Biotechnol. Bioprocess Eng. **2002**, 7: 6-9

Enhanced Virus Removal by Flocculation and Microfiltration

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Abstract In this work we have investigated the feasibility of virus clearance by flocculation and tangential flow microfiltration. Chinese hamster ovary cell feed streams were spiked with minute virus of mice and then flocculated using cationic polyelectrolytes prior to tangential flow microfiltration. Our results indicate that flocculation prior to microfiltration leads to more than 100 fold clearance of minute virus of mice particles in the permeate. Today, validation of virus clearance is a major concern in the manufacture of biopharmaceutical products. Frequently new unit operations are added simply to validate virus clearance thus increasing the manufacturing cost. The results obtained here suggest that virus clearance can be obtained during tangential flow microfiltration. Since tangential flow microfiltration is frequently used for bioreactor harvesting this could be a low cost method to validate virus clearance.

Keywords: flocculation, hollow fibres, membranes, microfiltration, virus clearance

INTRODUCTION

The use of cell lines containing autonomously replicating vectors, *e.g.* retroviruses and animal derived cell culture media carries with it the risk of contamination of the final product by viruses associated with the raw materials. The United States Food and Drug Administration (FDA) requires that the maximum level of any possible virus contamination be quantified and that clearance (removal or inactivation) in excess of this level be demonstrated in the purification process [1]. Virus clearance is defined as the ratio of virus particles in the fed to particles in the product stream from a given unit operation. The required level of virus clearance can be as high as 10^{15} fold [2]. As viruses can not be grown to a sufficiently high titre to show this level of clearance, even if a unit operation could achieve 10^{15} fold clearance it could not be validated. Often clearance in excess of 10^4 fold is difficult to validate. Therefore virus clearance from a number of different unit operations must be summed in order to determine the overall clearance for the purification train.

The FDA also specifies acceptable virus clearance methods. Virus clearance should be validated using FDA approved model viruses. Reduction factors for two unit operations with the same mechanism of action may not be added. Further, due to the high variability of infectivity assays, reduction factors of less than 10 fold are not included in the overall reduction factor. Consequently new unit operations are frequently added to the purification train simply to validate virus clearance resulting in an increased manufacturing cost. The aim of this research is to validate virus clearance by flocculation and microfiltration.

Tangential flow microfiltration (TFM) is often the first of the purification operations in the downstream processing of biopharmaceutical products. It is used to separate cells, cell debris and other insoluble material (typically 0.02-10 μm in diameter) from the growth medium which usually contains the desired product. In TFM, the feed flows tangential to the membrane. The driving force for permeate flow through the membrane is the transmembrane pressure (TMP) drop.

Biological feeds are notoriously difficult to filter either because they are highly non-Newtonian or because the cake formed is highly compressible and highly fouling [3]. During filtration, these cakes deform into an impermeable mat resulting in very low permeate fluxes and high TMPs. The filterability of the feed may also depend on the cell viability at the time of harvesting. A low cell viability means a large number of the cells present are dead. These dead cells lyse, producing smaller particles that can easily plug the membrane pores. The filterability of biological feeds is often improved by pretreatment with polyelectrolytes prior to filtration [3]. Thus, not only is TFM an existing unit operation, addition of flocculants to remove virus particles represents a minor deviation from current practice.

In this study, we have used Chinese hamster ovary (CHO) cell feed streams. These feed streams were spiked with minute virus of mice (MVM) and then flocculated using a cationic flocculant. CHO cells are frequently used in the biotechnology industry. MVM is an FDA recommended model parvovirus. It is a nonenveloped, icosahedral virus [4]. Validating clearance of MVM is often very difficult.

Polyelectrolytes are water soluble charged polymers with weight-average molecular weights ranging from

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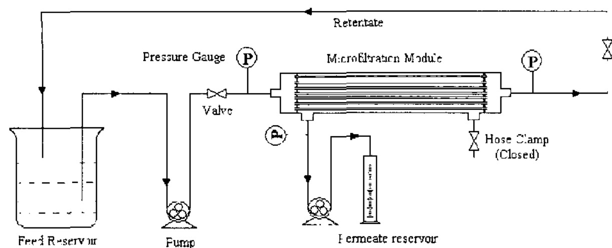

Fig. 1. Experimental set up.

10^3 to 5×10^6 [5]. Past investigators have shown that the addition of polyelectrolytes leads to reduced virus loads in the supernatant after centrifugation [6-7].

MATERIALS AND METHODS

Fig. 1 shows our experimental set up. The feed stream consisting of CHO cells spiked with MVM was placed in the feed tank. CHO cells have a particle size around 20 μm while MVM particles are 18-26 nm in size [4]. Flocculant was added to the feed reservoir and the suspension stirred. CYSEP 4052, a cationic polyacrylamide with molecular weight of $1\text{-}5 \times 10^6$, charge density of 75-80% (percentage of monomer units that are charged), provided by Cytec Industries (Stamford, CT, USA) was used in this study. This cationic polyacrylamide is supplied as a viscous liquid containing 30% polymer.

The flocculant dose was 10 g of CHO cells/g flocculant. The suspension was stirred at 300 rpm for 3 min prior to microfiltration. Based on earlier studies [8] it was determined that these conditions maximized the permeate flux. A/G Technology (Needham, MA, USA) hollow fibre microfiltration modules were used. These modules contain 6 hollow fibres, 10.9 cm in length and 1 mm ID. The membrane surface area was 20 cm^2 with a nominal pore size of 0.2 μm. Permeate was withdrawn from the shell side and collected in the permeate reservoir. The particle size distribution of the unflocculated and flocculated CHO cells was measured using a Coulter (Miami, FL, USA) LS 230 laser diffraction particle size analyzer.

The virus concentration was determined using the $TCID_{50}$ assay [9]. Briefly, this assay involves the determination of the number of dilutions ($TCID_{50}$ number) for a given infective solution that is required in order to inactivate 50% of a given batch of indicator cells. Thus the difference of the Log_{10} ($TCID_{50}$) between the feed and permeate streams will give the 10 fold clearance of MVM.

RESULTS AND DISCUSSION

Virus clearance results are presented in Figs. 2, 3, 5 and 6. Initially a control experiment was run in order to ensure that MVM was not inactivated by exposure to the atmosphere. This experiment consisted of placing an MVM solution in a fume hood for the duration of the microfiltration experiments. The solution was then assayed to determine the residual viral activity. From Fig. 2 it can be seen that the $TCID_{50}$ number of this control does not decrease as a consequence of exposure to the atmosphere.

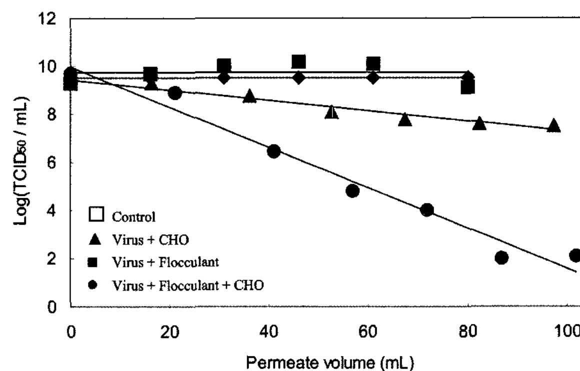

Fig. 2. Virus clearance during microfiltration. The diamonds represent a control experiment and indicate no MVM inactivation when exposed to the atmosphere. Squares, triangles, and circles represent the variation of virus concentration in the permeate for: a) virus and flocculant, b) virus and CHO cell, and c) virus, flocculant, and CHO cell fed streams, respectively.

The remaining three sets of data shown in Fig. 2 give the variation of the $TCID_{50}$ number of the permeate during microfiltration. The initial feed volume was 1 L. The squares show that there is no change in the $TCID_{50}$ number of the permeate when flocculant was added to an MVM solution and the suspension filtered. This is expected since the virus particles are much smaller than the membrane pore size. In addition, we show that the virus does not adsorb on to the membrane surface and that the flocculant does not inactivate the virus.

The triangles show that there is a decrease in the $TCID_{50}$ number of the permeate when a CHO cell suspension is spiked with MVM and filtered in the absence of flocculant. A reduction in the log($TCID_{50}$) value gives the 10 fold reductions (log clearance) of virus in the permeate (as it exits the hollow fibre module). Since the virus particles are much smaller than the membrane pore size, viruses are being rejected by the deposited cake layer. Moreover, when flocculant is added to a feed stream containing CHO cells and MVM the degree of virus clearance in the permeate is much greater (circles). Thus the interaction between flocculant, MVM particles and CHO cells leads to enhanced clearance of MVM from the permeate.

The effect of flocculant on the level of virus clearance is studied in more detail in Fig. 3. This figure shows the variations of both the virus concentration in the permeate as it leaves the module, and the permeate flux as a function of permeate volume collected. Results are

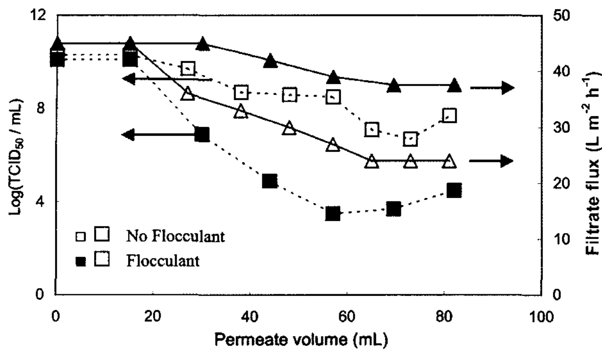

Fig. 3. Variation of permeate flux and virus load in the permeate as it exits the module. Solids concentration 0.5% (weight of CHO cell to weight of suspending medium).

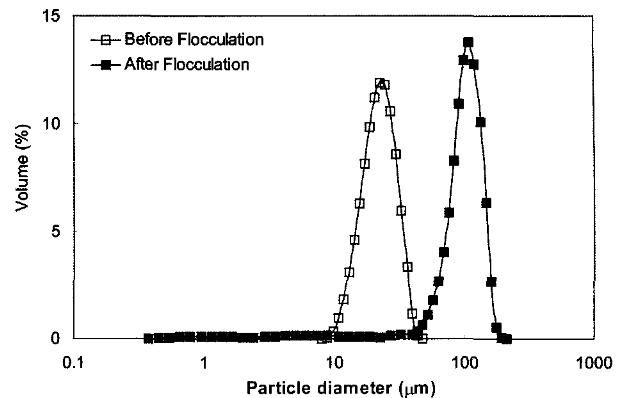

Fig. 4. Particle size distribution before and after flocculation.

shown for feed streams in the presence and absence of flocculant. The initial solids concentration was 0.5% CHO cells (weight of CHO cell to weight of suspending medium) and the feed volume was 130 mL. The total permeate volume collected was 85 mL, and thus the feed suspension was significantly concentrated during TFM. The x-axis shows the permeate volume in mL. The virus concentration in the permeate is shown on the left hand side y-axis as log($TCID_{50}$/mL). The solid and unfilled squares give the variation of virus concentration in the presence and absence of flocculant, respectively. By comparing these results, it can be seen that there is a significant decrease in the virus concentration in the permeate when the feed stream is flocculated. As microfiltration continues, the virus concentration in the feed will start to increase. Thus, eventually the virus concentration in the permeate should also start to increase. As expected, the virus concentration in the permeate starts to increase after removing about 60 to 70 mL of permeate as shown in Fig. 3.

The solid and unfilled triangles in Fig. 3 give the variation of permeate flux during TFM in the presence and absence of flocculant, respectively. The permeate flux is given in L m^{-2} h^{-1} and should be read using the right hand side y-axis. Further, the permeate flux decreases as the feed solution is concentrated. Fig. 3 shows that after about 60-70 mL of permeate have been removed the permeate flux becomes approximately constant for a short period. A constant permeate flux indicates a constant resistance to permeate flow. Thus the properties of the deposited cake layer are likely to be constant suggesting a constant rejection coefficient for viruses. If the rejection coefficient for virus is constant as the virus concentration in the feed increases, the virus concentration in the permeate should also increase. Fig. 3 shows that the virus concentration in the permeate starts to increase for flocculated and unflocculated feed streams when the permeate flux becomes approximately constant.

The results obtained here show that flocculation leads to an increase in permeate flux. The permeate flux during microfiltration depends on the average particle size [8,10]. An increase in average particle size will lead to an increase in the permeate flux. In order to verify this, we measured the particle size distribution of our CHO cell suspensions before and after flocculation. The results are shown in Fig. 4. In Fig. 4, the volume percent of a given particle diameter is plotted against particle diameter in micron. Before flocculation, the modal diameter is around 20 μm corresponding to single CHO cells. After flocculation, the modal particle size is around 100 μm.

From a practical perspective, it is the virus load in the bulk permeate not the virus concentration in the permeate as it exits the hollow fibre module that is important. The bulk permeate is the product of TFM that moves on to the next unit operation. In Fig. 5, the virus concentration in the bulk permeate is plotted as a function of permeate volume. As can be seen in the absence of flocculant, the level of virus clearance in the bulk permeate is small being less than 10 fold. However, when the feed stream is flocculated, 1,000 fold virus clearance is obtained.

Our results show that the addition of flocculant to a CHO cell feed solution spiked with MVM virus leads to an increase in permeate flux and virus clearance in the permeate. By measuring the particle size distribution before and after flocculant addition, we see that the flocculant destabilized the CHO cells leading to an increase in the average particle size which in turn leads to higher permeate fluxes. However, improved MVM clearance could be due to interactions between the flocculant, MVM particles and CHO cells or simply entrapment of the MVM particle by the CHO cell/flocculant cake layer that forms on the membrane surface.

A series of centrifugation experiments was conducted in order to determine the effect of the flocculant on the virus particles. In these experiments, various solutions containing MVM were centrifuged at 3,000 rpm for various times. After centrifugation, the infectivity of the supernatant was determined. In Fig. 6, the log ($TCID_{50}$/mL) is plotted against the centrifugation time. The infectivity of the supernatant decreases with increasing centrifugation time. The diamonds represent a

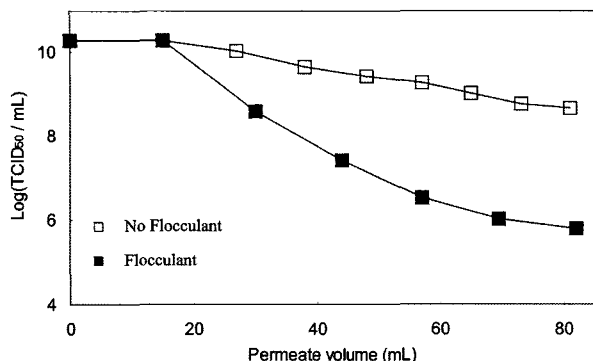

Fig. 5. Variation of virus load in the bulk permeate. Solids concentration 0.5% (weight of CHO cell to suspending medium).

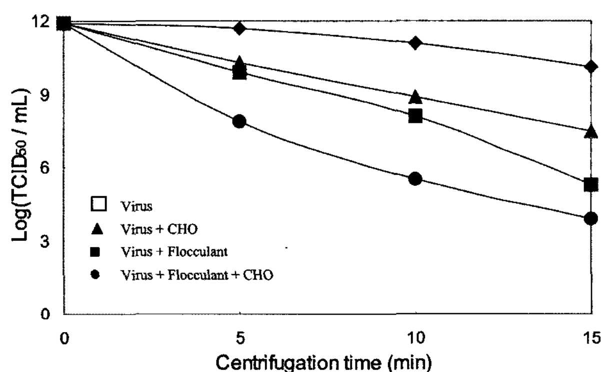

Fig. 6. Virus clearance by centrifugation.

feed solution containing only MVM particles. We chose the centrifugation speed to ensure little concentration of the virus particle in solution as shown in Fig. 6. The triangles represent a feed solution containing MVM and CHO cells, while the squares represent a feed solution containing MVM and flocculant. We can see that there is an interaction between the flocculant and virus particles since the infectivity of the supernatant is lower for all centrifugation times than the supernatant obtained from the MVM and CHO cell feed stream. We also see that MVM, flocculant and CHO cells gives the lowest supernatant infectivity. Further, since centrifugation is sometimes used for cell harvesting in biotechnological manufacturing processes, our results show that virus clearance by flocculation and centrifugation is also feasible.

CONCLUSION

Our experimental results show that cationic flocculants are successful in reducing the MVM load by 100-1,000 fold in the permeate. These results show that the level of virus clearance depends upon the permeate flux and the properties of the cake layer that forms on the membrane. Thus careful control of the permeate flux will be essential in order to ensure optimized virus clearance. By combining flocculation and microfiltration it is possible to obtain virus clearance during bioreactor harvesting. Given the need to validate virus clearance in the manufacture of biopharmaceutical products, flocculation and microfiltration could lead to a low cost method for validating virus clearance. In addition, virus clearance by flocculation and centrifugation may also be feasible.

Acknowledgements Financial support was provided by Biogen Inc. (Cambridge, MA, USA), Colorado State University (CO, USA), The Colorado Institute for Research in Biotechnology (CO, USA) and the National Science Foundation of USA (CAREER Program Grant no. BES 9984095).

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[Received July 25, 2001; accepted December 3, 2001]