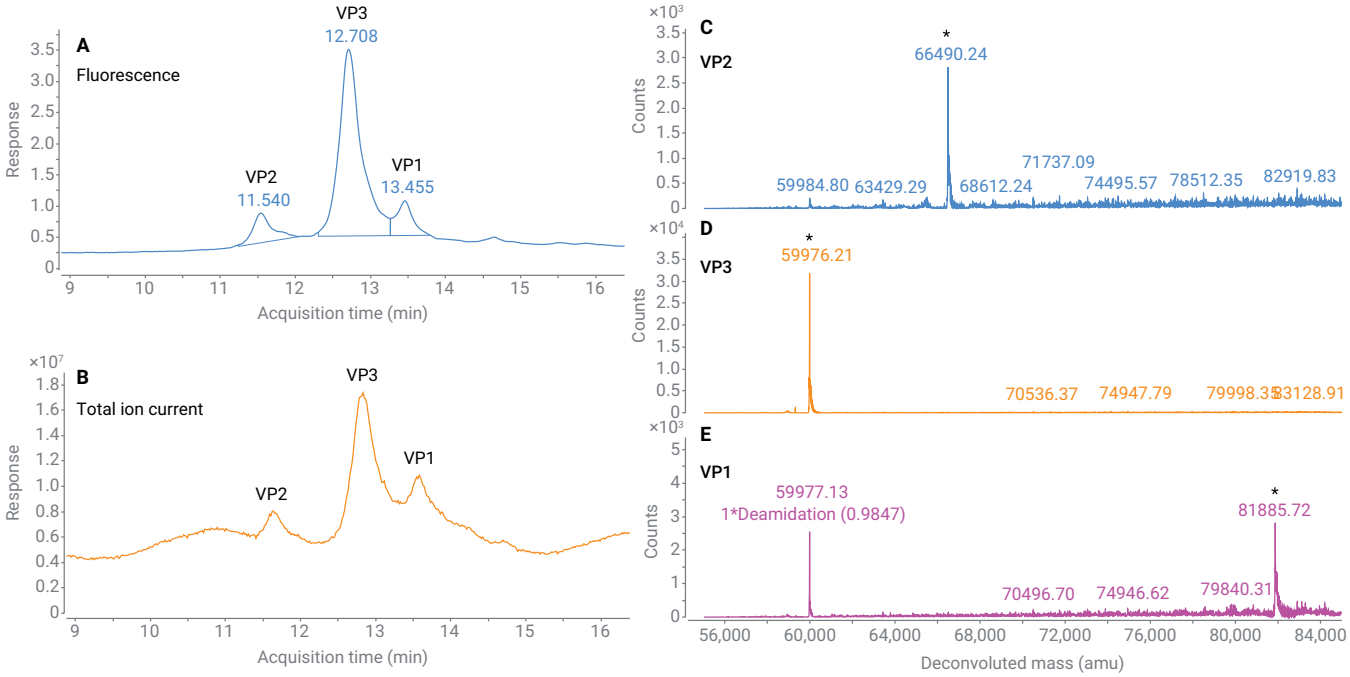


Figure 4. LC/MS of denatured AAV-2 capsid proteins using the optimized method on an Agilent ZORBAX RRHD 300Å SB-C18 column. (A) Fluorescence chromatogram depicting the three capsid proteins. (B) Total ion current. (C to E) Deconvoluted mass spectra of the three capsid proteins, with the relevant mass peaks marked by asterisks.

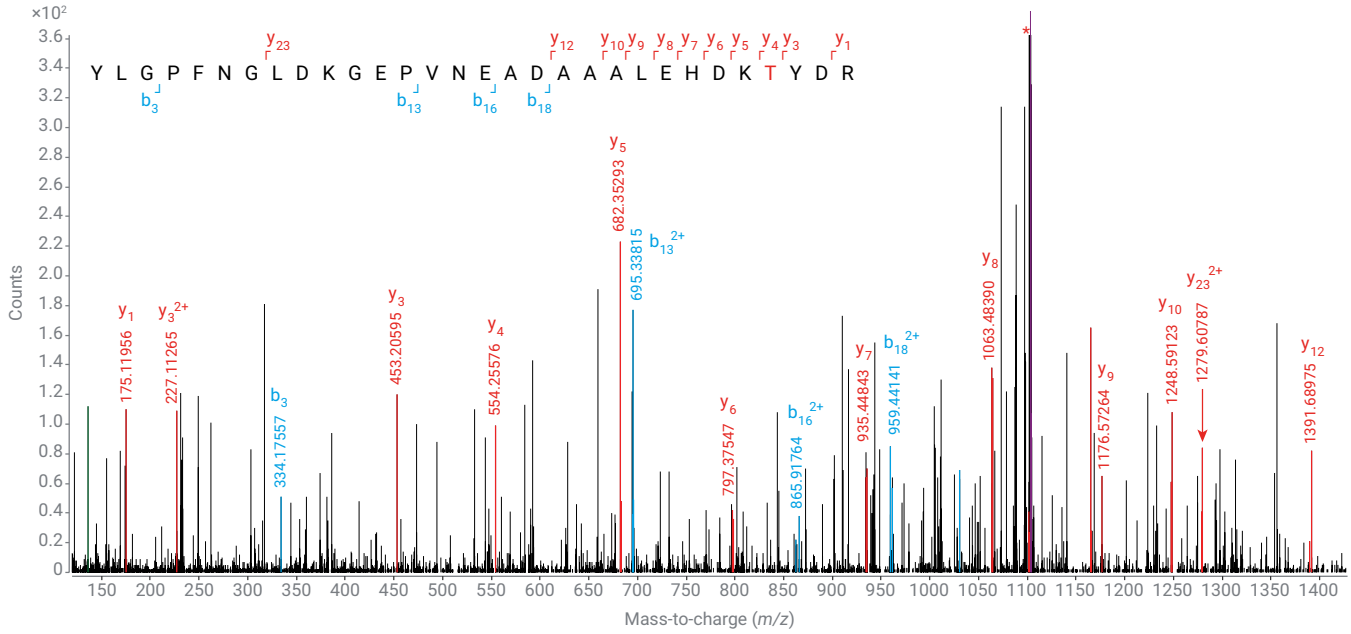


Figure 5. Confirmation of alanine & threonine substitution in VP1 by MS/MS. The substituted amino acid is shown in red font, and the precursor ion is marked by a red asterisk.

Orthogonal confirmation of this mutation was obtained by Sanger sequencing of the plasmid used for AAV-2 expression, which showed that a G & A substitution had altered a GCC codon to ACC (data not shown).

Analysis of six additional AAV serotypes

To assess the generalizability of this LC/MS method to the analysis of other AAV serotypes, AAV-7, 7m8, DJ, 9, rh10, and Anc80 were analyzed using the optimized SB-C18 method described above. AAV-7, 7m8 and DJ separated well using this method (Figures 6A to 6C), but AAV-9, rh10, and Anc80 separated as only two peaks, with VP1 and VP3 coeluting as single peaks.

Agilent diphenyl stationary phases have altered selectivity compared to alkyl chain stationary phases such as C3, C8, and C18. The phenyl groups permit separation based on π-π interactions with additional affinity for aromatic amino acids and double bonds.[9] An optimized SB-Diphenyl method (Table 1) was developed to achieve successful separation of AAV-9, rh10, and Anc80 (Figures 6D to 6F). The identity of each capsid protein was automatically matched by BioConfirm software using the deconvoluted mass spectrum from each peak, which showed minimal interference from neighboring peaks (Figures 7 and 8).

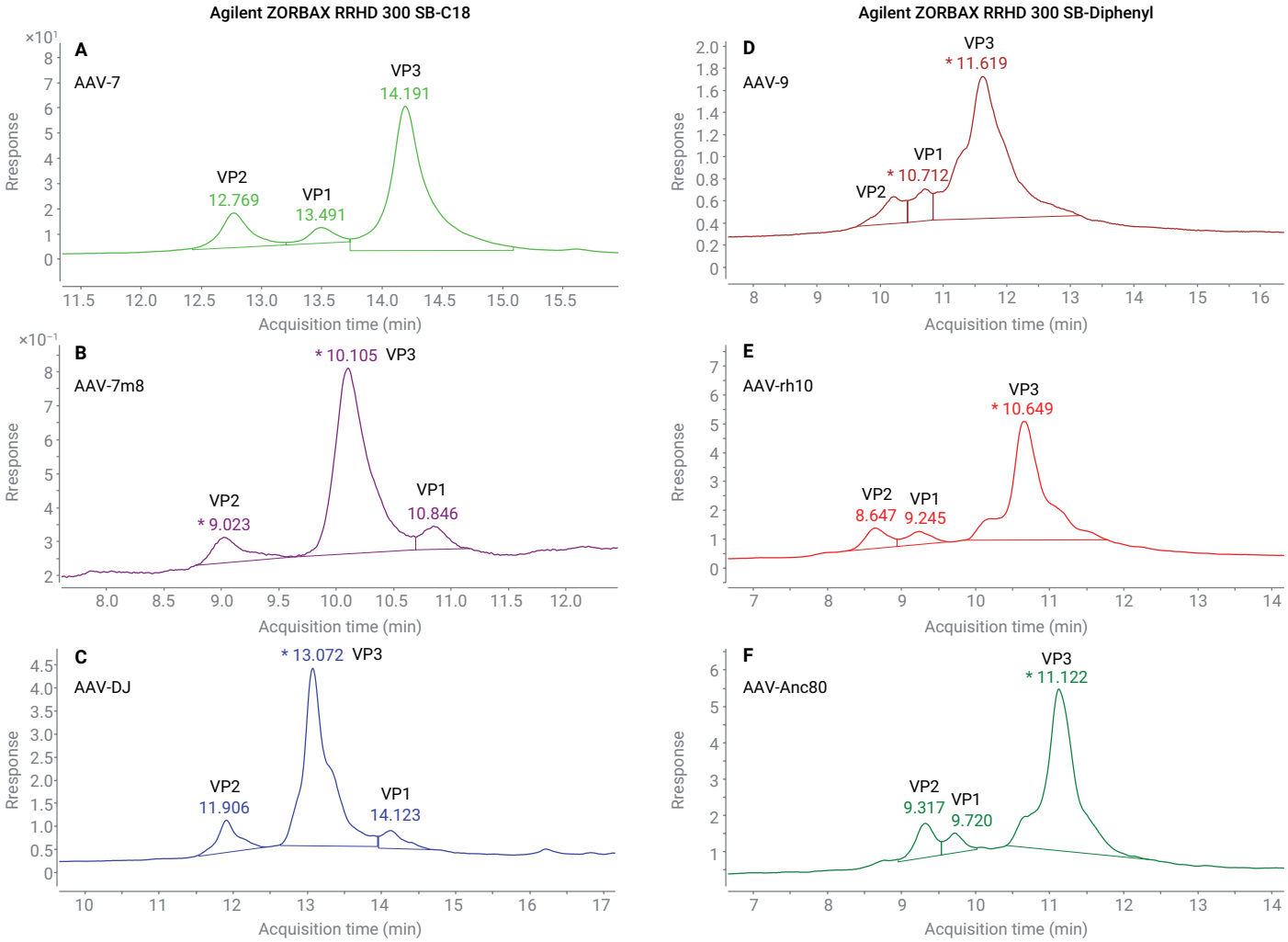


Figure 6. LC/MS of denatured AAV capsid proteins of six different serotypes on (A to C) Agilent ZORBAX RRHD 300 SB-C18, and (D to F) Agilent SB-Diphenyl columns.

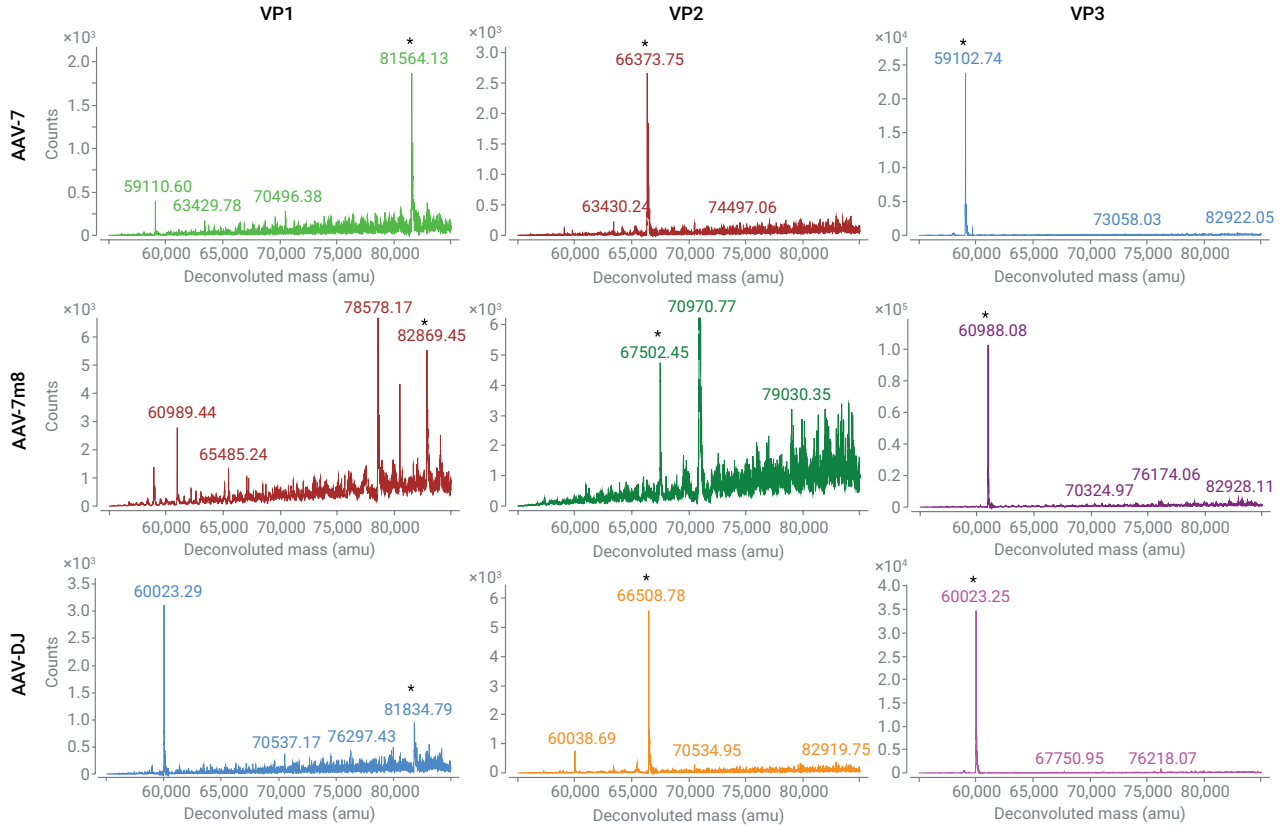


Figure 7. Deconvoluted mass spectra of AAV capsid proteins separated on an Agilent ZORBAX RRHD 300 SB-C18 column. Some interference from coeluting host cell proteins was seen in AAV-7m8 (70971.71, 78578.01, and 80495.10 Da).

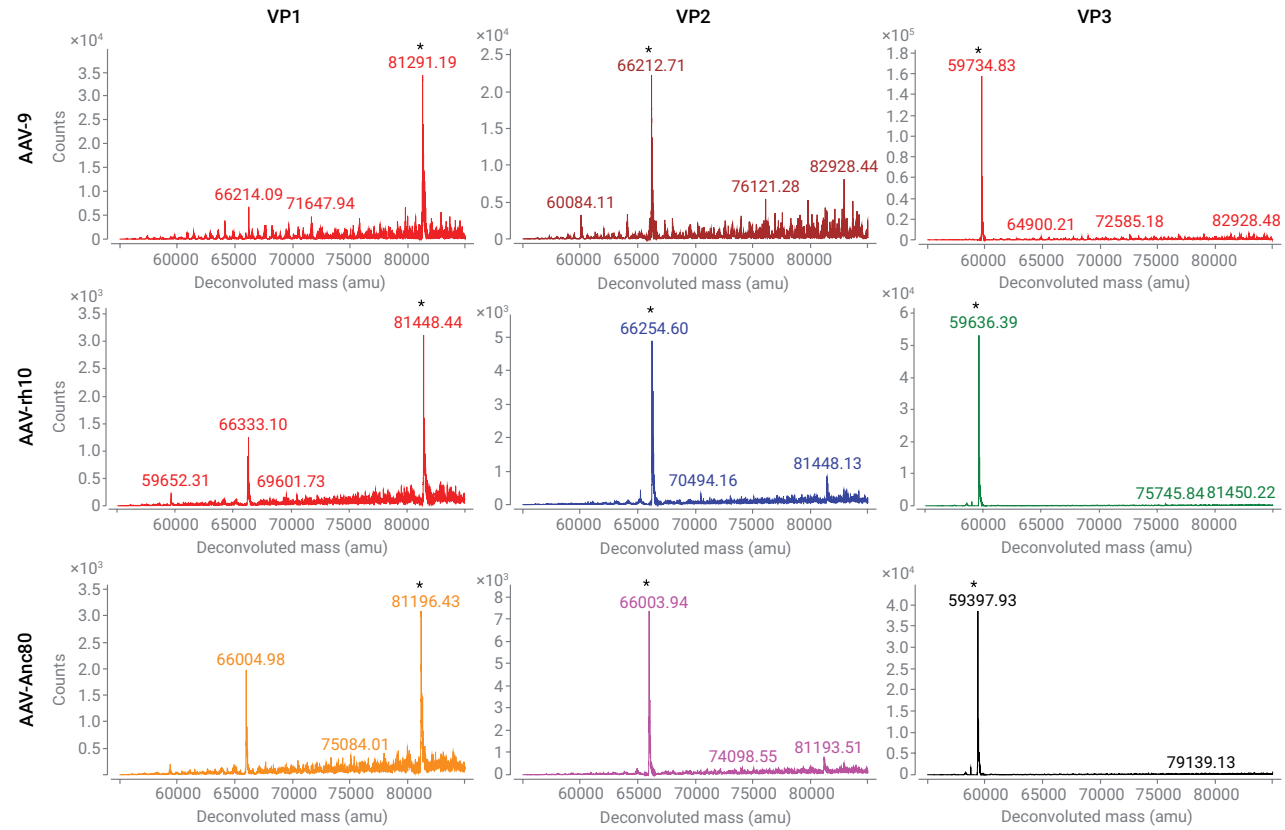


Figure 8. Deconvoluted mass spectra of AAV capsid proteins separated on an Agilent ZORBAX RRHD 300 SB-Diphenyl column.

Deamidations were putatively assigned to VP1 proteins of AAV-DJ and rh10, which could be due to cell culture conditions or generated during sample preparation as artifacts of heating. Except for AAV-2, the theoretical and observed masses for each capsid protein (Table 6) differed by ≤3 Da, corresponding to ≤32 ppm.

The relative quantities of VP1–3 in Table 7 were measured by integrating the fluorescence chromatogram peaks (Figure 6) corresponding to each capsid protein. While these results generally conformed to the expected VP1:2:3 stoichiometry of 1:1:10, the abundance of VP1 appeared to be more variable than VP2 across the different samples tested. As VP1 is known to be a critical nuclear localization factor,[10] the lower abundance of VP1 in some samples may partially account for differences in infectivity.

Table 6. Deconvoluted masses of capsid proteins.

AAV Capsid Protein Masses									
	VP1			VP2			VP3		
Serotype	Theoretical	Observed	Mass Accuracy (ppm)	Theoretical	Observed	Mass Accuracy (ppm)	Theoretical	Observed	Mass Accuracy (ppm)
2	81886.16	81885.72	5.37	66488.91	66490.24	20.00	59974.70	59976.21	25.18
7	81564.08	81564.13	0.61	66372.17	66373.75	23.81	59101.05	59102.74	28.60
9	81291.57	81291.19	4.67	66210.77	66212.71	29.30	59733.54	59734.83	21.60
7m8	82868.27	82869.45	14.24	67501.05	67502.45	20.74	60986.83	60988.08	20.50
DJ	81835.21	81834.79	5.13	66508.06	66508.78	10.83	60021.86	60023.25	23.16
rh10	81445.85	81448.44	31.80	66252.96	66254.60	24.75	59634.58	59636.39	30.35
Anc80	81195.42	81196.68	15.52	66002.53	66003.80	19.24	59396.22	59397.97	29.46

Conclusion

This application note shows the development of the method for the efficient separation of intact AAV capsid proteins using the 1290 Infinity II LC in conjunction with ZORBAX RRHD 300Å SB-C18 or SB-Diphenyl columns, followed by subsequent detection and analysis using a 1260 Infinity II fluorescence detector in tandem with a 6545XT AdvanceBio LC/Q-TOF. This application note demonstrates the capabilities of this method with seven different AAV serotypes. Only a relatively short denaturation step was required for sample preparation, and no buffer exchange was required because the method was robust to high salt conditions as well as the common detergent additive Pluronic F-68.

This method significantly outperformed recent published efforts[5] in terms of chromatographic separation, and

Table 7. Relative quantities of capsid proteins.

Capsid Protein Quantification (FLD %)			
Serotype	VP1	VP2	VP3
2	10.67	10.61	78.72
7	7.16	9.81	83.03
9	8.75	8.04	83.21
7m8	7.62	9.70	82.68
DJ	8.62	11.72	79.66
rh10	6.58	9.18	84.24
Anc80	5.64	10.62	83.74

represents a more general approach to the efficient separation of AAV capsid proteins, which may coelute with one column chemistry, but resolve well on another. In comparison to another recent effort using a ZipChip CE/MS system for a similar analysis,[11] this approach achieves mass accuracy of ≤32 ppm compared to ≤60 ppm for the ZipChip. In addition, the relatively long retention time differences achieved by liquid chromatography facilitated accurate and

reproducible mass spectral deconvolution, and the inclusion of a fluorescence detector permitted sensitive and accurate relative quantitation of capsid protein abundance. In contrast, capsid proteins were shown to elute within a narrow 0.1-minute time window on the ZipChip, with the relatively slow scan rate of the mass spectrometer used in the experiment precluding accurate quantitation.

It is anticipated that this method will be useful for rapid confirmation of AAV product identity before release, as well as in screening AAV mutant libraries in discovery/development laboratories.

References

1. Keeler, A. M.; Flotte, T. R. Recombinant Adeno-Associated Virus Gene Therapy in Light of Luxturna (and Zolgensma and Glybera): Where Are We, and How Did We Get Here? *Annu. Rev. Virol.* **2019**, *6*, 601–621.
2. Backovic, A. *et al.* Capsid Protein Expression and Adeno-Associated Virus like Particles Assembly in Saccharomyces Cerevisiae. *Microb. Cell Fact* **2012**, *11*, 124.
3. Chemistry, Manufacturing, and Control (CMC) Information for Human Gene Therapy Investigational New Drug Applications (INDs) - Guidance for Industry. *US Food and Drug Administration* **2020**.
4. Kuck, D.; Kern, A.; Kleinschmidt, J. A. Development of AAV Serotype-Specific ELISAs Using Novel Monoclonal Antibodies. *Journal of Virological Methods* **2007**, *140*, 17–24.
5. Jin, X. *et al.* Direct Liquid Chromatography/Mass Spectrometry Analysis for Complete Characterization of Recombinant Adeno-Associated Virus Capsid Proteins. *Human Gene Therapy Methods* **2017**, *28*, 255–267.
6. Bosma, B. *et al.* Optimization of Viral Protein Ratios for Production of RAAV Serotype 5 in the Baculovirus System. *Gene Therapy* **2018**, *25(6)*, 415–424.
7. Rehder, D. S. *et al.* Reversed-Phase Liquid Chromatography/Mass Spectrometry Analysis of Reduced Monoclonal Antibodies in Pharmaceutics. *J. Chromatog. A* **2006**, *1102(1–2)*, 164–175.
8. Matuszewski, B. K.; Constanzer, M. L.; Chavez-Eng, C. M. Strategies for the Assessment of Matrix Effect in Quantitative Bioanalytical Methods Based on HPLC- MS/MS. *Analytical Chemistry* **2003**, *75(13)*, 3019–3030.
9. Long, W. J.; Mack, A. E. Comparison of Selectivity Differences Among Different Agilent ZORBAX Phenyl Columns Using Acetonitrile or Methanol. *Agilent Technologies application note*, publication number 5990-4711EN, **2009**.
10. Popa-Wagner, R. *et al.* Impact of VP1-Specific Protein Sequence Motifs on Adeno-Associated Virus Type 2 Intracellular Trafficking and Nuclear Entry. *Journal of Virology* **2012**, *86*, 9163–9174.
11. Zhang, Y. *et al.* Identification of Adeno-Associated Virus Capsid Proteins Using ZipChip CE/MS. *Analytical Biochemistry* **2018**, *555*, 22–25.

Mary Ann Liebert, Inc. publishers

REVIEW

Chemical Modifications of the Capsid for Redirecting and Improving the Efficacy of Adeno-Associated Virus Vectors

Anh K. Lam,[1,*,i] Dylan Frabutt,[1] Lei Li,[2] and Weidong Xiao[1]

[1]Department of Pediatrics, Herman B Wells Center for Pediatrics Research, Indiana University School of Medicine, Indianapolis, Indiana, USA; [2]Department of Chemistry, Georgia State University, Atlanta, Georgia, USA.

[i]ORCID ID (https://orcid.org/0000-0002-8830-9349).

Adeno-associated virus (AAV) vector-directed gene therapy is one of the most exciting modalities of biotechnology as more applications enter clinical stage. Although AAV vectors generally feature low toxicity, high stability, and long- lasting transgene expression, potential challenging issues of AAV include high vector dose, limited tissue tropism, and the host immune response and inflammation, which are all related to the capsid protein. To overcome these challenges, various strategies have been developed to engineer AAV capsids. Apart from widely employed genetic engineering of capsid protein, powerful and versatile chemical modification strategies are underexploited. This minireview summarizes recent advances and our perspectives for future direction in AAV capsid chemical modification to enhance its therapeutic use for gene therapy.

Keywords: adeno-associated virus, AAV, chemical modification, capsid engineering, capsid modification

Introduction

FOR THE PAST three decades, recombinant adeno-associated virus (rAAV) vectors have been proven to be successful tools for gene therapy against many genetic diseases. Wild-type (WT) adeno-associated virus (AAVs) are icosahedral single-stranded (ss) DNA parvoviruses with around 4.7 kilobase (kb) genome.[1,2] They are nonpathogenic and contain nonenveloped protein capsid. rAAV vectors have been used to conduct >200 clinical trials since 1989, with several achieving Food and Drug Administration (FDA) approval.[3] WT AAV's genome consists of three open reading frames: rep, cap, and X genes. *rep* encodes for four proteins for regulation, replication, and assembly. *cap* encodes for three overlapping capsid proteins: VP1 (90kDa), VP2 (72kDa), and VP3 (60kDa), with an abundance ratio of 1:1:10. Within an alternate reading frame of *cap*, AAP (assembly-activating protein) gene is

*Correspondence: Dr. Anh K. Lam, Department of Pediatrics, Herman B Wells Center for Pediatrics Research, Indiana University School of Medicine, Indianapolis, IN 46202- 5114, USA. E-mail: aklam@iu.edu

Reprinted with permission from: Human Gene Therapy, Vol. 32, No. 23-24, 2021; p. 1433-1437; DOI: https://doi.org/10.1089/hum.2021.124

also known to help with the capsid assembly. X gene is responsible for encoding replication supportive proteins.[4] Viral capsid sequence is responsible for attachment to host cell receptors and subsequent internalization, which determines vector tropism.[5] Owing to the requirement of high viral titers for productive transduction of the target tissue, the therapeutic index of AAV vectors is still relatively low compared with adenovirus or lentiviral- based gene therapy vectors. Their broad tissue tropism also provides an incentive for the development of vectors that can target rAAV to a specific tissue for more efficient transduction, which would reduce the titer of virus required for a therapy and also reduce adverse side effects caused by capsid immunogenicity. Although progress is underway, strategies for capsid modification to improve the clinical efficacy of AAV vectors have shown with strong potentials.

Genetic modification of AAV'S capsid

A comprehensive review by Büning and Srivastava summarizes genetic approaches on AAV capsid modification.[6] As a rational approach, incorporation of known ligands can be performed in packaging cell lines using genetic means as a VP2-fusion protein or using biochemical means such as split intein-mediated protein trans-splicing.[7] These methods can be disruptive for viral packaging, which may require further optimization for the successful production of the vector in relevant quantities for laboratory or clinical studies. Directed evolution approaches utilize AAV vectors that code for their own rep and cap and apply a selection pressure in the growth environment. Combinations of rational design and directed evolution have also been performed, where a feature

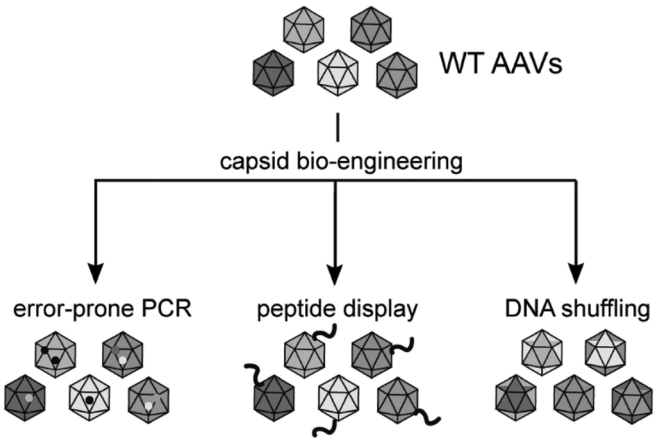


Figure 1. Summary of genetic modification approaches for AAV capsids. Generation of highly diverse mutants from either error-prone PCR, peptide display library, or DNA shuffling among different capsid serotypes offer *in vivo* selection screenings. Enhanced AAV transduction and/or evasion of neutralizing antibodies can be achieved through these approaches. AAV, adeno-associated virus.

such as the binding sites of neutralizing antibodies against AAV8 can be identified, randomized, and then selected for packaging and transduction.[8] Selection screens of AAV peptide display, error-prone PCR, or DNA shuffling library can also be a high-throughput approach for identifying targeting ligands (Fig. 1). Genetic modifications of the AAV capsid allow for selections to be performed and the amino acid sequence to get determined from the vector DNA. However, the process is often time consuming. Selecting for tissue tropism specificity with genetic engineering also comes at the expense of packaging efficiency and short- term or nonreproducible vector infectivity.[1,3,6]

Protein chemical modification

Among many approaches that have been developed to improve viral vectors in gene therapy, chemical modification has recently been exploited and gained a promising role. Chemical modification of proteins is an important approach in research and development of new and improved products

from food, agriculture, to medicine. Proteins are predominantly modified through amino acids with the most reactive side chains. These modifications often rely on the chemical reactions of oxidation/reduction, and nucleophilic/electrophilic substitutions, which is well summarized previously.[9] Specifically, the thiol group found on the amino acid, cysteine (Cys), is the most reactive target for protein chemical modification, followed by the primary amino functionality found on side chain of lysine (Lys). The average abundance of Cys residues (~3%) on proteins is less than that of Lys (~7%), which enables relatively site- specific modification or chemical labeling using thiol-reactive probes.[10] The unprotonated amino groups of lysine are nucleophilic, which makes lysine the next key target for chemical modification. Depending on the availability of such functional groups exposed on a protein as well as its binding activity, either Cys or Lys residues can be targeted for specific modification. Some common chemical labeling reactions are shown in Fig. 2. The conjugation addition of thiol groups can be carried out using Michael acceptors such as maleimides or with alkyl halides and iodoacetamides to form thioethers (Fig. 2a). Maleimides and iodoacetamides can react with the free base form of amino groups (such as Lys) if the condition is above pH 8.[11] The primary amines can react with isothiocyanates to form thioureas, or with *N*-hydroxysuccinimide (NHS) esters to form carboxamides with stable amide bonds (Fig. 2b).[12]

In addition, tyrosine (Tyr) residues can be modified by either at the hydroxyl group or by aromatic ring substitution. One common method is esterification of the hydroxyl group, but hydroxyl modifications are often reversible during reagent removal. The guanidinium group of arginine (Arg) can react with a-di-carbonyl compounds such as methylglyoxal (Fig. 2c) to form hydroimidazolone products.[13,14] Other functional groups such as the indole of tryptophan (Trp) and imidazole of histidine (His) can also be modified using substitutions and alkylations or nucleophilic attacks.[10] Epoxides are known for efficient labeling

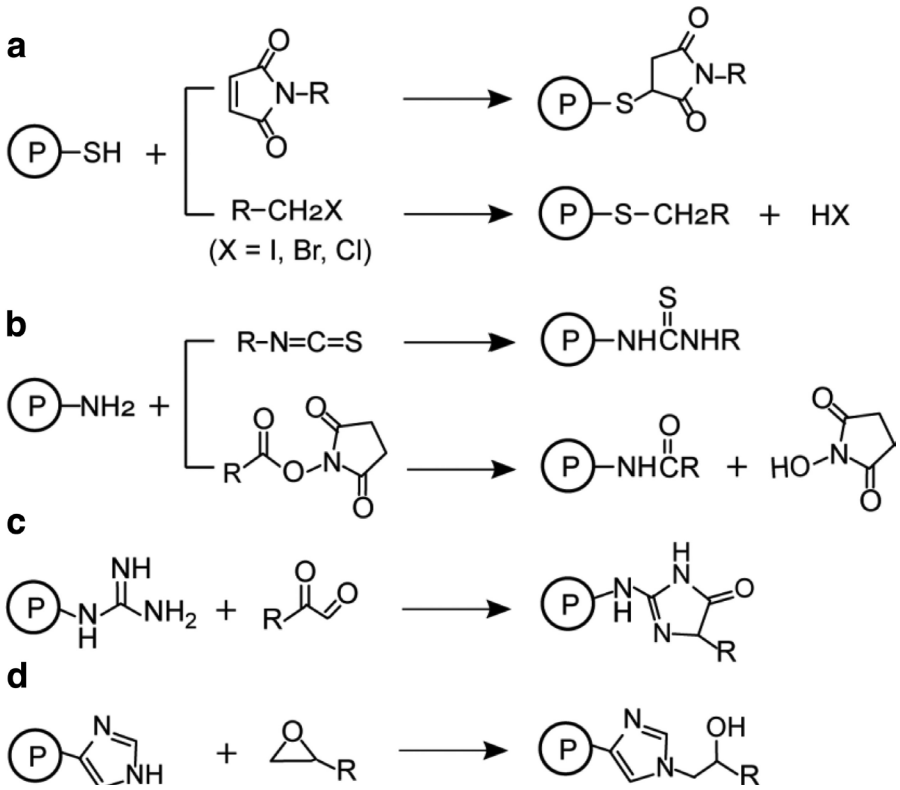


Figure 2. Common chemical modifications of proteins at cysteine (**a**), lysine (**b**), arginine (**c**), and histidine (**d**). P denotes protein; (**a**) shows the reaction of cysteine thiol group with either maleimides (*top reaction*) or with alkyl halides (*bottom reaction*) to form thioethers; (**b**) shows the reaction of lysine amino group with isothiocyanates to form thioureas (*top*) or with NHS esters to form carboxamides; (**c**) shows the reaction of arginine guanidinium group with a-dicarbonyl compounds to form hydroimidazolones; (**d**) shows the reaction of histidine imidazole group with epoxides under alkaline condition. NHS, *N*-hydroxysuccinimide.

of histidine in alkaline condition (Fig. 2d). However, the reagents or conditions that are required to modify these functional groups are also reactive toward the thiol or amino groups of Cys and Lys residues, making the chemo- selectivity challenging.[15]

There are 60 monomeric proteins forming the icosahedral capsid that encases AAV genetic information and determining the tropism of the viral vector. The capsid can be chemically tailored to manipulate its therapeutic function. However, each rAAV serotype contains different capsid peptide sequences exposing unique amino acids. These exposed amino acids on the capsids are keys for selective chemical modifications. A minor change or addition to surface amino acids can ultimately alter the receptor binding motif of the vector or how it interacts with the host's immune response. However, a knowledge gap regarding the interaction of AAV capsid sequences and their binding receptors adds more challenges for chemical modification of AAV. The need for investigations of AAV's chemical modification is clearly unmet. This minireview focuses on current studies regarding rAAV capsid chemical engineering to enhance its therapeutic use in gene therapy.

AAV capsid chemical modifications

Nonselective PEGylation on Lys

Polymeric chemical moieties have long been utilized by cross-linking to the surface of viral vectors, which can act as a shield to evade neutralizing antibodies. They are covalently attached to the viral capsid using chemical reactivity of nucleophilic groups of exposed amino acids such as Lys and Cys residues. Conjugated addition of polyethylene glycol (PEG) has extensively been carried out on adenoviruses to reduce immune response and extend circulatory half-life.[16–19] Lee *et al.* used biotin- PEG-NHS with functional group of electrophilic succinimidyl propionic acid to cross-link with Lys residues on AAV2 capsid. They examined three molecular weights of PEG-2000, -5000, and -20000 Da. Biotin acts as a label for the detection of PEGylated AAVs using horseradish peroxidase conjugated streptavidin through Western blotting. For each PEG molecular weight, the molar ratio of PEG:Lys also vary for systemic comparison in transduction efficiency and antibody neutralization. All three polymer sizes did not enhance the infectivity of the AAV virus, in fact, infectivity decreased significantly at high molar ratio of PEG:Lys. However, protection of AAV2 by PEG-2000 (molar ratio of 1,000: 1) from serum neutralization moderately increased ~2.3-folds. Modification with a larger PEG- 20000 alter AAV morphology significantly, and was proposed to block the cell receptor binding hence leading to low infectivity of the virus.[20]

Nonselective small molecule modification on Arg and Lys

Without any peptide insertions, Horowitz *et al.* chemically glycated exposed arginine (Arg) residues on the capsid of AAV2 (which clustered around the primary receptor heparan sulfate) by using methylglyoxal. Methylglyoxal (MGO) is a a-dicarbonyl compound that is known to be highly reactive to form covalent adducts with Arg (chemical reaction as in Fig. 1d) and Lys residues. Glycated AAV2 was found to be significantly lower in heparin binding as well as more than twofold decrease in binding to monoclonal antibody A20. However, transduction efficiency was lowered 1,000-fold compared with unmodified AAV2. Intravenous tail vein injection of glycated AAV2 into mice showed expanded viral tissue

tropism compared with unmodified AAV2. Interestingly, the transgene expression of luciferase found in cardiac and skeletal muscles was 100-fold more by glycated AAV2 than by AAV2 vectors. These results highlighted the feasibility of altering AAV morphology and tropism by simple chemical modification of AAV capsid.[13] Pearce *et al.* also glycate the Arg residues on the capsid of AAV6 with 4-azidophenyl glyoxal (APGO). This APGO was then further covalently conjugated with a single-chain antibody (scFv) that is actively targeting inflamed endothelia through vascular cell adhesion molecule (VCAM-1). With optimized modification, the natural tropism of WT AAV6 was removed, and its transduction of endothelial cells was significantly enhanced about four- to fivefolds *in vitro*, highlighting the feasibility of retargeting AAV to a specific host cell by chemical modification of the capsid.[21]

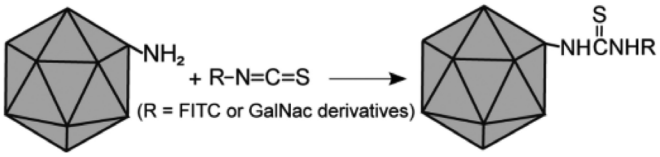


Figure 3. Modification of the AAV capsid Lys residues with isothiocyanate by Mével *et al.*[22]

In a different study, Mével *et al.* exploited surface Lys residues on the AAV capsid to react with amine-reactive molecules. Proof of concept was done using a fluorophore FITC (fluorescein isothiocyanate) and a control of fluorescein without the reactive isothiocyanate group (Fig. 3). Dot blot and Western blot analyses were performed to confirm the chemical reaction using either anti-AAV antibody to recognize the capsid, anti-FITC antibody, or direct fluorescence emission. Buffer TBS at pH 9.3 was chosen for labeling the primary amine that does not affect the AAV infectivity. The density of FITC molecules on the capsid was also modulated to fine-tune the viral therapeutic index. Using this concept, *N*-acetylgalactosamine (GalNAc)—a monosaccharide ligand that binds to a highly expressed receptor of hepatocytes—was coupled with the reactive isothiocyanate group (-NCS). The GalNAc-NCS molecule was chemically attached to AAV8 capsid and was shown, *in vivo*, to reduce serotype-specific neutralizing antibodies. Although significant increase in transduction efficiency was found for GalNAc-AAV at 10 days postadministration on mice, no difference showed at 21 days, when compared with unmodified virus. This study highlights a promising therapeutic advancement by using chemical modification on AAV capsid for redirecting the tropism as well as enhancing the transduction efficiency.[22]

Site-specific modification through peptide insertion

For a more site-specific modification, Liu *et al.* implemented a genetic insertion of 13-amino-acid (including Cys) sequence into the cap gene of AAV2 (amino acid 587 within VP1/VP2/VP3 regions). Then inserted Cys was going under chemical modification to convert into an aldehyde tag. This aldehyde tag was further chemically modified with covalent conjugation of hydrazide- or hydroxylamine-functional fluorophore such as Alexa- hydrazide. Figure 4 shows the schematic flow of their approach. Monoclonal A20 antibody, which is specific for intact AAV2, was subsequently used to confirm the chemical reaction of the viral vector and the aldehyde tag by overlaid imaging.[23] To examine the transduction efficiency of this method, AAV-aldehyde-tag was conjugated with a monoclonal antibody against human leukocyte antigen (anti-HLA). HepG2 cells have limited permissiveness to WT AAV2,

but anti-HLA-AAV2 expanded this tropism and significantly increased the transduction from ~2% to ~12%. This study indicated an active targeting approach for expanding AAV tropism using chemically modified AAV capsid bearing specific ligands, which could be a promise for cancer gene therapy.[23] In a similar concept, Chandran *et al.* inserted a tetracysteine- peptide into the capsid (amino acid 138 of VP1 and VP2) of AAV9 without compromising the viral infectivity.[24] The functional thiol groups of this tetracysteine allowed for further incorporation of a fluorescent dye to the capsid through a thiol-maleimide reaction. Fluorescent-labeled viruses enabled monitoring of the modified particles *in vivo*, leading to the generation of two capsid interactomes: blocking integrin aVb6 receptor decreased AAV9 transduction, and lower expression of histone deacetylase 4 (HDAC4) increased its transduction.

Site-specific modification through unnatural amino acid incorporation

Unnatural amino acids (UAAs) bearing bio-orthogonal chemical groups (*e.g.,* azide group) can be incorporated into the AAV capsid during transfection to produce tagged-AAV particles for subsequent chemical modifications through specific cross-linking chemistries (Fig. 5). Specifically, Zhang *et al.* expanded the genetic code of AAV2 by adding a plasmid vector pAAV-RC-TAG (TAG, stop codon at different sites on the *cap* gene) and a UAA N-2-azideoethyloxycarbonyl-L-lysine to a typical triple- plasmid transfection.[25] This step allowed them to harvest azide-AAV2, and the azide functional group allowed for further bio-orthogonal conjugation of cRGD (cyclic tri- peptide of Arg-Gly-Asp that is known as a ligand of integrin avB3 molecules highly expressed in a wide variety of tumors) through azide-alkyne Huisgen cycloaddition in a site-specific manner. AAV2-N587+1/UAA/RGD was chosen for *in vivo* study since it best enhanced the transduction of the suicide agent Herpes simplex virus thymidine kinase (KT), which is most widely used in cancer gene therapy in response to the prodrug ganciclovir. At day 15, this modified AAV2-N587+1/UAA/RGD significantly suppressed the tumor volume in rats by ~8-folds compared with the WT AAV2, and ~3.5-folds compared with the AAV2-N587RGD (mutation without using UAA).

The same concept of altering the tropism of AAV2 by incorporating azido-modified UAA to the capsid site R588 for attaching the cRGD motif was also investigated by Kelemen *et al.*[26] *In vitro* infectivity of AAV2-R588/UAA/cRGD was 20% lower than the WT control on the ovarian tumor-derived cells and completely lost on HEK293 cells.[25] Through a similar concept, Yao *et al.* modified AAV2 with azido-modified UAA at the capsid sites Q325+1, S452+1, and R585+1, for a subsequent site- selective PEGylation (20kD PEG) into these azide groups. Viral titers of these modified viruses were several folds

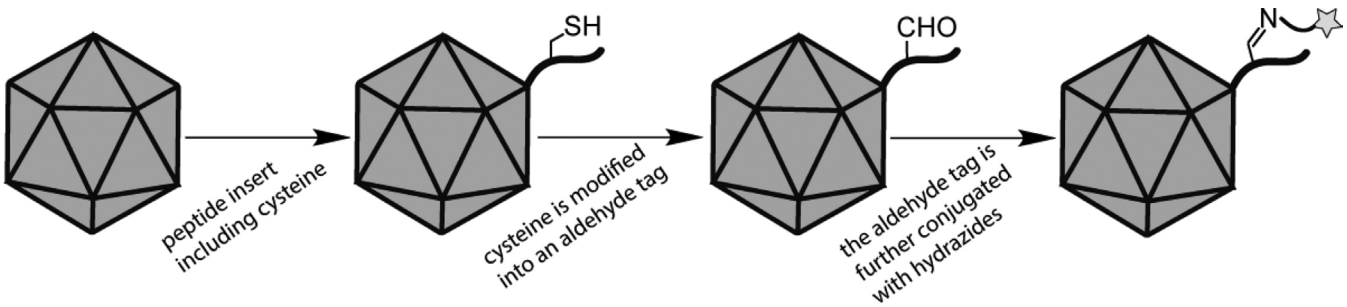


Figure 4. Schematic representation of Liu *et al.* modification process.[23]

lowered than the WT control, but their protection against antibody recognition in rats was moderately improved (~20%). However, the transduction of these modified viruses *in vivo* was not discussed.[27] In a different study, Katrekar *et al.* engineered AAV2 and AAV-DJ by combining the insertion of UAA with a reactive group and further chemical conjugation of that group. Four surface capsid residues R447, S578, N587, and S662 were co-translationally substituted with an azide-modified pyrrolysine derivative, which again allow specific chemical conjugation through azide-alkyne Huisgen cycloaddition.[28] Since AAV-DJ is a chimera of AAV serotypes 2, 8, and 9, its titer and infectivity outperform AAV2 about 10-folds in multiple cell lines and naive mice. Mutant AAV-DJ-N589UAA produced a titer 15-fold higher than AAV2-N587UAA; both mutants did not alter the virus infectivity. Later azido-alkyne click chemistry added an oligonucleotide of 10kDa, which could be conjugated with multifunctionality probe such as biotin. Conjugated biotin was used to confirm the UAA-modified AAV. The oligo-AAV was masked with lipofectamine as a shield that protected the virus from neutralizing antibodies. One limitation is that the UAA- modified capsid leads to several folds lower in viral titer.[28] These studies show the successful incorporation of a UAA insert into the AAV capsid and using specific chemistry of the insert to tether the AAV capsid to a spectrum of different molecules for different purposes.

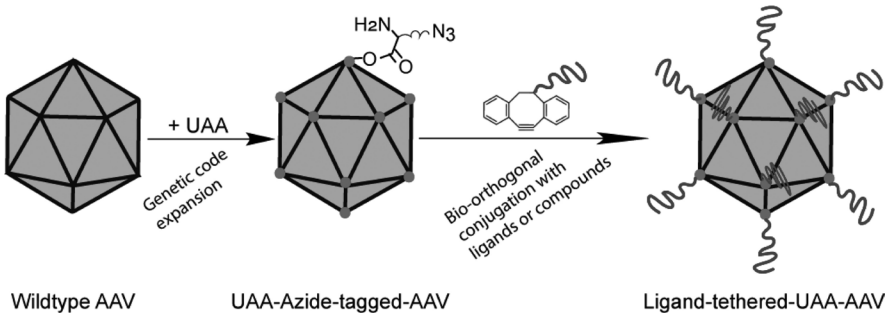


Figure 5. Representation of AAV modification through UAA approach. UAA, unnatural amino acids.

Conclusion

The rising knowledge of AAV biology has enabled more directed design of chemical modification for AAV capsid. Whether to protect the virus from neutralizing antibodies or to redirect its tropism to specific tissues, chemical modification is undoubtedly capable of. Future directions are not limited there, as chemical engineering can also tailor the capsid to be more controllable therapeutic agent. For instance, capsid was tethered with a fluorophore for *in vivo* imaging monitor[24] or to be altered the capsid geometry for enhancing its stability.[29] Although more and more AAV gene therapy drugs have entered clinical trials, challenges exist. One of them is the lack of a standard system for testing the efficacy and safety of AAV vectors on humans. Engineered AAV vectors could show great potentials in animal models or even humanized animals but might not transfer to other kinds of animals and humans. If AAV capsid can be engineered so that it becomes more specific tropism for a targeted tissue, its required doses would be reduced, leading to greater transduction efficiency and lower unwanted immunogenicity. Although investigations on AAV chemical modification are underexploited, the rate of novel innovation in gene and cell therapy will quickly produce more and more promising engineered candidates. As more and more complete structures of WT AAV capsids have been discovered and published, better and clearer strategies in chemical modification can be implemented and developed. Efforts

are underway with one common goal—to benefit patients.

Author disclosure

No competing financial interests exist.

Funding Information

No funding was received for this review.

References

1. Grimm D, Zolotukhin S. E pluribus unum: 50 years of research, millions of viruses, and one goal— tailored acceleration of AAV evolution. Mol Ther 2015;23:1819–1831.
2. Xiao W, Chirmule N, Berta SC, et al. Gene therapy vectors based on adeno-associated virus type 1. J Virol 1999;73:3994–4003.
3. Li C, Samulski RJ. Engineering adeno-associated virus vectors for gene therapy. Nat Rev Genet 2020;21:255–272.
4. Cao M, You H, Hermonat PL. The X gene of adeno-associated virus 2 (AAV2) is involved in viral DNA replication. PLoS One 2014;9:e104596.
5. Zincarelli C, Soltys S, Rengo G, et al. Analysis of AAV serotypes 1–9 mediated gene expression and tropism in mice after systemic injection. Mol Ther 2008;16:1073–1080.
6. Buning H, Srivastava A. Capsid modifications for targeting and improving the efficacy of AAV vectors. Mol Ther Methods Clin Dev 2019;12: 248–265.
7. Reul J, Muik A, Buchholz CJ. Ligand coupling to the AAV capsid for cell-specific gene transfer. Methods Mol Biol 2019;1950:35–50.
8. Havlik LP, Simon KE, Smith JK, et al. Coevolution of adeno-associated virus capsid antigenicity and tropism through a structure-guided approach. J Virol 2020;94:e00976–20.
9. Boutureira O, Bernardes GJ. Advances in chemical protein modification. Chem Rev 2015;115:2174– 2195.
10. Feeney RE, Yamasaki RB, Geoghegan KF. Chemical modification of proteins: an overview. Adv Chem 1982;198:3–55.
11. Chalker JM, Bernardes GJ, Lin YA, et al. Chemical modification of proteins at cysteine: opportunities in chemistry and biology. Chem Asian J 2009;4: 630–640.
12. Basle E, Joubert N, Pucheault M. Protein chemical modification on endogenous amino acids. Chem Biol 2010;17:213–227.
13. Horowitz ED, Weinberg MS, Asokan A. Glycated AAV vectors: chemical redirection of viral tissue tropism. Bioconjug Chem 2011;22:529–532.
14. Lo T, Westwood ME, McLellan AC, et al. Binding and modification of proteins by methylglyoxal under physiological conditions. A kinetic and mechanistic study with N alpha-acetylarginine, N alpha-acetylcysteine, and N alpha-acetyllysine, and bovine serum albumin. J Biol Chem 1994;269: 32299–32305.
15. Reddy NC, Kumar M, Molla R, et al. Chemical methods for modification of proteins. Org Biomol Chem 2020;18:4669–4691.
16. Croyle M, Le H, Linse K, et al. PEGylated helper- dependent adenoviral vectors: highly efficient vectors with an enhanced safety profile. Gene Ther 2005;12:579–587.
17. Hofherr SE, Mok H, Gushiken FC, et al. Polyethylene glycol modification of adenovirus reduces platelet activation, endothelial cell activation, and thrombocytopenia. Hum Gene Ther 2007;18:837–848.
18. O'Riordan CR, Lachapelle A, Delgado C, et al. PEGylation of adenovirus with retention of infectivity and protection from neutralizing antibody in vitro and in vivo. Hum Gene Ther 1999;10: 1349–1358.
19. Espenlaub S, Wortmann A, Engler T, et al. Reductive amination as a strategy to reduce adenovirus vector promiscuity by chemical capsid modification with large polysaccharides. J Gene Med 2008;10:1303–1314.
20. Lee GK, Maheshri N, Kaspar B, et al. PEG conjugation moderately protects adeno-associated viral vectors against antibody neutralization. Biotechnol Bioeng 2005;92:24–34.
21. Pearce HA, Qian H, Connell TU, et al. Site-specific glycation and chemo-enzymatic antibody sortagging for the retargeting of rAAV6 to inflamed endothelium. Mol Ther Methods Clin Dev 2019;14:261–269.
22. Mével M, Bouzelha M, Leray A, et al. Chemical modification of the adeno-associated virus capsid to improve gene delivery. Chem Sci 2020;11:1122–1131.
23. Liu Y, Fang Y, Zhou Y, et al. Site-specific modification of adeno-associated viruses via a genetically engineered aldehyde tag. Small 2013;9:421–429.
24. Chandran JS, Sharp PS, Karyka E, et al. Site specific modification of adeno-associated virus enables both fluorescent imaging of viral particles and characterization of the capsid interactome. Sci Rep 2017;7:14766.
25. Zhang C, Yao T, Zheng Y, et al. Development of next generation adeno-associated viral vectors capable of selective tropism and efficient gene delivery. Biomaterials 2016;80:134–145.
26. Kelemen RE, Mukherjee R, Cao X, et al. A precise chemical strategy to alter the receptor specificity of the adeno-associated virus. Angew Chem Int Ed Engl 2016;55:10645–10649.
27. Yao T, Zhou X, Zhang C, et al. Site-specific PEGylated adeno-associated viruses with increased serum stability and reduced immunogenicity. Molecules 2017;22:1155.
28. Katrekar D, Moreno AM, Chen G, et al. Oligonucleotide conjugated multi-functional adeno- associated viruses. Sci Rep 2018;8:3589.
29. Stone NP, Demo G, Agnello E, et al. Principles for enhancing virus capsid capacity and stability from a thermophilic virus capsid structure. Nat Commun 2019;10:1–13.

Received for publication May 24, 2021;
accepted after revision July 5, 2021.

Published online: July 12, 2021.

Resolution of Adeno-Associated Viral Vector Aggregates and Fragments with Agilent Bio SEC-5

Author | Drs. Brian Liau, Anne Blackwell, and Matthew L. Turner Agilent Technologies, Inc.

Introduction

Adeno-associated viruses (AAVs) are a promising new class of biotherapeutic with many proven applications.[1] AAVs are large molecular complexes consisting of approximately 60 copies of capsid protein(s) encapsulating a single stranded DNA genome. Individual AAV virions may exceed 5 MDa[1], and are approximately 250 Å in size. AAV aggregates are therefore challenging to resolve using standard SEC columns, which typically have pore sizes ≤300 Å. Conventional wisdom indicates that an SEC pore size at least three times larger than the molecule of interest should be chosen, which would indicate a pore size ≥750 Å.

This application note demonstrates chromatographic resolution of AAV aggregates and fragments on an Agilent Bio SEC-5 column with 1,000 Å pore size. Bio SEC-5 columns are packed with 5 µm silica particles coated with a proprietary hydrophilic layer for efficiency and stability, and are thus well suited for analysis of large, complex biological molecules such as viral particles.

Methods

AAV samples of serotypes 2 and 9 were purchased from Vigene Biosciences, and had particle concentrations of 1.27×10^{12} and 1.76×10^{12} VP/mL respectively as determined by ELISA. Agilent AdvanceBio SEC 300Å Protein Standard (part number 5190-9417) was used for calibration and confirmation of relative mass of each AAV serotype.

Size-exclusion chromatography was conducted on an Agilent 1290 Infinity II Bio LC equipped with a binary pump and an Agilent 1260 Infinity II fluorescence detector set to Ex = 280 nm, Em = 340 nm. The mobile phase consisted of 50 mM phosphate buffer + 400 mM NaCl, pH 7.4. An Agilent Bio SEC-5 column (4.6 × 300 mm, 5 µm, 1000 Å) was chosen, with a constant flow rate of 0.4 mL/min.

Prior to analysis, 15 µL of unstressed AAV-2 or AAV-9 was diluted in 100 mM phosphate-buffered saline, pH 7.4, to a final volume of 65 µL. Injections of 20 µL were then performed in triplicate.

Published reports indicate that AAV aggregation is driven primarily by electrostatic interactions and is favored by low ionic strength and the presence of residual DNA.[2] Aggregation was induced by diluting 15 µL of AAV-9 in deionized water to a final volume of 65 µL in 0.5 mL Eppendorf Protein LoBind tubes. Two microliters of PCR-amplified dsDNA (length = 3,800 nt, concentration = 0.2 µg/ µL) was then added, and the samples were incubated overnight at

37 °C. Injections of 20 µL were then performed in triplicate the following day.

Results

Based on the reported hydrodynamic radius of monomeric thyroglobulin[3] (85.8 Å), AAV virions were expected to elute between thyroglobulin monomers and their aggregates, which was indeed observed (Figure 1).

Unstressed AAV-2 and AAV-9 had similar purity values of 98.7% and 98.8% respectively, each containing 1.2 to 1.3% aggregates.

In contrast, stressed AAV-9 showed extensive formation of aggregates and substantial levels of degradation. Stressed AAV-9 was 83.6% pure, containing 7.5% aggregates and 8.8% fragments. Notably, the total peak area of stressed samples was only approximately 60% that of unstressed samples, possibly indicating the formation of insoluble aggregates (Figure 2).

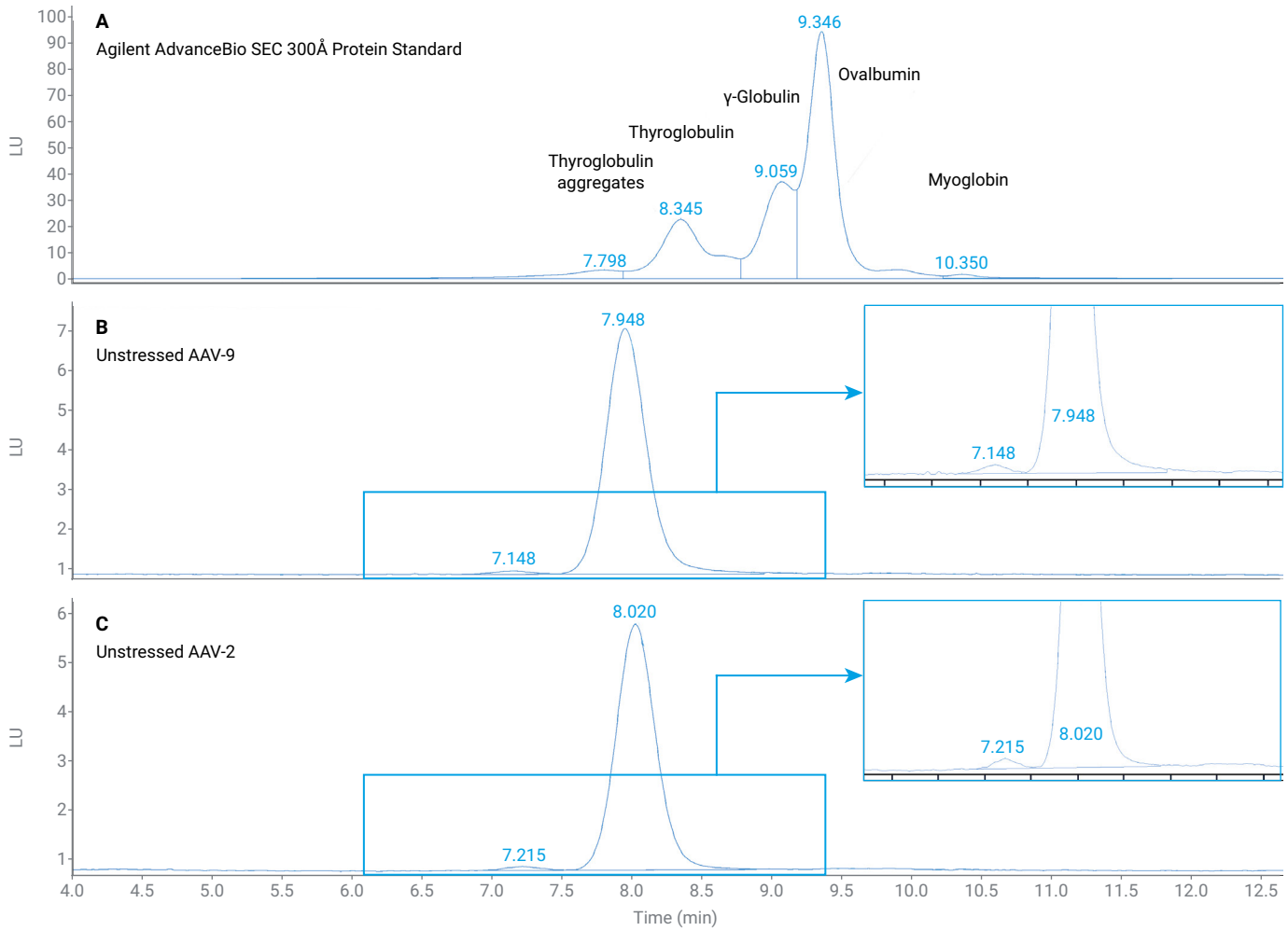


Figure 1. Fluorescence chromatograms of Agilent AdvanceBio SEC 300Å Protein Standard and unstressed AAV-9 and AAV-2. Note that the protein standard also contains insulin, which is not shown because it contains no tryptophan amino acids and is therefore nonfluorescent under the chosen excitation and emission settings.

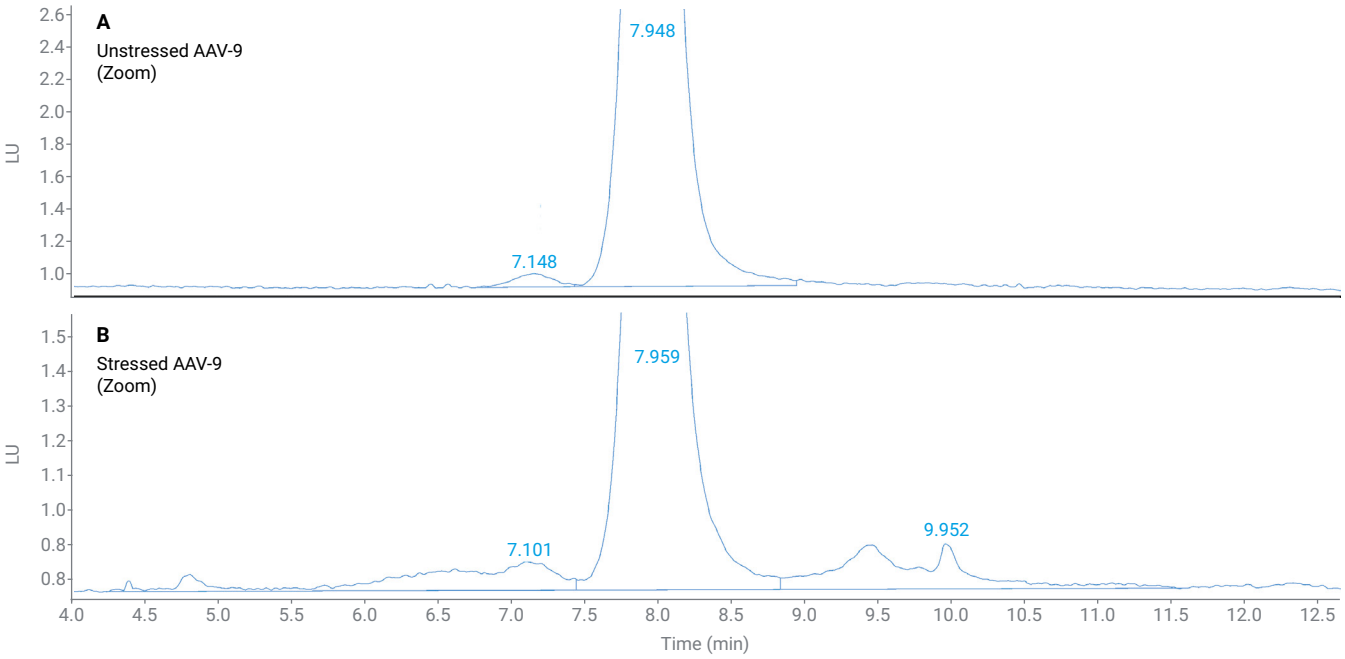


Figure 2. Fluorescence chromatograms of unstressed and stressed AAV-9.

Conclusion

Agilent Bio SEC-5 columns contain particles with 1,000 Å pore size making them suitable for AAV aggregate and fragment analysis, which is essential during AAV purification and subsequent formulation stability testing. Orthogonal methods such as bulk dynamic light scattering or analytical ultracentrifugation may be useful for addressing large, insoluble aggregates that may inevitably form under stress conditions along with the demonstrated SEC aggregate analysis for routine testing.

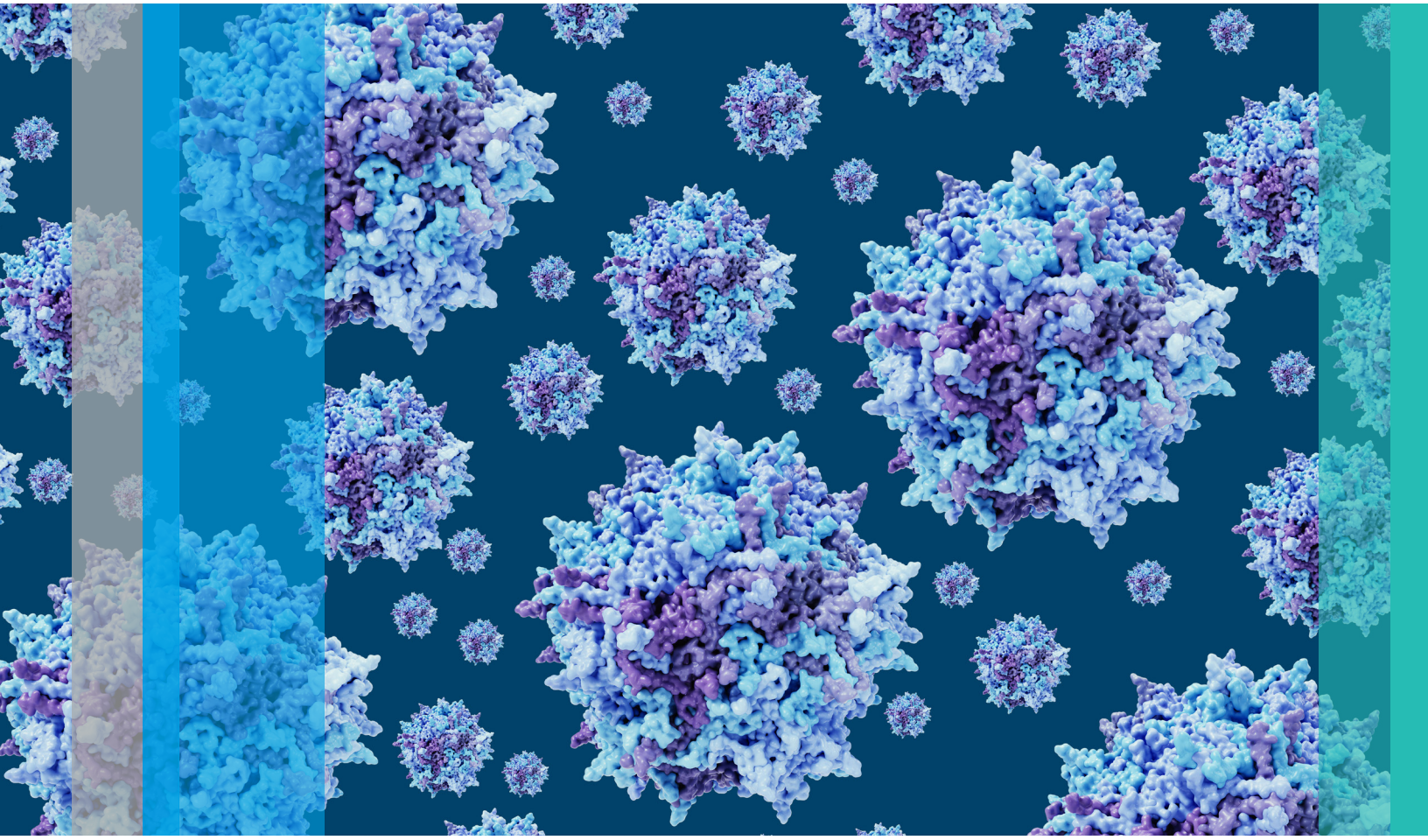
References

1. Pierson, E. E. *et al.* Resolving Adeno-Associated Viral Particle Diversity with Charge Detection Mass Spectrometry, *Analytical Chemistry* **2016**.
2. Wright, J. F. *et al.* Identification of Factors That Contribute to Recombinant AAV2 Particle Aggregation and Methods to Prevent Its Occurrence During Vector Purification and Formulation. *Molecular Therapy* **2005**.
3. Nobuo, Ui. Electrophoretic Mobility and Isoelectric Point of Hog Thyroglobulin. *BBA* **1972**.

RA44770.4420138889 5994-7772EN May 2022

Agilent

Trusted Answers



Confirm the Identity of Your AAV

High-resolution reversed phase separations for intact or digested capsid proteins

Verifying the correct sequence of VP1, VP2, and VP3 is critical—especially in a facility producing more than one serotype. Agilent AdvanceBio HPLC columns enable high-resolution LC/MS analysis of intact proteins for molecular weight confirmation, or peptide mapping for post-translation modification (PTM) analysis.

www.agilent.com/chem/advancebio-aav

Agilent ZORBAX RRHD Wide Pore columns for intact protein separations.

Agilent AdvanceBio Peptide Mapping columns for sequence confirmation and PTM identification.