*Glycoviruses*

# 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.[1] 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,[2] whereas others accommodate transgene expression within nondiseased cells such as muscle cells or liver hepatocytes in a so-called "cell factory" approach[3,4] 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.[5]

The adenovirus (AV) is a commonly used vector for therapeutic gene therapy.[6] It has an icosahedral structure with 12 protruding fiber proteins,[7] each of which comprise a knob domain that binds through a three-way interaction with the coxsackie adenovirus receptor (CAR) of target cell membranes.[7,8] CAR binding is currently the major route of infection, although nonspecific integrin-mediated uptake is also known.[8] In both cases, critical lysine residues exposed on

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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).

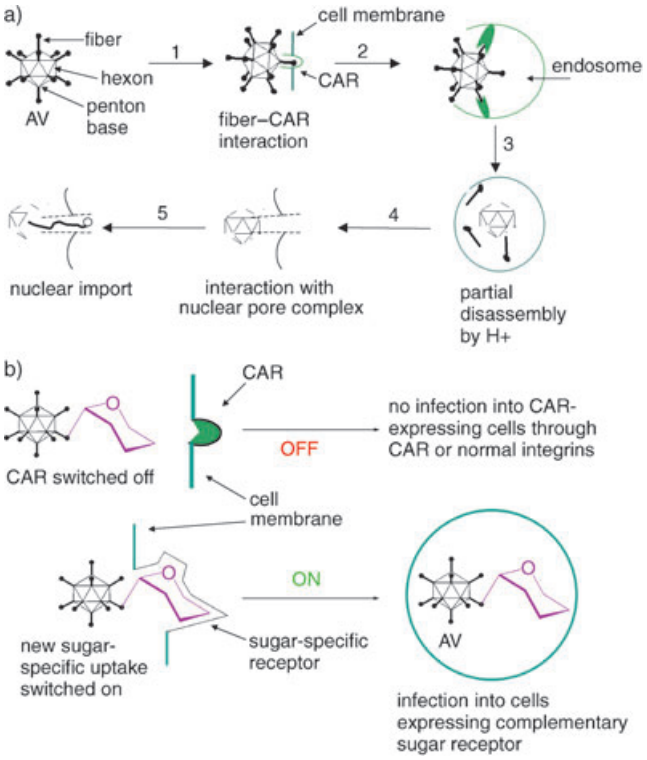


***Figure 1.*** a) Currently accepted mechanism of adenovirus (AV) transfection through coxsackie adenovirus receptor (CAR).[7b] Lysine residues on fiber proteins are believed to be highly involved in the protein–protein interaction.[9] 1) Interaction with CAR-expressing cell; 2) receptor-mediated endocytosis; 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.

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: $Man_H$-AV, $Man_M$-AV, $Man_L$-AV, $Gal_H$-AV, $Gal_M$-AV, and $Gal_L$-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 $Man_H$-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 $Man_H$-AV (or $Gal_H$-AV) which is consistent with the conversion of surface lysines ($pK_a \approx 11$) into amidines, which are also basic ($pK_a \approx 15$–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 ($Man_H$-AV and $Gal_H$-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 3 b).[33] Concanavalin A (ConA) affinity chromatography[34] revealed a high affinity for $Man_H$-AV but not $Gal_H$-AV[35] or AV. Indeed, only upon addition of a mannose-rich buffer did the

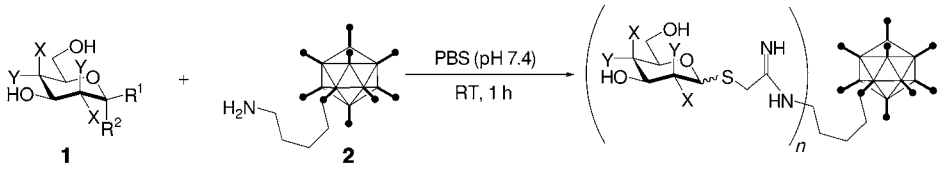


***Figure 2.*** Glycosylation of adenovirus (**2**) with IME reagents (**1**). (**1a** = Gal: X = OH, Y = H, $R^1 = SCH_2C(NH)OMe$, $R^2 = H$; **1b** = Man: X = H, Y = OH, $R^1 = H$, $R^2 = SCH_2C(NH)OMe$). PBS = phosphate-buffered saline solution.

  

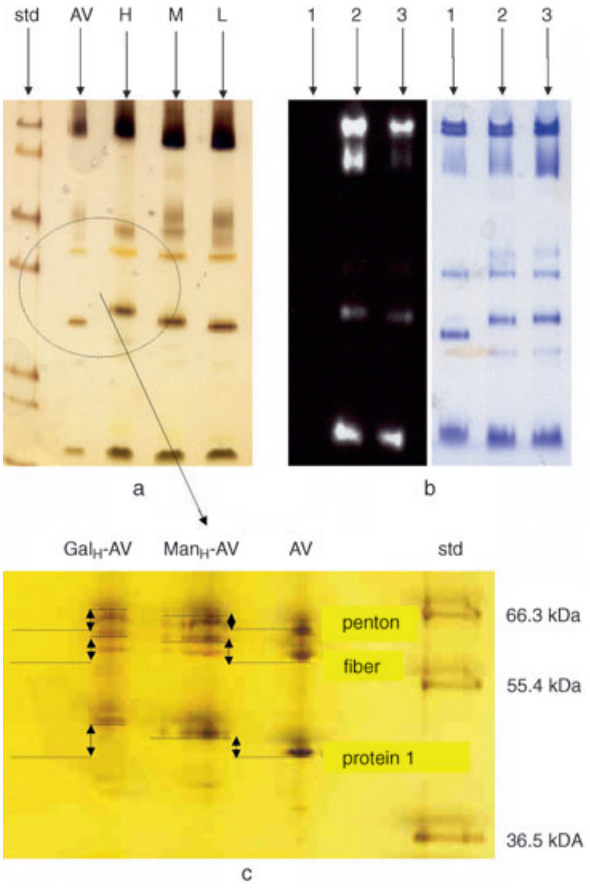


***Figure 3.*** a) Characterization of glycosylation of Man-AV by SDS-PAGE with silver stain (std = standard, AV = adenovirus, H = high, M = medium, L = low). b) Glycoproteins, 1 = AV, 2 = $Gal_H$-AV, 3 = $Man_H$-AV, revealed with Pro-Emerald 488 stain (left) and with coomassie brilliant-blue protein stain (right). c) Expanded view shows more-detailed levels of progressive, tuneable glycosylation. $Man_H$-AV particles were also retained on a ConA affinity column which is consistent with mannosylation.[31]

mannosylated $Man_H$-AV particles elute.[31] 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[23–26] with the calculated increases in weight and the percentage of glycosylated lysines showed that $Gal_H$-AV = 93 ± 3%, $Gal_M$-AV = 40 ± 1.5%, and $Gal_L$-AV = 7 ± 0.2%. $Man_H$-AV displayed a percentage that is comparable with $Gal_H$-AV, whereas $Man_M$-AV and $Man_L$-AV showed slightly lower values compared to the corresponding Gal-modified structures.[36]

As lysine residues that are present on AV fiber proteins are required for effective interaction of AV with CAR and membrane integrins,[9] 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.[37] $Man_H$-AV and $Gal_H$-AV (shown as H in Figure 4a) showed a dramatic reduction with no significant transfection ability above the background signal in A549 cells.

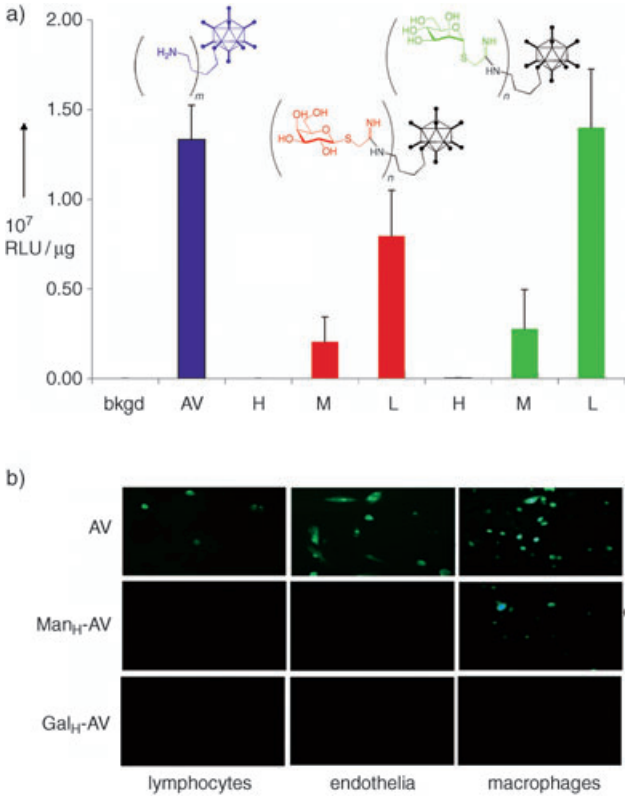


***Figure 4.*** a) Effect of glycosylation in vitro on usual transduction mechanism of adenovirus in coxsackie adenovirus receptor (CAR)-expressing A549 (lung carcinoma) cell lines (RLU = relative light units). Red bars indicate galactosylated AV and green bars indicate mannosylated AV. H, M, and L indicate the high, medium, and low levels, respectively, of glycosylation within sugar modification. The left-hand column indicates the level of background fluorescence (bkgd). The number of naturally occurring lysine residues per virus particle is 22200. b) The retargeting of mannosylated adenovirus ($Man_H$-AV) is selective for macrophages, which express the mannose receptor. Unmodified virus (AV) is used as a positive control and galactosylated virus ($Gal_H$-AV) is used as a negative control.

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

| Protein | Number of repeats/virus | Estimated number of lysine residues | Estimated number of sugars/protein (for Gal-AV) H | M | L |
|---|---|---|---|---|---|
| Fiber | 24 | 15 | 14 | 5 | 2 |
| Penton | 12 | 20 | 14 | 3 | nd[a] |
| Hexon | 720 | 30 | 28 | 12 | 2 |
| Total | | 22200 | 20700[b] ± 700 | 8800[b] ± 300 | 1490[b] ± 40 |

[a] Not detected. [b] Based on calculated increases in weight. See main text for details.

With the successful modulation of transfection in A549 cells observed, we next examined the retargeting of $Gal_H$-AV and $Man_H$-AV green fluorescent protein[37]

  

(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 $Gal_H$-AV and $Man_H$-AV was removed by modification. Endothelial cells[38] were not transfected by either, whereas AV remained active. Lymphocytes, which are not transfected by AV,[39] 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 $Man_H$-AV. Excitingly, significant transduction was observed with $Man_H$-AV.[42] AV also showed transfection of macrophages possibly through integrin binding.[8] $Gal_H$-AV showed no transduction of macrophages which suggests that $Man_H$-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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[42] Relative transgene expression levels for $Man_H$-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 $1.2 \times 10^7$, $Man_H$-AV 0; macrophage: AV $1.1 \times 10^9$, $Man_H$-AV $4.5 \times 10^8$).

  

[43] Given the similarly high glycosylation levels of $Man_H$-AV (90 ± 3%) and $Gal_H$-AV (93 ± 3%), we believe that the possibility of low-level normal mode infection being present in $Man_H$-AV but not in $Gal_H$-AV is unlikely. Nonetheless, to discount the possibility of low-level normal mode infection, we confirmed the mannose-dependent nature of $Man_H$-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.

  