

REVIEWS

Bioprocess Engineering Issues That Would Be Faced in Producing a DNA Vaccine at up to 100 m³ Fermentation Scale for an Influenza Pandemic

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The risk of a pandemic with a virulent form of influenza is acknowledged by the World Health Organization (WHO) and other agencies. Current vaccine production facilities would be unable to meet the global requirement for vaccine. As a possible supplement a DNA vaccine may be appropriate, and bioprocess engineering factors bearing on the use of existing biopharmaceutical and antibiotics plants to produce it are described. This approach addresses the uncertainty of timing of a pandemic that precludes purpose-built facilities. The strengths and weaknesses of alternative downstream processing routes are analyzed, and several gaps in public domain information are addressed. The conclusion is that such processing would be challenging but feasible.

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Introduction

The World Health Organization (WHO) has expressed increasing concern that a bird flu characterized as H5N1 poses a serious risk of causing a human pandemic (1), and the issue has been highlighted in the scientific press (2, 3). At present a small number of fatalities have occurred by infection from chickens to humans and rare human to human transfer (4). If a form allowing ready infection of humans were to occur, according to WHO the virus has the potential to infect at least 20% of the global population. Efforts are being made to develop a conventional virus-based vaccine, but the production process is relatively slow and the number of specialist producers small (5). The expectation from past pandemics is that global spread would occur in 6 months (6). This is approximately the period required for vaccine production in hen eggs under favorable conditions together with the brief clinical studies that are required in Europe (7). World capacity for influenza vaccine has been 900 million units of a monovalent vaccine (1). Some 65% of this is produced in Europe. It will be difficult for countries without capacity to put virus-based vaccine production in place just for a possible pandemic, and increased production will probably occur slowly as wider general vaccination for inter-pandemic periods develops. The 900 million doses will be reduced if more than one injection of the normal amount is required or if the need for protection against new human strains such as the California H3N2 type becomes pressing. In addition a candidate virus-based vaccine prepared in 2003 was not usable because by 2004 antigenic differences had occurred (8) so that applicability of stocks remains uncertain. Potential drugs are available but on an even smaller scale and of variable effectiveness against different

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strains. Against this background a DNA vaccine is of global interest since in principle it might be possible to produce this on many more industrial sites.

At present there is no commercial influenza DNA vaccine, though human trials for this virus have been successful at Phase 1 (9). Ulmer (10) (2002) has noted that a wide variety of small animal studies of DNA vaccines for flu have been promising, and since this review Soboll and colleagues (11) have reported that particle-mediated influenza DNA vaccination in the horse, a large animal, was successful though it activated different responses compared with viral infection. As discussed later, the issue of what size of dose of DNA vaccine is needed for humans with their considerable mass is crucial. A particularly detailed patent application on the concept of an influenza DNA vaccine has been published (12). Human trials on DNA vaccines for HIV (13), Ebola virus (14), and malaria (15) also are under way.

Kodihalli and colleagues (16) showed that a DNA vaccine encoding hemagglutinin could provide protective immunity against H5N1 influenza virus infection in mice. They (16) also noted that the plasmid DNA could be defined at any time from the latest infected tissue and estimated a matched DNA vaccine could be prepared (as a culture) in 1 month, with isolation of the initial DNA from the virus requiring BSL3 containment. In normal circumstances it would take a number of years for a novel form of a vaccine to progress to a point where it would be applied to a human population. However, accelerated human trials could be contemplated if heavy fatalities from a virulent strain threatened and the pandemic showed the potential for continuing to outrun conventional vaccine supply. There would be major medical, social, regulatory and political issues raised, but equally critical would be the supply of DNA vaccine on the kilogram scale necessary. In an influenza pandemic a DNA vaccine would not need to be cheaper or better than a conventional vaccine but it must be capable of fast production. The particular aim of this Review is to explore the feasibility of using existing plants for manufacture.

A handful of companies in the United States and Europe have the expertise, facilities, and platform strains to manufacture DNA vaccines to clinical grade standards. Probably only one or two currently have the capacity to produce quantities that could address whole populations. Experience of the 1976 swine flu potential pandemic in the U.S. (17) suggests that it would be extremely difficult for any government to supply flu vaccine to others in a pandemic before its own nationals were fully covered. Similarly it may be possible to license strains, patents, and know-how, but given the relevance of these to other commercial vaccine targets, that will require more negotiation than seems likely to occur until a pressing emergency exists. The purpose of this paper is to address the process issues that would be faced outside the small group of current companies producing plasmid DNA for trials and to identify the most relevant literature so that the technical challenges are discussed. The publications and patents cited give a great deal more detail than can be summarized here and would be an essential basis for any process planning. Because there are significant gaps in the published knowledge the present paper also either provides a foundation for making choices or defines the gaps. Finally, because there are so few companies with capacities for large scale manufacture specifically for DNA vaccine and potentially no time to build tailored capacity, it has been necessary to identify existing types

of facilities that might be switched. Neither of the most likely, biopharmaceutical protein or nonpenicillin antibiotics plants, are ideal, but both could in principle be applied. Most biopharmaceutical plants are located in the U.S. and Europe. The great majority are for recombinant protein production rather than plasmid DNA. In that situation the front end fermentation and recovery systems will be partly applicable, though the oxygen and heat transfer capacity may not always be geared to the usual *E. coli* source of plasmid DNA. The purification stages of current biopharmaceutical facilities are heavily chromatographic, but because of the relatively much larger size of plasmid DNA, the capacity of columns will not match the need. It also is possible in principle that some of the mammalian cell facilities for protein production could be utilized for virus-based vaccines. This option will not be considered further here, though such a vaccine would be the first choice because it is closer to existing safe products. The technology is even more demanding than that for DNA vaccines and so harder to transfer.

Beyond biopharmaceutical facilities the main pharmaceutical capacity globally will be for antibiotics, and here fermenters of 100 m³ will be common. The relevant downstream processing will be dominated by liquid-liquid extractors, resin-based ion exchange, and hydrophobic sorption-desorption. In addition it is regulatory practice that penicillin production facilities are not used for other products because of the concern for cross contamination and the impact on penicillin-allergic patients. The numbers actually testing allergic are much smaller than patient numbers reporting it (18), but at 0.015–0.04% cases of anaphylaxis the 150,000–400,000 per 100 million becomes very significant with potential pandemic vaccination. Other antibiotics pose a lesser problem but it remains an issue (19), and plant revalidation would be required as indeed it would formally in other facilities. Significant antibiotics capacity would need to be retained not least to deal with microbial infections secondary to influenza, but relatively speaking global capacity is high.

There are very large uncertainties in the potential dose and probably in the plasmid DNA titers that would be achieved in practice with nontailored plants, at much larger scales than currently used and with less experienced operators. These are addressed in the discussion. Finally the uptake of the vaccine by the population will be hard to predict and will be likely to depend on the risk perceived by individuals of the pandemic virus versus a previously unused vaccine type. For this reason the paper comments on issues at both 10 and 100 m³ fermentation scales. The 100 m³ scale is principally relevant to antibiotics facilities, but these would be the only ones available on some continents. They could also potentially process large amounts quickly. To be of value the facilities to be used would need to be operating in the pharmaceutical sector and with Good Manufacturing Practice (GMP). Equally only staff experienced in all issues of producing pharmaceutical products could address the challenge, and even then it would be difficult unless there was detailed input from those experienced in commercial plasmid processing. The greatest scale challenge would occur in the initial plasmid DNA recovery operations, but it is also the case that conventional purification methods would be stretched.

Several publications have reviewed aspects of the larger scale preparation of plasmid DNA (e.g., refs 20–24 and particularly ref 25). However, none have explored the specific bioprocess engineering issues raised by production at 10–100 m³ fermentation scale utilizing

existing industrial plants. Regulatory issues are at the heart of all pharmaceutical processes and have been reviewed for plasmid DNA (20, 26–28). They will be addressed here as they bear on the use of existing facilities. The process and material it produces will need to meet regulatory guidelines such as those of the FDA (<http://www.fda.gov/cber/gdlns/plasnavac.htm>). There would also be some parallels in assessing safety with current guidelines for virus-based vaccines for a pandemic (<http://www.emea.eu.int/pdfs/human/vwp/471703en.pdf>).

Laboratory Procedures, Methods of Analysis, and Properties of Plasmid DNA

Because those who might be required to implement a switch in plant use will not necessarily have knowledge of the field of DNA vaccines, the properties of the plasmid DNA involved and its key contaminants are briefly summarized. The main alternative process routes to extraction are also summarized. Two methods with many variants have been used to prepare plasmid DNA in the laboratory. In the first, lysozyme is used to prepare a spheroplast from *E. coli*, which is then lysed by brief heating to near boiling point in the presence of a detergent, usually Triton X-100 (29). A second major route utilizes sodium hydroxide and the detergent sodium dodecyl sulfate (SDS) for lysis (30). The resulting material is neutralized by potassium acetate, which leads to a flocculant precipitate. By choosing the correct pH or equivalent sodium hydroxide concentration the chromosomal DNA together with protein are irreversibly denatured while neutralization recovers the plasmid largely in its desirable supercoiled form. The clarified liquor from the suspension represents the feedstock for further purification. The process in the laboratory is performed with cells from fermentation that have been harvested and resuspended typically at a concentration of 125 g/L wet weight. Following these two alternative methods of cell lysis a variety of laboratory methods are used to produce pure plasmid DNA. Classical methods involved 2-propanol precipitation, cesium chloride gradient centrifugation, and *n*-butanol or phenol extraction. These are not susceptible to scale-up.

The methods of analysis of plasmid DNA and its contaminants have been addressed recently (20, 31–35). The form of the plasmid required is the covalently closed supercoiled one and the contaminants include the molecule nicked on one strand (open circle forms) or on both strands at or near the same junction (linear forms). To give some idea of the size, a linear plasmid of 3000 base pairs (3 kb) has a molecular weight of 2×10^6 Da and a length of 1 μ m. A supercoiled plasmid probably of 5–10 kb would be involved in a DNA vaccine for influenza. Such a plasmid would contain a pathogen-derived sequence, though it could, like the proposed H5N1 viral-based vaccine, be an altered sequence. Given that more than 50% of the cell solids are protein, more than 20% RNA, and that the plasmid DNA level will be 1–3%, the challenge of purification is large. The *E. coli* chromosomal DNA, which represents about 3% of the cell solids, is a contaminant with properties potentially close to those of the plasmid DNA. In addition, with a level of *E. coli* lipopolysaccharide of about 4%, reducing endotoxin from 10^7 EU/mL to less than 1 EU/mL will be demanding. The *E. coli* strain has a considerable bearing on the outcome, and ideally this will have been selected for high productivity. In a situation where large numbers of patients might eventually be treated, it will be especially crucial that potential DNA integration into human chromosomes

be avoided. Therefore, as Prather and colleagues (25) noted, care should be taken in the choice of the insert and in avoiding homologous sequences between the human genome and the plasmid DNA. Promoters and terminator regions should also be carefully selected to restrict their biological activities on the sequences inserted in the plasmid. In a global pandemic situation the organism will be likely to come from an agency or company experienced in the appropriate DNA science and technology and, like virus-based vaccines, represent an international consensus.

Plasmid DNA is a much larger molecular structure than proteins and less compact so that it is more susceptible to shear forces (36) depending on the plasmid size. For plasmid DNA of 20 kilobase (kb) or smaller the effect is less serious for most operations than the shear-induced damage that the very large chromosomal *E. coli* DNA suffers. If chromosomal DNA is degraded to approach the size of the plasmid DNA, this material is difficult to remove. One virtue of alkaline lysis is that the denatured chromosomal *E. coli* DNA is mostly in the form of single strands that differ significantly from supercoiled double-stranded plasmid DNA in some separation properties.

Pandemic Considerations and Process Choice

For clinical trials or for the preparation of plasmid DNA for small selected groups, any well described method of preparation of clinical grade material will suffice, though a subsequent change to address larger quantities will pose regulatory problems. When whole populations are the target, fermentation scales of 10 m^3 or above will mean that the downstream processing steps must have a good capacity and, as discussed below, operations such as chromatography and expanded bed adsorption become less attractive. In addition, at large scale and with a potentially simultaneous global demand, the consumables such as reagents and chromatographic media must be available commercially on a substantial scale. Preferably they should be produced by manufacturers in a number of countries because commandeering by some governments cannot be ruled out. Though cost will be less of an issue in a pandemic, simple processes and reagents are attractive because they facilitate rapid progress. All large scale purification routes to plasmid DNA (Figure 1) are the subject of patents, so it will probably be sensible to choose the most attractive with respect to available plant. However, an early capacity to license know-how and patents in an uncertain pre-pandemic period when patent concerns apply could influence the choice. There are emerging technologies that could improve individual processing steps considerably, such as better centrifuge designs, superior chromatographic and expanded bed media, and alternative membrane-based anion exchange adsorbents. Their performance has not been described in as much detail here because current distribution is limited, but their local availability will influence process choice.

Fermentation

The first challenge is to grow the plasmid DNA-containing *E. coli* efficiently on a very large scale. That this is possible is indicated by the commercial production of recombinant insulin using the organism, though details of the fermentation are not publically available. *E. coli* has been grown at a 30 m^3 scale on a defined medium (37), and other engineering studies have been reported at a 12 m^3 scale (38, 39) and 3 m^3 scale (40). A particularly detailed account of scale-up has been given

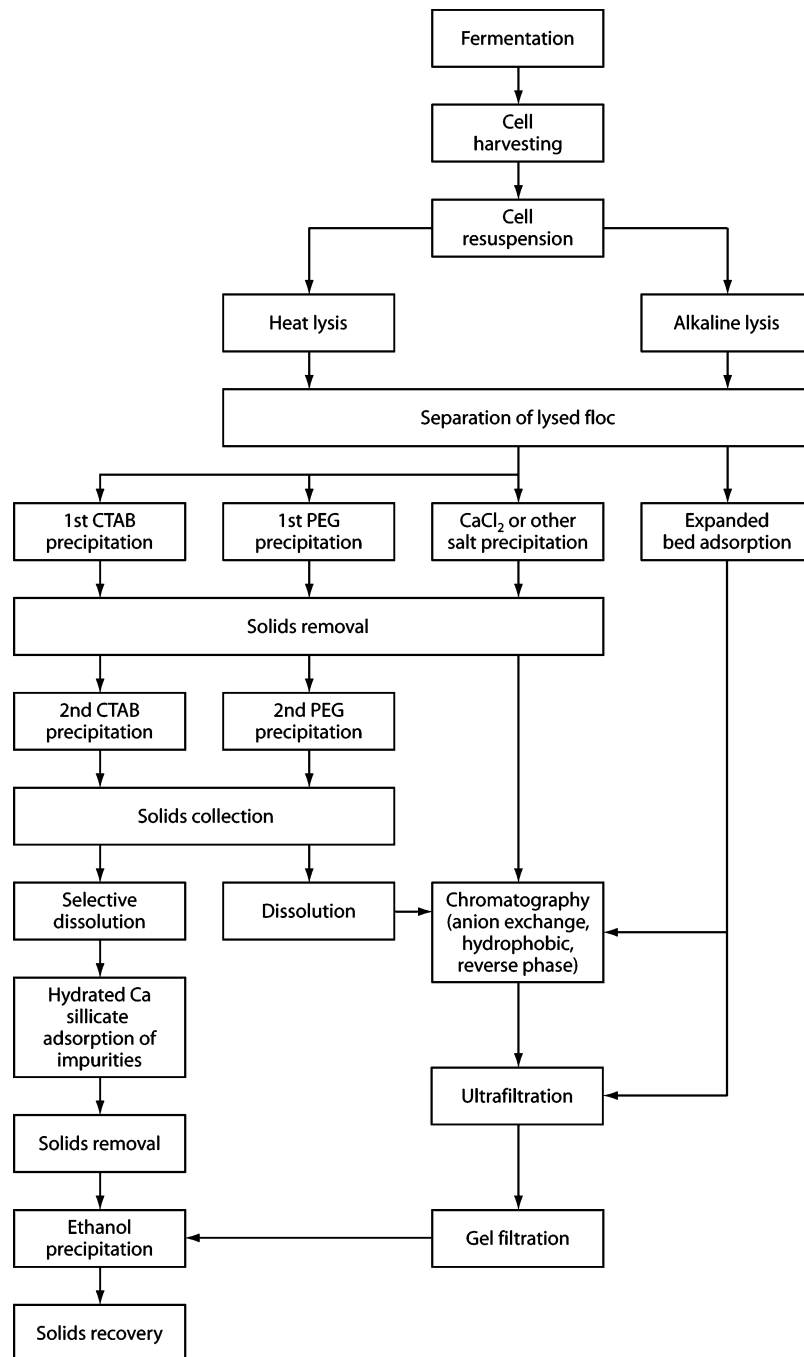


Figure 1. Alternative processing routes for DNA vaccine production.

in Junker (41) specifically with regard to *E. coli*. It covered scales from 30 L to 19 m³ and noted that by comparison with mycelial broths (which will be common with some antibiotics plants that might be used) the rheology is less important. Scale-up based on achieving a minimum dissolved oxygen level was recommended. Though the 30 m³ scale fermentation above was conducted with a defined medium, the majority of smaller scale studies have used yeast extract with simple salts and carbon and nitrogen sources. The general balance of advantage and disadvantage of defined media for commercial fermentations has been reviewed (42).

Fed-batch mode has been shown to yield higher cell densities and as a consequence higher volumetric productivities of plasmid DNA (43–47). There may be some facilities where this is less easily achieved, and Voss and colleagues (48) have noted relatively high productivity

for batch fermentation of 50 mg/L with an ammonium chloride supplemented medium. Chartrain (49) noted earlier use of monosodium glutamate but also its replacement due to quality concerns by “NH₄OH” functioning for pH control and nitrogen source with a feed of 60% glycerol solution and a medium containing neomycin. Here oxygen uptake and carbon dioxide evolution rates were measured online, and thiamine, ammonium, and glycerol offline. Changing the carbon:nitrogen balance can increase the plasmid yield 10-fold in a semidefined medium, and the medium can have an effect on cell resuspension and alkaline lysis (50). Fed-batch mode has the advantage that production of supercoiled plasmid is stable over a longer period than for batch fermentation. This is helpful in terms of downstream processing at large scale where harvesting occupies longer periods: Chartrain (49) showed a relatively constant specific yield

Table 1. Centrifugation Performance and Product Stream Quality for Cell Harvesting, Alkaline Lysate Separation, CTAB Precipitate Recovery, and Hydrated Calcium Silicate plus Impurity Removal

% solids sedimented	flow rate/equivalent settling area ^a (L/m ² /h)					
	whole <i>E. coli</i> cells	heat-treated clarified lysate ^c	neutralized whole <i>E. coli</i> lysate ^c	CTAB plasmid DNA ppt with cellulose ^c	homogenized <i>E. coli</i> ^b	NSO mammalian cell ^e
99.99	0.027 ^b		0.090			
99.9	0.038 ^b		0.180			
99.0	0.057 ^b (0.039) ^c 0.015 ^d	(0.003)	0.270	>0.16	(0.004)	0.018
95.0	0.081 ^b 0.076 ^c 0.032 ^d	0.006		>0.16	0.010	0.065
fraction of solids holding space ^g	0.4 ^b	0.2	0.5	0.1 ^f	0.6	

^a Users and their centrifuge suppliers are familiar with equivalent settling area (Σ) to characterize machines. The values given can simply be multiplied by the equivalent settling area in m² to give the flow rate in L h⁻¹ corresponding to the % solids sedimented in the left-hand column. Because many biopharmaceutical plants process mammalian cells, a value is included as are values for homogenized *E. coli* cells with which some biopharmaceutical users will be familiar. The data are all from pilot scale intermittent discharge disk machines so that allowance for shear is inherent. Values obtained by extrapolation appear in parentheses; all others were obtained by interpolation of probability functions. ^b Higgins et al. (54) *E. coli*. ^c UCL, unpublished, for rec *E. coli*. ^d UCL, unpublished for high cell density *E. coli*. ^e Hutchison et al., unpublished. ^f Dominated by cellulose, which absorbs about 10 times its weight in liquor. ^g Packed solids capacity before breakthrough as a fraction of the total solids holding capacity.

between 30 and 40 h cultivation time of a 1300 L fermentation fed from about 12 h. Rozkov and colleagues (51) using continuous culture methods have shown that resistance genes on the plasmid vector can place a significantly higher oxygen demand or necessitate a lower feed rate in high-density cultures than for plasmid-free cultures. This will become a bigger issue at scales of 10–100 m³ for *E. coli* at high cell density. Productivity for plasmid DNA reflects differences in cell density, especially between batch and fed-batch of 1–8 g/L dry weight (batch) to 60–120 g/L (fed-batch) (25). Prather and colleagues (25) summarize plasmid DNA productivity values in laboratory and small pilot plants ranging from 4 to 225 mg/L.

Companies specializing in plasmid DNA production can attain yields of as much as a gram per liter of fermentation broth. This is only achieved by equal attention to the construction of the strain, the medium, and the fermentation conditions. The higher level also rests on use of a defined medium tailored to the strain. If the specific yield of plasmid per mass of cells is very high, the total dry weight of cells per liter need not be exceptional and this reduces the downstream solids loading. Ideally in a pandemic situation the best strain would be available and the medium known. If this is not in place, a lower yield of plasmid DNA will be a necessary assumption. If the strain available is not so highly selected, a medium with yeast extract may be more appropriate, and global supplies of yeast extract are fairly widely distributed.

Cell Harvesting

With 10–100 m³ of fermentation broth the main harvesting options are centrifugation and membrane-based cross-flow microfiltration or ultrafiltration. Classical filtration with a bacterium such as *E. coli* is constrained by cell size. Flocculating agents have been applied but tend to interfere with the product (52). Some lysis of *E. coli* harvested by disk centrifuges has been observed (53, 54). Premature lysis is more pronounced with defined media (53), and this may be another factor favoring a yeast extract medium. More detailed studies indicate that premature lysis is principally associated with cell discharge in the underflow stream. It is accompanied by additional intracellular damage as revealed

by flow cytometry measurements (Gerard Chan et al., unpublished). In addition, holding cells at 13 °C for 2–6 h reduced supercoiled content substantially and increased contamination due to premature cell membrane lysis. Larger disk machines may produce better results and new designs of disk machine also will reduce the effect. An automated discharge tubular centrifuge produces less damage, but such machines are also less common. Centrifuge performance data for *E. coli* cell harvesting are shown in Table 1. Most laboratory and pilot studies are conducted with frozen pastes and these can differ in robustness from fresh cells direct from a fermenter, which is the form of material likely to be essential with 10 m³ fermentation scale and above. The freezing of cells has previously been used as an opportunity to assess the quality of the fermentation product.

Cross-flow microfiltration has become increasingly common in both biopharmaceutical and antibiotics plants. In each case it is used near the front end of the process so that it should have the capacity for cell harvesting. Several studies of cross-flow microfiltration of bacteria have been published (55–57). A plasmid DNA patent (58) mentions concentrating the cells from the fermenter 4-fold using tangential flow “filtration” across a 500 kDa nominal molecular weight cutoff membrane. The concentrated cells were then diafiltered with three equivalent volumes of sterilized saline and resuspended to an OD of 30 at 600 nm (1 OD₆₀₀ = 0.5 g/L dry cell weight). Stratton and Meagher (59) observed that ultrafiltration membranes gave higher flux rates with *E. coli* than microfiltration membranes. From the above papers and our own measurements with *E. coli* the flux, leaving aside the atypical initial rate, is likely to be about 25 L/m²/h. This will, of course, be membrane-specific. Meyer and colleagues (60) observed a flux reduction of 15% when cells were stored for 5 h at 4 °C and 30% reduction with freezing and thawing.

Cell Lysis

Alkali-Based Chemical Process. Both alkaline and heat lysis will be described because they each have advantages and disadvantages on a very large scale. Alkali lysis becomes more difficult to engineer as scale increases. That is because as soon as the SDS-alkali

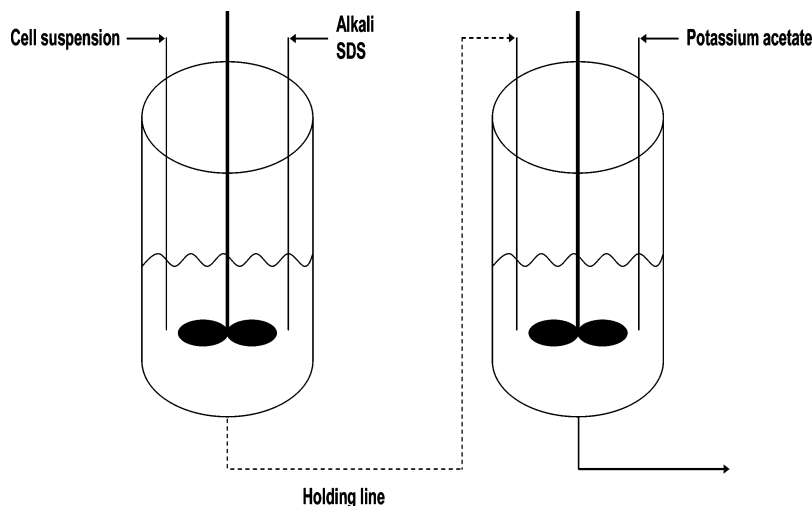


Figure 2. Possible tank-based continuous alkaline lysis.

penetrates the cell there is a potential for pH damage to supercoiled plasmid DNA. If batch mixing intensity is increased, the precipitated chromosomal *E. coli* DNA is susceptible to shear damage. Several papers on the kinetics of the process have been published (61–63). Ciccolini and colleagues (63) indicated that a shear rate of about 460 s^{-1} with neutralization after 300 s was optimal in terms of maximum plasmid DNA release while avoiding excessive chromosomal DNA floc breakdown and maximum SDS precipitation of contaminants. The results were obtained in laminar flow shear fields. The neutralizing potassium acetate stream needs to be as cold as possible to insolubilize the contaminants, and at a m^3 scale this will demand significant chilling capacity. Recently the impact of lysis time, pH, and mixing has been examined (64). The study demonstrated several interesting features related to large industrial scale operation:

- The pH changes very little above 12 as alkali of 0.2 M or less is added, and it may be feasible to use overall alkali molarity rather than potentially unreliable pH as a measure of the condition desired, provided the cell concentration is maintained constant.
- There is a reasonable NaOH molarity gap between irreversible chromosomal denaturation (below 0.06 M) and damage to supercoiled plasmid DNA (generally above 0.10 M).
- To achieve the best outcome, the effective pH and time need to be within an operating window that these variables together define.

The observations bear on the adoption of existing industrial plants in several ways. At large scales, batch mixing will involve increasingly difficult compromises. At pilot level these can to a degree be ameliorated by tailored mixer design (65, 66). Where feasible they are desirable but at 1–10 m^3 scale and in a pandemic situation with existing plants being taken over, this would generally not be an option. However, elements of the ideal batch mixer may be feasible in individual facilities. First, the use of low power number impellers is desirable, and if possible several on the same shaft. Helical ribbon mixers are also an option and large marine impellers are reasonably effective. Individual impeller diameters should preferably be greater than half the vessel diameter. Second, there should be several feed points into well-mixed regions around the distal end of one or more impellers. A particular challenge of alkaline lysis is the sudden increase in viscosity and the formation

of a sticky precipitate that can envelop the agitators. It is essential that agitators are not underpowered such that they are slowed by the high viscosity. Baffles will help but must stand free of the vessel sides to avoid dead zones. In the absence of specialist impellers, several separate mixers may be applied. Because higher pH strongly enhances shear-induced DNA degradation (67), it will be necessary to err on the side of stronger mixing to avoid such extremes. Ideal mixing would be continuous with a brief period of high shear followed by conditions of low shear, though as the scale increases even mixing in a continuous system will take longer. As recognized by Wan and colleagues (68), the full gains come with potassium acetate also added subsequently in continuous mode, but this increases the engineering complexity. A patent linked to that of Wan (69) described the small pilot continuous flow system. The continuous injection of the neutralized lysate into a batch collection vessel will agitate preformed floc with consequences for solid–liquid separation by flotation (see below). Companies do manufacture very large scale in-line mixers of the kind that would be needed to treat multi- m^3 volumes. However, the degree of engineering modification of existing plants is nontrivial. A similar result could be achieved by the setup shown in Figure 2 where existing pipelines and tanks are used. This also potentially allows the alkali and potassium acetate streams to be smaller and more concentrated. The degree of agitation in the two tanks must be adequate to avoid significant bypassing of fluid. We have explored the approach recently at pilot scale, and though there is still the need to manage the mixing on filling of the first tank, the system is workable. Most published procedures have a period of holding prior to neutralization of a few minutes, and this means interposing a holding line between the tanks.

Heat-Induced Lysis. In addition to the early literature (29) there is a patent (70) that described continuous heat treatment in a stainless steel tube maintained at 70–77 °C followed after 30 min or less by cooling in the same tubular system. No visible clogging of the tube occurred. Industrial scale heat exchangers will be common in many plants, though the complexity of their internals may lead to a higher risk of blockage. One of the advantages of the method is that the volume of lysate is not increased. A disadvantage for some purification methods is that the chromosomal DNA produced is mostly in a double-stranded form. In addition the patent (70) noted that the yield of plasmid DNA was reduced

by 4–5 times if lysozyme was omitted. It also seems to be the case that there is a 6-fold reduction in throughput because of a need for a longer holding time. In a pandemic situation the availability of recombinant lysozyme that was used would be an issue. Watson (71) noted that heat lysis represented 25% of the processing costs, with lysozyme accounting for the majority of the cost. The process needs to be controlled well because above a temperature of 93 °C there is plasmid degradation and below 75 °C the *E. coli* deoxyribonuclease that can degrade genomic DNA evidently is not fully denatured. The viscosity of the cell suspension, initially 1.94 cp (0.00195 Ns/m²) at 24 °C rose to 40 cp (0.040 Ns/m) on lysis. This procedure used Triton X-100 rather than the SDS employed with alkali. Kresheck and Altschuler (72) in filing a patent on an alternative detergent noted that Triton X-100 and SDS can degrade with time. A plate heat exchanger system of a relevant type is described in Higgins and colleagues (54) for treating mechanically disrupted *E. coli* to remove RNA. It was found necessary to use widely spaced plates and more of them to increase the heat transfer area so that hot water rather than steam could be used as the heating medium. A three-stage arrangement was used in which heating to 55 °C was achieved in two stages with a 30 s residence time, using in the first the previously heated process liquor (regeneration stage) and in the second, water at 65 °C. After the regeneration stage further cooling was achieved with chilled water. The heating and cooling media were passed in cross-flow. Wang et al. (73) made a detailed study of the heating method without lysozyme and concluded that it was capable of a comparable yield to the alkali/SDS procedure. The paper provided viscosity data and kinetic models of lysis.

Harvesting of the Lysed Floc

Neutralized Alkaline Lysate. Achieving harvest at 1–10 m³ scale of a shear-sensitive floc of about 100 g/L wet weight is difficult. Thatcher and colleagues (74) described the use of bag filters with 100 and 25 μm woven nylon in stainless steel housings, and such filters are available for the scales contemplated. Nienow et al. (2002) (66) indicated that this was followed by 5 μm filtration via a depth filter. A patent (75) described mixing 1200 g of diatomaceous earth with 13.5 L of neutralized lysate and filtering on precoated paper in 15 min. The 12 L recovered was a measure of the very large amount of body feed (88 g/L versus the more usual 15–30 g/L). It was also gravity-fed, whereas pressured feeds of 0.5 barg are normal (76, 77). Precoat would typically be 1 kg/m² of filter area. When a total neutralized lysate suspension was filtered (78), the flux rates with a variety of filter cloths, meshes, and filter aids were relatively low if solids extrusion was to be avoided (average about 100 L/m²/h but ceasing in an hour) and filter blinding was observed with or without filter aid precoat. The process would be improved by body feed of filter aid and to a degree by rotary vacuum filtration with a high knife cutting rate, as observed (79) for cell debris. To meet biopharmaceutical cGMP, a rotary vacuum filter would need to be of an enclosed design as used in handling solvent and sometimes employed in blood plasma fractionation. Adsorption of plasmid DNA on the diatomaceous earth will not occur between pH 7 to 10, and this is further aided by avoiding the presence of divalent ions or masking them by complexing agents. Even then levels above 20 mM for potassium ions, 50 mM for sodium ions, and 150 mM for ammonium ions will lead to loss by adsorption (80). The requirements of process revalidation

mean that the filtration medium, if it is cloth, should ideally be disposable because there is much inaccessible surface area.

In terms of large scale filtration it is possible that lessons can be learned from human plasma fractionators, a number of whom use rotating vertical leaf filters. De Jonge and colleagues (81) described the background to this though not the large scale detail. Wolter (82) described the use of a vertical rotating discharge precoat filter with body feed of between 20 and 80 g/L diatomaceous earth with 1–3 mm of precoat for a heat-ethanol isolation of albumin from plasma. This yielded flux rates of 77–120 L/m²/h with 80 μm pore filter elements. The suspension was recirculated until clarity was achieved with a filtration surface of 2.2 m². Some 180 L of the total volume of 220 L feed was recovered as filtrate, and recovery could be increased to 90% by washing. Contamination was not a problem, and its gentle nature was stressed. Johnston and colleagues (83) have described the use of cellulose filter aids in human plasma fractionation. They found that concentrations of cellulose between 0.5% to 2.0% (w/v) were best. They noted the need to allow time for the cellulose to swell. The patent showed filtration to achieve a turbidity 4-fold lower than centrifugation, though even with washing yield was about 10% less. Pressure drops across the filter rose according to the fineness of the cellulose from 0.07 to 0.4 bar. The results for plate and frame filters and for rotating vertical leaf filters were very similar, and the data related to filters up to 18 m² area. Given the scouring problems of diatomaceous earth with pumps, centrifuges, and electropolished surfaces, this is relevant also to potential centrifugal separation. Disposal of filter aid and recombinant *E. coli* residues on the scale contemplated would be nontrivial.

Floc can be separated by flotation at a tens of liters scale (76, 84). Wright and colleagues (85) observed on the other hand that their equivalent floc sank. However, they observed breakup of the floc in a strongly agitated reactor, and air salted out by the cold potassium acetate and associated with the floc will be driven out by strong mixing. The bottom liquor from flotation studies (76) was low in particulates (0.2–2.5 g/L). However though it filtered at a higher rate than an unsettled floc, the percentage of contaminant chromosomal DNA also was higher. This is probably because the full floc deposit on a filter acts as a secondary filter aid. At 15 L scale about 80% of the bottom liquor could be collected. If the product in the floc is lost, it is significant in a pandemic situation where speed of production would be critical, but there will also be loss into the solids stream if disk centrifuges are used (about 15%). Breul (86) recently reported a flotation procedure enhanced by application of a vacuum for which a patent is pending and noted pilot scale (700 and 1200 L) operations. The enhancement of floc flotation by air followed by vacuum deaeration is used on a large scale in other industries, and pressure vessels with vacuum lines are available in many pharmaceutical facilities.

We have recently examined the use of a disk centrifuge for recovery. Neutralized alkaline lysate was readily recovered to yield a supernatant with low solids (OD₆₀₀ = 0.08). The centrifugal performance data are given in Table 1 and show a comparatively good outcome in terms of sedimentation, but the fraction of the holding space that is usable is modest. It will be important to achieve a very high clarity here if subsequent stages are to be effective. A small increase in degraded plasmid isoforms and lower molecular weight species was observed with

increasing flow rates to the centrifuges. Here, shear on discharge of the solids is not a problem and discharge of the liquid phase containing the plasmids does not cause significant damage. The earlier observation of substantial damage to plasmid DNA in an automated discharge tubular centrifuge (21) related to discharge of the supernatant. Watson (71) indicated that centrifugation was being considered as potentially a preliminary to polishing filtration in the heat lysis process (70). One other option that would apply to the liquor obtained beneath a floated floc is centrifugation by a multichamber machine. Given a solids content of 0.2 g/L at 15 L scale (76) and their large solids capacity, these machines may have the ability to polish substantial volumes. In these machines, shear on entry to the rotor does rise substantially with scale (87).

In recovery of the liquid phase, cross-flow microfiltration has the advantage over centrifugation because it avoids loss with the centrifuge underflow. Preliminary studies we have made of cross-flow microfiltration with unclarified neutralized lysate in a small pilot system have suggested this was not promising. However Sandberg and colleagues (88) reported a flux of 13 L/m²/h with a 100,000 MW nominal cutoff ultrafiltration hollow fiber cartridge with a fairly dilute lysate from a 48 g/L cell paste. The use of a cross-flow microfiltration (0.45 μm) membrane on prefiltered material has been examined (89), though this was of nitrocellulose, which deliberately absorbs single-stranded genomic DNA. Given the size of the pores it is likely that plasmid DNA fluxes found are still relevant in relation to nonadsorptive membranes. Retentate recirculation rates at linear velocities of 0.025–0.9 m/s and transmembrane pressure between 200 and 2000 Pa gave permeate flux rates of about 20 L/m²/h after the initial rapid fall in flux. Recovery of plasmid DNA was 100%. However, sustaining such a low pressure drop at a large scale would probably require a vibrating membrane system.

Heat Lysate. The inherently continuous nature of the large scale heat lysis process suggests direct linkage to continuous flow solid–liquid separation. The nature of heat coagulated material will favor subsequent separation provided shear does not degrade it. Higgins and colleagues (54) reported the recovery of heat-treated *E. coli* homogenate material by disk centrifuge. By comparison with whole *E. coli* the heat-treated material rapidly interfered with the efficiency of the machine and only a part of the sludge space could be filled after each bowl opening to achieve good clarification (see Table 1). However, most of the debris had been removed prior to this step, which may have led to it being stickier. Lander and colleagues (90, 91) used filtration here and in subsequent CTAB and hydrated calcium silicate adsorption steps, and their recirculation of filtrate to achieve clarity and use of pressurized air to displace liquor are key features in the high performance achieved.

Post-Lysate Recovery Options

Following lysis and solid–liquid separation, there is a need to purify the supercoiled plasmid DNA product from impurities, which include nicked and linear forms as well as remaining chromosomal DNA, protein, RNA, and endotoxins. At the laboratory scale this has been accomplished by either immediate progression to chromatography or at pilot scale to a variety of lower resolution separations steps prior to chromatography. These include precipitation, membrane separation, and batch sorption–desorption. Most of the data on these operations, even in patents claiming scale-up relevance, involve solid–

liquid separation that uses high-speed centrifugation. Therefore, the quality of the supernatants will be much higher than achieved if industrial scale centrifugation is used for lysate harvesting, and this will affect all subsequent steps. Caution is thus required in using the data for assessing large scale performance. In particular, colloid fouling will reduce cross-flow membrane rates and adsorbent or chromatographic capacities. Filtration (90, 91) will give a clarified lysate closer to that achieved by laboratory centrifugation.

Fractional Precipitation

Given a capacity to conduct solid–liquid separation of the shear-sensitive solids, precipitation is a method that can deal with very large volumes. In plasmid DNA purification the method may precipitate either the plasmid or some of its contaminants. If the former, then a resultant dissolution step is needed that may represent a point of potential shear-induced yield loss because of the intensity of mixing required. Plasmid DNA has been precipitated by 2-propanol, poly(ethylene glycol), and cetyltrimethylammonium bromide. 2-Propanol is unattractive at large scale because of flammability. Precipitation by poly(ethylene glycol) (PEG) is sensitive to the size of the molecules, which makes this agent valuable in separating plasmid DNA from larger chromosomal DNA (92). For the selective precipitation of impurities a range of salts such as lithium chloride, sodium acetate, ammonium acetate, and sulfate and calcium chloride have been used, and the most promising are described below. Lithium chloride would not be favored because of the potential toxicity of the metal ion.

CTAB Fractional Precipitation. The reagent CTAB has been used for many years in plasmid purification. Recently a patent was published on its use following the lysozyme/heat lysis procedure (91) followed by a paper from the same company with complementary information (90). The reagent after heat lysis was found capable of first precipitating chromosomal, linear, and open circle plasmid DNA, and following its removal, plasmid DNA could be precipitated prior to RNA, endotoxin, and protein. The precipitation was done in the presence of filter aid, and the level of CTAB needed for chromosomal DNA and later plasmid DNA precipitation was assessed by a particle size probe analyzer, which detected the formation of large aggregates of filter aid and precipitate. This was important because the precise level of CTAB varied with, for example, the level of lysis agent Triton-X100. With a 2% w/v CTAB precipitate containing 10 g/L of diatomaceous earth and a batch that yielded 20 g of plasmid, filtration in a Nutsche-type stirred filter tank was used with a 50 cm diameter and 25 μm stainless steel mesh (93). The cake was washed by reslurrying and refiltering. Watson (2005) (71) showed an overall yield for the step of about 83%. It was found critical in removing RNA to wash with 2-propanol and 50 mM NaCl prior to dissolution of the DNA. This allowed the subsequent hydrated calcium silicate adsorption to remove remaining traces of RNA. The stirred Nutsche has the advantages of easy resuspension of the cake and with stainless steel mesh is cleanable in a way cloth is not. Such devices are produced with up to at least 12.5 m² filtration area and some pharmaceutical facilities will have a capacity for large batch filtration suited to this approach. Lander and colleagues (90, 91) described a subsequent extraction of the second CTAB precipitate containing principally plasmid DNA with an increasing sodium chloride concentration. Here by measuring viscosity it was possible to detect the point where just the

plasmid DNA was solubilized leaving contaminants insoluble. The step yield was about 76% (71).

The CTAB patent (91) noted that the alternative alkaline lysis step may be used but that "SDS is omitted from the alkaline step to prevent interference with CTAB-induced DNA precipitation". However, SDS contributes to the speed of alkaline lysis and to the formation of the insoluble floc of irreversibly denatured chromosomal DNA and proteins on addition of potassium acetate. Gani and colleagues (94) analyzed the conditions of formation of an SDS-CTAB-DNA ternary complex. It showed that though this complex is soluble in the case of double-stranded DNA at neutral pH, it is insoluble at higher pH. In the case of heat lysis, 95% of the chromosomal DNA remains double-stranded (64) and therefore soluble in the ternary complex. In a study of batch CTAB precipitation of clarified, neutralized alkali/SDS lysate, we observed precipitation of the total nucleic acid and a subsequent filtration rate of 160 L/m²/h with diatomaceous earth. We then examined centrifugation as an alternative to filtration using cellulose powder as a coprecipitant to avoid scouring (Table 1). The level of cellulose had to be adjusted to about 1–2 g/L to avoid occupying too much of the bowl space that occurred at 5 g/L or foaming on discharge at 0.5 g/L.

Other Precipitants. Eon-Duval and colleagues (95) compared the effectiveness of ammonium acetate and sulfate, sodium sulfate, tripotassium citrate, and calcium chloride as precipitants of RNA and showed that the latter was superior. It gave up to 94% RNA removal from lab centrifuge clarified lysate as well as 96% protein reduction, 98% removal of chromosomal DNA, and 91% endotoxin reduction. The pH was critical and needed to be below 8 to avoid large plasmid loss. The conditions of 1.4 M calcium chloride at pH 8 for 10 min were found optimal. In this and an accompanying paper (96) they showed the complementarity of a cross-flow ultrafiltration procedure in removing low molecular weight RNA. A patent (69) described the use of solid ammonium sulfate to 80% saturation. This would be difficult to centrifuge in industrial machines due to the salt density. The step was followed logically by reverse phase chromatography. It is possible to precipitate genomic DNA at 4% PEG and plasmid DNA at 10% (92, 97).

Fractionation Using Cross-Flow Ultrafiltration

Cross or tangential flow microfiltration separation has been referred to for lysate harvesting, but other publications have focused on the use of ultrafiltration for molecular fractionation (96, 98, 99). Eon-Duval and colleagues (96) have published a laboratory scale description of RNA removal by this method showing the need for a low ionic strength during diafiltration to fractionate the RNA to permeate and retain the plasmid DNA. At least 50 diafiltration volumes were required for RNA clearance. They showed that with a plasmid DNA load increased from 111 to 939 mg/m² the percentage of residual RNA rose from 60% to 80% of that in the lysate. They explored both 300 and 500 kDa (protein) membranes but found no advantage in the larger pore. As in a patent (98) it was evident the degree of upstream concentration polarization had a strong influence on the composition of the permeate, and even with the lower molecular weight cutoff membrane, plasmid was lost without there being a polarized layer. They concluded that prior calcium chloride precipitation was necessary to achieve good removal of RNA by cross-flow ultra-

filtration. The membrane was useful in removing contaminants including protein and calcium chloride. The patent (98) employed earlier treatment with ribonuclease so that easier removal of degraded RNA occurred than in the above study (96). The latter authors observed higher plasmid retention in some experiments on a calcium chloride pretreated liquor with a 100 kDa membrane and noted that this was used because calcium chloride compacts the plasmid DNA. Both groups noted the need for considerable washing to recover plasmid from the membrane. Both (98, 96) stated a plasmid load of about 1 g/m² of membrane area. Neither gave flux versus time profiles. However Kahn and colleagues (99) using a 500–1000 kDa membrane operating at a constant transmembrane pressure of 10–15 psi (0.7–1 bar) and a retentate flow of 320 L/m²/h observed a flux rate of about 16 L/m²/h. Bussey and colleagues (98) used lower molecular weight ultrafiltration (below 100 kDa) in later stage diafiltration, and it is possible that with the prior removal of high molecular weight RNA this may allow fluxes higher than 300–500 kDa membranes used with deliberate gel polarization. A 30 kDa UF membrane has been used to concentrate 25-fold after expanded bed anion exchange purification. (100)

Batch and Expanded Bed Adsorption and Chromatography

The feasibility of using more conventional biopharmaceutical methods of purification rests on the scale of available systems. The earliest point at which such procedures could be applied is to the clarified neutralized lysate. Here expanded beds have the advantage that they are tolerant of more particulate material.

Hydrated Calcium Silicate Adsorption. Further purification of CTAB-treated material using hydrated calcium silicate has been described (58, 101). Chromosomal DNA and open-circular plasmid bind with greater affinity than the more conformationally constrained supercoiled plasmid. With a level of 36 g/g of total DNA at 0.6 M NaCl the plasmid, which in a pure state can bind, was largely displaced back into solution. The chromosomal DNA was reduced to below 0.1% w/w of plasmid over at least 6 h in an operation that must be slow to allow the required balance of adsorption. Impurities such as endotoxin and reagents such as the CTAB also were removed by selective binding to the hydrated calcium silicate with the solids removed by filtration (101). Watson (71) recorded a level of less than 1 µg/mL CTAB in the final product and a step yield of 72%. Though several global manufacturers list hydrated calcium silicates, they will not necessarily all meet the pharmaceutical specifications and this applies also to diatomaceous earths. The plasmid DNA was precipitated by ethanol, sterile filtered to yield with the CTAB procedures a clinical grade material of >99% purity. The hydrated calcium silicate does not scour in the way that diatomaceous material does so that disk centrifugation in principle is an option.

Anion Exchange. A patent (74) described anion exchange binding to an expanded bed with bag filtered extract with 7.5 L of anion exchange medium. The feed was 32 L of lysate treated with ribonuclease. The linear flow was 105 cm/h and the patent described a possible need for recycle to complete plasmid DNA binding. The bed was washed to remove particulates, and then elution was done with downward flow. If further chromatography was used, 7–14 L of eluate were diluted 13-fold and

applied to two 10 L columns, and optionally detergents such as Triton X-100 or Tween 20 (0.1–1% v/v) were added to aid removal of endotoxins. Ferreira and colleagues (102) also examined expanded bed recovery with anion exchangers. They operated with an upward linear flow of 300 cm/h and an expanded bed height of 50 cm. Elution was with 1 M NaCl in downward flow at 120 cm/h. Most of the RNA and protein was in the breakthrough, and high molecular weight RNA, chromosomal DNA, and plasmid DNA forms were eluted later as a single peak. If loading with more than one sedimented column volume equivalent of lysate was attempted there was medium clogging and channeling. This was eased by insertion of an intermediate propanol precipitation step, but they concluded that in this situation there was less justification for expanded bed operation. The difference in perspective from refs 74 and 100 may be due to the latter's use of ribonuclease and the consequent degradation of RNA and possibly to lower ionic strengths in loading. Published examples of really large scale expanded bed separation are rare, e.g., ref 103, where a column of 60 cm diameter \times 100 cm was fed with 1377 L of fermentation broth containing a recombinant protein diluted to reduce the ionic strength. Feed rate was 850 L/h, equivalent to 300 cm/h linear flow rate.

Eon-Duval and Burke (104) have conducted a detailed laboratory study of anion exchange chromatography media. As noted by others (21, 105), a tenticular medium gained by its capacity to allow access of large macromolecules. Media designed for high porosity were the next most effective forms. By careful adjustment of conditions, resolution of plasmid DNA and RNA was achieved with a maximum dynamic capacity of 3 g/L. The paper emphasized the need for prepurification and proposed calcium chloride precipitation and cross-flow membrane separation. In a patent, Bhikhabhai (106) used calcium chloride precipitation prior to two stages of anion exchange. The presence of the calcium chloride altered the charge on the RNA so that it was more readily eluted, leaving DNA bound. The plasmid was separated from chromosomal DNA on a second anion exchanger by using a shallower gradient. A linear flow rate of 150 cm/h has been noted in loading a strong anion exchanger and 60 cm/h in eluting plasmid (107). The step was preceded by alkaline lysis, filtration, diafiltration, PEG, ammonium acetate, and isopropyl precipitations and centrifugal separations. A silica-based anion exchanger has been described, and its application to clinical trial scale purification has been summarized (27). With the use of a proprietary composite depth filter, this produced acceptable material evidently at least up to 25 g of plasmid and utilized about 1.4 kg/kg of wet weight biomass. Breul (86) has indicated that this material can completely separate RNA from plasmid DNA. In all such chromatographic and expanded bed methods, capacity is the key issue particularly with molecules as large as plasmid DNA. Dynamic capacities at 10% breakthrough in packed bed anion exchangers have been given that are below 0.4 g/L of supercoiled plasmid DNA for clarified lysate with no prepurification (105). However, with a PEG fractional precipitation the binding capacities were 2-fold higher. The paper noted a capacity of conventional expanded bed adsorbents of 200 μ m diameter of about 50 mg/L for plasmid DNA. In a patent (108) results were given to support use of poly(ethylene glycol) as a compacting agent during anion exchange and plasmid DNA with 1% PEG was more effectively retained. The feed was lysate preprecipitated sequentially by PEG, ammonium acetate, and propanol.

Recently a new generation of ion exchange membranes has been developed that have higher capacities for plasmid DNA (84, 109, 110). These are likely to be less widely available globally in the event of a pandemic than anion exchange chromatography media but where accessible will represent an attractive alternative in terms of capacity. Haber and colleagues (110) showed that the dynamic capacity of DNA was reduced at increasing flow rates. Membrane capacity in volume per volume terms was approximately an order of magnitude higher than corresponding resins. If only classical ion exchange material is available, and without chromatography systems of appropriate scale, batch adsorption in a stirred system followed by recovery of exchanger by filtration or basket centrifugation is an option, and a basic study of batch adsorption of plasmid DNA has been made (111).

Gel Filtration. Horn and colleagues (75) applied gel filtration to lysate previously purified by filtration, PEG-8000 precipitation, ammonium acetate precipitation, concentration by diafiltration, and propanol precipitation. Feed to a small pilot scale column was 0.2 μ m filtered and fed at 17 cm/h. The loading volume was less than 1% of the total column. Supercoiled product emerged after 0.3–0.4 column volumes. Ferreira and colleagues (112) compared two gel filtration media. Prepurification was by PEG and ammonium acetate precipitation with lab centrifugation. Without effective prepurification the presence of contaminants such as RNA reduced flow rate and resolution. Varley and colleagues (100) using ribonuclease applied gel filtration with a loading of about 11% of the column volume and achieved good resolutions of plasmid DNA and RNA. The stage was preceded by ultrafiltration-based concentration.

Other Chromatographic Methods. Though affinity methods have generally been excluded from this analysis on the grounds that they could not be widely available quickly, metal affinity (113) may be an exception in that the basic chelating adsorbent is available from a major producer and the metal salts are simple reagents. The adsorbents bind single-stranded oligonucleotides and RNA. Hydrophobic interaction chromatography has also been occasionally applied (114). Recently plasmid DNA chromatography has been reviewed (22, 115, 116) and is also summarized elsewhere (23, 25).

Secondary and Finishing Operations and Formulation

The shear sensitivity of plasmid DNA (36) and chromosomal DNA and especially that of the precipitates containing them means that components such as pumps and throttle valves can cause great damage. The behavior with respect to precipitates will vary between pump types in the same manner as for protein precipitates (117), with peristaltic pumps causing least damage and those such as screw pumps and especially centrifugal pumps with throttle valves causing much more. Lander and colleagues (58) note that there can be problems in filtering the ethanolic final precipitate and suggest the precipitated paste is centrifuged and added to 100% ethanol. This is mixed with a high speed homogenizer such as a rotor stator. The paste is simultaneously dehydrated and wet milled into hard particles that are amenable to filtration and drying. They also noted that material produced with the aid of hydrated calcium silicate will contain calcium and indicated its removal by EDTA in conjunction with ultrafiltration or precipitation. Finishing and formulation will be heavily influenced by the means of delivery to be used. The issues of shear

Table 2. Data Extrapolated Linearly from Lander and Colleagues (90, 91) to a 1 m³ Starting Cell Resuspension Scale

stage	vol (m ³)	sc plasmid cumulative yield (%)	sc plasmid purity (%)	sc plasmid concn (mg mL ⁻¹)	sc plasmid mass (g)
lysis (heat) ^a	1.0				
filtrate ^b	0.84	100	84	0.28	
CTAB filtrate ^c	1.09	100	84	0.22	
CTAB filtrate ^d	1.20				
redissolved ppt after 0.5M NaCl	0.13	93	93	1.80	236
filtrate from hydrated Ca silicate ^e	0.07	53	97	1.75	125

^a 7.3 mL of lysozyme (Epicentre 30 ku/ μ L). ^b Diatomaceous earth (Cellpure P300), 25 kg used. Filtrate area, 3 m². ^c CTAB, 2.5 L; diatomaceous earth, 5.7 kg. ^d CTAB, 3.6 L; diatomaceous earth, 4.9 kg. ^e Hydrated calcium silicate, 11.1 kg. The original publication was based on 6 L of suspended cells with an OD₆₀₀ of 30.

sensitivity of the plasmid DNA can loom as large in operations such as injection into a vial as they do during purification, and losses in steps such as sterile filtration with such a large and flexible macromolecule can be substantial. Kong et al. (unpublished) found that plasmid DNA loss on sterile filtration with a 0.22 μ m poly(vinylidene difluoride) membrane varied linearly with molecular weight from 2% to 80% for sizes between 6 and 116 kb. The loss was not significantly changed with flux through the membrane (140–1400 L/m²/h) or concentration (10–1000 μ g/mL). Watson (71) noted a similar trend and observed that for a 5 g/L plasmid solution the filter area needed dropped from about 1 to 0.01–0.1 m² as supercoiled plasmid content rose from 65% to 95% for plasmids of 5.5–11 kb. Stability in shipping and storage (35, 118) will also be important. Lyophilization can be applied, but shear damage must be avoided (Schleef, private communication). Volkin and colleagues (119) described formulation conditions and materials in detail.

Overall Process

The logistics of alternative processes can be illustrated by examples from the literature (Tables 2 and 3). The data have been linearly extrapolated to 1 m³ scales of suspended cells from the small pilot scale volumes reported. Direct comparisons must be made with caution, particularly because the data in Table 3 relates to an initial cell concentration that is nearly twice as high as that in Table 2. The processes may also have been superseded. However, they give some indication of the volumes involved and the quantities of reagents, adsorbents, and chromatographic media. The impact on process volumes of the alkaline lysis route is clear comparing Tables 2 and 3. In alternative routes beyond lysis that used precipitation these volumes could be further doubled. However, the initial downstream volume is about 15% of the original fermentation volume so that after harvesting there is a step change in scale. Table 4 is of interest because, though it is based on a pre-CTAB process patent, the data and the footnotes that have now been added indicate some column volumes based on a simple linear extrapolation to 1 m³ of original lysate. Because the clarified lysate is diafiltered with a 100 kDa membrane, treated with ribonuclease, rediafiltered, and 0.45 μ m filtered, the load on the anion exchanger is reduced versus that of the expanded bed of Table 3. This and the use of a newer generation of porous chromatographic media is reflected in a much smaller required volume of absorbent. All of the purification methods would benefit from use of ribonuclease degradation of high molecular weight RNA. Bovine ribonuclease is excluded on safety grounds, but it is possible to use known strains of *E. coli* expressing a ribonuclease (120).

It is clear that fed-batch is the desirable mode of fermentation. For lysis the value of using lower volume heat lysis rests on a capacity to achieve very large scale

Table 3. Data Extrapolated Linearly from Thatcher and Colleagues (74) and Varley and Colleagues (100) to a 1 m³ Starting Cell Resuspension Scale

stage	vol (m ³)
pre lysis	1.0
post alkali lysis	4.0
post expanded bed ^a	1.6
post ultra filtration	0.01
post gel filtration ^b	0.25

^a Expanded bed anion exchanger, 1.25 m³. ^b Gel filtration medium, 1.16 m³. Fermenter yield, 15.8 mg/m³. Final mass supercoiled plasmid, 39 g. Published scale based on 6 L of suspended cells at 120 g/L (probable OD₆₀₀ = 55), where the total process took 3 days.

treatment with heat exchangers and preferably access to recombinant lysozyme. The alternative alkaline lysis could be applied in most plants but at very large scales may be hard to control. In terms of preferred solid–liquid separation, cell harvesting by cross-flow microfiltration avoids cell damage caused by disk centrifuge discharge and any problems of cell resuspension. Centrifugation of neutralized lysate has proved workable and is also applicable to liquor from beneath a floated floc. Filtration is attractive for CTAB-diatomaceous earth recovery and hydrated calcium silicate removal, but disk centrifuges can be used. In newer process schemes of more conventional types the use of membrane-based methods for fractionation and diafiltration is allowing reduction to a single anion exchange chromatographic step.

Perhaps the most relevant factor for the use of existing cross-flow microfiltration or centrifugation systems in the pandemic context is the expected flux compared with that for the materials these machines will normally operate on. In antibiotics plants this may be large cellular aggregates such as fungal pellets or smaller cells such as Streptomycetes. In biopharmaceutical plants the largest cells will be mammalian. Table 1 allows some centrifuge comparisons. Though many disk centrifuges in antibiotics plants are involved in liquid–liquid separations, most are three-phase machines and can be converted to solid–liquid (clarifiers) by changing the ring dam diameter, though the riser channels in the disks may not be optimal. Postlethwaite and colleagues (121) noted previous microfiltration reports of steady-state fluxes with baker's yeast of 20–50 L/m²/h at concentrations from 0.5 to 20 g/L yeast and with bovine serum albumin present as a soluble component (0.75 g/L). The membrane pore sizes were 0.02–0.45 μ m, and the yeast is representative of existing biopharmaceutical processing with eukaryotes.

Both biopharmaceutical and antibiotics plants have large continuous harvesting capabilities. Antibiotics plants as noted have a majority of downstream operations geared to small molecules. However, microfiltration (122, 123) and ultrafiltration (124) are used for some cell harvesting on a production scale at relevant fluxes. The

Table 4. Purification Data from Lee and Sagar (70)

stage	plasmid product (mg)	% step yield	genomic DNA (mg/mg)	protein (mg/mg)	RNA (mg/mg)	LAL (EU/mg)
clarified lysate ^a	6750	100	0.52	7.6	196	1.1 × 10 ⁷
concentration/RNase ^b /diafiltration/dead-end filtration	6500	93	0.50	1.6	2.21	3.4 × 10 ⁶
anion exchange ^c	4000 of 5000	80	0.41	0.3	0.1	1.2 × 10 ⁴
reversed phase ^d	2300 of 3200	77	0.029	<0.01 ^f	<0.01 ^f	62
concentration/diafiltration into final buffer	2110	100	0.029	<0.01 ^f	<0.01 ^f	2.8
final process yield ^e		54				

^a The original lysate was 4.5 L of paste in 33.7 L. ^b Ribonuclease was used. ^c The anion exchanger (POROS PI/M) would have a volume of 80 L based on 1 m³ of unclarified lysate. ^d The reversed phase medium (POROS R2/M) would have a volume of 154 L based on 1 m³ of unclarified lysate. ^e The yield from 1 m³ of clarified lysate would be 95 g. The contaminants are given as mg per mg plasmid DNA. ^f Below detection limits of assay method.

availability of centrifuges and filters for later stages will vary between products and plants. Depth filtration with recombinant proteins has been principally for secondary clarification where the solids are a residual contaminant rather than the product (125). Because protein processes use disk centrifuges and large cross-flow microfiltration just at the front end, the train of solid-liquid separations implied in harvesting, separation of the post-lysis precipitate and potentially CTAB, hydrated calcium silicate and other precipitates will have to be achieved by reuse of the same centrifuges and microfilters. Chromatographic systems will be undersized for plasmid DNA, and this will be especially so if an existing initial step for protein production uses affinity chromatography, so reducing required subsequent capacity. It seems likely with less than ideal operations in non-purpose-designed plants, especially for solid-liquid separation, that the purities per stage will be poorer and it will be necessary to add steps based on different separation mechanisms. Expanded bed and chromatographic methods rest on the type of media available and older less sophisticated materials will produce poorer separation. Though cost would not be the primary consideration in a pandemic, Watson (71) noted a 10-fold reduction in capital by using the CTAB and hydrated calcium silicate procedures, which reflects the simplicity of equipment used. The reduced cost is divided between lysis, precipitation, adsorption, and final diafiltration, with the latter somewhat larger than the others. In his comparable conventional process, chromatography represented half of the cost.

Discussion

Probably the upper fermentation yield will be 1 g/L and the maximum recovery downstream 40% of this or 0.4 kg/m³. An effective dose of 4 μg was observed in a recent phase 1 trial of a routine influenza DNA vaccine using gold particle delivery (126). However, with a virus previously not experienced by the human population a higher dose may be required, so 10 μg per dose may be a reasonable estimate. There are indications that up to milligrams of plasmid DNA are needed without special delivery agents (15), but this is not a level that could be contemplated and there are now a range of options in terms of particulate and other adjuvants (127, 128) that should reduce this. Wong and colleagues (129, 130) have shown that a DNA vaccine for influenza can be delivered to mice intranasally using liposome-encapsulated plasmid DNA. (For comparison, the dose of each of three hemagglutinin antigens in a virus-based vaccine is 15 μg. The expectation with virus-based vaccines is that a single dose is unlikely to be suitable considering the naivety of the population with regard to a pandemic virus and adjuvants may be needed (Fedson (5); <http://www.emea.eu.int/pdfs/human/vwp/471703en.pdf>)). There is evi-

dence that priming with DNA followed by a boost with a protein-based vaccine is an effective combination (131), and if there is a need for two doses, this could be a way to use virus-based vaccine sparingly. From the above, for a DNA vaccine in a best case, 40 million doses/m³ of fermentation broth and related processing may be possible. However, without all the detailed know-how and tailored facilities an overall yield of one-fifth of the above is perhaps reasonable. If a 10 μg dose is again assumed with an overall yield of 80 g/m³, then 8 million doses/m³ would be available. The inference from these figures and the uncertainties in them is that overall fermentation capacities in many countries would need to be of the order of tens of m³ to quickly address populations rather than just front line groups. With a process cycle of 2–3 weeks, a distribution and immunization time of perhaps 5 weeks and a period of 3–5 weeks for development of immunity the overall cycle is quite long in relation to a pandemic. It suggests that antibiotics plants, commonly of 100 m³ fermentation capacity, could represent a way to achieve the necessary speed, as well as being the only facilities in many countries. If they could address harvesting, lysis, clarification, and CTAB precipitation and recovery, the plasmid solids could be further purified in other smaller facilities. Filtration equipment capable of processing m³ quantities is more common in chemical pharmaceutical facilities and could play a role. In the biopharmaceutical sector, media for mammalian cell culture are now shipped in m³ quantities where sterility is also a critical issue. The one significant difference with the *E. coli* material would be that there could be viable recombinant cells present; however, such material can be shipped with due precaution. On all of these issues it would be much preferable if the best process and delivery technologies were available by negotiation.

The present analysis has assumed a single plasmid coding for one antigen. However, it is known that other genes of the influenza virus can have a very strong impact on the outcome so that more than one gene may be beneficial (132). This poses the challenge of either repeated processes and careful blending or of larger plasmids with higher shear sensitivity (36) which both set new process constraints. The use of di- or polycistronic constructs coexpressing multiple antigens may ease this problem. A recent study suggests oxygen demand in fermentation will be unaffected by plasmid size (133). There have been concerns that the intrinsic DNA structure, which may differ between plasmids, would have an impact on processing, but recent studies indicate a serious effect only with Z-DNA (134) though a triplex form did reduce the relative amount of supercoiled plasmid by 5% in a 3.8 kb plasmid and by up to 36% after freeze thaw.

As noted virtually all steps are the subject of patents, as is the case for new technology associated with the

production of virus-based vaccines. The World Health Organization has stressed the need to resolve intellectual property issues prior to any pandemic. The analytical requirements, though similar in approach to those for biopharmaceutical proteins, are distinctive. For companies unfamiliar with them, analytical services by, for example, contract research organizations could be necessary to achieve speed. Throughout the process the local availability of high grade raw materials will be a key issue (135), and the possibility of commandeering of key materials by governments in some countries must be borne in mind.

At present almost all of the focus of pandemic planning is on virus-based vaccines because they are at the heart of international policies on managing the strain changes in interpandemic years and also represent the established technology. It is unlikely that this position will change unless a low percentage vaccine cover of the global population becomes an issue. The virtue of the virus-based vaccine is that the organization of production, regulation, and clinical use is established. Its disadvantage is that its capacity to respond will be slow and cover may be modest. The challenge for a DNA vaccine at present is the process setup time and the greater need for clinical testing. From our analysis we conclude that processing on the necessary scale would be challenging but feasible. Short of a more imminent threat it will be hard for biopharmaceutical and antibiotics companies to devote time to the issue unless governments take major initiatives. If the potential gains from fast production of DNA vaccines in relation to a pandemic were to be achieved it would be essential that such initiatives were taken. In this uncertain situation the public sector bioprocess engineering community can at least help to prepare the ground for large scale production in case it is needed. The most pressing bioprocess priorities that emerge from the analysis are

1. More detailed comparisons of the virtues of heat versus alkaline lysis as related to large scale operation.

2. Information bearing on the performance of large scale solid-liquid separation in the several stages where this is central.

3. Additional information on the effect of shear forces in secondary but all-pervasive operations such as pumping and in finishing operations such as lyophilization.

4. Studies at all stages with test materials that reflect real process conditions in earlier steps.

Much of the biochemical engineering involved will apply if DNA vaccines come to be used in other major diseases such as malaria or HIV prevention. The authors would welcome comment from all quarters on the issues that have been raised.

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