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Amphiphilic Polyphenylene Dendron Conjugates for Surface Remodeling of Adenovirus 5

Jessica Wagner,[a,b] Longjie Li,[c] Johanna Simon,[a] Lea Krutzke,[d] Katharina Landfester,[a] Volker Mailänder,[a,e] Klaus Müllen,[a] David Y.W. Ng,*[a] Yuzhou Wu*[a,c] and Tanja Weil*[a]

Abstract: The arrangement of amphiphilic surface groups plays an important role in many biological processes such as protein folding or biorecognition. We report the synthesis of amphiphilic polyphenylene dendrimer branches (dendrons) providing alternating hydrophilic and lipophilic surface groups and one reactive enthynyl group at the core. The amphiphilic surface groups serve as biorecognition units that bind to the surface of adenovirus 5 (Ad5), which is one of the most common vectors in gene therapy. The Ad5/dendron complexes showed high gene transduction efficiencies in coxsackie-adenovirus receptor (CAR)-negative cells. Moreover, the dendrons offer incorporating new functions at the dendron core by *in situ* post-modifications even when bound to the Ad5 surface. Surfaces coated with these amphiphilic dendrons were analyzed for their blood protein binding capacity, which is essential to predict their performance in the blood stream. In this way, we provide a new platform for introducing bioactive groups to the Ad5 surface without the necessity to chemically modify the virus particles.

Introduction

Amphiphilicity plays an important role in the formation of biological architectures such as the structure of proteins, the self-assembly of peptides, or the build-up of biological membranes.[1] Due to the characteristics of amphiphiles to organize into higher ordered structures[2], their interactions with other biomolecules is a complex process of high biological relevance, which is still not fully understood. For example, the exposure of nanomaterials like polymers, liposomes, or nanoparticles to biological fluids, such as human blood plasma, gives rise to a protein corona around nanoparticles that also directs their transport *in vivo*.[3] It has been demonstrated that the variation of surface charges[4] or coating of nanoparticles e.g. with polymers like polyethylene glycol[5] has an impact on the protein corona and often controls their aggregation[6], biodistribution[7] as well as cellular uptake properties[5]. By employing amphiphilic surface patterns on nanoparticles, their influence on biological systems was studied.[8] It is still very challenging to control the surface contour of nanoparticles[8b] and to impart distinct amphiphilic surface patterns with molecular precision that maintain their perfect nanosize definition in various biological environments[9]. Therefore, highly branched macromolecules with precise structures and molecular weights such as dendrimers have emerged as a monodisperse platform providing characteristic features of proteins.[10] Hence, they are often referred to as "artificial proteins"[11] and their applications in biomedicine range from drug delivery of serum albumin mimicking polyphenylene dendrimers[12] to multivalent dendrimers as antiviral drugs[13] and gene delivery agents[14]. For example, it has been demonstrated that dendrons bind to a virus capsid via supramolecular interactions leading to an electrostatically driven self-assembly into dendron-virus complexes. These complexes could be disassembled by an optical trigger to release the virus.[15]

Amphiphilic polyphenylene dendrimers (PPDs) are macromolecules with given surface patterns consisting of e.g. alternating sulfonic acid and *n*-propyl groups.[16] These dendrimers are internalized into cells while showing low toxicity both *in vitro* and *in vivo* and they possess the ability to transport lipophilic drugs within their unpolar inner cavities.[12] PPDs are unique due to the rigidity of their sterically demanding and space filling pentaphenyl-benzene scaffold and therefore provide persistent three-dimensional structures.[17] This class of dendrimer has the advantage that surface patterns can be exactly positioned since no backfolding of single dendritic arms (dendrons) can occur.[18] Furthermore, we have shown previously that out of a set of amphiphilic PPDs, only one type of PPDs with high density of amphiphilic surface patterns was able to bind to adenovirus 5 (Ad5).[19] Less branched amphiphilic PPDs showed a significantly lower binding to Ad5 and a negatively charged PPD-surface did not lead to any binding. These findings indicated that the dense amphiphilic surface motif is required for Ad5 binding.[19]

Adenovirus (Ad) is a non-enveloped double stranded DNA virus with an icosahedral capsid infecting respiratory epithelial cells.[20] Ads are the most common vectors in gene therapy due to their significant advantages such as genetic stability, well-characterized biology, and high transduction efficiency in cells.[20a, 21] They enter cells by specific interaction with the coxsackie-adenovirus receptor (CAR) and integrins limiting applications to such cell types.[22] Moreover, the three major capsid proteins — hexon protein, penton base, and fiber — bind to antibodies, which lead to immunogenic responses or neutralization, which needs to

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be reduced for *in vivo* applications.[20a] One strategy focuses on shielding the Ad surface from antibody binding by covalent attachment of polymers like polyethylene glycol, which could also lead to reduced transduction efficacies.[23]

We have discovered recently that the formation of an amphiphilic PPD corona promotes cellular internalization into CAR-negative cells that cannot be intrinsically targeted by Ad5.[19] In human blood serum, neutralization by antibodies and binding of coagulation factor X (FX), the primary transport mechanism of Ad5 to the liver, have been altered significantly after PPD adsorption. We could show that there are no electrostatic interactions between the positively charged fibers of Ad5 and negatively charged sulfonic acids of the PPD. In addition, FX could not bind to Ad5 when shielded with the dendrimer indicating that the PPD blocks the binding site for FX. As the amphiphilic PPDs bind to the virus capsid proteins, they also impart a novel surface pattern onto Ad5 controlling their various interactions with other blood serum proteins.[19] In consequence, reduced gene transduction in liver tissue and an enhanced transduction in heart tissue was observed *in vivo*.[19] Thus, amphiphilic PPDs provide a novel platform for virus redirection into different cells and tissues due to their ability to coat and protect Ad5 from FX binding, which influences cellular uptake and biodistribution of Ad5 *in vivo*. Enhanced structural variability of the dendrimer scaffold is a prerequisite to further advance the applicability of amphiphilic PPDs in biomedicine. Therefore, a dendritic structure is required that enables the incorporation of additional functionalities (Fig. 1).

Consequently, we combine the defined amphiphilic pattern of PPD3 for biorecognition with a novel synthetic handle enabling post-modifications (Fig. 1). Since PPDs are symmetric macromolecules, the incorporation of an additional feature is challenging while retaining the desired amphiphilic surface structures. Therefore, we desymmetrized the PPD3 structure and synthesized the first amphiphilic polyphenylene dendron, i.e. a dendrimer branch corresponding to one quarter of the entire PPD3 that should combine Ad5 binding features with the potential for post-modifications. We demonstrate a novel multistep synthesis of an amphiphilic polyphenylene dendron with a propargyl-modified triethylene glycol linker at the core. By applying this linker, high water solubility as well as the possibility to introduce functional units like a fluorophore for imaging or a bio-orthogonal group via copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) are envisioned. Additionally, upon complexation with Ad5, the alkyne group can act as a functional handle for *in situ* CuAAC to serve as a versatile platform for introducing chemical modifications on the viral surface. Furthermore, we analyzed the binding of blood proteins in order to understand the influence of amphiphilic dendron-coated surfaces in the blood stream.

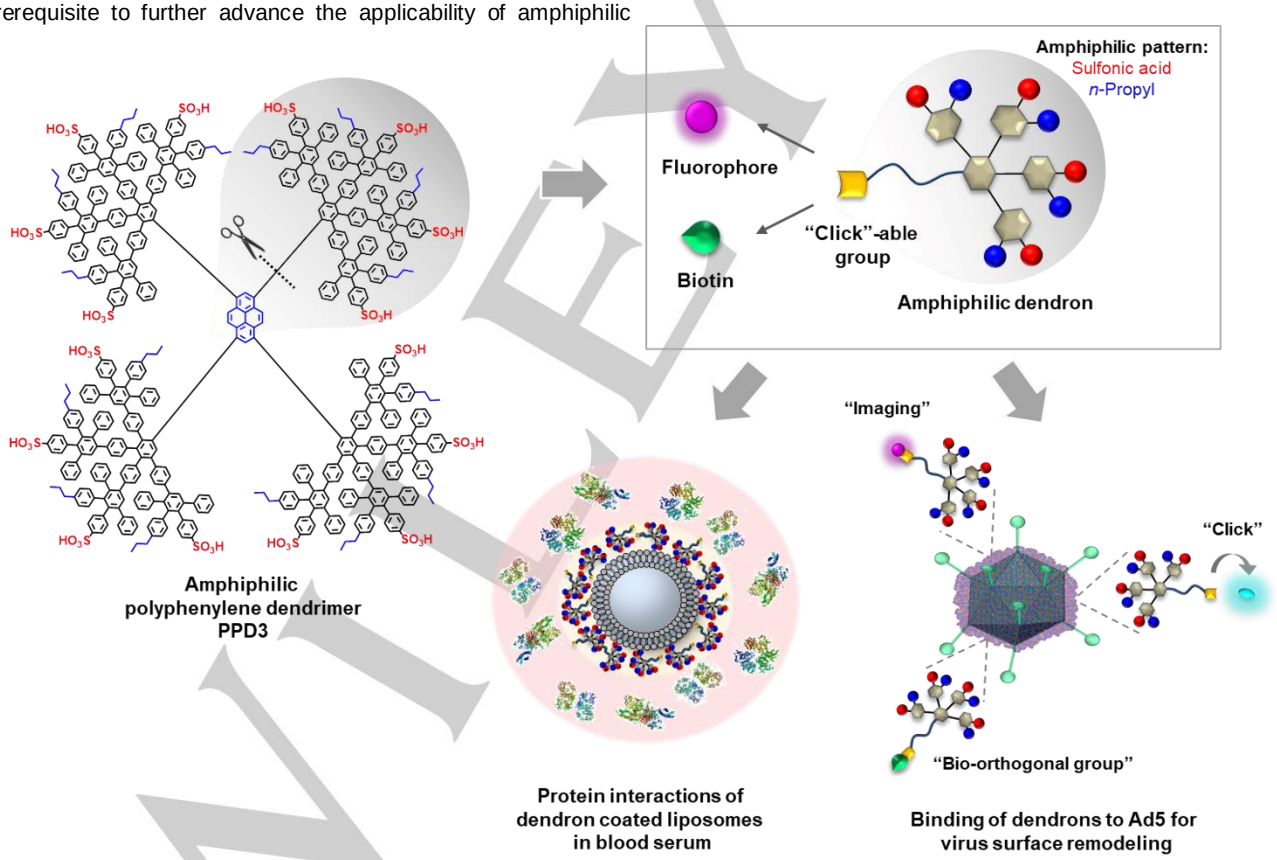

Figure 1. Structural design of an amphiphilic polyphenylene dendron by desymmetrization of amphiphilic dendrimer PPD3. By the employment of a "click"-able ethynyl group, the introduction of a second function by post-modification was achieved. The amphiphilic pattern of these dendron-conjugates interacts with biological structures like proteins which was verified by interactions of dendron-coated liposomes with blood serum proteins (protein structures: PDB-files 4NHH[24], 1FZC[25], 5Z0B[26]) as well as binding to Ad5 for re-direction into CAR-negative cells.

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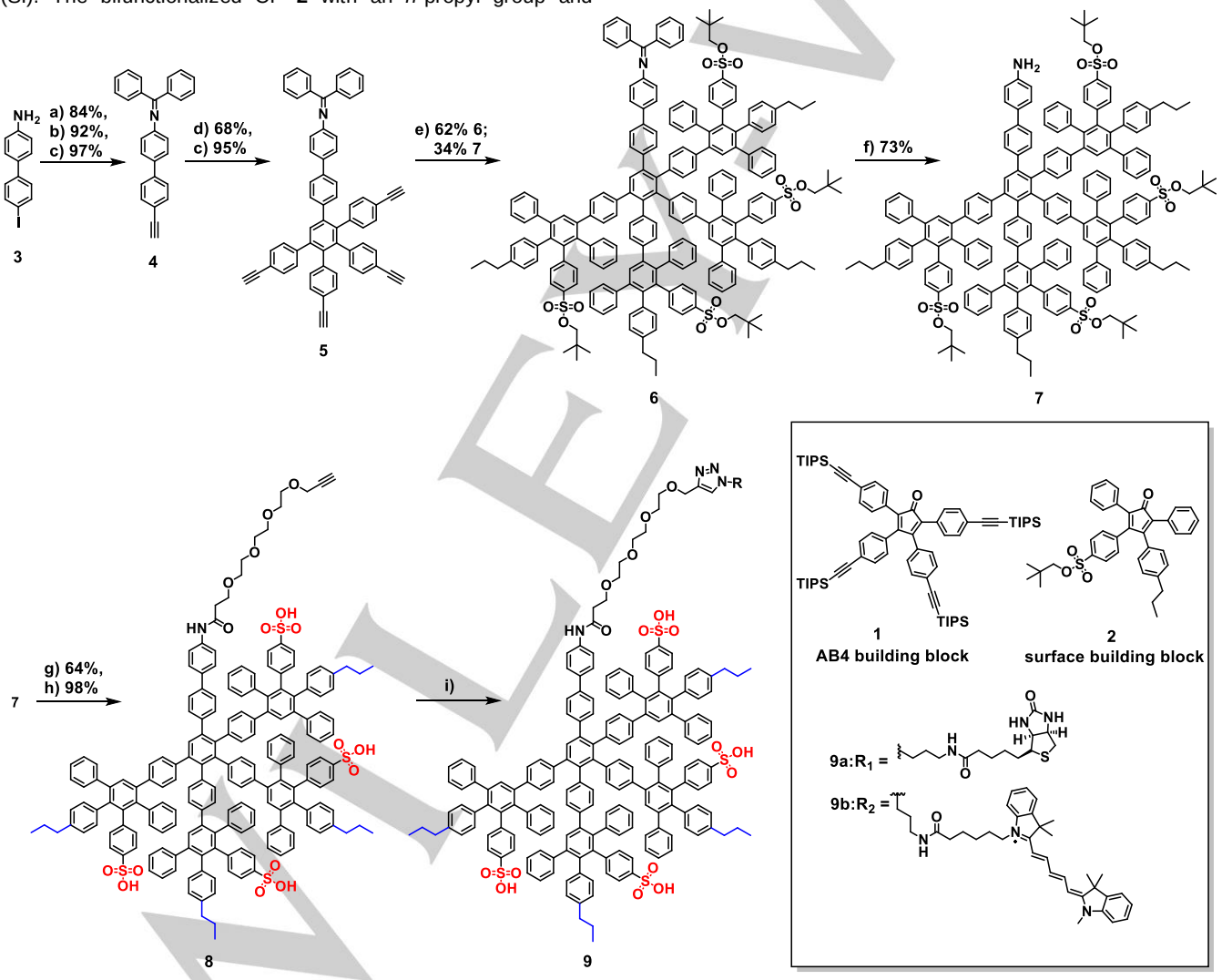
Results and Discussion

The divergent synthesis of a second-generation amphiphilic dendron (Scheme 1) is based on iterative [4+2] Diels–Alder cycloadditions of an ethynylated core with tetraphenylcyclopentadienones (CPs). The CP either determines the branching or serves as an end-capping unit introducing the surface patterns.[27] As previously reported, a high density of amphiphilic surface groups resulting from a highly branched dendrimer scaffold leads to Ad5 binding.[19] In order to integrate this dense surface pattern in a dendron scaffold, we synthesized a dendritic branch with similar amphiphilic surface groups, representing one quarter of the entire dendrimer. Therefore, the AB4 building block **1** with four branching points was used, which was synthesized based on modified protocols from Morgenroth *et al*.[28] The synthesis scheme of the AB4 building block **1** (SI, Fig. S1A) as well as all reaction conditions are summarized in the Supporting Information (SI). The bifunctionalized CP **2** with an *n*-propyl group and neopentyl-protected sulfonic acid group was applied as end-capping unit and was synthesized based on modified protocols from Stangenberg *et al*.[16] The syntheses (Fig. S1B) and reaction conditions are summarized in the SI.

In this study, neopentyl-protected amphiphilic polyphenylene dendron **7** was synthesized and post-modified with a propargyl-TEG-linker followed by attachment of D-biotin or Cyanine 5 (Cy5)-moiety *via* CuAAC ("Click" reaction) (Scheme 1). Cy5 was introduced for cellular uptake experiments and co-visualization of dendron uptake and Ad5 gene delivery. D-Biotin was attached as an example of a bio-orthogonal group to study the influence of a functional group on Ad5 binding.

The detailed structure of the dendron core is crucial since its accessibility as well as peripheral functionality after dendron growth are required. Dendron **7** was synthesized from a bifunctional biphenyl dendron core **3**, which allowed minimizing steric hindrance at the post-modification step. The iodo group of



Scheme 1. Synthesis of amphiphilic polyphenylene dendron conjugates. a) Benzophenone, toluene, molecular sieve 4Å, reflux, 15 h; b) TIPS-acetylene CuI, $Pd(Ph_3P)_2Cl_2$, THF/ NEt_3 (5:1), RT, 15 h; c) TBAF, THF, 0 °C, 0.5 h; d) AB4 building block, *o*-xylene, 160 °C, 24 h; c) TBAF, THF, 0 °C, 0.5 h; e) Surface building block, *o*-xylene, 145 °C, 48 h; f) HCl, THF, 20 min; g) propargyl-TEG-COOH, EDC·HCl, DMAP, DMF, RT, 24 h; h) DMF, 180 °C, 36 h; i) R–N_3, $CuSO_4$, sodium ascorbate, TBTA, DMF/H_2O, RT, 24 h.

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dendron core **3** enabled the coupling with an ethynyl group that is required for dendron growth while the aniline group offered the possibility for post-modification at the focal point after dendron synthesis. In a condensation reaction, the aniline group was protected with benzophenone resulting in an imine to prevent side reactions of the amine group during the harsh conditions of PPD synthesis. In the next step, the tri*iso*propylsilyl (TIPS) protected ethynyl group was introduced by Sonogashira–Hagihara coupling followed by removal of the silyl groups to afford dendron core **4** in good yields (75% over three steps). Further dendron growth was conducted by utilizing branching unit **1** (AB4[28]) in a [4+2] Diels–Alder cycloaddition under standard conditions. Since dendron core **4** only features one dienophile (ethynyl group), the reaction time was reduced from 48 h[12] to 24 h. After deprotection of the ethynyl groups, the first generation dendron **5** was obtained in 65% yield over two steps. Subsequently, the next [4+2] Diels–Alder reaction with amphiphilic end capping unit **2** was performed for 48 h at reduced temperatures of 145 °C to avoid deprotection of the sulfonic acid groups.[16] Under these conditions, the imine protective group was partially cleaved so that imine-dendron **6** (62%) as well as amine-dendron **7** (34%) were isolated by column chromatography. The remaining imine protective group of dendron **6** was removed by acidic treatment to afford amine-dendron **7**. After ligation of **7** to a triethylene glycol (TEG) derivative (propargyl-TEG-linker) by amide coupling, the sulfonic acid groups were deprotected thermally to obtain propargyl-TEG-dendron **8**. The removal of the neopentyl group requires high temperatures so that the heat-sensitive bioactive groups were attached after deprotecting the sulfonic acid groups. D-Biotin and Cy5 derivatives were ligated by ligand-accelerated CuAAC, applying tris((1-benzyl-4-triazolyl)methyl)amine (TBTA) as ligand. The crude products were purified by gel permeation chromato-

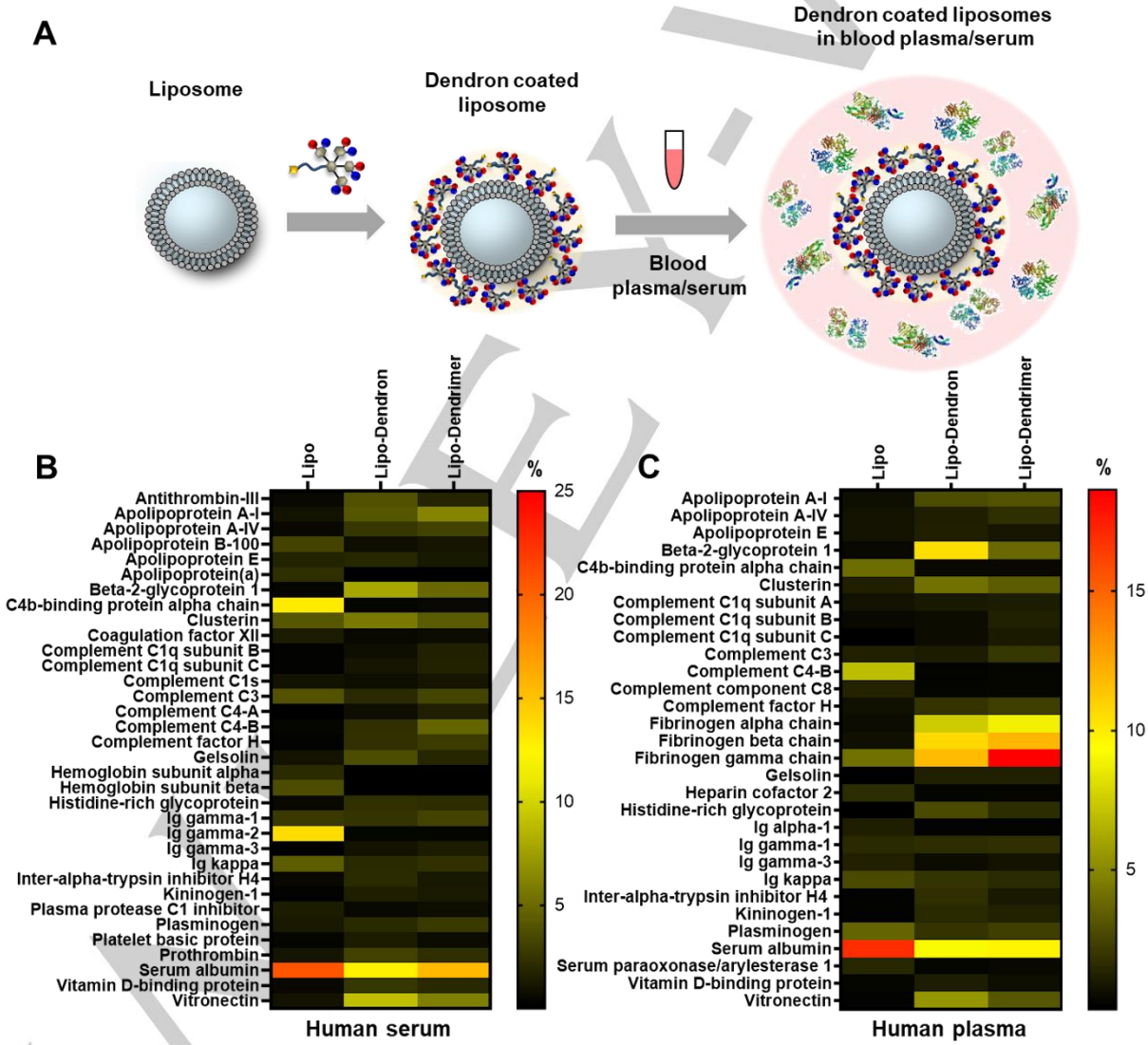

Figure 2. Dendron-coated liposomes form a protein corona in blood serum/plasma. (**A**) Coating of liposomes with dendron **8** and incubation with blood serum or blood plasma leading to protein corona (protein structures: PDB-files 4NHH[24], 1FZC[25], 5Z0B[26]). Heat map of adsorbed proteins to dendron **8** and dendrimer PPD3-coated liposomes in (**B**) blood serum and (**C**) blood plasma. The amount of each protein is given in % based on all identified corona proteins. A list of all identified proteins is provided in the SI (Fig. S34-37).

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graphy (GPC) in dimethylformamide to remove CuAAC reagents and unreacted starting materials to afford biotinylated dendron **9a** and Cy5-dendron **9b**. A detailed reaction scheme is provided in the Supporting Information (SI) (Fig. S2).

Due to the asymmetry of surface building block **2,** second generation dendrons were obtained as constitutional isomers, as reported previously.[16, 29] The constitutional isomers were confirmed by ^{1}H NMR spectroscopy, where they are most notably visible in the spectra of the neopentyl-protected dendrons (SI, Fig. S5). NMR spectroscopy and MALDI-TOF spectrometry demonstrate the successful synthesis of propargyl-modified dendron **8** (SI Fig. S6-7 and S13) and post-modification by CuAAC to achieve biotinylated dendron **9a** and Cy5 labelled dendron **9b** (SI Fig. S8-S12 and S14-S15). The signals in the ^{1}H NMR spectrum can be assigned to propargyl-modified dendron **8**, biotinylated dendron **9a** and Cy5 labelled dendron **9b**. The detailed synthesis description as well as characterization of dendron intermediates and final products are summarized in the SI.

It is well known that Ads are involved in several protein interactions in the blood stream.[30] However, after PPD3 complexation, blood coagulation factor X (FX) could not bind to Ad5.[30] In order to shed light on the potential interaction partners of amphiphilic dendrons in the blood stream, we analyzed proteins binding to surfaces coated with **8**. The virus capsid was simplified by applying nanocarriers as already validated model systems with less complexity.[31] Thus, liposomes[31a] prepared from 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), L-*α*-phosphatidylcholine (egg PC) and cholesterol (Chol) (PC:DOPE:Chol = 1:1:1, Ø = 242 ± 6 nm) as well as polystyrene particles (PS-NH_2, Ø= 98 ± 10 nm)[31b] with comparable sizes to Ad5 were coated with the amphiphilic dendrimer PPD3 as well as dendron **8** (for details see SI). Then, the protein adsorption in blood plasma as well as in blood serum was analyzed. As mentioned above, changes of surface charges as well as polymer coatings can influence the protein corona of nanoparticles.[4b] As previously reported, amphiphilic patterned PPDs seem to bind to a lipid monolayer via electrostatic interactions between negatively charged sulfonic acid groups and positively charged headgroups of the lipid (SI, Fig. S22).[32] Here, the dendron/dendrimer-coated liposomes and polystyrene (PS) particles were prepared following a standard protocol.[5, 33] Briefly, liposomes or nanoparticles were incubated with excess dendron or PPD3 and purified by centrifugation. The coating was verified by DLS and zeta potential (SI, Table S1). Then, blood serum or plasma was added (Fig. 2A). After centrifugation, washing steps and desorption of corona proteins, the isolated proteins were analyzed qualitatively by SDS-PAGE (SI, Fig. S17-20) and quantitatively by Pierce Assay (Fig. S21) and LC-MS/MS (Fig. 2B, C and SI Fig. S16).

Coating with dendron **8** or PPD3 had a major impact on protein corona formation of liposomes (Fig. 2) and PS nanoparticles (SI, Fig. S16). For unfunctionalized liposomes and PS nanoparticles, we detected high amounts of albumin (~26 ± 3%) and immunoglobulins (e.g. Ig kappa, ~6 ± 1%) in the protein corona after serum incubation (Fig. 2B). However, after coverage with dendron **8** (lipo-dendron) the amount of Ig kappa is significantly lower (~2 ± 0.2%). Immunoglobulins belong to the protein class of opsonins and can mediate the interaction with phagocytic cells[34]. The adsorption of IgG can dramatically reduce the blood circulation time and hereby, also reduce the interaction with targeted cells.[35] As reported in the literature, the protein source additionally shapes the protein corona formation.[36] This is in line with our findings as we have observed differences in the protein corona composition after serum and plasma incubation (Fig. 2B *versus* C).

For serum preparation, blood was clotted and then centrifuged to remove the clot. The resulting supernatant no longer contained all proteins. Due to this preparation, fibrinogen and other clotting factors were removed. In contrast, for plasma preparation, an anticoagulant was added to prevent blood clotting and therefore plasma contained all blood proteins including the clotting factors. For dendron **8** and PPD3-covered liposomes, the amount of fibrinogen was higher than for the unfunctionalized ones after plasma incubation. Comparable results were obtained for dendron **8** and PPD3-coated PS (Fig. S16-18) indicating that the protein interactions are governed by the dendron **8** and PPD3 coating and not by the underlying base material. In addition, minor differences were observed for the protein corona composition of

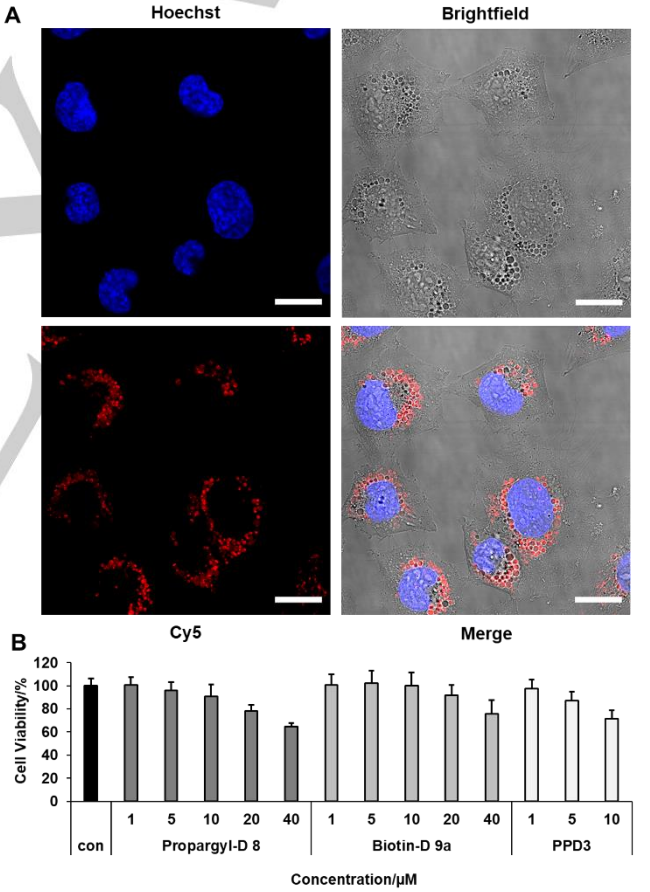

Figure 3. Cellular internalization of amphiphilic dendrons and cell viability on CHO-K1 (**A**) Confocal image of CHO-K1 cells incubated with 1 µM Cy5-Dendron **9b** for 24 h and nucleus staining with Hoechst 33258 (scale = 20 µM) (**B**) Cellular viability of propargyl-dendron **8** and biotin-dendron **9a** compared to PPD3 by applying four times higher dendron concentration to achieve approximate same quantities of surface patterns. Cell viability was tested with CellTiter-Glo®-Assay. Data from three independent experiments with quadruplets (total n = 12) is shown.

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lipo-dendron and lipo-dendrimer after serum and plasma incubation. This effect was most prominent for β-2 glycoprotein 1, also known as Apolipoprotein H (ApoH).[4b] The corona of lipo-dendron was enriched with ApoH (8 ± 0.5% serum, 11 ± 1.5% plasma), whereas lower amounts were detected for lipo-dendrimer (5 ± 0.3% serum, 4 ± 0.3% plasma) or unfunctionalized liposomes (0.5 ± 0.2% serum, 0.3 ± 0.5% plasma). It has been previously shown that due to coating of PS nanoparticles with ApoH, the interaction with human mesenchymal stem cells is favored.[4b] A similar trend was observed for the adsorption of vitronectin, which increased in case of lipo-dendron compared to PPD3-coated or unfunctionalized liposomes. Here, we found that the protein corona of lipo-dendron was enriched with vitronectin (9 ± 0.8% serum, 6 ± 0.5% plasma), whereas lower amounts were adsorbed on lipo-dendrimer (6 ± 0.5% serum, 3 ± 0.2% plasma) or unfunctionalized liposomes (1 ± 0.7% serum, 0.2 ± 0.3% plasma). Interestingly, vitronectin was also detected in the protein corona of DOTAP/DNA lipoplexes and it was demonstrated that vitronectin could mediate a selective uptake of the lipoplexes towards MDA-MB-435S cancer cells, which have a high expression level of the vitronectin $\alpha_v\beta_3$ integrin receptor.[37] Taken together, these results indicate that coating with dendron **8** favors the interactions with specific blood proteins, which eventually also determine the interactions with cells and cellular uptake behavior.

Since amphiphilic dendrimers with alternating sulfonic acid and *n*-propyl groups are internalized into cells and transported *via* vesicles,[12] we tested the cellular uptake of the dendron to assess whether the surface pattern of a desymmetrized dendron is similar to the symmetric PPD3 dendrimer. Chinese Hamster ovary cell line CHO-K1 was selected, since it possesses low CAR-expression rendering it also suitable for gene transduction experiments within this study. The cells were incubated with 1 µM Cy5-dendron **9b** for 24 h, washed with phosphate buffered saline (PBS), and the nucleus was stained with Hoechst 33258. The cellular internalization was followed by confocal laser scanning microscopy. As depicted in Fig. 3A, Cy5-dendron **9b** was internalized into CHO-K1 cells. We observed that dendron **9b** was located in vesicles in a similar way reported for amphiphilic PPDs.[12] The comparison with the blank control is provided in the SI (Fig. S23).

Cell compatibility of dendron conjugates **8** and **9a** was compared to PPD3 in CHO-K1 cells by a cell viability assay. Cells were treated with 1–40 µM dendron conjugates **8** and **9a** as well as 1–10 µM PPD3 for 24 h. We used four equivalents of dendron conjugates compared to PPD3 to adjust the numbers of surface patterns to approximately similar quantities. The cell viability was determined by quantification of ATP levels applying CellTiter-Glo®-Assay. Both dendron conjugates and PPD3 displayed no

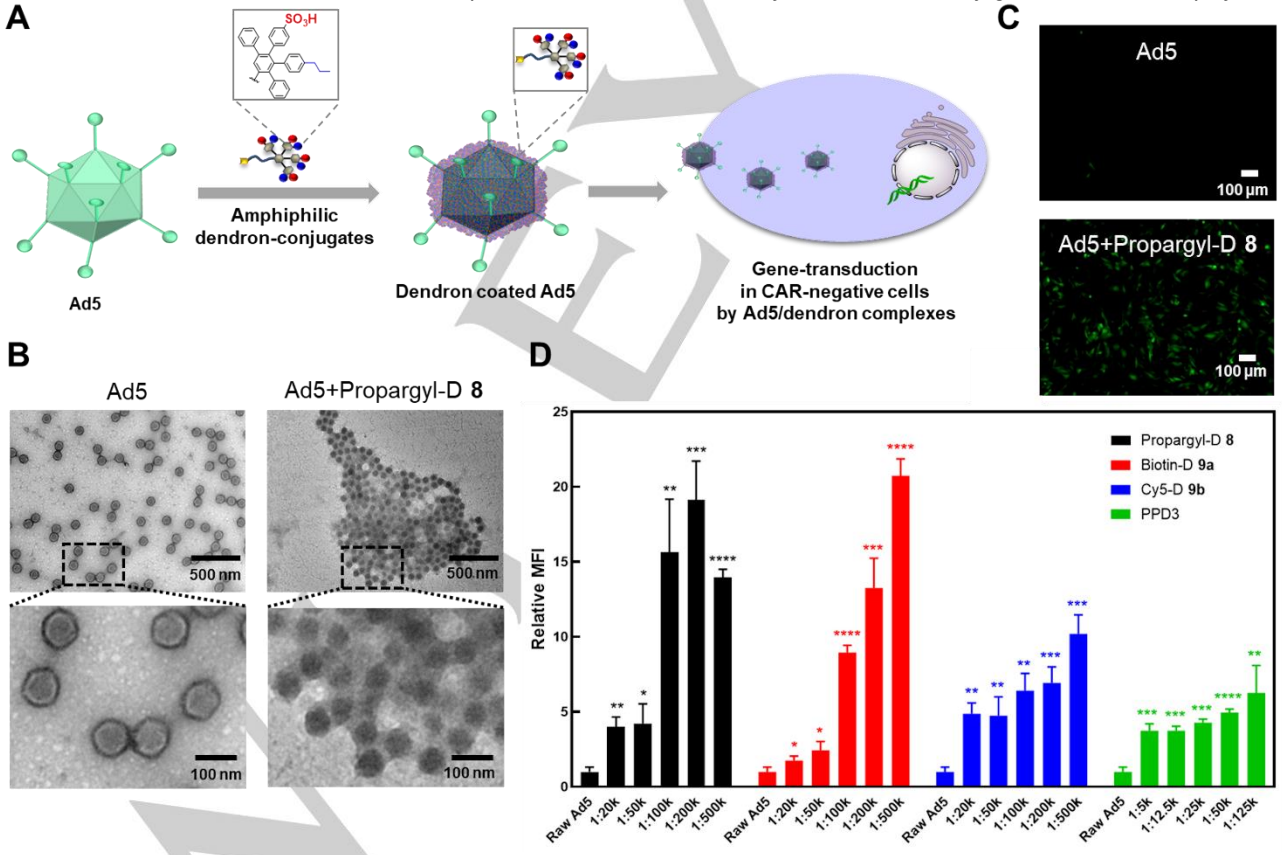

Figure 4. Dendron-Ad5 complex formation leads to EGFP-transduction in CAR-negative cells. (**A**) Coating of Ad5 by dendrons and the potential for cellular uptake into CAR-negative cells resulting in gene transduction; (**B**) TEM images show binding of dendrons to Ad5 (Ad5: Dendron = 1: 100k); (**C**) Fluorescent microscopy image of EGFP-transduction in CAR-negative CHO-K1 cells (Ad5: Dendron = 1: 500k); (**D**) Flow cytometry of CHO-K1 cells incubated with Ad5-dendron complexes with ratios of Ad5: Dendron = 1:20k–1:500k and Ad5: PPD3 = 1:5k–1:125k respectively (n = 3, * represents p-value <0.05, ** represents p-value <0.01, *** represents p-value <0.001, **** represents p-value <0.0001).

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significant cytotoxicity up to 20 µM and 5 µM, respectively.

Next, we studied the performance of the dendron conjugates in Ad5-assisted gene transduction (Fig. 4A). The formation of a dendron corona was studied by transmission electron microscopy (TEM), dynamic light scattering (DLS) and zeta potential, and we performed a functional assay to assess its capability to transport Ad5 into CAR-negative cells monitored by fluorescence microscopy. The influence of surface patterns on gene transduction was compared with PPD3 using flow cytometry. Consequently, dendron **8**, **9a** and **9b** as well as PPD3 were mixed with Ad5 at the ratios of 1:20k to 1:500k (Ad5:dendron) and 1:5k to 1:125k (Ad5:PPD3), respectively, in PBS and complex formation as well as gene transduction of Ad5 in low CAR expressing cell line CHO-K1 were tested. For all experiments, four equivalents of dendron related to PPD3 were used to compare their properties while maintaining approximately the same amount of surface patterns. Here we used an eGFP-expressing Ad5 and analyzed its interaction with the dendrons. TEM analysis of vector particles incubated with the dendron for 40 min at a molar ratio of 1:100K was performed. Results clearly confirmed that dendrons **8**, **9a** and **9b** bound to and formed complexes with Ad5 (Fig. 4B, Fig. S24). To further analyze this interaction, DLS measurements were conducted. Our data shows that dendrons alone self-assemble in solution due to their intrinsic amphiphilicity (Table S5 and Fig. S26B). Nevertheless, in presence of Ad5, all dendrons demonstrably bound to the vector particles, which was clearly indicated by an increase of the vector particle size (Table S3, Table S4, Fig. S25 and Fig. S26A). To further confirm this interaction, we measured the surface charge of dendrons alone, Ad5 alone and dendron-coated Ad5 by zeta potential analysis. Dendron-coated vector particles showed a ratio-dependent increase of the negative surface charge compared to dendrons alone or Ad5 particles alone (Table S3, Table S4, Table S5). By this means, we ancillary confirmed the binding of Ad5 and analyzed dendrons. Additionally, we could show that at ratios beyond 1:1000K for Ad5/propargyl-dendron **8** and 1:200K for Ad5/biotin-dendron **9a**, saturation of Ad5 particles is reached, which results in free, unbound dendron molecules in solution (Table S4, Fig S26).

By biolayer interferometry (BLI) we previously studied the binding strength of an amphiphilic dendrimer binding motif to Ad5 [19]. This method required the immobilization of the dendrimer on a streptavidin-coated surface (SI, Fig. S32). Thus, we used biotin-dendron **9a** and an equilibrium dissociation constant value of K_D = 1.27 pM was determined (SI, Fig. S33 and Table S6). Since the dendron binds to Ad5 in a multivalent way, this value does not present a single binding event of the dendron to Ad5, but point towards strong interactions between Ad5 and dendron. [19] We observed green fluorescence for all cells treated with Ad5/dendron complexes by fluorescence microscopy whereas Ad5 alone led to low gene transduction (Fig. 4C and Fig. S27). Therefore, all dendron conjugates as well as the control dendrimer PPD3 bound to Ad5, and they transported Ad5 into cells via a CAR-independent pathway leading to EGFP-expression. We quantified the gene transduction of Ad5 in low CAR cell line CHO-K1 via flow cytometry by measuring the fluorescence intensity of EGFP. We found that gene transduction was enhanced when increasing the molar ratio of dendron to Ad5 (Fig.4D and Fig. S28). A double-positive signal of EGFP and Cy5 was observed when infecting the cell with Cy5-dendron **9b**/Ad5 (Fig. S29). We found a significantly higher gene transduction of Ad5 when coated with four molar equivalents of propargyl-dendron **8** and biotin-dendron **9a** compared to coating with one molar equivalent of sterically more demanding PPD3. Cy5-labelled dendron **9b** showed lower gene transduction efficiency than dendrons **8** and **9a**, which could be due to the attachment of the fluorophore that might also influence Ad5 binding and its cellular uptake. These results indicate that four molar equivalents of dendrons **8** and **9a** increase Ad5 transport into these CAR-negative cells compared to one molar equivalent of PPD3. As reported for amphiphilic PPDs[19], distinct amphiphilic surface patterns of the dendrons are crucial for biorecognition of Ad5. Even though the amphiphilic dendrons only represent one quarter of the full dendrimer PPD3, they retain both Ad5 binding capacity and gene transduction into CAR-negative cells, while providing a second functionality for post modifications (Fig. 4A, Fig. 5A).

In the next step, we studied whether the functionality at the focal point of the dendron was still accessible after complexation with Ad5. Consequently, we covalently modified the dendrons *in situ*

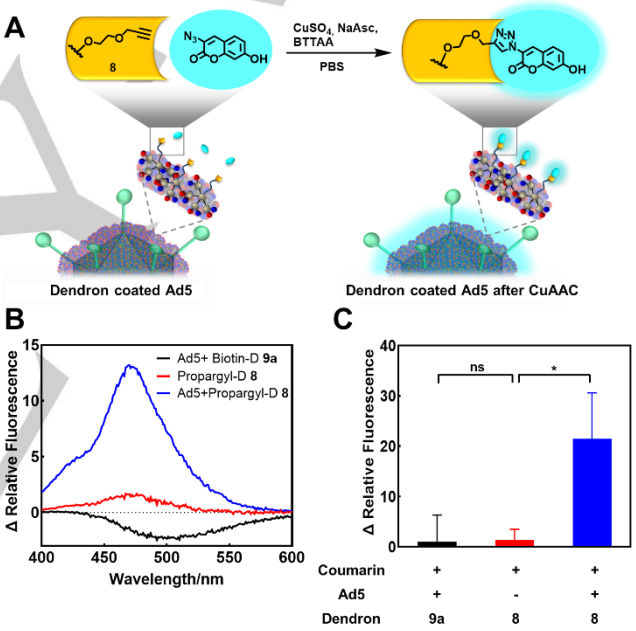

Figure 5. CuAAC on dendrons bound to Ad5. (**A**) CuAAC of propargyl-dendron **8** with 3-azido-7-hydroxycoumarin on the Ad5 surface leads to fluorescence of the dendron-fluorophore conjugate (λ_{abs}= 404 nm, λ_{em}= 477 nm after click reaction). (**B**) Fluorescence spectra of dendron conjugates after treatment with CuAAC reagents. Ad5 was mixed with propargyl-dendron **8** or biotin-dendron **9a** (negative control). After incubation for 40 min, unbound dendron was removed by ultrafiltration. Propargyl-dendron **8** alone was treated under same conditions. Then, CuAAC reagents were added and fluorescence spectra were recorded after incubation for 1 h. 3-azido-7-hydroxycoumarin was subtracted as background. (**C**) The change in relative fluorescence of Ad5+Biotin-D **9a** group, Propargyl-D **8** group (with ultrafiltration) and Ad5+Biotin-D **9a** group at 477nm (emission) after treatment are described in (**B**). 3-azido-7-hydroxycoumarin was subtracted as background and the change in relative fluorescence of Ad5/propargyl-dendron after CuAAC (blue column) is relative to the Ad5+Biotin-D **9a** group (black column) that serves as negative control (n=3, * represents p<0.05, ns means not significant).

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by CuAAC on the Ad5 surface. The reaction was performed using propargyl-dendron **8** with 3-azido-7-hydroxycoumarin that is known to become highly fluorescent when forming a 1,2,3-triazole product (Fig. 5A).[38] As negative control, we used biotin-dendron **9a** attached to Ad5 that cannot undergo a CuAAC. We first investigated if the CuAAC proceeded in the micromolar range as well as the necessity of a ligand like TBTA to stabilize the copper(I) species[39]. After 1 h of incubation of propargyl-dendron **8** and 3-azido-7-hydroxycoumarin with click reagents at a concentration of 10 µM dendron, we only found high fluorescence for the TBTA treated sample. This indicates the importance of adding a ligand like TBTA to the reaction mixture (Fig. S30). Additionally, it is reported that this class of ligands protect biomolecules from reactive oxygen species (ROS) during the ligation.[40] For CuAAC on the Ad5 surface, we used the water-soluble (4-{[bis-(1-*tert*-butyl-1H-[1,2,3]triazol-4-ylmethyl)-amino]-methyl}-[1,2,3]triazol-1-yl)-acetic acid (BTTAA), which is even more efficient in aqueous solutions than TBTA.[41] Briefly, propargyl-dendron **8** was incubated with Ad5 for 1 h, and unbound dendrons were removed by ultrafiltration. Subsequently, the CuAAC reagents were added. The negative control, biotin-dendron **9a** coated Ad5, was treated under the same conditions. In addition, ultrafiltered propargyl-dendron **8** without Ad5 was prepared and click reagents were added to the supernatant to demonstrate that free dendrons could be removed by ultrafiltration. After incubation for 1 h, fluorescence spectra were recorded (Fig. 5B). Since free 3-azido-7-hydroxycoumarin is slightly fluorescent, we subtracted it as background. We observed a 21-fold increase in relative fluorescence intensity at 477 nm compared to the biotin-dendron coated Ad5 that served as negative control (Fig. 5C). These results indicate successful CuAAC at the focal points of the propargyl-dendron **8** after formation of Ad5/dendron complexes. Therefore, we demonstrate that the functionalities of the dendron core are still accessible for post-modifications even after complexation with Ad5. This model reaction proves that our structural dendron concept represents a promising tool for future applications in terms of *in situ* attachment of cell targeting groups or drug molecules.

Conclusion

Ad5 is a common vector in gene therapy but its clinical usage has limitations due to mistargeting of plasma protein-coated Ad5 and acute toxicity. We present the synthesis of amphiphilic polyphenylene dendrons that bind to the surface of Ad5 by their polar and unpolar surface groups and facilitate transport of the Ad5/dendron complexes into CAR-negative cells. In this way, these dendrons maintained the crucial biorecognition features of the full dendrimer PPD3. As the dendrons form a new, outer layer at the Ad5 surface, their interaction with blood plasma proteins might be crucial for future applications. Dendron-coated liposomes were found to interact with specific proteins of the blood serum and plasma proteins such as vitronectin and ApoH, which could promote uptake into cancer and mesenchymal stem cells. In contrast to dendrimer PPD3, the dendrons provide an additional functionality for post-modifications such as a fluorophore for imaging and D-biotin as a bio-orthogonal group. By desymmetrization of the dendrimer structure, we opened up a new platform for introducing bioactive groups to the Ad5 surface without the necessity to covalently modify the virus particles. These reactive groups were well accessible at the Ad5 surface as shown by CuAAC reactions. Moreover, this new concept of forming a supramolecular dendron corona at virus surfaces opens up exciting opportunities for attaching e.g. cell targeting groups or drug molecules and paves the way to rational control of Ad5 biodistribution to ultimately improve its capacity in virus-assisted gene therapy.

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Keywords: Adenovirus-dendron complex • adenovirus coating • amphiphilic surfaces • protein corona • gene delivery

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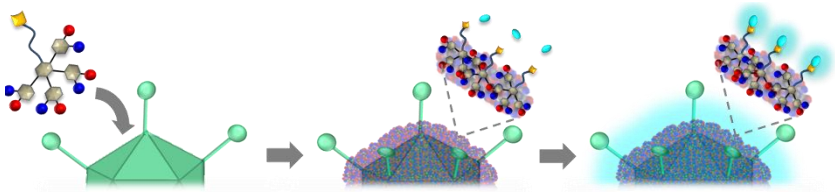

Amphiphilic polyphenylene dendrons were prepared that recognize gene vector adenovirus 5 (Ad5) due to polar and unpolar surface groups that also control its cellular uptake. The new dendron layer at the Ad5 surface provides reactive groups accessible for post-modifications at the virus surface.

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Amphiphilic Polyphenylene Dendron Conjugates for Surface Remodeling of Adenovirus 5