Disruption of protein–protein interactions using nanoparticles: inhibition of cytochrome c peroxidase†

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Functionalized gold nanoparticles bind selectively to cytochrome c or cytochrome c peroxidase and inhibit enzyme turnover.

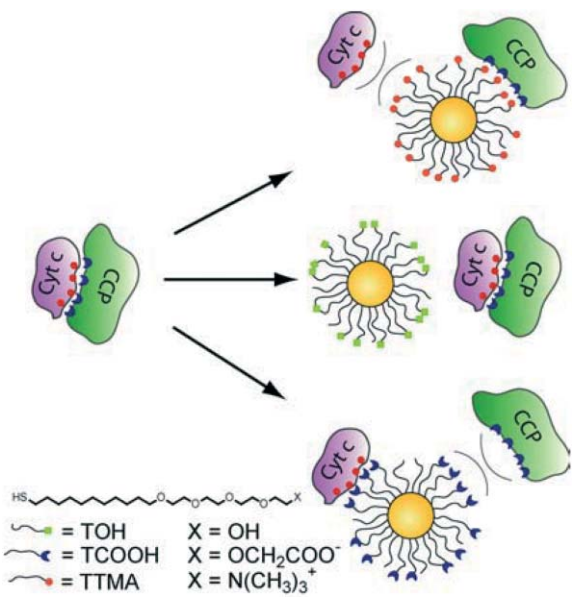
Protein–protein recognition is a key aspect of many complex cellular functions, including apoptosis[1] and angiogenesis.[2] Control over surfaces involved in interprotein recognition holds the potential for therapeutic applications,[3] however this approach is challenging due to the difficulty of designing molecules that selectively bind to the surface of a target protein.[4,5] We have recently demonstrated that mixed-monolayer protected colloids (MMPCs) are effective at recognizing protein surfaces.[6,7] These MMPCs are commensurate in size with proteins, biocompatible, and possess surfaces that are easily imparted with functional groups.[8] To extend this recognition motif to the interruption of protein–protein interactions, we have chosen the well-defined interaction between cytochrome c peroxidase (CCP) and cytochrome c (Cyt c) as a target. This communication reports the selective inhibition of protein–protein recognition under turnover conditions in the CCP–Cyt c system.

CCP catalyzes the reduction of hydrogen peroxide to water, utilizing two electrons supplied by two equivalents of Cyt c in an ordered mechanism.[9] Cyt c binding to CCP has been shown by mutagenesis,[9] co-crystallization of the proteins,[10] and ITC[11] to involve a single moderate-affinity site ($K_D \sim 10$ μM). Steady-state kinetics indicate that the 1 : 1 adduct is relevant for the reaction of equine Cyt c with CCP.[12] There remains some debate over the potential relevance of a second, low-affinity Cyt c binding site observed with yeast Cyt c,[12] however it is clear that Cyt c from either species must bind to the moderate-affinity site of CCP prior to any electron transfer.

The moderate-affinity site is defined by salt bridges between the basic Cyt c (p*I* = 10.3)[13] and acidic CCP (p*I* = 5.3).[14] Specifically, the CCP residues Asn[38], Glu[35], and Glu[290] present a negative patch on the surface of CCP involved in the electron transfers with Cyt c.[10] The surface of Cyt c is rich in basic residues, with Lys[72], Lys[73], and Lys[8] found at the electron-transfer interface. These charged surface patches present ideal targets for inhibiting protein–protein recognition in the CCP–Cyt c system. As MMPCs recognize charged protein surfaces, we asked whether such recognition could be made selective, and competitive, in a protein recognition pair under turnover conditions.

Surface-functionalized MMPCs with gold cores (2 nm) were prepared utilizing thiolates with biocompatible TEG groups terminated in alcohol (**Au-TOH**), carboxylate (**Au-TCOOH**), or trimethyl-amine (**Au-TTMA**) functionalities. **Au-TCOOH** and **Au-TOH** nanoparticles were synthesized according to previous methods.[15,16] **Au-TTMA** nanoparticles were synthesized and purified by related methods (see supporting information†). Recombinant yeast CCP (MKT CCP) was expressed and purified as described by Goodin, *et al.*[17] Horse heart Cyt c (Sigma) was reduced with dithionite and purified on a G-75 column.[17] These surface-functionalized MMPCs were tested for their ability to interact specifically with recombinant CCP and horse heart Cyt c (Scheme 1).

Native gels were used to monitor binding between proteins and MMPCs in 10 mM Tris-HCl, pH 7.4, 0.7% agarose (Fig. 1A). CCP (11.8 μM) and Cyt c (14.3 μM) were loaded together in lanes 3–6. Lanes 1 and 2 are controls showing that CCP and Cyt c migrate as anticipated for their p*I* values. These concentrations of CCP and Cyt c were sufficient to favor substantial formation of the CCP–Cyt c adduct, as shown in lane 3. Protein bands for both CCP and Cyt c were shifted from the control lanes due to rapid equilibration between the bound and unbound states.



Scheme 1 Protein surface recognition by CCP, Cyt c, and MMPCs.

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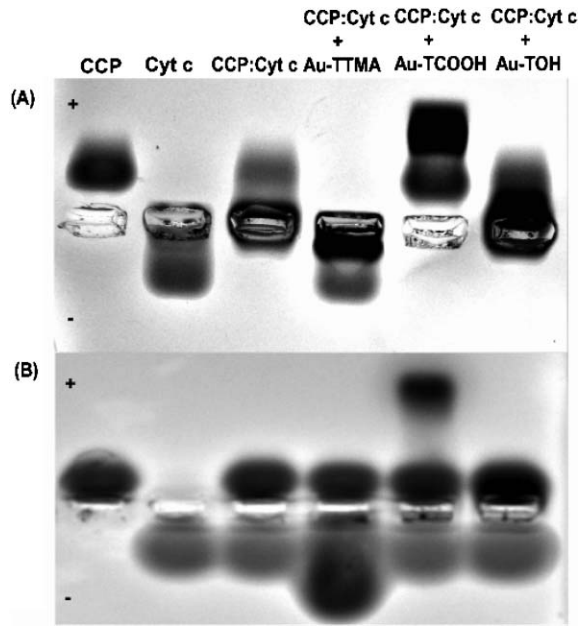

Fig. 1 Native protein gels (0.7% agarose). (A) 10 mM Tris, pH 7.40. (B) 10 mM Tris, 300 mM NaCl, pH 7.40. The cathode (+) is on the top.

The effect of added MMPC on CCP–Cyt c is shown in lanes 4–6, in which surface functional groups on the MMPCs lead to selective binding. Addition of **Au-TTMA** (11.4 μM) to CCP–Cyt c (lane 4) caused the CCP band to shift, but Cyt c migrates as if unbound, indicating that **Au-TTMA** selectively binds to CCP, disrupting the CCP–Cyt c adduct. Addition of **Au-TCOOH** (11.5 μM) to CCP–Cyt c leads to the appearance of free CCP, but shifted Cyt c, indicating that **Au-TCOOH** binds selectively to Cyt c. Addition of **Au-TOH** (11.5 μM) has no effect on the migration of CCP–Cyt c, indicating that the neutral particle binds to neither CCP nor Cyt c. Notably, **Au-TTMA** outcompetes Cyt c for the surface of CCP, and **Au-TCOOH** outcompetes CCP for the surface of Cyt c, indicating that the K_D for MMPC–protein binding is lower than the K_D for Cyt c–CCP ($K_D < 10$ μM).

To test the importance of charge complementarity, native gels were run at elevated ionic strength (Fig. 1B). Addition of 300 mM NaCl to the gel buffer disrupted the CCP–Cyt adduct (lane 3), causing both proteins to run the same as their control lanes. Furthermore, the NaCl also disrupted the MMPC–protein interactions, as Cyt c and CCP migrated as free proteins, with a separate band observed for the MMPC (lanes 4–6). These results indicate that electrostatics are largely responsible for the MMPC–protein recognition.

As the functionalized MMPCs selectively bind to CCP or Cyt c, they were tested for their ability to act as enzyme inhibitors. CCP (2.1 nM) and reduced Cyt c (5.4 μM) were thermally equilibrated in the presence of varying concentrations of MMPC, and the CCP activity assayed in 10 mM Tris-HCl, pH 7.40, 25.0 °C. Nearly saturating H_2O_2 (100 μM) was added to initiate turnover, with the rate of CCP turnover defined as half the rate of Cyt c oxidation determined spectrophotometrically.[12]

Under low-ionic strength conditions, nanomolar concentrations of **Au-TTMA** inhibited CCP turnover, but **Au-TCOOH** and **Au-TOH** did not (Fig. 2). The inhibition with **Au-TTMA** was fitted with the Hill equation: $V = V_{min} + (V_{min} - V_{max})/(1 + 10^{([I] - \log IC50)H})$, where H is the hill slope, the concentration of inhibitor, I, causing 50% inhibition is IC_{50}.[18] The fit to the data indicated that the **Au-TTMA** was a potent inhibitor of CCP, with $IC_{50} = 13$ nM, and exhibited positive cooperativity, with $H = -2.1$. High ionic strength (150 mM NaCl) caused **Au-TTMA** to be less potent as an inhibitor, $IC_{50} = 211$ nM, $H = -2.9$ (Fig. 3).

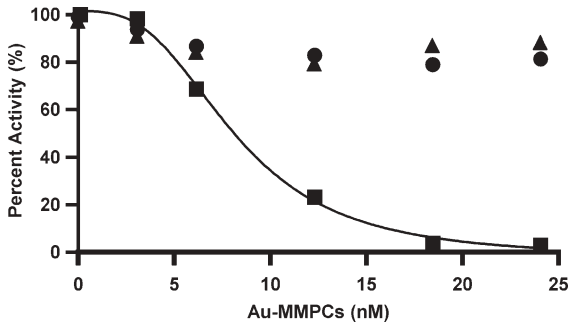

Fig. 2 Inhibition of CCP (2.1 nM) by **Au-TTMA** (squares); and lack of interaction with **Au-TCOOH** (circles), and **Au-TOH** (triangles) in 10 mM Tris, pH 7.40. The line is a fit to the Hill equation; see text for details.

That **Au-TTMA** inhibits CCP suggests that the **AuTTMA**–CCP adduct is unable to bind Cyt c productively, consistent with our native gel data. The inability of **Au-TCOOH** to inhibit turnover, despite its competence to bind Cyt c, likely reflects the high Cyt c–**Au-TCOOH** ratio (*ca.* 500 : 1) employed for our steady-state assays. The inability of **Au-TOH** to inhibit turnover is entirely consistent with the lack of binding between either protein and this particle.

The binding stoichiometry for the **Au-TTMA**–CCP and **Au-TCOOH**–Cyt c adducts were determined by circular dichroism.‡ Binding protein to nanoparticle led to insignificant changes in far-UV CD band at 222 nm, indicating that both CCP and Cyt c retain their dominantly α-helical secondary structure. The CD feature at 208 nm arising from a hemin transition[19] became more intense upon adduct formation, and was analyzed to evaluate the binding stoichiometry. Assuming that the MMPC has n identical and independent binding sites governed by a common dissociation constant (K_D), the binding isotherm is described by eqn (1), in which $[P]_o$ and $[Au]_o$ are the initial concentrations of protein and MMPC, respectively. MMPC–protein binding was fit by nonlinear least-squares curve-fitting (Fig. 4).

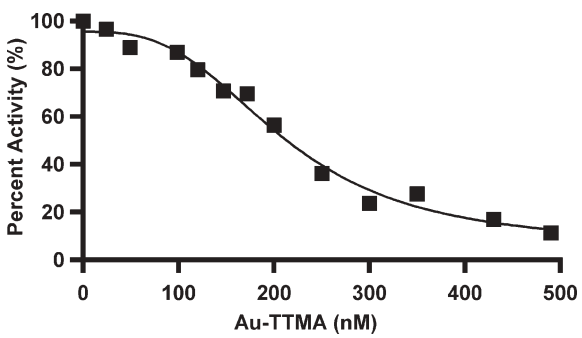

Fig. 3 Inhibition of CCP (2.1 nM) by **Au-TTMA** in 10 mM Tris, pH 7.40. The line is a fit to the Hill equation; see text for details.

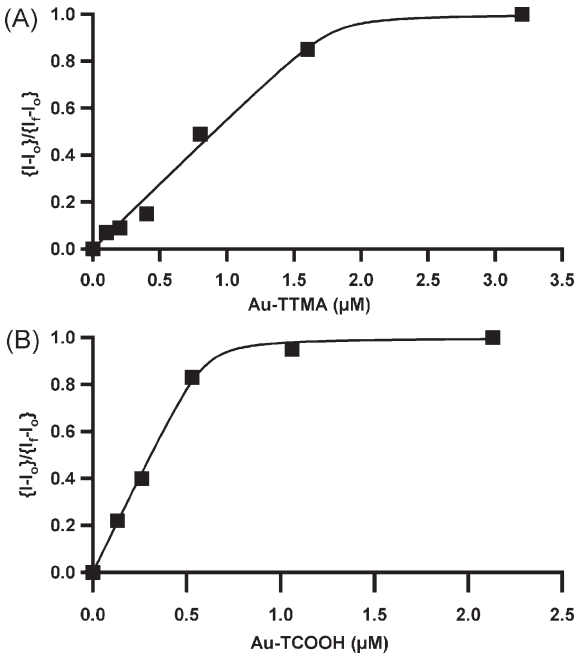

Fig. 4 Binding isotherms in 10 mM Tris, pH 7.40, as measured by CD at 208 nm. Lines are fits to eqn (1). (A) CCP (3.9 μM) titrated with **Au-TTMA**. (B) Cyt c (2.0 μM) titrated with **Au-TCOOH**.

$$\frac{I-I_o}{I_f-I_o} = \frac{\left([P]_o+n[Au]_o+K_D\right)-\sqrt{\left([P]_o+n[Au]_o+K_D\right)-4n[P]_o[Au]_o}}{2[P]_o} \quad (1)$$

The binding of CCP to **Au-TTMA** was fit with n = 2.1 ± 0.1 and K_D = 25 ± 30 nM. The binding ratio and K_D of **Au-TCOOH**–Cyt c was calculated as 4.0 ± 0.2 and 40 ± 31 nM respectively. The binding ratios may reflect the larger size of CCP (34 kDa) relative to Cyt c (12 kDa). The substantial error in the K_D is simply due to the fact that the concentrations of protein and MMPC is much greater than the K_D. Nevertheless, it is clear that both the **Au-TTMA**–CCP and **Au-TCOOH**–Cyt c adducts bind with about 3 orders of magnitude greater affinity ($K_D \sim 10^{-8}$ M) than the CCP–Cyt c adduct ($K_D \sim 10^{-5}$ M). To confirm the binding ratios, native gel shift assays were also performed. The native gels corroborate the formation of **Au-TTMA**–CCP and **Au-TCOOH**–Cyt c with a ratio of 1 : 2 and 1 : 4 respectively.†

Dynamic light scattering studies assessed the aggregation state of the MMPC–protein adducts. The diameters of **Au-TTMA** and CCP were measured as 11.6 ± 3 nm and 4.9 ± 2 nm, respectively. Mixing CCP (2 μM) with **Au-TTMA** (0.9 μM) formed an adduct with a diameter of 21 ± 5 nm, as expected for a simple 1 : 2 **Au-TTMA**–CCP adduct. The diameters of **Au-TCOOH** and Cyt c were measured as 10.1 ± 3 nm and 2.6 ± 1 nm respectively. The addition of Cyt c (20 μM) to **Au-TCOOH** (1.2 μM) formed an adduct with a diameter of 13.4 ± 3 nm, consistent with the 1 : 4 **Au-TCOOH**–Cyt c adduct anticipated from the CD data. In both cases, MMPC binding to protein formed discrete complexes, as opposed to extended aggregates.

In conclusion, we have demonstrated that surface functionalized MMPCs selectively interact with CCP and Cyt c based upon charge complementarity. The proteins retain their native structure upon binding MMPC, and binding is reversed by high ionic strength. Yeast CCP is inhibited by **Au-TTMA** in the low nM concentration range under turnover conditions. This inhibition of activity is consistent with **Au-TTMA** competing with Cyt c for the CCP surface. Further studies to characterize the inhibition mechanism are underway.

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Notes and references

‡ CD experiments were performed on a Jasco J-720 spectrometer, using a quartz cuvette with a 1 mm path length. Three scans were taken for each sample from 190 to 250 nm at a rate of 20 nm min^{-1}. All the experiments were performed at 25 °C with a 5 min equilibration before the scans.

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