Chemical Conjugation

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A Precise Chemical Strategy To Alter the Receptor Specificity of the Adeno-Associated Virus

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Dedicated to Professor Peter G. Schultz on the occasion of his 60th birthday

Abstract: *The ability to target the adeno-associated virus (AAV) to specific types of cells, by altering the cell-surface receptor it binds, is desirable to generate safe and efficient therapeutic vectors. Chemical attachment of receptor-targeting agents onto the AAV capsid holds potential to alter its tropism, but is limited by the lack of site specificity of available conjugation strategies. The development of an AAV production platform is reported that enables incorporation of unnatural amino acids (UAAs) into specific sites on the virus capsid. Incorporation of an azido-UAA enabled site-specific attachment of a cyclic-RGD peptide onto the capsid, retargeting the virus to the $\alpha_v\beta_3$ integrin receptors, which are overexpressed in tumor vasculature. Retargeting ability was site-dependent, underscoring the importance of achieving site-selective capsid modification. This work provides a general chemical approach to introduce various receptor binding agents onto the AAV capsid with site selectivity to generate optimized vectors with engineered infectivity.*

Harnessing the ability of viruses to efficiently deliver genetic cargo to human cells is one of the most successful strategies for gene therapy.[1] The intrinsic preference of a virus for infecting a certain subset of human cells, which is frequently at odds with the therapeutic need, is a key challenge in developing effective therapeutic vectors, and the ability to precisely alter the host tropism of a virus is highly desirable.[2] Adeno-associated virus (AAV), a small non-enveloped human virus, is one of the most promising vectors for human gene therapy owing to its naturally replication deficient nature, lack of associated pathogenicity, the ability to elicit long-term gene expression in vivo, and low immunogenicity.[3] Herein we describe a chemical approach to site-specifically introduce synthetic targeting groups onto the AAV capsid, which enables the virus to use non-native cell-surface receptors to enter human cells.

While a number of AAV-based gene therapies are currently undergoing clinical trials, all rely upon the natural tropism of various AAV serotypes.[3b] However, several important therapeutic targets remain resistant to available AAV serotypes, and their systemic delivery is often associated with off-target infectivity.[3b] Significant efforts have been made to both broaden the tropism of AAV and specifically target it to desired cells.[3a,4] Such strategies include directed evolution of novel capsid proteins with altered receptor binding properties,[5] incorporating peptide sequences into permissive capsid sites that either directly bind a specific receptor or can be enzymatically functionalized with binding agents,[6] and fusing antibody fragments to the N-terminus of the minor capsid protein VP2.[7] While useful, the directed evolution approach often fails to produce a specific vector for a desired therapeutic target. Owing to the complex and delicate architecture of the AAV capsid, composed of 60 intricately interlocked capsid proteins (Figure 1 A), strategies

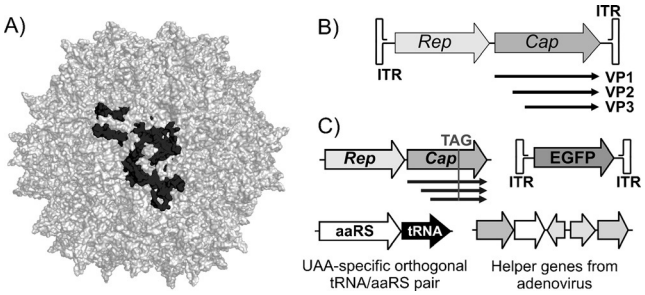

Figure 1. A) Structure of the AAV2 capsid showing a single capsid protein in black. B) Genome organization of AAV2. C) Genetic elements necessary to produce AAV2 site-specifically incorporating UAA residues into its capsid proteins (for further details, see the Supporting Information, Materials and Methods and Figure S1).

such as peptide insertion or antibody fusion are often poorly tolerated, leading to defects in virus assembly or entry.[8] Consequently, such modifications are associated with drastically limited site selection, which in turn precludes any optimization of how the binding motif is displayed relative to the capsid.

Chemical conjugation is an alternative approach to introduce synthetic receptor binding motifs onto the AAV capsid, and it offers a number of significant advantages including the ability to use targeting agents that cannot be genetically encoded (such as small molecules and aptamers), flexible control over the stoichiometry of virus labeling, and an opportunity to take advantage of synthetic linkers of

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varied chemical properties to optimize the display of the binding motif relative to the virus capsid. However, conjugation reactions currently available to modify the AAV capsid are targeted to natural amino acid side chains such as arginine or lysine.[9] Owing to an abundance of these amino acid residues on the AAV capsid, this strategy intrinsically lacks site specificity, and runs the risk of modifying residues functionally important in viral entry. Metabolic incorporation of azido sugars into protein glycosylation sites, or replacement of methionine residues with its structural surrogate azidohomoalanine, have been used for chemical capsid modification of adenovirus.[10] However, the absence of glycosylation precludes the extension of the first strategy to AAV, while the presence of multiple methionine residues on AAV capsid compromises the site specificity associated with the second approach.

Expansion of the genetic code has enabled site-specific co-translational incorporation of unnatural amino acids (UAAs) into proteins expressed in living cells in various domains of life.[11] This technology takes advantage of an engineered tRNA/aminoacyl-tRNA synthetase (aaRS) pair to deliver the UAA of interest in response to a repurposed nonsense codon (typically TAG). Over 100 UAAs have been genetically encoded, including those harboring functionalities that allow bio-orthogonal chemical conjugation reactions.[11,12] Incorporation of such UAA residues into recombinant proteins has enabled their site-specific modification with a variety of molecules.[13] The ability to extend this technology for incorporating UAAs into chosen sites on the AAV capsid will provide a powerful new approach for its precise chemical modification.

Relative to the envelope proteins of enveloped viruses (encapsulated by a lipid membrane), which have been recently subjected to UAA mutagenesis,[14] capsids proteins of non-enveloped viruses (no lipid membrane) such as AAV are more challenging targets for structural modifications. Unlike the former, which is embedded in a lipid bilayer, the latter is engaged in an intricate network of protein-protein interaction that can be perturbed from even small modifications (Figure 1 A).[15] However, we envisioned that the availability of a crystal structure of the AAV serotype-2 (AAV2) capsid should facilitate rational site selection to minimize the chances of such perturbation.[16] The AAV2 virion consists of sixty proteins arranged in an icosahedral symmetry, packaging a 4.7 kb single-stranded DNA genome.[15] The genome harbors two open reading frames (ORFs), *rep* and *cap*, each encoding multiple overlapping genes necessary for AAV2 replication and capsid protein synthesis, respectively (Figure 1 B). The *cap* ORF encodes three capsid proteins, VP1, VP2, and VP3, expressed approximately in a 1:1:10 ratio, all of which share the same 533 C-terminal amino acids, while the minor capsid proteins VP1 and VP2 have additional N-terminal extensions (Figure 1 B).[15] Since these N-terminal extensions are buried within the AAV2 capsid, we targeted the common C-terminal region for UAA incorporation, which will modify all 60 capsid proteins (Figure 1 C). Five distinct surface-exposed sites in three different regions were chosen as sites of UAA incorporation (Figure 2 A).[15,16] G453 and T454 are on the tip of the highly surface-exposed threefold

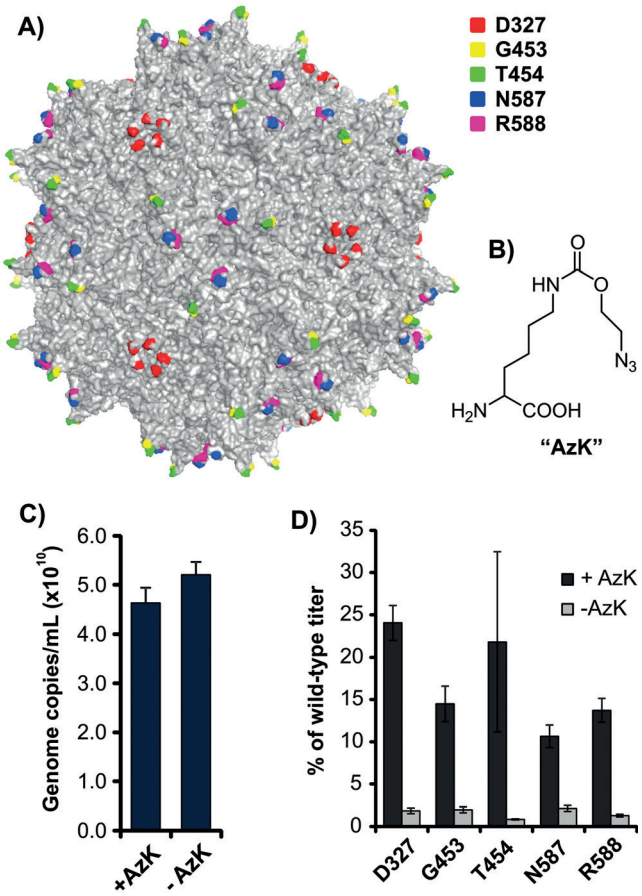

Figure 2. A) Distribution of the residues targeted for UAA-mutagenesis on the AAV2 capsid. B) Structure of AzK. C),D) Production of C) wild-type or D) site-specific UAA-mutants of AAV2 in the presence or absence of 1 mM AzK in the medium, measured as packaged genome copies/mL by qPCR.

proximal spike. N587 and R588 residues are located in a basic cluster used to bind heparan sulfate proteoglycan (HSPG), the primary receptor for AAV2 cell entry. Lastly, the D327 residue resides around the pore at the fivefold symmetry axis. The tight packing of this residue from five different capsid proteins in a functionally important region makes it a more challenging target for UAA mutagenesis relative to the other chosen sites, which are known to tolerate insertion of foreign peptide sequences.[3a,4]

The pyrrolysyl-tRNA/aaRS pair was selected to drive UAA incorporation into the AAV2 capsid, since it boasts the largest collection of genetically encoded UAAs in eukaryotic cells.[11] We incorporated expression cassettes of the *Methanosarcina barkeri* pyrrolysyl-tRNA-synthetase (MbPylRS) and *Methanosarcina mazei* pyrrolysyl $tRNA_{CUA}$ ($tRNA^{MmPyl}_{CUA}$), expressed from CMV and U6 promoters, respectively, into a commercially available plasmid system for AAV2 production (Figure 1 C; Supporting Information, Figure S1). Additionally, the five aforementioned amino acid residues in the common C-terminal region of the capsid proteins were individually mutated to TAG. We selected the amino acid AzK (Figure 2 B) for capsid modification, the azido functionality of which should enable a facile bioconju-

gation reaction with high specificity via strain-promoted azide-alkyne cycloaddition (SPAAC).[12d,17] Additionally, the long and lean structure of this UAA should minimally perturb the AAV2 capsid and display the azido functionality effectively for conjugation reactions.

The efficiency of AAV2 production for each of the five mutants was determined by transfecting HEK293T cells with our AAV2 production plasmids in the presence or absence of 1 mM AzK, and then measuring the number of capsid-packaged viral genomes using qPCR, three days post-transfection. It confirmed efficient production of all AzK-containing mutants (Figure 2C,D) only in the presence of AzK. Our plasmid system introduces a wild-type EGFP reporter in the AAV2 genome, which enables a convenient analysis of its infectivity. To ensure that the incorporation of AzK does not negatively influence the AAV capsid structure or perturb its native infectivity, HEK293T cells were individually infected with wild-type as well as all five mutant viruses at a fixed MOI (multiplicity of infection, or genome copies per cell) of 50 and EGFP reporter expression was analyzed 48 hours post-infection using fluorescence microscopy, as well as the measurement of EGFP-fluorescence in cell-lysate (Figure 3A; Supporting Information, Figure S2). Viruses harboring AzK at G453, T454, and N587 sites had infectivity similar to wild-type, while the D327AzK mutant was slightly less infective. The R588AzK mutation, which eliminates one of the arginine residues essential for binding the primary receptor HSPG,[15] resulted in a near-complete loss of infectivity as expected. This analysis also revealed that in the absence of AzK, the TAG mutants of AAV2 produced no detectable infective virus (Supporting Information, Figure S2), even though the qPCR analysis suggested attenuated production of packaged genomes (Figure 2D).

We purified the wild-type AAV2 as well as the T454AzK mutant by HSPG-affinity chromatography. An SDS-PAGE analysis of both viruses revealed the presence of the three capsid proteins in an appropriate ratio (Figure 3C). To confirm the presence of the azido residue on the surface of the mutant virus, both the wild-type and the T454AzK AAV2 were treated with a strained alkyne–fluorophore conjugate (DBCO-Cy5; Figure 3B). SDS-PAGE resolution followed by fluorescence imaging confirmed selective labeling of all three capsid proteins of T454AzK (Figure 3C), but not wild-type AAV2, confirming the presence of the azido functionality on the virus capsid, as well as our ability to functionalize it using SPAAC. An analogous labeling experiment of R588AzK AAV2 capsid proteins using DBCO-Cy5 was also successful (Supporting Information, Figure S3).

Next, we explored the possibility of retargeting AAV2 to a new cell-surface receptor by chemically attaching a synthetic receptor-targeting motif onto the site-specifically introduced AzK residues. A cyclic peptide with a RGD motif (cRGDFC; Figure 4C; Supporting Information, Figure S4), which is known to bind the $\alpha_v\beta_3$ integrin receptors expressed highly on tumor vasculature,[18] was chosen as the targeting agent. Ovarian tumor-derived SK-OV-3 (high $\alpha_v\beta_3$ integrin expression) cells were used as the target cancer cell line, while the HEK293T cells (weak $\alpha_v\beta_3$ integrin expression) were used as

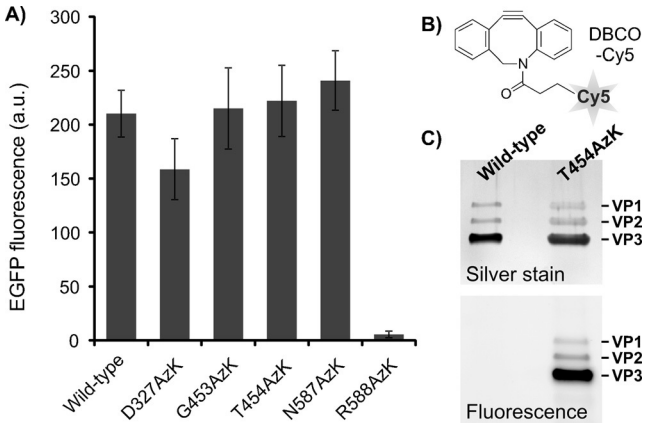

Figure 3. A) Infectivity of wild-type and the site-specific AzK-mutants of AAV2, measured as EGFP fluorescence in the lysate of a constant number of HEK293T cells infected with 50 genome copies/cell of each virus. B) Structure of DBCO-Cy5. C) Silver stain (top) or Cy5-fluorescence imaging (bottom) analysis of SDS-PAGE-resolved capsid proteins associated with purified wild-type or T454AzK AAV2, following treatment with 5 μM DBCO-Cy5.

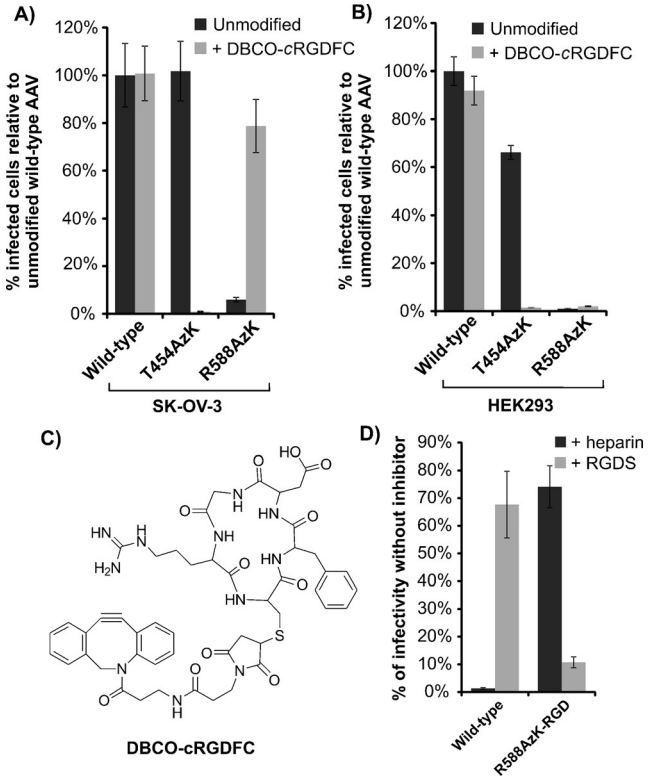

Figure 4. A),B) Infectivity of wild-type, T454AzK, and R588AzK AAV2 towards A) SK-OV-3 and B) HEK293T cells, before and after functionalization with C) DBCO-cRGDFC. Infectivity was measured as the percentage of EGFP-expressing cells (FACS), and was normalized relative to unmodified wild-type AAV2 (at a fixed genome-copy/cell). D) Infectivity of wild-type and DBCO-cRGDFC-modified R588AzK AAV2 towards SK-OV-3 cells in the presence of RGDS peptide or heparin. Infectivity for each virus was measured as the percentage of EGFP-expressing cells (FACS), and was normalized relative to the corresponding no inhibitor control.

a control.[6e] Sites T454 and R588 on the AAV2 capsid were selected for a preliminary evaluation of cRGDFC attachment mediated retargeting to the $\alpha_v\beta_3$ integrin receptor. Upon treatment with 20 μM DBCO-cRGDFC, the R588AzK virus, which is non-infective owing to the absence of the primary receptor binding Arg588 residue, regained significant (80% of wild-type AAV2; Figure 4A; Supporting Information, Figure S5B) infectivity towards SK-OV-3 cells, but not HEK293T cells (Figure 4B; Supporting Information, Figure S5A), indicating successful retargeting to the $\alpha_v\beta_3$ integrin receptor. The enhanced infectivity of the modified virus was strongly inhibited by the RGDS peptide (which blocks binding to $\alpha_v\beta_3$ integrin), but not heparin sulfate (which inhibits HSPG binding), further confirming its $\alpha_v\beta_3$ integrin-dependent cellular entry (Figure 4D; Supporting Information, Figure S8). Meanwhile, analogous functionalization of the T454AzK (Figure 4A,B, Supporting Information, Figure S5A,B) virus resulted in a complete loss of its infectivity towards both HEK293T and SK-OV-3 cells. However, mutating the primary receptor-binding residues of T454AzK AAV2 provided a vector that behaved similarly to its R588AzK counterpart: the unmodified virus was non-infective but gained infectivity towards SK-OV-3 cells, but not HEK293T cells, upon modification with DBCO-cRGDFC (Supporting Information, Figure S7). The retargeting efficiency of this modified virus was somewhat lower than its R588AzK counterpart. These observations underscore the importance of achieving site-specific modification to generate optimally active virus-targeting agent conjugates.

In conclusion, we demonstrate for the first time the site-specific incorporation of an UAA into the capsid of a non-enveloped eukaryotic virus and its use for attaching a synthetic ligand that successfully retargets the virus to a new cell surface receptor. Since the AzK residue was well-tolerated in multiple distinct regions of the capsid, including seemingly challenging areas such as around the pore, our approach significantly expands the selection of sites that can be evaluated for attaching a variety of retargeting motifs. Efforts are currently under way to extensively characterize the retargeting efficiency associated with a large selection of additional sites, as well as evaluating the optimal degree of capsid-functionalization. We are also adapting this technology to enable UAA incorporation only into a subset of the three capsid proteins, which will provide further control over the number and the display of attachment sites on the AAV capsid. Finally, AAV is a model system to understand the assembly, cell entry, and the genome release of *parvoviridae* family of viruses,[15] and the platform described here will greatly facilitate such studies by enabling site-specific incorporation a number of powerful genetically encoded biochemical and biophysical UAA-probes such as fluorophores and photo-crosslinkers.[11b,c,19]

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