Asian Journal of Chemistry; Vol. 33, No. 8 (2021), 1929-1934

ASIAN JOURNAL OF CHEMISTRY

https://doi.org/10.14233/ajchem.2021.23372

Investigating the Chaperoning Effect of Nanoparticles in Chemically Denatured zDHFR: An *in vitro* Study

P. GUPTA[1], A.K. VERMA[2] and P. CHAUDHURI (CHATTOPADHYAY)[1,*]

[1]Molecular Biophysics Lab, Amity Institute of Biotechnology, Amity University, Sector 125, Noida-201313, India
[2]Department of Zoology, Kirori Mal College, University of Delhi, New Delhi-110007, India

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

Received: 5 June 2021; *Accepted*: 7 July 2021; *Published online*: 26 July 2021; AJC-20448

Maintenance of native structure and function of the protein is a major concern for industrial production of aggregation prone therapeutically important recombinant proteins. Aggregation may results due to change in the native conformation of proteins under different stress conditions. To overcome the problem of protein aggregation, role of silver and gold nanoparticles have been investigated. The nanoparticles owing to their affirmative interaction with the proteins possess chaperoning activities and protect the native state from denaturation. In the present study, through performing chemical denaturation of zebrafish dihydrofolate reductase using denaturants like guanidine hydrochloride and urea in the presence and absence of gold and silver nanoparticles and monitoring the process through enzyme activity assay and intrinsic tryptophan fluorescence, we have demonstrated the impact of nanoparticles in maintaining native conformation of proteins. Further, the outcome of refolding studies of DHFR protein with nanoparticles monitored by UV-visible spectroscopy was also reported.

Keywords: Aggregation, Zebrafish dihydrofolate reductase, Nanoparticles, Enzyme activity assay, Intrinsic tryptophan fluorescence.

INTRODUCTION

Commercial scale production of medicinally important recombinant protein is very much effected by the instability of protein. The recombinant protein production is caused by the homologous and heterologous overexpression of the protein in *Escherichia coli* which is a prevalent method [1]. The three-dimensional conformation of protein is known as stable conformation [2]. Changes in native conformation of protein can be caused by the effect of chemical denaturants such as chaotropic agents, alcohols, detergents, pH, heat shocks. *etc*. that causes unfolding of protein and because of this protein lost its stability. To attain increased level of functional protein for different research purposes, the recombinant protein expression in *E. coli* has become necessary procedure [3]. Although this method is simple but correctly folded, biologically active protein production is always restricted by misfolding and aggregation of recombinant proteins [4]. The inherent nature of recombinant protein, the rate of protein expression or the concentration of intermediates during protein folding are among the different factors which effects the process of folding and causes protein instability by forming aggregates, also known as inclusion body, which are non-native in nature and can create problems in structural and biochemical analysis [5].

To increase the production of essential recombinant proteins, it is mandatory to develop the methods that would help in inhibiting protein aggregation. For the production of proteins, *E. coli* is one of the best suited model as its genetics is intensively studied and shows a high rate of growth such that high cell density is achieved quickly. Protein refolding causes formation of correct 3D conformation which is a complex procedure and are not understood clearly. The process of refolding is regulated to inhibit aggregation which may result in correct refolding of protein, leading to increased production of the biologically active form of protein [6]. For the eradication of this problem, researchers have used different methods including osmolytes, nanoparticles, *etc*.

In present study, we have used dihydrofolate reductase (DHFR) (EC 1.5.1.3), which is a common enzyme in all the organisms [7,8]. It performs a significant function in thymidylate synthesis, purines and many other amino acids like glycine, methionine and serine. It catalyzes the reaction in which

dihydrofolate reduces to form tetrahydrofolate [9-11]. The activity of DHFR if inhibited, affect the processes which involves the purine and thymidylate synthesis [12]. In addition, dihydrofolate reductase's inhibition also disrupts DNA replication [13]. This type of interruption causes cell death. DHFR has been studied as an advantageous target for designing of inhibitors that could assist in treatment of various deadly diseases *e.g.* cancer [7].

Improper protein folding is caused by the protein's transition state that is very crucial. It can happen in both *in vivo* and *in vitro* conditions. Misfolding and aggregation of proteins are the main factors which provide protein instability that leads to a variety of protein folding diseases and many neurodegenerative diseases [14]. Nanoparticles are materials at sub-micrometer scales, usually 1-100 nm, so they possess a large surface to volume ratio. Due to this property, nanoparticles have significant adsorption capacities, therefore they are able to bind or carry other molecules, chemical compounds, drugs, probes and proteins. These molecules are attached to the nanoparticle's surface by covalent bonds or by adsorption. These nanoparticles have the ability to influence protein folding and aggregation [15]. Herein, we have used gold and silver nanoparticles, which acts like chaperones and helps in enhancing the stability of chemically denatured protein and monitored using UV-visible spectrophotometer and intrinsic fluorescence spectrometry.

EXPERIMENTAL

Zebrafish DHFR was over-expressed and purified from BL21 (DE3) Rosetta *Escherichia coli* strains. zDHFR gene was incorporated in the plasmid, pET 43.1a vector which was obtained from Dr. Tzu-Fun, Taiwan. Nicotinamide adenine dinucleotide phosphate (NADPH), dihydrofolate (DHF), isopropyl-*d*-1-thiogalactopyranoside (IPTG), urea, guanidine hydrochloride (GdnHCl), oxidized glutathione (GSSG) and reduced glutathione (GSH) were purchased from Sigma Chemical Company (USA). Imidazole with high purity grade, Tris HCl, potassium chloride and sodium chloride were purchased from HiMedia, India. Analytical grade reagents were used all the time. Experimentation was done with Milli-Q (Merck Millipore) or double distilled water.

Preparation of recombinant zDHFR protein: Expression and purification of recombinant zDHFR was performed with BL21 (DE3) Rosetta *E. coli* strain that consist of the plasmid, zDHFR-His/pET43.1a which encodes the DHFR gene. Immobilized metal ion affinity chromatography (IMAC) was done in single step purification with Ni^{2+} as chelating ion [16]. A linear gradient of 0-500 mM imidazole was used and the elution of His-tagged zDHFR protein was done around 150 mM imidazole. The aliquots which have purity higher than 95% were collected and dialysis was done and further the concentration of purified protein was checked using Bradford assay. The expression and purification of zDHFR was confirmed with 12% SDS-PAGE [17].

Enzyme assay of zDHFR: zDHFR catalyzes the reaction in which DHF converted to THF which was monitored on a UV-vis spectrophotometer by observing the decrease in absorbance of NADPH at 340 nm. Each assay mixture contains 140 μM NADPH, 100 μM DHF and 0.2 μM Zebrafish DHFR, pH 7.4 [18,19]. All the enzyme assays were executed in triplicate. Freshly prepared NADPH, DHF which were incubated in ice, used within 2 h of experimentation.

Synthesis of silver and gold nanoparticles: An aqueous solution was form by mixing sodium citrate with tannic acid then the solution was heated for 15 min under brisk stirring then added $AgNO_3$ solution into this solution after boiling. Bright yellow colour develops in the solution immediately. Now centrifugation (10000 g) was used to purify the AgNPs for the eradication of excess tannic acid which was followed by redispersal in milli-Q-water before characterization the sample [20].

Gold nanoparticles were amalgamated using the widely known Turkevich method [21]. In a round-bottom flask attached to a condenser, 0.1 mg/mL $HAuCl_4$ solution was boiled while stirring vigorously. Upon adding 1% sodium citrate trihydrate, the $HAuCl_4$ was then reduced, turning the solution from dark purple to red wine colour. It was then continuously stirred bringing it to room temperature.

Interaction of Ag and AuNPs with zDHFR: The zDHFR was mixed with silver nanoparticles to prepare the conjugates in Tris-KCl buffer, pH 7.4. Then the incubation was done for 1 h at 4 ºC in test tubes. Experiment was executed in different fractions in which concentration of AgNPs (5 nM) remains constant while the protein concentration was kept different (0-500 nM). The UV-visible spectra of different zDHFR-AgNPs conjugates were monitored at different wavelengths (300-1100 nm). The errors of the background were removed by Tris-KCl buffer, which was used as blank [22].

The gold nanoparticle and zDHFR conjugates were prepared by incorporating the solution of AuNPs and zDHFR in Tris-KCl buffer. Then the mixture was incubated at 4 ºC in test tubes for 1 h. A set of conjugate system was prepared with different amount-of-substance ratios of AuNPs/zDHFR. The concentration of AuNPs (5 nM) remains constant while different concentrations of protein (0-90 nM) was taken. The absorption spectra of various AuNPs-zDHFR conjugate solutions were recorded from 300-1100 nm. Tris-KCl buffer was used as a blank, which was subtracted from the absorption spectra of the specific samples [23].

Equilibrium unfolding of GdnHCl and urea induced zDHFR in presence and absence of Ag and AuNPs by intrinsic tryptophan fluorescence spectroscopy: Intrinsic fluorescence measurements were executed using Perkin-Elmer LS 55 spectrofluorimeter for unfolding studies using an optical quvette of 1 cm path-length. Protein samples were prepared by incubating with different concentration of Gdn HCl (0-5 M) and Urea (0-11 M) at 25 ºC for 30 min in Tris-KCl buffer, pH 7.4. Excitement of samples was done at 295 nm and spectra of emission were recorded between 310-400 nm, with excitation and emission slit width 5 and 7.5 nm, respectively with and without Ag and AuNPs.

Equilibrium unfolding of GdnHCl and urea induced zDHFR in presence and absence of Ag and AuNPs by enzymatic assay: Different concentrations of GdnHCl (0-5 M) and urea (0-11 M) in 20 mM Tris, 25 mM KCl buffer, pH 7.4 was taken for measuring enzyme activity of zDHFR samples in

presence and absence of Ag and AuNPs at 340 nm using UV-visible spectrophotometer.

Refolding studies by enzymatic assay with and without Ag and AuNPs: zDHFR protein was refolded using denatured protein, which was diluted 100-fold into the refolding buffer composed of 25 mM KCl, 20 mM Tris HCl, 10% glycerol, 1 mM GSSG, pH 7.4 and then incubate it for 2 h at 25 ºC. The regaining of activity was the indicator of refolding process.

RESULTS AND DISCUSSION

Protein expression and purification: The over-expression (Fig. 1a) and the purification profile (Fig. 1b) of zDHFR protein were validated at 21.6 kDa by 12% SDS-PAGE.

Interaction of Ag and AuNPs with zDHFR: The interaction of AgNPs with zDHFR protein has been investigated by UV-visible spectroscopy. The absorption spectrum of AgNPs (5 nM) with different concentrations of zDHFR (0-500 nM) revealed that the solutions with lower concentration of zDHFR protein (0-200 nM) shows an acute drop in the strength of the absorption band, which indicates the existence of aggregation while a narrow peak at 405nm was shown by 250 nM concentration of protein which shows maximum interaction of AgNPs with protein (picture not given, please refer ref. [22]).

The conjugation of AuNPs with zDHFR was also analyzed by using UV-visible spectroscopy. It was analyzed that with a fixed concentration of AuNPs (5 nM) with variable concentrations of zDHFR (0-90 nM), the absorption band gradually shifted and widened as the zDHFR content in mixture reduces which indicates that AuNPs have aggregated. It was also observed that the narrow peak of spectrum with concentration of zDHFR shows maximum conjugation of AuNPs (5 nM) with the protein (picture not given, please see ref. [23]).

Equilibrium unfolding of GdnHCl and urea induced zDHFR in presence and absence of Ag and AuNPs by intrinsic tryptophan fluorescence spectroscopy: Unfolding of zDHFR with and without Ag and AuNPs was studied by performing the intrinsic tryptophan fluorescence spectroscopy. The relative intrinsic fluorescence of denatured enzyme and the concentration of urea and GdnHCl were plotted against each other (Fig. 2a-b). It was analyzed that during the GdnHCl induced unfolding process, the emission intensity increased upto 0.5 M concentration of GdnHCl, this change may be because of the internal quenching of protein fluorescence and lower concentration of denaturant which is dequenching the fluorophores. Beyond 0.5 M concentration of GdnHCl, tryptophan emission intensity reduces gradually, becoming almost constant after 2 M concentration of GdnHCl and after that there is no change in fluorescence intensity. Hence, the protein got denatured at 2 M concentration of GdnHCl without nanoparticles while with Ag and AuNPs it denatures around 3.5 and 4 M, respectively. For urea induced unfolding, there is high reduction in fluorescence intensity upto 2 M but after this concentration, there is low reduction in fluorescence intensity upto 7 M and there is no change in fluorescence intensity after this concentration. Thus, the protein shows complete denaturation at 7 M in absence of nanoparticles while with Ag and AuNPs; the protein denatures around 8 and 10 M, respectively. It was also noticed that gold nanoparticles show significant reduction in the extent of aggregation of alcohol dehydrogenase (ADH) and insulin by increases their stability [24]. Thus, it may be concluded that nanoparticles assist in enhancing the zDHFR protein stability and in this way reduce its aggregation to some extent.

Equilibrium unfolding of GdnHCl and urea induced zDHFR in presence and absence of Ag and AuNPs by enzymatic assay: Unfolding of zDHFR was studied by performing the enzymatic assay for the denatured enzyme and the percentage (%) enzymatic activity of the denatured enzyme was plotted against the GdnHCl (Fig. 3a) and urea concentration (Fig. 3b). During the unfolding process, the enzyme denatures at 2 M concentration of GdnHCl completely without nanoparticles while with Ag and AuNPs, enzyme retains about 8% and 16% of activity at this concentration of GdnHCl and lost its complete activity at around 3.5 and 4 M, respectively. In urea induced unfolding, the protein lost its activity at around 7 M concentration of urea in absence of nanoparticles. While with Ag and AuNPs, the enzyme retains around 5% and 10% of activity at this urea concentration and completely denatures at 8 and 10 M, respectively. Thus, nanoparticle helps in incre-

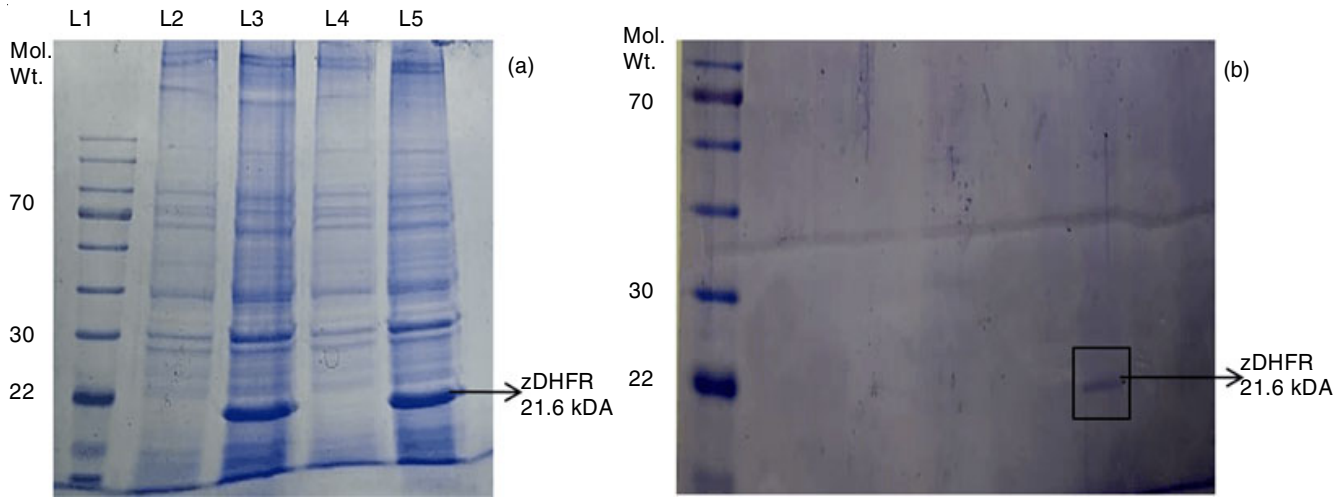

Fig. 1. (a) Expression profile of zebrafish DHFR protein analyzed by 12% SDS-PAGE: L1: low molecular weight protein marker; L2, L4: Uninduced cells; L3, L5: Induced cells with IPTG; (b) Purification profile of zDHFR

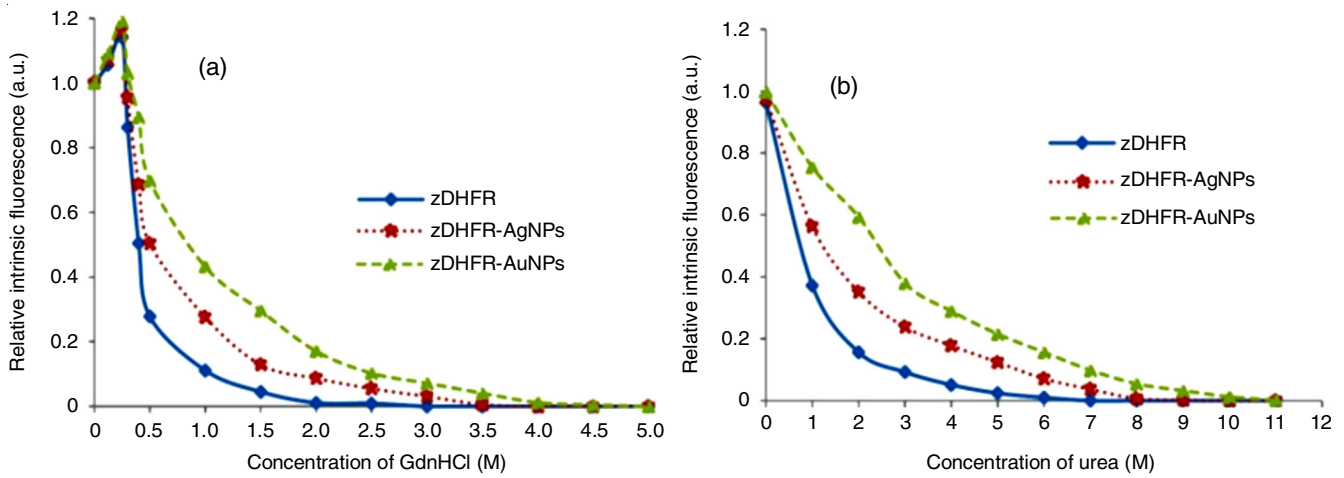

Fig. 2. (a) Equilibrium unfolding of GdnHCl induced zDHFR in presence and absence of Ag and AuNPs by Intrinsic tryptophan fluorescence spectroscopy (b) Equilibrium unfolding of urea induced zDHFR in presence and absence of Ag and AuNPs by Intrinsic tryptophan fluorescence spectroscopy

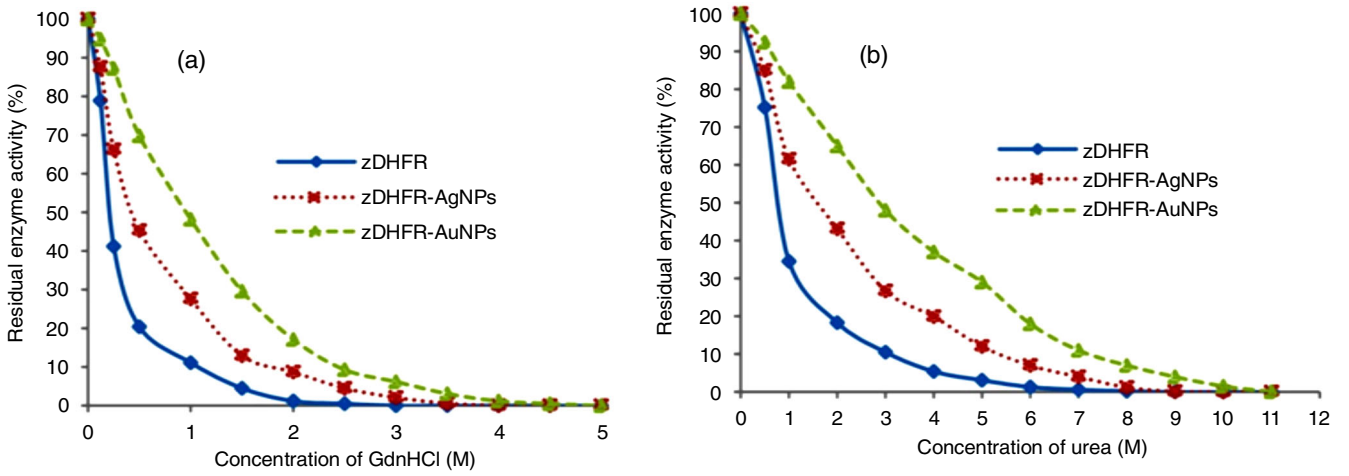

Fig. 3. (a) Equilibrium unfolding of GdnHCl induced zDHFR in presence and absence of Ag and AuNPs by enzyme activity assay (b) Equilibrium unfolding of urea induced zDHFR in presence and absence of Ag and AuNPs by enzyme activity assay

asing the zDHFR protein's stability. It was studied that the human placental cystatin (HPC) protein completely denatures in between the 4-6 M range of GdnHCl, whereas in case of urea complete inactivation was studied in between the 6-8 M concentration range [25]. The unfolding behaviour was also observed in case of chicken DHFR which completely inactivates at 2 M concentration of GdnHCl and with urea, it shows complete denaturation when the concentration is higher than 3 M [26]. The unfolding behaviour of dihydrofolate reductase from Chinese hamster in solutions of GdnHCl was also studied [27].

Refolding studies by enzymatic assay with and without Ag and AuNPs: Refolding of zDHFR was studied by performing enzyme activity assay with UV-vis spectrophotometer. For refolding, dilution of sample was done using refolding buffer to reduce the GdnHCl and urea concentration. The GdnHCl denatured protein regain its activity to 42.6% spontaneously while in presence of Ag and AuNPs, the rate of recovery was increased to 71.2% and 84.7%, respectively as compared to spontaneous refolding (Fig. 4a-b).

The urea denatured protein recovered spontaneously by 32.5% as compared to assisted refolding, which was 55.4% and 75.9% for Ag and Au, respectively (Fig. 4c-d). Hence, nanoparticles help in increasing the recovery rate of the native protein (stable) as compare to spontaneous refolding, thus enhancing the stability of protein at significant level. The protein is said to be functionally active, when it is folded into its native conformation. This observation also helps in knowing the relationship between structure and function of the protein. Thus, nanoparticles are capable to improve the stability of protein by preventing it from being aggregated.

Conclusion

There is a need of recombinant protein production commercially in functionally active form for different research purposes. Current research focuses on the chemically denaturation of zDHFR protein followed by refolding with Ag and AuNPs. Unfolding of protein was induced by GdnHCl and urea. Present data indicates that nanoparticles help in enhancing the stability of protein which is very important component in the production

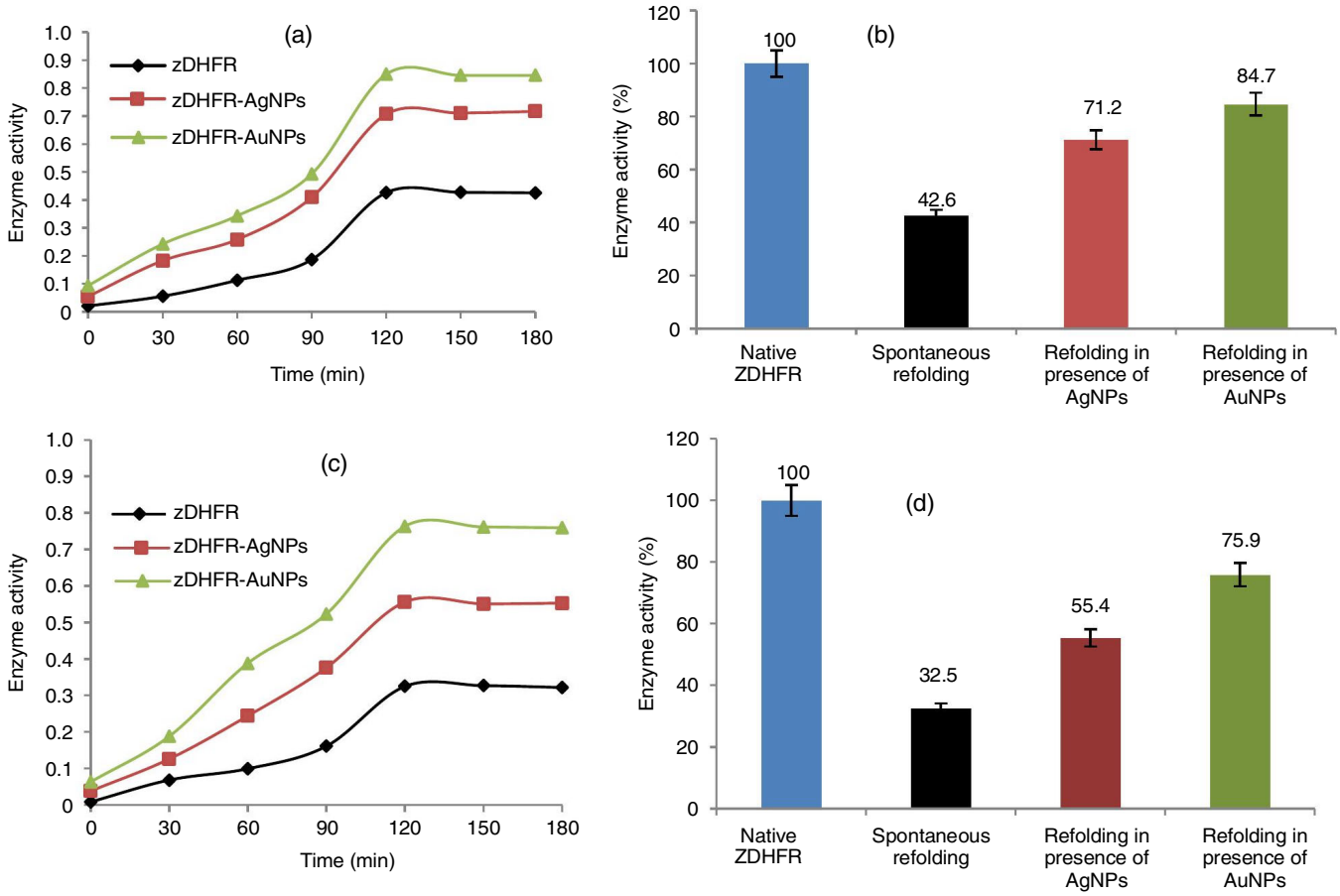

Fig. 4. (a) & (b) Refolding studies of GdnHCl denatured zDHFR protein in presence of Au and AgNPs (c) & (d) Refolding studies of urea denatured zDHFR protein in presence of Au and AgNPs

of biologically important recombinant proteins. Further, refolding was done under the influence of nanoparticles which also indicates that Ag and Au nanoparticles assist in increasing the refolding yield to stabilize the protein at significant level. Hence, nanoparticles may helps in attaining the functionally active protein (stable) in adequate amount commercially.

ACKNOWLEDGEMENTS

One of the authors, Prof. Pratima Chaudhuri acknowledges the funding from Department of Biotechnology, DBT, Govt. of India (Sanction No. BT/PR2193/NNT/28/1225/2017). The authors thanks Dr. Neetu Singh from Centre for Biomedical Engineering, Indian Institute of Technology, New Delhi, India for providing gold and silver nanoparticles. The authors also thankful to Amity Institute of Biotechnology, Amity University, Noida, India for providing the financial assistance.

CONFLICT OF INTEREST

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

REFERENCES

1. K. Terpe, *Appl. Microbiol. Biotechnol.*, **72**, 211 (2006); https://doi.org/10.1007/s00253-006-0465-8
2. N. Rashid, C. Thapliyal and P. Chaudhuri (Chattopadhyay), *Int. J. Biol. Macromol.*, **111**, 186 (2018); https://doi.org/10.1016/j.ijbiomac.2017.12.129
3. C. Thapliyal, N. Jain, N. Rashid and P. Chaudhuri (Chattopadhyay), *Arch. Biochem. Biophys.*, **637**, 21 (2018); https://doi.org/10.1016/j.abb.2017.11.006
4. T.K. Chaudhuri, G.W. Farr, W.A. Fenton, S. Rospert and A.L. Horwich, *Cell*, **107**, 235 (2001); https://doi.org/10.1016/S0092-8674(01)00523-2
5. A. De Marco, L. Vigh, S. Diamant and P. Goloubinoff, *Cell Stress Chaperones*, **10**, 329 (2005); https://doi.org/10.1379/CSC-139R.1
6. C. Thapliyal, N. Jain and P. Chaudhuri (Chattopadhyay), *Int. J. Biol. Macromol.*, **91**, 736 (2016); https://doi.org/10.1016/j.ijbiomac.2016.06.017
7. B.S. Askari and M. Krajinovic, *Curr. Genomics*, **11**, 578 (2010); https://doi.org/10.2174/138920210793360925
8. J.R. Schnell, H.J. Dyson and P.E. Wright, *Ann. Rev. Biophys. Biomol. Struct.*, **33**, 119 (2004); https://doi.org/10.1146/annurev.biophys.33.110502.133613
9. B.I. Schweitzer, A.P. Dicker and J.R. Bertino, *FASEB J.*, **4**, 2441 (1990); https://doi.org/10.1096/fasebj.4.8.2185970
10. K.M. Frey, K. Viswanathan, D.L. Wright and A.C. Anderson, *Antimicrob. Agents Chemother.*, **56**, 3556 (2012); https://doi.org/10.1128/AAC.06263-11
11. W. Hong, Y. Wang, Z. Chang, Y. Yang, J. Pu, T. Sun, J.C. Sacchettini, S. Kaur, H. Jung, W. Lin Wong, L. Fah Yap, Y.F. Ngeow, I.C. Paterson and H. Wang, *Sci. Rep.*, **5**, 15328 (2015); https://doi.org/10.1038/srep15328
12. N. Rashid and P. Chaudhuri (Chattopadhyay), *Protein J.*, **37**, 301 (2018); https://doi.org/10.1007/s10930-018-9784-8

13. J. Neradil, G. Pavlasova, M. Sramek, M. Kyr, R. Veselska and J. Sterba, *Oncol. Rep.*, **33**, 2169 (2015); https://doi.org/10.3892/or.2015.3819
14. N. Rashid, C. Thapliyal and P. Chaudhuri (Chattopadhyay), *Int. J. Biol. Macromol.*, **103**, 1044 (2017); https://doi.org/10.1016/j.ijbiomac.2017.05.143
15. L. Fei and S. Perrett, *Int. J. Mol. Sci.*, **10**, 646 (2009); https://doi.org/10.3390/ijms10020646
16. T.T. Kao, K.C. Wang, W.N. Chang, C.Y. Lin, B.H. Chen, H.L. Wu, G.Y. Shi, J.N. Tsai and T.F. Fu, *Drug Metab. Dispos.*, **36**, 508 (2008); https://doi.org/10.1124/dmd.107.019299
17. U.K. Laemmli, *Nature*, **227**, 680 (1970); https://doi.org/10.1038/227680a0
18. B. Hillcoat, P. Nixon and R. Blakley, *Anal. Biochem.*, **21**, 178 (1967); https://doi.org/10.1016/0003-2697(67)90179-0
19. C. Thapliyal and P. Chaudhuri (Chattopadhyay), *J. Protein Proteomics*, **6**, 211 (2015).
20. N.G. Bastús, F. Merkoçi, J. Piella and V. Puntes, *Chem. Mater.*, **26**, 2836 (2014); https://doi.org/10.1021/cm500316k
21. K. Wagers, T. Chui and S. Adem, *ISOR J. Appl. Chem.*, **7**, 15 (2014).
22. P. Gupta, R. Verma, A.K. Verma and P. Chaudhuri (Chattopadhyay), *Biochem. Biophys. Rep.*, **24**, 100856 (2020); https://doi.org/10.1016/j.bbrep.2020.100856
23. R. Verma, N. Singh and P. Chaudhuri (Chattopadhyay), *Int. J. Biol. Macromol.*, **167**, 987 (2021); https://doi.org/10.1016/j.ijbiomac.2020.11.053
24. A. Das, A. Chakrabarti and P.K. Das, *RSC Adv.*, **5**, 38558 (2015); https://doi.org/10.1039/C4RA17026A
25. F. Rashid, S. Sharma and B. Bano, *Protein J.*, **24**, 283 (2005); https://doi.org/10.1007/s10930-005-6749-5
26. Y. Fan, M. Ju, J. Zhou and C. Tsou, *Biochem. J.*, **315**, 97 (1996); https://doi.org/10.1042/bj3150097
27. J.W. Wu, Z.X. Wang and J.M. Zhou, *Biochim. Biophys. Acta*, **1343**, 107 (1997); https://doi.org/10.1016/S0167-4838(97)00089-7