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Trimeric SARS-CoV-2 spike proteins from CHO-cells in bioreactors are high quality antigens

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Abstract: The spike protein of SARS-CoV-2 is essential for virus entry into human cells and is also the main antigenic determinant of the virus, being therefore essential to induce and detect antibodies. As a consequence, the global demand for spike proteins has rapidly increased and could exceed hundreds of grams to kilograms annually. Coronavirus spikes are large, heavily glycosylated, homotrimeric complexes, with inherent instability. Their poor manufacturability now threatens the availability of these proteins for large scale manufacture of vaccines and diagnostic tests. Here, we outline a scalable, GMP-ready, chemically defined process for production of a stabilized form of the trimeric spike protein. The process is chemically defined and based on a clonal, suspension-CHO cell line and purification of the protein via a two-step, scalable down-stream process. The trimeric conformation was confirmed using Cryo-EM and HPLC analysis. Binding to SARS-CoV-2 host cells was shown using a virus-inhibition assay. The diagnostic sensitivity and specificity for detection of serum SARS-CoV-2 specific IgG1 was investigated and found to exceed that of spike fragments (S1 and RBD).

Keywords: SARS-CoV-2; trimeric spike; CHO cells; manufacturability; vaccine; diagnostics.
1. Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the virus responsible for the 2019 coronavirus disease (COVID-19) pandemic [1, 2] which presents an unprecedented challenge to societies globally. Effective vaccines and sensitive diagnostic tools for COVID-19 are urgently needed, and systems to produce and deliver these tools in sufficient quantities are needed. It has become clear that currently used protein production technologies for Spike proteins are not able to meet the unprecedented global demand for ingredients for these. We believe that the SARS-CoV-2 trimeric Spike complex could be a useful ingredient for vaccine and diagnostic applications. Stabilized trimeric Spike proteins are considered the antigen of choice for RNA and virus-vector based vaccine candidates currently under development (Moderna, Novavax, Pfizer, J&J etc.). The efficacy of these vaccines is hoped or predicted to be between 50 and 70%. To boost the immune response, it is likely that follow-up vaccinations will be required. An adjuvated subunit vaccine based on the stabilized trimeric spike protein would be ideal for this purpose. Subunit vaccines can be produced in a cost-effective way and after lyophilization they can be transported and stored at ambient temperature. Also, whether any vaccination approach has elicited a sufficiently protective immune response against SARS-CoV-2 and its perpetuity needs to be verified. To address this, the quantification of antibody levels against SARS-CoV-2 in serum is the most practical approach. For this reason, there will be a high demand for diagnostic tests for quantification of SARS-CoV-2-specific antibodies. The antigen which provides the best sensitivity and specificity for detection of SARS-CoV-2 antibodies is the trimeric form of the spike protein [3].

The viral spike (S) protein complex is a surface-exposed homo-trimeric structure, that mediates entry into host cells. The spike engages the cellular host receptor and mediates virus-host membrane fusion. It is critical in the viral life cycle and thus the spike complex is considered the primary target of neutralizing antibodies [4-7]. The ideal diagnostic test for SARS-CoV-2 antibodies would detect all antibodies directed against the trimeric S protein complex. Production of such diagnostic tests implies the production of large quantity of highly purified S protein similar to its natural prefusion conformation [8]. The Corona virus Spike protein is a large, heavily glycosylated complex, with inherent structural flexibility and instability. In addition, the S protein is processed by Furin and another protease, named TMPRSS2 [6, 8]. Poor manufacturability and pre-fusion instability of the S protein have hampered its use for development of vaccines and diagnostic tests. We explored the manufacturability of S protein trimer in a soluble and secreted form using widely used Chinese Hamster Ovary (CHO) cells. We wished a process that can be used to produce high quality trimeric S protein for diagnostic tests as well as for vaccine applications. We used the fully characterized and CMC-compliant CHOExpress™ cell host, single use equipment, chemically defined media and additives. Also, regulatory requirements from DNA construction to production in bioreactors were strictly followed. The clonally derived cell line and the scalable production process outlined here, should allow manufacture of trimeric S protein in grams and even kilograms, should the demand rise to such a level.

2. Material and Methods
2.1. Construction of spike protein encoding DNA

SARS-CoV-2 derived spike proteins were constructed and codon-optimized for CHO cells according to manufacturer recommendations (https://www.excellgene.com/). A total of 11 DNA constructions were designed (Figure 1) and synthetized by ATUM, Menlo Park, USA. An IgG leader sequence was added to mediate efficient signal peptide cleavage. Histidine (8mer) encoding DNA was added for carboxyterminal expression. Where mentioned, the furin cleavage site RRAR is mutated to RGSA. The transmembrane domain and the C terminal intracellular tail were removed and replaced by a T4 foldon sequence [9] in trimer designs. For the RBD-fragment (aa319-541) IgG leader and histag sequences were added. After appearance of the D614G mutation in spike proteins of the virus, this mutation was also incorporated into the eventually chosen best construct.

2.2. Expression and purification of SARS-CoV-2 proteins

Manufacturer’s protocols were followed for transfection and culturing of CHOExpress™ cells (ExcellGene SA, Monthey). An optimized transfection reagent mix (CHO4Tx®, ExcellGene SA) was used to transfect cells in animal-component free media with the vector pXLG-6 (ExcellGene SA) containing SARS-CoV-2 spike DNA sequences, driven by a strong promoter/enhancer complex and associated elements, including an expression cassette for a puromycin resistance maker. Supernatants of high-density transient transfections under suspension culture were harvested after 14 days, mimicking a fed-batch process with ExcellGene’s animal component-free and protein-free CHO4Tx® PM (production medium) in 50 mL TubeSpin® bioreactor 50 tubes (TPP, Trasadingen, Switzerland). Cultures were shaken in a humidified ISF1-X/ISF3-X shaker (Kuhner, Birsfelden, Switzerland). Cultures were shifted from 37ºC to 31ºC during the production phase. Supernatants were harvested on day 10.

Recombinant CHO cell lines: Suspension cells were stringently selected with puromycine. Resulting stable pools of recombinant CHO cells with satisfying yields were kept. Clonal cell lines were obtained by image-assisted cell distribution into single wells of 96 well plates (b-sight, Cytena GmbH, Freiburg). Expanded cell populations with high level expression for the trimers were frozen in a mini-banks. Both, a recombinant pool cell line for the RBD spike fragment and the lead clonal cell line for the spike trimer were used for scale-up in an optimized fed-batch process at the 0.2 L, 10 L and 50 L bioreactor scale of operation.

The basic production medium employed EX-CELL® Advanced™ CHO Fed-batch medium (Merck-Sigma). Bioreactors were seeded at 5x10^5 cells/mL at 37ºC. On day 4 the cell culture was shifted to 31º C, and animal component free 7a and 7b feeds (HyClone Cell Boost, Cytiva) were used as feeds according to an ExcellGene optimized procedure. Culture fluids were harvested, clarified and subjected to purification by affinity chromatography by pumping them through layered (5 µm, 0.6µm, 0.2 µm) harvest filters (Cytiva, Ultrapure) to remove cells. Loading onto, washing and elution from a Ni-Sepharose column (Cytiva) were optimised, following the resin producers’ suggestions. The eluted product stream was loaded on a Size-Exclusion column (SEC, Superdex 200 pg, Cytiva) for further purification, following a tangential flow filtration with a cut-off of 100’000 Dalton for the trimer spike product. This step was omitted for the RBD fragment.
2.3. Inhibition test of infection by SARS-CoV-2

Vero E6 cells maintained in adherent format were infected at an MOI of 0.01 with preparations which either were used as such or were mixed with 2-fold serial dilutions of RDB or spike trimer, ranging from 240 µM to 0.244 µM. Cells were checked for infection 48 hours post infection, using a virus nucleocapsid antigen specific staining (red) and by a cell nucleus specific staining (blue), as described in [13]. Quantification of fluorescence was done as described [14].

2.4. Detection of SARS-CoV-2 antibodies using RDB, monomeric spike and spike trimer.

A bead-based serological assay [15] was used. Briefly, patient sera were collected and reacted with commercially available RBD and monomeric S1 domain protein, as well as S trimer and S RBD. Relative optical readings were taken for each protein specific assay using the control sera and the COVID 19 sera. The relative reading for individual samples ranged from 0.01 AU/mL to about 1000 AU/mL. The sensitivity of the assay with each of the tested protein components was plotted in a Receiver Operating Characteristic (ROC) plot.

2.5. Negative stain electron microscopy

Frozen aliquots of SARS-CoV-2 S-2P and S-D614G were thawed and purified by size exclusion chromatography (Superose 6 Increase 10/300, GE Healthcare) using 2 mM Tris pH 8.0, 200 mM NaCl and 0.02% (w/v) NaN₃. Elution fractions containing S trimer were collected and diluted to 0.05 mg/mL. Samples were deposited onto plasma-cleaned, carbon-coated copper grids (CF400 mesh, Electron Microscopy Sciences), and stained in 2% (w/v) uranyl acetate at pH 7.0. Grids were imaged at a resolution of 92.000X in a Talos F200C transmission electron microscope equipped with a Ceta 16M detector (ThermoFisher Scientific). The pixel size was 1.63 Å. Contrast transfer function estimation and particle picking were performed in cisTEM [16]. Extracted particles were exported to cryoSPARC-v2 (Structura Biotechnology Inc.) for 2D classification, ab initio 3D reconstruction and homogeneous refinement. Three-fold symmetry (C3) was imposed during the final round of refinement.

3. Results and discussion

3.1. Design and selection of a CHO manufacturable SARS CoV-2 S trimer

In order to define the optimal construct design to produce a soluble S trimer we evaluated the expression with various S protein-variants (Figure 1) by transient expression. The transmembrane domain and the C terminal intracellular tail were removed and replaced by a T4 foldon DNA sequence [9] with or without (GGGS)ₙ linker and a 8xHis tag encoding DNA. The furin cleavage site RRAR is mutated to RGSA. WT spike and amino-acid mutations K986P/V987P (“2P”) for locking the protein into a prefusion conformation [10] were also used. In addition, a receptor binding domain truncation of the S trimer was used (RBD-His). S proteins variants with a scrambled furin cleavage site were found better expressed. Mutation of the furin cleavage site seemed to increase trimer assembly and/or stability, suggesting that trimer assembly via T4 foldon occurs in the Golgi (Trans-Golgi network where furin is active). The same construct with above modifications were also applied for the D614G mutation in the spike protein which has been shown up recently in Europe.
**Figure 1**: Schematic representation of SARS-CoV-2 spike protein designs. SP: signal peptide, NTD: N terminal domain, RBD: Receptor binding domain, FCS: Furin Cleavage Site, FP: Fusion peptidpe, HR1: heptad repeat 1, HR2: heptad repeat 2, TM: transmembrane domain, CT: C terminal tail.

**Table 1**: Overview of the relative expression levels of the different SARS-CoV-2 spike protein designs evaluated under fed batch conditions, as well as their ability to form trimers in culture supernatants. ΔCter in the table and the following text refers to a construct that has also the transmembrane section of the protein deleted.
Based on the relative expression levels in transient expressions with CHO cells combined with the efficiency of trimerization (Table 1), the Spike_ΔCter_ΔFurin_2P_T4_His design was selected for further evaluation. For reasons of clarity, this design will be referred to as S trimer.

### 3.2. CHO expression and purification of proteins

Stable recombinant cell pools expressing S trimer and RBD were generated using puromycin selection. From a total of 300 clonally derived cell populations, those with the highest expression levels of S trimer and RBD were expanded and a fed-batch production process was developed. Cell culture process conditions, such as medium formulations, feeds, feed and temperature shift timing were evaluated at 10mL scale in TPP® TubeSpin 50 bioreactor tubes as previously described [11, 12]. Optimal conditions were selected on the basis of product yield, product quality, viable cell density (VCD) and cell viability. The production process was scaled up to 200 mL shake flasks, to 10 L and to 40 L stirred tank bioreactors (STR). Viable cell density (VCD) and viability remained high for at least 10 days (Figure 2). In addition, viable cell density (VCD) and viability profiles were comparable between shake flasks, 10L and 40L STRs, indicating process scaleability.

**Figure 2.** Cell culture performance in shake flasks and Bioreactors. Viable cell densities (cells/mL) and viability (%) are shown at various scales of operation for both S trimer (a) and RBD (b).

S trimer and RBD from cell-free culture supernatant were purified using an immobilized metal affinity chromatography (IMAC) capture step followed by preparative size exclusion. Eluates were concentrated and formulated at 1mg/mL in PBS pH7.4. Purity of the final products was estimated to be over 95% as analysed by HPLC-SEC (Figure 3 a and b).

HPLC-SEC (non-denaturing conditions) indicates that the Spike trimer is trimeric (about 460 kDa) and the RBD is monomeric. The expected size of 170 and 29 kDa for the S trimer and RBD monomers were confirmed by reducing SDS-PAGE (Figure 3 c, d). Trimeric confirmation was confirmed using Cryo-EM analysis (Figure 3 e - h).
Figure 3. Characterization of the recombinant trimeric Spike Protein and RBD. Size-exclusion chromatography (SEC) analysis plot with a single peak at 4.0 minutes and 6.4 minutes. SDS-PAGE gel showing bands for S trimer at different dilutions (c) and (d) for RBD monomer, NR=non-reduced, R=reduced. Negative stain electron microscopy of SARS-CoV-2 S trimer (Wuhan, e – f, D114 G, g-h). White bars 50 nm. Six 2D class averages are shown below each representative micrograph. 3D reconstructions are shown in (f) and (h).

3.3. Inhibition of infection of susceptible cells

SARS-CoV-2 virus was provided to serially diluted RBD or spike trimer and the mixture was used to infect Vero E6 cells. 48 hours later SARS-CoV-2 infection was visualized and quantified (Figure 4 a and b). The presence of both the RBD spike fragment and spike trimer reduced infection of Vero cells. For the RBD, reduction of infectivity was observed at a concentration of 20 µM and higher. For the spike trimer, a near 100-fold lower concentration reduced infection (Figure 4 b).

Figure 4 Inhibition of SARS-CoV-2 infection in Vero E6 cells

(a) Inhibitory effect of S trimer and RBD on infection of Vero E6 cells. SARS-CoV-2 antigen-positive cells were visualized by immunofluorescent staining. Virus nucleocapsid antigen staining (red), cell nucleus staining (blue), non-infected 1, 5, positive control 2,6, 250 µM trimeric spike 3,7, receptor binding domain (RBD) 4,8. All methods as described previously[13].
3.4. Sensitivity and specificity for SARS-CoV-2 antibodies

Obtaining a preliminary insight into spike trimer reactivity against sera of patients (n=9, 30 days after symptom onset) and non-infected volunteers (n=5), a simple ELISA showed for patient sera a moderate to strong reactivity whereas pre-pandemic volunteer sera showed only weak reactivity (data not shown).

Subsequently, SARS-CoV-2–specific IgG were quantified with a bead-based serological assay [15]. HEK293-produced monomeric S1 protein was used as positive control. We used the test to analyze a panel of sera from a diverse range of illness presenting patients (n = 151). 79 sera were collected from patients before the emerged pandemic, but who had shown non-corona influenza-like symptoms (n=28), and patients that had seasonal corona-induced (not SARS-CoV-2) influenza like illness (n = 35). These control sera were compared to sera from PCR-confirmed COVID-19 patients’ samples.
Sera were collected 4-40 days after disease onset, from hospitalized (n=16) and non-hospitalized (n=56) patients.

**Figure 5.** Performance of RBD, S1 and S trimer in a diagnostic assay to identify COVID-19 patients. a) Control sera (n = 151) and COVID-19 sera (n = 72) collected at day 4-40 of symptoms were tested and compared for concentrations of IgG. Median concentration and 95% confidence intervals are shown. b) The sera tested in (a) were analyzed by ROC. Abbreviations: AU, arbitrary unit; IgG, immunoglobulin G; RBD, receptor binding domain; ROC, receiver operator characteristic; S1, spike protein subunit 1; S, full-length spike protein.

Within the control and within the COVID-19 sample sets, for RBD, S1 monomer and trimeric S no significant differences were found (P > 0.05) in average signal-intensity (Figure 5 a). The mean values for control and COVID samples however showed a near 100 fold difference, while the individual data for reactivity had a large range, as has been reported by other groups as well. With Receiver Operator Characteristic (ROC) analysis a higher test-sensitivity and specificity when using S trimer compared to HEK293-produced S1 monomer and CHO-produced RBD (Figure 4 b) was found. The ROC curves for S1 and RBD were similar. Together, these results indicate that CHO-produced Trimeric Spike can deliver higher diagnostic specificity and sensitivity than HEK 293 produced S1 monomer, in line with previous observations [3].

3. Conclusion

Using an optimized CHO expression system and corresponding scalable production approach with chemically defined ingredients from transfection to purification, near wildtype trimeric SARS CoV-2 spike protein preparations could be provided in sufficient quantities. The S trimer was purified to high purity and proved to be efficient in blocking virus infectivity in an in-vitro model and highly specific and sensitive towards sera of COVID-19 patients.

**Author Contributions:** For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used “Conceptualization, P.P., D.K., M.J.W., J.K. and F.M.W.; methodology, P.P., J.K., R.D., V.A., G.P., J.M., P.M.; validation, P.P., J.K., and M.J.W.; formal analysis, F.M.W., P.P., J.K., J.M.; writing—original draft preparation, F.M.W.; writing—review and editing, P.P., J.K., F.M.W.; supervision, P.P., J.K.; project administration, M.J.W.; All authors have read and agreed to the published version of the manuscript.”

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Conflicts of Interest: ExcellGene declares that some material as the result of this work is being provided commercially to interested parties. ExcellGene authors declare that the interpretation of the results is done with the highest standards of objectivity. Conclusions and interpretation of results of non-ExcellGene authors (block of virus infectivity, reactivity against patient sera, etc.) were entirely based on the judgement of the non-ExcellGene authors.

References


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