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Polypyrrole nanoparticles as promising enzyme mimics for sensitive hydrogen peroxide detection

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Received (in XXX, XXX) Xth XXXXXXXXX 200X, Accepted Xth XXXXXXXXX 200X
DOI: 10.1039/b000000x

We first discover that the polypyrrole nanoparticles possess intrinsic peroxidase-like activity, which can be employed to quantitatively monitor the H$_2$O$_2$ generated by macrophages.

H$_2$O$_2$ detection is of critical importance in the fields of food chemistry, biology, clinical, pharmaceutical, industrial and environmental protection. These far-ranging impacts of H$_2$O$_2$ provide motivation to devise various methods for detecting and quantifying its production. The commonly used optical H$_2$O$_2$ detection involves peroxidase (HRP) catalyzing a chromogenic substrate such as 3,3',5,5'-tetramethylbenzidine (TMB) to form the colored product. Although this assay is effectively, the inherent drawbacks of the natural enzyme, including easy denaturation by environmental changes, digestion by proteases, time-consuming and expensive preparation and purification, limit their application for biosensors. Recently, many nanomaterials, such as Fe$_3$O$_4$ nanoparticles, graphene oxide, carbon nanotubes, gold nanoparticles, silver nanoparticles, gold nanoclusters, carbon nanodots, graphitic carbon nitride nanosheets, and TiO$_2$ nanotube arrays, have been reported to possess an intrinsic enzyme mimetic activity similar to that found in natural peroxidase and been utilized to detect H$_2$O$_2$. The lower cost and higher chemical stability of nanoparticles or nanosheets toward peroxidase make the assays more robust. Despite of the enormous progress in the optical probes and sensors for H$_2$O$_2$, there is still a demand to improve the available probes for utilization in biological samples such as cells, tissue or body fluids, which require high water solubility, good biocompatibility, excellent stability, and high sensitivity.

As an organic conductive polymer, polypyrrole (PPy) has been widely used in organic electronics owing to its high conductivity and outstanding stability. Recently, PPy nanoparticles have also received tremendous attention in biomedical application. Due to their excellent solubility, controllable size and good biocompatibility, PPy nanomaterials have been used in drug delivery and tissue engineering. With strong NIR absorbance, PPy nanoparticles have also been explored as a new photothermal therapy agent for photothermal treatment of cancer in vitro and in vivo. For instance, by taking advantage of the strong NIR absorbance of PPy, Liu and co-workers reported the use of PPy for efficient photothermal ablation of cancer both in vitro and in vivo. In addition, PPy nanoparticles have been applied as a novel contrast agent for photoacoustic and optical coherence tomography. Despite these burgeoning developments, to the best of our knowledge, the PPy-based enzyme mimics have not been explored until now. Herein, we make the surprising discovery that PPy nanoparticles possess intrinsic peroxidase-like activity, which can catalyze the reaction of peroxidase substrate TMB in the presence of H$_2$O$_2$ to produce a blue color reaction. As compared to natural enzyme, PPy may serve as promising candidates of artificial enzymes with advantages of controlled synthesis in low cost, tunability in catalytic activities, and a high stability against stringent conditions. A simple, cheap, highly sensitive and selective colorimetric method for H$_2$O$_2$ detection has been developed and been used in buffer solution or even in living cells, which will expand the applications of PPy in medical diagnostics and biotechnology.

Fig. 1 The SEM image (a) and size distribution analysis (b) of PPy. The total number of PPy nanoparticles counted for the histogram was 100. (c) FTIR spectrum of PPy nanoparticles. (d) UV-vis-NIR absorbance spectrum of PPy nanoparticles.

PPy nanoparticles were prepared according to previous reports, which were synthetized through a microemulsion method using polyvinyl alcohol (PVA) as the stabilizer and ferric chloride (FeCl$_3$) as the catalyst. Excess free PVA and FeCl$_3$ were then removed by centrifugation and repeated water washing. After that, the PPy nanoparticles were characterized by scanning electron microscope (SEM) imaging, Fourier transform infrared (FTIR) spectroscopy and UV-vis-NIR spectrum. As can be seen in Fig. 1a, the PPy nanoparticles were discrete and uniform with...
an average diameter of 58.8 nm (Fig. 1b). The FTIR spectrum of the PPy samples (Fig. 1c) had the main absorption peaks of PPy at 3400 cm$^{-1}$ (stretching vibration of N–H bonds), 1560 cm$^{-1}$ (stretching vibration of C=C bonds), 1045 cm$^{-1}$ and 1304 cm$^{-1}$ (in-plane vibrations of C–H bonds), 940 cm$^{-1}$ (C=C bond vibration), and 790 cm$^{-1}$ (out-of-plane vibrations of the C–H bonds).$^{15}$ In addition, the absorbance spectrum showed that PPy nanoparticles exhibited a strong NIR absorbance band, which was characteristic of the bipolaronic metallic state of doped polypyrrole (Fig. 1d).$^{14}$

In the following, the peroxidase-like activity of PPy nanoparticles was evaluated in the catalysis of peroxidase substrates TMB. PPy nanoparticles could catalyze the oxidation of TMB in the presence of H$_2$O$_2$, and produce a deep blue color, with maximum absorbance at 652 nm. In contrast, PPy or H$_2$O$_2$ alone did not produce significant color change (Fig. 2a). These results confirmed that the PPy nanoparticles exhibited peroxidase-like activity toward TMB. EDS analysis confirmed the presence of C, N, O, Cl and Fe in the PPy nanoparticles (Fig. S1), which indicated that the peroxidase-like activity of PPy might come from a trace amount of metal residue.$^{16}$ Meanwhile, the changes in enzyme activity of PPy after treated with 2% NaBH$_4$ or 2% NaIO$_4$ further demonstrated that Fe was determinant for the peroxidase-like activity of PPy (Fig. S2).$^{4b}$

The activity of PPy in different batches is reproducible (Fig. S3 and S4). In addition, Fig. S5 showed the time-dependent absorbance changes (at 652 nm) against different concentrations of PPy. Dramatic improvement of catalytic activity could be observed with the steady increase of PPy concentration. Furthermore, like other nanomaterial-based peroxidase mimics, the activity of PPy was also dependent on pH (Fig. S6), temperature and H$_2$O$_2$ concentration (Fig. 2b-2d). Most notably, the PPy nanoparticles exhibited high activity at low H$_2$O$_2$ concentrations. As shown in Fig. 2d, the peroxidase-like activity of PPy remained about 40% in the presence of 1 mM H$_2$O$_2$ compared to that in the presence of 50 mM H$_2$O$_2$.

For further analyzing the catalytic mechanism and acquiring kinetic parameters, the catalytic activity of the PPy nanoparticles was studied by enzyme kinetics theory and methods. Typical Michaelis–Menten curves (Fig. S7a-d) were received in a certain range of H$_2$O$_2$ or TMB concentrations. With the Lineweaver–Burk equation, the important enzyme kinetic parameters such as Michaelis–Menten constant (K$_m$) and Maximum initial velocity (V$_{max}$) were obtained in Table 1. K$_m$ was identified as an indicator of enzyme affinity to substrates. A low K$_m$ represented a strong affinity and vice versa.$^{17}$ Surprisingly, one feature of PPy as the enzyme mimic was that the K$_m$ value (0.027 mM) of PPy with H$_2$O$_2$ as the substrate was about eight times lower than that of the natural enzyme HRP (0.214 mM), showing that PPy had higher activity to H$_2$O$_2$ than HRP. Additionally, the double-reciprocal plots (Fig. S7e, f) revealed the characteristic parallel lines of a ping-pong mechanism$^{16,40}$ and implied that, like HRP, PPy bound and reacted with the first substrate, then released the first product before reacting with the second substrate.

**Table 1.** Comparison of the apparent Michaelis–Menten constant (K$_m$) and maximum reaction rate (V$_{max}$) between PPy and HRP.$^{7a}$

<table>
<thead>
<tr>
<th>Catalyst</th>
<th>Substance</th>
<th>$K_m$ [mM]</th>
<th>$V_{max}$ [10$^3$ M·s$^{-1}$]</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPy</td>
<td>TMB</td>
<td>0.2157 ± 0.038</td>
<td>1.29 ± 0.037</td>
</tr>
<tr>
<td>PPy</td>
<td>H$_2$O$_2$</td>
<td>0.027 ± 0.003</td>
<td>1.17 ± 0.013</td>
</tr>
<tr>
<td>HRP</td>
<td>TMB</td>
<td>0.275 ± 0.013</td>
<td>1.24 ± 0.12</td>
</tr>
<tr>
<td>HRP</td>
<td>H$_2$O$_2$</td>
<td>0.214 ± 0.014</td>
<td>2.46 ± 0.32</td>
</tr>
</tbody>
</table>

Inspired by the high activity to H$_2$O$_2$ of PPy, the PPy/TMB kit was used to monitor the extracellular H$_2$O$_2$ burst generated by macrophages, which would provide a drastic positive effect on diagnosis and prognosis. It was known that the RAW264.7 cells could produce H$_2$O$_2$ upon stimulation with lipopolysaccharide (LPS) or phorbol-12-myristate-13-acetate (PMA).$^{18}$ Fig. 3a showed the UV absorbance calibration curve for H$_2$O$_2$ in presence of the PPy/TMB kit in PBS buffer, with a detection range from 5 to 100 µM (Fig. 3a and S8). Afterwards, PMA was used to stimulate macrophage activation and PPy was utilized to detect the extracellular H$_2$O$_2$. In this assay, Raw 264.7 cells were first washed with PBS (pH = 7.4, 20 µL) and then PMA with different concentrations (5 µL) were added into cells and incubated for 20 min. After that, the PPy/TMB kit containing 80 mM TMB in 10 µL DMSO and 200 µL PPy (8 µg) in PBS (pH = 4.0) was added into above solution, followed by monitoring the absorbance changes at 652 nm. Fig. 3b illustrated the UV absorption changes upon incubation with a series of concentrations of PMA. As can be seen, a dramatic increase in UV absorbance at 652 nm was observed with the increase in the PMA concentration from 0.5 to 5 µg/mL. The absorption intensity related directly to TMB oxidation by H$_2$O$_2$ released from PMA-stimulated Raw 264.7 cells. We could see that with increased concentration of PMA, the H$_2$O$_2$ concentration increased accordingly, consistent with the results obtained from the HRP/TMB assay (Fig. S9). The H$_2$O$_2$ concentration could be reached to 10 µM when the PMA concentration was 0.5 µg/mL. These results were consistent with the previous reports that PMA...
could induce H₂O₂ generation through a cellular inflammation response. In addition, LPS could also be efficiently used for stimulation of H₂O₂ release (Fig. 3b).

In summary, for the first time, we discover that the PPy nanoparticles possess intrinsic peroxidase-like activity and the catalysis is strongly dependent on pH, temperature, and H₂O₂ concentration, similar to HRP. Kinetic analysis indicates that the catalysis follows a ping-pong mechanism. PPy, as a mimic peroxidase, shows several unprecedented advantages. First of all, as compared to natural enzymes, PPy is a promising candidate as enzyme mimics with advantages of ease of preparation, low-cost, and high stability. Second, PPy can serve as a high-efficiency peroxidase-mimic for a sensitive H₂O₂ assay. Kinetic analysis indicates that PPy has higher activity to H₂O₂ than HRP. Notably, PPy has been successfully employed to quantitatively monitor the H₂O₂ generation by macrophages. Our work will facilitate the utilization of the intrinsic peroxidase activity of PPy in medical diagnostics and biotechnology.

Financial support was provided by the National Basic Research Program of China (Grant 2011CB936004 and 2012CB720602) and the National Natural Science Foundation of China (Grants 2120002, and 91213302).

Notes and references

† Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/b000000x/


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