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# Production of nano-protein particles in living cells

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ABSTRACT

The escalating demand for therapeutic proteins calls for advanced biomanufacturing and formulation strategies to ensure efficient production and timely supply. Productions of therapeutic proteins, as well as many other proteins, require high purity and stability. However, the manufacturing process is bottlenecked by the purification process. We have established a novel platform leveraging the capabilities of living *E. coli* cells as cell factory, for efficient synthesis (protein expression) and easy separation (protein particle formation) of a model protein, Red Fluorescent Protein (mRFP). With the designed plasmid, the target protein (mRFP) was heterologously expressed inside cells, and directed to form protein particles, and the red fluorescence of these particles proved their correct folding and bioavailability. Target protein production was validated through SDS-PAGE and fluorescent microscopy. The cells with protein particles and the isolated protein particles were characterized by SEM, optical and fluorescent microscopic. The new cell factory technology developed for mRFP production has exhibited broad potential in advancing biomanufacturing, which can be further developed to enable the efficient bioproduction of other therapeutic proteins and peptides.

## 1. Introduction

Many chemical engineering research are inspired by nature. The production of the solid form of protein, protein particles, can enhance the efficient biomanufacturing processes by replacing the expensive chromatographic technology, which is the bottleneck in the purification of biopharmaceuticals or biomaterials (Hekmat, 2015). In nature, protein particles are found inside biological organisms, macrobiota, such as butterfly photonic crystal, kidney stone, coccolith calcite (McClelland et al., 2017; Tang et al., 2023; Welch and Vigneron, 2007). Some protein particles are protein crystals inside microorganisms. Viral capsids and multi-protein complexes were reported to naturally formed inside cells (Casjens and King, 1975; Fromont-Racine et al., 2003) as well as cry protein crystals formed inside Bacillus thuringiensis were used as pesticide (Nickerson, 1980; Schnepf et al., 1998). The functions of the protein crystallization for the cells include protein storage and catalysts, and some proteins are disease-associated (Fromont-Racine et al., 2003; Koopmann et al., 2012; Schönherr et al., 2018, 2015). Some protein particles are inclusion bodies, and the amorphous protein aggregates consist of misfolded or partially folded proteins. The formation was due to over expression (Carvajal et al., 2011; Wang et al., 2015), and they have little or limited bioavailability. Many research have been focused on refolding the misfolded inclusion body and recovering the bioavailability (Betton and Hofnung, 1996; Singh et al., 2015). However, the refolding efficiency and yield are still very low.

Despite the challenges in the refolding of proteins, the formation of the solid protein particles within cells offers the benefit of easier separation, and if the protein particles are crystals (highly ordered arrangement of protein molecules with bioavailability) or other solid forms with bioavailability, the separation process can be cost-effective and easy to scale up. However, the suitable conditions for obtaining the protein crystals or protein particles with bioavailability were still challenging for researchers and industrials (Singh et al., 2015; Tian et al., 2023, 2021; Yang et al., 2019). This work has demonstrated the initial developments of a novel biotechnological technique to efficiently produce target protein particles with high bioavailability. There is no requirement for a huge amount of work on screening the suitable crystallization conditions with this technology. The *E. coli* (Escherichia coli) cell functions as a cell factory to produce mRFP protein particles inside through T7 expression system (Fuerst et al., 1986). The live cells were observed under a bright and fluorescent microscope during the cell culture. Further isolated protein particles were characterized by SEM (Scanning Electron Microscope) and SDS-PAGE (Sodium dodecyl-sulfate polyacrylamide gel electrophoresis).

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## 2. Experimental

For recombinant protein production as shown in Fig. 1A, BL21 (DE3) (Promega, US) cells were used to over-express a designed gene after induction with IPTG (isopropyl β-D-1-thiogalactopyranoside) solution (Sigma-Aldrich, US). The gene sequence of mRFP was fused with Cry1Ac (CS-PDB 4W8J 609–872 (Evdokimov et al., 2014)), which was developed as a crystal scaffold (CS). After introducing the CS-RFP plasmid, cultures of E. coli were grown in Luria-Bertani (LB) broth (Invitrogen, US) at 100 ml, supplemented with 50 μg/ml of kanamycin solution (ThermoFisher scientific, US), over a period of 10 h at 30 °C. Distilled water was supplied by the Millipore Water System with a resistivity of 18.2 mX cm at 298 K.

Cells were recovered by centrifugation at 8000*g* for 10 min, then washed with Tris-HCl (50 mM pH 7.8) buffer. After the cell lysis was conducted in lysis buffer (50 mM TrisHCl pH = 7.8, 200 mM NaCl and lysozyme 3 mg/ml) for 1 h at room temperature and an additional 30 min with DNase1 at 37 °C. The lysed cells were centrifugated at 15,000 g for 20 min at 4 °C and the pellets were washed 5 times with 1 ml of the lysis buffer and 0.5 % Triton X-100 to get protein particles impure fraction. The lysed cell fraction was loaded on a three-layer liquid-liquid-phase-separation solution, with a composition of about 30 %, 45 % and 60 % of OptiPrep Density Gradient Medium (Sigma, UK) and settled for an hour. The solution from each layer was separated and then centrifugated at 5000 g for at least an hour. The protein particles were separated from the middle layer of the solution and washed three times with distilled water. The separated protein particles were further observed under an optical microscope, fluorescence microscope and SEM (JSM-7100 F). The protein particles were dissolved in 4x lamilli buffer, and boiled for 10 min for SDS-PAGE analysis (Mini-PROTEAN Tetra Cell, Bio-Rad).

## 3. Results and discussion

Fig. 2A shows the red color culture solution, which proved that these recombinant proteins with the fused mRFP correctly folded inside *E. coli* cells. The color became darker with time, as shown in Fig. 2B, indicating the successful protein expression of CS-RFP during the cell culture period and the increase in the quantity of the target protein. Without the plasmid, there was no red color cell culture solution during the cell culture as shown in Fig. 2B. The cell pellet in Fig. 2C shows the isolated cell pellet after centrifugation and multiple washes, proving the target protein inside *E. coli* cells, and cells without the plasmid appeared normal cell color, shown in Fig. 2F.

In Fig. 3A, the optical microscope images show that the control experiment with cell culture of *E. coli* cells after 6 h without plasmid induction. Fig. 3C shows the *E. Coli* cells 6 h after introduction of the plasmid, and there were protein particles inside cells, most of the cells had one or more protein particles inside. The examples of protein particles inside cells are shown in Fig. 3E and F.

The fluorescence microscope image in Fig. 3B shows the *E. Coli* in the control experiment without plasmid induction. There were fluorescence signals, indicating the locations of *E. Coli* cells. However, there were no clear fluorescence images for *E. Coli* cells, due to no fluorescence protein and overall low protein concentration inside cells. Fig. 3D shows strong fluorescence of the E. Coli cells 6 h after the introduction of the plasmid, and the cell images were clear due to high RFP protein expression and high protein concentration inside cells. The protein particles inside cells were much brighter than the rest part of the cells, as the mass density of the protein particles was much higher than the soluble protein in other parts of the cytoplasm. The bright protein particles were mostly located near the pole of the cells, and examples are shown in Fig. 3G and H. This was consistent with the literature that due to macromolecular crowding in bacterial cytosol, the over expressed protein tends to be squeezed to the pole side (Coquel et al., 2013). The protein particles inside cells, as the lump parts of the cells shown in Fig. 2D, have been harvested after cell lysis and separation from the cell debris, which were observed by SEM images of the cells. Further investigation by TEM (transmitted electrons microscope) on nano meter thick cell sample might be helpful to identify more details inside *E. coli* cells (Carvajal et al., 2011; Wang et al., 2015).

Fig. 4A shows the diameters of protein particles are all about 500 *nm*, which was smaller than the diameter of the *E. coli* cells. The size distribution of these nano protein particles is relatively uniform. Most protein particles tended to agglomerate together due to their small size at nano scale, as shown in Fig. 4B. It is noted that these particles are not spherical and some of them had clear edges.

In Fig. 4C, the results of the SDS-PAGE revealed protein profiles following cell lysis, with a notable band appearing near 60 kDa and most of the proteins in the isolated fraction were in the range of 30–120 kDa. For protein particles after dissolving shown in Fig. 4D, there was a band of about 60 kDa, which was in agreement with the size of the recombinant CS-RFP protein. The additional band observed near 120 kDa may represent the dimer formation of the target protein.

As the cell culture was under antibiotic, ensuring only the cell with successful plasmid induction survived, the results of SDS-PAGE have proved that the protein particles were the target CS-mRFP protein. The fluorescent protein inclusion bodies would lose the fluorescent features and bioavailability due to protein denaturation and polymer formation (Waldo et al., 1999). These protein particles exhibited red fluorescence, proving the correct folding rather than forming inclusion bodies. In addition, the inclusion body is commonly hard to be dissolved, but these CS-mRFP protein particles can be dissolved in a basic solution. All the SEM and microscope images indicate the formation of protein crystals inside the cells. Nevertheless, all evidences proved that these protein particles were not inclusion bodies, it would require additional X-ray diffraction patterns to identify the crystalline structure of the protein crystals.

In this work we have firstly demonstrated the new biotechnology, inspired by nature, to harvest target protein particles with bioavailability, and these protein particles were crystalline indicated by the characterizations, with further investigations for identification. Based on the chemical engineering principles, a certain level of supersaturation is key for the crystallisation or formation of the protein particles inside cells. Too high supersaturation would drive the protein aggregated or misfolded to form inclusion body particles with little bioavailability. The protein particles with bioavailability or protein crystals can lead to a cost-effective and efficient strategy for producing

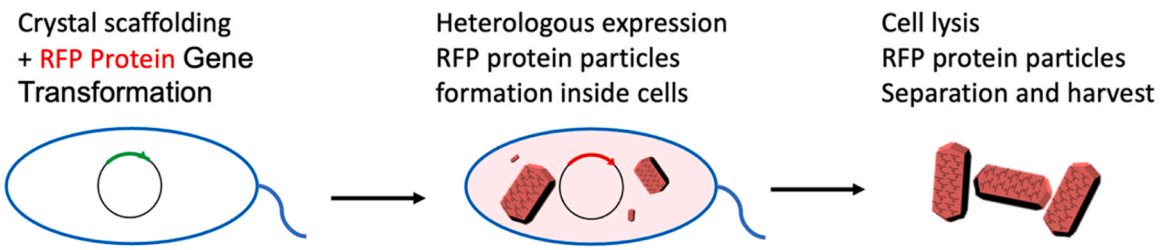


**Fig. 1.** (A) Schematic diagram for the formation of CS-RFP protein particles inside *E. coli* cell and separation of protein particles.

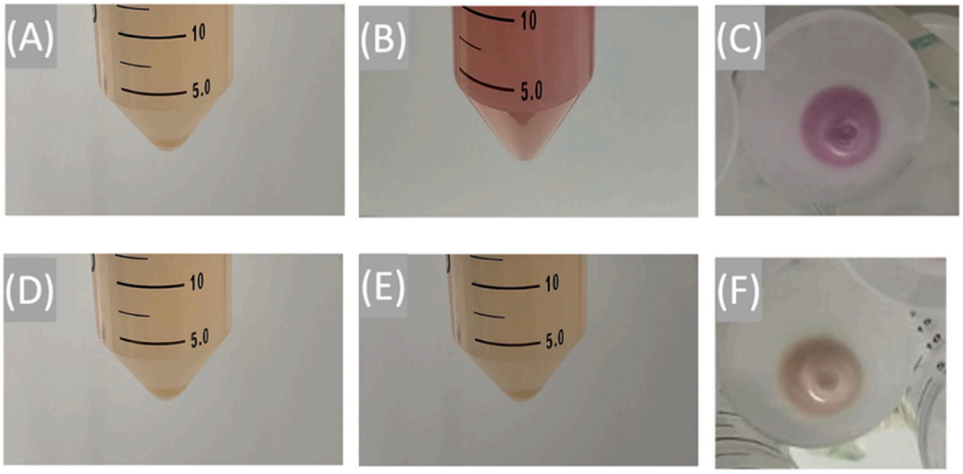


**Fig. 2.** *E. coli* cell culture solution during the cell culture process with induction of CS-RFP plasmid in the beginning of cell culture (A) and middle period of cell culture (B) and without plasmid in the beginning of cell culture (D) and middle period of cell culture (E), and the cell pellet after centrifugation with the plasmid (C) and without (F).

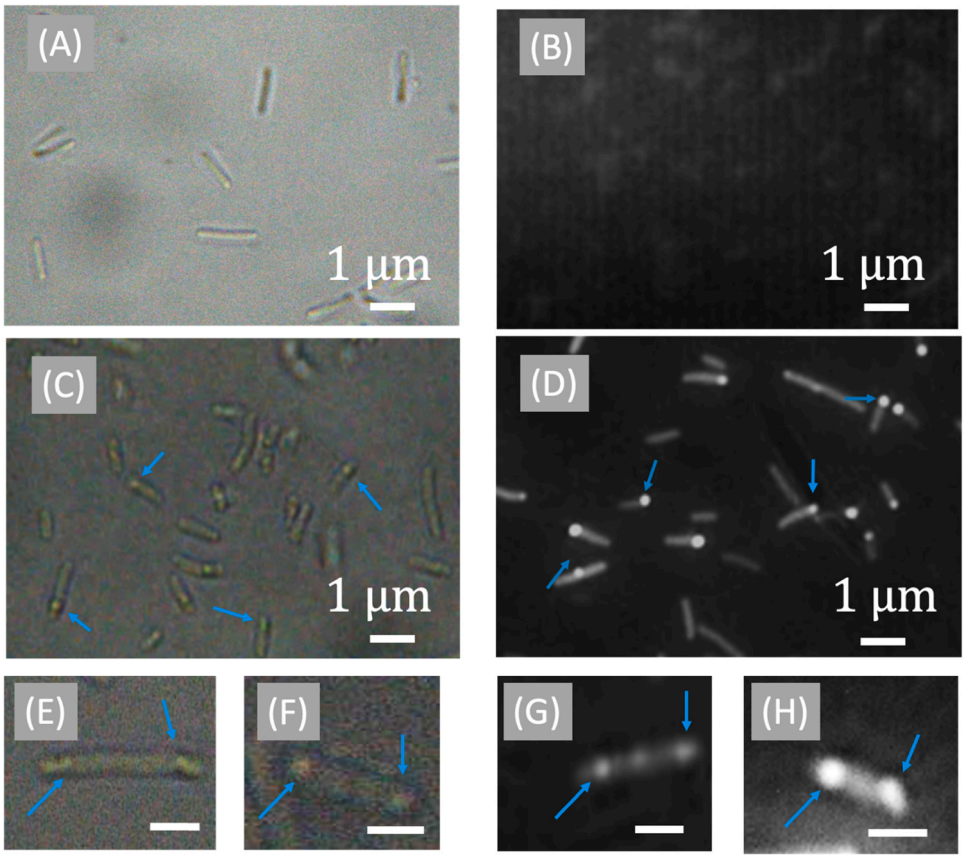


**Fig. 3.** Optical microscope (A) and fluorescence microscope image (B) for *E. coli* cells after 6 h cell culture without plasmid. Optical microscope (C) and fluorescence microscope image (D) for *E. coli* cells after 6 h cell culture with plasmid. Examples of multiple protein particles formation inside cells (E) and (F) under the optical microscope, and (G) and (H) under the fluorescence microscope.

and purifing proteins. The method is also easy to scale up by larger-scale cell culture, with further potential in optimisation of the bioprocess (Zhang et al., 2011; Kasemiire et al., 2021). With cell culture at a larger scale, more crystalline protein could be harvested, which will help to perform more characterizations to identify the protein structure, particle size distribution, stability and bioavailability. In addition, the design cell factory for producing protein particles / protein crystals need the biochemical engineering skills, biotechnology skills combined with chemical engineering skills, requiring further investigations to understand complex mechanisms.

## 4. Conclusion

This work demonstrated the ability of this novel cell factory to produce the protein particles with bioavailability inside living *E coli* cells. The RFP protein, fused with crystal scaffold, was correctly heterologous

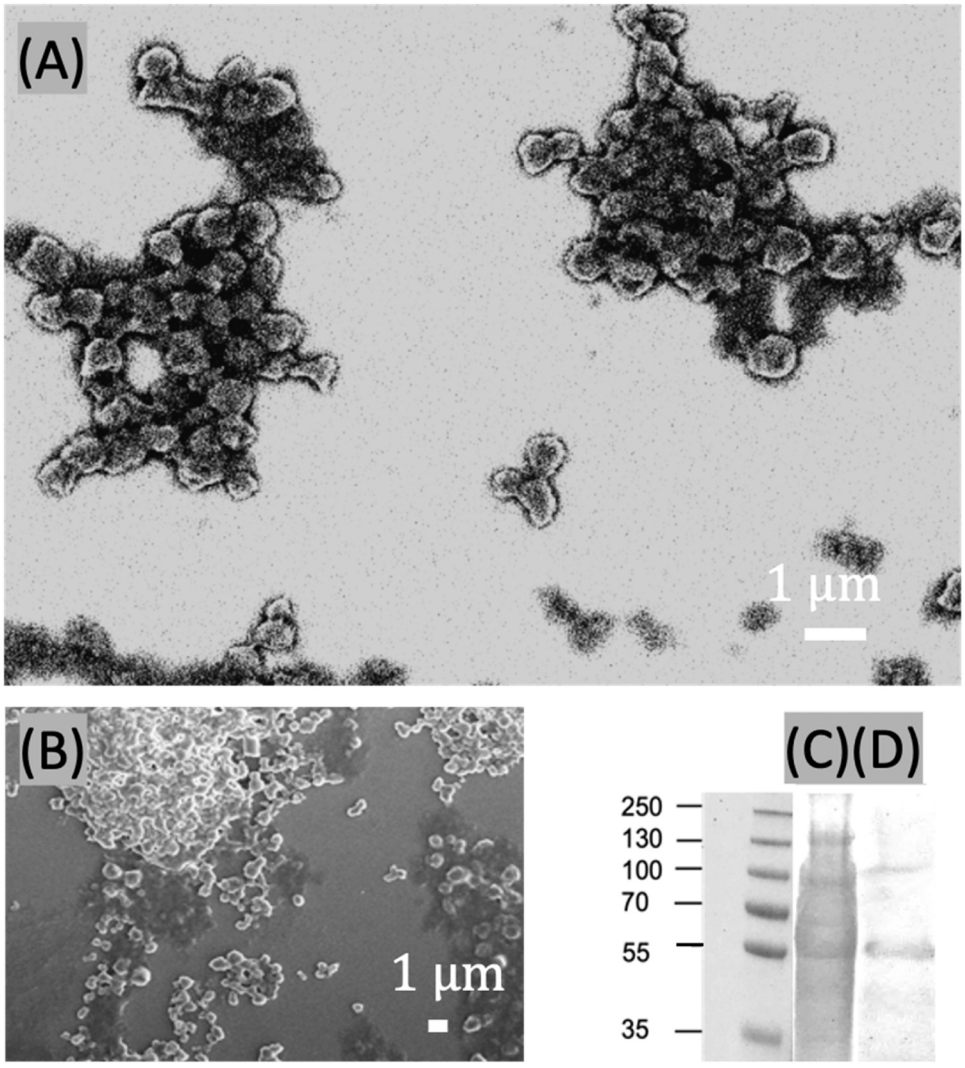


**Fig. 4.** SEM of protein particles (A and B) and SDS-PAGE results of all proteins after cell lysis (C) and the protein particles after separation and dissolving (D).

expressed with *E coli* cells, proved by the red color colonies, which was also validated by SDS-PAGE. The protein particles were collected after cell lysis, and the size of all protein particles had a relatively uniform size distribution at about 500 nm. All bright, fluorescent microscope, SEM images and soluble properties reveal that the protein particles are distinct from inclusion bodies, suggesting a crystalline structure within the cells, which still require further investigations to confirm the crystal structure. Nevertheless, these protein particles with correct folding show red fluorescent feature with demonstration of good bioavailability. This solid form of protein particles or crystals inside cells introduces an innovative strategy for protein synthesis and purification, with demonstrated success in its implementation.

## CRediT authorship contribution statement

**Vikash Kumar Yadav:** Investigation, Formal analysis, Data curation. **Huaiyu Yang:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Data curation, Conceptualization. **Kavita Yadav:** Writing – original draft, Visualization, Validation, Resources, Methodology, Investigation, Formal analysis, Data curation.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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