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# Mitigating the Immunogenicity of AAV-Mediated Gene Therapy with an Immunosuppressive Phosphoserine-Containing Zwitterionic Peptide

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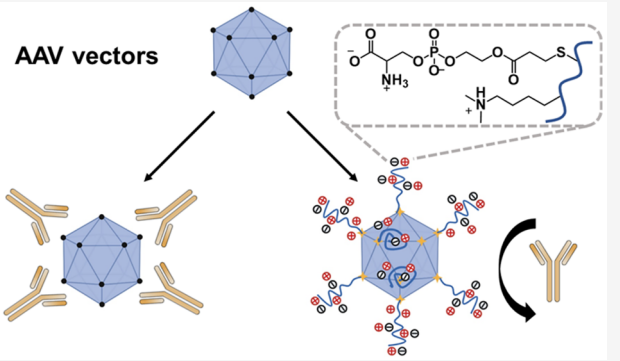
**ABSTRACT:** Although recombinant adeno-associated viruses (AAVs) are considered low immunogenic and safe for gene delivery, the immunogenicity of capsids still represents a major obstacle to the readministration of AAV vectors. Here, we design an immunosuppressive zwitterionic phosphoserine (PS)-containing polypeptide to induce AAV-specific immune tolerance and eradicate the immunological response. AAVs modified with the zwitterionic PS polypeptide maintain their transduction activity and tissue tropism but suppress the induction of AAV-specific antibodies. In a hemophilia A mouse model (FVIII knockout mice), the readministration of zwitterionic PS polypeptide-modified AAV8-FVIII vectors successfully evades immunological response, corrects blood FVIII levels, and stops blood loss in tail-bleeding experiments. This potent and safe technology mimics the natural tolerance of apoptotic cells and controls the immunosuppressive, zwitterionic, and degradable polypeptide precisely, reducing the concern of toxicities upon readministrations. This work presents a new concept and a platform of engineered viral vectors by chemically linking immunosuppressive materials to AAV vectors, enabling the readministration of AAV vectors while maintaining their transduction efficiency to a considerable degree.






## INTRODUCTION

Recombinant adeno-associated virus (AAV) vector-mediated gene therapy is the only FDA-approved DNA-based gene therapy. Many AAV serotypes targeting different organs have been explored for a variety of chronic and genetic diseases.[1] Despite huge clinical outcomes to date, AAV vector gene delivery has been limited due to the durability of the effect.[2] Single AAV administration usually lasts from months to several years of gene expression above therapeutic levels.[3] However, many inherited diseases require lifelong treatments to avoid irreversible tissue damage.[4] Increasing administration dosage may extend the therapeutic windows, but the concern about potential toxicities rises.[5,6] Thus, the ability to readminister AAV is crucial to achieving sustained therapeutic efficacy over time. Although AAVs are considered low immunogenic and safe as compared with other viral vectors, the immunogenicity of capsids still represents a major obstacle to the readministration of AAV vectors.[3] Both humoral and cell-mediated immunities are observed in preclinical animal studies and human patients.[7,8] A high level of neutralizing antibodies (NAbs) is triggered after vector administration and lasts for a long time, making it impossible or risky to re-administer AAV vectors. The induction of capsid-specific CD8+ T cell responses[9] clears transduced patient cells and causes the efficacy loss even for the first AAV dose. Poly(ethylene glycol) (PEG) polymer was utilized before to chemically modify AAV serotype 2 (AAV2) capsids to block AAV2-specific neutralizing antibodies.[10,11] However, while PEG and other hydrophilic "inert" materials may reduce humoral immunity, it is not effective against cell-mediated immunity. A variety of approaches that aim to induce immune tolerance and recover immune balance have been proposed such as coadministration of clinically approved immunosuppressive drugs.[12,13] In a typical case, rapamycin, an mTOR inhibitor that can drive tolerogenic antigen-presenting cells (APCs) and regulatory T (Treg) cell induction, has been employed as an immunosuppressive drug to modulate the adaptive immune response induced by AAV-based gene vectors.[14,15] However, the systemic administration of these small-molecule drugs may bear some potential toxicities in addition to their suppression of overall immunity.[16,17] In view of pharmaceutics, hydrophobic rapamycin is also hard to formulate with many biological drugs that usually are dispersed in aqueous buffers.

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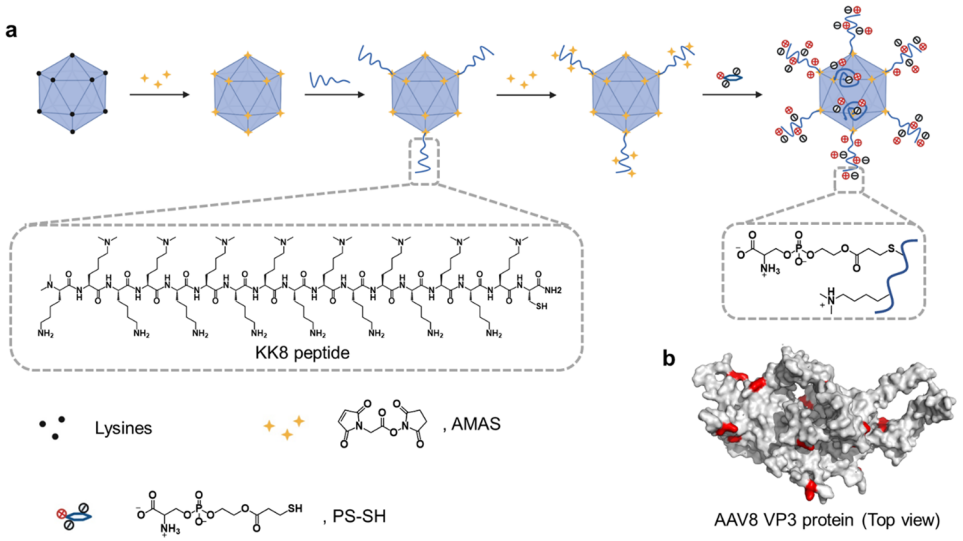
**Scheme 1. (a) Illustration of the Conjugation Strategy for Preparing PS-Containing Peptide-Conjugated AAV Vectors; (b) Top View of the Structure of VP3 Protein from AAV Serotyped 8 (PDB #: 2qa0)[a]**




[a]Red moieties indicate lysine residues.

Encapsulating and delivering them in separate formulations could be a compromised strategy, leading to inevitably high usage of drugs and potential off-target organ accumulation. Theoretically, a direct conjugation strategy could facilitate the precise co-delivery of the antigens and immune modulators to immune cells. Hydrophilicity and engineerable property will be the prerequisites in identifying a suitable immune-tolerant agent. Endogenous immune-tolerant structures are far more favorable than exogenous synthetic drugs. Phosphatidylserine, a natural lipid with phosphoserine (PS) as the headgroup, serves as an immunomodulatory signal under physiological conditions.[18] During cell apoptosis, PS is exposed on the cell surface from the inner membrane and then recognized as an "eat-me" signal to phagocytes, promoting self-clearance.[19−21] Meanwhile, PS elicits anti-inflammatory signal cascades in the engulfing phagocyte, induces immune tolerance to components of the apoptotic cell, and thus prevents the aberrant immune response to self-antigens.[22] Interestingly, many natural enveloped viruses mimic a similar apoptotic mechanism for their entry, binding, and immune evasion.[23] For example, lentiviruses produce a large amount of phosphatidylserine-containing pseudotyped lentiviral particles during replication, which dampens the innate immune responses in the host.

In our recent work, we prepared PS-containing zwitterionic polymers and conjugated them to uricase, an immunogenic enzyme drug for refractory gout treatment.[24] The PS structure carries one net negative charge, which will break the charge balance of protein surfaces and alter the overall drug pharmacokinetics. Thus, by adding one positive charge next to every PS moiety, we improved the blood half-life of uricase conjugated with PS-containing zwitterionic polymers, which were able to fully get rid of unwanted immune responses and achieve multiple drug administrations without any efficacy loss compared to unconjugated uricase. Encouraged by this foundational work, we are exploring the idea of "immunosuppressive" zwitterionic PS materials that can mitigate the immunogenicity of AAV vectors and allow readministration while maintaining their tissue tropism and transduction efficacy. Here, we redesign the structure of the PS-containing polymer to maximize the immune tolerance capacity and maintain the transduction potency. A peptide backbone is used here to replace the nondegradable polymer backbone, which will impact its translational potential. To maximize the biological activity of the PS structure while maintaining the overall zwitterionic property, we retain the native PS structure and compensate an equivalent positive charge next to each PS moiety, resulting in a mixed charged (or zwitterionic) peptide form.

## RESULTS AND DISCUSSION

AAVs infect host cells by receptor-mediated interactions.[25] Modifying AAVs with polymeric materials may block these interactions and lead to compromised transfection efficiency. Previous studies on PEGylation of AAV vectors showed that the large molecular weight and high density of conjugated PEG significantly reduced the transfection of AAV vectors. Thus, we first did an in vitro screening to identify a suitable peptide length, which could balance the amount of carrying PS and AAV transfection capacity. A series of alternating Glu-Lys (EK) peptides with different lengths were first prepared and conjugated onto AAV8 encoding firefly luciferase (AAV8-Fluc). Choosing the inert EK peptide as a starting peptide is due to its zwitterionic characteristic and previously established conjugation method,[26] which makes it a simplified analog to the PS-containing peptide. In vitro transfection results revealed a clear relationship between the peptide length and luminescence signal. As shown in Figure S1, 50% to 70% of transfection was maintained when the peptides were not longer than 21 amino acids (EK10 plus one terminal cysteine). A significant efficacy drop (>70% loss) was observed when the peptide length reached 25 amino acids (or EK12 with one terminal cysteine). Based on those results, we prepared the KK8 peptide consisting of eight primary and eight tertiary amines alternatively. KK8 is used instead of KK10 because the bulky PS moiety will further increase the peptide size in this work to enable the conjugation between the PS moiety and the AAV vector. The KK8 peptide was prepared on a microwave-assisted solid phase synthesizer starting from a KK dimer,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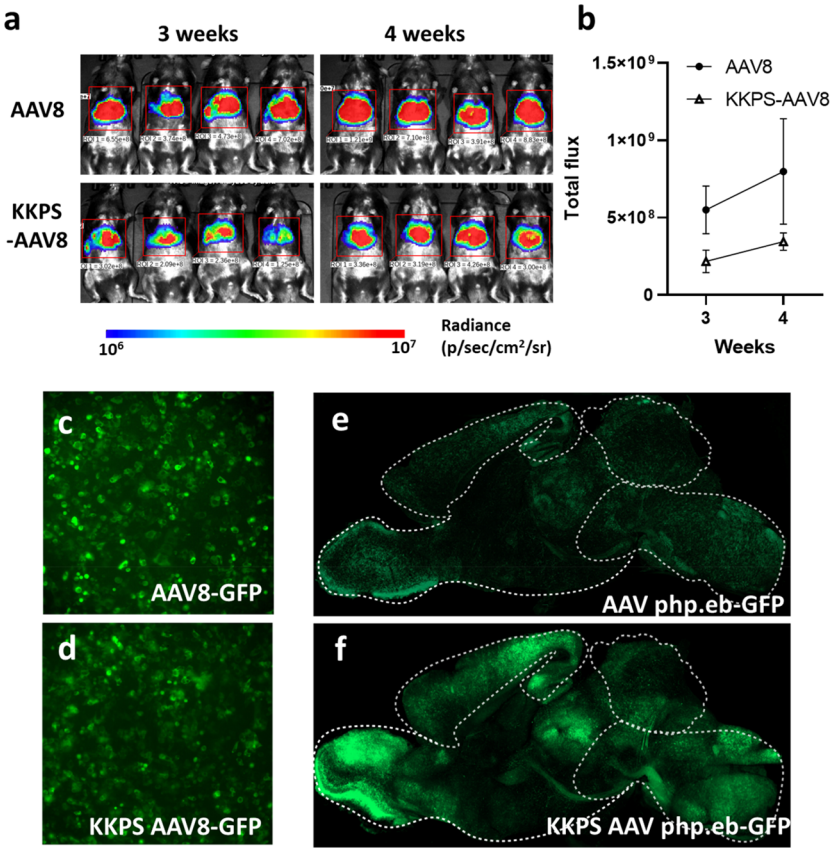


**Figure 1.** (a) IVIS images of luciferase expression in C57bl/6 mice using native (unmodified) AAV8 and KKPS-AAV8, respectively. Mice were i.v. injected with a single dose of native or modified AAV8-CMV-Fluc ($4 \times 10^{12}$ vg/kg) on day 1. On days 21 and 28, mice were i.p. injected with D-luciferin (150 mg/kg) and imaged in an IVIS system (PerkinElmer). (b) Summarized data of total luminescence in Figure 1a; GFP expression in mouse liver sections that were taken after 3 weeks of a single-dose injection of (c) AAV8-CAG-GFP and (d) KKPS-AAV8-CAG-GFP. A single dose of i.v. injection of AAV php.eb-hSYN-eGFP (e) and KKPS- AAV php.eb-hSYN-eGFP (f) showed the whole brain transduction of eGFP in C57bl/6 mouse brain after 6 weeks.

which had two orthogonal protection groups (the structure shown in Scheme S1). By selective deprotection and reductive amination, every other primary amine was converted to a tertiary amine and the remaining primary amines were retained for PS moiety conjugation (Scheme S2). Such a design maintains the zwitterionic property of the final structure. A similar conjugation strategy according to our previous study was adopted to prepare PS-containing peptide-conjugated AAV vectors (KKPS-AAV) as shown in Scheme 1. Generally, lysine residues on the AAV8 surface were first activated by a bifunctional cross-linker, i.e., *N*-$\alpha$-maleimidoacet-oxysuccinimide ester (AMAS), which converted amine groups into maleimide moieties. KK8 peptide containing one C-terminal cysteine was then linked to maleimide moieties on the AAV surface via thiol−ene click chemistry. In the second round of conjugation, amine groups from the KK8 peptide were also converted to maleimide groups and then reacted with a customized thiol-terminated PS compound (Scheme 1) to obtain the final product of KKPS-AAV (Scheme 1a). In vitro transfection evaluation was performed in 293T cells using KKPS-modified AAV8-Fluc (Figure S1). About 54.6% luciferase expression relative to that of native AAV8 was maintained by KKPS-AAV8-Fluc, which matched the performance of EK8- or EK10-modified AAV8.

In vivo expression of KKPS-modified AAV8 was evaluated in C57BL/6 mice to determine if KKPS peptide modification would alter the tissue tropism of AAV8 (Figure 1a). Three weeks after the single-dose treatment, all mice showed liver-specific luciferase expressions. The total flux of the bioluminescence signal was measured and showed about half of the expression rate of native AAV8 was maintained after modification (Figure 1b). It should be pointed out that PS is known as an "eat-me" signal and will be recognized by phagocytes, promoting clearance via the lymphatic system, resulting in efficacy loss, especially for liver-targeting AAV serotypes. Our recent study showed a shift in delivery targets from the liver to secondary lymphoid organs when a natural PS lipid has been added to a conventional lipid nanoparticle (LNP) formulation.[27] Native and KKPS-modified AAV8 vectors encoding GFP were prepared for further in vivo transfection. Both vector-treated mice showed GFP expression in liver sections, but a slight reduction of GFP expression was detected after conjugation/modification (Figures 1c and 1d). To verify if the KKPS conjugation strategy could be applied to other AAV vectors, we also modified a brain-targeting AAV vector, Serotype php.eb,[28] with our peptide and tested it in C57BL/6 mice. A neuron-specific promoter, human synapsin 1 gene promoter (hSYN), was constructed upstream of the open reading frame (ORF) to evaluate of the neuron-specific transduction ability. The single-dose intravenous injection of native AAV php.eb-hSYN-eGFP and KKPS-modified vector showed the whole brain transduction of eGFP in the whole mouse brain after 6 weeks (Figures 1e and 1f). Neuron cells in the brain cortex, hippocampus, and thalamus showed strong


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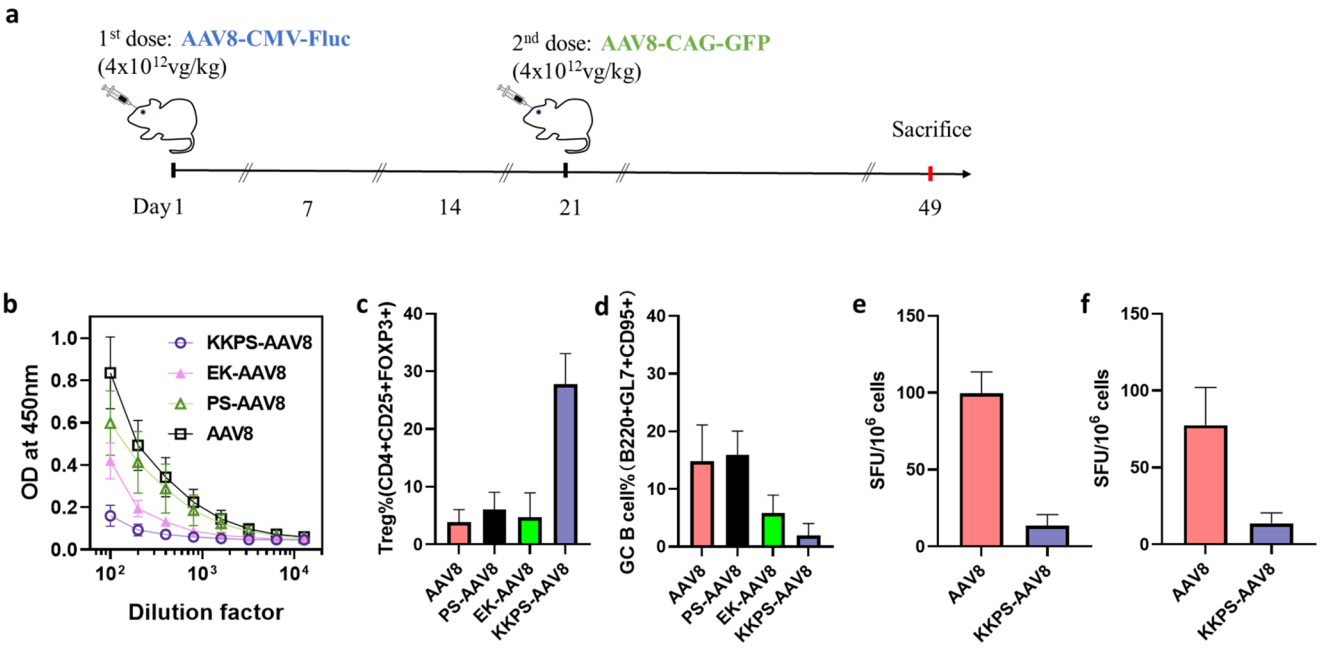


**Figure 2.** (a) Administration route of the two-dose cohort for immunogenicity studies. Two doses of AAV8 vectors ($4 \times 10^{12}$ vg/kg each, with the same capsids, but different expression cassettes) were i.v. injected into C57bl/6 mice on day 1 and day 21, respectively. Animals were sacrificed on day 49, and sera were separated for the ELISA test. The mice spleens were harvested for the extraction of splenocytes, which were cultured in the presence of native AAV8, PS-AAV8, EK-AAV8, or KKPS-AAV8 for 72 h. Then cells were stained for flow cytometry analysis. (b) Anti-AAV8 IgG titers. Conjugation of KKPS successfully mitigated the generation of anti-AAV8 antibody (titers: 800), while native AAV8 showed the highest antibody titers (>6400). (c) Summary of the percentage of Treg phenotype (Foxp3+) cells among CD4+CD25+ splenocytes. (d) Summary of the percentage of activated Gemina center B cells. (e) AAV8-specific mouse interferon gamma ELISPOT. (f) An nti-AAV8 IgG secreting B cell ELISPOT.

eGFP expressions (Figure S3). Those results confirmed that KKPS modification maintained the transduction ability of brain/neuron tropistic AAV serotype. Higher transduction efficiency based on fluorescence density analysis was observed in the mouse brain treated with KKPS-modified AAV php.eb than its unmodified counterpart (Figure S3e), which is worth further study.

Although AAV-based gene therapies represent substantial advances in the treatment of chronic and genetic diseases, they carry an inherent risk of eliciting a strong immune response that can adversely affect the long-term efficacy and safety of the treatment. Currently, most FDA-approved AAV therapies or ongoing clinical trials can only achieve single-dose administration.[2] Patients who are prescreened with anti-AAV Abs are excluded from the therapies or trials, and those patients who generated an adaptive immune response to the AAV drugs may not be able to benefit from the therapies again in the future. In another aspect, although AAV vector-based gene therapies are expected to have a long-term therapeutic outcome, results of the ongoing clinical trial of valoctocogene roxaparvovec, AAV-mediated FVIII gene therapy for hemophilia A, show that a significant decrease in factor VIII activity levels occurs 4 years after the initial injection in the high-dose cohort.[29] Therefore, it is crucial if second and even multidose AAV administrations can be achieved. Therefore, we performed in vivo tests of the antigen-specific immunosuppression capacity of the KKPS peptide as proposed in this work. To evaluate the immunogenicity of modified and native AAV carriers, but avoid interference coming from the immunogenicity of encoding proteins, two types of AAV8 vectors encoding luciferase (Fluc) and green fluorescence protein (GFP) were prepared (Figure 2a, labeled in blue and green colors, respectively) and further modified with KKPS peptide. As a non-PS analog of immunosuppressive KKPS-AAV8, inert zwitterionic peptide EK10-modified AAV8 was used to evaluate the influence of steric hindrance coming from the peptide architecture. Small PS molecules were also directly conjugated to AAV8 vectors as a control group without the peptide linker. Each group of C57bl/6 mice was i.v. injected with one capsid type of AAV8-CMV-Fluc on day 1, while a booster dose with the same capsid modification (encoding GFP) was injected on day 21 (Figure 2a). After animals were sacrificed on day 49, AAV-specific IgG generation in mice serum was measured by ELISA. As expected, native AAV8 induced strong capsid-specific IgG (Figure 2b, titers >6400), while KKPS conjugation mitigated the generation of anti-AAV8 IgG (titers = 800). Single PS molecule modification also failed to reduce the immunogenicity of AAV8, but the EK10 peptide showed mitigation (titers = 1600). Inert EK10 was previously reported by us to block antibody binding based on steric hindrance. We further tested the immune cell activation by incubating harvested splenocytes with previously injected AAV vectors. As shown in Figure 2c, only splenocytes from the KKPS-AAV8-treated group were found to have a high percentage of activation of Treg cells, indicating that high-density PS moieties are essential for the immunosuppression of AAV8. Conjugation of a zwitterionic peptide could reduce antibody generation against underlying proteins to a certain degree via steric shielding, but not in an active suppression way, which is needed for blocking cell-mediated immune response. The results on germinal center B cell activation were consistent with those of IgG titers shown in Figure 2d. Interferon-gamma T cell ELISpot (Figure 2e) and anti-AAV8 IgG secreting B cell (Figure 2f) ELISpot experiments showed


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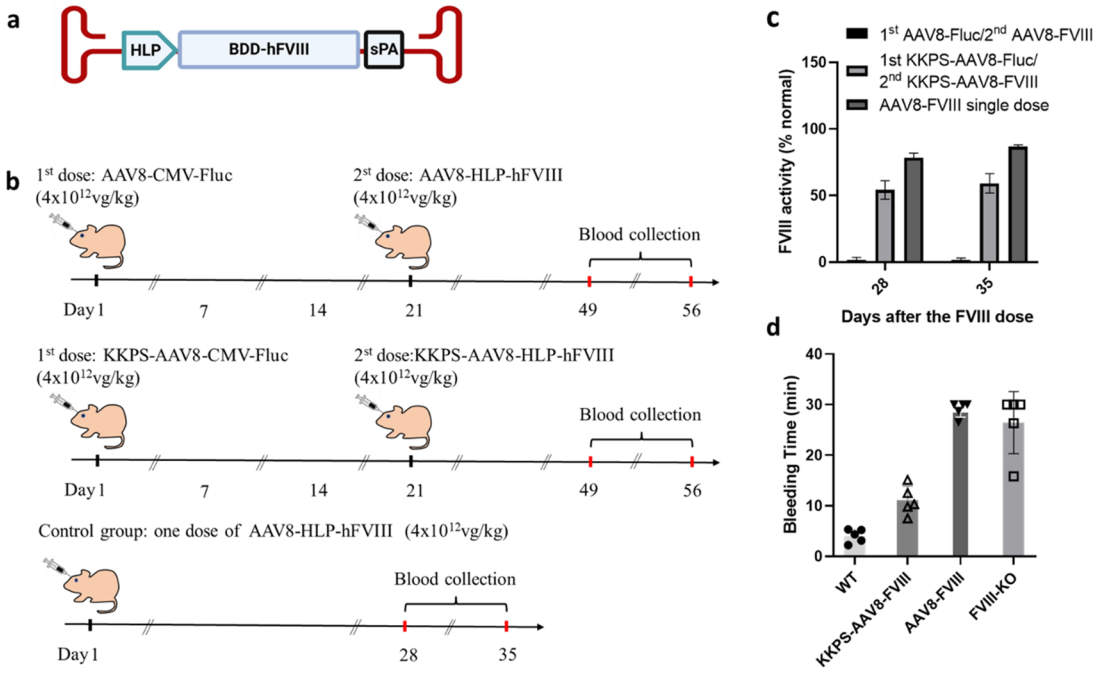


**Figure 3.** (a) rAAV constructs encoding human B domain depleted FVIII. (b) FVIII gene delivery in FVIII-knockout hemophilia A mice. A model AAV8 vector encoding luciferase ($4 \times 10^{12}$ vg/kg) was i.v. injected into mice on day 1. Mice received the second AAV8 vector encoding hFVIII ($4 \times 10^{12}$ vg/kg). Plasma was collected on days 49 and 56 (or days 28 and 35 after the injections of AAV8 vector encoding hFVIII). Gene knockout mice only receiving one dose of AAV8-HLP-hFVIII were set as the positive control group and plasma was collected on days 28 and 35. A tail bleeding test was also performed on day 56. After that, all the mice were sacrificed immediately. (c) FVIII activities in plasma 28 and 35 days after the injections of AAV8 vector encoding hFVIII. Data were normalized with the standard FVIII activity tested from pooled health human plasma. (d) Tail bleeding test. The distal part of the tail (at 6 mm) of FVIII knockout mice was cut off and immersed in 37 °C saline for 30 min. The experiment ended at 30 min, and bleeding time was recorded or written as 30 min if the bleeding does not stop.

strong immune activation by two doses of AAV8 lacking KKPS modification.

Hemophilia A (HA) is a common inherited bleeding disorder, caused by a deficiency of clotting factor VIII. Compared with the direct protein replacement, AAV-mediated FVIII gene delivery is believed to be a long-term potent therapy for those with severe bleeding incidences. The current phase III clinical trial of AAV5-mediated FVIII gene therapy achieves more than 3 years of blood human FVIII correction. As gene copies of the AAV cassette would be lost over time, readministration of AAV vectors is still highly desirable and demanded. To verify if KKPS peptide could enable the readministration of AAV8 vectors in an animal model, we prepared rAAV8-HLP-hFVIII-loaded AAV8 vectors and tested their potency in FVIII knockout mice. The gene construct of rAAV8-HLP-hFVIII is a B-domain depleted human FVIII with an HLP promoter (Figure 3a), which is constructed according to a published design.[30] In the animal study design, we adopted the specific antibody-inducing route of an AAV capsid in FVIII knockout mice. Administration routes are shown in Figure 3b. Generally, $4 \times 10^{12}$ vg/kg of native or KKPS-modified AAV8 vectors encoding a nonrelevant model protein firefly luciferase were first injected to induce adapted immunity in animals. Three weeks later, the same dose of native or KKPS-modified AAV8-HLP-hFVIII was administrated. The activity of hFVIII in murine plasma was monitored at two time points after the second dose injection. As hFVIII is considered exogenous in FVIII knockout mice, such a dosing pattern will exclude the effects from the encoding proteins other than AAV vectors. Data are shown in Figure 3c. In a positive control group, hFVIII levels in gene knockout mice that only received one dose of AAV8-HLP-hFVIII reached 78.3% and 86.5% of normal FVIII levels after 4 and 5 weeks after the injection. KKPS-modified AAV8 achieved 54.1% and 59.1% of normal FVIII levels after 4 and 5 weeks after the dose encoding hFVIII injection, while unmodified AAV8 has no significant FVIII activity observed. Tail bleeding tests were also performed on day 35 after the second dose injection (data shown in Figure 3d). FVIII knockout mice treated with KKPS-modified AAV8-HLP-hFVIII stopped bleeding at an average time of 11.04 min, while WT mice had an average bleeding time of 4.03 min. In the cohort of knockout mice receiving unmodified AAV8, three of them could not stop bleeding in 30 min (the presetting end point) and the others have an average bleeding time of 21.05 min. Three untreated FVIII knockout mice could not stop bleeding, and the rest of the mice in the cohort stopped bleeding at an average time of 29.04 min. Results of the tail bleeding test reflected the same scenario in blood FVIII activities. Immunogenicity of the capsid protein of AAV8 blocked the therapeutic efficacy of a subsequent dose, suggesting that the immunomodulation of AAV vectors via redosing is required for long-term viral vector-based gene therapies.

## CONCLUSIONS

Inspired by the natural tolerance of apoptotic cells, we designed a zwitterionic PS-containing peptide and chemically conjugated it to AAV vectors. This modification technology could induce AAV-specific immune tolerance and eradicate the immunological response. By optimizing the polypeptide, we maintained the transduction efficiency of AAV8 vectors to a considerable degree and kept their original tissue tropism but


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suppressed the induction of AAV8-specific antibodies. We further tested our platform in FVIII knockout mice. Succeeding readministration of the zwitterionic PS polypeptide-modified AAV8-FVIII corrected blood FVIII levels and stopped blood loss in tail-bleeding experiments. The current work presented a new concept and a platform of engineered viral vectors by chemically linking immunosuppressive materials to AAV vectors, enabling the readministration of AAV vectors. We expect that more immunosuppressive moieties could be applied in this platform to increase gene delivery efficiency further and broaden tissue tropism.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.2c09484.

Experimental procedures, supplementary contents, and supplementary tables and figures (PDF)

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### Author Contributions

Z.Y. and B.L. contributed equally.

### Notes

The authors declare the following competing financial interest(s): Z.Y., B.L., W.G., S.L., R. Li, and S.J. are authors of a patent application related to this work (PCT/US2022/021929) filed by Cornell University and the University of Washington.

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