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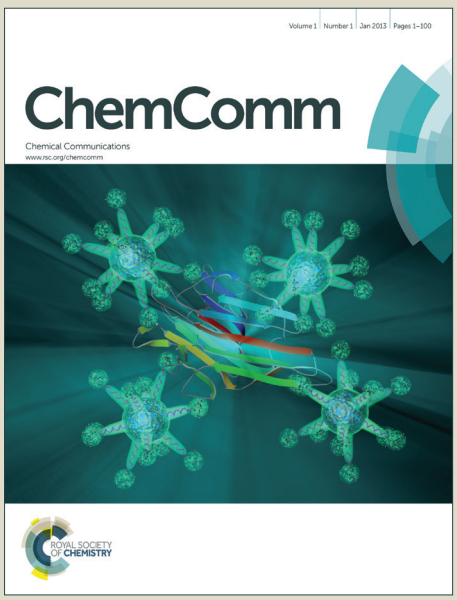

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ARTICLE TYPE

Polypyrrole nanoparticles as promising enzyme mimics for sensitive hydrogen peroxide detection

Yu Tao, Enguo Ju, Jinsong Ren,* and Xiaogang Qu*

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We first discover that the polypyrrole nanoparticles possess intrinsic peroxidase-like activity, which can be employed to quantitatively monitor the H_2O_2 generated by macrophages.

H_2O_2 detection is of critical importance in the fields of food, chemistry, biology, clinical, pharmaceutical, industrial and environmental protection.[1] These far-ranging impacts of H_2O_2 provide motivation to devise various methods for detecting and quantifying its production. The commonly used optical H_2O_2 detection involves peroxidase (HRP) catalyzing a chromogenic substrate such as 3,3',5,5'-tetramethylbenzidine (TMB) to form the colored product.[2] 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.[3] Recently, many nanomaterials, such as Fe_3O_4 nanoparticles,[4] graphene oxide,[5] carbon nanotubes,[6] gold nanoparticles,[7] silver nanoparticles,[8] gold nanoclusters,[9] carbon nanodots,[10] graphitic carbon nitride nanosheets,[11] and TiO_2 nanotube arrays[12] et al., have been reported to possess an intrinsic enzyme mimetic activity similar to that found in natural peroxidase and been utilized to detect H_2O_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_2O_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.[13] Recently, PPy nanoparticles have also received tremendous attention in biomedical application.[14] Due to their excellent solubility, controllable size and good biocompatibility, PPy nanomaterials have been used in drug delivery and tissue engineering.[14h-j] 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.[14a-c] 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.[14c] In addition, PPy nanoparticles have been applied as a novel contrast agent for photoacoustic and optical coherence tomography.[14e,14g] 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_2O_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_2O_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.

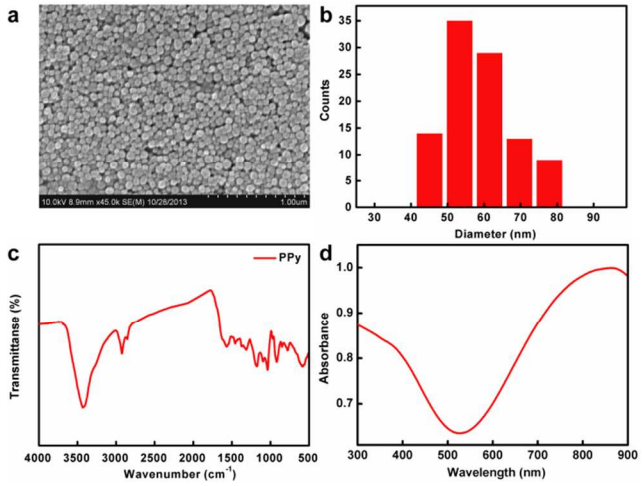

Fig. 1 The SEM image (a) and size distribution analysis (b) of PPy. The total number of PPy nanoparticles counted for the histogram was 100. (c) FTIR spectrum of PPy nanoparticles. (d) UV-vis-NIR absorbance spectrum of PPy nanoparticles.

PPy nanoparticles were prepared according to previous reports,[13b,14d] 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 absorption band, which was characteristic of the bipolaronic metallic state of doped polypyrrole (Fig. 1d).[14d]

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_2O_2, and produce a deep blue color, with maximum absorbance at 652 nm. In contrast, PPy or H_2O_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_2O_2 concentration (Fig. 2b-2d). Most notably, the PPy nanoparticles exhibited high activity at low H_2O_2 concentrations. As shown in Fig. 2d, the peroxidase-like activity of PPy remained about 40% in the presence of 1 mM H_2O_2 compared to that in the presence of 50 mM H_2O_2.

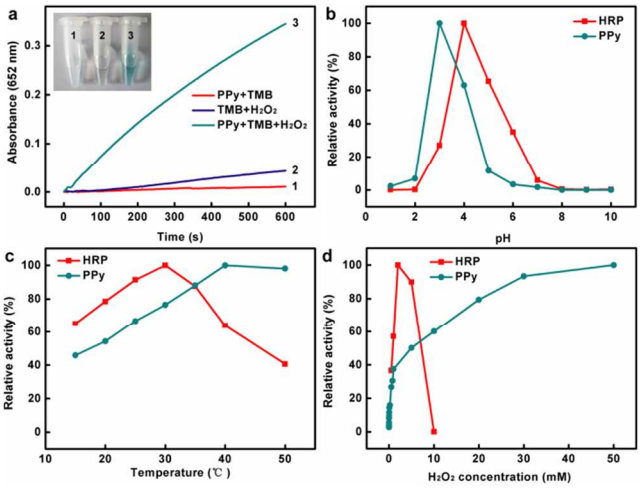

Fig. 2 (a) Time-dependent absorbance changes at 652 nm of TMB in different reaction systems: (1) PPy + TMB, (2) TMB + H_2O_2 and (3) PPy + TMB + H_2O_2. (b, c, d) The peroxidase-like activity of the PPy nanoparticles is dependent on pH (b) and temperature (c) and H_2O_2 concentration (d).

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_2O_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_2O_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_2O_2 than HRP. Additionally, the double-reciprocal plots (Fig. S7e, f) revealed the characteristic parallel lines of a ping-pong mechanism[3a,4b] 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.[3a]

Catalyst	Substance	K_m [mM]	V_{max} [10^{-8} M·s^{-1}]
PPy	TMB	0.2157 ± 0.038	1.29 ± 0.037
PPy	H_2O_2	0.027 ± 0.00244	1.17 ± 0.0126
HRP	TMB	0.275 ± 0.013	1.24 ± 0.12
HRP	H_2O_2	0.214 ± 0.014	2.46 ± 0.32

Inspired by the high activity to H_2O_2 of PPy, the PPy/TMB kit was used to monitor the extracellular H_2O_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_2O_2 upon stimulation with lipopolysaccharide (LPS) or phorbol-12-myristate-13-acetate (PMA).[18] Fig. 3a showed the UV absorbance calibration curve for H_2O_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_2O_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_2O_2 released from PMA-stimulated Raw 264.7 cells. We could see that with increased concentration of PMA, the H_2O_2 concentration increased accordingly, consistent with the results obtained from the HRP/TMB assay (Fig. S9). The H_2O_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 H_2O_2 generation through a cellular inflammation response.[19] In addition, LPS could also be efficiently used for stimulation of H_2O_2 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 H_2O_2 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 H_2O_2 assay. Kinetic analysis indicates that PPy has higher activity to H_2O_2 than HRP. Notably, PPy has been successfully employed to quantitatively monitor the H_2O_2 generated by macrophages. Our work will facilitate the utilization of the intrinsic peroxidase activity of PPy in medical diagnostics and biotechnology.

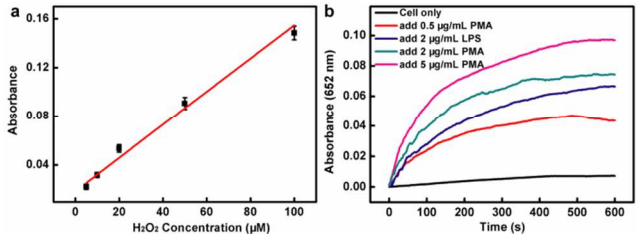

Fig. 3 (a) H_2O_2 concentration dependent UV absorbance containing the PPy/TMB kit. The background absorbance from the same solutions but without H_2O_2 have been subtracted. Error bars represent standard deviation of three independent measurements. (b) Time-dependent absorbance changes at 652 nm of TMB from PBS solution containing PPy/TMB kit and RAW264.7 cells (3×10^5 cells) in the presence of different concentrations of PMA and LPS.

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Notes and references

[a] *Laboratory of Chemical Biology and, State Key Laboratory of Rare Earth Resource Utilization, Graduate School of the Chinese Academy of Sciences, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun, Jilin 130022, China.*
E-mail: jren@ciac.ac.cn; xqu@ciac.ac.cn

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