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Effect of L-Arginine Modified Magnetic Nanoparticles (RMNPs) on Lysozyme in the Presence or Absence of Urea

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ABSTRACT

L-Arginine (Arg or R) is a non-toxigenic, metabolically versatile and conditionally essential amino acid. Single-pot synthesis of Arg modified magnetic nanoparticles (RMNPs) was achieved using magnetite [Fe_3O_4] nanoparticles (MNPs) and binary function of Arg as a functional group and an alkali precipitator. This modification is supposed to offer several advantages to the MNPs from a bio-application viewpoint, including improved stabilization effect, bio-safety, biocompatibility and surface functionalization. Here, the influence of RMNPs in the structure and function of the model protein hen egg white lysozyme (HEWL) (EC 3.2.1.1) is reported. After synthesis of RMNPs, vibrating sample magnetometry (VSM), X-Ray Diffraction (XRD), Fourier Transform Infrared (FT-IR), Zetasizer, Transmission Electron Microscopy (TEM), and Scanning Electron Microscopy (SEM) were used to characterize the nanoparticles. The impact of RMNPs was investigated on lysozyme structure and activity during storage and in the processes of denaturation and refolding by dilution or dialysis using fluorescence, circular dichroism (CD) and UV-Vis spectroscopies. RMNPs revealed structural ordering or disordering effects on lysozyme in a RMNP:HEWL ratio dependent manner. Accordingly, a concentration ratio of threshold (CRT) was determined at 0.296. At ratios lower than the CRT the protein gained more ordered structure with increased helicity. Inversely, HEWL was increasingly unfolded and helicity was decreased at ratios higher than the CRT, rendering the protein more disordered after interaction with RMNPs. At RMNPs:HEWL concentration ratios above and even below the CRT, 6 M urea had a further disordering effect. Nevertheless, significant improvements were observed in the refolding of the protein due to dilution or dialysis, by courtesy of the RMNPs. The presented data helps to expand the thriving applications of RMNPs in biotechnology and biomedicine.

Abbreviation: Nanoparticles: NPs; L-Arginine: Arg or R; Magnetic [Fe_3O_4] nanoparticles: MNPs; L-Arginine modified magnetic [Fe_3O_4@Arg] nanoparticles: RMNPs; Hen egg white lysozyme: HEWL; Concentration Ratio of Threshold: CRT; *Micrococcus lysodeikticus*: *M. luteus*

Keywords: L-Arginine modified magnetic nanoparticle (RMNP), Hen egg white lysozyme (HEWL), Stability, Denaturation, Refolding, Concentration ratio of threshold (CRT)

INTRODUCTION

Magnetic nanoparticles (MNPs) and Arg modified magnetic nanoparticles (RMNPs) as aminated MNPs have widespread applications in areas such as laboratory diagnostics [1], cell sorting [2], DNA sequencing [3], Detecting minimal traces of DNA [4], medical drug targeting [5] and cardiovascular disease [6] therapies.

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Biological entities of a commensurate size ranging from cells to macromolecules can be targeted by MNPs [7]. They can be used to label said entities following surface functionalization using the appropriate bioligands [8]. Various modification methods have been developed to produce biocompatible MNPs for magnetic resonance imaging [9], immunoassays [10], immobilization of proteins, peptides or enzymes [11], and drug or gene delivery [12]. The superparamagnetic behavior of MNPs as well as being non-toxic, non-cytotoxic and non-immunogenic, are some of the many attractive features of MNPs, making them an ideal candidate for many applications since they can be controlled and manipulated by an external magnetic field gradient. The impressive developments in nano-biotechnology have facilitated the adjustment and customization of their composition, size, surface functionalization and magnetic properties [7,13,14].

MNPs can be functionalized with different organic or inorganic coating agents, including surfactants, polymers, silica, or carbon. Addition of these functional groups to MNPs provides a chance for engineering the surface properties regarding several targeted applications [13,14]. Coating MNPs with amino acids, for example, L-Arginine (Arg or R), is a resourceful strategy for developing a new generation of MNPs [15] which are more biocompatible and eco-friendly. This also helps to protect MNPs against agglomeration and allows them to play a role as antibody connectors [16], catalysts [17], drug loaders, stability modifiers [15] and to directly interact with proteins/peptides through the side chains of amino acid residues and peptide bonds.

Positively charged guanidinium groups (*pKa*, 12.48) along with three aliphatic methylene groups in Arg make it the most basic amino acid [18] and an alkali precipitator with the highest isoelectric point (pI, 10.76) [19]. In general, Arg modified magnetic nanoparticles [Fe_3O_4@Arg] or RMNPs are produced by direct and simple bonding upon Arg attachment to the surface of the MNPs through α-COOH groups [14,17]. In this way, guanidinium groups are added as a functional group to the surface of RMNPs. A common strategy for driving protein refolding or suppression of aggregation is the addition of small molecules known as kosmotropic co-solvents [20]. Both kosmotropic [21] and chaotropic effects [22] have been reported for free Arg. Moreover, we have recently reported the dual concentration-dependent effect of MNPs on a well-characterized glycoprotein, hen egg white lysozyme (HEWL) (EC 3.2.1.1) [23,24], a single subunit model protein consisting of 129 amino acid residues (with a molecular mass of 14.3 kDa) [25]. Nevertheless, the possible ambivalent effect of RMNPs on the urea-induced unfolding and refolding of HEWL structure and function which is an important issue has not been reported and the study of the interaction between RMNPs and HEWL in various fields helps to describe the interactions between bio-macromolecules and RMNPs.

Therefore, in this study, we report the concentration-dependent effects of RMNPs on ordering (structure making effect) or disordering (structure breaking effect) HEWL structure as a model protein. It was found that higher efficiency in biological processes can be achieved by a low density of RMNPs.

MATERIALS AND METHODS

Materials

HEWL from hen egg white (HEWL) (EC 3.2.1.17, MW: 14.5kDa), $FeCl_2.4H_2O$ (99%), $FeCl_3.6H_2O$ (99%), 9,10-phenanthrenequinone and ammonium hydroxide (25 wt% NH_3 in water) were obtained from Sigma-Aldrich. Arginine hydrochloride (Arg) and all organic solvents were from Merck (Darmstadt, Germany). Dried bacterial cell wall as the substrate from Gram-positive *Micrococcus lysodeikticus* (*M. luteus*) was prepared according to Surekha *et al*. [26].

Synthesis and Characterization RMNPs

RMNPs were synthesized by dissolving Iron(III) chloride hexahydrate ($FeCl_3.6H_2O$) and Iron(II) chloride tetrahydrate ($FeCl_2.4H_2O$) at 2:1 molar ratio. Then, 1 mM Arg, as a precipitator, was added, under a nitrogen atmosphere while being vigorously stirred. After 1 h, synthesis was completed by adding 7 ml ammonium hydroxide (25 wt% NH_3 in water) according to the coprecipitation method in the presence of Arg [14]. The product was washed with deionized water and ethanol six times to remove residual Arg and solvent. Finally, a magnet was used to separate RMNPs from the suspension, then, the

black sediments of the RMNPs were dried at 40 °C. After completion of the synthesis, the RMNPs were characterized using various analytical techniques. Confirmation of the attachment of Arg on MNPs was achieved by a quick and simple method using 9-10-phenanthrenequinone [27]. The amount of Arg capped on RMNPs synthesis products was determined to be 1.62 µg (0.0093 µmol) per mg of RMNPs using a colorimetric method [28].

After synthesis of RMNPs, they were characterized using various analytical methods as follows. X-ray diffraction (XRD) was performed using a Rigaku-Dmax 2500 diffractometer, with Cu Kα radiation (λ = 1.5406 nm) and 2θº from 5 to 90° in which the rate of warming-up was adjusted at 0.02° min^{-1}. The Fourier-transform infrared (FT-IR) spectra were obtained using a Bruker VERTEX 70 model. The RMNPs were directly monitored by scanning electron microscopy (SEM) with a CamScan MV2300, with 20000 accelerating voltage for the electrons and magnification of 40000. The saturation magnetization (M_s) of RMNPs was measured by applying vibrating sample magnetometry (VSM) in a vibrating sample magnetometer (VSM) (Meghnatis Daghigh Kavir Co.; Kashan Kavir; Iran) at room temperature. The zeta potential (ζ mV) of RMNPs was determined in phosphate-buffered saline (PBS, 100 mM, pH = 7.4) using the dynamic light scattering (DLS) instrument from Malvern ZS-Nano series.

Enzyme Treatment by RMNPs

Phosphate-buffered saline (PBS, 100 mM, pH = 7.4) was used to prepare the HEWL (lyophilized powder, EC 3.2.1.1; Sigma-Aldrich) stock solution and concentrations were adjusted using absorbance at 280 nm extinction coefficient [29]. In order to study the effect of RMNP on HEWL at three representative RMNP:HEWL ratios (0, 0.025-1.250), appropriate amounts of RMNPs were added after ultrasonication at a power of 28 kHz for 10 min to 0.2 mg ml^{-1} protein without or with urea treatment. The mixtures were incubated for 1 hour at 25 °C with shaking at 300 rpm to maintain homogeneity.

Effect of RMNPs on Urea-induced Unfolding and Refolding of HEWL

In order to study the effect of RMNPs on urea-induced HEWL unfolding three representative ratios of RMNP:HEWL as 0, 0.025 and 1.25, in the absence (-Urea) or presence (+Urea) of 6 M urea were investigated. Accordingly, three samples of 0.2 mg ml^{-1} HEWL were prepared in the absence (as control) or presence of RMNPs at 0.005 mg ml^{-1} and 0.250 mg ml^{-1}, in the absence or presence of 6 M urea.

In continue, the effect of RMNPs on the efficiency of dilution and dialysis strategies on refolding of HEWL treated by 6 M urea were explored. The efficiency of dilution strategy on refolding of urea denatured/dissolved protein was investigated in three RMNP:HEWL ratios as 0, 0.025 and 1.25 after performing 30 fold dilution using PBS, 100 mM, pH = 7.4 [30]. Also, the effect of RMNPs (at RMNP:HEWL ratio of 0.025) on the refolding of urea denatured/dissolved HEWL was explored using dialysis strategy. Dialysis was performed against PBS (100 mM, pH 7.4) under continuous stirring for 24 h at 4 °C in dialysis tubes (6-8 kDa cut-off) [31]. In brief, dialysis of urea treated HEWL samples in the absence or presence of RMNPs were carried out and results were compared against two control samples as "*-Urea, -Dialysis"* and "*+Urea, -Dialysis"*.

HEWL Assay

For the enzyme assay dried bacterial cell wall from the Gram-positive bacterium, *M. luteus* was prepared [32] and used as a substrate in 100 mM potassium phosphate pH 7.4 at 25 °C using a UV-Vis spectrophotometer [Jenway 6500 series] [33,34]. Briefly, after addition of 40 µl HEWL at 0.2 mg ml^{-1} to 1 ml of *M. luteus*, the lysis of *M. luteus* cell wall was monitored by measuring the decrease in absorbance at 450 nm.

Spectroscopic Analysis

Tryptophan fluorescence emission spectra of HEWL as control and samples with different concentrations of RMNPs were recorded using a spectrofluorimeter (BioTek, Synergy™ H4 Hybrid microplate reader) in a 96-well quartz plate at room temperature. The fluorescence spectrum of HEWL at 0.2 mg ml^{-1} was obtained in the wavelength range of 300-400 nm after excitation at 290 nm at 25 °C. In order to obtain the protein spectra in the presence of RMNPs, the suspensions were shaken for 1 h at 200 rpm under ambient conditions.

The helicity of the treated HEWL at 0.2 mg ml^{-1} in the

absence or presence of RMNPs at two representative concentrations of 0.005 and 0.250 mg ml^{-1} was also studied using the AVIV circular dichroism spectrometer Model 215, with and without urea (6 M) treatment followed by dialysis. The results were analyzed using CDNN deconvolution software by mean residual ellipticity (MRE) in deg cm^2 $dmol^{-1}$. Our reported data are the mean of three measurements for each test.

RESULTS AND DISCUSSION

Although the Arg as a kosmotropic agent is used in various protein recovery/refolding protocols [35], the modulation of this application for Arg at the surface of MNPs is the subject of debate. The characterization of synthesized nanoparticles (NPs) including MNPs and RMNPs was performed using X-ray diffraction (XRD), Fourier-transform infrared (FT-IR), vibrating sample magnetometry (VSM), scanning electron microscopy (SEM) and finally, the surface zeta potential of the particles was estimated using a Zeta-sizer (Fig. 1).

The Fourier-transform infrared (FT-IR) spectra provided a direct proof for the synthesis of a Fe_3O_4 core that was successfully functionalized by Arg. The FT-IR spectra measured the RMNPs' bonds between different ingredients in the mid and near IR regions [14] in the range of 500-4000 cm^{-1}, as can be seen in figure 1a. The bonds of Fe-O at about 520-580 cm^{-1} represent the formation of magnetite The absorption band at 2950 cm^{-1} is assigned to the (OH) stretching mode and overlaps with the C-H and N-H stretching vibration peaks and the weak absorption band observed at 1606 cm^{-1} is due to the bending vibration of absorbed water [36] which indicates the presence of water molecules linked to RMNPs. Stretching vibrations seen at ~1600 cm^{-1} to ~1650 cm^{-1} are because of the C=O and C-O bonds and the bonds of NH_2 and OH at around 2800-3300 cm^{-1} indicate that the Arg has completely modified the surface of the RMNPs [14,37]. According to the XRD analysis data, and the diffraction pattern of the RMNPs according to Bragg's reflections the representative peaks were observed in (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 the cubic phase of Fe_3O_4 (Fig. 1b), in agreement with the JCPDS card No. 19-629 [38]. Figure 1c shows scanning electron microscopy (SEM) micrograph of RMNPs and its relative distribution sizes. According to this figure, the morphology of the RMNPs is clearly spherical with the average particle size of 32.14 nm. The saturation magnetization (M_s) of RMNPs by applying vibrating sample magnetometry (VSM) was estimated to be 69.9 emu g^{-1} at 25°C (Fig. 1d). RMNPs present super-paramagnetic behavior due to being smaller than the critical size and they have no coercivity and remanence [39,40]. According to Fig. 1e, the biological applications of RMNPs are most profoundly impacted by their surface chemistry. Since the surface charge of the RMNPs is thought to be influenced by electrostatic interactions, the zeta potential (ζ mV) of RMNPs was determined in phosphate-buffered saline (PBS) 100 mM, pH = 7.4. The average zeta potential value of RMNPs is -34.3 mV. This measurement exhibits a large negative charge that can attract positively charged targets in the water shell that surrounds the protein molecules.

The effect of RMNPs on HEWL structure and activity in the absence or presence of urea was explored. Intrinsic protein fluorescence is due to aromatic amino acids, mainly tryptophan, which can be selectively measured by excitation at 280 nm [41]. Changes in emission spectra of tryptophan are due to the protein conformational transitions, subunit association, ligand binding or denaturation, which affect the local environment.

Figure 2 reveals the cumulated functions of RMNP:HEWL including changes in emission spectra of tryptophan intrinsic fluorescence, secondary structure alteration, and percent of remaining activity to investigate the effect of RMNPs on protein structure and function. The intrinsic fluorescence emission of HEWL is mainly due to Trp 62 and Trp 108 residues which makes it a useful model to study protein conformational changes [42]. The resulting spectra indicated that the environment in the vicinity of the tryptophan residues of the HEWL molecules was changed during the binding and decreased in a polar hydrophilic environment [43]. The fluorescence emission peaks resulted as a function of RMNP:HEWL ratios, using 0.2 mg ml^{-1} HEWL as a model protein to study the effect of RMNPs concentration on HEWL conformational structure (Fig. 2a). RMNPs change the protein hydration pattern, resulting in increasing or decreasing Trp emission after excitation due to the protein folding or unfolding, respectively, depending on

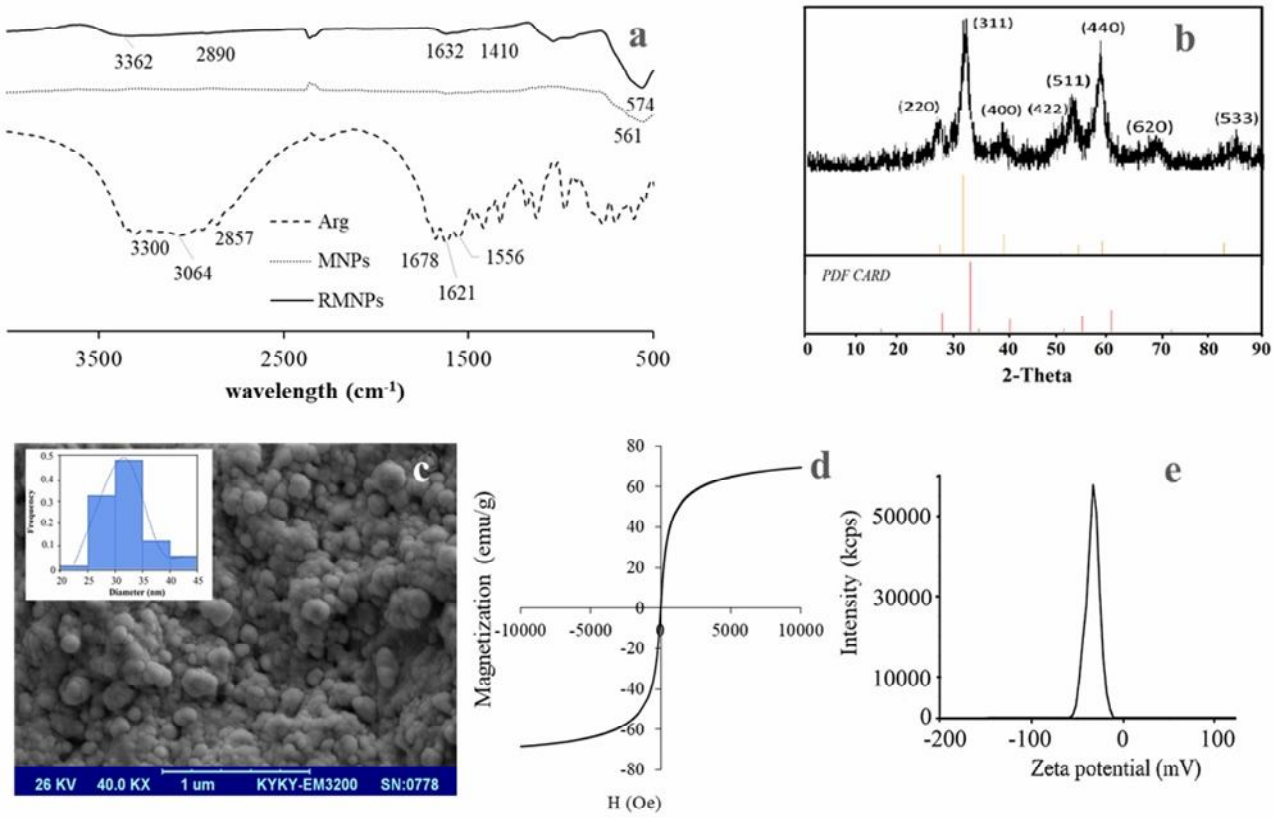

Fig. 1. (a) FT-IR spectra of MNPs, Arg and RMNPs, (b) X-ray diffraction, (c) SEM image and distribution size with particle analysis using ImageJ (inset to Fig.), (d) VSM graph and (e) Zeta potential (DLS) of RMNPs.

the molar ratios of RMNP:HEWL. The aforementioned RMNP-induced HEWL structural change brings about increasing or decreasing of the quantum yield of protein fluorescence emission with an inflection point at the concentration ratio of threshold (CRT) of 0.296 as depicted in Fig. 2a. Therefore, RMNPs represent two opposing effects including structure-making (or kosmotropic-like) and structure-breaking (or chaotropic-like) effects at lower and higher ratios than the CRT, respectively. A large body of results for various samples shows that low concentration of RMNPs acts as stabilizing agents (kosmotropic co-solvents) through improving protein hydration, increasing protein stability and consequently maintaining biological activity of macromolecules [44]. To explain the effect of high concentrations of RMNPs on the stability of HEWL, it is anticipated that a vast number of RMNPs are not covered by protein corona, negatively affecting protein stability and structure, and acting as chaotropic agents.

The effect of RMNP:HEWL ratios of 0.025 and 1.25 on HEWL helicity in the absence or presence of urea have been presented (Fig. 2b). Accordingly, using CD analysis as a quite sensitive method to study the secondary structure of HEWL, the protein α-helicity is affected by the RMNPs content of the solvent system. In accordance with fluorescence results in the absence of urea (-Urea), the helicity is increased at the RMNP:HEWL ratio of 0.025 (< CRT) displaying even higher helicity than the control sample as depicted in Fig. 2b. However, when using lower or higher ratios (0.025 and 1.25, respectively) of RMNP:HEWL (< CRT or > CRT, respectively) in the presence of 6 M urea (+Urea), the helicity was severely diminished. Thus, low concentrations of RMNPs

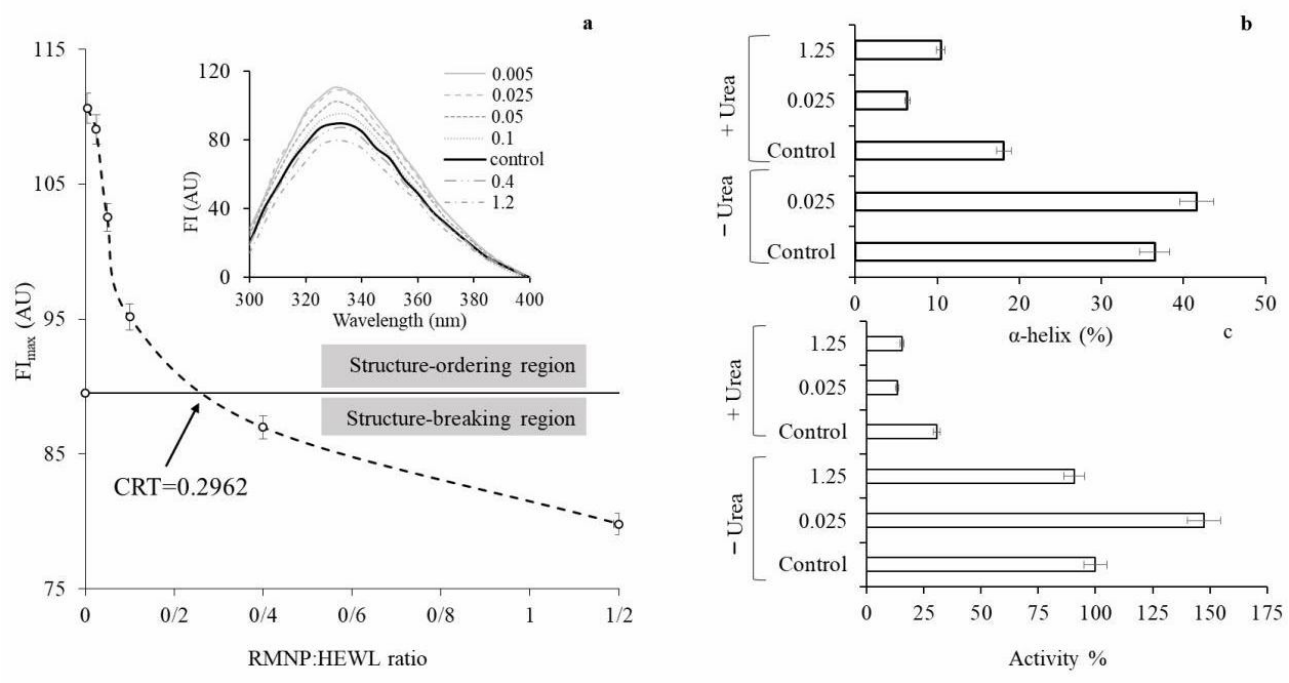

Fig. 2. Study of the structure-ordering and structure-breaking effects of RMNPs on HEWL after treatment with 0.025 (< CRT) and 1.25 (> CRT) RMNP:HEWL ratios in the absence and presence of urea in phosphate-buffered saline (PBS, 100 mM, pH = 7.4). (a) Effects of RMNP:HEWL ratios on changing the emission spectra of tryptophan intrinsic fluorescence of HEWL samples as revealed by fluorescence spectroscopy, (b) Alterations in α-helix structure content of HEWL after treatment with RMNPs and (c) evaluation of the remaining HEWL activity in the presence of RMNPs.

(0.005 mg ml^{-1}) lead to HEWL denaturation even more than the control sample (Control, +Urea). Moreover, the enzymatic activity of HEWL in the absence of urea rose sharply at RMNP:HEWL ratios of 0.025 (< CRT) and decreased minimally at RMNP:HEWL ratios of 1.25 (> CRT) (Fig. 2c). As with helicity, despite using lower or higher ratios of RMNP: HEWL (< CRT or > CRT, respectively) in the presence of 6 M urea (+Urea), the protein activity is decreased significantly, which is in good accordance with structural observations. It can thus be deducted that, additives act to reduce the denaturing effect of the denaturant chiefly by altering the balance between preclusion of additives, which results in enhanced hydration of the protein [45]. Correspondingly, pertaining to HEWL, it is anticipated that the addition of RMNPs shifts the equilibrium between the preferential binding of urea and preferential exclusion of NPs, leading to a reduction in the hydration of the protein and aiding the destabilizing action of the denaturant. Several models have explained the mechanism of action of denaturants (such as urea) for destabilizing proteins by migrating into the interior of the protein and forming hydrogen bonds to atoms in the backbone or decreasing the solvent polarity and dielectric constant [46].

In continuance, the effect of RMNPs on the functional stability of HEWL was assessed (Fig. 3). The remaining activity of HEWL as a model protein was monitored for 64 h in the absence of RMNPs (as control) and presence of RMNPs at the RMNP:HEWL ratios of 0.025 and 1.250 at 25 °C. As shown in figure 3a, the remaining activity of samples has been provided which shows that the functional stability of the enzyme has improved by the decline in the amount of RMNPs (RMNP:HEWL ratios of 0.025 (< CRT)) and vigorously reduced at RMNP:HEWL ratios of 1.25 (> CRT) (Fig. 3a).

In biological fluids, proteins bind to the surface of NPs

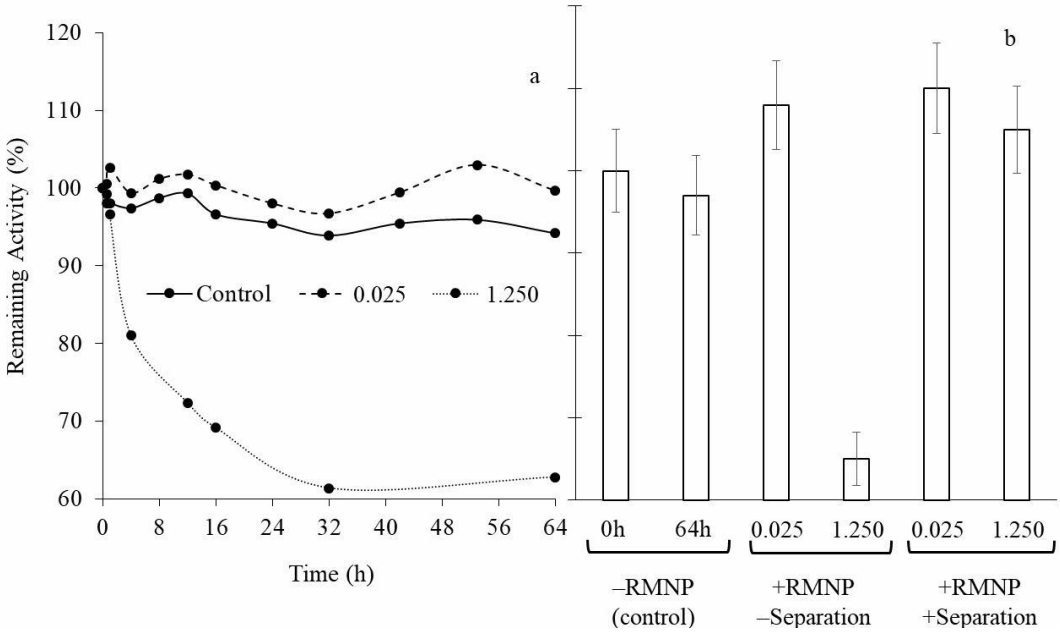

Fig. 3. Concentration-dependent effect of RMNPs on the HEWL remaining activity at RMNP:HEWL ratios of 0.025 (< CRT) and 1.250 (> CRT) in phosphate-buffered saline (PBS, 100 mM, pH = 7.4), (a) during 64 h and (b) at 64 h before and after separation of RMNPs.

to form a protein coating or corona which can critically affect the interaction of the NPs with its nearby environment including adjacent living systems [47]. The positive charge of the enzyme at neutral pH could be effectively adsorbed by the negatively charged RMNPs. Effective corona formation occurs in the far lower concentrations of RMNPs (<< CRT), and RMNPs are well saturated by proteins which in turn cooperatively brings about protein hydration and has a positive effect on the protein structure. Figure 3b illustrates the remaining activities of HEWL in three groups: in the absence of RMNPs at the beginning and after 64 h (controls); in the presence of RMNPs at two representative RMNP:HEWL ratios including 0.025 (< CRT) or 1.25 (> CRT) after 64 hours with RMNPs separation; in the presence of RMNPs at two representative RMNP:HEWL ratios including 0.025 (< CRT) or 1.25 (> CRT) after 64 h without RMNPs separation. It is obvious that the enzyme is preserved from loss of activity in the absence of RMNPs after 64 h. The remaining activity of the enzyme in the presence of RMNPs at RMNP:HEWL ratios of 0.025 (< CRT) or 1.25 (> CRT) without separation is improved and declined in comparison with the control sample, respectively. Moreover, the remaining activity of the enzyme after 64 hours incubation in the presence of RMNPs at both RMNP:HEWL ratios of 0.025 (< CRT) and 1.25 (> CRT), followed by RMNPs separation is effectively improved although the extent of improvement is reported to be higher for a representative ratio of <CRT than a representative ratio of > CRT. Upon separation of the RMNPs from the protein solution, due to the remaining single domain RMNPs, the ratio of RMNP:HEWL decreases significantly, which leads to a meaningful improvement in the structure-making effect (or kosmotropic-like) of RMNPs due to the falling RMNP:HEWL ratio to far less than the CRT (<< CRT), presumably to make an effective improvement in protein hydration. Additionally, these results can be suggestive that even high ratios are not detrimental to protein structure, and the negative effect of high ratios (>> CRT) is reversed upon their removal.

As lower final protein concentration in low concentrations of denaturant provides a greater efficiency of protein refolding, the unfolded urea treated protein is commonly diluted or dialyzed against a buffer to achieve

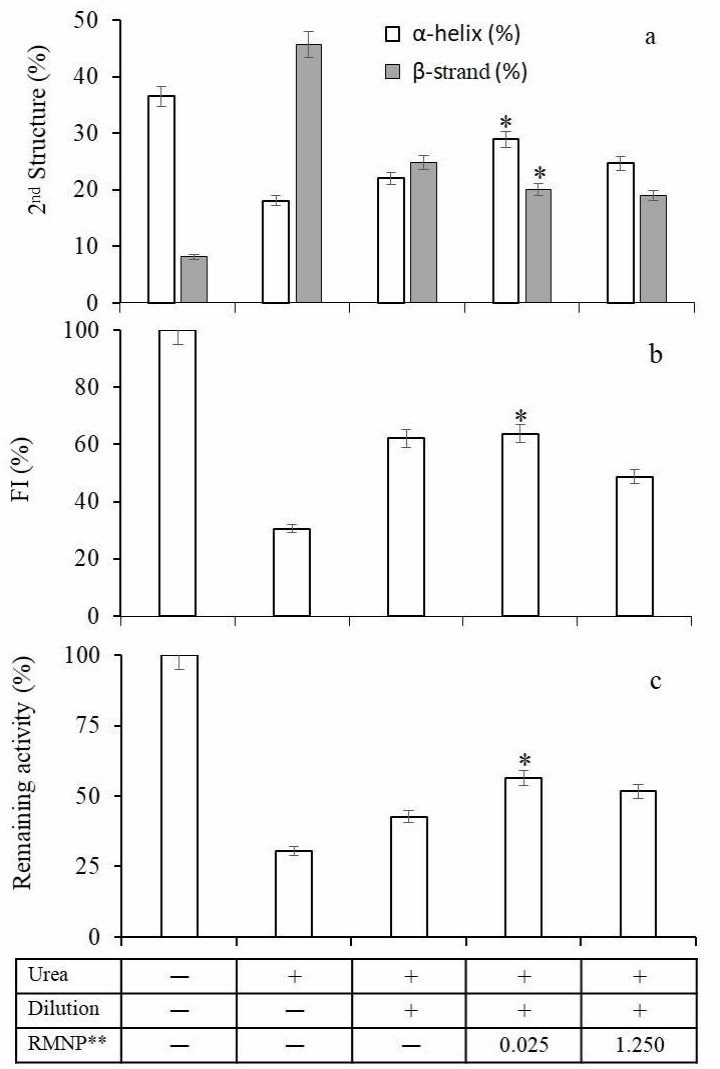

Fig. 4. Secondary and tertiary structure of HEWL in the absence and presence of RMNPs. Samples are native protein (0.2 mg ml^{-1} HEWL in PBS, 100 mM, pH = 7.4), unfolded protein and protein treated with 30-fold diluted urea in the absence and presence of RMNPs at RMNP:HEWL ratios of 0.025 (< CRT) and 1.25 (> CRT), evaluated by (a) Far-UV CD spectra (b) Intrinsic fluorescence emission spectra, and (c) Remaining activity.

protein refolding [48]. The efficiency of dilution of urea treated HEWL was investigated in the absence and presence of two representative RMNP:HEWL ratios of 0.025 and 1.025 as < CRT and > CRT in PBS (100 mM, pH = 7.4), at room temperature upon examining the secondary structure, intrinsic fluorescence and activity analysis (Fig. 4).

Circular dichroism analysis (CD) was used to study the secondary structures of the HEWL as an indicative parameter of protein refolding [49]. As depicted in Fig. 4a, by performing dilution in the presence of RMNPs, the percentage of α-helix presents a sharp rise and β-strand conformations are more effectively decreased than in the absence of RMNPs. Accordingly, in comparison with the absence of RMNPs α-helix structure shows a further 7% and 2.6% increase in the presence of RMNPs at 0.025 (< CRT) and 1.25 (> CRT), respectively. Thus, the protein will have a more regular and stable structure. Moreover, the β-conformation is also more effectively

improved by diluting the HEWL in the presence of RMNPs rather than in their absence (Fig. 4a).

According to the fluorescence data provided in Fig. 4b, the achieved refolding efficiency by dilution was 62% in the absence of RMNPs which was increased to 64% at the RMNP:HEWL ratio of 0.025 (< CRT) and decreased to 48.8% at the RMNP:HEWL ratio of 1.250 (> CRT). A higher increase in the efficiency of dilution for the refolding process is clearly seen at a low concentration of RMNPs, which is in good accordance with CD results. According to Fig. 4c, the remaining activity of the refolded HEWL shows an increase in activity in the presence of RMNPs in both concentrations: A remaining activity of 42.6% by dilution in the absence of RMNPs was increased to 56.4% in the presence of RMNPs at a ratio of 0.025 and 51.7% in the presence of RMNPs at a ratio of 1.25. Therefore, the ordering concentration of RMNPs in the kosmotropic-like ratios shows that the protein has more regular, stable conformation and also displays improved activity. In other words, at the RMNP:HEWL ratio of 0.025 (< CRT) the RMNPs can provide a considerable improvement in the refolding of the unfolded urea treated protein species using dilution.

As well as HEWL refolding using the dilution strategy, the influence of the RMNPs on the refolding of denatured HEWL in a dialysis system was also examined using intrinsic fluorescence and activity analysis (Fig. 5). The dialysis text was done overnight against PBS (100 mM, pH 7.4) in the absence and presence of RMNPs at the RMNP:HEWL ratio of 0.025 (< CRT) with continuous stirring for 24 h at 4 °C in dialysis tubes (6-8 kDa cut-off). In Fig. 5a, the effect of the RMNP:HEWL ratio of 0.025 has been considered and the results of fluorescence spectroscopy are depicted. According to the fluorescence spectra presented in Fig. 5a, the HEWL denatured by urea illustrates a great rise in the fluorescence spectra which is a witness for refolding by performing dialysis. Moreover, improved refolding is reported in the presence of a 0.025 ratio of RMNPs as a representative of the structure-ordering range of RMNP:HEWL ratios (< CRT). The effect of RMNPs on dialysis performance was also studied using HEWL remaining activity. A significant increase in the recovered HEWL activity is reported for dialysis of unfolded HEWL in the absence of RMNPs (†) compared to the samples, which were dialyzed in the presence of RMNPs (N) from 56.5 to 63 percent (Fig. 5b). Accordingly, the RMNPs, at the RMNP:HEWL ratio of 0.025 (< CRT), effectively ameliorate the dialysis-based refolding of the unfolded urea-treated protein species. Thus, for increasing efficiency, using very small amounts of RMNPs (in the structure-ordering range) is recommended in the dilution and dialysis process.

Various RMNPs characteristics such as superparamagnetism, high surface area, large surface-to-volume ratio, and easy separation under external magnetic fields, has attained great recognition for RMNPs, putting them in the spotlight for various applications in the biological industries. We endeavoured to study this new generation of MNPs modified by Arg as they influenced a model protein (HEWL) structure and function in the presence and absence of a denaturant (Urea), in a concentration-dependent fashion, since it is essential to comprehend the influence of RMNPs on HEWL storage, denaturation and refolding by dilution or dialysis. The interaction between RMNPs with HEWL was explored by a combination of structural and functional analysis. Although the mechanism of intercommunications between RMNPs 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 RMNPs at RMNP:HEWL ratios lower or higher than the resulting CRT, respectively. A concentration ratio threshold (CRT) of RMNP:HEWL at 0.2962 was calculated and is suggested to be calculated independently prior to the utilization of NPs in any biological application, in order to assess the positive or negative effects of any kind of NPs.

CONCLUSIONS

In this paper, Arg modified magnetic nanoparticles (RMNPs) were synthesized and their influence on the structure and function of the model protein hen egg white lysozyme (HEWL) (EC 3.2.1.1) is reported. We have described how RMNPs affect the structure and activity of HEWL in a concentration-dependent manner, both in the presence and absence of a denaturing agent, urea. RMNPs act as a double-edged sword which is exhibited by either a

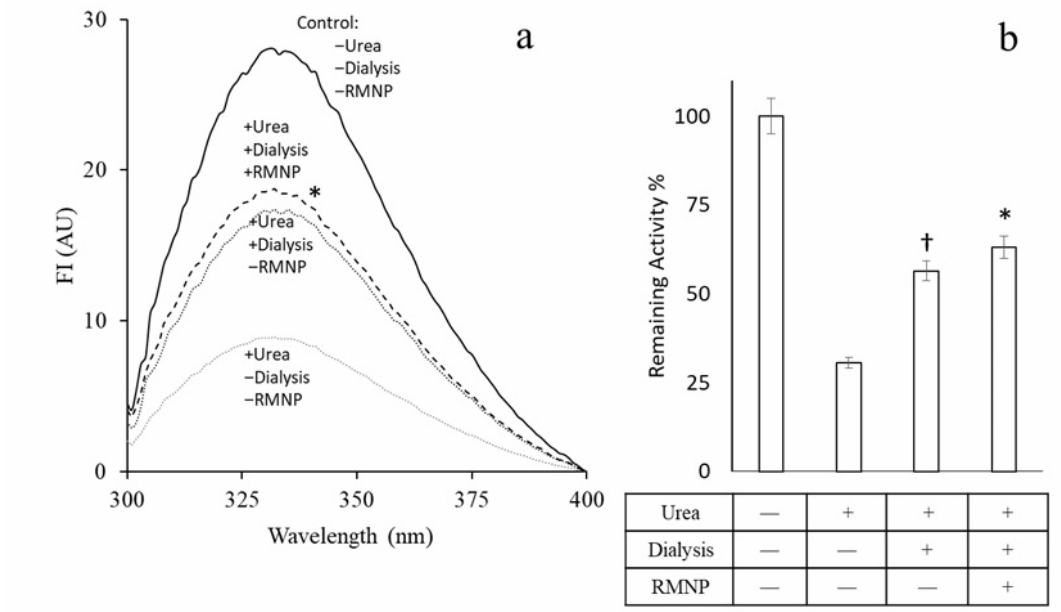

Fig. 5. Study of tertiary structure and activity of HEWL in the absence and presence of RMNPs in phosphate-buffered saline (PBS, 100 mM, pH = 7.4). Untreated HEWL (0.2 mg ml^{-1}) in three different states (native protein, unfolded protein and refolded protein treated with dialysed urea) in the absence (†) and presence (*) of RMNPs a t the RMNP:HEWL ratio of 0.025 (< CRT) evaluated by (a) Intrinsic fluorescence emission spectra, and (b) Remaining activity.

kosmotropic-like or chaotropic-like effect. When the ratio of RMNPs to HEWL is below the CRT (< CRT), the RMNPs assert a positive effect on the structure of the protein, and as stands to reason, at higher ratios than the CRT (> CRT) they break down the protein structure. Conversely, during the refolding of HEWL, unfolded due to the presence of urea, which is achieved through dilution or dialysis (urea removal), RMNPs show a curative effect in all RMNP:HEWL ratios. To conclude, based on our observations it is recommended to utilize RMNPs at ratios below the CRT whereupon HEWL structure and functions were preserved. Recovery of protein folding through dialysis or dilution subsequent to denaturation/solubilization by urea was also enhanced by RMNPs. These processes are extensively employed in biotechnology, nanomedicine and nanodiagnostics, hence emphasizing the use of RMNPs and studies to optimize their performance.

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REFERENCES

[1] A. Choi, K.D. Seo, B.C. Kim, D.S. Kim, Lab on a Chip. 17 (2017) 591.

[2] F Shen, JK. Park, Analytical chemistry. 2017 Dec 6.

[3] H. Teymourian, A. Salimi, S. Khezrian, Electroanalysis 29 (2017) 409.

[4] M. Fuentes, C. Mateo, A. Rodriguez, M. Casqueiro, J.C. Tercero, H.H. Riese, R. Fernández-Lafuente, J.M. Guisán, Biosensors and Bioelectronics 21 (2006) 1574.

[5] O. Veiseh, J.W. Gunn, M. Zhang, Advanced Drug Delivery Reviews 62 (2010) 284.

[6] H.Y. Tsai, C.F. Hsu, I.W. Chiu, C.B. Fuh, Analytical Chemistry 79 (2007) 8416.

[7] A.H. Latham, M.E. Williams, Accounts of Chemical Research. 41 (2008) 411.

[8] S. Laurent, D. Forge, M. Port, A. Roch, C. Robic, L. Vander Elst, R.N. Muller, Chemical Reviews 108 (2008) 2064.

[9] Y.W. Jun, Y.M. Huh, J.S. Choi, J.H. Lee, H.T. Song, S. Kim, S. Kim, S. Yoon, K.S. Kim, J.S. Shin, J.S. Suh, Journal of the American Chemical Society 127 (2005) 5732.

[10] D. Tang, R. Yuan, Y. Chai, The Journal of Physical Chemistry B 110 (2006) 11640.

[11] W. Xie, N. Ma, Energy & Fuels 23 (2009) 1347.

[12] T. Neuberger, B. Schöpf, H. Hofmann, M. Hofmann, Journal of Magnetism and Magnetic Materials 293 (2005) 483.

[13] F. Kashanian, G. Kokkinis, J. Bernardi, M.R. Zand, A. Shamloo, I. Giouroudi, Sensors and Actuators A: Physical 270 (218) 223.

[14] A.R. Bagherpour, F. Kashanian, S.S. Ebrahimi, M. Habibi-Rezaei, Nanotechnology 29 (2018) 075706.

[15] D. Rehana, A.K. Haleel, A.K. Rahiman, Journal of Chemical Sciences 127 (2015) 1155.

[16] Y. Lai, W. Yin, J. Liu, R. Xi, J. Zhan, Nanoscale Research Letters NRL, 2010.

[17] K. Azizi, M. Karimi, H.R. Shaterian, A. Heydari, RSC Advances 4 (2014) 42220.

[18] S.H. Lacerda, J.J. Park, C. Meuse, D. Pristinski, M.L. Becker, A. Karim, J.F. Douglas, ACS Nano 4 (2009) 365.

[19] M. Lombardo, S. Easwar, F. Pasi, C Trombini, D.D. Dhavale, Tetrahedron 64 (2008) 9203.

[20] D. Rozema, S.H. Gellman, Journal of the American Chemical Society 117(1995) 2373.

[21] Y.Y. Wang, W.Y. Qiu, Z.B. Wang, H.L. Ma, J.K. Yan, RSC Advances 7 (2017) 11067.

[22] T. Arakawa, D. Ejima, K. Tsumoto, N. Obeyama, Y. Tanaka, Y. Kita, S.N. Timasheff, Biophysical Chemistry 127 (2007) 1.

[23] F. Kashanian, M. Habibi-Rezaei, A.R. Bagherpour, A. Seyedarabi, A.A. Moosavi-Movahedi, RSC Advances 7 (2017) 54813.

[24] F. Kashanian, M. Habibi-Rezaei, A.A. Moosavi-Movahedi, A.R. Bagherpour, M. Vatani, Biomedical Materials 13 (2018) 045014.

[25] L. Stevens, Comp. Biochem. Physiol. Part B Biochem. 100 (1991) 1.

[26] P.Y. Surekha, P. Dhanya, MK SJ, S. Pradeep, S. Benjamin, Electronic Journal of Biology 12 (2016).

[27] S. Yamada, HA. Itano, Biochimica et Biophysica Acta (BBA)-General Subjects 130 (1966) 538.

[28] R.E. Smith, R. MacQuarrie, Analytical Biochemistry 90 (1978) 246.

[29] K.C. Aune, C. Tanford, Biochemistry 8 (1969) 4586.

[30] B.R. Zhou, Y. Liang, F. Du, Z. Zhou, J. Chen, J. Biological Chem. 279 (2004) 55109.

[31] V. Olieric, A. Schreiber, B. Lorber, J. Pütz, Biochemistry and Molecular Biology Education 35 (2007) 280.

[32] Z. Liu, B. García-Díaz, B. Catacchio, E. Chiancone, H.J. Vogel, Biochim. Biophys. Acta-Biomembr, 1848 (2015) 3032.

[33] D.H. Li, Y.J. Chi, China Dairy Industry 30 (2002) 128.

[34] S.H. Hoseinifar, R. Safari, M. Dadar, General and Comparative Endocrinology 243 (2017) 78.

[35] K. Shiraki, S. Tomita, N. Inoue, Current Pharmaceutical Biotechnology 17 (2016) 116.

[36] T. Ozkaya, M.S. Toprak, A. Baykal, H. Kavas, Y. Köseoğlu, B. Aktaş Journal of Alloys and Compounds 472 (2009) 18.

[37] Z. Wang, H. Zhu, X. Wang, F. Yang, X. Yang, Nanotechnology 20 (2009) 465606.

[38] E. Courvoisier, P.A. Williams, G.K. Lim, C.E. Hughes, K.D. Harris, Chem. Commun. 48 (2012) 2761.

[39] P. Pradhan, J. Giri, G. Samanta, HD. Sarma, KP. Mishra, J. Bellare, R. Banerjee, D. Bahadur, Biomedical Materials Research Part B: Applied Biomaterials 81 (2007) 12.

[40] B. Ünal, Z. Durmus, A. Baykal, H. Sözeri, M.S. Toprak, L. Alpsoy, Journal of Alloys and Compounds 505 (2010) 172.

[41] L. Pellegrino, A. Tirelli, International Dairy Journal 10 (2000) 435.

[42] T. Imoto, L.S. Forster, J.A. Rupley, F. Tanaka, Proceedings of the National Academy of Sciences 69 (1972) 1151.

[43] V.I. Teichberg, N. Sharon, FEBS Letters 7 (1970) 171.

[44] R. Joshi, R.G. Kumar, Effect of Sugars on the

Thermal Stability of Hen Lysozyme both in the Absence and Presence of Denaturants (Doctoral Dissertation).

[45] Y. Kita, T. Arakawa, T.Y. Lin, S.N. Timasheff, Biochemistry 33 (1994) 15178.

[46] W. Smith, P.S. Kishnani, B. Lee, R.H. Singh, W.J. Rhead, L.S. King, M. Smith, M. Summar, Urea Cycle Disorders: Critical Care Clinics 21 (2005) S9.

[47] V. Mirshafiee, M. Mahmoudi, K. Lou, J. Cheng, M.L. Kraft, Chemical Communications 49 (2013) 2557.

[48] A.D. Guise, S.M. West, J.B. Chaudhuri, Molecular Biotechnol. 6 (1996) 53.

[49] B Ranjbar, P. Gill, Chemical Biology & Drug Design 74 (2009) 101.