Methods of enhancing the recovery of plasmid genes from neutralised cell lysate

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I. Theodossiou, O.R.T. Thomas, P. Dunnill

Abstract In this study we have investigated the use of flotation and filtration, singly and combined, to enhance the separation of plasmid containing liquors from neutralised lysates with very different levels of solids. Filtration of crude neutralised lysates, containing roughly 100 g l^{-1} solids, through various diatomaceous earth and cellulose precoat materials was invariably accompanied by severe loss of plasmid through adsorption and/or absorption. The use of more refined and inert filter aids did not alleviate these problems. The finest filter aid, Celatom FP-1SL, gave the best compromise of filtrate clarity (solids content of 0.05 g l^{-1}) and plasmid purity (71%) and was selected for further studies involving combined use of flotation and filtration. Removing the vast bulk of solids prior to filtration by flotation of the floc and draining of the plasmid liquor beneath, impacted dramatically on the filtration performance. Though systematic reductions in the solids challenge per unit filter area were accompanied by increased flux, elevated levels of solids extrusion, chromosomal DNA and protein contamination were also observed, and losses of plasmid to filter aids were still high. We have observed that increasing the scale of operation during lysis and neutralisation from 0.3 or 0.6 l to 15 l is accompanied by significant improvements in separation of cell debris solids from the plasmid and increased recoveries of the plasmid containing liquor. At the latter scale, the drained liquor contained ~80% of the plasmid and the solids content was only 0.2 g l^{-1}.

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1 Introduction

In the next century it may be possible to prevent, cure or significantly modify the course of many serious genetic ailments by introducing heterologous genetic information into defective or damaged tissues [1, 2]. Thus far, some 4,000 specific genetic disorders arising from the action of single mutant genes have been identified [3]. Although the majority of these are rare, some are quite common, for example, cystic fibrosis, a lethal inherited disorder affecting 1 in 2,000 Caucasian children [3]. Currently, the options for gene transfer can be broadly divided into those that use plasmid DNA vectors and those employing viral vectors. To date, most clinical work has focused upon the use of attenuated replication deficient viruses as the vehicles for gene transfer [4]. However, reservations concerning the use of viral vectors as therapeutic agents exist, primarily over safety in respect of replicative reversion and immunogenicity, as well as contained scale up and reproducibility in manufacture [5]. Furthermore, large scale production of viral vectors may be limited by the need to use eukaryotic packaging cell lines [6]. These concerns have driven growing interest in the design of innovative, modified, safer vehicles that are as efficient as adenoviral vectors, but lack the extra genetic information [7] and also in non viral delivery systems based on plasmid DNA, which have been used in naked form and in condensed states with cationic lipids and/or peptide polymers [8–12]. Non viral transfection methods, though generally regarded as safer than viral transduction, are currently less efficient. Consequently, thousands of plasmids must be presented to the target cell in order to achieve successful gene transfer [13]. Given the potential patient numbers and high dose requirements, there is a need to be able to manufacture large quantities of highly purified clinical grade plasmid DNA in a reproducible and scaleable manner. To date, however, no commercial processes have been developed.

The initial stages of downstream processing of plasmid offer several distinct challenges. Many laboratory scale methods for isolation of plasmid DNA have been described but most of these employ techniques and chemicals that make them unsuitable for development as manufacturing processes [14, 15]. The extraction of plasmid DNA from *E. coli* requires gentle techniques that impart minimum shear given the size, associated viscosity and physical/chemical fragility of DNA. Processes that generate high shear forces, such as mechanical disruption of cells [16], and use of continuous industrial centrifuges [15, 17] are

not attractive options. Lee and Sagar [18] recently described a scaleable method, which involved passing *E. coli* suspensions, containing a non ionic surfactant, through a flow-through heat exchanger to lyse the cells and release the plasmid DNA. However, most research groups and emerging companies employ lysis with sodium hydroxide and SDS [19]. Under these conditions chromosomal DNA and globular proteins denature and then on neutralisation precipitate together with cell debris, RNA, degraded plasmid and other impurities as a gel-like floc, leaving intact plasmid DNA in the soluble fraction. The processing of this neutralised cell lysate presents a significant challenge because the floc is extremely susceptible to mechanical shear forces and if degradation occurs the separation of solids from the plasmid is made difficult [15].

In previous work [14, 15], we examined the primary separation of a 7 kb plasmid DNA from neutralised *E. coli* lysates in a nutsche filter operated under pressure. Operating pressure, pore size and the material of the filter membranes exerted considerable impact on purity and yield of plasmid DNA. Filtration through membranes precoated with Celatom FP-1SL diatomaceous earth resulted in very pure plasmid DNA and virtually complete suspended solids and protein removal, but losses of plasmid DNA due to adsorption on to diatomaceous earths were at significant levels [15]. In the same paper we also noted that the floc formed by neutralisation of the alkaline lysate floats and that recovery of the liquor from beneath the floc by draining may be an attractive process option.

In this paper we extend our previous studies by evaluating a comprehensive range of more refined and inert precoat materials for the primary separation of plasmid from neutralised *E. coli* lysates by filtration and we also present results from flotation experiments carried out at both laboratory and pilot scales.

2
Materials and methods

2.1
Materials

The filter precoat materials used in this study (Table 1) were received as gifts from Mr. P. Denyer of British Filters Limited (Plymouth, Devon, UK) and Mr. T.E. Sulpizio of World Minerals Inc. (LaGrande, OR, USA). PX 587-09 filter cloth and HP^{2TM}/cellulose composite filter sheet were also gifts from P & S Filtration (Rossendale, Lancashire, UK) and Carlson Filtration Ltd., (Colne, Lancashire, UK), respectively. Miracloth® filter material was purchased from Calbiochem (La Jolla, CA, USA).

The plasmid pCH110 (7,128 base pairs) was obtained from Pharmacia Biotech (St. Albans, Herts, UK). Bovine pancreatic ribonuclease A (type XII-A), deoxyribonucleic acid from *E. coli* strain B (type VIII: sodium salt), diphenylamine (free base purified), bovine albumin (fraction V, 96–99% albumin), phenol: chloroform: isoamyl alcohol (25:24:1; saturated with 10 mM Tris, pH 8, 1 mM EDTA), sodium acetate buffer solution (3 M, pH 7 ± 0.05 at 25 °C), agarose (molecular biology grade), tris-borate-EDTA buffer (TBE), ethidium bromide and gel loading solution (with EDTA to inactivate nucleases) were purchased from Sigma-Aldrich Company Ltd. (Poole, Dorset, UK). BCA Protein Assay Reagent was supplied by Pierce & Warriner (UK) Ltd., (Chester, Cheshire, UK). Perchloric acid, acetaldehyde and sodium dodecyl sulphate (SDS) were purchased from BDH Laboratory Supplies (Poole, Dorset, UK).

All other chemicals were obtained from BDH Laboratory Supplies and Sigma–Aldrich Company Ltd.

2.2
Production of cells and release of plasmid DNA

The procedures for production of *E. coli* cells containing plasmid pCH110 and release of the plasmid DNA have been discussed in detail recently [15] and only a very brief description of the methods is presented here. Routinely, cells were grown in Terrific broth [20] containing ampicillin in a 30 l working volume batch fermenter (LH Engineering, Reading, Bucks, UK), and were harvested at an OD_{600nm} of ~6 by centrifugation in a Sharples 1P tubular bowl centrifuge (Sharples Pennwalt Ltd., Camberley, Surrey, UK). The cell pastes obtained (~500 g packed wet weight) were either stored at 4 °C overnight for use the next morning in pilot scale lysis experiments, or at –20 °C for laboratory experiments.

Cells (fresh or defrosted) were resuspended to a final concentration of ~120 g l^{-1} with 10 mM Tris/HCl, 1 mM EDTA pH 8. Plasmid was released from cells by lysis with NaOH and SDS. The alkaline cell lysates where then neutralised by addition of potassium acetate and left on ice for 1 h. The ratio of volumes of cell suspension to alkaline lysis solution (0.2 M NaOH; 1% w/v SDS) to neutralising solution (3 M potassium acetate pH 5.5) was 1:1:1 in all experiments.

Alkaline lysis mixtures typically contained plasmid DNA, total DNA, total protein and wet solids at the following concentrations: 65.1 ± 4.3 mg l^{-1} (n = 11),

Table 1. Filter materials and manufacturer's specifications

Filter materials	Description	Permeability (darcies)
Celatom FP-1SL	calcined diatomaceous earth	0.07
Chromosorb® FC	purified dehydrated diatomaceous earth	0.1
Celite 521 (A/W)	calcined diatomaceous earth	0.3
Celite Hyflo® Super-Cel (A/W)	flux calcined diatomaceous earth	1
HP^{2TM} 1000	flux calcined diatomaceous earth	1
Fibra-Cel® BH-100	α-cellulose	1.2
HP^{2TM}/cellulose composite	filter sheet composed of 50% HP^{2TM} and 50% α-cellulose	0.83

0.97 ± 0.14 g l^{-1} (n = 11), 4.77 ± 0.63 g l^{-1} (n = 10) and 95.3 ± 4.6 g l^{-1} (n = 15) respectively. All values are given as the mean ± sample standard deviation (n = number of separate analyses).

2.3 Filtration experiments

For filtration studies a stainless steel nutsche laboratory filter "60" (Schenk Laboratory Systems Limited, Oxton, UK) was employed [14, 15].

In experiments employing compressed filter sheets composed of filter aid materials, a supporting septum was not used. In all other cases the filter was fitted with a 60 mm diameter polypropylene filter cloth of 5 μm pore size (PX 587-09), which was selected on the basis of previous studies [14, 15]. In most experiments the cloth was precoated with a variety of filter aid materials (see Table 2). Diatomaceous earth and α-cellulose were applied to 5 μm septa as slurries in distilled water using the quantities of material per unit filter area recommended by Schweitzer [21], i.e. 100 mg cm^{-2} for the diatomaceous earths and 225 mg cm^{-2} for α-cellulose. The filter aids were dewatered by applying a pressure of 0.4 barg and the depths of the precoats formed on top of the 5 μm septum, were ~3 mm and ~9 mm for the diatomaceous earths and cellulose respectively. Neutralised *E. coli* lysates (described in Sections 2.2 and 2.4) were poured into the filter unit, which was then tightly sealed. Filtration was carried out using a variable pressure regime, since we have previously shown that a 'soft' start followed by step increases in pressure gives the best combination of filtration rate and filtrate clarity [15]. No pressure was applied for the first 5 min. Thereafter, the pressure was increased in steps every 5 min in the following sequence: 0.4, 0.8, 1.0 and 1.4 barg, and filtrate fractions were collected manually every 0.25–3 min, depending on the flow rate, and were assayed for turbidity, plasmid DNA, total DNA and protein contents as described in Section 2.5. At the end of the experiment, the filter unit was dismantled and the retentate and filter materials were collected and subjected to analysis for plasmid DNA, total DNA and protein (see Section 2.5).

2.4 Flotation experiments

Flotation experiments were carried out at a variety of scales (0.3, 0.6 and 15 l) in purpose-designed transparent polycarbonate vessels (0.5, 1 and 25 l; with aspect ratios of ~1.5:1) fitted with spigots for draining. Alkaline cell lysis and neutralisation were performed in situ and the neutralised cell lysates left to age at 10 °C for 1 h. At the end of the ageing period the large off-white aggregates formed a 'bed' of floating material leaving the plasmid containing liquor underneath. This liquid was drained by opening the spigot. As the level of floating debris approached that of the tap, the valve was closed and draining stopped. Drained liquors were then subjected to various further polishing filtration steps. These included: filtration through one layer of Miracloth® (synthetic non woven rayon fabric with an average pore size of 22 to 25 μm) to remove floating fine debris; and pressure filtration through a 5 μm polypropylene cloth (PX 587-09) with or without a precoat material of the lowest permeability (Celatom FP-1SL).

Table 2. Peak and average filtration rates during filtration experiments (Fig. 1) for the recovery of plasmid DNA from alkaline lysis mixtures using various filter aids

Filter aids	Peak flow at 0.4 barg (cm h^{-1})	Average flow rate (cm h^{-1})
Celatom FP-1SL	118	5.4
Chromosorb® FC	83	9.7
Celite 521 (A/W)	407	22.2
Celite Hyflo® Super-Cel (A/W)	572	21.7
HP^{2TM} 1000	676	21.0
Fibra-Cel® BH-100	400	19.1
HP^{2TM}/cellulose composite	393	27.6

2.5 Analysis

A detailed description of the analytical methods for determining the solids, protein, total DNA and plasmid DNA contents has been described recently [15] and is only mentioned briefly here. The concentrations of solids in filtrates and drained liquors, arising from filtration and flotation experiments respectively, were determined by turbidimetric measurements at 600 nm, and from the weights of solids following centrifugation at 6,450 g_{av} for 30 min. Soluble and insoluble DNA contents were measured by the diphenylamine assay [22] and protein contents were determined by the BCA assay. Insoluble protein samples were solubilised immediately prior to assay by incubation at 50 °C in 10 mM Tris/HCl, 1 mM EDTA, pH 8 containing 1% SDS [15]. Retentates were centrifuged at 6,450 g_{av} for 30 min and the resulting solid and supernatant fractions were then assayed for solids, DNA and protein content.

Following filtration with filter aids, separation of the fragile wet cakes from the supporting septa was achieved by freezing the assemblies at −20 °C and then teasing the polypropylene membranes away from the frozen filter aid cakes with the aid of a spatula. Three samples were taken from both the top and bottom surfaces of the filter aid cakes and were analysed for DNA and protein contents as described above.

Relative amounts of plasmid and chromosomal DNA were determined by densitometric scanning of ethidium bromide-stained agarose gels, following extraction of the DNA and separation by electrophoresis [15].

3 Results and discussion

3.1 Filtration experiments

Figure 1 and Table 2 show results for the filtration rates and filtrate solids contents arising from the filtration of neutralised cell lysates through the different filter materials presented in Table 1. Regardless of filter material employed we have observed a number of common features in the filtration behaviour of neutralised cell lysates both in

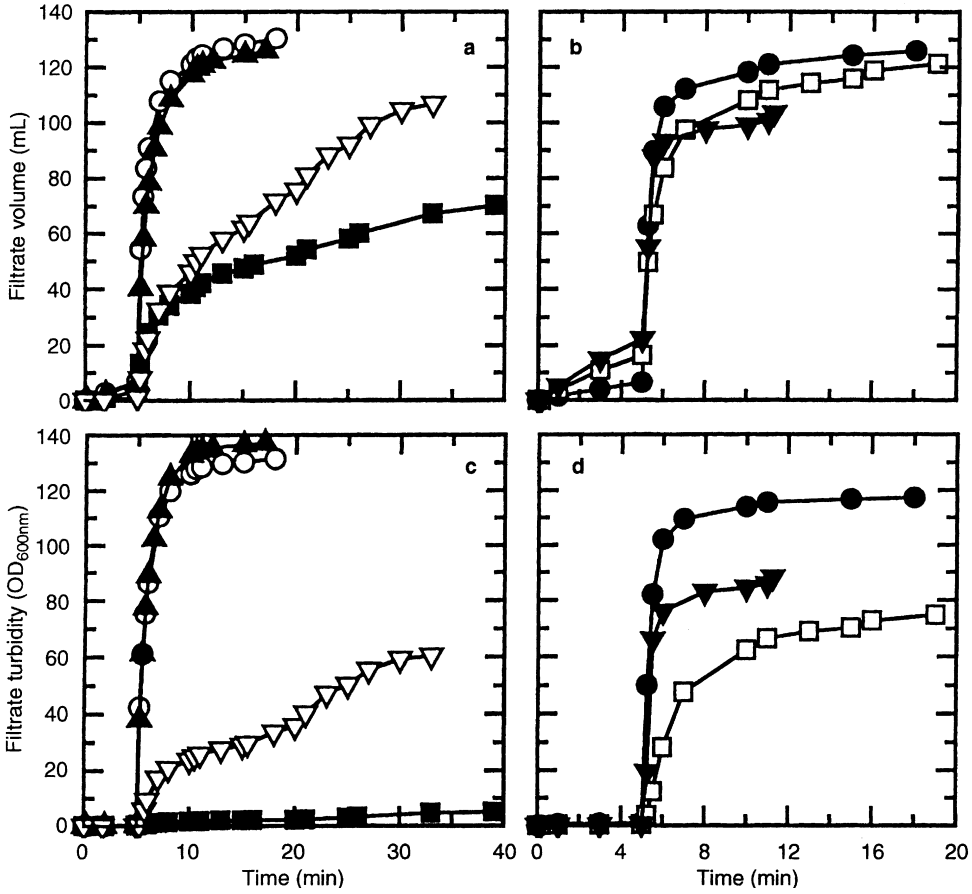

Fig. 1a–d. Filtrate volume and solids content vs. time profiles for the filtration of neutralised *E. coli* lysate mixtures through different filter materials. Panels (**a**) and (**c**): (■) Celatom FP-1SL; (▽) Chromosorb^{R} FC; (▲) Celite 521 (A/W); (○) Celite Hyflo^{R} Super-Cel (A/W); Panels (**b**) and (**d**): (●) HP^{2TM} 1000; (□) Fibra-Cel^{R} BH-100; (▼) HP^{2TM}/cellulose composite

this and our previous study [15]. Peak flow occurs after a pressure of 0.4 barg is applied, and this is accompanied by transmission of solids into the filtrate by an extrusion mechanism. Thereafter, the rates of filtration and solids transmission drop dramatically with the onset of severe blinding effects. The volume vs. time profiles and average filtration rates for filtration of crude neutralised cell lysate mixtures through septa precoated with Celite 521 (A/W), Celite Hyflo® Super-Cel (A/W), HP^{2TM} 1000 and Fibra-Cel® BH-100 were very similar. Moreover, the average rates were very close to those observed previously [15] for the same 5 μm septum lacking a precoat material (22.1 cm h^{-1}). However, the peak flow rates were more variable (see Table 2) and lower than those obtained in the absence of a precoat (880 cm h^{-1}). The highest average filtration rate was obtained with the compressed filter sheet composite of HP^{2TM} 1000 and cellulose. This material, however, blinded more rapidly than all other filter aids tested and filtration stopped at 11 min after only 103 ml of filtrate had been collected. Though the quoted permeabilities for Celatom FP-1SL and Chromosorb® FC were very similar, significant differences in their filtration behaviour were noted. Chromosorb® FC, a more refined material than Celatom FP–1SL, gave a very unsatisfactory combination of flux and filtration turbidity. Celatom FP-1SL, though delivering by far the lowest flow rate, yielded a filtrate of exceptional clarity. The other filter materials tested had darcy numbers falling between 0.3 and 1.2, and although these permitted average flow rates of >20 cm h^{-1}, gave much more turbid filtrates. However, an absolute correlation between the manufacturer's quoted darcy number and filtrate turbidity was not evident.

Profiles for the appearance of protein, chromosomal DNA and plasmid DNA recovered in the filtrate over time are presented in Fig. 2a–g. Somewhat surprisingly the overall performance of all the 'more refined' filter materials was markedly inferior to that of Celatom FP-1SL. Though improvements in plasmid yield were noted (see Table 3), the levels of all principal contaminants were very much higher and losses of plasmid to the filter materials remained at significant levels. During filtration we have observed that a thin tightly adhering layer of solids builds up on some precoat materials (Celite 521 (A/W), Celite Hyflo® Super-Cel (A/W), HP^{2TM} 1000 and Fibra-Cel® BH-100) but not on others (Celatom FP-1SL, Chromosorb® FC and HP^{2TM} 1000/cellulose composite sheet). Rough values for contents of protein, nucleic acid and plasmid in these solids were estimated from difference measurements of samples taken from the top and bottom surfaces of the filter cakes (see section 2.5). Although the skin layers contain appreciable quantities of protein and chromosomal DNA, they do not appear to contain plasmid DNA. Therefore plasmid loss occurs specifically within the precoat by a combination of adsorption and absorption.

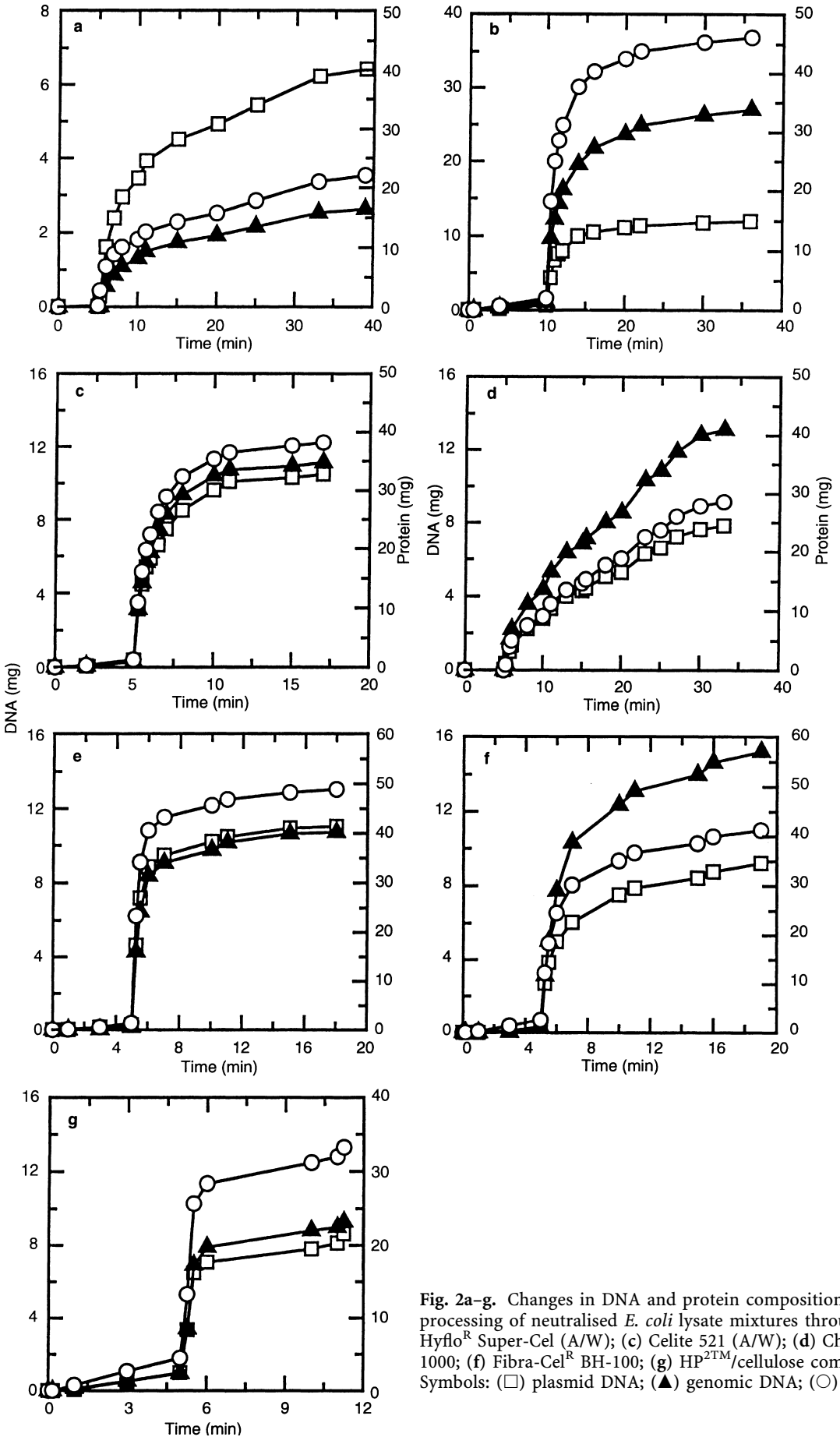

Fig. 2a–g. Changes in DNA and protein composition of filtrates during the processing of neutralised *E. coli* lysate mixtures through: (**a**) FP1-SL; (**b**) Celite Hyflo^{R} Super-Cel (A/W); (**c**) Celite 521 (A/W); (**d**) Chromosorb^{R} FC; (**e**) HP^{2TM} 1000; (**f**) Fibra-Cel^{R} BH-100; (**g**) HP^{2TM}/cellulose composite. Symbols: (□) plasmid DNA; (▲) genomic DNA; (○) protein

Table 3. Summary of data from filtration experiments in Fig. 1 for the recovery of plasmid DNA from neutralised alkaline lysis mixtures

Filter material	% Recovery in the filtrate					% Overall purity of plasmid DNA in the filtrate	% Loss of plasmid DNA in the filter aid
	Solids	Liquid	Protein	Total DNA	Plasmid DNA		
Celatom FP-1SL	0.05	52.3	3.1	6.2	44.0	71.0	8.4
Chromosorb® FC	0.6	79.2	4.0	14.3	54.1	37.4	22.3
Celite 521 (A/W)	1.3	93.2	5.3	14.7	74.1	48.5	17.4
Celite Hyflo® Super-Cel (A/W)	1.3	95.9	6.4	26.4	81.5	30.6	13.4
HP^{2TM} 1000	1.1	90.9	6.6	14.5	72.9	50.7	14.2
Fibra-Cel® BH-100	0.7	90.5	5.9	17.0	59.8	37.8	22.3
HP^{2TM}/cellulose composite	0.9	77.3	5.3	13.9	68.5	48.1	nd

3.2 Flotation experiments

Figure 3 shows photographs of lysis experiments conducted at 15 l scale. Immediately on addition of 3 M potassium acetate, a large mass of off-white gelatinous solids appeared in solution (Fig. 3a). With increasing time, the majority of these solids float upward, and after 1 h at 10 °C, ~80% of the 'cleared' plasmid containing liquid, with a solids content of only 0.2 g l^{-1} (Fig. 3b and Table 4), could be recovered from beneath the floating floc by draining. We have repeated this procedure at a variety of scales (0.3, 0.6 and 15 l) and have found that the flotation process works best at the largest scale investigated (see Table 4). For example, at 0.3 and 0.6 l scales, recoveries of plasmid liquor are less than 50% compared to ~80% at 15 l scale. Moreover, the solids contents of the drained liquors are very much higher (approximately 24 and 12 g l^{-1} at 0.3 and 0.6 l scales respectively). The effi-

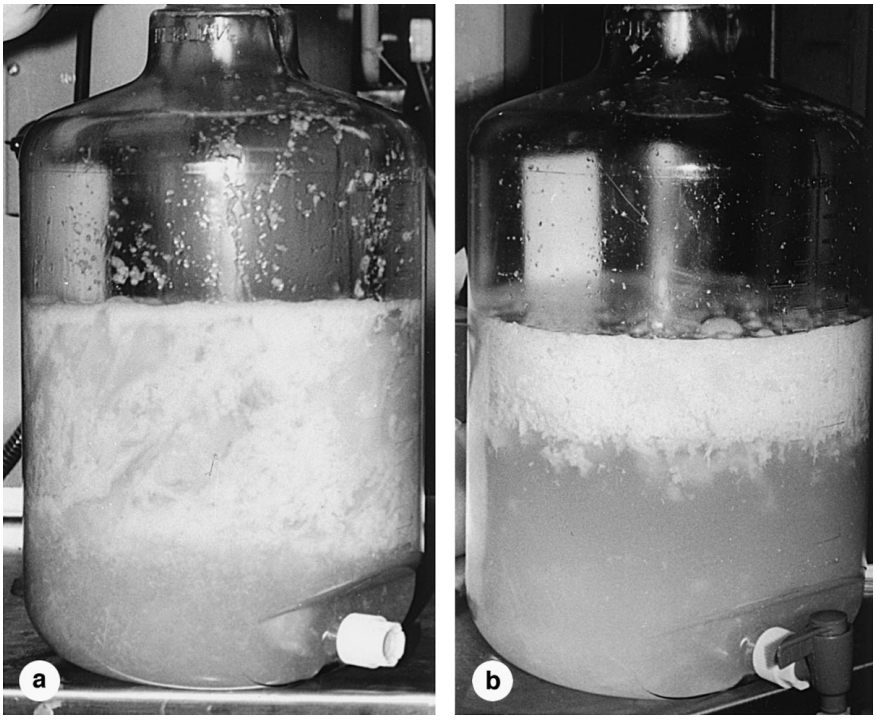

Fig. 3a,b. Photographs of 15 l scale lysis experiments; (**a**) immediately following neutralisation; (**b**) following ageing at 10 °C for 1 h

Table 4. Flotation at different scales of cell lysis. Data are from two separate experiments at each scale. All values are given as the mean ± standard deviation (σ_{n-1})

Scale of lysis (L)	% liquid recovered by draining	Solids content of drained liquid (g L^{-1})	Drained liquid filtered through Miracloth®	
			Solids content (g L^{-1})	Turbidity (OD_{600})
0.3	49.9 ± 5.4	24.10 ± 0.85	2.45 ± 0.07	0.83 ± 0.03
0.6	45.9 ± 2.4	12.15 ± 0.21	1.20 ± 0.13	0.43 ± 0.04
15	79.4 ± 7.1	0.20 ± 0.01	0.17 ± 0.01	0.06 ± 0.01

ciency of flotation at 15 l scale with respect to 'clearing' of the liquor and formation of a tight floating layer of solids at the air-liquid interface appears to be assisted by entrapment of air within the solids. In Fig. 3b air blisters can clearly be seen at the surface, but these are essentially absent at 0.3 and 0.6 l scales. The efficiency of flotation is inextricably linked to mixing and clearly to make this approach scaleable both upward and downward will require precise control of the mixing conditions. However, the efficiency of cell lysis does not appear to have been impaired with increase in scale since the plasmid concentrations in the drained liquors remained constant. A detailed rheological characterisation of alkaline lysis and its impact on flotation is currently under investigation in our laboratories [23].

Drained plasmid liquors from flotation experiments were passed through one layer of Miracloth® to retain residual floating solids. Although this crude filtration step reduced the solids content of liquors obtained from small scale lysis and flotation experiments by ~90%, the final solids contents were still very much higher than those produced by just lysis and flotation at 15 l scale (see Table 4). Filtration of this liquor through Miracloth® reduced the solids content slightly, from 0.20 to 0.17 g l^{-1}, and the turbidity of this filtered liquor was very low.

3.3 Combined flotation and filtration experiments

Results of combined flotation and filtration experiments are presented in Fig. 4 and Table 5. The effects of systematically reducing the solids content of neutralised *E. coli* lysates on the volume vs. time profiles for filtration through 5 μm polypropylene septa with and without a precoat of Celatom FP-1SL are quite striking. An ~8-fold drop in the challenge of solids per unit filtration area (from 1.49 to 0.18 g cm^{-2}) evidently reduced blinding effects on filtration of lysates through the 5μm cloth as judged by a ~2.7-fold increase in average flow rate (Table 5) and change in the shape of the volume vs. time profile (compare Fig. 4a and 4b). In stark contrast, a similar drop (10-fold) in challenge of solids per unit filter area apparently exerts little effect on the volume vs. time profile for filtration through the Celatom FP-1SL precoated filter (compare Fig. 4d and 4e) and only a small increase in average filtration rate was observed (Table 5). For filtration through the 5 μm filter, a further ~11-fold reduction in solids challenge per unit filter area (moving from Fig. 4b to 4c) was accompanied by substantial increases in both peak and average flow rates (see Table 5). The equivalent transition (from 0.15 to 0.014 g cm^{-2}) for filtration of lysates through Celatom FP-1SL clearly alleviated severe blinding, because the filtration rate was more or less constant with respect to time, but even so the average filtration rate was only twice that observed with >100 times the solids challenge per cm^2 of filter area.

Reducing the solids content of the lysate (by first flotation and then filtration through Miracloth®) prior to filtration through the 5 μm filter is accompanied by increased turbidity of the filtrate. In contrast, during filtration through Celatom FP-1SL no adverse effects on filtrate turbidity were noted on shifting from a solids challenge of 1.46 to 0.15 g cm^{-2} of filter area. However, a further reduction to 0.014 g cm^{-2} met with a dramatic increase in filtrate turbidity and <25% of the solids were retained. Clearly, the solids content impacts heavily on the resulting filtrate clarity. A certain level of solids is necessary to prevent loss through the filter, and this level will depend on the effective pore size of the filter material. Previously we have shown that solids passage is greatest with larger pore sizes [14, 15].

Figure 5 shows the effects of reducing solids challenge on the appearance of plasmid DNA, and principal contaminants, protein and chromosomal DNA, in the filtrate with increasing time for the filtration of lysates through FP-1SL. During filtration of the crude neutralised lysate, the purity of plasmid DNA (with respect to total DNA) was exceptionally high (~95%) over the first 10 min, but fell gradually to a final value of ~65%, as increasing quantities of chromosomal DNA appeared in the filtrate. In line with the observations above regarding solids extrusion (in Fig. 4) reducing the solids content in the lysate was accompanied by significantly elevated levels of chromosomal DNA and protein contaminants in the filtrate. A shift in solids challenge per unit filter area from 1.46 to 0.15 g cm^{-2} was accompanied by a marked reduction in plasmid purity to 35%, and an approximate doubling in the levels of contaminating protein (from 3.4 to 6.3 mg per mg plasmid). Moreover, though a further 11-fold lowering of the solids challenge to 0.014 g cm^{-2} exerted little further effect on the final plasmid purity (34%) with respect to DNA, contaminating protein levels rose further to 7.8 mg per mg plasmid. Reduction in the solids content prior to filtration did not alleviate loss of plasmid to the filter aid. For example, when expressed as percentages of the plasmid recovered in the filtrate and filter aid combined, the

Table 5. Peak and average filtration rates during filtration experiments in Fig. 4 (see legend of Fig. 4 for details). Peak flow rate determined at [i] 0.4 barg and [ii] at 0.2 barg

Experiment	Volume of lysate (mL)	Solids content (g L^{-1})	Solids/filter area (g cm^{-1})	Filter material	Peak flow rate (cm h^{-1})	Average flow rate (cm h^{-1})
Fig. 4a	300	99.3	1.49	PX587-09	881[i]	22.1
Fig. 4b	150	23.5	0.18	PX587-09	871[i]	58.6
Fig. 4c	135	2.4	0.016	PX587-09	3342[i]	84.5
Fig. 4d	300	97.3	1.46	Celatom FP1-SL	96[ii]	8.0
Fig. 4e	250	12	0.15	Celatom FP1-SL	>92[ii]	13.3
Fig. 4f	250	1.1	0.014	Celatom FP1-SL	>76[ii]	17.8

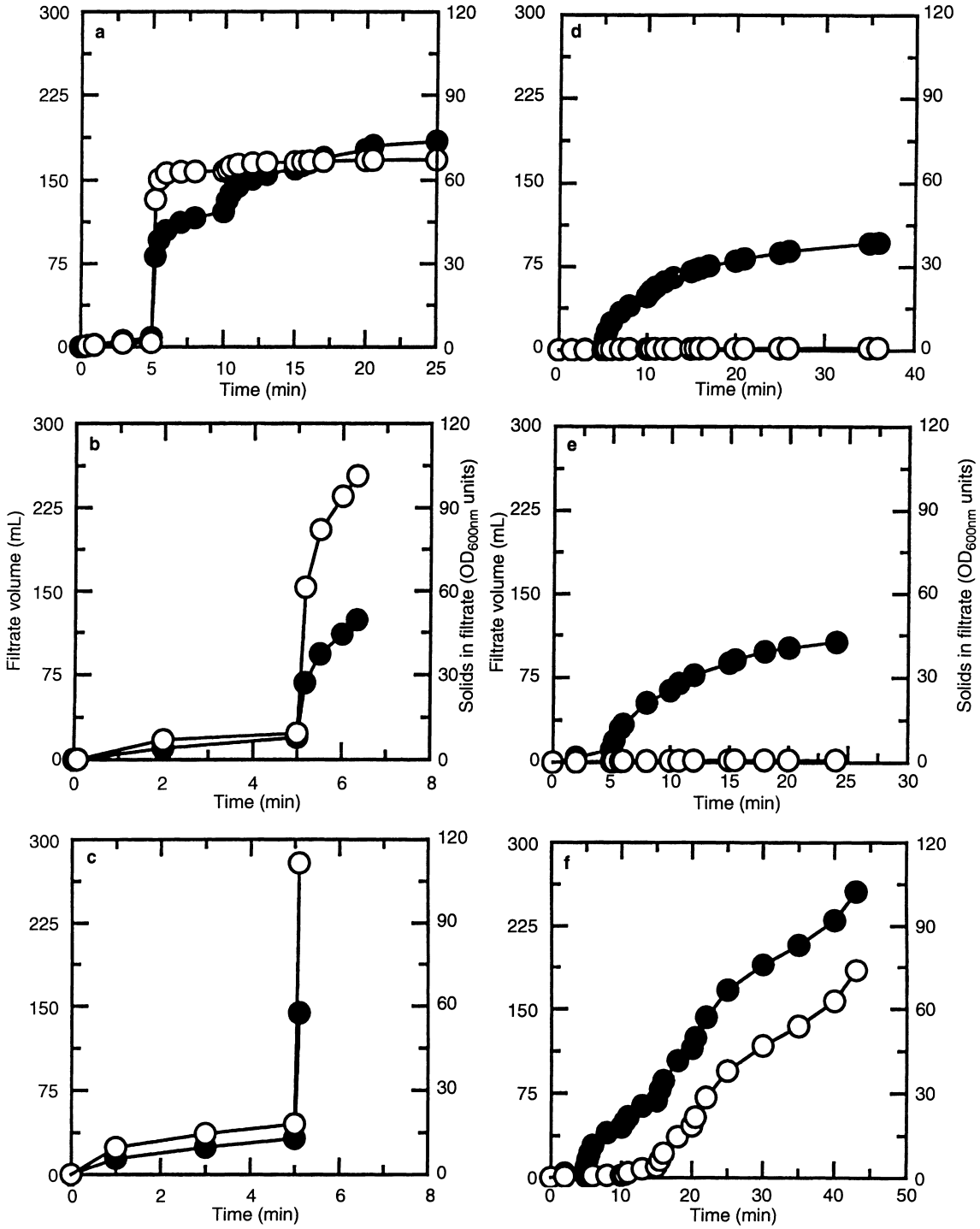

Fig. 4a–f. Filtrate volume and solids content vs. time profiles for the filtration of neutralised *E. coli* lysate mixtures with different solids contents through PX587-09 cloth and filter aid, Celatom FP1-SL. Panels: (**a**)–(**c**) PX587-09; (**d**)–(**f**) Celatom FP1-SL supported on a PX587-09 septum. Panels: (**a**) 300 ml of crude neutralised lysate with a solids content of 100 g l^{-1}; (**b**) 150 ml of lysate drained from beneath floc with a solids content of 23.5 g l^{-1}; (**c**) 135 ml of lysate drained from below floc and filtered through Miracloth^{R} with a solids content of 2.4 g l^{-1}; (**d**) 300 ml^{-1} of crude neutralised *E. coli* lysate with a solids content of 100 g l^{-1}; (**e**) 250 ml of lysate drained from beneath floc with a solids content of 12 g l^{-1}; (**f**) 250 ml of lysate drained from beneath floc and filtered through Miracloth^{R} with a solids content of 1.1 g l^{-1}. Symbols: (●) volume; (○) solids

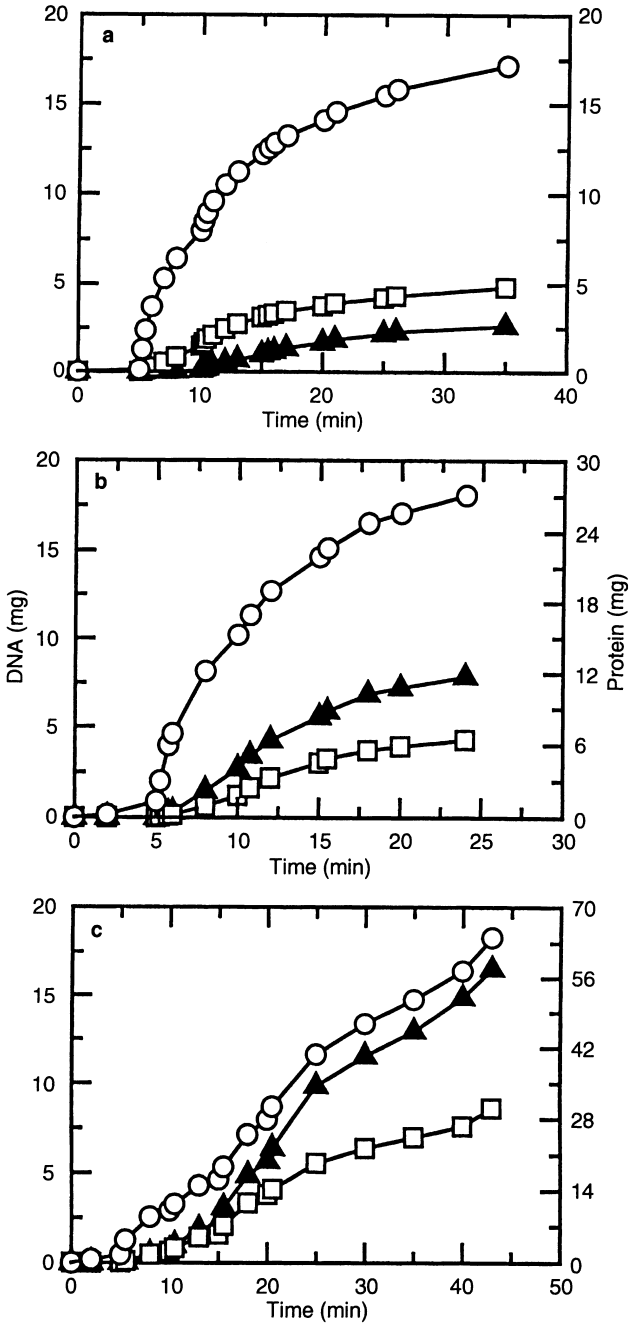

Fig. 5a–c. Changes in DNA and protein composition of filtrates during the processing of neutralised *E. coli* lysate mixtures with different solids contents through Celatom FP1-SL supported on PX587-09 septum. Panels: (**a**) 300 ml of crude lysate with a solids content of 100 g l^{-1}; (**b**) 250 ml of lysate drained from beneath floc with a solids content of 12 g l^{-1}; (**c**) 250 ml of lysate drained from beneath floc and filtered through Miracloth^{R} with a solids content of 1.1 g l^{-1}. Symbols: (□) plasmid DNA; (▲) genomic DNA; (○) protein

plasmid losses corresponding to the above experiments in Fig 4d–f were 19, 34 and 12% respectively.

4 Conclusions

These studies have highlighted a number of unique challenges facing the processing of neutralised lysates containing plasmid DNA. Clearly separation of the solid cellular debris from the plasmid containing liquor by flotation is a simple and promising approach. However, it would appear that subsequent polishing of the fine solids haze may be more difficult to achieve in view of the extreme sensitivity of this material to attrition, and the consequent formation of both soluble and very fine insoluble contaminants. Though a thorough understanding of the mechanisms of floc formation and its flotation properties may make it possible to deliver a 'cleared' plasmid liquor of exceptional clarity combined with high recovery, further polishing prior to application to packed bed chromatographic columns will undoubtedly be required.

In this and our previous studies [14, 15] we have investigated the use of different filter materials to enhance the separation of plasmid containing liquors from neutralised lysates with very different levels of solids (i.e. from whole crude lysates and drained liquors following flotation). The highest plasmid purities and lowest levels of contaminating protein, chromosomal DNA and solids were obtained by filtration of crude neutralised lysates through a 5 μm polypropylene filter cloth precoated with Celatom FP-1SL. Horn and co-workers [24] advocated the use of filter aids to selectively remove RNA from plasmid containing liquors, but they did not present data concerning possible plasmid loss[1]. Our experiments clearly illustrate that loss of plasmids through adsorption and/or absorption mechanisms, to all diatomaceous earth and cellulose filter materials tested, is a significant problem. Moreover, the use of more refined filter aids (e.g. Celite acid washed grades-521 & Hyflo® Super-Cel, and Celite HP^{2TM} 1000) does not appear to reduce this risk, nor does removing the vast bulk of solids prior to filtration (e.g. by draining the cleared liquor from beneath the floating floc).

Other methods for polishing the drained plasmid liquor following lysis and flotation are clearly required in view of the acutely shear sensitive nature of the residual fine solids and significant levels of plasmid loss experienced with filter aids. Work on control of the flotation process and subsequent operations relevant to the scaleable production of plasmid based genes for gene therapy and vaccine applications is on going in our laboratories.

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[1]Laboratory scale plasmid DNA purification kits employing diatomaceous earths are available (e.g. Bio-Rad's Quantum Prep Kits). These rely on initial clarification of the neutralised lysate and subsequent adsorptive capture from the highly clarified liquor.

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