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Effects of silica nanoparticle supported ionic liquid as additive on thermal reversibility of human carbonic anhydrase II

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ABSTRACT

Silica nanoparticle supported imidazolium ionic liquid [SNImIL] was synthesized and utilized as a biocompatible additive for studying the thermal reversibility of human carbonic anhydrase II (HCA II). For this purpose, we prepared additive by modification of nanoparticles through the grafting of ionic liquids on the surface of nanoparticles (SNImIL). The SNImIL were fully characterized by Fourier transform infrared spectroscopy, scanning electron microscopy and thermo gravimetric analysis. The characterization of HCA II was investigated by various techniques including UV–vis and ANS fluorescence spectrophotometry, differential scanning calorimetry, and docking study. SNImIL induced disaggregation, enhanced protein stability and increased thermal reversibility of HCA II by up to 42% at pH 7.75.

1. Introduction

Irreversibility causes misfolding and aggregation of proteins in cells and under in vitro conditions. Most importantly, it is involved in a wide range of diseases, including some of the most prevalent neurodegenerative disorders [1]. Human carbonic anhydrase (HCA II) is a zinc metalloenzyme which catalyzes the reversible hydration of carbon dioxide to bicarbonate and hydrogen ions [2,3]. At least 14 different CA isoforms have been isolated in higher vertebrates. These isozymes have diverse tissue distribution and subcellular localizations, and they exist in archaea, eubacteria, animals and plants [4]. Many of these isozymes are significant targets for inhibitors with clinical applications [5,6]. HCA II has a single polypeptide chain of 259 amino acid residues with a molecular mass of about 29 kDa [7]. This enzyme is known as a cytoplasmic isozyme and has a high catalytic activity with very high affinity for sulfonamides. The inhibition of CA II aggregation is important in the treatment of glaucoma, marble brain syndrome, CA II deficiency syndrome [8], altitude sickness, and obesity. More than its medical relevance, its tractability and simplicity are what make CA II particularly an attractive model enzyme [9].

Aggregation processes are important challenges while working with proteins. Biopharmaceutical products with low quality can potentially affect drug activity, immunogenicity and pharmacokinetics. All living organisms are potentially exposed to such stress conditions as non-optimal environmental temperatures and osmotic pressures, as well as the presence of toxic chemicals at certain stages of their life. These conditions pose a serious threat to the living organism often by causing the cellular proteins to unfold, which may lead to aggregation [10]. Neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis and prion diseases are increasingly being realized to have common cellular and molecular mechanisms including protein aggregation and inclusion body formation [1].

HCA II serves as a good model system for the study of enzymes; however, its denaturation is irreversible. Thus, any efforts to improve the thermal reversibility of HCA II could be important. Due to the aforementioned principles, several attempts to control aggregation and refolding of CA, as well as other proteins, have been made [11–22]. Some of these reports used nanoparticles of

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hydrophobized poly (vinyl alcohol), silica and hydrogel [23–25], imidazole [26], ionic liquid [27–32] or β-casien [33–40] to control aggregation and refolding of proteins. Over the past decade, nanotechnology has become one of the fastest growing research fields, mainly due to the unique large surface area, small size, physicochemical properties and successful usage of nanoparticles in different fields including imaging, diagnosis, catalysts, biocatalysis and drug delivery [41–46].

Generally, the development of silica nanoparticles as tunable supporting material in nanostructure design is of great significance for both academia and industry [47,48]. Ionic liquids, which are low temperature melting salts, have also been considered as eco-friendly alternatives to volatile organic solvents because of their negligible vapor pressure, nonflammable nature, chemical stability, relatively high ionic conductivity and wide potential window [49–51]. These properties have opened up many applications such as solvent in synthesis, catalysis, biocatalysis and in electrochemistry [52–58]. Their applications in biology such as ionic liquids (ILs) as refolding additives have been recently reported [27–32]. Although the ability of ILs in refolding has been successfully demonstrated in many fields the heterogeneous systems are still preferred, because of their easy handling and separation. Thus, supported ILs are highly desirable. Supported ILs improved the efficiency versus homogenous ones [59–61].

The combinations of silica nanoparticle and ionic liquid properties have already been probed by some researchers. Nanoparticles have significant adsorption capacities due to their relatively large surface area, therefore, they are able to bind or carry other molecules such as chemical compounds, drugs, probes and proteins attached to their surface by covalent bonds or by adsorption. Hence, the physicochemical properties of nanoparticles, such as hydrophobicity and charge, can be altered by attaching specific chemical compounds, peptides or proteins to their surface [62–65].

The efficacy of nanoparticles for any application depends on the physicochemical characteristics of both their material and surface modifiers. The composition of the nanoparticles, together with their surface properties, also determines their biocompatibility and their ability to be biodegraded. Apart from the impact on function, modifying the surface properties of nanoparticles are also used to reduce their toxicity [24].

In this report, we investigate the structural studies of HCA II reversibility assisted by silica nanoparticle supported imidazolium ionic liquids as a biocompatible and favorable additive for disaggregation and growing thermal reversibility of HCA II.

2. Materials and methods

2.1. Chemicals

All starting materials were of reagent grade. Tetraethoxysilane (TEOS) was from Fluka, chloropropyltrimethoxysilane, sodium iodide, imidazole and methanol were from Aldrich. The 8-anilino-1-naphthalene sulfonic acid (ANS) and other chemicals of the analytical grade were obtained from Sigma–Aldrich. All the solutions were prepared in doubly distilled water and all experiments were carried out in 20 mM Tris–sulfate, pH 7.75 at 27 °C, which is optimum [11].

2.2. Protein purification

HCA II isoenzyme was purified from red blood cells according to Nyman's method [66] with some minor modifications. The enzyme purity was confirmed by SDS-PAGE. The concentration of HCA II was determined by absorbance at 280 nm with an extinction coefficient (ε_{280}) $5.7 \times 10^{-4}\,M^{-1}\,cm^{-1}$ [18] or confirmed by the Lowry's method [67].

2.3. Synthesis of chloropropyltrimethoxysilane modified silica nanoparticles

In a 250 ml round bottom flask, 60 ml (10 mmol) ammonia solution (32%) and 1.98 g (110 mmol) water were added to 100 ml of absolute methanol. The solution was stirred for 5 min before adding 10.41 g (50 mmol) TEOS drop-wise. The final solution was stirred for three days at ambient temperature. Approximately, 35 ml of the prepared silica nanoparticle suspension was degassed using vacuum for several minutes to remove excessive ammonia. One gram (5 mmol) 3-chloropropyltrimethoxysilane was then added drop-wise, and the suspension was refluxed for 72 h. After cooling, the particles were collected by filtration or centrifuging, and exhaustively washed with ethanol and water, and dried under vacuum to give chloropropyl-silica nanoparticles. The synthesized silica nanoparticles (1 g) was stirred with 1-methyl imidazole (5 mmol) in toluene (30 ml) and refluxed for 24 h. After cooling, the formed silica nanoparticle supported imidazolium ionic liquid (SNImIL). The nanoparticles were then collected by centrifuging, repeatedly redispersed, washed with ethyl acetate, diethyl ether and water, and then dried under vacuum at 110 °C and isolated as SNImIL.

2.4. Aggregation study

The aggregation of thermally induced HCA II (0.1 mg/ml) was monitored at 360 nm at 60 °C for 10 min using a Cary Varian UV–vis spectrophotometer model 100 Bio. In order to reduce HCA II aggregation, 0.06 mg/ml of SNImIL was added and transferred to a 600 μl cuvette.

2.5. ANS fluorescence spectrophotometry

The ANS fluorescence of HCA II was recorded using Cary Eclipse Varian fluorescence spectrophotometry. A solution of HCA II (0.1 mg/ml in Tris–sulfate buffer at pH 7.75) in the presence of SNImIL (0.06 mg/ml) was prepared, transferred to a 0.4 ml cuvette and fluorescence intensity was measured in the presence of 5 mM ANS at λ excitation 350 nm at 58 °C with a 10 nm band width.

2.6. Calorimetric study

Calorimetric study was carried out by Nano-DSC differential scanning calorimeter (Setaram, France) equipped with a 0.348 ml cells. All DSC experiments were performed under 2 atm pressure. The concentration of SNImIL was 0.06 mg/ml and HCA II 1 mg/ml in 20 mM Tris–sulfate buffer (pH 7.75). The experiments were performed at a scan rate of 1 °C/min. To check the reversibility of denaturation, the sample was heated to 1–2 °C above the T_m, then cooled and reheated. If the original curve completely or partially reproduced, it is evident that denaturation is fully or partially reversible; otherwise it is an irreversible process. The extent of reversibility depends on a number of factors including the temperature to which the protein was heated during the first scan [68].

2.7. Docking studies

The 3D structure of HCA II, which was used for the docking studies as receptor, was obtained from Protein Data Bank (PDB) with PDB ID: 1CA2. The Discovery Studio Visualizer (version 2.5 Accelrys Software Int.) and AutoDock Tools (Version 1.5.6rc1) [69] were used to obtain the representative forms of the ligand and receptor structures, and visualization of docking results. The Kollman

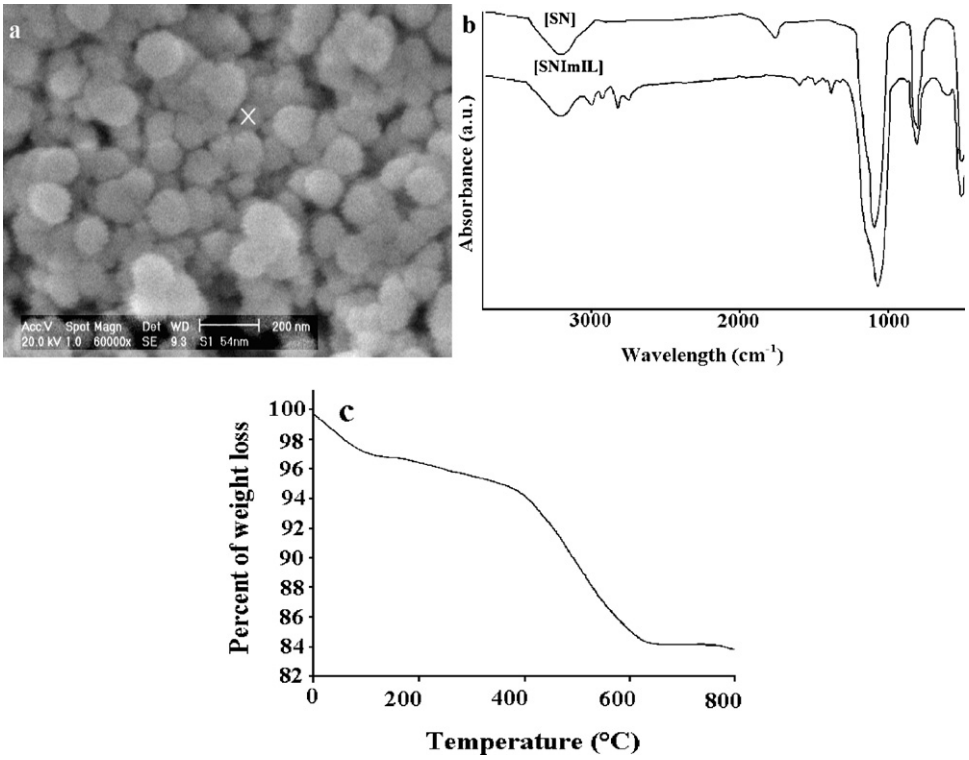


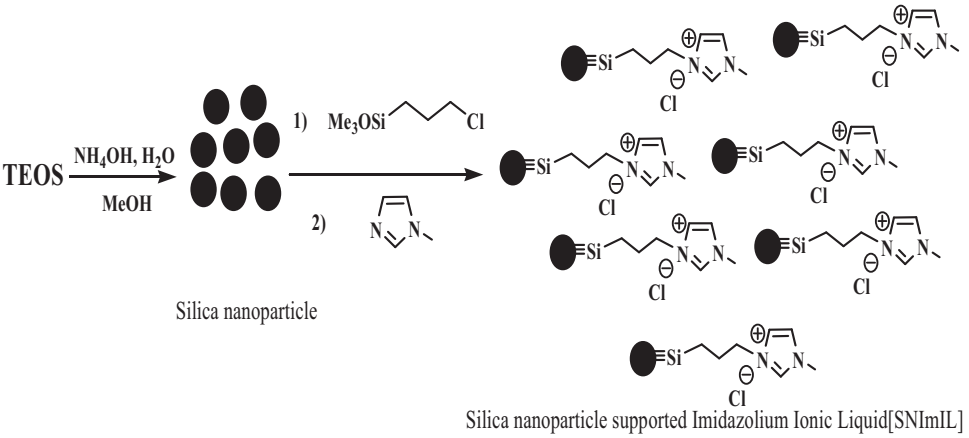
Fig. 1. (a) Scanning electron microscopy (SEM); (b) FT-IR spectrum; (c) thermo-gravimetric analysis (TGA) of silica nanoparticle supported ionic liquid.

charges were added as receptor residue charges and Gasteiger was computed to set the partial charges of the ligand. In this theoretical method, molecular docking was performed using AutoDock 4.2 and Autodock Vina as a new generation of AutoDock, which predicts binding mode more accurately than the Autodock 4.2. Vina has an improved scoring function compared with AutoDock 4.2, and generates an automatic grid map. This generation of AutoDock is more accurate and faster than AutoDock 4.2 [70]. For using AutoDock 4.2 selected conformational search algorithm was Lamarckian genetic algorithm [71].

3. Results and discussion

Here the HCA II solution at pH 7.75 has 2 positive charges and a p*I* of 6.86 as the target protein. The silica nanoparticles supported imidazolium ionic liquid (SNImIL) was prepared via grafting of organic spacers on the surface of synthetic silica nanoparticles, and in the second step the modified silica nanoparticles were connected by ionic liquid like linkers (Scheme 1).

The SNImIL were fully characterized by SEM, N_2 adsorption–desorption isotherm (BET), FT-IR and TGA (Fig. 1). The SEM results showed that the silica nanoparticles have a uniform size of less than 100 nm (Fig. 1a). The presence of the IL functional groups on the surface of silica nanoparticles following post-synthesis functionalization was confirmed by FTIR spectra (Fig. 1b). The typical Si—O—Si peaked around 1100 and 480 cm^{-1}, and the bands around 3500 and 814 cm^{-1} were attributed to stretching and out-of plane bending of free-silanol O—H groups, respectively. These were present in both of the synthesized and functionalized samples. In the FT-IR spectrum of functionalized samples, the absorption bands at 2847, 2934 and 2960 cm^{-1} were due to the asymmetric and symmetric vibration absorptions of (C–H). The weaker absorption peak at 1656 cm^{-1} could be attributed to (C=N). Thus, the FTIR confirmed that the ionic liquid



Scheme 1. Synthesis of silica nanoparticle supported imidazolium ionic liquid [SNImIL].

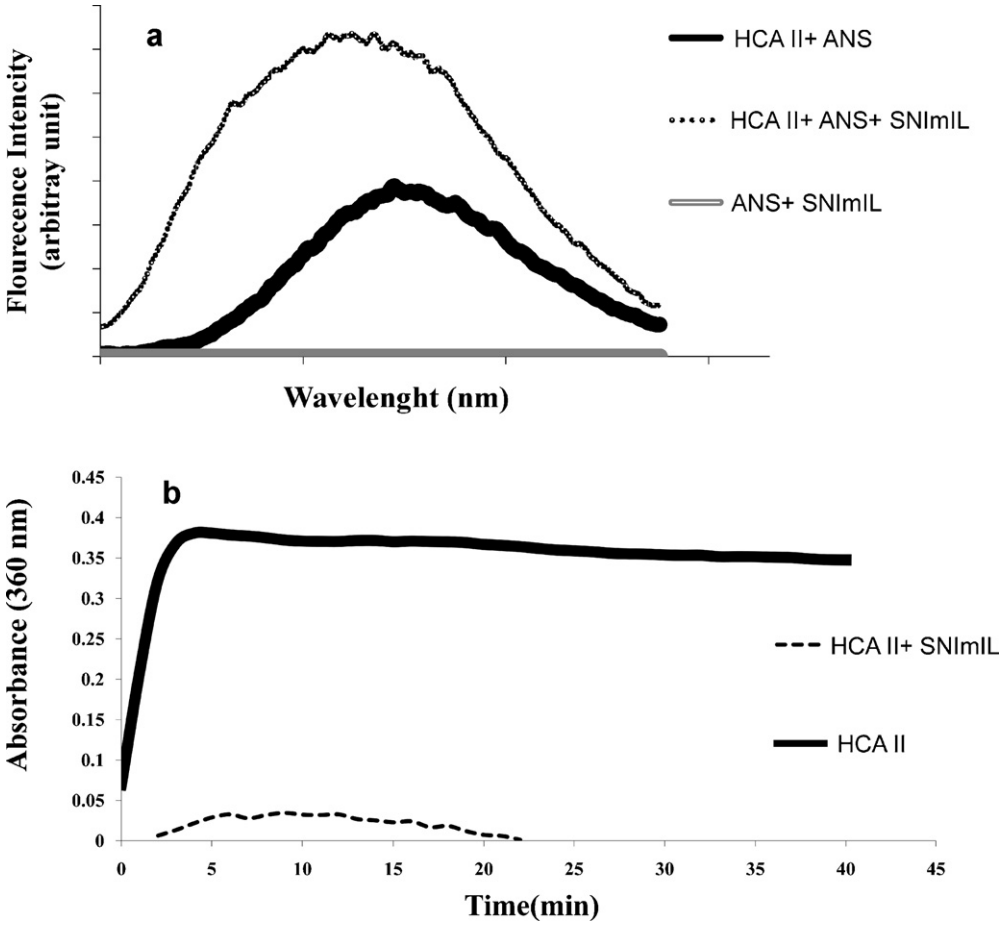


Fig. 2. (a) ANS fluorescence of HCA II (0.1 mg/ml in 20 mM Tris–sulfate buffer at pH 7.75, at 58 °C) alone and in the present of SNImIL (0.06 mg/ml); and (b) UV–vis spectroscopy to aggregation test of HCA II (0.1 mg/ml in 20 mM Tris–sulfate buffer at pH 7.75 at 60 °C) alone and in the present of SNImIL (0.06 mg/ml).

groups were covalently bond to the surface of silica nanoparticles, and the functionalization of the silica surface was successful.

The TGA showed that the loading of organic functions was 20% weight related to the imidazolium based ionic liquid modified silica nanoparticles (Fig. 1c). The immobilized ionic liquid was apparently stable up to 220 °C. From the N_2 adsorption–desorption isotherms of the modified and unmodified silica nanoparticles, one can conclude that the modified sample exhibited an isotherm shape similar to that of unmodified silica nanoparticles. Thus, suggesting no obvious changes in the structure after modification. Following surface modifications, a decrease in surface area was observed compared to the parent silica nanoparticles, which could likely be due to the presence of the functional groups.

In an initial screening experiment, the amount of [SNImIL] was optimized to achieve the best transparency in spectroscopy techniques. To determine the effect of [SNImIL] on HCA II, applied ANS fluorescence, firstly. Fig. 2a shows that the intensity of ANS fluorescence increased after addition of [SNImIL] to HCA II after incubation at 58 °C for 5 min incubation. This increase could be a reliable proof for the enhancement of hydrophobic patches on the protein surface. It appears that the protein unfolding caused an increase in hydrophobicity and led to aggregation. In spite of this fact, SNImIL induced disaggregation of HCA II (Fig. 2b). We found that SNImIL provides a hydrophobic microenvironment which protects the enzyme by inhibiting the mixing of the enzymes [72].

To determine whether disaggregation affects thermal reversibility, we examined differential scanning calorimetry (DSC) at pH 7.75 and selected [SNImIL] of 0.06 mg/ml (Fig. 3).

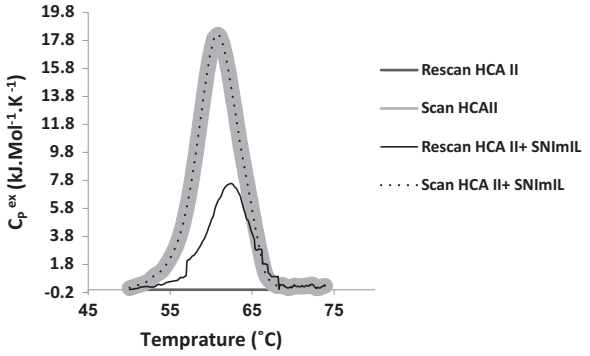


Fig. 3. The DSC profiles of scan of HCA II (1 mg/ml in 20 mM Tris–sulfate buffer at pH 7.75) in the absence/presence of SNImIL (0.06 mg/ml) and the rescan of HCA II in the absence/presence of SNImIL heated up 61 °C.

The percent reversibility was calculated from the enthalpies of first (ΔH_{first}) and second (ΔH_{second}) scans of the same sample using the equqtion [73]:

$$\text{Reversibility}\,\% = \frac{\Delta H_{second}}{\Delta H_{first}} \times 100\%$$

These results showed that the reversibility of HCA II-SNImIL was equal to 42%. SNImIL enhanced the percentage of reversibility and the amount of Tm. The increased Tm was a powerful proof for successful achievement of higher protien stability. Thus, these

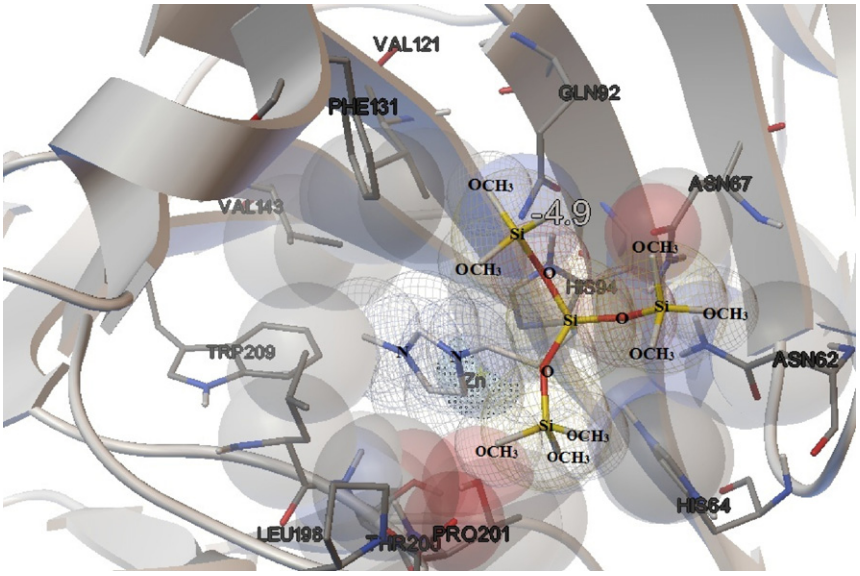


Fig. 4. Best predicted pose with AutoDock Vina for HCA-II as receptor and modified ionic liquid as ligand. Amount of predicted free energy of binding illustrated in gray digits as −4.9. Dimension of predicted free energy is kcal/mol. Zn ion from receptor structure is illustrated in central point of this figure in blue grid form. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

results indicate SNImIL is an effiecient biocompatible additive for the enhancemet of thermal reversibility and stability of HCA II.

SNImIL as an ionic liquid-modified silica nanoparticle consists of bulky organic cations that are combined with inorganic and organic anions which operate as a good additive for reversibility of HCA II. The surface-immobilized ILs led to enhance the hydrophobicity, which showed excellent effect on the thermal reversibility and stability of the protein, because of its surface interaction with the enzyme. A possible mechanism for stabilizing the enzyme in this system might be to provide a "protective" coating for the enzyme. It has previously been suggested that coating an enzyme in a hydrophobic IL can improve protein refolding [29]. We know that SNImIL may interact with the enzyme's microenvironment and affect its activity through different mechanisms including hydrogen bond, hydrophobic interactions, and charge–charge interactions. Here we propose that SNImIL provides a hydrophobic microenvironment by coating the enzyme. These results were confirmed by theoretical investigation via computational ligand–enzyme analysis.

The computational ligand–enzyme analysis was used to demonstrate the proposed mechanism for SNImIL improving the reversibility of HCA II. This plausible mechanism briefly expressed that hydrophobic interactions of SNImIL with HCA II interferes with protein–protein hydrophobic interactions, which resulted in disaggregation. Additionally, in these interactions, mantling of some critical residues with important roles in folding processes might protect them from participation in aggregation.

In the computational studies, HCA II (PDB ID: 1CA2) and SNImIL were used as receptor and ligand, respectively. Best-predicted pose obtained from AutoDock Vina, which is illustrated in Fig. 4. Ligand binding pocket is the HCA II active site pocket. The distance of labeled residues is up to 1.2 Å from atoms of the ligand. Thus, these residues are in contact and interaction with ligand. Five of these residues are from hydrophobic group of amino acids. Here it is demonstrated that Trp209, which is one of these five residues, has an important role in the native folding of HCA II [74]. As a plausible mechanism, covering of hydrophobic areas of HCA II could interfere with HCA II aggregation.

It has been confirmed that hydrophobic interactions between protein molecules cause aggregation. Thus, this suggested mechanism could be responsible for decreased aggregation and increased reversibility of HCA II in the present of modified ionic liquid in solution. In addition, using AutoDock 4.2 to get an analytical view of predicted free energy of binding illustrated more than 90% of ionic liquid affinity for HCA II caused by van der Waals interactions. It is clear that the van der Waals interactions create hydrophobic interactions and support the suggested mechanism. These results imply that SNImIL interacts with HCA II mainly through hydrophobic interactions leading to disaggregation.

The ILs are attached to silica nanoparticles with average diameters of 100 nm. Because of this linked particle to the ligand, the vast area of the active site face of the enzyme mantles with modified ionic liquids. Regarding this fact and best pose of the computational ligand–receptor analysis, many other residues including some other hydrophobic ones, including Pro-237, mantled with our ligand. Pro-237 has a critical role in HCAII folding [75]. Silica nanoparticles attached to ionic liquid may help the ligand to show anti-aggregation properties. As a result, the suggested mechanism from computational ligand–enzyme interaction analysis additionally denoted mantling of this area could protect the enzyme from aggregation and interference from involving critical residues for folding in interaction with other molecular enzymes during the denaturation process. These interactions prohibited the involvement of critical folding residues such as Trp209 and Pro237, in interactions with other molecular enzymes during the process of partial denaturation.

In the creation of the 3D model for modified ionic liquid, 7 atoms of silica nanoparticles inserted in ligand structure. This was enough for accepting the role of nanoparticles as a barrier for penetration of ligand and accepting other roles of nanoparticles in interaction with enzyme. The immobilized imidazolium based ionic liquid residue is illustrated in central point of Fig. 4.

4. Conclusion

This report indicates the enhancement of thermal reversibility and stability of HCA II assisted by silica nanoparticle supported imidazolium based ionic liquid. The biocompatibility properties of these nanoparticles make them extraordinary materials for biotechnology applications. Our data indicate that this additive is able to enhance the reversibility and protect HCA II against thermal aggregation and induce thermal reversibility and stability.

The computational ligand–receptor analysis cofirmed that SNImIL, which penetrated the active site pocket of the enzyme, mantled a vast area of hydrophobic residues.

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