Journal of Biotechnology 166 (2013) 152–165

Contents lists available at SciVerse ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

Generation of HIV-1 Gag VLPs by transient transfection of HEK 293 suspension cell cultures using an optimized animal-derived component free medium

Laura Cervera[a], Sonia Gutiérrez-Granados[a], Marta Martínez[a], Julià Blanco[b], Francesc Gòdia[a], María Mercedes Segura[a,*]

[a] Departament d'Enginyeria Química, Universitat Autònoma de Barcelona, Campus Bellaterra, Cerdanyola del Vallès 08193, Barcelona, Spain
[b] IrsiCaixa AIDS Research Institute – HIVACAT, Hospital Universitari Germans Trias i Pujol, Badalona, Spain

ARTICLE INFO

Article history:
Received 3 January 2013
Received in revised form 29 April 2013
Accepted 2 May 2013
Available online 17 May 2013

Keywords:
Animal cell culture
Media supplementation
Design of experiments (DoE)
Virus-like particles (VLP)
HIV-1 vaccine
Transient gene expression (TGE)

ABSTRACT

Virus-like particles (VLPs) offer great promise as candidates for new vaccine strategies. Large-scale approaches for the manufacturing of HIV-1 Gag VLPs have mainly focused on the use of the baculovirus expression system. In this work, the development and optimization of an HIV-1 Gag VLP production protocol by transient gene expression in mammalian cell suspension cultures is reported. To facilitate process optimization, a Gag-GFP fusion construct enabling the generation of fluorescent VLPs was used. The great majority of Gag-GFP present in cell culture supernatants was shown to be correctly assembled into virus-like particles of the expected size and morphology consistent with immature HIV-1 particles. Medium optimization was performed using design of experiments (DoE). Culture medium supplementation with non-animal derived components including recombinant proteins and lipids of synthetic or non-animal-derived origin resulted in improved HEK 293 cell growth and VLP production. The maximum cell density attained using the optimized Freestyle culture medium was 5.4×10^6 cells/mL in batch mode, almost double of that observed using the unsupplemented medium (2.9×10^6 cells/mL). Best production performance was attained when cells were transfected at mid-log phase ($2–3 \times 10^6$ cells/mL) with medium exchange at the time of transfection using standard amounts of plasmid DNA and polyethylenimine. By using an optimized production protocol, VLP titers were increased 2.4-fold obtaining 2.8 μg of Gag-GFP/mL or 2.7×10^9 VLPs/mL according to ELISA and nanoparticle tracking quantification analyses, respectively.

1. Introduction

Upon expression in heterologous systems, the human immunodeficiency virus type 1 (HIV-1) Gag polyprotein is able to self-assemble giving rise to non-infectious VLPs in the absence of any other viral protein or virus RNA (Buonaguro et al., 2001; Rovinski et al., 1995; Sakuragi et al., 2002). HIV-1 Gag VLPs have shown great promise as platforms for the presentation of envelope antigens (Deml et al., 2005). However, the complexities associated with their manufacturing have hindered their evaluation beyond early-pre-clinical testing (Hammonds et al., 2007). Large-scale approaches for the manufacturing of HIV-1 Gag VLPs have mainly focused on the use of the baculovirus-expression system (Cruz et al., 1998; Pillay et al., 2009; Visciano et al., 2011). The generation of HIV-1 Gag VLPs using mammalian cells is less reported in the literature (Hammonds et al., 2007; Jalaguier et al., 2011), with no reports describing their production in suspension cell cultures.

The development of a scalable HIV-1 Gag VLP production strategy in human embryonic kidney 293 (HEK 293) suspension cell cultures is reported in this work. An improved culture medium supplemented with non-animal derived components was developed. The need for removal of serum from industrial manufacturing processes was recognized decades ago. In order to ensure cell line growth and productivity, early developments in serum-free media (SFM) resulted in formulations containing components normally supplied by serum such as insulin, transferrin and lipids of animal origin as well as other poorly defined mixtures (extracts, hydrolysates) (Keenan et al., 2006). However, during the past few decades there has been increasing safety concerns associated to the emergence of new viruses and prion strains prompting regulatory authorities to recommend the use of not only SFM but also animal-derived component free (ADCF) media formulations for the manufacture of biopharmaceutical products. The increasing

* Corresponding author. Tel.: +34 93 581 4794; fax: +34 93 581 2013.
E-mail addresses: mersegura@gmail.com, mersegura@hotmail.com (M.M. Segura).

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http://dx.doi.org/10.1016/j.jbiotec.2013.05.001

number of ADCF and chemically defined (CD) media formulations available in the market and the relatively recent commercial availability of recombinant versions of key serum proteins produced in *Escherichia coli* or yeast (e.g. albumin and transferrin), as well as supplements of plant origin or synthetic nature should facilitate the switch to efficient animal-derived component-free production processes.

Design of experiments (DoE) has been chosen as a valuable tool for medium optimization. Statistically relevant information can be extracted from experimental designs with a minimum number of experiments. Non-animal derived additives evaluated in this work include three recombinant proteins (r-albumin, r-transferrin and r-insulin) and an *in-house* designed animal-component free lipid mixture containing synthetic cholesterol and fatty acids. A two-phase experimental design was used. Screening of components was performed in a first phase using a Plackett–Burman experimental design (Plackett and Burman, 1946). In a second phase, a response surface methodology (Box–Behnken design) was used to determine optimal concentration levels for each component showing a significant effect on HEK 293 cell growth (Box and Behnken, 1960). The performance of the optimized cell culture medium for the production of HIV-1 Gag VLPs was evaluated.

The selected production method was transient gene expression (TGE) as it offers a convenient means for the generation of recombinant products for pre-clinical and early clinical phases (Baldi et al., 2007; Geisse, 2009; Pham et al., 2006). TGE becomes particularly attractive when a large number of product variants needs to be tested and/or in cases where the expression of cytotoxic genes complicate the generation of stable cell clones, which is the case for HIV-1 VLPs. Considerable progress has been made in the past several years toward establishing large-scale transient transfection protocols (Backliwal et al., 2008a; Durocher et al., 2002; Geisse and Henke, 2005; Pham et al., 2003; Tuvesson et al., 2008). HEK 293 is the preferred host system due to the many industrially relevant features this cell line offers including ease of genetic manipulation, ability to grow in suspension culture, ability to grow to high cellular densities and adaptation to serum-free culture conditions. In addition, the HEK 293 cell line and its variants (e.g. HEK 293T, HEK 293E) are used for the production of many virus-based products including viral vaccines and most viral vectors (Durocher et al., 2007; Ghani et al., 2006; Kamen and Henry, 2004; Le Ru et al., 2010; Segura et al., 2007). Moreover, HEK 293 cells are rapidly gaining industry acceptance as they have been approved for the production of the first adenovirus-based gene therapy product (Gendicine®) in China and a therapeutic recombinant protein (Xigris®) by FDA and EMA.

2. Materials and methods

2.1. Cell line, media and culture conditions

The cell line used in this work is a serum-free suspension-adapted HEK 293 cell line (HEK293SF-3F6) kindly provided by Dr. Amine Kamen at the National Research Council of Canada (Montreal, Canada). It was derived from a cGMP master cell bank that is available for manufacturing of clinical material. Five commercial serum-free media formulations for HEK 293 were tested for cell growth, transfection efficiency and VLP production. These include HyQ SFM4 Transfx293 from HyClone Thermo Scientific (Logan, UT, USA), ExCell 293 from SAFC Biosciences (Hampshire, UK), Freestyle 293, CD 293 and 293 SFM II from Invitrogen (Carlsbad, CA, USA). All formulations were supplemented with GlutaMAX™ (4–6 mM) (Invitrogen, Paisley, UK) with the exception of Freestyle 293 media that already contains GlutaMAX™ in its formulation. Freestyle 293 medium and 293 SFM II medium were also supplemented with 0.1% Pluronic® (Invitrogen). Cell cultures were pre-adapted to each formulation prior to experimentation. An YSI 7100 MBS glucose/lactate/glutamine analyzer (YSI, Yellow Springs, OH, USA) was used to measure the concentrations of the major nutrients and by-products in cell culture supernatants. Cells were routinely maintained in 125-mL disposable polycarbonate Erlenmeyer flasks (Corning, Steuben, NY, USA) in 20 mL of culture medium. Flasks were shaken at 110 rpm using an orbital shaker (Stuart, Stone, UK) placed in an incubator maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air. Cell count and viability were determined using trypan blue and a microscope counting chamber.

2.2. Transient transfection

The pGag-EGFP plasmid used in this work codes for a Rev-independent HIV-1 Gag protein fused in frame to the enhanced GFP (Hermida-Matsumoto and Resh, 2000). The plasmid from the NIH AIDS Reagent Program (Cat 11468) was constructed by cloning the Gag sequence from pCMV55M1-10 (Schwartz et al., 1992) into the pEGFP-N1 plasmid (Clontech, Palo Alto, CA, USA). The plasmid was prepared and purified as previously described (Segura et al., 2007). HEK 293 suspension cells were transiently transfected using 25-kDa linear polyethylenimine (PEI) (PolySciences, Warrington, PA, USA). Transfections were performed using a final DNA concentration of 1 μg/mL of media unless otherwise stated and a DNA to PEI mass ratio of 1:2. PEI/DNA complexes were formed by adding PEI to plasmid DNA diluted in fresh culture media (10% of the total volume of the culture to be transfected). The mixture was incubated for 15 min at room temperature to allow complex formation prior to its addition to the cell culture. The percentage of GFP positive cells was assessed using a BD FACS Canto flow cytometer (BD Biosciences, San Jose, CA, USA) at different hours post-transfection (hpt).

2.3. Fluorescence confocal microscopy

The visualization of VLP producer cells was achieved using a Fluoview® FV1000 confocal microscope (Olympus, Tokyo, Japan). Transfected cells were mixed with 0.1% of Hoechst (Invitrogen, Eugene, OR, USA) and 0.1% of CellMask™ (Invitrogen) in order to stain the cell nucleus and lipid membrane, respectively. Two washes were performed by centrifuging the cells at 300 × *g* for 5 min and resuspending the pellets in PBS. Samples were placed in 35 mm glass bottom petri dishes with 14 mm microwell (MatTek Corporation, Ashland, MA, USA) for visualization.

2.4. Transmission electron microscopy

Gag-GFP VLP samples were prepared by air-dried negative staining method at the Servei de Microscòpia (UAB, Spain). Briefly, 5 μL of the mixture were placed on carbon-coated copper grids and incubated at room temperature for 5 min. Excess sample was carefully drained off the grid with the aid of filter paper. Samples were negatively stained with 5 μL of uranyl acetate (2%) by incubation for 1 min at room temperature. Excess stain was drained off as before and grids were dried for a minimum of 50 min at room temperature before the examination in a Jeol JEM-1400 transmission electron microscope equipped with a Gatan ES1000W Erlangshen CCD Camera (Model 785).

2.5. VLP quantitation

The concentration of Gag-GFP VLPs was assessed by fluorimetry using an *in-house* developed and validated quantification assay (Gutiérrez-Granados et al., 2013). VLP containing supernatants were recovered by cell culture centrifugation at 1000 × *g* for 5 min. Green fluorescence was measured at room temperature using a