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The ambivalent effect of Fe_3O_4 nanoparticles on the urea-induced unfolding and dilution-based refolding of lysozyme

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Abstract

Due to the numerous biological applications of magnetite (Fe_3O_4) nanoparticles (MNPs), it is essential to identify the influence of these nanoparticles on basic biological processes. Therefore, in this research, the effect of MNPs on the structure and activity of hen egg white lysozyme (HEWL) (EC 3.2.1.1) as a model protein was examined using tryptophan intrinsic fluorescence, UV/Vis, and circular dichroism spectroscopy. Moreover, enzyme activities were analyzed by a turbidometric approach in the presence of MNPs at concentrations providing MNPs/HEWL ratios in the range of 0.04–1.25. As-synthesized MNPS were characterized by Fourier transform infrared spectroscopy, x-ray diffraction, scanning electron microscopy, transmission electron microscopy, vibrating sample magnetometry and the zeta potential of MNPs was measured to be −29 mV. The goal of this work was investigating the ordering or disordering effect of MNPs on protein structure at ratios lower or higher than 0.918 as concentration ratio of threshold (CRT), respectively, in order to answer the question: 'How can the denaturation and refolding of a model protein (HEWL) be affected by MNPs?' As has been reported recently, the protein folding, helicity, and half-life were improved at <CRT to make the protein more ordered and conversely, HEWL was unfolded, and the helicity and half-life were decreased at >CRT to make the protein more disordered upon interaction with MNPs. The disordering effect of urea at >CRT and even at <CRT in the denaturation buffer (urea 6 M) increased and at <CRT the MNPs can provide a significant improvement in the refolding of the unfolded urea treated protein. These observations provide a new perspective on the growing applications of MNPs in biotechnology and biomedicine.

Abbreviations

MNPs	Magnetite (Fe_3O_4) nanoparticles	XRD	X-ray diffraction
HEWL	Hen egg white lysozyme (EC 3.2.1.1)	VSM	Vibrating sample magnetometry
ML	*Micrococcus lysodeikticus*	SEM	Scanning electron microscopy
CRT	Concentration ratio of threshold	TEM	Transmission electron microscopy
		FT-IR	Fourier transforms infrared spectroscopy

DLS	Dynamic light scattering
CD	Circular dichroism spectroscopy

1. Introduction

Nanotechnology has recently become one of the most exciting forefront fields in analytical chemistry [1]. Magnetite (Fe_3O_4) nanoparticles (MNPs) with good stability are one of the great points of interest in biotechnological and bio-medicinal applications due to their nontoxicity, biocompatibility, chemical stability, and substantial potential to reach the target tissue or organ [2]. MNPs have been used in many applications such as magnetic resonance imaging [3], hyperthermia for tumor treatment [4], cell labeling and sorting [5], separation and purification of nucleic acids [6] or proteins [7], and drug delivery [8]. Nowadays, approximately 50% of all new medications are biopharmaceuticals, and in comparison with chemical-based drugs, the demand for innovative protein based treatments continues to soar due to their targeted and specific action, in order to achieve better efficacy for fighting against diseases [9].

Determining the nature of the interactions between proteins and MNPs can open a new field of research related to the study of MNPs in proteomics [10]. The functional structure of proteins is formed due to the systematic and precise interactions of the protein surface with its aqueous environment. When the balance of interactions between protein molecules and their solvents are disrupted, proteins are unfolded. NPs could potentially impact protein–solvent interactions, helping to gain more insight into the function, stability, and dynamics of proteins in water environments [11]. MNPs act as a double-edged sword, influencing the structure and function of proteins in a concentration-dependent manner through electrostatic adsorption on NPs. Therefore, NPs can act on proteins either as a friend or foe depending on their concentration [12].

Therefore, an important safety concern for the application of MNPs to biological systems remains that the structure and activity of proteins can be changed upon interaction with MNPs [13, 14]. As the biological activities of proteins depend on their conformations, it is essential to study the structural effects of MNPs on protein conformation for avoiding any threats to their biological activities [15]. Hen egg white lysozyme (HEWL) (EC 3.2.1.1) is a monomeric globular model protein, comprised of 129 amino acid residues including six tryptophan, three tyrosine, and eight cysteines residues to form four disulfide bonds [16]. It acts to hydrolyze the glycosaminoglycan in bacterial cell walls. It is a strongly basic protein with an isoelectric point (pI) of 10.7, therefore effectively is absorbed on the negatively charged MNPs [17, 18]. Study of the interaction between MNPs and HEWL helps to elucidate the chemical essence of the interactions between bio-macromolecules and MNPs. In this study, HEWL was used as a model protein to investigate the effect of MNPs on protein stability, function and refolding (by dilution) after denaturation by urea. It was found that the effect of MNPs on ordering or disordering the protein structure is concentration-dependent.

2. Materials and methods

2.1. Materials

Lysozyme from hen egg white (HEWL) (EC 3.2.1.17, MW: 14.5 kDa) and 9,10-phenanthrenequinone were obtained from Sigma-Aldrich. Other chemicals were from Merck (Darmstadt, Germany) unless otherwise stated. As the substrate, *Micrococcus lysodeikticus* cell wall was prepared according to Surekha *et al* [19].

2.2. Magnetic nanoparticles (MNPs) synthesis and characterization

Fe_3O_4 nanoparticles as MNPs were synthesized according to the Massart method [20, 21]. In a typical synthesis procedure, 10 ml NH_3 (25%) was added dropwise to the 190 ml solution, containing 16 mmol of $FeCl_3.6H_2O$ and 8 mmol of $FeCl_2.4H_2O$ under an N_2 stream. The precipitates were washed with the mixture of ethanol and distilled water several times and dried at 40 °C in a vacuum oven for 1 h. Then the synthesized MNPs were characterized using various methods as follows. Fourier transform infrared (FT-IR) spectra of the MNPs were recorded by a Magna Nicolet 550 spectrophotometer in KBr pellets. Transmission electron microscopy (TEM) images were obtained using a Philips EM208S TEM with an accelerating voltage of 100 kV. Scanning electron microscopy (SEM) with a CamScan MV2300, was used for monitoring MNPs size with 20 000 accelerating voltage for the electrons and a magnification of 70 000 folds. The magnetic property of the sample was measured in a vibrating sample magnetometer (VSM) (Meghnatis Daghigh Kavir Co.; Kashan Kavir; Iran) at room temperature. The zeta potential of MNPs was estimated using dynamic light scattering (DLS) Malvern ZS-Nano series. X-ray diffraction (XRD) patterns were recorded by a Philips-X'PertPro, x-ray diffractometer using Ni-filtered Cu Kα radiation at scan range of $5 < 2\theta < 90$.

2.3. The enzyme assay

HEWL assay was performed using *Micrococcus lysodeikticus* (ML) cell wall as a substrate using a turbidometric approach [22, 23]. In brief, after adding 40 μl HEWL at 0.2 mg ml^{-1} on 1 ml of ML, the lysis of ML cell wall was monitored upon absorbance decrease at 450 nm. The activity of lysozyme in the absence of MNPs is taken 100, then all resulting values for lysozyme activity in the experiments were reported as the percent of the remaining activity.

2.4. The Enzyme treatment by MNPs

Stock solutions of the enzyme were prepared in sodium phosphate buffer (0.1 mM, pH 7.4) and concentrations were adjusted using absorbance at 280 nm [24]. To study the effect of MNPs on HEWL, the HEWL solutions at 0.2 mg ml^{-1} were treated in the presence of different concentrations of ultra-sonicated MNPs (0.008–0.24 mg ml^{-1}) to provide MNPs:HEWL ratios in the range of 0.04–1.25 in sodium phosphate buffer 0.1 mM, pH 7.4. A HEWL solution with the same concentration without any MNPs was used as a control. The controls and test samples then were incubated under magnetic stirring for an hour.

2.5. Urea-induced unfolding and dilution-based refolding

To investigate the effect of MNPs on the urea-induced unfolding of HEWL, 0.2 mg ml^{-1} HEWL solutions in sodium phosphate buffer 0.1 mM, pH 7.4 were treated with 6 M urea in the presence of two concentrations of ultra-sonicated MNPs yielding MNPs:HEWL ratios between 0.4 and 1.25. Untreated HEWL (0.2 mg ml^{-1}) in two different states, native protein (Control, −urea), unfolded protein (Control, +urea) and protein treated in the presence of 0.4 and 1.25 MNPs: HEWL were used as controls. The controls and test samples then were incubated in the shaker for an hour.

In order to explore the structure ordering effect of MNPs on dilution-based refolding of HEWL, the HEWL solutions (0.2 mg ml^{-1} in sodium phosphate buffer 0.1 mM, pH 7.4) were treated in the presence of MNPs with different ratios to the HEWL. Untreated HEWL (0.2 mg ml^{-1}) in three different states, native protein (Control, −urea), unfolded protein (Control, +urea) and protein treated with 30× diluted urea in the absence of MNPs (Control, diluted) and protein treated with 30× diluted urea and incubated for an hour in the presence of MNPs with a ratio of 0.4 to HEWL.

2.6. Spectroscopic analysis

Tryptophan dependent fluorescence intensities of the HEWL (0.2 mg ml^{-1}) in a sodium phosphate buffer (0.1 M and pH 7.4) were measured in the absence of MNPs as control, and in the presence of different concentrations of MNPs resulting in MNPs:HEWL ratios in the range of 0.4–1.25. The fluorescence spectra were recorded in the wavelength range of 300–400 nm after excitation at 290 nm using a spectrofluorimeter (BioTek, Synergy™ H4 Hybrid microplate reader) [25]. Both excitation and emission slits were set at 5 nm. Afterward, protein and MNPs were dispersed under stirring, for an hour, to establish adsorption–desorption equilibrium. Furthermore, the fluorescence emission of the MNPs in the same buffer at corresponding concentrations was recorded as the blank. Alteration in the helicity of HEWL (0.2 mg ml^{-1}) in the absence or presence of MNPs was studied using circular dichroism (CD) spectroscopy. The dichrographs were obtained using the AVIV Circular Dichroism Spectrometer Model 215. The far UV region was scanned between 190 and 250 nm and the test was repeated thrice. The final spectra were corrected using the buffer as the blank. The CD data were expressed in terms of mean residual ellipticity in deg cm^2 dmol^{-1} and the CDNN software was used for deconvolution of the far-CD spectra to measure secondary structure changes and percentage composition of the different secondary structural elements. Each reported data in this paper is the average of three experiments.

3. Results and discussion

The concentration-dependent ordering and disordering effects of MNPs on HEWL as a model protein were investigated through analyzing the protein structure and function in the absence and presence of urea as a denaturing agent. The XRD pattern of the as-synthesized MNPs is shown in figure 1(a). Bragg's reflections are observed for the MNPs in the XRD pattern at values of 30, 36, 43, 54, 58, 63, 71, and 74, representing (2 2 0), (3 1 1), (4 0 0), (4 2 2), (5 1 1), (4 4 0), (6 2 0), and (5 3 3) planes of cubic phase of Fe_3O_4, respectively, which is in agreement with the JCPDS card No. 19-629 [26]. The FT-IR spectra of MNPs in the range 400–4000 cm^{-1} are shown in figure 1(b). The absorption band at 3400 cm^{-1} is assigned to the ν(OH) stretching mode and the weak absorption band observed at 1633 cm^{-1} is due to the bending vibration of absorbed water [27] which indicates the presence of physisorbed water molecules linked to MNPs. The most profound impact of biological applications of MNPs with regard to protein adsorption is their surface chemistry and zeta potential (ζ). The zeta potential measurement is essential due to the fact that the changes in the surface of the MNPs are the origins of electrostatic interactions [28, 29]. According to figure 1(c), the zeta potential of MNPs measured −29 mV. Carrying a positive charge in a 100 mM PBS solution at pH = 7.4, HEWL can adequately be absorbed on the MNPs which carry a negative charge. This alters the pattern of protein–water interactions, in turn, leading to changes in the structure and activity of the protein, in the presence or absence of urea.

According to the SEM and TEM images, the MNPs mainly consisted of spherical particles with the average particle size of 30–40 nm (figures 1(d) and (e), respectively). The VSM of MNPs was studied to examine their magnetic properties (figure 1(f)). The magnetization at saturation (Ms) is estimated to be 74 emu g^{-1} at 300 K (the saturation magnetization Ms was determined through extrapolation of the curve of H/M versus H) [30]. Moreover, the size and morphology of MNPs were examined by SEM and TEM analysis.

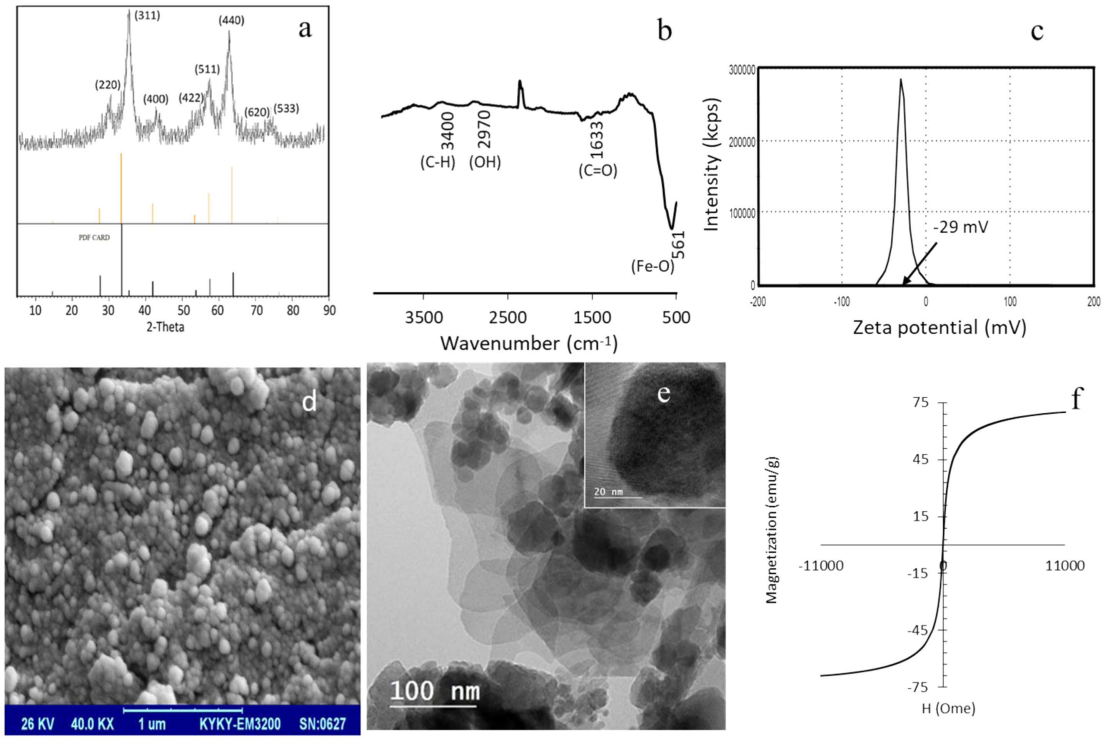

Figure 1. (a) X-ray diffraction (b) FT-IR spectra (c) zeta potential (DLS) (d) SEM image (e) TEM images and (f) VSM graph of MNPs.

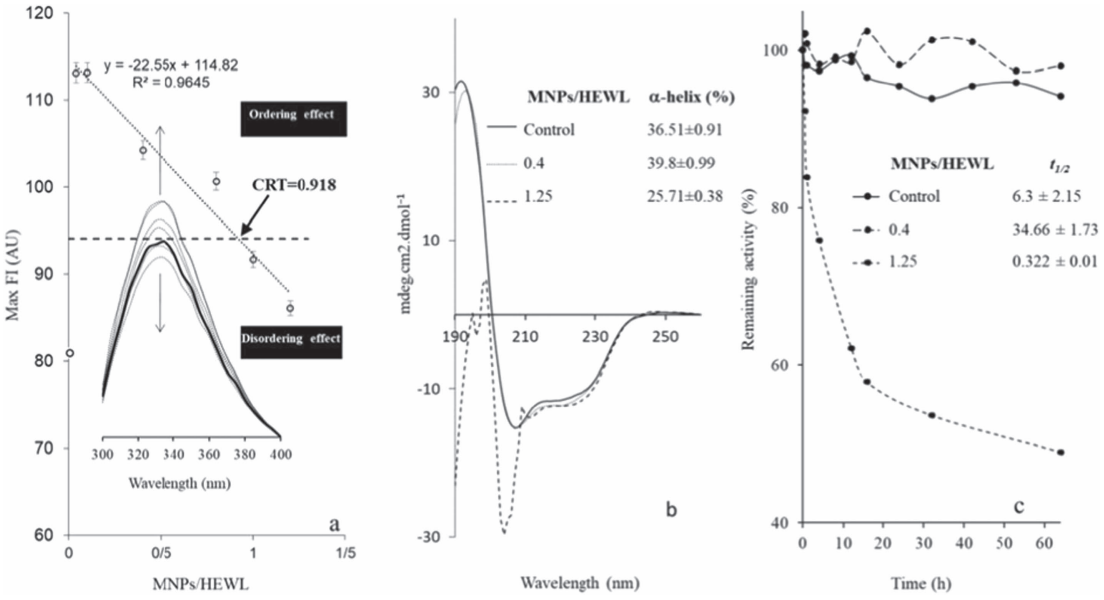

Figure 2. (a) Effects of MNPs:HEWL ratio on the intrinsic fluorescence of HEWL samples as revealed by fluorescence spectroscopy, (b) far CD spectra of HEWL after treatment with 0.4 and 1.25 MNPs:HEWL ratios. Inset to figure shows the presence of alpha helix structures after treatment with MNPs and (c) assessment of the HEWL remaining activity in the presence of MNPs at low (0.4) and high (1.25) MNPs:HEWL ratios until 64 h.

Intrinsic tryptophan (Trp) fluorescence is effectively influenced by the environs of the indole ring and has therefore been recognized as a useful tool to study protein conformational changes. Accordingly, the emission, as the maximum fluorescence intensity (FI), is increased or decreased when buried in a hydrophobic core or exposed to the polar surface environment, respectively. In the native HEWL, Trp 62 and Trp 108 are suggested to be dominant emitters and their fluorescence emission intensity is reduced upon HEWL denaturation [31]. Figure 2(a) shows the effect of MNPs on HEWL structure at different MNPs:HEWL ratios. Accordingly, MNPs have mutual contrast effects on the Trp fluorescence emission of HEWL to increase or decrease the

quantum yield of fluorescence emission HEWL due to ordering or disordering effects, respectively. As depicted in figure 2(a), MNPs exhibit structure ordering or disordering effects at ratios lower or higher than 0.918, respectively, which is reported as the ratio of threshold (CRT). The value CRT can be obtained according to the intersection of the linear function of Max FI (AU) versus MNPs/HEWL ratio at the corresponding Max FI (AU) value achieved in the absence of nanoparticles. Inspired by the concepts of action of kosmotropic and chaotropic agents, MNPs should most probably improve model protein hydration, and consequently increase protein stability at ratios lower than CRT. Conversely, MNPs diminish HEWL hydration and consequently decrease protein folding at ratios higher than CRT in which a vast number of MNPs are not covered by protein corona that has a negative effect on the protein stability and structure, similar to chaotropic agents. More recently, the stabilizing effect of Fe_3O_4 nanoparticles on HEWL at the ratio range of 0.025–0.75 (<CRT) has been reported [10].

CD spectroscopy was also applied to study the secondary structure of HEWL in the absence of MNPs (as the control), a representative MNPs:HEWL ratio lower than CRT (0.4), and a representative MNPs:HEWL ratio higher than CRT (1.25) (figure 2(b)). Change in the CD spectra of HEWL (0.2 mg ml^{-1}) after treatment with MNPs at 0.40 and 1.25 MNPs:HEWL ratios are related to change in the secondary structure of the HEWL due to interaction with MNPs in sodium phosphate buffer (0.1 M and pH 7.4). The inset to figure 2(b) shows the α-helix contents before and after treatment of HEWL with corresponding ratios. Accordingly, the helicity is significantly increased at the ordering region of the MNPs:HEWL ratio (<CRT), but in the disordering region of the MNPs:HEWL ratio (>CRT), the helicity is significantly decreased.

Functional stability of the HEWL as a model protein was investigated to study the mutual effects on HEWL activity under treatment by two representative ordering or disordering MNPs:HEWL ratios for 64 h at 300 K (figure 2(c)). As a result, at regions of MNPs:HEWL ratios lower than CRT or higher than CRT, the functional stability and the half-life of the enzyme were improved or aggravated, respectively. The half-life is defined as the time required for 50% activity loss. The folds of $t_{1/2}$ improvements in the presence of the ordering MNPs:HEWL ratio was found to be 5.5 times. In this regard, a mechanism was proposed about the concentration-dependent effects of MNPs on a model protein; MNPs indirectly influence the structure and dynamics of the water surrounding proteins, which in turn affects the structure and function of proteins in a concentration-dependent manner. Therefore, MNPs enhance protein hydration at low concentrations, resulting in an ordering effect. Moreover, at higher concentrations, MNPs interfere adversely with protein hydration and present a disordering effect. Nevertheless, the mechanism has not yet been fully understood and remains to be further explored.

There is a strong demand for the large-scale production of proteins through heterologous expression of desired genes in bacteria using recombinant technology which often leads to the formation of inclusion bodies through aggregation of unfolded conformations. These inactive aggregates are comprised of almost pure protein, so their recovery is essential in the process of obtaining active proteins from them. The recovery process encompasses solubilization using denaturants, mainly urea, and refolding into an active conformation by reducing the concentration of the denaturant through strategies such as dilution which competes with protein aggregation to form inactive protein species. Hence, we investigated the interference of MNPs in both the disordering effect of urea on folded proteins, and protein refolding by dilution. Urea is a known structure disordering agent which lowers protein hydration upon addition to the protein–solvent interface [32]. Figure 3 reveals the exacerbating effect of MNPs on the disordering effect of urea in a model protein, lysozyme, using intrinsic fluorescence (figure 3(a)), CD (figure 3(b)), and functional stability (figure 3(c)) analyses. The intrinsic fluorescence emission spectra of HEWL was decreased in the presence Urea (6 M in sodium phosphate buffer 0.1 mM, pH 7.4) due to the structure disordering effect of urea. Interestingly, the MNPs dramatically exacerbated the disordering effect of urea even at the ordering region of the MNPs:HEWL ratio (ratios lower than CRT) (figure 3(a)). Moreover, the observed exacerbation effect of MNPs was also confirmed at the secondary structure level (figure 3(b)). Accordingly, the MNPs also dramatically exacerbated the helicity decreasing effect of urea even at 0.4, as a representative for the ordering region of the MNPs:HEWL ratio. Figure 3(c) shows the percentages of the remaining activities of HEWL after 1 h incubation in the absence or presence of urea and MNPs at a ratio that provides ordering or disordering effects on the structure of HEWL in the absence and presence of urea (MNPs:HEWL = 0.4), respectively. As expected, the remaining activity of the model enzyme was increased 43.11% and decreased 84.7% in the absence and presence of urea (6 M in sodium phosphate buffer 0.1 mM, pH 7.4), respectively. This observation is in good agreement with their observed structural effects using intrinsic fluorescence and CD. Although, the ordering effect of MNPs at ratios lower than CRT is explained by probable increasing in the protein solvation effect, urea disorders both the native and aggregated states of protein which are considered as ordered structures [33, 34]. The concentration-dependent ambivalent effect of MNPs on the structure and activity of HEWL with a CRT at 0.918 was shown in figure 2. In the absence of urea, the MNPs exhibited ordering and disordering effects on folded native protein species at

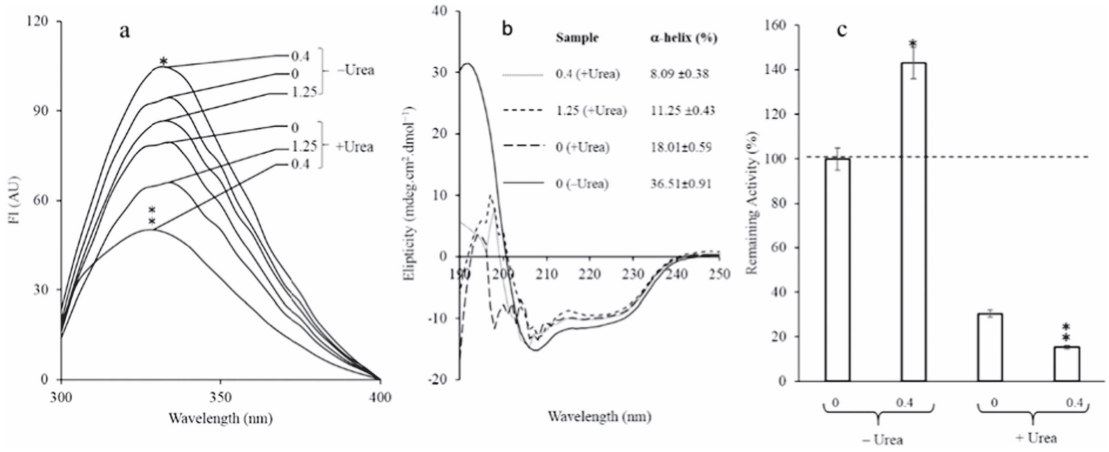

Figure 3. (a) Intrinsic fluorescence emission spectra, (b) far CD spectra of HEWL after treating with 0.4 and 1.25 MNPs:HEWL ratios in the denaturation buffer including 6 M urea. Inset to (b) shows the presence of Alpha helix structure and (c) evaluation of the HEWL remaining activity (%) in the presence of MNPs:HEWL ratio of 0.4 in sodium phosphate buffer 0.1 mM, pH 7.4 in the presence or absence of Urea (6 M) after 1 h ambient incubation and 200 rpm shaking.

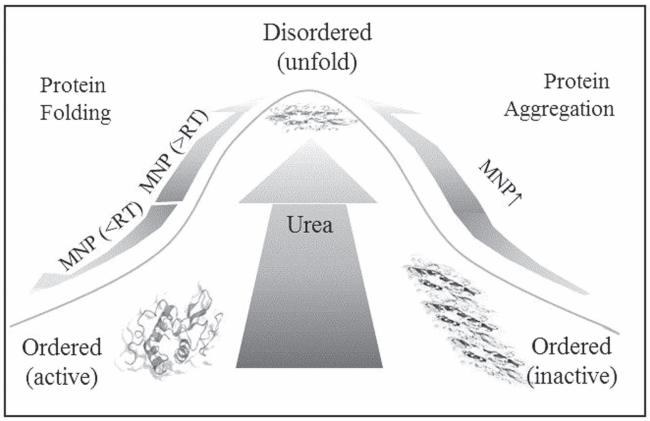

Figure 4. A hypothetical mechanism of the disordered/unfolded proteins through two competing routes to generate active folded or inactive aggregated protein.

<CRT and >CRT, respectively. While, in the presence of urea or starting from unfolded denatured protein species, they intensified the disordering effect of urea at >CRT and even at <CRT (0.4, as a representative MNPs:HEWL ratio). The reason for such an interesting observation remains to be elucidated. The schematic presentation of the above mentioned observation has been provided in figure 4. By decreasing the urea concentration using dilution approach, disordered/unfolded proteins can be ordered through two competing routes to generate active folded or inactive aggregated protein species (figure 4). Routinely, the addition of co-solutes is often essential to enhance protein folding and/or suppress protein aggregation [35].

To continue our investigations on the interference of MNPs on the disordering effect of urea on native HEWL (figure 3) and protein refolding by dilution, the interference of MNPs in the dilution process was studied at an MNPs:HEWL ratio with proven ordering effects at <CRT (0.4), by following the structural and functional features of HEWL. The secondary and tertiary structure of HEWL was studied by CD spectropolarimetry and fluorescence spectroscopy, respectively. Moreover, the interfering effect of MNPs in the diluted urea treated HEWL was examined by determining the remaining activities. Unique and definitive interactions occur during aggregation between molecules [11, 36]. During the refolding process, generation of intermediate structures with partial folding plays a key role in aggregate formation [37]. The ability of the MNPs to reduce aggregate formation is due to interactions with the denatured lysozyme, and stabilization of the folded conformation of the protein. Accordingly, figure 5 shows the samples of the native protein (Control, −urea), unfolded protein (Control, +urea), and 30× diluted urea treated protein in the absence of MNPs (Control, diluted) and in the presence of MNPs at the final MNPs:HEWL ratio of 0.4, respectively. According to intrinsic fluorescence

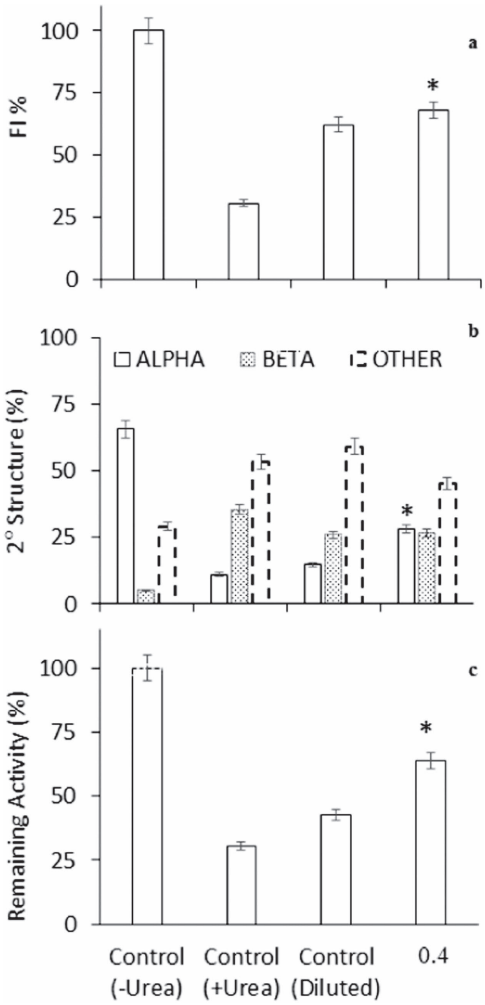

Figure 5. Evaluation of the secondary and tertiary structure of HEWL in the absence and presence of MNPs. Untreated HEWL (0.2 mg ml^{-1}) in three different states, native protein (Control, −urea), unfolded protein (Control, +urea) and protein treated with 30× diluted urea in the absence of MNPs (Control, diluted) and protein treated with 30× diluted urea and incubated in the presence of MNPs with a ratio of 0.4 to HEWL were evaluated by (a) intrinsic fluorescence emission spectra, (b) far CD spectra and (c) the percent of remaining activity.

results, the achieved refolding efficiency was 31.59% by dilution in the absence of MNPs that was increased to 37.41 by dilution at an MNPs:HEWL ratio of 0.4 (<CRT) (figure 5(a)). As depicted in the figure 5(b), the helicity is improved in the presence of a 0.4 MNPs:HEWL ratio as established to be the ordering ratio of MNPs:HEWL using CD analysis. As can be seen in the figure 5(c), measurement of the remaining activity of the refolded HEWL shows an increase in activity that causes further destruction of the cell membrane of ML. Therefore, the MNPs at <CRT can provide a significant improvement in the refolding of the unfolded urea treated protein species which is considered to be helpful from a biotechnological point of view.

For increasing efficiency, using small amounts of MNPs is recommended in the dilution process. Evaluation of secondary structures shows a significant improvement in the refolding of urea-induced unfolded protein species. According to our computations, the proportions of the helical content of HEWL display an increase in the presence of MNPs at a MNPs:HEWL ratio of 0.4 (<CRT) (figure 5(b)) which is in accordance with the results provided by fluorescence spectroscopy and the percentage of remaining activity (figures 5(a), (c)). Thus, the ordering concentration of MNPs helps the protein to have a more regular, stable conformation and to display improved activity.

In this work, the dual concentration-dependent effect of MNPs on HEWL was observed in which the concentration threshold shifts between two opposing effects, namely, structure-making and structure-breaking. Ordering and inversely, disordering effects of MNPs on HEWL are reported at lower and higher MNPs/HEWL ratios, respectively. This perspective on the concentration-dependent effect of magnetite nanoparticles on the structure and function of lysozyme in solution has not been evaluated with regards to storage conditions and the denaturation and refolding of the protein.

4. Conclusion

In conclusion, MNPs impact lysozyme structure and function in a concentration-dependent manner in the absence and presence of urea as a denaturing agent. However, we showed the possibility and necessity of determining a CRT of MNPs for a desired specific protein for biotechnological or biomedical applications with an especial emphasis on biopharmaceuticals. Regarding HEWL as a model protein, we determined an MNPs:HEWL ratio threshold at 0.918 which is supported by spectroscopic analysis of the enzyme structure and furthermore by the enzyme activity. Lysozyme shows an increase and decrease in the structure and function at the ordering (<CRT), and disordering (>CRT), a region of the MNPs:HEWL ratio, respectively. The disordering effects of urea were significantly intensified in the presence of MNPs even at ratios lower than the CRT in which ordered structures, both the native and aggregated forms of the protein, exist. MNPs:HEWL ratios lower than the CRT were proven to drive the protein conformation towards more ordered structures, and the MNPs interfered with the dilution process, even at these ratios. It was thus concluded that at these 'ordering' concentrations, MNPs promote protein activity, as well as stability and regular structure.

Although the mechanism of intercommunications between MNPs and proteins has not been well understood and continues to be investigated, we discussed a protein hydration mechanism in the protein corona to support the structure ordering and disordering effects of MNPs at concentrations lower or higher than the resulting CRT, respectively.

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