Video Article

Efficient Mammalian Cell Expression and Single-step Purification of Extracellular Glycoproteins for Crystallization

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Abstract

Production of secreted mammalian proteins for structural and biophysical studies can be challenging, time intensive, and costly. Here described is a time and cost efficient protocol for secreted protein expression in mammalian cells and one step purification using nickel affinity chromatography. The system is based on large scale transient transfection of mammalian cells in suspension, which greatly decreases the time to produce protein, as it eliminates steps, such as developing expression viruses or generating stable expressing cell lines. This protocol utilizes cheap transfection agents, which can be easily made by simple chemical modification, or moderately priced transfection agents, which increase yield through increased transfection efficiency and decreased cytotoxicity. Careful monitoring and maintaining of media glucose levels increases protein yield. Controlling the maturation of native glycans at the expression step increases the final yield of properly folded and functional mammalian proteins, which are ideal properties to pursue X-ray crystallography. In some cases, single step purification produces protein of sufficient purity for crystallization, which is demonstrated here as an example case.

Video Link

The video component of this article can be found at https://www.jove.com/video/53445/

Introduction

Understanding protein structure at an atomic level is key to uncovering the molecular basis of biological pathways and diseases. X-ray protein crystallography is the most widely used/applicable method for determining macromolecular structures. The main challenge of this method is obtaining sufficient amounts of properly folded, pure protein. This becomes an issue particularly when working with secreted mammalian proteins, which undergo specific post-translational modifications.

Bacterially-expressed proteins are the primary source of crystallized proteins deposited in the Protein Data Bank[1]. Bacterial expression systems are largely preferred because they are fast, inexpensive and typically produce high yields of protein. However, extracellular domains of mammalian proteins expressed in bacteria are often not properly folded, in which case refolding and extensive purification steps are required for obtaining homogeneously folded protein. Additionally, many mammalian proteins require post-translational glycosylation to achieve proper folding[2]. Although expression and glycosylation in yeast or insect cells can overcome the folding problem, post-translational modifications, including glycosylation, differ significantly from those of mammalian cells[3], yielding proteins with incorrect or non-homogeneous modifications.

Mammalian cells express all the required molecular machinery to ensure proper post-translational modifications and folding; however, these expression systems are not typically preferred by most labs, due to limited yields and high costs of reagents and consumables. Polyethyleneimine (PEI), a standard transfection reagent is relatively cheap but imposes considerable cytotoxicity and low transfection efficiency, resulting in increased costs in cell media, DNA, and culturing equipment. Many alternatives to PEI are prohibitively expensive. We address these issues by describing a combination of improved cell culture tools and chemically modified PEI for the quick and relatively inexpensive method for the expression of secreted mammalian proteins, followed by single-step purification. This robust method gives sufficient yields for functional and biochemical studies[4], and in some cases, results in protein amenable to crystallization without further purification.

This protocol describes several techniques to maximize expression and yield for secreted mammalian proteins in human embryonic kidney (HEK) 293F cells grown in suspension. Transfection efficiency (and cost), protein production and purification are all greatly enhanced by following this protocol. PEI modified by the addition of carbamates through a single-step ring-opening reaction (PEI-TMC-25, synthesis and properties described in detail in ref [5]) greatly improves transfection efficiency, reduces the cytotoxicity from cationic membrane disruption and accordingly reduces experiment costs. Furthermore, cell viability and protein expression are greatly improved with the addition of culture supplements to supply glucose and vitamins. Importantly for the production of glycosylated proteins, treatment with kifunensine, a non-toxic chemical inhibitor of Mannosidase I, produces proteins with defined, immature glycans, which can be removed by the endoglycosidase EndoHf to yield proteins with a single N-acetylglucosamine in place of a full-length N-linked glycan[6]. Finally, the secretion of proteins into a serum-free, chemically defined medium allows rapid and facile purification for structural and biochemical studies. Single-step nickel-nitrilotriacetic acid (Ni-NTA) resin purification removes the majority of contaminating species in the supernatant and, in some cases, can yield protein of sufficient purity for crystallization.

Protocol

1. Production of Milligram Quantities of Plasmid DNA for Large-scale Transient Transfection

1. Clone the protein of interest into a high copy number mammalian expression vector using restriction site cloning, or other appropriate technique.
    1. For optimal results, use pHLsec [7] vector, which has a built-in C-terminal 6His-tag, a strong promoter Kozak sequence and an optimized secretion signal.
2. Transform the plasmid onto competent cells.
    1. Add 20 µl of competent E. coli cells onto 1 µg of plasmid DNA and incubate on ice for 30 min.
    2. Heat shock cells at 42 °C for 35 sec, then incubate on ice for 2 min.
    3. Add 300 µl of microbial growth medium (SOC) and incubate at 37 °C for 45 mins, shaking at 220 rpm.
    4. Plate cells on agar plate with appropriate antibiotic selection.
        1. Use 100 µg/ml carbenicillin if the plasmid is in the pHLsec vector.
3. Culture colonies in 250 ml of Luria Broth (LB) Media supplemented with 100 µg/ml antibiotic (carbenicillin) O/N at 37 °C, shaking at 220 rpm.
4. Purify DNA from culture using Hi-Speed Plasmid Maxi Kit according to manufacturer's protocol.
    1. Elute DNA in buffer EB (10 mM Tris-Cl, pH 8.5), instead of buffer TE.
    2. Aliquot the purified plasmid at amount needed for transfection and store at -20 °C.

2. Large-scale Culture and Transient Transfection of 293F Cells

1. Supplement 1 L 293F media with 10 ml of glutamine and 5 ml Pen/Strep (both 100x). Store at 4 °C. 5 ml Pen/Strep is a sufficient strength in serum-free conditions and the reduced antibiotic concentration improves cell viability during transfection, which improves protein yields.
2. Culture 293F cells in 300 ml media in 1 L polycarbonate baffled Erlenmeyer flasks with vented caps at 37 °C with 8% CO_2, while shaking in a standard tissue culture incubator.
3. Dilute cells to 5 x 10^5/ml density one day before transfection.
4. On the day of transfection, supplement culture medium by adding 10% volume of 2% w/v Cell Boost in 293F media.
    1. Measure glucose concentration using a glucose monitor according to the manufacturer's instructions and use supplements as needed to achieve a glucose concentration of 500 mg/dL.
5. Add kifunensine (1 µg/ml final concentration) at this step to control protein glycosylation.
6. Calculate volume of DNA required for 1 µg plasmid per 1 x 10^6 cells. Under sterile conditions, dilute DNA in 5 ml serum-free medium.
7. Calculate volume of transfection reagent required for 1 µg plasmid per 2 µl transfection reagent. Under sterile conditions, dilute transfection reagent (PEI-TMC-25) in 5 ml serum-free medium.
8. Add transfection reagent into DNA solution in 1 ml increments, mixing gently. Incubate for 30 min at RT for reagent-DNA complexes to form. Then add the solution onto the cells in a drop-wise fashion.
9. Allow transfected cells to express protein for 72-96 hr. Supplement with ~10% volume Cell Boost Media daily, or as necessary to keep glucose reading 400-600 mg/dl.

3. Purification

1. Decant culture into a centrifuge flask, centrifuge for 20 min at 1,300 x g to pellet cells and then collect the supernatant. If necessary, spin a second time and/or use 0.22 µm filter to clarify supernatant.
2. Add 10% volume 10x Ni-NTA binding buffer (1.5 M NaCl, 0.5 M K_2HPO_4, 0.1 M Tris pH 8.5, 50 mM imidazole).
3. Prepare a gravity column by adding 2 ml of Ni-NTA slurry in a column and equilibrating with 10 column volumes (CV) of 1x binding buffer. If possible, do all column steps in a 4 °C room. Alternatively, chill protein and all buffers on ice before column step, and keep protein and collected flow-through on ice.
   Note: Ni-NTA slurry is 50% resin by volume and the manufacture's stated binding capacity is 50 mg/ml. Ni-NTA beads can be re-charged for multiple uses
4. Flow the supernatant over the resin and collect flow-through. Repeat this step.

5. Wash with 10 CV of wash buffer (300 mM NaCl, 50 mM K_2HPO_4, 20 mM imidazole pH 8).
6. Elute the protein in 5 CV of elution buffer (300 mM NaCl, 50 mM K_2HPO_4, 250 mM imidazole pH 8).
7. If deglycosylation is required:
    1. For a final volume of 0.5 ml, concentrate eluate to 0.43 ml using a centrifugation concentrator. If precipitates form, pellet any debris by centrifugation at 16,000 x g and 4 °C.
    2. Add 50 µl of 500 mM Na-Citrate pH 5.5.
    3. Add 20 µl of EndoHf (1 x 10^6 U/ml). Incubate at RT for 2 hr.
    Note: The enzyme works optimally at 37 °C, which may cause the concentrated protein to aggregate. Extend the RT incubation, if deglycosylation, assessed by SDS-PAGE or immunoblotting, is incomplete. The enzyme does not have activity at 4 °C.
    4. To remove EndoHf: Wash Amylose Resin 3x in phosphate buffered saline (PBS) or final storage buffer. Incubate protein with resin for 1 hr at 4 °C. Spin 5 min at 1,000 x g to pellet beads and collect the supernatant.
    5. Concentrate protein using appropriate molecular weight cutoff centrifugation filter and buffer exchange into storage buffer (150 mM NaCl and 20 mM HEPES pH 7.5).

Representative Results

Herein follows the results of this expression system applied to a secreted 13 kDa immunoglobin (Ig) domain from the human protein triggering receptor expressed on myeloid cells 2 (hTREM2, residues 19-132). TREM2 is a type I transmembrane protein containing a single extracellular Ig domain that has two disulfide bonds and two N-linked glycosylation sites. Unlike many other Ig domain proteins[8], TREM2 was not amenable to refolding from bacterial inclusion bodies[9]. Subsequent mutagenesis confirmed N-linked glycans are required for proper expression and folding. To facilitate structural and functional studies, TREM2 was introduced into the pHLsec vector with a C-terminal 6His-tag[7] using standard molecular biology techniques. Transient expression in HEK293F cells treated with kifunensine yielded protein that was purified by Ni-NTA chromatography (**Figure 1B, lane 2**) and the sample was then deglycosylated to produce natively folded, homogenous protein (**Figure 1B, lane 3**). 293F expression of TREM2 produced 5-10 mg/L (5-10µg/million transfected cells). After buffer exchanging into the storage buffer, this protein was crystallized (**Figure 1C**). Despite the final purity of about 80%, these crystals reliably reproduced, diffracted, and were shown by silver-stain to only contain TREM2 protein (**Figure 1D**). This observation suggests the biochemical homogeneity of the protein (*i.e.,* folding and post-translational modifications) can, in some cases, be more critical than overall purity for crystallization success.

In addition to crystallization, this system offers a robust tool for structural and functional studies. It is exploited to produce natively glycosylated protein and achieve >95% purity by size-exclusion chromatography (**Figure 1E, F**). This additional purification step, which shows the solution behavior of the protein, is also an ideal way to monitor protein quality. The purified protein should elute at a volume corresponding to its molecular weight and should be the most abundant species in the sample. Abnormally large amounts of aggregated protein may indicate unstable protein and provide clues to optimize protein production and purification. Furthermore, this final purified protein is superior for use in quantitative biophysical experiments such as circular dichroism spectroscopy, thermal stability, and investigating protein-ligand interactions. Lastly, controlling the extent of glycosylation provides a robust tool to study glycan-dependent functions.

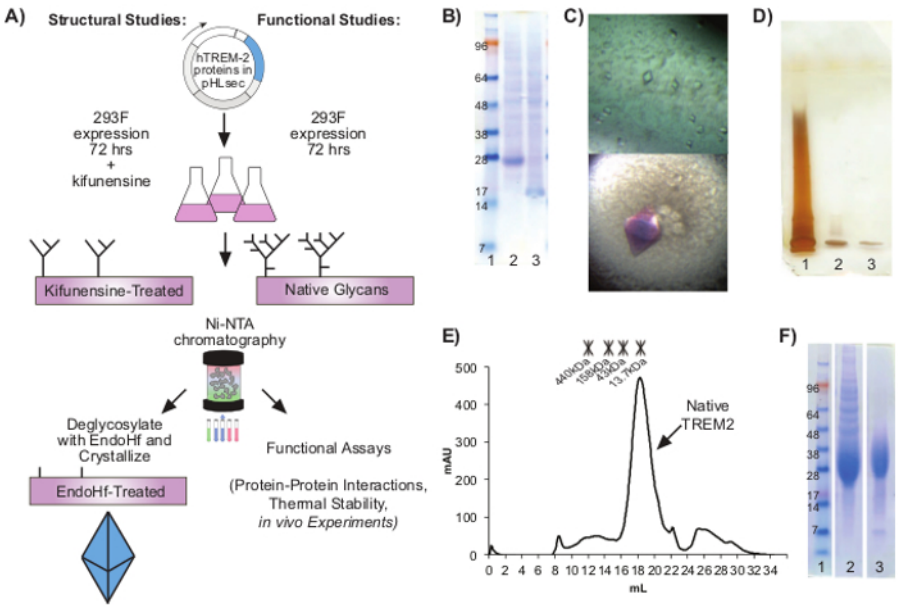

Figure 1. Mammalian Expression System: (**A**) Scheme for mammalian expression of proteins with controlled or native glycosylation in HEK 293F cells. (**B**) SDS-PAGE of NiNTA-purified hTREM2 expressed under kifunensine shown before (lane 2) and after (lane 3) deglycosylation with EndoHf. (**C**) Crystals produced from NiNTA-purified and deglycosylated protein. (**D**) Silver stain of crystallization input (lane 1) and harvested crystals (lanes 2 and 3) reveals crystals only contain TREM2. (**E**) Further purification of TREM2 was achieved using size-exclusion chromatography of NiNTA-purified TREM2 produced in 293F cells without Kifunensine. Asterisks (*) indicate elution volumes of molecular weight standards. Proteins eluted in TBS using an analytical S200 column (GE). (**F**) SDS-PAGE analysis of size exclusion-purified TREM2: input protein (lane 2) and folded peak (lane 3). Note how full, heterogeneous glycans run at a higher apparent molecular weight with a broad smear by SDS-PAGE. Please click here to view a larger version of this figure.

Discussion

HEK 293F cells offer robust production of proteins requiring post-translational modifications. This system allows rapid and scalable expression of natively folded proteins containing disulfides, glycosylation, and phosphorylation that would otherwise be absent using more routine expression tools. In addition, this system can be used for the expression and purification of multi-protein complexes simply by co-transfection of multiple plasmids. Besides TREM2, this system has been extensively used for functional studies with other proteins of interest in the lab[10,11]. Mammalian cells also offer endotoxin-free protein expression optimal for *in vivo* experiments or for production of natively folded antigens to generate conformation-dependent antibodies.

Optimal cell viability and transfection conditions are the most crucial for efficient protein production. For better cell viability, low-passage cell cultures are boosted with cell culture supplements and the antibiotic concentration in the growth media is reduced. The modified PEI-TMC-25 has suitable transection efficiency with most proteins; however, there are other options available at moderate price that offer increased transfection efficiency and reduced cytotoxicity. Hype 5 (Oz Bioscience) increases yields compared to PEI or other reagents, at a considerably reduced cost compared to more expensive transfection reagents, such as 293fectin. The reagent type and DNA: reagent ratios can be optimized for individual expressions and the needs of the desired experiment.

The method described here has several advantages over other methods of protein production. Mammalian cells offer native chaperone folding and post-translational modifications unavailable to bacteria and yeast. The reduced time for plasmid construction and preparation, along the use of media, the pH of which can be directly modified, make it superior to baculovirus-based production in insect cells; and finally, the serum-free media offers facile scaling (higher cell density) and purification not attainable to adherent 293T cells. The chief limitation of this system is only the time and scale the individual researcher is able to commit to protein expression.

Included below are troubleshooting options for the most common issues encountered.

Troubleshooting

If there is low protein expression, avoid passaging 293F cells longer than 2-3 months. Prolonged passaging results in decreased cell health reflected in slower doubling times and significantly reduced protein expression. Cell growth should be monitored daily and cultures no longer doubling daily should be discarded. Cell viability can be improved by using culture supplements while passaging cells. Monitor glucose to ensure a culture concentration of 400-600 mg/dl Cell density should not exceed 2 million/ml and cell viability should be ≥98% by trypan blue exclusion. Dilute dense cells to 0.5 million/ml and allow to double O/N before transfection. Optimize transfection using different ratios of transfection reagent: DNA (within the range of 1 µg DNA: 1.5-6 µl reagent). This can be done using 2 ml cultures in a 6-well plate and the output measured by immunoblotting. Doing 12 hr time points can indicate if the protein is being expressed and degraded rapidly; check the cell lysate if protein is not observed in the supernatant fraction. Misfolded proteins, which fail to be secreted, will still be apparent in the cell lysate by immunoblotting.

If Ni-NTA retention is low, *i.e.,* protein remains in flow-through after column binding, use fresh resin. Increasing pH of sample to 8.5 will result in stronger binding to the resin, although this will also increase non-specific binding. Concentrating the sample and batch binding to resin O/N at 4 °C before Ni-NTA purification may result in ~5x increase in recovered protein. If the 6His-tag is inaccessible due to tertiary structure of the protein, try cloning it on the other terminus. Alternatively, use Cobalt resin, which has a higher affinity for His residues.

If the protein aggregates and/or precipitates during purification, increase solubility by adding 10% glycerol into the Ni-NTA wash and elution buffers. Run the nickel affinity columns at RT instead of 4 °C. Use differential scanning fluorimetry[12] to screen for pH and salt/additives that increase stability of the protein in solution.

Disclosures

The authors declare that they have no competing financial interests.

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