S.4.1 ROS and Live/Dead assay test

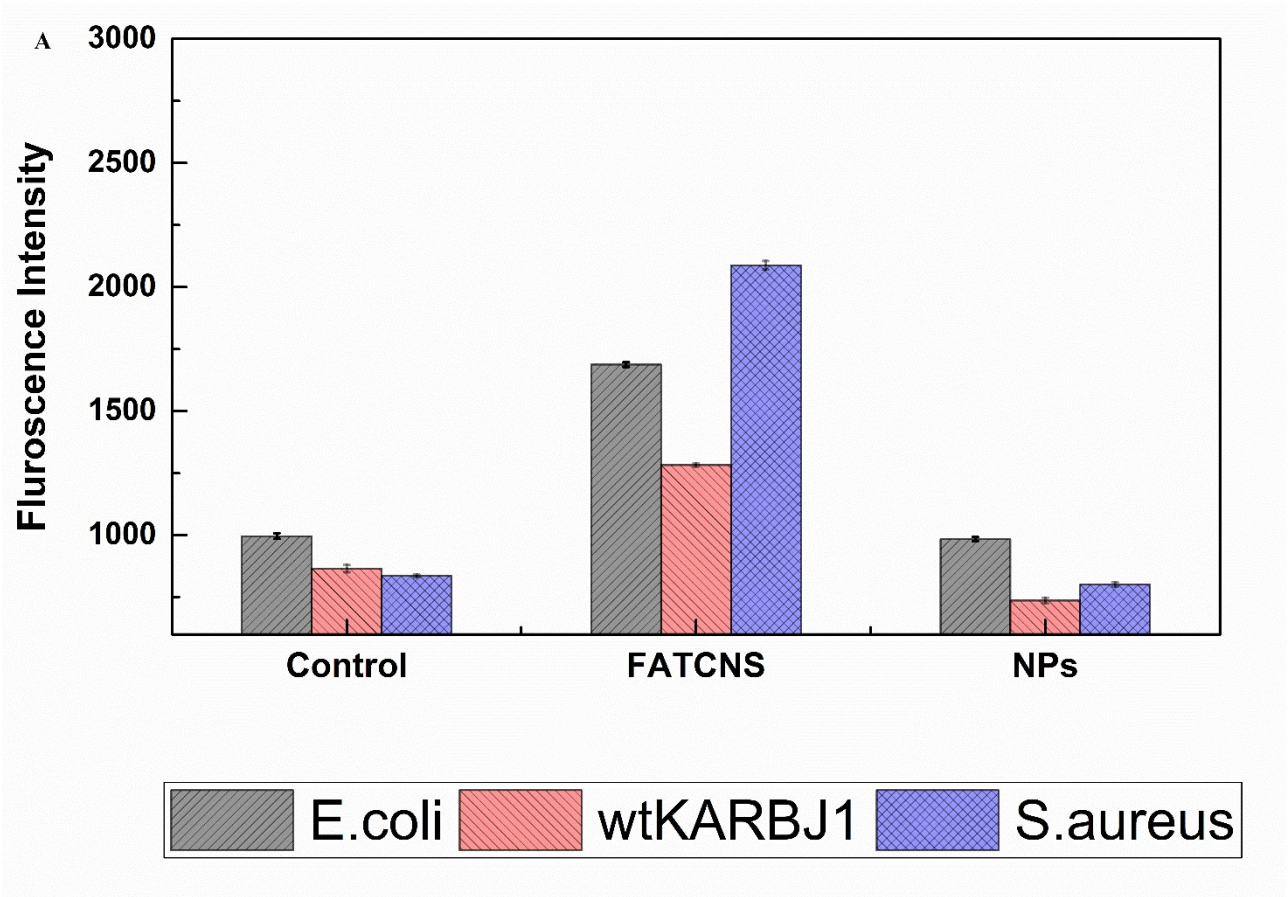
Experimental

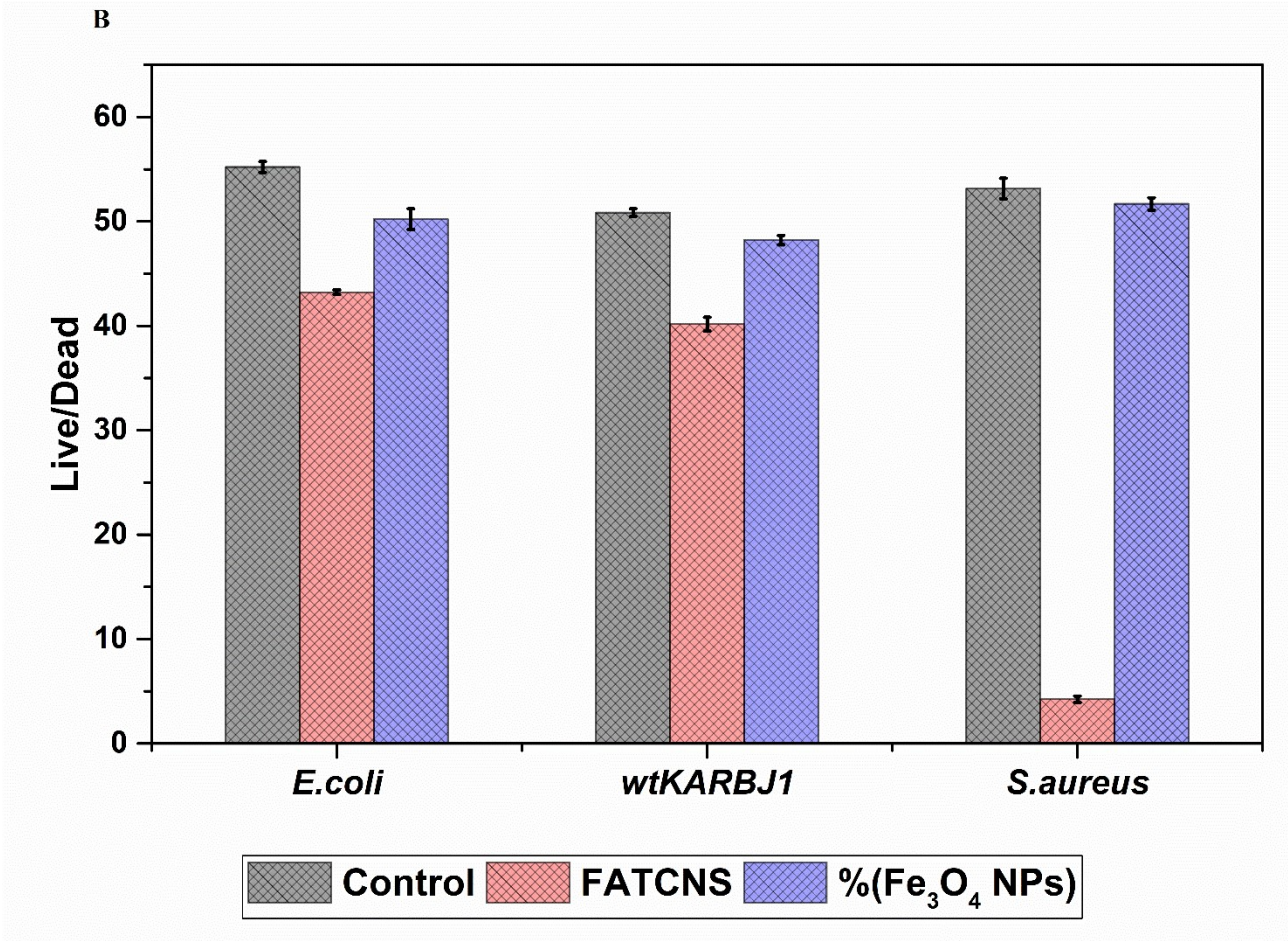
The production of oxidative stress by bacteria after treatment with (sub-MIC) FATCNS and (3000 μg ml-1) Fe_3O_4 NPs was evaluated using, 2′-7′-Dichlorofluorescein (DCF) (Sigma-Aldrich). DCF is a peroxynitrite indicator which can detect ROS.[1] The bacteria were incubated with NSs and NPs in presence of DCFH-DA at a final concentration of 5 μM in Tris and incubated at 37 °C for 30 min and compared with control. The ROS activity was measured using fluorescence intensities of DCF emission at λex 504 nm; λem 529 nm. Subsequently, after 15 min and 30 min of incubation of bacteria with (sub-MIC) FATCNS and (3000 μg ml^{-1}) Fe_3O_4 NPs, 100 μL of the bacterial-NAs suspension was transferred into a 96-well plate. A Live/Dead assay was performed to determine the rapidity of the effect of NSs in bacteria, and the procedure was followed according to the manufacturer's instructions (Live/Dead, L7007; Invitrogen). Briefly, two solutions with a specific SYTO 9 dye and propidium iodide (PI) were added to a bacterial solution in 100 μL per well. The plate was incubated in the dark for 15 min at room temperature, as per the manufacturer's instructions. Fluorescence intensities for live cells (excitation: 485 nm, emission: 530 nm) and dead cells (excitation: 485 nm, emission: 630 nm) were measured using a micro-plate reader. The ratio of Live/Dead bacteria after the treatment of FATCNS was determined using fluorescence intensities.

Results

It is known that higher the concentration of NAs in bacterial suspension and increase in contact time between bacteria and NAs influence the bactericidal and removal efficiency. The bactericidal effect of FATCNS in bacteria is due to increase in the cellular levels of ROS causing cell death. It is known that the DCF readily crosses the cell membrane along with the NAs through passive diffusion followed by deacetylation. This deacylated product is further oxidized to form fluorescent DCF, which is measured.[1] After 30 min of NAs and bacteria incubation, the ROS assay results (Figure S 4.1.A) confirm that the ROS production in *bacteria* is enhanced and higher when treated with FATCNS compared to control and NPs. This suggests that the ROS production has an indirect effect on the growth of *bacteria and one of the caused cellular death. Further*, the higher fluorescent intensity is observed in *S.aureus* compared to other two bacteria sugessting that the interaction of NSs with S.aureus is rapid and strong which was confirmed by Live/Dead assay results.

After 30 min of NAs and bacteria incubation, the Live/Dead assay results (Figure S 4.1. B) confirm that the ratio of Live/Dead bacteria is significantly lower compared to control even when the sub-MIC of FATCNS (sub MIC for *E.coli*- 50 µg (10 ml^{-1}), wtKARBJ1- 110 µg (10 ml^{-1}) and *S.aureus* 30 µg (10 ml^{-1})) is used in the assay. On the other hand, there is hardly any effect of NPs (3000 µg ml^{-1}) on the Live/Dead ratio of bacteria after 30 min of incubation time. However, for *S.aureus*, the ratio drastically decreased after 15 min of incubation time compared to other two bacteria, that is, interaction time required for NSs to inactivate *S.aureus* is less than other two bacteria as seen in Figure S 4.1.C. This may indicate that FATCNS may be selective towards *S.aureus*, Gram-positive bacteria. Overall, the results suggest that the FATCNS reacts with the intracellular oxygen in bacteria, leading to oxidative stress and cell membrane disruption. Moreover, FATCNS is expeditious in inactivating *S.aureus*, Gram-positive bacteria even at sub-MIC.




B
Live/Dead
60
50
40
30
20
10
0
E.coli
wtKARBJ1
S.aureus
Control
FATCNS
%(Fe3O4 NPs)

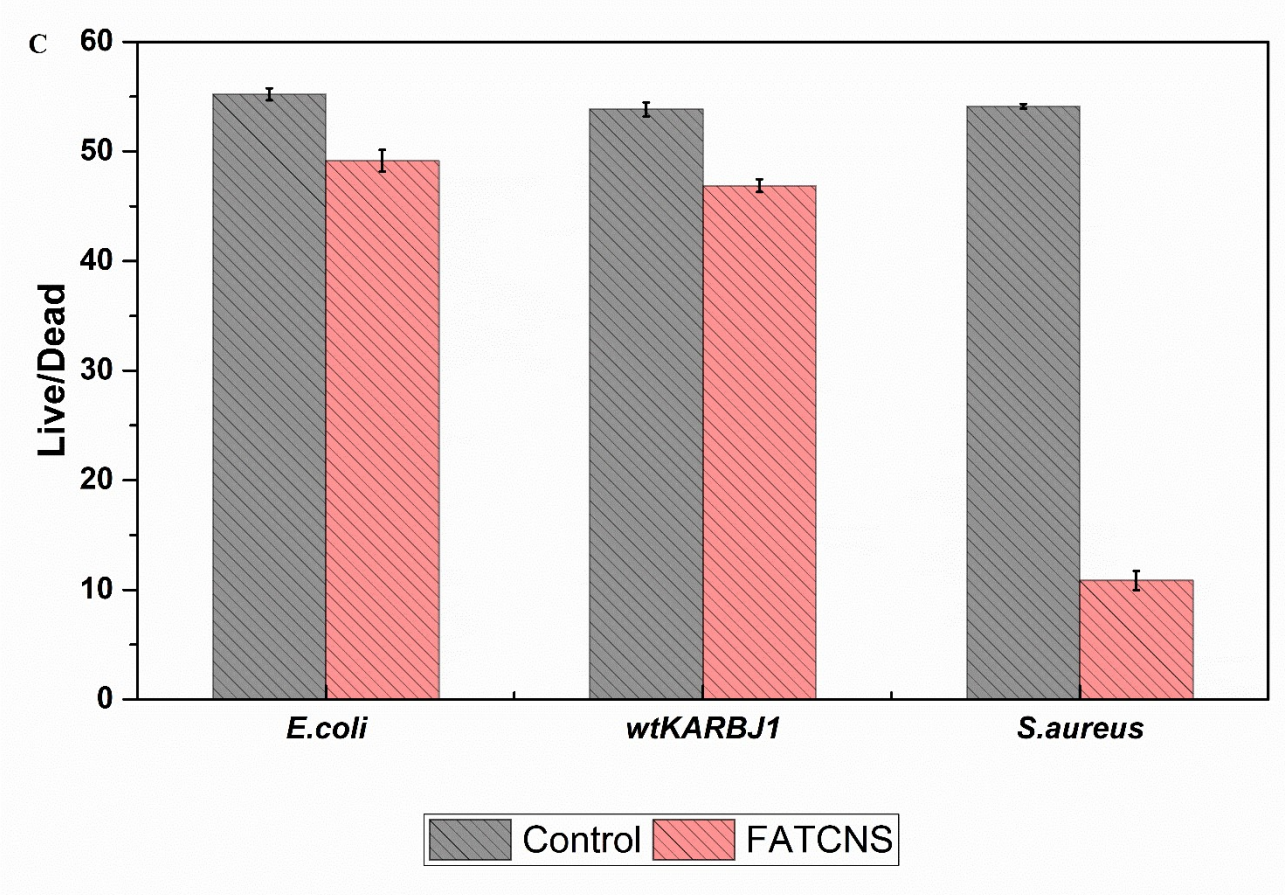


Figure S 4.1. (A) Quantitation of intracellular ROS production bacteria with FATCNS and Fe_3O_4 NPs after 30 min treatment. Results are mean fluorescence intensity ± SD (n = 3). Live/Dead assay results show lower Live/Dead ratios in the presence of (B) the sub-MIC of FATCNS and Fe_3O_4 NPs (3000 µg ml-1) solution after 30 min incubation and (C) the sub-MIC of FATCNS after 15 min incubation with SYTO 9 dye and propidium iodide (PI). Data = mean ± SD; n = 3. *P <0.05 compared with control sample.

S 4.2. Reusability of FATCNS

Experimental

The characterization of the NSs after performing the reusability tests of FATCNS for ten cycles.

Results

The FATCNS show remarkable reusability even after ten cycles of bacterial inactivation experiments. The removal capacity of FATCNS was nearly 100% for the bacteria. SI-4 Figure 2 show the FESEM images and XRD graphs of NAs after the repeatable cycles. The FESEM image in SI- 4 Figure 2 A shows aggregation of the NPs while the XRD measurements show

denaturation of the peaks after the 2nd use. However, SI- 4 Figure 2 B depicts no change in the FESEM image and the XRD spectrum of the NSs recovered after the 10th cycle.

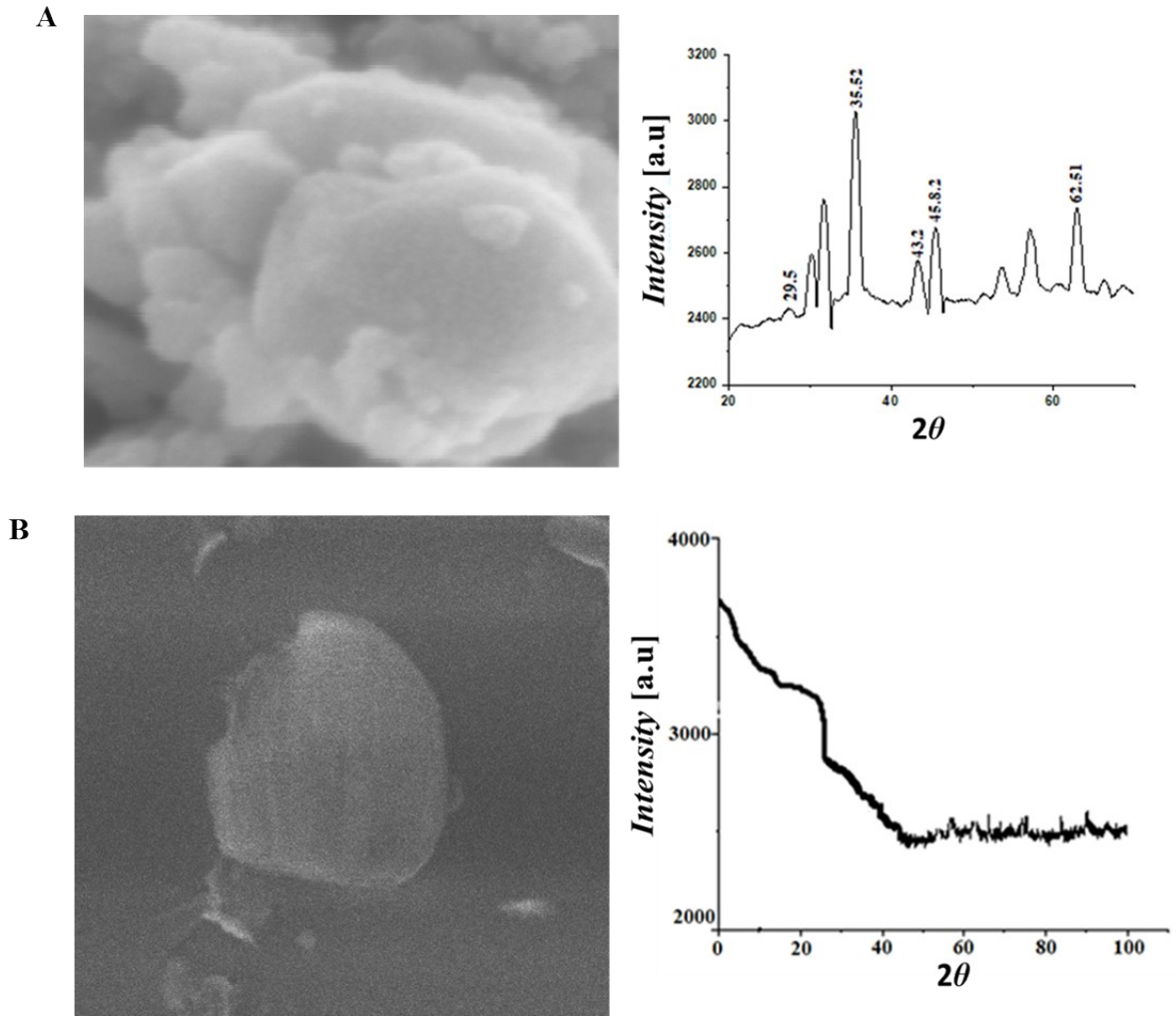


Figure. S 4. 2. (A) FESEM image and XRD spectrum of Fe_3O_4 NPs after the 2nd reusability cycle. (B) FESEM image and XRD spectrum of FATCNS after the 10th cycle of the reusability test against *S.aureus*.

Table S 4. 1. Reusability of FATCNSs and Fe_3O_4 NPs at different cycles.

Removal Efficiency (%)	4th cycle	6th cycle	10th cycle
River water + NSs	98	96	96
Kitchen wastewater + NSs	94	93	93
Tap water + NSs	95	93	90
River water + NPs	26	25	25
Kitchen wastewater + NPs	35	32	29
Tap water + NPs	30	29	28

References

(1) Miura, M.; Murai, N.; Hattori, T.; Nagano, T.; Stuyvers, B. D.; Shindoh, C. Role of Reactive Oxygen Species and Ca2 + Dissociation from the Myofilaments in Determination of Ca2 + Wave Propagation in Rat Cardiac Muscle. *J Mol Cell Cardiol* 2013, **56 (1)**, 97–105.