Regulation of α-chymotrypsin activity on the surface of substrate-functionalized gold nanoparticles†

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A gold nanoparticle functionalized with substrates for α-chymotrypsin was fabricated to afford an enzyme modulator that exhibited enzyme-specific activation coupled with general inhibition of other proteases.

Regulation of enzyme activity in biological systems plays a central role in governing cell behavior.[1] Likewise, controlled release/activation of drug molecules is an important tool for therapeutic applications. External stimuli such as pH-response,[2] enzymatic degradation,[3] light irradiation[4] and redox agents[5] have been employed to trigger drug release. Nevertheless, a particularly attractive strategy for modulating biological processes would be to have the target enzyme directly digest the prodrug, releasing an inhibitor. This target-activated inhibition would provide a responsive system featuring a form of feedback control. Proteases provide a practical target for this strategy, as release of inhibitor can be triggered by amide hydrolysis. Moreover, proteases are central in the etiology of a diverse range of diseases,[6] making their inhibition of great importance in therapeutics.[7]

Our recent studies have demonstrated that mixed monolayer-protected clusters (MMPCs) with anionic functions inhibit the activity of α-chymotrypsin (ChT, pI = 8.75) through surface binding.[8] We envisioned that this interaction could be used to provide target-activated inhibition *via* conversion of a neutral substrate-functionalized particle to its anionic analog. As illustrated in Fig. 1, the substrate-functionalized MMPC would be digested by ChT to afford an anionic derivative, which then acts as a receptor of the enzyme. The binding of ChT onto the product would then block its active pocket, inhibiting enzyme activity. In this system, the MMPC is not only a substrate, but also a promodulator for ChT. An additional advantage of this system is the specificity in the hydrolysis process and hence activation can be imparted through choice of substrates, which possesses potential applications in chemical biology.[9]

N-Succinyl-L-phenylalanine *p*-nitroanilide (SPNA), a commercially available chromogenic substrate of ChT, was chosen as the functional unit for target-activated inhibition. Cluster **NP_SPNA** (Fig. 2) was prepared by place-exchange of 1-pentanethiol protected 2 nm gold nanoparticle[10] with SPNA-functionalized thiol ligand in dichloromethane (see Supporting Information†). Based on the UV absorption of SPNA moiety at 320 nm, it is estimated that there are *ca.* 90 SPNA-functionalized thiol ligands on each MMPC and the SPNA ligand coverage is thus around 90%.[11] A carboxylic acid-functionalized gold nanoparticle, **NP_TCOOH**, was prepared according to the reported procedure,[12] which serves as an anionic model of fully cleaved **NP_SPNA**.

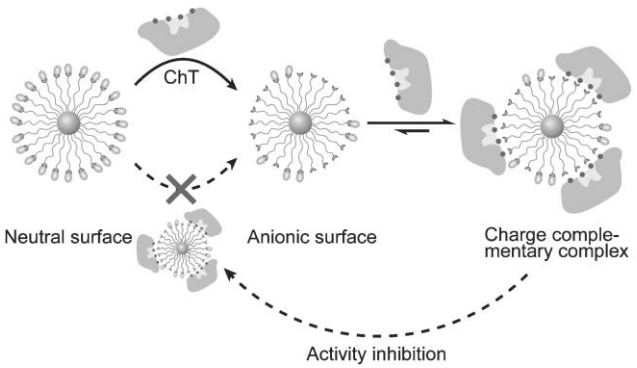

Fig. 1 Schematic illustration for the interaction of ChT with substrate-functionalized MMPCs. The neutral MMPC is transformed to a negatively charged derivative by ChT, blocking the active site of ChT and inhibiting its activity toward either the surface substrates or external substrates through complementary electrostatic interaction.

The nanoparticle **NP_SPNA** is highly soluble in DMSO, moderately soluble in dichloromethane and methanol, but insoluble in water. To employ it in biological studies, **NP_SPNA** was dissolved in DMSO–ethanol (1 : 2) and subsequently diluted

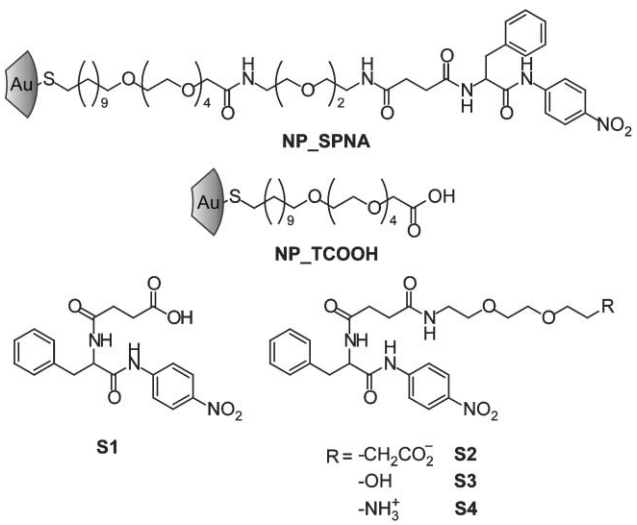

Fig. 2 Structure of MMPCs and the substrates examined.

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with 5 mM Tris–HCl buffer (pH 7.4), where the final concentration of DMSO–ethanol $< 5\%$ (v/v). UV–vis studies showed that upon incubation with ChT the initial absorption maximum of **NP_SPNA** at 320 nm disappeared with a peak appearing at 400 nm arising from 4-nitroaniline (Fig. S2†). Zeta-potential studies showed that **NP_SPNA** has a potential of 1.0 ± 0.6 mV in Tris–HCl buffer (pH 7.4, 100 mM). After incubation with ChT, a new peak appeared at -42.1 ± 2.5 mV, demonstrating that the MMPC surface was transformed from neutral to negative. These observations confirm that the SPNA-functionalized nanoparticle is a viable substrate of ChT.

Activity assays were carried out to follow the activity of ChT towards various substrates in the presence of **NP_SPNA**. As shown in Fig. 3a, the progress profiles for the generation of 4-nitroaniline are critically dependent on the substrate used. For negatively charged substrates **S1** and **S2**, the generation rate of 4-nitroaniline significantly decreases over time, indicating that the activity of ChT is depressed along with the complex formation as a result of hydrolysis of **NP_SPNA**. For neutral substrate **S3**, less pronounced inhibition was observed over time. For positively charged **S4**, however, no inhibition was observed within the monitoring period. This profile is consistent with previous studies using anionic particles, where the substrate selectivity of enzyme was attributed to the combination of steric hindrance and electrostatic interactions between substrates and the MMPC.[12] Briefly, the complex formation blocks the active site of ChT and thereby reduces the accessibility of substrates to the active pocket due to the evident steric hindrance. On the other hand, the bound protein is located at the periphery of the MMPC and is surrounded by negatively charged carboxylates. The encapsulation of negatively charged substrates like **S1** and **S2** by ChT must overcome additional unfavorable electrostatic repulsion, leading to the significant inhibition of ChT activity towards substrates of this type. For positively charged **S4**, however, the additional favorable electrostatic attraction between the substrate and the MMPC surface makes up for the steric hindrance, resulting in the maintenance of the reaction rate. Significantly, such substrate-selectivity demonstrates that particle–protein surface interactions are responsible for the reduction in activity, differentiating this process from normal competitive or noncompetitive product inhibition.

The inhibition ability of the current **NP_SPNA** was further proven by time course studies of activity upon incubation of ChT and excess **NP_SPNA**. The inactivation of ChT towards **S1**, **S2** and **S3** increased over time (Fig. 3b), indicating that the inhibition is based on the carboxylate coverage on the surface of MMPCs.‡ As before, the essentially unaltered activity of ChT towards positively charged **S4** (less than 10%) arises from the balance between favorable electrostatic attraction and unfavorable steric hindrance.

The enzymatic activity of various concentrations of ChT and constant concentration of **NP_SPNA** were further examined to evaluate the regulatory ability of **NP_SPNA**. As shown in Fig. 4, the generation rate of 4-nitroaniline increases linearly with the increase of ChT concentration. Upon incubation with **NP_SPNA**, however, the rate of 4-nitroaniline formation exhibits saturated enzymatic activity at higher concentrations. Obviously, this behavior arises from the increasing concentration of ChT expediting the rate of inhibitor production, which in turn inhibits more enzymes. Such nanoparticle–protein interaction provides a form of feedback control (see also Fig. S4†). Thus, **NP_SPNA** serves as a regulator of ChT concentration/activity, although the regulatory effect is still limited.

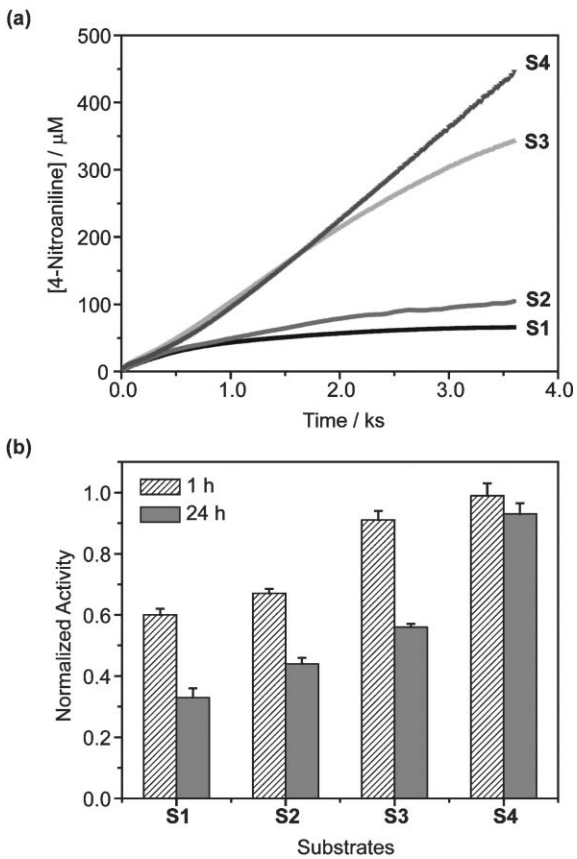

Fig. 3 (a) Progress curves for the hydrolysis of SPNA (**S1**) and its derivatives (**S2**–**S4**) in the presence of both ChT (3.2 μM) and **NP_SPNA** (0.8 μM). Concentration of substrates was kept at 2 mM. (b) Normalized residual activity of ChT (3.2 μM) towards various substrates (**S1**–**S4**) upon incubation with **NP_SPNA** (0.8 μM) for 1 h and 24 h, respectively.

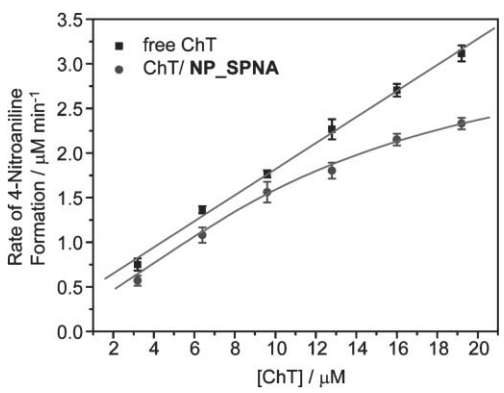

Fig. 4 Generation rate of 4-nitroaniline from substrate **S1** (2 mM) in the presence of various concentrations of ChT and ChT with **NP_SPNA** ([**NP_SPNA**] = 3.2 μM, incubated for 30 min). The absorption changes at 405 nm were monitored for a period of 10 min to get the rate of 4-nitroaniline formation.

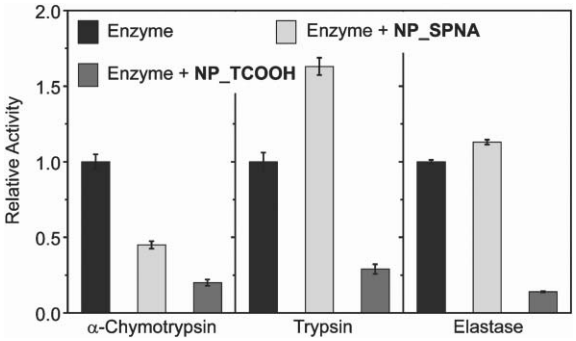

Fig. 5 Relative activity of (a) α-chymotrypsin (3.2 μM), (b) trypsin (1 μM), and (c) elastase (250 nM) in the absence and presence of **NP_SPNA** (2 μM) and **NP_TCOOH** (2 μM). SPNA (2 mM), N_{α}-bezoyl-DL-arginine 4-nitroanilide (BANA, 1 mM) and *N*-succinyl-Ala-Ala-Ala-4-nitroanilide (SAAANA, 0.5 mM) were used as substrates, respectively. The incubation period is 2 h.

As mentioned above, choice of substrate on the particle should provide selectivity in terms of activation. Control experiments showed that anionic **NP_TCOOH**[12] nonspecifically depressed the activity of ChT, trypsin (pI = 10.5) and elastase (pI = 8.5), three positively charged serine proteases in the chymotrypsin superfamily. The incubation of **NP_SPNA** with trypsin or elastase did not reduce their activity (Fig. 5).§,[13] In contrast to that, ChT catalyzes the hydrolysis of peptide bonds involving bulky aromatic side chains; trypsin and elastase are known to preferentially cleave peptide bonds adjacent to positively charged side chains and small uncharged side chains, respectively. Therefore, **NP_SPNA** cannot be digested by these two enzymes to provide the anionic inhibitors. With **NP_SPNA**, a specific activation process (ChT-mediated hydrolysis) generates a general inhibitor, since the product generated by ChT is a receptor for all three enzymes. Indeed, when **NP_SPNA** was incubated with a mixture of ChT, trypsin and elastase, the activity of three enzymes was commonly depressed (see Fig. S5†).

In summary, we have constructed an artificial system to mimic the self-regulation of biomacromolecular function in natural systems. The SPNA-functionalized gold nanoparticle acts herein as a proinhibitor of ChT, that blocks the active site of ChT through surface complementary electrostatic interaction after digestion by this protease. Thus, the MMPC and ChT form a self-regulation system where the concentration of the enzyme controls the generation rate of the inhibitor. Moreover, the inhibitor is chemoselective in terms of activation, and general in terms of inhibition. Such specific activation has great potential in biological systems as a controlled trigger.

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Notes and references

‡ Control experiments showed that the complex formation can be prohibited by increasing the concentration of Tris–HCl buffer to 30 mM or higher. In addition, when a mixed monolayer-protected nanoparticle functionalized with only 30% coverage of SPNA ligand on the surface was exploited, no inhibition on ChT activity towards all substrates was detected.

§ A different level of superactivation was observed with trypsin and elastase, presumably due to the micellar effect of the SPNA-functionalized nanoparticle, which can afford micellization of the hydrophobic substrates and thereby increase the enzyme activity.

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