



Semliki Forest Virus replicon particles production in serum-free medium BHK-21 cell cultures and their use to express different proteins

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Abstract The production of biopharmaceuticals as vaccines in serum-free media results in reduced risk of contamination and simpler downstream processing. The production of enveloped viruses and viral vectors such as Semliki Forest Virus (SFV) typically requires lipids that are provided by supplementation with animal serum, so production under serum-free conditions is challenging. In this work, the capacity to deliver genetic material of SFV-viral replicon particles (SFV-VRPs) produced in BHK-21 cells adapted to serum-free medium (BHK/SFM) was evaluated. Three transgenes were evaluated: GFP used as a model

protein, while hepatitis C virus nonstructural protein 3 protease domain (HCV-NS3p) and rabies virus glycoprotein (RVGP) were selected based on their distinct nature (enzyme and glycoprotein, respectively). BHK/SFM cells produced a sevenfold higher number of SFV-VRPs, as determined by qRT-PCR. These particles showed similar capacities of infecting BHK/FBS or BHK/SFM cells. GFP expression was evaluated by flow cytometry, HCV-NS3p activity by enzymatic assay, and RVGP expression by ELISA and Western Blot. Expression analysis revealed higher levels of GFP and HCV-NS3p in BHK/SFM, while the levels of RVGP were similar for BHK/SFM and BHK/FBS. In conclusion, the BHK/SFM cells showed increased SFV-VRP production yields, without affecting vector infectivity or heterologous gene expression, hence validating the use of BHK/SFM for industrial applications.

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Keywords BHK-21 cells · Hepatitis C virus nonstructural protein 3 protease domain · Rabies virus glycoprotein · Serum-free medium · Semliki Forest Virus · Viral replicon particles

Abbreviations

SFV-VRPs	Viral replicon particles of Semliki Forest Virus
BHK/FBS	BHK-21 cells grown in DMEM supplemented with 10% of fetal bovine serum

BHK/SFM	BHK-21 cells adapted to serum free medium
VRP-GFP-SFM or VRP-GFP-FBS	Viral replicon particle carrying the GFP gene produced in BHK/SFM or BHK/FBS respectively
VRP-NS3p-SFM or VRP-NS3p-FBS	Viral replicon particle carrying the NS3p protease domain gene produced in BHK/SFM or BHK/FBS, respectively
VRP-RVGP-SFM or VRP-RVGP-FBS	Viral replicon particle carrying the RVGP gene produced in BHK/SFM or BHK/FBS

Introduction

The development of an effective and safe recombinant vaccine depends on the efficient presentation of antigens to the immune system. A variety of molecular biology technologies and expression platforms are available to facilitate the expression of different antigens. Vaccine technologies based on nucleic acids represent important new platforms for the development of vaccines. Consequently, considerable research efforts and investments are being directed towards the development of DNA vaccines (Wolff et al. 1990; Ulmer et al. 1993; Hasan et al. 1999) and RNA-based vaccines (Geall et al. 2013; Sahin et al. 2014; Mogler and Kamrud 2015).

Recent technology improvements have presented opportunities for employing RNA-based approaches, mainly as mRNA or RNA virus-based drugs and vaccines. Moreover, the vectors can be applied in various forms such as recombinant viral particles, naked replicon RNA or layered RNA–DNA vectors (Lundstrom 2018). The vectors are usually obtained by genetic engineering techniques from positive single-stranded RNA viruses, such as alphaviruses, picornaviruses, and flaviviruses (Sahin et al. 2014).

Alphaviruses are commonly used as highly effective viral vectors and have been fundamental in the development of platforms to obtain efficient self-replicating RNA, due to the presence of their strong promoters (Geall et al. 2013; Mogler and Kamrud 2015). Alphaviruses are used for protein expression

due to their simple genomic organization and efficient propagation in different cells cultures, with the generation of high levels of the heterologous protein due to self-amplification of the replicon RNA (Rayner et al. 2002). Several features of alphaviruses make them suitable for vaccine development: they are stimulators of the immune response; they allow the construction of multivalent vaccines; and pre-existing immunity to alphaviruses in humans is neglectable. Furthermore, the cytoplasmic replication of alphavirus confers an additional safety level by eliminating the possibility of integration into the host genome (Riezebos-Brilman et al. 2006; Vander Veen et al. 2012).

The main alphaviruses used as vectors for vaccine production are SFV, Sindbis virus, and VEE (Venezuelan equine encephalitis). These can be administered in the form of naked RNA, vectors containing DNA–RNA hybrid molecules (Zimmer 2010), or virus replicon particles (VRPs) (Lundstrom 2003). VRPs are fully assembled viral particles harboring a self-replicating RNA genome in which the structural genes were replaced by a gene of interest. Consequently, VRPs can infect the cells and express the gene of interest but they cannot generate new VRPs (Spurgers and Glass 2011). VRPs provide increased safety when compared with conventional life-attenuated or inactivated vaccines (Slovin et al. 2013).

Vaccination of animals with VRPs based on alphaviruses, employing rats, rabbits, pigs, calves, sheep, non-human primates, and fish have validated the efficiency and safety of the VRP vaccination platform (Ljungberg and Liljeström 2015). The results suggest that this approach generates cellular and humoral responses and, in many cases, protection against challenge with lethal doses of viruses and other non-viral agents (Lundstrom 2012a; Astray et al. 2014; Ljungberg and Liljeström 2015; Ajbani et al. 2015).

The SFV system enables rapid expression of high levels of heterologous proteins. Therefore, vaccination with SFV-VRPs enables to increase the dose of the antigen (recombinant protein) without increasing the number of administered VRPs. Alternatively, to reduce the number of VRPs while maintaining the dose of the antigen hence potentially making SFV vaccines safer by reducing the number of vectors administered.

An important way to increase the safety of biological products used for vaccination is to eliminate poorly-defined and potentially harmful components, such as animal serum, from the cell culture medium. Several serum-free medium (SFM) formulations have been developed for different cell lines. The identification of a suitable serum-free formulation for a given animal cell line and culture condition should be determined empirically (Sinacore et al. 2000). The assessment of different media is advisable, in order to achieve an efficient and economically viable process (Rodrigues et al. 2012).

Serum-free media are commonly used in the production of monoclonal antibodies, vaccines, and other biological products (Lohr et al. 2010; Peschel et al. 2013; Rourou et al. 2014; Genzel 2015; Manna et al. 2015; Zhang et al. 2017). Serum-free platforms have been described for viral vaccine production, with examples including the use of rabies virus in Vero, BHK-21 and HEK-293 cells (Merten et al. 1994, 1999; Perrin et al. 1995; Kallel et al. 2002; Frazzatti-Gallina et al. 2004; Peschel et al. 2013; Fontana et al. 2015); foot-and-mouth disease virus in BHK-21 cells (Barteling and Vreeswijk 1991); influenza virus in MDCK cells and HEK293SF-3F6 cells (Brands et al. 1999; Petiot et al. 2011; Huang et al. 2015), and poliovirus in Vero cells (Cinatl et al. 1993; Merten et al. 1994).

The impact of the adaptation of mammalian cell lines to SFM for the production of vaccines is not yet fully understood. The adaptation of cells to new culture conditions promotes changes not only in growth characteristics and the dynamics of virus replication, but also in protein expression and biochemical characteristics (Kluge et al. 2013). In the case of an enveloped virus such as SFV, the host-derived membrane is a structural component of the virus particle. So the composition of this membrane plays a fundamental role in the production of VRPs. Fetal serum supplementation provides an external source of lipids, and studies of enveloped virus (retrovirus) have found that serum deprivation results in lower infectious virus titers (Rodrigues et al. 2009, 2012). In addition to protein expression, the production of SFV-VRPs also represents a challenge under serum-free conditions.

The goal of this work was to study the influence of animal serum in the production of SFV-VRPs and its impact in the expression of 3 proteins [green fluorescent protein (GFP), hepatitis C virus nonstructural

protein 3 protease domain (HCV-NS3p), and rabies virus glycoprotein (RVGP)] selected based on their distinct nature and the strong interest in their use as vaccine antigens. The performance of the BHK cells adapted to SFM was evaluated by measuring cell culture kinetic parameters, SFV-VRP production, SFV-VRP infection, and expression of different heterologous proteins.

Materials and methods

Cell culture

Baby Hamster Kidney 21 (BHK-21) cells were first cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Glasgow, UK) containing 10% (v/v) of FBS (Sigma, St. Louis, USA). The BHK-21 cells were then adapted to a serum-free medium (CHO-S-SFM II, Life Technologies), using a sequential adaptation method (Sinacore et al. 2000). The cells were maintained in 25 or 75 cm² T-flasks in an incubator at 37 °C, under 5% CO₂, and were subcultured 2–3 times per week.

Cell growth assay

The cell growth kinetic parameters of adherent cells were determined during five days, using 6-well plates with 2 mL of medium containing an inoculum of 2.5×10^5 cells/mL. All experiments were carried out (in duplicate) at 37 °C, under 5% CO₂. Cell growth was followed for 120 h, with samples being harvested daily for cell counts and metabolite determinations.

Viable cells were determined by trypan blue exclusion, with a 1:10 (v/v) mixture of the cell sample and 0.4% (w/v) trypan blue, using a hemocytometer (Improved Neubauer, Brand). Concentrations of glucose, glutamine, and lactate were determined using a YSI 2700 analyzer (Yellow Spring Instruments, USA).

Generation of SFV-VRPs

SFV-VRPs expressing the genes of interest were generated using BHK-21 cells, based on a production system described previously (Benmaamar et al. 2009; Lundstrom 2012b). For obtaining SFV-VRPs, the genes coding for structural proteins were supplied by

SFV-Helper2 plasmid (Berglund et al. 1993). The SFV vectors used were previously obtained: SFV-RVGP and SFV-Helper2 (Benmaamar et al. 2009); SFV-GFP (Puglia et al. 2013) and SFV-NS3p (Lemos et al. 2018). Briefly, the helper plasmid (pSFV-Helper2) and expression plasmids (pSFV-GFP, pSFV-NS3p, and pSFV-RVGP) were linearized using *SpeI* (for the helper plasmid) and *NruI* (for the expression plasmids) restriction enzymes and were confirmed by restriction digestion analysis. Linearized plasmids were transcribed in vitro using the MAXIScriptSP6 kit (Life Technologies, Carlsbad, USA), according to the manufacturer's instructions. The pSFV transcripts were capped during transcription using Cap analog (Invitrogen, Carlsbad, USA). The transcription mix was incubated at 40 °C for 2 h. The concentrations of synthesized RNAs were determined by fluorimetry, using the Quant-iT RNA Assay Kit (Life Technologies, Eugene, USA).

Expression and helper RNAs were used for co-transfection into 8×10^6 BHK/SFM or BHK/FBS cells/800 μ L by electroporation using a Gene Pulser II system (Bio-Rad, Hercules, CA, USA), with two pulses of 850 V/25 mF. The medium containing the SFV-VRPs (VRP-GFP, VRP-NS3p, or VRP-RVGP) was collected 24 h after co-transfection, clarified to remove cellular debris by centrifugation at 12,000g for 30 min at 4 °C, and stored at -80 °C until analyzed.

Titration of SFV-VRPs

Recombinant virus titration was performed as described previously (Puglia et al. 2013). The SFV-RNA extraction was carried out using the QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA, USA), following the manufacturer's protocol. The RNA was treated with DNase I enzyme (Promega, Madison, WI, USA). The synthesis of cDNA was performed by reverse transcription with M-MLV enzyme (Thermo Fisher, Waltham, MA, USA), using the SFV-R-E-2, 5'-CTCAATGATGAC GTGGAGCT-3' primer.

Quantification of the SFV-VRPs was performed by quantitative PCR (qPCR), using a StepOne Real-Time PCR System (Applied Biosystems, Foster City, USA). The reaction was set up using the PowerSYBR[®] Green kit (Life Technologies, Foster City, USA). The sequences of the primers used were as follows: SFV-F-I-2, 5'-ACAGACTGTCACTGAGCAG-3' and SFV-R-I-2, 5'-TCTCTGCAGTAGATGGTCAC-3'.

The cDNA of the samples was quantified using a standard curve obtained using serial dilutions of a linearized SFV-RVGP plasmid containing from 6×10^7 molecules/ μ L to 6×10^2 molecules/ μ L. The samples and standards were submitted to qPCR cycling, using the following conditions: 5 °C for 10 min, $40 \times$ (95 °C for 15 s, 53 °C for 15 s, 60 °C for 15 s). The fluorescence was measured at 60 °C. After amplification, the melting curve (60–95 °C) was constructed. The total SFV RNA copy number present in the original sample was calculated by multiplication of the cDNA copy number by a conversion factor specific to each sample, which considered all the dilutions performed. The virus titer was expressed as the number of virus replicon particles per milliliter (VRP/mL). The titers presented are the averages for three replicates.

Protein expression using SFV-VRPs

Protein expression was evaluated using adherent cultures of BHK/SFM or BHK/FBS in 6-well plates. On the previous day, the cells were seeded at a concentration of 4×10^5 cells/well. The cells were infected in 0.5 mL of serum-free medium, using different VRP:cell ratios. In order to achieve infectivity, the SFV-VRPs were treated with α -chymotrypsin (Sigma-Aldrich, St. Louis, MO, USA), at 1.6 mg/mL, for 30 min at room temperature, followed by inactivation of the protease activity for 5 min with aprotinin (Sigma, USA), at 1 mg/mL. After 2 h of adsorption, fresh medium was added (2 mL). Samples of the supernatant and cells were collected several times post-infection, for subsequent determination of protein expression.

Flow cytometry analysis

Following infection with VRP-GFP-SFM or VRP-GFP-FBS, the BHK/SFM cells were harvested by pipetting, while the BHK/FBS cells were harvested in PBS containing 5 mM EDTA. The cells were centrifuged at 700g for 5 min and suspended in $1 \times$ PBS. Fluorescence analyses were performed by counting 10,000 events using a FACS Calibur flow cytometer (Becton–Dickinson, San Jose, USA). GFP fluorescence was excited at 488 nm and emission was measured at a wavelength of 530 nm. Non-infected BHK/SFM and BHK/FBS cells were used as controls.

The geometric mean values were used for comparisons of the fluorescence intensities of the different samples and the percentages of fluorescent cells.

NS3p-mRNA and RVGP-mRNA analysis

The BHK/FBS and BHK/SFM cells infected with VRP-NS3p or VRP-RVGP were harvested and pelleted by gentle centrifugation at various post-infection times. The cell pellets were immediately stored at -80°C . Quantification of mRNA, RNA extraction, DNase treatment, reverse transcription, and qPCR procedures were performed as described previously (Benmaamar et al. 2009).

HCV-NS3p protease assay

Analysis of the activity of HCV-NS3p expressed by the BHK/SFM or BHK/FBS cells infected with VRP-NS3p-SFM was performed using the SensoLyte[®] 520 HCV Protease Assay Kit (AnaSpec, Fremont, CA, USA). Briefly, BHK/SFM or BHK/DMEM cells infected with SFV-NS3p were harvested 24 h post-infection, centrifuged at 300g for 5 min, and resuspended in PBS. The washed cells were again pelleted to exchange PBS for a hypotonic buffer (10 mM Tris-HCl, pH 7.8, 10 mM NaCl), using a ratio of 100 μL of buffer for every 2.0×10^6 cells. After 30 min incubation at 4°C , the cells were lysed using three freeze-thaw cycles, followed by centrifugation at 900g for 5 min. The supernatant containing intracellular virus particles was collected and used according to the manufacturer's protocol. Fluorescence signals were acquired every 10 min, during 2 h, using an Infinite[®] 200 PRO NanoQuant microplate reader (Tecan, Männedorf, Switzerland), with excitation and emission wavelengths set at 490 and 520 nm, respectively.

RVGP detection

RVGP ELISA The RVGP concentrations in the cell cultures were determined using an ELISA kit (Institute Pasteur, Paris), as described previously (Astray et al. 2008). For this, BHK/SFM or BHK/FBS cells infected with VRP-RVGP-SFM or VRP-RVGP-FBS were harvested, centrifuged at 700g for 5 min, and resuspended in 1 mL of lysis buffer (25 mM Tris, pH 7.4, 25 mM NaCl, 5 mM MgCl_2) containing 0.2% Nonidet P-40 detergent (Fluka, Cat. No. 74385). The

samples were mixed every 15 min during the incubation period (1 h at 4°C), followed by centrifugation for 5 min at 10,600g for sedimentation of the cell debris. ELISA was performed (in duplicate) using 200 μL volumes of the supernatants. The samples were measured at 492 nm using a LabSystems MultiSkan MS photometer. The RVGP content was calculated using a calibrated standard curve obtained using purified RVGP (supplied in the RVGP ELISA kit).

RVGP Western Blot assays The RVGP was also detected using Western Blot assays. For this, the cell pellets were treated with RIPA lysis buffer (10 mM Tris, 140 mM NaCl, 1% Igepal, 0.5% deoxycholate, 0.1% SDS, 1 mM PMSF, pH 8.0), followed by centrifugation of the lysates at 15,000g for 10 min and recovery of the supernatant. The samples were loaded onto an SDS 12% polyacrylamide gel, under non-denaturing conditions, using LDS loading buffer (Thermo, São Paulo, Brazil), and were blotted onto a nitrocellulose membrane. The membrane was blocked with low-fat milk and was then incubated with anti-rabies virus glycoprotein antibody (LS-C75309, LSBio, Seattle, WA, USA) and anti-mouse HRP conjugated antibodies (Invitrogen, Carlsbad, CA, USA). Detection was performed using the Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL, USA) and the Alliance 2.7 chemiluminescence imaging system (Uvitec, Cambridge, UK).

Statistical analysis

The ANOVA test with a confidence interval of 95% ($p < 0.05$) was used for analysis of the kinetic parameters and the results of the RVGP expression studies.

Results

Adaptation of cell cultures to a serum-free medium

Adaptation of the BHK-21 cells to SFM was performed according to the sequential adaptation method (Sinacore et al. 2000). A preliminary analysis of different commercial SFMs indicated CHO-S-SFMII as the one enabling higher SFV-VRP production (data

not shown). Adaptation of the cells was achieved using decreasing concentrations of DMEM supplemented with 10% FBS. The cells were first grown in a medium containing 25% SFM and 75% DMEM with FBS (2–3 passages with 90% viability), in which the cells remained adherent. The cells were then progressively transferred to a medium containing 75% SFM (3–4 passages with over 90% viability), 90% and 95% SFM (3 passages with viability above 90%). The cells were then transferred to 100% SFM, under these culture conditions the cells become semi-adherent and showed visible morphological changes which were maintained during several passages and after thawing (data not shown). Consequently, it was not necessary to use trypsin for detachment. The BHK/SFM cells were submitted to at least ten passages before establishing a cell bank and performing the analytic and kinetic tests.

Growth kinetics of the BHK-21 cells in SFM

In order to characterize the metabolism of the BHK-SFM cells, growth kinetic studies were performed during 120 h, using the BHK-SFM and BHK-FBS cells in 6-well plates. As shown in Fig. 1a, the BHK/SFM and BHK/FBS cells exhibited similar growth. The BHK/SFM cells reached a maximum cell concentration of 2.16×10^6 cells/mL at 96 h of culture, with a maximum specific growth rate of 0.036 h^{-1} , while the BHK/FBS cells reached a maximum cell concentration of 2.5×10^6 cells/mL at 72 h of culture, with a maximum specific growth rate of 0.043 h^{-1} . For both cell types, the exponential growth phase occurred during the period between 24 and 72 h, with cell viability remaining above 90% until the third day of the culture, with 80% of viable cells at the end of the experiment.

Figure 1b shows the evolution of glucose–glutamine consumption and lactate production. The BHK/SFM cells consumed 88% of the glutamine, reaching a final concentration of 0.07 g/L, while the BHK/FBS cell cultures showed complete depletion of this nutrient after 120 h. The BHK/SFM and BHK/FBS cells consumed 84% and 40% of the glucose, respectively. Consequently, lactate production was twofold higher for the BHK/SFM cells (3.31 g/L), compared to the BHK/FBS cells (1.84 g/L).

Statistical analysis ($p < 0.05$) of the kinetic parameters (Fig. 1c) showed no significant differences

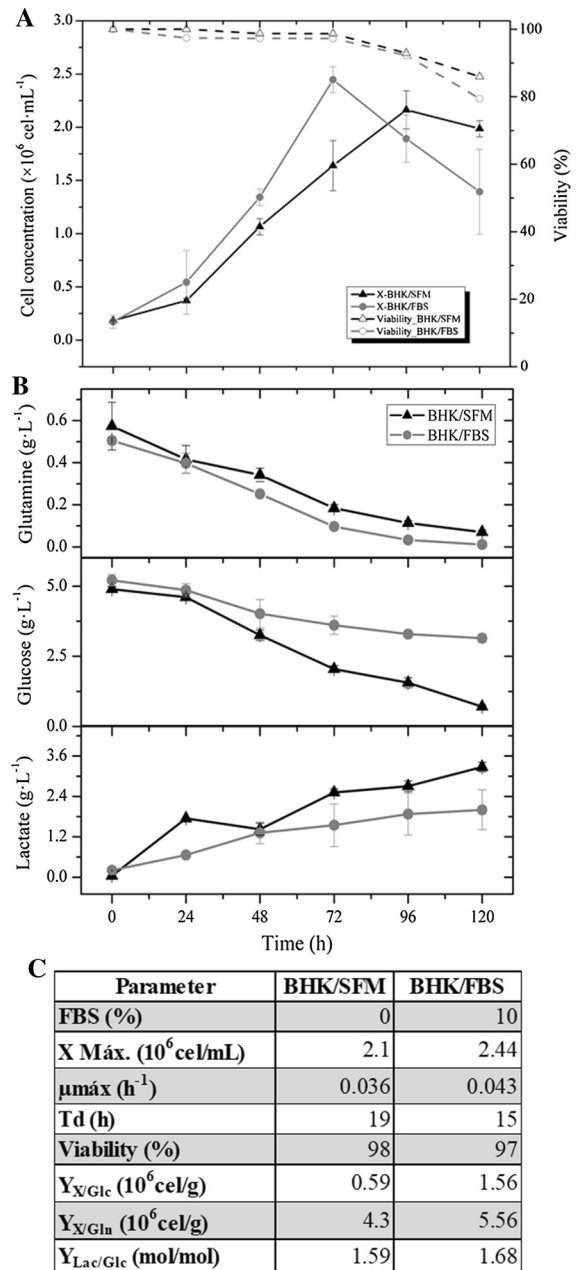


Fig. 1 Kinetic parameters of BHK/SFM and BHK/FBS cells. BHK/SFM cells (black line) and BHK/FBS cells (gray line) were cultured in a 6-well plate with an initial concentration of 2×10^5 cells/mL. Growth and metabolic status were monitored for 5 days. **a** Viable cell concentration and percentage viability. **b** Consumption of glutamine and glucose and lactate production. The bars represent the standard deviation of three independent experiments. **c** Summary of main kinetic parameters of BHK/SFM vs BHK/FBS cells

between the BHK/SFM and BHK/FBS cells in terms of growth, viability, doubling time, or maximum

specific growth rate. Important indicators of the metabolic states of cells are the ratio between the number of cells produced and the glucose/glutamine consumed ($Y_{X/Glc}/Y_{X/Gln}$), as well as the ratio between the amount of lactate produced and the amount of glucose consumed ($Y_{Lac/Glc}$). Among these parameters, differences were observed for $Y_{X/Glc}$, which suggested better use of glucose in the BHK/FBS cells, while the other parameters were similar for the two cell types.

These results indicated that adaptation of the BHK/SFM cells was successful without major changes in cell growth and metabolic profiles.

SFV-VRPs production and titration

To evaluate the ability of the BHK/SFM cells to produce functional SFV-VRPs the expression of different heterologous genes (VRP-GFP, VRP-NS3p, and VRP-RVGP) was quantified. The VRP/mL unit refers to the quantity of RNA-SFV copies present in the sample.

Efficient production was observed for all the SFV-VRPs, with titers ranging from 4.2×10^9 to 4.8×10^9 VRP/mL for SFV-VRPs produced in BHK/SFM, and from 0.5×10^9 to 1.8×10^9 VRP/mL for SFV-VRPs obtained in BHK/FBS (Fig. 2). The values for all the SFV-VRPs produced in BHK/SFM cells were

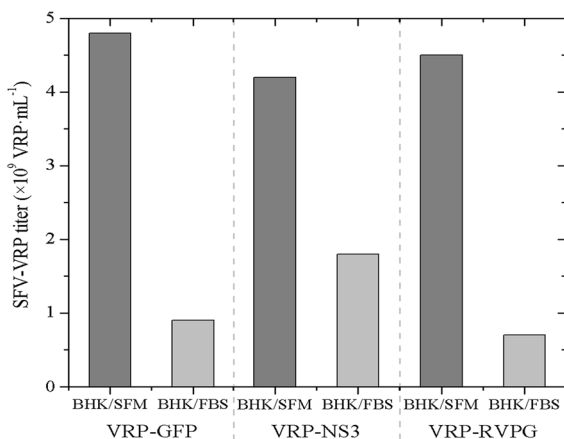


Fig. 2 SFV-VRPs production in BHK/SFM vs. BHK/FBS cells. BHK/SFM or BHK/FBS cells were co-transfected with RNA-SFV-GFP or RNA-SFV-NS3p or RNA-SFV-RVGP and RNA Helper. The supernatant was collected 24 h after transfection and RNA copies were quantified by qPCR assay and indicated as VRP-SFV (VRP/mL). The results represent the mean of technical replicates

approximately 4.6-fold higher than the titers obtained using BHK/FBS cells. These findings showed that despite being enveloped virus, the production of SFV-VRPs was more efficient in the BHK/SFM cells.

Recombinant protein expression in BHK/SFM cells using SFV-VRPs

Gfp

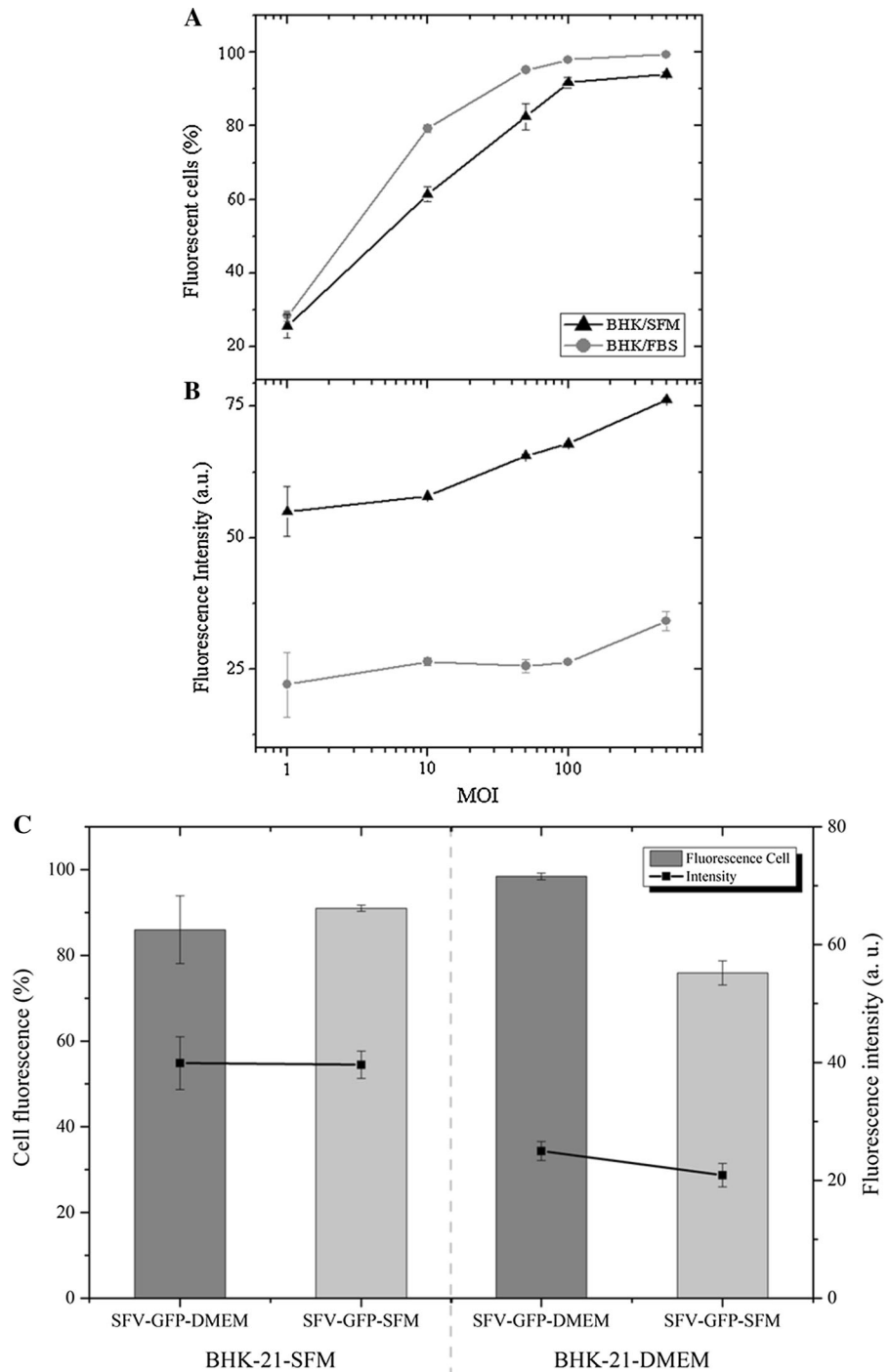
The influence of the amount of SFV-VRPs used for cell infection (Multiplicity of infection, MOI) on recombinant protein expression was investigated by infecting BHK/SFM and BHK/FBS cells with VRP-GFP-SFM or VRP-GFP-FBS, at different MOIs. The percentage of fluorescent cells and the fluorescence intensity were measured at 6, 12, and 24 h post-infection. For both BHK/SFM and BHK/FBS cells, the results showed increase in percentage of fluorescent cells using MOIs from 1 to 50, while at MOIs of 100 and 500 resulted in no significant increase, likely a plateau was reached where all the cells should be infected (Fig. 3a). The peak numbers of fluorescent cells were obtained using a MOI equal to 100, with 91% and 97% of fluorescent cells for BHK/SFM and BHK/FBS, respectively.

Initial GFP-expression and emission was detected 6 h post-infection, indicating efficient infection and successful processing of the SFV-VRPs in the BHK/SFM cells. The fluorescence intensity was twofold higher for the BHK/SFM cells, compared to the BHK/FBS cells (Fig. 3b), suggesting that BHK/SFM expressed more GFP molecules per cell, compared to BHK/FBS.

In order to compare the infection efficiencies of the SFV-VRPs produced with or without FBS, BHK/SFM and BHK/FBS cells were infected in parallel with VRP-GFP-SFM and VRP-GFP-FBS (Fig. 3c). The results showed the same pattern as observed in Fig. 3a, b, indicating that the VRPs seem to infect with higher efficiencies when using the same media they were produced in.

These results demonstrated that the BHK/SFM cells were effectively infected and that higher GFP expression (measured as fluorescence intensity) was obtained in SFM media despite the origin of the VRPs (i.e. particles produced in BHK/FBS or BHK/SFM).

Fig. 3 Expression of GFP protein in BHK/SFM vs BHK/FBS cells. BHK/SFM or BHK/FBS cells were infected with VRP-GFP-SFM or VRP-GFP-FBS respectively. Different MOIs were used (1; 10; 100; 500) for A and B. The experiments were performed in a 6-well plate and expression was measured at 24 h after infection by flow cytometry; in **a** percentage of fluorescent cells and in **b** fluorescence intensity. **c** BHK/SFM and BHK/FBS cells were infected in parallel with VRP-GFP-SFM or VRP-GFP-FBS, using a MOI of 100. Cells were analyzed 24 h post-infection. The bars represent the standard deviation of duplicate



HCV-NS3p

The expression of HCV-NS3p was determined quantifying both mRNA (through RT-qPCR amplification) and NS3p protease activity. The expression levels of

mRNA-NS3p (at 3, 6, and 12 h post-infection) were similar for the BHK/SFM and BHK/FBS cells (Fig. 4a), confirming efficient delivery of VRP-NS3p-SFM transgene into the BHK/SFM cells.

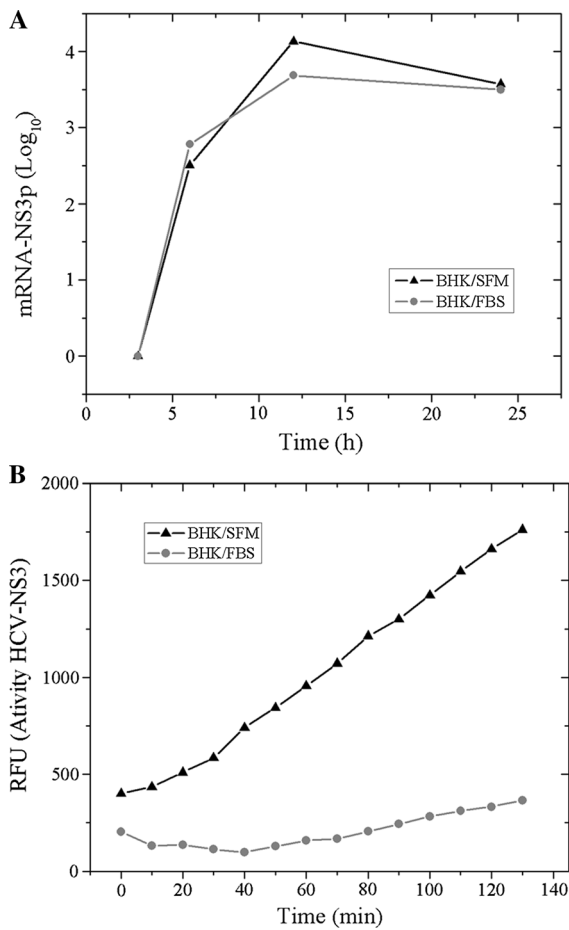


Fig. 4 mRNA-NS3p expression and HCV-NS3p activity from BHK/SFM vs BHK/FBS cells. BHK/SFM and BHK/FBS cells were infected in 6 well plates, collected 3; 6; 12 and 24 h post-infection for **a** and 24 h post-infection for **b** and Sensolyte520 kit respectively. In **a** mRNA-RVGP expression was evaluated by qPCR in cells with 50:1 VRP: Cell of SFV-NS3p-SFM. The results are average of duplicate. In **b** proteolytic cleavage of the 5-FAM/QXL™ 520 FRET substrate by NS3p expressed on BHK/SFM or BHK/SFB cells infected with MOI of 100. The fluorescence signal was monitored every 10 min at an excitation/emission ratio of 490/520 nm. Samples were normalized from the control of the assay containing substrate without NS3p. The results represent the mean of technical replicate

The functionality of NS3p expressed in BHK/SFM and BHK/FBS cells was evaluated by determining its proteolytic activity. As shown in Fig. 4b, the cleavage of a reporter peptide by NS3p increased considerably over time. NS3p expressed in BHK/SFM showed 4.8-fold higher activity at 130 min when compared to NS3p expressed in BHK/FBS cells. These results could reflect differences in activity of the proteins

expressed in the BHK/SFM or BHK/FBS cells as a result from different protein processing such as post-translational modifications (Liefhebber et al. 2010; Hundt et al. 2013).

RVGP

RVGP expression was evaluated using mRNA-RVGP amplification, ELISA, and Western Blot assays. The mRNA-RVGP amplification pattern was similar for the BHK/SFM and BHK/FBS cells (Fig. 5a), with an increase in mRNA-RVGP expression from 3 to 12 h post-infection, followed by a decrease at 24 h post-infection.

The expression of mRNA-RVGP in BHK/SFM was superior, however, the ELISA results revealed no significant differences in RVGP expression among the different experimental conditions. The values obtained for RVGP expression were in the range 0.8–1.2 $\mu\text{g}/10^6$ cells (Fig. 5b). The difference showed between mRNA expression and ELISA results could reflect variance in protein processing.

The Western Blot results (Fig. 5c) showed that RVGP synthesized in the BHK/SFM and BHK/FBS cells exhibited the expected molecular mass of 65 kDa, corresponding to the size of the protein monomer. These results suggested that the protein expressed in the BHK/SFM cells had the correct conformation which is essential for antibody recognition.

Overall, SFV-VRPs produced in BHK/SFM or BHK/FBS cells had the same infection capacity, with effective amplification of recombinant mRNA, indicative of correct delivery and processing of the VRPs. Furthermore, the qualitative and quantitative levels of protein expression were also similar.

Discussion

VRPs represent attractive vectors for vaccine development, whose use for immunization has resulted in strong immune responses and generation of neutralizing antibodies in various animal models (Lundstrom 2016). The development of serum-free platforms is important for streamlining the bioprocess and obtaining a high-quality product suitable for clinical use, contributing to improvement in the production of modern vaccines. In this study, three different SFV-

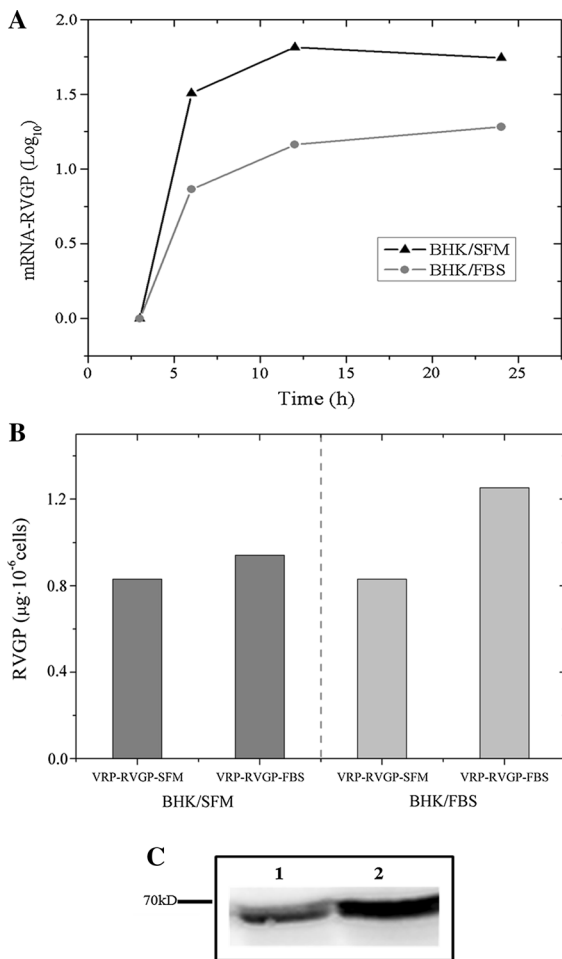


Fig. 5 RVGP expression in BHK/SFM vs BHK/FBS cells. BHK/SFM and BHK/FBS cells were infected in parallel with VRP-RVGP-SFM or VRP-RVGP-DMEM, using a MOI of 50 for **a** and 100 for **b**, **c**. The experiments were performed in 6-well plates, the cells were collected 3; 6; 12 and 24 h post-infection for **a** and 24 h post-infection for **b**, **c**. In **a** mRNA-RVGP expression was evaluated by qPCR. The results are average of duplicate. In **b** RVGP expression was assessed by ELISA. The results are average of three independent experiments. No statistically significant differences were detected. In **c** samples were analyzed by WB assay. Lane 1: BHK/SFM cells; lane 2: BHK/FBS cells

VRPs were obtained in serum free conditions. One was obtained using VRP-GFP, during preliminary characterization of the system. The other two SFV-VRPs carried viral antigens (VRP-NS3p or VRP-RVGP). The NS3p protein is an enzyme responsible for the cleavage of Hepatitis C virus proteins. RVGP is a glycoprotein responsible for promoting the entry of the rabies virus into the target cell. This enabled

assessing the expression of complex proteins and important targets for diagnosis and vaccine strategies, using cells cultured in serum-free media. This condition is very important for the development of new vaccine platforms, since it avoids the use of FBS throughout the production process.

Initial characterization of the system was performed using VRP-GFP. The first step as to adapt cells to serum free medium to obtain a serum-free cell line. This, was achieved using BHK-21 cells that are widely used for the propagation of viruses and protein expression by infection and transfection (Barteling 1976; Cruz et al. 1998; Kallel et al. 2002; Frazatti-Gallina et al. 2004; Hernandez and Brown 2010). The ability of BHK-21 cells to grow in serum-free media has been reported previously (Barteling 1976; Cruz et al. 1998; Kallel et al. 2002; Frazatti-Gallina et al. 2004). Despite accumulated literature on serum-free formulations, one must test different available SFM in order to determine which is the most suitable for a specific application/cell line (Sinacore et al. 2000). To this end, several commercial SFMs were tested, evaluating the growth kinetic parameters and VRP-SFV production, resulting in the selection of CHO-S-SFMII, which provided better results in terms of VRPs production (results not shown).

Most commercial serum-free culture media are unable to provide the necessary conditions for cell growth after an internal adaptation (Link et al. 2004). Hence, the sequential adaptation technique was selected, which has been used in several previous studies (Cruz et al. 1998; Costa et al. 2013; Huang et al. 2015; do Amaral et al. 2015). Major morphological alterations were observed in the cells, due to the new culture conditions, including the formation of cell aggregates, loss of adhesion capacity (semi-adherent growth), and rounded morphology (data not shown). These features are common in adaptation processes and have been reported for BHK cells (Cruz et al. 1998; Hesse and Wagner 2000; Kallel et al. 2002).

Growth kinetic parameters including cell viability, density, and maximum specific growth rate were evaluated in several passages, with similar results observed for the BHK/SFM and BHK/FBS cells. The values obtained were in agreement with results reported previously for BHK-21 cells cultured in DMEM supplemented with FBS (Cruz et al. 1998; Fernández-Núñez et al. 2015), and were higher than

those reported for BHK-21 cells adapted to same SFM using the multi-step adaptation (Moreira et al. 1995; Cruz et al. 1998).

The development of a process in which animal components are replaced must include its validation by comparison with the process using serum-containing media, in order to ensure that the original endpoints are not affected (van der Valk et al. 2010). Therefore, a safe and reproducible method for quantification of SFV-VRPs is a crucial requirement for comparative purposes. The qRT-PCR technique is an important tool for virus titration (Mackay et al. 2002; Sastry et al. 2002; Hitchman et al. 2007; Hoffmann et al. 2009; Slomka et al. 2010). Here, the production of SFV-VRPs was quantified using a qRT-PCR protocol previously standardized in our laboratory (Puglia et al. 2013). This assay is based on RNA quantification of the SFV nsP3 region, enabling quantification of SFV-VRPs carrying different genes. The results indicated that under serum-free conditions, the cells produced higher quantities of SFV-VRPs, irrespective of the heterologous gene (VRP-GFP, VRP-NS3p, or VRP-RVGP). Furthermore, the values obtained were superior to those reported by Puglia et al. (2013) for SFV-VRPs produced under conditions employing serum, demonstrating an improvement in the production process.

The BHK/SFM cells showed to have potential for the production of SFV-VRPs carrying different genes. Furthermore, the findings demonstrated that the BHK/SFM cells could be used as a platform for the production of other viruses or viral vectors. The qRT-PCR technique proved to be effective for the quantification of different SFV-VRPs and showed good correlation with protein expression, indicating the reliability and feasibility of this methodology for predicting biological results.

The SFV-VRP system can be used directly as a vaccine or for gene therapy, or indirectly for the *in vitro* expression of heterologous proteins. Analysis of the expression of three different proteins (GFP, HCV-NS3p, and RVGP) delivered using the SFV-VRP system was used to evaluate the performance of the BHK/SFM cells as a production platform. Determination was made of the infection efficiency of SFV-VRPs produced under serum-free conditions, as well as the capacity of the BHK/SFM cells to receive, replicate, and translate the genetic material delivered by the SFV-VRPs.

The results for GFP suggested that the BHK/FBS cells were infected more efficiently, since an 80% population of fluorescent cells could be achieved using a low MOI (10). In comparison, BHK/SFM cells required a MOI of 100 to reach 90% of fluorescent cells, but exhibited twofold higher fluorescence intensity for all MOIs tested, indicating that the BHK/SFM cells could express more protein per cell, compared to the BHK/FBS cells. Therefore, it is important to determine the best MOI, since very high virus concentrations may impair protein expression, due to cell death caused by the virus (Rhême et al. 2005; Fernández-Núñez et al. 2015).

Two viral antigens of clinical interest were selected to evaluate heterologous protein expression using the VRP-SFV-SFM system, one corresponding to a non-structural protein (HCV-NS3p) and the other to a structural protein (RVGP). The expression of NS3p was confirmed by RNA and enzymatic assays, showing that NS3p was expressed in both systems (serum and serum-free) and was biologically active. The NS3p expressed in the BHK/SFM cells presented higher proteolytic activity towards the specific substrate, compared to the NS3p produced in the BHK/FBS cells. As shown in the present work, the evaluation of viral particles produced under serum-free conditions is a valid strategy for the establishment of new vaccine platforms. The findings indicated that BHK/SFM cells can be used as an effective system for the expression of nonstructural proteins.

RVGP is a structural membrane glycoprotein, so it requires post-translational modifications to enable it to function as an activator of the immune response (Burger et al. 1991). The BHK/SFM cells were shown to be capable of transiently expressing this protein with the correct conformation and in amounts equivalent to those found in the BHK/FBS cells and in previous studies using the SFV system (Benmaamar et al. 2009; Fernández-Núñez et al. 2015). SFV-RVGP are able to induced levels of antibodies in mice comparable to those values obtained with a commercial rabies vaccine (Astray et al. 2014). Therefore VRP-RVGP-SFM system is a promising system for the production of Rabies vaccine candidates under serum-free conditions.

Overall, compared to BHK/FBS cells, the BHK/SFM cells showed superior or similar performance in terms of the production of SFV-VRPs, cell infection capacity, and protein expression. Importantly, the

price of SFM is lower (\$47/L) in comparison with DMEM supplemented with 10% of FBS (\$171/L), this together with a cleaner downstream process makes the particles produced under in serum-free conditions a platform attractive for industrial production.

To sum up, the serum-free platform provided production of virus particles, and complex and functional proteins that was at least as efficient, and sometimes more efficient, compared to platforms that involve the use of serum. It will be crucial to undertake in vivo follow-up studies to evaluate the immune response, as well as purification studies. Since the entire process is performed under serum-free conditions, the VRPs could be concentrated, purified, and used directly for immunization.

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Compliance with ethical standards

Conflict of interests The authors declare that they have no conflict of interests.

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