Site-specific Modification of AAV Vector Particles With Biophysical Probes and Targeting Ligands Using Biotin Ligase

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We have developed a highly specific and robust new method for labeling adeno-associated virus (AAV) vector particles with either biophysical probes or targeting ligands. Our approach uses the *Escherichia coli* enzyme biotin ligase (BirA), which ligates biotin to a 15-amino-acid biotin acceptor peptide (BAP) in a sequence-specific manner. In this study we demonstrate that by using a ketone isotere of biotin as a cofactor we can ligate this probe to BAP-modified AAV capsids. Because ketones are absent from AAV, BAP-modified AAV particles can be tagged with the ketone probe and then specifically conjugated to hydrazide- or hydroxylamine-functionalized molecules. We demonstrate this two-stage modification methodology in the context of a mammalian cell lysate for the labeling of AAV vector particles with various fluorophores, and for the attachment of a synthetic cyclic arginine–glycine–aspartate (RGD) peptide (c(RGDfC)) to target integrin receptors that are present on neovasculature. Fluorophore labeling allowed the straightforward determination of intracellular particle distribution. Ligand conjugation mediated a significant increase in the transduction of endothelial cells *in vitro,* and permitted the intravascular targeting of AAV vectors to tumor-associated vasculature *in vivo*. These results suggest that this approach holds significant promise for future studies aimed at understanding and modifying AAV vector–cellular interactions.

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INTRODUCTION

Biophysical probes such as fluorophores have been very useful for investigating the process of adeno-associated virus (AAV) infection,[1–5] but technological hurdles have limited their use, and their chemical conjugation to viral particles is often associated with a significant loss of titer. Green fluorescent protein (GFP) is an attractive option because of its high-labeling specificity and ease of use, but it is a large tag (238 amino acids) and can be used only for fluorescent imaging. While GFP has been fused to the amino-terminus of AAV2 VP2, and particles incorporating this fusion have been generated,[6,7] it is unclear whether this approach will be universally adaptable to the other AAV serotypes, and the influence of such a large tag on AAV biology has yet to be thoroughly evaluated. Furthermore, the low valency of VP2 display significantly lessens the signal intensity obtained using this approach. Other protein-based tags that either react covalently or form high affinity complexes with small-molecule probes have yet to be introduced into AAV particles, but the problem of size remains.[8,9] Labeling approaches that use peptides rather than proteins as targeting sequences are less invasive but generally sacrifice specificity and efficiency. For instance, the FlAsH technology, which targets arsenic-functionalized fluorophores to tetracysteine motifs displayed on recombinant proteins, requires complex washout procedures to remove the probe from nontarget monothiols, and the labeling is unstable.[10] Fluorophore-binding peptide aptamers similarly show reduced specificity as compared to protein tag-based methods[11] and, while a recently developed hexahistidine-based labeling approach may offer higher specificity, it also relies on a noncovalent interaction that deteriorates within minutes.[12]

In recent years, there have been intensive efforts in many laboratories to generate targeted AAV vectors by modifying the cell-binding characteristics of these particles. The primary strategy has been to genetically modify the AAV capsid proteins,[13–18] while an alternative strategy has been to use soluble bifunctional crosslinkers that bind both to the vector particle and to a cell-surface receptor, thereby providing a molecular bridge to anchor the vector to a targeted receptor.[19] A combined approach has been the display of an immunoglobulin binding domain on the vector as a genetic fusion to the capsid protein, and then the use of a monoclonal antibody to crosslink the vector with the targeted cell.[20] While the genetic insertion of targeting peptide motifs into the AAV capsid has met with the greatest success, this approach is often problematic, and does not lend itself to the high-throughput evaluation of different targeting ligands. Often, the genetic insertion dramatically reduces vector titer or DNA packaging

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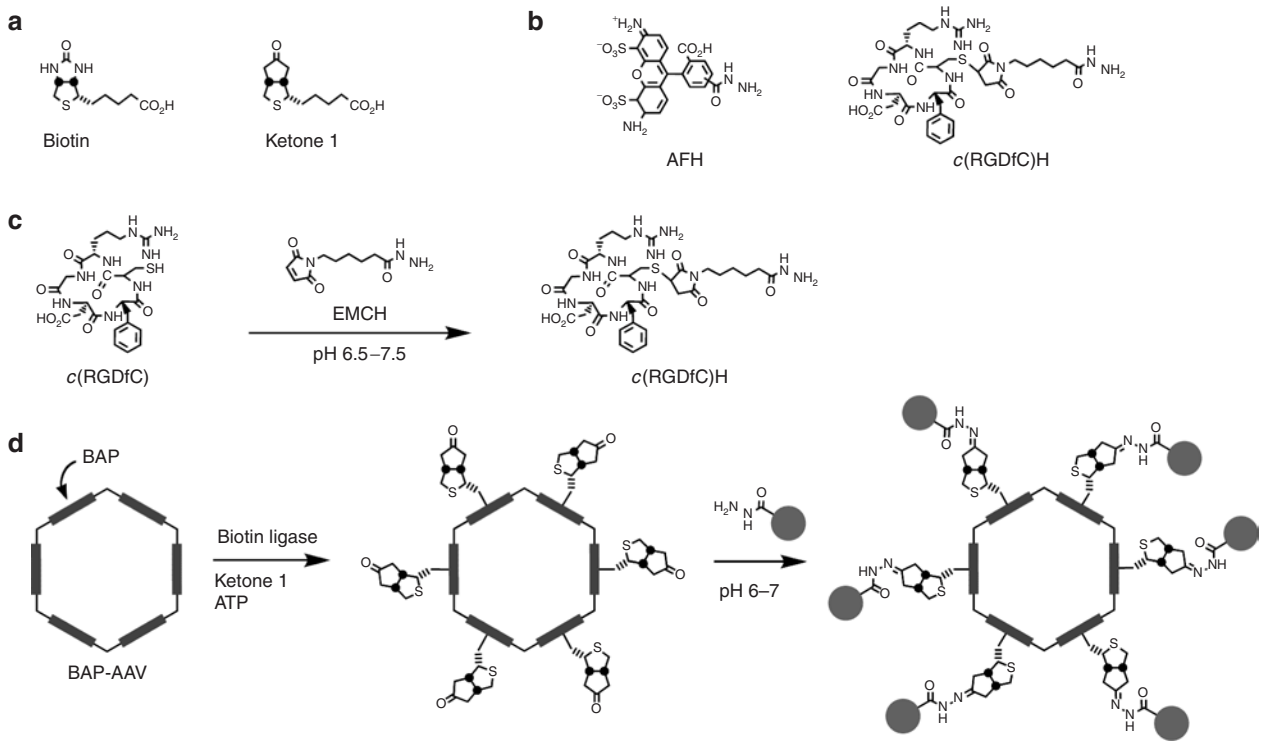

Figure 1 Site-specific labeling of adeno-associated virus (AAV) particles using biotin ligase and a ketone analog of biotin. (**a**) Structures of biotin and ketone 1. (**b**) Structures of Alexa Fluor 488 hydrazide (AFH) and cyclic arginine–glycine–aspartate (RGD) peptide hyrazide (*c*(RGDfC)H). (**c**) Addition of a hydrazide group to the cyclic RGD peptide (*c*(RGDfC)) using EMCH (3,3′-N-[e-Maleimidocaproic acid] hydrazide, trifluoroacetic acid salt). (**d**) General method for modification of biotin acceptor peptide (BAP)-tagged recombinant AAV particles (BAP-AAV) with biophysical probes or targeting ligands. Biotin ligase (BirA) catalyzes the ligation of ketone 1 to the BAP (blue); a subsequent bio-orthogonal ligation between ketone and hydrazide (or hydroxylamine) introduces the probe (green). ATP, adenosine triphosphate. The figure is available in color in the online version of the article.

efficiency, or has other deleterious effects on vector function.[14,15,18] Furthermore, some peptide epitopes are inefficiently or inappropriately displayed when engineered into AAV capsids, or are rendered unable to bind their targeted receptor in the context of AAV virions,[17] further reducing the effectiveness of this approach.

To date, all AAV vector–targeting strategies have been based on the assumption that an engineered interaction between the vector and a cell-surface receptor will result in more efficient gene delivery by promoting enhanced attachment or altering intracellular trafficking pathways. However, given the myriad different entry mechanisms employed by naturally occurring viruses, it is not surprising that this simple assumption has not always proven correct. Although the engineered interactions mediated by capsid modification have invariably proven necessary for enhanced transduction, the mechanisms by which vector modification can increase transduction have been shown to vary, and have required a detailed intracellular analysis for their full characterization (M.D.S. and J.S.B., unpublished observation). Therefore, a single technology that allows vector modification with both targeting ligands and biophysical probes to monitor intracellular events would be of great benefit to the field. Ideally such a technology should permit straightforward incorporation of targeting ligands into the AAV vector system, allow their rapid evaluation without the need for genetic manipulation, and combine the minimal invasiveness of a small peptide tag with the excellent labeling specificity of GFP for intracellular particle localization. Moreover, the modification strategy should be covalent, so that probe or ligand dissociation is not a concern. In order to address this problem, we capitalized on the *Escherichia coli* enzyme BirA, which catalyzes the biotinylation of a lysine side-chain within a 15-amino acid consensus "biotin acceptor peptide" (BAP) sequence. BirA has already been used for the specific biotinylation of BAP-modified AAV vectors.[21] The mechanism of biotinylation involves activation of biotin as an adenylate ester, followed by its trapping by the lysine side-chain of the BAP. In order to harness the specificity of BirA for ligation of other biophysical probes or targeting ligands to BAP-modified AAV particles, we utilized ketone 1, a biotin isostere with the ureido nitrogens replaced by methylene groups (**Figure 1a**). Ketone 1 also serves as a substrate for BirA.[22] Because the ketone group is absent from natural proteins, carbohydrates, and lipids, it can be selectively derivatized on the vector surface with hydrazide- or hydroxylamine-bearing compounds under physiological conditions.[23] In this study we describe the development of a new site-specific vector modification methodology (**Figure 1**) based on the ability of BirA to use ketone 1 efficiently in place of biotin. We show that this approach permits the ligation of either targeting ligands or biophysical probes to vector particles, and demonstrate the usefulness of the technology for both AAV vector–targeting and intracellular particle–trafficking studies.

RESULTS

Site-specific labeling of AAV particles with biophysical probes

In order to test the use of BirA and ketone 1 for the labeling of recombinant AAV vectors, we generated BAP-modified AAV by

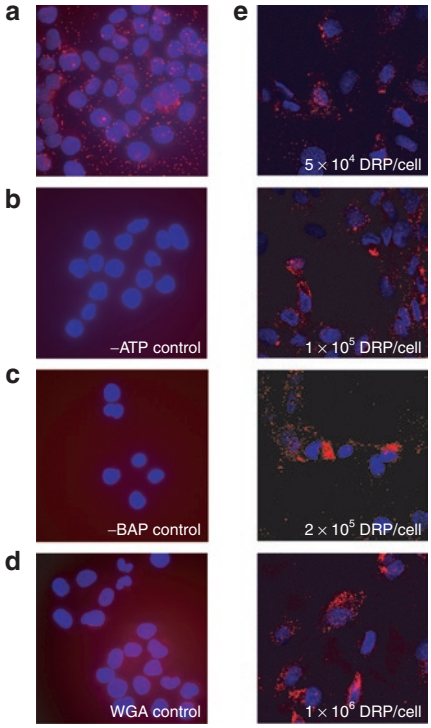

Figure 2 Site-specific labeling of adeno-associated virus (AAV) particles with biophysical probes. (**a**) Fluorescence image of human HeLa C12 cells incubated with biotin acceptor peptide (BAP)-modified AAV1eRFP particles (red) labeled with ketone 1 and Alexa Fluor 555 hydrazide using BirA (4′,6-diamidino-2-phenylindole nuclear stain, blue). (**b**) Labeling was dependent on adenosine triphosphate (ATP), since omission of ATP in the reaction eliminated labeling. (**c**) Labeling was specific to BAP-modified AAV particles, as shown by the fact that particles lacking the BAP modification were not labeled, and (**d**) labeling was specific to AAV, as shown by the fact that cell-associated fluorescence was blocked by competition with 10 mg/ml wheat germ agglutinin (WGA). (**e**) Fluorescence images of human umbilical vein endothelial cells incubated with Alexa Fluor 555-labeled particles, demonstrating the sensitivity of BirA labeling. Visualization of particle binding and intracellular distribution was possible at very low particle concentrations (50,000 vector genomes/cell; ~2 transducing units/cell). DRP, DNase-resistant particle; RFP, red fluorescent protein.

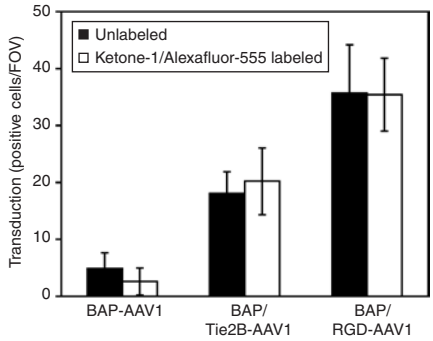

Figure 3 Ketone modification and fluorophore conjugation do not alter vector titer on human umbilical vein endothelial cells (HUVECs). Gene transduction mediated by equal numbers [3 × 10^4 DNase-resistant particle (DRP)/cell] of unmodified AAVeRFP particles or AAVeRFP particles labeled with ketone 1 and Alexa Fluor 555 was assessed on HUVEC. Biotin acceptor peptide (BAP)-modified AAV1 (BAP-AAV1), and BAP-modified AAV1-based vectors engineered for enhanced tranduction of human endothelial cells through the targeting of either Tie2 (BAP/Tie2B-AAV1) or integrin receptors (BAP/RGD-AAV1) were individually labeled and compared with unlabeled vectors. The data are presented as the mean values ± SD (n = 10). FOV, field of view. Gene transduction mediated by modified vectors is not significantly different from that mediated by unmodified vectors ($P > 0.05$, t-test). AAV, adeno-associated virus; RGD, arginine–glycine–aspartate.

genetically inserting the BAP into the coding sequence of the AAV1 capsid proteins.[21] Partially purified BAP-modified AAV1 was first enzymatically labeled with ketone 1 using BirA, and then Alexa Fluor hydrazides were added to derivatize the ketones. The resulting product was either used directly, or reduced with sodium cyanoborohydride to improve its stability, and separated from excess fluorophore by gel filtration. Fluorophore-labeled AAV particles produced in this manner could be readily detected within infected cells by fluorescence microscopy (**Figure 2**). Ketone modification required adenosine triphosphate, thereby showing that conjugation was dependent on enzyme activity and on the presence of the BAP in the viral particle and further indicating that the labeling was site-specific (**Figure 2b** and **c**). Specificity was further assessed by competing cellular binding of fluorophore-labeled AAV1 particles with wheat germ agglutinin, a specific inhibitor of AAV1 cellular attachment (**Figure 2d**). Using this approach, the sensitivity was found to be significantly greater than that of previous fluorescent labeling methodologies,[1,2] and allowed the determination of intracellular particle distribution at much lower multiplicities of infection than had been previously possible[1,2] (**Figure 2e**). Because the coupling was performed enzymatically, the reaction conditions are quite gentle and perhaps more appropriate for the modification of complex biomolecules such as viruses. In fact, there was no loss of vector titer after either ketone modification or fluorophore conjugation (**Figure 3**). In order to ensure that labeling would not affect alternative receptor usage, AAV1-based vectors engineered to infect human endothelial cells through alternative receptors (Tie2, the cell-surface receptor for angiopoietin; or integrin) were also labeled with fluorophores using this approach. In each case the enhanced transduction properties of these vectors was maintained after probe conjugation (**Figure 3**).

In order to explore the utility of BirA-catalyzed AAV particle modification, we examined the intracellular distribution of tropism-modified AAV vectors as compared to unmodified AAV vectors by labeling particles with different fluorophores. AAV1 vector particles comprised of AAVCap1.D590_P591ins*BAP* capsid proteins, and integrin-targeted mosaic AAV1 vector particles comprised of both AAVCap1.D590_P591ins*BAP* and AAVCap1.D590_P591ins*RGD* capsid proteins (1:4 ratio) were individually enzymatically labeled with ketone 1, and then derivatized with Alexa Fluor hydrazide 555 or Alexa Fluor hydrazide 488, respectively. The functionalized particles were then added together to cultured human endothelial cells and the particle distribution was assessed at 60 minutes after infection using fluorescence microscopy. This approach allowed multiple wavelength imaging and the straightforward determination of different intracellular localizations exhibited by tropism-modified vectors as compared to unmodified vectors (**Figure 4**). Importantly, the labeling reactions could be carried out with only small amounts of vector and did not require extensive vector purification either pre- or

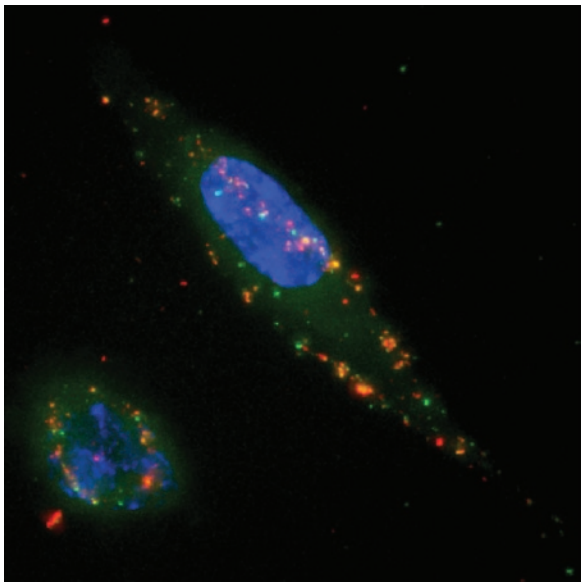

Figure 4 Multiple wavelength imaging of labeled adeno-associated virus (AAV) particles for determination of various intracellular trafficking pathways. Intracellular location of unmodified AAV1 vectors and integrin-targeted arginine–glycine–aspartate (RGD)-modified AAV1 vectors was assessed on human umbilical vein endothelial cells (nuclear stain, blue) 60 minutes after infection. Alexa Fluor 555 hydrazide (red) was conjugated to ketone 1–modified AAV1eRFP particles comprised of AAV1.D590_P591ins*BAP* capsid proteins, and Alexa Fluor 488 hydrazide (green) was conjugated to ketone 1–modified mosaic AAV1eRFP particles comprized of AAV1.D590_P591ins*RGD* and AAV1.D590_P591ins*BAP* capsid proteins (4:1 ratio), as described. The image was recorded using an Olympus BX61 microscope and Hamamatsu ORCA-ER digital camera controlled by Slidebook v4.0 (Intelligent Imaging Innovations, Denver, CO). The image stack was deconvolved using a constrained iterative algorithm in Slidebook v4.0. A merged image of "x-y" planes through the middle of the cells is presented. AAV, adeno-associated virus; BAP, biotin acceptor peptide; RFP, red fluorescent protein.

postlabeling, thereby overcoming the two significant drawbacks to current labeling technologies.[1,2] Furthermore, because of its size in comparison to GFP, the 15-amino-acid BAP tag is less likely to alter the trafficking of AAV particles. We have shown earlier that the BAP tag does not alter vector-mediated gene transduction or *in vivo* tropism.[21] Therefore this labeling approach seems to be minimally invasive and should allow the introduction of a wide range of probes with which to study AAV distribution and function in live cells.

Addition of targeting ligands to AAV vector particles with biotin ligase

The targeting of gene delivery to tumors is a central challenge for improving existing cancer gene therapies, because it is likely to enhance the efficacy and decrease the side-effects of these approaches. While various strategies have been pursued to achieve this goal, the most commonly used vehicles for targeting nongene-based therapies to tumors are "engineered antibodies," *i.e.*, bispecific antibodies or antibody fragments.[24,25] The incorporation of these agents into gene delivery systems has been problematic and has led to only limited success, especially with AAV-based vectors. "Homing" peptides are promising

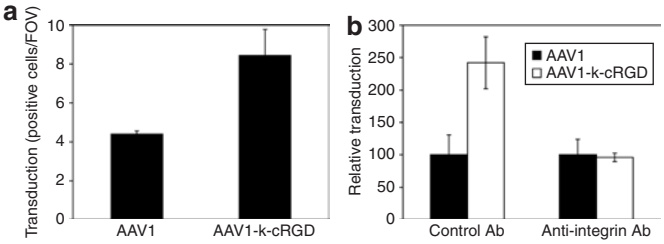

Figure 5 Targeted gene transduction mediated by AAV vector modification. Transduction of (**a**) human umbilical vein endothelial cells (HUVECs) [30,000 DNase-resistant particle (DRP)/cell] and (**b**) Hela C12 cells (2,500 DRP cell) mediated by AAV1dsRed2 vector or cyclic arginine–glycine–aspartate (cRGD)-modified AAV1dsRed2 vector. At 48 hours (HeLa C12) or 90 hours (HUVEC) after transduction, the cells were enumerated for red fluorescent protein expression using fluorescence microscopy. For the competition experiment, vectors were added to HeLa C12 cells in the presence of either a control antibody (Ab) or an anti-integrin specific monoclonal Ab (LM609; 1:200 dilution). The data are presented as the mean number of positive cells per field of view (FOV) ± SD ($n = 10$) (HUVEC; ~20 total cells/FOV) ($P < 0.05$, *t*-test), and as the relative number of cells transduced with cRGD-modified vector as compared to unmodified vector, ± SD ($n = 10$) (HeLa C12). AAV, adeno-associated virus.

alternatives, because they bind to surface molecules that are specific to organ or tumor cells,[26,27] are smaller than the antibody fragments, and can be genetically incorporated into AAV capsid proteins.[14,16,17] A prominent example of a homing peptide is the arginine–glycine–aspartate (RGD) motif.[26,28] This motif is present in many extracellular matrix components such as fibronectin and vitronectin, and binds to integrins. RGD-analogs have been extensively used for tumor imaging,[29] in antiangiogenesis approaches,[30,31] and in tumor targeting with radionucleotides[31] or chemotherapeutic drugs.[27] We have shown earlier that AAV1- and AAV2-based vectors that display genetically incorporated RGD motifs mediate significantly better gene transfer to endothelial cells and tumor cells than unmodified vectors do,[17,32,33] and that RGD-modified AAV1 vectors can effectively target gene delivery to tumor-associated vasculature when administered intravenously to tumor-bearing animals (M.D.S. and J.S.B., unpublished observation).

Here we assessed the use of BirA and ketone 1 for conjugation of a synthetic cyclic RGD peptide (c(RGDfC)) to the surface of BAP-modified recombinant AAV1 vectors. Partially purified BAP-modified AAV1dsRed2 particles (AAV1.D590_P591ins*BAP* capsid proteins) were first enzymatically labeled with ketone 1 (**Figure 1**) and then derivatized with cRGD peptide that had been modified with EMCH to create a free hydrazide. The cRGD-modified AAV1dsRed2 vector demonstrated significantly enhanced transduction efficacy in cultured human endothelial cells as compared to unmodified AAV1dsRed2 vector (**Figure 5**). Enhanced transduction was specific for the targeted integrin receptor, because it could be effectively competed using an anti-integrin antibody (**Figure 5**). Importantly, the cRGD-modified AAV1eGFP vector was able to mediate efficient gene transfer to tumor-associated vasculature in a murine model of peritoneal ovarian cancer after intravascular administration (**Figure 6**). These data clearly demonstrate that BirA and ketone 1 have the potential to add synthetic-targeting ligands to AAV particles for modifying vector tropism.

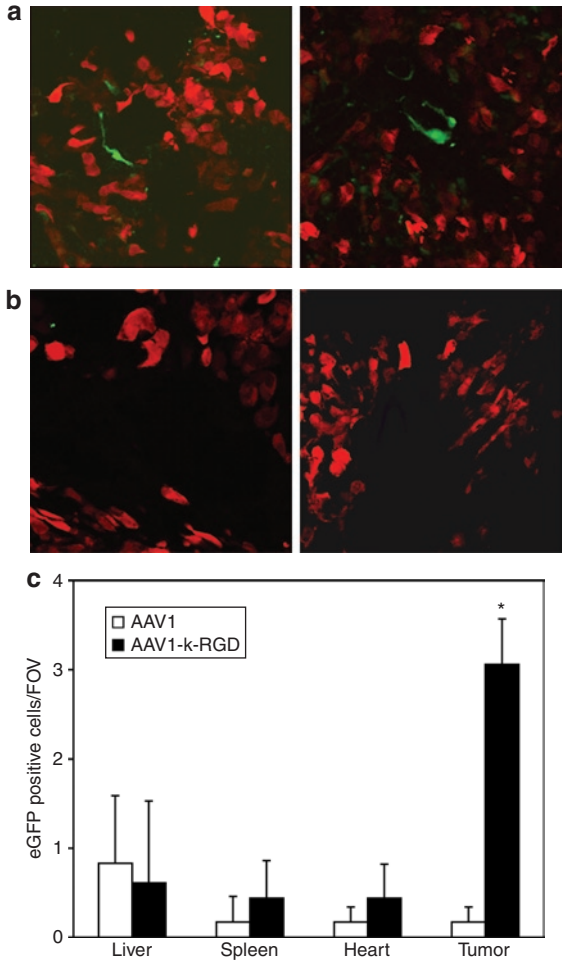

Figure 6 The cyclic arginine–glycine–aspartate (cRGD)-modified AAV1eGFP vector mediates effective gene transfer to tumor-associated vasculature. The ability of BirA to attach a cRGD-targeting ligand to adeno-associated virus (AAV) particles that could be used for systemic targeting of vector to tumors was assessed in a murine model of peritoneal ovarian cancer. Tumors were established by intraperitoneal injection of DsRed2-expressing SKOV-3 cells. Two weeks later the mice were injected intravenously through the tail vein with 1×10^{10} DNase-resistant particle (DRP) of cRGD-modified AAV1eGFP vector, or 1×10^{10} DRP of unmodified AAV1eGFP vector. Gene transduction was assessed 4 weeks later by staining tissue sections obtained from liver, spleen, heart, and tumor with anti-eGFP antibody (green). Tumor cells were visualized by direct DsRed2 fluorescence (red). (**a**) Tumor-bearing animals that had received cRGD-modified vector displayed clear tumor-restricted expression of GFP. Expression was evident in tumor-associated stroma, morphologically suggestive of endothelial cells, but not in the tumor cells. (**b**) In contrast, animals treated with unmodified AAVeGFP vector displayed little tumor-specific gene transfer. Representative sections from each experimental group are shown. (**c**) eGFP-positive cells enumerated in various tissues 4 weeks after vector administration. *Significantly different from all groups ($P < 0.001$, analysis of variance). eGFP, enhanced green fluorescent protein; RGD, arginine–glycine–aspartate.

DISCUSSION

We have developed a new methodology for tagging AAV vectors with biophysical probes using a 15-amino-acid BAP sequence and the enzyme BirA. The method is highly specific because it capitalizes on the sequence-specificity of BirA, and it is versatile because the ketone platform allows the introduction of a wide range of probes. BirA-based labeling is superior to existing labeling approaches that are based on conjugation of small-molecule fluorophores. Conventional succinimidyl ester forms of common dyes such as fluorescein isothiocyanate, carbocyanine dyes, and the Alexa dyes, react with amines on the capsid surface; this means that they are selective for lysine side chains, as well as for the N-termini of the capsid protein monomers. Reactivity of the amines requires that they be deprotonated, so these reactions are typically carried out at pH values between 8.3 and 9.3,[2,5] depending upon the stability of the dye. While the variations in pH may be attributable to historical precedent, as opposed to careful optimization, it is clear that AAV does not tolerate these conditions for extended periods of time. Therefore, conjugation of small-molecule fluorophores to AAV is often associated with loss of titer, particle aggregation, or low-labeling efficiency. Because BirA-based labeling is enzymatic and carried out at a more neutral pH, it is considerably more gentle and efficient than the amine-reactive chemistries used earlier. Furthermore, BirA-based labeling is site-specific, allowing far greater control over probe conjugation and minimizing the effects on viral biology.

To our knowledge this method is the only reported example of enzyme-mediated site-specific modification of a viral particle. A unique advantage of this method is that the ketone platform allows the introduction of a wide variety of different probes or targeting ligands. Earlier, targeting ligands incorporated into AAV vectors were limited to either small peptides[13–18] or antibodies.[19,20] Our method should provide a considerably more robust platform for the incorporation of not only peptide and protein ligands, but also of natural lectins, carbohydrates, glycolipids, polymers, and synthetic ligands into AAV particles. Similarly, although we show data only for the conjugation of fluorescent probes to AAV particles, modification of vectors with other biophysical probes should also be possible. Such probes could be used to monitor properties such as vesicular pH, capsid disassembly, or phospolipase activity.

In conclusion, we have developed a new AAV-labeling and modification methodology that combines the specificity of GFP tagging with the minimal invasiveness of a small peptide. We have demonstrated the labeling of AAV particles with fluorophores and the use of this approach to study vector trafficking in live cells. We have also shown that this method could be used to specifically attach targeting ligands to AAV vector particles, and that vectors modified in this manner could mediate enhanced gene transfer to target cells both *in vitro* and *in vivo*. Future efforts will focus on extending this methodology to additional biophysical probes for studying AAV vector entry and intracellular processing, and to different targeting ligands for targeted gene delivery.

MATERIALS AND METHODS

Recombinant AAV production. Low passage number (passage number 20–40) HEK 293 cells[34] and HeLa C12 cells[35] were grown in Dulbecco's modified Eagle's medium supplemented with 10% cosmic calf serum (Hyclone, Logan, UT), penicillin (100 U/ml), and streptomycin (100 U/ml) at 37 °C and 5% CO_2. Plasmid pXR1-Cap1.D590_P591ins*BAP*,[21] pXR1-Cap1.D590_P591ins*RGD*,[33] or pXR1 (ref. 36) DNA was triple transfected with the previously described pXX6-80 (containing the adenovirus *VA*, *E2A*, and *E4* genes)[37] and either pTR-UF5 (containing the enhanced GFP (eGFP) gene driven by the cytomegalovirus enhancer/promoter in a standard AAV plasmid vector) or pTrsSKcmvRFP (containing the dsRed2 fluorescent

protein gene driven by the cytomegalovirus enhancer/promoter and in a self-complementary AAV plasmid vector) into HEK 293 cells.[33] A 1:1:1 molar ratio of plasmids was used. For generating mosaic AAV1 virions containing the two different modified capsid proteins, the different helper constructs were mixed at 1:4 molar ratio before transfection. However, the total amount of helper plasmid used for vector production remained constant. Transfections were carried out at 37 °C using the calcium phosphate transfection system (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's specifications. Sixty hours after transfection, the cells were harvested by centrifugation at 500 *g* for 10 minutes and resuspended in 100 mmol/l NaCitrate, 2.5 mmol/l $MgCl_2$, 10 mmol/l Tris pH 8.3. The vector was then released by three cycles of freeze–thaw. The crude lysate was clarified by centrifugation at 500 *g* for 10 minutes, viscosity was reduced by the addition of Benzonase (50 U/ml) and incubation at 37 °C for 30 minutes, and the lysate was then fractionated on an iodixanol step gradient as previously described.[38] DNase-resistant particle (DRP) values were determined using real-time PCR assay and infectious titers were determined by gene transduction assay on HeLa C12 cells, as described previously.[16,39]

Probe ligation and hydrazide conjugation. Racemic ketone 1 was synthesized and attached to BAP-modified AAV vector particles as described earlier for ligation to synthetic peptides and BAP-modified cyan fluorescent protein (CFP).[22] Briefly, iodixanol purified BAP-modified AAV1 was added to an equal volume of bicine buffer (50 mmol/l bicine, 5 mmol/l magnesium acetate, pH 8.3) containing 8 mmol/l adenosine triphosphate, 200 µmol/l racemic ketone 1, and 2 µmol/l BirA. Reactions were incubated at 30 °C for 3 hours, and 0.1 mol/l HCl was added to adjust the pH to 6.5–7. For fluorescent labeling, Alexa Fluor 488 hydrazide or Alexa Fluor 555 hydrazide (Invitrogen) was added to a final concentration of 500 µmol/l, and the reaction was incubated at 30 °C for 8–18 hours. Excess unreacted hydrazide was removed by gel filtration (HiTrap; Amersham Biosciences, Piscataway, NJ). Cyclic-RGD peptide (c(RGDfC)); Peptides International, Louisville, KY) was modified with EMCH (3,3′-N-[e-Maleimidocaproic acid] hydrazide, trifluoroacetic acid salt; Pierce) in accordance with the manufacturers recommendations, creating a hydrazide that was then conjugated to a ketone-modified vector overnight at 30 °C. For the most part, reaction mixtures containing modified AAV vectors were used without subsequent treatment or purification. However, in some instances, sodium cyanoborohydride (15 mmol/l) was added to reduce the hydrazone, for 1.5 hours at 4 °C.[22]

Intracellular particle distribution studies. Human HeLa C12 cells or umbilical vein endothelial cells were grown on chambered microscope slides in Dulbecco's modified Eagle's medium (Hyclone) supplemented with 10% fetal bovine serum or complete EBM-2 media (Clonetics, Walkersville, MD) respectively at 37 °C in a 5% CO_2 atmosphere. Nonspecific binding was blocked by incubating the cells for 30 minutes at 4 °C with serum-free and supplement-free medium containing 5% bovine serum albumin and image-iT FX reagent (Invitrogen). Fluorophore-conjugated AAV (5×10^4–1×10^6 DRP/cell) was added to the blocking solution and left on the cells for 1 hour at 4 °C. Where indicated, wheat germ agglutinin (10 mg/ml; Vector Laboratories, Burlingame, CA) was added at the same time as the virus. The cells were then washed three times with phosphate-buffered saline and fresh growth medium was added. The cells were incubated at 37 °C in a 5% CO_2 atmosphere for 15 minutes. Noninternalized particles were removed with an acid wash (50 mmol/l 2-(N-morpholino)ethanesulonic acid, 280 mmol/l sucrose, pH 5.0)[2] and either fixed immediately with 4% paraformaldahyde for 15 minutes, or returned to 37 °C for 60 minutes prior to fixing. Slides were mounted using Vectashield mounting medium (Vector Laboratories) and images were collected using an Olympus BX61 microscope and Hamamatsu ORCA-ER digital camera controlled by Slidebook v4.0 software (Intelligent Imaging Innovations, Denver, CO). When indicated, image stacks were deconvolved using a constrained iterative algorithm in Slidebook v4.0. Merged images of "x-y" planes through the middle of cells are presented.

Gene transduction assays. For analysis of enhanced gene transduction mediated by vector modification, human umbilical vein endothelial cells were seeded onto 48-well plates and treated with vector preparations (3×10^4 DRP/cell) at ~80% confluency. The cells were maintained at 37 °C for 16 hours and washed three times with phosphate-buffered saline before fresh growth media was added. For the determination of ligand-dependent enhancement of gene transduction, vectors were added to target cells for 2 hours at 4 °C in the presence of an anti-integrin monoclonal antibody (LM609), or an isotype-matched control antibody. The cells were then washed three times with fresh medium to remove unbound vector and returned to 37 °C. Gene transduction was determined 90 hours after treatment by counting red fluorescent protein–positive cells using an inverted fluorescent microscope (10 fields of view/well). All transduction assays were performed in triplicate, and the results are presented as mean values ± SD. Statistical analysis was performed using GraphPad Prism version 4.0b for Macintosh (GraphPad Software). Analysis was performed using Student's *t*-test for comparison of two groups. Results were considered significant when $P < 0.05$.

Ovarian carcinoma model and immunostaining for targeted gene delivery. Tumors were established by injecting 2×10^6 DsRed2-expressing SKOV-3 cells intraperitoneally into female athymic nude (nu/nu) mice. Two weeks later, tumor growth was confirmed by whole-body fluorescent imaging, and the mice were injected intravenously through the tail vein with 1×10^{10} DRP of cRGD-modified AAV1eGFP vector, 1×10^{10} DRP AAV1eGFP vector, or phosphate-buffered saline control. In order to determine the targeting efficiency of cRGD-modified AAV vectors, the mice were killed two weeks after vector administration, and the tumor, liver, spleen, and heart tissues were isolated, frozen, cut, and stained for eGFP expression, using a rabbit anti-GFP antibody (Molecular Probes, Invitrogen) overnight at 4 °C, and donkey anti-rabbit IgG-Alexa-488–conjugated antibody (Molecular Probes, Invitrogen) for 1 hour at room temperature. The number of eGFP-positive cells was determined using stained sections observed under ×400 original magnification fluorescence microscopy. Gene transfer was assessed as the average number of positive (green) cells per field of view in five random frozen sections. The data are presented as mean values ± SD. Statistical analysis was performed using GraphPad Prism software.

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REFERENCES

1. Bartlett, JS and Samulski, RJ (1998). Fluorescent viral vectors: a new technique for the pharmacological analysis of gene therapy. *Nat Med* **4**: 635–637.
2. Bartlett, JS, Wilcher, R and Samulski, RJ (1999). Infectious entry pathway of adeno-associated virus and adeno-associated virus vectors. *J Virol* **74**: 2777–2785.
3. Ren, C, White, AF and Ponnazhagan, S (2006). Notch1 augments intracellular trafficking of adeno-associated virus 2. *J Virol* **81**: 2069–2073.
4. Sanlioglu, S, Benson, PK, Yang, J, Atkinson, EM, Reynolds, T and Engelhardt, JF (2000). Endocytosis and nuclear trafficking of adeno-associated virus type 2 are controlled by rac1 and phosphatidylinositol-3 kinase activation. *J Virol* **74**: 9184–9196.
5. Xiao, W, Warrington, KH Jr., Hearing, P, Hughes, J and Muzyczka, N (2002). Adenovirus-facilitated nuclear translocation of adeno-associated virus type 2. *J Virol* **76**: 11505–11517.
6. Lux, K, Goerlitz, N, Schlemminger, S, Perabo, L, Goldnau, D, Endell, J *et al.* (2005). Green fluorescent protein-tagged adeno-associated virus particles allow the study of cytosolic and nuclear trafficking. *J Virol* **79**: 11776–11787.
7. Warrington, KH Jr., Gorbatyuk, OS, Harrison, JK, Opie, SR, Zolotukhin, S and Muzyczka, N (2004). Adeno-associated virus type 2 VP2 capsid protein is nonessential and can tolerate large peptide insertions at its N terminus. *J Virol* **78**: 6595–6609.
8. Keppler, A, Pick, H, Arrivoli, C, Vogel, H and Johnsson, K (2004). Labeling of fusion proteins with synthetic fluorophores in live cells. *Proc Natl Acad Sci USA* **101**: 9955–9959.
9. Miller, LW, Sable, J, Goelet, P, Sheetz, MP and Cornish, VW (2004). Methotrexate conjugates: a molecular *in vivo* protein tag. *Angew Chem Int Ed Engl* **43**: 1672–1675.
10. Adams, SR, Campbell, RE, Gross, LA, Martin, BR, Walkup, GK, Yao, Y *et al.* (2002). New biarsenical ligands and tetracysteine motifs for protein labeling *in vitro* and *in vivo*: synthesis and biological applications. *J Am Chem Soc* **124**: 6063–6076.
11. Marks, KM, Rosinov, M and Nolan, GP (2004). *In vivo* targeting of organic calcium sensors via genetically selected peptides. *Chem Biol* **11**: 347–356.
12. Guignet, EG, Hovius, R and Vogel, H (2004). Reversible site-selective labeling of membrane proteins in live cells. *Nat Biotechnol* **22**: 440–444.

13. Girod, A, Ried, M, Wobus, C, Lahm, H, Leike, K, Kleinschmidt, J *et al.* (1999). Genetic capsid modifications allow efficient re-targeting of adeno-associated virus type 2. *Nat Med* **5**: 1052–1056.
14. Grifman, M, Trepel, M, Speece, P, Gilbert, LB, Arap, W, Pasqualini, R *et al.* (2001). Incorporation of tumor-targeting peptides into recombinant adeno-associated virus capsids. *Mol Ther* **3**: 964–975.
15. Nicklin, SA, Buening, H, Dishart, KL, de Alwis, M, Girod, A, Hacker, U *et al.* (2001). Efficient and selective AAV2-mediated gene transfer directed to human vascular endothelial cells. *Mol Ther* **4**: 174–181.
16. Shi, W, Arnold, GS and Bartlett, JS (2001). Insertional mutagenesis of the adeno-associated virus type 2 (AAV2) capsid gene and generation of AAV2 vectors targeted to alternative cell-surface receptors. *Hum Gene Ther* **12**: 1697–1711.
17. Shi, W and Bartlett, JS (2003). RGD inclusion in VP3 provides adeno-associated virus type 2 (AAV2)-based vectors with a heparan sulfate-independent cell entry mechanism. *Mol Ther* **7**: 515–525.
18. Wu, P, Xiao, W, Conlon, T, Hughes, J, Agbandje-McKenna M, Ferkol, T *et al.* (2000). Mutational analysis of the adeno-associated virus type 2 (AAV2) capsid gene and construction of AAV2 vectors with altered tropism. *J Virol* **74**: 8635–8647.
19. Bartlett, JS, Kleinschmidt, J, Boucher, RC and Samulski, RJ (1999). Targeted adeno-associated virus vector transduction of nonpermissive cells mediated by a bispecific F(ab'gamma)2 antibody. *Nat Biotechnol* **17**: 181–186.
20. Gigout, L, Rebollo, P, Clement, N, Warrington, KH Jr., Muzyczka, N, Linden, RM *et al.* (2005). Altering AAV tropism with mosaic viral capsids. *Mol Ther* **11**: 856–865.
21. Arnold, GS, Sasser, AK, Stachler, MD and Bartlett, JS (2006). Metabolic biotinylation provides a unique platform for the purification and targeting of multiple AAV vector serotypes. *Mol Ther* **14**: 97–106.
22. Chen, I, Howarth, M, Lin, W and Ting, AY (2005). Site-specific labeling of cell surface proteins with biophysical probes using biotin ligase. *Nat Methods* **2**: 99–104.
23. Mahal, LK, Yarema, KJ and Bertozzi, CR (1997). Engineering chemical reactivity on cell surfaces through oligosaccharide biosynthesis. *Science* **276**: 1125–1128.
24. Chames, P and Baty, D (2000). Antibody engineering and its applications in tumor targeting and intracellular immunization. *FEMS Microbiol Lett* **189**: 1–8.
25. Hudson, PJ (1999). Recombinant antibody constructs in cancer therapy. *Curr Opin Immunol* **11**: 548–557.
26. Pasqualini, R (1999). Vascular targeting with phage peptide libraries. *Q J Nucl Med* **43**: 159–162.
27. Arap, W, Pasqualini, R and Ruoslahti, E (1998). Cancer treatment by targeted drug delivery to tumor vasculature in a mouse model. *Science* **279**: 377–380.
28. Pasqualini, R, Koivunen, E and Ruoslahti, E (1997). Alpha v integrins as receptors for tumor targeting by circulating ligands. *Nat Biotechnol* **15**: 542–546.
29. Haubner, R, Wester, HJ, Reuning, U, Senekowitsch-Schmidtke, R, Diefenbach, B, Kessler, H *et al.* (1999). Radiolabeled alpha(v)beta3 integrin antagonists: a new class of tracers for tumor targeting. *J Nucl Med* **40**: 1061–1071.
30. Pasqualini, R, Koivunen, E, Kain, R, Lahdenranta, J, Sakamoto, M, Stryhn, A *et al.* (2000). Aminopeptidase N is a receptor for tumor-homing peptides and a target for inhibiting angiogenesis. *Cancer Res* **60**: 722–727.
31. van Hagen, PM, Breeman, WA, Bernard, HF, Schaar, M, Mooij, CM, Srinivasan, A *et al.* (2000). Evaluation of a radiolabelled cyclic DTPA-RGD analogue for tumour imaging and radionuclide therapy. *Int J Cancer* **90**: 186–198.
32. Shi, W, Hemminki, A and Bartlett, JS (2006). Capsid modifications overcome low heterogeneous expression of heparan sulfate proteoglycan that limits AAV2-mediated gene transfer and therapeutic efficacy in human ovarian carcinoma. *Gynecol Oncol* **103**: 1054–1062.
33. Stachler, MD and Bartlett, JS (2006). Mosaic vectors comprised of modified AAV1 capsid proteins for efficient vector purification and targeting to vascular endothelial cells. *Gene Ther* **13**: 926–931.
34. Graham, FL, Smiley, J, Russell, WC and Nairn, R (1977). Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J Gen Virol* **36**: 59–74.
35. Clark, K, Voulgaropoulou, F and Johnson, P (1996). A stable cell line carrying adenovirus-inducible rep and cap genes allows for infectivity titration of adeno-associated virus vectors. *Gene Ther* **3**: 1124–1132.
36. Rabinowitz, JE, Rolling, F, Li, C, Conrath, H, Xiao, W, Xiao, X *et al.* (2002). Cross-packaging of a single adeno-associated virus (AAV) type 2 vector genome into multiple AAV serotypes enables transduction with broad specificity. *J Virol* **76**: 791–801.
37. Xiao, X, Li, J and Samulski, R (1998). Production of high-titer recombinant adeno-associated virus vectors in the absence of helper adenovirus. *J Virol* **72**: 2224–2232.
38. Zolotukhin, S (1999). Recombinant adeno-associated virus purification using novel methods improves infectious titer and yield. *Gene Ther* **6**: 973–985.
39. Clark, KR, Liu, X, McGrath, JP and Johnson, PR (1999). Highly purified recombinant adeno-associated virus vectors are biologically active and free of detectable helper and wild type viruses. *Hum Gene Ther* **10**: 1031–1039.