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Reversible regulation of esterase activity *via* host–guest molecular recognition at the nanoparticle surface

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Abstract. Precise and controlled regulation of enzymes is an important aspect to understand their fundamental complex biological molecular mechanism. While many synthetic receptors were developed to inhibit enzymatic activity, they lack reversible control over their function. Herein, we present an engineered nanoparticle (NP) surface that synergistically combines host–guest assembly with protein surface targeting to reversibly control enzyme activity. We demonstrate the effective inhibition of anionic esterase enzyme activity upon electrostatic binding to dimethyl-benzyl ammonium terminated positively charged gold NPs. The NP surface upon threading by non-covalent host–guest interactions with CB[7] moiety has enabled the reactivation of enzyme catalysis. This reactivation has been further reversed by disrupting NP–CB[7] complex by employing a competitive orthogonal guest, hence leading to the controlled reversibility of enzyme activity. Tuning of NP surfaces by different supramolecular interactions and concomitant protein recognition on the NP surface can thus be emphasized as a significant tool for biotechnology applications.

Keywords. Enzymatic activity; reversible regulation; host–guest recognition; gold nanoparticle; esterase.

1. Introduction

Precise control over the activity of an enzyme is a useful tool for both fundamental studies and applied biomedical research. Either initiating or terminating enzyme function *via* synthetic means is remarkably beneficial to gain control over nearly all cellular processes like catalysis, metabolic pathways, DNA replication and signal transduction [1–5]. Within the biological milieu of a living cell, the regulatory aspects of enzymes are controlled by various internal chemical signals that block or alter the active domain of proteins. In recent years, various synthetic receptors, including small-molecule ligands [6–9], macrocyclic motifs [10–13] and self-assembled materials [14,15], have been fabricated to recognize the active site or the surface of enzymes. Structural engineering of these systems *via* chemical means has resulted in a number of efficient and specific receptors with the capability to modulate enzymatic activity. An important constrain with the majority of these receptors remains to be the fact that they irreversibly alter protein function. However, reversible modulation serves as a better control for many aspects. For example, by using reversible modulation, the same system can be studied over a short period of time with and without the altered protein activity. Additionally, reversible modulation minimizes downstream effects, contributing to a better understanding of the specific protein function in a biological system with a minimized secondary effect.

To achieve reversibility, it is essential to integrate stimuli–responsive functionality in the receptor or ligand design [16]. Towards this direction, in recent years, strategies to regulate enzyme activity were achieved *via* the incorporation of photoresponsive molecules into the receptor design [17–19]. For example, the light-induced *cis-trans* isomerization of azobenzene has been adopted to achieve reversible surface photoregulation of α-chymotrypsin enzyme,

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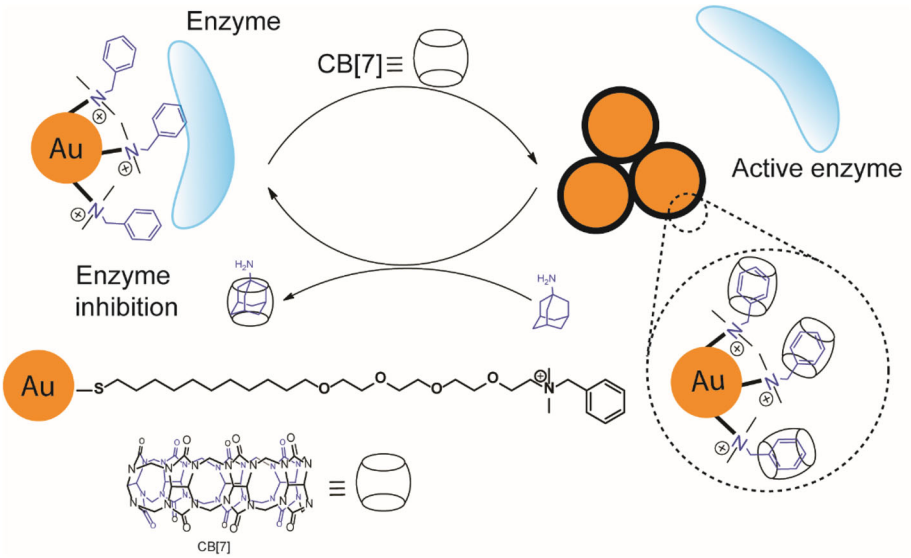
which utilizes phenylalanine-based photoswitchable inhibitor [20]. Although these light-responsive synthetic systems have attracted great interest owing to their unique advantages of non-invasiveness and spatiotemporal specificity, natural regulatory mechanisms that function in a living system generally utilize chemical signals for the reversible control of protein activity. In this article, we attempted to mimic the natural regulatory mechanism with a synthetic supramolecular system and created a synthetic receptor that utilizes chemical fuel to modulate protein activity reversibly.

Synthetic host–guest-based supramolecular systems exploit various non-covalent interactions, including hydrogen bonding, dipolar interaction, ion–ion, pi–pi stacking and van der Waals interactions, for their specific molecular recognition process. Importantly, the weak and reversible nature of these interactions enables the host–guest system to assemble/disassemble spontaneously in response to a range of orthogonally presented guest molecules. This dynamic and reversible nature of such host–guest assemblies has enabled a number of recent advances in biology [21–23]. For example, it has been elegantly employed to design indicator displacement assays (IDAs) for sensitive detection of various analytes [24–27]. Therapeutic activation strategies, including the stimuli–responsive release of pharmaceutical drug molecules and actuation of therapeutic gold nanoparticles (NPs), were established based on the reversible nature of the host–guest interactions [28–30]. Additionally, a capture and release strategy for membrane proteins at mild conditions was realized using host–guest binding motifs [31]. In the context of enzymatic regulation, Ghosh and Isaacs [32] developed a two-faced inhibitor that contains both active site and host binding domains to achieve reversible control over enzyme activity. This strategy represents an effective approach to regulate enzymatic activity *via* active site targeting. In this article, we demonstrate an alternative approach, where we synergistically combined host–guest assembly with protein surface targeting to reversibly control enzyme activity. Extending beyond the traditional small molecule-based inhibitor design that targets an active site, surface-recognition-based inhibition provides new targets for the control of enzymatic processes as well as allows the regulation of proteins that do not have a defined active site.

To achieve reversible protein regulation based on surface targeting, a preorganized receptor presenting complementary functional elements is required to bind with the solvent-exposed exterior of proteins. In recent years, various mixed monolayer protected gold NP scaffolds [14,33] have been employed to efficiently bind biomolecular surfaces due to their large surface area with a size comparable to proteins. Moreover, these particles provide well-controlled multivalent surface functionality that complements the structural complexity of proteins and allows them to mimic protein–protein surface interactions [15,34,35]. These divergently engineered NPs provide a level of selectivity as well as a unique opportunity to control the surface interactions by their complementary electrostatic nature and preorganized surface [36]. Likewise, supramolecular host–guest chemistry has been exploited to modulate NP surface property [30,37]. The supramolecular non-covalent threading of complimentary host/guest molecules on the NP surface can alter the physicochemical properties of the surface monolayer on NP. It has been demonstrated that this post-synthetic strategy for surface modulation of NPs, where the host/guest molecules are imparted as a monolayer, regulates the associated macromolecular recognition properties and binding strength of biomolecule-NP assemblies [38]. In the present work, we report the reversible modulation of esterase activity *via* surface recognition of enzyme with host–guest engineered gold NPs (scheme 1). We synthesized dimethyl-benzyl ammonium bearing cationic gold NP (Au-benzyl NP) to bind with the anionic surface residues of esterase enzyme by complementary electrostatic interaction. This surface binding resulted in inhibition of the catalytic activity of esterase, which is orchestrated by enzymatic hydrolysis of chromogenic substrate 2,4-dinitrophenyl butyrate (DNPB) to release 2,4-dinitrophenol (DNP) as a product. Besides protein surface binding, the dimethyl-benzyl ammonium headgroup on the AuNP surface acts as a recognition unit for complexation with cucurbit[7]uril (CB[7]) host. The capping of the Au-benzyl NP surface with CB[7] provided a supramolecular assembly, which showed a negligible effect on the enzymatic activity of the esterase. Strategically, we then reversed this assembly by providing orthogonal guest molecule 1-adamantylamine (ADA), having a high binding affinity towards CB[7] as compared to dimethyl-benzyl ammonium [39]. The removal of CB[7] from the NP surface leads to the restoration of surface binding property of the Au-benzyl NP with a concomitant enhancement in the inhibitory effect on the catalytic property of the enzyme. This study suggests NP surface tuning by reversible supramolecular interactions and its influence on protein activity represents a significant tool for future applications in biology and medicine.

2. Results and discussion

In our studies, we selected esterase from the porcine liver as a model enzyme. It is a highly active serine-based carboxylic-ester hydrolase that cleaves a variety of ester substrates [40]. It consists of a negatively charged surface at physiological pH due to pI (isoelectric point) value at 5 [41]. We used a surface-functionalized NP scaffold with a core diameter of $\sim$2 nm for the receptor design. To make Au-benzyl NP, pentanethiol-coated AuNP was used as a precursor NP. The precursor NP was decorated with thiolated dimethyl-benzyl ammonium ligand *via* place exchange reaction. The resultant Au-benzyl NP was found to be water-soluble and stable over months. With a pI of $\sim$5, esterase is expected to possess an electrostatically



Scheme 1. Schematic showing supramolecular tailoring of Au-benzyl gold NPs surface to achieve reversible modulation of esterase enzymatic activity.

complementary surface at pH 7.4 for binding with the cationic surface of Au-benzyl NP. We performed fluorescence titration studies to understand the binding efficiency of esterase protein with Au-benzyl NP. For this purpose, esterase was taken as a fluorescent probe due to its tryptophan residues, which shows emission maximum at 325 nm with excitation at 270 nm. To execute this experiment, 2 µl of esterase enzyme (1.19 µM) in 98 µl of 5 mM phosphate buffer (pH = 7.4) was taken. This esterase solution was then titrated with Au-benzyl NP of varying concentrations (0.238–1.90 µM) with the continued addition of 2 µl of Au-benzyl NP from the NP stock solution (11.9 µM). The fluorescence emission from esterase was recorded from 300 to 370 nm throughout the titration experiment. The intrinsic fluorescence of the esterase was found to be completely quenched upon the addition of Au-benzyl NP (figure 1a). This indicates that the protein is quantitatively bound to the NP surface where inherent fluorescence of the esterase is quenched *via* proximity to the Au core. The normalized fluorescence intensity of the esterase was plotted against the ratio of NP to esterase to analyse the binding isotherm (figure 1b). Association constant (K_s) of 2.11 × 10^6 M^{-1} and association stoichiometries (esterase/NP) of 2.79 were estimated from the non-linear least square curve-fitting analysis.

We performed an enzymatic assay using DNPB as a substrate to study the effect of Au-benzyl NP-mediated surface recognition on esterase activity. To conduct the activity assay, esterase (1.19 µM) was incubated with the varying concentrations of Au-benzyl NP (0.1–1 eq) for 15 min in 96-well microplates. As a control, only enzyme solution was also taken with the same experimental conditions.

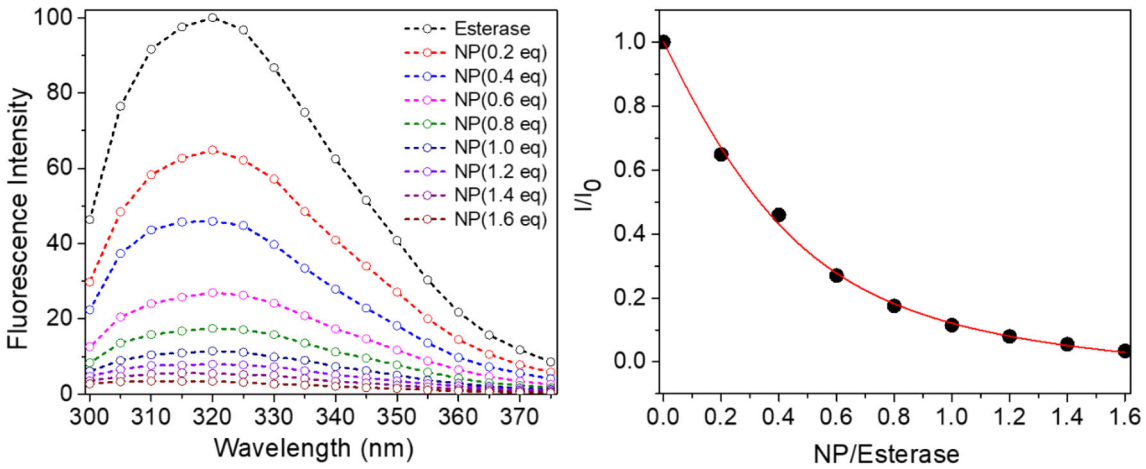


Figure 1. (**a**) Fluorescence titration of esterase with Au-benzyl NP. (**b**) Binding isotherm analysis of normalized fluorescence intensity *vs*. the ratio of NP to the enzyme. The red line shows the fitted curve.

Finally, to all these prepared solutions, 5 µl of chromogenic substrate DNPB from a stock solution in acetonitrile was added by a multichannel pipette for enzyme–substrate reaction studies. The total reaction volume was kept at 100 µl with acetonitrile concentration of 5% in 5 mM phosphate buffer, and the temperature was held constant at 25°C. The enzyme kinetics was monitored by UV–Vis spectroscopy through the release of DNP products at a wavelength of 420 nm. The reaction kinetics was recorded for 20 min duration with a 40 s time interval using a microplate reader. Control experiments, such as only substrate hydrolysis in 5 mM phosphate buffer (pH = 7.4), were also carried out in a similar manner. The formation of DNP with respect to time was calculated from the absorbance value, where it was converted to a concentration scale by a molar absorption coefficient of 6400 M^{-1} cm^{-1} for DNP [42]. The initial period of enzyme kinetics is given in figure 2a. Notably, in the presence of Au-benzyl NP, the rate of esterase-catalysed hydrolysis of DNPB significantly decreased, clearly suggesting the enzymatic activity inhibition through complex formation. We anticipate that the activity inhibition results from the obstruction of the active site by the esterase–NP complex formation, thereby reducing the accessibility of the substrates. We calculated the initial reaction rate from the slope of the DNP concentration *vs.* the reaction time. Figure 2b shows the initial reaction rate for different NP/esterase ratio. The inhibition effect of NPs on the enzymatic activity of esterase was further validated by varying the concentrations of the DNPB substrate. The auto-hydrolysis of the substrate DNPB, which is investigated as a control, is presented in supplementary figure S1. Overall, we observed a ∼60–80% decrease in activity in the presence of Au-benzyl NP (figure 2c). These observations demonstrated that Au-benzyl NP receptor can inhibit the activity of esterase *via* surface recognition.

We used MALDI-MS spectroscopy as a tool to study reversible host–guest complexation with NP. First, Au-benzyl gold NP was analysed using the α-cyano-4-hydroxycinnamic acid matrix to find the signatures of the pure NPs. Au-benzyl NP exhibited characteristic molecular ion peaks at *m/z* of 498.52 and 600.55, where *m/z* of 498.52 corresponds to benzyl thiol ligand and *m/z* of 600.55 represents disulphide ligand, which is formed between pentanethiol and benzyl thiol ligand (figure 3a). To find the signature of supramolecular assemblies, first, a host–guest

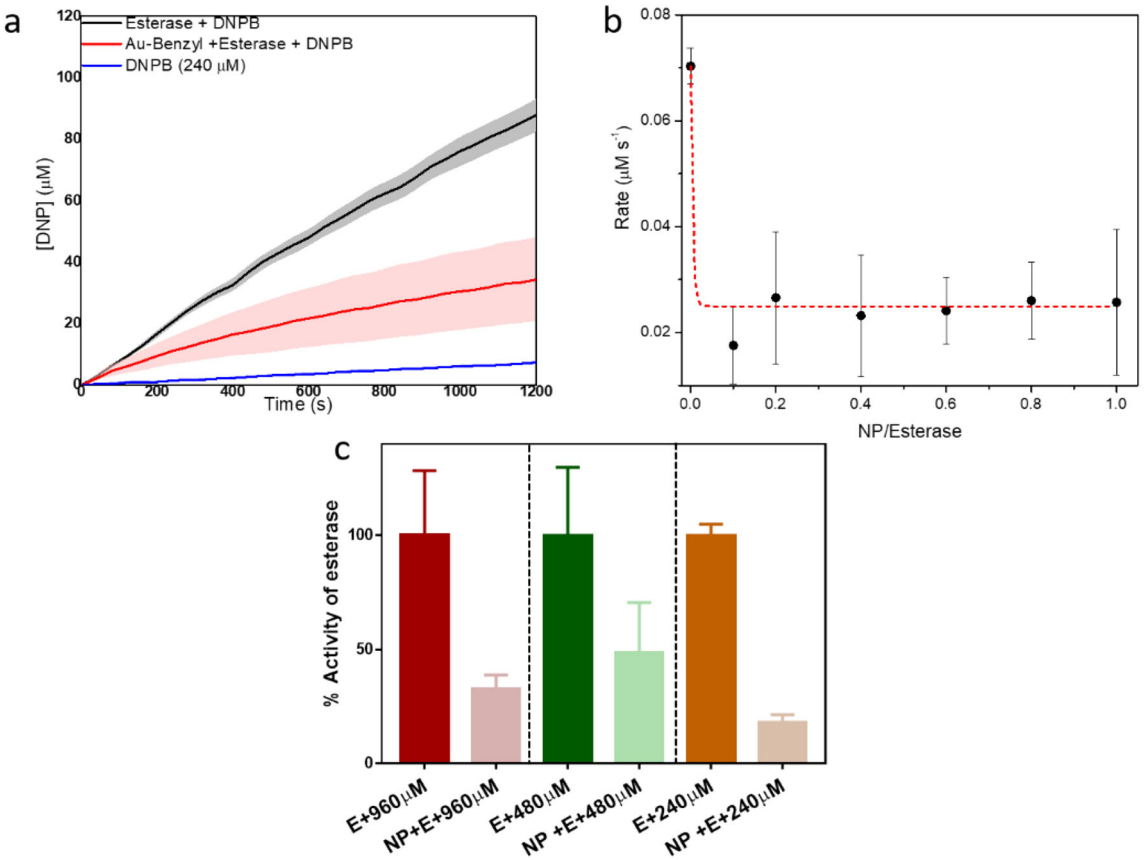


Figure 2. (**a**) Enzymatic inhibition assay of esterase hydrolysis of DNPB in the presence of Au-benzyl NP. (**b**) A plot of the initial reaction rate of DNPB hydrolysis *vs.* the ratio of NP–esterase complex. (**c**) Percentage activity of esterase with and without Au-benzyl NP at different DNPB concentrations (the plots have been normalized against esterase hydrolysis).

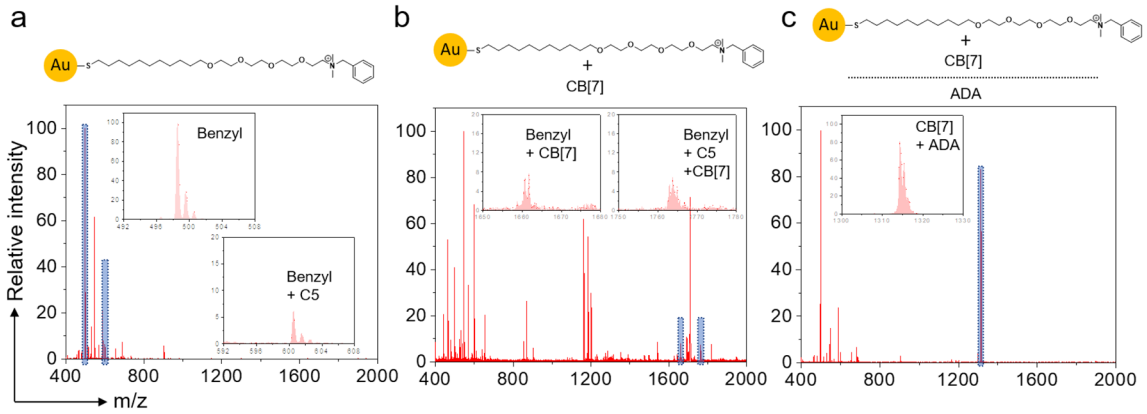


Figure 3. MALDI-MS characterization of (**a**) Au-benzyl gold NPs, (**b**) NP–CB[7] complex, (**c**) dissociation of NP–CB[7] complex by the addition of ADA.

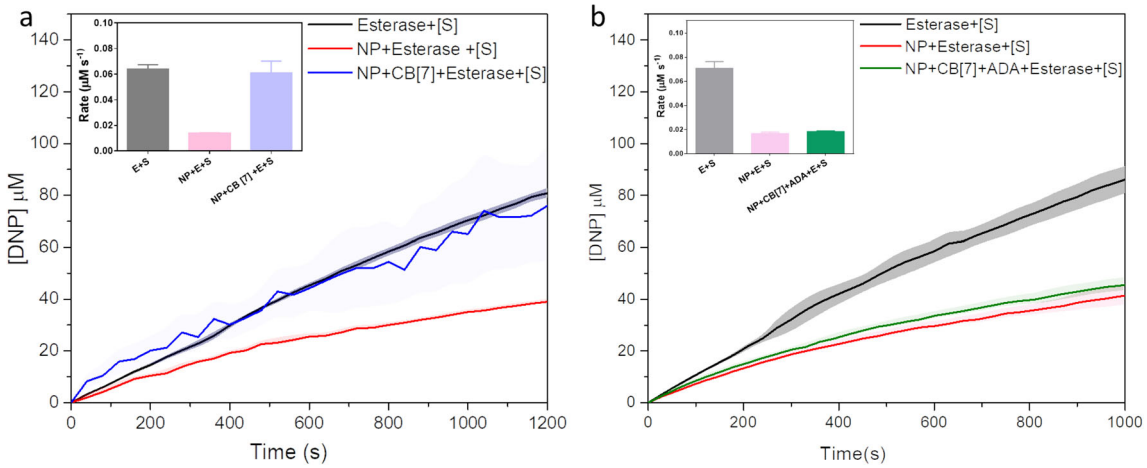


Figure 4. (**a**) Supramolecular host–guest mediated tuning of enzymatic activity using CB[7]-capped NP. (**b**) Enzyme kinetics showing retain of inhibitory effect *via* ADA trigger (inset: bar graph showing initial reaction rate of modified NP–esterase complexes, error bar represents the standard deviation of triplicate measurements).

complex was formed *via* mixing of 1 μM of Au-benzyl with 250 equivalent of CB[7] in aqueous solution. Analysis of this mixture *via* MALDI-MS displayed molecular ion peaks at *m/z* of 1660.79 and 1763.05, corresponding to the signature of host–guest complex formation. Molecular ion peak at *m/z* of 1660.79 is assigned to the complex of benzyl thiol ligand with CB[7], whereas *m/z* of 1763.05 is attributed to the complex of disulphide ligand of benzyl thiol and pentane thiol with CB[7] (figure 3b). Next, to stimulate the dissociation of NP–CB[7] assemblies, we used ADA as an orthogonal guest. To accomplish this, the same amount of ADA (250 μM, molar ratio of CB[7]/ADA = 1:1) was added to NP–CB[7] complexed solution and the resultant aqueous solution was analysed *via* MALDI-MS. The signature peaks of the host–guest complex of CB[7] with surface ligands on gold NPs disappeared in the MALDI spectra, indicating the reversal of the supramolecular assembly (figure 3c). Moreover, the MALDI-MS spectra showed the reappearance of ligand molecular ion peak at *m/z* of 498.44 with an additional new peak at *m/z* of 1314.41 corresponding to the ADA–CB[7] complex, further indicating efficient reversibility of the system.

We next attempted to expand the effect of host–guest complexation to enzymatic regulation. To understand the effect of assembly, we first determined the esterase enzyme activity in the presence of CB[7]-capped Au-benzyl NP. Subsequently, we compared this kinetics data against the kinetics of NP-inhibited enzyme and native enzyme. The release of DNP with respect to time for all these systems is shown in figure 4a. The kinetics data suggest that CB[7]-capped NP does not have any inhibitory effect on the enzymatic function. The initial rates are presented in the inset of figure 4a, which showed that ∼80% decrease in activity was achieved with only NP where a comparable rate of hydrolysis was observed for native enzyme or in the presence of CB[7]-capped Au-benzyl NP. On the one hand, this data demonstrated the effect of host–guest assembly on the NP surface in modulating the activity of the esterase

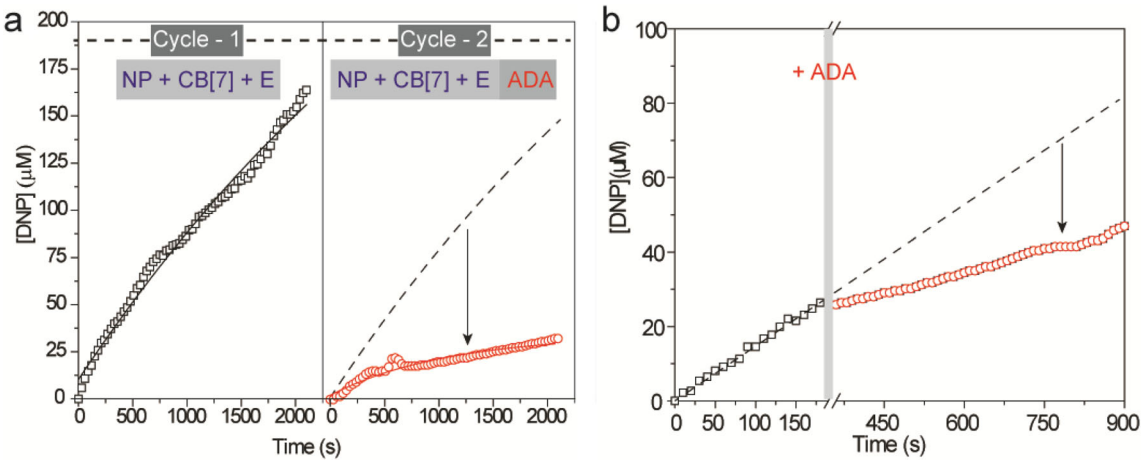


Figure 5. (**a**) *In situ* inhibition during the 2nd cycle of enzymatic activity. (**b**) Kinetic data showing immediate enhancement of inhibition upon the addition of the ADA trigger.

enzyme. On the other hand, this modulation presents an opportunity to achieve reversible tunning of enzymatic activity *via* the disassembly of the host–guest complex. To study this, we incubated 20 μl of Au-benzyl gold NPs (0.238 μM) with 20 μl of CB[7] (23.8 μM, 100 eq) for 10 min resulting into NP–CB[7] complex. In another case, we incubated this preassembled NP–CB[7] system with 10 μl of ADA (47.6 μM, 200 eq) for 10 min to release the CB[7] from NP surface. Subsequently, 2 μl of native esterase (1.19 μM) was added to both the systems and incubated further for 15 min. Finally, to all these prepared solutions, 5 μl of 4.8 mM of the DNPB substrate (final concentration = 240 μM) was added in phosphate buffer. The enzymatic activity which was kinetically monitored by recording the absorbance at 420 nm for the release of the DNP is shown in figure 4a and b. The kinetics data clearly suggest that the release of CB[7] *via* ADA trigger enhanced the inhibitory effect. The initial rate calculation demonstrates that the competitive disruption of NP–CB[7] complexes by ADA re-inhibited the enzymatic hydrolysis with a ∼75% decrease in enzymatic activity (figure 4b). Additionally, inhibition from the disassembled system was comparable to only NP where inhibition was ∼80%, suggesting a highly efficient reversible nature of the system (figure 4b). Once the reversibility of the system was established, we tested if we could regain the inhibitory effect of NP *in situ*. Two strategies were studied: (1) ADA trigger was provided to the NP + CB[7] system after the first cycle of the catalysis and (2) ADA trigger was added to the NP + CB[7] system while it was performing catalysis. The time-dependent release of the DNP is shown in figure 5. The kinetics data highlighted that ADA addition significantly inhibited the enzyme function during the 2nd cycle (figure 5a). Additionally, an immediate enhancement of inhibition was observed upon the addition of the ADA trigger, while the NP + CB[7] was performing catalysis (figure 5b). Both of these results successfully demonstrate the realization of *in situ* activation strategies.

3. Conclusion

In summary, we have developed a host–guest modified NP system that can act as a highly effective and reversible modulator for the enzymatic activity of esterase. We have shown that the strong electrostatic interaction between the cationic terminal group of gold NPs and anionic residues of esterase located around the periphery of the active site leads to ∼80% inhibition of enzymatic reactions. We further established that enzyme activity could be efficiently tailored by threading CB[7] host onto the NP surface, which can be further reversed by competitive complexation. The timescale of activation and deactivation of enzymatic activity, which includes benzyl NP–CB[7] and ADA–CB[7] complexation, is fast as a result of high association rate constant of host–guest complex formation. Overall, this approach provides a strategy to fabricate synthetic host–guest supramolecular nano-scaffolds capable of regulating and controlling versatile biomolecules for biotechnology and delivery applications.

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