"Cleaning" of nanoparticle inhibitors *via* proteolysis of adsorbed proteins

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Cytochrome *c* adsorbed to anionic nanoparticles is selectively proteolyzed by trypsin, providing a mechanism for the catalytic degradation of proteins.

The binding of synthetic molecules to large areas on protein surfaces provides an alternate approach to active site targeting for enzyme inhibition,[1] as well as an effective means of regulating protein–protein interactions. This surface recognition approach relies upon the interaction between amino acid residues on the surface of the protein and synthetic receptors. With this strategy, adsorption to nanoparticle surfaces can inhibit protein function by binding and sterically blocking the surface of the protein. Alternatively, inhibition can arise through binding-induced changes in the protein conformation.[2] These changes in the conformation of a protein may make it susceptible to proteolysis through the accessibility of the backbone amide bonds to the active site of a protease.[3] Proteolysis would then provide a means for "cleaning" these receptors, greatly enhancing their ability to regulate, as opposed to simply inhibit biological processes.

Denaturation of proteins can be achieved in a variety of ways, such as high temperatures, high concentrations of denaturants or the addition of surfactants. Although denaturation in this manner is easily attained, it is inherently non-selective when compared to cellular-based proteolytic pathways.[4] Selective targeting of proteins for proteolytic degradation has been successfully demonstrated by synthetic scaffolds such as the use of metalloporphyrins to bind and induce proteolysis *via* denaturation.[5] However, gold nanoparticles present an alternate and advantageous synthetic scaffold for targeting protein surfaces,[6] and have been demonstrated to bind bio-macromolecules,[2,7] facilitate DNA transfection[8] and reversibly inhibit enzymes.[9] The ease of fabrication and tunability of the organic monolayer makes them attractive tools for selective binding and denaturation of proteins, thereby allowing a control over the proteolytic degradation of the target proteins by proteases.

Recently, we have used nanoparticles to target protein surfaces through complementary electrostatic interactions.[2,10] Here, we report the use of mercapto-undecanoic acid nanoparticles (MUA) and thioalkylated tetra(ethylene glycol) functionalized nanoparticles (TCOOH, Fig. 1), featuring a 2 nm gold core diameter, to target cytochrome *c* (cyt *c*) for modulation of catalytic proteolysis. These nanoparticles were synthesized using previously published procedures[11] and are 100% functionalized with carboxylate groups. The binding and subsequent proteolysis depicted in Fig. 1 is achieved by incubating the protein with the nanoparticles for 1 h at room temperature and thereafter incubating with trypsin for another hour at 37 °C.

The binding ratios of cyt *c* (equine heart) with both MUA and TCOOH nanoparticles were determined by native gel electrophoresis. Nanoparticles were added to increasing concentrations of protein and run on a 1% agarose gel. The binding stoichiometry was found to be ~1 : 4 nanoparticle to protein (Fig. 2).

Circular dichroism (CD) was employed to determine the change in protein structure upon binding to the nanoparticles. Cyt *c* was incubated with both MUA and TCOOH nanoparticles for 1 h before the CD spectra were recorded. Cyt *c* displayed a change in the CD spectra around 222 nm and 208 nm when bound to MUA nanoparticles. A subtle change in the CD spectra of cyt *c* with MUA as compared to spectra of cyt *c* with TCOOH was observed (Fig. 3A). When trypsin was added to both MUA–cyt *c* (MUA digest) and TCOOH–cyt *c* (TCOOH digest), there was a loss of α-helical secondary structure, as displayed by a loss of intensity centered around 222 nm (Fig. 3B). The change in secondary structure around 222 nm with the TCOOH digest was found to be smaller as compared to the MUA digest. This loss of α-helical content suggests that there is degradation of cyt *c*. Both the MUA

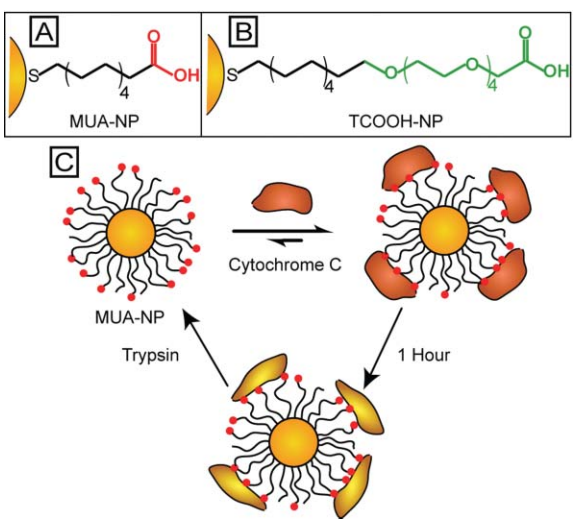

Fig. 1 The monolayer composition of (A) MUA and (B) TCOOH nanoparticles. (C) Nanoparticles were added to a solution of cyt *c* at a stoichiometric binding ratio and incubated for 1 h at room temperature. Trypsin was then added (1 : 150 cyt *c* : trypsin) and incubated at 37 °C for 1 h.

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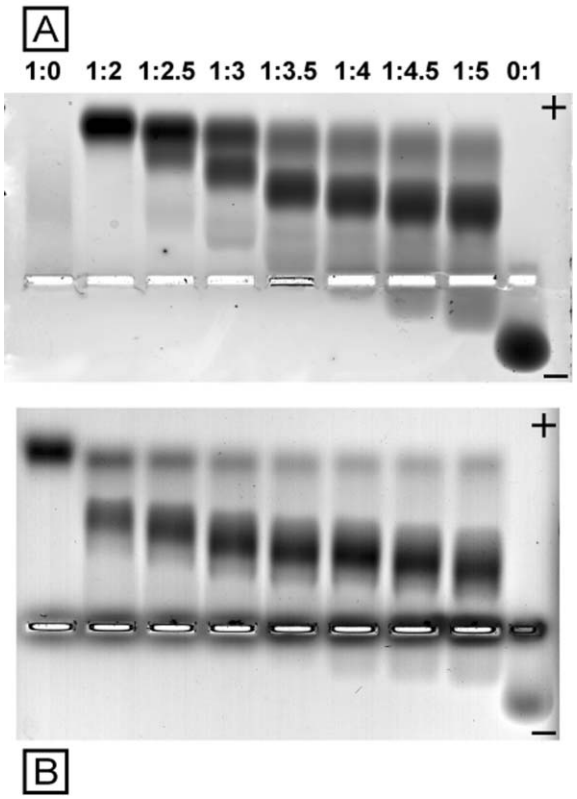

Fig. 2 Gel electrophoresis displaying different nanoparticle : protein ratios for (A) MUA and (B) TCOOH nanoparticles indicating ~1 : 4 nanoparticle–protein binding.

digest and the TCOOH digest were subsequently monitored at 222 nm for 1 h to determine the loss of secondary structure. By 60 min, the signal at 222 nm for the MUA digest had decreased by roughly half when compared to the control (cyt *c* incubated with trypsin only). There was only a slight change in signal of the TCOOH digest in comparison to the control, indicating that MUA particles have a much greater effect on proteolysis than their TCOOH counterparts.

To obtain further evidence of proteolysis, a discontinuous SDS-PAGE was run and analyzed for both the MUA digests and the TCOOH digests at 0, 5, 10, 15, 30 and 60 min.[12] The band corresponding to cyt *c* had disappeared by 30 min in the MUA digest but was still present at 60 min for the TCOOH digest. This indicates that cyt *c* was digested by trypsin to small peptides undetectable by the SDS gel. The bands present on the gel were quantified by using NIH software Image J.

The combined studies suggest that while the MUA nanoparticles make cyt *c* greatly susceptible to proteolysis, the TCOOH nanoparticles do not enhance trypsin-mediated proteolysis. The difference in behavior between the two nanoparticles can be explained by the difference in their monolayers. The MUA nanoparticles have a hydrophobic alkyl chain terminated with a carboxylate moiety while the TCOOH nanoparticles have a tetraethylene glycol linker between the hydrophobic alkyl chain and the carboxylate moiety. Cyt *c* can interact with the hydrophobic portion of the monolayer in the MUA nanoparticles but not with the TCOOH nanoparticles due to the intervening ethylene glycol linker. This accessibility of the hydrophobic MUA monolayer may

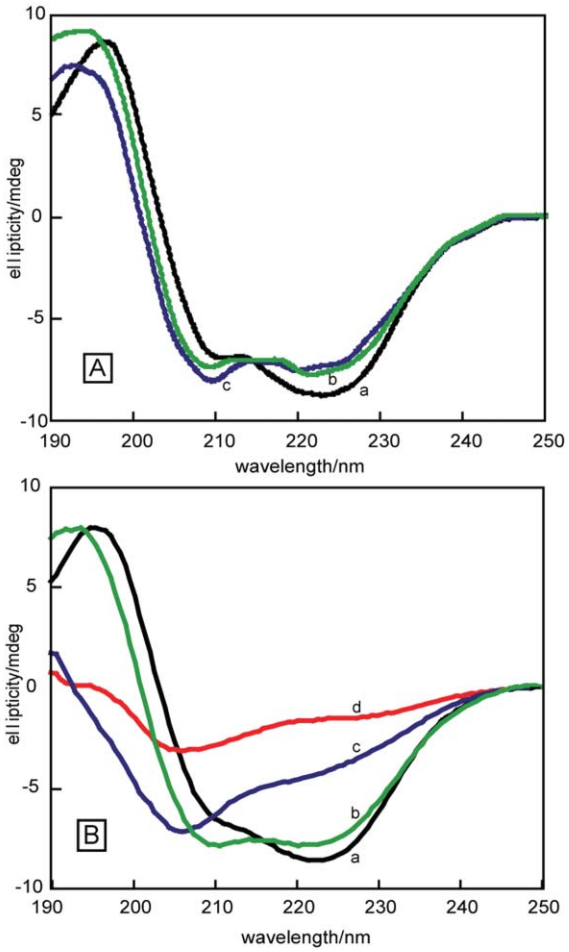

Fig. 3 CD spectra of (A) (a) native cyt *c* (b) cyt *c* with TCOOH and (c) with MUA; and (B) cyt *c* and nanoparticle digests: (a) native cyt *c* (b) TCOOH digest (c) MUA digest and (d) thermally denatured cyt *c* (95 °C). Reaction conditions are as follows: cyt *c* = 8 μM; nanoparticles = 2 μM; trypsin = 0.06 μM in 5 mM potassium phosphate buffer (pH 7.4).

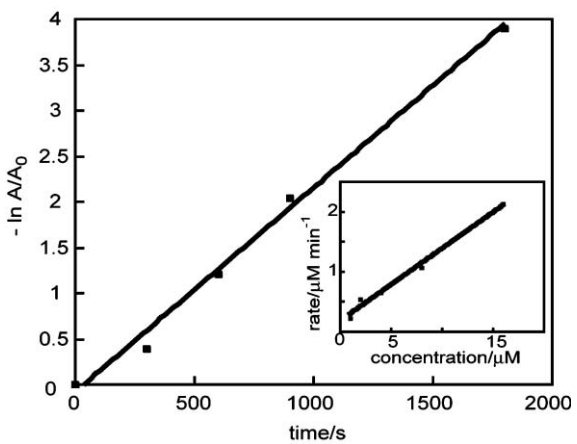

Fig. 4 First-order plot of cyt *c* digestion. A_o and A represent the Image J derived concentrations in the gel lanes for the digests of cyt *c* and MUA-bound cyt *c* respectively. Inset shows rate as a function of MUA concentration, indicating that proteolysis is first-order with respect to MUA concentration.

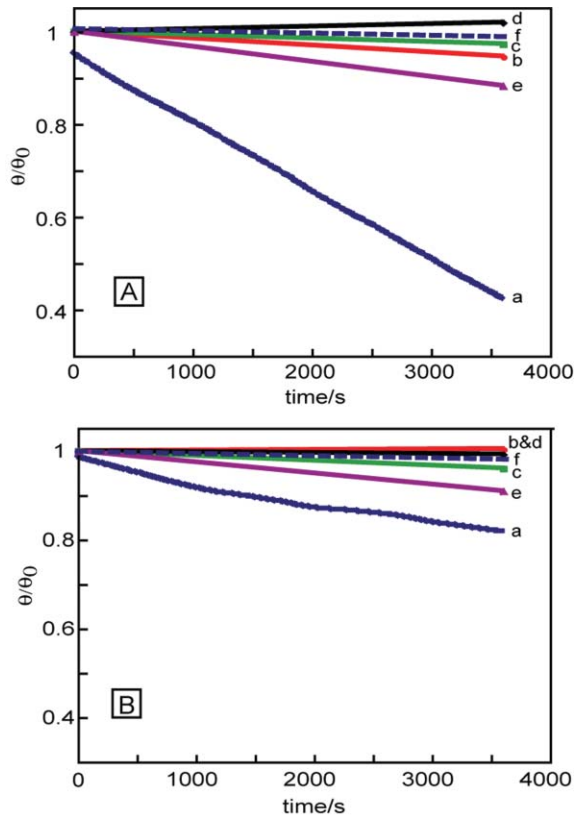

Fig. 5 Normalized (A) MUA and (B) TCOOH digestion of cyt *c* as compared to other proteins at 222 nm: (a) cyt *c*, (b) RNase, (c) myoglobin, (d) α-amylase and (e) BSA. (f) Digestion profile of cyt *c* without nanoparticles present.

induce subtle conformational changes in the protein, thereby allowing exposure of the peptide bonds in the protein for degradation by trypsin.[13]

The nanoparticle : protein ratio was then decreased from the binding stoichiometry and incubated with trypsin to determine whether the protein could be cleaned from the surface of the nanoparticle at lower stoichiometries, to explore the possibility of catalytic degradation (Fig. 1). CD experiments were conducted at ratios 1 : 4, 1 : 8 and 1 : 16, which also displayed the decrease in signal at 222 nm. The experiment was repeated with SDS-PAGE experiments at ratios of 1 : 4, 1 : 8, 1 : 16, 1 : 32 and 1 : 64 (nanoparticle : protein). Samples were taken every hour for 6 h and the bands corresponding to cyt *c* were quantified using NIH software Image J. There was a steady decrease in intensity for each of the samples with the lowest ratio being completely digested by 6 h. This suggests that the bound cyt *c* is being digested on the MUA nanoparticle surface which consequently enables the MUA nanoparticle to bind other proteins in solution. This allows for catalytic quantities of nanoparticles to be used in protein degradation reactions. A plot of $-\ln A/A_o$ *versus* time indicates that the reaction is first-order with respect to cyt *c* (Fig. 4) and the rate of digestion increases with increasing nanoparticle concentration (Fig. 4 inset), once again following a first-order rate profile.

A total of four other proteins were also chosen to determine if the mediation of proteolysis by MUA nanoparticles was selective. Native agarose electrophoresis experiments were conducted under the same conditions as with cyt *c*. RNase (bovine pancreas) was the only other protein in which binding was observed, also at a 1 : 4 ratio (nanoparticle : protein). Only weak binding was observed for bovine serum albumin (BSA), myoglobin (equine heart) and α-amylase (*Bacillus licheniformis*).

CD experiments were conducted under the same conditions as cyt *c* with each of the other four proteins. Each of the proteins changed only subtly or not at all when incubated with both the MUA and TCOOH nanoparticles. Upon incubation with nanoparticle and subsequently trypsin, little or no loss of CD signal was observed when compared to that of cyt *c* (Fig. 5). This indicates that the MUA nanoparticles are a highly efficient, selective catalyst for cyt *c* proteolysis.

In summary, we have demonstrated that MUA nanoparticles make cyt *c* susceptible to proteolysis. This ability to bind, denature, and degrade proteins indicates that these systems are capable of regulating protein levels in a catalytic fashion, analogous to cellular regulatory processes.

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