Supplementary material for

**Synthesis and synergistic antibacterial efficiency of chitosan-copper oxide nanocomposites**

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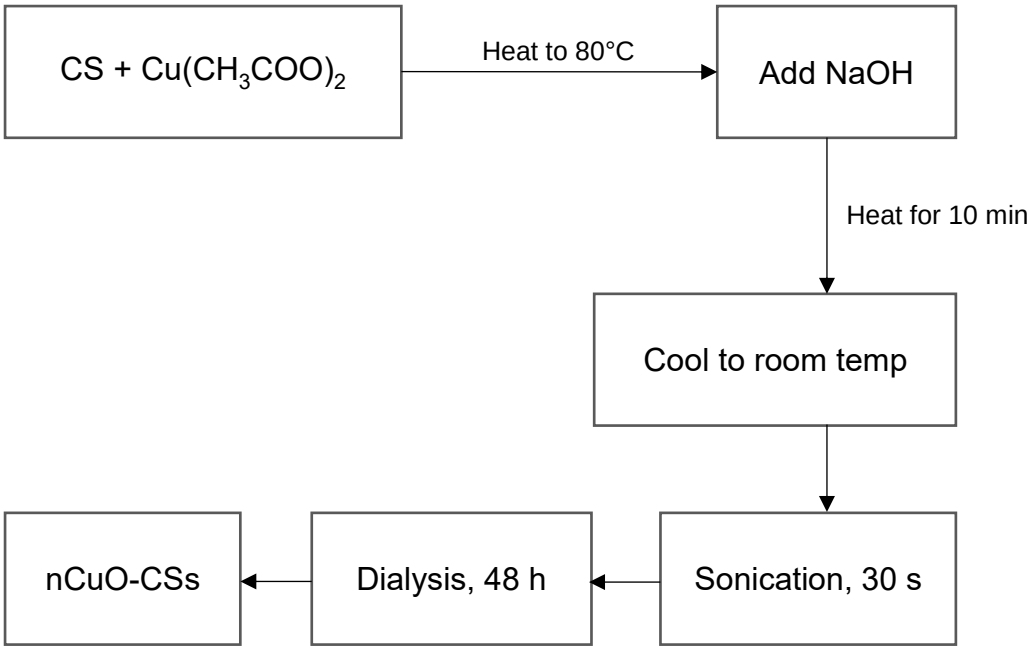


**Figure S1.** Schematic representation of the synthesis of chitosan-copper oxide nanocomposites (nCuO-CSs). Chitosan (CS) and copper(II) acetate are mixed in three Cu:CS ratios (1:0.3, 1:1, 1:3) on a magnetic stirrer with temperature control. Based on Cu:CS mass ratio, approximately 0.55, 0.8 or 1.1 mL of 1M NaOH is added drop-wisely. Dialysis (cut-off 12 000 kDa) is performed against 2 L of deionized water (changed after 24 h).

*S1. Measurement of abiotic and biotic reactive oxygen species (ROS)*

The tests in abiotic (i.e., without bacteria) conditions were performed as described by Aruoja *et al*. [1]. In short, a 1.3 mM solution of DCFH2-DA was prepared in ethanol, deacetylated with 0.01 M NaOH at a ratio of 1:4 (DCFH2-DA:NaOH) and incubated for 30 min in the dark at room temperature. The reaction was stopped by diluting the solution 6-fold with 25 mM sodium phosphate buffer (pH 7.4). The mixture was stored on ice and protected from light until further use. 100 µL of that solution was added to the wells of a black 96-well microplate (Greiner Bio-One, Frickenhausen, Germany) containing 100 µL of the studied NCs in DI water. The plates were incubated for 30 min in the dark at room temperature. Fluorescence was recorded as described below. DI water was used as a negative control. Manganese(II, III) oxide ($Mn_3O_4$) nanoparticles (synthesized previously [1]) were used as a positive control.

The potential of the NCs to induce intracellular (biotic) ROS generation was determined, as detailed by Käosaar *et al*. [2]. Briefly, the bacteria were cultured and exposed to NCs in accordance to section 2.5. However, black 96-well microplates were used in this analysis. A 5 mM solution of DCFH2-DA was prepared in ethanol, diluted 10x with 25 mM sodium phosphate buffer (pH 7.4), and 50 µL added to each of the 96-well microplate wells. The plates were covered with a gas-permeable microplate film (nerbe plus GmbH & Co. KG, Winsen (Luhe), Germany) and incubated for 30 min in the dark at room temperature. DI water was used as a negative and $H_2O_2$ as a positive control. Each test was carried out twice.

Fluorescence (excitation at 485 nm and emission at 527 nm) was recorded with a Fluoroskan Ascent FL (Thermo Labsystems, Helsinki, Finland) microplate reader. The abiotic and biotic ROS levels were presented via the following calculation:

$$F = \frac{F_{t30\ (sample)}}{F_{t30\ (control)}}$$

where $F_{t30}$ (sample) and $F_{t30}$ (control) are the recorded fluorescence values of the studied NC suspension and negative control, respectively, after 30 min incubation with the fluorescent dye. Fluorescence is presented in relative units (RFU).

**References**


[1] V. Aruoja, S. Pokhrel, M. Sihtmäe, M. Mortimer, L. Mädler, A. Kahru, Toxicity of 12 metal-based nanoparticles to algae, bacteria and protozoa, Environ. Sci. Nano 2 (2015) 630–644. https://doi.org/10.1039/C5EN00057B.

[2] S. Käosaar, A. Kahru, P. Mantecca, K. Kasemets, Profiling of the toxicity mechanisms of coated and uncoated silver nanoparticles to yeast Saccharomyces cerevisiae BY4741 using a set of its 9 single-gene deletion mutants defective in oxidative stress response, cell wall or membrane integrity and endocytosis, Toxicol. In Vitro 35 (2016) 149–162. https://doi.org/10.1016/j.tiv.2016.05.018.

*S2. Visualization of bacterial cell membrane damage*

To visualize the impact of nCuO-CSs on the integrity of bacterial cell membrane, the cells were stained after a 1-h exposure to the nanocomposites at a concentration of 1 mg Cu/L and comparatively to copper(II) acetate at 1 mg Cu/L and low molecular weight chitosan at 1 and 3 mg/L, with propidium iodide (PI, 81845; Sigma-Aldrich, Steinheim, Germany), which penetrates only cells with damaged membranes, and SYTO 9 (S34854; Thermo Fisher Scientific, Waltham, MA, USA), which can enter both damaged and intact cells. A 0.1% (w/vol) sodium dodecyl sulfate (SDS) solution was used as a positive control for membrane-compromised cells (PI-positive cells). The final working concentrations of the PI and SYTO 9 were 30 and 5 µM, respectively. After a 10-minute incubation in the dark at room temperature, the stained bacterial suspensions were observed on a 1.3% agarose pad using a Zeiss LSM800 widefield fluorescence microscope (Zeiss, Jena, Germany) equipped with Zeiss Filter Set 10. PI and SYTO 9 signals were detected at 575–640 nm and 515–565 nm, respectively. In the case of nCuO-CS_1 and nCuO-CS_3, due to the tendency to form large cell aggregations, a Zeiss LSM800 confocal laser scanning microscope (CLSM) was applied using excitation/emission track settings of 561 nm/550–700 nm for the PI signal and 488 nm/450–550 nm for the SYTO 9 signal.

**Table S1.** The change of hydrodynamic size, polydispersity index, and ζ-potential with storage at 4 °C in the dark. The dynamic light scattering and electrophoretic light scattering analyses of chitosan-copper oxide nanocomposites (nCuO-CS) were carried out at a concentration of ~40 mg Cu/L in deionized water.

| Sample | Cu:CS* ratio | 8.05.2023 | 4.09.2023 | 31.10.2023 | 31.01.2024 | Average | SD |
|---|---|---|---|---|---|---|---|
| | | Hydrodynamic size, nm | | | | | |
| nCuO-CS_0.3 | 1:0.3 | 91.7 | 90.2 | 90.6 | 103 | 93.8 | 5.97 |
| nCuO-CS_1 | 1:1 | 122 | 124 | 118 | 154 | 130 | 16.7 |
| nCuO-CS_3 | 1:3 | 180 | 178 | 168 | 205 | 183 | 15.5 |
| | | Polydispersity index | | | | | |
| nCuO-CS_0.3 | 1:0.3 | 0.24 | 0.23 | 0.23 | 0.35 | 0.26 | 0.06 |
| nCuO-CS_1 | 1:1 | 0.25 | 0.27 | 0.26 | 0.41 | 0.30 | 0.08 |
| nCuO-CS_3 | 1:3 | 0.33 | 0.35 | 0.27 | 0.39 | 0.33 | 0.05 |
| | | ζ-potential, mV | | | | | |
| nCuO-CS_0.3 | 1:0.3 | 46.2 | 45.0 | 43.1 | 40.0 | 43.6 | 2.72 |
| nCuO-CS_1 | 1:1 | 45.8 | 46.0 | 41.5 | 44.0 | 44.3 | 2.08 |
| nCuO-CS_3 | 1:3 | 44.7 | 43.5 | 41.8 | 42.0 | 43.0 | 1.36 |

* CS – chitosan

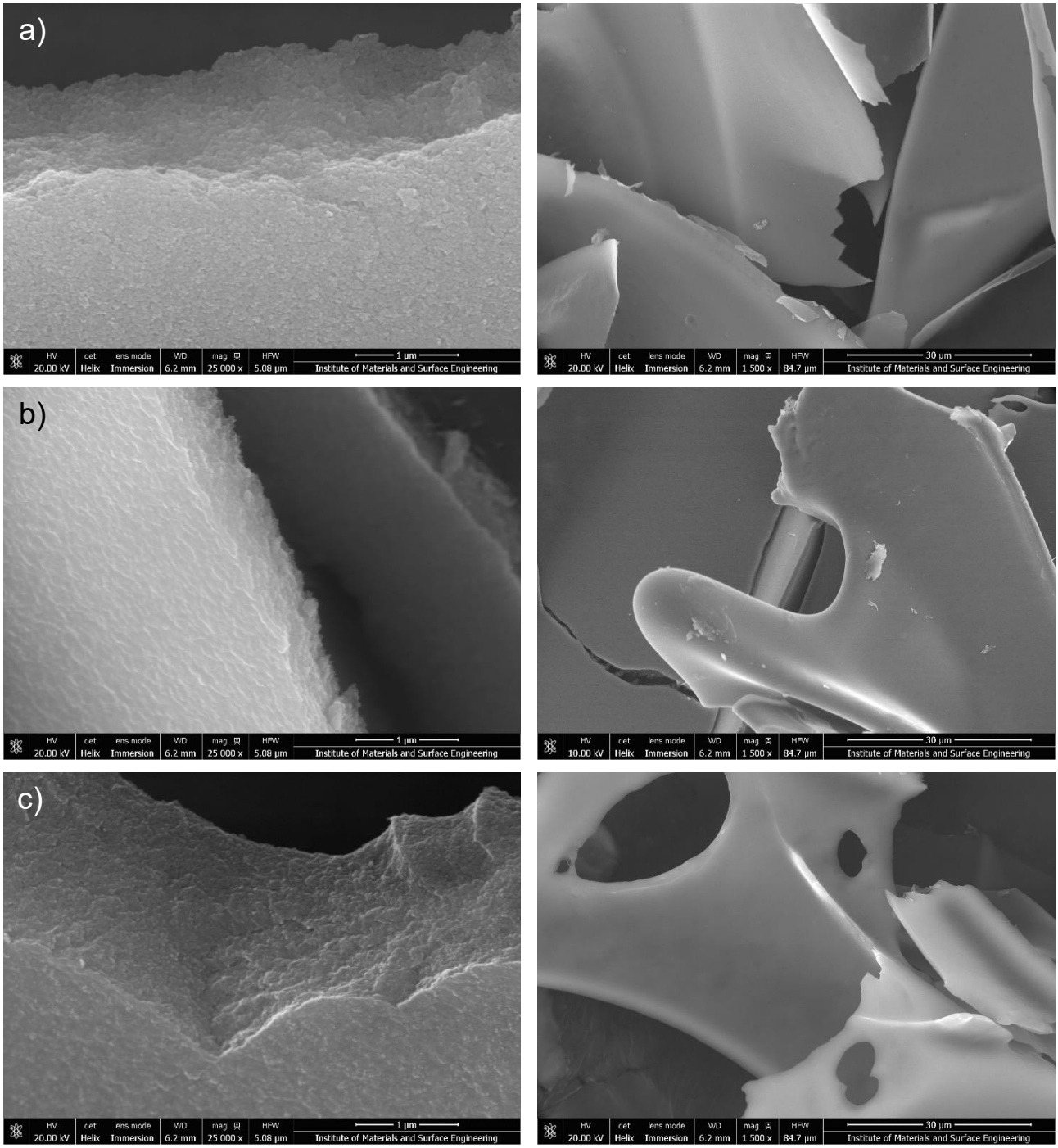


**Figure S2.** SEM images of chitosan-copper oxide nanocomposites (nCuO-CS). (a) nCuO-CS_0.3, (b) nCuO-CS_1, (c) nCuO-CS_3. nCuO-CS_0.3, nCuO_CS_1 and nCuO-CS_3 designate nanocomposites with different Cu:chitosan mass ratios (1:0.3, 1:1 and 1:3, respectively).

**Table S2.** The elemental composition (at%) of chitosan-copper oxide nanocomposites (nCuO-CS) as measured by energy-dispersive X-ray spectroscopy (EDS) and X-ray photoelectron spectroscopy (XPS), respectively. The nCuO-CS samples were lyophilized prior to analyses.

| EDS | Cu:CS* mass ratio | Cu | N | O | C |
|---|---|---|---|---|---|
| Chitosan | - | 0 | 12.9 | 28.0 | 59.1 |
| nCuO-CS_0.3 | 1:0.3 | 17.0 | 6.0 | 33.4 | 43.5 |
| nCuO-CS_1 | 1:1 | 10.7 | 8.3 | 29.5 | 51.5 |
| nCuO-CS_3 | 1:3 | 15.3 | 4.1 | 24.3 | 56.2 |
| XPS | Cu:CS* mass ratio | Cu | N | O | C |
| Chitosan | - | 0 | 7.4 | 31.4 | 61.2 |
| nCuO-CS_0.3 | 1:0.3 | 3.2 | 5.4 | 33.0 | 58.4 |
| nCuO-CS_1 | 1:1 | 1.7 | 6.2 | 30.3 | 61.8 |
| nCuO-CS_3 | 1:3 | 1.0 | 4.3 | 27.7 | 67.0 |

* CS – chitosan

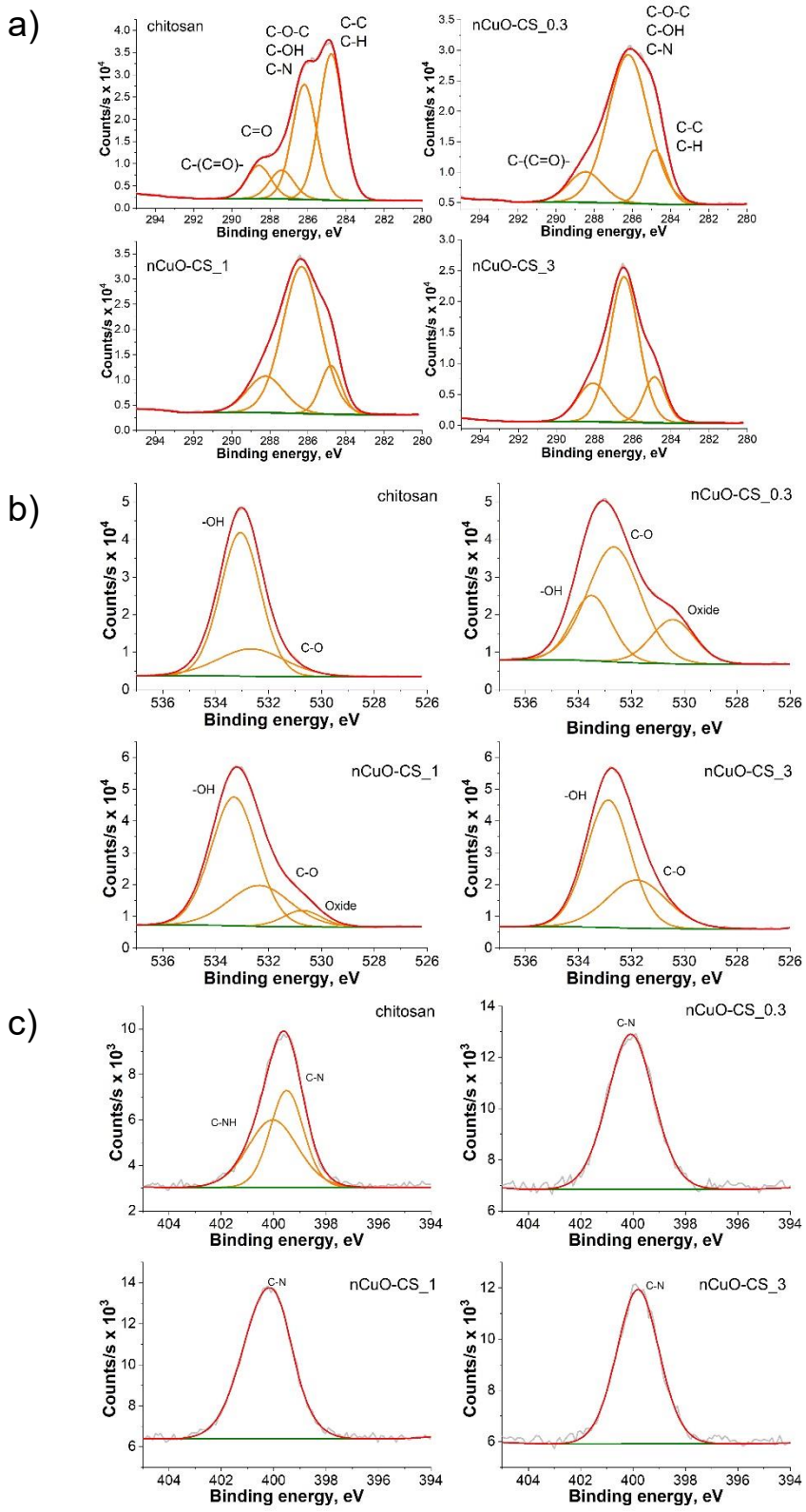


**Figure S3.** X-ray photoelectron spectroscopy narrow scan spectra of chitosan-copper oxide nanocomposites (nCuO-CS). (a) Carbon 1s, (b), oxygen 1s, (c) nitrogen 1s. nCuO-CS_0.3, nCuO_CS_1 and nCuO-CS_3 designate nanocomposites with different Cu:chitosan mass ratios (1:0.3, 1:1 and 1:3, respectively).

**Table S3.** Minimum biocidal concentration (MBC) of chitosan-copper oxide nanocomposites (nCuO-CSs). nCuO-CS_0.3, nCuO_CS_1 and nCuO-CS_3 designate nanocomposites with different Cu:chitosan mass ratios (1:0.3, 1:1 and 1:3, respectively), nCuO refers to commercially available CuO nanoparticles (Sigma-Aldrich, St. Louis, MO, USA) and $Cu^{2+}$ to copper(II) acetate. Low molecular weight chitosan (50–190 kDa, 75–85% deacetylated) was used for additional comparison.

| Bacteria | MBC, mg Cu/L | 1 h | SD | 4 h | SD | 24 h | SD |
|---|---|---|---|---|---|---|---|
| *E. coli* | nCuO-CS_0.3 | 0.96 | 0.10 | 0.29 | 0.10 | 0.24 | 0.03 |
| ATCC 25922 | nCuO-CS_1 | 0.83 | 0.20 | 0.31 | 0.10 | 0.22 | 0.05 |
| (G-) | nCuO-CS_3 | 0.75 | 0.27 | 0.25 | 0.00 | 0.17 | 0.05 |
| | $Cu^{2+}$ | 1.67 | 0.52 | 0.46 | 0.10 | 0.32 | 0.11 |
| | nCuO | 160 | 0.00 | 10.0 | 4.71 | 1.04 | 0.29 |
| | chitosan | 1.42 | 0.60 | 1.02 | 0.26 | 0.92 | 0.25 |
| Bacteria | MBC, mg Cu/L | 1 h | | 4 h | | 24 h | |
| *E.coli* | nCuO-CS_0.3 | 0.50 | 0.00 | 0.30 | 0.11 | 0.21 | 0.06 |
| MG1655 | nCuO-CS_1 | 0.50 | 0.00 | 0.25 | 0.09 | 0.16 | 0.06 |
| (G-) | nCuO-CS_3 | 0.24 | 0.02 | 0.18 | 0.05 | 0.12 | 0.04 |
| | $Cu^{2+}$ | 0.90 | 0.14 | 0.35 | 0.14 | 0.24 | 0.09 |
| | nCuO | 160 | 0.00 | 10.42 | 5.10 | 2.86 | 1.35 |
| | chitosan | 1.56 | 0.66 | 1.10 | 0.14 | 1.10 | 0.14 |
| Bacteria | MBC, mg Cu/L | 1 h | | 4 h | | 24 h | |
| *P. aeruginosa* | nCuO-CS_0.3 | 1.50 | 0.58 | 0.44 | 0.13 | 0.25 | 0.00 |
| ATCC 27853 | nCuO-CS_1 | 1.00 | 0.00 | 0.44 | 0.13 | 0.25 | 0.00 |
| (G-) | nCuO-CS_3 | 0.63 | 0.14 | 0.38 | 0.14 | 0.25 | 0.00 |
| | $Cu^{2+}$ | 3.25 | 0.96 | 0.63 | 0.25 | 0.25 | 0.00 |
| | nCuO | >250 | | 24.24 | 3.68 | 1.39 | 0.27 |
| | chitosan | 3.00 | 1.41 | 2.00 | 0.00 | 2.00 | 0.00 |
| Bacteria | MBC, mg Cu/L | 1 h | | 4 h | | 24 h | |
| *S. aureus* | nCuO-CS_0.3 | 14.0 | 2.31 | 0.25 | 0.00 | 0.15 | 0.04 |
| ATCC 6538 | nCuO-CS_1 | 16.0 | 0.00 | 0.43 | 0.28 | 0.15 | 0.04 |
| (G+) | nCuO-CS_3 | 16.0 | 0.00 | 0.53 | 0.16 | 0.22 | 0.06 |
| | $Cu^{2+}$ | 8.00 | 3.27 | 0.47 | 0.06 | 0.22 | 0.04 |
| | nCuO | >250 | | 6.51 | 0.00 | 0.98 | 0.00 |
| | chitosan | >500 | | >500 | | 250 | 0.00 |

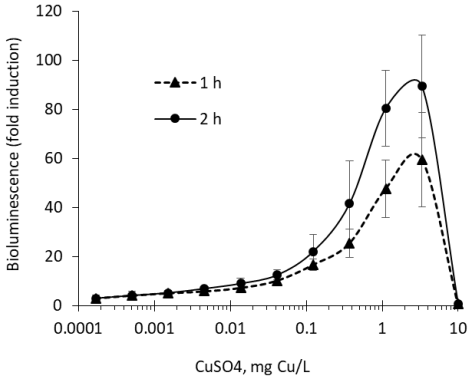


**Figure S4**. Induction (fold) of Cu-sensing bacteria *Escherichia coli* MC1061(pSLcueR/pDNPcopAlux) by $CuSO_4$ after 1 and 2 h incubation at 30°C in the medium composed of 20 mM MOPS (pH 6.5), 0.05% acid hydrolyzed casein, and 0.05% glucose.

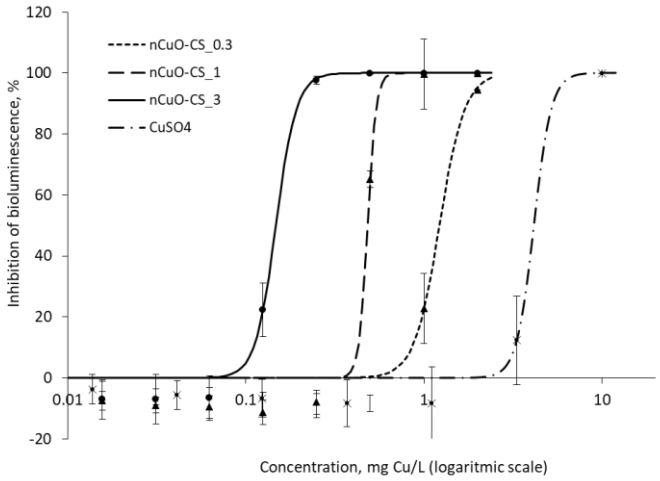


**Figure S5.** Inhibition of bioluminescence (% of control) of *Escherichia coli* MC1061(pDNlux) (control bacteria) by chitosan-copper oxide nanocomposites with mass ratios of 1:0.3 (nCuO-CS_0.3), 1:1 (nCuO-CS_1), 1:3 (nCuO-CS_3), and $CuSO_4$ after 2-h incubation at 30°C in the medium composed of 20 mM MOPS (pH 6.5), 0.05% acid hydrolyzed casein, and 0.05% glucose.

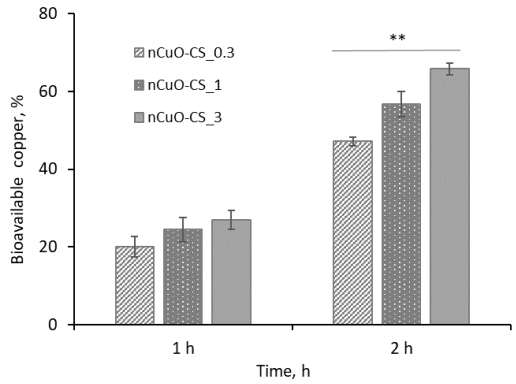


**Figure S6.** Bioavailable (intracellular) copper from chitosan-copper oxide nanocomposites (nCuO-CS) at a concentration of 0.016 mg Cu/L after 1 and 2 h exposure. Bioavailable copper was measured by Cu-inducable bacteria *Escherichia coli* MC1061(pSLcueR/pDNPcopAlux) at 30°C in the induction medium composed of 20 mM MOPS (pH 6.5), 0.05% acid-hydrolyzed casein, and 0.05% glucose. nCuO-CS_0.3, nCuO_CS_1 and nCuO-CS_3 designate nanocomposites with different Cu:chitosan mass ratios (1:0.3, 1:1 and 1:3, respectively). Asterisks designate statistically significant differences, ** $p < 0.01$ (calculated by t-test in Microsoft Excel 2019).

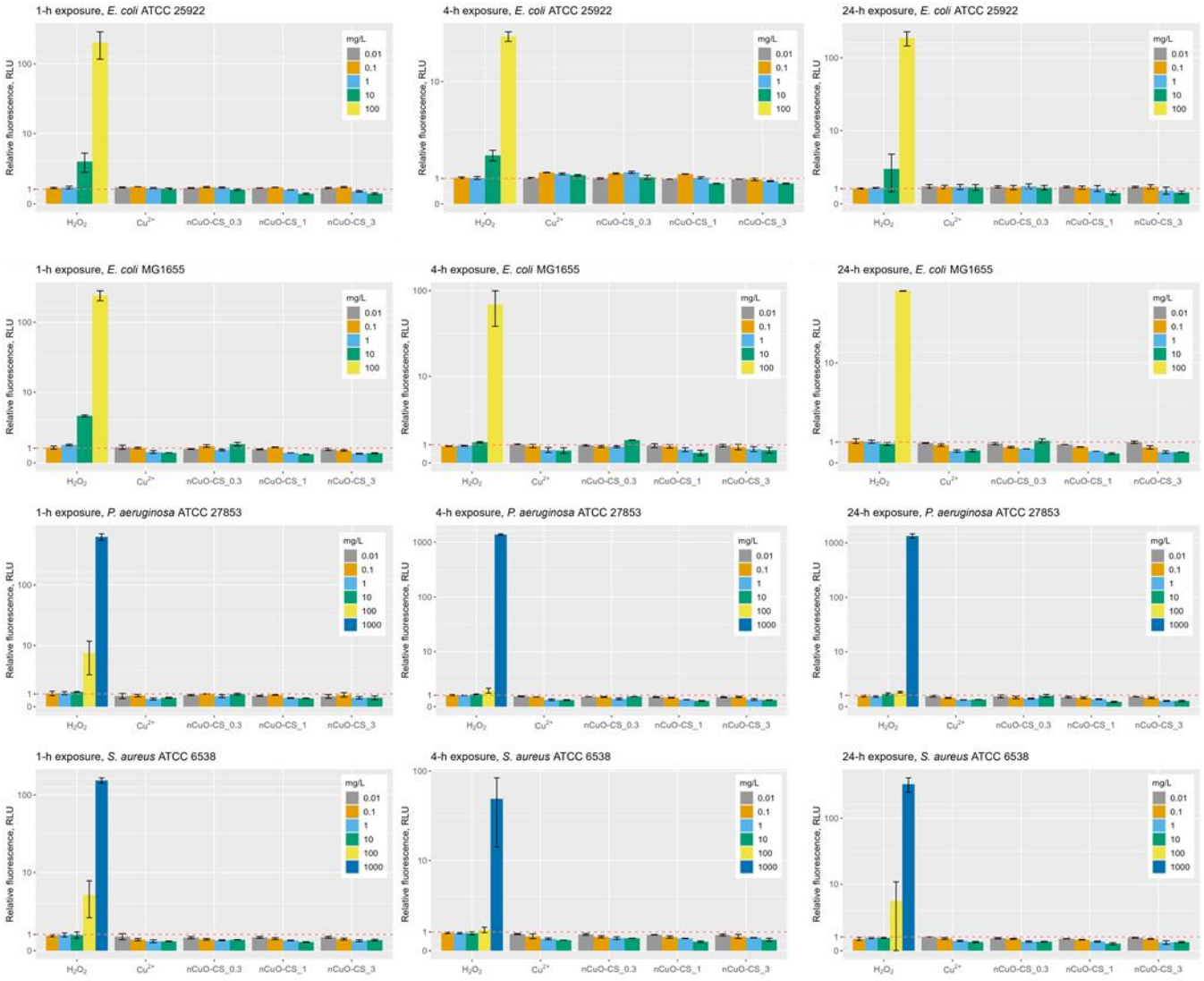


**Figure S7.** Generation of reactive oxygen species (ROS) by chitosan-copper oxide nanocomposites (nCuO-CS). The values are based on the increase in fluorescence in the presence of nCuO-CSs compared to the negative control (deionized water), hydrogen peroxide ($H_2O_2$) was used as a positive control, the mean of two experiments with standard deviation is presented, the dashed line represents ROS level in deionized water. nCuO-CS_0.3, nCuO_CS_1 and nCuO-CS_3 designate nanocomposites with different Cu:chitosan mass ratios (1:0.3, 1:1 and 1:3, respectively).

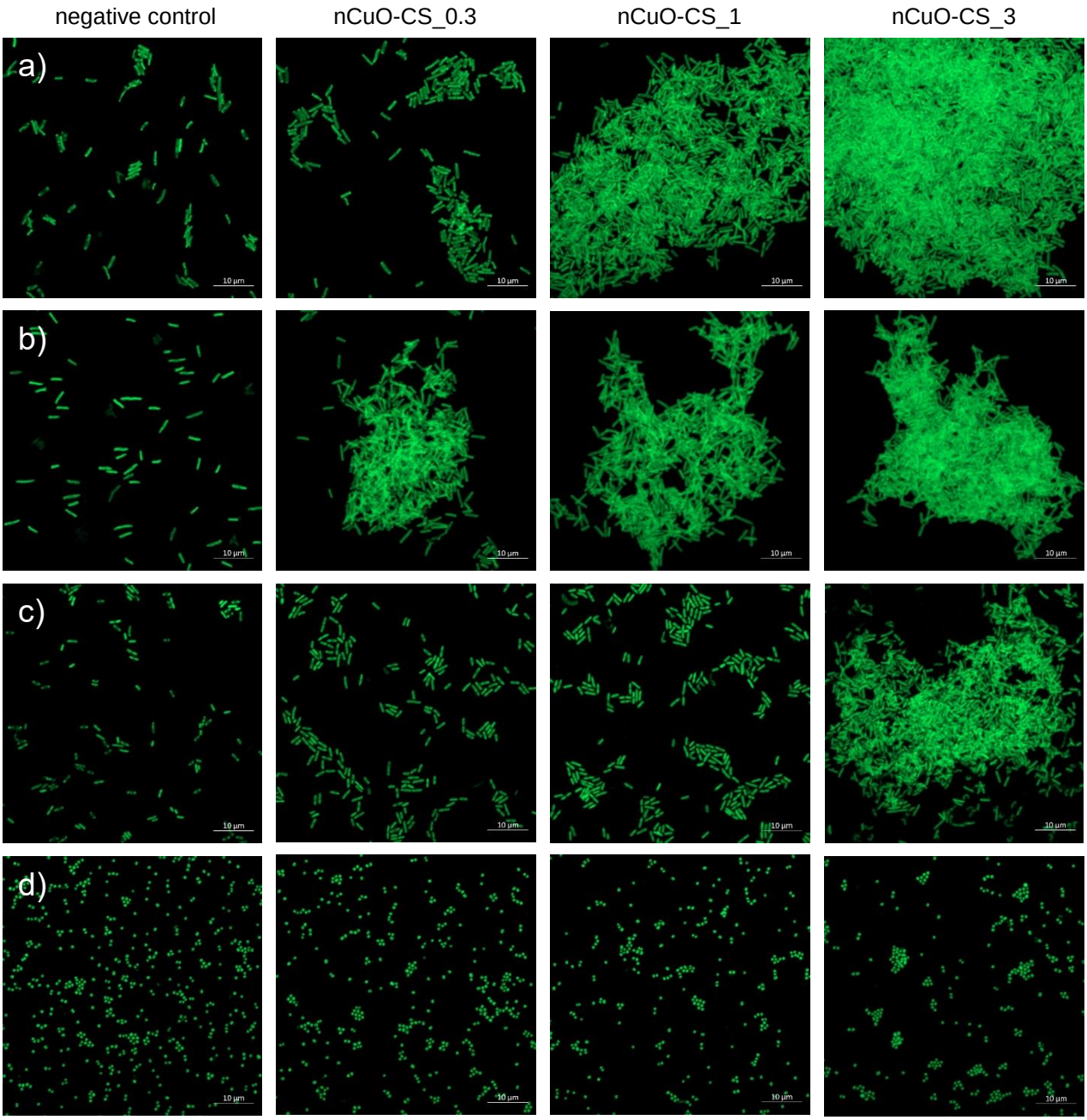


**Figure S8.** Confocal laser scanning microscopy images of bacterial cells. (a) *Escherichia coli* ATCC 25922, (b) *E. coli* MG1655, (c) *Pseudomonas aeruginosa* ATCC 27853, and (d) *Staphylococcus aureus* ATCC 6538 were exposed to chitosan-copper oxide nanocomposites (nCuO-CS) in deionized water at a concentration of 1 mg Cu/L for 1 h at 30°C in the dark. nCuO-CS_0.3, nCuO-CS_1, and nCuO-CS_3 designate nanocomposites with different Cu:chitosan mass ratios (1:0.3, 1:1, and 1:3, respectively). All scale bars are 10 µm.

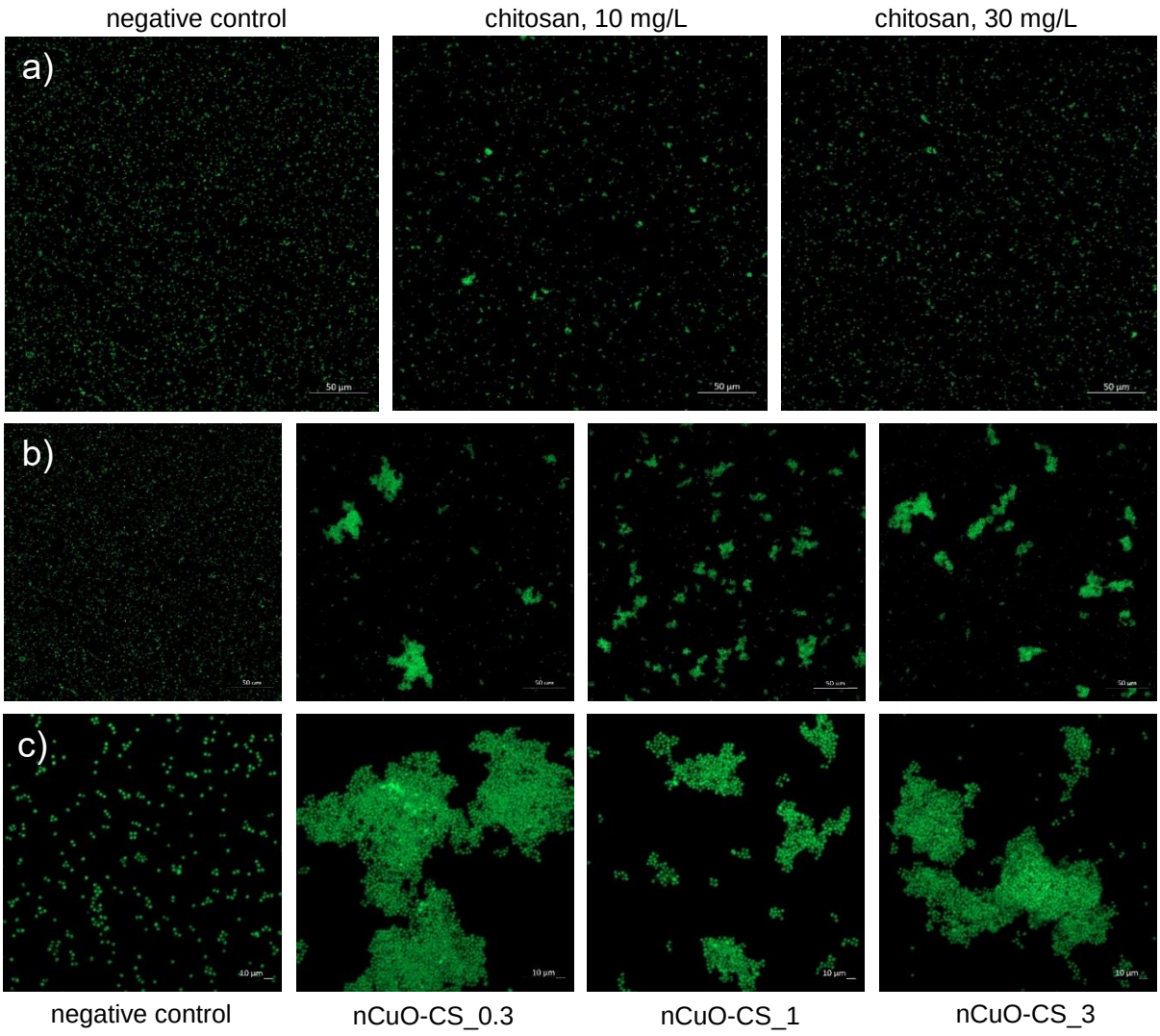


**Figure S9.** Confocal laser scanning microscopy images of the Gram-positive *Staphylococcus aureus* ATCC 6538. (a) *S. aureus* was exposed to low molecular weight chitosan at two concentrations – 10 and 30 mg/L – and (b), (c) to chitosan-copper oxide nanocomposites (nCuO-CS) in deionized water at a concentration of 10 mg Cu/L for 1 h at 30°C in the dark. nCuO-CS_0.3, nCuO-CS_1, and nCuO-CS_3 designate nanocomposites with different Cu:chitosan mass ratios (1:0.3, 1:1, and 1:3, respectively). The scale bars are 50 (a, b) or 10 µm (c).

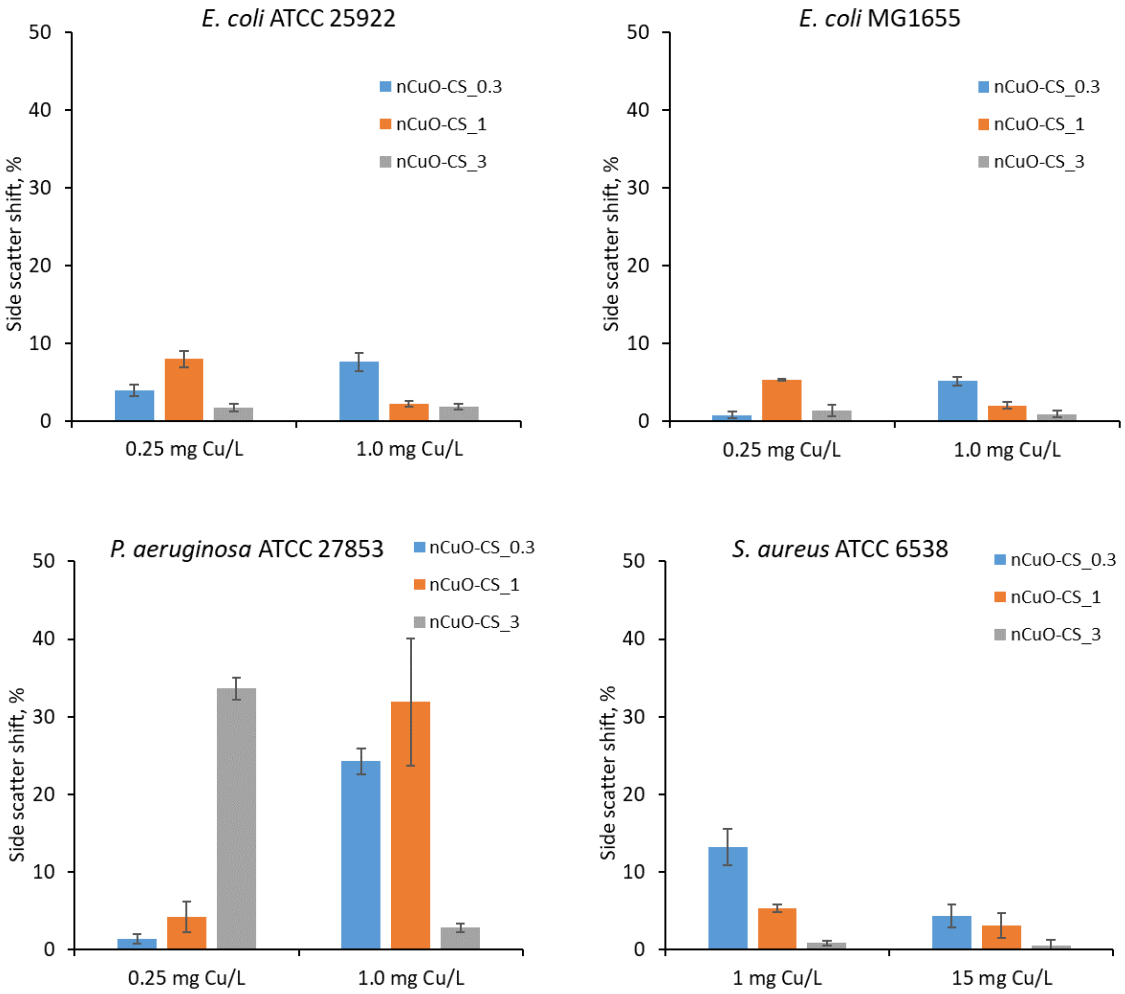


**Figure S10.** Flow cytometry studies of bacteria-nanocomposite interactions. *Escherichia coli* ATCC 25922, *E. coli* MG1655, and *Pseudomonas aeruginosa* ATCC 27853 were exposed to chitosan-copper oxide nanocomposites (nCuO-CS) in deionized water at two concentrations – 0.25 and 1 mg Cu/L – for 1 h at 30°C in the dark; *Staphylococcus aureus* ATCC 6538 was similarly exposed but at concentrations of 1 and 15 mg Cu/L. 20 000 cells were analyzed from each sample, and interactions between bacteria and nCuO-CSs attributed to the increase in side scattered light intensity compared to the (unexposed) control cells. nCuO-CS_0.3, nCuO-CS_1, and nCuO-CS_3 designate nanocomposites with different Cu:chitosan mass ratios (1:0.3, 1:1, and 1:3, respectively).

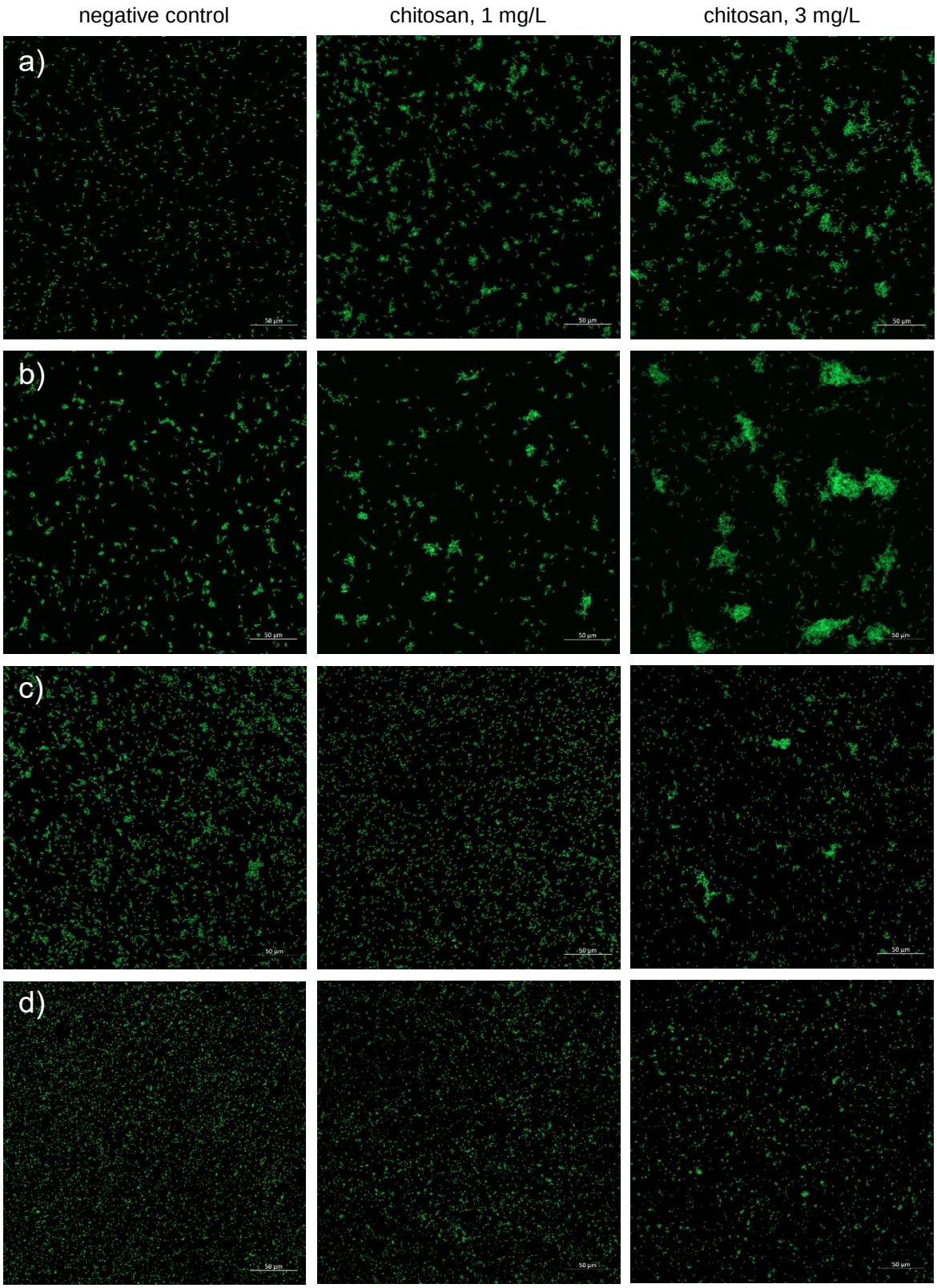


**Figure S11.** Confocal laser scanning microscopy images of bacterial cells. (a) *Escherichia coli* ATCC 25922, (b) *E. coli* MG1655, (c) *Pseudomonas aeruginosa* ATCC 27853, and (d) *Staphylococcus aureus* ATCC 6538 were exposed to low molecular weight chitosan at two concentrations – 1 and 3 mg/L – for 1 h at 30°C in the dark. All scale bars are 50 µm.

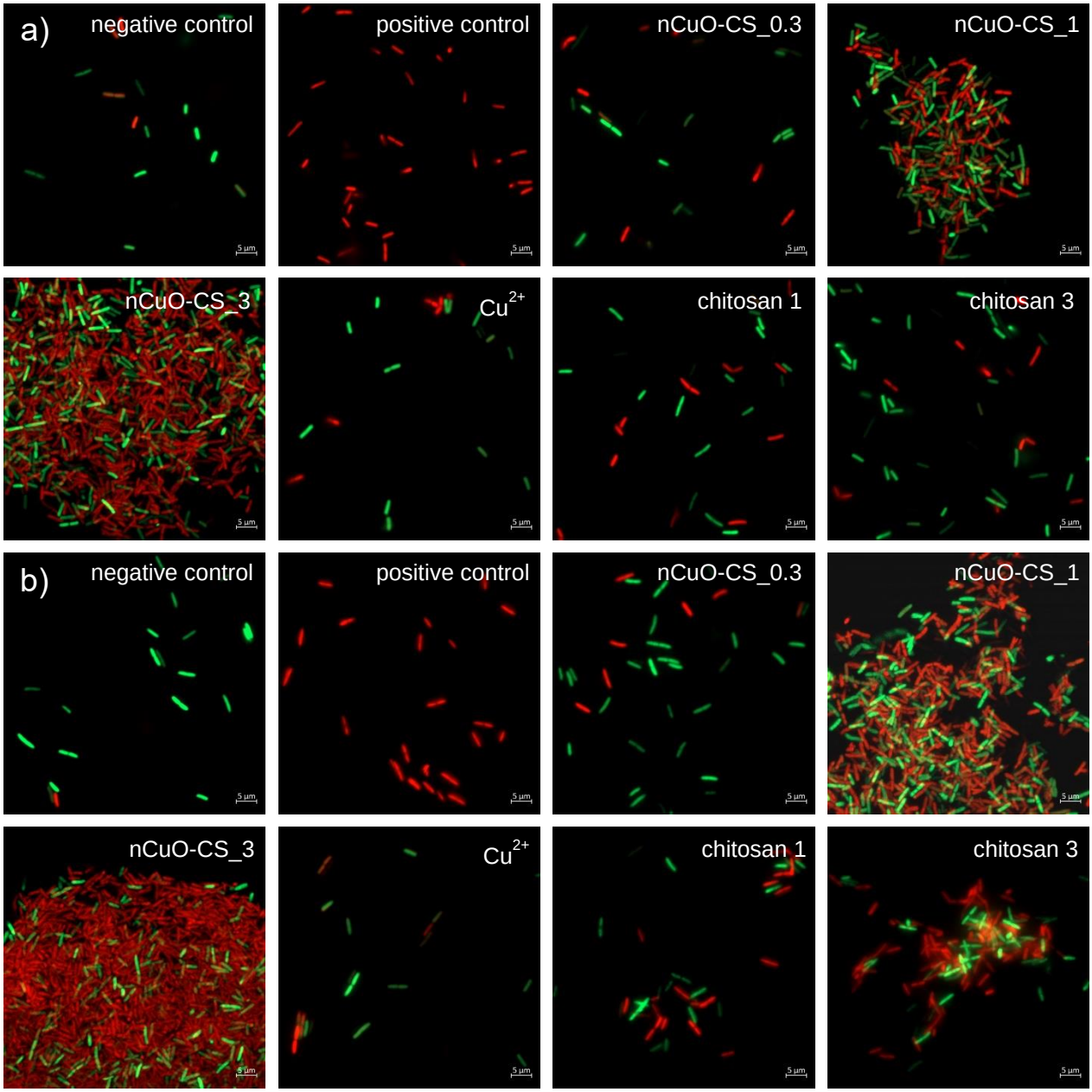


**Figure S12.** Microscopy images of bacterial cells. (a) *Escherichia coli* ATCC 25922 and (b) *E. coli* MG1655 were exposed to chitosan-copper oxide nanocomposites (nCuO-CS) in deionized water at a concentration of 1 mg Cu/L for 1 h at 30°C in the dark. nCuO-CS_0.3, nCuO-CS_1, and nCuO-CS_3 designate nanocomposites with different Cu:chitosan mass ratios (1:0.3, 1:1, and 1:3, respectively). A 0.1% (w/vol) sodium dodecyl sulfate solution was used as a positive control for membrane-compromised cells. Copper(II) acetate (1 mg Cu/L) and low molecular weight chitosan (1 and 3 mg/L) were analyzed for comparison. After exposure, the cells were stained with propidium iodide and SYTO 9. All scale bars are 5 µm.

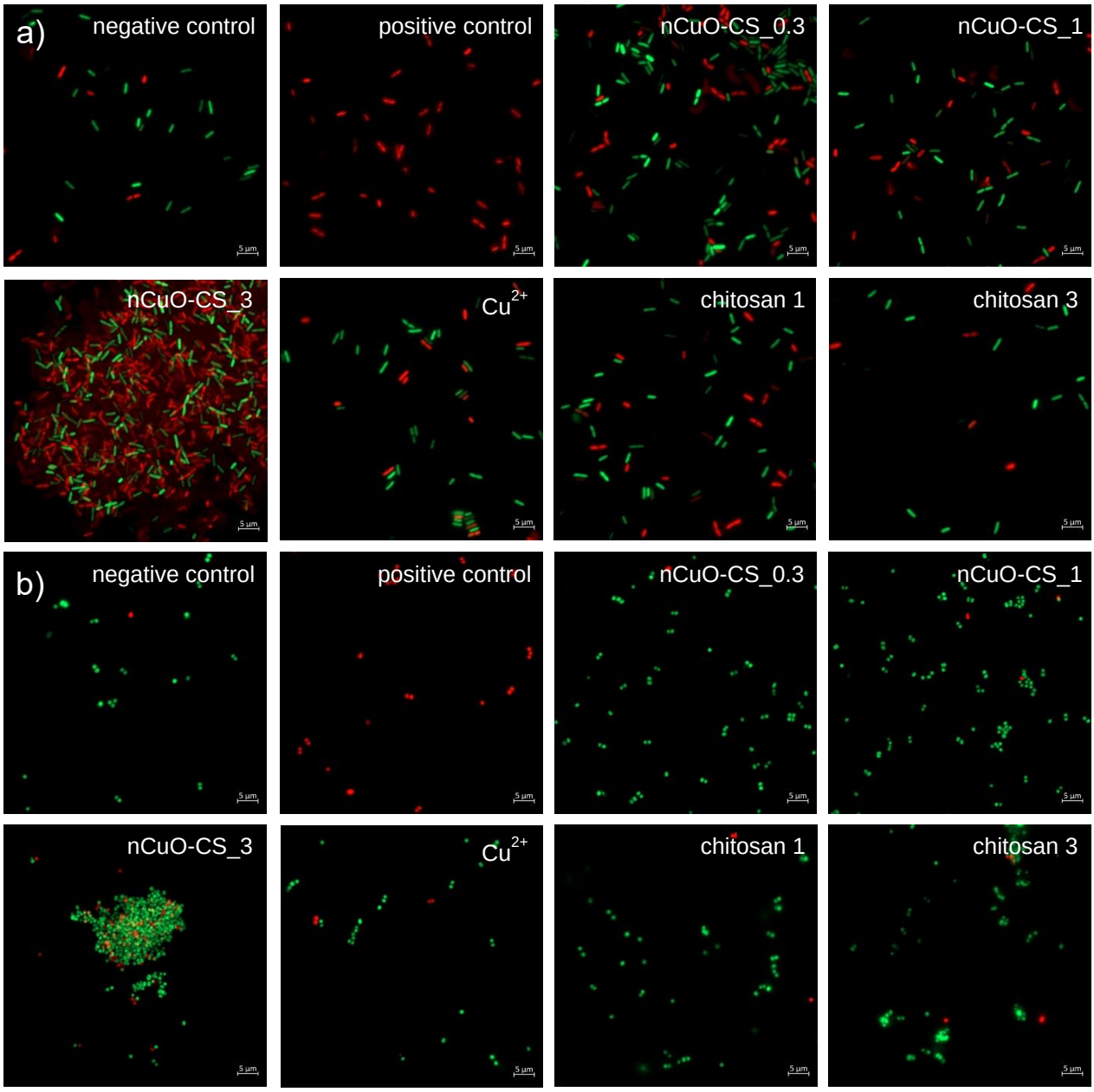


**Figure S13.** Microscopy images of bacterial cells. (a) *Pseudomonas aeruginosa* ATCC 27853 and (b) *Staphylococcus aureus* 6538 were exposed to chitosan-copper oxide nanocomposites (nCuO-CS) in deionized water at a concentration of 1 mg Cu/L for 1 h at 30°C in the dark. nCuO-CS_0.3, nCuO-CS_1, and nCuO-CS_3 designate nanocomposites with different Cu:chitosan mass ratios (1:0.3, 1:1, and 1:3, respectively). A 0.1% (w/vol) sodium dodecyl sulfate solution was used as a positive control for membrane-compromised cells. Copper(II) acetate (1 mg Cu/L) and low molecular weight chitosan (1 and 3 mg/L) were analyzed for comparison. After exposure, the cells were stained with propidium iodide and SYTO 9. All scale bars are 5 µm.