Synthetic "chaperones": nanoparticle-mediated refolding of thermally denatured proteins†

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Thermally denatured chymotrypsin, lysozyme and papain are substantially refolded towards their native conformation by gold nanoparticle bearing dicarboxylate sidechains.

Protein refolding is the process by which a denatured protein reconfigures to its characteristic functional native state. Denatured proteins are responsible for numerous diseases such as Creutzfeldt-Jakob disease, bovine spongiform encephalopathy (mad cow disease), and amyloid-related illnesses such as Alzheimer's disease.[1,2] Additionally, numerous medical and biotechnological applications require the rescue of misfolded proteins produced by *in vitro* or *in vivo* genetic expression.[3] A practical solution to the issue of *in vitro* misfolding would be of great importance to biotechnology and would provide leads for the creation of *in vivo* therapeutics.

In general, native proteins have a hydrophobic core and a charged and/or polar group on the surface. The hydrophobic core helps to stabilize the tertiary structure of the protein by hydrophobic or π-stacking interaction[4] while the outer polar surfaces preferentially interact with the exterior aqueous medium. In cells, chaperones such as GroEL and GroES are used to stabilize unfolded proteins and hinder aggregation, allowing subsequent folding in a controlled manner.[5] From the established mechanism of the molecular chaperone machinery, a variety of biomimetic refolding strategies have been introduced. Rozema and Gellman presented an elegant two-step protein refolding process: (i) the capture step, in which a denatured protein binds with an artificial host molecule to prevent aggregation and (ii) the release step, where host molecules are removed from refolded proteins using competitive guests.[6] In this protocol the hosts are hydrophobic in nature like GroEL or GroES, allowing interaction with the hydrophobic domains of proteins to prevent aggregation. Other examples of this approach include using linear dextrins, hydrophobized carbohydrate nanogels, stimuli-responsive polymers, and liposomes to effect refolding.[7] Alternatively, hydrophilic/amphiphilic additives have been used to effect refolding, including polyamines, amino acids, and polyethylene glycol.[8]

Selective binding to the hydrophobic regions of proteins provides one route to protein refolding. Recognition of polar residues provides an alternative paradigm, with refolding dictated by complementary electrostatic interaction with the exposed charged residues of the denatured protein. The large surface area and surface tunability of nanoparticles[9] make them excellent candidates for this approach. In our previous studies we bound and denatured α-chymotrypsin (ChT) using anionic gold nanoparticles and then released the protein by the addition of cationic surfactants as a releasing agent.[10] Based on these studies, we hypothesized that highly charged nanoparticle based hosts could serve as refolding agents by interacting with charged residues on denatured proteins, facilitating refolding and preventing aggregation. After the partial refolding of the proteins, they could then be released from the nanoparticle by increasing the ionic strength of the solution,[11] thereby attenuating electrostatic protein–particle interactions (Fig. 1b). We have recently shown that 2-(10-mercaptodecyl)malonic acid functionalized 2 nm core gold nanoparticles (**AuDA**) form a high affinity complex with ChT due to its high negative charge density,[12] making it an excellent candidate for assisting protein refolding. To test this hypothesis, we used three cationic proteins, ChT, lysozyme and papain, as model proteins (Fig. 1c). In our studies, the proteins were denatured thermally, with **AuDA** then added to refold the denatured proteins. The enzymatic activities of papain and ChT were studied to determine the extent of refolding.

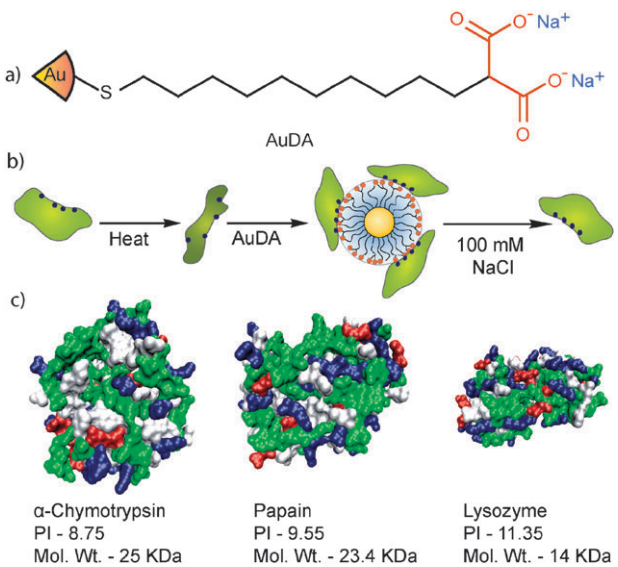

Fig. 1 (a) Schematic representation of the structure of the **AuDA** (2 nm core) and (b) thermal denaturation followed by nanoparticle mediated refolding of proteins. (c) Surface structural features of three positively charged proteins used in the refolding study. Colour scheme for the proteins: basic residues (blue), acidic residues (red), polar residues (green) and nonpolar residues (grey).

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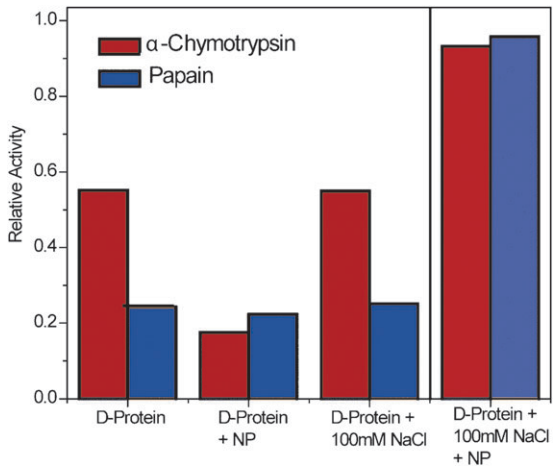

Fig. 2 Enzymatic activity of thermally denatured ChT and papain (3.2 μM) in the presence of **AuDA** (0.8 μM) and 100 mM NaCl solution in 5 mM sodium phosphate buffer (pH = 7.4).

Additionally, circular dichroism (CD) was used to assess the refolding process of all three proteins.[13,14]

The proteins were denatured by heating in 5 mM sodium phosphate buffer at pH 7.4 for 30 min (ChT and papain at 80 °C, lysozyme at 60 °C). The **AuDA** was then added and the mixture was allowed to stand for one hour. 100 mM NaCl was then added to release the refolded protein through disruption of the electrostatic interaction.

The activities of ChT and papain were measured before and after release of the protein by increased ionic strength. The activity of ChT was monitored using *N*-succinyl-l-phenylalanine *p*-nitroanilide (SPNA). Upon release by 100 mM NaCl solution highly efficient (~93%) restoration of activity was observed with **AuDA**, whereas only ~50% activity was observed with salt alone (Fig. 2). Similar enzymatic studies were performed with papain using *N*-α-benzoyl-L-arginine *p*-nitroanilide (BAPNA) as a substrate in the presence of β-mercaptoethanol as an activator. Similarly to ChT, the enzymatic activity of the thermally denatured protein increases to ~97% in the presence of **AuDA** compared to the native protein.

Further insight into the refolding process was obtained by using CD spectra to estimate the secondary structure of proteins and monitor the conformational changes during refolding. To estimate the protein conformation during binding and release from **AuDA**, we performed CD measurements with the denatured and refolded proteins (Fig. 3). CD spectra of protein with 100 mM NaCl could not be monitored at wavelengths shorter than 200 nm due to its high absorbance from the increased salt concentration.

The CD spectrum of native ChT has two characteristic minima at 202 and 230 nm.[15] Denaturation of ChT results in the blue shift of the 202 nm feature, and disappearance of the minimum at 230 nm (Fig. 3a).[16] The addition of 100 mM NaCl to thermally denatured ChT does not result in any spectral changes. In contrast, after incubation with **AuDA** the minimum at 202 nm was regenerated, consistent with the observed restoration of activity. The minimum at 230 nm was not restored, which is indicative of localized misfolding.[17] Estimation of secondary structures (helices, strand, turn and random coil) using DICHROWEB indicates significant refolding of the protein structure. According to the calculation, the proportions of each of the secondary structure motifs are far closer to the native structure (Fig. 3d). Similar experiments were also performed with the other two cationic proteins, lysozyme[18,19] and papain[20] with consistent results.

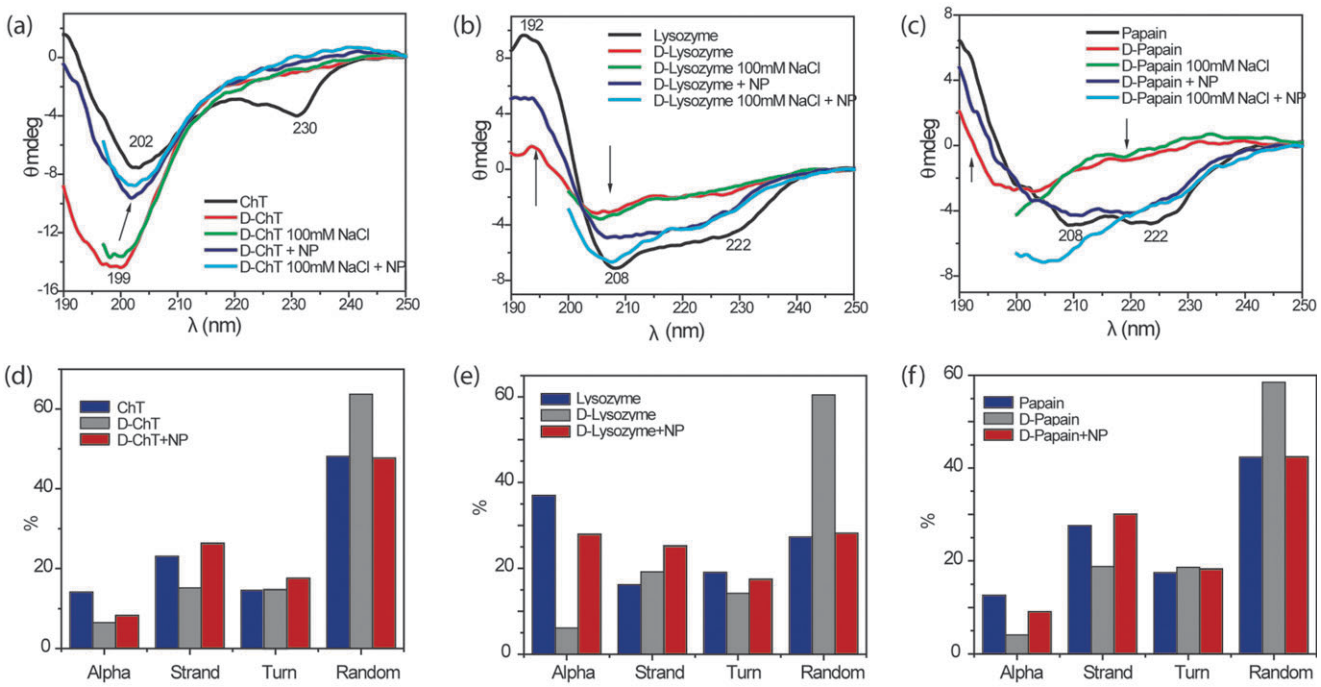

Fig. 3 CD spectra of proteins, thermally denatured proteins (5 μM), denatured proteins with NP (1.25 μM) and in the presence of 100 mM NaCl after 4 h incubation. (a), (b) and (c) are CD spectra for α-chymotrypsin, lysozyme and papain, respectively.

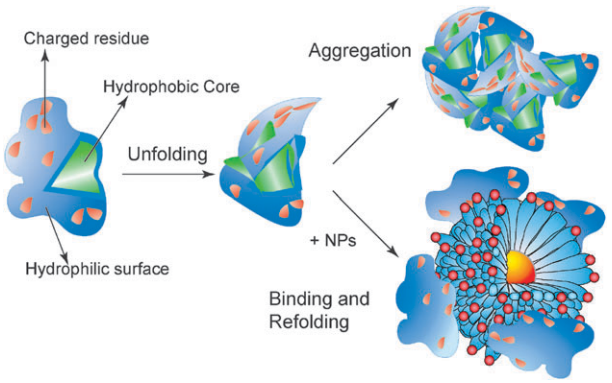

Fig. 4 Illustration of the thermally induced protein unfolding process and the exposure of the hydrophobic core followed by either aggregation in the absence of nanoparticle or binding and refolding in the presence of nanoparticle.

In our study the cationic proteins have positive residues (*i.e.* arginine and lysine) on the surface which prevent protein aggregation. During the thermal denaturation, the hydrophobic inner core becomes exposed, causing aggregation by intermolecular association of the hydrophobic core.[21,22] Employment of **AuDA** nanoparticles results in a nanoparticle–protein complex by binding with unfolded proteins through the electrostatic interaction with positive residues. The overall high negative charge of these complexes, as evident from the zeta potential measurement (Table S2†), prevents the aggregation, thereby promoting correct refolding (Fig. 4). Upon removal of negatively charged nanoparticles by increasing the salt concentration, the released partially refolded proteins are poised to fold to a native-like structure. During this refolding process the protein passes through several intermediate structures as established by NMR analysis.[23] Interestingly, for some proteins an intermediate structure is present in equilibrium with other structures.[24] In the case of lysozyme, an isosbestic point at 202 nm observed in the CD study clearly suggests an equilibrium between the intermediate states.

In conclusion, we have demonstrated the refolding of denatured proteins using nanoparticle hosts. In the present study we used highly anionic **AuDA** nanoparticles. It is likely that this process is general, and refolding of anionic proteins will be aided by cationic nanoparticles. We are exploring this possibility along with applying this strategy for the rescue of misfolded proteins during expression in biotechnology applications.

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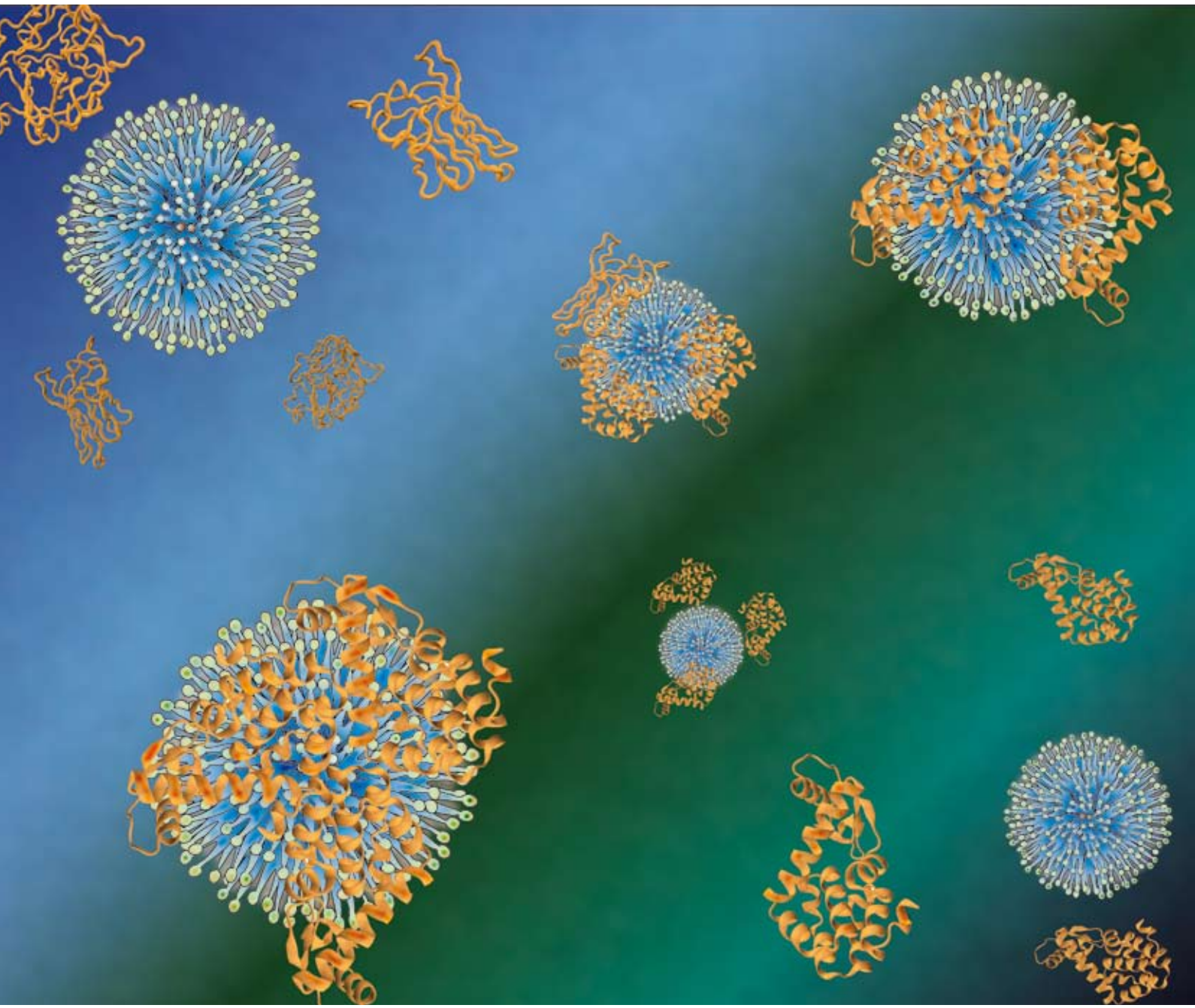

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