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Highly active fluorogenic oxidase-mimicking NiO nanozymes†

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Oxidase-mimicking nanozymes are attractive since they do not require H_2O_2, but such examples are quite rare. In particular, few can catalyze oxidation of fluorogenic substrates. We herein communicate that NiO nanoparticles are an oxidase nanozyme at physiological pH for fluorogenic Amplex red. Its activity is much higher than that of the commonly used nanoceria. This finding fills an urgent gap for biosensor development and intracellular imaging for the nanozyme field.

Nanozymes are nanoparticle-based enzyme mimics.[1–5] With low cost and high stability, nanozymes have attracted extensive interest. This field is experiencing a rapid growth,[6] and nanozymes have already found interesting applications in therapy,[7–9] environmental remediation,[10,11] and biosensor development.[12–16] Yan and coworkers articulated the concept of nanozymes using iron oxide as a peroxidase mimic.[3] In fact, most reported nanozymes have peroxidase-like activities,[1–4,17–20] meaning that they require H_2O_2 to oxidize their substrates.

On the other hand, few nanozymes possess oxidase-like activities,[11,21–25] although oxidases are often more desirable due to simpler reaction conditions (*e.g.* no H_2O_2 needed). CeO_2 is a popular oxidase-mimicking nanozyme.[9,26–28] Mn_2O_3 also has oxidase-like activity,[29] while gold nanoparticles have glucose oxidase-like activity.[22] A copper-nucleotide coordination nanoparticle has laccase-like activity.[11] Overall, the examples of oxidase nanozymes are quite limited.

Another limitation of oxidase nanozymes is the type of substrate. CeO_2 and other oxidase nanozymes mainly used chromogenic substrates such as 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), 3,3′,5,5′-tetramethylbenzidine (TMB), and dopamine,[27] while fluorogenic substrates were rarely demonstrated.[30] This has limited their bioanalytical applications such as imaging. Finally, most oxidase and peroxidase nanozyme reactions were performed at an acidic pH (*e.g.* pH 4.0). These factors have limited oxidase nanozymes to simple colorimetric biosensors, whereas fluorescent sensors and cell imaging are more difficult to realize. We are interested in expanding oxidase nanozymes for producing fluorescence signals. Amplex red (AR) is a commonly used fluorogenic substrate.[31–33] In this work, we communicate that NiO has excellent oxidase-like activity for AR at neutral pH.

The intended reaction is shown in Fig. 1A, where non-fluorescent AR is converted to red fluorescent resorufin after oxidation.[30,34] We first performed a screening experiment by mixing AR with a few common metal oxide nanoparticles.

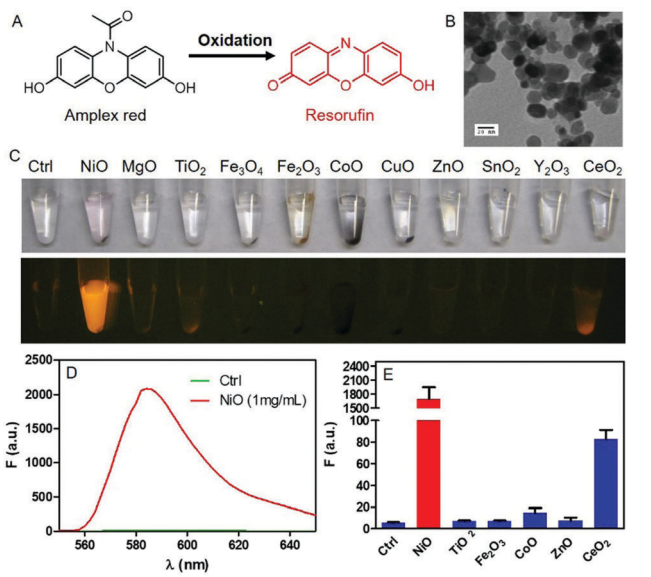

Fig. 1 (A) Oxidation of AR to fluorescent resorufin. (B) A TEM micrograph of the NiO nanoparticles; scale bar = 20 nm. (C) A photograph of 1 μM AR reacted with various metal oxides (1 mg mL^{-1}) in 50 mM HEPES buffer (pH 7.4) for 30 min under ambient light (top) and in the dark with 470 nm excitation. (D) Fluorescence spectra of the reaction product with and without NiO excited at 540 nm. (E) The 580 nm emission intensities of some samples in (C).

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After a 30 min at pH 7.4, the samples were centrifuged to precipitate the oxides. Under normal light, most samples appeared water-like (Fig. 1C, top panel). Under 470 nm excitation, the NiO sample showed the highest fluorescence, indicating oxidation of AR (Fig. 1C, bottom panel). The fluorescence at 580 nm increased 301-fold compared to the control sample without NiO (Fig. 1D). CeO_2 also had a fluorescence signal (only ~5% of that from NiO, Fig. 1E), consistent with its reported low oxidase-like activity.[30] The size of our CeO_2 (~5 nm) was much smaller than that of NiO (~20 nm, Fig. 1B). At the same concentration of 1 mg mL^{-1}, the higher activity of NiO cannot be attributed to its larger surface area. Unlike CeO_2 with oxidative Ce^{4+} sites, NiO itself does not have oxidation activity (*e.g.* Ni^{2+} is not a strong oxidant). Therefore, NiO must serve as a catalyst for this reaction, and it is a real nanozyme instead of an oxidizing reagent.

Since a promising response was observed with NiO, we further characterized it. Our NiO nanoparticles were spheres of ~20 nm (Fig. 1B). Its XRD pattern matched with that of NiO (Fig. S1, ESI†). The surface property of NiO was probed by zeta-potential measurement (Fig. S2, ESI†). It was positively charged at neutral pH, but became negatively charged at pH 9.2.[35] The surface charge is determined by the (de)protonation of the surface hydroxyl groups. The size of our NiO was measured also in dispersion by dynamic light scattering (DLS) showing extensive aggregation reaching over 500 nm (Fig. S3, ESI†). This was much larger than the individual particle size observed under TEM, and such aggregation can be attributed to the lack of strong capping ligands (we wanted to study the native oxide surface).

In addition to AR, we also tested a few other typical substrates, such as ABTS (Fig. 2A and B) and TMB (Fig. 2C and D) to gain further insights. The reactions were performed at both pH 4 and pH 7 using a few metal oxides. At pH 4.0, we observed blue and green colors with CoO and CeO_2 indicative of oxidation of ABTS (Fig. 2A) and TMB (Fig. 2C), respectively.[17] However, the response of NiO was very weak for these two substrates. At pH 7, none of the samples showed color change (Fig. 2B and D). The fact that pH 4 had better oxidation for TMB and ABTS with CeO_2 was consistent with the literature.[21] Therefore, NiO was specific for AR. Oxidation of AR takes place on its phenol oxygen, while for TMB and ABTS, the oxidation products are nitrogen-based radical cations stabilized by the conjugated systems (Fig. S4, ESI†). This chemical difference might be the origin for selective AR oxidation with NiO.

As a further control, we tested whether the oxidation was really due to NiO nanoparticles, or from the dissolved Ni^{2+} ions. For this purpose, we centrifuged our NiO sample and confirmed that the supernatant was inactive (Fig. S5, ESI†). We then mixed AR with various concentrations of Ni^{2+} ($NiCl_2$ solution up to 10 mM) under the same buffer condition (Fig. 2E), and no fluorescence was observed either. This confirmed that dissolved Ni^{2+} was inactive and the origin of activity was from the NiO nanozyme.

Since NiO can be produced by condensation of $Ni(OH)_2$, an interesting question is whether $Ni(OH)_2$ has activity. To test this, we added NaOH to $NiCl_2$ and green $Ni(OH)_2$ precipitants were obtained (Fig. 2F). At various $Ni(OH)_2$ concentrations, however, the samples remained dark (Fig. 2G). Thus NiO was the active species and it cannot be replaced by $Ni(OH)_2$.

Our above AR oxidation was performed at neutral pH. Since many nanozymes worked better at acidic pH, we also studied the effect of pH. From direct fluorescence reading, the yield was quite stable from pH 7.5 to 9.2 (Fig. 3A). At acidic pH,

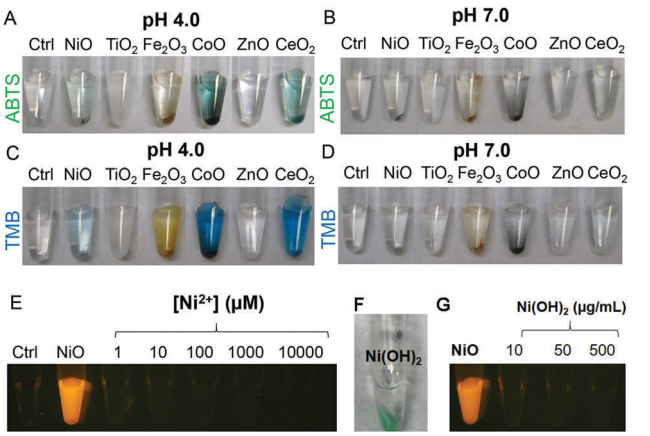

Fig. 2 Photographs of oxidation of 1 μM ABTS (A and B) and 1 μM TMB (C and D) at pH 4 (A and C) and pH 7 (B and D) in dark for 30 min with 1 mg mL^{-1} of various metal oxides. (E) Free Ni^{2+} cannot oxidize AR at pH 7.4. (F) A photograph of $NiCl_2$ (1 M) mixed with NaOH (1 M) after centrifugation. (G) $Ni(OH)_2$ cannot catalyze AR oxidation at pH 7.4.

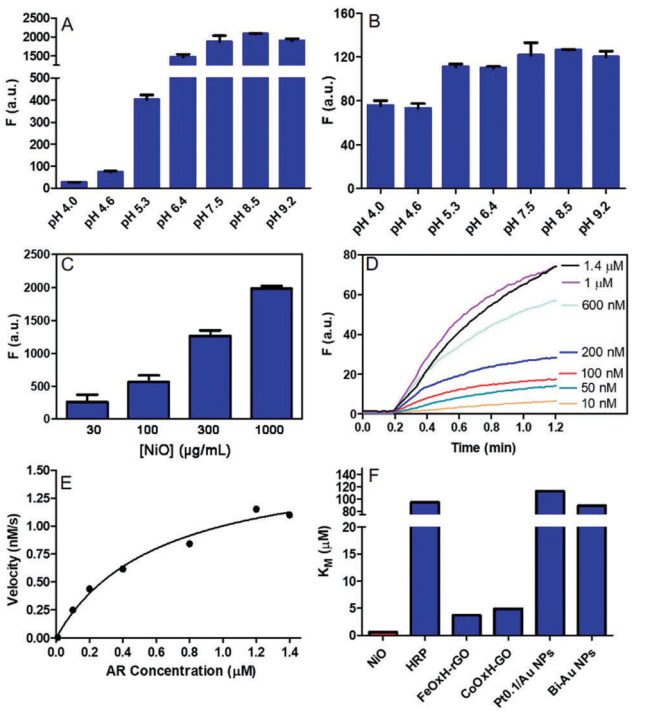

Fig. 3 (A) The fluorescence of AR (1 μM) oxidation products by NiO at various pH's for 30 min in the dark. (B) The samples in (A) after diluting 20-fold into a pH 7.4 buffer. (C) Fluorescence at 30 min of AR oxidation by various concentrations of NiO. (D) Kinetics of oxidation at various AR concentrations with 0.2 mg mL^{-1} of NiO. (E) Fitting the kinetic data to the Michaelis–Menten equation. (F) K_m values of our NiO nanozyme and some other enzymes and nanozymes taking from ref. 37–39.

the fluorescence was lower. When pH was lower than 5, the fluorescence dropped to almost zero. Since the quantum yield of AR is dropped at low pH,[30] the lower fluorescence may not necessarily mean low conversion. To test this, we diluted all the samples by 20-fold to neutral pH (Fig. 3B). In this case, the fluorescence intensity was quite similar for all the samples. Therefore, NiO worked at all these pH values, but care needs to be taken to interpret the data at low pH.

Since NiO has interesting oxidase-like activity for AR, we then characterized it as an enzyme.[36] We first varied the concentration of NiO. Faster oxidation was observed with higher concentration of NiO (Fig. 3C), and good activity was observed with 30 μg mL^{-1} of NiO. We then fixed the NiO concentration and measured the reaction kinetics at various AR concentrations (Fig. 3D). By fitting these data to the Michaelis–Menten equation (Fig. 3E), we calculated the k_{cat} (0.14 s^{-1}) and K_m (0.62 μM) of our NiO nanozyme. Our reaction was finished in just a few minutes, while using CeO_2 for AR oxidation under similar conditions required a few hours.[30] We compared the K_m values of our NiO and a few other enzymes and nanozymes for AR oxidation (Fig. 3F), and our NiO had the highest substrate binding affinity (*i.e.* lowest K_m). A low K_m indicates a strong affinity between AR and NiO. Due to the oxidation reaction, we could not directly measure the adsorption isotherm, but in general all the metal oxides in Fig. 3F had low K_m values indicating a strong metal-related interaction. Its k_{cat} was also compared, but we cannot find oxidase nanozymes for AR and thus mainly peroxidases were listed (Table S1, ESI†).

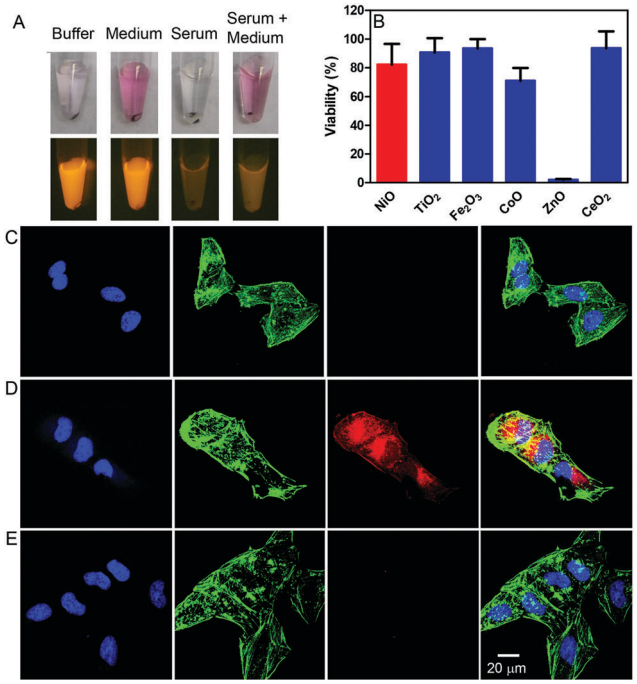

Fig. 4 (A) NiO (1 mg mL^{-1}) oxidizes AR (1 μM) in cell culture medium and in 10% serum. (B) MTT assay of HeLa cells when expose to 0.2 mg mL^{-1} of metal oxides. Confocal fluorescence micrographs of HeLa cells incubated with (C) AR; (D) 0.2 mg mL^{-1} of NiO and AR; and (E) resorufin. The cell nuclei were stained by DAPI (blue), the cell skeleton was stained in green, and the red channel was the AR oxidation product.

Given the activity of NiO for AR oxidation, an important application is bio-imaging.[40] For this, we first measured its activity in cell culture medium and in serum (Fig. 4A). The medium did not affect its activity, but serum decreased its activity by ~90%. Despite this, AR oxidation was still observed in serum containing medium. We then measured the cytotoxicity of NiO using the MTT assay (Fig. 4B), and the cells remained >80% viable with 0.2 mg mL^{-1} of NiO. Among the tested metal oxides, only ZnO showed high toxicity.[41] Finally, using 0.2 mg mL^{-1} of NiO, we tested its intracellular oxidation of AR. HeLa cells were first incubated with AR and NiO before analyzed by confocal fluorescence microscopy. Without NiO, no red fluorescence was observed (Fig. 4C), while NiO produced strong intracellular red fluorescence (Fig. 4D). As a control, we also incubated the cells with resorufin (the AR oxidation product), and no red fluorescence was detected (Fig. 4E). Therefore, the observed fluorescence in Fig. 4D was due to oxidation of AR inside cells.

NiO is a well-known oxidizing catalyst. For example, it was used to oxidize formaldehyde, but the reaction was carried out at 90 °C or higher.[42] NiO was also used for oxidizing olefins,[43] including styrene,[44] and quinolin compounds.[45] Again, these reactions were performed at higher than 100 °C. Our AR oxidation performed efficiently at room temperature is highly attractive and fits the scope of enzyme mimics at near physiological conditions. Other Ni containing materials were also studied as nanozymes. For example, it was reported that for peroxidase-like activity, porous $LaNiO_3$ with Ni^{3+} was about 58-fold more active than NiO (Ni^{2+}) and 22-fold higher than Ni nanoparticles (Ni^0).[46] NiO was recently reported to have very good DNA adsorption properties in biological samples.[35,47] Its nanozyme property adds more excitement to its bio-related applications.

In summary, we communicated a new oxidase-mimicking nanozyme, NiO. It can highly effectively oxidize fluorogenic AR at physiological conditions, making NiO unique and useful for intracellular imaging. AR is the most commonly used fluorogenic substrate for nanozyme and immunoassays. Most previously reported AR oxidation required H_2O_2, and thus relied on its peroxidase activity. In this work, we eliminated the unstable and toxic H_2O_2 and explored the oxidase activity. It will find important bioanalytical and imaging applications considering the low background and high sensitivity of fluorescence detection.

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Conflicts of interest

There are no conflicts to declare.

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