Exploring multiple effects of $\text{Zn}_{0.15}\text{Mg}_{0.85}\text{O}$ nanoparticles on *Bacillus subtilis* and macrophages

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The increasing number of multidrug resistant bacteria raises a serious public-health concern, which is exacerbated by the lack of new antibiotics. Metal oxide nanoparticles are already applied as an antibacterial additive in various products used in everyday life but their modes of action have remained unclear. Moreover, their potential negative effects to human health are still under evaluation. We explored effects of mixed metal oxide $\text{Zn}_{0.15}\text{Mg}_{0.85}\text{O}$ on *Bacillus subtilis*, as a model bacterial organism, and on murine macrophages. $\text{Zn}_{0.15}\text{Mg}_{0.85}\text{O}$ killed planktonic bacterial cells and prevented biofilm formation by causing membrane damages, oxidative stress and metal ions release. When exposed to a sub-inhibitory amount of $\text{Zn}_{0.15}\text{Mg}_{0.85}\text{O}$, *B. subtilis* up-regulates proteins involved in metal ions export, oxidative stress response and maintain of redox homeostasis. Moreover, expression profiles of proteins associated with information processing, metabolism, cell envelope and cell division were prominently changed. Multimode of action of $\text{Zn}_{0.15}\text{Mg}_{0.85}\text{O}$ suggests that no single strategy may provide bacterial resistance. Macrophages tolerated $\text{Zn}_{0.15}\text{Mg}_{0.85}\text{O}$ to some extend by both the primary phagocytosis of nanoparticles and the secondary phagocytosis of damaged cells. Bacterial co-treatment with ciprofloxacin and non-toxic amount of $\text{Zn}_{0.15}\text{Mg}_{0.85}\text{O}$ increased antibiotic activity towards *B. subtilis* and *E. coli*.

Providing an efficient and safe treatment for bacterial multi-drug resistant strains is a major health challenge worldwide. Some bacterial strains have the potential to adhere on any surfaces and form slimy layer known as a biofilm. The formation of biofilms enhances the bacterial resistance to current treatments by slowing penetration of the antibiotic into the biofilm, altering chemical microenvironment of bacterial cells and by enabling cell differentiation similar to spore formation. There is an urgent need to develop novel pharmacological approaches to fight multidrug-resistance pathogenic bacteria and to destroy or prevent their biofilm formation or sporulation. Metal oxide nanoparticles (NP), such as ZnO, CuO and TiO$_2$, have already been proven as a good candidate to fight various bacteria. However, their therapeutic applications as antibacterial agents are still limited as these metal oxides at nanoscale may exhibit high cytotoxicity on mammalian cells. Thus, new insights into the complex tri-part interactions, bacterial cells-metal oxide nanoparticles-mammalian cells, are required to rationally design novel biocompatible antibacterial agents. We hypothesis here that mixed metal oxide NPs with synergic effects of two oxides may provide a new solution for an infectious disease treatment.

MgO NP is a commonly used model system for studying surface reactions at nanoscale, mainly due to its simple rock-salt crystal structure and purely inorganic nature. Unlike other NPs with antibacterial activity, such as colloidal silver NPs, which release cytotoxic Ag$^+$ ions, or photocatalytic nanoparticles that demand intense irradiation to be efficient, nanostructured MgO are low cost, easy to manipulate and show intrinsic biocompatibility. Two strategies have been proposed to improve the antibacterial activity of MgO NPs. First, antibacterial efficiency of MgO can be significantly enhanced by decreasing the size of MgO nanocubes to less than 10 nm.

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The lowering the particles' size facilitates NP penetration into bacterial cells and biofilms and results in high crystal surface area which enhances particles' reactivity. Second, the admixing of another cation into the MgO NPs may significantly improve particle's antibacterial efficiency. Doping MgO with Ca, Zn or Li was shown to modify MgO morphology, surface structure and physicochemical properties. For instance, while MgO nanocubes only partially inhibited bacterial growth, mixed biphase ZnMgO NPs, of a similar size, showed a high antibacterial efficiency.

Herein, we used small monophasic MgO NPs decorated at edges with Zn (15 at. %), called Zn$_{0.15}$Mg$_{0.85}$O. Zn$_{0.15}$Mg$_{0.85}$O were regular cubic NPs of a narrow particle size and shape distribution with a high surface specific area and a large number of low-coordinated ions and/or surface defects. In consequence, Zn$_{0.15}$Mg$_{0.85}$O NPs are expected to show enhanced surface reactivity and, thus, a high antibacterial efficiency.

*Bacillus subtilis* was used as a model bacterial specie to evaluate Zn$_{0.15}$Mg$_{0.85}$O NP effects on planktonic bacterial cells, biofilms and spores. In the depth, the NP size, interfacial potential and ROS production in solutions, antibacterial efficiency as well as effects on cellular morphology and cytotoxicity were all considered. The viability and NP intake by mammalian cells were studied in macrophage cells exposed to Zn$_{0.15}$Mg$_{0.85}$O at various concentrations. Macrophages were chosen as they act scavenger’s role to eliminate pathogens and harmful particles from the body. Taking together, we expect that this study sheds light on molecular mechanisms involved in metal oxide interactions with bacteria and immunological cells and reveals potentials for improving design of doped metal oxide NPs for biomedical applications.

## Results

**Zn$_{0.15}$Mg$_{0.85}$O nanoparticles characterization in solution.** Zn$_{0.15}$Mg$_{0.85}$O were produced as monophasic nanocubes with a 4 nm average size (Fig. S-1). The low Zn/Mg ratio was chosen to assure single crystal structure of doped MgO nanocubes and to prevent enhanced cytotoxicity. Indeed, ZnO nanomaterials are instable in aqueous solutions and have tendency to release highly toxic Zn$^{2+}$ ions.

Zn$_{0.15}$Mg$_{0.85}$O NPs tended to aggregate and precipitate in water. To check whether the solubility of Zn$_{0.15}$Mg$_{0.85}$O NPs increased when ions, detergent or proteins were added to the water, DLS analysis was performed to measure the mean hydrodynamic radius (R$_H$) of NPs in aqueous solutions (Fig. 1A). The R$_H$ value of Zn$_{0.15}$Mg$_{0.85}$O were found to shift from about 1 µm in water to 0.1 µm in PBS containing 150 mM NaCl. Such sizes were much higher than the primary nanoparticle size indicating aggregation of particles in both water and PBS. However, the possibility that NPs were dissolved to some extend cannot be roll out. DLS cannot detect small sizes were much higher than the primary nanoparticle size indicating aggregation of particles in both water and PBS.

To verify whether interfacial potential of Zn$_{0.15}$Mg$_{0.85}$O NPs differed in various solutions, zeta potential of Zn$_{0.15}$Mg$_{0.85}$O were examined against pure H$_2$O and (0.33 ± 0.01) μM H$_2$O$_2$ was detected in LB medium. The concentration of O$_2$ in all solutions tested increased with increasing Zn$_{0.15}$Mg$_{0.85}$O concentration and reached saturation at 1 mg/mL (Fig. 1B). To check whether Zn$_{0.15}$Mg$_{0.85}$O NPs can generate ROS, the XTT assay was performed (Fig. 1F). The concentration of H$_2$O$_2$ in solutions was calculated using a calibration curve obtained with pure H$_2$O$_2$. Upon 5h incubation, 0.5 mg/mL of Zn$_{0.15}$Mg$_{0.85}$O generated O$_2$ in all solutions tested while no absorption peak was observed in the absence of NPs (Fig. 1D). The highest concentration of O$_2$ was detected in LB medium. The concentration of O$_2$ increased with increasing Zn$_{0.15}$Mg$_{0.85}$O concentration and reached saturation at 1 mg/mL (Fig. 1F).

To verify whether Zn$_{0.15}$Mg$_{0.85}$O NPs were dissolved in water solution containing BSA, Tween20 or NP40 indicating that protein and non-ionic detergents adsorb on metal oxide NPs and stabilize them in solutions. Two main populations of NP aggregates with R$_H$ of about 50 and 500 nm were found for Zn$_{0.15}$Mg$_{0.85}$O admixed with LB bacterial medium, while only a single peak with R$_H$ ~10 nm was observed for Zn$_{0.15}$Mg$_{0.85}$O added to mammalian cell culture medium (MEM, completed with 10% serum). The dissolution of Zn$_{0.15}$Mg$_{0.85}$O NPs in MEM was probably enhanced by BSA from serum.

**Zn$_{0.15}$Mg$_{0.85}$O nanoparticles generate ROS.** Antibacterial activity of metal oxide NPs is thought to be mediated by reactive oxygen species (ROS). To verify whether Zn$_{0.15}$Mg$_{0.85}$O produced O$_2$ in aqueous solutions, the XTT assay was performed. When reduced by O$_2$, XTT forms water soluble XTT-formazan which absorbs light at 470 nm. Figure 1C shows the increase in XTT absorption peak intensity obtained in various solu-

**Antibacterial activity of Zn$_{0.15}$Mg$_{0.85}$O on *B. subtilis* planktonic cells.** The antibacterial activity of Zn$_{0.15}$Mg$_{0.85}$O was examined against *B. subtilis*, a representative of Gram (+) bacteria. Growth curves of *B. subtilis* exposed to 0.1 or 1 mg/mL of NPs were determined by monitoring the optical density (OD) at 600 nm over time. We observed that growth of *B. subtilis* decreased significantly with the increasing concentration of NPs (Fig. 2A). Cell viability evaluated by the colony counting method was about 100-fold reduced as compared to the initial concentration of cells (10$^6$ CFU/ml) upon incubation with 1 mg/mL Zn$_{0.15}$Mg$_{0.85}$O NPs after 30 min (Fig. 2B). The minimum inhibitory concentration (MIC) value of Zn$_{0.15}$Mg$_{0.85}$O NPs against *B. subtilis* evaluated by broth microdilution method with 10$^4$ cfu/mL was 450 mg/L. In comparison, the MIC of Zn$_{0.15}$Mg$_{0.85}$O NPs against *E. coli* was 710 mg/L (see Supplementary information S-2 for details).

To visualize bacterial morphology upon treatment with Zn$_{0.15}$Mg$_{0.85}$O, TEM measurements were performed on tin cross-section of *B. subtilis* cells incubated with Zn$_{0.15}$Mg$_{0.85}$O over only 60 min. As depicted in Fig. 2C
B. subtilis cultured in the absence of NPs showed intact rod-shaped and round-shaped cells protected with smooth and well-structured membrane layer. All cells were viable and no membrane damage could be observed. After exposing to sub-inhibitory concentration of Zn$_{0.15}$Mg$_{0.85}$O (0.1 mg/mL) bacterial cells still maintained their integrity. However, upon exposure to 0.5 mg/mL Zn$_{0.15}$Mg$_{0.85}$O NPs the attachment of nanoparticles to the bacteria was observed (Fig. 3C). The shape of bacterial cells changed to more irregular suggesting that Zn$_{0.15}$Mg$_{0.85}$O NPs injured the membrane of B. subtilis. Membrane damage further caused membrane leakage and increased cell permeability leading ultimately to bacterial death, as illustrated in Fig. 3C.

Figure 1. Characterization of Zn$_{0.15}$Mg$_{0.85}$O in solutions. (A) DLS measurements showing size distributions of 0.1 mg/mL Zn$_{0.15}$Mg$_{0.85}$O in pure water, bacterial LB medium, mammalian cell medium (MEM), in PBS, pH 7.4, and PBS containing 0.05% NP40, 0.05% Tween-20 or 2 mg/mL BSA. All solutions were incubated for 24 h at room temperature before measurements. (B) Zeta–potential analysis of Zn$_{0.15}$Mg$_{0.85}$O NPs in different solutions used in this work; (C) O$_2$•$^-$ generation of Zn$_{0.15}$Mg$_{0.85}$O NPs (0.1 mg/mL, initial concentration) in various solutions, at 25 °C obtained by measuring reduction of 200 µM XTT; (D) No reduction of 200 µM XTT was observed in control experiments without NPs. (E) Generation of O$_2$•$^-$ by different concentrations of NPs in PBS; (F) Production of H$_2$O$_2$ by Zn$_{0.15}$Mg$_{0.85}$O NPs in PBS and LB obtained by the Amplex red assay.
Impact of Zn\textsubscript{0.15}Mg\textsubscript{0.85}O on B. subtilis proteome. To understand the physiological state of B. subtilis cells upon exposition to Zn\textsubscript{0.15}Mg\textsubscript{0.85}O, a comparative proteomic analysis of the membrane fractions of untreated and treated bacteria was performed. To preserve cell integrity, the bacterial culture were treated with low concentration of NPs, 0.05 mg/mL or 1 mg/mL Zn\textsubscript{0.15}Mg\textsubscript{0.85}O NPs in LB, as quantified by the colony counting method. (C) TEM cross-section observations of untreated B. subtilis (mock) or treated with 0.1 mg/mL or 0.5 mg/mL Zn\textsubscript{0.15}Mg\textsubscript{0.85}O NPs.

**Figure 2.** Antimicrobial effects of Zn\textsubscript{0.15}Mg\textsubscript{0.85}O NPs. (A) Growth curves of B. subtilis in LB medium in the absence (mock) and in the presence of 0.1 mg/mL or 1 mg/mL of Zn\textsubscript{0.15}Mg\textsubscript{0.85}O nanoparticles. (B) B. subtilis cell viability obtained upon bacterial cell incubation with 0.1 mg/mL or 1 mg/mL Zn\textsubscript{0.15}Mg\textsubscript{0.85}O NPs in LB, as quantified by the colony counting method. (C) TEM cross-section observations of untreated B. subtilis (mock) or treated with 0.1 mg/mL or 0.5 mg/mL Zn\textsubscript{0.15}Mg\textsubscript{0.85}O NPs.
AhpC or bacilliredoxins BrxA and BrxB, which perform redox switch in response to oxidative stress. By contrast, we observed down-regulation of the mini-ferritins Dps and MrgA, which act as internal iron metal chelators. Proteomic analysis additionally revealed a wide and prominent effect of Zn$_{0.15}$Mg$_{0.85}$O NPs on proteins associated with information processing, metabolism, cell envelope dynamics or cell division (Fig. 3C; Table 2).

Zn$_{0.15}$Mg$_{0.85}$O affects B. subtilis biofilm formation. The ability to form biofilms is an important characteristic of many environmental bacteria, including B. subtilis. The specific structure of biofilms increases bacteria resistant to antibiotics and chemicals. The B. subtilis 168 strain and its derivatives show a reduced biofilm forming ability because of mutations accumulated during laboratory propagation 26. For this reason, the direct effect of Zn$_{0.15}$Mg$_{0.85}$O NPs on biofilm formation was tested on the undomesticated strain NDmed 27. To assess the time course of biofilm formation in the presence of NPs, microtiter plates were inoculated with NDmed strain. After 24 h of incubation, biofilms were stained with crystal violet, which stains bacterial cells and biofilm matrix components 28 (Fig. 4A). Measurable amounts of biofilm were detected when NDmed was cultivated in the absence of NPs in both LB and biofilm-promoting MSgg medium (Fig. 4). In the presence of non-lethal concentration of Zn$_{0.15}$Mg$_{0.85}$O NPs (0.1 mg/mL), the biofilm was 2.7- and 10.4-fold reduced in LB and MSgg, respectively (Fig. 4A and B). Since B. subtilis forms a floating film 29,30, the detached films might not be quantified with the crystal violet staining. We, thus, compared the dynamics of pellicle formation of NDmed biofilm in MSgg medium while in the presence of Zn$_{0.15}$Mg$_{0.85}$O NPs (Fig. 4C). It appears thus that Zn$_{0.15}$Mg$_{0.85}$O NPs have a drastic inhibitory effect on the formation of biofilms by B. subtilis.

Figure 3. Comparative proteome analysis of B. subtilis membrane fractions of untreated bacterial cells and cells treated with 0.05 mg/mL Zn$_{0.15}$Mg$_{0.85}$O NPs in LB for 1 h. (A) Statistical analysis indicated that 62 proteins had modified abundances in treated and untreated bacteria. Two modes of label free quantification were used: spectral counting (SC) and eXtracted Ion Current ion chromatograms of each peptide (XIC). (B) Representation of the proteome response of B. subtilis to Zn$_{0.15}$Mg$_{0.85}$O NPs. The pie chart shows the size of functional categories according to the SubiWiki database. (C) Heatmap presentations of upregulated and downregulated proteins. Left panel, proteins quantified by XIC statistic method; right panel, proteins quantified by SC. Only proteins showing significant abundance change between untreated (mock) and treated bacteria (ANOVA, adjusted p value < 0.05) are displayed.
Table 1. Proteins up- or down-regulated in *B. subtilis* cells treated with Zn$_{0.15}$Mg$_{0.85}$O.

$$\text{Zn}_{0.15}\text{Mg}_{0.85}\text{O did not effectively inactivate } B.\text{ subtilis spores. }$$ We also examined whether Zn$_{0.15}$Mg$_{0.85}$O NPs destroy *B. subtilis* spores. Comparing, the viability of *B. subtilis* spores pre-incubated with 1 or 5 mg/mL NPs for 1 to 24 h to that of untreated spores by plate count experiments indicated that Zn$_{0.15}$Mg$_{0.85}$O had no effect on viability of *B. subtilis* spores. Considering the complex structure of spores, which are encased in a thick multilayered coat surrounded by the exosporium, their resistance to Zn$_{0.15}$Mg$_{0.85}$O NPs was expected.

**Zn$_{0.15}$Mg$_{0.85}$O cytotoxicity towards macrophages.** To apply a manufactured NP as an efficient and safe agent for antibacterial applications, it should specifically kill pathogenic bacteria without being toxic to mammalian cells. To test cytotoxicity of Zn$_{0.15}$Mg$_{0.85}$O NPs we explored their effects on the RAW 264.7 macrophage cell line. The MTT test measures the cellular reduction of the tetrazolium dye MTT as an indicator of the cell viability$^{31}$. No significant reduction in cell viability was observed in macrophages treated with 0.025–0.25 mg/mL Zn$_{0.15}$Mg$_{0.85}$O NPs (Fig. 5A). In contrast, MTT reduction dropped drastically to an average of 10% to that of control cells after an exposure to 1 mg/mL Zn$_{0.15}$Mg$_{0.85}$O NPs (Fig. 5A). These data suggest that Zn$_{0.15}$Mg$_{0.85}$O NPs are highly cytotoxic at concentrations $\geq$ 1 mg/mL.

To verify whether Zn$_{0.15}$Mg$_{0.85}$O NPs damage cell membrane in macrophages, a FACS analysis was performed on treated cells stained with acridine orange (Fig. 5B). Macrophages were harvested and analyzed for necrosis after their incubation with Zn$_{0.15}$Mg$_{0.85}$O NPs. Acridine orange dye easily enters the cell membrane and accumulates in lysosomes. During necrosis lysosomes are ruptured due to the loss of the membrane integrity decreasing the dye fluorescence$^{31}$. A shown in Fig. 5B no reduction of acridine orange derived fluorescence intensity was observed in cells treated with 0.1 mg/mL Zn$_{0.15}$Mg$_{0.85}$O NPs. However, 1 mg/mL Zn$_{0.15}$Mg$_{0.85}$O NPs induced 100% of cell mortality of treated macrophages. The quantitative membrane damage FACS analysis is, therefore, consistent with the cell viability test obtain with MTT assay.

TEM analysis was done on thin sections of treated macrophages to analyze effects of Zn$_{0.15}$Mg$_{0.85}$O on cellular and subcellular morphology. Untreated macrophages served as control (Fig. 5C). Ultrastructural analysis of macrophages incubated with a non-toxic dose of NP (0.1 mg/mL) revealed that cellular and organelle architecture of most treated cells changed (Fig. 5D). The electron dense areas were observed within cells suggesting that Zn$_{0.15}$Mg$_{0.85}$O NPs were internalized. The localization of NPs inside cells was rather dispersed showing a different degree of aggregation. Typically, electron dense aggregates were in a vicinity of membrane-rich regions. Some treated macrophages displayed features of cell death: loss of cell membrane specialization like pseudopodia, scroll-like arrangement of a lipid bilayer called myelin bodies, ballooning degeneration, swelling of mitochondria, shrunken or fragmented nucleus. Those dead cells were often in contact to pseudopodia of neighbor healthy macrophages. This suggests that dead cells were phagocytosed. The healthy macrophages also displayed intracytoplasmic vacuoles with debris suggesting phagocytosis of extracellular debris or autophagocytosis. Autophagy...
is a major mechanism by which macrophages eliminate intracellular pathogens or noxious particles. Together, cytotoxic and structural observations indicate that Zn$_{0.15}$Mg$_{0.85}$O at a nontoxic dose induced death of some macrophages that are secondary phagocytyzed by the remaining healthy ones.

Zn$_{0.15}$Mg$_{0.85}$O potentiates the activity of ciprofloxacin. We finally examined whether Zn$_{0.15}$Mg$_{0.85}$O has an additive effect when applied together with ciprofloxacin. Ciprofloxacin is a fluoroquinolone antibiotic, widely used for human and livestock treatments because of its broad-spectrum activity against both Gram (+) and Gram (−) bacteria. The contributing effect of Zn$_{0.15}$Mg$_{0.85}$O was tested against B. subtilis and E. coli by disk diffusion method. As shown in Fig. 6, sub-inhibitory levels of Zn$_{0.15}$Mg$_{0.85}$O (0.32 µg to 10 µg/disk) increased the zone of inhibition by ciprofloxacin against both bacterial strains. This implies that Zn$_{0.15}$Mg$_{0.85}$O has a

| Protein name | Fold change ZnMgO vs control | Gene name | Product and Function |
|--------------|-------------------------------|-----------|---------------------|
| LTLD         | 0.62                          | lytD      | Peptidoglycan N-acetylglucosaminidase, major autolysin, cell separation |
| PGDS         | 1/4                           | pgdS      | Gamma-DL-glutamyl hydrolase, polyglutamic acid degradation |
| SEPF         | 1.58                          | sepf      | Part of the divisome, recruits FtsZ to the membrane |
| SP5G         | 1.63                          | spoVG     | RNA-binding regulatory protein, negative effector of asymmetric septation at the onset of sporulation |

### Metabolism

| Protein name | Fold change ZnMgO vs control | Gene name | Product and Function |
|--------------|-------------------------------|-----------|---------------------|
| ATPE         | 2.24                          | atpE      | ATP synthase (subunit c) |
| BGLH         | 0.45                          | bglH      | Phospho-beta-glucosidase, salicin utilization |
| GC5H         | 2/0                           | gc5H      | Glycine cleavage system H protein for lipoyc acid biosynthesis |
| KAD          | 2.03                          | adk       | Adenylate kinase, ADP formation |
| ODIP         | 1.58                          | pdlC      | Pyruvate dehydrogenase, links glycolysis and TCA cycle |
| PTVJ3B       | 0.32                          | bglP      | Beta-glucoside uptake and phosphorylation, control of LicT activity |
| THIO         | 1.72                          | thiO      | FAD-dependent glycine oxidase, biosynthesis of thiamine |
| TP15         | 3.33                          | tpiA      | Triose phosphate isomerase, glycolytic/ gluconeogenic enzyme |

### Information processing

| Protein name | Fold change ZnMgO vs control | Gene name | Product and Function |
|--------------|-------------------------------|-----------|---------------------|
| ABRB         | 1.71                          | abrB      | Transcriptional regulator of transition state genes |
| PP1B         | 4.13                          | pp1B      | Peptidyl-prolyl cis-trans isomerase (protein folding) |
| PRPC         | 1.85                          | prpC      | Protein phosphatase (protein modification) |
| RPOZ         | 3/0                           | rpoZ      | Omega subunit of RNA polymerase |
| YUKB         | 7/2                           | yukB      | Membrane FtsK/SpoIIIE-like ATPase |
| CTC          | 3.09                          | ctc       | Similar to ribosomal protein L25 |
| IF3          | 1.92                          | infoC     | Translation initiation factor IF-3 |
| RL6          | 2.08                          | rplF      | 50S ribosomal protein L6 |
| RL9          | 1.87                          | rplI      | 50S ribosomal protein L9 |
| RL13         | 1.77                          | rplM      | 50S ribosomal protein L13 |
| RL18         | 1.58                          | rplR      | 50S ribosomal protein L18 |
| RS11         | 3.02                          | rplK      | 30S ribosomal protein S11 |
| RS9          | 2.35                          | rplI      | 30S ribosomal protein S9 |
| RS12         | 2.00                          | rplL      | 30S ribosomal protein S12 |
| RS15         | 3.19                          | rplO      | 30S ribosomal protein S15 |
| RS18         | 2.63                          | rplR      | 30S ribosomal protein S18 |
| RS19         | 2.95                          | rplS      | 30S ribosomal protein S19 |

### Proteins of unknown function

| Protein name | Fold change ZnMgO vs control | Gene name | Product and Function |
|--------------|-------------------------------|-----------|---------------------|
| YUTI         | 2.56                          | yutI      | Putative iron-sulfur scaffold protein |
| TTSP         | 2.57                          | ytsP      | Unknown |
| YRPD         | 0.46                          | yrpD      | Unknown |
| YUAE         | 1.7                           | yuaE      | Unknown |
| YXNE         | 2.11                          | xenE      | Unknown |
| YXXC         | 0.64                          | yxkC      | Unknown |
| YTKA         | 1.49                          | ytkA      | Unknown |
| YHHA         | 1.61                          | yhaA      | Unknown |
| YSDC         | 0.49                          | yudC      | Similar to endo-1.4-beta-glucanase |
| YTTP         | 0.58                          | ytoP      | Similar to glutamyl aminopeptidase |
| YERC         | 1.73                          | yerC      | Unknown |
| YNCG         | 0.65                          | yncM      | Unknown |

Table 2. Proteins up- or down-regulated in B. subtilis cells treated with Zn$_{0.15}$Mg$_{0.85}$O.
potentiation effect on ciprofloxacin activity. However, Zn$_{0.15}$Mg$_{0.85}$O (up to 10 $\mu$g/disk) had no effect on the activity of penicillin and vancomycin towards 
B. subtilis and E. coli.

**Discussion**

The innovative nanomaterials that kill multidrug-resistant bacteria and disturb antibiotic resistant biofilm are needed for industry, agriculture and healthcare. Here, we showed that (i) Zn$_{0.15}$Mg$_{0.85}$O killed planktonic B. subtilis cells (MIC 450 mg/L), and E. coli cells (MIC 710 mg/L), (ii) sub-inhibitory concentration of Zn$_{0.15}$Mg$_{0.85}$O (100 mg/L) prevented biofilm formation by NDmed strain, (iii) sub-inhibitory concentration of Zn$_{0.15}$Mg$_{0.85}$O (50 mg/L) modified expression of 62 membrane proteins in B. subtilis and (iv) sub-inhibitory amounts of Zn$_{0.15}$Mg$_{0.85}$O potentiated antibacterial efficiency of ciprofloxacin towards B. subtilis and E. coli in a dose-response manner.

Zn$_{0.15}$Mg$_{0.85}$O NPs aggregated in water and PBS but tended to dissolve in biological media when coated with proteins and non-ionic surfactants. MgO NPs were shown to mainly produce ROS when their electrons localized in crystal structure defects and holes, that have high oxidation and reduction energies, reduce molecular oxygen to superoxide ion (O$_2^{-}$). Subsequently, O$_2^{-}$ can become a precursor of highly cytotoxic species as hydroxyl radicals (‘OH) or singlet oxygen (1O$_2$). In contrast, ZnO NPs were shown to generate mainly H$_2$O$_2$ and OH$^*$. Hydrogen peroxide is usually generated upon water oxidation by photo-generated holes forming hydroxyl radicals. Interestingly, we observed that Zn$_{0.15}$Mg$_{0.85}$O NPs produced both O$_2^{-}$ and H$_2$O$_2$ when admixed in aqueous solutions. The production of both ROSs increased with increasing NP concentration but saturated at 1 mg/mL Zn$_{0.15}$Mg$_{0.85}$O. Likely, NPs aggregated at high concentrations, which reduced their surface reactivity, and thus the production of ROS.

Oxidative stress in bacteria induced by ROS is considered to play a key role in molecular mechanism of metal oxide NP antibacterial activity. Our proteomic data highlight that ROS generation in combination with Zn$^{2+}$- and Mg$^{2+}$-ion release from Zn$_{0.15}$Mg$_{0.85}$O NPs triggered a broad oxidative stress response (Table 1). For instance, Spx and YraA proteins related to thiol oxidative stress, which interferes with zinc metabolism$^{35}$ were up-regulated. Synthesis of both proteins are under the control of the regulator CzrA, which is an indicator of Zn-ions excess. This response suggests an intracellular dissolution of up-taken Zn$_{0.15}$Mg$_{0.85}$O. Remarkably, upregulated GcsH is involved in lipoic acid biosynthesis but also acts as an antioxidant and free-radical scavenger$^{36}$. The exposure to

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**Figure 4.** Effect of Zn$_{0.15}$Mg$_{0.85}$O NPs on biofilm formation by NDmed B. subtilis in LB and MSgg medium (A,B). Bacterial cells were incubated in the absence of NPs and in the presence of 0.1 mg/mL Zn$_{0.15}$Mg$_{0.85}$O. Microtiter plates were stained with crystal violet after incubation at 30 °C without agitation for 24 h. Quantification of biofilm density was obtained by measuring OD at 595 nm of solubilized crystal violet formed in microtiter plate assay. The error bars represent mean ± SD from at least four independent experiments. (C) Pellicle biofilm formation by NDmed strain in MSgg medium alone and with added Zn$_{0.15}$Mg$_{0.85}$O NPs at 0.1 mg/mL and 0.5 mg/mL.
NPs induced other stress response-related proteins, such as RsbW, which is involved in control of the general stress sigma factor SigB activity, and DnaK, a chaperone protein activated in response to heat shock. Several of up-regulated proteins have a function related to cation-dependent cellular processes. For instance, the phosphatase PrpC requires divalent metal cations such as Mg$^{2+}$ or Mn$^{2+}$ to be active. The SepF protein is a part of the divisome and recruits FtsZ to the membrane. It has been shown that Mg$^{2+}$ impact cell division of bacilli due to its involvement in FtsZ assembly$^{37}$. 

In addition, *B. subtilis* recruited proteins that participate in translation and transcription cellular processes that depend on Zn and Mg availability (Table 2). The levels of at least 10 ribosomal proteins were affected by the NPs. In *B. subtilis*, composition of ribosomal sub-units can be modified in response to zinc availability$^{38}$. Moreover, up-regulated RpoZ protein is part of the RNA polymerase. Structure and assembly of RNA polymerase multisubunits require Zn$^{2+}$ while its catalytic activity is assisted by Mg$^{2+}$$^{39}$. Similarly, an addition of zinc markedly increased yields of active RNA polymerase in *Escherichia coli$^{40}$. Zn$_{0.15}$Mg$_{0.85}$O also impacted metabolic pathways. Both up-regulated proteins BglH and BglP (also named Ptv3b, Table 2) are involved in the specific carbon source utilization. In *Streptococcus pyogenes*, shifts in metabolic pathways occurred in response to zinc excess$^{41}$. This further suggests that Zn$_{0.15}$Mg$_{0.85}$O NPs disrupt *B. subtilis* zinc homeostasis leading to central carbon metabolism adjustments. Together proteomic findings indicate that *B. subtilis* activated multiple mechanisms to recompense for the damage caused by Zn$_{0.15}$Mg$_{0.85}$O NPs to the cell physiology.

Proteomic data, thus, strongly suggest difficulties for bacteria to become resistant to Zn$_{0.15}$Mg$_{0.85}$O NPs, which makes Zn$_{0.15}$Mg$_{0.85}$O a promising antibacterial agent. Indeed, the multiple simultaneous mechanisms of action against bacterial cells would require multiple simultaneous gene mutations to make them resistant. Recently was shown that Gram (-) bacteria might develop resistance to silver NPs after repeated exposures$^{42}$. The mechanism

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**Figure 5.** Cytotoxic effect of Zn$_{0.15}$Mg$_{0.85}$O NPs on macrophage cells. (A) MTT reduction in macrophages incubated with Zn$_{0.15}$Mg$_{0.85}$O NPs at various concentrations overnight. The % of MTT reduction relative to that of control cells incubated with PBS is plotted. The error bars represent SD of the means over total of 8 replicates, ** correspond to P-value < 0.01, and ***P < 0.001. (B) Viability of macrophages incubated with 0.1 mg/mL or 1 mg/mL Zn$_{0.15}$Mg$_{0.85}$O NPs overnight was estimated by acridine orange staining and flow cytometry analysis. Note that there was no significant difference in acridine orange fluorescence between untreated (mock) and cells treated with 0.1 mg/mL Zn$_{0.15}$Mg$_{0.85}$O. (C) Representative thin section electron micrographs of untreated macrophage cells and (D) macrophages cells incubated with 0.1 mg/mL Zn$_{0.15}$Mg$_{0.85}$O for 24h. m, mitochondria; er, endoplasmic reticulum; Lys, lysosome; S-m, swelling mitochondria.
seems to involve the production of the adhesive flagellum protein flagellin, which binds and extracellularly aggregates NPs. We also observed the accumulation of NPs at the external surface of \( B. \) subtilis (Fig. 2C). Since \( \text{Zn}_{0.15}\text{Mg}_{0.85} \) NPs were of negative surface potential, their accumulation on the negatively charged bacterial surface suggests that \( B. \) subtilis made efforts to sequestrate extracellularly NPs, probably to prevent their entry into the cell.

Macrophages are a canonical model of immune-competent cells that are likely to afford the first-line of defense responsible for clearing, processing and degrading foreign materials from circulation. As expected, macrophages phagocytized \( \text{Zn}_{0.15}\text{Mg}_{0.85} \) NPs. Upon 24 h of incubation with macrophages, NPs were observed segregated into membrane rich region or dispersed within electron dense area. Such localization suggests that macrophages preceded their transformation as previously observed with \( \text{Fe}_3\text{O}_4 \) NPs43. Interestingly, many treated macrophage cells that showed loss of pseudopodia, swelling mitochondria or fragmented nucleus where linked to pseudopodia of neighbor healthy macrophages suggesting that damaged cells were eliminated by the secondary phagocytosis. However, increasing concentration of \( \text{Zn}_{0.15}\text{Mg}_{0.85} \) NPs to 1000 mg/L impeded biodegradation mechanism and led to macrophage death.

We show that sub-inhibitory amounts of \( \text{Zn}_{0.15}\text{Mg}_{0.85} \) applied with ciprofloxacin had higher antibacterial efficiency compared to ciprofloxacin alone towards \( E. \) coli and \( B. \) subtilis. This finding suggests a synergistic bacterial killing that may result from the additive bactericidal activity of ROS generated by \( \text{Zn}_{0.15}\text{Mg}_{0.85} \) NPs with that of ciprofloxacin, which inhibits bacterial DNA gyrase and cell division. Our proteomics data suggest that \( \text{Zn}_{0.15}\text{Mg}_{0.85} \) affected bacterial physiological state, which may also increase bacterial susceptibility to antibiotics. Previously was shown that nano-\( \text{ZnO} \) enhanced activity of ciprofloxacin and ceftazidime against \( A. \) baumannii by modifying bacterial morphology from rod to cocci forms and by inducing bacterial filamentation44. Similarly, we observed that \( \text{Zn}_{0.15}\text{Mg}_{0.85} \) NPs modified bacterial morphology and damaged cell membrane. The increased permeability of bacterial cell membrane facilitates ciprofloxacin uptake, which is expected to enhance its efficiency. Nevertheless, metal oxide NPs as well as divalent metal ions were reported to complex antibiotics and improve their antibacterial affinity45–47. For instance, protonated nitrogen atoms of ciprofloxacin quinolone ring may directly bind hydroxylated \( \text{Zn}_{0.15}\text{Mg}_{0.85} \) NPs by ionic bonds as evidenced for some divalent metal ions by spectroscopic and X-ray analyses48. In addition, the oxygen from the carbonyl group of the ciprofloxacin ring was shown to bind \( \text{Mg}^{2+} \)-ions forming stable complexes49. Such interactions between ciprofloxacin and divalent metal ions were shown to facilitate ciprofloxacin interaction with bacterial DNA50. Moreover, the efficiency of the \( \text{Zn}^{2+} \)-ciprofloxacin complex was shown to be additionally increased by addition of \( \text{H}_2\text{O}_2 \). To elucidate the exact mechanism of \( \text{Zn}_{0.15}\text{Mg}_{0.85} \) enhancing effect on ciprofloxacin activity a deep structural-functional study remains to be done.

In conclusion, \( \text{Zn}_{0.15}\text{Mg}_{0.85} \) NPs are a promising antibacterial agent as exert multiple effects on bacterial cells. The efficiency of \( \text{Zn}_{0.15}\text{Mg}_{0.85} \) may be inhibited by particle aggregation in solution that reduce ROS production and metal ion release, and probably by their aggregation at the bacterial surface. The activation of multiple cellular mechanisms by \( \text{Zn}_{0.15}\text{Mg}_{0.85} \), suggests that bacteria need multiple simultaneous gene mutations to acquire resistance to mixed metal oxide NPs. We expect that further sustainable development of antibacterial metal oxide

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**Figure 6.** \( \text{Zn}_{0.15}\text{Mg}_{0.85} \) potentiates antibiotic activity of ciprofloxacin against \( B. \) subtilis and \( E. \) coli. Paper disk diffusion assay was performed on BHI agar plates with disks loaded with 5 µg ciprofloxacin alone or co-loaded with various amount of \( \text{Zn}_{0.15}\text{Mg}_{0.85} \) NPs. Disks loaded only with corresponding amounts of \( \text{Zn}_{0.15}\text{Mg}_{0.85} \) NPs were tested as controls. Left panel illustrates the distribution of disks per plate and two scanned plates showing the effect of \( \text{Zn}_{0.15}\text{Mg}_{0.85} \) NPs on the activity of ciprofloxacin. For both strains tested, the inhibition zone around disk co-loaded with \( \text{Zn}_{0.15}\text{Mg}_{0.85} \) and ciprofloxacin was broader than that around disk loaded only with ciprofloxacin. Bars indicates the percent increase in zone of inhibition for ciprofloxacin with NPs relative to that of ciprofloxacin alone. Error bars are 95% confidence intervals.
NPs will combine various doping and coating of particles to deliver safe nanomaterials that kill infection agents at high efficiency. Since effects of metal oxide NPs are additive with that of other compounds, the combination of Zn$_{0.15}$Mg$_{0.85}$O NPs with currently used antibiotics could be helpful to prevent new antibiotic resistance crises. In addition, different surfaces can be coated with highly stable and uniform Zn$_{0.15}$Mg$_{0.85}$O NPs to, which opens the way for a wide range of applications in agriculture, industry and medicine.

**Methods**

**Synthesis of Zn$_{0.15}$Mg$_{0.85}$O NPs.** Nanoparticles were prepared and characterized as previously described (see Supporting Information S1, for details)\(^{18,19}\).

**Bacterial strains, growth conditions and antibiotics.** Bacillus subtilis 168 strain (lab’s collection), Escherichia coli TGI strain, and Bacillus subtilis NDmed strain\(^2\) (a kind gift from Roman Briandet) were cultivated in LB medium (10 g/liter tryptone, 5 g/liter yeast extract, 5 g/liter NaCl). Biofilm formation was studied in M5g medium (5 mM potassium phosphate (pH 7), 100 mM MOPS (pH 7), 2 mM MgCl$_2$, 700 μM CaCl$_2$, 50 μM MnCl$_2$, 50 μM FeCl$_3$, 1 μM ZnCl$_2$, 2 μM thiamine, 0.5% glycerol, 0.5% glutamate, 50 μg mL$^{-1}$ tryptophan, 50 μg mL$^{-1}$ phenylalanine). A Penicillin, ciprofloxacin and vancomycin were from Sigma.

**Antibacterial activity and Minimum Inhibitory Concentration (MIC) estimation.** Overnight cultures of B. subtilis 168 were diluted in fresh LB medium to initial OD$_{600}$ of 0.1 and incubated in flasks with shaking (200 rpm) at 37 °C. Zn$_{0.15}$Mg$_{0.85}$O NPs were added at final concentration of 0.1 or 1 mg/ml. Bacterial growth was measured by following the optical density at 600 nm. A blank containing the equivalent concentration of nanoparticles in LB medium incubated under the same conditions was used as control. After 0, 150 and 330 min of incubation in LB medium, bacterial cultures were taken, successively diluted in LB medium and plated onto LB plates. Colonies forming units (CFU) were counted after incubation at 37 °C overnight. All experiments were performed in triplicate and averaged. To determine the MIC, bacterial cells (10$^4$ cfu/mL) were inoculated into fresh brain heart infusion (BHI) broth in 96-microtitre plates containing varying concentrations of NPs (0.01–10 mg/L) and grown overnight at 37 °C. The wells containing no NP were positive controls while wells containing no bacteria served as a negative control. The MIC was found as a minimal concentration of NPs preventing the culture to be turbid.

**Quantitative biofilms assays.** The microtiter plate assay quantitates the cells attached to the wells. Precultures of B. subtilis NDmed at OD$_{600}$ of 1.0 were diluted in fresh LB or in M5g to a final OD$_{600}$ of 0.01. Samples of 125 μl of the diluted cells were inoculated in wells of a 96-well polystyrene chloride (PVC) microtiter plate (Falcon 35911). Zn$_{0.15}$Mg$_{0.85}$O NPs were added at final concentration of 0.1 mg/ml. Microtiter plates were incubated without agitation at 30 °C. Biofilm amount was measured by discarding the medium, rinsing the wells with phosphate buffered saline (PBS) once, and staining bound cells with a 1% crystal violet solution at room temperature for 20 min. The wells were then washed with PBS buffer three times. The dye was solubilized with acetonitrile:ethanol 20:80, and absorbance at 595 nm was determined using a microtiter plate reader. For each experiment, background staining was corrected by subtracting the crystal violet bound to control wells. To perform pellicle assay 2 μl of bacterial culture grown at 37 °C upon agitation to an OD$_{600}$ ~ 0.6 was added to 2 ml of M5g (alone or with admixed NPs) in a well of 6-well microtiter plate. The plates were incubated without agitation at 30 °C for 72 h and 144 hours. Photographs were required with Samsung Galaxy smartphone. Each assay was performed at least in three independent experiments.

**Preparation of B. subtilis spores.** B. subtilis 168 cells were induced to sporulate by nutrient exhaustion in Difco sporulation medium (DSM)\(^3\). A single colony was picked from a fresh agar plate and used to inoculate 25 ml of DSM and allowed to grow at 37 °C for 48 h. Spores and other cells/cellular debris were collected by centrifugation and washed twice with distilled water. The pellet was then resuspended in 1 ml of distilled water. The pellet was then resuspended in 1 ml of distilled water. A heat treatment (20 min at 80 °C) was applied to eliminate vegetative cells. Zn$_{0.15}$Mg$_{0.85}$O NPs were added to spores suspension at final concentration of 1 or 5 mg/ml. After 24 h of incubation, the number of CFU in the presence and in the absence of NPs was determined by plating dilutions on agar plates.

**Disk diffusion assays.** One ml of exponentially growing cells (OD$_{600}$ = 0.8–1) of the strain being tested was spread on the Petri plates containing BHI agar medium. The plates were allowed to dry briefly in a laminar flow before 6.6 mm filter paper disks (Whatman) containing the antibiotics and/or nanoparticles (20 μl volume) were placed on the plates (ciprofloxacin 250 μg/ml, penicillin 16 mg/ml, vancomycin 1 μg/ml). The plates were incubated at 37 °C overnight and the zones of inhibition were measured. The values in Fig. 6 are an average of three independent experiments.

**Cell culture and MTT test.** The immortalized murine peritoneal macrophage cell line RAW 264.7 from the American Type Culture Collection was utilized to determine cytotoxic effect of Zn$_{0.15}$Mg$_{0.85}$O. Macrophages were grown in complete Dulbecco’s Modified Eagle Medium (DMEM) medium (Eurobio, France) supplemented with 2 mM glutamine, antibiotics (100 U/ml penicillin A and 100 U/ml streptomycin) and 10% heat-inactivated fetal bovine serum and maintained in a humidified incubator at 37 °C under 5% CO$_2$. Macrophages were plated at a density of 30,000 cells per well on 96-well plates. After 24 h, cell medium was exchanged and various concentrations of Zn$_{0.15}$Mg$_{0.85}$O were loaded per well and incubated for 1 h. Afterwards, a freshly prepared 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) at final concentration of 0.8 mg/ml was added and incubated for a further 1 h. Then, the cell layer was dried and MTT formazan produced by conversion of the water soluble MTT was suspended in 100 μl of dimethyl sulfoxide. Cell survival was quantified by measuring
Resorufin fluorescence was measured using a spectrofluorometer (Tecan infinite M200PRO) with excitation and 10. Gilbert, B.

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Author Contributions
S.A. and J.V. conceived and designed the study. S.St. and S.Su. provided nanoparticles. S.A., C.H, C.P., N.L., N.B. and J.V. performed experiences. S.A., C.H., C.P., T.L., and J.V. carried out data analysis, interpretation and contributed to the redaction of the manuscript. All authors read and approved the final manuscript.

Additional Information
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