Nanoporous Metal-Phenolic Particles as Ultrasound Imaging Probes for Hydrogen Peroxide

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Hydrogen peroxide ($\text{H}_2\text{O}_2$) is a reactive oxygen species (ROS) produced endogenously that is required for various physiological processes (e.g., host defense). $\text{H}_2\text{O}_2$ is also an important second messenger in signal transduction pathways. These beneficial aspects of $\text{H}_2\text{O}_2$ are counter-balanced by its hormetic effect, where $\text{H}_2\text{O}_2$ at higher concentrations is linked to various pathologies, such as diabetes, cardiovascular disease, renal injury, retinopathy, and cancer. The overproduction of $\text{H}_2\text{O}_2$ can damage DNA, RNA, cellular lipids, and proteins, thereby inhibiting their normal physiological roles.

The detection of specific ROS, especially $\text{H}_2\text{O}_2$, has been achieved by using fluorescent molecular probes, near-infrared imaging, and electrochemical sensors. However, limitations of these conventional methods, such as poor specificity, toxicity, complicated fabrication, non-biodegradable materials, as well as use of not readily available imaging modalities, and high-cost in clinical applications, have hampered further clinical translational applications. This has resulted in a lack of easy and efficient methods to detect specific ROS in vivo. The lowering of pathological levels of $\text{H}_2\text{O}_2$ is also seen as a desirable therapeutic goal that may offer a means for lessening the pathological burden. However, large scale clinical trials (HOPE, GISSI and Physicians Health Study II) failed, where the non-specific vitamins C and E demonstrated no additional benefits, and were actually linked to a worsening of cardiovascular disease outcomes. Therefore, the development of improved methods for $\text{H}_2\text{O}_2$ detection is highly desirable, not only for the visualization of $\text{H}_2\text{O}_2$ production, but also for the development and testing of novel therapeutics capable of reducing $\text{H}_2\text{O}_2$ under pathological conditions.

Among the different imaging modalities available in current clinical settings, ultrasound imaging technology (US) offers significant advantages, such as easy access, non-ionizing radiation, low-cost, portability, and real-time imaging, thereby making it well suited to routine clinical applications such as screening and early disease detection. US imaging has been improved with the use of contrast agents, such as microbubbles (MBs), and research into molecular US imaging is ongoing. Recently, we showed that a common natural phenolic,
Tannic acid (TA, Figure S1), can be used to coordinate different metals, across the periodic table, through a rapid assembly process, thereby establishing a broad library of metal-phenolic network (MPN) films and capsules. The functional properties of MPN materials, such as biodegradation, fluorescence, and multimodal imaging, can be easily tailored through the choice of the metals used for complexation. These MPNs represent a platform for engineering advanced US contrast agents, including the detection of H$_2$O$_2$ in biological and/or biomedical applications.

Herein, we report the development of nanoporous metal-phenolic particles that are capable of probing endogenous levels of H$_2$O$_2$ by US imaging in vitro, ex vivo and in vivo. These particles (nanoporous Fe$^{III}$-TA replica particles, denoted npFe$^{III}$-TA RPs) are engineered by the nanostructural replication of nanoporous calcium carbonate (npCaCO$_3$) particles filled with Fe$^{III}$-TA complexes (Figure 1a). The Fe$^{3+}$ chelated by the phenolic ligand possesses catalytic activity that converts H$_2$O$_2$ into oxygen (O$_2$) molecules under physiological pH (Figure 1b). Under pathological conditions, tissues produce elevated levels of H$_2$O$_2$, and this H$_2$O$_2$ can be activated by the npFe$^{III}$-TA RPs to generate MBs of O$_2$, thereby changing the acoustic impedance and creating specific non-linear oscillations, which can be detected by US imaging in real time (Figure 1c). Wild-type (WT) and glutathione peroxidase-1 knockout (GPx1-/-) mice were chosen as models to examine the potential of these npFe$^{III}$-TA RPs in the detection of endogenous H$_2$O$_2$ ex vivo and in vivo. Compared with the previously reported lipopolysaccharide (LPS)-treated inflammatory model, GPx1-/- mice are more clinically relevant, as they have been shown to be more susceptible to oxidant-mediated damage associated with endothelial dysfunction, cardiovascular diseases (eg., atherosclerosis), diabetic complications, stroke, traumatic brain injury, and retinal diseases. This study provides one of the few examples of the facile yet efficient formation of highly biocompatible nanostructured particles that are capable of rapidly detecting elevated levels of H$_2$O$_2$, and also demonstrates the potential use of metal-phenolic particles for advanced ultrasound imaging in biomedical research.
Figure 1. Schematic representation for the formation of npFe^{III}-TA RPs for the formation of chemically generated microbubbles (MBs) to probe H$_2$O$_2$ *ex vivo* and *in vivo* by ultrasound (US) imaging. (a) The formation of npFe^{III}-TA RPs by replication of npCaCO$_3$ template particles filled with Fe$^{III}$-TA complexes. (b) The interfacial reaction of H$_2$O$_2$, splitting to H$_2$O and O$_2$ with the catalytic assistance of chelated Fe$^{3+}$ in the npFe$^{III}$-TA RPs. (c) Gas nucleation of O$_2$ forms MBs, leading to changes of acoustic impedance in tissue; these changes can be detected by scanning the region of interest using US imaging.

The npFe$^{III}$-TA RPs were formed by complexing TA and Fe$^{3+}$ in npCaCO$_3$ particles, followed by removal of the templates (Figure 2a). The npCaCO$_3$ particles (Figure S2) were synthesized by calcination of poly(sodium styrene sulfonate) (PSS)-stabilized CaCO$_3$ particles, according to modified literature methods.$^{[22]}$ To facilitate the efficient filling of Fe$^{III}$-TA complexes into the npCaCO$_3$, TA solution was used to infiltrate the template particles, and then FeCl$_3$ solution and 3-(N-morpholino)propanesulfonic acid (MOPS) buffer solution (pH 8.0) were sequentially added. Finally, the npCaCO$_3$ templates were removed by washing with ethylenediaminetetraacetic acid (EDTA) solution. The UV-Vis spectrum of the obtained npFe$^{III}$-TA RPs showed a characteristic band at around 520 nm, which can be attributed to the charge transfer from the galloyl group of TA to chelated Fe$^{3+}$ (Figure S3).$^{[23]}$ npFe$^{III}$-TA RPs showed a spherical structure (Figure 2b), without collapse, after air drying, confirming that they are particles, and dissimilar to our previously reported Fe$^{III}$-TA capsules.$^{[14]}$ This difference can be the result of different nanostructures in the capsules and replica particles: Fe$^{III}$-TA capsules were formed from thin films of thickness $\sim$10 nm,$^{[14]}$ while the npFe$^{III}$-TA RPs were constructed with dense Fe$^{III}$-TA complex clusters (Figure S4). As shown in Figure 2c-e, the pore size of npFe$^{III}$-TA RPs was measured to be 50.1 ± 18.8 nm (Figure S5), and this nanoporous structure can
facilitate the highly efficient interfacial reaction between the particles and H$_2$O$_2$, which results in a quick and highly sensitive reaction to breakdown H$_2$O$_2$ to O$_2$. High-angle annular dark-field (HAADF) images further confirmed the hollow and highly porous structures of the $np$Fe$^{III}$-TA RPs (Figure 2f). The corresponding energy dispersive X-ray (EDX) mapping analysis showed that the Fe distribution patterns matched well with the HAADF image and the distribution patterns of C and O maps. This confirmed that the Fe$^{3+}$ were associated with TA and highly distributed in the particles acting as a series of highly efficient catalytic sites. The cytotoxicity of Fe$^{III}$-TA materials was found to be negligible.[14] Because of the highly porous structure, $np$Fe$^{III}$-TA RPs were also shown to have potential for the loading and delivery of therapeutic drugs (Figure S6).

Figure 2. Structural characterization of $np$Fe$^{III}$-TA RPs. (a) Differential interference contrast (DIC) images of $np$CaCO$_3$ template particles and $np$Fe$^{III}$-TA RPs. (b) and (c) Scanning electron microscope images of $np$Fe$^{III}$-TA RPs with hollow structures. Inset of (c) shows the nanoporous networks. (d) and (e) Transmission electron microscopy images of $np$Fe$^{III}$-TA RPs with dense nanoclusters of Fe$^{III}$-TA complexes. (f) HAADF microscope image and corresponding EDX mappings of $np$Fe$^{III}$-TA RPs: dark field is represented as DF, carbon (C), oxygen (O), and iron (Fe). The scale bars are (a) 5.0 μm, (b) 1.0 μm, (c) 500 nm and 100 nm (inset), (d) 500 nm, (e) 100 nm, and (f) 1.0 μm.

To examine the ability of $np$Fe$^{III}$-TA RPs to generate O$_2$ MBs in the presence of H$_2$O$_2$, a H$_2$O$_2$ solution (pH 7.4, PBS buffer) was added into a suspension of $np$Fe$^{III}$-TA RPs (Figure 3a). Using the naked eye, it could be observed that small bubbles formed and merged into
bigger bubbles after 10 min. The generation process of the MBs was observed using differential interference contrast (DIC) microscopy. Initially, the MBs were ~5 μm in diameter, however as more O₂ was generated, the small MBs merged to form larger MBs with a diameter of ~40 μm. We also noted a color change of the npFe³⁺-TA RPs suspension, which could be the result of oxidative degradation of TA, which led to the disassembly of the Fe³⁺-TA complexes (Figure S7). Bubbles were not observed in the H₂O₂ solution without npFe³⁺-TA RPs. To further prove the existence of O₂, npFe³⁺-TA RPs (~0.043 mM Fe³⁺) were mixed with different concentrations of H₂O₂ (pH 7.4, PBS buffer), and the generated O₂ was measured using an extracellular flux (XF) analyzer. Within the first 10 min, the generation of O₂ could not be measured due to the initialization process of the XF. Thereafter, the O₂ level increased dramatically for the next 20 min, reaching a maximum level of 315 mmHg (in 100 mM H₂O₂), and minimum level of 148 mmHg (in 0.5 mM H₂O₂). The generation of O₂ decreased 35 min after starting the experiment, and steady levels of O₂ were generated until the experiment ended at 70 min (Figure 3b,c). No increase of O₂ could be observed in npFe³⁺-TA RPs without H₂O₂. The cumulative O₂ production was linearly correlated within a wide range of H₂O₂ concentrations (0–100 mM, R² = 0.96, and 0–5 mM, R² = 0.99).

Figure 3. H₂O₂-dependent generation of O₂ MBs by npFe³⁺-TA RPs in vitro. (a) npFe³⁺-TA RPs suspension (i), mixture of npFe³⁺-TA RPs and H₂O₂ in PBS buffer (ii), growth of MBs under DIC microscopy after mixing the npFe³⁺-TA RPs and H₂O₂ for 30 s (iii) and 150 s (iv).
(b) and (c) Kinetics of O$_2$ production with different concentrations of H$_2$O$_2$: (b) full H$_2$O$_2$ concentration range (0 to 100 mM); and (c) low H$_2$O$_2$ concentration range (0 mM to 5 mM). The dashed lines represented the standard deviations. Data represent means ± SD, n = 3. (d) Linear increase of the cumulative O$_2$ production based on H$_2$O$_2$ concentration. Scale bars are 5.0 μm in the microscopy images.

GPx1$^{-/-}$ mice were chosen as a clinically relevant model to examine the potential of npFe$^{III}$-TA RPs in the detection of endogenous H$_2$O$_2$ ex vivo and in vivo (Figure 4). WT mice were used as a negative control. GPx1 is an important antioxidant enzyme that forms part of the second-step of the antioxidant pathway in the conversion of H$_2$O$_2$ to H$_2$O.$^{[24]}$ GPx1$^{-/-}$ mice have a reduced capacity to remove H$_2$O$_2$, due to the complete knockout of GPx1 within both the mitochondria and the cytosolic compartments of the cell (Figure 4a)$^{[25]}$. Studies in WT mice have demonstrated high levels of GPx1 expression in organs such as the liver, and therefore GPx1$^{-/-}$ mice demonstrate elevated levels of H$_2$O$_2$ in various organs, particularly the liver. The elevated liver H$_2$O$_2$ levels make these mice an ideal model to investigate the npFe$^{III}$-TA RPs for detecting H$_2$O$_2$. Initially, livers were isolated from the mice and placed on an imaging platform (Figure 4b,c). The npFe$^{III}$-TA RPs (50 μL, ~0.86 mM Fe$^{3+}$) were then injected steadily into the ex vivo liver tissue by a micro-injector (Figure S8). An increased spot of US brightness was observed after injection, demonstrating the production of O$_2$ MBs resulting from the npFe$^{III}$-TA RPs interacting with the elevated liver H$_2$O$_2$ levels for the GPx1$^{-/-}$ mice (Figure 4d). In contrast, no increased brightness was observed in the control WT mice, as determined by measuring the grayscale intensity in the region of interest for the acquired images (Figure 4e,f). For the GPx1$^{-/-}$ mice, the grayscale intensity increased after injection and reached a peak at 15 min, followed by a moderate decrease after 20 min. The decrease of grayscale intensity after the initial peak could be due to the diffusion of the MBs and the degradation of the npFe$^{III}$-TA RPs after imaging. Lower concentrations of npFe$^{III}$-TA RPs (50 μL, ~0.43 mM Fe$^{3+}$) were also injected into the liver, and the increased grayscale intensity correlated well with the injected dose (Figure S9).

With these promising ex vivo results, next we examined an in vivo model where we intravenously injected the npFe$^{III}$-TA RPs (100 μL, ~0.86 mM Fe$^{3+}$) into WT and GPx1$^{-/-}$ mice (Figure 4g). To facilitate the injection, an incision was performed on the leg of the mice to allow the insertion of a catheter into the femoral vein. Similar to our previous study on MPNs,$^{[14b]}$ the npFe$^{III}$-TA RPs accumulated in the liver and high brightness spots were visible 10 min after injection using US (Figure 4h). The intensity of these spots increased after
approximately 10 min, peaked at 25 min, and diminished at approximately 35 min post injection. No bright spots were observed in the WT control mice. Similar to our ex vivo experiments, we analyzed the acquired images and measured the grayscale intensity at the region of interest (Figure 4i). A significant increase in grayscale intensity was noted at 15, 20, and 25 min post injection in the GPx1−/− mice, while there was no increase in grayscale intensity for the control WT mice. The enhancement of US contrast agreed well with the in vitro XF experiments, which showed a dramatic increase of O2 production in the initial 20 min, followed by a gradual decrease (Figure 3b,c).

**Figure 4.** Enhanced ultrasound contrast resulting from the interaction of the npFeIII-TA RPs with oxidative stress in mouse livers ex vivo and in vivo induced by GPx1 deficiency. (a) H2O2 concentrations in the GPx1−/− deficiency and WT mice, ***P < 0.001 (Student’s t-test). (b) Schematic representation for the isolation of livers and the US probing of H2O2. (c) Photograph of the ex vivo US imaging setting. (d) Representative brightness mode (B-mode) and contrast
enhanced ultrasound (CEU) images of the mouse livers over time ex vivo. (e) Corresponding mean grayscale changes of the mouse livers ex vivo show the enhanced US contrast after 5 min injection. *** $P < 0.001$ (two-way ANOVA). (f) Quantitative image analysis of the CEU images of the mouse livers ex vivo. *** $P < 0.001$ (Student’s t-test). (g) Schematic representation of the US probing of H$_2$O$_2$ in vivo. (h) Representative B-mode and CEU images of the mouse livers over time in vivo. The inset shows the area of interest with enhanced brightness and contrast. (i) Corresponding mean grayscale changes of the mouse livers in vivo shows enhanced US contrast 15 min after injecting the npFe$^{III}$-TA RPs into the GPx1$^{-/-}$ mice. ** $P < 0.01$, *** $P < 0.001$ (two-way ANOVA). Data represents means ± SD, n = 3.

In summary, we have reported the facile and efficient formation of nanoporous metal-phenolic particles (npFe$^{III}$-TA RPs) through the coordination complexation of a polyphenol (TA) and Fe$^{3+}$. The npFe$^{III}$-TA RPs have highly nanoporous structures, formed by the nanostructural replication of nanoporous CaCO$_3$ template particles. The Fe$^{3+}$ chelated in the phenolic ligands possess catalytic activity to breakdown H$_2$O$_2$ into O$_2$, accompanied by disassembly of the Fe$^{III}$-TA complexes at physiological pH. The cumulative production of O$_2$ is linearly related over a wide concentration range of H$_2$O$_2$ (0 to 100 mM), which ensures the nucleation of O$_2$ microbubbles (MBs). The npFe$^{III}$-TA RPs were used to rapidly detect endogenous levels of H$_2$O$_2$ by US in GPx1$^{-/-}$ mice ex vivo and in vivo. Given the high biocompatibility of these materials (TA is an antioxidant and Fe$^{3+}$ is an essential mineral), the npFe$^{III}$-TA RPs provide various opportunities for advanced ultrasound imaging; from facilitating the preclinical investigation of physiological and pathological processes in biological systems to new theranostic methods in the process of clinical translation.

Supporting Information
Supporting Information is available from the Wiley Online Library. HOPE denotes an antioxidant trial of Heart Outcomes Prevention Evaluation, and GISSI denotes an antioxidant trial of Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico (an Italian group for the study of the survival of Myocardial Infarction).

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Nanoporous metal-phenolic particles are fabricated through the nanostructural replication of dense Fe$^{III}$-TA complexes in nanoporous CaCO$_3$ template particles. The npFe$^{III}$-TA RPs have potential for the diagnostic detection of endogenous levels of H$_2$O$_2$ ex vivo and in vivo by ultrasound imaging, which is based on the catalytic activity of the coordinated Fe$^{3+}$ in the particles to breakdown H$_2$O$_2$ to O$_2$ microbubbles.

Keywords: Hydrogen peroxide, metal-phenolic particles, and ultrasound imaging

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