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Chemical approaches to probe and engineer AAV vectors

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Abstract

Adeno-associated virus (AAV) has emerged as the most promising vector for *in vivo* human gene therapy, with several therapeutic approvals in the last few years and countless more under development. Underlying this remarkable success are several attractive features that AAV offers, including lack of pathogenicity, low immunogenicity, long-term gene expression without genomic integration, the ability to infect both dividing and non-dividing cells, etc. However, the commonly used wild-type AAV capsids in therapeutic development present significant challenges, including inadequate tissue specificity and the need for large doses to attain therapeutic effectiveness, raising safety concerns. Additionally, significant preexisting adaptive immunity against most natural capsids, as well as the development of such anti-capsid immunity after the first treatment, represent major challenges. Strategies to engineer the AAV capsid are critically needed to address these challenges and unlock the full promise of AAV gene therapy. Chemical modification of the AAV capsid has recently emerged as a powerful new approach to engineer its properties. Unlike genetic strategies, which can be more disruptive to the delicate capsid assembly and packaging processes, “late-stage” chemical modification of the assembled capsid — whether at natural amino acid residues or site-specifically installed noncanonical amino acid residues — often enables a versatile approach to introducing new properties to the capsid. This review summarizes the significant recent progress in AAV capsid engineering strategies, with a particular focus on chemical modifications in advancing the next generation of AAV-based gene therapies



Introduction

Adeno-associated virus (AAV) is a small, non-enveloped virus belonging to the parvoviridae family, which has exhibited tremendous potential as a vector for the development of human gene therapies.[1-7] With 12 distinct human serotypes, none of which are linked to any human disease, AAV efficiently transduces a broad spectrum of both dividing and non-dividing cells, allowing for the prolonged expression of transgenes. Furthermore, AAV triggers minimal innate immune responses and is inherently replication deficient. Thanks to these attractive features, AAV stands out as a highly favorable vector for gene delivery in both *in vitro* and *in vivo* settings. To date, the FDA has approved five AAV-based drugs for gene therapy that address various genetic diseases:[8-13] Luxturna, an AAV2 drug for inherited retinal disease; Zolgensma, an AAV9 drug for spinal muscular dystrophy; Hemgenix, an AAV5 drug for hemophilia B; Roctavian, an AAV5 drug for hemophilia A; and Elevidys, an AAVrh74 drug to treat Duchenne muscular dystrophy. Numerous other clinical trials are underway, showing promising results.[3]

AAV possesses a compact genome, approximately 4.7 kb of single-stranded DNA flanked by two inverted terminal repeats (ITR).[6,14] The native genome features two main open reading frames —rep and cap — each of which produces multiple non-structured and structured proteins through alternative splicing and the use of alternative start codons. The capsid comprises three proteins, VP1, VP2, and VP3, approximately in a 1:1:10 ratio, forming an icosahedral particle with extensive interactions related to two-, three-, and five-fold symmetry.[14] The initial step in AAV's infectious pathway involves binding to a primary cell-surface receptor, followed by interaction with a secondary receptor.[15-17] This secondary interaction initiates diverse internalization pathways, resulting in trafficking to endosomal and Golgi compartments. Subsequently, AAV particles escape into the cytoplasm, accumulating in the perinuclear space before ultimately entering the nucleus for genome release and replication. These processes entail complex changes in capsid conformation and intricate interactions between the capsid and cellular factors, many of which remain poorly characterized. One of the main reasons behind this knowledge gap is the lack of appropriate tools to probe the biology underlying the entry of this virus. Many established tools used to probe protein localization and interaction network involve tagging the target with other proteins (e.g., proteins with intrinsic fluorescence or proximity labeling capabilities), which are typically incompatible with the delicate and complex AAV capsid. Consequently, new tools are



needed to elucidate the biology of AAV, which are associated with minimal perturbation to the target. In addition, recent advances in mass-spectrometry-based platforms have also revealed the presence of many novel post-translational modifications on the AAV capsid residues across multiple serotypes.[18,19] However, the distribution of these modifications is quite heterogeneous and, except for a few well-documented cases such as phosphorylation mediated capsid degradation,[20-22] and their physiological significance remains poorly understood.

Despite its remarkable success over the last decade, the wild-type AAV capsids that the industry and most clinical studies continue to rely on face several considerable disadvantages. One such key drawback is the limited control over the tissue tropism of native AAV serotypes. The broad tropism of AAV, affecting various tissues such as lung, liver, spleen, muscle, brain, kidney, etc., makes it challenging to deliver a transgene selectively to a target tissue. The liver, a common target for many AAV serotypes, acts as a sink, consuming the majority of delivered particles. Consequently, high doses of viral vectors are typically required to achieve sufficient transgene expression at the desired tissues, leading to potential toxicity in the liver and possibly other organs. Additionally, a large percentage of human population have AAV-neutralizing antibodies due to prior exposure to this virus, excluding them from AAV vector-based treatments. Furthermore, treatment of eligible patients with a particular therapeutic AAV vector typically triggers significant adaptive immune response, and the resulting neutralizing antibodies prevent subsequent redosing. To overcome these limitations, various strategies, including genetic engineering and chemical modification, have been employed to engineer the viral capsid of AAV.[1,2,6,23-29] In this review, we summarize these strategies, with a particular focus on the chemical approaches that have been utilized to both probe and engineer the biology of AAV.

An overview of the genetic approaches to engineer AAV

In this section we seek to provide a brief overview of the genetic approaches employed to engineer the properties of the AAV capsid, representative example of which are also summarized in Table 1. These examples can be broadly divided into two categories, those relying on rational engineering and on directed evolution. Rational design offers a more direct approach, and relies on prior knowledge of the structure and the function of the capsid to engineer the capsid proteins in a hypothesis-dependent manner. Such approaches typically involve rational mutation of residues



known to negatively impact AAV (e.g., phosphodegrons),[20-22] or the insertion of a peptide or a protein domain into permissive sites of the capsid proteins to introduce novel properties such as tissue specificity. Insertion of novel peptide sequences, such as those identified from *in vitro* selection experiments, into permissive loops of the AAV capsid proteins is straightforward, and has been used with some success for targeting the virus to specific cell-types.[30-35] However, permissive sites in the capsid that withstand such insertions are limited, and only small peptides are typically tolerated. Fusion of larger proteins – such as antibody fragments and DARPINs – has also been used, but these are largely restricted to the N-terminus, or into the variable domain IV of the minor capsid protein through extensive linker optimization.[36-43] Such strategies have also been used to improve the selectivity of AAV vectors. However, fusion of a foreign protein to the N-terminus of the minor capsid protein VP2 disrupts the natural capsid architecture, since VP2 N-terminus is normally tucked inside the capsid. Similarly, the disruptive effect of internal fusion on the capsid has also significantly limited the size and complexity of proteins that can be amenable to such fusion. Although there are numerous examples of using peptide and protein fusion strategies to engineer AAV, potential disruption to the complex and delicate capsid architecture represents a potential limitation of this strategy. Another notable rational approach involves the use of bispecific antibodies, which can mediate the interaction between an AAV capsid and a target receptor.[44,45] Insertion of peptide tags into the capsid, which can be subsequently covalently (e.g., formylglycine, HUH etc.), or noncovalently (e.g., asymmetric leucine zippers) labeled with function-altering entities with high specificity, has also been employed to engineer the properties of the capsid. [46-50]

Directed evolution approaches have been extensively utilized to create AAV variants with enhanced capabilities such as selective transduction of specific tissues and evading neutralizing antibodies. Several approaches have been used to generate the libraries of capsid variants for such directed evolution experiments, including error-prone mutagenesis of the cap gene,[51-53] DNA shuffling of the cap gene from various existing AAV serotypes,[54-57] as well as the insertion of randomized peptide libraries into a permissive site of the capsid (e.g., at the variable domains IV and VIII of the capsid).[58-66] Facilitated by the available structural information of the AAV capsid, and the rapid development of DNA synthesis and next-generation sequencing capabilities, numerous AAV directed evolution studies have been reported in both cell cultures and animal models. This process has successfully generated variants capable of efficiently transducing



challenging targets such as the brain, muscles, and T cells while exhibiting lower immunogenicity, which are excellent potential candidates for gene therapy. Despite these success stories, directed evolution of AAV capsid can be a time- and labor-intensive process, which is not guaranteed to yield desirable results. Moreover, mutant capsids with novel properties selected using a specific setup (in cell culture or non-human animal models) may not always translate to humans, due to subtle differences in receptor structures other factors *in vivo*.

Finally, *in silico* engineering of the AAV capsid has recently emerged as a promising alternative approach. For example, machine learning strategies, that capitalize large sequence-function correlation datasets, such deep-mutation profiling data of the AAV capsid gene, are being used to design novel capsid mutants.[67,68] Computational approaches have also been used to resurrect extinct ancestral AAV capsid sequences, which may have limited preexisting immunity.[69,70]

Table 1: Summary of major genetic strategies to engineer AAV

Strategies	Selected Methods	Advantages	Disadvantages	References
Directed evolution	Error-prone PCR	Enables diversification across the whole capsid. Can find unexpected mutations which improve function	Low mutation rate in library generation, Limited coverage of the possible sequence space	[51-53]
	Serotype shuffling	Combines the benefits of multiple serotypes. Enables protein fusions at logical break points	Unlikely to acquire novel and unique functions	[54-57]
	AAV display	Allows <i>in vivo</i> selection of cell-binding peptides incorporated in the capsid	Selections are technically demanding, outcome is unpredictable	[58-66]
	Structure-guided approach	Can be used on any serotype to retarget virus to tissue of interest Evolved variants can gain altered tissue tropism and immune evasion	Requires relevant structural information.	[71,72]
Rational Design	Point mutation to ablate phospho-	Improves both <i>in vitro</i> and <i>in vivo</i> transduction abilities via point	Limited scope	[20-22]



	degrons on the Capsid Surface	mutations (such as tyrosine residues)		
	Peptide Loop Insertion	Can be well tolerated at specific sites, Straightforward approach	May destabilize the capsid in most sites, limited to short peptides,	[30-35]
	N-terminal fusion and loop insertion to Minor Capsid Proteins	Can be used to attach larger, folded protein domains (such as affibodies and DARPin)	Can disrupt capsid, and thus infectivity and viral titer	[36-43]
	Non-covalent modification using biochemical tags	Can be used to attach small to large, folded proteins (antibodies, protein vault nanoparticles)	Some bacteria-derived tags can be immunogenic <i>in vivo</i> Non-covalent conjugation can cause loss of the attached molecules.	[46-50]
<i>In silico</i> design	Computational reconstruction of Ancestral AAVs	Allows discovery of ancestral capsids that are not recognized by the immune system	Unpredictable tropism. Acquires adaptive immune response after first dose	[69,70]
	Machine-learning assisted capsid evolution	Diverse, synthetic capsid variants can be generated with higher throughput than rational design and random mutagenesis approaches	Requires necessary machine learning models and algorithms Require library cloning capable of covering the vast amount of generated variants	[67,68]

Chemical modification of the AAV capsid

Genetic approaches to engineer AAV are typically associated with significant sequence alterations (mutation or insertions) which may have unanticipated impacts on the complex structure and biology of AAV. These concerns are further amplified by our limited understanding of AAV biology, which compromises our ability to fully map the associated impacts of such sequence changes. Chemical modification of native AAV capsids, or those harboring site-specifically incorporated bioorthogonal conjugation handles, has emerged as an alternative approach for introducing function-altering entities on them to engineer their properties. Chemical modification



has also been used to introduce small biophysical probes on the capsid to study the biology of AAV. Since the modification is introduced on packaged capsids, this strategy largely maintains the native capsid architecture and packaging behavior of AAV. The degree of capsid modification can also be carefully controlled by modulating reaction conditions to avoid introducing perturbation to its structure and function. Furthermore, this approach allows the introduction of entities with practically unlimited chemical diversity on the AAV capsid, enabling the creation of diverse AAV conjugates with novel properties. This includes user-defined synthetic molecules, fluorescent dyes, quantum dots, DNA, RNA, peptides, and even proteins. The resulting conjugates have many potential applications such as: 1) aiding the virus in evading the immune system, 2) retargeting the virus to tissues of interest with high efficiency and selectivity, 3) studying the biology of the virus through real-time imaging and controlling its behavior with light, and 4) using the virus as a tool for the directed evolution of biomolecules. Below, we discuss the advances in this area, focusing first on the modification of native AAV capsids at canonical amino acid residues, and then bioorthogonal site-specific modification of AAV capsids harboring precisely incorporated noncanonical amino acid residues.

Modification of the AAV capsid at canonical amino acid residues

Advances in protein-labeling chemistries provide numerous ways to modify the capsid proteins at various canonical amino acid residues with high chemoselectivity, including lysine, arginine, cysteine, and tyrosine residues with no genetic manipulation needed.

Utilizing lysine residues

Earlier work on chemical modification of AAV has centered around the surface exposed lysine residues on the viral capsid.^{33–37} [73–77] Due to the nucleophilicity of the lysine side-chain, it is possible to acylate the amino groups with either N-hydroxysuccinimide (NHS) esters or isothiocyanates in buffered aqueous solutions near physiological pH. In an effort to understand how AAV invades the cell, early research used lysine-selective NHS esters to introduce fluorescent cyanine dyes on the capsid. These probe-labeled virus particles allowed the real time imaging of viral entry into live cells.[73–76] Such experiments provided initial knowledge about the kinetics of AAV entry and trafficking. This strategy has also been used to introduce other probes such as



quantum dots, which are less susceptible to photobleaching, as well as radioactive isotopes (which was attached to AAV by NHS-tetrazine, followed by TCO ligation), enabling other ways to probe AAV entry and trafficking.[78-81]

Additionally, lysine-targeted NHS ester chemistry was also used by the Schaffer group to attach polyethylene glycol (PEG) to AAV to shield the virus from the immune system.[82] Various PEG lengths were appended to uncover the optimal shielding effect against neutralizing antibodies in serum. By testing multiple virus:PEG ratios, the study underscored the importance of regulating capsid modification levels to achieve optimal shielding without compromising viral infectivity. Similarly, the Jiang group tethered an immunosuppressive zwitterionic phosphoserine (PS)-containing polypeptide as an immunomodulatory signal to the viral capsid.[83] The resulting conjugates were observed to significantly diminish anti-AAV immune responses without substantially altering viral transduction and tropism. Kwon *et al.* conjugated AAV with eosin (a photo-initiator) at surface-exposed lysine residues using isothiocyanate, [84] which was subsequently used to initiate a polymerization reaction to create an acid-degradable polyketal shell around the virus, which was further embedded with siRNAs. This polymer-coated virus evaded immune response while co-delivering both the virus and the siRNA into cells. In a different approach, Ayuso *et al.* employed isothiocyanate chemistry to attach carbohydrates as hepatocyte-targeting moieties.[85]

To develop a more universal platform, streptavidin or biotin was conjugated to the AAV capsid using NHS reagents. Taking advantage of the rapid and near-irreversible association between biotin and avidin, the platform allowed the attachment of various biomolecules of interest to the AAV capsids which was chemically conjugated with either of the two. For example, Lee and Ahn demonstrated that AAV2 modified with streptavidin–NHS ester can be noncovalently linked to biotin-tagged antibodies.[86] The anti-EpCAM-AAV2 conjugates exhibited remarkable specificity in targeting and slowing tumor progression in mouse models by transporting EGFR shRNA. In an alternative approach, the Castillas, Jr. team engineered AAV2 by introducing biotin-NHS, then coupling it with EGF-Streptavidin fusion, resulting in over a 100-fold increase in transduction in SK-OV-3 cells. Similarly, Rao *et al.* employed biotin-labeled AAV along with T4 phage, connected through a streptavidin bridge, yielding a hybrid vector capable of carrying unprecedented payloads of up to 170 kb DNA genome and up to 1025 protein molecules. Although



this approach is valuable, a downside of this strategy includes the poor control over attachment site and stoichiometry. Potential immunogenicity associated with bacteria-derived avidin is also a concern.

Utilizing arginine residues

Many serotypes of AAV utilize positively charged arginine residues to bind heparan sulfate proteoglycan for cellular entry. Alterations to these arginine residues can ‘detarget’ the virus from its primary receptor for reengineering AAV tropism. Asokan *et al.* demonstrated the feasibility of masking surface-exposed arginines of AAV2 using methylglyoxal, generating charge-neutral hydroimidazolones and thereby disrupting viral binding to heparan sulfate.[87] This led to the observed ability to evade neutralizing antibodies, along with introducing novel tissue tropism towards cardiac and skeletal muscle. Similarly, Pearce *et al.* glycated arginine residues with 4-azidophenyl glyoxal, introducing a click chemistry handle to the AAV6 capsid.[88] The azide group reacts with BCN through strain-promoted azide-alkyne cycloaddition (SPAAC), facilitating the crosslinking of anti-VCAM1-scFv to the virus as a retargeting agent. Similar to lysine residues, targeting arginine residues also faces intrinsic challenges in controlling the site of attachment and the right degree of modification to optimize AAV conjugate. Disruption of the endogenous net charge of AAV, as well as the cross-reactivity of methylglyoxal with other nucleophilic residues can also be problematic.

Utilizing tyrosine residues

In addition to modifying lysine and arginine, tyrosine-selective conjugation reactions have also been used to modify AAV. Targeting tyrosine residues is advantageous because, unlike lysine and arginine-selective reactions, tyrosine modification is typically not associated with a change in charge, minimizing the chances of perturbing virus structure and function. In addition, tyrosine residues are less abundant than lysine and arginine on the capsid, affording a lower degree of heterogeneity when chemically modified. Leveraging the unique redox properties of the tyrosine phenol, Asokan and colleagues used a mild metal-catalyzed oxidation reaction to crosslink a pair of neighboring tyrosine residues bridging two capsid proteins together.[89] This selective crosslinking reaction was used to probe the role of interfacial dynamics at the AAV capsid's two-

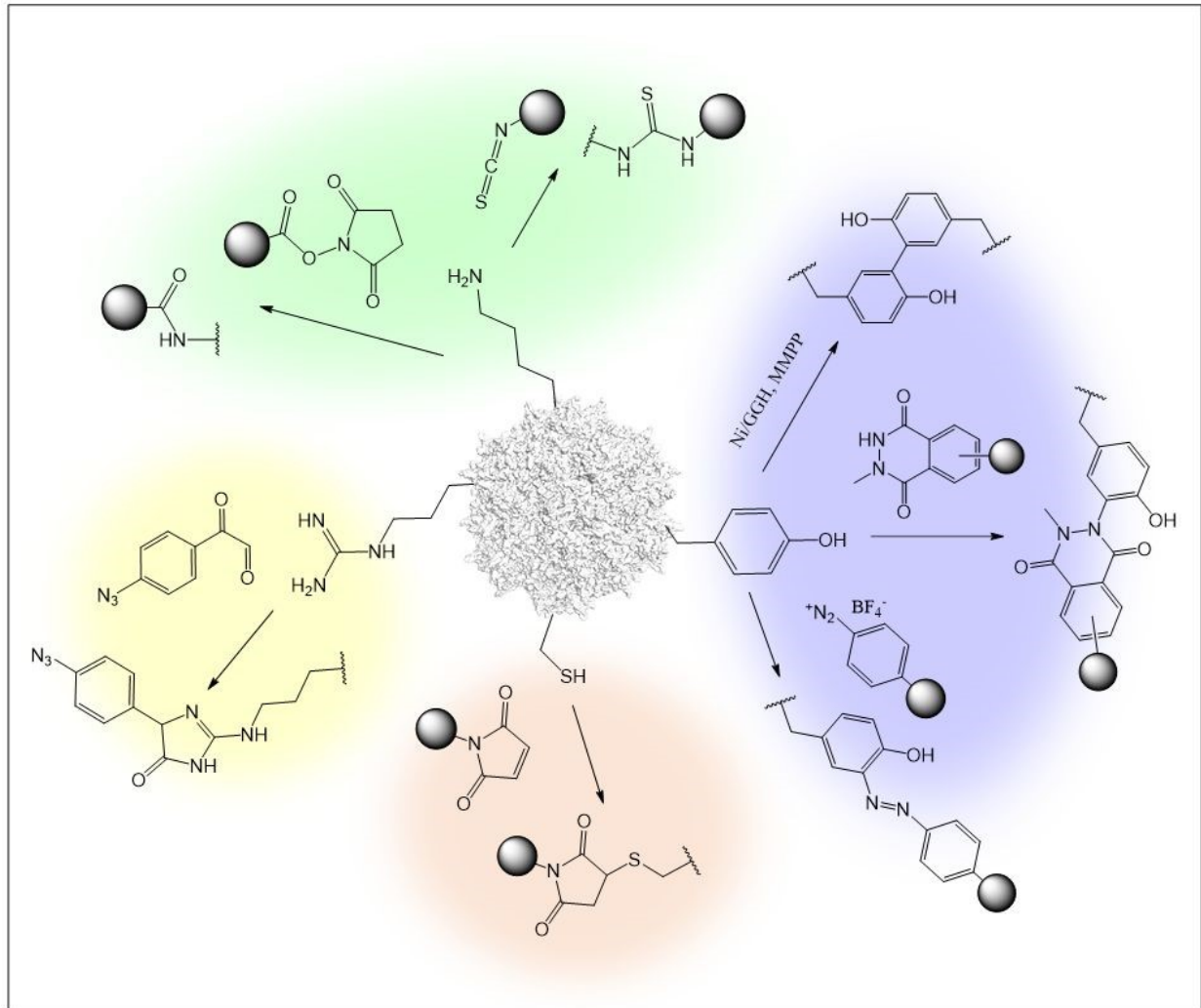


fold symmetry axis, in the externalization of N-terminal domain of a capsid protein during infection. Recently, a novel tyrosine-selective electrochemical conjugation strategy was developed by Gouin *et al.* using N-methyluminol derivatives.[90] The tyrosine residues of AAV2 were used to attach carbohydrates such as acetylgalactosamine (GalNAc) or mannose (Man) as retargeting moieties.[91] Alternatively, Mével *et al.*, have utilized the azo-coupling reaction to label tyrosine residues on the AAV capsid to attach carbohydrates for selective transduction of liver and retinal targets.[92]

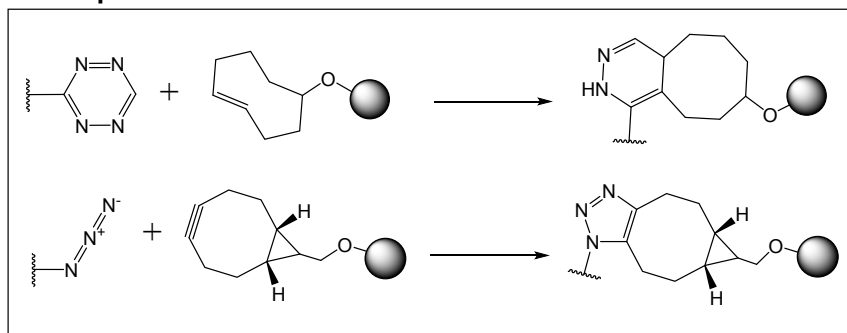
Utilizing cysteine residues

A key limitation of targeting canonical amino acids for protein labeling is the limited control over site and stoichiometry. Due to the relative scarcity of cysteines in proteins, coupled with its unique reactivity as a potent nucleophile, engineered cysteine residues have been frequently used for labeling proteins with improved site-specificity. Given the lack of exposed cysteine residues in AAV capsids, the introduction of engineered cysteine residues represents an attractive strategy for site-specific and stoichiometry control capsid modification. However, use of this strategy has remained relatively rare for AAV capsid engineering, possibly due to associated technical challenges such as oxidation of the exposed cysteines, as well as potential capsid aggregation. The Azzouz lab incorporated a 12-amino acid tetracysteine motif into the interface of VP1/VP2 proteins of AAV9.[93] After packaging, the disulfide bonded cysteine residues were reduced with TCEP, yielding nucleophilic thiols, followed by labeling with maleimide-containing molecules. Using this strategy, fluorescent dyes were attached to the capsid, enabling real-time *in vivo* imaging of viral particles in the mouse brain. Additionally, by conjugating the virus with biotin to enable streptavidin enrichment, it was possible to identify potential host interaction partners, including actin/cytoskeletal proteins, proteins involved in RNA splicing/processing, chromatin modification, intracellular trafficking, etc.





Subsequent chemical reactions to attach molecule of interest



- Modification of Lys
- Modification of Arg
- Modification of Cys
- Modification of Tyr
- Molecule to be attached

Figure 1: Various chemistry on canonical amino acids to engineer the AAV capsid



Table 2: Strategies for chemical modification of AAV at canonical amino acid residues

Amino acid to modify	AAV serotype	Reagent and chemistry to modify	Molecule to be attached	Application	Reference
Lys	AAV2 AAV3 AAV9 AAVrh .10	N-Hydroxy-succinimide (NHS)	Fluorescence dyes	FACS analysis, real time imaging, Real-time single particle tracking during infection	[73-76]
			Quantum dot	Long-term live-cell imaging	[78]
			Radioactive isotope	Imaging in animal models	[79-81]
			Polyethylene glycol	Vector protection against neutralizing antibodies	[82]
			Paclitaxel	Delivery of taxol	[94]
			Biotin	Noncovalently conjugate with streptavidin – EGF fusion protein, or with T4 phage	[95, 96]
			Streptavidin	Noncovalently conjugate with biotin tagged antibody for retargeting	[86]
AAV8	NHS followed by maleimide	zwitterionic phosphoserine	Suppress preexisting immunity by evading anti-AAV antibodies	[83]	
AAV8	3,3'-dithiobis(sulfo-succinimidyl propionate) (DTSSP)	L-fucose, cross-linked AAV	Targeted gene delivery in pancreatic cancer cells	[97]	
AAV2, AAV8	Isothiocyanates	FITC, GalNac, Mannose	Live-cell imaging, Targeted delivery to hepatocytes	[85]	
		Eosin to trigger photopolymerization	Encapsulate AAV in an acid-degradable polymeric shell embedded with siRNA for 1) gene and siRNA co-delivery and 2) immune-evasion	[84]	
AAV2, AAV8, AAV9	N-ethyl Maleimide (NEM)		Retargeting to murine bone marrow	[98]	



Arg	AAV2	Methylglyoxal	Hydroimidazol -ones	- Retarget from liver to skeletal and cardiac muscle - Immune evasion	[87]
	AAV2	4-azidophenyl glyoxal, followed by BCN (via SPAAC)	Anti V-CAM1 scFv	Retargeting to endothelial cells	[88]
Tyr	AAV2	MMPP	Cross-linked VP protein	Investigation the impact of inter- subunit protein dynamics on externalization of VP N-terminus	[89]
	AAV2	Diazonium Salts	GalNac, Mannose	Targeted delivery to hepatocytes and retina	[92]
	AAV2	N- methylluminol	GalNac, Mannose	Improved transduction of hepatocyte carcinoma	[91]
Cys	AAV9	TCEP, followed by maleimide	Fluorescence dye, biotin	<i>In vivo</i> imaging Characterization of the capsid interactome	[93]

Chemical modification of AAV capsid on non-canonical amino acids

Although labeling canonical amino acid residues have been used extensively, this approach provides limited control over the sites and stoichiometry of capsid modification, and typically results in heterogeneous mixture of conjugates. The lack of site-specificity also may be problematic due to modification of functionally important residues. The genetic code expansion technology (GCE) provides an exciting solution to these challenges by enabling site-specific incorporation of a noncanonical amino acid (ncAA) into the virus capsid with a bioorthogonal conjugation handle, which can be subsequently labeled with high specificity.

Production of AAV incorporating genetically encoded ncAAs

Using the genetic code expansion (GCE) technology, numerous ncAAs have been genetically encoded in various domains of life.[99-101] This technology uses an engineered aminoacyl-tRNA synthetase (aaRS)/tRNA pair to incorporate a ncAA in response to a repurposed nonsense codon (typically the amber stop codon UAG). With access to >200 ncAAs in mammalian cells, including those suitable for diverse applications such as bioorthogonal conjugation and photo-crosslinking. Their small structural footprint minimizes potential perturbation to the virus capsid, while providing powerful new capabilities to probe and engineer its properties.



Using the GCE technology, our lab and others have generated AAVs site-specifically decorated with ncAAs, through transient transfection of AAV2 genes, adenovirus helper genes, and a suitable aaRS/tRNA pair in mammalian cells.[102-105] To incorporate ncAA into the capsid protein, a stop codon is strategically placed at a surface-exposed site, which is suppressed by the engineered aaRS/tRNA pair. Since all capsid proteins VP1, VP2, VP3 share the same coding sequence with different splicing and alternative starts, introducing a stop codon at the common VP3 region results in the ncAA incorporation at all 60 capsid proteins. Using the pyrrolysyl synthetase (PylRS)/tRNA pair, it has been possible to incorporate azido-lysine (AzK) at various sites on the AAV capsid, without perturbing its properties. The azide-containing side chain of this ncAA enables subsequent bioorthogonal modification of the resulting capsids.

Recently, we developed a split-cap system to selectively incorporate the ncAA into a chosen subset of the capsid proteins (VP1, VP2, and VP3), providing further control over the number of ncAA handles present per capsid; capsids with 5 or 10 or 60 ncAAs can be generated by selectively incorporating them into capsid proteins VP1/VP2, VP1+VP2 or all VP1+VP2+VP3. [106] Additionally, the incorporation of ncAAs at minor capsid proteins was shown to provide wild-type-like titer of the recombinant virus, while also showing a greater tolerance to chemical modification with more defined conjugates.

Retargeting AAVs using site-specific capsid modification

It has been possible to retarget AAV to distinct cell surface receptors by attaching targeting ligands at ncAA residues on the capsid. For example, bioorthogonal attachment cyclic-RGD peptides onto AzK-modified AAV2 capsids using strain-promoted azide-alkyne cycloaddition has been used to selectively redirect them to cancer cell lines overexpressing integrin receptors.[102-106] A key advantage of this strategy is the ability to systematically modulate the site and stoichiometry of ligand attachment to fine-tune the properties of the resulting conjugate. We have shown that the site of attachment indeed has a significant impact on the efficacy of the resulting capsid conjugates. The number of retargeting ligands attached per capsid is also critically important. For example, cyclic-RGD mediated retargeting was found to be optimal at a labeling density of approximately 12 ligands/capsid.[106] Lower stoichiometry was insufficient for strong retargeting, likely due to inefficient binding, while excessive modification with cRGD was detrimental for infectivity. Kay *et al.* has also functionalized various AAV serotypes at site-



specifically incorporated AzK residues with aptamers as retargeting ligands, and demonstrated significant improvement in transduction in cancer cell lines *in vitro* [107]. However, the efficacy of the aptamer-AAV conjugates was modest *in vivo* in animal models, perhaps due to the aptamers' instability in blood. There is thus a need to further explore the possibility of attaching retargeting moieties with high receptor binding affinity and stability. Additionally, Wang *et al.* demonstrated that incorporation AzK into specific sites of the AAV5 capsid alone enhanced their lung-specific transduction.[108] This work highlights how the incorporation of diverse chemistries represented in the GCE toolbox – even without further modification – can impact the properties of AAV.

Lower the immunogenicity of AAV

GCE technology has significantly enhanced the control over the site and stoichiometry of capsid modification. Using this strategy, PEG molecules with varying molecular weights have been attached onto different sites on the AzK-containing virus particle.[109] The resulting viruses exhibited an approximately 50% reduction in anti-AAV antibody generation upon intravenous administration. In a different approach, the Mali *et al.* incorporated AzK into the AAV-DJ capsid for further conjugation with oligonucleotides using copper click chemistry.[110] By complexing the resulting oligonucleotide-labeled capsids with lipofectamine (a cationic lipid based transfection agent), they showed that the resulting lipofectamine-coated capsids were effectively shielded from neutralizing serum.

Probing the biology of viruses

Using GCE, we have incorporated photo-activatable ncAAs into the capsid of AAV2. Incorporating a photocaged lysine (NBK), replacing key arginine residues (R588 or R585) crucial for binding the primary receptor heparan sulfate proteoglycan (HSPG) resulted in non-infective mutants which were unable to bind HSPG.[111] However, a brief irradiation fully uncaged the positively charged lysine residue, restoring the HSPG receptor binding and nearly full-infectivity of these mutants. Using this strategy, it should be possible to reversibly perturb distinct molecular interactions between the virus particle and various host factors to probe their roles during viral entry. Additionally, AzK-labeled AAV capsids have also been bioorthogonally labeled with fluorescent dyes, enabling real-time imaging of virus particles during cellular entry and intracellular trafficking.[112]



Despite its remarkable potential, functionalizing AAVs with ncAAs also have some drawbacks. This approach is technically demanding, and requires significant manipulation of the standard genetic machinery needed for AAV production. Incorporating ncAAs using nonsense suppression can also lead to a reduction in viral yield, particularly when the ncAA is incorporated across all 60 capsid proteins. In addition, despite their limited footprint, not all ncAAs are tolerated at all exposed sites on the AAV capsid. In fact, only AzK and its analogs have been successfully integrated into AAV to date using the pyrrolysyl pair. Expanding the variety of ncAAs that can be incorporated into AAVs will significantly broaden the scope of applications achievable through modifying ncAAs on AAVs.

Virus as a tool for directed evolution

Beyond exploring the biology and engineering of AAV for enhanced gene therapy, we recently showed that AAV can be used to perform directed evolution of tRNAs (and potentially other biomolecules) in mammalian cells.[113,114] In this process, the activity of an AAV-encoded library of UAG-suppressing tRNA variants is coupled to the expression of a UAG-encoding AAV Cap gene. Consequently, tRNA variants with higher activity enable the production of ncAA-labeled Cap protein, facilitating the packaging of the progeny virus displaying the bioorthogonal ncAA handle on its capsid, which are further enriched through bioorthogonal biotinylation, followed by avidin enrichment. This chemical modification/enrichment strategy to improve the stringency of the selection was crucial for its success. This strategy has already been used for successful engineering of pyrrolysyl[113,115] and bacterial leucyl tRNAs,[116] and it has tremendous potential to facilitate directed evolution of additional biomolecules in mammalian cells. Gratifyingly, the engineered tRNAs generated by this method significantly improved the efficiency of ncAA incorporation into the capsid of AAV, providing wild-type-like yields.



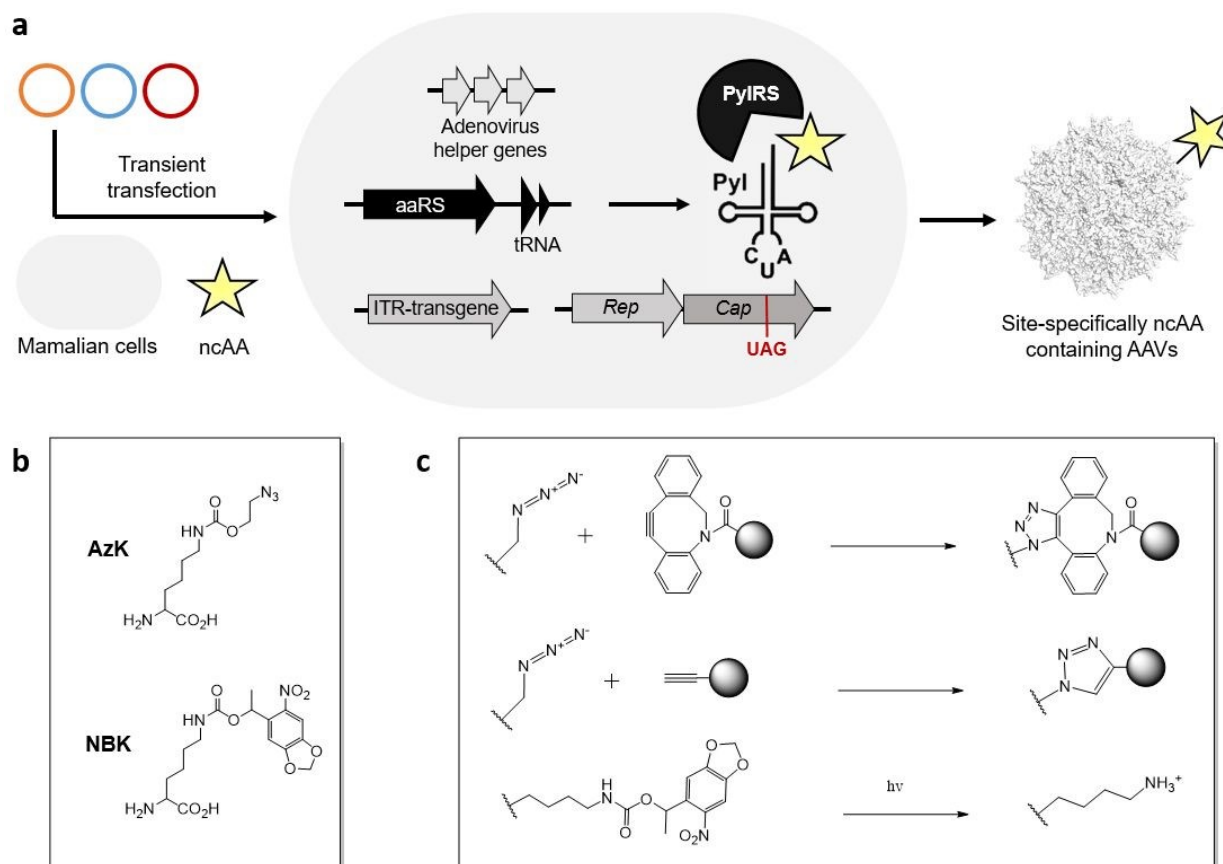


Figure 2: a) A general scheme demonstrating the site-specific incorporation of ncAAs into AAV capsid protein in mammalian cells using a nonsense-suppressing engineered aaRS/tRNA pair. b) Structure of ncAAs incorporated in the AAV capsid. c) Different chemistry on noncanonical amino acids to engineer the AAV capsid

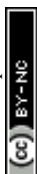


Table 3: Chemical modification of AAV using GCE technology

Serotype	ncAA	Tested sites	Reagents and chemistry to modify	Application	References
AAV2	AzK	S261, S264, A266, Q325, Q325+1, D327, N381, Y444, R447, T450-R459, S452+1, G453+1, S492, Y500, F534, E548, E548+1, T573, S578, R585+1, N587, N587+1, S662, T454 (VP1/VP2 only or VP1+VP2)	DBCO (via SPAAC) DIBO (via SPAAC)	- Attach cRGD peptides for retargeting to ovarian cancer cell lines - Biotin attachment for VADER selection in mammalian cells - Attach cRGD peptides to improve transduction of glioblastoma cells - Optimizing PEG linker size and site of incorporation to reduce immunogenicity - Attachment of fluorescent probes for imaging	[103,104,106] [113-116] [105] [109] [112]
	NBK	R585, R588	Irradiation with 365 nm deca	- Perturbation of HSPG binding to control AAV infection with light	[111]
AAV5	AzK	D374, E381, T444, G455, V481, S485, S518, T539, S576, T577	DBCO (via SPAAC)	-Attachment of fluorescence dyes - Improve transduction of many cell lines (HEK293, HUH7, C2C12, A549) and tissues (lung, liver, heart, TA muscle)	[108]
AAV-DJ	AzK	R447, S578, N587, S662	Alkyne (via Cu-click)	-Attachment of fluorescence dye for imaging - Attachment of oligonucleotide to coat AAV with lipofectamine, shielding AAV from neutralizing serum	[110]
		T456, D555, A587, N589	DBCO (via SPAAC)	- Attachment of biotin for streptavidin blot	[107]



		N589 (VP2/VP3 only)		- Attachment of folic acid or aptamer for retargeting	
AAV8/ AAVL KO3	AzK	AAV8 (E330, T457, T499, N590, N590+1, AAV-LK03 (N588, T455, T456)	DBCO- (via SPAAC)	- Attach folic acid for improved transduction of HeLa cells	[107]

Conclusion

In this review, we have outlined various exciting strategies for modifying the capsid of AAV to understand and engineer its biology, especially for developing next-generation gene therapy vectors. Among these methods, we've particularly emphasized on the chemical approaches to modify the AAV capsid, either at canonical amino acid residues, or at site-specifically incorporated ncAA residues using the GCE technology. This approach allows the capsid to be decorated with diverse entities, including biophysical probes, peptides, proteins, sugars, oligonucleotides, carbohydrates, etc. to probe and manipulate AAV biology. Modification of the canonical amino acid residues provides a technically simple, yet enabling approach to modify the AAV capsid. However, this strategy is typically associated with a lack of control over the site of modification. Although technically more demanding, the site-specific ncAA incorporation technology offers a complementary strategy with an exquisite level of control over the site and stoichiometry of capsid modification. Recent results have underscored how such controlled modification is crucial for producing AAV conjugates with optimal properties. Further development of these strategies has the potential to overcome the existing hurdles in AAV gene therapy, including retargeting the virus to specific tissues and protecting it from the immune system, thereby fully harnessing the remarkable potential of this exciting new therapeutic modality.



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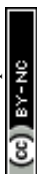
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