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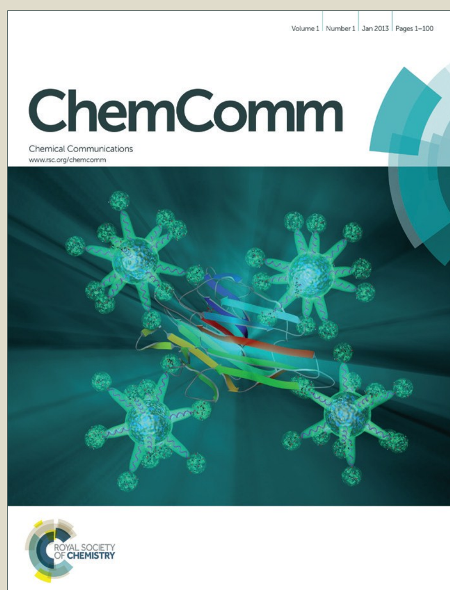

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Reversible pH-controlled Switching of An Artificial Antioxidant Selenoenzyme Based on Pseudorotaxane Formation and Dissociation

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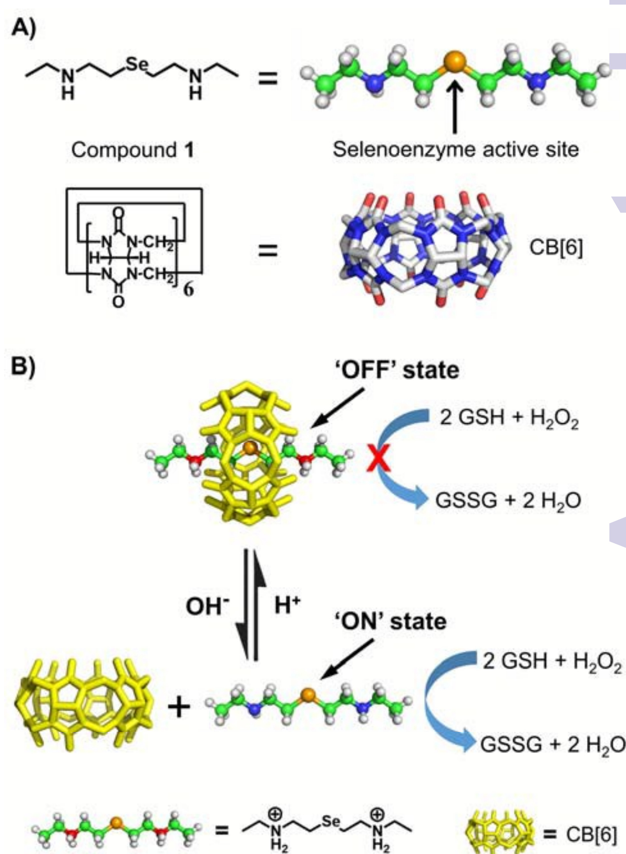
A pH-responsive artificial selenoenzyme was constructed by reversible binding between organoselenium compound 1 and CB[6] to form pseudorotaxane-based molecular switch in response to pH stimuli. The glutathione peroxidase (GPx) activity of the artificial selenoenzyme can be switched on/off at a mild and body suitable environment between pH=7 and pH=6.

Glutathione peroxidases (GPx) are antioxidant selenoenzymes that protect various organisms from oxidative stresses by catalyzing the reduction of hydroperoxides (ROOH) at the expense of tripeptide glutathione (GSH) and maintain the metabolic balance of reactive oxygen species (ROS) in *vivo*.[1,2] For exploring enzyme mechansim and developing highly efficient antioxdative drugs, imitating GPx functions has aroused high interests. Mimicking the functions of this important antioxidant enzyme was first focused on producting organoselenium/tellurium compounds.[3,4] The most successful one among these compounds is ebselen (2-phenyl-1,2-benzoisoselenazol-3(2H)-one) which is being used in clinical trials as a potential antioxidant medicine.[5,6] For further promoting the catalytic efficiency of the artificial GPx system, a series of chemically operated host molecules including cyclodextrins, catalytic antibodies and natural enzymes with high substrate specificity and pre-orientated catalytic selenium moieties were developed.[7-9] Afterwards, a genetic engineering strategy was further exploited to obtain well-defined selenoenzymes.[10,11] Furthermore, various nanoenzyme models which combine biological, supramolecular and nanoscientific strategies were well demonstrated.[12-14]

In recent years, other than improving the activities of the enzyme mimics, the design of smart artificial enzymes had attracted increasing attention.[15,16] These smart artificial enzymes were constructed by stimuli-responsive materials through changing their structures and properties in response to external stimuli such as temperature[17], pH[18], light[19,20] and others[21-23]. For example, our group successfully constructed a Ca^{2+}-responsive artificial selenoenzyme with a GPx-like active center by engineering of Ca^{2+}-binding protein recoverin. The engineered selenoenzyme can be switched on/off by Ca^{2+}-induced allosterism of the recoverin.[22] We also constructed smart supramolecular nanoenzymes with temperature-driven switching property by the self-assembly of cyclodextrin-based



Scheme 1. A) Structures of artificial antioxidant selenoenzyme compound **1** and cucurbit[6]uril (CB[6]). B) Schematic representation of the pH-controlled switching of the artificial antioxidant selenoenzyme. The 'OFF' state of artificial antioxidant selenoenzyme was formed by 1:1 compound **1**-CB[6] pseudorotaxane complex when pH ≤ 6, while the 'ON' state was formed by dissociation of the pseudorotaxane complex when pH ≥ 7.

host–guest supra-amphiphiles. The on/off switches of the peroxidase activity had been achieved through changing the temperature to

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trigger the reversible transformation of nanostructures from tube to sphere.[17]

Among these stimuli-responsive methods, the pH-responsive artificial enzymes have a bright future as potential medicines because the pH level in human body is varied with different organs, e.g. cancer and normal cells. Especially the ones that can be switched on/off around the neutral conditions are very mild and suitable for human body. One important example is the design of pseudorotaxane-based pH-responsive molecular switch. One of the pioneers of this work is Mock et al., who utilized a cucurbit[6]uril (CB[6]) 'bead' and a polyamine 'string' to design cucurbit[6]uril-pseudorotaxane-based pH-responsive molecular switch.[24] Cucurbit[n]uril (CB[n] n=5-8) is a family of macrocyclic compounds with n-glycoluril units and has a hydrophobic cavity that is accessible through two identical carbony-fringed portals.[25-27] As a member of this family, CB[6] has been studied extensively to form very stable 1:1 host–guest complex with diprotonated diaminoalkanes, particularly diaminobutane and diaminopentane ($K = \sim 10^5$-10^6).[28] However, when the two nitrogen atoms are monoprotonated or deprotonated, the binding constant with CB[6] decreases significantly. That's why this kind of molecular switch has a strong respond to pH change.

Inspired by this work, we want to develop a conceptual smart GPx model by using simple selensium-containing compound and cucurbit[6]uril-pseudorotaxane-based molecular switch. Thus we designed an organoselenium compound **1** (Scheme 1A) as GPx model which contains not only catalytic selenium center but also two imino groups. In the presence of CB[6], the GPx mimic formed 1:1 host–guest pseudorotaxane complex when pH was below 6, because two nitrogen atoms were diprotonated. In this case, the compound **1** did not show GPx activity obviously as the active site of compound **1** was encapsulated into CB[6] and thereby cannot bind substrate (Scheme 1B). When pH was above 7, the binding ability of CB[6] with compound **1** decreased significantly as the two nitrogen atoms of compound **1** were monoprotonated or deprotonated. As a result, with the rise of pH the active site gradually exposed to solution that caused a gradually increased GPx activity. Thus, the designed selenoenzyme model can be switched on/off through the changing of pH at a mild environment (pH between 7 and 6).

The synthetic routes of compound **1** were shown in Supporting Imformation (Scheme S1). In order to prove the change of compound **1** between diprotonated state and monoprotonated or deprotonated state occurred at a mild environment, we first used acid-base titration to measure the pKa of the conjugate acid of compound **1** (Figure S6). The acid-base titration curve showed a typical titration curve of diamine which contains two titration end points. The pKa_1 of the conjugate acid of compound **1** was 6.5 and the pKa_2 was 9.3 (the detailed computing method is showed in supporting information). When $pH<pKa_1$ ($pH<6.5$), the two nitrogen atoms of compound **1** were diprotonated and thereby CB[6] and compound **1** had a strong host–guest interaction to form pseudorotaxane. With the rise of pH ($pKa_1<pH<pKa_2$, namely $6.5<pH<9.3$), the two nitrogen atoms of compound **1** became fully monoprotonated and even part of deprotonated gradually. As a result, the binding ability of CB[6] and compound **1** decreased and the pseudorotaxane dissociated gradually. So the pseudorotaxane-based molecular switch formed by CB[6] and compound **1** can be easy switched on/off at a mild environment.

The acid-base titration study showed that compound **1** was diprotonated when $pH<6.5$. In other words, compound **1** can form pseudorotaxane with CB[6] under acidic conditions. ^1H NMR titration experiment confirmed the formation of pseudorotaxane at pH=6 (Figure 1A). Upon the addition of CB[6] into compound **1** in D_2O, progressive disappearance of the signals corresponding to H_1-H_4 of compound **1** occurred, followed by the growth of a group of new peaks attributed to the encapsulated of compound **1**. After 1 equiv of CB[6] was introduced, the original peaks corresponding to H_1-H_4 of compound **1** disappeared completely and all of them had an obviously movement (H_1 moved from 2.91ppm to 1.88ppm, H_2 moved from 3.35ppm to 3.09ppm, H_3 moved from 3.14ppm to 3.32ppm, H_4 moved from 1.30ppm to 1.49ppm). This indicates that CB[6] and compound **1** can form 1:1 host–guest pseudorotaxane complexes under acidic conditions. The binding constant between compound **1** and CB[6] was calculated to be $1.77\pm0.23\times10^4$ M^{-1} at pH=6. The binding constant between compound **1** and CB[6] was similar to that between compound **1** analogs and CB[6] which has been well studied by Isaacs[26] and Kim[28]. ESI mass spectrometry was

Figure 1. A) Partial ^1H NMR spectra (500 MHz) of compound **1** and mixtures of compound **1** (1mM) with different amounts of CB[6] at pH=6. B) Partial ^1H NMR spectra (500 MHz) of mixtures of compound **1** (1mM) with 1 equiv of CB[6] at different pH. C) Mass spectrum of the pseudorotaxane formed by CB[6] and compound **1**.

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also used to probe the formation of the pseudorotaxane. We found the peaks of the pseudorotaxane from the mass spectrum (calculated 1220.3, found 1221.3 $(M+H)^+$). The mass spectrum (Figure 1C) contained typical isotopic peaks of selenium, suggesting the formation of the pseudorotaxane by CB[6] and compound **1** clearly. This experiment gave the strong evidence for the pseudorotaxane formation between CB[6] and compound **1,** and also demonstrated that the binding ability of CB[6] and compound **1** was strong and the pseudorotaxane was stable enough under acidic conditions indirectly.

With the rise of pH, the binding ability of CB[6] and compound **1** had a significant decrease followed by pseudorotaxane dissociation. This was also studied by ^1H NMR spectrums of compound **1** and 1 equiv of CB[6] in D_2O at different pH (Figure 1B). Under acidic conditions, compound **1** formed pseudorotaxane with 1 equiv of CB[6] as mentioned above. With the rise of pH, peaks attributed to the encapsulated of compound **1** had a significantly reduction. Through comparing the integral of original and new peaks of compound **1**, we calculated that about 50% of the pseudorotaxane was dissociated when pH rose to 7. ESI mass spectrometry was also used to probe the partial dissociation of the pseudorotaxane at pH=7 (Figure S7). Moreover, when pH rose to 9, the ratio of the dissociated pseudorotaxane increased to more than 60%. Finally the ratio of remained pseudorotaxane was less than 10% when pH goes up to 11 (Figure S8). Therefore, under acidic conditions, the active site of compound **1** was encapsulated into the hydrophobic cavity of CB[6] as compound **1** and CB[6] formed pseudorotaxanes. However, under neutral and alkaline conditions, most of the pseudorotaxanes were dissociated and the active site of compound **1** was exposed to solution. Thus the activity of the selenoenzyme compound **1** can be switched ON/OFF through the formation and dissociation of the pseudorotaxanes under acidic and neutral conditions.

The catalytic activity of compound **1** was first investigated in an 3-carboxy-4-nitrobenzenethiol (TNB) assay system using TNB as a GSH alternative at 37°C.[29] The initial rates (υ_0) for the reduction of H_2O_2 by TNB was determined by monitoring the UV absorption at 410nm for a few minutes due to the disappearance of the thiolate (TNB) absorption. The activity of compound **1** at this system is 1.93×10^{-3} μmol•min^{-1}•μmol^{-1} (Figure S9). In contrast to diphenyl diselenide (PhSeSePh), a typical GPx mimic, the efficiency of compound **1** increases by an order of magnitude. To further investigate the enzymatic kinetics of compound **1**, double-reciprocal plots were evaluated using a GSH reductase-reduced nicotinamide adenine dinucleotide phosphate (NADPH) coupled assay.[30] The double-reciprocal plots of the initial velocity versus substrate concentration yielded a series of intersecting linear plots for both substrates (Figure 2), which indicated a sequential mechanism.[11] The apparent kinetic parameters are listed in Table S1. The second-order constant $k_{cat}/K_{m\,H2O2}$ and k_{cat}/K_{mGSH} are at the magnitude of 10^{-2} M^{-1} min^{-1}.

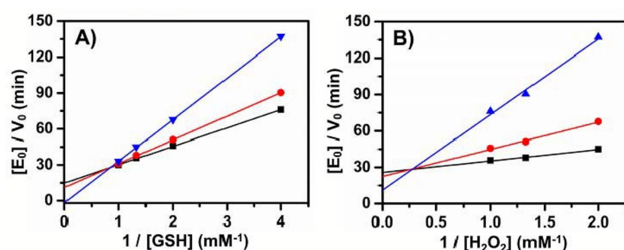

Figure 2. Double-reciprocal plots of the reduction of H_2O_2 by GSH under the catalysis of compound **1** ($[E_0]$ = total enzyme concentration). A) $[E_0]$ / V_0 versus 1 / [GSH] (mM^{-1}) at $[H_2O_2]$=0.5mM (▼), 0.75mM (●) and 1.00mM (■). B) $[E_0]$ / V_0 versus 1 / [H2O2] (mM^{-1}) at [GSH]=0.25mM (▼), 0.5mM (●) and 0.75mM (■).

After detailed evaluating the catalytic property of model compound **1**, we tested the ON−OFF switches of the peroxidase activity by tuning pH. We first tested the decrease of the peroxidase activity of compound **1** under the formation of the pseudorotaxane by CB[6] and compound **1** at pH=6. Upon the addition of CB[6], the peroxidase activity of compound **1** decreased progressively (Figure S11). There was almost no activity when 1 equiv CB[6] was added, because the active site of compound **1** was fully encapsulated into the hydrophobic cavity of CB[6]. There was also a progressive decrease of the peroxidase activity of compound **1** upon the addition of CB[6] at pH=7. However, after 1 equiv CB[6] was added, there was still more than 50% activity compared to compound **1** without CB[6] (Figure 3A, S12). That was because the binding ability of CB[6] and compound **1** decreased significantly compared to that at pH=6 and only part of the compound **1** formed pseudorotaxane with CB[6] under this condition. We also synthesized another organoselenium compound **2** (Scheme S2) as control which contains two hydroxyl groups instead of the two imino groups in compound **1**. Because the oxygen atoms cannot be protonated, compound **2** has no specific binding with CB[6] no matter under acidic conditions no acidic conditions. After 1 equiv CB[6] was added, the catalytic activity of compound **2** almost had no change (Figure S13, S14).

The investigation above demonstrated that the pH-switched GPx activity of pseudorotaxane formed by CB[6] and compound **1** can be switched on/off through changing the pH between 7 and 6, which is a mild environment and may be suitable for human body. The pseudorotaxane was stable because of the high binding ability of CB[6] and compound **1** at pH=6. The active site of compound **1** was encapsulated into CB[6] and thereby no GPx activity was displayed. In contrast, when pH rose to 7, a part of the pseudorotaxane was dissociated. The active site of compound **1** exposed to solution and GPx activity was displayed at this time. As a switchable GPx model, the reversibility of the catalytic activity of the pseudorotaxane was also tested. The results revealed that the catalytic activity of the enzyme is completely reversible after multiple change of pH between 6 and 7 (Figure 3B).

We also evaluated the GPx activity of the pseudorotaxane at a more extensive range of pH (from pH=5 to pH=9). The results indicated that there was almost no activity when pH≤6, because the active site of compound **1** was encapsulated into CB[6] (Figure 4A). When pH≥7, the GPx activity started to display. As the pH changed from 7 to 9, the binding ability of CB[6] and compound **1** had a slight decrease, because only part of compound **1** changed from monoprotonated state to deprotonated state according to the acid-base titration curve of compound **1**. In this case, more active sites exposed to the solution with the rise of pH, thus the relative activity had a slow increase when pH changed from 7 to 9. As a result, the GPx activity had a significant increase when pH changed from 7 to 9

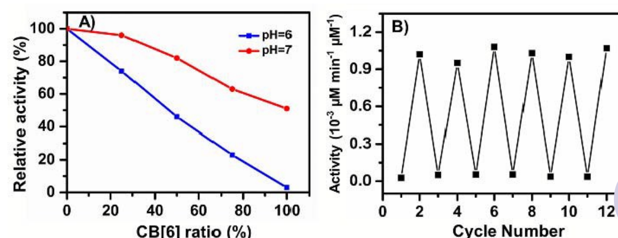

Figure 3. A) Decrease of relative activity versus mixtures of compound **1** with different amounts of CB[6] at pH=6 and pH=7. The activity of compound **1** without any CB[6] was defined as 100%. B) The ON−OFF switch of catalytic activity of the pseudorotaxane formed by CB[6] and compound **1** at the change of pH between 6 and 7.

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(Figure 4B). However, it's not the only reason that cause the increase of GPx activity. The other reason is that the catalytic efficiency of the reaction is higher under acidic conditions.

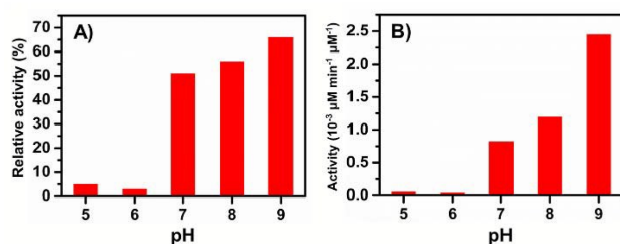

Figure 4. A) Relative catalytic activities and B) Catalytic activities of the pseudorotaxane formed by CB[6] and compound **1** at a range of pH from 5 to 9.

Conclusions

In conclusion, we constructed a pH-responsive artificial selenoenzyme by CB[6] and organoselenium compound **1** with pseudorotaxane-based molecular switch. This conceptual smart GPx model shows typical seleoenzyme GPx behaviors. Moreover, its activity can be switched on/off at a very mild and body suitable environment between pH=7 and pH=6. It is anticipated that such smart artificial selenoenzymes have a bright future which could be controlled according to the needs of the human body.

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Notes and references

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† Electronic Supplementary Information (ESI) available: Synthesis and characterization of CB[6], compound **1** and compound **2**, acid-base titration curve of compound **1**, enzymatic analysis of compound **1** and the pseudorotaxane formed by compound **1** and CB[6]. See DOI: 10.1039/c000000x/

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Table of Contents (TOC):

A conceptual smart GPx model that had a response to pH stimuli was developed by using simple selensium-containing compound and cucurbit[6]uril-pseudorotaxane-based molecular switch.

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