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Surface modification of Adenovirus type 5 vectors via two-step metabolic labeling for PET imaging

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Jai Inder Pal

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Abstract of the Thesis

Surface modification of Adenovirus type 5 vectors via two-step metabolic labeling for PET imaging

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Surface modification of viruses is of great interest for therapeutic gene delivery. Numerous viruses have been, and continue to be engineered in an attempt to optimize them for targeted *in vivo* gene therapy. Adenovirus has been shown by a myriad of publications to be the most efficient delivery vehicle in different cell types. But while it is the preferred delivery agent in many gene therapy studies, drawbacks such as off-targeting, immune response and toxicity still hinder it from being used in clinical studies. In an attempt to modify the surface of Adenovirus efficiently, our lab developed a simple two-step labeling protocol to modify Adenovirus particles with specific ligands in a time efficient manner without affecting the virus infectivity. We have shown that our method is able to produce site specific surface modified infectious Adenoviruses with high titers. For future studies, we intend to use this approach to engineer dually modified Adenovirus particles with a targeting ligand and a PET scanning radioactive moiety (^{18}F or ^{89}Zr) to help visualize distribution *in vivo*. The results from our studies will provide useful insight into

the biodistribution of Adenovirus, which can eventually help contribute to the future development of next generation of Adenovirus vectors better suited for gene delivery.

Dedication Page

I would like to dedicate this to my parents and siblings for always motivating me.

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List of Abbreviations

AdV	Adenovirus
AdV5	Adenovirus Type 5
Aha	Azidohomoalanine
Ac_4GalNAz	Peracetylated N-azidoacetylgalactosamine
BCS	Bovine Calf Serum
CAR	Coxsackievirus and Adenovirus Receptor
CsCl	Cesium Chloride
CuAAC	Copper (I)-Catalyzed Azide-Alkyne Cycloaddition
DMEM	Dulbecco's Modified Eagle's Medium
^{18}F	Radioactive Fluorine-18
GlcNAz	N-azidoacetylglucosamine
HEK 293	Human Embryonic Kidney 293 cells
iPSC	Induced Pluripotent Stem Cells
MOI	Multiplicity of Infection
OD	Optical Density
PEG	Polyethylene Glycol
PET	Positron Emission Tomography
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SPAAC	Strain-Promoted Azide-Alkyne Cycloaddition
^{89}Zr	Radioactive Zirconium-89

Acknowledgments

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Introduction

Viral Vectors and Gene Therapy

Viruses are evolved biological gene delivery vehicles that use their host's cellular components to replicate and proliferate. As a result, when manipulated and packaged with genes of interest, they are ideal candidates for delivering specific genes to cells. Because of this attribute, there has been, and continues to be great interest in modifying infectious viruses for therapeutic purposes including vaccine development, gene therapy to treat oncolytic tumors and genetic disorders [1].

Gene therapy aims to supplant a gene in a cell in order to restore proper protein production, or alter it. In theory, the concept of gene therapy is quite simple but in order for gene therapy to be successful *in vivo*, there needs to be an efficient and non-toxic gene delivery system. Viruses, by nature, are carriers of genetic information and consequently are currently the most popular and effective delivery agents [2]. Over the years, numerous virus vectors including retrovirus, adenovirus (AdV), adeno-associated virus and a myriad of others have been developed and are being used to treat diseases. To date, 70 % of gene therapy clinical trials have used viral vectors as the preferred means of gene transfer [3, 4]. As a result of this significant potential, there continues to be extensive efforts to engineer viral vectors for gene therapy.

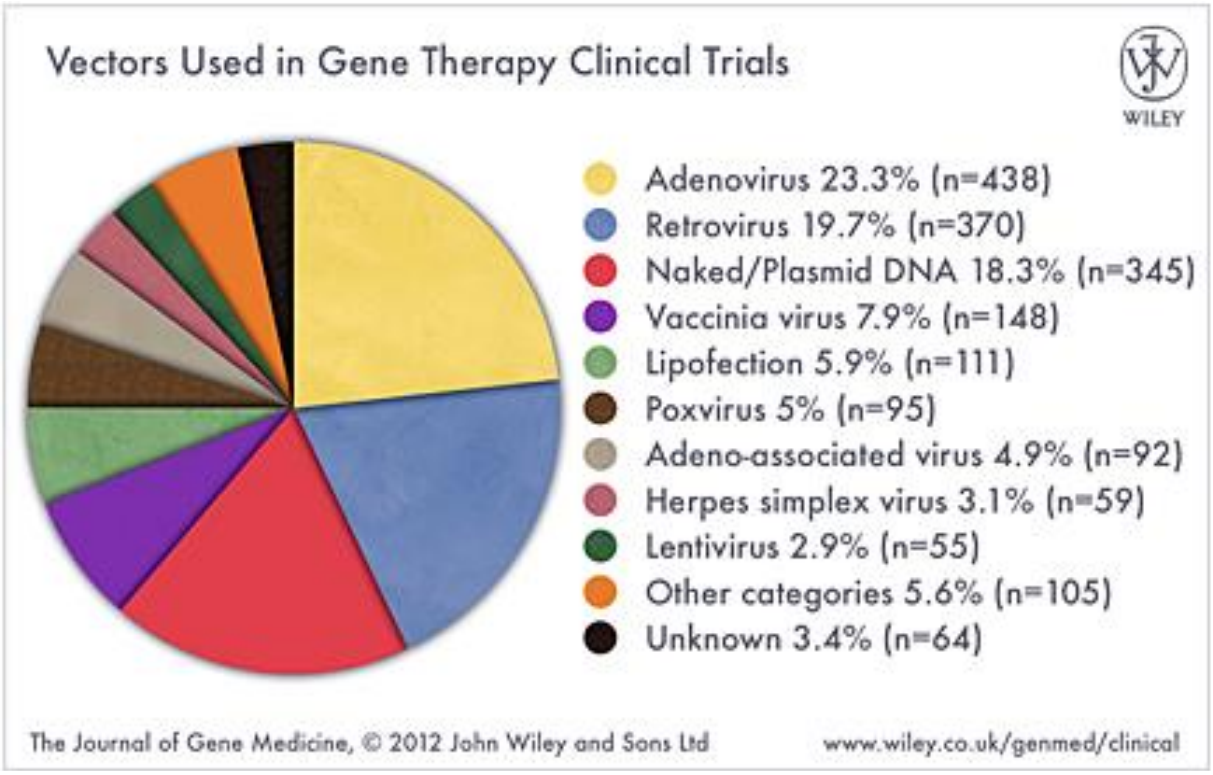

Figure 1: Viral vectors used in gene therapy clinical trials. A worldwide statistic till 2012. Adenovirus represent one of the most widely used viral vectors for gene therapy trials [5].

Adenovirus virotherapy and Adenovirus serotypes

Currently, human AdV are the most widely employed vectors for gene therapy. AdVs have several unique features that make them ideal candidates for use in gene therapy. They are highly prevalent, can transduce a variety of cell types including non-dividing and quiescent cells with high efficiency and high levels of gene expression, they have low levels of pathogeniety in humans, rarely integrate into the host genome, can be easily manipulated and are produced with high titers [6, 7, and 8]. In fact, the advantageous characteristics of AdV have led to its licensing to be used as an approved gene therapy agent for the treatment of head and neck cancers in China [9].

There are over 60 different AdV serotypes that have been discovered and characterized into groups (A-F). Serotypes from the same group usually exhibit similar structures and pathogenies. Out of the abundant serotypes, serotypes 2 and 5 of species C are the most intensively studied and constitute the majority of the vectors used in disease treatment. Serotype 5 has been most widely used but drawbacks such as targeting, immune response and liver enzyme secretion which leads to destruction of the virus particles limits its *in vivo* efficacy and, prevents its clinical trials and use in common gene therapy treatments. As a result, much effort has been focused on developing strategies to tamper the immune response and target the virus to specific cell types [8, 10, and 11].

Need for engineered viruses

Although AdV is regarded as a leading delivery agent for gene therapy, targeting specific tissues by surface modification is still a challenging task. The success of gene therapy depends on the availability of efficient gene delivery vehicles that can specifically administer genes to target cells without affecting other cells in close proximity.

The most notable obstacle with using AdV is that it exhibits off-target interactions with cells that express the CAR receptors which are the entry sites of AdV into the cells. Most oncolytic cells are known to only express low levels of the CAR receptor which makes it difficult to direct targeted delivery of therapeutic genes using the wild-type AdV [8]. This results in a lower therapeutic index of AdV, which poses a problem for clinical trials. Because the CAR receptor is broadly distributed, the ideal strategy would be to modify the surface of AdV to produce AdV particles that can be targeted to specific cells independent of the CAR receptor [8,

12]. As a consequence numerous, transductional and transcriptional approaches have been developed to modify AdV vectors for targeted delivery of therapeutics to specific cells.

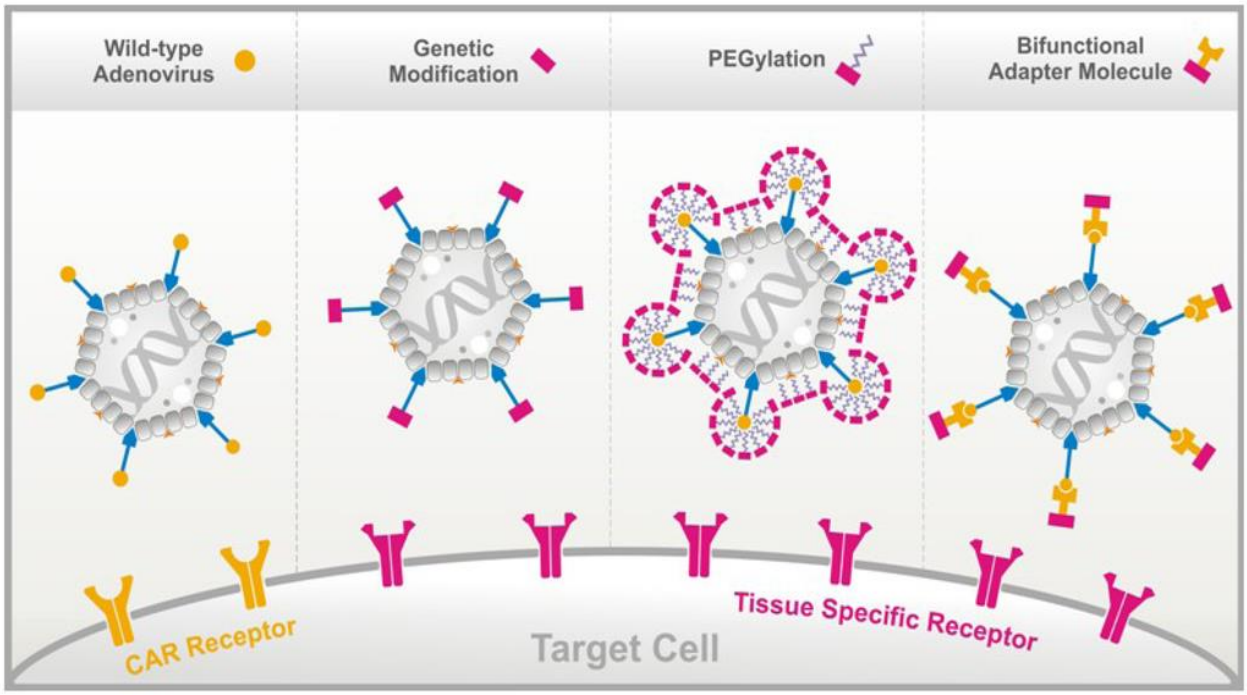

Figure 2: Strategies for surface modification of AdV vectors for enhanced *in vivo* targeted gene delivery [13]. A) Wild-type AdV enters the cell via the CAR receptor. B) Genetic integration of peptide into the virus fiber. C) Chemical conjugation using polyethylene glycol (PEG). D) Bridging the cell and virus using bispecific adaptors [13].

AdV surface modification approaches have aimed to reduce transduction to nonspecific organs and minimize immune response. The strategies include pseudotyping AdV using different serotypes or AdV from different species, genetic modification of the virus fiber proteins at different regions, addition of a targeting peptide ligand onto AdV surfaces by chemical conjugation using PEGylation or non-covalent adapter linkage, which bears specificity to both AdV and the target the cells [13]. Although these approaches have some advantage of increased specificity of AdV for the targeted cells, their drawbacks cannot be overlooked. Poor specificity of ligands, standardization of virus production, disruption of viral stability and infectivity remain

the major concerns [12, 8]. Hence, these approaches are not the most optimum for producing modified infectious viruses that can be used in clinical trials.

An ideal strategy for the production of chemically modified AdV should allow us to produce viruses efficiently with high titers while retaining the viral fitness and infectivity, and without the need for genetic modifications. The approach must be highly reproducible, efficient, and technically straightforward. In an effort to produce surface modified viruses, our lab has come up with a simple two-step approach to chemoselectively modify AdV particles.

Two step labeling approach

In order to increase the selectivity of AdV5 surface modification, our lab has utilized bioorthagonal click chemistry to develop a two-step metabolic labeling protocol for virus surfaces. In the two step click chemistry reaction, substrates are first modified with unique functional groups and then a reporter probe or small molecule of interest is covalently attached to the specific functional group via click reaction. An azide group is one of the most widely used click reagents as it is small, biologically stable, induces minimal perturbance in biological systems and can modify proteins and other classes of biomolecules [14].

In our two-step approach we use azides as the uniquely reactive functional groups for surface modification of the virus particles. Initially, viruses are produced in the presence of unnatural amino acids or sugars substituted with an azide functional group. The unnatural substrates are analogs of the natural substrates which are utilized by the aminoacyl-t-RNA and the protein manufacturing machinery of the cell, and are eventually incorporated into the surface proteins of the virus. Finally, the azides are modified with selective bioorthagonal chemistry for different experimental purposes. Incorporation of azides allows access to highly selective

reactions such as azide-alkyne cycloaddition (Cu-catalyzed or strain promoted) or Staudinger ligation, to covalently attach probes or other small molecules of interest on to the virus surface [14, 15].

The two-step method is particularly advantageous. First, because the labeling utilized the natural metabolic pathway, it has a minimal impact on the virus infectivity and titer. Additionally, a wide array of effector ligands can be used. And lastly, it is simple, fast, reliable and readily accessible to use [15, 16].

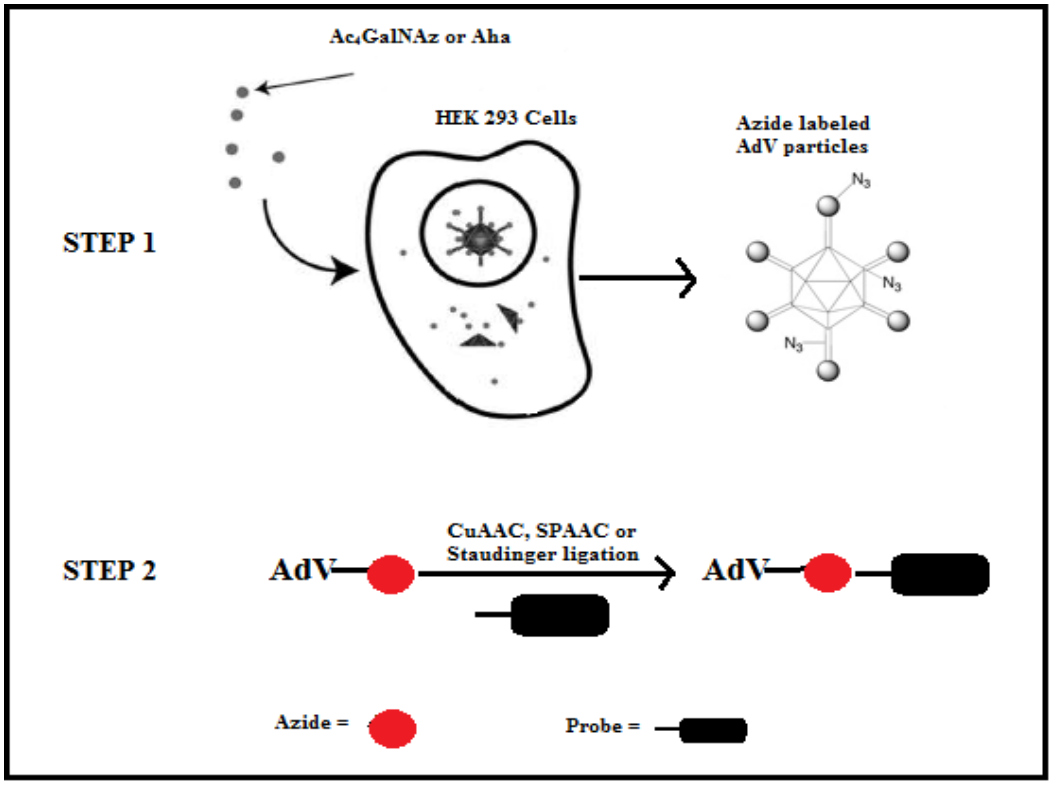

Figure 3: Two-step labeling protocol developed in our lab. 1) In the first step AdV particles are charged with reactive azide groups via metabolic labeling by using unnatural amino acid such as azidohomoalanine (Aha) or an unnatural sugar, such as peracetylated N-azidoacetylgalactosamine (Ac_4GalNAz). 2) In the second step, the azide can be used for highly specific click reactions such as Copper (I)-Catalyzed Azide-Alkyne Cycloaddition (CuAAC), Strain-Promoted Azide-Alkyne Cycloaddition (SPAAC) or the Staudinger ligation to ligate specific probes/ligands onto the virus surface.

Lack of biodistribution data for AdV and engineering of PET radioisotope labeled AdV5 particles

One of the setbacks in the attempt to develop novel AdV vectors for specific targeting is the lack of understanding of the biodistribution and *in vivo* targeting capacity of the virus vectors to specific cell types. The lag in the development of imaging techniques is one of the reasons for this gap of knowledge. Conventional studies for tracking of AdV vectors use reporter genes such as green fluorescent protein (GFP), luciferase,somatostatin receptor type 2 (SSTR-2) and sodium iodide symporter (NIS), as well as soluble marker peptides such as human carcinoembryonic antigen (hCEA) and bhuman chorionic gonadotropin (b-hCG) [17]. These approaches require viral infection and gene transduction for imaging. Since their detection is limited on the expression of the genes, they do not truly represent the physical distribution and pharmodynamic data of the virus. The drawbacks of these reporter gene and markers prevent them from being the optimum candidates to be used for gathering physical bio-distribution data. These approaches do not give a direct quantification of the viral vector, which makes them not very reliable [17, 18].

Positron emission tomography (PET) is a leading method for particle tracking in real time and in living systems. As evident, it would be plausible to use PET studies for viral tracking *in vivo*. Labeling the surface of AdV with a PET imaging probe using our two-step protocol should allow us to produce virus particle that can be visualized in living systems and increase our knowledge of biodistribution of the particles.

In our study we are interested in labeling AdV particles using our two-step protocol with a radioisotope and a targeting ligand to track the modified virus in *vivo*. Using the metabolic

labeling method should lead to generation of high titer virus production with minimal loss of virus infectivity. ^{18}F is the preferred radioisotope used in PET tracking studies, as it exhibits great sensitively, is a pure positon emitter, generates low energy positons and is substantially stable under physiological conditions. While ^{18}F is an attractive candidate for PET studies, it has a relatively short half-life (110 minutes) which would not be ideal for studies of deep penetrated tumors. Consequently, it would be beneficial to use alternative radioisotope with a relatively longer half-life and would provide an extended imaging time span. ^{89}Zr is a radioisotope with a longer half-life (3.27 days), and it also allows a sensitive analysis of particle tracking making it a more applicable isotope for deeply penetrated tumors. So in our study we intend to engineer an AdV surface labeled with the ^{89}Zr radioisotope and a folate ligand to track and visualize the virus *in vivo.*

The successful labeling and visualization of the PET moiety labeled virus in an *in vivo* model would provide important input into the emerging field of AdV engineering. The results will be an important contribution to the current research of efficient surface modifying AdV particles and optimizing virus targeting to specific locations in tissues. The insights gained from this study will help us further our knowledge of targeted viral vector engineering and help to design future viral vectors and labeling strategies.

Materials and Methods

HEK 293 cell culture: Dulbecco's modified Eagle's medium (DMEM), penicillin-streptomycin, and 0.5 % trypsin EDTA were purchased from Gibco (Grand Island, NY). Bovine calf serum (BCS) was purchased from HyClone (Logan, UT). HEK 293 cells were maintained in DMEM supplemented with 10 % BCS and 1 % Pennstrep. All cells were maintained in 100 mm tissue culture dishes obtained from BD Biosciences and incubated at 37 °C and 5 % CO_2.

AdV5 production and harvest: All AdV5 replication was carried out in HEK 293 cells in 10 (100 mm) tissue culture plates. Each plate was infected with 1 ml of the AdV5 infection buffer (2% (v/v) bovine calf serum, 1× TC solution, AdV5 in storage buffer, adjusted final volume using TD buffer) with a MOI of 200 PFU/cell . Plates were incubated for 1 hour at 37 °C degree, rocking every 15 minutes. After the 1 hour infection period 9 ml of DMEM (regular or supplemented with labeling media) was added to the infected cells and the plates were incubated at 37 °C at 5 % CO_2 (incubation time and media change dependent on the type of labeling). After the growth period, virus infected cell suspension (~100 ml) was collected into two 50 ml falcon tubes and centrifuged at 2000 g for 10 minutes at 4 °C**.** The supernatant was discarded and the cell pellet was resuspended in 8 ml of TD buffer. The virus was then harvested with three repeated freeze thaw cycles using liquid nitrogen and a 37 °C water bath. The suspension was again centrifuged under the same conditions and the supernatant was purified to collect the virus.

AdV5 purification with CsCl gradient ultracentrifugation: All AdV5 purification was carried out using CsCl gradients. 2.5 ml of 1.25 g/ml was added into an ultracentrifuge Beckmann tube and then 2 ml of 1.40 g/ml of CsCl solution was added on the bottom of the tube by carefully placing the tip of the pipette on the bottom of the tube and releasing the solution slowly. The virus suspension was then added on top of the CsCl gradient. The centrifuge was balanced with

an equal volume of TD buffer and the tubes were placed into the SW41 rotor in a Beckman Coulter Optima L-90K Ultracentrifuge to carry out the centrifugation at 32,000 rpm for 1 hour at 15 °C.

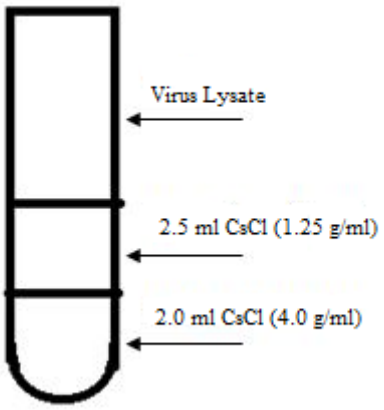

Figure 4: CsCl density gradient set up for AdV5 purification using SW41 rotor with ultracentrifuge.

Metabolic labeling of AdV5 with Azidohomoalanine: 10 plates of 80-90 % confluent HEK 293 cells were infected with wild-type AdV5 particles at a MOI of 200 PFU/cell for one hour (as described earlier). Regular HEK 293 growth media was added to each plate after the 1 hour infection and incubated for 18 hours. After18 hours, the growth medium was removed, and cells were washed with 4-5 ml of TD buffer (25 mM Tris, 125 mM NaCl, 5 mM KCl, and 1 mM Na_2HPO_4, pH 7.5). After washing with TD buffer, 10 ml of 4 mM Aha-supplemented DMEM (lacking Met and supplemented with 2 mM cysteine, 2 mM glycine, and 10 % BCS) medium was added to each plate of the infected cells and incubated for 6 hours at 37 °C for labeling. At the end of the 6 hour labeling period, the labeling media was removed and cells were supplemented with complete DMEM medium and incubated for another 18 hours to grow. The cells were harvested after a total of 44 hours post virus infection. The virus particles were then

purified over CsCl gradients. The white virus band at the junction of the two CsCl bands was collected, dialyzed and stored at -20 °C.

Metabolic labeling of AdV5 with Ac_4GalNAz: 10 plates of 80-90 % confluent HEK 293 cells were infected with wild-type AdV5 particles at a MOI 200 PFU/cell. After the cells were infected for 1 hour (as described under the AdV5 production section) 10 ml of Ac_4GalNAz (100 µl of 50 mM stock Ac_4GalNAz in methanol was added to 100 ml of DEK 293 DMEM growth media) supplemented labeling media was added to the cell plates and the plates were incubated for 44 hours at 37 °C and 5 % CO_2. The cells were then harvested and purified over the CsCl gradients. The virus band was collected, dialyzed against storage buffer and stored at -20 °C.

Adenovirus dialysis and storage: CsCl purified AdV5 particles were further dialyzed overnight against the AdV5 storage buffer (5 mM Tris-HCl buffer, pH 8.0, containing 0.5 mM $MgCl_2$, 50 mM NaCl, 25 % (v/v) glycerol, and 0.05 % (w/v) Bovine Serum Albumin (BSA)) to remove the excess CsCl salts. AdV5 particles in CsCl salt solution was loaded into a dialysis cassette and allowed to dialyze against 150 ml of dialysis buffer. The buffer was replaced after 60 minutes and 120 minutes. The dialysis was then allowed to proceed overnight at 4 °C. After dialysis, AdV particles in storage buffer were stored at -20 °C.

Adenovirus OD 260 titer assay: To quantify virus DNA particles/ml, an aliquot of AdV5 particles stored in CsCl or storage buffer was diluted to 1:20 with 0.1 % SDS solution (0.1 g in 100 TE solution (1M Tris-HCl, pH=8, 0.5 M EDTA)). The mixture was then heated for 10 minutes at 56 °C. 100 µl aliquots were then used to take a UV spectrum reading using a Beckman Coulter DU 730 at 260 nm using the appropriate blanks.

Conjugation of DyLight 488-Phosphine to Ac_4GalNAz labeled AdV5 particles via Staudinger ligation: The Dylight 488-Phosphine was purchased from Thermo Scientific

(product number: 88907). Virus particles containing metabolically labeled incorporated Ac_4GalNAz were treated with Dylight 488-Phosphine at a concentration of 500 μM for 3 hours at room temperature for the Staudinger ligation to proceed. The Dylight 488-Phosphine stock was stored in a PBS at -20 °C.

10 % SDS-PAGE gel analysis of DyLight 488-Phosphine ligated AdV5 particles: Prestained protein marker, broad range (# P7708) was purchased from New England Biolabs. Samples were diluted 1:5 with 5X loading dye and boiled for 10 minutes. The samples were then vortexed and centrifuged briefly and 10 μl aliquots were loaded onto a 10 % SDS-PAGE gel. Gel was run for 1 hour at 200 V (keep the electrophoresis apparatus covered with aluminum foil during the entire period if using a light sensitive probe). After the 1 hour, gel was scanned under a Typhoon fluorescent gel scanner with an emission filter at set at 530 DF 30.

Results

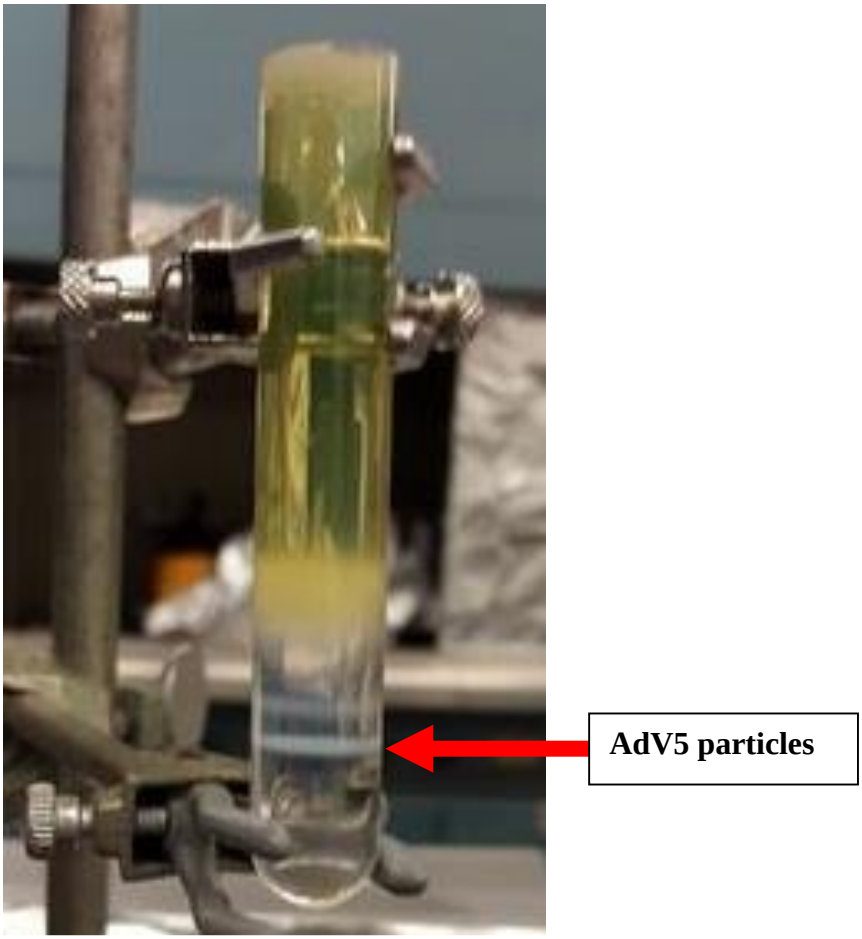

Figure 5: Visible white band of AdV5 particles after purification with CsCl density gradients in the ultracentrifuge at 32,000 rpm, for 1 hour at 15 °C.

After approximately 44 hours post infection (including labeling time), the AdV5 particles were harvested by three cycles of freeze thaw. The virus lysate, which was pal yellow in color was added on top of the CsCl gradients and was purified by ultracentrifugation for 1 hour at 32,000 rpm at 15 °C. After ultracentrifugation, the viral particles were separated and can be seen as a thick white band between the junction of the CsCl gradient.

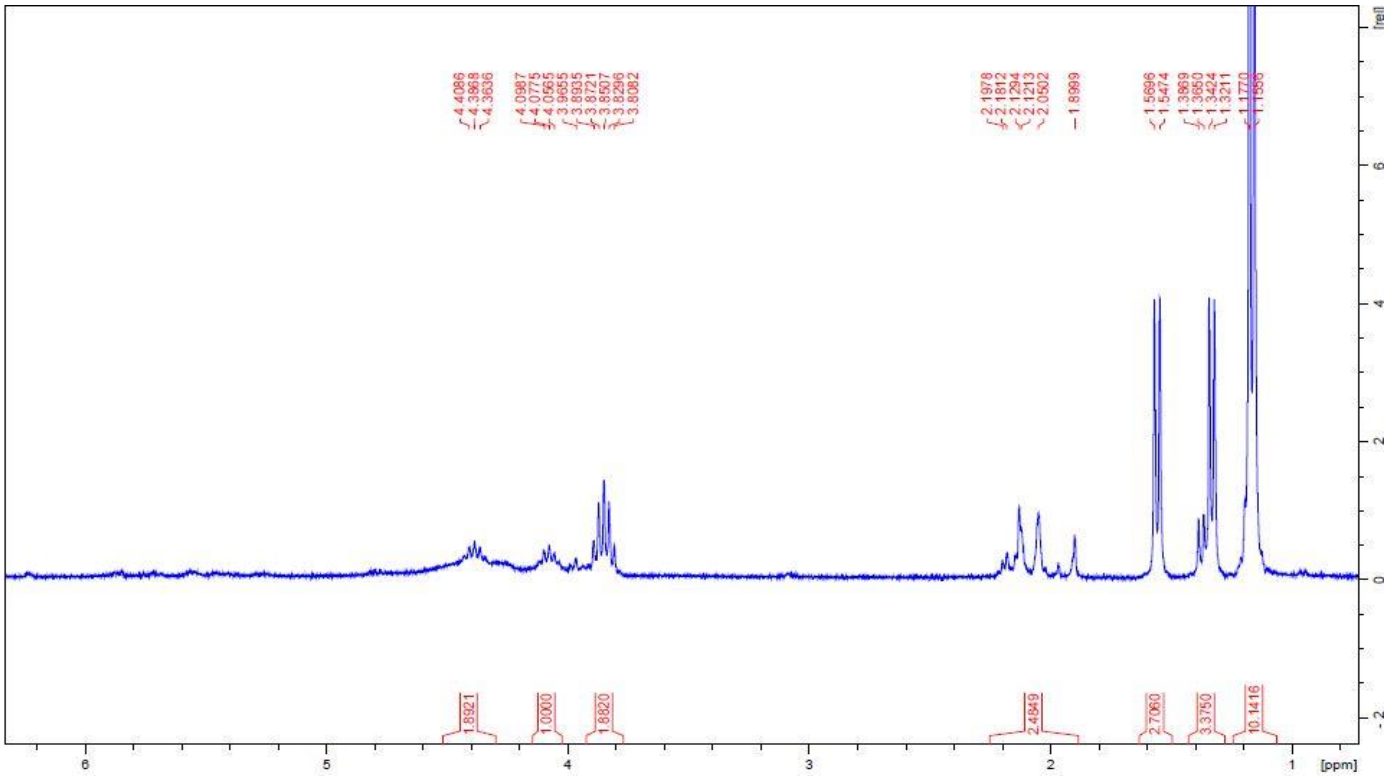

Figure 6: Proton NMR spectrum of synthesized and purified $Ac_4GalNAz$.

$Ac_4GalNAz$ was synthesized and purified as outlined in the protocol by Laughlin and Bertozzi. A proton NMR spectrum was taken to characterize the purified $Ac_4GalNAz$. The characteristic peak of the azide at 3.92 (2H, s) and other typical peracetylated sugar peaks as dictated in the protocol were evident in the NMR spectrum indicating the complete syntheses and purification of the peracetylated azido sugar.

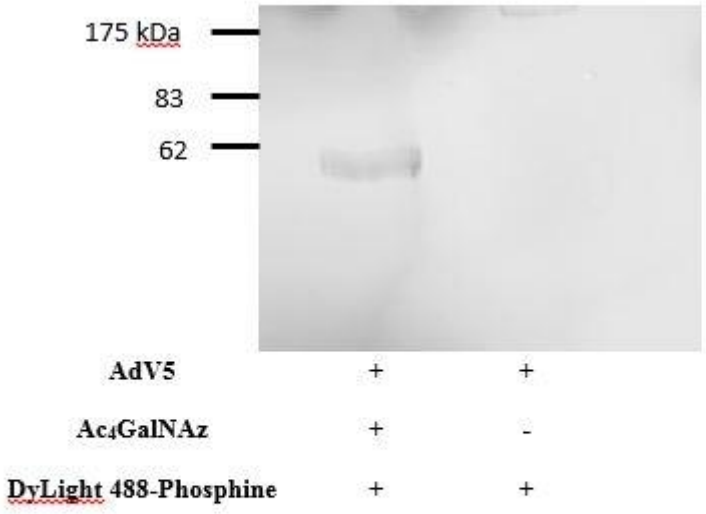

Figure 7: Fluorescent gel scan of Ac_4GalNAz labeled AdV5 particles. AdV5 were labeled with Ac_4GalNAz as described and a DyLight 488-Phosphine probe was conjugated onto the azide group via Staudinger ligation. The Typhoon fluorescent gel scan illustrate that only one of the capsid proteins was labeled with Ac_4GalNAz.

AdV5 particles were metabolically labeled with Ac_4GalNAz. The Staudinger ligation reaction at room temperature, was used to conjugate a DyLight 488-Phosphine probe onto the virus surface protein azide. The fluorescent gel scan was taken using a Typhoon scanner with validate the azide was incorporated onto one of the surface proteins of the AdV5 as evident in the gel image.

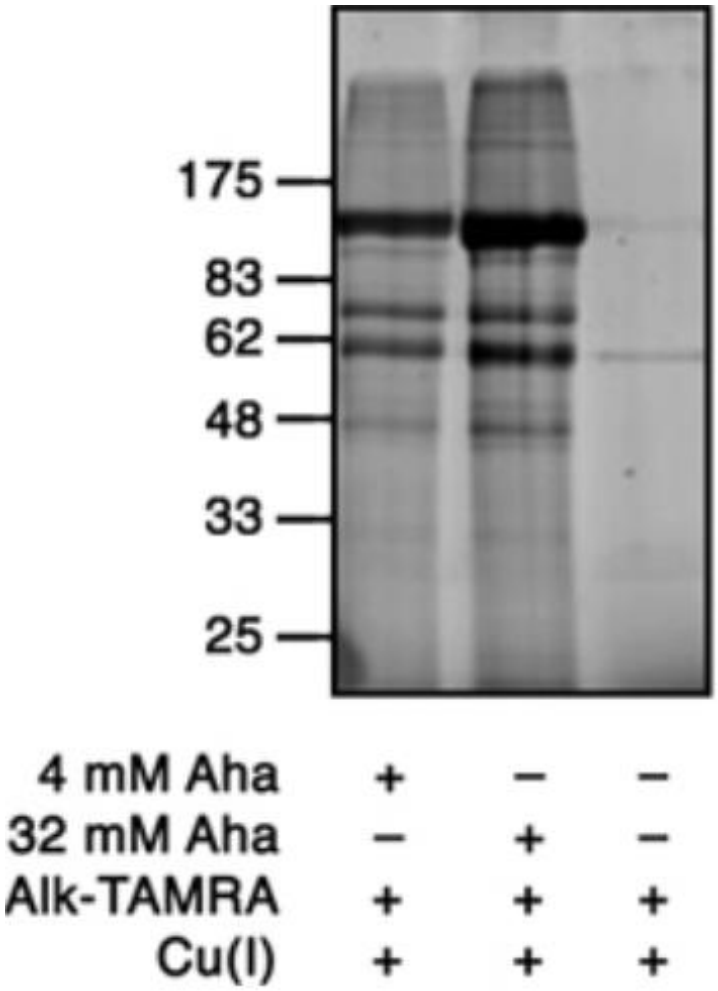

Figure 8: Representative fluorescent scans of 10% SDS-PAGE gel of Aha incorporated AdV5 particles after conjugated with TAMRA-alkyne using CuAAC as previously reported by our group. Two different concentrations of Aha were used and the hexon, penton and fiber proteins were labeled as apparent in the figure [15].

Figure 8 is a representation of Aha labeling results as previously reported by our group. Azide labeled AdV5 particles were produced as described and the TAMRA probe was conjugated via CuAAC as described in Rubino *et al.* Previously by our group. The representative fluorescent gel scan illustrate that the unnatural amino acid was incorporated into the hexon, penton and fiber capsid proteins of AdV5.

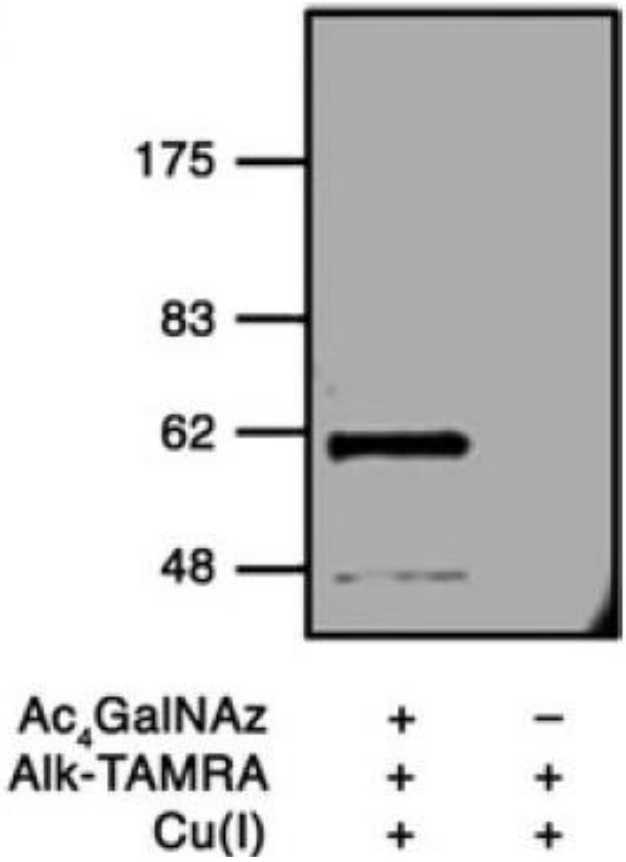

Figure 9: Representative fluorescent scans of 10 % SDS gel of Ac_4GalNaz labeled AdV5 particles. Azido sugar labeled virus particles were ligated with a TAMRA-alkyne probe using CuAAC as reported previously by our group [15].

Figure 9 is a representation of fluorescent gel scan of Ac_4GalNAz labeled AdV5 as previously done in our lab. The TAMRA-alkyne was conjugated to the Ac_4GalNAz labeled virus using the protocol outlined in Rubino *et al.* by our group. The representative figure suggests that only the fiber protein of AdV5 is labeled with Ac_4GalNAz.

Discussion

Originally gene therapy was thought to be a treatment only for heredity single gene defects but today, an array of acquired diseases including cardiovascular disease, neurodegenerative disorders and infectious diseases are a subject of research as well [10]. Gene therapy can introduce suicide genes to make cells more sensitive to chemotherapeutic drugs; and more recently, it's been combined with human iPSCs to reverse a genetic defect and produce cells to be used for autologous cell therapy [19, 20]. As evident, with its expanding horizon, gene therapy holds great promise for future treatment of different diseases.

There are numerous protocols for AdV surface labeling. In the current study, we have used the two-step metabolic labeling process to incorporate unnatural amino acid Aha and unnatural sugar Ac_4GalNAz onto the virus surface. Because these unnatural substrate are fed to the virus replicating cells during their metabolic process, the protein building machinery utilized these unnatural substrates that are analogs of the natural substrates, for protein production. This results in minimum perturbance of viral infectivity and titer. It is a simple approach that can potentially be used to modify different virus particles with ligands of interest under psychological conditions.

Aha is an unnatural surrogate of methionine with an azide function group. The azide group can be used to carry out very specific chemistries with ligands containing alkynes, aldehydes or other azide reactive groups. After the infection and labeling time period as described, the cells showed cytotoxic affects which are typical of virus producing cells and is a physical evident that the infection was complete. During the 6 hour labeling period, the virus

proteins are being assembled and Aha is incorporated into the onto the methionine sites on the virus surface under the normal physiological conditions and does not have any impact on the virus fitness which is why the two-step method is so simple and convenient to use as it requires no special equipments and environment. It takes approximately two days to generate AdV5 particles modified with the unnatural substrate which can further be modified with desired dyes, ligands or small proteins of interest for characterization purposes. The ease of production and time efficiency makes the two step protocol quite plausible to be used for generating surface modified AdV5 particles.

Ac_4GalNAz is a precursor to GlcNAz in the metabolic pathway. Since they have similar structures, cells use peracetylated N-azidoacetylgalactosamine (Ac_4GalNAz) and convert it to GlcNAz during the process and it is incorporated into the virus proteins. The peracetylated azido sugar was synthesized in our lab following the Laughlin and Bertozzi protocol [21] which has reported a detailed protocol for synthesizing azido sugars, and peracetylating and purifying the azido sugars. The Ac_4GalNAz labeling is quite simple compared to the Aha labeling as no change of media is required. Cytotoxic affects were evident in the plates after 44 hours of incubation indicating virus infection. As there are no time restrictive media changes required for this, it is a fairly simple method to efficiently modify the AdV for surface modifications at capsid proteins.

To purify the virus, the most widely used technique of ultracentrifugation against CsCl gradients was used. It is important to have a neat setup of the gradients at this point as it might affect the subsequent purification and visualization of the virus band. After centrifugation, in both the Aha and Ac_4GalNAz labeling attempts, the broad white virus band was clearly visible as seen in Figure 5. The test tube was punctured with a needle and the virus band was collected.

The collected faction was approximately 300 µl, which was consistent with the previous results in our lab. As evident in the figure, two white bands were present in the tube but only the bottom band consisted the virus particles, the top band constituted mostly empty capsids and was not collected with the virus fractions.

An OD 260 assay was run to determine the virus titer of the collected fractions. This is a fairly quick and easy approach to determine the titer of the virus in a short amount of time and usually requires a very small sample size. The UV readings taken at 260 nm represents the total number of DNA and protein particles present in the sample. In the Aha and Ac_4GalNAz labeling attmpts, the virus titers determined using this assay were $2.31X\ 10^{12}$ partcles/ml and $2.28\ X10^{12}$ particles/ml respectively. The observed titers were consistent with our previous lab results and with other publications. The results from this assay validate the fact that our two step approach is perfectly capable of generating high titer viruses. AdV5 is known to be stable in high salt concentrated solutions for a few days but for longer storage time the virus solutions need to be dialyzed against the virus storage buffer and stored at -20 °C to avoid the risk of losing virus titer.

There are multiple azide specific click reactions that can be carried out to conjugate ligands or probes to the azide on the surface of the virus. Figure 10 illustrates three different approaches which are commonly used for azide specific click reactions. In the current study, Staudinger ligation was used to ligate a DyLight 488-Phosphine probe on the Ac_4GalNAz modified AdV5 particles. The Staudinger ligation was utilized as the specific click reaction to conjugate the probe only on to the azide functional group, and the gel scan validate theoretical result. As visible in Figure 7, only AdV5 particles that were labeled with azide with the aid of the unnatural sugar Ac_4GalNAz were able to be ligated with the DyLight 488-Phosphine probe.

Also, only one band was visible in the azide labeled AdV5 indicating that only the fiber capsid proteins were labeled with the azide functional group. This result was consistent with the previous results reported by our group as illustrated in Figure 9.

Alternatively, different probes and reaction chemistries can be conjugated to the azide group via the SPAAC, CuAAC or a phosphine-FLAG Staudinger ligation. The protocols for these methods are outlined in Rubino *et al.* [15] and Banerjee *et al.* [18]. The conjugated particles can then be analyzed and characterized using gel scanning or western blot techniques.

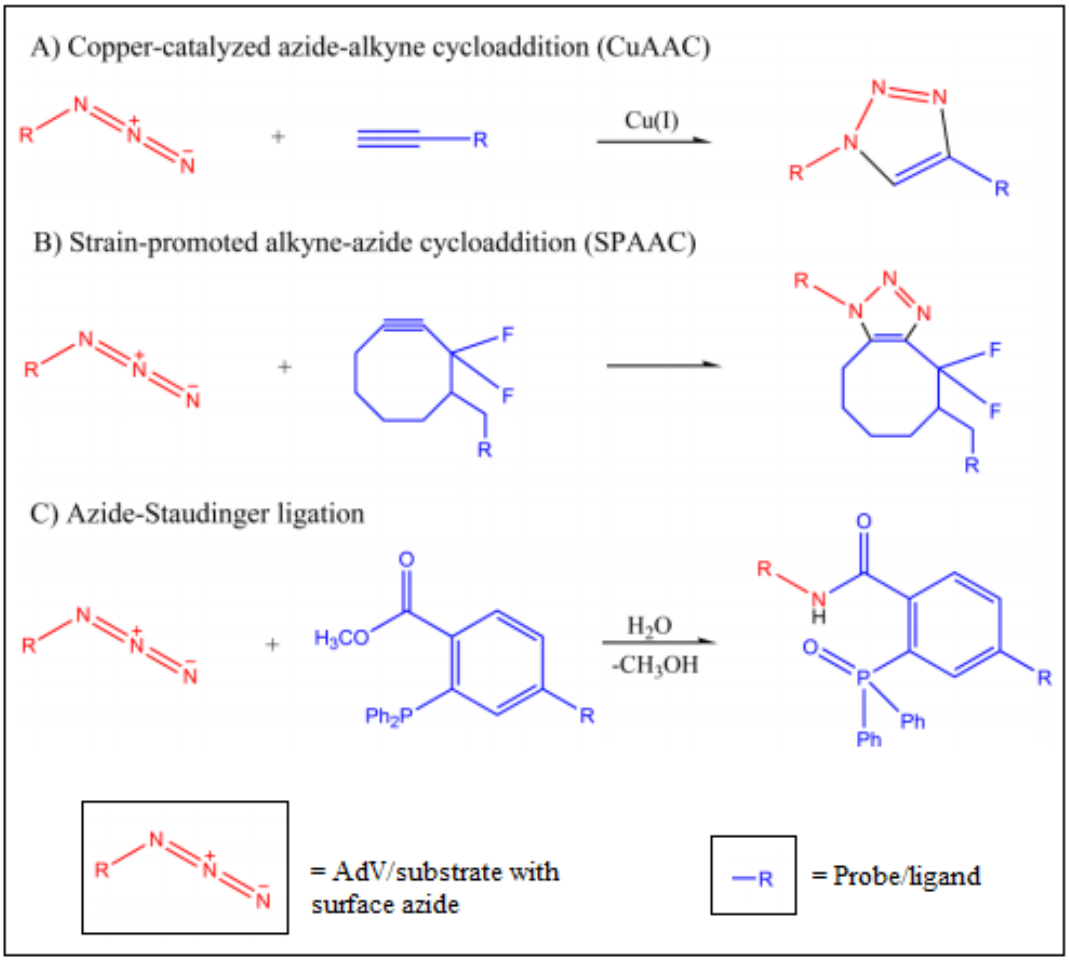

Figure 10: Azide specific reaction chemistries used to ligate probes/small molecules. A) Copper (I)-Catalyzed Azide-Alkyne Cycloaddition (CuAAC). B) Strain-promoted azide-alkyne cycloaddition. C) Azide-Staudinger ligation [14].

Previous results of ligation of a TAMRA probe with CuAAC are presented in this work. Figures 6 and 7 represent images of fluorescent scanned SDS gels of Aha and AC_4GalNAz labeled AdV5 particles respectively. As evident from the figures the unnatural amino acid, Aha, is incorporated into the fiber, penton and the hexon of the virus whereas the unnatural sugar, Ac_4GalNAz, is only incorporated into the fiber capsid proteins of the virus. The integration of these unnatural substrates at specific sites on the viral surface provide us with a tool to modify the virus at specific fiber, hexon and penton proteins for the purposes of different studies. For example different fiber proteins are known to interact with different kinds of receptors on tumor cells by identifying and specifically modifying these fiber proteins we can enhance the targeting capability of the AdV5 vectors.

In general the results from our lab group suggest that the two step metabolic labeling approach is a simple and convenient method to generate surface modified viruses at specific locations. The metabolic integration of azide has no effect on the virus physiology and there are different click chemistries that can be utilized to further modify these azide functional groups with desired substrates. The results are replicative and in theory, we should be able to use this approach with different virus types and substrates.

Future Directions

Potential improvements of targeted gene therapy dependent on AdV vectors are greatly indebted to the ability and knowledge of visualizing real-time biodistribution of AdVs. Keeping this in perspective, the future studies will focus on engineering dually modified AdV5 virus particles; labeled with a targeting moiety and a radioisotope such as ^{18}F and ^{89}Zr. Since ^{18}F has a shorter half- life than ^{89}Zr, so in an effort to increase imaging time span, we will attempt to use ^{89}Zr as the preferred radioisotope. AdV5 particles will be dually modified with a caged ^{89}Zr radioisotope and a folate targeting ligand. A folate ligand will enables us to target tumor cells specifically and the^{89}Zr cage will provide an *in vivo* PET scanning modality. Employing the two step approach provides the ability to produce these specifically modified AdV particles in a proficient manner.

For the purpose deep manifested tumor cells, an ^{89}Z radioisotope cage will be used rather than other available radioisotopes, as it has a significantly longer half life, is cheaper to produce and emits low positron energy compared to other radioisotopes used for PET scans. First the AdV5 virus particles will be loaded with the two unnatural substrates during the metabolic viral assembly process and consequently, the surface proteins with the unnatural moieties will then be modified with specific click chemistries. Homopropargylglycine (HPG) an unnatural surrogate of methionine will be used to add the ^{89}Zr cage via CuAAC modification. And GlcNAz will be used to conjugate the targeting folate ligand via the Staudinger ligation.

The impact of the surface modifications on viral infectivity and gene delivery will be analyzed in vitro. And the engineered virus will be purified and optimized for *in vivo* murine model studies to visualize and track the virus. The successful engineering and *in vivo*

visualization results will be an important contribution to current AdV biodistribution and vector development knowledge.

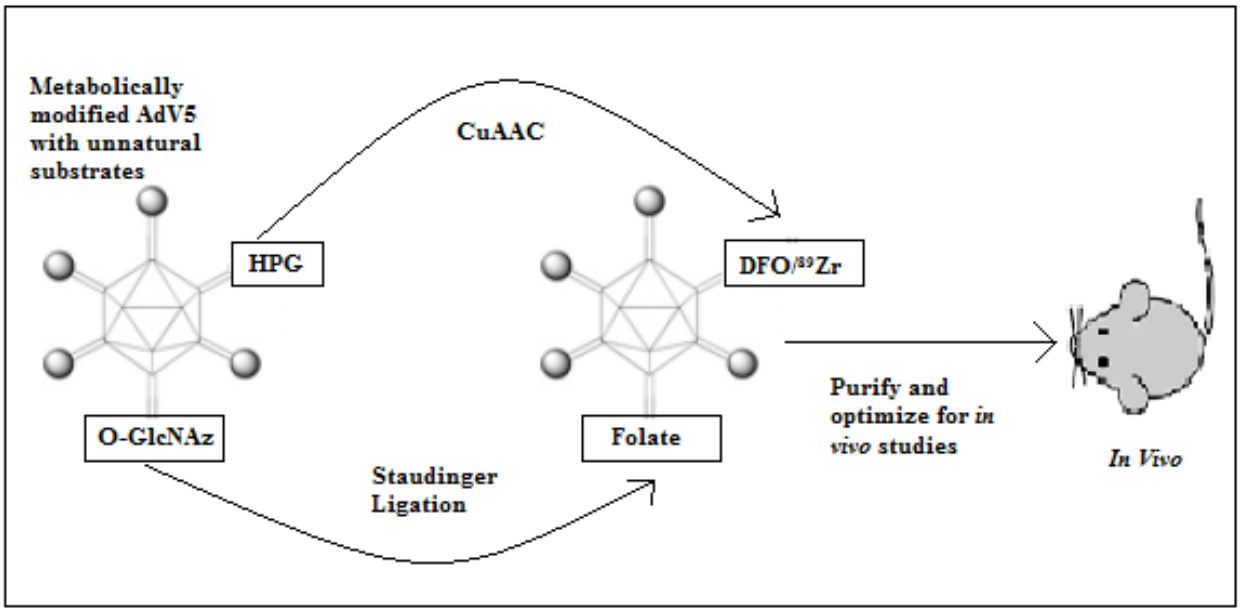

Figure 11: Systematic representation of future studies to dually modify AdV5 particles for *in vivo* study with ^{89}Zr. AdV5 particles incorporated with unnatural substrates HPG and O-GlcNAz will be used to ligate ^{89}Zr (via CuAAC) and folate ligand (via Staudinger ligation) respectively. After analysis for virus infectivity, stability and optimization the particles will be injected into tumor implanted mice for analysis of biodistribution via PET scanning.

Although AdV5 is widely used as the preferred vector for gene therapy studies, it still poses a few drawbacks such as low specificity, immune response and toxicity, in respect to research. As a result, there is a significant interest in using different AdV serotypes in order to circumvent these hurdles. Since most tumor cells lack the CAR receptor which is used by AdV5 to enter the cells and transduce the delivery gene, there is immense interest in using chimeric AdV serotypes that can bypass the low efficiency of the virus. Numerous studies have suggested that AdV5 incorporated with AdV3 fiber knob protein increases the targeting of the virus to tumor cells in CAR independent manner [22, 23, 24 and 25].

As a future progression of this study, using one of these chimeric AdV5/3 chimeric vectors to label and visualize *in vivo* tracking might be of great benefit. It may result in the increased targeting of the vector being used and will also present a route to broaden the applicability of our two step labeling approach to different serotypes. Since the chimeric vector has already been used in different tumor models and groups have reported that it has a higher potential for gene transduction in prostate cancer models [25], perhaps using our approach in the same tumor context may help further validate the benefits of the vector and our approach.

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Conclusions

Gene therapy holds a tremendous potential for treatment of diseases that are yet to be cured with synthetic drugs. With thousands of articles and a significant number of clinical trials with AdV vectors, it is plausible to say that AdV vectors are the most efficient means of gene delivery *in vivo* [26]. And there is continuous effort to make these vectors better for specific cell targeted future therapeutics by surface modifications. There are different strategies that are proposed for such modification and there continuous to be growth in the field. In an attempt to modify AdV5 at specific sites in a time efficient manner we have a two-step labeling approach.

Our studies will provide valuable results for better understanding of the targeting and biodistribution of the AdV which can be used to optimized therapeutic delivery and enhance future gene therapy. Consequently, the most valuable information will be garnered from *in vivo* and preclinical studies of the modified virus particles.

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