BIOTECHNOLOGY

Determination of Particle Heterogeneity and Stability of Recombinant Adenovirus by Analytical Ultracentrifugation in CsCl Gradients

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ABSTRACT: Recombinant adenoviruses (rAd), widely used as vectors for gene therapy, are generally purified by column chromatography and frequently contain empty capsids and other aberrant forms of virus particles. To determine particle heterogeneity we utilized analytical ultracentrifugation (AUC) in CsCl density gradients. Preparations of three different rAd vectors were assessed. AUC was able to resolve multiple density forms including two empty capsid types in various virus preparations. One unusual density form (form V), was noninfectious and lacked protein VI. AUC was able to quantify empty capsids and monitor their removal during process development. Their relative concentrations were reduced by either addition of an immobilized zinc affinity chromatography (IZAC) step or by extension of the infection time. The Adenovirus Reference Material (ARM), a wild-type Ad5, had 2.2% empty capsids and no other detectable minor particle forms. Finally, AUC was utilized to monitor the thermal instability of the three rAd vectors via the transformations of different density forms. The vector and empty capsids containing protein IX were more stable than those without IX. Together, these results exemplify AUC in CsCl density gradients as a valuable technique for evaluating product particle heterogeneity and stability. © 2007 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 97:746–763, 2008

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INTRODUCTION

Recombinant adenovirus (rAd) particles are used extensively to deliver genes into cells for vaccines or gene therapy.[1–4] Adenovirus is a nonenveloped DNA virus of icosahedral structure consisting of an outer protein capsid and an inner DNA/protein core. The capsid primarily consists of the hexon trimers, proteins III (penton base) and IIIa, fiber, and the accessory proteins VIII and IX, while the core is composed of viral DNA and proteins V, VII, and mu, with protein VI as a tether between the core and the inner capsid.[5,6] Other lower concentration proteins are also present including the adenovirus proteinase and smaller peptides resulting from cleavage of certain virus protein precursors. During the production of adenovirus, incomplete or aberrant particle forms containing less viral DNA and/or incompletely processed viral proteins are generated together with the complete mature virions.[7]

The classic studies on the separation and characterization of different buoyant density

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forms of adenoviruses utilized preparative ultracentrifugation in CsCl density gradients.[8–13] The lightest forms of these particles were identified by electron microscopy and polypeptide/DNA analysis as empty capsids as they lack the viral genome. Adenovirus particle forms have also been observed at positions in CsCl density gradients intermediate between the empty capsids and mature virus, as well as at positions of higher density than the mature virus.[7] The quantitative analysis of the various particles following the fractionation of the preparative ultracentrifugation gradients is cumbersome, laborious, and requires a large amount of sample. Moreover, the resolution of certain forms might be obscured during the centrifugation deceleration and especially by the fractionation process.

Large-scale purification of rAds for use in gene therapy studies requires chromatographic procedures rather than the traditional virus purification method of preparative ultracentrifugation on CsCl density gradients. However, empty capsids or incomplete particles are often observed in these column-purified vectors.[14–16] The final amount of these particles can differ between the vector types and with the host cell culture and process condition variables. Quantification of these different particle forms is essential for process development and quality control of the final purified products.

Here we report the use of analytical ultracentrifugation (AUC) in CsCl density gradients to assess the particle heterogeneity of rAd preparations. Its primary advantage is that the particle separation is monitored during the centrifugation *in situ* and in real time without disturbing the details of the absorbance profile. Although velocity centrifugation using AUC is applied extensively for protein size characterization,[17,18] this equilibrium centrifugation technique using AUC to observe adenovirus particle heterogeneity is rarely reported.[7] AUC demonstrated that the column-purified virus preparations contained different species of virion particles with distinct densities, the particle heterogeneity could vary from vector to vector as well as from preparation to preparation, and changes in infection or purification process could alter this heterogeneity. This technique was also used to examine the virus stability, particularly the thermal instability intermediates of virus particles, and detected subtle structural changes that other techniques failed to uncover. AUC in CsCl density gradients should become a routine tool to evaluate the quality of rAd preparations.

EXPERIMENTAL PROCEDURES

rAd Vector Production and Purification

Three different column-purified rAd vectors were utilized for this study. The production and purification procedures for each vector also varied as detailed below. All vectors were in 14 mM Tris, 11 mM sodium phosphate, 2 mM $MgCl_2$, 2% sucrose, 10% (w/v) glycerol, pH 8.1 at 4°C (Buffer A), unless otherwise noted. The Adenovirus Reference Material (ARM) is a column-purified wild-type 5 adenovirus. It was produced and vialed by Introgen Therapeutics, Inc., in Houston, Texas under the guidance of the Adenovirus Reference Material Working Group (ARMWG), and is from American Type Culture Collection, Manassas, VA (catalog no. VR-1516). Information regarding ARM is published on the Williamsburg BioProcessing Foundation (WilBio) website at: http//www.wilbio.com.

Vector 1

This replication-deficient type 5 rAd contained the E1/E3 region deletion and carried the human p53 transgene.[19] This vector was produced in HEK 293 cells grown in bioreactors on Cytodex 3 microcarriers; the infected cells were separated from the beads by a fluidized bed and lysed and cleared of cell debris by microfiltration. The lysate was benzonase-treated, concentrated, and diafiltered. The rAd was purified by anion exchange on DEAE-Fractogel and by gel filtration on Superdex 200 as previously described.[14] Vector 1 lacks the adenovirus protein IX gene, and lacks the protein as confirmed by RP-HPLC.

Vector 2

This replication-deficient type 5 rAd shared the same backbone with vector 1 but carried the transgene for human p21.[20] This vector was produced in a HEK293 subclone (D) which has higher surface expression of integrin $\alpha_V\beta_3$.[21] The production, recovery, and purification procedures were similar to those described above except the diafiltration process was extended approximately twofold, a second benzonase treatment was performed for 30 min halfway through the diafiltration process, and 20% (w/v) glycerol was