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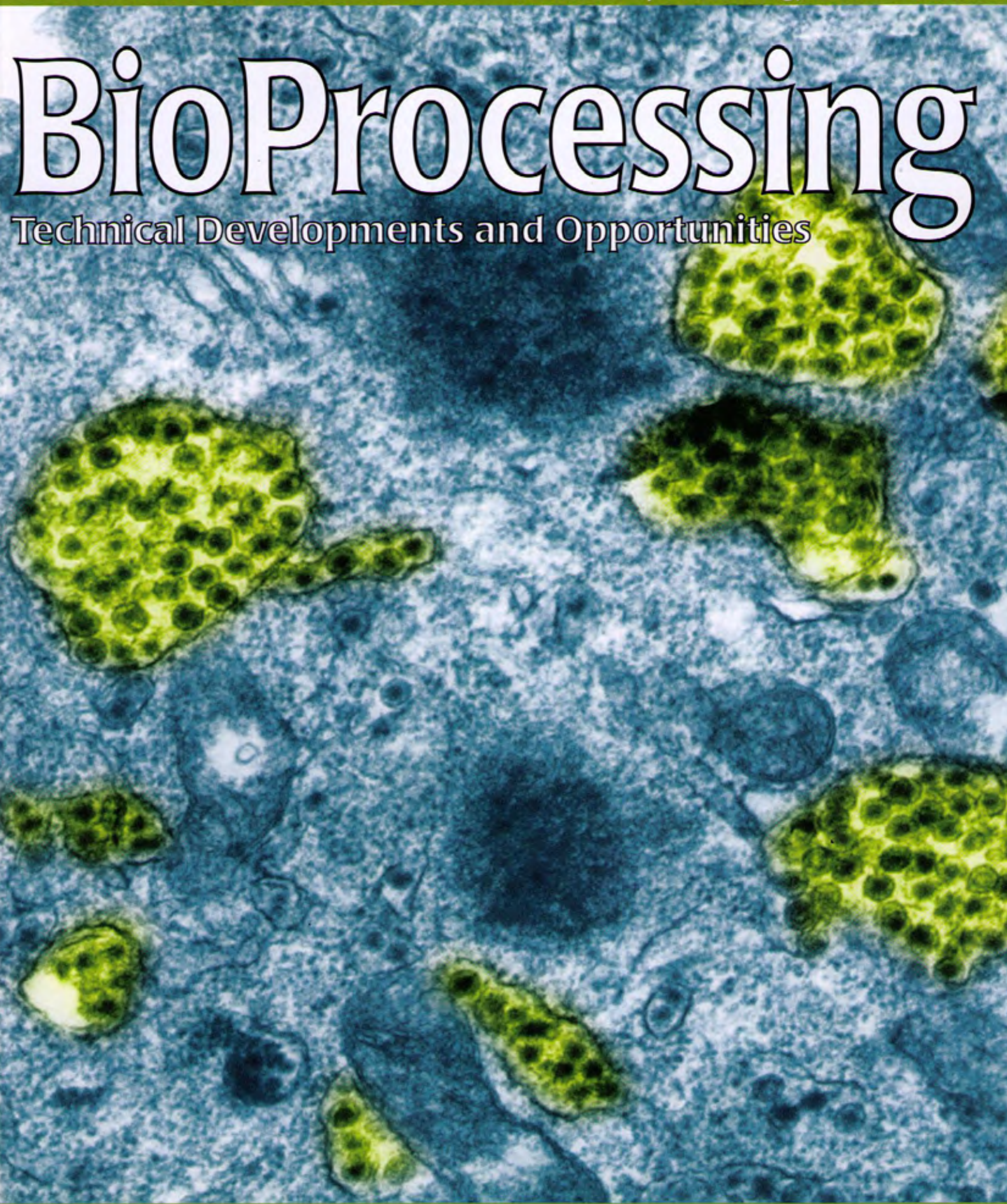

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Chemical Cell Lysis For Large-Scale Adenoviral Vector Production

BY M.I. FITCHMUN, C. LANIO, B. GRIMM, E. PROKOPENKO, J. ZHOU, AND S. JOSEPHS

Production of non-enveloped viruses generally requires a cell lysis procedure to liberate mature particles trapped within their host cells. The standard bench-scale practice of using freeze/thaw cycles is simple and effective, but heat transfer limitations restrict the technique to relatively small applications. Here we show that a ten-minute treatment with a dilute mixture of polysorbate-80 and tri-butyl phosphate effectively liberates adenovirus from host cells. Virus recovery is excellent and relative volumes are low, with concentrations exceeding 10^{12} vp/mL. Also, in contrast to other techniques, treatment with polysorbate and tri-butyl phosphate yields lysates that are non-viscous and relatively low in cell debris. We have used this technique successfully with cell suspensions ranging in volume from 100 microliters to 10 liters. Virus has also been effectively recovered from cells trapped by microcarriers.

Introduction

Over the last several decades, advances in animal virus culture techniques have facilitated developments in prophylactic vaccines, basic and applied research, and recently gene therapy.[1] In general, *in vitro* virus expansion involves introducing virus to an established culture of permissive host cells, followed by an incubation period, and then a harvest of the virus containing cell supernatant or cell lysates. Production of non-enveloped viruses, in both laboratory and large scale manufacturing settings, often requires a lysis procedure to liberate mature particles that are trapped within their host cells well after the conclusion of virus biosynthesis. Notable examples include adenovirus (Ad) and parvovirus, or adeno-associated virus (AAV).[2] The standard bench-scale practice of using freeze/thaw cycles is simple and effective, but heat transfer limitations restrict the technique to relatively low-volume applications. Mechanical cell lysis techniques such as micro-fluidization have proven to be effective in high volume manufacturing settings.[3] However, mechanical techniques involve cumbersome equipment that requires maintenance and cleaning. Moreover, micro-fluidization is generally unsuitable for small-scale applications, necessitating additional study when transitioning from bench to pilot-scale development. Detergent cell lysis techniques have also been described.[4] These techniques have the advantage of scalability, but tend to liberate large quantities of cell debris and DNA, which result in viscous solutions that complicate vector purification.

It has been known for some time that treatment with tri-butyl phosphate, and a nonionic surfactant such as Triton™ X-100 or Tween™-80, can disrupt lipid bilayers and thereby inactivate enveloped viruses.[5,6] Today, researchers use these agents to inactivate potential pathogens during clinical manufacturing of blood-derived, and protein-based, therapeutics. However, such chemical treatments do not typically inactivate non-enveloped viruses, even though scientists hypothesize that these treatments may prove useful during the manufacturing of Ad and AAV, among others.[5,7] Specifically, they might be used to disrupt plasma membranes and thereby release virus from cells upon completion of biosynthesis.

This report outlines a new chemical lysis technique that utilizes a mixture of polysorbate-80 and tri-butyl phosphate (TnBP) to effectively and quickly liberate recombinant Ad from host cells. The technique has been used successfully with cell suspensions ranging in volume from 100 microliters to 10 liters. Benefits include the facts that the resulting lysates exhibit low viscosity, and the virus is unaffected by prolonged exposure to the chemical agents. Virus has also been effectively recovered from cells trapped by microcarriers. Importantly, we employ chemical agents familiar to regulatory agencies in the US, Europe, and Japan. The assessment techniques utilized make residual chemical levels easy to measure, and these materials have proven easy to clear during downstream processing without having to modify established manufacturing procedures.

Corresponding author ***Mark Fitchmun, Ph.D.*** *is a manager at GenStar Therapeutics Corp., San Diego, California; fitchmun@san.rr.com.*

Materials and Methods

Preparation of chemical lysis stock solution

A concentrated stock of lysis solution was prepared by combining NF grade polysorbate-80 (JT Baker, Phillipsburg, NJ) and UPS/NF grade tri-butyl phosphate (JT Baker) in a 3:1 ratio.

This solution was stored in an amber glass bottle, since many plastic formulations are soluble in TnBP. Actually, TnBP, as well as the undiluted chemical lysis stock solution, should not be allowed to contact materials other than glass or polypropylene. When diluted to working concentrations, the mixture no longer degrades laboratory plastic ware.

Virus culture

Early generation (DE1) recombinant Ad5-based vectors, which contain a ß-gal expression cassette, were propagated in the AdE1-complimenting cell line (A549E1) described previously.[8] Briefly, the cells were grown in either tissue culture flasks (Corning Inc., Corning, NY) or Cell Factories (Nalge Nunc, Naperville, IL). The growth medium used was DMEM (Invitrogen, Carlsbad, CA) supplemented with fetal bovine serum 10% (v/v) (Invitrogen). Confluent cultures were infected with virus and incubated until a cytopathic effect was observed, generally between 40 and 60 hours post-infection.

Cell lysis

Once the cytopathic effect was well progressed in the experiments conducted in tissue culture flasks and cell factories, cells were harvested and re-suspended in their conditioned media. Lysis stock solution was then mixed into the cell suspensions, in ratios ranging from 1:75 to 1:300, and then allowed to incubate at room temperature for times ranging from 60 seconds to 2 hours. During incubation, samples were mixed approximately every ten minutes. Controls were either freeze/thawed three times in a dry ice/ethanol slurry, or they received no treatment other than matched incubations. Samples were centrifuged at 16,000 g for 30 seconds, and the supernatants were collected for analysis.

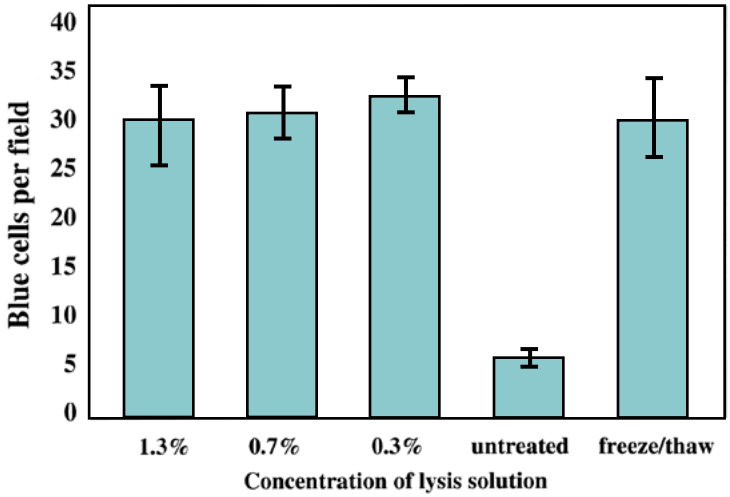

Figure 1: Virus yield as a function of the amount of lysis solution used. Various concentrations of lysis solution were added to A549E1 cells that were infected with virus containing a ß-gal reporter gene. Supernatants collected after a 10-minute incubation were diluted 1:1000 and used to infect HEK-293. The 293 cells were stained with X-gal 24 hours post infection, and the blue cells were counted. For comparison, both 293 and A549E1 cells were subjected to freeze/thaw lysis or were left untreated.

Virus purification and analysis

Cell lysate, either chemical or freeze/thaw derived, was layered on top of a CsCl step gradient with a density range of 1.4 g/mL to 1.25 g/mL. Following centrifugation at 113,000 g (60 minutes at 10 °C) in a SW28 rotor (Beckman, Fullerton, California), crude virus was collected from the interface between the lower two solutions, and then adjusted to a density of 1.33 g/mL. The density-adjusted virus was further purified using two equilibrium density centrifugations, each at 39,000 g for 20 hours at 10 °C in a VTi50 rotor (Beckman). Finally, purified virus was dialyzed into Dulbecco's PBS containing 10% glycerol by volume. Virus concentration was determined by anion exchange high performance chromatography, the residual polysorbate was measured with a wet chemical assay for ether groups, and the residual TnBP was detected via gas-liquid chromatography and flame-ionization.

ß-gal activity determination

Using 10^6 HEK-293 cells/well, 12-well plates were seeded and incubated overnight. Cells were then infected with lysates at a 1/1000 dilution, and then incubated over a second night. The following day, cells were fixed, stained with X-gal staining solution, and incubated at 37 °C for 30 minutes. Finally, the blue cells were counted.

Results

Virus was grown in tissue culture as described earlier. Once a cytopathic effect was observed, cells were harvested and re-suspended in a volume of spent media equal to that of the packed cells. Stock lysis solution was diluted into the cell suspensions at ratios of 1:75, 1:150, or 1:300, and then allowed to incubate at room temperature for ten minutes. The samples were then centrifuged to remove gross particulates, and finally assayed for ß-gal activity. For comparison, a lysate was prepared by substituting three freeze/thaw cycles for the chemical treatment. The negative control was handled in an identical manner, but lacked a lysis step. The three chemically treated samples, as well as the freeze/thawed sample, resulted in high virus yields that were not distinguishable from one another. Virus yield from untreated samples was low, yielding approximately 4 blue cells per field, as compared to an average of 31 for chemically treated and freeze/thawed samples (Fig. 1).

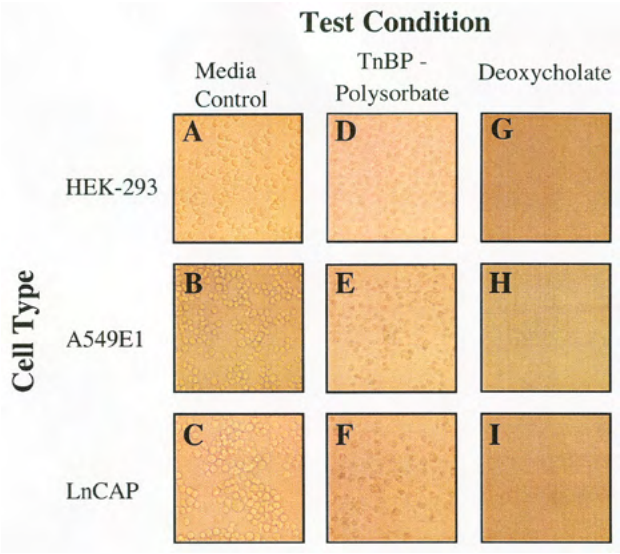

Figure 2: Comparison of Different Chemical Lysis Agents.
Different cell types used in the production of recombinant adenovirus were harvested, and then incubated for 15 minutes in DMEM (A-C), DMEM plus 1% TnBP/Polysorbate stock solution (D-F), or DMEM plus 0.5% deoxycholate (G-I). Cells were visualized using phase contrast microscopy.

During experiments used to compare different lysis techniques, it was observed that lysates generated with TnBP/polysorbate contained markedly fewer solid and oily precipitates than the samples prepared with either freeze/thaw or mechanical micro-fluidization techniques. Detergent lysis techniques, such as treatment with 0.5% deoxycholate, produced highly viscous solutions. Of the techniques tested, lysates generated with TnBP/polysorbate contained the lowest levels of gross contaminants, and were the easiest to purify with chromatography or density gradient centrifugation (data not shown). Microscopic observation revealed that lysates generated with TnBP/polysorbate were relatively free of particulates, but more interesting, the cells did not fully disintegrate during the lysis procedure. The fact that much of the cellular material remained intact seemed consistent with the observation that these lysates were cleaner and less viscous. The bulk of cellular material was easily removed via filtration or low speed centrifugation.

To better visualize this phenomenon, the wells of a 12-well plate were filled with one mL of media, or with media containing either 1% TnBP/polysorbate lysis solution or 0.5% deoxycholate (the latter, an agent often used for detergent-based lysis). HEK-293, A549E1, and LnCAP cells were then added into the various wells, and finally photographed after 15 minutes (Figure 2). Upon treatment with TnBP/polysorbate, all three cell types appeared to lose a well-defined plasma membrane, but retained their approximate size and shape. On the other hand, treatment with deoxycholate caused all three cell types to disintegrate and discharge their constituents into the solution.

To determine the rate of lysis, TnBP/polysorbate stock lysis solution was diluted into the suspensions of virus laden cells, at ratios of 1:75, 1:150, or 1:300, and was allowed to incubate at room temperature for 1, 3, 10, or 30 minutes with mixing every ten minutes, where applicable. The samples were then centrifuged to remove gross particulates, and finally assayed for ß-gal activity. In all cases, maximum recovery of activity was obtained within ten minutes, and statistically different yields were not observed among the samples treated with different concentrations of the lysis solution (Table 1, page 39).

To determine the effects of prolonged exposure of adenovirus to chemical lysis agents, stock lysis solution was diluted into the cell suspensions, at a ratio of 1:100, and were then allowed to incubate at room temperature for 30 or 120 minutes with mixing every ten minutes. For comparison, a lysate was prepared by substituting three freeze/thaw cycles for the chemical treatment.

The samples were then centrifuged to remove gross particulates, and finally assayed for ß-gal activity. Recovery of activity from the two chemically treated samples, as well as the freeze/thawed control, resulted in high virus yields that were not distinguishable from one another (Fig. 3).

In addition, we assessed the extent to which chemical lysis agents were removed during pilot scale virus production. To achieve an accurate assessment, lysates were prepared from a 1:100 mixture of stock lysis solution, and the cell suspension was incubated at room temperature for 30 minutes, with mixing every ten minutes. The lysate was then centrifuged briefly at low speed to remove gross particulates, a process that was followed by a step density gradient ultracentrifugation to isolate crude virus. Final purification was accomplished with equilibrium density ultracentrifugation, performed twice, and then by dialysis into phosphate buffered saline containing 10% glycerol. Post-production analysis revealed that final levels of both polysorbate and tributyl phosphate were well within safe levels for intravenous administration (Table 2), and that there was no significant difference in the virus activity from that of the freeze/thaw controls.

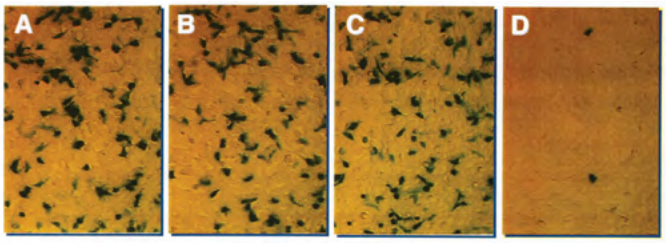

Figure 3: Virus infectivity after exposure to chemical lysis agents.
A549E1 cells infected with virus containing a ß-gal reporter gene were lysed with a 1:100 dilution lysis solution or three freeze/thaw cycles. Supernatants were collected at 30 minutes (A) or 120 minutes (B), diluted 1:1000 and used to infect 293 cells. The 293 cells were stained with X-gal at 24 hours post-infection. For comparison, in the same experiment cells were lysed with three freeze/thaw cycles (C) or were left untreated (D).

Table 1: Virus recovery as a function of concentration and exposure time to TnBP/polysorbate.

Dilution of Lysis Stock	Incubation Time: 1 min.	3 min.	10 min.	30 min.
1:75	20.8 ± 2.6	22.0 ± 4.0	30.0 ± 3.2	31.0 ± 2.6
1:150	23.8 ± 4.8	20.6 ± 5.4	30.6 ± 5.4	31.2 ± 4.4
1:300	16.6 ± 2.1	18.8 ± 3.6	29.8 ± 7.0	34.2 ± 7.1

Table 2: Removal of lysis agents during vector purification.

	Initial values	Final values	% Removal
Polysorbate	6667 ppm	132 ppm	98
Tri-butyl/ phosphate	3333 ppm	0.5 ppm	99.99

Discussion

The chemical-based cell lysis technique tested here is gentle and rapid, and as effective in liberating biologically active adenovirus from host cells as other methods we evaluated. However, this method is unique in its ability to be fully scalable. Other methods we tested included freeze/thaw techniques, which are poorly suited for large volume applications, and micro-fluidization, which is poorly suited for scaled down applications. Of all the techniques evaluated, including other detergent based techniques, the polysorbate-80 and TnBP method described here produced non-viscous lysates containing the lowest levels of gross contaminants, and thereby simplified downstream processing. Importantly, we employed chemical agents familiar to regulatory agencies in the US, Europe, and Japan. Assessment techniques made residual levels easy to calculate, and the material was easily cleared during downstream processing without modifying established manufacturing procedures. This technique provided consistent outcomes across a wide range of conditions, where neither the concentration of lysis agents nor exposure time significantly affected virus yield or activity.

Procedures were easily adapted for harvesting virus from microcarriers in a 10-liter stirred tank bioreactor. Briefly, once the cytopathic effect was well progressed, lysis stock solution was added directly into the reactor which was then stirred for 30 minutes. Microcarriers were removed using a coarse filter, and the product was concentrated by tangential flow ultrafiltration. The process took approximately 90 minutes and was accomplished in a completely closed, sterile system where all wetted parts were disposable. Virus recovery and biological activity were comparable to those seen at tissue culture flask and cell factory scales. Increasing the process volumes to hundreds of liters would not be expected to increase processing times.

Mechanistically, it appeared that in combination, polysorbate-80 and TnBP disrupt the host cell's plasma membranes and thereby allow trapped virions to freely diffuse into the surrounding media. The virus remained unharmed because the chemical agents neither disrupted protein structures nor protein-protein interactions. This result led us to expect that these agents would also be effective with other non-enveloped viruses such as AAV, and we encourage other groups to investigate the utility of this technique for their own applications. We used polysorbate-80 and tri-butyl phosphate during processing of therapeutic non-enveloped viruses, which might have the added benefit of inactivating pathogenic, lipid-containing contaminants such as HIV and mycoplasma. The ability to disrupt lipid structures, without disrupting protein structures, was likely responsible for the observation that these lysates were non-viscous and relatively free of cellular debris and greasy residues. We interpreted these observations as indicating that lipids are well solubilized, while many protein structures, including cytoskeletal and nuclear matrix structures, remain intact. Presumably, host chromosomal DNA remains trapped within the remains of the cells after lysis.

In conclusion, the cell lysis technique described here is well suited for adenoviral vector production at all scales, ranging from the bench-top to large-scale GMP manufacturing. Scaling up or scaling down necessitates no change in methodologies. The process eliminates inefficiencies and expense, is highly reproducible, and is unlikely to generate regulatory concerns in a clinical manufacturing settings.

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