Ferromagnetic nanoparticles with peroxidase-like activity enhance the cleavage of biological macromolecules for biofilm elimination†}

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an enormous medical challenge, responsible for millions of healthcare-associated infections annually worldwide. Biofilms consist of cells within a matrix of nucleic acids, proteins and polysaccharides, which connect and protect resident bacteria from external damage. Nanocatalysts that could potentiate the effects of H2O2 might have great utility for a
variety of applications, potentially including biofilm destruction in the sterilization of medical surfaces, surgical instruments, and indwelling medical devices.

MNPs have been used as a general anti-bacterial agent for hygienic and medical treatments. H2O2 generates free radicals which oxidize organic chemicals or biomolecules, but the process is slow with low efficiency and bacteria easily develop resistance, especially in a formed biofilm. Biofilms represent an enormous medical challenge, responsible for millions of healthcare-associated infections annually worldwide. Biofilms consist of cells within a matrix of nucleic acids, proteins and polysaccharides, which connect and protect resident bacteria from external damage. Nanocatalysts that could potentiate the effects of H2O2 might have great utility for a
variety of applications, potentially including biofilm destruction in the sterilization of medical surfaces, surgical instruments, and indwelling medical devices.

Results and discussion
To test the ability of an MNP–H2O2 system to degrade biofilms and kill resident bacteria, we hydrothermally prepared MNPs having a bulk morphological diameter of 500 nm, with a rough surface containing 5–10 nm diameter protrusions (Fig. S1†). These nanoparticles show very high peroxidase-like activity as demonstrated by the 3,3',5,5'-tetramethylbenzidine (TMB) colorimetric reaction (Fig. S1b†). We then used these MNPs to test whether the MNP–H2O2 system could degrade each of the 3 major components of biofilms. We used H2O2 at concentrations of 1–3%, consistent with domestic hygiene uses, to investigate first the MNP–H2O2 system’s ability to degrade nucleic acids. Long chain plasmid DNA was completely cleaved into small fragments (Fig. 1b). Plasmid DNA in the presence of H2O2 alone showed topological change from supercoiled structure to linearized form but catalysis into fragments was dependent upon the presence of MNPs (Fig. S2a†). Cleavage of plasmid DNA was also dependent upon concentrations of H2O2 and DNA, as well as the time and temperature (Fig. S2a–d†), but was not
influenced by the pH within the tested range of 4.5–9 (Fig. S2f). The only slight difference among the various forms of nucleic acids we tested was that total RNA showed slight cleavage by H2O2 alone (Fig. 1e). Our results indicated that the MNP–H2O2 system could be used as a universal reagent for cleaving or degrading nucleic acids, suggesting additional applications for this system beyond biofilm degradation.

We next investigated the cleavage of proteins by the MNP–H2O2 system using similar conditions as above (Fig. 2a). In our first trial, we found that BSA (80 µg, MW = 66 kDa) was cleaved into small fragments as seen in a SDS-PAGE gel stained with Coomassie blue R-250. In contrast, BSA treated with 3% H2O2 alone showed very limited cleavage (Fig. 2b). We next varied the concentrations of H2O2 or BSA while keeping the reaction buffer constant (Fig. S3†). Although higher concentrations of H2O2 led to some cleavage, complete cleavage was dependent upon the presence of MNPs (Fig. S3a†). Lowering the amount of protein, or increasing the time of reaction or the temperature from room temperature to 37 °C resulted in more cleavage as expected (Fig. S3b–d†). However, unlike the effect on DNA cleavage, protein cleavage by the MNP–H2O2 system showed obvious pH dependence with increased cleavage at lower pH (Fig. S3e†). We assessed the versatility of the cleavage using more complex proteins, including IgG and lysates from whole bacteria and mammalian cells. We found that cleavage was not protein specific; mixtures of the wide variety of proteins found in cell lysates were completely cleaved under the same reaction conditions, demonstrating the generality of the cleavage mediated by the MNP–H2O2 system.

Polysaccharides are the third major component in the biofilm matrix.44 We chose chitosan as a model polysaccharide to test the effect of MNP–H2O2 versus H2O2 alone. We prepared a chitosan hydrogel by glutaraldehyde crosslinking in a glass vial. The chitosan gel had a yellow color with high adherence and viscosity, enabling it to stay suspended upside down in a tube (Fig. 3a). Although H2O2 alone cleaved the crosslinked chitosan to a degree, MNP–H2O2 had a greater effect at reducing the mass that remained as a gel (Fig. 3). We also found that chitosan cleavage was independent of pH, with cleavage occurring under both acidic (pH 4.5) and neutral (pH 7.4) conditions (data not shown).

Fig. 1 MNP enhanced oxidative cleavage of nucleic acids. (a) Schematic of MNP–H2O2 cleaving nucleic acids. (b) Plasmid DNA cleavage with MNP–H2O2. Plasmid DNA (4 µg, ~6500 bp) was incubated with 3% H2O2 alone, MNP alone, or MNP–H2O2. "C" denotes control with DNA only. "M" denotes the DNA marker (1 kb DNA ladder, NEB). (c) Cleavage processes of a PCR DNA product (1 µg), (d) mouse genomic DNA (1 µg), and (e) mouse total RNA (from testis) were all performed under similar conditions but with 1% H2O2. All experiments were repeated in triplicate with representative images shown.

Fig. 2 MNP enhanced oxidative cleavage of proteins. (a) Schematic of MNP–H2O2 cleaving protein. (b) Cleavage of BSA with MNP–H2O2. 80 µg BSA was incubated with 3% H2O2 alone, MNP alone, or MNP–H2O2. "C" denotes control with BSA only. "M" denotes the protein molecular weight marker (Precision Plus Protein™ Dual Color Standards, BIO-RAD). (c) Cleavage processes of rabbit polyclonal IgG (10 µg), (d) E. coli lysate (40 µg), and (e) HEK293 mammalian cell lysate (20 µg) were all performed under similar conditions but with 1% H2O2. All experiments were repeated in triplicate with representative images shown.

Fig. 3 MNP enhanced cleavage of a polysaccharide (chitosan). A chitosan gel was prepared at 0.5% with glutaraldehyde crosslinking. Chitosan gels were mixed with 1% H2O2 alone, MNP alone, or MNP–H2O2 to test for cleavage. "C" denotes control with chitosan only. All experiments were repeated in triplicate with representative images shown. When individually compared against the control, H2O2 and MNP–H2O2 were found to be significantly different (p < 0.05, n = 3, Student’s t-test). When compared against each other, the amount of biofilm remaining after treatment with MNP–H2O2 was significantly less than that when treated with H2O2 alone (p < 0.05, n = 3, Student’s t-test). Error bars denote the standard deviation.
We next sought to verify the mechanism by which the MNP–H$_2$O$_2$ system was able to enhance cleavage of nucleic acids, proteins, and polysaccharides, hypothesizing that the benefits were obtained through production of additional free radical oxidants generated by MNP catalysis of H$_2$O$_2$. To test the hypothesis, we investigated the impacts on cleavage of an antioxidant reagent, hypotaurine, which specifically scavenges hydroxyl radicals. Fig. 4a shows plasmid DNA cleavage in the presence of varied concentrations of hypotaurine (1–8%). We observed that cleavage was retarded with increasing concentrations of hypotaurine until it was almost stopped with 8% hypotaurine. Fig. 4b shows a similar reduction in cleavage of BSA. These data suggested that enhanced cleavage was dependent on free hydroxyl radicals produced by MNPs with peroxidase-like activity (Fig. 4c).

Therefore, it should be possible to control the kinetics or extent of cleavage by adjusting the generation of these free radical oxidants. A simple way to do this would be to change the amount of the catalyst, MNPs. We varied the amounts of MNPs (0–20 μg) while keeping the other components constant in the cleavage reaction and found that 10–20 μg of MNPs cleaved 2 μg of plasmid DNA into small pieces below 100 bp, but 5 μg of MNPs resulted in fragments between 500 bp and 1000 bp (Fig. S4†). The effects on cleavage of BSA were similar in that 5 μg of MNPs only moderately reduced the intensity of the full-length band, with increasing cleavage by 10 or 20 μg MNPs (Fig. S5†).

To this point, our data were consistent with the Fenton reaction, in which iron and H$_2$O$_2$ interact, yielding radical oxidants. We wished to determine whether cleavage was initiated from the nanoparticles, or from the supernatant which might have leached free Fe ions. To investigate this, we pre-incubated the MNPs in the NaAc buffer (0.1 M, pH 4.5, 1 h) without adding H$_2$O$_2$ or DNA/BSA. We then collected the MNPs by centrifugation, removed the supernatant, and resuspended the MNPs in the same buffer. We separately conducted cleavage reactions as above by adding H$_2$O$_2$ and plasmid DNA or BSA to both the supernatant and the resuspended MNPs. We observed significantly higher cleavage for both DNA and BSA in the tubes with the MNPs (Fig. S6a and b†), suggesting that the MNPs were primarily responsible for cleavage, which is consistent with our previous research and the findings of others under similar experimental conditions.\textsuperscript{16,17,27}

Having demonstrated an ability to enhance cleavage of the individual components of biofilms, we next sought to test the effects of the MNP–H$_2$O$_2$ system on live bacteria. When E. coli were plated, 1% H$_2$O$_2$ in 50 μl NaAc buffer (0.1 M, pH 4.5) killed 98% of the bacteria within 5 min, in the presence or absence of MNPs (Fig. S7a†). However, we observed equivalent killing when incubated with 0.01% H$_2$O$_2$ in the presence of 20 μg MNPs, confirming enhanced bacterial killing by the MNP–H$_2$O$_2$ (Fig. S7b†). This result was consistent with prior studies using MNPs for anti-bacterial properties including prevention of biofilm formation.\textsuperscript{32}

As E. coli die, they release a complex mixture of cellular components including nucleic acids and proteins. The released biomass might accumulate around resistant cells and, therefore, contribute to biofilm formation and protection from disinfectants or antimicrobial treatments. To test whether the MNP–H$_2$O$_2$ system would degrade these complex mixtures, we collected the released nucleic acids and proteins and incubated them with MNP–H$_2$O$_2$ or controls, including either MNPs or H$_2$O$_2$. Fig. 5 shows that treatment with MNP–H$_2$O$_2$ was successful at degrading these released products, thus not only efficiently killing bacteria, but also degrading the biomass released from the dead cells.

![Fig. 4](image-url) **Mechanism of MNP-enhanced oxidative cleavage.** The antioxidant, hypotaurine, inhibited cleavage of 2 μg plasmid DNA (a) or 20 μg BSA (b) by MNP–H$_2$O$_2$ in a dose-dependent fashion. "M" denotes the DNA or protein marker, respectively. "C" denotes control with DNA or BSA only. All experiments were repeated in triplicate with representative images shown. (c) Schematic of MNP-enhanced cleavage of a nucleic acid, a protein, and a polysaccharide. MNPs catalyze H$_2$O$_2$ with high efficiency, making free radicals that attack these biomolecules.

![Fig. 5](image-url) **Killing of E. coli and cleavage of released nucleic acids and proteins by the MNP–H$_2$O$_2$ system.** (a) Schematic of MNP–H$_2$O$_2$ killing of E. coli and cleavage of released nucleic acids and proteins. (b) MNP–H$_2$O$_2$ cleaved nucleic acids released from killed E. coli cells. "C" denotes control with nucleic acids only. "M" denotes the DNA marker. (c) MNP–H$_2$O$_2$ cleaved proteins released from killed E. coli cells. "M" denotes the protein marker. These experiments show that the MNP–H$_2$O$_2$ system was able to degrade the complex mixture of organic components that results from bacterial death. Penetration of organic materials would be an important characteristic of a disinfectant. All experiments were repeated in triplicate with representative images shown.
These data led us to study whether the MNP–H₂O₂ system could penetrate and eliminate biofilms, facilitating killing of resident bacteria as well as planktonic bacteria. We addressed these questions using a biofilm from *Pseudomonas aeruginosa*, a common cause of nosocomial infection and a significant cause of nosocomial infection. We next assessed the viability of bacteria resident in biofilms formed under these conditions as well as planktonic bacteria. We performed a series of experiments designed to address these questions using a biofilm system. In particular, we investigated the intrinsic peroxidase-like activity of MNPs resulting in the enhanced cleavage of nucleic acids, proteins, and polysaccharides. The new features could be used for the degradation of and penetration into the formed biofilm, as well as the killing of planktonic bacteria and prevention of biofilm formation. Enhanced oxidative cleavage by the MNP–H₂O₂ system therefore provides a promising new approach for cleaning of medical surfaces and instruments that are often resistant to disinfection because of biofilms.

**Conclusions**

The combined results of our *in vitro* assays and biofilm quantification and cell viability assays suggested that the MNP–H₂O₂ system had marked advantages over the use of H₂O₂ alone. In particular, the intrinsic peroxidase-like activity of MNPs resulted in the enhanced cleavage of nucleic acids, proteins, and polysaccharides. The new features could be used for the degradation of and penetration into the formed biofilm, as well as the killing of planktonic bacteria and prevention of biofilm formation. Enhanced oxidative cleavage by the MNP–H₂O₂ system therefore provides a promising new approach for cleaning of medical surfaces and instruments that are often resistant to disinfection because of biofilms.

**Materials and methods**

Sodium acetate (NaAc), ethanol, and Coomassie blue R-250 were purchased from Fisher Scientific. Ethylene glycol was purchased from J.T. Baker. Iron(III) chloride (FeCl₃), bovine serum albumin (BSA), hydrogen peroxide (H₂O₂) (30% stock), chitosan (low molecular weight), ethidium bromide (EB), hypotaurine, HRP, 3,3’,5,5’-tetramethylbenzidine (TMB), mouse IgG, and anti-mouse IgG were purchased from Sigma-Aldrich (St. Louis, MO, USA). Plasmid DNA, testis RNA, and lysates from *E. coli* and HEK293 cells were prepared in our laboratory in the presence of protease inhibitors (Protease Inhibitor Cocktail Tablets, Roche Applied Science) using standard approaches.

**Preparation of ferromagnetic nanoparticles with peroxidase-like activity**

Ferromagnetic nanoparticles (MNPs) were prepared and used as catalysts in these experiments. Fe₃O₄ MNPs were synthesized in one-step in a solvothermal system by combining FeCl₃ and NaAc in ethylene glycol. Briefly, 0.82 g of FeCl₃ was dissolved in 40 ml of ethylene glycol to form a clear solution. Then, 3.6 g of NaAc was added to the solution with vigorous stirring for 30 min. The mixture was then transferred to a 50 ml teflon-lined stainless-steel autoclave and left to react at 200 °C for 12 h. After the autoclave cooled to room temperature, the black precipitate was collected, rinsed several times using ethanol and then dried at 60 °C. The synthesized nanoparticles were characterized using scanning electron microscopy (SEM; Philips XL-30 field, 15 kV). The peroxidase-like activity was tested in a mixture of 500 µl NaAc buffer (0.1 M, pH 4.5) containing 20 µg MNPs, 0.3% H₂O₂ and 100 µg TMB. The blue color produced was recorded with a spectrophotometer at 652 nm.

**Cleavage of nucleic acids and proteins**

Unless indicated otherwise, nucleic acid cleavage assays were performed at 37 °C for 3 hours in 50 µl NaAc buffer (0.1 M, pH 4.5) containing 20 µg MNPs and 1–3% H₂O₂. In these and later experiments, nucleic acid cleavage products were identified by agarose gel electrophoresis and ethidium bromide staining. Protein cleavage assays were performed at 37 °C for 3 hours in 50 µl NaAc buffer (0.1 M, pH 4.5) containing 20 µg MNPs.
and 1–3% H₂O₂ prior to separation by SDS-PAGE and Coomassie staining.

Cleavage of polysaccharides

A chitosan hydrogel was prepared by glutaraldehyde cross-linking. Briefly, chitosan at 0.5% was dissolved in 500 µl NaAc (0.1 M, pH 4.5) and incubated with 0.2% glutaraldehyde at 37 °C for 1 hour. The formed hydrogel had a yellow color in a glass vial and its weight was recorded as “before cleavage”. 120 µg of MNPs or 120 µg of MNPs with 1% H₂O₂ in 300 µl NaAc (0.1 M, pH 4.5) was added to cover the gel in the glass vial and incubated at 37 °C for 1 hour. Then the supernatant was discarded and the remaining gel was rinsed 3 times with water. Exposure to filter paper for 1 hour was used to remove water and uncrosslinked chitosan, and the mass of the remaining gel was recorded as “after cleavage”. The volume of the gel remaining in the glass vial was visualized after inversion.

Hyoptaurine inhibition of cleavage with MNPs

To investigate the effect of hyoptaurine on cleavage by MNP–H₂O₂, we incubated either 2 µg plasmid DNA or 20 µg of BSA at 37 °C for 3 hours in 50 µl NaAc buffer (0.1 M, pH 4.5) containing varying concentrations of hyoptaurine (1–8%), with 20 µg MNPs and 1% H₂O₂. To investigate the relative catalytic effects of leached Fe ions versus the MNPs, we incubated 20 µg MNPs in 50 µl NaAc (0.1 M, pH 4.5) for 1 hour at 37 °C, and then collected the MNPs by centrifugation. The supernatant was mixed with 2 µg plasmid DNA or 20 µg BSA and 1% H₂O₂ and incubated for 1 hour at 37 °C. The MNPs were resuspended in 50 µl NaAc (0.1 M, pH 4.5) containing 2 µg plasmid DNA or 20 µg BSA and 1% H₂O₂ and incubated for 1 hour at 37 °C. Efficacy of cleavage was then evaluated by electrophoresis.

Killing of E. coli and cleavage of released cellular components

Killing of E. coli by the MNP–H₂O₂ system was tested using TOP10 cells [Invitrogen]. TOP10 cells were cultured in liquid LB (with ampicillin, 100 µg ml⁻¹) medium and incubated at 37 °C overnight; OD600 measured approximately 2.0. Then 20 µl of TOP10 cells were spun down and the pellet was resuspended in 50 µl NaAc (0.1 M, pH 4.5), containing 20 µg of MNPs and 1% H₂O₂. After incubation at 37 °C, the mixture was centrifuged, the supernatant was removed, and the pellet was resuspended in 1 ml LB (ampicillin, 100 µg ml⁻¹) liquid media. OD600 was measured after incubation at 37 °C for 6 hours, reflecting the population size.

To investigate the degradation of DNA and protein released from dead bacteria, E. coli cells were boiled at 100 °C. The complex mixture of organic cellular components released by and representing the killed cells was incubated in 50 µl NaAc buffer (0.1 M, pH 4.5) containing 20 µg MNPs and 1% H₂O₂. Nucleic acids were identified by agarose gel electrophoresis and ethidium bromide staining. Protein products were identified via SDS-PAGE with Coomassie staining.

Biofilm elimination

Pseudomonas aeruginosa (PA01) was cultured in biofilm minimal media (M63, Amresco) supplemented with 0.2% glucose, 0.5% casamino acids (BD), and 1 mM MgSO₄ in a 96 well plate overnight at 37 °C. The wells were rinsed with ddH₂O and dried. Wells were then challenged with minimal media only (positive control), minimal media + MNPs (20 µg/50 µl), minimal media supplemented with 1% H₂O₂, or minimal media supplemented with 1% H₂O₂ + MNPs. Challenged plates were then incubated for 2 hours at 37 °C with periodic mixing. For the biofilm quantitation assay, the challenged wells were rinsed to remove any remaining planktonic cells and stained with 0.1% crystal violet for 15 minutes. Plates were rinsed to remove the stain and dried. Wells were imaged to visualize biofilm rings that were formed at the air/liquid interface. Subsequently, 10% acetic acid was used to solubilize the dye and then the acetic acid/CV mixture was quantitated using a UV spectrophotometer at 600 nm. The amount of CV present is directly proportional to the number of cells in the biofilm ring. For the viability assay in the biofilm, challenged wells were rinsed to eliminate any remaining planktonic cells. A sterile cotton swab was used to remove cells resident to the biofilm from the well and was subsequently used to inoculate 500 µl of M63 salts. Dilutions were made and 100 µl aliquots of each dilution were plated onto LB agar plates, and incubated overnight at 37 °C. Colonies were counted and the number of CFUs was compared for each sample.

Statistics

All experiments were performed in triplicate and data were analyzed using the paired Student’s t-test. When numerical data are presented as bar graphs, lines denote standard deviations. Additional details on methods can be found in the ESI.

Author contributions

L. G., K. M. G., H. S., and A. J. T. conceived of experiments. L. G. and K. M. G. performed the experiments. J. L. N. extracted and purified genomic DNA and RNA. L. G., J. L. N., K. M. G., H. S. and A. J. T. wrote and revised the manuscript.

Notes

The authors declare no competing financial interest.

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Notes and references