Using Continuous Integrated Micro Filtration for the Production of Pseudotype Vectors in a Fixed Bed Reactor

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Authors:

Dirk Nehring, Ralf Pörtner*, Peter Czermak

University of Applied Sciences Giessen-Friedberg, Department of Biotechnology, Wiesenstr. 14, 35390 Giessen, Germany

**Technische University Hamburg-Harburg, Department of Biotechnology I, Denickestraße 15, 21071 Hamburg, Germany

Abstract:

Retroviral pseudotype vector, derived from the murine leukaemia virus carrying the HIV-1 envelop protein MLV (HIV-1) were produced using a integrated, continuous cultivation and harvest process. A 200ml fix bed reactor was used to cultivate the anchorage dependent packaging cell line on macro-porous carriers until the maximum glucose uptake was reached. After starting the cultivation in batch mode, the reactor was either run in perfusion or repeated-batch mode. Parallel to the cell growth inside the fixed bed the medium in the conditioning vessel was harvested permanently. A cross flow filtration module including a commercial asymmetric micro filtration membrane was set up parallel. Filtration was carried out either continuously or batch wise.

To optimise the production and downstream processing of vector formation and concentration a holistic mathematical model including the complete cultivation and cross flow filtration process was developed and validated.

The integrated optimisation with the aid of the extended model lead one to the conclusion that there is an optimal time to start the filtration process and that there are optimal filtration parameters to achieve the highest concentration of colony forming unit per ml (cfu/ml).

Sensitivity analysis performed on the results of the optimisation showed that under optimal conditions the final concentration and the yield of the entire process is more sensitive to the parameters of the filtration than to the bioreaction due to the fast degradation of replication competent pseudo-type vectors.

High concentrated active vector supernatant can be produces in a semi-continuous way, combining continuous fix bed cultivation with the benefits of a cross flow filtration process. The cultivation and production process could be run stable for a long period of time such as 420h. The filtration as a part of the downstream process was successfully integrated into the cultivation system.

Comparing the results of different culture systems 15 therapeutic doses of 100 ml with titer from 10^6 cfu ml^{-1} according to literature can be produced with 22 l medium after 420h in the fixed bed reactor combined with a cross flow filtration module, while in standard flask culture or roller bottles the required vector titer can only be reached after additional batch filtration. To produce the same quantity of active vector particles in flask culture a medium quantity of 40 to 50l would be necessary.

Moreover, scale up of the production process is more applicable for the fixed bed reactor system in comparison to culture flasks. This process setup might be an

alternative to standard production procedures for the production of degradable bioactive products, because the product can be transferred from the culture medium into more stable conditions continuously.
However, while testing a continuous filtration during the entire cultivation the process of cell growth was not stable enough to perform a long-term cultivation. To perform continuous filtration for 24h and more the retention of nutrient need to be further investigate in order to avoid limitation of the cell culture.

The results of this study emphasises that not only optimisation of packaging cell lines but also optimisation of the cultivation and purification process is needed in order to achieve suitable concentrations for gene therapy application such as treatment with retroviral particles.

Keywords: retrovirus, gene therapy, purification, membrane filtration, mathematical model, fixed bed reactor, continuous filtration

Nomenclature

$c_{gluc_{Feed}}$	[mmol l^{-1}]	Glucose concentration in the feed
$c_{gluc_{CC}}$	[mmol l^{-1}]	Glucose concentration in the conditioning vessel
C_{AV_R}	[cfu ml-1]	Concentration of infective vector particles in retentate
C_{AV_P}	[cfu ml-1]	Concentration of infective vector particles in permeate
gur	[mmol $cell^{-1}$ h^{-1}]	Cell specific glucose uptake rate
$k_{aV(T)}$	[cfu cell-1 h-1]	Cell specific temperature dependant vector production
$k_{iV(T)}$	[h^{-1}]	Temperature dependant vector decay constant
N_{AV_P}	[cfu]	Amount of infective retroviral vector in permeate
N_{AV_R}	[cfu]	Amount of infective units of retroviral vector in retentate
$N_{IV\,R+P}$	[cfu]	Amount of inactive vector in retentate and permeate
$N_{aV_{CC}}$	[cfu]	Amount of active vector in the conditioning vessel
$N_{iV_{CC}}$	[cfu]	Amount of inactive vector in the conditioning vessel
$N_{gluc_{CC}}$	[mmol]	Amount of glucose in the conditioning vessel
N_X	[cells]	Number of cells
R	[-]	Retention of vector particles
t	[h]	Time
V_{CC}	[ml]	Volume of conditioning vessel
V_P	[ml]	Permeate volume
V_R	[ml]	Retentate volume
V_{FB}	[ml]	Medium volume of the fixed bed
$\dot{V}_{FB_{out}}$	[ml h^{-1}]	Flow from the fixed-bed to the conditioning vessel
$\dot{V}_{FB_{in}}$	[ml h-1]	Flow from the conditioning vessel into the fixed-bed
$\dot{V}_{Feed}$	[ml h^{-1}]	Feed flow

$\dot{V}_{Harvest}$	[ml h^{-1}]	Harvest flow
$\dot{V}_{TM}$	[ml h^{-1}]	Transmembrane flow
$\dot{V}_{P_{back}}$	[ml h^{-1}]	Backflow

Material and Methods

Cell lines

All cells used in this work were adherent human cell lines. Two retroviral packaging cell lines and one target cell line to detect the gene transfer were cultured under sterile conditions.

Retroviral packaging cell line K52S

The retroviral packaging cell line TELCeB6/pTr712-K52S (K52S) was derived from the *env*-negative MLV packaging cell line TELCeB6 by transfection of the HIV-1 *env*-gene with the plasmid pTr712 and was donated by Prof. Cichutek (Paul Ehrlich Institute, Langen, Germany) [16]. It produces permanent MLV(HIV-1)-vector particles containing the transfer vector MFGInslacZ. In addition the selection marker *bsr* was implanted to gain blasticidin resistance. The cell line K52S produces a maximum vector titer of $2x10^5$ cfu ml^{-1} in static cell culture flasks.

Target cell line Hela $CD4^+$

A clone of the anchorage dependent HeLa $CD4^+$ cell line, derived from a human epithelial cervix-carcinoma was used as target cell line. A gene sequence that expresses the membrane proteins $CD4^+$ gives the properties of human T-Lymphocytes to the HeLa cells. Thus the infection cycle is comparable with MLV or HIV-1 infection [15].

Media and solvents

Culture medium

Dulbecco's Modified Eagle's medium (DMEM) (Gibco/BRL, Eggenstein, Germany) with a glucose concentration of 4.5 g L^{-1} was used for cell cultivation. K52S and TELCeB6 cells were grown in DMEM containing 5% fetal calf serum (FCS) (PAA, Germany), 800 mg/l neomycin sulphate (Roth, Giessen, Germany), 5mg/l blasticidin S (ICN –Flow, Meckenheim, Germany) and 2 mM glutamine (ICN –Flow, Meckenheim, Germany) contents. All HeLa $CD4^+$ target cells were cultured in the same medium but without blasticidin.
The medium was produced from demineralised water under sterile conditions and stored at 4°C in the refrigerator. The fetal calf serum (FCS) was heated at 56°C for 45 min in order to inactivated components that might destroy active vector particles.

Static cell culture systems

Cells were grown in 75 cm^2, 150 cm^2 and 300 cm^2 culture flask (Biochrom AG, Germany).
For vector titer analysing and for small-scale experiments cell culture wells with 6 to 96 cavities (Costar, Germany) were applied.

Macro porous carriers

In order to immobilize the anchorage dependant cells inside the fix-bed reactor macro porous carries were used. Before use the carriers were washed thoroughly in demineralised water.

The ceramic carriers Sponceram® (Zellwerk GmbH, Germany) consist of a doped open porous ZrO_2-structure in a cylindrical shape with 3 mm in diameter and a height of 5 mm. Since the ceramic is resistant against steam sterilisation, treatment with acids and bases the carriers are suitable for cell culture. Furthermore, the carriers can be regenerated by cauterisation. Different anchorage dependent cell lines were successfully cultured on Sponceram and growth rates were comparable to typical growth rates in culture flasks according to the manufacturer. The surface area according to the manufacturer is approx. 100 cm^2/g.

Fixed-bed reactor

The set up of the fixed bed reactor was carried out in a two-vessel system similar to the system described by Fassnacht et al. [9].

An axial flow vacuum insulated fixed bed with a working volume of 200 ml and an inner diameter of 56 mm was packed with different carriers and connected to the conditioning vessel (Sartorius BBI Systems GmbH, Melsungen, Germany) in order to perfuse the immobilized cells. The circulating flow through the fixed bed was led in from the bottom in order to avoid trapped gas bubbles. The volume of the conditioning vessel was adjustable between 500 and 2000 ml. The temperature of the conditioning vessel was controlled with the aid of tempered water that was led through its double walls. The conditioning vessel was used for bubble aeration. Dissolved oxygen was measured (Mettler Toledo, Giessen, Germany) in the conditioning vessel and was controlled by the Biostat controller unit (B. Braun International, Germany). The pH was measured (Mettler Toledo, Giessen, Germany) and controlled by adjusting the CO_2-concentration in the gas mixture.

The medium in the conditioning vessel was changed either batch wise or continuously in perfusion mode. In continuous operation, fresh medium was pumped from a cooled 5 L bottle into the conditioning vessel, and product-containing medium removed to a harvest bottle. Sampling also took place in the conditioning vessel. In order to remove moisture the gas exhaust was cooled.

The fixed bed was inoculated with a suspension of exponentially growing cells by pumping them from the conditioning vessel into the fixed bed. The inoculum contained between $3x10^7$ and $7x10^7$ cells. According to the process mode feed and harvest flow were switched on or off. Both flow rates were controlled either by the setting of the peristaltic pump and by weighing.

Figure 1: Setup of the fixed-bed reactor system showing the fixed bed reactor in the middle in front of the double walled conditioning vessel. On the right hand side the control unit equipped with four peristaltic pumps for taking samples can be seen. On the lift side the circuit pump is place in the back. The peristaltic pump in the front was used for batch harvesting. Feed and harvest vessels are shown next to the fixed bed.

Analytical methods

Cell number

Two different methods were applied for cell counting.

The cell concentrations of all cell suspensions for inoculation were determined by haemocytometer. Viability was determined by trypan blue exclusion. 100 μL of 0.4% trypan blue in phosphate buffer were added to 200 μL samples.

For the flask culture experiments to analyse the cell related vector production cells were counted photographically on the flask surface. This method was applied in order to have permanent experimental data of cell number and vector of 60 h batch culture. While standard methods disturb the cell growth, this method was developed in order to determine a maximal cell growth under optimal cell culture conditions. Eight images at different positions of each cell culture dish or flask were taken under the microscope and the total cell number was counted for each image. The image area represents a flask surface area of 0.27 mm^2. Then the total cell number calculated proportional to the area ratio of the entire dish or flask.
Compared to the haemocytometer the photographically calculated cell number is about 5 to 10% higher. This can be explained by the loss of cells during the

trypsinisation process and because the cell density on the edges of the culture flask or dish is inhomogeneous.

The total number of cells in the carriers of the fixed bed was determined with the crystal violet method at the end of the cultivation (modified after Clarke and Griffiths [4]). 20 ml of 0.1 % crystal violet (Sigma, Germany) in 0.1 M citric acid was added to 10 ml carriers and incubated at 37°C in a flask for one hour. The solution was then thoroughly shaken to remove all nuclei from the carriers, and the cell number was determined by counting the nuclei in a haemocytometer.

Furthermore, the cell number in running a fixed bed cultivation was estimated by glucose uptake rate. Since there was no method to measure the cell number in a fixed bed without breaking up the cultivation process, the glucose uptake rate was taken as an indicator for the number of cells. Since the correlation between glucose uptake and cell numbers is dependant on the metabolism of the cells, the glucose uptake rate was only used for a rough estimation of cell numbers. The modelling was base on common mathematical models approaches [7][8].

Glucose

To measure glucose concentration in the medium Infinity-Glucose-reagent (Sigma, Germany, Cat. No. 17-100P) was applied. The method is based on the hexokinase/glucose-6-phosphate-dehydrogenase. The enzyme hexokinase catalyses the phoshorylisation of glucose by adenosine triphosphate (ATP) and glucose-6-phosphat and adenosine diphosphate (ADP) is produced. In the presence of glucose-6-phosphate-dehydrogenase glucose-6-phospate is oxidised by NAD^+ while $NADH_2$ is produced. The quantity of $NADH_2$ can be measured photometrical at 340 nm (Sigma, Germany). In the range of 0 to 5 g/L the standard deviation of the method was 3-5% of the measured value.

Vector titer

To calculate the vector titer a X-Gal staining procedure was applied. The vector containing supernatant of the K52S cells was used to incubate $CD4^+$ HELA cells. Due to the transduction a Lac-Z reporter gene is transferred from the vector particle into the genome of the target cell [2]. The expression of the Lac-Z gene results in production of the enzyme β- Galactosidase. Due to the reaction of the substrate 5-Bromo-4-chlor-3-indolyl-β-D-Galactopyranoside to a blue colour complex driven by the β- Galactosidase, infected cells can be detected by intracellular colourisation [6].
Endpoint titration of pseudotype vector stocks was performed using various dilutions with a total volume of 1ml to infect adherent $CD4^+$ HELA cells. Adherent target cells were grown at an initial density of 1.6×10^4 cells per 24-well culture dish (Nunc, Wiesbaden, Germany) 24 hours prior to transduction. All target cells were exposed to vector particles for 4 hours followed by washing with phosphate buffer (PBS). Transduced cells were then further expanded for two days before *X-Gal staining* detected *lacZ-positive* cells. The blue coloured cells were counted under the microscope (magnification 100-times) and related to the complete area of the well. The infective units (cfu) per ml were calculated by multiplying the counted numbers with the dilution factor and dividing by the volume of supernatant that was exposed to the target cells [2].

The standard deviation of the cfu per ml was +/- 9.5 to 12.5 % of the measured value. Eight samples of each probe were analyzed at different diluted concentrations. A similar standard deviation was found by Cosset et al. [6].
The initial number of target cells and the supernatant incubation time of 4 hours was always kept constant to make the results of this cell-based assay comparable.
Comparison of the vector titer of different packaging cell lines is difficult due to the differences of the bioreaction and infection pathway of other cell based targeting systems [12]. However, the status of the transduction efficiency seems to be comparable if basic parameters such as cell number and volumes of the assay are fixed [13].

Cell cultivation

All cell culture handling was carried out under a laminar flow clean bench to avoid contamination. Medium and buffer solutions were preheated to 37°C. The cultivation of all cells was done at 37°C and 5% CO_2 atmosphere.

Filtration

In order to increase the concentration of the vector particles filtration was applied with a ceramic membrane.

Cross flow filtration was applied in the experiments either as batch filtration or as continuous filtration. For batch filtration a certain amount of supernatant was placed on the concentrate side and filtrated.

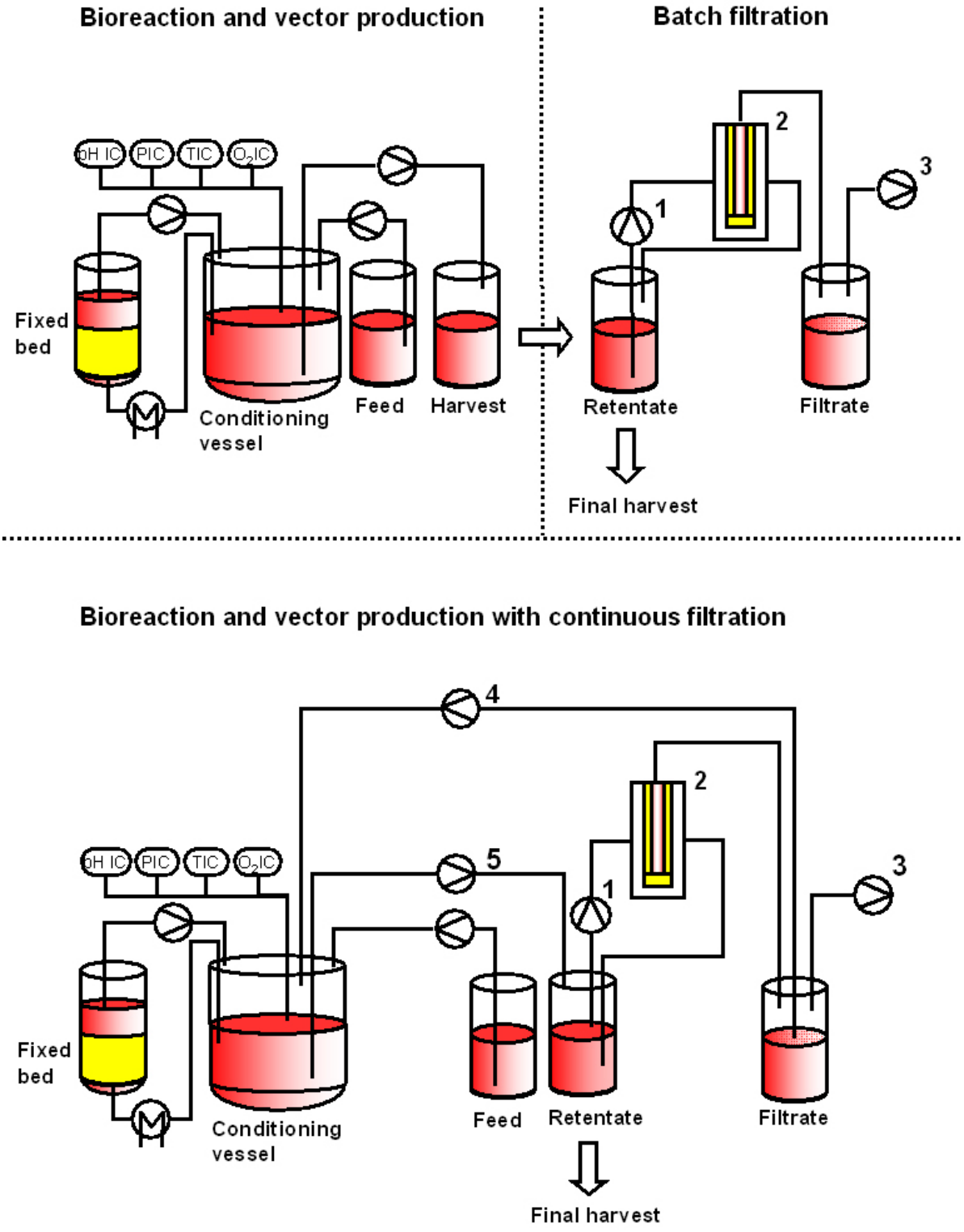

Figure 2: Schematic structure of batch filtration and continuous filtration mode. In batch operation the concentrate side is filled once with vector containing supernatant coming from the vector production and the filtration is performed with the aid of a cross flow pump (1), a membrane module (2) and a vacuum pump (3) until most of the batch is filtered. In case of continuous operation the filtrate is transferred back by the peristaltic pump (4) to the conditioning vessel and the retentate side is fed continuously with vector containing supernatant by peristaltic pump (5).

For continuous filtration the filtrate was transferred back to the conditioning vessel of the bioreactor and the concentrate was fed permanently with vector containing supernatant form the bioreactor. Both operation modes are shown in Figure 2.

Batch cross flow filtration

Cylindrical ceramic membrane with an outer diameter of 25 mm and 19 inner tubes with a diameter of 3 mm (Atech, Gladbeck, Germany) were used for cross flow filtration. As shown in Figure 3 the membrane consists of an Al_2O_3 support material and two asymmetric layers. The first layer is built of zirconium oxide while the second layer, that gives the cut off molecular size, consists of TiO_2. The manufacturer specified the membrane cutoff with 20 kDa.

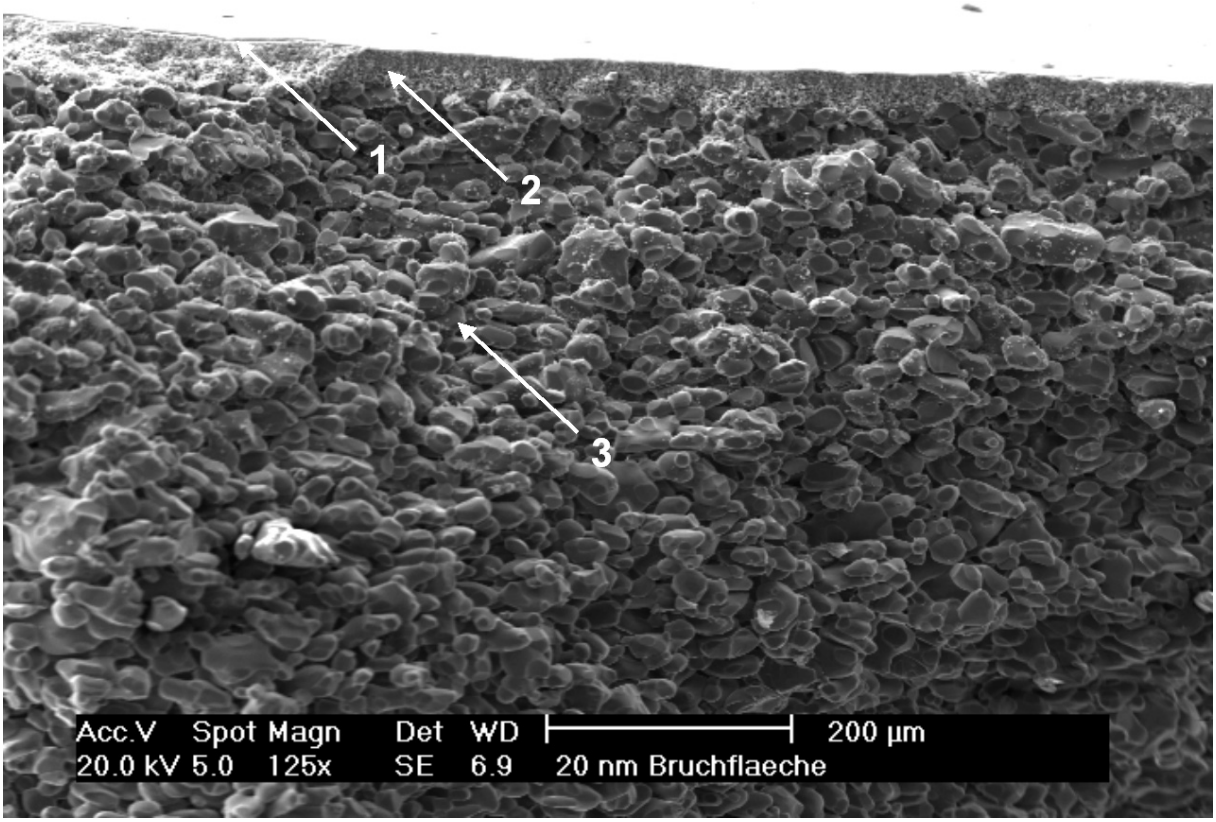

Figure 3: Cross section of a ceramic membrane showing three different layers. The one at the bottom is the support layer (3), in the middle (2) is a layer with a smaller core diameter and on the top (1) the functional membrane surface that is responsible for the membrane separation properties.

On the concentrate side of the membrane module a 1 l glass bottle was connected to the inner side of the membrane. A peristaltic pump (Watlow, USA) with a flow rate of 2 l min^{-1} was used to circulate the vector containing supernatant. On the vacuum side of the device a 1 l glass bottle was also installed and connected to the outer side of the filtration module. Furthermore a vacuum of 200 to 1000 mbar absolute pressure was applied to the filtration chamber while experiments with vector free media were carried out. The batch filtration experiments to concentrate vector particles were carried out at 400-mbar transmembrane pressures. All chambers and tubes were cooled with crushed ice. By opening the valve of the vacuum tube the filtration was

started and the weight of the filtrate was measured over time with a scale (Sartorius, Germay).

Continuous cross flow filtration

For continuous cross flow filtration as shown in Figure 2 the same inorganic membrane module as specified in Table 1 and displayed in Figure 3 (manufactured by Atech, Germany) was used.

Table 1: Technical parameters of cross flow filtration module [11].

Support material	Aluminium oxide
Layer material	Titanium oxide
Cut off	20 KD
Length	450 mm
Outer diameter	25 mm
Filtration surface	Approx. 0.1 m^2
Design	Tubular module 19 channels
Channel diameter	3 mm
pH-value	1-14

Model of inline filtration and vector production

To set up the integrated filtration process further balance equations of active vector were required for the filtrate and retentate vessels. The bioreaction and the filtration process was set up in a holistic model.

All flow to the conditioning vessel including the backflow of glucose content in the filtrate to the conditioning vessel were implemented into equation Eq. 1. The balance of glucose in both conditioning vessel and fixed bed reactor is given by:

$$\frac{d\ N_{gluc_{CC}}}{dt} = \dot{V}_{Feed} \cdot c_{gluc_{Feed}} + \dot{V}_{P_{back}} \cdot c_{gluc_{CC}} - gur \cdot N_X - \dot{V}_{Harvest} \cdot c_{gluc_{CC}} \qquad \text{Eq. 1}$$

The equation Eq. 1 takes into account the glucose containing feed as well as the harvest flow. $N_{gluc_{CC}}$ is the amount of glucose in the conditioning vessel, $\dot{V}_{Feed}$ is the flow of fresh feed medium, $c_{gluc_{Feed}}$ is the glucose concentration of the feed, $c_{gluc_{CC}}$ is the glucose concentration of the conditioning vessel, gur is the cell specific glucose uptake rate, $\dot{V}_{P_{back}}$ the permeate flow back to the conditioning vessel and $\dot{V}_{Harvest}$ the harvest flow.

Due to the complete retention of the vector particles at the retentate of the membrane the backflow did not contain any vector particles and active vector concentration is described by:

$$\frac{d\ N_{aV_{CC}}}{dt} = k_{aV(T)} \cdot N_{X_V} - k_{iV(37°C)} \cdot N_{aV_{CC}} - \dot{V}_{Harvest} \cdot \frac{N_{aV_{CC}}}{V_{CC}} + \dot{V}_{P_{back}} \cdot \frac{N_{aV_P}}{V_P} \qquad \text{Eq. 2}$$

with $N_{aV_{CC}}$ as the amount of active vector in the conditioning vessel, $k_{aV(T)}$ as cell specific vector production rate and $k_{iV(T)}$ the temperature dependent vector decay. V_{CC} is the medium volume of the conditioning vessel.

The medium volume of the conditioning vessel was calculated by:

$$\frac{dV_{CC}}{dt} = \dot{V}_{Feed} + \dot{V}_{FB_{out}} + \dot{V}_{P_{back}} - \dot{V}_{Harvest} - \dot{V}_{FB_{in}} \qquad \text{Eq. 3}$$

$\dot{V}_{FB_{out}}$ is the flow coming from the fixed bed to the conditioning vessel and $\dot{V}_{FB_{in}}$ the flow that is leading from the conditioning vessel to the fixed bed and $\dot{V}_{P_{back}}$ is the flow coming back from the filtrate to the conditioning vessel.

Moreover, the retentate volume is given by:

$$\frac{dV_R}{dt} = \dot{V}_{Harvest} - \dot{V}_{TM} \qquad \text{Eq. 4}$$

with $\dot{V}_{TM}$ as the transmembrane flow. Thus, the permeate volume yields to:

$$\frac{dV_P}{dt} = \dot{V}_{TM} - \dot{V}_{P_{back}} \qquad \text{Eq. 5}$$

The balance of active vector particles in the retentate is described by:

$$\frac{d\,N_{aV_R}}{dt} = k_{iV(4°C)} \cdot N_{aV_R} + \dot{V}_{Harvest} \cdot \frac{N_{aV_{CC}}}{V_{CC}} - \dot{V}_{TM} \cdot (1-R)\frac{N_{aV_R}}{V_R} \qquad \text{Eq. 6}$$

with R as retention coefficient and the active vector amount in the retentate N_{aV_R}.
Furthermore, the balance of active vector amount in the permeate chamber leads to:

$$\frac{d\,N_{aV_P}}{dt} = k_{iV(4°C)} \cdot N_{aV_P} - \dot{V}_{P_{back}} \cdot \frac{N_{aV_P}}{V_P} + \dot{V}_{TM} \cdot (1-R)\frac{N_{aV_R}}{V_R} \qquad \text{Eq. 7}$$

However, assuming a complete vector retention of the membrane (R = 1) equation Eq. 6 and Eq. 7 lose the transmembrane term.

Continuous vector production and inline filtration in a fixed bed reactor

The cultivation was carried in batch mode for the first 60 to 70 h. Then the perfusion mode was started. The process was kept stable for 420h and inline filtration was applied five times after 70 h, 169.5 h, 263.75 h, 316.5 h and 407.75 h for certain periods of time ranging from 6.5 to 21.75 h as can be seen in Figure 4.

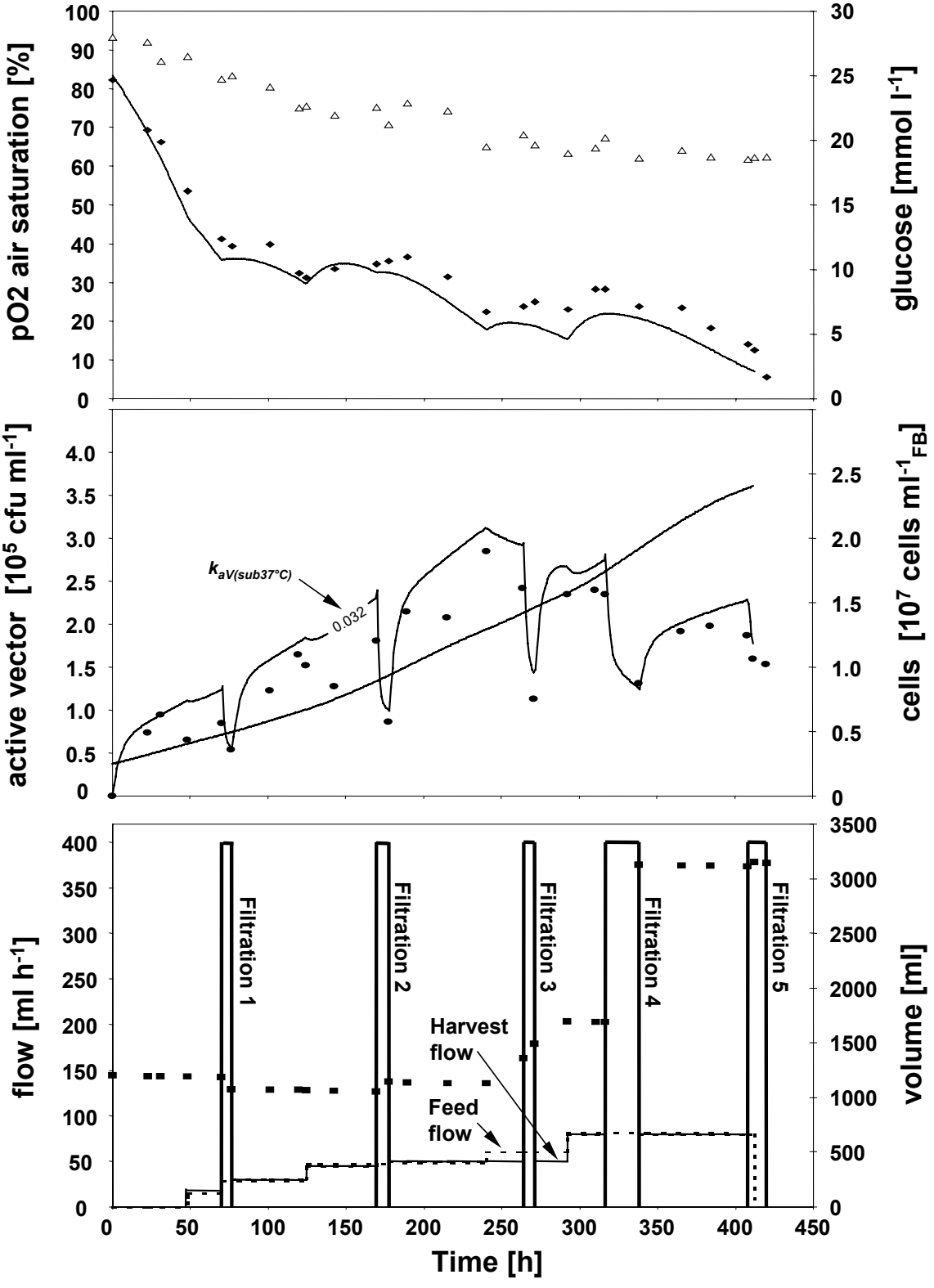

Figure 4: Time course of cultivation in a 200 ml fixed bed reactor showing experimental data of pO_2 (Δ), glucose (♦), filtration flow rate (-), medium volume in the conditioning vessel (■), feed flow rate (---), harvest flow rate (-) and active vector (•) concentrations measured in conditioning vessel. Inline filtration was carried out five times. Vector titer, glucose and oxygen concentration was predicted by the simulation (-) data. In the conditioning vessel a vector titer ranging from 1.1 10^5 cfu ml^{-1} to 2.8 10^5 cfu ml^{-1} was measured after 200 h of cultivation.

While the feed flow to the conditioning vessel was switch on after 48h and increased stepwise until the maximum rate of 80 ml h^{-1} was reached after 292.25 h, the harvest flow was switched off during the inline filtration process.
The model was able to fit the experimental data of glucose and vector concentration in the conditioning vessel fairly well. At the end of the cultivation the number of cell nuclei of the entire fixed bed was counted by the crystal violet method described in chapter 0 to 2.2 10^7 cells ml_{FB}^{-1}, which corresponds to the final simulated value of 2.4 10^7 cells ml_{FB}^{-1} as indicated in Figure 4.

Table 2: Experimental results of cultivation with in-line filtration

Total time [h]	420
Total medium amount [l]	21.43
Inoculate cell number [-]	1.01 10^8
Final cell number [-]	4.13 10^9
Total vector in retentate [cfu]	2.08 10^9
Total vector production [cfu]*	5.45 10^9
Maximal vector titer [cfu ml^{-1}]	2.8 10^6
Volume retentate 1-5 [ml]	Each harvest: 300
Vector titer retentate 1 [cfu ml^{-1}]	4.8 10^5
Vector titer retentate 2 [cfu ml^{-1}]	9.1 10^5
Vector titer retentate 3 [cfu ml^{-1}]	1.2 10^6
Vector titer retentate 4 [cfu ml^{-1}]	2.8 10^6
Vector titer retentate 5 [cfu ml^{-1}]	1.6 10^6

There were no infective vector particles detectable in the filtrate. However, in the retentate of the inline filtration an active vector titer of 4.8 10^5 cfu ml^{-1} to 2.8 10^6 cfu ml^{-1} was reached at the end of each filtration procedure. In Table 2 the final active vector concentrations of each batch of retentate is shown. The total vector harvested production including both retentate and the direct harvest from the conditioning chamber of the entire process was 5.45 10^9 cfu.
The model was used to predict the experimental data of the vector concentration. Comparing the vector titer of the conditioning vessel to the retentate a maximum concentration factor of 10 was reached.

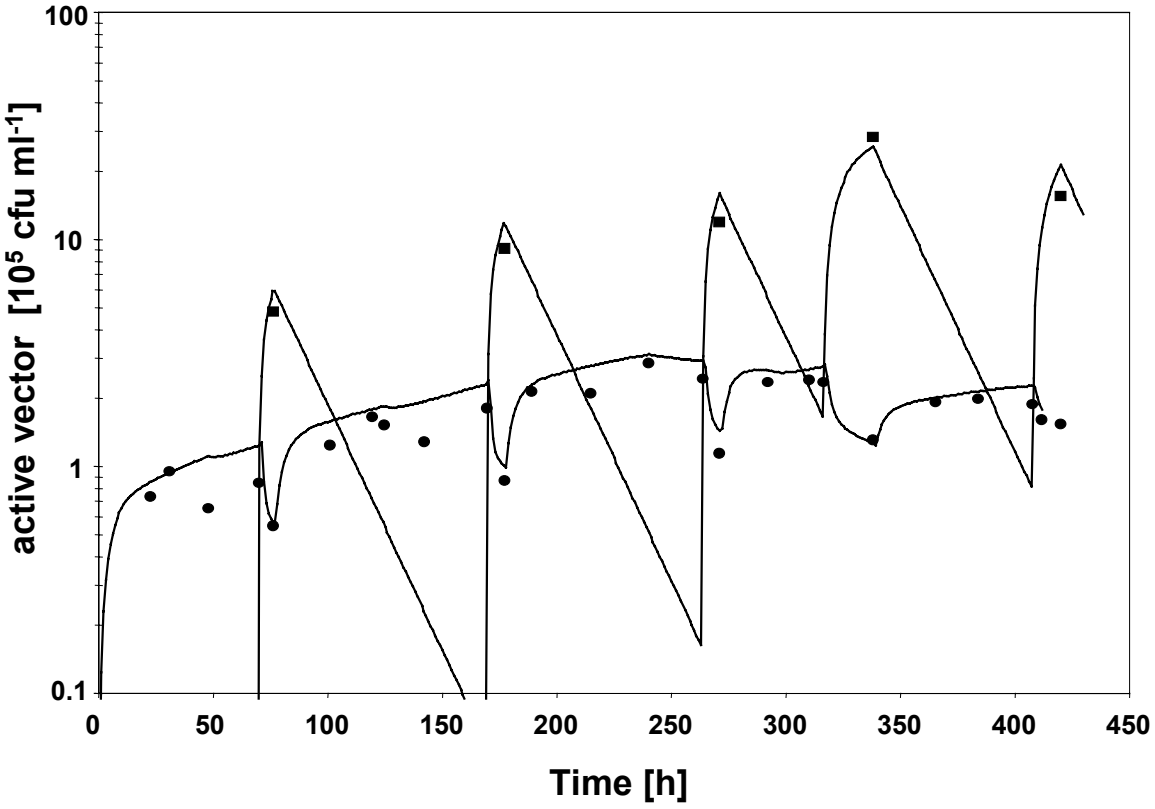

Figure 5: Experimental data of active vector titer in the conditioning vessel (●) and retentate (■) from the same cultivation of K52S cells as shown in figure Figure 4. Inline filtration was performed 5 times while the cultivation process was kept in a continuous mode. The lines show the simulation of both titers. The retentate was measured at the end of each filtration. The highest vector titer of 2.8 10^6 cfu ml^{-1} was reached at the end of the fourth continuous filtration step. Titer in the retentate was 10 fold higher in comparison to the conditioning vessel.

As can be seen in Figure 5 the model predicts the experimental data quite well. Compared to culture flask with a maximum active vector titer of 1.3 to 2.0 10^5 cfu ml^{-1} (results not shown) and publication [16]) the inline filtration experiment proofs that in continuous cultivation higher productivity can be reached. The retentate of each filtration was taken in order to perform another batch cross filtration with the same system. In Table 3 vector titer, concentration factor and the yield of the filtration are displayed.

Table 3: Results of final batch filtration of in-line filtrated supernatant

Process conc. factor*	Feed			Retentate				
	Volume [ml]	cfu ml^{-1} ($x10^5$)	Std dev. ($x10^5$)	Volume [ml]	cfu ml^{-1} ($x10^6$)	Std dev. ($x10^5$)	Recovery**	Filt. Time [min]
2.91	300	4.80	0.04	85	1.39	1.41	82.34%	23
2.98	300	9.11	0.06	82	2.72	1.48	81.56%	21
2.58	300	11.83	0.12	91	3.05	0.96	78.32%	24
2.38	300	27.98	0.32	94	6.65	1.83	74.45%	23
2.68	300	15.51	0.24	86	4.16	1.30	76.93%	23

* Process concentration factor: Theoretical increase factor of vector concentration in the retentate.
** Recovery: Amount of active vector particles in final retentate divided by the initial amount in the feed.

Five retentate batches with a vector titer ranging from 1.39 10^6 to 6.65 10^6 cfu ml^{-1} with volumes from 82 to 94 ml were produced in 420h. According to literature this could be enough for five therapeutic doses with a total vector amount of 1.18 10^8 to 6.25 10^8 cfu depending on the efficiency of the gene-transfer [1][3][5][10].

Conclusion

High concentrated active vector supernatant can be produced in a semi-continuous way, combining continuous fix bed cultivation with the benefits of a cross flow filtration process. The cultivation and production process could be run stable for a long period of time such as 420 h. The filtration as a part of the downstream process was successfully integrated into the cultivation system.
Comparing the results of different culture systems 15 therapeutic doses of 100 ml with titer from 10^6 cfu ml^{-1} according to literature can be produced with 22 l medium after 420h in the fixed bed reactor combined with a cross flow filtration module, while in standard flask culture or roller bottles the required vector titer can only be reached after additional batch filtration. To produce the same quantity of active vector particles in flask culture a medium quantity of 40 to 50 l would be necessary considering the production methods described above.
Moreover, scale up of the production process is more applicable for the fixed bed reactor system in comparison to culture flasks. This process setup might be an alternative to standard production procedures for the production of degradable bioactive products, because the product can be transferred from the culture medium into more stable conditions continuously and less handling is required compared to culture flask cultivation.
However, while testing a continuous filtration during the entire cultivation the process of cell growth was not stable enough to perform a long-term cultivation. To perform continuous filtration for 24 h and more the retention of nutrient need to be further investigate in order to avoid limitation of the cell culture (results not shown).

A holistic mathematical model describes the entire process including vector formation and filtration process. The model can be used for further optimisation of optimal cultivation parameters to achieve higher yields and concentration. Furthermore, the model can be applied for other packaging cell lines with different kinetic parameter in order to predict the production of vector particles. Since long-term experiments are

time consuming and expensive the model can speed up process development and safe costs.

In future experiments with recently optimised MLV packaging cell lines that produce a 10 to 100 fold higher vector titer than the K52S cell line in static flask culture [14], higher yields and vector concentrations might be achieved by this method.

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