



Synthesis of reduced graphene oxide-iron nanoparticles with superior enzyme-mimetic activity for biosensing application



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ABSTRACT

Development of enzyme-mimetic catalysts with sustainability and environmental benignancy has gained considerable attention with the growing demands for large-scale applications in recent years. Here, we demonstrate that the reduced graphene oxide (RGO)-iron nanoparticles (INs) can be utilized as the highly active and cost-effective enzyme-mimetic catalysts for the first time, which have been successfully synthesized by a facile iron-self-catalysis process at room temperature. Benefitting from synergetic effects between RGO and INs, the RGO-INs could efficiently catalyze the oxidization of 3,3',5,5'-tetramethylbenzidine (TMB) in the presence of H₂O₂ to produce a typical color reaction, showing the much better peroxidase-like activity than that of each individual part. The mechanistic insight into the enhanced peroxidase-like activity of the RGO-INs was investigated systematically. On the basis of the enzyme-mimetic activity of the RGO-INs, the simple, sensitive, selective and cost-effective colorimetric assays for the detection of hydrogen peroxide and glucose with naked eyes were successfully established. The RGO-INs showed several prominent advantages, such as facile preparation, low cost, tunability in catalytic activity, and low detection limit, over natural peroxidase or other nanomaterial-based alternatives, holding great potential as enzymatic mimics for biosensing applications.

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1. Introduction

Natural enzymes are well-known class of biological catalysts with high substrate specificities and high activity under mild reaction conditions, which have found significant and widespread applications in medicine, chemical industry, food processing and agriculture [1,2]. However, they intrinsically own some drawbacks, including limited natural sources, inherent instability, and requiring expensive and time-consuming purification, restricting their practical applications greatly. To overcome these limitations of natural enzymes while maintain the high reactivity, numerous efforts have been focused on the design and construction of artificial enzymes that can mimic the complexities and functions of natural enzymes. Particularly, the merging of nanotechnology with biology has ignited research interest for designing and seeking functional nanomaterials mimicking catalytically active enzymes [3,4]. For instance, inorganic metal oxides (Fe₃O₄ [5], MnO₂ [6], Co₃O₄ [7], CuO [8], TiO₂ [9], V₂O₅ [10]), metal sulfides (CuS [11], FeS [12], CdS [13], MoS₂ [4]) and carbon (carbon nanodots [14], graphene oxide [15], carbon nanotube [16]) nanomaterials have

been discovered to possess intrinsic peroxidase-like activity and shown promising potential in environmental remediation [17] and biosensing applications. Meanwhile, noble metal nanoparticles have been emerging as another type of the most promising materials with excellent peroxidase-mimetic activity because of their unique physical and chemical properties. For example, Au [18], Pt [19], Ag [20] nanoparticles have been reported separately to possess an intrinsic peroxidase-like activity. Their nanostructured alloys such as Au@Pt [21], AgM (M = Au, Pd, Pt) [22], Au@PtAg [23], and AuPd [24] achieve excellent ability toward peroxidase mimicry for catalytic acceleration of specific reaction as well. However, their practical large-scale applications are restricted dramatically by the inevitable disadvantages of high price and scarcity. Towards this end, it is of considerable interest and great significance to develop cheap and earth-abundant alternatives to noble metal catalyst to achieve high efficiency at low cost.

Iron is an earth abundant metal, and its oxides have been demonstrated as highly active peroxidase mimetics [25,26]. Apparently, utilization of metallic iron as peroxidase mimetics for biosensing application is remarkably attractive because of the anticipated sustainability and environmental benignancy. Very recently, Huang and his coworkers have been demonstrated a non-noble metal-based bimetallic Fe-Co nanoparticles indeed

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possesses peroxidase-like activity which exhibits high affinity to H_2O_2 and found that the combination with metallic cobalt generates the synergistic effect to enhance the peroxidase-like activity of monometallic iron [27]. However, to a certain extent, it is still not fully understood or very limitedly what dominates the catalytic mechanism and how metallic iron interacts with substrates during biosensing application. More surprisingly, to the best of our knowledge, there are no reports in the literature on the peroxidase mimicking behaviors of other metallic iron-based nanoparticles.

To maximize the reactivity and stability of metal NPs, a robust support is usually required to protect them against dissolution and aggregation. Owing to its large specific surface area, great mechanical strength, and low manufacturing cost, graphene has been considered a promising candidate as a new two-dimensional (2D) carrier to support metal nanoparticles [28,29]. Moreover, graphene-family materials represent the interesting properties which would be favorable for the design of nanomaterial-based enzyme mimics [30]. The opened surface areas of graphene are readily accessible to substrates with a small diffusion and transport barrier, while its rich surface chemistry ensures the stability of the supported systems. Thus, it is reasonably expected that graphene would be an effective 2D carrier for loading metal nanoparticles to create highly active enzyme mimetics and to further realize the cooperatively enhanced performances by the combination of the respective properties of each component.

Bearing these issues in mind, we herein report a facile and rational synthesis of reduced graphene oxide-iron nanoparticles (RGO-INS) by an iron-self-catalysis process and demonstrate the resulting RGO-INS can be utilized as the highly active and cost-effective enzyme-mimetic catalysts for the first time. The synthetic strategy is simple, inexpensive and scalable, and the whole processing is completely at room temperature. Benefitting from synergistic effects between INS and RGO, the RGO-INS could catalyze the oxidation of different peroxidase substrates in the presence of H_2O_2 to produce typical color reactions, which showed the better peroxidase-like activity than that of each individual part. Based on the peroxidase-like behavior of the RGO-INS, the simple, sensitive, selective and cost-effective colorimetric assays for the detection of H_2O_2 and glucose with naked eyes were successfully established.

2. Experimental

2.1. Materials

Graphite powder, KMnO_4 , NaNO_3 , H_2SO_4 (98%), $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, NaBH_4 , acetic acid (HAc), sodium acetate (NaAc) and H_2O_2 (30 wt%) were purchased from Kelong Chemical Reagents Company (Chengdu, China). Na_2HPO_4 , KH_2PO_4 , polyvinylpyrrolidone (PVP) and 3,3',5,5'-tetramethylbenzidine dihydrochloride (TMB) were purchased from Aladin Ltd (Shanghai, China). Glucose, fructose, lactose, maltose, and glucose oxidase (GOx, 340 U mg^{-1}) were purchased from Sangon Biochemical Engineering Technology Co., Ltd (Shanghai, China). All chemicals used in this study were analytical reagent grade. Freshly deionized water was used to prepare all solutions and conduct all of the tests. Graphite oxide was synthesized from oxidation of nature graphite powder by a modified Hummers method.

2.2. Preparation of reduced graphene oxide-iron nanoparticles (RGO-INS)

The preparation procedure for the RGO-INS was schematically illustrated in Fig. 1. In a typical synthesis, the required amount of graphite oxide was dispersed in deionized water by ultrasonication for 1 h. 5 mL of aqueous solution containing $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (0.6757 g) and PVP (0.111 g) was poured into GO solution and sonicated for another one hour. Afterward, 20 mL of NaBH_4 (1.25 g) solution was added dropwise into the above mixture solution under constant stirring at room temperature. After the reaction of 3 h, the black solid was collected by filtration, washed with ethanol several times, and dried in flowing nitrogen. The catalysts with different ratios of RGO to INS for the designed RGO-INS were listed in Table S1 (see Supporting Information). Among all the samples, the RGO-INS-5% showed the best peroxidase-like activity; therefore, if no further notification is provided, the RGO-INS notation in this study refers to RGO-INS-5%.

2.3. Characterization

The powder X-ray diffraction (XRD) measurements were recorded on a Rigaku Dmax/Ultima IV diffractometer with monochromatized $\text{Cu K}\alpha$ radiation ($\lambda = 0.15418$ nm). The morphology was observed with a JEOL JSM-6510LV scanning electron microscope (SEM) and transmission electron microscope (TEM, FEI Tecnai G20). The elemental composition of the samples were characterized by energy-dispersive X-ray spectroscopy (EDS, Oxford instruments X-Max). The Fourier transform infrared (FTIR) spectrum was measured on a Nicolet 6700 FTIR spectrometric Analyzer using KBr pellets. Raman measurements were carried out by a confocal laser micro-Raman spectrometer (Thermo DXR Microscope, USA). The laser was 633 nm with a 5 mW.

2.4. Peroxidase-like catalytic activity of the RGO-INS

To evaluate the peroxidase-like catalytic activity of the RGO-INS, the catalytic oxidation of the peroxidase substrate TMB in the presence of H_2O_2 was tested. The measurements were carried out by monitoring the absorbance change of TMB at 652 nm. In a typical experiment, 80 μL of the RGO-INS dispersion (1 mg mL^{-1}) was mixed in 1600 μL of NaAc buffer solution (pH 3.0), followed by adding 400 μL of TMB solution (1 mM, ethanol solution). Then, 20 μL of H_2O_2 with various concentrations was added into the mixture. The mixed solution was incubated at 40 $^\circ\text{C}$ for 30 min. For comparison, the control experiments were also conducted under the same conditions by using bare RGO, INS or their physical mixture as catalysts. In addition, the influences of reaction buffer pH and incubation temperature on the peroxidase-like catalytic activity of the RGO-INS were also investigated.

2.5. Bioassay

Kinetic measurements were carried out in time course mode by monitoring the absorbance change at 652 nm. To investigate the mechanism, assays were carried out by varying concentrations of TMB at a fixed concentration of H_2O_2 or vice versa. Experiments were performed using 38 $\mu\text{g mL}^{-1}$ RGO-INS in 1600 μL of reaction buffer (0.2 M NaAc, pH 3.0) with 0.09 mM TMB as substrate, or 0.47 mM H_2O_2 , unless otherwise stated. The apparent kinetic parameters were calculated using Lineweaver–Burk plots of the double reciprocal of the Michaelis–Menten equation: $1/v = K_m/V_{\text{max}}(1/[S] + 1/K_m)$, where v is the initial velocity, V_{max} is the maximal reaction velocity, $[S]$ is the concentration of substrate, K_m is the Michaelis constant [14,31].

2.6. Detection of glucose using the RGO-INS as peroxidase-like mimetics

Glucose detection was carried out as follows: firstly, 100 μL of GOx aqueous solution (1.0 mg mL^{-1}) and 100 μL of D-glucose with various concentration were mixed in 500 μL of NaH_2PO_4 buffer (0.5 mM, pH 7.0) and incubated at 37 $^\circ\text{C}$ for 1 h; then 200 μL of TMB (5 mM, ethanol solution), 100 μL of the RGO-INS stock solution (1 mg mL^{-1}) and 4.00 mL of NaAc buffer (0.2 M, pH 3.0) were successively added to the glucose reaction solution; finally, the mixed solution was incubated at 40 $^\circ\text{C}$ for 30 min for standard curve measurement.

3. Results and discussion

Our synthesis strategy was based on the *in situ* simultaneous reduction of Fe^{3+} and GO at room temperature. Graphite oxide with abundant oxygenous groups was highly negatively charged, which was firstly liquid exfoliated by ultrasonication to form a stable aqueous solution. When Fe^{3+} ions were introduced, the electrostatic interactions between positively charged Fe^{3+} and GO provided a necessary driving force for the effective enrichment of Fe^{3+} onto GO. During the chemical reduction process, iron nanoparticles (INS) were then *in situ* formed on the surface of the reduced GO. Importantly, reduction of GO in our case can be achieved in a mild condition (e.g. room temperature and short reaction time). As previously reported, the reactive hydrogen atoms released from the NaBH_4 hydrolysis can reduce the oxygenous groups on the GO, which is a critical step toward the chemical reduction ability of NaBH_4 [32]. It is believed that *in situ* formed INS can act as the catalyst to accelerate the NaBH_4 hydrolysis to release active hydrogen. To support this hypothesis, we further monitored the amounts of hydrogen generation during the synthesis process (Fig. 2). It is interestingly found that GO was incapable for hydrogen generation, while both the INS and RGO-INS could induce *in situ* fast hydrogen generation, confirming that the formation of

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