Target-triggered cascade assembly of a catalytic network as an artificial enzyme for highly efficient sensing†

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Determining the catalytic activity of artificial enzymes is an ongoing challenge. In this work, we design a porphyrin-based enzymatic network through the target-triggered cascade assembly of catalytic nanoparticles. The nanoparticles are synthesized via the covalent binding of hemin to amino-coated gold nanoparticles and then the axial coordination of the Fe center with a dual-functional imidazole or pyridine derivative. The network, which is specifically formed by coordination polymerization triggered by Hg2＋ as the target, shows high catalytic activity due to the triple amplification of enzymatic activity during the cascade assembly. The catalytic dynamics are comparable to those of natural horseradish peroxidase. The catalytic characteristics can be ultrasensitively regulated by the target, leading to a selective methodology for the analysis of sub-attomolar Hg2＋. It has also been used for "signal-on" imaging of reactive oxygen species in living cells. This work provides a new avenue for the design of enzyme mimics, and a powerful biocatalyst with signal switching for the development of biosensing protocols.

Introduction

In nature, enzymes often participate in self-assembly processes to form “soft” nanostructures, such as spheres and tubes, demonstrating their catalytic functions. Many synthetic catalysts that behave as enzymes can mimic the functions of natural systems. However, the performance of some artificial enzymes is very inferior to that of corresponding natural enzymes, possibly as a result of the aggregation of metal macrocycles, such as porphyrins and phthalocyanines, to form inactive dimers that are arranged in a disordered manner. To improve the catalytic efficiency, much effort has been devoted to the self-assembly of porphyrins into ever-larger structures involving porphyrinic dendrimers, nanocrystals, and square nanosheets, and the incorporation of porphyrin-containing hemin in a guanine quadruplex to dissociate the aggregate. The assembly of porphyrins on nanomaterials has been demonstrated to be a simple and efficient approach for achieving high catalytic activity, specific recognition and even signal transduction, and it was used to produce multifunctional nanoscale systems for artificial photosynthesis, catalysis, and biosensing. Porphyrinic metal–organic frameworks as crystalline molecular materials have shown unique enzyme-like activities. Inspired by the promising applications of porphyrin assemblies as artificial enzymes, this work designs a target-triggered cascade assembly mechanism for the formation of a porphyrin-based enzymatic network, which leads to an ultrasensitive signal switch for target-related bioanalysis. The assembly of porphyrins on nanomaterial surfaces is generally performed through chemisorption, π–π stacking interactions, covalent binding and axial ligation. Owing to the lack of functional groups on biocompatible gold nanoparticles (AuNPs), which are extensively used in biomedicine, it is difficult to immobilize porphyrinic catalysts on AuNPs. Thus, this work describes the preparation of amino group modified AuNPs (N-AuNPs) using a rationally designed ligand, pamidronic acid disodium salt (PADS), for effective chemical combination with metalloporphyrins (hemin) via an amide reaction (Fig. 1A). The Au-Hem conjugates then assemble with two dual-functional small molecules: 5-(1H-imidazol-4-ylmethylene)-pyrimidine-2,4,6-trione (MPT) via the model shown in Fig. 1B, and 5-(pyridin-4-ylmethylene)-pyrimidine-2,4,6-trione (PMPT) through axial coordination. In the presence of Hg2＋ as a target, the coordination polymerization of functionalized AuNPs (Au-Hem-MPT or Au-Hem-PMPT) via N-Hg2＋-N bond connectivities produces enzyme-active networks (Au-Hem-Net and Au-Hem-pNet). Both of the networks show catalytic activities that are much higher than that of free hemin due to the triple amplification of enzymatic activity during the cascade assembly (Fig. 1C). The ordered network provides catalytic dynamics that are comparable to those of the natural enzyme horseradish...
peroxidase (HRP). Moreover, the catalytic characteristics can be flexibly regulated by trace amounts of Hg^{2+}, a highly toxic metal ion that damages the central nervous and endocrine systems.\textsuperscript{12,13} This work provides a powerful protocol for the ultrasensitive sensing of sub-attomolar Hg^{2+}. More importantly, the network showed low cytotoxicity and it can be conveniently used for the sensitive imaging of intracellular enzyme-related species, such as reactive oxygen species (ROS). Thus, the cascade synthesis of target-controllable catalytic systems provides a new concept for the design and application of enzyme mimics.

Results and discussion

Construction and characterization of enzyme-active networks

The N-AuNPs were synthesized via the reaction of HAuCl\textsubscript{4} with PADS as both the reducing agent and the protection ligand in a boiling aqueous solution. At different mass ratios of PADS:HAuCl\textsubscript{4}, the obtained N-AuNPs showed different colors related to size and dispersion (Fig. 2A). The sharp absorption peak in their UV-vis spectra (Fig. 2B) and dynamic light scattering measurements (Table S1\textsuperscript{†}) indicated a relatively narrow size distribution at ratios from 3.2 : 1 to 11.0 : 1. Transmission electron micrographs (TEMs) and zeta-potential analysis further demonstrated that the best dispersion of N-AuNPs was at a ratio of 5.0 : 1 (Fig. S1 and Table S1\textsuperscript{†}), which showed the largest zeta potential, a diameter of around 17 nm, and the characteristic surface plasmon band centered at 525 nm.

The formation mechanism of N-AuNPs was studied using 1-hydroxyethane-1,1-diphosphonic acid (HEDP), which does not contain an amino group at the tertiary carbon, and 3-amino-propylphosphonic acid (APPA), which does not contain a hydroxyl group at the carbon adjacent to the phosphonic group, as controls. An obvious color change occurred in the mixtures of HAuCl\textsubscript{4} with PADS and HEDP, while no reaction and no plasmon absorption of the AuNPs were observed in the mixture of HAuCl\textsubscript{4} with APPA (Fig. S2\textsuperscript{†}), demonstrating that the hydroxyl group at the tertiary carbon was involved in the oxidation reaction of PADS or HEDP by HAuCl\textsubscript{4}.\textsuperscript{34,35} The oxidation reaction led to a shift in the \textsuperscript{31}P NMR peak to a higher magnetic field, accompanied by the signal disappearance of two multiple peaks that were attributed to two methylene groups in the \textsuperscript{1}H NMR spectrum (Fig. 2C and D), helping to identify the formation of a C=O bond via the cleavage of another group from the carbon, as shown in the electrospray ionization mass spectra (Fig. S3\textsuperscript{†}). The N-AuNPs prepared with PADS remained as a deep red suspension for several months and exhibited better stability and dispersion (Fig. S2\textsuperscript{†}) due to the presence of an excess amount of PADS on their surfaces. The presence of excess PADS on the nanoparticles was supported by the characteristic vibration frequencies of primary amines at 3228.9 and 3406.1 cm\textsuperscript{-1} for N-AuNPs in the infrared absorption spectrum (Fig. 3A). More importantly, the adsorbed PADS not only protected the AuNPs against aggregation, but also provided the −NH\textsubscript{2} group for covalent binding with hemin, which was characterized by the infrared absorption of the amide bond at 1698.4 and 1298.6 cm\textsuperscript{-1} (Fig. 3A).

The formed Au-Hem showed a single Soret band at around 400 nm (Fig. 3B). The disappearance of the dimeric absorption of hemin at 385 nm and the presence of monomeric absorption at 400 nm and around 350 nm (Fig. S4A\textsuperscript{†}) suggested that the hemin on the N-AuNPs was in its monomeric state, the preferred form for obtaining a high catalytic ability.\textsuperscript{40} The number of hemin complexes on each Au nanoparticle was calculated to be 73 from the Soret band intensity of hemin using UV-vis spectroscopy (see the ESI Experimental section and Fig. S4B\textsuperscript{†}), indicating an acceptable capacity of the enzyme mimic system for amplifying the catalytic activity.

The Au-Hem was further modified with MPT or PMPT via the axial coordination of their imidazole or pyridine group with the
Fe center of hemin. Both MPT and PMPT were synthesized to contain a pyrimidinetrione moiety for the preparation of the enzyme-active network by coordination polymerization. The MPT and PMPT structures were firstly characterized using MS and NMR information (see the ESI Experimental section, and Fig. S5 and S6†). The Fe 2p X-ray photoelectron spectra (XPS) (Fig. 3C and S7A†) demonstrate that the axial coordination interaction of hemin with both MPT and PMPT increased the Fe 2p1/2 binding energy due to a change in the electron density on the Fe atom.27 The coordination numbers and the binding constants (n and K) were determined via the titration of the hemin solution with MPT and PMPT,27 which led to a slight red shift of the Soret band of hemin at 385 nm (Fig. 3D and S7B†). The Fe 2p XPS spectra of hemin and the hemin–MPT complex were synthesized to form a 1 : 1 pyrimidinetrione–Hg2+ coordination reaction (Fig. 3F). Thus the structure of the MPT–Hg2+ coordination polymer is represented in the inset of Fig. S9A†. The matrix-assisted laser desorption/ionization time-of-flight mass spectrum of the mixture of MPT and Hg2+ further verified the formation of the 1 : 1 MPT–Hg2+ coordination polymer (Fig. S9A†), in which the mass peaks at m/z 406.6, 612.6, 655.9 and 847.6 could be attributed to the characteristic fragments MPT + Hg-1H, 2MPT + Hg-2H, 2MPT + 2Hg-2 imidazole groups-3H and 2MPT + 2Hg + Cl-3H, respectively.

The interaction between the imide site of thymine and Hg2+ to form a “N–Hg(th)–N” structure is well known and is used for bioanalysis.40,41 The designed pyrimidinetrione moiety in both MPT and PMPT contained two imide sites. Using MPT as an example, the absorption peak of MPT was red-shifted from 260 nm to 265 nm upon the addition of Hg2+ in the MPT solution, while other ions did not change the absorption peak (Fig. 3E), demonstrating the specific recognition of the two imide sites of MPT to Hg2+ to form a “N–Hg(th)–N” like binding motif.42 Using the absorbance ratio of A265 nm to A260 nm in the UV-vis spectra, the Job’s plot analysis revealed a 1 : 1 binding stoichiometry of the coordination reaction (Fig. 3F). Thus the structure of the MPT–Hg2+ coordination polymer was synthesized at 37 °C, the physiological temperature, for ensuring effective enzymatic catalytic activity.
Catalytic performance

To evaluate the catalytic performance of the enzyme mimics proposed in this work, a fluorescence assay of the tyramine oxidation reaction was performed, in which the nonfluorescent tyramine (0.14 mM) was oxidized by hydrogen peroxide (0.4 mM) to give fluorescent dityramine, thus the fluorescent signal represented the catalytic activity of these enzymes as catalysts. The amplified catalytic activity was demonstrated by the strong fluorescent signal of the oxidation product of tyramine by H$_2$O$_2$ in the presence of Au-Hem, when compared to the N-AuNPs and free hemin in aqueous solution (Fig. 5A).

On increasing the MPT concentration in Au-Hem, the catalytic activity was slightly enhanced (Fig. 5B), suggesting that the axial coordination of MPT to the Fe center of hemin changed its electronic and geometric structure, which increased the rate of the oxidation-reduction reaction. However, the catalytic activity greatly increased upon the addition of MPT in the mixture of Au-Hem and Hg$^{2+}$, and reached a maximum at a hemin/MPT concentration ratio of 1 : 1. At this concentration ratio, the formed mimic produced a 2.6, 3.1 and 14.9 times higher fluorescence intensity than that of Au-Hem-MPT, Au-Hem and hemin, respectively, (Fig. 5A), suggesting an improved peroxidase activity in each assembly step. As a control, different concentrations of Hg$^{2+}$ were added in the Au-Hem solution, and the catalytic oxidation product did not show any fluorescence change (Fig. 5C). More interestingly, this method showed high selectivity. Other metal ions did not produce an obvious fluorescence change (Fig. 5D). Thus, high peroxidase activity resulted from the Hg$^{2+}$-induced coordination polymerization of Au-Hem-MPT. That is, Hg$^{2+}$-triggered nanoparticle aggregation results in ordered multiscale organization and the strong interparticle coupling of surface plasmons between neighboring nanoparticles. Thus, the plasmon enhancement effect accelerates the electronic transfer between H$_2$O$_2$ and Fe$^{3+}$ hemin with the production of an initial intermediate (a ferrylporphyrin radical cation), leading to the improved catalytic performance of the Hg$^{2+}$-triggered network. In addition, the networks could be formed by the different sized N-AuNPs with average diameters of 26.1–6.4 nm, which was deduced from the increased fluorescence intensity after the addition of Hg$^{2+}$, among which small-sized particles had less of a tendency to form the network (Fig. S10†).

The Hg$^{2+}$-regulated catalytic characteristics of Au-Hem-MPT could be applied to the sensitive detection of Hg$^{2+}$ using the catalytic fluorescence signal. Using the optimized incubation (9 min) and catalytic reaction (15 min) time (Fig. 6A and B), a remarkable fluorescence increase was observed upon addition of Hg$^{2+}$ in the mixture of Au-Hem-MPT, tyramine and H$_2$O$_2$ at 37 °C (Fig. 6C). The plot of fluorescence intensity vs. the logarithm of Hg$^{2+}$ concentration showed good linearity in the range 1.0 aM to 10 pM with a correlation coefficient of 0.992 (Fig. 6D). It is worth noting that the detection limit at 3σ was 0.30 aM, which was 7 orders of magnitude lower than that of conventional analytical methods, and also the most sensitive method available for detection of Hg$^{2+}$ (Table S3†). Since Hg$^{2+}$ is very difficult to biodegrade and can accumulate in organisms to cause various human diseases, the coordination polymerization-amplified catalytic reaction is of great importance in monitoring Hg$^{2+}$ at ultralow abundances and the slow accumulation of Hg$^{2+}$ in organisms.

The Hg$^{2+}$-triggered formation of Au-Hem-pNet could also be used for Hg$^{2+}$ detection with the same detection system, and showed a detectable concentration range of 1.0 aM to 100 pM with a detection limit of 0.78 aM (Fig. S11A and B†). Similarly,
the formed highly catalytic network could be applied to the detection of H$_2$O$_2$. Using Au-Hem-Net as an example, the mixture of Au-Hem-Net and tyramine showed linearly increasing fluorescence intensity with increasing H$_2$O$_2$ concentration in the range 4.4 to 100 μM (Fig. S11C and D).

To evaluate the catalytic dynamics of the designed network, the oxidation product of pyrogallol by H$_2$O$_2$ was detected using UV-vis spectroscopy. At pyrogallol concentrations that were much higher than those of the enzyme mimics, three enzyme mimics, Au-Hem, Au-Hem-PMPT and Au-Hem-pNet, all showed a linear Lineweaver–Burk plot at the initial oxidation stage (Fig. 7), which led to two important kinetic parameters, $K_M$ (the Michaelis constant) and $k_{cat}$ (the catalytic kinetic constant), as listed in Table 1. The $K_M$ value of Au-Hem was a surprisingly low value of 0.40 mM, which was less than that of HRP (0.81 mM), indicating a good affinity of pyrogallol to the Au-Hem conjugate. Meanwhile, Au-Hem-PMPT and Au-Hem-pNet showed comparable affinities to HRP. The $k_{cat}$ values of Au-Hem (419.6 min$^{-1}$), Au-Hem-PMPT (714.3 min$^{-1}$) and Au-Hem-pNet (1054.5 min$^{-1}$) were 2–3 orders of magnitude higher than that of free hemin (2.4 min$^{-1}$) and higher than those of other carrier-supported hemin composites. Moreover, the kinetic constant of Au-Hem-pNet was comparable to that of natural HRP (1750 min$^{-1}$), highlighting the excellent catalytic activity of the enzyme-active network, which was greatly facilitated by the substrate being close to the active site of the enzyme and cooperative binding.

Fluorescence imaging of intracellular reactive oxygen species

In view of the high catalytic activity towards the substrate oxidation by different ROS (Fig. 8A), the cascade-assembled network, Au-Hem-Net, was used for the fluorescence imaging of ROS in living cells with o-phenylenediamine as the substrate. The cytotoxicity of Au-Hem-Net along with the substrate was examined using MTT assay (a colorimetric assay for assessing cell metabolic activity, see the ESI materials and reagents, Experimental section†) by incubating HeLa cells with the

Table 1 Kinetic parameters for the pyrogallol oxidation catalyzed by enzyme mimics at room temperature

| Mimics          | $K_M$ (mM) | $k_{cat}$ (min$^{-1}$) | Ref.     |
|-----------------|-----------|------------------------|---------|
| Au-Hem          | 0.40      | 419.6                  | This work |
| Au-Hem-PMPT     | 0.97      | 714.3                  | This work |
| Au-Hem-pNet     | 1.36      | 1054.5                 | This work |
| Hemin           | 87.1      | 2.4                    | 52      |
| Hemin–graphene  | 1.22      | 246                    | 21      |
| Hemin–hydrogel  | —         | 19.9                   | 20      |
| Hemin–polymer   | —         | 7.6                    | 20      |
| FeTMPyP$^{a}$   | —         | 83                     | 53      |
| FeTMPyP–graphene| 0.96      | 545                    | 21      |
| FeTMPyP–antibody| 8.6       | 680                    | 53      |
| HRP             | 0.81      | 1750                   | 53      |

$^a$ FeTMPyP: tetramethylpyridylporphyrin iron.
We present a highly catalytic network using axial coordination and coordination polymerization to achieve target-triggered cascade assembly based on the specific recognition of pyrimidinetrione moieties to the target. Two dual-functional pyrimidinetrione-containing derivatives of imidazole (MPT) and pyridine (PMPT) are designed to link the target Hg$^{2+}$ and the Fe center of hemin in a covalently assembled amino group modified AuNPs. The high catalytic activity of the network results from the triple amplification of the enzymatic activity during the cascade assembly: monomeric hemin loading on the N-AuNPs, axial coordination of MPT or PMPT to the Fe center of hemin to form a five-coordinated hemin structure, and coordination polymerization via a 1:1 binding stoichiometry to form a “N-Hg$^{2+}$-N” like binding motif for the ordered aggregation of the catalytic monomers. The catalytic activity of the network can be flexibly controlled by Hg$^{2+}$, which leads to an extremely sensitive strategy for Hg$^{2+}$ detection. The sub-attomolar detection limit is 7 orders of magnitude lower than those of conventional analytical methods. This catalytic system shows low cytotoxicity and has been successfully utilized in sensitive fluorescence imaging of intracellular ROS. The proposed catalytic network showed excellent affinity and high catalytic kinetics that are comparable to those of natural HRP. The cascade synthesis of the target-controllable catalytic system opens up new perspectives for not only the assembly of metalloporphyrin in the discovery and design of enzyme mimics, but also the development of biosensing protocols.

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**Notes and references**

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