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Nanoparticles Mediated Protein Stability in Comparison with Osmolytes: *in vivo* Approach

R. VERMA[1], N. SINGH[2] and P. CHAUDHURI (CHATTOPADHYAY)[1,*]

[1]Molecular Biophysics Lab, Amity Institute of Biotechnology, Amity University, Sector 125, Noida-201313, India
[2]Centre for Biomedical Engineering, Indian Institute of Technology, Hauz Khas, New Delhi-110016, India

*Corresponding author: E-mail: pchaudhuri@amity.edu

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The native three-dimensional structure of protein is quite unstable under critical destabilizing conditions. In order to enhance the stability and activity for a proper folded environment of a protein, many stabilizing materials are added such as nanoparticles and osmolytes to an unfolded state of protein. Osmolytes are the important group of molecules which are engaged by the cell as an adaption in the severe conditions. In this communication, a comparative *in vivo* study is reported for imparting the status of stability and folding ability of zebrafish dihydrofolate reductase (zDHFR) protein with gold nanoparticles and various osmolytes (glycerol, glucose and betain). Present observations revealed that the interaction of gold nanoparticles (AuNPs) with bacteria at the cellular level helps in maintaining the stability of protein more effectively than osmolytes which could be used for many biological and pharmacological approaches although glycerol as an osmolyte also stabilizes the protein at a significant level.

Keywords: Stability, Osmolytes, Nanoparticles, Zebrafish dihydrofolate reductase (zDHFR), Solubility, Cellular Folding.

INTRODUCTION

In the era of modern biotechnology, functionality as well as solubility of recombinant proteins are of immense interest and are on high demand. Recombinant protein production is the most challenging technique nowadays. The rising application of recombinant proteins from the fields of proteomics, biotechnology and drug development programs are generally attained by the protein expression and purification using *Escherichia coli* system as a host due to its integrity and simplicity [1]. *E. coli* is famous as a most suitable expression platform which helps in the administration of expeditious, augmented and cost-effective manufacturing of recombinant proteins. It also aids in speedy development in a modest medium along with the accessibility of the genetic data and establishment of numerous cloning vectors [2]. Although the procedure is simple, absence of proper folding climate and post translational alterations in *E. coli* system affects many proteins and restricts their expression [1]. Improper folding of protein forms aggregates which influences the folding process of protein. These protein aggregates have complicated biochemical and structural characteristics, which are non-active in nature. Formation of aggregates depends on many elements like the quantity of expressed protein, the genetic nature of recombinant protein and the concentration or intermediates formed during protein folding [2]. In order to maintain convenient folding environment, bacteria have defence mechanisms which help the proteins to attain its structure and function in native form. Other than this defending property, use of external supplements such as osmolytes and nanoparticles also prevent protein aggregation.

Osmolytes, which are also known as chemical chaperones, occur naturally as low-molecular weight organic compounds [3]. The presence of osmolytes under stress conditions regulates the level of solubility of a protein *in-vivo* and thus encourages proper refolding of unfolded polypeptides [1-4]. Osmolytes are divided in to five parts such as amino acids, carbohydrates, methylamines, carbamides and salts [4]. Several researchers have investigated the presence of osmolytes in the growth media aids in keeping the stability of proteins which causes proper folding and thus increasing the solubility of protein [5]. Osmolytes play an important role in almost all the organisms and help in maintaining their cellular environment and metabolic functions. They are an essential class of versatile compounds possessing numerous biological roles which can be

scrutinized for different purposes like drug development, over production of recombinant proteins, increasing the productivity of plants, and treating diseases such as Alzheimer's disease [3]. Osmolytes have significant safeguarding functions for proteins, such as sugars [6].

Other than using osmolytes, nanotechnology has also raised their bars in the field of biological science, especially in the clinical fields [4]. The growth in the aspects of life is primarily because of the potential advancement and execution in the area of nanotechnology. Development of new unique diagnostic, sensing and treatment efficiencies such as advanced gene therapy, targeted drug delivery, biological warfare agent detection and magnetic resonance imaging contrast can be formed by progressive understanding of interaction between nanoparticles and biological cell [7]. Nanoparticles possess large surface to volume ratio and have significant adsorption capacities, which help them to form strong nanoparticle-protein interactions and thus helps in maintaining the stability of particular proteins which prevent it from aggregation also [8]. Nanoparticles have been extensively adapted in numerous fields of biomedical sciences such as immunoassay technique and *in vivo* cancer targeting and imaging [9].

Dihydrofolate reductase enzyme (DHFR 5,6,7,8-tetrahydrofolate:$NADP^+$ oxidoreductase), EC (1.5.1.3) act as a catalysts in the reaction, which involves the reduction of dihydrofolate (H_2F) to tetrahydrofolate (H_4F) employing NADPH to work as a cofactor [10]. Tetrahydrofolate acts as a precursor for cofactors, which is essential in the production of amino acids which are important for the synthesis of DNA, RNA and proteins such as purine, thymidylate, glycine, methionine, serine and nucleotides. Inhibition in the enzymatic activity of DHFR, caused due to decreased level of folate in the cell, obstructs the mechanism involved in the synthesis of purine and thymidylate. Moreover, hindrance in DHFR functioning mechanism causes termination of DNA replication process and ultimately results into cell death. DHFR serves as a target in the developing inhibitors and this helps in curing many lethal diseases [11]. DHFR have also supported for treating numerous non-malignant diseases [12,13].

Zebrafish dihydrofolate reductase (zDHFR) serves as an exemplary model for research in the fields of developmental biology. Its similarity with human dihydrofolate reductase (DHFR) indicates its use in an *in vitro* study used for folate synthesis and drug discovery [13]. In the present investigation related to zDHFR in *E. coli* cells, gold nanoparticles (AuNPs) and osmolytes (chemical chaperones) were employed on the expression profile and cellular protein folding. Nanoparticles (NPs) represents chaperonin activity [8], hence, we have done a comparative study of AuNPs with numerous osmolytes like glycerol [14], glucose and betain on the zDHFR protein. An investigation with the influence of AuNPs and osmolytes on the production of folded and functional zDHFR protein during its over-expression in the *E. coli* host was also done.

EXPERIMENTAL

Over-expression and the purified zebrafish dihydrofolate reductase (zDHFR) protein were obtained using strains of *E. coli*. BL21 (DE3) possessing vector pET 43.1a, which contain zDHFR gene gifted by Dr. Tzu-Fun, Taiwan. Tetrachloroauric acid ($HAuCl_4$), sodium citrate trihydrate ($C_6H_{11}O_{10}Na_3$), Luria-Bertani broth, glucose, glycerol, betain, isopropyl-d-1-thio-galactopyranoside (IPTG), dihydrofolic acid (DHF), high purity grade imidazole, nicotinamide adenine di-nucleotide phosphate (NADPH), magnesium chloride, lysozyme, sodium phosphate buffer, pH 7.4 containing sodium dihydrogen phosphate (NaH_2PO_4) and disodium phosphate (Na_2HPO_4), Tris base, phenylmethylsulfonyl fluoride (PMSF), potassium chloride, sodium chloride, Tris hydrochloride (Tris HCl), coomassie blue R-250 and dialysis membrane were ordered from HiMedia, India. Reagents used were of analytical grade. Milli-Q (Merck Millipore) or double distilled water was used throughout.

Over-expression and purification of zebrafish DHFR: For over-expressing zDHFR protein, transformation process was done using BL21-DE3 cells with recombinant plasmid vector. Ampicillin (100 µg/mL) containing Luria-Bertani (LB) media were used for growth of cells at 37 ºC. After the absorbance reaches to ~0.6, IPTG (1 mM) was added to induce protein expression for 6 h at 25 ºC [15]. Cells after induction were centrifuged at 6000 rpm for time interval of 30 min. Lysis buffer containing 0.2M sodium phosphate buffer with pH 7.4, 500 mM NaCl, 0.2 M $MgCl_2$, 0.1 mg/mL lysozyme and 1 mM PMSF were supplemented into the pellet attained after centrifugation. Cells were lysed by sonication after the incubation of 30 min. Lysate was centrifuged for 40 min at 13000 rpm at 4 ºC. Supernatant was then filtered using 0.22 µm Millipore filter. Filtered supernatant were passed through a column of nickel-nitrilotriacetic acid (Ni-NTA) by the use of immobilized metal ion affinity chromatography (IMAC). Histidine-tagged zDHFR protein elutes out with using imidazole (0-500 mM). The protein elutes out at 150 mM Imidazole concentration. Collection of different protein fractions were monitored using 12% SDS-PAGE. Fractions with >99% purity were pooled and then dialyzed using Tris KCl buffer, pH 7.4 [16].

Enzyme activity: Enzyme activity of purified protein was monitored using U-5100 HITACHI spectrophotometer at 340 nm at 25 ºC, which was monitored by the reduction in absorbance of NADP. The content of assay buffer was 140 µM NADPH, 100 µM DHF, 0.2 µM zDHFR in Tris-KCl buffer (pH 7.4). Freshly prepared reagents were made to avoid any degradation and experiments were performed in triplicates.

Preparation of gold nanoparticles (AuNPs): AuNPs were amalgamated using a well known process called as Turkevich method. Addition of 0.1 mg/mL of $HAuCl_4$ was done into a round-bottom flask which was attached to a condenser. This solution was boiled while stirring simultaneously in a vigorous manner. Reduction of $HAuCl_4$ turns the solution to red wine in colour from dark purple by adding of 1% sodium citrate trihydrate. The solution was brought to room temperature by continuously stirring [16].

Optimization of AuNPs concentration and various osmolytes: An extensive range of AuNPs and osmolyte concentrations were applied to optimize the level of enhancement on zDHFR protein. The LB media contains varying concentration

of AuNPs and osmolytes (glucose, glycerol and betain) and further the expression was analyzed by 12% SDS-PAGE.

Growth profile of zDHFR with AuNPs and various osmolytes: The LB media was inoculated with 1% inoculum from the culture grown overnight along with optimized concentration of AuNPs and osmolytes (data not shown) (glucose, glycerol and betain) and further incubated at 37 ºC in an incubator shaker at 250 rpm. Collection of samples was done after specified interval of time and absorbance was monitored at 595 nm.

***in vivo* Production of zDHFR with AuNPs and various Osmolytes:** *E. coli* transformed cells containing zDHFR gene was inoculated in LB ampicillin medium with addition of known concentrations of gold and osmolytes (glucose, glycerol and betain). Absorbance was monitored for every culture at 595 nm. When the $O.D_{595nm}$ reaches to 0.8-1.0, every culture was induced using 0.1 mM IPTG and further the incubation proceeds for 6 h at 25 ºC. The level of zDHFR expression was confirmed by 12% SDS-PAGE for each sample.

zDHFR protein production with AuNPs and osmolytes under heat shock conditions: zDHFR protein were applied to heat shock conditions with AuNPs and osmolytes (glucose, glycerol and betain) to monitor their defensive role under heat stress conditions. Cells were incubated overnight in existence and non-existence of 0.1M NaCl at 37 ºC [1,4]. Dilution was proceeded using fresh LB medium (1:100) and further incubated at 37 ºC with AuNPs (using culture without NaCl) and osmolytes (using culture with NaCl) till $O.D_{595nm}$ reaches to 0.8-1.0. Incubation of these cultures was done using water bath at selected temperatures (25, 32, 37 and 45 ºC) to optimize heat shock temperature. The cultures were kept at specific temperature till it reaches to the temperature of water bath, then incubated with 100 µM IPTG and kept in an incubator shaker at 250 rpm for 30 min at selected temperatures (25, 32, 37 and 45 ºC). At 45 ºC, the cells acquired a heat shock condition. Further incubation was done at 25 ºC for 6 h. Similar growth conditions were retained for gold nanoparticles and all osmolytes.

***in vivo* Folding study of zDHFR with AuNPs and various osmolytes:** IPTG induced cells with AuNPs and osmolytes (glucose, glycerol and betain) were harvested by centrifugation at 13000 rpm for 40 min at 4 ºC. The supernatant (soluble part) obtained in each case was filtered using a 0.22 µm Millipore syringe filter. The soluble and insoluble (pellet) level of the entire cell extracts with AuNPs and osmolytes were analyzed by 12% SDS-PAGE. IMAC was used to analyze the level of purified protein and was quantified using Bradford's assay.

RESULTS AND DISCUSSION

Over-expression and purification of zDHFR: Over-expression of zDHFR was analyzed using 12% SDS PAGE (Fig. 1). Molecular mass of zDHFR was estimated at 21.6 kDa. Purified fraction of zDHFR accessed as described earlier [16].

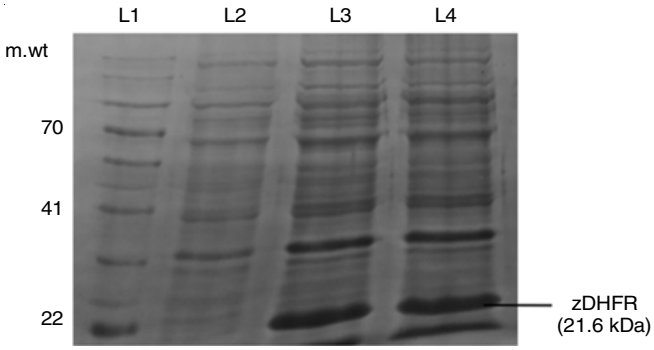


Fig. 1. Over-expression of zebrafish DHFR protein analyzed by 12% SDS-PAGE: L1: low molecular weight protein marker, L2-L3: Uninduced cells, L4-L6: Induced cells with IPTG

Gold nanoparticles synthesis: Gold nanoparticles were synthesized by a well-established Turkevich methodology [17]. The synthesized AuNPs were then characterized by transmission electron microscopy (TEM) and UV-visible absorbance spectroscopy and of ~20 nm in size (Fig. 2a-b). AuNPs shows absorbance maxima with a peak at 520 nm [16].

Optimization of AuNPs concentration and osmolytes: zDHFR over-expression was obtained after inducing in the mid-exponential phase with IPTG. Various concentrations of AuNPs and osmolytes (glycerol, glucose and betain) were used in order to optimize the enhanced level of zebrafish DHFR expression. The level of expression with different concentrations of AuNPs (0-0.8 nM) was monitored using 12% SDS-PAGE (Fig. 3).

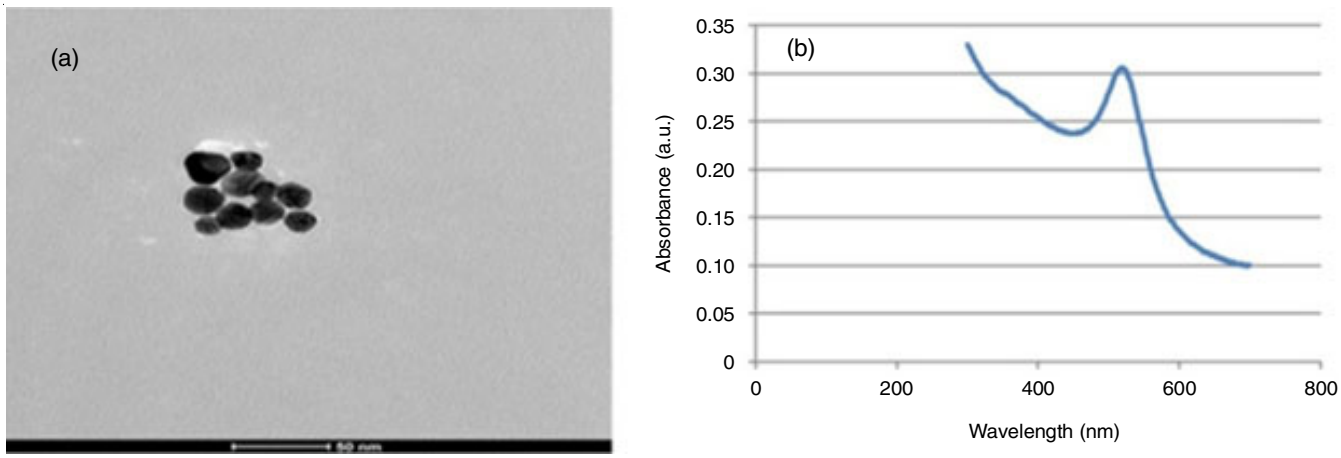


Fig. 2. (a) TEM picture depicting uniform size distribution of synthesized AuNPs, (b) UV-visible absorbance spectra of AuNPs

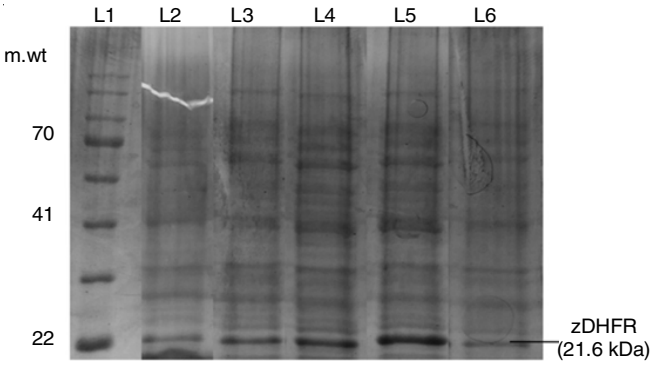


Fig. 3. Level of zDHFR over-expression using different concentration of AuNPs (0-0.8nM) confirmed by 12% SDS-PAGE. L1: molecular marker, L2: Control, L3: 0.2 nM, L4: 0.4 nM, L5: 0.6 nM, L6: 0.8 nM

The optimized concentration of AuNPs was observed around 0.6 nM. There was no further enhancement beyond this range. The optimized concentration of glucose, glycerol and betain was found to be 0.2%, 2 M and 1 mM, respectively (data not shown). It was investigated that AuNPs with concentration of 0.6 nM shows the highest level of expression as compared to those of osmolytes used.

Effect of AuNPs and osmolytes on the growth rate of zDHFR protein: The specific growth rate of zDHFR protein with optimized concentration of AuNPs and osmolytes (glucose, glycerol and Betain) was monitored and shown in Fig. 4. The growth rate of cells without any AuNPs and osmolyte was studied as a control experiment. The growth curve of zDHFR clearly depicted the lag, log, stationary and death phase under normal condition. Growth level of recombinant cells with AuNPs was monitored to be the maximum amongst all cell types. Growth of zDHFR with glycerol also raises the level of expression but comparatively less than AuNPs. Cells with glucose and betain shows reduced rate of growth level when compared to control. This bacterial growth curve represents the number of live cells in a bacterial population over a period of time. The slower growth rate may be because of lack of oxygen and crowding environment of the cells [18].

***in vivo* Expression of zDHFR with AuNPs and osmolytes:** The concept of folded or native protein in soluble fraction may be utilized to confirm the enhancement of expression of

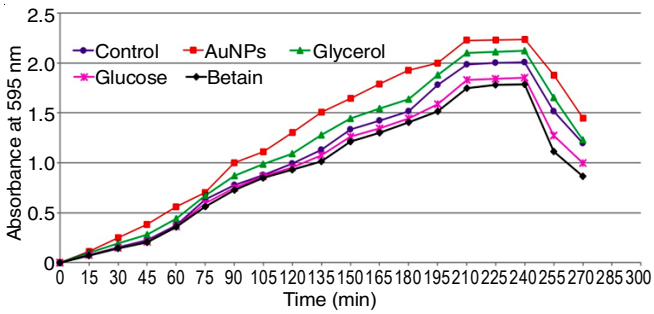
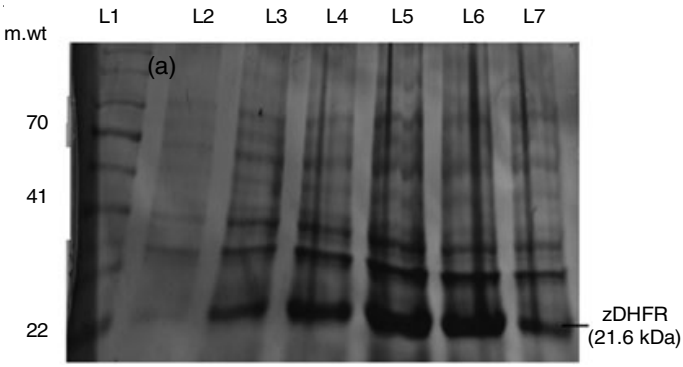


Fig. 4. Growth profile of zebrafish DHFR protein with AuNPs and osmolytes at 37 °C

zDHFR by AuNPs and various osmolytes. The comparative over-expression of zDHFR with AuNPs and osmolytes has been represented by Fig. 5a. The result shows that AuNPs and glycerol have enhanced the expression of zDHFR protein to a significant level whereas betain and glucose showed repressed level of expression. The highest level of expression was shown by AuNPs. The level of *in vivo* zDHFR expression with various osmolytes and AuNPs is shown in Fig. 5b.

Level of expression of zDHFR protein with AuNPs and osmolytes under heat shock conditions: In order to optimize heat shock conditions, the cells were incubated at varying temperatures of 25, 32, 37 and 45 ºC. It was observed that cells received a heat stress by impeding the expression level at 45 ºC (Fig. 6a). For monitoring the protective role of AuNPs and osmolytes (glucose, glycerol and betain) on improving the expression at 45 ºC, AuNPs and osmolytes were added to the LB media, which represents maximal level of protein in presence of 0.6 nM AuNPs followed by 2 M glycerol. But, betain and glucose were not helpful in providing stability against heat stress and shows inefficiency in increasing the expression level of zDHFR protein (Fig. 6b).

***in vivo* Folding assay of zDHFR protein with AuNPs and osmolytes:** Over-expression of recombinant proteins in *E. coli* often leads to generate misfolded and aggregated form known as inclusion bodies. Methods to improve the solubility and gain folded form of protein are the implementation of several additives such as osmolytes and nanoparticles. Osmosis can be influenced by the osmolytes and they conserve the organisms under stressful conditions. Correctly folded proteins are gene-



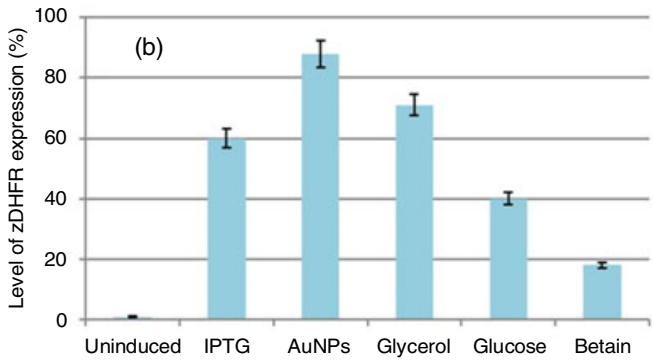


Fig. 5. (a) Over-expression of zebrafish DHFR protein with AuNPs and various osmolytes confirmed by 12% SDS-PAGE. L1: protein marker, L2: uninduced cells, L3: 0.2% Glucose, L4: induced cells, L5: 0.6 nM AuNPs, L6: 2 M Glycerol, L7: 1 mM Betain, (b) *in vivo* expression level of zebrafish DHFR protein using an optimized concentration of AuNPs and various osmolytes (bar-graph)

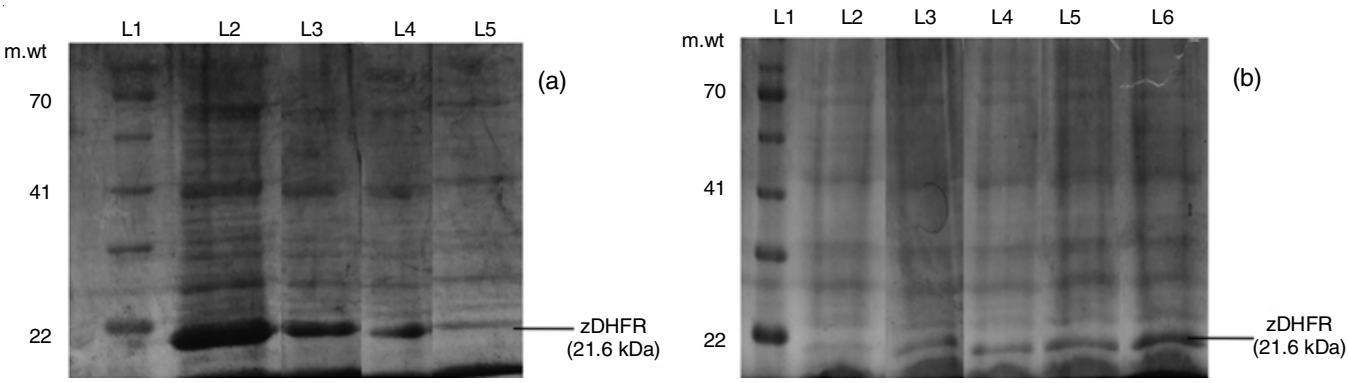


Fig. 6. (a) Level of zDHFR expression at selected temperatures for optimizing heat shock. L1: molecular marker, L2: at 25 °C, L3: at 32 °C, L4: at 37 °C, L5: at 45 °C, (b) Expression level of zDHFR protein underwent heat shock at 45 °C with AuNPs and various osmolytes. L1: protein marker, L2: IPTG induced zDHFR, L3: zDHFR with Betain, L4: zDHFR with glucose, L5: zDHFR with glycerol, L6: zDHFR with 0.6 nM AuNPs

rally soluble in cytoplasm and buffer in aqueous medium while the incorrect form of protein are insoluble and form aggregates. The enhancement in the solubility of zDHFR protein with AuNPs and osmolytes (glucose, glycerol and betain) were monitored. The soluble part and insoluble (pellet) part of zDHFR protein with AuNPs and osmolytes (glucose, glycerol and betain) was monitored by 12% SDS-PAGE, which represents that the cells growing in presence of AuNPs and glycerol produced greater quantity of the total protein in soluble form in comparison with the cells growing without any additives (control) whereas cells in presence of glucose and betain were not able to enhance the solubility of the zDHFR protein (Fig. 7a). The maximum portion of soluble protein was raised to 79% in presence of 0.6 nM AuNPs, 67% with 2M glycerol, 30% with 0.2% glucose, 22% in presence of 1mM betain and 45% in control. This represents that AuNPs shows an important role in preserving aggregation prone, zDHFR protein. From Fig. 7b, it is very clear that the *in vivo* role of glucose and betain on the folding of zDHFR is lower as compared to traditional IPTG induced expression resulting in lower activity of protein meanwhile glycerol shows highest level of expression as well as activity with zDHFR having three H-bond acceptor and donor count [2,18]. On the other hand, zDHFR form conjugation with AuNPs by forming covalent bonds with surface cysteine [20-23].

Purified protein was attained by IMAC and the solubility of zDHFR protein was quantified using Bradford's assay by UV-visible spectroscopy along with AuNPs and various osmolytes (Fig. 8).

From the above figure it was observed that the solubility of protein with AuNPs and glycerol were much more in comparison to control (only IPTG) but glucose and betain were showing lower amount of soluble protein.

Conclusion

The present study is a unique demonstration towards the *in vivo* folding of zDHFR protein using AuNPs and various osmolytes. This comparative study is relevant for the stabilization of protein. The observed results signify that the folding of zDHFR without any external supplement differs from the zDHFR which is conjugated with AuNPs and osmolytes. The most significant observations made on the stability of protein is that the AuNPs have the capability of rectifying aggregation and promoting proper folding of proteins, which can play an important role in reforming various diseases that are prevalent

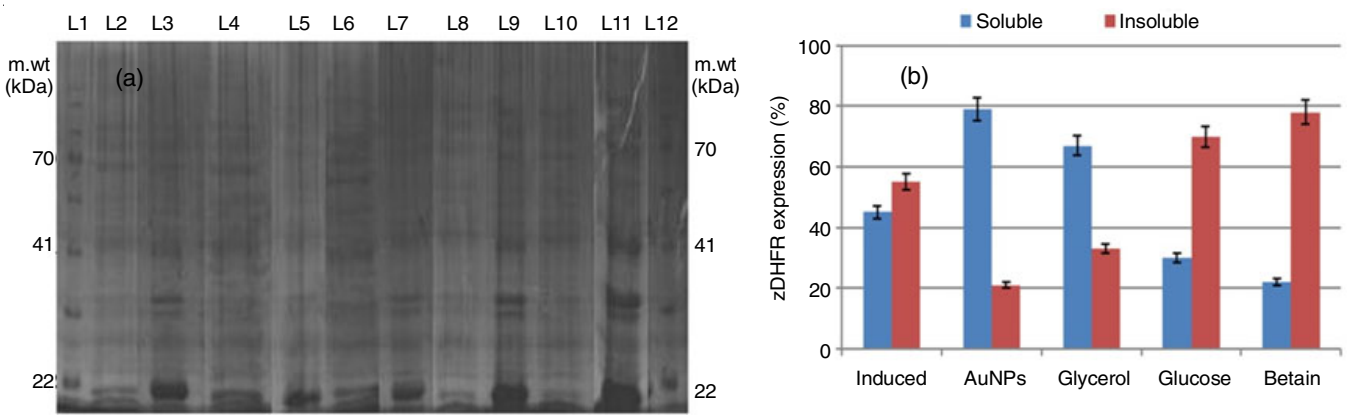


Fig. 7. (a) *in vivo* folding of zebrafish DHFR protein. L1: molecular marker, L2: supernatant of zDHFR (control), L3: pellet of zDHFR (control), L4: supernatant of zDHFR with AuNPs, L5: pellet of zDHFR with AuNPs, L6: supernatant of zDHFR with Glycerol, L7: pellet of zDHFR with Glycerol, L8: supernatant of zDHFR with Glucose, L9: pellet of zDHFR with Glucose, L10: supernatant of zDHFR with Betain, L11: pellet of zDHFR with Betain, L12: molecular marker, (b) Effect of AuNPs and various osmolytes on the folding of zDHFR protein (bar-graph)

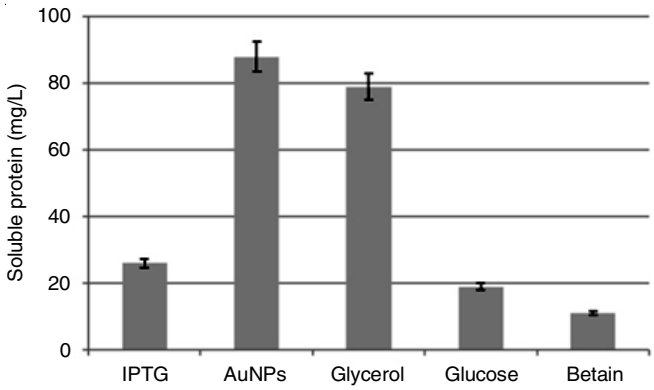


Fig 8. Quantification of soluble zDHFR protein with AuNPs and various osmolytes using Bradford's assay by UV-visible spectrophotometer at 595 nm

due to the misfolding of proteins. Other than AuNPs, glycerol, also improves the stability of protein due to its protecting nature which increases the free energy of the unfolded form by interacting with the peptide bond in an unfavourable manner and hence, favouring the folding of zDHFR [3,24].

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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