ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Enhanced Preparation of Adeno-Associated Viral Vectors by Using High Hydrostatic Pressure to Selectively Inactivate Helper Adenovirus

Joshua N. Leonard,[1] Peter Ferstl,[2] Antonio Delgado,[3] David V. Schaffer[1]

[1]Department of Chemical Engineering and Helen Wills Neuroscience Institute, University of California, Berkeley, California 94720; telephone: (301) 435-6442; fax: (301) 496-0887; e-mail: schaffer@berkeley.edu; josh.leonard@stanfordalumni.org

[2]Chair of Fluid Mechanics and Process Automation, Technische Universität München, Freising 85350, Germany

[3]Chair of Fluid Mechanics, Friedrich-Alexander-Universität, Erlangen 91058, Germany

Received 7 November 2006; accepted 17 January 2007

Published online 24 January 2007 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/bit.21355

ABSTRACT: Gene delivery vectors based on adeno-associated virus (AAV) have significant therapeutic potential, but much room for improvement remains in the areas of vector engineering and production. AAV production requires complementation with either helper virus, such as adenovirus, or plasmids containing helper genes, and helper virus-based approaches have distinct advantages in the use of bioreactors to produce large quantities of AAV vectors for clinical applications. However, helper viruses must eventually be inactivated and removed from AAV preparations to ensure safety. The current practice of thermally inactivating adenovirus is problematic as it can also inactivate AAV. Here, we report a novel method using high hydrostatic pressure (HHP) to selectively and completely inactivate helper adenovirus without any detectable loss of functional AAV vectors. The pressure inactivation kinetics of human adenovirus serotype 5 and the high-pressure stabilities of AAV serotypes 2 and 5 (AAV2, AAV5), which were previously unknown, were characterized. Adenovirus was inactivated beyond detection at 260 MPa or higher, whereas AAV2 was stable up to ~450 MPa, and surprisingly, AAV5 was stable up to at least 700 MPa. The viral genomic DNA of pressure-inactivated AAV2 was made sensitive to DNAse I digestion, suggesting that gross changes in particle structure had occurred, and this hypothesis was further supported by transmission electron microscopy. This approach should be useful in the laboratory- and clinical-scale production of AAV gene delivery vectors. Moreover, HHP provides a tool for probing the biophysical properties of AAV, which may facilitate understanding and improving the functions of this important virus.

Biotechnol. Bioeng. 2007;97: 1170–1179.

Correspondence to: J.N. Leonard or D.V. Schaffer

Contract grant sponsor: Bavaria-California Technology Center
Contract grant number: HP-IKAAV
Contract grant sponsor: NIH
Contract grant number: EB003007

KEYWORDS: adeno-associated virus (AAV); adenovirus; stability; inactivation; pressure; purification

Introduction

Adeno-associated virus (AAV), which does not cause any known human disease, it is of great interest because it may be re-engineered into a gene delivery vector to help in *treating* a host of human diseases. Each viral particle is a highly ordered nanostructure (~20–25 nm in diameter), composed of 60 protein subunits arranged in an icosahedral capsid shell that encloses a single-stranded 4.7 kb DNA genome. Gene delivery vectors based on AAV are highly promising because AAV is inherently safe, and vectors based on AAV can mediate long-term expression of transgenes in numerous target cell types in vitro and in vivo (Bueler, 1999; Choi et al., 2005; Kaspar et al., 2002; Lai et al., 2003; Muzyczka and Warrington, 2005). Furthermore, clinical trials are currently underway to begin translating these technologies into viable therapeutic products (Carter, 2005).

AAV is usually produced in mammalian cell cultures, though insect cell-based techniques have also been developed recently (Aucoin et al., 2006; Urabe et al., 2002). Vector production always requires complementation with "helper" virus gene products. This can be accomplished by infecting the producer cells with a helper virus, such as adenovirus or

herpes simplex virus, by transfecting helper plasmid constructs that drive the expression of helper virus genes (Xiao et al., 1998), or through the use of hybrid vectors based on both adenovirus and AAV (Goncalves et al., 2002). While helper-virus-free techniques are highly promising, helper adenovirus is well-suited for some applications since the latter approach has already been adapted for commercial processes and is very amenable to large-scale production in suspension cultures where plasmid transfection is problematic (Farson et al., 2004). Whenever helper virus is used, however, it must eventually be both inactivated and removed in order to avoid helper virus-induced toxicity or adverse immune responses.

Downstream separations processes, including cationic exchange and affinity chromatography (e.g., matrices incorporating heparin, which mimics the cellular receptor for AAV serotype 2) and density-equilibrium centrifugation (using either CsCl or iodixanol gradients), are able to separate AAV from the majority of the adenovirus components, but significant amounts of adenoviral protein and live adenovirus can remain in the final vector stock (Zolotukhin et al., 1999). Recently, a combination of cation and anion exchange chromatography that significantly improved the removal of adenovirus was described (Gao et al., 2000), but since even trace amounts of live adenovirus may cause adverse immune responses, a separate inactivation step must complement downstream separation processes.

The most commonly used helper inactivation process is heat treatment (typically at 56°C for 1 h), but such an incubation has been reported to cause a concomitant inactivation of ~50% of the vectors based on the widely used AAV serotype 2 (AAV2) (Turnbull et al., 2000). In addition to reducing the yield of functional vectors, inactivation of AAV during any production step is problematic because inactivated AAV particles may compete with active particles for cellular receptors and therefore necessitate the injection of more overall vector protein in order to achieve a given level of transgene expression. This increases the risk of eliciting an adverse immune response (Manno et al., 2006; Zaiss and Muruve, 2005). Unfortunately, inactive AAV particles cannot be readily separated from active virions. For example, cesium chloride gradient ultracentrifugation can separate full AAV particles from empty ones, but thermally inactivated AAV particles may have the same density as active particles, rendering separation by ultracentrifugation ineffective. Moreover, cesium chloride ultracentrifugation itself tends to substantially decrease the ratio of active-to-inactive particles (Auricchio et al., 2001; Gao et al., 2000; Zolotukhin et al., 1999). Although cation-exchange and affinity chromatography can successfully purify AAV vectors without adversely impacting the active-to-inactive particle ratio, they do not improve this ratio (Zolotukhin et al., 1999), so such techniques would probably not be useful for removing thermally inactivated particles. Therefore, from both process optimization and safety standpoints, it is desirable to inactivate helper adenovirus while fully preserving the activity of AAV vectors.

In this study, we investigated whether high hydrostatic pressure (HHP) could be used to selectively inactivate adenovirus while leaving the AAV particles intact and functional. HHP is currently used to sterilize various commercial products, ranging from food to human blood products (Bradley et al., 2000; Makita, 1992), and pressure has previously been shown to effectively inactivate viruses including HIV-1, poliovirus, and adenovirus (Masson et al., 2001; Pontes et al., 1997; Wilkinson et al., 2001). Furthermore, high-pressure treatment makes it possible to manipulate biomolecules in unique manners (Doster and Gebhardt, 2003). In contrast to thermal or chemical denaturation, high-pressure treatment changes protein structures by a small and defined set of mechanisms. For example, elevated pressure reduces the free volume of a protein and changes the hydration by solvent water molecules. Such changes tend to weaken electrostatic interactions (such as salt bridges) and hydrophobic interactions in biomolecules, whereas hydrogen bonds are stabilized due to decreased inter-atomic distances (Mozhaev et al., 1996). HHP thus provides a useful tool for investigating a variety of biophysical phenomena, including protein–protein interactions (Gebhardt et al., 2006), protein folding dynamics (Scharnagl et al., 2005; Silva et al., 2001) and the protein misfolding that leads to amyloidosis disease (Randolph et al., 2002). Pressure manipulation has also been used to illuminate various aspects of virus structure and assembly (Silva et al., 1996, 2002). Studies on the high-pressure stability of the icosahedral virus bacteriophage P22 led to the identification of amino acid residue that are crucial for stabilizing the macromolecular assembly (Foguel et al., 1995; Prevelige et al., 1994). High-pressure studies have also contributed insights into the assembly and disassembly of another icosahedral virus, bromegrass mosaic virus (BMV) (Leimkuhler et al., 2000).

We hypothesized that HPP might be used to investigate and potentially exploit particle stability differences between adenovirus and AAV. Furthermore, such information may contribute future insights into the physical properties of this important gene delivery vehicle. Prior to this study, the stability of AAV at high pressure had not been investigated. In addition, previously investigated properties of adenovirus inactivation were difficult to attribute to pressure alone, since the elevated temperatures that accompany water compression may also contribute to viral inactivation, an effect not investigated (Makita, 1992; Pontes et al., 1997; Wilkinson et al., 2001). Accordingly, we first characterized the high-pressure stabilities of human adenovirus type 5 (Ad5) and AAV serotypes 2 and 5 (AAV2, AAV5), while controlling for temperature. Using this information, we propose a novel method for using HHP to efficiently and completely inactivate helper adenovirus while retaining the full activity of recombinant AAV gene delivery vectors.

Materials and Methods

Cells

HeLa and HEK 293T cells (ATCC; Manassas, VA) were cultured in Isocove's Modified Dulbecco's Medium (IMDM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. AAV293 cells (Stratagene, La Jolla, CA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing the same levels of FBS and antibiotics. All cells were incubated at 37°C, 5% CO_2.

Viral Vector Production

An E1, E3-deficient human adenovirus serotype 5 vector encoding GFP under the control of the CMV promoter (Ad5-GFP) was amplified on HEK-293T cells. As previously described (Lai et al., 2003; Lee et al., 2005; Maheshri et al., 2006), helper-free AAV2 vectors expressing either GFP or luciferase under the control of the CMV promoter were produced by triple transfection of AAV2-GFP or AAV2-Luc vector backbone with AAV2 Rep-Cap helper plasmid pXX2 (Xiao et al., 1998) and adenovirus helper plasmid pHelper (Stratagene) into AAV293 cells. Helper-free AAV5-GFP was produced as previously described (Rabinowitz et al., 2002). Briefly, AAV2-GFP vector backbone, pHelper, and pXR5 (which expresses AAV5 Cap and an AAV5/AAV2 chimeric Rep) were triple transfected into AAV293 cells. Where indicated, AAV was purified by Optiprep iodixanol (Sigma Aldrich, St. Louis, MO) gradient centrifugation, according to the manufacturer's instructions and as previously described (Zolotukhin et al., 1999). Tween-20 (Fisher Scientific, Hampton, NH) was added to purified AAV to a final concentration of 0.01%. For helper-dependent AAV production, pHelper was omitted from the transfection, and helper gene products were supplied by Ad5-GFP co-infection at an MOI of 2.

Infectious Viral Titer Determination

Infectious titers of GFP-expressing AAV and Ad5 vector samples were determined by infecting HeLa cells and assessing the transduction efficiencies (% GFP-positive) at 72 h post-infection by flow cytometry. The detection limit of our assay was a 10^5-fold reduction in Ad5 titer for some experiments (untreated Ad5-GFP titers were $\sim 10^7$ infectious units, or IU, per mL, and untreated AAV titers were $\sim 10^8$ IU/mL). Therefore, for the sake of consistency, reductions in Ad5-GFP titer below the limits of detection were conservatively reported here as 10^5-fold reductions. The titers of pressure-treated vectors were normalized to those of untreated controls from the same vector preparations. The infectious titers of luciferase-expressing vectors were determined similarly, except that at 72 h after infection, cell lysate was collected, and luciferase assays were performed as previously reported (Ignowski and Schaffer, 2004). These luciferase signals, which varied linearly with viral titer (data not shown), were used to normalize the infectious titers of pressure-treated samples to those of untreated control samples.

High Hydrostatic Pressure (HHP) Treatment

Viral samples were sealed in leak-proof containers that allowed for moderate volume change, and pressure treatment was performed using a hyperbaric research system, model "Mini Food Lab" (Stansted Fluid Power Ltd, Essex, UK). Briefly, 1 mL of sample was placed in a 1.2 mL Nalgene cryovial (Nalge Nunc International, Rochester, NY), and a silicone stopper (US size 000; Fisher Scientific) was inserted in the top. The stopper was held in place by a custom-milled aluminum cap with a hole bored in the center to allow direct exposure of the stopper to the pressure transduction fluid. Finally, the assembled container was submerged in an ethanol/oil mixture in the pressure transduction chamber of the hyperbaric device.

AAV DNAse I Digestion and Quantitative Real-Time PCR (QPCR)

Five microliters of iodixanol-purified and HHP-treated AAV was added to 40 μL water, 5 μL of 10× DNAse I buffer (500 mM KCl, 20 mM $MgCl_2$, 200 mM Tris HCl pH 8.3), and 10 units of DNAse I (Roche, Basel, Switzerland). The mixture was incubated for 1 h at 37°C, after which DNAse I was inactivated at 75°C for 10 min. Hundred micrograms Proteinase K (New England Biolabs, Ipswich, MA) was added, and the mixture was incubated for 2 h at 37°C. Proteinase K was then inactivated at 95°C for 20 min. The concentration of viral genomic DNA (vgDNA) in each sample was then determined by QPCR of the CMV promoter, as previously described (Rohr et al., 2002) using SYBR Green dye (Invitrogen, Carlsbad, CA) and a Bio-Rad iCycler. Primers used were CMV_forward (5′-TGAACCGTCAGATCGCCTGGA-3′) and CMV_reverse (5′-CATCAAGCGTCCCATAGACTCACCC′3′), and thermocycling conditions were 95°C for 5 min, then 40 cycles of: 95°C for 30 s, 60°C for 30 s, 72°C for 10 s.

Transmission Electron Microscopy (TEM)

Formalin-coated carbon grids were activated by glow discharge. One microliter of iodixanol-purified AAV ($\sim 5 \times 10^{10}$ physical particles per mL) was added to the grid for 5 s, and 4 μL of PBS was then added. Grids were stained with 1% uranyl acetate for 1 min, and TEM imaging was performed on an FEI Tecnai 12 120 KV.

DOI 10.1002/bit

Results

HHP Process Characterization

It is known that during pressurization of water to levels required to inactivate microorganisms (usually up to several hundred megapascals, MPa), temperature rises significantly as a direct thermodynamic consequence of adiabatic compression (Makita, 1992). For example, compression from ambient conditions to 800 MPa can raise the temperature of water as much ~25°C *above* room temperature. However, this temperature spike can be controlled and alleviated by an adaptive process control (Hartmann et al., 2004), for example, by modulating the pressurization rate. While rapid compression leads to a larger temperature spike, slow compression yields a smaller rise in temperature since heat has more time to conduct and dissipate through the metal chamber walls during compression. Since AAV2 is stable up to at least 37°C, but begins to inactivate at significantly higher temperatures (Turnbull et al., 2000), we first identified pressurization conditions that avoided temperature spikes over 37°C.

A sealed sample container that allows for moderate volume change under pressure was devised (Fig. 1A). Initially, a thermocouple was inserted into the vial to monitor the temperature of the sample during compression. In other HHP systems, it was reported that a compression rate of 5 MPa/s or less was required to stay below the temperature increase predicted by thermodynamics with no thermal dissipation (Makita, 1992). With a sample temperature of 20°C and pressurization rate of 2 MPa/s, we observed that pressure could be increased to at least 800 MPa without the sample temperature rising above 37°C (Fig. 1B). We also found the same result for compression rates of up to 5 MPa/s (data not shown). Since the peak temperature inside the vial was consistently at least ~2°C lower than the temperature in the chamber (which can be monitored non-invasively), the chamber temperature was hereafter considered suitable for tracking the maximum sample temperature. Finally, we found that when the sample was held at elevated pressures for extended times, its temperature returned to 20°C within ~10 min (data not shown). These results were used to ensure that all subsequent experiments were conducted under conditions that did not exceed 37°C.

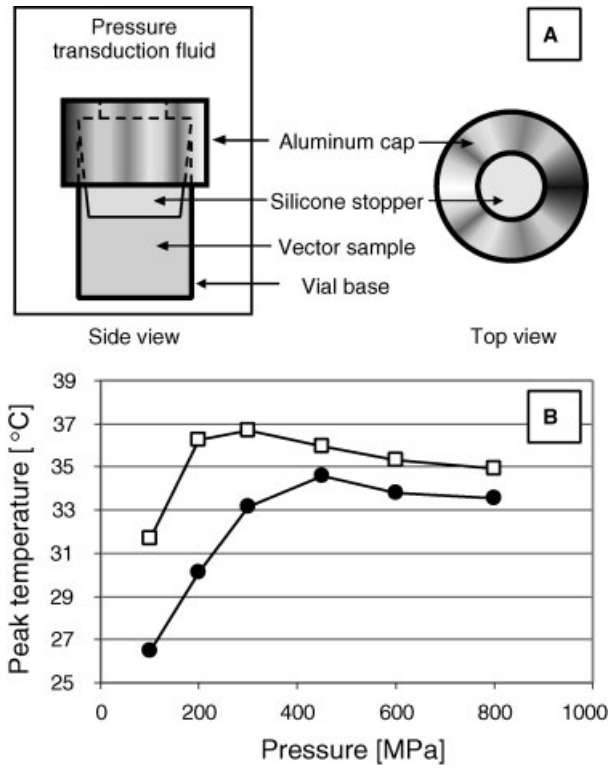

Figure 1. High-pressure experimental system. Leak proof containers were devised to allow for pressure transduction and moderate volume change. A cryovial was filled with sample, plugged with a silicone stopper, and held together with an aluminum cap. A hole in the cap exposed the top of the stopper directly to pressure transduction fluid (**A**). The temperature inside the vial (●) and in the surrounding chamber (□) was tracked over three independent compression series for each data point (**B**).

Pressure Inactivation Kinetics

We initially investigated the feasibility of using HHP to selectively inactive helper virus by determining the relative stabilities of recombinant vectors based on either human adenovirus serotype 5 (Ad5) or AAV serotype 2 (AAV2), the first and still most broadly utilized AAV vector. For these initial experiments, helper-free triple transfection was used to produce an AAV2 vector that expresses the green fluorescent protein (GFP) under the cytomegalovirus (CMV) immediate early constitutive promoter. Separately, we also produced an E1, E3-deficient helper Ad5 vector also containing a CMV-GFP insert.

HHP treatments were performed on vectors in their crude cell lysates at the completion of the vector packaging step of production, as Ad5 inactivation is often carried out at this point (Clark et al., 1999). Furthermore, HHP-mediated helper inactivation immediately following packaging would be compatible with all types of downstream purifications. Following pressure treatment, the remaining infectious titer of each vector was determined by a single-step infection of HeLa cells. Samples were exposed to a range of elevated pressures for 15 min, and interestingly Ad5-GFP was effectively completely inactivated at 260 MPa (undetectable in the infectious titer assay, corresponding to at least a 10^5-fold reduction, as described in Materials and Methods Section), consistent with previous reports (Wilkinson et al., 2001). In stark contrast, AAV2-GFP remained fully active up to ~450 MPa, though it also began to lose activity at higher pressures (Fig. 2A).

We next investigated viral inactivation kinetics under longer holding times at elevated pressures, since extending

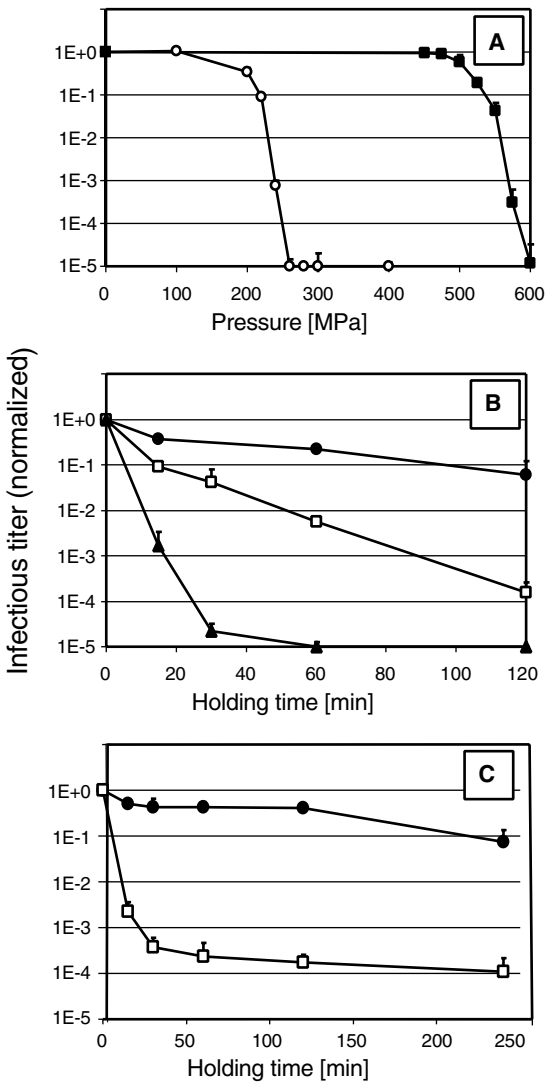

Figure 2. Ad5 and AAV2 stability at high pressure. The kinetics of AAV and Ad5 inactivation were assessed over a range of pressures and holding times. Samples of Ad5-GFP (○) or helper-free AAV2-GFP (■) vectors were treated with HHP for 15 min, after which remaining active titer was assessed by single-step infection of HeLa cells (**A**). Ad5-GFP was held at 200 MPa (●), 220 MPa (□), or 240 MPa (▲) for extended times (**B**). AAV2-GFP was held at 500 MPa (●) or 550 MPa (□) (**C**). Experiments were performed in triplicate, error bars indicate one standard deviation, and reductions in titer beyond detection limits are reported as 10^5-fold reductions.

the treatment time could reduce the minimum pressure required to inactivate Ad5. Ad5-GFP was progressively inactivated at lower pressures as the holding time was increased (Fig. 2B). By contrast, at pressures high enough to inactivate AAV, AAV2-GFP exhibited more complex inactivation kinetics (Fig. 2C). Specifically, at both 500 and 550 MPa, the active AAV2-GFP titer appeared to plateau at a finite value, though at 550 MPa the viral activity eventually resumed its decline after remaining stable for ~90 min. Interestingly, qualitatively similar inactivation kinetics were previously reported for the *thermal* inactivation of AAV2 (Turnbull et al., 2000).

Selective Inactivation of Ad5

We next tested whether selective inactivation of Ad5 would be possible in an Ad5/AAV2 mixture, as our initial results with the separate viruses supported this possibility. In order to individually assay for active Ad5 and AAV2 in a mixed preparation, the firefly luciferase gene (Luc) was inserted into the AAV2 vector in place of GFP. One potential complication is that adenovirus co-infection has been shown to increase AAV transduction efficiency by enhancing AAV second strand DNA synthesis (Fisher et al., 1996). Since this effect could complicate the interpretation of results of simultaneous viral inactivation, we co-infected HeLa cells with Ad5-GFP and AAV2-Luc (helper-free) at various ratios and assayed for Luc expression. AAV2-Luc transduction efficiency was not significantly increased by Ad5-GFP co-infection as long as the multiplicity of infection (MOI) for Ad5-GFP was less than 1 (data not shown). This condition was therefore maintained for all subsequent experiments.

A mixed vector stock was produced using Ad5-GFP as a helper virus for AAV2-Luc production. As anticipated from the stabilities of the vectors tested individually, 15 min treatments at either 300 or 400 MPa were sufficient to completely inactivate Ad5-GFP (no active Ad5-GFP was detectable in our assay, corresponding to a titer reduction of $>10^5$-fold) while leaving AAV2-Luc fully intact (Fig. 3A). We attempted to achieve a comparable selective inactivation at the lowest pressure possible by treating samples at 240 MPa, since this was the lower limit at which Ad5-GFP was previously inactivated by increasing the holding time (Fig. 2B). As before, Ad5-GFP was inactivated more than 10,000-fold after 30 min at 240 MPa, whereas AAV2-Luc remained fully active for similar treatments of up to 2 h (Fig. 3B). However, the very small residual of Ad5-GFP infectivity after 2 h at 240 MPa (compared to Fig. 2) indicates a small amount of variability in the extent of inactivation achieved under these conditions. In practice, the exact process limits would need to be defined for each implementation of this technique, but possible contributing factors include variations in sample composition and imperfect control of pressure treatment conditions (e.g., slight variations in the rate at which samples are pressurized and depressurized).

Mechanism of AAV2 Inactivation

Since HHP treatment can reveal interesting biological insights into viral structure (Chauvin et al., 1978; Foguel et al., 1995; Leimkuhler et al., 2000; Lucas et al., 2002; Prevelige et al., 1994; Silva et al., 2001, 2002), we next investigated the mechanism by which pressure inactivates AAV2. We hypothesized that AAV2 inactivation at 600 MPa could result from either severe disruption of particle structure or more subtle structural changes that disrupt some step of the infectious pathway without causing dissociation of capsid proteins from one another and from

DOI 10.1002/bit

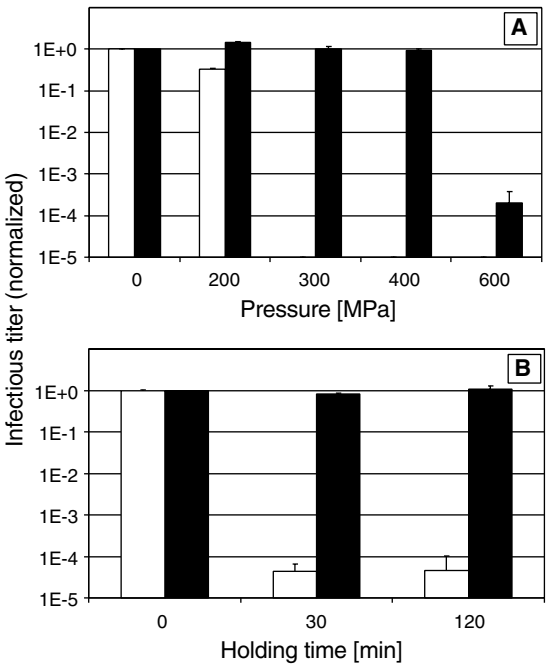

Figure 3. Selective inactivation of Ad5. AAV2-Luc (black bars) was produced using Ad5-GFP (white bars) as a helper virus. The mixture was treated at various pressures, and parallel infections of HeLa cells were assayed separately for the two reporter gene products to determine infectious titers. Treatments lasting 15 min at various pressures (**A**) and extended treatments at 240 MPa (**B**) were used. Experiments were performed in triplicate, error bars indicate one standard deviation and reductions in titer beyond detection limits are reported as 10^5-fold reductions.

vgDNA. For example, HHP could induce conformational changes in the structure of individual monomers that prevent cellular receptor binding while leaving the overall particle structure essentially intact. To differentiate between these possibilities, we assessed the sensitivity of vgDNA to DNAse I digestion after pressurization. To ensure that residual cellular or plasmid DNA in the crude lysate would not significantly affect the analysis, AAV2 samples were first purified using equilibrium density centrifugation on an iodixanol gradient, as previously described (Zolotukhin et al., 1999), prior to HHP treatment. This separation removes cellular debris but does not separate full viral particles (containing vgDNA) from empty ones (containing assembled capsid proteins but no DNA). The enriched AAV2 samples were diluted to a viral concentration comparable to that in crude lysate, HHP was applied, and the resulting active titer was assayed to confirm that purified vector stability was identical to that previously observed with vectors in crude lysate (Figs. 2A and 4). Subsequently, HHP-treated samples were incubated with DNAse I for 1 h at 37°C, and the remaining concentration of vgDNA was subsequently determined using quantitative real-time PCR (QPCR).

The untreated control and 450 MPa-treated sample showed a slight reduction in vgDNA following DNAse I digestion, consistent with previous reports using DNAse I in AAV titering (Rohr et al., 2002), whereas the 600 MPa-treated sample exhibited a striking reduction in detectable vgDNA that mirrored the reduction in infectious titer (Fig. 4). This observed increase in susceptibility of vgDNA to DNAse I suggests that at 600 MPa, AAV2 particles become at least partially disassembled. Interestingly, similar digestions of these samples with Benzonase (Sigma Aldrich), which hydrolyzes both double- and single-stranded DNA and is used routinely to digest residual cellular and plasmid DNA in AAV preparations, did not significantly reduce the amount of vgDNA detectable by QPCR in the 600 MPa-treated sample (data not shown; Benzonase activity was confirmed by agarose gel electrophoresis, also not shown).

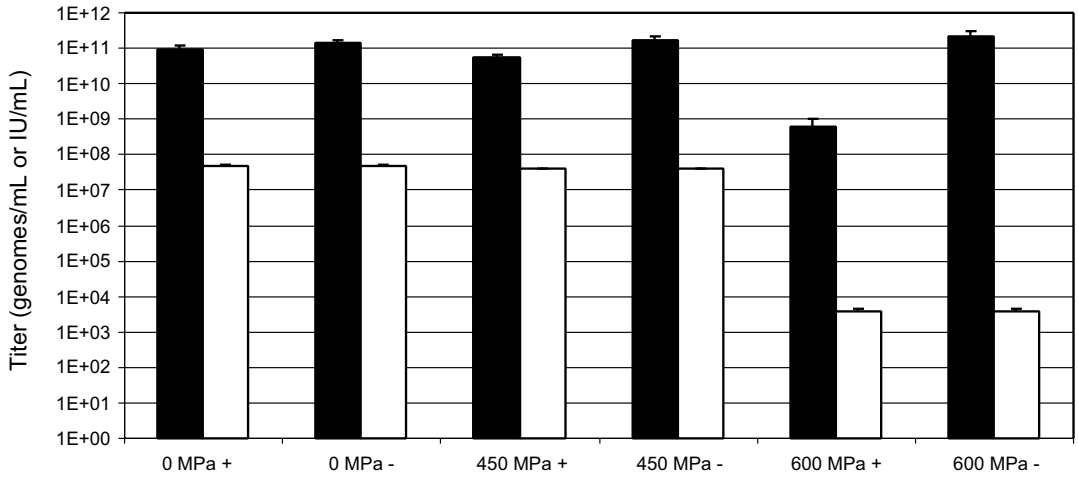

Figure 4. DNAse I sensitivity of HHP-treated AAV. Iodixanol-purified AAV2-GFP was HHP-treated. Infectious titer was determined by single-step infection of HeLa cells (white bars), and genomic DNA titer was determined by QPCR following DNAse I digestion (+) or mock digestion (−) (black bars). Experiments were performed in triplicate, and error bars indicate one standard deviation.

This could indicate that in these samples, AAV vgDNA remained associated with proteins or in aggregates that excluded Benzonase but not DNAse I by an unknown mechanism.

To confirm that AAV2 inactivation by HHP occurs due to gross particle disruption, as suggested by the DNAse I sensitivity analysis, we next visualized HHP treated AAV2 particles using transmission electron microscopy (TEM). We attempted to capture TEM images of HHP-treated AAV2 in crude lysate but were unable to do so, potentially because excess cellular debris prevented the attachment of viral particles to the grids. TEM grids were therefore prepared from the same iodixanol purified, HHP-treated vector stocks used for the DNAse I analysis in order to avoid possible effects due to changes in either vector composition or particle concentration prior to HHP treatment. Whereas intact AAV2 particles were readily distinguishable in both the control and 450 MPa-treated samples (see representative images in Fig. 5A and B), absolutely no viral particles could be identified in the 600 MPa-treated samples, even after sample preparation was repeated several times. These observations support the argument that AAV2 inactivation at 600 MPa occurs due to gross and irreversible changes in particle structure not induced at 450 MPa or below.

Extension to Other AAV Serotypes

Finally, we tested the pressure stability of a vector based on another AAV serotype, AAV5, to analyze the potential generality of the approach. While AAV serotypes 2, 3, 4, 6 are ~75%–78% identical at the level of DNA sequence, AAV5 shares only 54–56% sequence identity with this group (Bantel-Schaal et al., 1999), making AAV5 is the most genetically distant of the primate AAV serotypes 1–6 (Lukashov and Goudsmit, 2001). In addition, AAV5 vectors are capable of enhanced gene delivery to tissues in which AAV2 transduction efficiency is low, such as airway epithelia (Zabner et al., 2000). In these experiments, the initial AAV5 vector concentration and buffer composition were comparable to those used in the AAV2 experiments. Intriguingly, the AAV-GFP vectors pseudotyped with AAV5 capsid (AAV5-GFP) (Rabinowitz et al., 2002) were significantly more stable than their AAV2 counterparts and retained full activity after treatments of up to 700 MPa (Fig. 6).

Discussion

We report a robust technology for enhancing the safety of AAV vector preparations by selectively inactivating contaminating helper viruses while retaining full activity of the AAV. AAV vectors are useful vehicles for gene delivery in research settings and increasingly in clinical applications (Bueler, 1999; Carter, 2005; Kaspar et al., 2002; Lai et al., 2003), and several important considerations arise in moving toward the large-scale production and purification of AAV. While the use of helper adenovirus is well-suited to large-scale AAV production (Xie et al., 2004), the helper must be completely inactivated prior to downstream purifications to minimize the risk of adverse immune responses (Monahan et al., 1998). In addition, an important goal for maximizing therapeutic safety and efficacy is minimizing the amount of viral protein injected into a patient.

Although AAV vectors are safe, as evidenced by the fact that ~90% of the human population has been exposed to wild-type AAV with no associated disease (Berns and

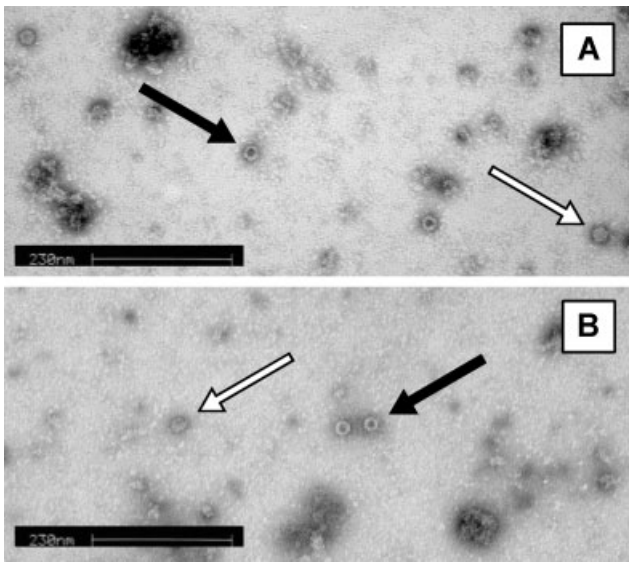

Figure 5. TEM analysis of HHP-treated AAV. Iodixanol-purified AAV was HHP-treated, applied to formalin-coated carbon grids, and negatively stained with 1% uranyl acetate. Representative TEM images of AAV2 control (**A**) or following 450 MPa HHP (**B**) are shown. Particles containing viral DNA exhibit a light core (white arrows), and empty particles have a dark core (black arrows).

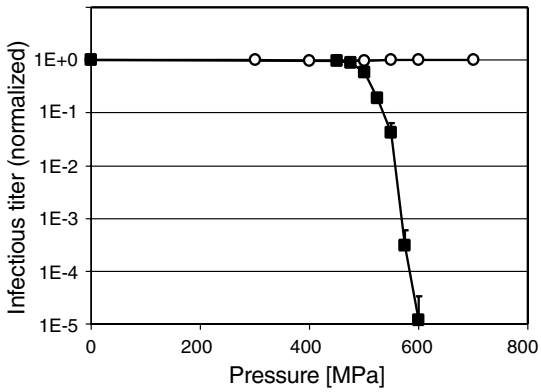

Figure 6. HHP stability varies by AAV serotype. AAV2-GFP (■) was produced by helper-free plasmid transfection, and AAV5-GFP (○) was produced by helper-free cross packaging (Rabinowitz et al., 2002). Each was treated with HHP, and the remaining active titer was assessed by single-step infection of HeLa cells. Experiments were performed in triplicate, and error bars indicate one standard deviation.

DOI 10.1002/bit

Linden, 1995; Moskalenko et al., 2000), the broad pre-existing immunity to AAV in the human population poses another challenge. A large fraction of the human population harbors neutralizing antibodies against many AAV serotypes, and while AAV vectors have been engineered to evade pre-existing immunity (Maheshri et al., 2006), such vectors may also become less effective during subsequent treatments if the initial injection contains excess viral protein that enhances the generation of novel neutralizing antibodies. In addition, it has become increasingly recognized that AAV also elicits cellular immune responses (Manno et al., 2006). To minimize the amount of viral protein injected and avoid these adverse reactions, all steps of clinical AAV production should be optimized to retain maximal vector activity.

We have developed a method for using HHP to selectively inactivate Ad5 while leaving AAV fully active. This represents a significant improvement over thermal inactivation of helper adenovirus, which can concomitantly inactivate ~50% of the AAV2 particles (Turnbull et al., 2000). While Ad5 can be completely inactivated by 15 min at ~260 MPa, AAV2 remains stable up to ~450 MPa (Fig. 2). We found that a somewhat higher pressure was required to inactivate Ad5 than was previously reported by Pontes et al. (1997) (220 MPa for 60 min), which might indicate that concurrent thermal inactivation occurred in this previous study (Pontes et al., 1997). Nonetheless, a wide practical window exists for achieving the desired selective inactivation with HHP. To test whether this approach could be extended to other serotypes, we also characterized the stability of vectors based on the genetically distant AAV5 (Lukashov and Goudsmit, 2001). Surprisingly, AAV5 was significantly more stable than AAV2 (Fig. 6), even though the particle structures are similar (Chiorini et al., 1999). This result suggests that high-pressure studies of AAV might contribute physical insights into overall particle structure.

HHP has previously proven useful for studying a variety of biophysical processes, including analysis of protein folding and aggregation (Randolph et al., 2002; Silva et al., 2001), as well as characterization of the structure of viruses (Foguel et al., 1995; Prevelige et al., 1994; Silva et al., 1996, 2002). For example, the monomeric capsid proteins of bacteriophage P22 were found to be relatively unstable under pressure, whereas the assembled icosahedral particle was significantly more stable (Prevelige et al., 1994). When temperature was lowered, the assembled particles became pressure-labile as well, indicating that particles are stabilized by intra-monomer entropic interactions. Subsequent mutational analysis identified two separate amino acids that do not affect monomer pressure-stability but are crucial for stabilizing the assembled particles (Foguel et al., 1995). High-pressure studies have also yielded insights into the assembly and disassembly of BMV, a ~28 nm icosahedral virus comprised of 180 identical protein subunits and one or two segments of its multipartite RNA genome (Lucas et al., 2002). Since protein–RNA interactions dominate over protein–protein interactions in stabilizing particles (Chauvin et al., 1978), it was hypothesized that such interactions govern particle assembly. This hypothesis was bolstered by demonstrating that high pressure-induced viral particle disassembly was reversible only if viral RNA was present (Leimkuhler et al., 2000). Furthermore, this study demonstrated that the assembled capsid subunits can undergo irreversible conformational changes *before* dissociating, which leads to aggregation, but that this conformational change is inhibited by interactions with viral RNA.

We too observed a number of interesting biophysical phenomena using HHP. First, although Ad5 inactivation by HHP was characterized primarily by simple exponential decay, AAV2 inactivation kinetics were more complex. Specifically, AAV2 decay was not monotonic but instead appeared to plateau (Fig. 2). Since this behavior was also reported for the thermal decay of AAV2 (Turnbull et al., 2000), it may reflect an inherent heterogeneity in AAV2 populations. The capsid of each AAV particle is a 60 subunit complex composed of three structural proteins: 3–6 subunits of either VP1 or VP2, and the remainder of the 60 comprised by VP3 (Muzyczka and Warrington, 2005). However, since AAV particles lacking VP2 are still infectious (Warrington et al., 2004), it is possible that subunit stoichiometry might vary somewhat between individual viral particles in a given population. We hypothesize that these different particle compositions might result in different pressure stabilities. Such a distribution of particle stabilities within a single viral population may indicate the existence of competing evolutionary selective forces favoring alternatively higher stability (which might enhance viral survival during passage between hosts) or lower stability (which might improve the efficiency with which viral particles disassemble after infecting a cell).

The greatly enhanced stability of AAV5 over AAV2 is an example of dramatic variation in the properties of AAV serotypes (Fig. 6). This difference may be due to increased resistance of individual capsid subunits to conformational change, stronger interactions between capsid subunits, or different interactions between subunits and vgDNA. As recent studies with bacteriophage Φ29 have demonstrated, viral genomic nucleic acids are sometimes packed into particles under intense pressure (Smith et al., 2001), which have the potential to resist an externally applied pressure. In BMV, however, viral RNA–protein interactions stabilize the overall particle structure (Chauvin et al., 1978). Particle stability may therefore depend upon the extent to which vgDNA is loaded into the viral capsid, or DNA conformation within the particle. Both of these mechanisms are other potential sources of the observed population heterogeneity in AAV pressure stability (Fig. 2).

Significant advances continue to be made in the design of both therapeutic cargoes and gene delivery vectors capable of carrying them to numerous target tissues. This study demonstrates that HHP treatment can be useful for AAV vector production, and the reported results should help to develop processes for selectively sterilizing helper adenovirus (or other helper viruses) as well as other contaminating

microorganisms, without compromising AAV vector activity. In addition, pressure-mediated characterization of AAV stability should prove useful for probing other aspects of the virion macromolecular assembly. HHP can thus be added to the repertoire of useful tools for producing and improving AAV gene delivery vectors.

We thank William Halford (Department of Veterinary Molecular Biology, University of Montana) for generously providing Ad5-GFP and James T. Koerber for helpful discussions. This work was funded in part by the Bavaria-California Technology Center (grant HP-IKAAV) and by NIH EB003007.

References

Aucoin MG, Perrier M, Kamen AA. 2006. Production of adeno-associated viral vectors in insect cells using triple infection: Optimization of baculovirus concentration ratios. Biotechnol Bioeng 95(6):1081–1092.

Auricchio A, Hildinger M, O'Connor E, Gao GP, Wilson JM. 2001. Isolation of highly infectious and pure adeno-associated virus type 2 vectors with a single-step gravity-flow column. Hum Gene Ther 12(1):71–76.

Bantel-Schaal U, Delius H, Schmidt R, zur Hausen H. 1999. Human adeno-associated virus type 5 is only distantly related to other known primate helper-dependent parvoviruses. J Virol 73(2):939–947.

Berns KI, Linden RM. 1995. The cryptic life style of adeno-associated virus. Bioessays 17(3):237–245.

Bradley DW, Hess RA, Tao F, Sciaba-Lentz L, Remaley AT, Laugharn JA, Jr., Manak M. 2000. Pressure cycling technology: A novel approach to virus inactivation in plasma. Transfusion 40(2):193–200.

Bueler H. 1999. Adeno-associated viral vectors for gene transfer and gene therapy. Biol Chem 380(6):613–622.

Carter BJ. 2005. Adeno-associated virus vectors in clinical trials. Hum Gene Ther 16(5):541–550.

Chauvin C, Pfeiffer P, Witz J, Jacrot B. 1978. Structural polymorphism of bromegrass mosaic virus: A neutron small angle scattering investigation. Virology 88(1):138–148.

Chiorini JA, Kim F, Yang L, Kotin RM. 1999. Cloning and characterization of adeno-associated virus type 5. J Virol 73(2):1309–1319.

Choi VW, McCarty DM, Samulski RJ. 2005. AAV hybrid serotypes: Improved vectors for gene delivery. Curr Gene Ther 5(3):299–310.

Clark KR, Liu X, McGrath JP, Johnson PR. 1999. Highly purified recombinant adeno-associated virus vectors are biologically active and free of detectable helper and wild-type viruses. Hum Gene Ther 10(6):1031–1039.

Doster W, Gebhardt R. 2003. High pressure—unfolding of myoglobin studied by dynamic neutron scattering. Chem Phys 292(2–3):383–387.

Farson D, Harding TC, Tao L, Liu J, Powell S, Vimal V, Yendluri S, Koprivnikar K, Ho K, Twitty C, et al. 2004. Development and characterization of a cell line for large-scale, serum-free production of recombinant adeno-associated viral vectors. J Gene Med 6(12):1369–1381.

Fisher KJ, Gao GP, Weitzman MD, DeMatteo R, Burda JF, Wilson JM. 1996. Transduction with recombinant adeno-associated virus for gene therapy is limited by leading-strand synthesis. J Virol 70(1):520–532.

Foguel D, Teschke CM, Prevelige PE, Jr., Silva JL. 1995. Role of entropic interactions in viral capsids: Single amino acid substitutions in P22 bacteriophage coat protein resulting in loss of capsid stability. Biochemistry 34(4):1120–1126.

Gao G, Qu G, Burnham MS, Huang J, Chirmule N, Joshi B, Yu QC, Marsh JA, Conceicao CM, Wilson JM. 2000. Purification of recombinant adeno-associated virus vectors by column chromatography and its performance in vivo. Hum Gene Ther 11(15):2079–2091.

Gebhardt R, Doster W, Friedrich J, Kulozik U. 2006. Size distribution of pressure-decomposed casein micelles studied by dynamic light scattering and AFM. Eur Biophys J 35(6)503–509.

Goncalves MA, van der Velde I, Janssen JM, Maassen BT, Heemskerk EH, Opstelten DJ, Knaan-Shanzer S, Valerio D, de Vries AA. 2002. Efficient generation and amplification of high-capacity adeno-associated virus/adenovirus hybrid vectors. J Virol 76(21):10734–10744.

Hartmann C, Schuhholz J-P, Kitsubun P, Chapleau N, Le Bail A, Delgado A. 2004. Experimental and numerical analysis of the thermofluiddynamics in a high-pressure autoclave. Innovative Food Sci Emerg Technol 5(4):399.

Ignowski JM, Schaffer DV. 2004. Kinetic analysis and modeling of firefly luciferase as a quantitative reporter gene in live mammalian cells. Biotechnol Bioeng 86(7):827–834.

Kaspar BK, Vissel B, Bengoechea T, Crone S, Randolph-Moore L, Muller R, Brandon EP, Schaffer D, Verma IM, Lee KF, et al. 2002. Adeno-associated virus effectively mediates conditional gene modification in the brain. Proc Natl Acad Sci USA 99(4):2320–2325.

Lai K, Kaspar BK, Gage FH, Schaffer DV. 2003. Sonic hedgehog regulates adult neural progenitor proliferation in vitro and in vivo. Nat Neurosci 6(1):21–27.

Lee GK, Maheshri N, Kaspar B, Schaffer DV. 2005. PEG conjugation moderately protects adeno-associated viral vectors against antibody neutralization. Biotechnol Bioeng 92(1):24–34.

Leimkuhler M, Goldbeck A, Lechner MD, Witz J. 2000. Conformational changes preceding decapsidation of bromegrass mosaic virus under hydrostatic pressure: A small-angle neutron scattering study. J Mol Biol 2961:295–305.

Lucas RW, Larson SB, McPherson A. 2002. The crystallographic structure of brome mosaic virus. J Mol Biol 317(1):95–108.

Lukashov VV, Goudsmit J. 2001. Evolutionary relationships among parvoviruses: Virus-host coevolution among autonomous primate parvoviruses and links between adeno-associated and avian parvoviruses. J Virol 75(6):2729–2740.

Maheshri N, Koerber JT, Kaspar BK, Schaffer DV. 2006. Directed evolution of adeno-associated virus yields enhanced gene delivery vectors. Nat Biotechnol 24(2):198–204.

Makita T. 1992. Application of high-pressure and thermophysical properties of water to biotechnology. Fluid Phase Equilibria 76:87–95.

Manno CS, Arruda VR, Pierce GF, Glader B, Ragni M, Rasko J, Ozelo MC, Hoots K, Blatt P, Konkle B, et al. 2006. Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response. Nat Med 12(3):342–347.

Masson P, Tonello C, Balny C. 2001. High-pressure biotechnology in medicine and pharmaceutical science. J Biomed Biotechnol 1(2):85–88.

Monahan PE, Samulski RJ, Tazelaar J, Xiao X, Nichols TC, Bellinger DA, Read MS, Walsh CE. 1998. Direct intramuscular injection with recombinant AAV vectors results in sustained expression in a dog model of hemophilia. Gene Ther 5(1):40–49.

Moskalenko M, Chen L, van Roey M, Donahue BA, Snyder RO, McArthur JG, Patel SD. 2000. Epitope mapping of human anti-adeno-associated virus type 2 neutralizing antibodies: Implications for gene therapy and virus structure. J Virol 74(4):1761–1766.

Mozhaev VV, Heremans K, Frank J, Masson P, Balny C. 1996. High pressure effects on protein structure and function. Proteins 24(1):81–91.

Muzyczka N, Warrington KH, Jr. 2005. Custom adeno-associated virus capsids: The next generation of recombinant vectors with novel tropism. Hum Gene Ther 16(4):408–416.

Pontes L, Fornells LA, Giongo V, Araujo JRV, Sepulveda A, Vilas-Boas M, Bonafe CFS, Silva JL. 1997. Pressure inactivation of animal viruses: Potential biotechnological applications. In: Heremans K, editor. High pressure research in the biosciences and biotechnology. Leuven: Leuven University Press. p 91–94.

Prevelige PE, Jr., King J, Silva JL. 1994. Pressure denaturation of the bacteriophage P22 coat protein and its entropic stabilization in icosahedral shells. Biophys J 66(5):1631–1641.

Rabinowitz JE, Rolling F, Li C, Conrath H, Xiao W, Xiao X, Samulski RJ. 2002. Cross-packaging of a single adeno-associated virus (AAV) type 2 vector genome into multiple AAV serotypes enables transduction with broad specificity. J Virol 76(2):791–801.

DOI 10.1002/bit

Randolph TW, Seefeldt M, Carpenter JF. 2002. High hydrostatic pressure as a tool to study protein aggregation and amyloidosis. Biochim Biophys Acta 1595(1–2):224–234.

Rohr UP, Wulf MA, Stahn S, Steidl U, Haas R, Kronenwett R. 2002. Fast and reliable titration of recombinant adeno-associated virus type-2 using quantitative real-time PCR. J Virol Methods 106(1):81–88.

Scharnagl C, Reif M, Friedrich J. 2005. Stability of proteins: Temperature, pressure and the role of the solvent. Biochimica Et Biophysica Acta-Proteins and Proteomics 1749(2):187–213.

Silva JL, Foguel D, Da Poian AT, Prevelige PE. 1996. The use of hydrostatic pressure as a tool to study viruses and other macromolecular assemblages. Curr Opin Struct Biol 6(2):166–175.

Silva JL, Foguel D, Royer CA. 2001. Pressure provides new insights into protein folding, dynamics and structure. Trends Biochem Sci 26(10): 612–618.

Silva JL, Oliveira AC, Gomes AM, Lima LM, Mohana-Borges R, Pacheco AB, Foguel D. 2002. Pressure induces folding intermediates that are crucial for protein-DNA recognition and virus assembly. Biochim Biophys Acta 1595(1–2):250–265.

Smith DE, Tans SJ, Smith SB, Grimes S, Anderson DL, Bustamante C. 2001. The bacteriophage straight phi29 portal motor can package DNA against a large internal force. Nature 413(6857):748–752.

Turnbull AE, Skulimowski A, Smythe JA, Alexander IE. 2000. Adeno-associated virus vectors show variable dependence on divalent cations for thermostability: Implications for purification and handling. Hum Gene Ther 11(4):629–635.

Urabe M, Ding C, Kotin RM. 2002. Insect cells as a factory to produce adeno-associated virus type 2 vectors. Hum Gene Ther 13(16):1935–1943.

Warrington KH, Jr., Gorbatyuk OS, Harrison JK, Opie SR, Zolotukhin S, Muzyczka N. 2004. Adeno-associated virus type 2 VP2 capsid protein is nonessential and can tolerate large peptide insertions at its N terminus. J Virol 78(12):6595–6609.

Wilkinson N, Kurdziel AS, Langton S, Needs E, Cook N. 2001. Resistance of poliovirus to inactivation by high hydrostatic pressures. Innovative Food Sci Emerg Technol 2(2):95.

Xiao X, Li J, Samulski RJ. 1998. Production of high-titer recombinant adeno-associated virus vectors in the absence of helper adenovirus. J Virol 72(3):2224–2232.

Xie Q, Hare J, Turnigan J, Chapman MS. 2004. Large-scale production, purification and crystallization of wild-type adeno-associated virus-2. J Virol Methods 122(1):17–27.

Zabner J, Seiler M, Walters R, Kotin RM, Fulgeras W, Davidson BL, Chiorini JA. 2000. Adeno-associated virus type 5 (AAV5) but not AAV2 binds to the apical surfaces of airway epithelia and facilitates gene transfer. J Virol 74(8):3852–3858.

Zaiss AK, Muruve DA. 2005. Immune responses to adeno-associated virus vectors. Curr Gene Ther 5(3):323–331.

Zolotukhin S, Byrne BJ, Mason E, Zolotukhin I, Potter M, Chesnut K, Summerford C, Samulski RJ, Muzyczka N. 1999. Recombinant adeno-associated virus purification using novel methods improves infectious titer and yield. Gene Ther 6(6):973–985.