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Electronic Supplementary Information

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

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Materials

1,2-distearoyl-*sn*-glycero-3-phosphocholine (18:0 (Cis) PC), 1,2-dilinolenoyl-*sn*-glycero-3-phosphocholine (18:3 (Cis) PC) and cholesterol were obtained from Avanti Polar Lipids, USA. All other chemicals were purchased from Sigma-Aldrich, UK unless otherwise stated.

Synthesis of magnetite (Fe_3O_4) nanoparticles (MNPs)

The synthesis was based on the established protocol of oxidative hydrolysis, i.e., the precipitation of an iron salt ($FeSO_4$) in basic media (NaOH) with a mild oxidant.[1] In brief, nitrogen was bubbled through a solution of sodium hydroxide (0.1 M) and potassium nitrate (0.1 M) dissolved in deionised water at 90 ˚C for 1 h. Iron sulphate (0.03M) dissolved in sulphuric acid (0.01M) was added to the reaction and the mixture was stirred for 24 h at 90 ˚C under nitrogen. After this time the reaction was rapidly cooled on ice and the particles were washed 6 times in deionised water and magnetically separated from solution. The resultant particles (MNP-core) were re-suspended in water and stored at 4 ˚C.

Coating and characterization of MNPs

Fe_3O_4-core nanoparticles (2 mL) were sonicated in poly(ethylenimine) (PEI, 750 kDa) solution (5 mg mL^{-1}) for 2 h. The particles were then washed 6 times with deionised water and magnetically separated from solution (MNP-PEI). The MNP-PEI were re-suspended in 10 mL deionised water and stored at 4 ˚C. Freeze dried particles were run on the FTIR using a diamond tipped attenuated total reflectance attachment (Nicolette iS5 with iD5 ATR, Thermo-Fisher UK) to determine whether PEI coating was successful.[2] The MNPs were dispersed in deionised water and sonicated for 10 min before all measurements. Nanoparticle concentration was determined using ICP analysis (Optima 7000V DV, Perkin Elmer, UK).[2] TEM was performed by diluting MNP samples in deionised water and dropping 2 μL of the MNP suspension onto copper grids allowing to dry at room temperature. The grids were loaded into the TEM and directly imaged using a JEOL 1200 EX- FDL5000 microscope (Jeol, Japan). High resolution transmission electron microscopy (HRTEM) images were obtained on a JEOL-2011 electron microscope operating at 200 kV. Powder X-ray

diffraction (XRD) was performed on a PANalytical Empyrean diffractometer with Cu Kα radiation. Zeta potential measurements were carried out using a photon correlation spectrometer (Zetasizer Nano-ZS, Malvern Instruments, UK).

Liposome preparation

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 molar ratio (1:1:1) were dissolved or diluted in chloroform. In some experiments, triphenylphosphine (TPP) was mixed with the lipids at this stage (final concentration 35 μM in the liposome preparation of 1.0 mM total lipids). The lipid mixture was introduced into a round bottom flask attached to a rotary evaporator. The flask was rotated and evacuated in water bath at 60 ˚C until all the liquid has evaporated. The dry lipid film was vacuumed for 1h further before dissolved in pre-warmed (45 ºC) 200 mM sodium acetate (NaAc, pH4.8) or 50 mM PBS/150 mM NaCl (pH7.4). The sample was sonicated in water bath for 1 h before dialysed in corresponding buffer solution for 1 h. The liposome preparation was used immediately for lipid peroxidation experiments.

Lipid peroxidation in liposomes measured by thiobarbituric acid reactive substance (TBARS) assay

Liposomes (1.0 mM of total lipids) were incubated in either 200 mM NaAc pH4.8 or 50 mM PBS/150 mM NaCl pH7.4 buffer for 0.5, 1 or 2 h at 37°C in sealed eppendorf tubes with appropriate testing samples (MNP-core and MNP-PEI, iron leaching solution, HRP, H_2O_2) at appropriate concentrations. All MNPs were sonicated for 30 s prior incubation. The total reaction volume was 500 μL. 0.05% butylated hydroxytoluene (BTH) was then added to the samples followed by sonication for 5 s for three times at 40V on ice. Malondialdehyde bis(dimethyl acetal) (MDA) standard solutions (0-7.5 μM) were prepared. 100 μL of samples or standards were mixed with 100 μL 2% sodium dodecyl sulphate (SDS) in eppendorf tubes and were incubated for 5 min at room temperature. Thiobarbituric acid (250 μL) was added to each tube before incubation at 95 ˚C for 1 h. The samples were cooled on ice and centrifuged at 3000 rpm for 15 min at 4 ˚C. The supernatants were pipetted into

wells of a 96-well plate and fluorescent intensity measurement was taken at 530 nm (excitation) and 550 nm (emission) using a multi-mode plate reader (Tecan infinite M200, Austria) under optimal gain mode. The results were calculated as μmol MDA/mmol lipids, using appropriate buffer solution as the blank.

Recycle and repeated use of MNPs in the lipid peroxidative reaction

Liposomes (1.0 mM) were incubated for 1 h at 37°C in 200 mM sodium acetate pH4.8 in sealed tubes with 25 μg mL^{-1} MNP-core or MNP-PEI. At the end of the incubation, 1 ml reaction mixture was taken from each tube and proceeded for the TBARS assay as described above. MNPs in the remaining mixture were pulled down by a magnet and the supernatant (liposomes) decanted. The MNP pellet was re-suspended in fresh liposomes in a volume 1.0 ml less than that of the previous incubation and sonicated for 30 s before next round of incubation. The "incubation - MNP pull down - MNP resuspension" procedure was repeated 5 times, and all were followed immediately by the TBARS assay.

Examination of the effect of H_2O_2 on TBARS assay

To rule out the possibility of interference of H_2O_2 on TBARS assay, solutions of post MNP-liposome-H_2O_2 reactions were incubated with catalase (500U/ml) for 5 min at room temperature to eliminate the remaining H_2O_2 before proceeding to the TBARS assay. No difference between the reactions with or without catalase was observed (data not shown).

Effect of hydroxyl radical scavenging on lipid peroxidation by MNP- H_2O_2 in lipid hydroperoxide-deprived liposomes

Liposomes with TPP (35 μM) incorporated in during synthesis were co-incubated with 25 μg mL^{-1} of MNPs and varying concentrations of H_2O_2 (0-513 mM) in 200 mM NaAc pH4.8. In a separate set of the experiments, mannitol (10 mM) was added to the liposome-MNP-H_2O_2 mixture. Lipid peroxidation was analysed and measured by TBARS.

Analysis of HRP-like activity of MNPs

MNPs (25 μg mL^{-1}) were incubated in 500μL reaction buffer (200 mM NaAc, pH4.8) at 37 ˚C for 15 min with 3,3,5,5-tetramethylbenzidine (TMB, 816 μM) in the absence or presence of H_2O_2 (0-513 mM). The absorbance of reaction mixtures was measured using a multi-mode plate reader (Tecan M200) at 652 nm.

Detection of iron leaching and its effect on lipid peroxidation in liposomes

MNPs (250 μg/mL) were incubated in 200 mM NaAc pH4.8 for 2 h at 37 ˚C. The dissolved free iron in solution was separated from intact MNPs by a magnet. The leaching solution was incubated with liposomes in 200 mM NaAc pH4.8 buffer upon 1:10 dilution and lipid peroxidation was measured by TBARS as described above. The total ionic iron in leaching solution was measured, upon 1:10 dilution, by addition of 20 μL of 100 mM ascorbate to 1 mL of the solution and incubated the sample for 15 min to completely reduce the Fe^{3+}. 40 μL of 4.95 mM bathophenanthroline disulfonic acid was then added to the sample and the absorbance was measured at 535 nm after 90 s incubation (Tecan M200 microplate reader). The concentration of free Fe^{2+} was calculated with respect to a standard curve (R^2 = 0.9995).

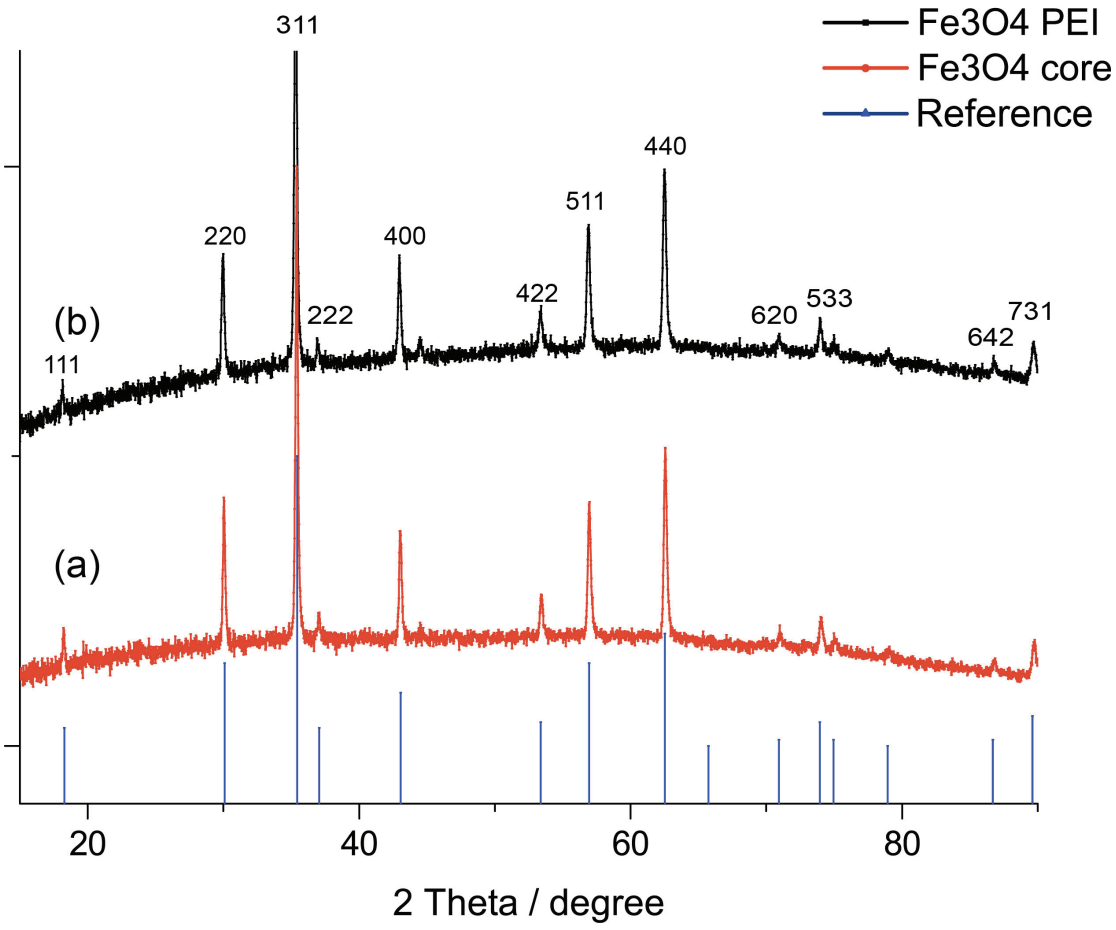

Fig. S1 Powder X-ray diffraction (XRD) pattern of (a) MNP-core (Fe_3O_4 core, red) and (b) MNP-PEI (Fe_3O_4 PEI, black). Nanoparticles are freeze-dried and XRD is performed on a PANalytical Empyrean diffractometer with Cu Kα radiation. Blue vertical lines indicate the peak position of reference Fe_3O_4 (JCPDS 00-019-0629).

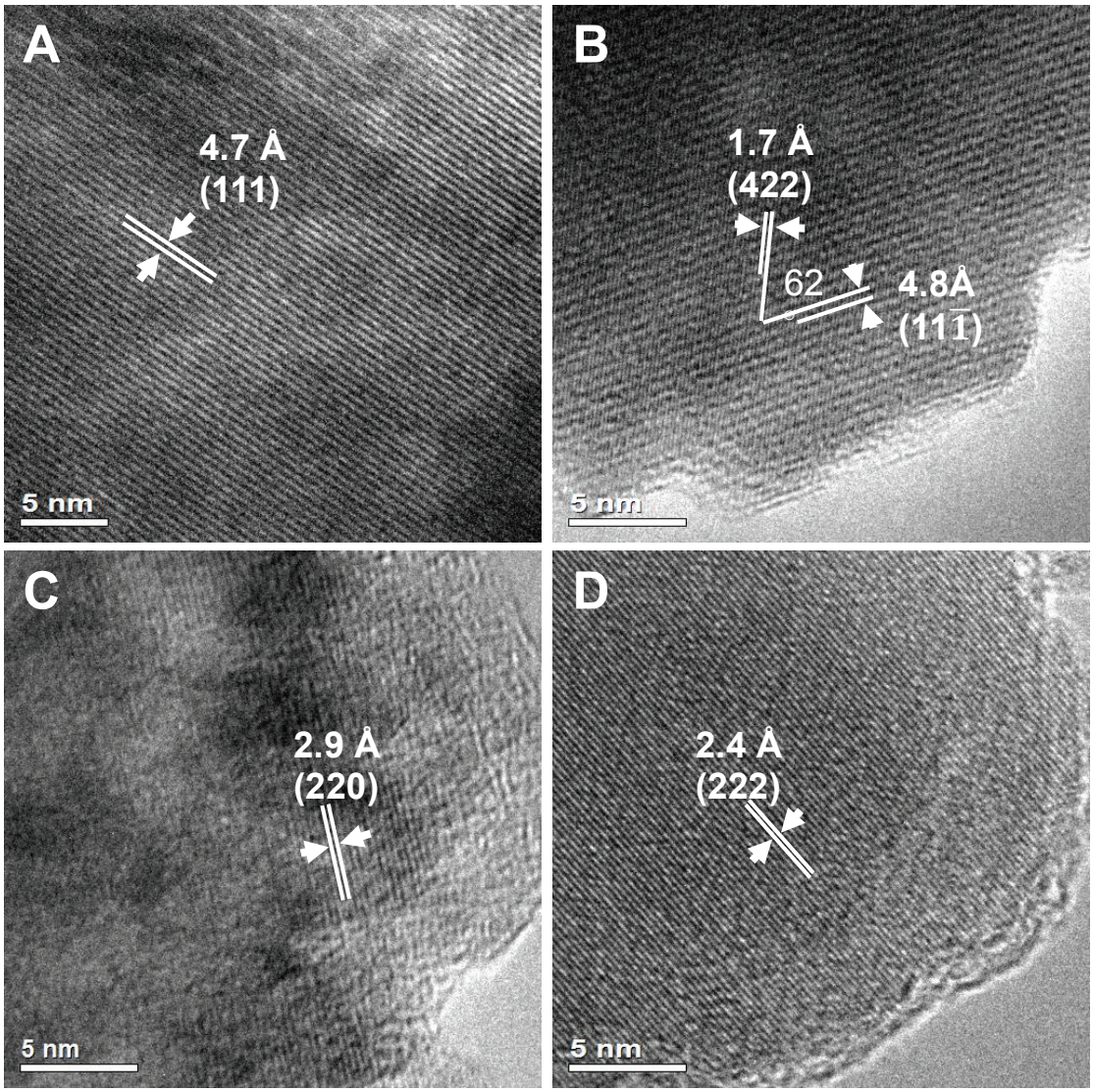

Fig. S2 High resolution electron microscopy (HRTEM) images of MNPs before (A, B) and after (C, D) reaction with the liposomes. MNP-core (A, C) and MNP-PEI (B, D) are incubated with liposomes and the reactions are repeated for five times. The nanoparticles are collected and are examined by HRTEM on a JEOL-2011 electron microscope operating at 200 kV. Representative *d*-spacing in the Fe_3O_4 crystal structures are indicated in the images and are accorded with the crystalline planes as indicated in the XRD pattern in Fig. S1.

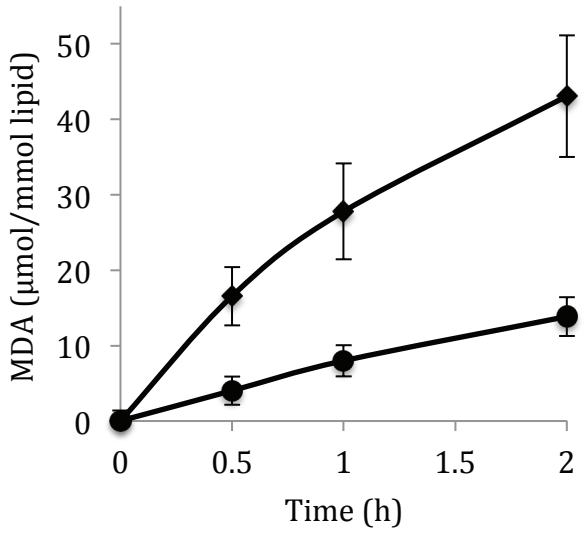

Fig. S3 Time-dependent oxidation of PUFAs in liposomes by MNPs at pH4.8. Liposomes (1.0 mM) are incubated with 25 µg mL^{-1} of MNP-core (◆) and MNP-PEI (•) in 200 mM NaAc pH4.8 for the duration as indicated and oxidised PUFAs' product MDA is measured by TBARS. Data are presented as the total amount of MDA in MNP-treated samples subtracted by that from liposome controls at each time point.

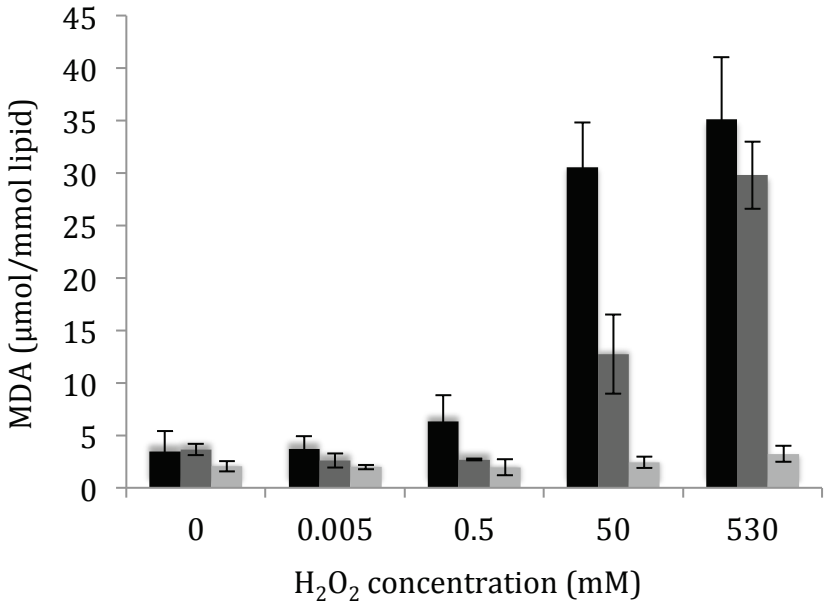

Fig. S4 Oxidation of PUFAs in liposomes by high concentration H_2O_2 in neutral pH is inhibited by MNPs. Liposomes (1.0 mM) are incubated with H_2O_2 at the concentrations as indicated (mM) in the absence (dark) and presence of MNP-core (dark grey) and MNP-PEI (light grey) in 50 mM PBS/150 mM NaCl pH7.4. Oxidised PUFAs' product MDA is measured by TBARS. The concentration of MNPs is 25 μg mL^{-1}.

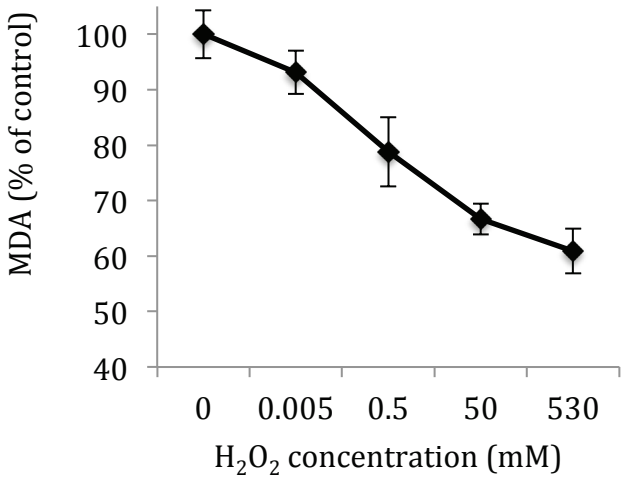

Fig. S5 Autoxidation of PUFAs in liposomes is inhibited by H_2O_2 in pH4.8. Liposomes (1.0 mM) are incubated with H_2O_2 at the concentrations as indicated (mM) in 200 mM NaAc pH4.8. Oxidised PUFAs' product MDA is measured by TBARS.

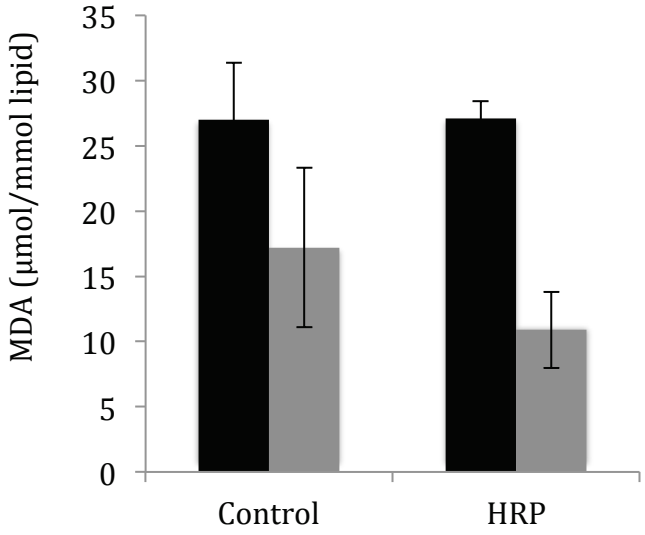

Fig. S6 HRP does not catalyse oxidation of PUFAs at pH4.8. Synthesised liposomes (1.0 mM) are incubated with HRP (10 ng mL^{-1}) in 200 mM NaAc buffer pH4.8 in the absence (dark) and presence (grey) of H_2O_2 (8.8 mM). MDA in the reaction mixtures is measured by TBARS assay.

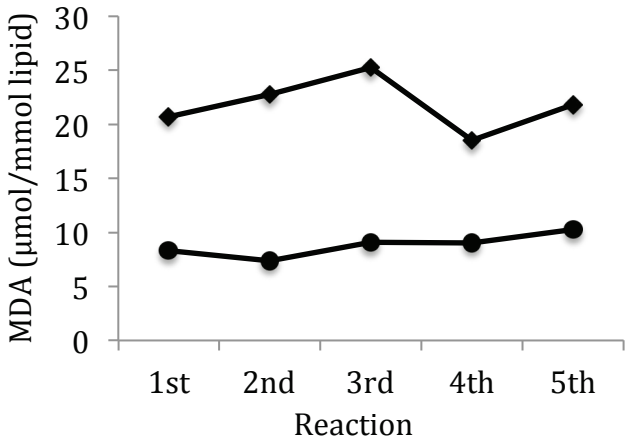

Fig. S7 Recycle and repeated use of MNPs in the catalysis of peroxidative reaction of PUFAs. Liposomes (1.0 mM) are incubated with 25 µg mL^{-1} of MNP-core (⬥) and MNP-PEI (•) in 200 mM NaAc pH4.8 for 1 h and oxidised PUFAs' product MDA is measured by TBARS. At the end of each reaction cycle, the MNPs are separated from the reaction mixture by a magnet and the reaction is repeated for five times with fresh liposomes under the same conditions. Data are presented as the total amount of MDA in MNP-treated samples subtracted by that from liposome controls and are means of two experiments.

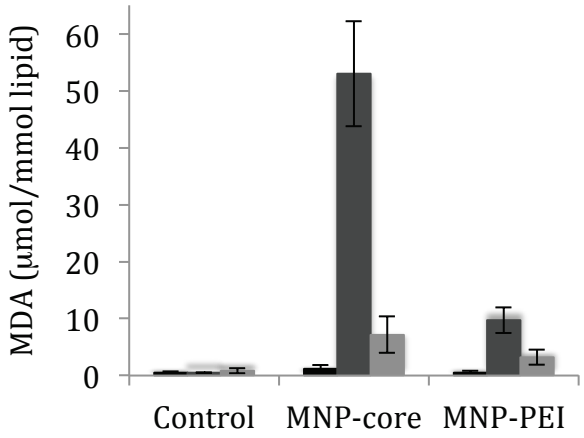

Fig. S8 Effect of mannitol on lipid peroxidation of LOOH-deprived liposomes by MNPs-H_2O_2 at pH4.8. Liposomes (LOOH deprived by TPP) are incubated with MNPs (25 μg mL^{-1}) in the absence (dark) and presence (dark grey) of H_2O_2 (530 mM). Mannitol (10 mM) is added in another set of incubations that contains H_2O_2 (light grey). MDA in the reaction mixtures is measured by TBARS.

Proposed reaction models involved in lipid peroxidation by MNPs in acidic pH

The fact that the MNPs could catalyse both the decomposition and formation of lipid hydroperoxides (LOOH) in the initiation and chain reaction loop of lipid peroxidation (see the TOC) may render the kinetic of lipid peroxidation by the MNPs extremely complex. Molecular oxygen cannot be incorporated directly into unsaturated lipids (LH) to form LOOH because the ground state of lipids is of singlet-multiplicity whereas that of oxygen is of triplet-multiplicity (spin-forbidden). Lipid peroxidation must therefore occur via reactions that by-pass the spin barrier between lipids and oxygen. The activation energy is thought to be generated by the formation of free radicals, lipid alkoxyl (LO·) or hydroxyl (HO·) radicals, by the decomposition of LOOH or H_2O_2, respectively. LO· or HO· free radicals act as the "initiator" that overcome the dissociation energy of an allylic bond and thus cause hydrogen abstraction and formation of a lipid alkyl radical (L·). Lipid alkyl radical can rapidly add oxygen to form lipid peroxyl radical (LOO·) which eventually liberate LOOH via hydrogen abstraction from a neighbouring allylic bond.[3]

The LO· or HO· free radicals may be produced by the following reactions:

H_2O_2 is required for the Fenton's reaction by iron,[4] and similar reactions may also occur on the surface of MNPs (MNP-Fe^{2+}/ MNP-Fe^{3+}):

$$\text{MNP-Fe}^{2+} + H_2O_2 + H^+ \rightarrow \text{MNP-Fe}^{3+} + HO\cdot + H_2O \quad (1)$$

$$\text{MNP-Fe}^{3+} + H_2O_2 \rightarrow \text{MNP-Fe}^{2+} + HOO\cdot + H^+ \quad (2)$$

At low pH, reaction (1) may be predominant over reaction (2), leading to the H_2O_2-dependent lipid peroxidation by MNPs in the absence of LOOH (see below). On the other hand, the effect of H^+ on the balance of above reactions may slightly change the redox chemistry of the MNP surface. This may help to explain the inhibition of MNP catalysed PUFA oxidation by H_2O_2 at high concentrations, at least in the case of MNP-PEI.

It was thought that iron ions catalyse LOOH in the way similar to the Fenton's reactions,[5] and similar reactions may also occur on the surface of MNPs (MNP-Fe^{2+}/ MNP-Fe^{3+}):

$$\text{MNP-Fe}^{2+} + LOOH \rightarrow \text{MNP-Fe}^{3+} + LO\cdot + OH^- \quad (3)$$

$$\text{MNP-Fe}^{3+} + LOOH \rightarrow \text{MNP-Fe}^{2+} + LOO\cdot + H^+ \quad (4)$$

In acidic environment, reaction (3) could occur like reaction (1):

$$MNP\text{-}Fe^{2+} + LOOH + H^{+} \rightarrow MNP\text{-}Fe^{3+} + LO\cdot + H_2O \quad (5)$$

Two reasons may decide that reaction (5) is predominant over reaction (4): (a) it is much faster than reaction (4);[6] (b) in acidic pH, the equilibrium in reaction (5) moves further towards the production of LO· and H_2O. Reaction (1) may compete with reaction (5) over the oxidising substrate (H_2O_2 vs LOOH) for MNPs, providing another possible explanation of H_2O_2' inhibitory effect.

The resultant LO· or HO· could then initiate lipid peroxidation by the following reactions:[7]

$$LO\cdot + LH \rightarrow L\cdot + LOH \quad (6) \quad \textbf{or} \quad HO\cdot + LH \rightarrow L\cdot + H_2O \quad (7)$$

The following reactions constitute a chain leading to the propagation of lipid peroxidation:

$$L\cdot + O_2 \rightarrow LOO\cdot \quad (8)$$

$$LOO\cdot + LH \rightarrow L\cdot + LOOH \quad (9)$$

The reaction product LOOH could then act as the oxidising substrate again for MNPs in reaction (5) to produce LO· for another round of initiation-propagation of lipid peroxidation.

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