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ABSTRACT

Vero cells are nowadays widely used in the production of human vaccines. They are considered as one of the most productive and flexible continuous cell lines available for vaccine manufacturing. However, these cells are anchorage dependent, which greatly complicates upstream processing and process scale-up. Moreover, there is a recognized need to reduce the costs of vaccine manufacturing to develop vaccines that are affordable worldwide. The use of cell lines adapted to suspension growth contributes to reach this objective.

The current work describes the adaptation of Vero cells to suspension culture in different serum free media according to multiple protocols based on subsequent passages. The best one that relies on cell adaption to IPT-AFM an in-house developed animal component free medium was then chosen for further studies. Besides, as aggregates have been observed, the improvement of IPT-AFM composition and mechanical dissociation were also investigated.

In addition to IPT-AFM, three chemically defined media (CD293, Hycell CHO and CD-U5) and two serum free media (293SFMII and SFM4CHO) were tested to set up a serum free culture of the suspension-adapted Vero cells (VeroS) in shake flasks. Cell density levels higher than 2×10^6 cells/mL were obtained in the assessed conditions. The results were comparable to those obtained in spinner culture of adherent Vero cells grown on Cytodex 1 microcarriers.

Cell infection with LP-2061 rabies virus strain at an MOI (Multiplicity of Infection) of 0.1 and a cell density of $8 \pm 0.5 \times 10^5$ cells/mL resulted in a virus titer higher than 10^7 FFU/mL in all media tested. Nevertheless, the highest titer equal to $5.2 \pm 0.5 \times 10^7$ FFU/mL, was achieved in IPT-AFM containing a reduced amount of Ca^{++} and Mg^{++}. Our results demonstrate the suitability of the obtained VeroS cells to produce rabies virus at a high titer, and pave the way to develop VeroS cells bioreactor process for rabies vaccine production.

1. Introduction

Harrison's pioneering achievement marks the beginning of cell culture technology, a long-established discipline older than one century [1,2]. Since the late 1940s, the introduction of animal cell culture for virus production was considered as the mainstay of modern vaccine technology [3]. This was initiated by Enders et al. [4] who demonstrated that poliomyelitis virus can be produced in non-nervous cells.

Cell culture technology, first developed for vaccine production, is currently of tremendous importance for biopharmaceutical production. More than 50% of the marketed therapeutic bioproducts are produced in mammalian cells [5,6]. Consequently, animal-cell derived products market accounts for US $100 bn in 2018, and shows an increasing share of the global pharmaceutical market [2,7].

Since 1987, a WHO study group published the acceptability of Continuous cell Lines (CCLs) having the potential for an infinite lifespan, for biological production [8]. Thereby, the collection of cell lines used in biomanufacturing was enriched with immortalized cells initially not recommended for human biopharmaceuticals production. This was achieved by dint of various studies based on advanced and perceptive screening technologies [2,9].

The choice of cell substrate being an integral component of viral vaccine preparation is crucial. It has an impact on process productivity and performance, as well as the quality of the manufactured product. It should be susceptible to various viruses, able to grow at high cell densities and easy to scale-up. Likewise, it has to be highly characterized according to international regulatory standards [10,11].

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Vero cell is a continuous cell line (CCL) derived from the kidney of a normal adult African Green Monkey (*Cercopithecus aethiops*) that had aroused by spontaneous immortalisation [12]. Vero cells at low passage (not higher than 150) are widely used for human vaccine manufacturing owing to their safety and permissiveness to different viruses [9,13]. Vero cells at low passage have been approved by World Health Organization since 1986 to manufacture different viral vaccines for human use [14,15].

As biopharmaceutical industry is always concerned with increasing productivity while decreasing costs, researchers are competing to bring novelties to the field of cell culture technology. For these reasons, the use of cell lines adapted to suspension growth is highly encouraged. This is because suspension culture offers easier scale-up and higher cell densities. Consequently, cheaper products more accessible to Low and Middle Income Countries (LMICs) can be obtained [3,16,17].

The interest to suspension-adapted cells began in the early 1950s with the pioneering research of Owens et al. [18] who succeeded in growing mouse fibroblast cells in suspension using the "tumbling tube" device. Subsequently, Earle et al. [19] cultured 929-L cells in modified roller tubes rotated in different drums [20]. These findings concurred with the first industrial application of a cell line growing in suspension, which was development of the foot-and-mouth disease virus veterinary vaccine in BHK-21 cells [21].

Despite this success, the first work describing Vero cells adaptation to suspension growth was only reported in 1992 by Litwin [22]. He demonstrated that suspension culture of Vero cell aggregates might be an alternative method for large-scale cultivation for vaccine production. This work opened the door to researchers to adapt cell lines used for biologicals production such as Vero [23,24], MDCK [25], and CHO [26] to suspension culture under serum free conditions.

Moreover, new cell lines growing in suspension like PER.C6, HEK293, AGE1.CR and CAP cells were designed as new cell substrate candidates for biopharmaceuticals manufacturing [27].

Rabies is one of the oldest widespread and most scary neglected zoonosis caused by lyssavirus infection. Despite the availability of various licensed vaccines, rabies still causes 59,000 human deaths per year globally as reported by WHO [28–31]. International health organizations have set up the global coordinated initiative "zero by 2030" to eradicate dog-mediated rabies by 2030 [29,32–35].

Cell culture derived rabies vaccines are considered among the safest and most efficient vaccines for human rabies prevention [36]. In this respect, Vero cells are widely used for rabies vaccine manufacturing [36]. Nevertheless, no published work describes the use of Vero cells suspension culture for rabies virus replication. The current work reports the adaptation of Vero cells to suspension growth in various serum-free media, using different protocols. As our group is familiar with rabies vaccine development [37–42], kinetics growth of adapted cells (VeroS) and their ability to produce LP-2061 rabies virus strain, were investigated in shake flasks, and in different serum free media.

Our ultimate goal is the development of a new human rabies vaccine candidate that could be more accessible to LMICs. The use of this new cell line combined with IPT-AFM an in-house developed animal-component free medium will highly contribute to reduce the vaccine cost.

2. Material and methods

2.1. Cell line

Vero cells at passage 131 were provided by the National Laboratory for Control of Biologicals (Tunis, Tunisia) and originally obtained from ATCC (CCL-81).

2.2. Culture media & chemicals

Adherent Vero cells were initially grown either in Minimum Essential Medium (MEM) (Gibco, Life technologies, USA, #61100-087) supplemented with 10% Foetal Bovine Serum (FBS) (Gibco, Life technologies, USA, #10270-106) or in IPT-AFM, an in-house developed animal component free medium [41,43]. M199 and M199 without calcium ($CaCl_2$) and magnesium ($MgSO_4$) (kindly provided by Dr Amine Kamen (McGill University, Department of Bioengineering, Montreal, Canada)) were manufactured by Gibco (Life Technologies, USA, #31100).

For suspension culture, five commercial serum free/chemically defined media were tested next to IPT-AFM. These media were Hycell CHO and SFM4CHO supplied by Hyclone (GE Healthcare, Logan, USA); 293SFMII and CD293 provided by Gibco (Life Technologies, MD, USA) and finally CD-U5 (ProBioGen, Germany) custom manufactured by Becton Dickinson (MD, USA). Cell Boost 5 Supplement (Hyclone, GE Healthcare, Logan, USA, #SH30865.01) was also used.

All chemicals used in this work were purchased from Sigma (USA).

2.3. Virus strain

Louis Pasteur 2061 rabies virus strain adapted to Vero cells (LP 2061/Vero) was provided by Institut Pasteur (Paris, France).

2.4. Cell dissociation

Adherent Vero Cells cultivated in serum containing medium (SCM) or in IPT-AFM were subcultivated using a recombinant trypsin called TrypLE Select Gibco (Life Technologies, New York, USA, #12563). Cell subcultivation was performed as described by the manufacturer. Briefly, after two washes with phosphate buffer saline (PBS), a thin layer of enzyme solution was added to the culture flask and the cells were incubated for 2–5 min at room temperature. Dislodged cells were then centrifuged at 1000 rpm for 10 min. Static cultures either in serum containing or animal component-free media were carried out at 37 °C and 5% CO_2 and a seeding density of 7×10^4 cells/cm^2.

2.5. Cell growth and infection in shake flasks

Vented, nonbaffled shake flasks of 125–500 mL (Corning, Acton, MA) were used. Cultivations in shake flasks were started at 0.5–1×10^6 cells/mL and passaged after 2–4 days with 10–50 vol (%) of medium from the previous culture. Cultures were conducted at 37 °C; 7% CO_2 and 150 rpm in an orbital shaker (Infors, Switzerland). 0.1% (v/v) pluronic acid (F-68) (Sigma, USA, #P1300) was added to the medium to protect the cells from shear forces.

Cells were infected at an MOI of 0.1 without proceeding to a medium exchange. After cell infection, temperature was switched to 34 °C and pH was maintained at 7.4 by daily addition of $NaHCO_3$ at 88 g/L, as described in [39].

2.6. Adaptation of Vero cells to suspension growth

As detailed in Fig. 1, three protocols were tested to adapt adherent Vero cells to suspension growth. In brief, the first protocol is based on sequential adaptation of Vero cells to 80% IPT-AFM in monolayer before moving to shake flask culture. Whereas, the second method relies on starting from one ampoule of cells cryoconserved under serum containing conditions, then performing one passage in monolayer, and finally moving to shake flask culture prior to progressive adaptation to IPT-AFM. Finally, the third adaptation procedure was performed starting from one ampoule of cells

cryoconserved in IPT-AFM then moving to agitated (shake flask) culture within 2 passages in static culture.

The cells were monitored daily and the kinetic parameters (Cd: Cell division number and μ: specific growth rate) were calculated.

The suspension adapted cells were cryopreserved with 7% Dimethlsulfoxyde (DMSO) and 0.1% methylcellulose in liquid nitrogen.

2.7. Cell counting

For viability assessment, cells were stained with trypan blue (0.2% (w/v) in phosphate buffered saline (PBS). For nucleus count (total cell count), five millilitres of cell culture were washed three times with PBS then treated in 5 mL of 0.1 M citric acid (Sigma, USA, C0706) containing 0.1% crystal violet (Sigma, USA, #C3886) and 0.1% Triton X-100 (Applichem Panreac, Germany, # A4975) and incubated at least for 1 h at 37 °C. The released nuclei were counted using a hemacytometer.

Viable cell density was calculated by multiplying the total cell density by the viability.

2.8. Monitoring of cell infection

Aliquots of 2 mL samples of rabies infected culture supernatant were first washed twice with PBS prior to cell resuspension in a minimum volume. Thereafter, 2 drops of ~50 μL were deposited on a microscope slide and let to dry at room temperature in a laminar flow cabinet. Then, the slide was fixed with chilled 80% acetone and stained with fluorescein-labelled anti-rabies nucloeocapsid antibodies (Biorad, France, #3572114).

2.9. Rabies virus titration

The virus titration protocol was carried out in accordance with a modified Rapid Fluorescence Focus Inhibition Test (RFFIT) described by Rourou et al. [44] and expressed in Fluorescent Focus Units per ml (FFU/mL). This method allows the assessment of intracellular rabies virus before its budding into the culture medium.

2.10. Calculations

– Cell division number, Cd was calculated according to the equation:

$$Cd = \log_2 \frac{Final\ cell\ density\ level\ (at\ the\ end\ of\ culture)}{Starting\ cell\ density\ (at\ inoculation)}$$

– Specific growth rate, μ (h^{-1}) was estimated by the following equation:

$$\mu = \frac{(\ln Xn - \ln Xn - 1)}{(tn - tn - 1)}$$

where X represents the viable cell density per mL, t represents the time point of sampling expressed in hours, the subscripts n and n – 1 stand for two succeeding sampling points.

3. Results

3.1. Adaptation of adherent Vero cells to suspension growth in IPT-AFM

Preliminary studies were first carried out to select an appropriate adaptation protocol for the continuity of the experiments. In this context, cells were adapted to suspension growth in IPT-AFM according to the 3 protocols described in material & methods and Fig. 1. Both protocols 1 and 2 started with the use of Vero cells cryopreserved in serum containing medium. Sequential adaptation of cells to grow in IPT-AFM in static culture was conducted for protocol 1, and then the cells were adapted to suspension culture once

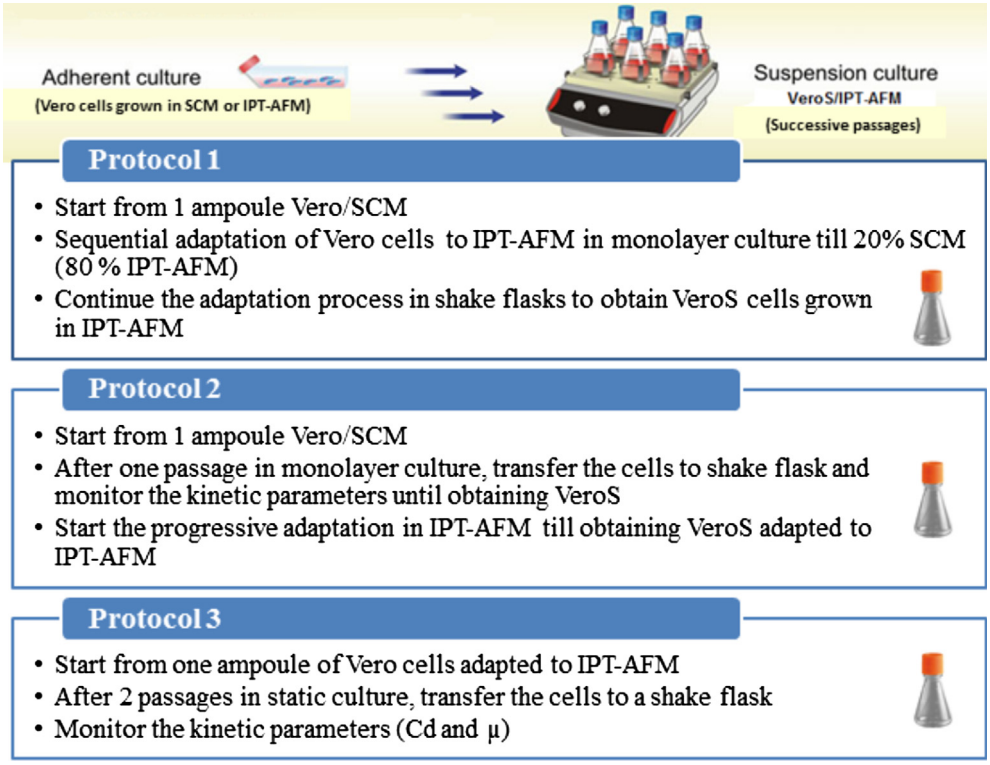

Fig. 1. Adaptation of adherent Vero cells to suspension culture according to the three protocols.

they were cultivated in 80% IPT-AFM. Whereas for protocol 2, cells were first adapted to suspension growth in serum containing medium in shake flasks, the obtained VeroS were then adapted to IPT-AFM. For protocol 3, Vero cells were already adapted to IPT-AFM (as detailed in Smith et al. [43]), and only adaptation to suspension growth was performed.

Data shown in Fig. 2 indicate that after 9 passages in shake flasks, a consistent cell growth was noticed. Nevertheless, higher cell density level was observed for cells adapted to suspension growth according to protocol 3. The highest cell density level reached $2.3 \pm 0.14 \times 10^6$ cells/mL versus $1.4 \pm 0.15 \times 10^6$ cells/mL and $1.65 \pm 0.12 \times 10^6$ cells/mL for protocols 1 and 2, respectively.

Hence, the third protocol was selected for further investigations. A new experiment to adapt Vero cells to suspension growth was performed according to protocol 3; 20 successive passages were therefore carried out. Cell growth performance of Vero cells adapted to suspension (VeroS) was checked; cells were grown in IPT-AFM in shake flasks (Fig. 3a). As indicated in Fig. 3b, VeroS cells were able to grow in suspension in IPT-AFM. As obtained previously, a consistent growth was observed after 9 to 10 passages. An average cell density of $2.1 \pm 0.4 \times 10^6$ cells/mL was obtained; C_d reached 1.53 ± 0.3 and the specific growth rate (μ) was 0.014 ± 0.003 h^{-1}.

VeroS cells grew mainly as individual cells in IPT-AFM. Nevertheless, small to medium aggregates (of about 5–20 cells) started to appear within 2 passages. Optimisation experiments were carried out to minimize/eliminate the aggregates. As calcium is involved in cell to cell adhesion, the amount of Ca^{++} was decreased from 500 μM to 100 μM by diluting 5-fold the basic medium which is M199 as a compromise between cell growth and cell aggregation. Moreover, mechanical dissociation was assessed by efficient pipetting and decantation. The use of an IPT-AFM free of Ca^{++} and Mg^{++} or with reduced amounts of these components next to mechanical dissociation, had allowed VeroS to grow as small aggregates (<10 cells) and/or single cells (Fig. 4).

3.2. Optimization of VeroS growth in IPT-AFM

We noted that the elimination of Ca^{++}, Mg^{++} contributing in aggregate removal, had slightly reduced cell density level. The maximum cell density reached $1.95 \pm 0.05 \times 10^6$ cells/mL versus $2.23 \pm 0.07 \times 10^6$ cells/mL in IPT-AFM (standard composition). To enhance cell density level, IPT-AFM depleted of Ca^{++}, Mg^{++} was enriched with 5% (v/v) of cell boost 5 supplement which is a chemically defined additive, designed to provide nutrients such as lipids, amino acids, vitamins, and growth factors. The enrichment of diluted IPT-AFM with cell boost 5 at 5% slightly improved cell growth; the highest cell density level was around $2.3 \pm 0.07 \times 10^6$ cells/mL which was comparable to that obtained in the standard IPT-AFM. In order to reach an acceptable cell density level with a minimum of aggregates, the use of a reduced amount of Ca^{++}, Mg^{++} was considered as the optimal solution. In addition, adding cell boost 5 to IPT-AFM without Ca^{++}, Mg^{++} didn't improve cell growth. As indicated in Figs. 4 and 5, a maximum cell density level of $2.9 \pm 0.1 \times 10^6$ cells/mL and a μ around 0.018 h^{-1} were reached within 4 days; furthermore growth as single cells was noticed in IPT-AFM with 20% Ca^{++}, Mg^{++}.

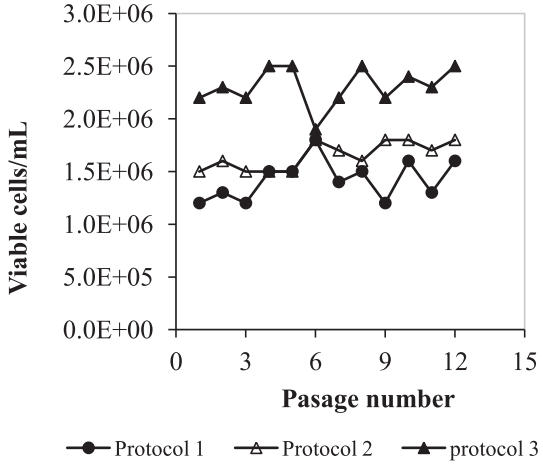

Fig. 2. Growth of Vero cells adapted to suspension culture in shake flasks according to 3 different protocols (data from 12 passages).

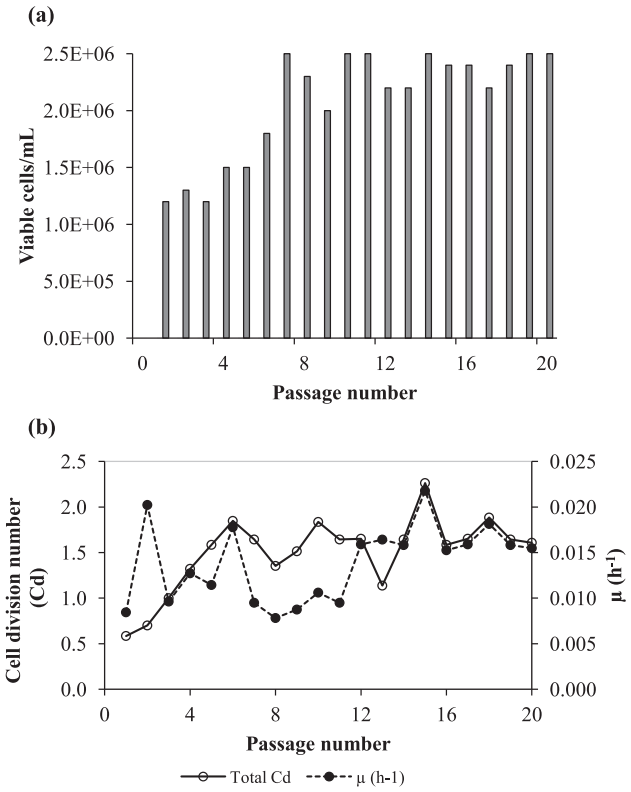

Fig. 3. (a) Growth profile of VeroS in IPT-AFM in shake flasks (data from 20 passages), (b) kinetic parameters of VeroS cells growth in IPT-AFM in shake flasks (data from 20 passages).

3.3. Study of VeroS growth in different serum free media

Kinetics of VeroS growth were also studied in commercially available serum free and chemically defined media. Cells were grown in the medium to be tested at least for 3 passages, prior to the kinetic study.

As displayed in Fig. 6, VeroS cells were able to reach $2.9 \pm 0.2 \times 10^6$ cells/ml and a specific growth rate (μ) of 0.018 $h^{-1} \pm 0.005$ in the chemically defined Hycell CHO medium, 4 days after starting the culture. Moreover, CD-U5 which is also a chemically defined medium, showed promising results; a maximum cell density of $2.5 \pm 0.5 \times 10^6$ cells/mL and μ around 0.017 h^{-1} were obtained. Besides, a cell density close to 2×10^6 cells/mL ($1.9 \pm 0.15 \times 10^6$ cells/mL) was achieved in the protein free medium SFM4CHO. These cell densities were higher than those obtained in the chemically defined CD293 and the serum free 293SFMII media ($1.7 \pm 0.2 \times 10^6$ cells/mL).

In addition, VeroS cell aspect was monitored over 10 passages in the different media (Fig. 7). IPT-AFM with 20% Ca^{++}, Mg^{++} next

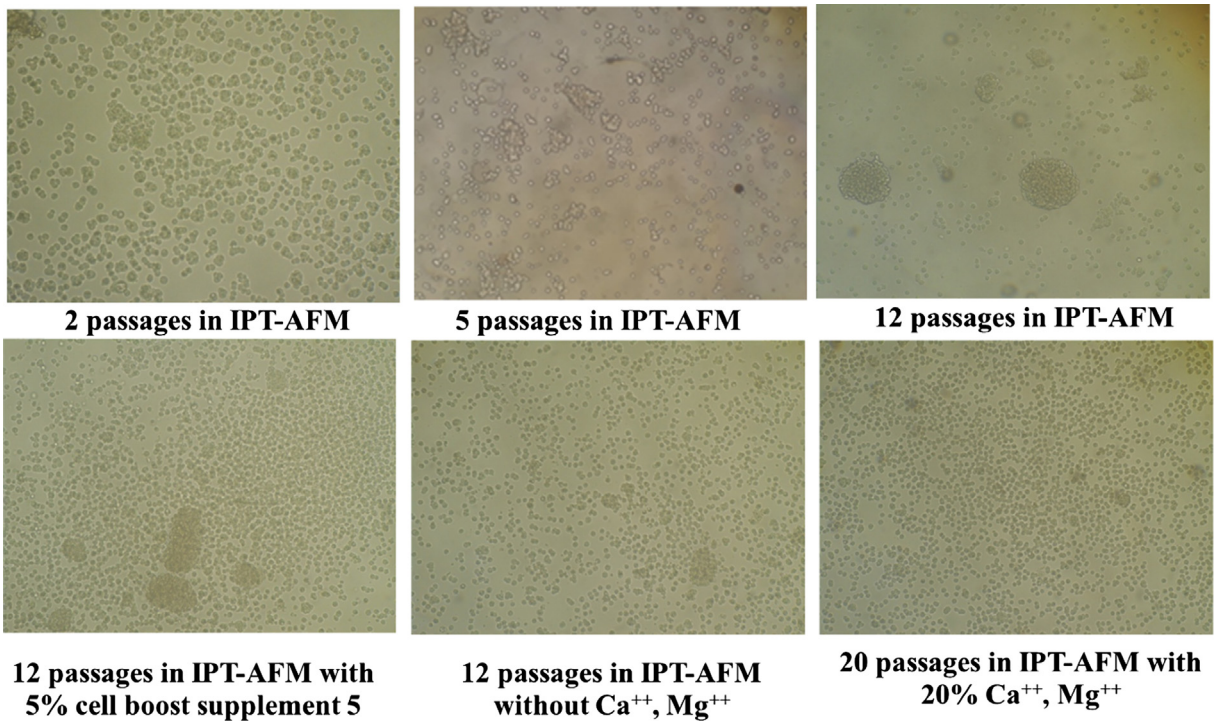

Fig. 4. Microscopic observation of VeroS grown in shake flasks in IPT-AFM during cell adaptation. Cells were observed with an inverted light microscope without staining at ×100.

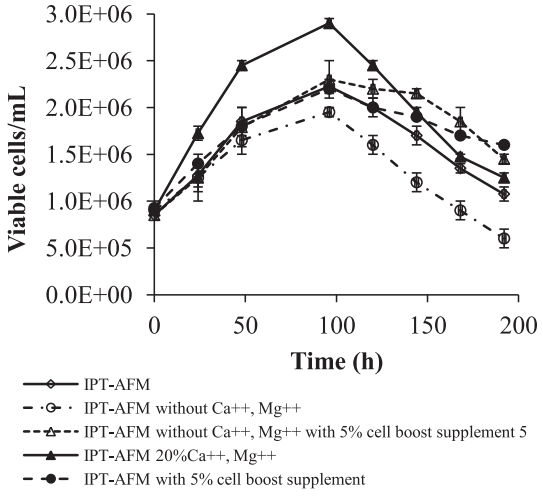

Fig. 5. Improvement of IPT-AFM formulation to sustain VeroS growth in shake flasks. The error bars represent the standard deviation of three experiments.

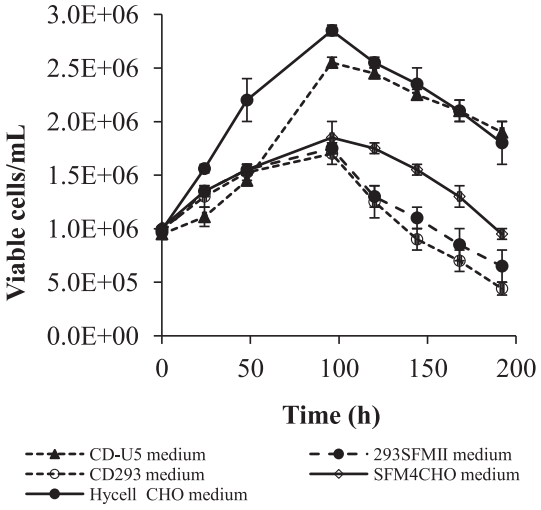

Fig. 6. Growth profile of VeroS grown in shake flasks in IPT-AFM and 5 commercial serum free/chemically defined media. The error bars represent the standard deviation of three experiments.

to Hycell CHO, CD-U5 and SFM4 CHO media were suitable for VeroS growth as single cells and small aggregates while CD293 and 293SFMII media enhanced aggregation.

Figs. 6 and 7 demonstrated that IPT-AFM with 20% Ca^{++}, Mg^{++} next to HyCell CHO, CD-U5 and SFM4-CHO media allowed the growth of VeroS as single cells and/or small aggregates (less than 10 cells). While CD293 and 293SFMII media showed a growth of VeroS cells with some aggregates in shake flasks. Cell growth as individual cells is desirable due to the fact that nutrient limitation may occur inside the aggregates leading to cell death [45]. For these reasons, Hycell CHO, SFM4 CHO and CD-U5 media were chosen next to IPT-AFM and IPT-AFM 20% Ca^{++}, $Mg^{++}$to perform rabies virus infection experiments.

3.4. Rabies virus production in VeroS cells

VeroS cells were first grown in shake flasks at 37 °C, 5%CO_2. Then at day 3 cells were infected with LP2061 rabies virus strain without medium exchange at an MOI equal to 0.1. As shown in Fig. 8, VeroS cells grown in the different media allowed LP-2061 rabies virus strain replication. Virus titer exceeded 10^7 FFU/mL in all tested media. The highest yield was obtained 2–3 days after cell infection, thereafter a progressive or a sharp decrease of virus titer was noticed depending on the medium used. The highest titer was obtained in IPT-AFM 20% Ca^{++}, Mg^{++} and was equal to $5.22 \pm 0.5 \times 10^7$ FFU/mL. The enrichment of IPT-AFM with cell

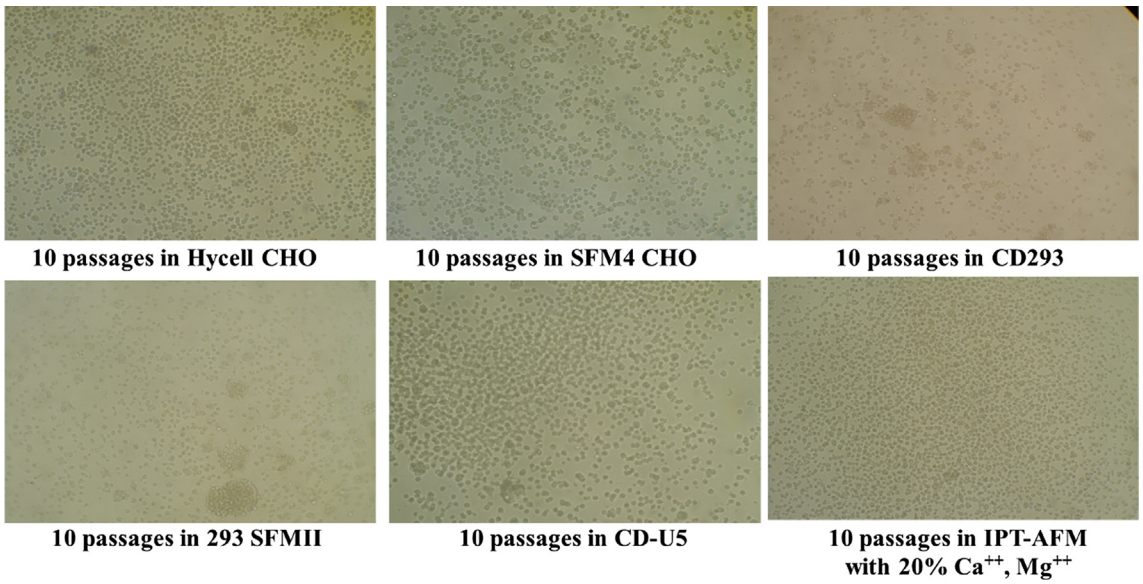

Fig. 7. Microscopic observation of VeroS grown in shake flasks in improved IPT-AFM and other serum free/chemically defined media. Cells were observed with an inverted light microscope without staining at ×100.

boost 5 supplement at 5% did not improve virus titer which was about 2.4×10^7 FFU/mL. Additionally, comparable virus titers were achieved in the other media. Virus titer levels reached 1.9, 2 and 2.4×10^7 FFU/mL in CD-U5, SFM4CHO and Hycell CHO media, respectively.

4. Discussion

Vero cells have an excellent safety profile and show permissiveness to various viruses. Hence, they were the first approved continuous cell line for human viral vaccines production [36,46]. Nowadays, numerous viral vaccines for human use on the market are produced in Vero cells (rabies, rotavirus, poliovirus, ...).

Since the adaptation of anchorage-dependent cells to suspension cultures solves scale-up limitations associated with monolayer cultures, it is considered as an attractive tool for industrial applications [17,23]. Various cell lines such as MDCK [25,47], HEK-293 [48,49], BHK-21 [21,50–52], CHO [53] and AGE1.CR [27,54] have been successfully adapted to suspension growth for biopharmaceuticals production. Nevertheless, only few studies describing the adaptation of Vero cells to suspension growth are published.

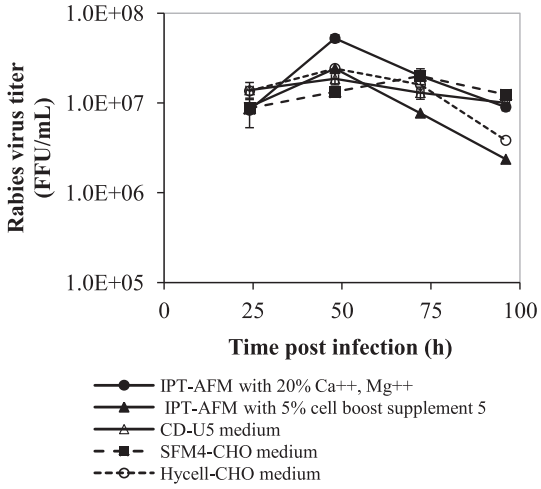

Fig. 8. Kinetics of rabies virus (strain LP 2061) production in VeroS cells grown in shake flask and in various serum free media. Cells were infected during exponential growth phase at an MOI of 0.1. Glucose level was not regulated. The error bars represent the standard deviation of three experiments.

In the current work, adherent Vero cells were first adapted to grow in suspension as individual cells. The adaptation procedure lasted 60 days which is shorter than the period reported by Paillet et al. [23] who needed 120 days to adapt Vero E6 cells to suspension growth in serum-free home-made medium using stepwise method. They obtained sVero cells growing as individual cells in suspension.

In our case, a preliminary study was first carried out to choose an adequate adaptation protocol. Once adapted to suspension growth, Vero cells no longer adherent, were named VeroS cells and they were able to grow in suspension as single cells and small aggregates. We showed that the growth profile of VeroS cells in IPT-AFM was maintained over 20 passages in shake flasks. In addition, growth kinetic parameters and cell yield were comparable to those reached with adherent Vero cells grown in VP-SFM, a commercially available animal component free medium [41,55,56]. Nevertheless, further studies should be conducted to enhance cell population doubling. In addition starting VeroS culture at lower cell density will be investigated.

The results reported in the current study are however different from those previously described by Litwin et al. [22] who only succeeded to grow Vero cells in suspension as cell-aggregates in an in-house developed serum-free medium containing insulin, bovine serum albumin and a commercial serum replacement concentrate. They also demonstrated that during the adaptation process, a long lag period of between 20 and 30 days was required to reach cell density levels over 10^6 cells/mL even though these cells had been adapted to a SFM in anchorage-dependent cultures. In our hands, VeroS cells were able to reach 2.9×10^6 cells/mL in IPT-AFM with 20% Ca^{++}, Mg^{++} 4 days after culture inoculation, although cell density level at inoculation was high (around 10^6 cells/ml).

As data describing Vero cells adapted to suspension growth are limited, it is valuable to quote some successful achievements related to other cell lines adapted to suspension growth. For instance, Van Wielink et al. [25] succeeded to adapt adherent MDCK cells to suspension growth in a commercial serum free

Table 1
Rabies virus yield in VeroS and adherent Vero cells cultivated in IPT-AFM.

Culture mode	Cell density at infection (cells/mL)	Maximum virus titer (FFU/mL)	Virus yield (FFU/cell/day)
Adherent/spinner	$2 \pm 0.2 \times 10^6$	$1 \pm 0.3 \times 10^7$	2.5 ± 0.8
VeroS/shake flask	$2 \pm 0.2 \times 10^6$	$3 \pm 1 \times 10^7$	3.75 ± 1

medium. They adopted a protocol based on gradual replacement of serum containing medium with a serum free one after 7–12 passages. They obtained a cell density of 2.2×10^6 cells/mL in shake flasks.

All studies described in the literature performed with various cell lines adapted to suspension growth indicated that cell behaviour is medium dependent. As an example, Merten et al. [57] developed MDSS2, a serum free medium sustaining the growth of BHK-21 cells as aggregates (BHK21-BRS) whereas Nikolay et al. [51] recently obtained BHK-21 cells grown as individual cells in suspension (BHK-21SUS). In the latter study, the cells were initially cultivated in the commercial Z-Medium before switching gradually to the chemically defined medium TCX6D.

In the last part of this work, the replication of LP-2061 rabies virus strain was studied. This was conducted in line with our continuous efforts to develop a rabies vaccine affordable for LMIC populations. The development of safe and cheaper vaccines can significantly reduce the economic burden of rabies vaccination programs worldwide leading to eventual success of rabies control globally [58].

Kinetics of rabies virus replication and production observed after infection of VeroS cells with LP-2061 virus strain were comparable to those obtained with adherent Vero cells in IPT-AFM [42] and VP-SFM [41] media on Cytodex 1 microcarriers. The use of perfusion culture during the virus production step will allow to avoid the decrease of virus titer as we showed in previous studies [41,42].

In addition, VeroS cell infection at a cell density of 2×10^6 cells/mL in IPT-AFM, and an MOI of 0.1 (data not shown), led to a slight enhancement of virus specific productivity when compared to adherent Vero cultures in IPT-AFM in spinner culture (Table 1).

The results described in the current study even though acceptable, are still preliminary; numerous improvements are possible. For instance, virus titer can be further enhanced once it is adapted to VeroS cells through several consecutive passages. This is in line with the data described by Perrin et al. [50] who demonstrated that the adaptation of the virus strain to BHK-21 cell aggregates by serial passages had significantly increased the virus titer from 10^5 to 8.5×10^8 FFU/mL.

Generally, cell adaptation to suspension growth results in an enhancement of the virus titer as reported in several studies described in the literature. It was shown that suspension adapted MDCK cells preserved their susceptibility to influenza strains. For example, the resulting virus yields obtained by Van Wielink et al. [25] for eight influenza virus strains (H1N1, H3N8, H5N2, H5N7, H5N9, H7N1, H7N7 and H9N2) were comparable to those obtained in adherent cells. Lohr et al. [47] also reported that suspension adapted MDCK (MDCK.SUS2) cells grown in suspension in a chemically defined medium gave similar performances to MDCK cells regarding cell proliferation and influenza virus production.

The current study demonstrates that VeroS cells are susceptible to rabies virus infection and are able to support effective production of rabies virus in batch shake flask culture. Nevertheless, the identity of the new VeroS cell line generated in this study should be checked. The safety of this new cell line should also be characterized according to the international regulatory guidelines before its acceptance as a cell substrate for human vaccine manufacturing. Tumorigenic potential of VeroS cells will be studied in vivo using the adequate animal model as described in Liu et al. [59]. On the other hand, cell growth performance of VeroS cell clones that will be obtained by the limit dilution method, will be also studied to demonstrate cell homogeneity. Additionally, further developments aiming to set up and to intensify the process will be conducted.

5. Conclusion

This work is a first step to adapt Vero cells to suspension growth; our results showed that Vero cells were successfully adapted to suspension culture in serum free media without the use of any disaggregation agent. Although these results are promising, further experiments are however required to enhance cell population doubling. A preliminary study was also carried out to investigate the replication of LP-2061 rabies virus strain in VeroS cells.

We showed that IPT-AFM with reduced amount of Ca^{++} and Mg^{++} next to the two chemically defined commercial media (Hycell CHO and CD-U5) were suitable for the replication of rabies virus in VeroS cells grown in shake flask. These media showed similar performance to serum-containing medium, in terms of cell growth and rabies virus titer. Moreover, VeroS cell infection showed a higher cell productivity compared to adherent Vero cells.

Ultimately, the establishment of suspension-adapted Vero cells (VeroS) grown in serum free medium will allow to develop an alternative cell cultivation method for the production of vaccines at large-scale.

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Declaration of Competing Interest

No conflict of interest is declared by the authors.

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