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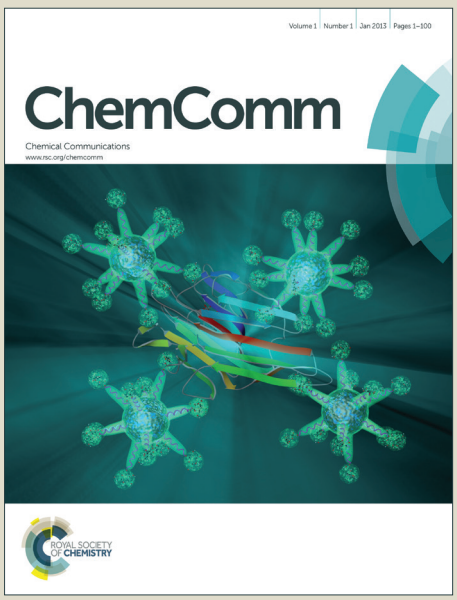

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Membrane lipid peroxidation by the peroxidase-like activity of magnetite nanoparticles

Cite this: DOI: 10.1039/xoxxoooooox

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Received ooth January 2012,
Accepted ooth January 2012

DOI: 10.1039/xoxxoooooox

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We report that Fe_3O_4 nanoparticles are able to catalyse lipid peroxidation in liposomes in acidic but not neutral pH. The oxidation is dependent on either pre-existing lipid peroxides or hydrogen peroxide as substrate. This is the first evidence for metal oxide nanoparticles as peroxidase mimetics oxidising biomolecules in relevant environment.

The catalytic activity of nanoparticles (NPs) with biomolecules is a fundamental issue that urgently needs to be addressed for better understanding of the mechanism(s) underlying the adverse effects and for biomedical applications of the nanomaterials. Natural peroxidases are a large family of enzymes that catalyse hydrogen peroxide (H_2O_2) or organic hydroperoxides to oxidise various substrates. Transition metal oxide NPs have been found to possess peroxidase- or catalase-like activities.[1] For example, magnetite (Fe_3O_4) nanoparticles (MNPs) can catalyse oxidation of a peroxidase substrate in an acidic solution in the presence of H_2O_2; whilst unlike natural peroxidases, MNPs almost lose peroxidase-like activity in neutral pH (with 3,3,5,5-tetramethylbenzidine, TMB, as substrate).[1a] Data from these studies were based on the catalytic reactions in NP–inorganic substrate mixtures and have attracted substantial attention in biological sensing, industrial detection and waste treatment.[2] The redox chemistry, i.e., the electron transfer capacity is thought to underlie the intrinsic catalytic activities of the NPs.[1e,3] Membrane lipid peroxidation is a well-known example of cellular damage under conditions of oxidative stress, a process in which molecular oxygen is incorporated into unsaturated lipids (LH) to form lipid peroxides (LOOH).[4] It is thought to occur via an initiator, which is commonly a free radical (X·), that overcomes the dissociation energy of an allylic bond and abstracts hydrogens from LH to form lipid alkyl radicals (L·) (**initiation (1)**). The lipid alkyl radicals can rapidly add oxygen to form lipid peroxyl radicals (LOO·) which then liberate LOOH via hydrogen abstraction from a neighbouring allylic bond (**chain reactions-propagation (2) & (3)**): [4a,c]

$$X\cdot + LH \rightarrow L\cdot + XH \quad (1)$$

$$L\cdot + O_2 \rightarrow LOO\cdot \quad (2)$$

$$LOO\cdot + LH \rightarrow L\cdot + LOOH \quad (3)$$

In this study we used synthesised liposomes that contain polyunsaturated fatty acids (PUFAs) in the phospholipids to investigate the potential oxidising activity of MNPs on biomolecules. The investigated MNPs have been shown in our previous studies to cause cellular oxidative stress and lipid peroxidation.[5] Our data demonstrate that MNPs catalyse the peroxidative reaction of the PUFAs by their peroxidase-like activity.

The magnetite NPs were synthesised (MNP-core), coated with polyethyleneimine (MNP-PEI) and characterised as previously reported.[5] The core size of the nanoparticles, as estimated by TEM, was approximately 30-40 nm (Fig. 1). More detailed structure analysis of the NPs by powder X-ray diffraction (XRD) and high resolution TEM (HRTEM) is provided in Supplementary Information (Fig. S1 and S2, ESI†). The zeta potential for MNP-core and MNP-PEI was -15.4 mV and +48.4 mV, respectively. The model liposomes used for the study were composed of lipids commonly present in biological membranes: 1,2-distearoyl-*sn*-glycero-3-phosphocholine (18:0 (Cis) PC), 1,2-dilinolenoyl-*sn*-glycero-3-phosphocholine (18:3 (Cis) PC) and cholesterol at the same molar concentration (molar ratio at 1:1:1). All liposomes were prepared in either 200 mM sodium acetate (NaAc) buffer (pH4.8) or 50 mM PBS/150 mM NaCl (pH7.4) and were immediately used for the experiments. The pH values were chosen to represent two biologically relevant microenvironments of cellular lysosome (pH4.8) and cytosol (pH7.4) in which NPs are commonly localised upon internalisation. Liposomes were incubated with MNPs in either pH 4.8 or pH 7.4 buffer at 37˚C and the resulting reaction mixtures were analysed by measuring the stable degradation product of PUFAs' hydroperoxides, malondialdehyde (MDA), by thiobarbituric acid reactive substance (TBARS) assay.

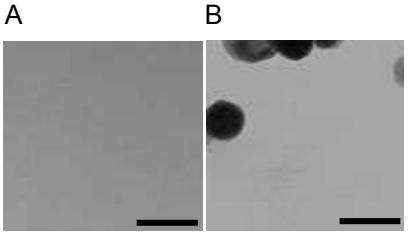

Fig. 1 TEM micrographs of (A) MNP-core and (B) MNP-PEI of Fe_3O_4 nanoparticles. Bar = 50 nm.

We found that both MNP-core and MNP-PEI dose-dependently oxidised PUFAs in the liposome membranes and produced MDA in acidic pH, in the absence of H_2O_2 (Fig. 2A). As commonly observed in any lipid preparations,[6] there were pre-existing LOO· and LOOH in the samples as the untreated liposomes also showed a basal amount of MDA (15.76±2.43 μmol/mmol lipid). The oxidising activity of the MNPs may rely heavily on the reactive sites of the particle surface[1d] as we observed a much lower MDA level in MNP-PEI treated membranes (Fig. 2A), although the electrostatic effect on the substrate affinity cannot be excluded.[7] The difference in the catalytic activity of uncoated and coated MNPs could also be seen in the time course of the reaction (Fig. S3, ESI†). The enzymatic activity was consistent with the reported peroxidase-like properties of MNPs in which the activity was maximal in acidic environment but diminished at neutral pH (Fig. 2B). At pH7.4 the measured MDA value in MNP-treated samples was not different from the basal level in the control (3.49±1.27 μmol/mmol lipid), which was much lower than that recorded in the acidic environment. Presence of MNP-PEI appeared to even reduce the autoxidation of PUFAs during the incubation (Fig. 2B). Further experiments at pH7.4 demonstrated that MNPs suppressed high concentration H_2O_2-induced lipid peroxidation in liposomes (Fig. S4, ESI†). This is not surprising as MNPs possess catalase-like activity, i.e., decompose H_2O_2 into H_2O and O_2, in neutral environment.[1b] Similar to its effect on basal autoxidation of PUFAs, MNP-PEI showed much stronger inhibition than MNP-core. This phenomenon should be further investigated. The pH-dependent lipid peroxidation may explain, at least partially, the recent finding that intracellular localisation and microenvironment may determine the cytotoxicity of MNPs.[1b]

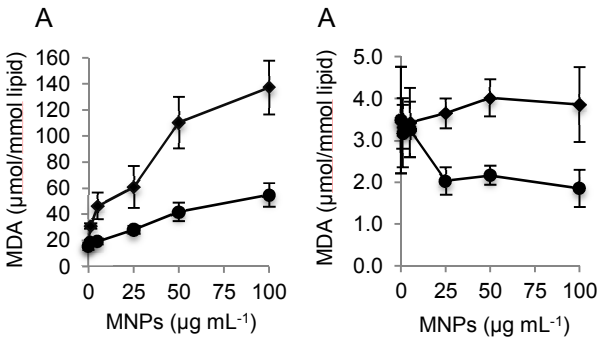

Fig. 2 Lipid peroxidation by MNPs at pH4.8 and pH7.4. MNP-core (⬥) and MNP-PEI (•) are incubated at concentrations as indicated with 1.0 mM liposomes in (A) 200 mM NaAc pH4.8 and (B) 50 mM PBS/150 mM NaCl pH7.4 for 2h. MDA production is measured by TBARS assay.

Unlike oxidation of other inorganic substrates, lipid peroxidation by MNPs in acidic pH was not dependent on H_2O_2 (Fig. 3A). H_2O_2 actually suppressed the autoxidation of PUFAs in control samples (Fig. S5, ESI†). Upon normalisation by this basal inhibition, MNP-core catalysed PUFA oxidation was largely unaffected by H_2O_2 at all concentrations tested, although the catalysis by MNP-PEI was further inhibited (Fig. 3A). The reactions were studied using H_2O_2 concentrations up to 530 mM, the optimal concentration of H_2O_2 for the peroxidase-like activity of the MNPs for other inorganic substrates (Fig. 3B).[1a] The activity of a natural peroxidase, horseradish peroxidase (HRP), could not be linked to the observed lipid peroxidation under the same experimental conditions (Fig. S6, ESI†). Similar to the catalysis of inorganic substrates, the MNPs could be recycled and reused for many rounds for peroxidative reaction of lipids without significant loss of the activity (Fig. S7 and Fig. S2, ESI†), demonstrating the robustness of these enzyme nano-mimetics.

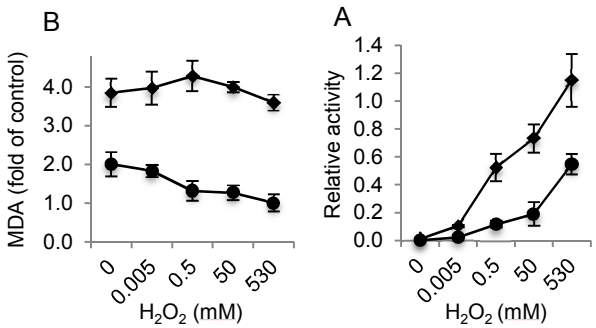

Fig. 3 Effect of H_2O_2 on liposome lipid peroxidation and oxidation of inorganic substrate by MNP-core (⬥) and MNP-PEI (•) (25 μg mL^{-1}) at pH4.8. (A) Liposome-MNP reaction mixtures are co-incubated with H_2O_2 at the concentrations as indicated. MDA is measured and expressed as fold of corresponding control liposomes incubated with same concentration of H_2O_2. (B) HRP-like activity of MNPs in the presence of H_2O_2 at the same range of concentrations, determined by the oxidation of 3,3,5,5-tetramethylbenzidine (TMB). The activity is expressed as absorbance at 652 nm.

Another important factor in the catalysis by iron oxide NPs is free ionic iron, which has been shown to effectively oxidise PUFAs.[8] MNPs were therefore incubated in the acidic reaction buffer for 2 h followed by separation by a magnet. The supernatants were examined for iron content and catalytic activity. The total iron concentration in MNP-core and MNP-PEI leaching solutions was 28.7 μM and 5.2 μM, respectively (the concentration of the MNPs was 25 μg mL^{-1} of total iron). Free iron ions in the leaching solutions indeed cause iron-dependent lipid peroxidation (Fig. 4). MDA values presented in this figure are the amount of MDA in the treated samples upon deduction by the MDA in controls, as such the contribution of the iron-dependent catalysis could be clearly demonstrated, which was 27.5% and 13.1% in MNP-core and MNP-PEI, respectively. Nonetheless, the intact MNPs acting as enzyme mimetics played a major role in the catalysis.

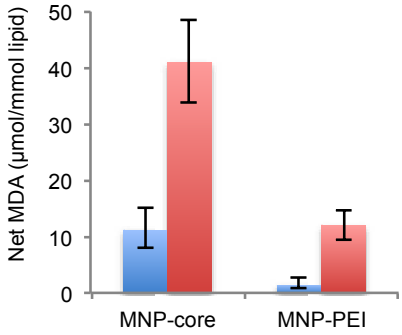

Fig. 4 Effect of iron leaching (blue) from MNPs (red) in acidic pH on liposome lipid peroxidation. MNPs are incubated in the reaction buffer (200 mM NaAc pH4.8) followed by separation by a magnet. The leaching solutions are assayed and compared with that of MNPs (25 μg mL^{-1}) for MDA by TBARS.

The independence of lipid peroxidation by MNPs on H_2O_2 prompted us to look to other peroxides that may be responsible for the process. When preformed LOOH was removed by incorporating triphenylphosphine (TPP) into liposomes, MNPs could no longer oxidise PUFAs (Table 1). These results indicate that the observed catalysis was dependent on the preformed LOOH in the liposomes.

Interestingly, when H_2O_2 was added into the reaction of LOOH-deprived liposomes, peroxidative reactions recurred, especially at high concentrations of H_2O_2 at which H_2O_2 alone was still unable to have any impact on the oxidation of PUFAs (Table 1). Together with the data presented in Fig. 3A, these results implicate the capability of MNPs of catalysing either LOOH or H_2O_2 as oxidising substrate for lipid peroxidation in acidic pH: MNPs (assumedly by ferrous iron on the particle surface)[1a] could decompose either LOOH or H_2O_2 to produce corresponding free radicals, lipid alkoxyl radicals (LO·) or hydroxyl radicals (HO·), respectively (for simplicity, only two key reactions are listed):

$$MNP(Fe^{2+}) + LOOH + H^+ \rightarrow MNP(Fe^{3+}) + LO\cdot + H_2O \quad (4)$$

or

$$MNP(Fe^{2+}) + H_2O_2 + H^+ \rightarrow MNP(Fe^{3+}) + HO\cdot + H_2O \quad (5)$$

The resultant LO· or HO· could then act as the initiator (X·) in reaction (1) to initiate the chain reactions ((2) and (3)).[4b] To verify if H_2O_2/HO· were involved in the initiation of lipid peroxidation in the absence of preformed LOOH, we added mannitol to the reaction mixtures to scavenge HO·. Elimination of this free radical effectively inhibited the lipid peroxidation by MNP-H_2O_2 (Fig. S8, ESI†), indicating the dual oxidising substrate catalysis mechanism. LOOH is concomitantly produced during the propagation and can be then catalysed again by MNPs to produce LO· for the initiation. This may add a further "propagation" loop to facilitate the catalytic reaction of PUFA oxidation by MNPs (detailed proposed reactions supplied in the ESI† and summarised in the Table of Contents (TOC)), although other reaction loop involving LOOH may also exist.[4b] The essentiality of preformed LOOH in the iron-dependent lipid peroxidation in liposomes, although complicated and still controversial, may explain a similar mechanism as we observed here.[8b] These observations tend to point to the potential substrate preference of MNPs to LOOH. It is not known how H_2O_2 reacts with various components in the reaction mixture when LOOH is available. H_2O_2 may affect the pre-existing oxidative products of lipids (LO·, LOO·, and etc) in liposomes (Fig. S5, ESI†), and may compete with LOOH as substrate for MNPs. The reason why we applied a wide range of H_2O_2 concentration in this study was that unlike natural peroxidase such as HRP, MNPs have a significantly higher K_m with H_2O_2, hence require much higher concentration of H_2O_2 than natural peroxidase to achieve their maximal activity.[1a] In the absence of LOOH, the peroxidative reaction reached a level comparable to that from "natural" liposomes (Fig. 1A) when H_2O_2 concentration was 530 mM (Table 1), suggesting that MNPs require similar optimal H_2O_2 concentration for the PUFA oxidation reactions. We should emphasise that the MNPs could exhibit much more complicated and wider spectrum of interfacing mechanisms than those we speculate based on these data. The substrate affinity of MNPs[2b,7] towards both LOOH/H_2O_2 and PUFAs in lipid membranes, and the pH- and LOOH-dependence of oxidation of PUFAs, which has been implicated in the lipid oxidation by haem proteins,[9] remits further extensive investigations.

Table 1. Lipid peroxidation by MNPs in LOOH-deprived liposomes at pH4.8.¶

H_2O_2 (mM)	MDA (μmol/mmol lipid)		
	Control	MNP-core	MNP-PEI
0	0.48±0.23	1.20±.86	0.52±0.41
0.005	0.38±0.27	1.62±0.53	0.87±0.38
0.5	0.97±0.54	10.02±3.64	4.04±1.45
50	0.64±0.25	34.09±3.98	7.78±2.73
530	0.53±0.04	53.47±9.89	10.06±2.40

¶TPP (35 μM) is incorporated into liposomes during the synthesis. The LOOH-deprived liposomes are incubated with MNPs (25 μg mL^{-1}) in the absence or presence of H_2O_2 at concentrations as indicated. Data are mean ± SD, n = 4.

Conclusions

We have reported a novel category of peroxidase-like activity of iron oxide NPs towards oxidation of PUFAs in liposomes. Our studies indicate that the lipid peroxidation occurs in acidic pH but not in neutral environment. MNPs catalyse either pre-existing lipid peroxides or, if lipid peroxides are removed from the liposomes, hydrogen peroxide, as substrate to initiate the chain reaction process. The substrate preference may be potentially determined by the redox chemistry of particle surface or NP-substrate interface. The results of the present study may shed light on the mechanisms of cellular oxidative damage induced by metal oxide NPs.

We acknowledge financial support from the MARVENE (ERA-NET NanoSci-E+ NAN092) EU project and the Engineering and Physical Sciences Research Council (EPSRC) UK (EP/H007040/1).

Notes and references

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† Electronic Supplementary Information (ESI) available: Experimental details and proposed model reactions involved in the peroxidation of lipids by MNPs. See DOI: 10.1039/c000000x/

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