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Enhanced Targeted Gene Transduction: AAV2 Vectors Conjugated to Multiple Aptamers via Reducible Disulfide Linkages

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Abstract

Enhanced targeted gene transduction by AAV2 vectors is achieved by linking the vector to multiple sgc8 aptamers, which are selective for cell membrane protein PTK7. Aptamer molecules are conjugated to multiple sites on a DNA dendrimer (G-sgc8), which is then linked to AAV2 via a dithiobis(succinimidyl propionate) cross-linker containing a disulfide group, which can facilitate the release of AAV2 vectors by reaction with the reduced form of intracellular glutathione. The G-sgc8-AAV2 vectors showed a 21-fold enhancement in binding affinity and an enhanced ability to protect sgc8 aptamers against nuclease degradation to cells expressing PTK7 compared to single aptamer-AAV2 conjugates. The transduction efficiency was tested by loading AAV2 with the gene for green fluorescent protein. Therefore, this modified recombinant vector is an attractive and promising tool for targeted biomedical applications.

Virus-mediated gene delivery has emerged as a powerful and promising option for treating a variety of human diseases,[1] including Parkinson's disease[2] and hemophilia B,[3] owing to the greater gene transfer efficiency of viral vectors compared to non-viral vectors. Among the viral vectors, AAV2 is particularly attractive because of its properties of non-pathogenicity,

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low immunogenicity, high transfection efficiency, and long-term transgene expression in vivo.[4] AAV2 vectors can autonomously transfer genes to a variety of cell types, such as muscle, brain, lung, blood progenitor, and liver.[8] However, by the requirement for primary receptor heparin sulfate proteoglycan (HSPG) in AAV2-based gene trans-duction,[5] AAV2 viral gene delivery is confronted with the challenge of inefficient delivery to non-permissive cell types, which has, as a result, continuously highlighted the need for significant advancements in developing safe and efficient targeted virus delivery systems.[6]

Small peptides[7] and antibodies[8] exhibit receptor-binding specificities that can be genetically incorporated into the AAV2 capsid to enhance gene transduction or expand the tropism of AAV2 vectors to a previously non-permissive cell type. Sometimes, however, the mutant viral capsid results in failed production of AAV2 vectors or lower vector infectivity. These limitations call for the development of highly efficient and stable methods for targeted AAV2 vectors. Thus, as an alternative binding moiety, aptamers comprise another class of molecular probes for selective disease tissue recognition in targeted delivery.[9] As emerging probes, aptamers exhibit the same advantages as antibodies, such as high affinity, excellent specificity, and low toxicity or immunogenicity, as well as the additional merits of easy synthesis and modification, inherent stability with long-term storage, high reproducibility at low cost, and design flexibility.[10] Many aptamers have been selected for different types of tumor cells with high specificity, strong affinity, and rapid tissue penetration.[11] All these advantages make aptamers ideal candidates for developing aptamer-mediated delivery vehicles.

In this study, we have designed an aptamer-based DNA dendrimer (G-apt) and investigated the reduction of G-apt-AAV2 vector conjugates by the reduced form of intracellular GSH for enhanced viral gene delivery efficiency. The DNA dendrimer provides a scaffold for mounting multiple copies of aptamers to generate multivalent aptamer-tethered AAV2 vectors with enhanced binding affinity, the ability to protect sgc8 aptamers against nuclease degradation, and gene delivery ability. As illustrated in Scheme 1a, the AAV2 capsid with lysine residue was conjugated with G-apt, followed by modification with dithiobis(succinimidyl propionate) (DSP) cross-linker, which contains a disulfide group able to release AAV2 vectors by reaction with the reduced form of intracellular GSH. The sgc8 aptamer, which selectively recognizes cell membrane protein PTK7, was chemically modified to hybridize with building blocks on the outer shell of DNA dendrimer. Consequently, our strategy allowed for selective internalization of AAV2 carrying the gene for green fluorescent protein (GFP) by target cancer cells (Scheme 1b), resulting in significantly enhanced gene delivery compared to individual sgc8 aptamers conjugated with AAV2 vectors, thus demonstrating the potential of aptamer-driven viral delivery for gene therapy applications.

DNA dendrimers linked to multiple sgc8 aptamers were prepared from Y-shaped DNA monomers using an enzyme-free step-by-step assembly strategy.[12] As shown in Figure 1a, formation of Y0, Y1, G1, and G-sgc8 was confirmed by agarose gel electrophoresis (Figure 1b) with G-sgc8 migrating the most slowly. DLS measurements (Figure 1c) showed that the average diameters of G1 and G-sgc8 are 14.6 and 24.8 nm, respectively. These results

confirmed that higher assemblies of DNA dendrimer are heavier in mass and larger in size, indicating successful self-assembly.

A number of lysine residues are positioned at, or near, the non-enveloped AAV2 capsid surface.[13] Thus, it is conceivable that the AAV2 capsid could be labeled with amino group-modified G-sgc8 (G-sgc8-AAV2). Our strategy utilizes a coupling reaction[14] that operates under mild conditions, as illustrated in Scheme 1a. Based on Western blotting,[15] as shown in Figure 1d, significant increases in molecular mass of the capsid proteins occurred upon conjugation with G-sgc8 aptamers, demonstrating stable and successful conjugation between G-sgc8 aptamers and AAV2. DLS measurements (Figure 1c) showed that the average diameters of AAV2 and G-sgc8-AAV2 are 75.3 and 150.2 nm, respectively. The zeta potential (Figure S1) of G-sgc8-AAV2 is more negative than that of AAV2 owing to the negative DNA sequences. We did the non-specific binding test by mixing G-sgc8 and AAV2 vectors to confirm the covalent conjugation between G-sgc8 and AAV2 vectors (Figure S2). All these results suggested that G-sgc8 had been efficiently conjugated to the AAV2 vectors. We also prepared individual sgc8 aptamers conjugated with AAV2 vectors (Figure S3), and Western blotting method was used to demonstrate the successful conjugation between individual sgc8 and AAV2 vector (Figure S4). A HiTrap Heparin HP column (Figure S5) was used to purify the conjugated sample, and the BSA method (Figure S6) was used to determine the concentration of the conjugated vectors for further study. Sulfo-Cy5-NHS was used to label G-sgc8-AAV2 or sgc8-AAV2 to do flow cytometry of confocal imaging (Figure S7).

After the successful assembly of G-sgc8-AAV2 was confirmed, the cancer cell recognition capability of G-sgc8-AAV2 was investigated. Figure S8 shows the flow cytometric analysis comparison of target CCRF-CEM cells and control K562 cells. A noticeable higher enhancement in the fluorescence signal was observed for CEM cells treated with G-sgc8-AAV2 than treated with individual sgc8-AAV2, while no significant change in fluorescence intensity was observed for negative K562 cells, confirming the multivalent specific recognition capability of G-sgc8-AAV2 for the target CEM cell line. The binding affinities of sgc8-AAV2 and G-sgc8-AAV2 were determined by incubating CEM cells with some range concentrations of sgc8-AAV2 and G-sgc8-AAV2 at 4 °C for 30 min. As Figure 2b shows, G-sgc8-AAV2 (K_d = 0.27 ± 0.03 nM) exhibited a 21-fold enhancement in binding affinity compared to that of sgc8-AAV2 (Figure 2a) (K_d = 5.83 ± 0.75 nM). This favorable influence of multivalence on binding affinity can be explained in one of three ways.[16] First, by attaching multiple copies of an aptamer to a single AAV2, it is easy to achieve a local concentration of aptamer which is higher than the equivalent solution concentration containing the same amount of AAV2 vector (Figure S9a). Second, from a statistical perspective, the chance of reattachment of the aptamer to the cell upon dissociation of the initial event is increased due to the higher local aptamer concentration (Figure S9b). Third, the chelate effect makes the possibility for a multivalent aptamer-AAV2 conjugate to perform multiple binding interactions, which can achieve greater stability than possible by the sum of the independent interactions (Figure S9c). We then performed the stability tests of G-sgc8 construct compared with individual sgc8 sequence in human serum (Figure 2c) and human serum with 0.25 U/mL DNase I (Figure 2d). The degradation assays illustrated that the G-sgc8 is more stable than individual sgc8 aptamer, demonstrating the enhanced

ability of the assembled DNA dendrimer nanostructure to protect sgc8 aptamers against nuclease degradation.

It is well-known that the primary receptor heparin sulfate proteoglycan (HSPG) is required in AAV2-based transduction. Therefore, we tested the expression of HSPG receptor and PTK7 receptor on the membrane of HEK293 cells, HeLa cells, CEM cells, and K562 cells. From the results in Figures S10 and S11, we established a set of cell lines containing a range of HSPG and PTK7 expression profiles for our subsequent gene transduction experiments. Next, cell internalization assays were performed in the presence or absence of soluble heparin sulfate. G-sgc8-AAV2 vectors were unable to internalize into HEK 293 cells in the presence of heparin sulfate, but G-sgc8-AAV2 vectors could internalize into HEK293 cells in the absence of heparin sulfate (Figure S12a), while G-sgc8-AAV2 vectors were still able to internalize into HeLa cells in the presence of heparin sulfate (Figure S12b). These internalization results strongly suggested that the G-sgc8-AAV2 vectors can possibly direct infection via an HSPG-independent receptor, since the sgc8 aptamer can be internalized into PTK7-positive cells by receptor-mediated internalization.[17]

Next we tested the size of G-sgc8-AAV2 before and after treatment with GSH. As the DLS results in Figure 3a,b shows that the post-treatment sample of G-sgc8-AAV2 separates into two peaks, AAV2 and G-sgc8. The sizes of sgc8-AAV2 via DSP cross-linker before and after treatment with GSH were also tested (Figure S13), these results demonstrated that the disulfide linkages on DSP cross-linker can be efficiently cleaved in the reductive environment. To illustrate the key role of DSP in designing GSH-responsive AAV2 vectors, a similar cross-linker disuccinimidyl suberate (DSS) without disulfide moiety was used to prepare G-sgc8-AAV2 vector conjugates (Figure S14). Subsequently, we tested whether gene transduction in CEM cells of AAV2 vectors carrying the gene for Green Fluorescent Protein (GFP) could be reliably regulated by DSP or DSS cross-linkers by incubating these DSP- or DSS-prepared G-sgc8-AAV2 vectors with CEM cells. The gene transduction of G-sgc8-AAV2 conjugates with DSP and DSS cross-linkers was 49.2% and 20.6%, respectively (Figure 3c). G-sgc8-AAV2 vectors conjugates with DSP cross-linker showed almost 2.4-fold enhancement in gene transduction compared with that of conjugates prepared with DSS cross-linker, which is a significant improvement in gene transduction. It is likely that several downstream steps are required for productive infection[18] after sgc8-mediated internalization of AAV2 vectors, such as escape from endosomes, migration to the nucleus, and removal of the viral capsid coat. Thus, infectivity may be affected by any of these steps after conjugation with G-sgc8. We also tested individual sgc8 aptamers conjugated with AAV2 vectors and tested their gene transduction efficiency with a result of 30.2% (Figure 3c). Finally, we studied the stability of disulfide bond in G-sgc8-AAV2 construct by doing degradation test of G-sgc8-AAV2 in Human serum at 37 °C with different incubation time (Figure S15). Thus, it can be concluded that G-sgc8-AAV2 conjugates with DSP cross-linker can enhance the effective gene infection efficiency because of both the intracellular release of G-sgc8 motif upon reduction by GSH in the intracellular environment and the multivalent aptamer conjugation strategy.

We next investigated the transduction efficiency of G-sgc8 aptamer-modified AAV2 vectors with GFP gene via DSP cross-linker in HSPG-negative CEM cells. PTK7-negative and

HSPG-negative K562 cells were used as negative control. HSPG-positive and PTK7-negative HEK293 cells and HSPG/ PTK7-positive HeLa cells were chosen as positive control. The green signal from the expression of GFP gene was collected by flow cytometry (Figure 4). The transduction efficiency of G-sgc8-AAV2 vectors was 49.2% for CEM cells, while that of K562 cells was only 3.3%. It should be noted that we observed a decrease in the AAV2 vector-mediated gene delivery to permissive HEK293 cell line, which in turn indicating that the sgc8 aptamer-mediated PTK7 receptor targeting approach was both specific and restrictive to its targeting CEM cells. We also repeated the infection experiments with G-lib-AAV2 conjugates via DSP cross-linker and G-sgc8 mixed with AAV2-GFP without DSP linkers. Neither sample showed obvious enhancement of GFP expression, as expected. These results demonstrated that targeted gene transfer of AAV2 vectors can be achieved by direct chemical modification with multiple aptamers. We also performed a cell viability test to determine the cytotoxic effect of G-sgc8-AAV2 on HEK293 cells, HeLa cells, CEM cells, and K562 cells, and the results indicate (Figure S16) that G-sgc8-AAV2 conjugates are not cytotoxic to any of these cells. Therefore, G-sgc8-AAV2 vectors can be safely used as targeted gene transduction vectors for gene therapy.

In summary, we have demonstrated GSH-responsive and multivalent aptamer-based AAV2 vectors incorporating DNA dendrimer structure and disulfide linkages with enhanced specific recognition behavior and selective gene transduction to target cancer cell lines. For biomedical applications, these specific vectors possess several remarkable features: (1) wider application for AAV2 vectors to HSPG-negative cell lines because of conjugation with aptamer, (2) enhanced gene transduction efficiency by incorporating multivalent aptamers and GSH-stimulated response, (3) enhanced ability to protect sgc8 aptamers against nuclease degradation because of DNA dendrimer structure, (4) multiple applications, since this multivalent aptamer-tethered DNA dendrimer can be equipped with different aptamers toward a variety of target cancer cell lines using oligonucleotide base pairing without complicated chemical modification, (5) efficient cellular uptake of AAV2 vectors (Figure S17), as indicated by rapid internalization into target cells within the first 30 min by PTK7 receptor-mediated endocytosis, (6) easy design and preparation, as the aptamer-tethered DNA dendrimer structure can be assembled at room temperature by mixing different building blocks at fixed ratio, and (7) potential multimodal cancer theranostics. These vectors can be designed as safe nanocarriers for the codelivery of drug or imaging agents, and AAV2 vectors carrying therapeutic genes can be conjugated to G-sgc8 to achieve synergistic cancer therapy. Collectively, these remarkable features make our G-sgc8-AAV2 vectors uniquely attractive for the development of targeted gene therapy and even targeted synergistic therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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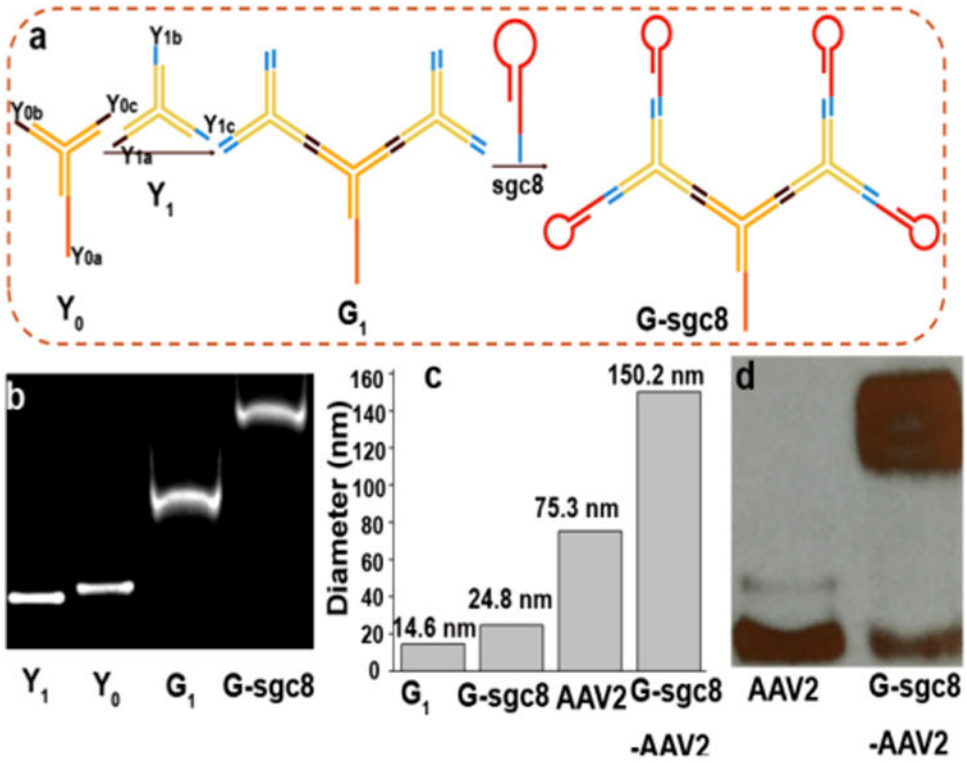


Figure 1.

(a) Preparations of Y0, Y1, G1, and G-sgc8. (b) Agarose gel electrophoresis results of Y0, Y1, G1, and G-sgc8. (c) DLS data for the diameter of G1, G-sgc8, AAV2, and G-sgc8-AAV2. (d) G-sgc8-AAV2 conjugate and AAV2 only were analyzed by Western blot.

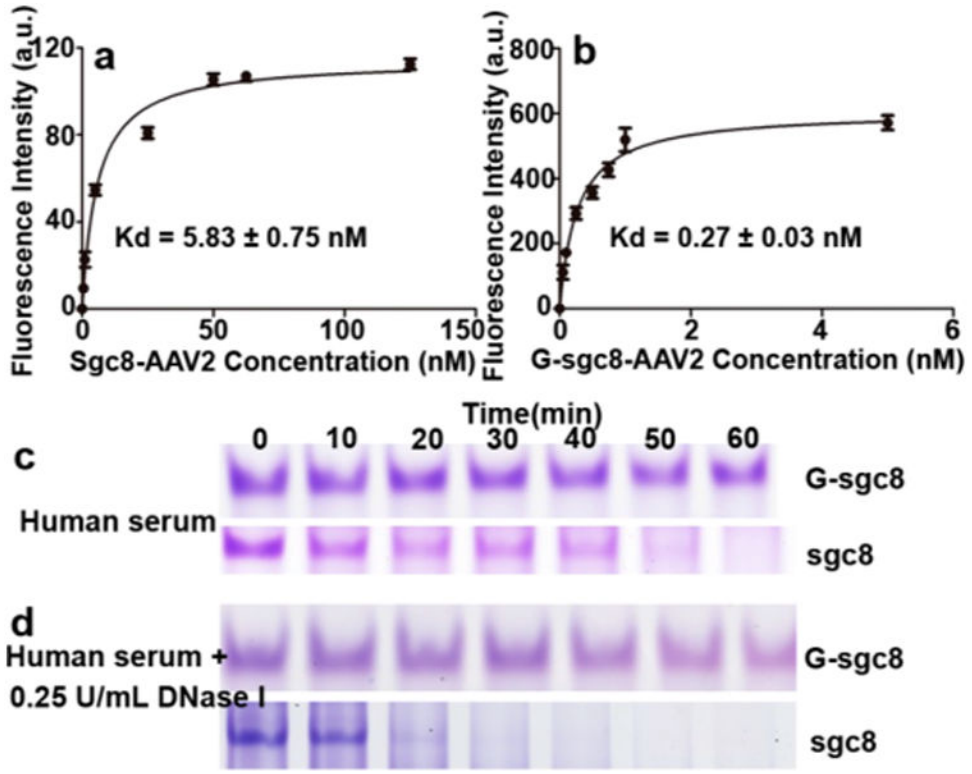


Figure 2.

Binding affinity tests of (a) sgc8-AAV2 and (b) G-sgc8-AAV2 to CEM cells. Native PAGE analysis of G-sgc8 construct and individual sgc8 aptamers in (c) human serum and (d) human serum with 0.25 U/mL DNase I at 37 °C with different incubation time.

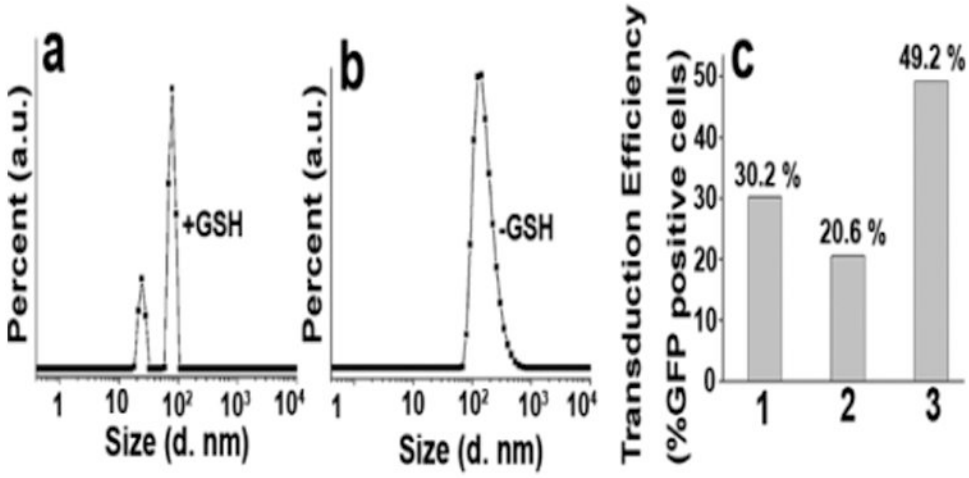


Figure 3.

DLS results for G-sgc8-AAV2 with DSP cross-linking (a) in the presence or (b) in the absence of GSH. (c) Transduction efficiency of AAV2 vectors containing GFP gene with different chemical modification to target CEM cells: 1, sgc8-AAV2 with DSP cross-linker; 2, G-sgc8-AAV2 with DSS cross-linker; 3, G-sgc8-AAV2 with DSP cross-linker.

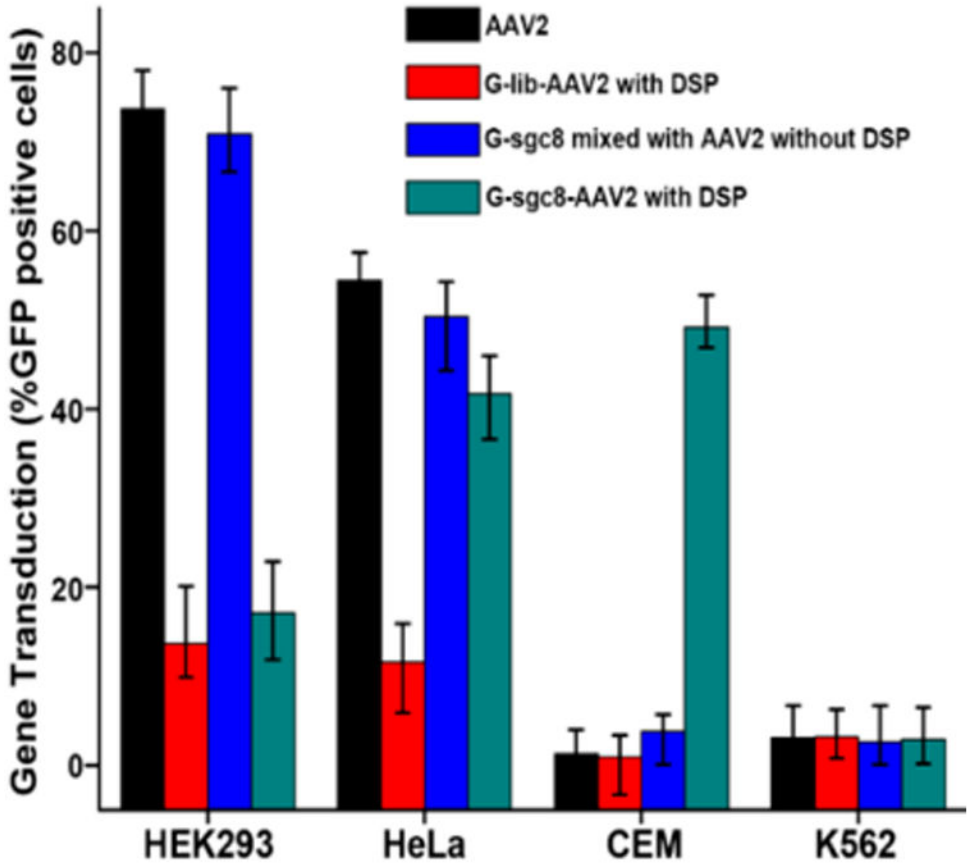
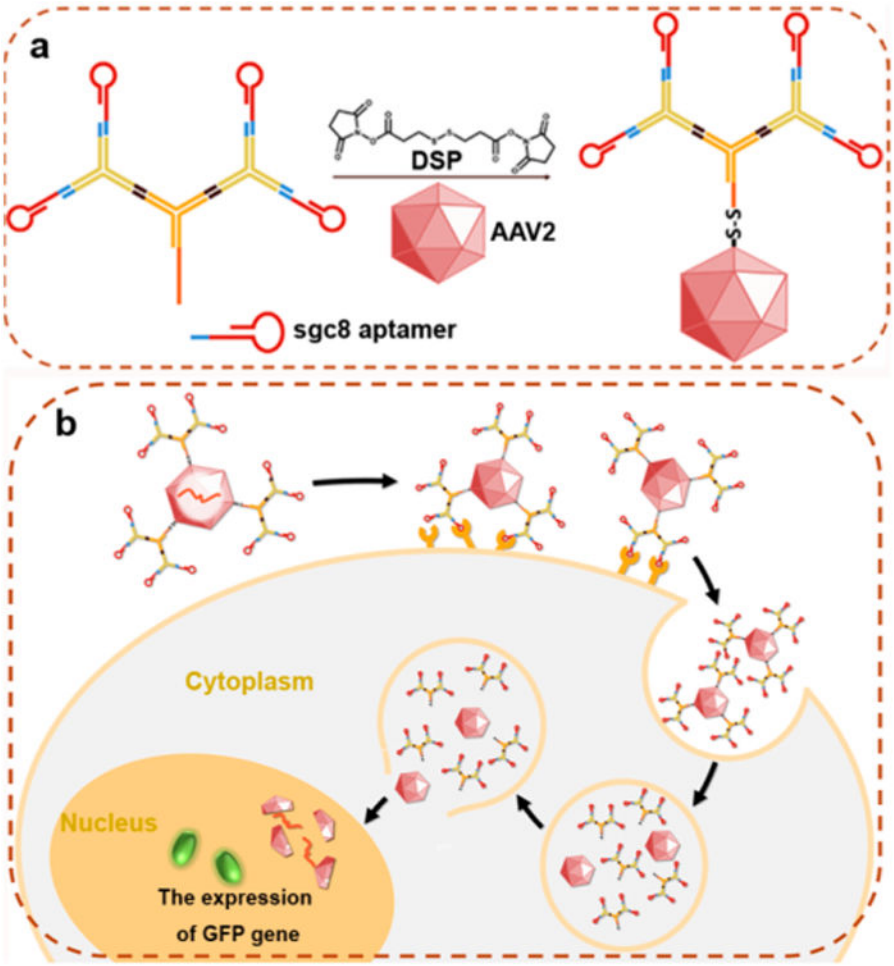


Figure 4.

Transduction efficiency of AAV2 vectors containing GFP gene with different chemical modification to different cell lines.



Scheme 1.

(a) G-sgc8-AAV2 Complexes Are Generated by DSP Cross-Linkers between the Amino Group on One Branch of G-sgc8 and Primary Amines from Lysine Residues on the AAV2 Capsid; (b) Intracellular GSH-Responsive Chemical Modification of AAV2 Vectors Carrying Green Fluorescent Protein Reporter Gene with Multiple Aptamers To Enhance Cell-Specific Transduction of AAV2-Based Vectors