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Mineralizing gold-silver bimetals into hemin-melamine matrix: A nanocomposite nanozyme for visual colorimetric analysis of H_2O_2 and glucose

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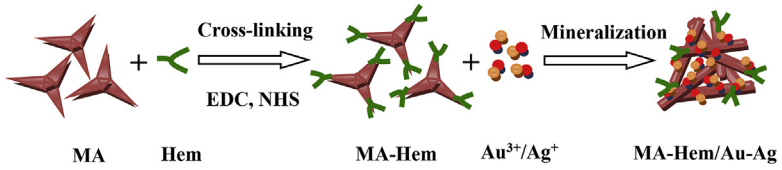
HIGHLIGHTS

- A rod-like nanocomposite nanozyme was fabricated by mineralizing Au–Ag bimetals into Hemin-melamine matrix.
- The mineralized Au–Ag bimetals could act as "nanowires" to promote the electron transferring within nanocomposites.
- The nanozyme with enhanced peroxidase-like catalysis was coupled with GOD towards a complex enzyme.
- High adsorption capacity could be expected for the substrates and targeting analytes.
- The catalysis-based colorimetric method can detect glucose with the level down to 1.8 μM.

GRAPHICAL ABSTRACT



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ABSTRACT

A nanocomposite nanozyme has been fabricated through mineralizing gold-silver bimetals into Hemin (Hem)-coupled melamine (MA) polymer matrix for visual colorimetric analysis of H_2O_2 and glucose. Catalytic Hem was cross-linked onto MA scaffold for the mineralization of Au–Ag bimetals yielding the rod-like nanocomposite of MA-Hem/Au–Ag. It was discovered that the resulting nanocomposite could present high aqueous stability and especially improved catalysis, which was more than four-fold higher than that of native Hem. Catalytic kinetics studies indicate that the prepared nanocomposite nanozyme could present much higher affinities to the substrates than those of native Hem or even horseradish peroxidase. Herein, the so mineralized Au–Ag bimetals with the "silver effect" would act as "nanowires" for promoting the electron transferring of nanocomposite nanozyme. Moreover, the Hem-coupled MA polymer matrix with high specific surface area could ensure the high adsorption capacity for the reactant substrates and targeting analytes. The application feasibility of the developed nanocomposite nanozyme was demonstrated subsequently by the colorimetric assays for H_2O_2 and glucose separately in milk and blood samples, with the linear ranges of 0.010–2.50 mM and 0.0050–2.0 mM, respectively. Such a

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bimetal mineralization-based fabrication route may open a new door toward the design of diverse nanocomposites nanozymes with improved catalysis and adsorption performances.

1. Introduction

Due to the high catalysis efficiencies and specificities, natural enzymes such as horseradish peroxidase (HRP) and glucose oxidase (GOD), have been widely applied in the catalysis, food, biomedicine, and environment fields [1]. Nevertheless, they may suffer from some inherent drawbacks, such as cost-ineffectiveness, storage instability and environmentally-affected catalysis [2]. Alternatively, many researchers have been focused on the fabrications of various artificial enzymes as more stable and low-cost alternatives to the natural ones by using porphyrins, supramolecules, biomolecules, and metal complexes to mimic natural enzymes [3–6]. As a representative, hemin (Hem), the heme-redox active sites of catalytic proteins such as HRP and hemoglobin, has been widely applied in catalysis field, but showing some disadvantages like low catalysis and poor aqueous solubility [7–11]. Also, increasing efforts have been devoted to the development of catalytic nanomaterials, known as nanozymes, such as Fe_3O_4 nanoparticles (NPs), carbon nanotubes, graphenes and ultra-small noble metals (Pt, Au and Ag) [6,12–20]. In particular, Au NPs have been utilized to label or anchor some enzymes (i.e., HRP) or catalytic derivatives to accelerate the electron transferring toward the improved catalysis [9,21–23]. Moreover, recent decades have witnessed the rapid development of nanocomposites-based nanozymes that are formed by noble metals or transition metal oxides showing enhanced peroxidase-like activities [14,24–30]. For example, Saeed Y and coworkers designed a nanocomposite nanozyme of C-dots/Fe_3O_4 for the determination of H_2O_2 in nanomolar levels [26]. Huang et al. have fabricated a graphene oxide-Se nanocomposite with glutathione peroxidase-like catalysis for cytoprotection [27]. Liu' group reported a nanocomposite nanozyme consisting of cobalt oxide and carbon for the colorimetric detection of glucose [28]. However, most of the nanocomposites-based nanozymes may suffer from a formidable limitation regarding the poor integration of different components or low environmental stability, which may greatly prevent them from being used on a large scale.

It is well recognized that with the synergetic effects, the integration of Au and Ag metals towards bimetallic Au/Ag NPs can achieve better electronic, optical and catalytic performances over the monometallic ones [17,31–34]. For example, Shi et al. discovered that a "silver effect" could be obtained for the bimetallic Au/Ag NPs presenting much higher catalysis than Au NPs alone [32]. Our group also established that bimetallic Au–Ag nanoclusters could display a "silver effect"-enhanced red fluorescence [17]. In addition, noble metals like silver could conduct the strong interaction with melamine (MA), a nitrogen-rich polymer molecule for forming nanocomposites [35], to yield the diverse functional nanocomposites [36–38]. For instance, Li's group synthesized the hierarchical silver nanochains by the Ag-MA self-assembly [36].

Inspired by the pioneering works above, in the present work, catalytic Hem was first covalently attached onto MA by the cross-linking chemistry to obtain the Hem-coupled MA polymer matrix. The in-site encapsulation of bimetallic Au–Ag was then conducted by the minerization route to yield the nanocomposite nanozyme. It was discovered that the resulting MA-Hem/Au–Ag nanocomposite could present the robust environmental stability and especially strong catalytic activities, which was more than four-fold higher than that of native Hem. Herein, the mineralized Au–Ag bimetals were thought to act as the "nanowires" for promoting the electron transferring of Hem-containing nanocomposites with the improved catalysis, in which the "silver effect" could be expected for Au NPs at a vital Au-to-Ag molar ratio (i.e., 5/2). Moreover, the catalysis performances of the MA-Hem/Au–Ag nanozyme were studied by catalyzing chromogenic reactions of 3, 3′, 5, 5′-tetramethyl benzidine (TMB) and H_2O_2. Also, steady-state kinetic studies were carried out to explore the catalysis and substrate affinities of MA-Hem/Au–Ag, of which the calculated parameters were compared with those of Hem and HRP. Subsequently, the feasibility of the developed catalysis-based colorimetric strategy for probing H_2O_2 and glucose was demonstrated with high sensitivity. To the best of our knowledge, this is the first report on the fabrication of nanocomposite nanozyme by the bimetallic mineralization into catalytic Hem-binding polymer matrix, showing greatly improved catalysis and adsorption capacity for the colorimetric assays of H_2O_2 and glucose.

2. Experimental section

2.1. Reagents and apparatus

Hemin (Hem) from bovine blood was purchased from Sigma to be used without further purification. Hydrogen tetrachloroaurate ($HAuCl_4$), silver nitrate ($AgNO_3$), hydrogen peroxide (H_2O_2), glucose, melamine (MA), 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide (EDC), triphenylphosphine (PPh_3), and N-Hydroxy succinimide hydrochloride (NHS) were obtained from Aladdin Reagent Co., Ltd. (Shanghai, China). Chromogenic substrates of 3, 3′, 5, 5' tetramethyl benzidine (TMB) and TMB-H_2O_2 were purchased from Sinopharm Chemical Reagent Co. (Shanghai, China). Glucose oxidase (GOD, 200 U mg^{-1}) was obtained from Sangon Biotech Co., Ltd (China). All other reagents are of analytical grade. Deionized water (>18 MΩ) was supplied from an Ultrapure water system (Pall, USA).

The colorimetric measurements of the catalytic reaction products were performed by a microplate reader (Infinite M200 PRO, Tecan, Austria) with 96-well plates (JET BIOFIL, Guangzhou, China). UV–vis absorption spectra were collected using UV-3600 spectrophotometer (Shimadzu, Japan), and scanning electron microscope (SEM, JSM-6700F, Japan) was employed to characterize the resulting nanocomposites.

2.2. Synthesis of MA-Hem/Au–Ag

Hem with carboxyl groups was first bound with amine-derivatized MA by the EDC-NHS cross-linking chemistry. Briefly, an aliquot of EDC (100 mM) and NHS (80 mM) were premixed and then added into the Hem solution (1.0 mL, 1.0 mg mL^{-1}) to be stirred for 1 h at room temperature. Next, an aliquot of MA (0.50 mL, 10 mM) was introduced into the solution of activated Hem to be vigorously stirred at 37 °C for 30 min, followed with the addition of an aliquot of $HAuCl_4$ (0.5 mL, 10 mM). After the mixture was stirred for 1.0 h, an aliquot of $AgNO_3$ (0.10 mL, 20 mM) was introduced into the mixture to be stirred for 8 h at 37 °C. After the products were further purified by dialysis, the resulting MA-Hem/Au–Ag

nanocomposites were collected and stored at 4 °C in dark for the future usage.

2.3. Colorimetric investigations of catalytic materials

The colorimetric investigations of peroxidase-like catalysis activities of catalytic materials were comparably conducted by using the TMB-H_2O_2 reactions. Typically, an aliquot of the prepared nanocomposites (4.31 mg mL^{-1}, 2.5 μL) or Hem (0.81 mg mL^{-1}, 2.5 μL) was introduced into the TMB-H_2O_2 reactions, of which the chromogenic reaction products were monitored at 652 nm using 96-well plates and a microplate reader. Moreover, the optimization of the main conditions for the synthesis of MA-Hem-Au-Ag nanocomposites were performed using different $AgNO_3$ amounts (10.0–60.0 mM), Hem dosages (0.20–1.8 mg mL^{-1}), pH values (2.0–12), and reaction time (2.0–12 h). Also, the optimization of the catalytic reaction conditions of nanocomposites were carried out with TMB-H_2O_2 reactions at different nanocomposites dosages (0.27–5.4 mg mL^{-1}), pH values (2.0–13), temperature (2.0–50 °C), and ion strengths in NaCl concentrations (0.83–41.67 mM). Additionally, catalysis kinetic studies were carried out for MA-Hem/Au–Ag nanocomposites by comparing with Hem (each containing 1.25 μM Hem), of which H_2O_2 of 8.82 mM or TMB of 0.42 mM was applied alternatively at a fixed concentration of one substrate versus varying concentration of the second substrate. The Lineweaver-Burk plots by the double reciprocal of the Michaelis-Menten equations were thus performed to calculate the Michaelis-Menten constants. Of note, the Hem concentrations in the MA-Hem/Au–Ag nanocomposites were measured from the data of UV–vis absorbance through referring to the plotted standard curve for Hem concentrations versus UV–vis absorbance values.

2.4. Colorimetric detection of H_2O_2 and glucose in samples

Under the optimized conditions, the colorimetric detections of H_2O_2 and glucose with different concentrations separately spiked in milk and blood samples were conducted. First, an aliquot of MA-Hem/Au–Ag nanocomposites (4.31 mg mL^{-1}) was added to TMB solutions with different concentrations of H_2O_2 spiked in milk samples (0.010, 0.050, 0.10, 0.25, 0.50, 1.0, 1.5, 2.0 and 2.5 mM). Then, the mixtures were incubated at 37 °C for 20 min with the UV–vis absorbance values to be measured at 652 nm using 96-well plates and a microplate reader. Herein, the developed method was applied to sensing H_2O_2 in the practical real samples, which were not pretreated to be diluted before usage. Besides, by following the similar analysis procedure, different concentrations of glucose spiked in blood (0.0050, 0.0010, 0.0020, 0.050, 0.10, 0.25, 0.50, 1.0, 1.5 and 2.0 mM) were analyzed. Briefly, an aliquot of the activated MA-Hem/Au–Ag nanocomposites (4.31 mg mL^{-1}) was mixed with GOD (10 mg mL^{-1}) under stirring for 1.0 h to form the MA-Hem/Au–Ag-GOD complex enzymes. An aliquot of the complex enzymes (containing 1.25 μM Hem) was then added to TMB solutions with glucose of different concentrations spiked in blood. After being incubated at 37 °C for 20 min, the product solutions were measured with UV–vis absorbance values recorded at 652 nm using 96-well plates and a microplate reader.

2.5. Preparation of blood samples

Blood samples, provided by the University Hospital by collecting from healthy volunteers with informed consent, were prepared through protein precipitation route. Briefly, an aliquot of 5.0 mL of collected blood was vigorously mixed with HCl (0.40 mL, 0.20 M) and PPh_3 (0.20 mL, 0.40 M) in water/acetonitrile of 20/80. After incubation for 15 min, the hydrolysed blood was mixed with 5.0 mL of acetonitrile to precipitate proteins, followed by centrifugation at 4000 rpm for 20 min. The so obtained supernatants were stored in 4 °C for future use. In addition, all the experiments were performed in compliance with the Ethical Committee Approval of China, and approved by the ethics committee at Qufu Normal University.

3. Results and discussion

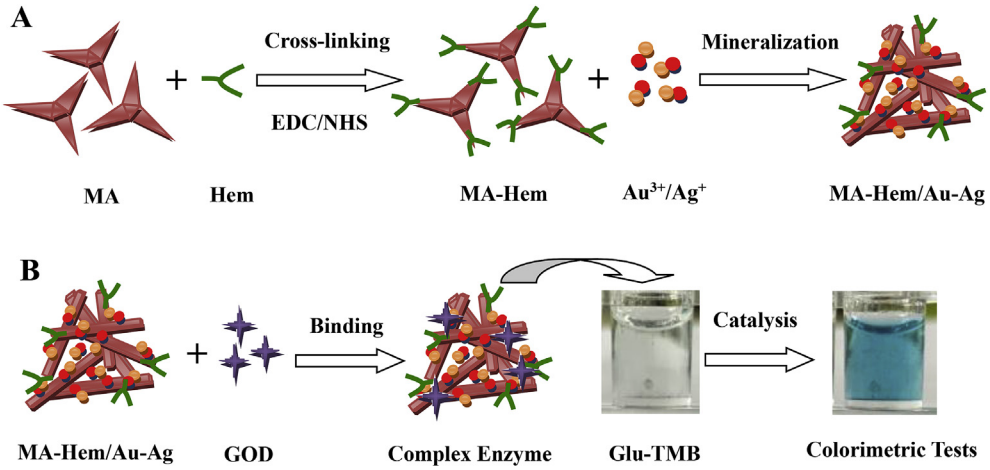
3.1. Synthesis and characterization of MA-Hem/Au–Ag

As illustrated in Scheme 1A, the catalytic Hem with carboxyl groups was first covalently cross-linked onto amine-derivatized MA scaffold through the EDC-NHS chemistry to yield the Hem-coupled MA polymer matrix, followed by the mineralization of Au–Ag bimetals. The resulting rod-like nanocomposite nanozyme of MA-Hem/Au–Ag was utilized for catalyzing the typical chromogenic TMB-H_2O_2 reactions. Herein, the Hem-coupled MA polymer matrix with some functional groups (i.e., amine, carboxyl) could act as the stabilizer and reducing agents for the bimetal mineralization. Importantly, the Au–Ag bimetals so mineralized would serve as “nanowires” to booster the electron transferring in Hem-containing nanocomposites leading to the greatly improved catalysis performances, in which a “silver effect” could be expected for increasing the “nanowiring” function of Au–Ag bimetals. In addition to the robust environmental stability, as a result, the as-prepared MA-Hem/Au–Ag nanocomposites could present the strong peroxidase-like catalytic activity to promise the colorimetric analysis applications. Furthermore, the MA-Hem/Au–Ag nanocomposites were attached with GOD to obtain the complex enzyme for the catalysis-based colorimetric analysis of glucose, as described in Scheme 1B. Herein, the GOD on the nanocomposites could catalyze the oxidation of glucose to gluconolactone and H_2O_2, of which H_2O_2 would conduct the oxidation of TMB catalyzed by the MA-Hem/Au–Ag nanocomposites, showing the blue reaction products (insert, Scheme 1B), thus achieving the colorimetric assays for glucose.

The topological structures of MA-Hem/Au–Ag nanocomposites were characterized using scanning electron microscope (SEM) in comparison with MA-Hem/Au nanocomposites without silver elements. One can note from Fig. 1A that the products of MA-Hem/Au–Ag nanocomposites could exhibit the uniform rod-like profile, of which the surfaces could be decorated with cloddy blocks, as more clearly shown in the amplified view (insert). In contrast, the MA-Hem/Au nanocomposites formed without silver elements could display the smooth surfaces (Fig. 1B), as disclosed in the amplified view (insert). Moreover, the characteristic UV–vis spectra of MA-Hem/Au–Ag nanocomposites were measured, taking Hem and MA as the controls (Fig. 2A). It was observed that the developed nanocomposites could include the absorbance peaks of Hem at about 395 nm and mineralized Au–Ag at about 330 nm, thus confirming the successful integration of Hem and mineralized Au–Ag bimetals into the nanocomposites.

3.2. Colorimetric investigations of peroxidase-like catalysis of MA-Hem/Au–Ag

The peroxidase-like catalysis of MA-Hem/Au–Ag nanocomposites was investigated by the colorimetric tests taking MA-Hem/Au and Hem for comparison (each containing Hem of similar concentration) (Fig. 2B). Accordingly, the MA-Hem/Au–Ag nanocomposites could display the strongest catalysis performance, which is more than four-fold and two-fold higher than that of native Hem and MA-Hem/Au, respectively. As aforementioned, here, the dramatically enhanced catalysis of MA-Hem/Au–Ag



Scheme 1. Schematic illustration of (**A**) the fabrication procedure of MA-Hem/Au–Ag nanocomposite nanozymes including MA-Hem cross-linking and Au–Ag mineralization, and (**B**) the catalysis-based colorimetric test for glucose using GOD-loading MA-Hem/Au–Ag nanozymes.

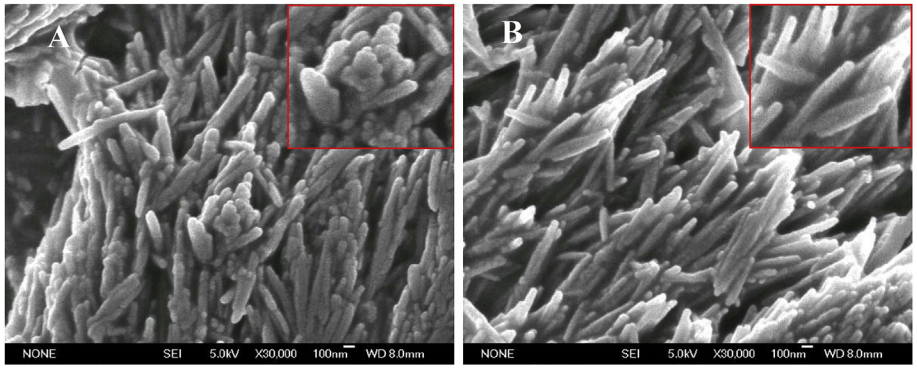


Fig. 1. SEM images of (**A**) MA-Hem/Au–Ag and (**B**) MA-Hem/Au nanocomposites (insert: the amplified views).

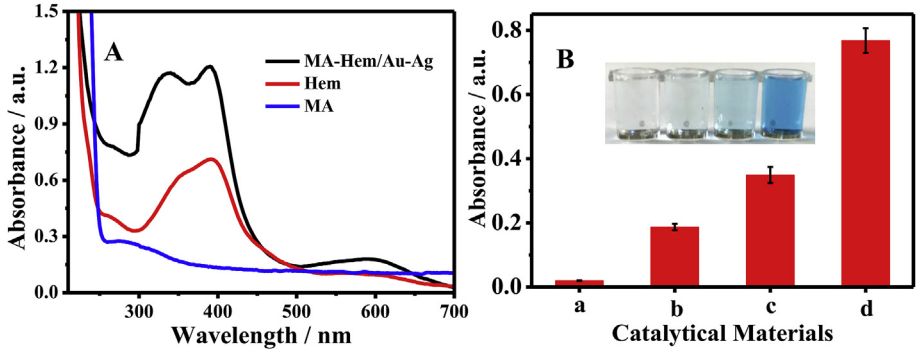


Fig. 2. Comparison of (**A**) UV–vis spectra among Hem, MA, and MA-Hem/Au–Ag and (**B**) the catalysis activities in catalyzing TMB-H_2O_2 reactions among (b) Hem, (c) MA-Hem/Au, (d) MA-Hem/Au–Ag, taking (a) TMB-H_2O_2 as the control (insert: the photographs of corresponding product solutions of catalytic TMB-H_2O_2 reactions).

nanocomposites over Hem was thought to result from the Au–Ag bimetals so mineralized that could increase the electron transferring by acting as the "nanowires". As aforementioned, herein, the introduction of silver elements would endow the Au–Ag bimetals with the "silver effect" achieving the better electronic and catalytic performances than the monometallic ones. The yielded bimetallic Au/Ag NPs in hemin-melamine matrix would act as the "nanowires" for the further enhanced function of accelerating the electron transferring of MA-Hem/Au–Ag nanocomposites, thus showing the stronger catalysis.

3.3. Main synthetic conditions of MA-Hem/Au–Ag

The main conditions for the synthesis of MA-Hem/Au–Ag nanocomposites were optimized (Fig. 3). First, the $AgNO_3$ concentrations could play a vital role in the preparation of MA-Hem/Au–Ag nanocomposites with the mineralization of Au–Ag bimetals. It was found that the catalytic activities of MA-Hem/Au–Ag nanocomposites could increase with the increasing concentrations of $AgNO_3$ till 20.0 mM with the Au-to-Ag ratios of 5/2 so calculated (Fig. 3A), over which a gradual decrease in the catalytic activities

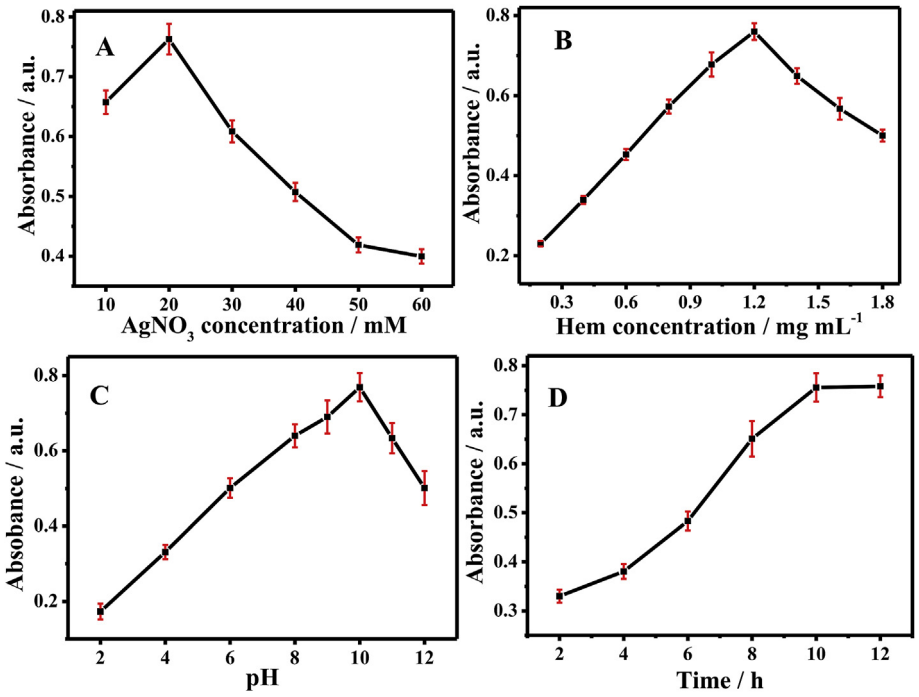


Fig. 3. Optimization of main synthetic conditions of MA-Hem/Au–Ag including (**A**) $AgNO_3$ concentrations, (**B**) Hem concentrations, (**C**) pH values, and (**D**) reaction time.

could be encountered. Second, the effects of Hem used on the fabrication of MA-Hem/Au–Ag nanocomposites were explored by using different Hem concentrations (Fig. 3B). Obviously, an aliquot of 1.20 mg mL^{-1} Hem in the synthesis reactions was suitable for yielding the MA-Hem/Au–Ag nanocomposites. Third, the pH values could influence the catalysis of MA-Hem/Au–Ag nanocomposites (Fig. 3C). Obviously, the highest response could be obtained at pH 10, which should be selected as the most suitable one. Finally, the reaction time for the formation of MA-Hem/Au–Ag nanocomposites was investigated (Fig. 3D), showing the optimum time of about 10 h.

3.4. Main catalytic reaction conditions of MA-Hem/Au–Ag

The main catalytic reaction conditions were explored for the MA-Hem/Au–Ag nanocomposites by using the TMB-H_2O_2 reactions, with the results shown in Fig. 4. As disclosed in Fig. 4A, the nanocomposites dosages play a vital role in the catalytic performances of MA-Hem/Au–Ag nanocomposites, with the optimal one at 4.31 mg mL^{-1}. Fig. 4B displays the pH value-depending catalysis performances of MA-Hem/Au–Ag nanocomposites. Apparently, the MA-Hem/Au–Ag nanocomposites could perform the best catalysis at pH 6.0. The results suggest that MA-Hem/Au–Ag nanocomposites might conduct the strong catalysis under the slightly acidic conditions. Furthermore, the temperature-dependent activities of MA-Hem/Au–Ag nanocomposites in catalyzing TMB-H_2O_2 reactions was explored (Fig. 4C). Accordingly, the developed nanocomposites nanozymes could exhibit the highest catalytic activities at about 37 °C, which is consistent with that of native Hem. Fig. 4D shows that the ion strength is another significant factor in the catalytic reactions with the optimal one at about 12 mM. These results indicate that MA-Hem/Au–Ag nanocomposites and Hem might conduct the catalysis under the basically similar reaction conditions. Therefore, the nanocomposites formed by the mineralized bimetallic route might ensure the main molecule structure and function of Hem, yet, dramatically improve their catalytic activities through promoting the electron-transferring.

Furthermore, the time-dependent catalytic performances for TMB-H_2O_2 reactions of the developed nanozymes of MA-Hem/Au–Ag nanocomposites was probed by monitoring the UV–vis absorbance changes of the TMB-H_2O_2 products with the increasing of reaction time (Fig. 5A). Accordingly, the MA-Hem/Au–Ag nanocomposites could well catalyze the TMB-H_2O_2 reactions within 22 min, showing a much faster catalysis rate than native Hem. In addition, studies were made on the environmental stability of the developed MA-Hem/Au–Ag nanocomposites stored over different time intervals (Fig. 5B). As expected, no significant change in the catalysis performances was witnessed for MA-Hem/Au–Ag nanocomposites even that they were stored in water up to seven months, thus confirming the high environmental stability, which is thought mainly from the Hem-coupled MA polymer matrix that might act as the scaffold stabilizer.

3.5. Kinetics studies on MA-Hem/Au–Ag catalysis

Catalysis kinetics studies were conducted for the developed nanozymes of MA-Hem/Au–Ag nanocomposites by using the Michaelis-Menten model, taking native Hem as the comparison (Fig. 6). One can observe that the reciprocal plotting of initial reaction rates versus varying reciprocal of TMB amounts (Fig. 6A) or H_2O_2 concentrations (Fig. 6B) could illustrate the typical Michaelis-Menten behavior. Importantly, the comparison of the colorimetric results revealed that the as-prepared MA-Hem/Au–Ag nanozymes could display much better catalysis performances than native Hem. Moreover, according to the regression of Lineweaver-Burk double reciprocal curves, the dynamic parameters were calculated including Michaelis constant (K_m) and the maximal reaction velocity (V_{max}). The results are summarized in Table 1 by comparing with those of HRP reported previously [39]. One can note that the apparent K_m value of MA-Hem/Au–Ag nanocomposites for H_2O_2 (2.70 mM) is much lower than that of native Hem (3.20 mM) or HRP

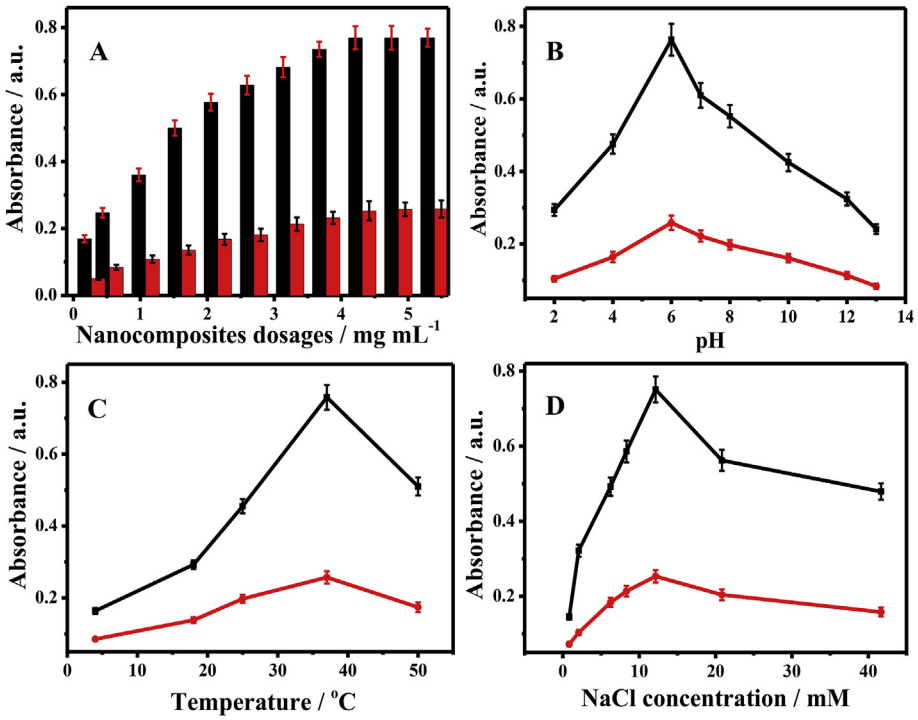


Fig. 4. Comparison of catalytic reaction conditions between MA-Hem/Au–Ag (black) and Hem (red) in catalyzing TMB-H_2O_2 reactions including (**A**) nanocomposites dosages, (**B**) pH values, (**C**) temperatures, and (**D**) ion strengths in NaCl concentrations. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

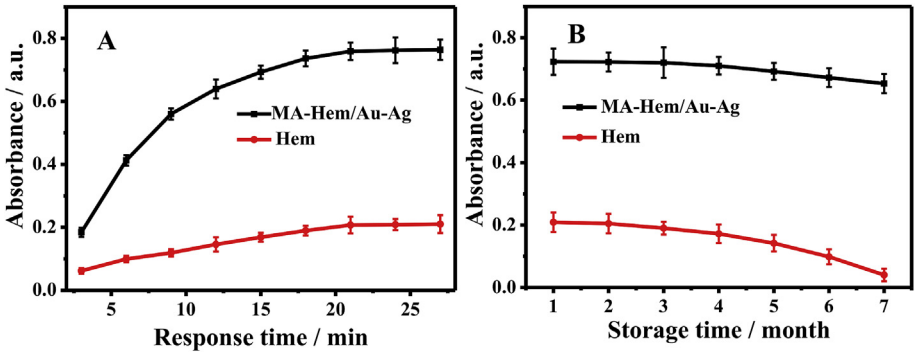


Fig. 5. Comparison of (**A**) time-dependent catalysis performances and (**B**) environmental stabilities between MA-Hem/Au–Ag (black) and Hem (red), which were stored over different time intervals to be applied for catalyzing the TMB-H_2O_2 reactions. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

(3.70 mM). Meanwhile, the K_m value of the developed peroxidase mimics for TMB substrate is 2.39 mM, which is also much lower than that of Hem (3.98 mM), but higher than that of HRP (0.434 mM). Moreover, MA-Hem/Au–Ag nanocomposites could present higher V_{max} values for H_2O_2 than native Hem or HRP. These data demonstrate that MA-Hem/Au–Ag nanocomposites could exhibit higher catalysis affinity to the substrates (i.e., H_2O_2 and TMB) over Hem, presumably due to that the mineralized Au–Ag bimetals could promote the electron-transferring aforementioned, and especially the Hem-coupled MA polymer matrix with functional groups (i.e., amine, carboxyl groups), which might build up a better catalytic reactivity pathways to accumulate more substrates towards their catalysis-active sites, so as to facilitate more efficient transformations of substrates [40–43]. Therefore, much stronger catalysis performances could be expected for the developed nanozymes of MA-Hem/Au–Ag nanocomposites, promising the catalysis-based colorimetric analysis of H_2O_2 afterwards.

3.6. Preliminary applications of the nanozyme-based colorimetric method for H_2O_2 and glucose in samples

Under the optimal conditions, the developed MA-Hem/Au–Ag-based colorimetric method was applied separately for the analysis of H_2O_2 and glucose in milk and blood samples, respectively (Fig. 7). Fig. 7A manifests the calibration curve of the nanocomposites-based colorimetric assays for H_2O_2. Accordingly, the detection of H_2O_2 in milk could be achieved with the concentrations linearly ranging from 0.010 to 2.50 mM. A detection limit (LOD) of about 2.5 μM was obtained as estimated by the 3σ rule, of which the

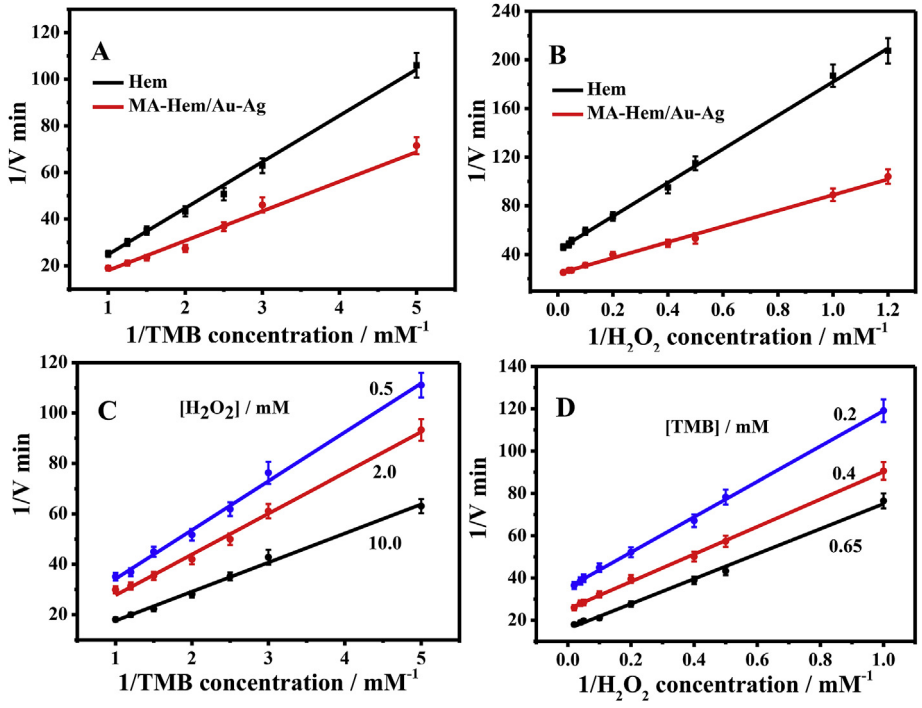


Fig. 6. Catalysis kinetics of double-reciprocal plots for (a) MA-Hem/Au–Ag and (b) Hem by using (**A**) various TMB concentrations at fixed 8.82 mM H_2O_2 and (**B**) various H_2O_2 concentrations at fixed 0.42 mM TMB; the double-reciprocal plots for kinetic catalysis of MA-Hem/Au–Ag composites using (**C**) various TMB concentrations at three fixed H_2O_2 concentrations, and (**D**) various H_2O_2 concentrations at three fixed TMB concentrations.

Table 1
Comparison of dynamic catalysis parameters among Hem, MA-Hem/Au–Ag, and HRP documented for TMB and H_2O_2 substrates.

Catalysis materials	Substrates	K_m (mM)	V_{max} (M s^{-1})
Hem	TMB	3.98	2.0×10^{-8}
HRP	TMB	0.434	10.0×10^{-8}
MA-Hem/Au–Ag	TMB	2.39	1.42×10^{-8}
Hem	H_2O_2	3.2	3.1×10^{-8}
HRP	H_2O_2	3.7	8.71×10^{-8}
MA-Hem/Au–Ag	H_2O_2	2.7	14.1×10^{-8}

performances (e. g. analysis range and LOD) are better than those of the other colorimetric methods for H_2O_2 reported previously [12,44]. Moreover, the MA-Hem/Au–Ag nanocomposites were bound with GOD to form the complex enzyme for the colorimetric analysis of glucose in blood (Fig. 7B). It was noted that glucose in blood could be detected with the linear concentration ranging from 0.0050 to 2.0 mM, with the LOD of about 1.8 μM. Such a LOD is lower than those of the other detection methods reported previously for glucose in blood [28,44–46]. Therefore, the developed catalysis-based colorimetric strategy with the nanocomposites nanozymes can allow for the analysis of H_2O_2 and glucose with high detection sensitivities and wide analysis ranges. In addition, the recovery tests were performed by using the developed colorimetric method to probe H_2O_2 in milk and glucose in blood samples, showing the recoveries obtained ranging from about 98.0 to 105.0% and 98.4–104.0%,respectively (Table 2).

4. Conclusions

To summarize, a nanozyme of MA-Hem/Au–Ag nanocomposite has been successfully fabricated with powerful catalysis and robust environmental stability by the in-site mineralization of Au–Ag bimetals into Hem-coupled MA polymer matrix for sensing H_2O_2 and glucose in milk and blood, respectively. As evidenced in the colorimetric assays, the obtained nanocomposite nanozyme could

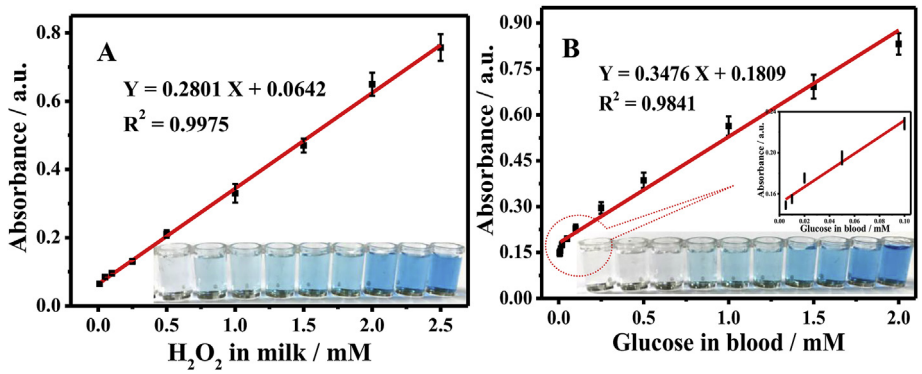


Fig. 7. The calibration curves for the MA-Hem/Au–Ag-based colorimetric assays for (**A**) H_2O_2 and (**B**) glucose with different concentrations spiked in milk and blood, respectively.

Table 2
Recovery test results of the developed colorimetric method in detecting H_2O_2 in milk and glucose in blood samples (n = 5).

H_2O_2 concentrations (mM)		Recoveries (%)
Added (mM)	Founded (mM)	
0.2	0.21±0.04	105.0
0.5	0.51±0.09	102.0
1.0	0.98±0.08	98.0
2.0	2.03±0.1	101.5
Glucose concentrations (mM)		Recoveries (%)
Added (mM)	Founded (mM)	
0.1	0.104±0.02	104.0
0.5	0.492±0.05	98.4
1.0	1.03±0.08	103
1.5	1.488±0.11	99.2

present the greatly improved catalytic activities, which are over four-fold higher than native Hem. Moreover, steady-state catalytic kinetics studies indicate that the developed nanocomposite nanozyme could achieve the higher substrate affinity and catalysis capacities than native Hem and even HRP, as demonstrated by lower K_m values. Herein, the introduction of Au–Ag bimetals mineralized into the Hem-coupled MA polymer matrix could not only functionalize as "nanowires" to accelerate the electron transferring, but also possess high adsorption capacity for the reactant substrates and targeting analytes towards the high substrate affinity. H_2O_2 and glucose in samples could be quantified by the MA-Hem/Au–Ag-based colorimetric method with the LOD down to about 2.5 μM and about 1.8 μM, respectively. Although the detailed mechanism should be investigated further, such a synthesis methodology with the bimetallic Au–Ag mineralization may be utilized for the fabrication of a variety of enzymes or catalytic mimetic elements (i.e., Hem) with improved catalysis performances, thus promising the extensive applications in the catalysis, biomedical analysis, and environmental treatment fields.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

[1] N. Lu, M. Zhang, L. Ding, J. Zheng, C. Zeng, Y. Wen, G. Liu, A. Aldalbahi, J. Shi, S. Song, X. Zuo, L. Wang, Yolk–shell nanostructured Fe_3O_4@C magnetic nanoparticles with enhanced peroxidase-like activity for label-free colorimetric detection of H_2O_2 and glucose, Nanoscale 9 (2017) 4508–4515.
[2] J. Xie, X. Zhang, H. Wang, H. Zheng, Y. Huang, J. Xie, Analytical and environmental applications of nanoparticles as enzyme mimetics, Trends Anal. Chem. 39 (2012) 114–129.
[3] R. Villalonga, R. Cao, A. Fragoso, Supramolecular chemistry of cyclodextrins in enzyme technology, Chem. Rev. 107 (2007) 3088–3116.
[4] R. Bonar-Law, J. Sanders, Polyol recognition by a steroid-capped porphyrin: enhancement and modulation of misfit guest binding by added water or methanol, J. Am. Chem. Soc. 117 (2002) 259–271.
[5] R. Lerner, S. Benkovic, P. Schultz, At the crossroads of chemistry and immunology: catalytic antibodies, Science 252 (1991) 659–667.
[6] H. Wei, E. Wang, Nanomaterials with enzyme-like characteristics (nanozymes): next-generation artificial enzymes, Chem. Soc. Rev. 42 (2013) 6060–6093.
[7] X. Jiang, Y. Chai, H. Wang, R. Yuan, Electrochemiluminescence of luminol enhanced by the synergetic catalysis of hemin and silver nanoparticles for sensitive protein detection, Biosens. Bioelectron. 54 (2014) 20–26.
[8] Y. Li, A. Townshend, Comparative study of some synthesised and commercial fluorogenic substrates for horseradish peroxidase and its mimetic enzyme hemin by a flow injection method, Anal. Chim. Acta 340 (1997) 159–168.
[9] S. Li, L. Zhang, Y. Jiang, S. Zhu, X. Lv, Z. Duan, H. Wang, In-site encapsulating gold "nanowires" into hemin-coupled protein scaffolds through biomimetic assembly towards the nanocomposites with strong catalysis, electrocatalysis, and fluorescence properties, Nanoscale 9 (2017) 16005–16011.
[10] T.C. Bruice, Reactions of hydroperoxides with metallotetraphenylporphyrins in aqueous solutions, Acc. Chem. Res. 24 (1991) 243–249.
[11] L. Zhang, S. Li, M. Dong, Y. Jiang, R. Li, S. Zhang, X. Lv, L. Chen, H. Wang, Reconstituting redox active centers of heme-containing proteins with biomineralized gold toward peroxidase mimics with strong intrinsic catalysis and electrocatalysis for H $_2O_2$ detection, Biosens. Bioelectron. 87 (2017) 1036–1043.
[12] H. Wei, E. Wang, Fe_3O_4 magnetic nanoparticles as peroxidase mimetics and their applications in H_2O_2 and glucose detection, Anal. Chem. 80 (2008) 2250–2254.
[13] H. Wang, S. Li, Y. Si, Z. Sun, S. Li, Y. Lin, Recyclable enzyme mimic of cubic Fe_3O_4 nanoparticles loaded on graphene oxide-dispersed carbon nanotubes with enhanced peroxidase-like catalysis and electrocatalysis, J. Mater. Chem. B 2 (2014) 4442–4448.
[14] H. Wang, S. Li, Y. Si, N. Zhang, Z. Sun, H. Wu, Y. Lin, Platinum nanocatalysts loaded on graphene oxide-dispersed carbon nanotubes with greatly enhanced peroxidase-like catalysis and electrocatalysis activities, Nanoscale 6 (2014) 8107–8116.
[15] J. Yu, S. Choi, R.M. Dickson, Shuttle-based fluorogenic silver-cluster biolabels, Angew. Chem. Int. Ed. 48 (2009) 318–320.
[16] B. Yoon, Charging effects on bonding and catalyzed oxidation of CO on Au_8 clusters on MgO, Science 307 (2005) 403–407.
[17] N. Zhang, Y. Si, Z. Sun, L. Chen, R. Li, Y. Qiao, H. Wang, Rapid, selective, and ultrasensitive fluorimetric analysis of mercury and copper levels in blood using bimetallic gold-silver nanoclusters with "silver effect"-enhanced red fluorescence, Anal. Chem. 86 (2014) 11714–11721.
[18] Z. Sun, N. Zhang, Y. Si, S. Li, J. Wen, X. Zhu, H. Wang, High-throughput colorimetric assays for mercury(II) in blood and wastewater based on the mercury-stimulated catalytic activity of small silver nanoparticles in a temperature-switchable gelatin matrix, Chem. Commun. 50 (2014) 9196–9199.
[19] H. Wei, C. Chen, B. Han, E. Wang, Enzyme colorimetric assay using unmodified silver nanoparticles, Anal. Chem. 80 (2008) 7051–7055.
[20] L. Jiao, H. Yan, Y. Wu, W. Gu, C. Zhu, D. Du, Y. Lin, When nanozymes meet single-atom catalysis, Angew. Chem. Int. Ed. (2019), https://doi.org/10.1002/anie.201905645 in press.
[21] F. Patolsky, Y. Weizmann, I. Willner, Long-range electrical contacting of redox enzymes by SWCNT connectors, Angew. Chem. Int. Ed. 43 (2004) 2113–2117.
[22] Y. Xiao, F. Patolsky, E. Katz, J.F. Hainfeld, I. Willner, Plugging into enzymes: nanowiring of redox enzymes by a gold nanoparticle, Science 299 (2003) 1877–1881.
[23] P. Xia, H. Liu, Y. Tian, Cathodic detection of H_2O_2 based on nanopyramidal gold surface with enhanced electron transfer of myoglobin, Biosens. Bioelectron. 24 (2009) 2470–2474.
[24] L. Zhang, C. Fan, M. Liu, F. Liu, S. Bian, S. Du, S. Zhu, H. Wang, Biominerized gold-Hemin@MOF composites with peroxidase-like and gold catalysis activities: a high-throughput colorimetric immunoassay for alpha-fetoprotein in blood by ELISA and gold-catalytic silver staining, Sens. Actuators, B 266 (2018) 543–552.
[25] S. Fan, M. Zhao, L. Ding, H. Li, S. Chen, Preparation of Co_3O_4/crumpled graphene microsphere as peroxidase mimetic for colorimetric assay of ascorbic acid, Biosens. Bioelectron. 89 (2017) 846–852.
[26] S. Yousefinejad, H. Rasti, M. Hajebi, M. Kowsari, S. Sadravi, F. Honarasa, Design of C-dots/Fe_3O_4 magnetic nanocomposite as an efficient new nanozyme and

its application for determination of H_2O_2 in nanomolar level, Sens. Actuators, B 247 (2017) 691–696.
[27] Y. Huang, C. Liu, F. Pu, Z. Liu, J. Ren, X. Qu, A GO-Se nanocomposite as an antioxidant nanozyme for cytoprotection, Chem. Commun. 53 (2017) 3082–3085.
[28] Y. Guo, L. Yan, R. Zhang, H. Ren, A. Liu, CoO-supported ordered mesoporous carbon nanocomposite based nanozyme with peroxidase-like activity for colorimetric detection of glucose, Process Biochem. 81 (2019) 92–98.
[29] Z. Moosavi-Movahedi, E. Kalejahi, M. Nourisefat, P. Maghami, N. Poursasan, A. Moosavi-Movahedi, Mixed SDS-Hemin-Imidazole at low ionic strength being efficient peroxidase-like as a nanozyme, Colloids Surf., A 522 (2017) 233–241.
[30] W. Liu, C. Gan, W. Chang, A. Qileng, H. Lei, Y. Liu, Double-integrated mimic enzymes for the visual screening of Microcystin-LR: copper hydroxide nanozyme and G-quadruplex/hemin DNAzyme, Anal. Chim. Acta 1054 (2019) 128–136.
[31] M. Hostetler, C. Zhong, B. Yen, J. Anderegg, S. Gross, N. Evans, M. Porter, R. Murray, Stable, monolayer-protected metal alloy clusters, J. Am. Chem. Soc. 120 (1998) 9396–9397.
[32] D. Wang, R. Cai, S. Sharma, J. Jirak, S. Thummanapelli, N. Akhmedov, H. Zhang, X. Liu, J. Petersen, X. Shi, "Silver effect" in gold (I) catalysis: an overlooked important factor, J. Am. Chem. Soc. 134 (2012) 9012–9019.
[33] R. Ferrando, J. Jellinek, R. Johnston, Nanoalloys: from theory to applications of alloy clusters and nanoparticles, Chem. Rev. 108 (2008) 845–910.
[34] P. Scodeller, V. Flexer, R. Szamocki, E. Calvo, N. Tognalli, H. Troiani, A. Fainstein, Wired-enzyme core-shell Au nanoparticle biosensor, J. Am. Chem. Soc. 130 (2008) 12690–12697.
[35] M. Tan, Y. Sum, J. Ying, Y. Zhang, A mesoporous poly-melamine-formaldehyde polymer as a solid sorbent for toxic metal removal, Energy Environ. Sci. 6 (2013) 3254–3259.
[36] J. Fei, J. Li, Controlled preparation of porous TiO_2-Ag nanostructures through supramolecular assembly for plasmon-enhanced photocatalysis, Adv. Mater. 27 (2015) 314–319.
[37] M. Liu, L. Zhang, Y. Hua, L. Feng, Y. Jiang, X. Ding, W. Qi, H. Wang, Mesoporous silver-melamine nanowires formed by controlled supermolecular self-assembly: a selective solid-state electroanalysis for probing multiple sulfides in hyperhaline media through the specific sulfide-chloride replacement reactions, Anal. Chem. 89 (2017) 9552–9558.
[38] Y. Hua, M. Liu, S. Li, F. Liu, Y. Cai, H. Liu, Y. Wan, X. Lv, H. Wang, An electroanalysis strategy for glutathione in cells based on the displacement reaction route using melamine-copper nanocomposites synthesized by the controlled supermolecular self-assembly, Biosens. Bioelectron. 124–125 (2019) 89–95.
[39] L. Gao, J. Zhuang, L. Nie, J. Zhang, Y. Zhang, N. Gu, T. Wang, J. Feng, D. Yang, S. Perrett, X. Yan, Intrinsic peroxidase-like activity of ferromagnetic nanoparticles, Nat. Nanotechnol. 2 (2007) 577–583.
[40] P. Dydio, J.N.H. Reek, Supramolecular control of selectivity in transition-metal catalysis through substrate preorganization, Chem. Sci. 5 (2014) 2135–2145.
[41] M. Raynal, P. Ballester, A. Vidal-Ferran, P. van Leeuwen, Supramolecular catalysis. Part 1: non-covalent interactions as a tool for building and modifying homogeneous catalysts, Chem. Soc. Rev. 43 (2014) 1660–1733.
[42] M. Raynal, P. Ballester, A. Vidal-Ferran, P.W. van Leeuwen, Supramolecular catalysis. Part 2: artificial enzyme mimics, Chem. Soc. Rev. 43 (2014) 1734–1787.
[43] Q. Wang, S. Gonell, S. Leenders, M. Durr, I. Ivanovic-Burmazovic, J. Reek, Self-assembled nanospheres with multiple endohedral binding sites pre-organize catalysts and substrates for highly efficient reactions, Nat. Chem. 8 (2016) 225–230.
[44] Y. Jv, B. Li, R. Cao, Positively-charged gold nanoparticles as peroxidiase mimic and their application in hydrogen peroxide and glucose detection, Chem. Commun. 46 (2010) 8017.
[45] C. Lu, X. Liu, Y. Li, F. Yu, L. Tang, Y. Hu, Y. Ying, Multifunctional Janus hematite-silica nanoparticles: mimicking peroxidase-like activity and sensitive colorimetric detection of glucose, ACS Appl. Mater. Interfaces 7 (2015) 15395–15402.
[46] A. Dalui, B. Pradhan, U. Thupakula, A.H. Khan, G.S. Kumar, T. Ghosh, B. Satpati, S. Acharya, Insight into the mechanism revealing the peroxidase mimetic catalytic activity of quaternary CuZnFeS nanocrystals: colorimetric biosensing of hydrogen peroxide and glucose, Nanoscale 7 (2015) 9062–9074.