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RESEARCH

Production of the SARS-CoV-2 nucleocapsid-human CD154 extracellular domain fusion protein in the EscoVacciXcell-CelCradle™ benchtop bioreactor

Hasel Aragón[1]*, Mayté Quintana[1]*, Williams Ferro[1], Airela Llamo[1], Grechen Menéndez[1], Marcos González[1], Cristina García[1], José García[1], Margarita Somoza[3], Yuleixis Barrisonte[3], Thailin LaO[2], Yamila Carpio[2], Mario Pablo Estrada[2], ✍Rodolfo Valdés[1]

[1]Department of Monoclonal Antibodies, Production Direction, Center for Genetic Engineering and Biotechnology, CIGB
Ave. 31, No. 15802 b/158 and 190, Cubanacán, Playa, CP 11600, Havana, Cuba
[2]Department of Animal Biotechnology, Direction of Agricultural Research, CIGB
[3]Department of Process Control, Production Direction, CIGB
✍rodolfo.valdes@cigb.edu.cu

ABSTRACT

The SARS-CoV-2 nucleocapsid protein (N) is abundantly expressed during the viral infection, and it can be used for vaccine development, serological assays and other purposes. Different strategies have been implemented for N-protein production in either suspension or anchorage cell systems. In this work, a fusion protein of the SARS-CoV-2 nucleocapsid to the extracellular domain of the human CD154 (N-hCD154) was produced in HEK-293 cells cultivated in the EscoVacciXcell CelCradle™ benchtop bioreactor, operated in continuous mode for 35 days. Effective operation parameters were: 0.88 ± 0.27 × 10^6 HEK-293 cells quantified per carrier (BioNOC™II), 547.9-898.2 × 10^6 cells/day, 89.4-98.2 % HEK-293 cell viability, 0.6 ± 0.01 pg/cell/day (specific secretion rate) and 22.38 ± 2.6 μg/mL N-hCD154 fusion protein concentration per harvest. A 75 % N-hCD154 fusion protein average purity was achieved in culture supernatant samples, and the downstream method combining ammonium sulfate precipitation and diafiltration step provided a N-hCD154 fusion protein with over 95 % of purity. The combination of the CelCradle™ benchtop bioreactor for HEK-293 cell culture operated in continuous mode with ammonium sulfate precipitation and a diafiltration step, made possible obtaining N-hCD154 fusion protein with a medium recovery and very high purity level as to be characterized for further research with medicinal purposes.

Keywords: SARS-CoV-2, COVID-19, nucleocapsid protein, human CD154 extracellular domain, HEK-293 cell, CelCradle™ bioreactor, diafiltration, ammonium sulfate precipitation

RESUMEN

Producción de la proteína de fusión de la nucleocápsida del SARS-CoV-2-dominio extracelular de CD154 humano en el bioreactor analítico EscoVacciXcell-CelCradle™. La proteína de la nucleocápsida (proteína N) del SARS-CoV-2 es altamente inmunogénica, se expresa abundantemente durante la infección, y puede usarse con fines vacunales, para el desarrollo de ensayos serológicos, y otros. Para tales fines, la misma se ha producido en células de suspensión o de anclaje. En este estudio, la proteína N del SARS-CoV-2 fusionada al dominio extracelular de la proteína CD154 humana (N-hCD154) se produjo en células HEK-293 cultivadas en el bioreactor analítico EscoVacciXcell CelCradle™ operado en modo continuo durante 35 días. Como resultados, la cantidad de células HEK-293 cuantificadas por BioNOC™II fue de 0,88 ± 0,27 × 10^6, la cantidad total de células HEK-293 osciló en el rango 547,9-898,2 × 10^6 células/día, su viabilidad entre 89,4-98,2 %, la secreción específica de 0,6 ± 0,01 pg/célula/día y la concentración de la proteína de fusión N-hCD154 por cosecha fue de 22,38 ± 2,6 μg/mL. La pureza promedio de la proteína N-hCD154 fue del 75 %, estimada en muestras de sobrenadante de cultivo. La aplicación del método de purificación con sulfato de amonio seguido de un paso de diafiltración, permitió incrementarla a más del 95 %. En resumen, la combinación del bioreactor analítico CelCradle™ empleado en el cultivo de las células HEK-293 operado en modo continuo con la precipitación con sulfato de amonio, seguido de un paso de diafiltración, permite obtener la proteína fusionada N-hCD154 con un recobrado medio y un nivel de pureza muy alto como para ser caracterizada en futuras investigaciones con fines medicinales.

Palabras clave: SARS-CoV-2, COVID-19, proteína de la nucleocápsida, dominio extracelular del CD154 humano, células HEK-293, fermentador CelCradle™, diafiltración, precipitación con sulfato de amonio

How to cite (Vancouver style):
Aragón H, Quintana M, Ferro W, Llamo A, Menéndez G, González M, *et al*. Production of the SARS-CoV-2 nucleocapsid-human CD154 extracellular domain fusion protein in the EscoVacciXcell-CelCradle™ benchtop bioreactor. Biotecnol Apl. 2022; 39(2):2211-6.

Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is an enveloped, positive-stranded RNA virus that causes COVID-19 [1]. COVID-19 killed about 16 millions of people since 2019 worldwide [2]. Therefore, there are still many ongoing biochemical and biophysical explorations to identify SARS-CoV-2 proteins as suitable targets for vaccine and diagnosis purposes [3-5].

In that sense, SARS-CoV-2 genome is composed of about 29 851 nucleotides, which encodes, among others, four structural proteins including the nucleocapsid protein (N-protein) [6]. The N-protein is a highly

*Both authors contributed equally to this work

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immunogenic and abundantly expressed protein during infection. Therefore, N-protein can be used to develop vaccines, serological assays and therapies [7]. Serological diagnosis detected antibodies against the N-protein in sera of SARS-CoV-2 patients of longer persistence than those of other viral structural proteins. Moreover, highly specific anti-N-protein antibodies can be found at early stages of infection [8].

At the same time, the human CD154 protein (hCD154), also known as TRAP (tumor necrosis factor-related activation protein) is a 32 kDa type II transmembrane molecule, member of the Tumor Necrosis Factor (TNF) superfamily [9]. It is a ligand for the human CD40 molecule, which is displayed on the surface of some human basophil and mast cells, and plays important regulatory roles in T and B lymphocytes functioning. The hCD154/CD40 interaction is essential for the of thymus-dependent humoral immune response development, the binding on B-lymphocytes by hCD154 present in T-lymphocytes promoting B-lymphocyte proliferation, immunoglobulin production, isotype switching, and memory B-lymphocyte generation [10, 11]. Therefore, these issues justify making fusion between the SARS-CoV-2 N-protein and hCD154 protein, to further increase the stimulation of the immune system for vaccine development. Furthermore, similar strategies have proven useful using the hCD154 extracellular domain fused to the classical swine fever virus E2 protein for veterinary vaccine development [12].

Moreover, several reports have described different strategies for successful N-protein production [13] using both suspension and anchored cells (including CHO, HEK-293 and insect cells) and different eukaryotic expression systems [14]. However, the production of the SARS-CoV-2 N-protein fused to the extracellular domain of hCD154 (N-hCD154) in bioreactors using mammalian cell culture such as EscoVacciXcell-CelCradle™ has not been reported yet.

There are available different bioreactors designed and used according to the host cells of choice to produce such complex heterologous proteins [15]. One of them is the CelCradle™ benchtop bioreactor (EscoVacciXcell, Singapore), which operates through the tide motion principle. It is based on cells attached to BioNOC™ II carriers, which are alternately exposed to aeration and nutrition via decompression/compression of the bellow holding culture medium. In general, the gentle vertical oscillation of culture medium creates a dynamic interface between air and culture medium on cell surface, providing cells with an environment of low shear stress, high aeration and nutrition levels, no foaming, and no O_2 limitation. This proper nutrient and oxygen transfer allows CelCradle™ benchtop bioreactor producing a relative high cell density [16].

Therefore, this study was aimed at studying the production of N-hCD154 protein in anchorage-dependent HEK-293 cells using the CelCradle™ benchtop bioreactor.

Materials and methods

Cell line

The HEK-293 cell line was used to produce N-hCD154 fused protein using as cell culture medium DMEM medium (GIBCO-BRL, Gaithersburg, USA) supplemented with 10 % fetal bovine serum (Capricorn Scientific, Ebsdorfergrund, Germany), 2 mM L-glutamine, 1 mM sodium pyruvate and 17 mM sodium bicarbonate.

Cellular inoculum propagation

Cells were seeded in six 162 cm^2-T-flasks at 0.2 × 10^6 cells/mL. After 72 h, a culture with the surface of the flasks covered with cells, was used for bioreactor inoculum preparation. Flasks were washed with 150 mM PBS, pH 7.2. Next, 5 mL of Trypsin/EDTA solution was added to be incubated for 10 min at 37 °C in a 5 % CO_2 atmosphere. The culture was resuspended and the trypsin/EDTA solution was replaced by fresh medium. Finally, cell concentration and viability were estimated by cell counting in a Neubauer chamber [17].

Culture of HEK-293 cells in the CelCradle™ benchtop bioreactor

DMEM medium (450 mL) supplemented with 10 % fetal bovine serum preheated to 37 °C was added to the CelCradle™-500. Then, 194.5 × 10^6 cells were resuspended in 50 mL and added to the bioreactor. The CelCradle™-500 bottle was transferred to an incubator at 37 °C with a 5% CO_2 atmosphere. To facilitate cell adhesion to BioNOC™ II, bioreactor parameters were adjusted as follows: rising rate (2 mm/s), top holding time (20 s), down rate (2 mm/s) and bottom holding time (0 s). After 5 h, bioreactor parameters were changed to rising rate (1 mm/s), top holding time (30 s), down rate (1 mm/s) and bottom holding time (20 s).

HEK-293 cell counting

Six BioNOC™II carriers located in random positions of the culture bottle were taken with sterile forceps and placed in three vials of 1.5 mL, at a rate of two carriers per vial. Two washes were done with 150 mM PBS, pH 7.2 and finally 1 mL of Trypsin/EDTA solution was added to each vial. Vials were incubated for 10 min at 37 °C under a 5 % CO_2 atmosphere. Subsequently, each vial was shaken to separate cells from BioNOC™II and cell concentration was estimated by the Neubauer chamber cell counting method using Trypan blue dye [17].

Downstream processing

The N-hCD154 protein molecules secreted to culture supernatants were firstly precipitated by adding ammonium sulfate at 30 % saturation overnight at 4 °C and centrifuged at 15 900 × *g* for 45 min at 4 °C (Beckman Coulter/Avanti J-265 XP). Subsequently, precipitated protein molecules were resuspended in 150 mM PBS, pH 7.6 ± 2. They were further diafiltered against 150 mM PBS, pH 7.6 ± 2, at 22 °C, with a 15 kDa-membrane cut-off and filtered under sterile conditions.

Total protein determination

Total protein concentration was determined according to Lowry *et al*. [18], using bovine serum albumin as standard material. The bovine serum albumin standard curve ranged from 10 to 100 µg/mL (10, 20, 40, 60, 80 and 100).

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Enzyme-linked immunosorbent assay

An enzyme-linked immunosorbent assay (ELISA) was done, with all incubations at 37 °C for 1 h, in a humid chamber. A 96-well PolySorp plate (Thermo Fisher Scientific Inc., Denmark) was coated with 10 µg/mL of CB.SCoV-2PN.14 anti-SARS-CoV-2 N-protein monoclonal antibody (100 µL/well) in 100 mM Na_2CO_3/$NaHCO_3$ buffer, pH 9.6. After incubation, the plate was blocked by adding 200 µL/well of 150 mM PBS/Tween-20 0.05 %/Skimmed milk 5 %, pH 7.6, and further incubated. Then, the plate was washed with 150 mM PBS, pH 7.2/0.05 % Tween-20, and 100 µL of each sample, standard curve or controls, were applied to single wells, and incubated. Subsequently, it was washed thrice with the same washing solution. An anti-SARS-CoV-2 N-protein rabbit polyclonal antibody preparation conjugated to horseradish peroxidase (Sigma-Aldrich, St. Louis, USA) was added at the 1:2000 optimal dilution and incubated. Then, the plate was washed five times with the washing solution and the reaction was developed with 100 µL of substrate solution (5 mg of Orthophenylenediamine, 5 µL of hydrogen peroxide, 10 mL of substrate buffer (90 mM $C_6H_8O_7 \cdot H_2O$/200 mM Na_2HPO_4, pH 5.5). The reaction was stopped after 10 min with 50 µL of 1.5 M H_2SO_4, and the absorbance was determined at 492 nm in a microELISA reader (Labsystems Multiskan MS, Finland).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Sample purity was estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [19] on 12.5 % (w/v) polyacrylamide gels. The gels were stained with Coomassie® brilliant blue R-250 (Bio-Rad) as described.

N-hCD154 protein detection by immunoblotting

A Hybond-C Extra nitrocellulose membrane (Sartorius, Germany) was activated in methanol for 20 min and placed in a dot-blot apparatus (Trans-Blot® SD, BioRad). Then, 100 µL of each sample and each point of the curve were applied. The membrane was blocked with 5 % non-fat dry milk (Oxoid Ltd) dissolved in 150 mM PBS, pH 7.6 at 4 °C overnight. Subsequently, the anti-SARS-CoV-2 N-protein CB.SCoV-2PN.14 monoclonal antibody was applied and incubated at room temperature for 1 h. Then, the membrane was incubated again with a HRP-labeled goat anti-mouse IgG conjugate (optimal dilution 1:2000) for 1 h, and the color development was further visualized using 150 mM PBS, pH 7.6/1,2'-diaminobenzidine and hydrogen peroxide in a volume of 12 mL. The reaction was stopped by adding purified water to the membrane.

Results and discussion

In mammalian cell culture, roller-bottles are commonly used for anchor-dependent cell multiplication [20]. Despite, a large number of roller-bottles would be used to obtain large amounts of cells, which require large facilities, heated rooms and an intensive culture manipulation, with the consequent risk of contamination. On the other hand, the yields achieved in roller-bottles are usually low, as compared to other cultivation systems [20-22]. To overcome these issues, bioreactor prototypes, as the CelCradle™ benchtop bioreactor (EscoVacciXcell, Singapore) have been designed. This type of bioreactor increases cell densities, by attaching cells to microcarriers, to be cultivated in suspension mode. It allows increasing protein yields, while reducing production costs [23, 24].

The CelCradle™ benchtop bioreactor is a new type of laboratory-scale disposable bioreactor, designed for cultivation of mainly anchorage-dependent cells (Figure 1). The principle of the tide motion in CelCradle™ contains packed bed (BioNOC™II carriers) and medium reservoir in the same bottle. This system is based on a tidal agitation system, following the flow and reflux principle, with a compression and decompression movement providing adequate oxygenation of cultures under gentile conditions [16]. It also avoids cell damage, due to the lack of shear forces observed in other types of bioreactors, such as marine propeller rotary stirring systems [25-27]. Therefore, CelCradle™ benchtop bioreactor can be used to replace ineffective systems such as roller-bottles for obtaining small amounts of target proteins for characterization studies, and also to evaluate different operation modes of bioreactors.

As abovementioned, the N-hCD154 fusion protein was produced in HEK-293 host cells. This cell line is very efficient for protein production and easy to transfect [28]. It supports the expression of complex proteins with similar post-translational modifications and at relative high yields. Hence, the CelCradle™-500 flask was inoculated with 194.5 × 10^6 cells (3.88 × 10^6 cells/mL) with a viability of 94.9 %. Strikingly, the amount of cells attached to the BioNOC™II in the first 72 h was as low as 16.9 % (equivalent to 0.16 × 10^6 cells/mL), with respect to the amount of cells inoculated.

Maybe this drastic decrease was caused by BioNOC™II carriers trapped right at the center of CelCradle™-500 flask, affecting cell adherences.

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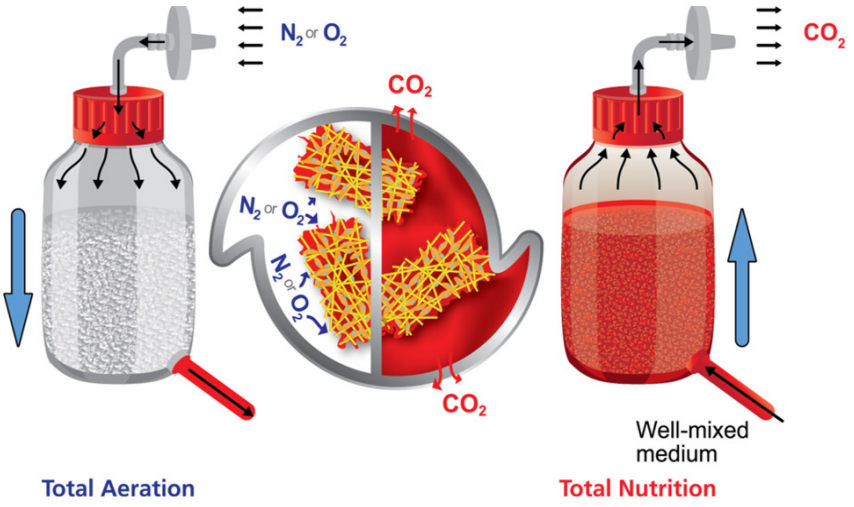

Figure 1. Diagram of the EscoVacciXcell-CelCradle™ benchtop bioreactor functioning.

This relatively low cell attachment issue is coincident with those observed by Pancawidyana *et al*. [29], who reported less than 30 % cell adhesion in the BioNOC™II system at the first 48 h of culture using the BHK-21 mammalian cell line [29]. On the contrary, there were reports on a higher cell attachment in the CelCradle™-500AP system. For instance, two types of cells, Vero and Primary Lamb Testis cells, were used to produce small ruminant morbillivirus, rift valley fever virus and lumpy skin disease virus, after an optimization procedure applied to the cell growth phase [30]. In that study, Vero cells could be grown to significantly higher cell densities of 3.04×10^9 cell/mL in CelCradle™-500A with a shorter doubling time. It represented a 19-fold increase in cell amounts as compared to seeding [30].

In the experimental setting described in this work, the maximum amount of cells per BioNOC™II measured was 1.44×10^6 cells at 26 days of culture, with 98.2 % viability. It was a 636.7 % increase in cell density with respect to the inoculated amount of cells. The carbon source consumption working parameter was 2.45 g average glucose consumption per day, with glucose concentration remaining in the medium range from 0.1 to 1.37 g, starting from the 10th day of culture. Such high glucose consumption measured on 10th day of the run was caused by medium renovation after 72 h and with the increase in cell concentration. Moreover, pH-values remained stable, in the range 6.43-7.5, with an average of 6.90 (Figure 2). The total amount of cells counted in the EscoVacciXcell CelCradle™ benchtop bioreactor was 1126×10^6 cells, while average from day ten of culture was 695×10^6 cells. It should be noted that from that day on, the cell culture started the growth exponential phase. The average cell viability estimated during the run was 94.3 %, which is a typical high value for mammalian cell culture in bioreactor operated under continuous mode (Figure 3). Equivalently, the amount of cells obtained using a single CellCradle™-500 flask was similar to that using 23 roller-bottles of 850 cm^2 surface area.

Regarding fusion protein production, up to 22.38 ± 2.6 µg/mL N-hCD154 protein in average were harvested per day, for a total amount of 157.55 mg (Figure 4). It could be regarded as low, most likely due to the low level of the protein expression by cells under the applied culture conditions. Another explanation could be difficulties to accurately capture the N-hCD154 fusion protein by the CB.SCoV-2PN.14 monoclonal antibody. This last is supported by other experimental findings using this monoclonal antibody, showing a significant reduction in the amount of N-hCD154 fused protein quantified. It seems to be that epitope recognized by this monoclonal antibody is close to the fusion site between both proteins, and, thus, the interaction could be affected. Moreover, some kind of aggregation of the N-protein has been also detected in bioreactor supernatants, which may affect the accurate quantification of the N-protein as well. Therefore, further experimentation is required for the fusion protein quantification using mainly polyclonal antibodies specific for the SARS-CoV-2 N-protein.

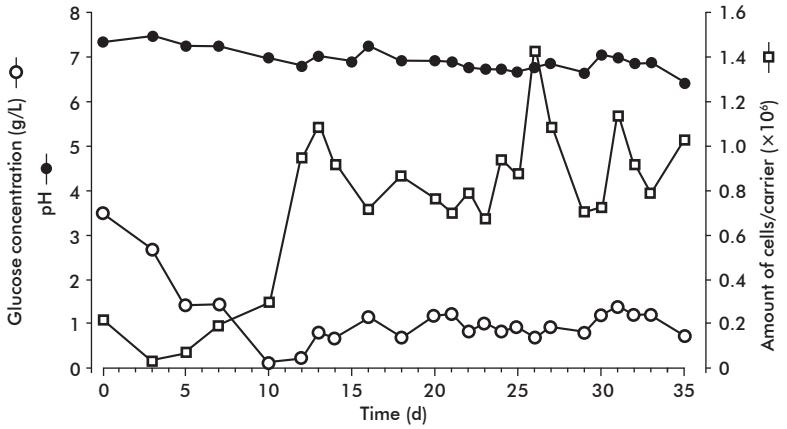

Figure 2. Glucose concentration, pH values in the cell culture supernatant and amount of cells per BioNOC™II carrier during the run of the EscoVacciXcell-CelCradle™ Benchtop bioreactor (EscoVacciXcell, Singapore).

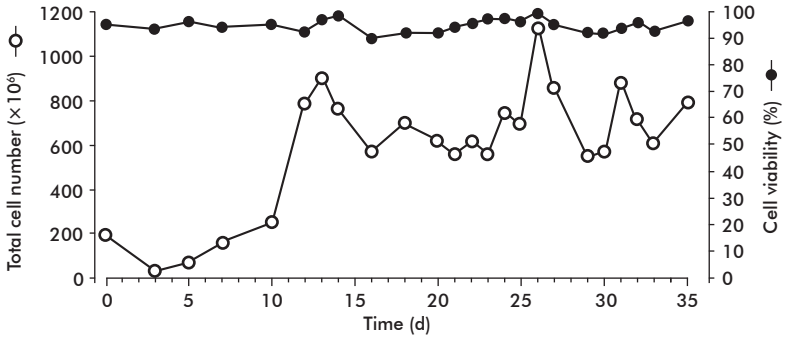

Figure 3. Total amount of cells and cell viability estimated in the CellCradle™ per day (EscoVacciXcell, Singapore) for HEK-293 cells expressing the SARS-CoV-2 N-human CD154 extracellular domain fusion protein.

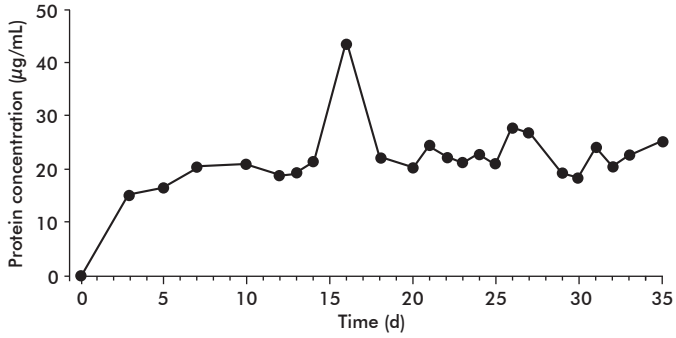

Figure 4. Total protein concentration of SARS-CoV-2 N-human CD154 extracellular domain fusion protein, quantified per cell culture supernatant harvest.

Regarding protein purification, protein precipitation by the salting-out method [31] is a low resolution fractionation tool, but powerful to concentrate proteins. The principle of salting-out is based on the preferential solvation, due to the exclusion of the salt from the water layer closely associated with the protein surface [31]. Usually, ammonium sulfate is the salt of choice, as it is inexpensive, highly soluble in water and can hydrate much more than almost any other ionic solvent. Therefore, ammonium

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sulfate precipitation was chosen to purify the N-hCD154 fusion protein, since, in a previous work, researchers demonstrated the effective use of this procedure in combination with size-exclusion chromatography to purify the IBV nucleocapsid protein [32]. The results of precipitation of the N-hCD154 fusion protein with ammonium sulfate at 30 % saturation and diafiltered with a 15 kDa-molecular weight cut-off membrane from the CelCradle™-500AP culture supernatant, are shown in the table. The average recovery was 54.23 %, which allowed obtaining 72.5 mg from 133.76 mg of N-hCD154 fusion protein. In general, all proteins can be fractionated between ~50-77 % saturation of salts [33]. For instance, low molecular weight proteins require higher salt concentration for precipitation than larger molecular proteins, which can often be salted-out with < 20 % saturation. In the case of N-hCD154 fusion protein, the percentage of ammonium sulfate should be optimized, since about 50 % of the amount of protein quantified by ELISA remained soluble. This aspect is less important for protein characterization, but remarkable for large-scale protein purification.

Figure 5 illustrates SDS-PAGE results of samples obtained after combining ammonium sulfate precipitation with diafiltration. As shown, the N-protein used as reference migrates approximately at the 45 kDa molecular weight, while the N-hCD154 fusion protein can be seen at a higher molar weight (~ 75 kDa). Hence, the differences in the molecular weight can be mainly attributed to the fusion to the CD154. Purity was higher than 95 %, except in the initial sample (75 %) due to contaminants in the cell culture supernatant. No differences in purity were observed among samples from two independent processing, and such high values supports using the N-hCD154 fusion protein for a further accurate characterization and pharmaceutical applications of the protein. Interestingly, no degradation and/or multimerization of the N-hCD154 fusion protein were observed in the SDS-PAGE. It could be elucubrated that such stability could be due to the lower accessibility of degradation sites in both molecules, caused by the fusion in the N-hCD154 protein, or the lack of the aggregation due to the fusion for the N-protein, which displays high aggregation when it is expressed as inclusion bodies in bacteria [34].

On the other hand, immunoblotting is a molecular technique highly sensitive for detecting biomolecules, based on the interaction with specific antibodies. It just requires a minimal amount of the target molecule applied directly to a membrane to be detected. Therefore, the immunoblotting technique applied confirmed the presence of the N-hCD154 fusion protein in the samples obtained, following the combination of ammonium sulfate precipitation with diafiltration (Figure 6). These results also confirmed a quantified amount of the N-hCD154 fusion protein lower than detected by ELISA in the cell culture supernatant.

Table. Results of Sars-Cov-2 N protein-human CD154 extracellular domain fusion protein (N-hCD154) precipitation with ammonium sulfate and diafiltration through a 15 kDa-molecular weight cut-off membrane*

Precipitation and dialfiltration samples	N-hCD154		Recovery (%)	Purity (%)
	Concentration at purification (mg/mL)	Amount (mg)		
I	0.69	34.5	51.58	96.0
II	0.61	38.0	56.88	97.0
Average	0.65	36.2	54.23	96.5

* Starting parameters of the samples: 5320 mL of supernatant, 0.019 mg/mL of protein at purification, and 66.88mg of total protein amount.

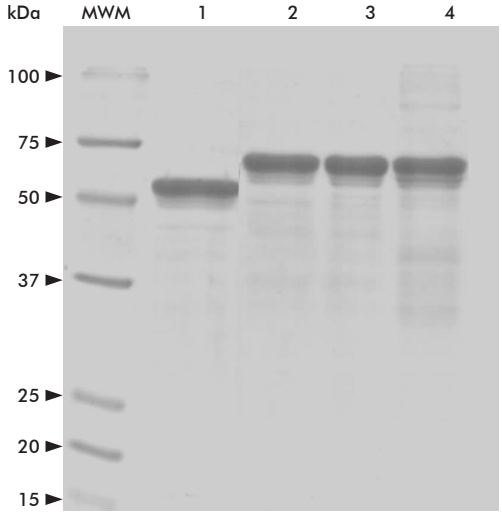

Figure 5. Purity of the SARS-CoV-2 N-human CD154 extracellular domain fusion protein (N-hCD154) estimated by SDS-PAGE. Lanes: MWM, molecular weight standard (Precision Plus Protein™ Standard; Catalog # 161-0373, BIO-RAD, USA); 1, N protein expressed in *E. coli* used as control (98 %); 2, N-hCD154 fusion protein precipitated and diafiltered (replica 1); 3, N-hCD154 fusion protein precipitated and diafiltered (replica 2); 4, sample of culture supernatant (75.0 % purity).

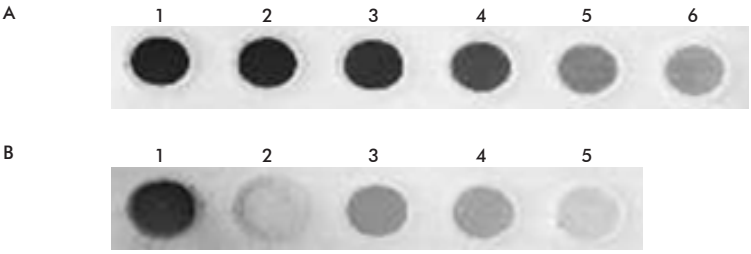

Figure 6. Immunoblotting of the SARS-CoV-2 N-human CD154 extracellular domain fusion protein (N-hCD154) samples produced in HEK-293 cells using the EscoVacciXcell-CelCradle™ benchtop bioreactor (EscoVacciXcell, Singapore) and purified by precipitation and diafiltration steps. A) N protein concentration curve (spots 1-6: 2.1, 1.06, 0.53, 0.26, 0.13 and 0.06 μg/mL, respectively). B) N-hCD154 samples: 1, culture supernatant sample (replicas 1 + 2). 2, negative control (cell culture medium). 3, positive control (N-protein expressed in bacteria). 4, precipitated and diafiltered N-hCD154 fused protein sample (replica 1). 5, precipitated and diafiltered N-hCD154 fused protein sample (replica 2).

Conclusions

Overall, these results demonstrate the feasibility of combining the EscoVacciXcell-CelCradle™ benchtop bioreactor with HEK-293 cells, operated in continuous mode, with ammonium sulfate precipitation and a diafiltration step for obtaining the N-hCD154 fused protein. This protein was successfully obtained with a medium recovery and over 95 % purity, enabling its

use for further benchtop experimentation, and in characterization studies of higher-scale culture and purification systems for experimental and/or vaccine purposes.

Acknowledgements

The authors kindly thanks to Yinet Cartaya Rodríguez, MSc. and Elias Nelson Rodríguez, PhD, Process Development Direction at the CIGB, for supplying the SARS-CoV-2 N-protein produced in bacteria used as control in the characterization experiments.

Conflicts of interest statement

The authors declare that there are no conflicts of interest.

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Received in January, 2022.
Accepted in May, 2022.