Glycoviruses: Chemical Glycosylation Retargets Adenoviral Gene Transfer

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Gene therapy of infectious, vascular, and multifactorial diseases employs a variety of viruses, which each have specific qualities that make them suitable for their chosen application. Gene therapy provides a means to exploit knowledge generated under the human genome project by the use of gene delivery vectors to supplement the function of missing or mutated genes. Some applications of gene therapy require therapeutic gene delivery to specific diseased cells, such as the cystic fibrotic epithelia for treatment of cystic fibrosis, whereas others accommodate transgene expression within nondiseased cells such as muscle cells or liver hepatocytes in a so-called “cell factory” approach. In both applications, successful delivery of the virus requires precise target-cell specificity, an ability to evade neutralizing antibodies, and increased blood circulation to the target cell or tissue. Control of these properties is one of the major challenges facing viral gene therapy today.

The adenovirus (AV) is a commonly used vector for therapeutic gene therapy. It has an icosahedral structure with 12 protruding fiber proteins, each of which comprise a knob domain that binds through a three-way interaction with the coxsackie adenovirus receptor (CAR) of target cell membranes. CAR binding is currently the major route of infection, although nonspecific integrin-mediated uptake is also known. In both cases, critical lysine residues exposed on
the virus capsid,[9] particularly on the knob domain and fiber protein, are responsible for successful interaction and cellular uptake and gives AV a broad tropism of infection.[10] We reasoned that a more-precisely targeted adenoviral vector might be possible if capsid lysine residues could be modified so that normal infection pathways (Figure 1 a) were disrupted and a new cell-specific infection was induced (Figure 1 b).

For this viral retargeting we chose carbohydrates, which play a critical role in cellular trafficking.[11] Interactions of carbohydrates with cellular receptors are often highly precise,[12] and important, elegant approaches have explored the potential of glycosylated nonviral gene vectors.[13–17] Control of glycosylation also influences protein delivery;[18–21] indeed, we recently showed that carbohydrates are powerful targeting moieties in a novel protein drug-delivery system called LEAPT (lectin-directed enzyme-activated prodrug therapy).[22] However, to the best of our knowledge, artificial viral glycosylation in gene delivery has not been explored and we show here that chemically glycosylated AVs are dramatically retargeted.

Careful control of conditions allowed three different levels of glycosylation—high (H), medium (M), and low (L)—of the approximately 1800 available surface lysines.[23–26] The use of different 2-imino-2-methoxyethyl-1-thioglycosides (IMEs, 1)[27,28] allowed both galactosylation (Gal) and mannosylation (Man) to create six novel glycosylated AV structures: ManH-AV, ManM-AV, ManL-AV, GalH-AV, GalM-AV, and GalL-AV (Figure 2).

Remarkably, adenovirus appears very robust under these conditions of chemical glycosylation, and following purification by means of a Microspin S-400HR column, yields of up to 91%[29] of intact adenovirus were obtained. PicoGreen analysis,[30] size-exclusion HPLC, photon correlation spectroscopy (PCS), and measurements of zeta potentials revealed that viral integrity is maintained in the purified virus after glycosylation and that size-exclusion spin column purification successfully removed degraded particles.[31] HPLC chromatograms[31] for purified, modified, and unmodified samples showed no differences in retention times which is consistent with the undisrupted virus and correlates with results of titrations using PicoGreen. PCS was used to examine the effects of modification of AV particles on their size and aggregation and showed a clear increase in diameter (for example, AV = 120 ± 6 nm, whereas ManH-AV = 204 ± 27 nm). Interestingly, this diameter (∼200 nm) is consistent with the diameter of adenovirus[31] if the diameter is measured from the tip of protruding fiber proteins, which are not usually detected by PCS. Glycosylation of AV fiber therefore appears to significantly enhance detection and particle measurement. Zeta potentiometry revealed similar levels of surface charges for AV and ManH-AV (or GalH-AV) which is consistent with the conversion of surface lysines (pKₐ ∼ 11) into amidines, which are also basic (pKₐ ∼ 15–17).[32]

SDS-PAGE (sodium dodecylsulfate polyacrylamide gel electrophoresis) was used to determine the levels and locations of glycosylation for all six glycoviruses. Heavily glycosylated structures (ManH-AV and GalH-AV) showed significant differences in protein mass for hexon, penton-base, and fiber proteins (Figure 3 a and b). The presence of sugars was confirmed by the cleavage of diols by periodic acid followed by staining with Pro-Emerald stain (Figure 3b).[33] Concanavalin A (ConA) affinity chromatography[34] revealed a high affinity for ManH-AV but not GalH-AV[35] or AV. Indeed, only upon addition of a mannose-rich buffer did the

![Figure 1](https://www.angewandte.org/article-pics/1057_44_1.png)

**Figure 1.** a) Currently accepted mechanism of adenovirus (AV) transfection through coxsackie adenovirus receptor (CAR).[6] Lysine residues on fiber proteins are believed to be highly involved in the protein–protein interaction.[9] 1) Interaction with CAR-expressing cell; 2) receptor-mediated endocytosis; 3) internalization; 4) trafficking to nuclear pore through microtubules; 5) further disassembly and import of viral DNA through interaction of terminal protein with host nuclear pore complex. b) Proposed effect of glycosylation of AV with carbohydrates acting as biological switches.

![Figure 2](https://www.angewandte.org/article-pics/1057_44_2.png)

**Figure 2.** Glycosylation of adenovirus (2) with IME reagents (1). (1a = Gal: X = OH, Y = H, R₁= SCH₂C(NH)OMe, R₂= H; 1b = Man: X = H, Y = OH, R₁= H, R₂= SCH₂C(NH)OMe). PBS = phosphate-buffered saline solution.
mannosylated ManH-AV particles elute. SDS-PAGE, which revealed the location of glycosylation, also allowed an estimation of the number of sugars that were attached to each protein (Table 1). For both Man- and Gal-modified structures, a comparison of the theoretical number of exposed lysine moities with the calculated increases in weight and the percentage of glycosylated lysines showed that GalH-AV = 93 ± 3%, GalM-AV = 40 ± 1.5%, and GalL-AV = 7 ± 0.2%. ManH-AV displayed a percentage that is comparable with GalH-AV, whereas ManM-AV and ManL-AV showed slightly lower values compared to the corresponding Gal-modified structures.

As lysine residues that are present on AV fiber proteins are required for effective interaction of AV with CAR and membrane integrins, we considered that increasing the level of glycosylation from low to high would in turn decrease AV transfection ability through CAR. We successfully demonstrated this reduction (Figure 4a) by using a luciferase-expressing AV mutant. ManH-AV and GalH-AV (shown as H in Figure 4a) showed a dramatic reduction with no significant transfection ability above the background signal in A549 cells. With the successful modulation of transfection in A549 cells, we next examined the retargeting of GalH-AV and ManH-AV green fluorescent protein.

Table 1: Predicted levels of glycosylation on major virus capsid proteins.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Number of repeats/virus</th>
<th>Estimated number of lysine residues</th>
<th>Estimated number of sugars/protein (for Gal-AV)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>H</td>
<td>M</td>
</tr>
<tr>
<td>Fiber</td>
<td>24</td>
<td>15</td>
<td>14 5 2</td>
</tr>
<tr>
<td>Penton</td>
<td>12</td>
<td>20</td>
<td>14 3 nd</td>
</tr>
<tr>
<td>Hexon</td>
<td>720</td>
<td>30</td>
<td>28 12</td>
</tr>
<tr>
<td>Total</td>
<td>22 200</td>
<td>20 700 [a] ± 700</td>
<td>8000 [b] ± 300</td>
</tr>
</tbody>
</table>

[a] Not detected. [b] Based on calculated increases in weight. See main text for details.
(GFP)-expressing reporter virus in three types of cell that are found in the human blood system, namely, lymphocytes, macrophages, and endothelial cells (Figure 4b). The CAR mechanism in Galβ1,3Manβ1,4Galβ1,3Galβ1,4GlcNAc2-AV and Manβ1,4Galβ1,3Galβ1,4GlcNAc2-AV was removed by modification. Endothelial cells[^30] were not transfected by either, whereas AV remained active. Lymphocytes, which are not transfected by AV[^30] were used as a negative control. A small amount of AV transfection was seen in the lymphocyte sample owing to the presence of contaminating monocytes[^40,41]. Finally, transfection of macrophages by means of retargeting through the mannose receptor[^18,20] was examined by using Manβ1,4Galβ1,3Galβ1,4GlcNAc2-AV. Excitingly, significant transduction was not observed by AV[^39], were used as a negative control. AV also showed transfection of macrophages possibly through integrin binding[^41,42]. Galβ1,3Manβ1,4Galβ1,3Galβ1,4GlcNAc2-AV showed no transduction of macrophages which suggests that Manβ1,4Galβ1,3Galβ1,4GlcNAc2-AV transduction is a specific, sugar-mediated uptake.[^43]

For the first time, by the use of controlled and precise glycosylation chemistry we have successfully modified the fragile structure of AV with carbohydrates and modulated its function. AV transfection can now be adapted to carbohydrate–protein receptor interactions as putative lysine glycosylation “switches off” normal receptor pathways and “switches on” specific sugar-mediated pathways; the clear potential in therapy is under investigation.

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[^8]: O. Meier, F. Greber Urs, J. Gene Med. 2004, 6, S152.
[^29]: Yields determined from the total DNA present in the sample by PicoGreen analysis.
[^30]: Adenoviral capsid proteins exist as homodimers or homotrimers that can be disrupted easily by the addition of a denaturant such as sodium dodecylsulfate (SDS) or guanidinium chloride. The level of destruction of the adenovirus was analyzed by the PicoGreen method (see Supporting Information), whereas spin column purification would have removed degraded viral particles. PicoGreen analysis of DNA solution levels is routinely used to indirectly calculate the number of viral particles present in solution and has the advantage of high sensitivity, which is useful when protein concentrations are too low for accurate analysis as is often the case with adenoviral titrations. PicoGreen analysis performed on intact viral particles showed only background fluorescence. By comparison of viral samples after glycosylation with the unglycosylated batch, we are able to determine the effect of glycosylation on the structure of adenoviruses.
[^31]: See Supporting Information.
[^33]: Purchased from Molecular Probes (www.probes.com).
[^34]: Purchased from Galab.
[^35]: The retention time for the galactosylated particles was slightly longer than that for the unmodified particles owing to a nonspecific interaction.
[^36]: See Supporting Information.
[^41]: This method was used to isolate lymphocytes and monocytes in high purity. The authors comment on the inevitability of contamination owing to both types exhibiting high heterogeneity for the method. Lymphocyte stimulation may also occur here.
[^42]: Relative transgene expression levels for Manβ1,4Galβ1,3Galβ1,4GlcNAc2-AV based on RLU (relative light units) are around 40% of the AV level in macrophage but 0% of AV transfection in A549 (A549: AV 1.2 × 10^3; Manβ1,4Galβ1,3Galβ1,4GlcNAc2-AV; macrophage: AV 1.1 × 10^3; Manβ1,4Galβ1,3Galβ1,4GlcNAc2-AV 4.5 × 10^3).
Given the similarly high glycosylation levels of Man<sub>hr</sub>-AV (90 ± 3%) and Gal<sub>hr</sub>-AV (93 ± 3%), we believe that the possibility of low-level normal mode infection being present in Man<sub>hr</sub>-AV but not in Gal<sub>hr</sub>-AV is unlikely. Nonetheless, to discount the possibility of low-level normal mode infection, we confirmed the mannose-dependent nature of Man<sub>hr</sub>-AV transfection through the use of 1% mannose solution, which completely ablated transgene expression to background levels. Transfection by unmodified AV was not affected by 1% mannose solution.