



Soft Matter

Electronic Supplementary Information

Influence of nanomechanical stress induced by ZnO nanoparticles of different shape on viability of cells

Kinga Matuła,^{a,†} Łukasz Richter,^{a,†} Witold Adamkiewicz,^a Bo Åkerström,^b Jan Paczesny,^a
Robert Holyst^a

^a*Institute of Physical Chemistry PAS, Kasprzaka 44/52, 01-224 Warsaw, Poland.
E-mail: jpaczesny@ichf.edu.pl, rholyst@ichf.edu.pl*

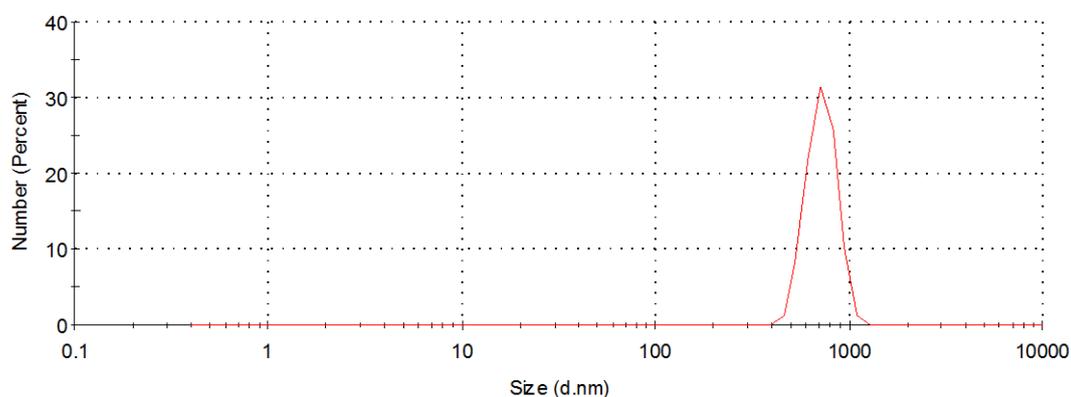
^b*Section for Infection Medicine, BMC Lund University, Sölvegatan 19, 221 84 Lund, Sweden.*

† *These authors contributed equally*

Zinc oxide nanoparticles and nanorods are denoted throughout the SI as **NP** and **NR**, respectively. The results obtained for control samples are depicted in black and the influence of Zn^{2+} in green, **NP** in red and **NR** in blue.

The hydrodynamic diameters measured by means of DLS (dynamic light scattering) were 322 ± 151 nm for **NP** and 732 ± 130 nm for **NR**. As **NR** are nonsymmetrical the rotation along short axis (width) increase the volume occupied by single NR. This reflected in much larger hydrodynamic diameter comparing to **NP**. The results are presented in **Figure S1**.

a)



b)

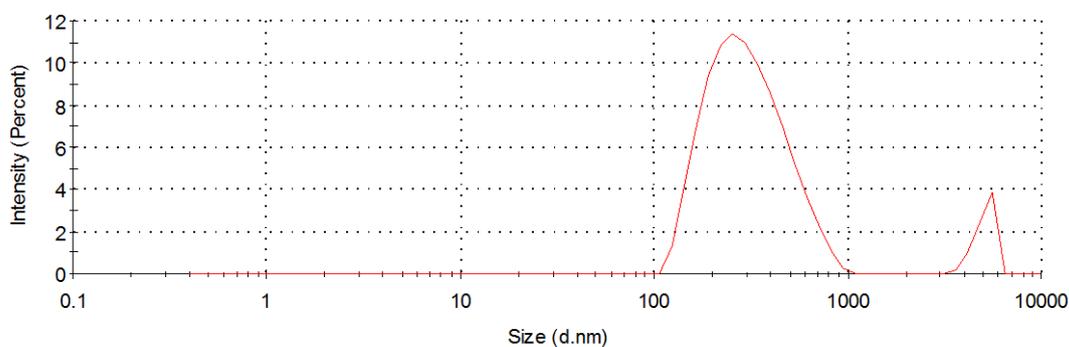


Figure S1. The hydrodynamic diameter of **a) ZnO NR**, **b) ZnO NP**, measured by DLS.

Below in **Figure S2** we provide results acquired by means of scanning electron microscope (SEM).

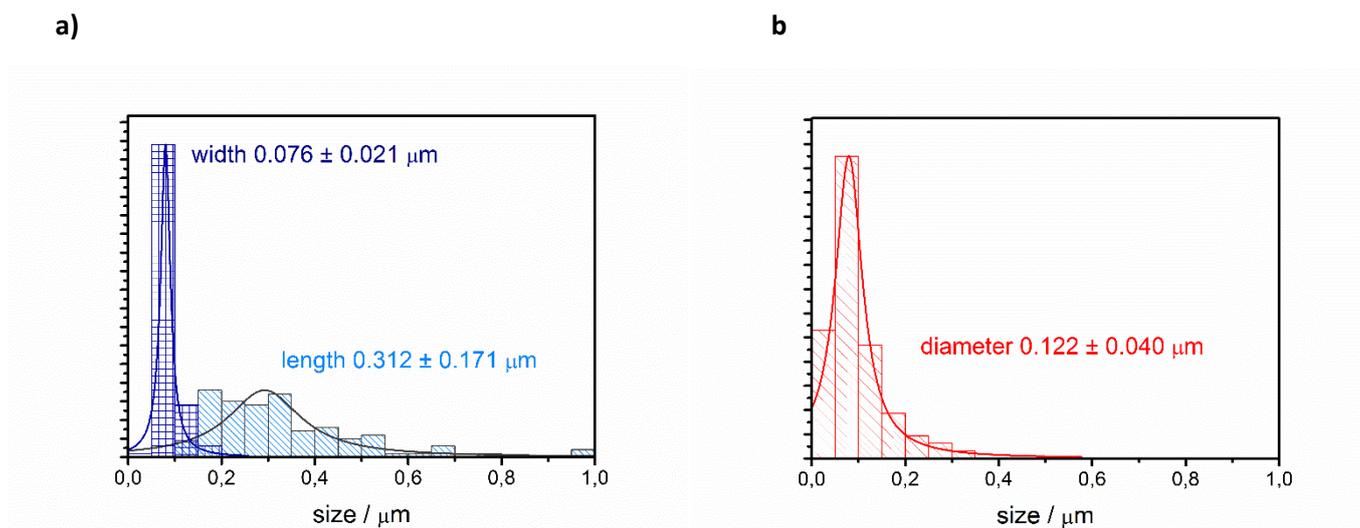
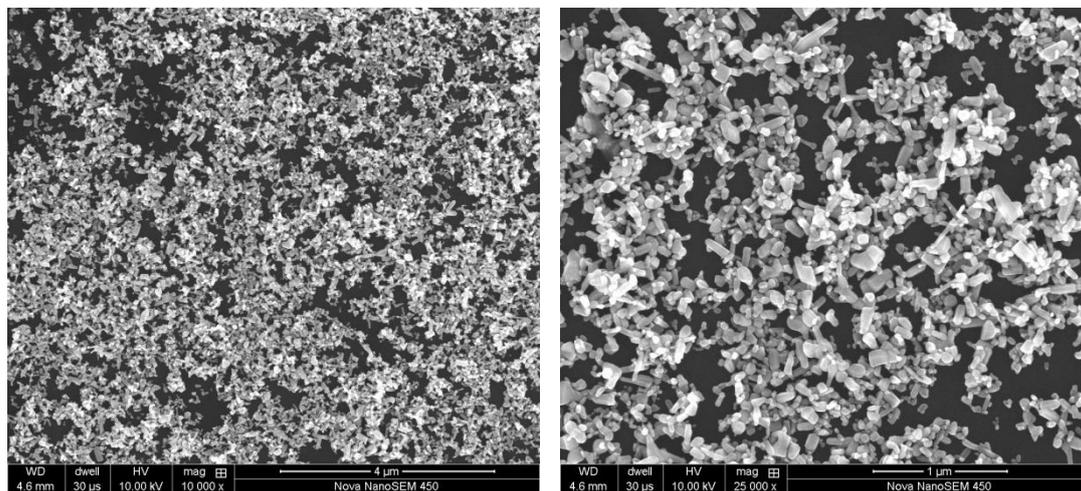
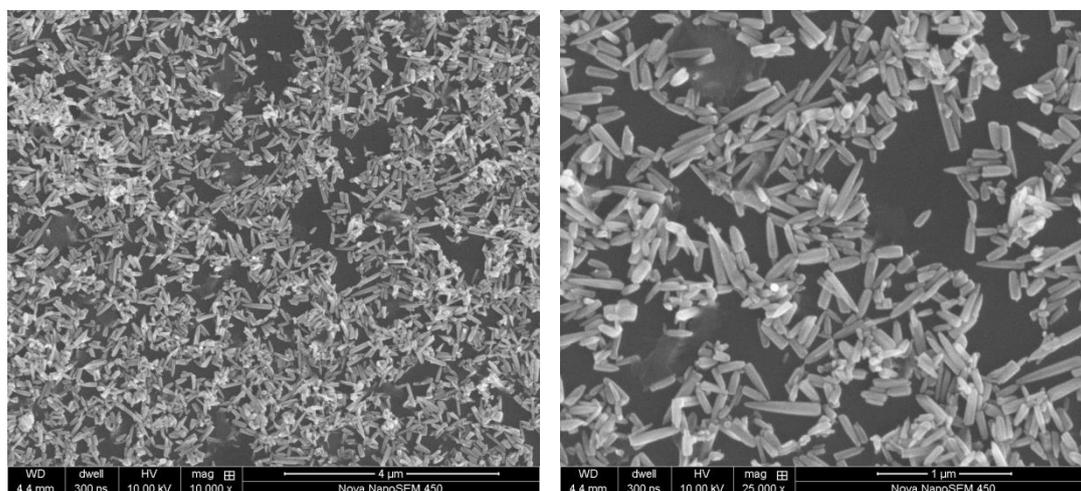


Figure S2. Size distribution of a) ZnO NR, b) ZnO NP, measured by SEM.

NP



NR



Influence of Zn²⁺ released from NP and NR

Number of publications focus on the effect of solubility of ZnO and dissolution of ZnO nanoparticles.

Paper by Reed *et al.* revealed solubility of nano-ZnO in ultrapure water and in biologically important matrices: RPMI-600 and Dulbecco's modified Eagle's medium.⁵¹ The highest concentration of Zn²⁺ found by authors was around 30 µg/mL and in most of the cases it did not exceed 10 µg/mL.

In the paper by Wong *et al.* the authors found the concentration of dissolved Zn²⁺ from nano ZnO in seawater to be smaller than 4 µg/mL.⁵² Seawater was chosen as the authors studied influence of nano ZnO on marine organisms.

Brunner *et al.* showed *in vitro* cytotoxicity of oxide particles using MSTO and 3T3 cells.⁵³ Authors gave value of 4.2 µg/mL of zinc ions due to dissolution of ZnO in 18°C.

Franklin *et al.* compared cytotoxicity of nanoparticulate ZnO, bulk ZnO and ZnCl₂ to freshwater microalga (*Pseudokirchneriella subcapitata*).⁵⁴ Authors found that in case of suspension of concentration of 100 mg/L of both bulk and nanoparticulate ZnO, the final concentration of Zn²⁺ coming from dissolution is only around 15 µg/mL in pH around 7.5.

David *et al.* showed the first application of the electroanalytical technique AGNES to measure the Zn²⁺ concentration in aqueous dispersions of ZnO nanoparticles.⁵⁵ The authors highlighted the large sensitivity of the solubility measurements to the particular experimental condition such as pH, temperature, medium composition, sample history and the procedures carried out to prior to elemental analysis.

Mudunkotuwa *et al.* showed the dependence between size and dissolution of well-characterized ZnO nanoparticle at circumneutral pH.⁵⁶ They also observed that the presence of citric acid significantly enhanced the extent of ZnO dissolution for all sizes of nanoparticles. The smaller the particles were the higher was amount of zinc ions in the solution.

The research from 2011 by Bian *et al.* compared the dissolution of ZnO nanoparticles of different nanoparticle diameters, including those near 15 and 240 nm at both, low and high pH.⁵⁷

We measured amount of Zn²⁺ ions released from suspensions of **NR** and **NP** of concentration of 1 mg/mL. LB medium and MiliQ water were used as solvents. The suspensions were mixed for 24 hours in the same conditions as bacteria cultures (37°C, 200 rpm). Afterwards, the suspensions of **NR** and **NP** were centrifuged at 10 000 rpm for 10 minutes. Supernatants were analyzed. We utilized spectrophotometric titration in presence of eriochrome black T indicator. All interfering ions were complexed by CN⁻ ions, whereas Zn²⁺ ions were released from such complexes upon addition of formaldehyde. The results are collected in **Table S1**. We examined the effect of even higher concentration of free zinc ions on living cells.

Table S1. Amount of Zn²⁺ ions released from the suspensions of **NR** and **NP**.

	NP	NR
MiliQ water	18.7 ± 0.9 µg/mL	16.6 ± 0.6 µg/mL
LB medium	44.6 ± 1.6 µg/mL	38.0 ± 1.5 µg/mL

Prokaryote

We performed number of control experiments. First, we simulated the influence of dissolution of **NP** and **NR** on the viability of cells. Based on the literature data on dissolution of ZnO the concentration of free Zn^{2+} ions in suspension of ZnO nanoparticles of diameter of around 150 nm of concentration of 1 mg/mL should not exceed 10 $\mu\text{g/mL}$ in pure water^{56,58} and around 60 $\mu\text{g/mL}$ in medium.⁵⁹ Mudunkotuwa *et al.* showed that the concentration of zinc ions decreases with an increase of nanoparticles size.⁵⁶ We analyzed the filtrate of the studied nanostructures by means of spectroscopic titration. The measured that concentration of Zn^{2+} ions was around $44.6 \pm 1.6 \mu\text{g/mL}$ for **NP** and $38.0 \pm 1.5 \mu\text{g/mL}$ for **NR** in LB medium. The obtained values were in good agreement with literature data. As control experiments we monitored the viability of bacteria upon addition of $ZnCl_2$ to final concentration of Zn^{2+} ions of 50 $\mu\text{g/mL}$. We decided to add excess of Zn^{2+} ions to be confident that the control experiment is valid also upon prolonged exposure of cell to ZnO nanostructures. *E. coli* appeared insensitive to presence of zinc ions (see **Figure S3a** and **Figure S4a**). In case of *S. epidermidis* the growth of the bacteria cultures was inhibited (**Figure S3b** and **Figure S4b**), however no bacteria killing was observed. The observed effect of zinc ions on Gram-negative and Gram-positive strains was in good agreement with the literature data.^{510,511} Li *et al.* showed that LPS layer gave Gram-negative strains additional protection against small toxic agents including ions.⁵⁸

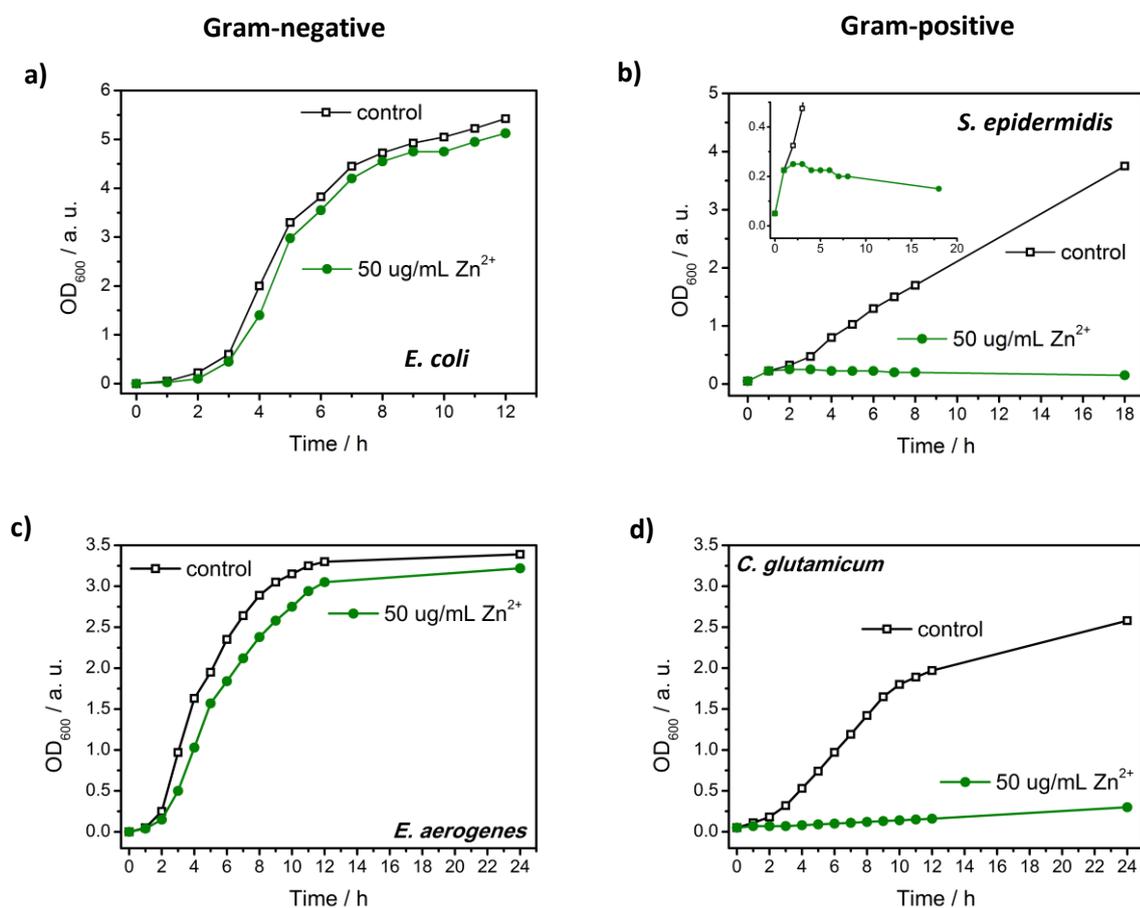


Figure S3. The influence of Zn^{2+} ions on **a)** *E. coli* and **b)** *S. epidermidis*, **c)** *E. aerogenes* and **d)** *C. glutamicum* depicted as changes of OD₆₀₀ of cultures in time. In case of Gram-negative strains small amount of overnight culture was added to medium with proper amount of $ZnCl_2$ (OD=0.01) In case of Gram-positive strains OD at the start of the experiment was higher (OD=0.03-0.05) due to vulnerability to presence of zinc ions. For OD=0.01 we did not observed any increase in OD during time. Zinc ions limited growth of Gram-positive strains, whereas they had small influence on Gram-negative bacteria.

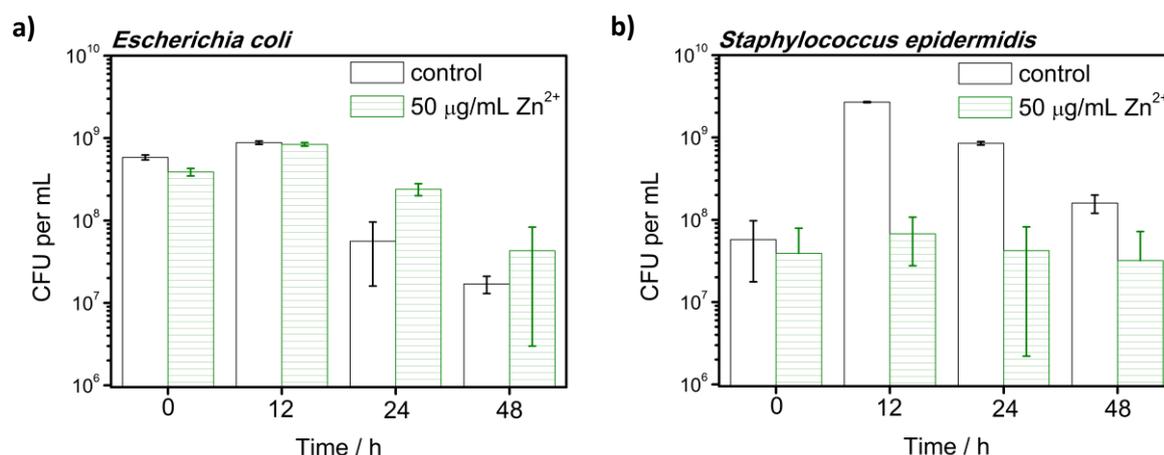


Figure S4. The influence of Zn²⁺ ions on **a)** *E. coli* and **b)** *S. epidermidis* depicted as changes of CFU of cultures in time. ZnCl₂ was added to culture of OD₆₀₀ equal 1 in both cases.

NP and NR embedded into agar plates

We were concerned whether dynamic interactions (induced by rotation) between cells and the ZnO nanostructures were of importance. To address this issue we performed static experiments. The most important results are described in the main text. Here, we provide additional experimental evidences. Agar plates with embedded **NP** and **NR** were prepared. The highest concentration of the ZnO nanostructures, that we succeeded to incorporate in a standard agar plate was around 1 mg/mL. In case of higher concentrations the material was not evenly distributed across the plate, with the majority of the ZnO concentrated at the bottom of the plate.

E. coli

No significant differences (within experimental error) in number of colonies between control and plates containing 1 mg/mL of the ZnO **NP** or **NR** were observed comparing to control (**Figure S5**).

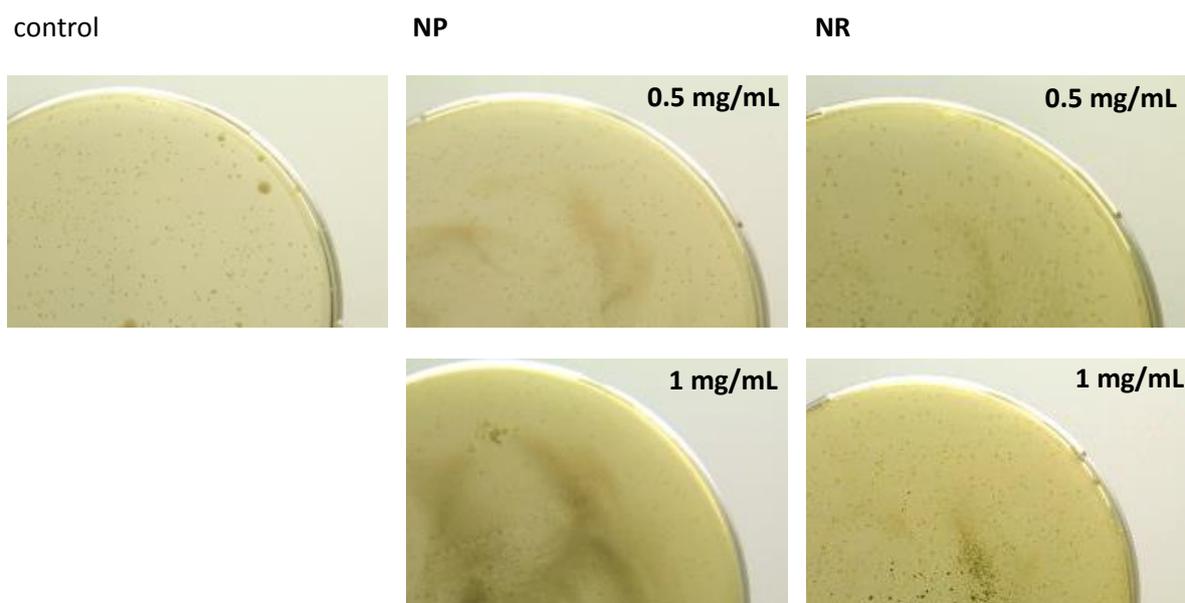


Figure S5. Pictures of agar plates showing that motionless **NP** and **NR** had small influence on Gram negative (*E. coli*) bacteria.

S. epidermidis

S. epidermidis appeared much more vulnerable to presence of zinc ions within the medium (due to lack of LPS layer, which scavenges small toxic agents and ions). When **NP** and **NR** were embedded into the agar plates the colonies were extremely small. They were hardly visible (it appeared impossible to make informative digital picture). This was in line with other experiments, which proved that zinc ions block the growth of the *S. epidermidis* bacteria.

0.1 mg/mL of ZnO nanostructures with mixing

Based on literature data presented by Yamamoto^{S12} for ZnO particles of diameter in range from 100 nm to 500 nm, the concentrations causing damage to bacteria are in the range 0.1 mg/mL to 10 mg/mL. The most interesting results were obtained for a final concentration of **NR** and **NP** of 1 mg/mL (presented in main text). Here we present results for concentration of 0.1 mg/mL for *Escherichia coli* and *Staphylococcus epidermidis*.

In low-concentration regime we observed an inhibition of the growth of bacteria associated with the presence of free zinc ions in the solution. Below we show plots for *E. coli* and *S. epidermidis* incubated with ZnO nanostructures of concentration of 0.1 mg/mL (**Figure S6**). The obtained results are in line with those presented by Jain,¹³ *i.e.* Gram-positive strains appeared more vulnerable to ZnO nanostructures. Maximal observed OD₆₀₀ of *S. epidermidis* culture was smaller than 1 a.u. Gram-negative bacteria possess protective LPS layer. Maximal OD₆₀₀ of *E. coli* culture in such conditions was around 4 a.u.

Only small differences between **NP** and **NR** were observed in this concentration regime in case of both Gram-positive and Gram-negative. At such a low concentration of ZnO physical contact of bacteria with the nanorods is infrequent and the influence of the puncturing of the cells on the entire population growth cannot be observed. The effect of Zn²⁺ or ROS release seemed to have major effect on the bacteria growth in regime of low concentrations of ZnO nanoparticles. This was in line with all previous experiments (*e.g.* with only ZnCl₂ added to a medium) and literature data, *i.e.* Gram-positive strains are much more vulnerable in such conditions.

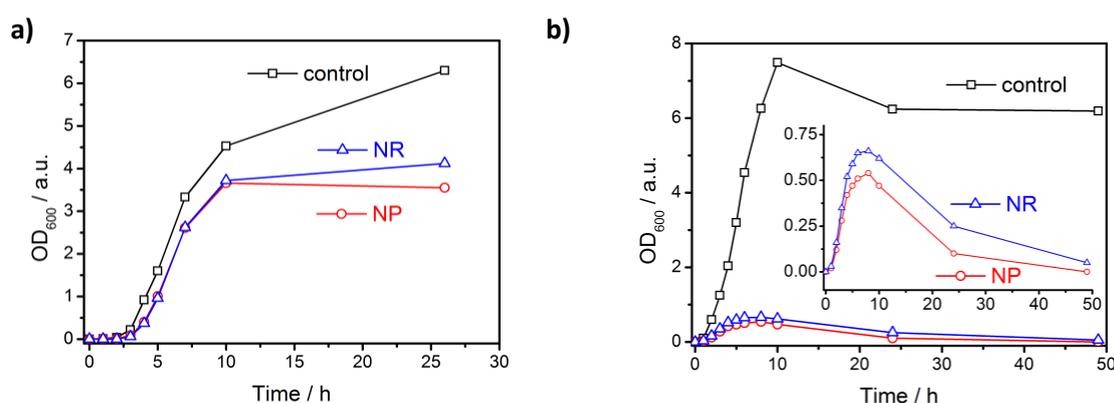
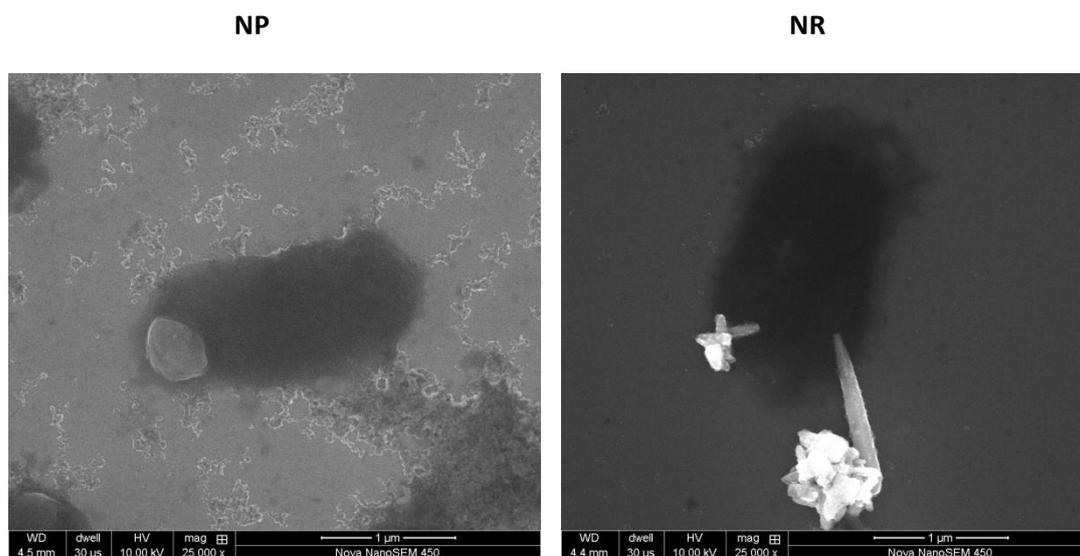
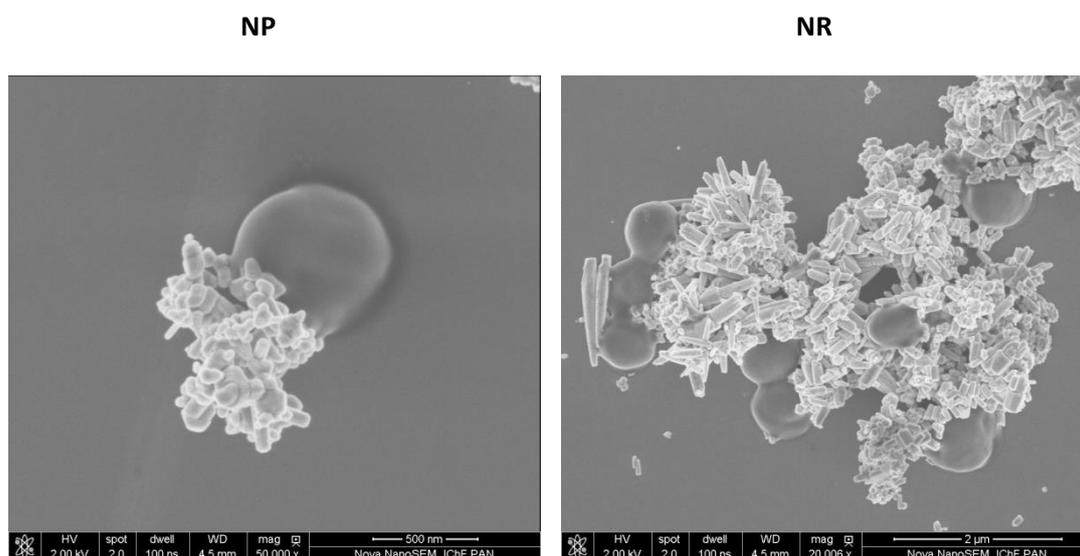


Figure S6. Graphs show changes of values of OD₆₀₀ of **a)** *E. coli* and **b)** *S. epidermidis* cultures in time. The ZnO nanorods (**NR**) and the nanoparticles (**NP**) were added to final concentration of 0.1 mg/mL. The cultures were incubated in 37°C and mixed gently (200 rpm) in course of the experiments.

Additional SEM pictures

E. coli*S. epidermidis***Eukaryote***S. cerevisiae*

We examined the viability of *S. cerevisiae* upon addition of $ZnCl_2$ to final concentration of Zn^{2+} ions of $50 \mu\text{g/mL}$ (see **Figure S7**). We did not observe any significant influence of zinc ions coming from dissolution of ZnO on *S. cerevisiae*. The general effects of zinc ions on yeast is positive and well described.^{S14}

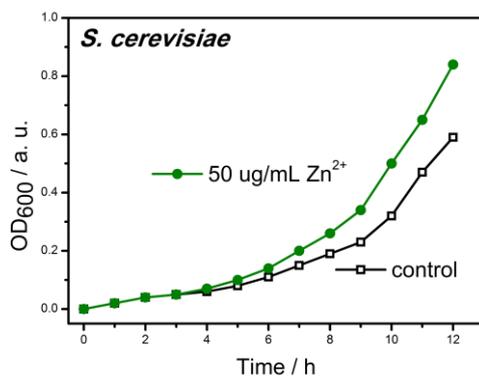
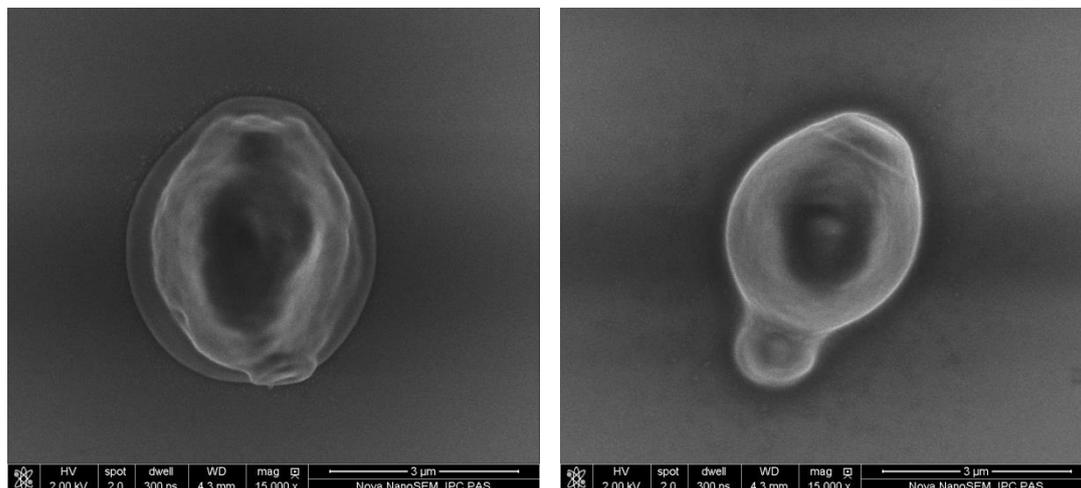


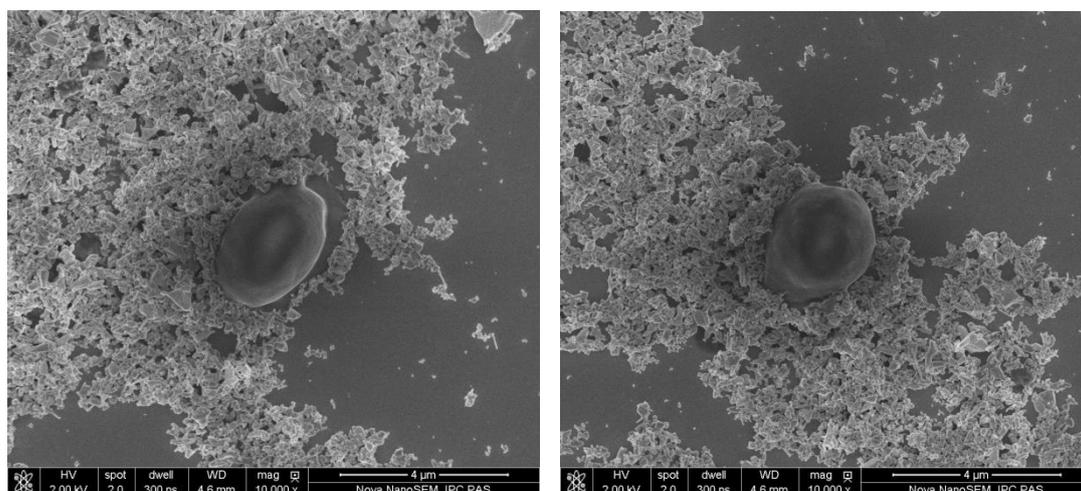
Figure S7. The influence of Zn²⁺ ions on *S. cerevisiae* depicted as changes of OD₆₀₀ of cultures in time.

Additional SEM pictures (*S. cerevisiae*)

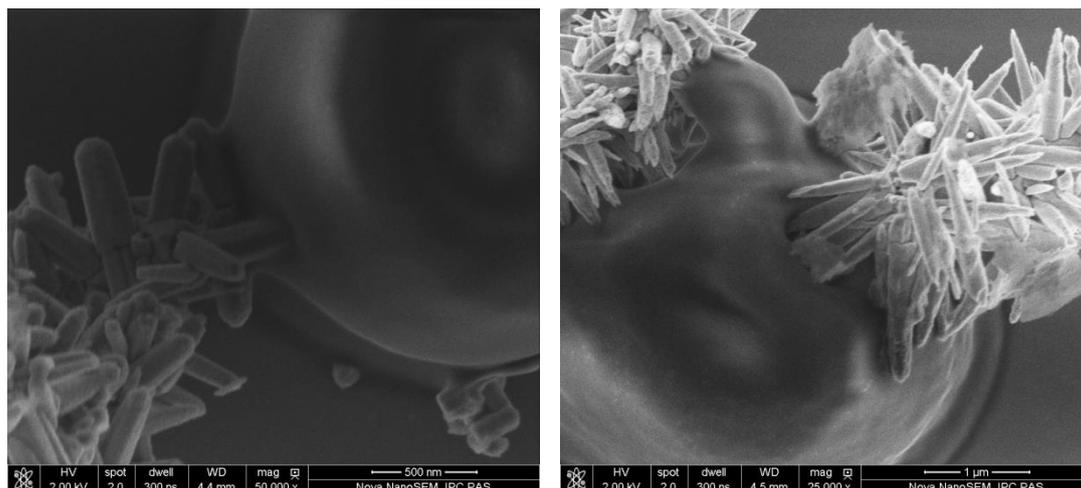
control



NP



NR

*Hep G₂*

NP and **NR** were added to final concentration of 1 mg/mL. Half of the culturing flasks were rotated, and half were incubated motionless. The concentrations of zinc ions were very similar in corresponding samples, thus only the effect of mechanical stress was evaluated. The LDH levels were much lower in motionless samples comparing to mixed ones.

The cells were observed under the fluorescence microscope after six hours of incubation with **NP** and **NR** (concentration of 1 mg/mL). Fluorescent dyes were added to monitor the death-rate of HepG₂ cells upon exposure to **NP** and **NR**. Nucleus-staining dye propidium iodide (PI) (maximum emission at 617 nm) and DNA binding dye Hoechst 33342 (maximum emission at 461 nm) were added to HepG₂ cells to final concentration of 10 μM and 1 μM, respectively. After 30 minutes, the cells were washed with medium without FBS. Hoechst is present in all cells, whereas PI is pumped out from the living cells, staining only dead cells. Both PI and Hoechst 33342 were bought from Invitrogen (USA). Nikon Eclipse TE300 (Japan) inverted fluorescence microscope equipped with a Hamamatsu C4742–95 cooled CCD camera (Japan) was used to collect the pictures of stained HepG₂ cells. More than ten random views were taken of each sample. No significant differences were visible in the various points of the culturing flask.

The cells were observed under the fluorescence microscope after six hours of incubation with **NP** and **NR**. Presence of the ZnO particles influenced morphology of the cells. They were smaller and more round comparing to the control samples. Additionally, cells were stained using Hoechst 33342 (present in all cells) and propidium iodide (present only in dead cells, denoted as PI). From pictures in **Figure S8** four main results were obtained. 1) Mixing did not influence control cells. Very little dead cells were observed in control samples. 2) ZnO nanostructures induced changes of morphology of the cells. 3) Relatively small fraction of cells was dead after six hours due to mechanical damage caused by **NR** or **NP**. LDH measurements gave insight into response of stressed cells before they were actually destroyed. 4) **NR** were more damaging comparing to **NP**.

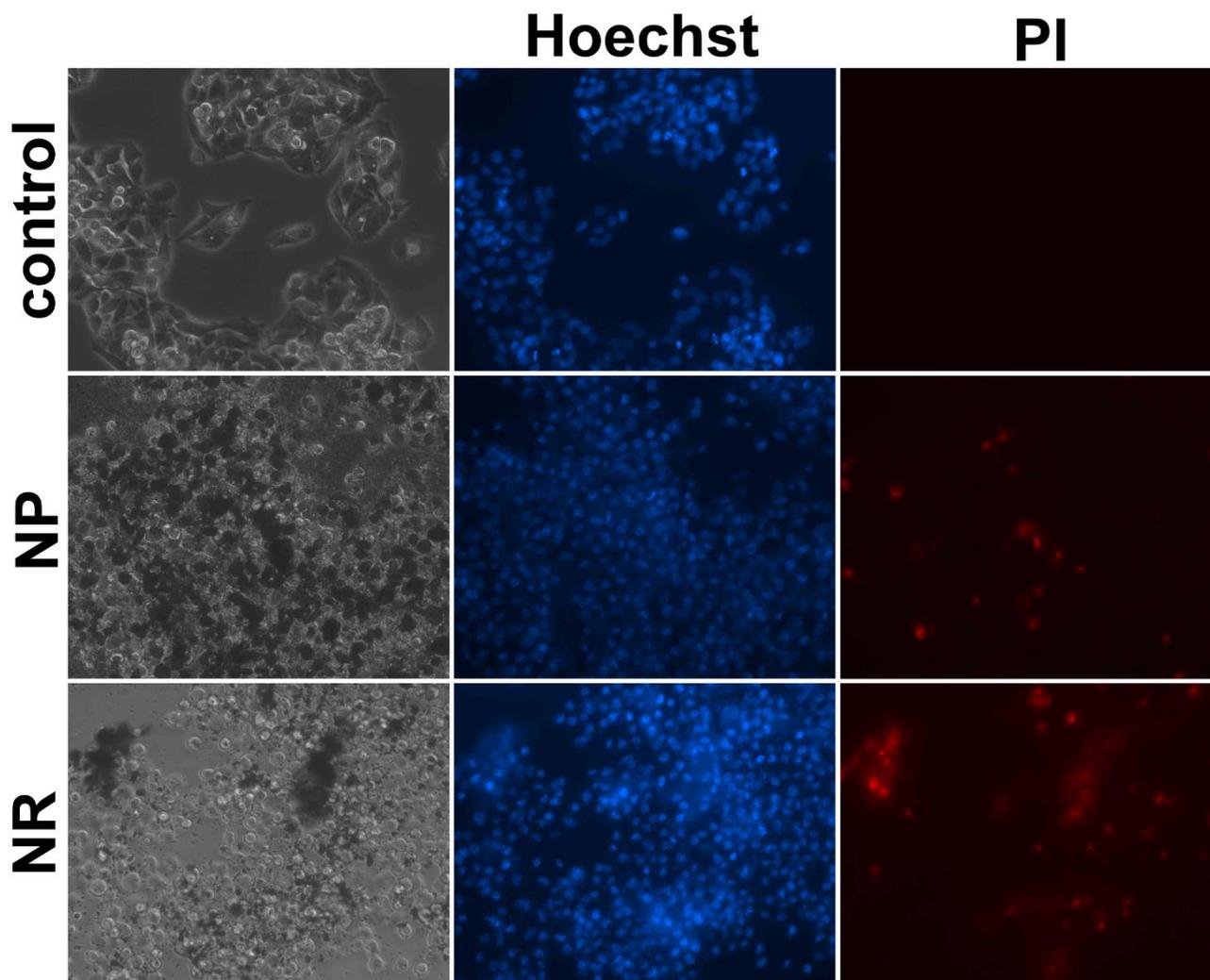


Figure S8. Fluorescence microscope images of HepG₂ cells stirred (100 rpm) for 6 hours are shown. Hoechst was present in all cells, whereas PI only in dead cells.

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