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The colorimetric assay of diamine oxidase activity with high sensitivity based on calixarene derivative-capped gold nanoparticles

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Diamine oxidase (DAO) is involved in regulating ingested or endogenous histamine, several types of human cancer, and the cell mass during embryonic development. Furthermore, DAO is a potential indicator of intestinal mucosa damage in inflammatory and neoplastic diseases or after chemotherapy. Therefore, developing a biosensor is urgently needed to assay DAO activity. In this work, a simple but sensitive colorimetric biosensor is developed for DAO assay and inhibitor screening. The biosensor is based on the host-guest interactions between the amine group on the terminal of 1,6-hexanediamine (HMD) and p-sulfonatocalix[6]arene (pSC\textsubscript{6}). These interactions may aggregate pSC\textsubscript{6} modified gold nanoparticles (AuNPs) and the corresponding color change of the testing solution. Specifically, AuNP-aggregation is affected because coordination reactivity does not exist between oxidized HMD and pSC\textsubscript{6} during HMD oxidation by DAO. Therefore, a simple colorimetric method with high sensitivity for the assay of DAO activity is proposed. A linear relation is presented under optimized experimental conditions in a range of 0.15 mU mL\textsuperscript{-1} to 4.5 mU mL\textsuperscript{-1} with the lowest detection limit of 0.062 mU mL\textsuperscript{-1}. Moreover, the inhibition effect of guanidine on DAO activity is tested with the IC\textsubscript{50} value of 2.4 μM using the proposed biosensor. Therefore, this biosensor has a great potential not only for the detection of DAO activity but also for inhibitor screening in the future.
1. Introduction

Diamine oxidase (DAO) is an enzyme found in the intestinal mucosa of humans, other mammalian species, and some plants, particularly in legumes, DAO catalyzes diamine oxidation with symmetric diamines, such as putrescine and cadaverine, as the best DAO substrate.[1,2] These symmetric diamines also weakly catalyze the oxidation of some monoamines. The highest expression of DAO has been observed in the intestine, kidney, and placenta.[3,4] DAO was involved in the regulation of ingested or endogenous histamine as it searched for extracellular histamine after a mediator release when functioning as a secretory protein. [5] A high DAO activity has also been found in some human tumors, such as medullary thyroid, lung, and granulosa cell carcinomas, as well as in effusion fluids in some human cancers.[6-10] Moreover, DAO regulates the cell mass during embryonic development because of the H$_2$O$_2$ production that occurs during polyamine catabolism.[11] Furthermore, the decreased activity of DAO is considered a potential indicator of the damage of intestinal mucosa in inflammatory and neoplastic diseases or after chemotherapy.[12,13] However, the detection methods of DAO are barely at present.[14] Therefore, developing an ultrasensitive detection strategy for DAO in clinical and bioscience research is urgently needed.

Colorimetric assays based on gold nanoparticles (AuNPs) have been receiving increasing attention because of their easily designable surface chemistry, technical simplicity, and high sensitivity, benefiting from the unique optical properties of AuNPs.[15,16] These assays have been used for detecting different kinds of substances, such as nucleic acids, proteins, and metal ions.[17-23] Macro cyclic molecules are important building blocks within supramolecular chemistry, particularly acting as efficient host molecules in host-guest chemistry for molecular recognition. The nature of these interactions is noncovalent and can be primarily classified into four types: hydrogen bonding, electrostatic interactions, van der Waals forces, and hydrophobic interactions. Calixarenes, along with cyclodextrins, cucurbiturils, cryptands, and crown ethers, are among the established host molecules with a unique three-dimensional surface. These host molecules have more conformational isomers and a larger number of cavities with different sizes and shapes that are involved in molecular recognition in comparison with polyethylene glycol and dextrin. Ghale et al. [24] have used cucurbituril [7] and acridine orange to build a protease and its inhibition model with a label-free technique. Calixarenes are available and easily functionalized at either the upper and/or lower rim of the molecular skeleton among potential building blocks. Thus, calixarenes have become important receptors as supramolecular platforms for the synthesis and application of molecular recognition, sensing and self-assembly, catalysis, nanotechnology, and drug discovery.[25,26] Therefore, the use of calixarenes has become increasingly popular in biochemical recognition and separation of bioactive molecules, such as amino acids, peptides, proteins, lectins, nucleotides, nucleosides, saccharides, and steroids.[27]

Recently, macrocycle molecules decorated with inorganic nanoparticles have drawn particular interests in optical, electronic, thermal, and catalytic performances because of their unique integration between the physical and chemical prosperities of nanoparticles and molecular recognition of macrocycles.[28-31] Han et al. synthesized p-sulfonatocalix[6]arene (pSC$_6$)-modified AuNPs based on water-soluble calixarenes, and these AuNPs were further utilized to recognize the aromatic amine isomers with a secondary amine.[32] However, the real application and commercial use of pSC$_n$ modified AuNPs combined with nanoparticles remain a challenge. The difficulties in synthesizing, as well as in the application of calixarene coated nanoparticles in detecting biomolecules, are due to the low aqueous solubility of most calixarene derivatives. The use of calixarene coated nanoparticle in detecting enzyme activity is not reported. In our previous work, calix[4]arene crown ether modified AuNPs were synthesized and further used to detect the aliphatic diamines successfully.[33]

Based on above studies, we designed a simple and sensitive strategy that combines the advantages of both supramolecule and AuNPs to detect DAO and its inhibitor through the color change of the pSC$_n$ modified (n=4, 6) AuNPs as shown in Fig. 1. Furthermore, this method can be used in a high-throughput fashion with the naked eye or a simple colorimetric reader, which may support a new strategy.
for other enzyme and protein detection methods in the future.

Fig. 1. Schematic presentation of the detection of DAO and its inhibitor.

2. Materials and methods

2.1 Materials and chemicals

The 1,6-hexanediamine (HMD), HAuCl₄, and NaBH₄ were purchased from Sigma-Aldrich (St. Louis, MO, USA). DAO, pSC₆ and guanidine hydrochloride were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). All chemicals were used as received, if not mentioned otherwise. Milli-Q water (>18 MQ; Branstead, USA) was used. The reagents were dissolved in water, if not mentioned otherwise. All the glassware used in AuNP synthesis were immersed in aqua regia for 30 min and washed over 10 times with distilled water (DW).

2.2 pSC₆-AuNP synthesis

pSC₆-AuNPs were synthesized according to previous methods. pSC₆ (2 mL, 10⁻² mol L⁻¹) and HAuCl₄ solutions (2 mL, 2.63x10⁻² mol L⁻¹) were added to a 94 mL water in turn at room temperature. After stirring for 20 min in the dark, freshly prepared NaBH₄ solution (2 mL, 10⁻² mol L⁻¹) was added into the reaction solution immediately and stirred for 2 h in the dark. A procedural colour change from bright yellow to light brown and brownish red was observed. The pSC₆-AuNP colloids were finally obtained. The resulting solution was deep red and stored at 4 °C in an amber bottle and ready for use.

UV-vis spectra (Shimadzu UV-2450 PC), zeta potential (Zetasizer 3000Hs, Malvern), and FTIR (Vertex 70, Bruker) were used to characterize the prepared AuNPs.

2.3 Assay of DAO activity

The assay of DAO activity was performed using the following procedure. First, the DAO was dissolved to 250 mU and then to different concentrations. Up to 20 µL of enzyme solution with different concentrations ranging from 0.01 mU mL⁻¹ to 11 mU mL⁻¹ was incubated at 37 °C for 30 min, and then 80 µL of dispersed pSC₆-AuNP solution was added to the mixed solution. The resulting mixture was reacted at room temperature. The reaction solutions were photographed after 20 min and used for UV-vis spectroscopic measurements.

2.4 Evaluation of inhibitor assay

For the inhibition assay, 5 µL of HMD was first premixed with DAO (5 µL, 9 mU mL⁻¹) for 30 min at 37 °C. Then, aqueous solutions of guanidine hydrochloride (10 µL) with different concentrations ranging from 0.05 mM L⁻¹ to 4.5 mM L⁻¹ were added to the mixed solution and further incubated for 20 min at room temperature. PSC₆-AuNPs (80 µL) was then added to the resulting solution at room temperature. The reaction solutions were photographed after 20 min and used for UV-vis spectroscopic measurements.

3. Results and discussion

3.1 Mechanism investigation of the assay of DAO activity

The mechanism of the aggregation process is shown in Fig. 1, in which pSC₆ holds the electron-rich cyclic cavity and the negative SO₃⁻ group. Furthermore, the amino residue of the diamine was partly protonated in neutral media. PSC₆ binds the aromatic and amino residues of the diamine via host-guest interaction, such as electrostatic forces, or cation-π and π-π interactions. The interactions between the amine group and two pSC₆ modified AuNPs result in the potential aggregation of pSC₆-AuNPs. PSC₆ presents a predefined host cavity with a stronger binding force because of its great conformational flexibility [34]. Therefore, HMD will aggregate pSC₆-AuNPs without DAO. The addition of DAO in this study prevented pSC₆-AuNPs from aggregating because DAO oxidases the amino group of HMD. Therefore, DAO was detected qualitatively and quantitatively. In addition, we also chose guanidine hydrochloride as the inhibitor of DAO and investigated the detection sensitivity based on the guanidine hydrochloride that competitively inhibited the DAO activity. In this system, DAO inhibitor was detected, and a positive result was obtained. Fig. 2 shows that upon the addition of DW into the pSC₆-AuNP solution in the control experiment, the red colour of the test solution remains unchanged. Furthermore, the solution showed an obvious absorption at 538 nm...
(black curve), showing the state of well-dispersed pSC₆-AuNPs. When DAO was added into the pSC₆-AuNPs, the pSC₆-AuNP solution did not aggregate; thus, the initial colour of the test solution and UV-vis spectrum of the well-dispersed pSC₆-AuNPs (red curve) remained unchanged. This result suggests that the addition of DAO alone does not induce the aggregation of pSC₆-AuNPs. After adding the HMD into the pSC₆-AuNPs, the solution caused an observable aggregation of pSC₆-AuNPs, thereby changing the color from the initial wine red to blue and producing a new absorption peak at 760 nm (blue curve). These results indicate that the specific interaction between HMD and pSC₆ was observed. Thus, the surface coordination interaction may have caused pSC₆-AuNP aggregation, which is responsible for the changes in both the colour and UV-vis spectrum of the dispersed pSC₆-AuNPs. Conversely, the pSC₆-AuNPs remained almost unchanged after adding both DAO and HMD (green curve). Moreover, both the colour and UV-vis spectrum of the solution changed (pink curve) after the simultaneous addition of DAO, HMD, and guanidine into the pSC₆-AuNP solution, suggesting pSC₆-AuNP aggregation. This phenomenon is attributed to the inhibition of the activation of DAO by guanidine, resulting in the combination of HMD and pSC₆-AuNPs again (Fig. 2).

3.2 pSC₆-AuNP characterization

The obtained pSC₆-AuNPs was characterized by FTIR and zeta potential. Fig. 3 shows the FTIR spectra, zeta-potential and dynamic light scattering spectra of pSC₆ and pSC₆-AuNPs. Compared with the pure spectrum of pSC₆ in Fig. 3(a), a significant feature was also observed in the pSC₆-modified AuNPs. The peaks for SO₃⁻ 1187 cm⁻¹ and 1049 cm⁻¹ found in pure pSC₆ were shifted to 1157 cm⁻¹ and 1038 cm⁻¹, respectively, suggesting the coordination of SO₃⁻ groups with the gold atoms on the AuNP surface. The pSC₆
was modified on the AuNPs from these spectra. The similar modification was found in a previous work.[34,35] pSC₆-AuNPs were also characterized by zeta potential with a mean size of 10.3 nm and −32.4 mV in potential to verify its stability and dispersity as shown in Fig 3(b) and 3(c). All these data confirmed that pSC₆-AuNPs is stable and in good disperse.

3.3 Kinetic analysis of HMD binding with pSC₆-AuNPs

![Kinetic plots of time-dependent A₇60 values versus those with different HMD concentrations. Digital photographs at a time point of 20 min after adding different HMD concentrations are shown on the right. Right, from vial 1 to vial 9: HMD concentrations of 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 1.5, and 2 mM. (b) Initial rate of coordinate reaction of pSC₆-AuNPs with different HMD concentrations.](image)

Fig. 4. (a) Kinetic plots of time-dependent A₇60/A₅38 values versus those with different HMD concentrations. Digital photographs at a time point of 20 min after adding different HMD concentrations are shown on the right. Right, from vial 1 to vial 9: HMD concentrations of 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 1.5, and 2 mM. (b) Initial rate of coordinate reaction of pSC₆-AuNPs with different HMD concentrations.

Fig. 1 shows the recognition principle between HMD and pSC₆-AuNPs. PSC₆ on the surface of AuNPs coordinated with HMD, causing AuNP aggregation. Hence, HMD concentration in the reaction solution affected the sensitivity of the detection. Therefore, the effect of HMD concentration on the detection system was investigated further. The UV-vis spectra of the reaction solution were successively recorded for 20 min after the addition of HMD with different concentrations. Fig. 4(a) shows the time-dependent A₇60/A₅38 values from the UV-vis spectra of pSC₆-AuNPs with different HMD concentrations to estimate the kinetics of the coordination between HMD and pSC₆-AuNPs. The A₇60/A₅38 values increased with the increasing HMD concentrations. Furthermore, the color of the aqueous of the pSC₆-AuNPs gradually changed from wine red to violet (right, from vial 1 to vial 9) at the time point of 20 min when HMD concentrations increased from 0.5 mM to 2.0 mM. These results indicate that pSC₆-AuNP aggregation becomes faster when HMD concentrations are increased in the testing solution. The initial reaction rate (A₇60/A₅38) was obtained by calculating the slopes of the initial part of the kinetic curves of different HMD concentrations (Fig. 4(b)). As shown in Fig. 4, with the initial reaction rate gradually increased with the increase of HMD concentrations from 0.5 mM to 2.0 mM. The changes in the rate become less evident when HMD concentration reached 1.0 mM. Moreover, the final A₇60/A₅38 values remained almost unchanged when HMD concentration varied from 1.0 mM to 2.0 mM (Fig. 4(b)). Therefore, the optimal reaction concentration of HMD was 1.0 mM. A high HMD concentration can cause pSC₆-AuNP to aggregate quickly and completely; thus the relatively high concentration of HMD was used to accelerate the assay in the detection system.

3.5 Optimization of DAO and HMD reaction time

The enzymatic activation is closely relative to the reaction time. The optimization of the reaction time is shown in Fig. 5. When the reaction time varied from 10 min to 30 min, the aggregation rate of pSC₆-AuNPs declined gradually and stabilized within 30 min to 60 min. Thus, the most appropriate time for DAO to catalyze HMD is 30 min, and this time was chosen as the reaction time in the following experiment.

3.6 Assay of DAO activity

As shown in Fig. 6(a), the increase of DAO concentrations from 0.15 mU mL⁻¹ to 4.5 mU mL⁻¹ turned the color of the dispersion from violet to wine red (from vial 1 to vial 6) gradually, and a dramatic change occurred in the UV-vis spectra of the dispersion for pSC₆-AuNPs. With the increasing concentration of DAO, A₇60 decreased and A₅38 increased. As shown in Fig. 6(b), A₇60/A₅38 showed good linearity in the range of 0.15 mU mL⁻¹ to 4.5 mU mL⁻¹. This result is attributed to the oxidation of the amine group on the HMD terminate
that is caused by DAO catalysis, leading to a decrease in AuNP aggregation. A linear correlation existed between $A_{760}/A_{538}$ and the DAO concentration ranged from 0.15 mU L$^{-1}$ to 4.5 mU L$^{-1}$ with a detection limit of 0.0621 mU mL$^{-1}$ (3 times signal to noise ratio). The linear equations are $A_{760}/A_{538} = 1.1509 - 0.1965 C$, and the coefficient of the linear fit is 0.9907.

Fig. 5. Initial rate of the coordination of HMD with pSC$_6$AuNPs in different reaction-time.

Fig. 6. (a) UV-vis spectra and photographs (inset) of the pSC$_6$AuNPs after adding the mixture of 1 mM HMD (10 µL) that reacted with the various concentrations of DAO (10 µL) from vial 1 to vial 6; the DAO concentrations are 0.15, 0.5, 1.5, 2.5, 3.5, and 4.5 mU, respectively. (b) The linear relationship of pSC$_6$AuNPs between the $A_{760}/A_{538}$ values and DAO concentrations.

3.7 Evaluation of inhibitor assay

Our system was further applied to screen DAO inhibitor. In this part, guanidine was chosen as the example. Guanidine has a diamine group that competitively binds with the active site of DAO with HMD, leading to the inhibition of the oxidation ratio. As shown in Fig. 7 (from vial 1 to vial 10), pSC$_6$AuNPs changed from wine to gray when the guanidine concentration increased from 0.05 µM to 5.4 µM, corresponding with the decrease and increase of absorbance at 538 nm and 760 nm, respectively. The responsible initial ratio is shown in Fig. 7(b). The addition of 4.8 µM guanidine and the...
activity of 5.5 mM mL\(^{-1}\) DAO were inhibited, and the UV-vis spectra or the color of the dispersion did not change (Fig. 7(b), vial 10). The result was based on this formula: inhibitory ratio (%) = \((A_{760}/A_{538}-A_{760}*/A_{538}*)/(A_{760}/A_{538}-A_{760}/A_{538}) \times 100\%\) (A = without adding guanidine, A* = with guanidine). The maximum inhibition ratio of guanidine is 54% with an IC\(_{50}\) value of 2.4 μM.

4. Conclusions

In summary, a simple, visible, and effective colorimetric method was developed for DAO activity detection based on the HMD binding to the pSC\(_6\) by utilizing the supramolecule coated AuNPs. Furthermore, the potential of this assay was used in inhibitor screening. Comparing with the other literature, the fabricated method transforms the detection events into colour changes, which is fast responding, low-cost and readable to the naked eye. Moreover, the method has a great potential for the practical detection of DAO activity and the effective screening of inhibitors because of its low technical and instrumental demands, and simple and rapid operation.

Acknowledgements

This work is supported by the National Natural Science Foundation of China (Grant No. 61275085).

References


