



Vaccine process development using Cytodex microcarriers in WAVE Bioreactor systems

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Vaccine process development using Cytodex™ microcarriers in WAVE™ Bioreactor systems

Key words: MDCK, Vero, cell culture, microcarrier, Cytodex, WAVE Bioreactor

Abstract

WAVE Bioreactor system was originally designed for culturing cells in suspension. However, several cell types used for cell therapy and vaccine production are anchorage-dependent and require a surface to grow on. In this study, Cytodex microcarriers were used in Cellbag™ bioreactors to expand Madine-Darby Canine Kidney (MDCK) and Vero cells. Different mixing conditions were tested for both cell lines in order to optimize cell attachment. We found that medium composition had a significant effect on cell attachment; for example, MDCK and Vero cells cultured in low serum containing medium were able to attach to the microcarriers with intermittent and continuous mixing. However, under serum-free conditions, high attachment rates for Vero cells could only be obtained under intermittent mixing. The MDCK cell growth in WAVE Bioreactor system was scalable to 2 l and 10 l working volumes. The cells reached a maximum concentration of 3×10^6 cells/ml. Vero cells were cultivated on Cytodex 1 and Cytodex 3 carriers at a working volume of 2 l and reached a maximum concentration of 1.4×10^6 cells/ml—the same concentration of Vero cells was obtained with experiments using spinner flasks. The data shows that WAVE Bioreactor system is a versatile platform for microcarrier cultivation of MDCK and Vero cells and it is also a fast and convenient alternative to conventional stirred-tank bioreactors or roller bottle systems.

Introduction

Unlike the mass cultivation of cells in roller bottles, the cultivation of adherent cells on microcarriers makes it relatively easy to scale up the production process. In addition, the use of microcarriers enables cell propagation in bioreactors, which provides greater process control and a reduced risk of contamination compared to multiunit operations in plastic ware. The use of disposable bioreactors reduces investment costs, increases flexibility (due to decreased turnover), and eliminates the need for cleaning validation of the production vessels.

WAVE Bioreactor system is a disposable fermentation equipment that is designed for the cultivation of human, animal, plant, and insect cells, and for bacterial and fungal cultures. WAVE Bioreactor system does not require cleaning or sterilization and this reduces the need for validation processes in biopharmaceutical productions. Although WAVE Bioreactor system was originally designed for suspension cells, we show that it is a suitable device for the cultivation of attachment-dependent cells on microcarriers.

This application note describes the cultivation of MDCK and Vero cells on Cytodex microcarriers in a WAVE Bioreactor system. We investigated parameters such as optimum mixing and working volumes necessary for microcarrier cultivations, cell attachment efficiency, maximum cell densities, and the feasibility of scaling from a working volume of 2 l to 10 l. We present a comparative evaluation of WAVE Bioreactor system and standard cultivation in spinner flasks using cell-specific parameters such as attachment, growth rate, and maximum cell concentration.



A general procedure for the cultivation of cells in a WAVE Bioreactor system is shown in Figure 1.

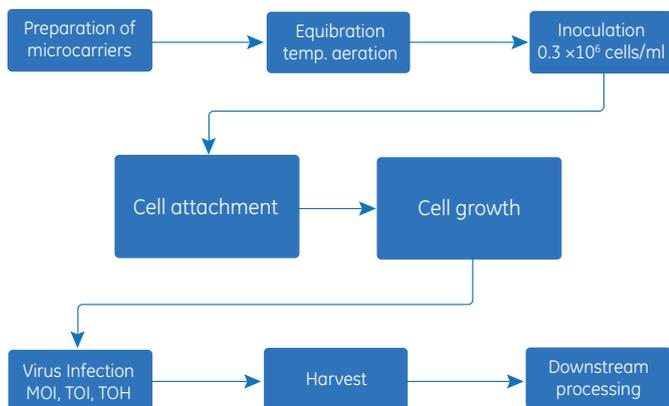


Fig 1. Schematic representation of process development in WAVE Bioreactor system.

Materials and methods

Cell line, medium, and stock solutions

MDCK cells were derived from ECACC (Nr. 84121903). The cells were cultured in UltraMDCK (12-749Q, Lonza) supplemented with 2% FBS (CH30160.03, HyClone). For the routine culture of cells in T-flasks and cell factories and also for the inoculation of microcarrier cultures, MDCK cells were first washed with PBS-EDTA (E8008, Sigma Aldrich) followed by detachment with accutase (L11007, PAA Laboratories).

Vero cells were derived from ATCC (Nr. CCL 81). The cells were cultivated in serum-free medium on DMEM/Ham's basis. The medium was supplemented with 0.2% Pluronic F68 (Sigma Aldrich) for cell propagation in WAVE Bioreactor systems. Trypsin (Gibco BRL) was used to detach the cells during routine culture in T-flasks and cell factories. Trypsin inhibitor (Sigma Aldrich) derived from soybean (1 mg/ml in PBS) and EDTA was used for trypsin inactivation. Vero cells were detached with 0.02% EDTA prior to inoculation of microcarrier cultures. The medium was supplemented with 5% serum, for serum containing Vero cultures.

Cultivation vessels

MDCK cells were grown in T-flasks and cell stacks (Corning Life Sciences, Sigma Aldrich) during routine culture and also for inoculum preparation.

Vero cells were grown in T-flasks and cell stacks during routine culture and also for inoculum preparation. Cell cultivations in spinner flasks were performed in 125 ml Techne spinner flasks (Bibby-Scientific Ltd) at a working volume of 60 ml. The flasks were stirred at 50 rpm.

Microcarriers

Cytodex 3 microcarriers were used for attachment to MDCK cells and Cytodex 1 and Cytodex 3 were used for Vero cells. The microcarriers were hydrated and sterilized in siliconized glass vessels. After three washings in Ca^{2+} and Mg^{2+} free PBS, the microcarriers were autoclaved for 15 min at 121°C and stored at 4°C. The microcarriers were washed with cultivation medium and transferred to the Cellbags 24 h before inoculation. Cytodex 1 and Cytodex 3 were used at a final concentration of 3 g/l. To prevent carrier adhesion to glass surfaces, transfer bottles and spinner flasks were treated with Sigma Cote (Sigma Aldrich).

Bioreactors for MDCK and Vero cells

WAVE Bioreactor system consists of a rocking platform with a disposable plastic Cellbag bioreactor with either a CO_2 mixer (CO2MIX20) or a WAVEPOD™ unit for controlling pH, temperature, oxygen, and mixing. MDCK cells were cultivated in WAVE Bioreactor System 20/50 with Cellbag 10L and Cellbag 50L. Vero cells were cultivated in WAVE Bioreactor System 20/50 with Cellbag 2L and Cellbag 10L. A WAVEPOD unit was used to monitor pH, temperature, oxygen, and mixing.

Staining solutions

A cell lysis buffer containing 0.1% crystal violet in 0.1 M citric acid was used for the nuclei-counting procedure for both MDCK and Vero cells. Trypan Blue (0.1% in PBS) was used to determine cell viability.

For Vero cells, hematoxylin stain (Carl Roth GmbH) for cell fixing and microscopic observation of carrier cultures was made of 0.9 g hematoxylin, 0.18 g NaIO_3 , 15.45 g $\text{AlK}(\text{SO}_4)_2 \times 12\text{H}_2\text{O}$, 45 g Chloralhydrate (Carl Roth GmbH) and 1 g citric acid monohydrate in 1 l distilled water.

Cell culture—MDCK cells

MDCK cells were passaged twice a week at split ratios of 1:5 to 1:6. For routine cell culture and also for the inoculation of microcarriers, the MDCK cells were first washed in PBS-EDTA followed by detachment from microcarriers by incubation with accutase at 37°C for 30 min. We drew a sample of the detached MDCK cells and determined cell viability with CEDEX (Innovatis) and this information was used to calculate the amount of cell suspension required to reach a target inoculum concentration. The cell suspension was added to the Cellbag using a transfer bottle. MPC connectors (Colly Sanitary Technologies) were used to connect the transfer bottle to the Cellbag.

Cell culture—Vero cells

Vero cells were passaged twice a week at split ratios of 1:3 to 1:4 for routine cell culture. In this case, inoculation of the microcarriers via trypsinization was not suitable because it weakened cell attachment and also prevented cell spreading on the carriers. Therefore, the cells were detached from the cell stacks with 0.02% EDTA in Ca^{2+} and Mg^{2+} free PBS. The 10-layer cell stacks were washed with PBS and the cells were detached with 100 ml EDTA solution followed by incubation at 37°C for 20 min. After incubation, we added 500 ml of cell culture medium and pooled the inocula from all the cell stacks. We drew a sample of the cells for counting in a hemocytometer. Trypan Blue exclusion (0.1% Trypan Blue in PBS) was used to determine cell viability. Next, we determined the amount of cell suspension required to attain a particular inoculum concentration and this was added aseptically by welding a transfer bottle to the Cellbag.

Sampling

The cells were sampled daily to determine concentration and morphology. The agitation rate of the base unit was increased to ensure a homogenous carrier suspension (16 rpm and 12° for Cellbag 2L and 18 rpm and 5° for Cellbag 10L, respectively), prior to sample removal. After 2 min at the increased agitation rate, 4 ml of carrier suspension was removed via the Clave port while the base unit was in continuous rocking motion. The agitation parameters of the base unit was then reverted to that for cell cultivation and the sample was transferred to a microcentrifuge tube. After the microcarriers had settled, the supernatant was decanted and microcarriers with Vero cells were suspended in 1 ml 0.1% crystal violet in 0.1 M citric acid followed by incubation for 1.5 h. The released nuclei were counted in a hemocytometer. For MDCK cells, the carriers were resuspended in 0.1% crystal violet in 0.1 M citric acid and Triton X-100. The carrier suspension was then vortexed for 30 s and the nuclei were counted in a Bürker chamber.

To microphotograph Vero cells, 0.5 ml microcarrier suspension was transferred to a microcentrifuge tube. After the microcarriers had settled, the supernatant was removed and Vero cells attached to microcarriers were fixed and stained with 0.3 ml hematoxylin solution. The carriers were viewed at 100-fold magnification.

Optimal working volume for microcarrier cultivations

Optimal working volumes were determined for Cellbag 10L and Cellbag 50L. The working culture volume in the bag was 40% of the maximum recommended volume (i.e., 2 l in Cellbag 10 and 10 l in Cellbag 50L). The microcarrier concentration was 3 g/l of Cytodex 3 and the inoculum concentration was 0.3×10^6 cells/ml. The following mixing rates were tested for each Cellbag and volume: 10 rpm and 5°, 12 rpm and 5°, 14 rpm and 5°, and 12 rpm and 6°.

Optimal mixing conditions for microcarrier cultivations

An optimal mixing study was performed for Cellbag 10L with a working volume of 2 l. The medium and microcarriers were equilibrated for 2 h in Cellbag 10L at a temperature of 37°C and 5% carbon dioxide. A cell inoculum of 0.3×10^6 cells/ml was added to the mixture and a continuous rocking speed was used during the cell-microcarrier attachment phase. The cell cultures were grown for 18 h and reached a cell density of approximately 0.6×10^6 cells/ml before mixing was increased in a stepwise manner. An initial rocking speed of 12 rpm was tested for 5°, 6°, and 7°. The rocking speed was increased hourly in a stepwise manner until the cells started to detach from the microcarriers.

Results and discussion

Determination of optimal working volume and mixing rates for microcarrier cultivations

Microcarrier cultivations produced a homogenous carrier distribution when we used 20% to 80% of the recommended working volume of the Cellbag (Fig 2).

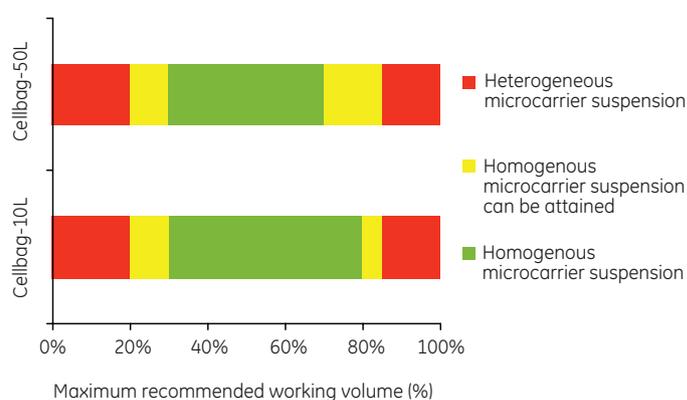


Fig 2. Working volume for microcarrier cultivations expressed as a percentage of the maximum recommended volume.

The microcarriers stuck to the Cellbag at a working volume of less than 20% of the recommended working volume of the Cellbag, and a working volume of more than 80% produced a nonhomogenous microcarrier suspension.

Figure 3 shows that a homogenous suspension of microcarriers was achieved for the rocking angles of 5°, 6°, and 7°. Rocking speeds above 8 rpm resulted in a homogenous microcarrier suspension. MDCK cells began to detach from the microcarriers at rocking speeds higher than 16 to 20 rpm.

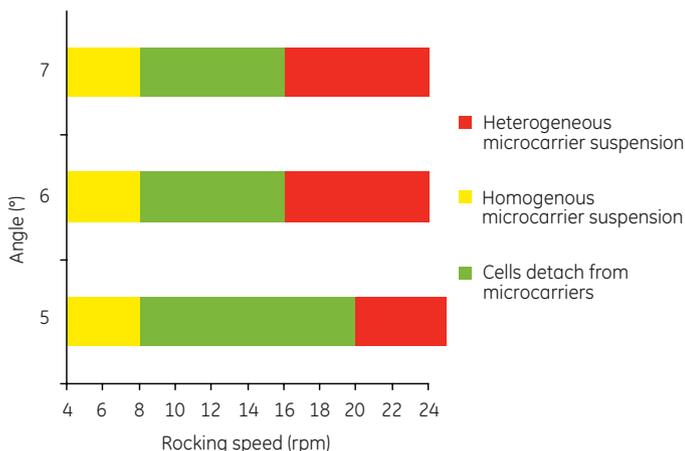


Fig 3. Determination of minimum mixing conditions required for a homogenous microcarrier solution and also, maximum mixing conditions prior to cell detachment from microcarriers using Cellbag 10L. The mixing conditions were similar for Cellbag 50L except that homogenous mixing could also be achieved with at 6 rpm and 7°.

MDCK cell attachment to microcarriers

To find optimal MDCK cell attachment conditions to Cytodex 3 in WAVE Bioreactor System 20/50 (for cell culture volumes up to 25 l), three mixing conditions were compared during the first 4 h of cultivation:

1) intermittent mixing for 2 min at 12 rpm and 6° for every 30 min (Fig 4);

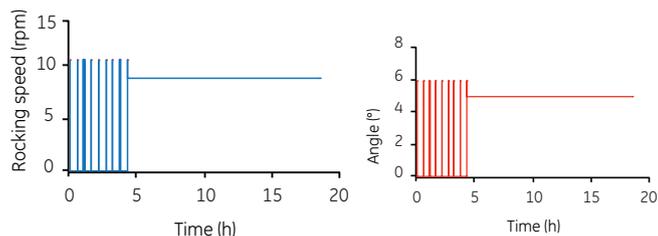


Fig 4. Intermittent mixing for 2 min (12 rpm and 6°) every 30 min for 4 h in Cellbag 10L. After 4 h, mixing was set to 10 rpm and 5°.

2) semi-stationary mixing (Fig 5) with increased mixing for 2 min at 12 rpm and 6° every 30 min with decreased mixing in between at 6 rpm and 3.3°;

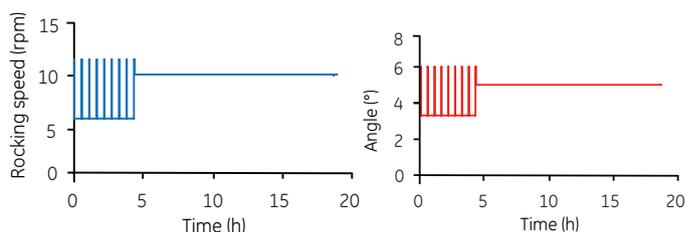


Fig 5. Semi-stationary mixing with increased mixing (at 12 rpm and 6°) for 2 min every 30 min followed by decreased mixing (at 6 rpm and 3.3°) for a duration of 4 h. After 4 h, the mixing was set to 10 rpm and 5°.

3) continuous mixing (rocking speed 8 rpm and 5°). After 4 h of cultivation, mixing was set to 10 rpm and 5° (Fig 6).

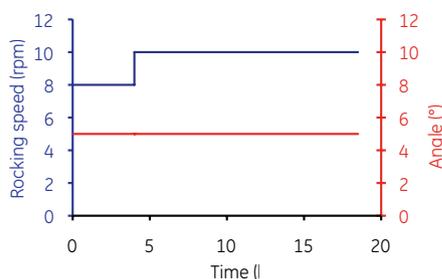


Fig 6. Continuous mixing at 8 rpm and 5° during the first 4 h of cultivation in Cellbag 10L. After 4 h, mixing was set to 10 rpm and 5°.

The microcarrier concentration was 3 g/l of Cytodex 3, and the inoculum cell concentration was 0.3×10^6 cells/ml. The culture conditions were 37°C, pH 7.3, 5% CO₂ and dissolved oxygen was monitored with an optical probe. A cell culture medium supplemented with 2% FBS was used. Samples were taken hourly to measure total cell density with crystal violet stain, and cells in suspension and dead cells were visualized with Trypan Blue.

MDCK cells began to attach to the microcarriers 1 h after inoculation (Fig 7A). After 3 h cultivation, 85% of the inoculum cells were attached (Fig 7B). After 18 h, cells were spread out and had started to grow (Fig 7C).

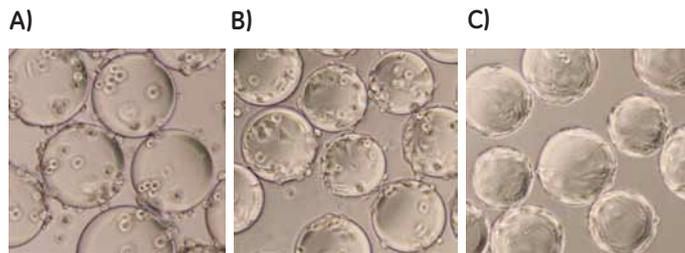


Fig 7. Phase contrast images of MDCK cell attachment to microcarriers. After 3 h cultivation, 85% of the inoculated cells were attached (7B). After 18 h, the cells were spread out and had started to grow (7C).

We did not observe any significant difference in cell attachment and growth between intermittent, semi-stationary, and continuous mixing protocols (Figures 8, 9, and 10), although continuous mixing could result in a more even distribution of the cells. The different mixing protocols showed similar results, and MDCK cell attachment was robust.

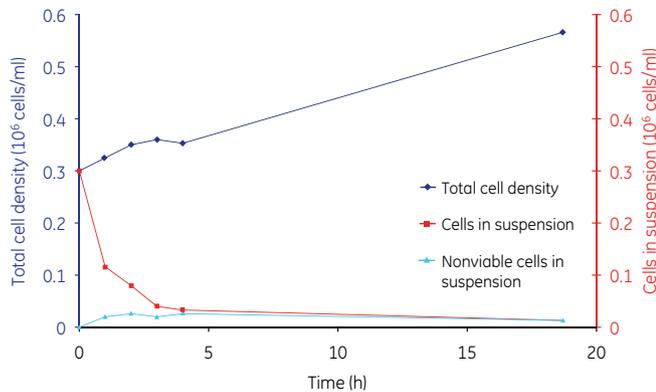


Fig 8. Intermittent mixing during cell attachment to microcarriers.

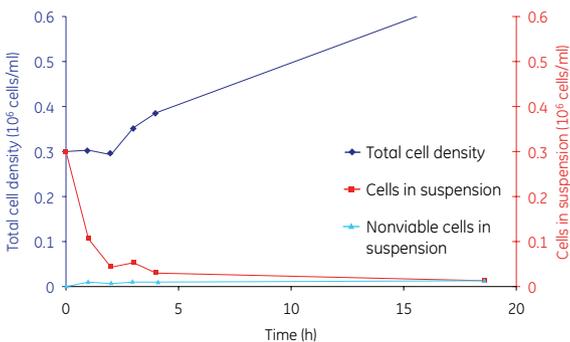


Fig 9. Semi-stationary mixing during cell attachment to microcarriers.

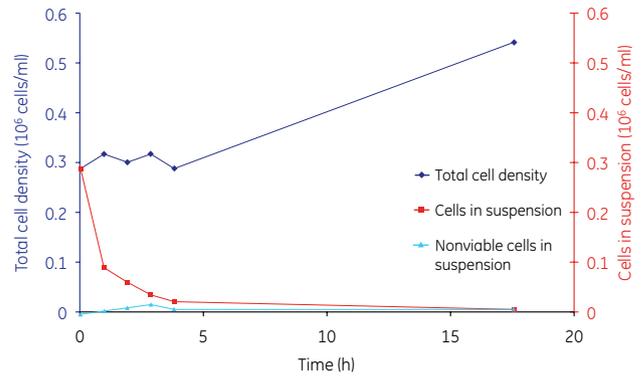


Fig 10. Continuous mixing during cell attachment to microcarriers.

Vero cell attachment to microcarriers

We used different shaking regimens and media compositions to study the attachment of Vero cells to Cytodex 3 microcarriers. Cell cultivations were performed in Cellbag 2L at a working volume of 600 ml. Continuous rocking was set to 12 rpm and 5.5°; intermittent rocking at 16 rpm and 5° for 2 min; followed by 0 rpm and 0° for 18 min. After 6 h, all the experiments were switched to continuous rocking mode at 12 rpm and 8°. Figure 11 shows that in the presence of serum, attachment of about 90% was achieved during continuous as well as intermittent rocking. Under serum-free conditions, the attachment was 80% during intermittent rocking and 30% during continuous rocking.

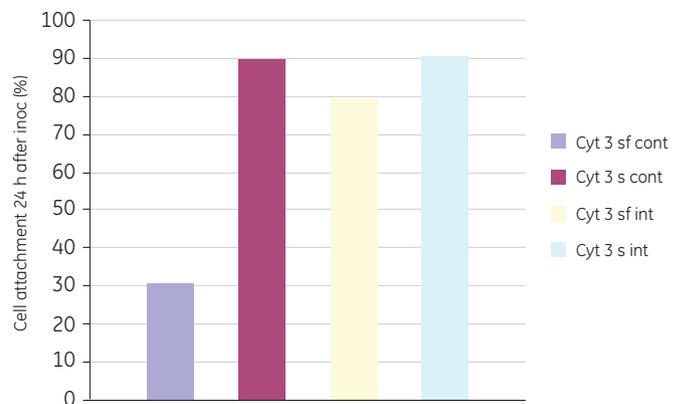


Fig 11. Influence of serum free (sf) and serum containing (s) medium on the attachment of Vero cells to Cytodex 3. The cultures were inoculated during continuous (cont) and intermitted (int) rocking. Cell attachment 24 h after inoculation is shown.

Growth and scale-up of MDCK cells

Maximum cell density and cell growth rate was compared between two bag sizes—Cellbag 10L and Cellbag 50L—in order to study the scalability of MDCK cell expansion in the Cellbag bioreactor. Working culture volume in the bag was 40% of the maximum recommended volume (i.e., 2 l in Cellbag 10L and 10 l in Cellbag 50L).

The microcarrier concentration was 3 g/l of Cytodex 3, and inoculum cell concentration was 0.3×10^6 cells/ml. MDCK cells were mixed at a rocking speed of 8 rpm and at an angle of 5° . The rocking speed was increased in a stepwise manner during cell cultivation from 8 to 14 rpm at a constant angle of 5° . The culture conditions were 37°C , pH 7.4, and dissolved oxygen was monitored. A cell culture medium supplemented with 2% FBS was used.

After an initial lag phase, cells grew to a final concentration of 3×10^6 cells/ml, which was similar to the maximum cell density obtained in a spinner flask. Cell growth was similar between the 2 l and 10 l working volume, thus showing good scalability within this range (Fig 12).

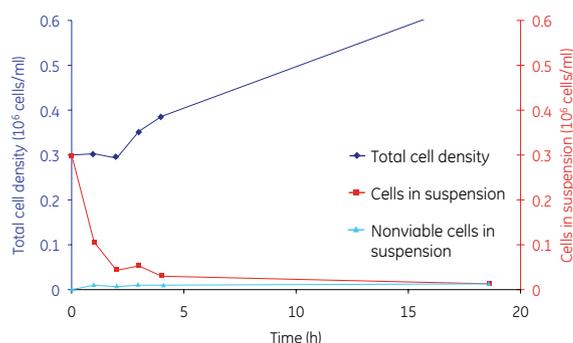
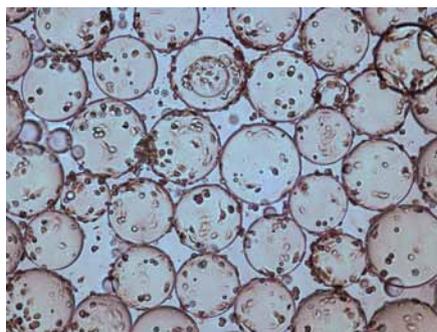


Fig 12. MDCK cell growth was similar for the 2 l and 10 l working volumes, demonstrating good scalability.

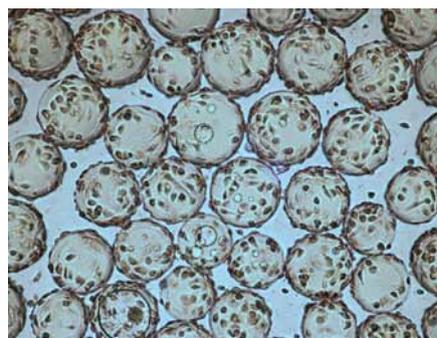
Growth of Vero cells on Cytodex 1 and Cytodex 3

Vero cells were grown on Cytodex 1 and Cytodex 3 in Cellbag 10L at a working volume of 2 l in serum-free medium with an inoculum of 4×10^5 cells/ml. The cells were mixed via intermittent rocking for 6 h (16 rpm and 4.5° for 2 min, 0 rpm and 0° for 18 min). Figure 13A shows that Vero cells were fully spread out on Cytodex 3 after 6 h of cultivation, but on Cytodex 1, the cells were not spread out (Fig 13B). To improve Vero cell spreading on Cytodex 1, the cultures were left unagitated for 2 h and this led to an improved cell spreading on Cytodex 1 (Fig 13C). Both cultures were then switched to continuous rocking (11 rpm and 4.5°) at 37°C .

A) Cytodex 1, 6 h p. i.



B) Cytodex 1, 8 h p. i.



C) Cytodex 3, 6 h p. i.

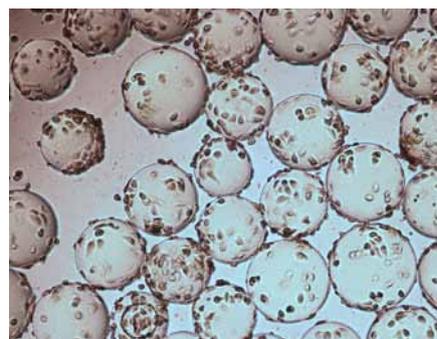


Fig 13. Morphology of Vero cells after attachment. The pictures were taken 6 and 8 h after inoculation.

Growth of Vero cells in spinner flasks

Vero cells were also cultivated in spinner flasks. As shown in Figure 14, the same maximum cell concentrations were achieved in both cultivation systems. The higher cell concentrations on Cytodex 1 are the result of the larger surface area of this carrier. Vero cells reached confluence four days after inoculation during cultivation in WAVE Bioreactor systems. We observed immediate cell growth on both microcarriers without a lag phase (Fig 14).

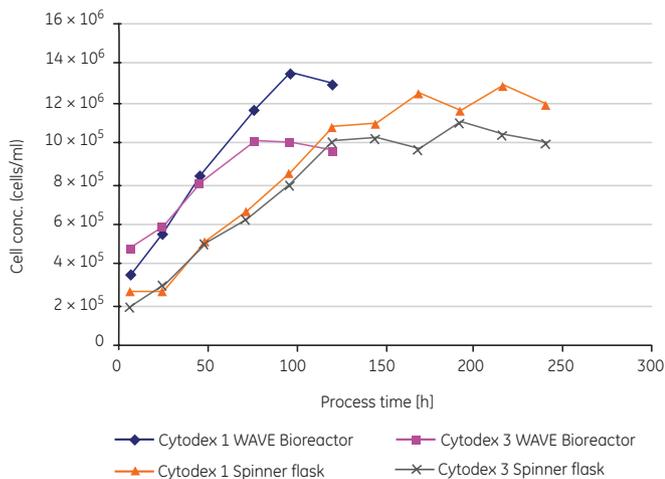
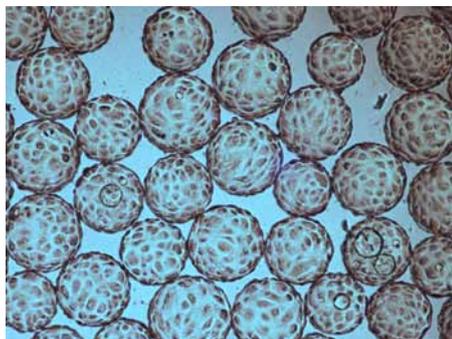


Fig 14. Growth of Vero cells on Cytodex 1 and Cytodex 3 in WAVE Bioreactor systems and spinner flasks.

Figure 15 shows cell morphology after four days.

A) Cytodex 1



B) Cytodex 3

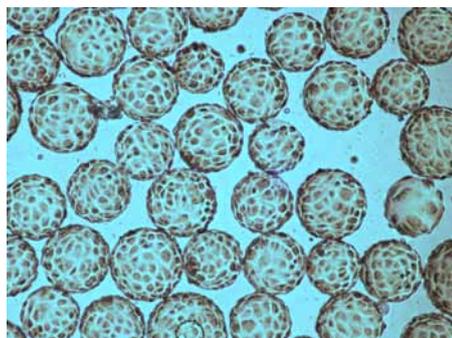


Fig 15. Morphology of Vero cells on Cytodex 1 and 3 after 4 d.

Trypan Blue staining was used to ascertain that the microcarriers were covered by a confluent layer of cells (Fig 16). An intact cell membrane would block Trypan Blue dye and prevent the microcarrier matrix from being stained. Uncovered Sephadex™ readily takes up the dye and this makes it possible to visualize any defects (holes) in the cell layer.

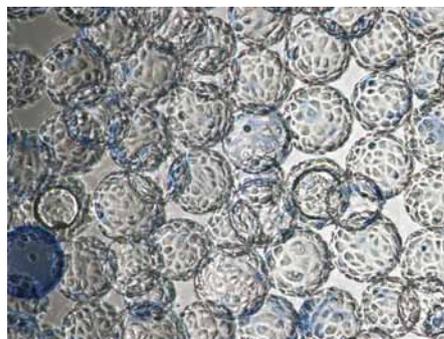


Fig 16. Trypan Blue staining of confluent Vero cells on Cytodex 3 in WAVE Bioreactor system.

Conclusions

In this study, we showed that WAVE Bioreactor system is a versatile tool for the production of MDCK and Vero cells with Cytodex microcarriers. We achieved high attachment rates for both MDCK and Vero cells to the microcarriers. We found that maximum cell concentrations for MDCK and Vero cells were similar between WAVE Bioreactor systems and alternative cultivations like spinner flasks. For microcarrier cultivations, we recommend an optimum working volume of 20% to 80% of the maximum recommended Cellbag volume.

MDCK cells

- We obtained a maximum cell density of 3×10^6 cells/ml, which was similar to the cell density obtained using spinner flasks under similar growth conditions.
- We successfully scaled up microcarrier cultures from a 2 to 10 l working volume using similar agitation conditions.

Vero cells

- We optimized inoculation procedures for Vero cells to achieve attachment of 80% under serum-free conditions.
- Vero cells were cultivated on Cytodex 1 and 3 in serum-free medium and we attained a homogenous distribution in which the cells reached confluence on the microcarriers.
- We grew Vero cells to maximum concentrations of about 1.4×10^6 cells/ml and the growth rate and final cell concentrations were the same for Vero cells grown in spinner flasks.

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