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Supplementary Information

Tailored Lysozyme-ZnO Nanoparticle Conjugates as Nanoantibiotic

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1. Experimental details

1.1. Materials

Zinc acetate dehydrate, hexamethylenetetramine (HMTA), lithium hydroxide (LiOH), phosphate-buffered saline (PBS), 3-aminopropyltriethoxysilane (APTES) and tetraethyl orthosilicate (TEOS), glutaraldehyde and hen egg lysozyme were purchased from Sigma Aldrich (St Louise, MO, USA). Kit used for viability test i.e. Live/Dead BacLight kit is purchased from Invitrogen (Carlsbad, CA, USA).

1.2. Synthesis of lysozyme-ZNPs conjugates

To synthesize ZNPs, zinc acetate dehydrate (0.1 M) and HMTA (0.05 M) was dissolved in distilled water (50 mL) with pH = 10 adjusted by 1M LiOH, then transferred into pyrex glass bottle and heated for 3 h at 80 ^{o}C. After completion of reaction, the products were washed, air dried and characterized.[1]

Firstly, aminated-ZNPs were synthesized by dispersing ZNPs (0.1 g) in 50 mL ethanol followed by addition of TEOS (15 μL), APTES (60 μL) (1:4 ratio) and stirred at room temperature for 3 h. Then, the product was washed with water and redispersed in PBS, to which 500μL glutaraldehyde was added and continuously stirred at room temperature for 1 h. In order to remove the unreacted glutaraldehyde, the product was washed with PBS several times. Finally, the L-ZNPs conjugates was prepared by adding a fixed amount of aminated-ZNPs to lysozyme solution prepared in PBS (pH= 6.0) with the weight ratio of lysozyme/ZNPs at 1:1, 10:1, 20:1, 30:1, 40:1, and 50:1, respectively. These mixtures were incubated overnight at room temperature with gentle stirring in dark, rinsed with PBS to remove unbound lysozyme and stored at 4 °C.

The morphology, particle size, surface groups and surface charge were analyzed by using transmission electron microscopy (TEM), field emission scanning electron microscopy (FESEM), Fourier transform infrared spectroscopy (FTIR), and zeta potential by a zeta potentiometer (Malvern Zeta Sizer 2000), respectively. Thermogravimetric analysis (TGA) was performed by TGA Q50 thermal analyzer in a flowing air atmosphere at the temperature range of 25-700 ^{o}C with a heating rate of 20 ^{o}C per min in air. The amount of loaded lysozyme was quantified by using UV-vis spectrophotometer (2550-Shimadzu, Japan). The amount of

immobilized lysozyme was calculated by deducting the lysozyme amount in the supernatant after adsorption from the known initial lysozyme concentrations.

The conjugation degree of lysozyme was also calculated using the following equation:

$$Lysozyme\ Conjugation\ Degree\ (\%) = 100 \times \frac{Total\ weight\ of\ conjuagted\ lysozyme\ to\ ZNPs}{Total\ lysozyme\ used\ for\ conjugation}$$

In order to quantify the released Zn ions, 200 µg/mL of each ZNPs and L-ZNPs were dispersed in acidic (pH = 5) and neutral (pH = 7.4) buffer solutions for 24 h at room temperature, followed by centrifugation of 1 mL aliquot and re-suspension (1:40, v/v) in DI water. Prior to analysis, nitric acid was added to a final concentration of 1% and the resulting Zinc ion concentration was measured using ICP-MS (SCIEX ELAN 5000 ICPMS, Perkin-Elmer, USA).

1.3. Antibacterial Tests

Antibacterial tests were performed both in liquid media and on solid agar plates with various concentrations of native lysozyme, pristine ZNPs and L-ZNPs conjugates concentrations ranging from 0-250 µg/mL. Prior to toxicology tests, *E. coli* (Gram-) and *S. aureus* (Gram+) strains were cultured in 100 mL nutrient broth medium and maintained at 37 ^{o}C shaking incubator up to the optical density (OD_{600}) reached 0.7-0.8. To check toxicological properties in liquid media, 0.1 % of inoculum was added to 100 mL freshly prepared nutrient broth medium supplemented with 50 µg/mL L-ZNPs conjugates (20:1 ratio), pristine ZNPs and native lysozyme; and maintained at 37 ^{o}C shaking incubator for 24 h. Further, the growth was monitored for 12 h using spectrophotometer. In order to perform bacteriological tests on solid agar, the total number of cells was further diluted to 10^6 cells/mL in sterilized distilled water and the cell cultures (100 µL) with L-ZNPs conjugates in PBS (900 µL) were spread over solid agar plates followed by incubation for 12 h at 37 ^{o}C. The antibacterial efficiency of the samples was measured by calculating colony-forming units (cfu) per plate (estimated as 200 cfu/per plate). Negative controls were prepared without nanoparticles. For further confirmation, the bacterial cells were exposed to L-ZNPs conjugates (100µg/mL) for 6 h on a shaker incubator (200 rpm) at 37 ^{o}C, followed by staining with LIVE/DEAD BacLight reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. The live and dead images were observed under confocal laser scanning microscopy (CLSM) at $\lambda_{excitation}$ = 488 nm and $\lambda_{emission}$ = 515 nm (LSM

510 META, Carl Zeiss). All the captured images were analyzed with image analysis software (LCS image browser).

1.4. Cytotoxicity Tests

HEK cells (Human Embryonic Kidney 293 cells) obtained from ATCC (Manassas, VA, USA), were cultured in DMEM containing L-glutamine (200 mg/L) supplemented with 10% (v/v) heat-inactivated FBS, 100U/mL penicillin, and 100μg/mL streptomycin, and maintained in a humidified incubator at 37 ^{o}C and 5% CO_2.

MTT assay was employed to measure the cytotoxicity induced by L-ZNPs conjugates. Briefly, cells (10^5/well) were incubated with different concentrations (50, 100, 150, 200, 250 and 300 μg/mL) of samples for 24 h. After the exposure, the medium was replaced with 200 μL/well of solution (0.8 mg/mL MTT in PBS) and incubated at 37 ^{o}C for 4 h. After formazan crystals formation, 200 μL/well acidified isopropanol was added and kept for 30 min at 4 ^{o}C to solubilize the crystals. The reaction product absorbance was then measured at 570 nm using a micro-plate reader (DYNEX Technology, Chantilly, VA, USA).

2. Structural Characterization

2.1. Characterizations of modified-ZNPs

The XRD pattern of pristine ZNPs displays good wurtzite crystallinity indicating that the nanoparticles have a single crystal structure (insert of a, top). The obtained diffraction peaks in the (100), (002), (101), (102), (110), (103), and (112) planes, with higher intensity in the (101) plane suggests the growth of ZNPs along the [0001] direction.

For precise assessment, FTIR spectra and zeta potentials of pristine ZNPs and aminated-ZNPs were studied, shown on Fig 1(c, d). The FTIR spectrum of pristine ZNPs (black line), showing a peak at 430 cm^{-1} is the stretching vibration of the ZnO. A broad absorption bands due to O-H (3420-3440 cm^{-1}) in samples is assigned to stretching vibration of O-H group, which may come from the adsorbed H_2O.[2] The peak at 1390-1410 cm^{-1} is attributed to the symmetric and asymmetric O-C-O stretching vibration of the adsorbed carbonate anion during synthesis of ZNPs.[3] Also, the peaks at 2937 and 2871 cm^{-1} correspond to C-H stretching vibration. In case of aminated-ZNPs (red line), peaks at 1564 and 1657 cm^{-1} are assigned to N-H bending vibrations.[4] Previously, the isoelectric point (IP) of ZnO NPs was reported to be ~9.5 although the value may

vary depending on the synthesis methods and also the presence of some residual acetate ions absorbed on the nanoparticles surfaces.[5] Herein the zeta potential of pristine ZNPs and aminated-ZNPs was compared at different pH values and found to be ~2.98 and ~10.93 at pH = 7, respectively. The isoelectric point of the pristine ZNPs was quite close to the previous study, which also implies that these particles were slightly positively charged. The change in IP of aminated-ZNPs demonstrates that the presence of amine groups on the surface of nanoparticles and a change in the surface electrostatic state of the particles (d). The measure DLS result suggests that the nanoparticles are prone to aggregation in solution (data not shown). Thus, prolonged sonication was performed to obtain mono-dispersed ZNPs aqueous solution.

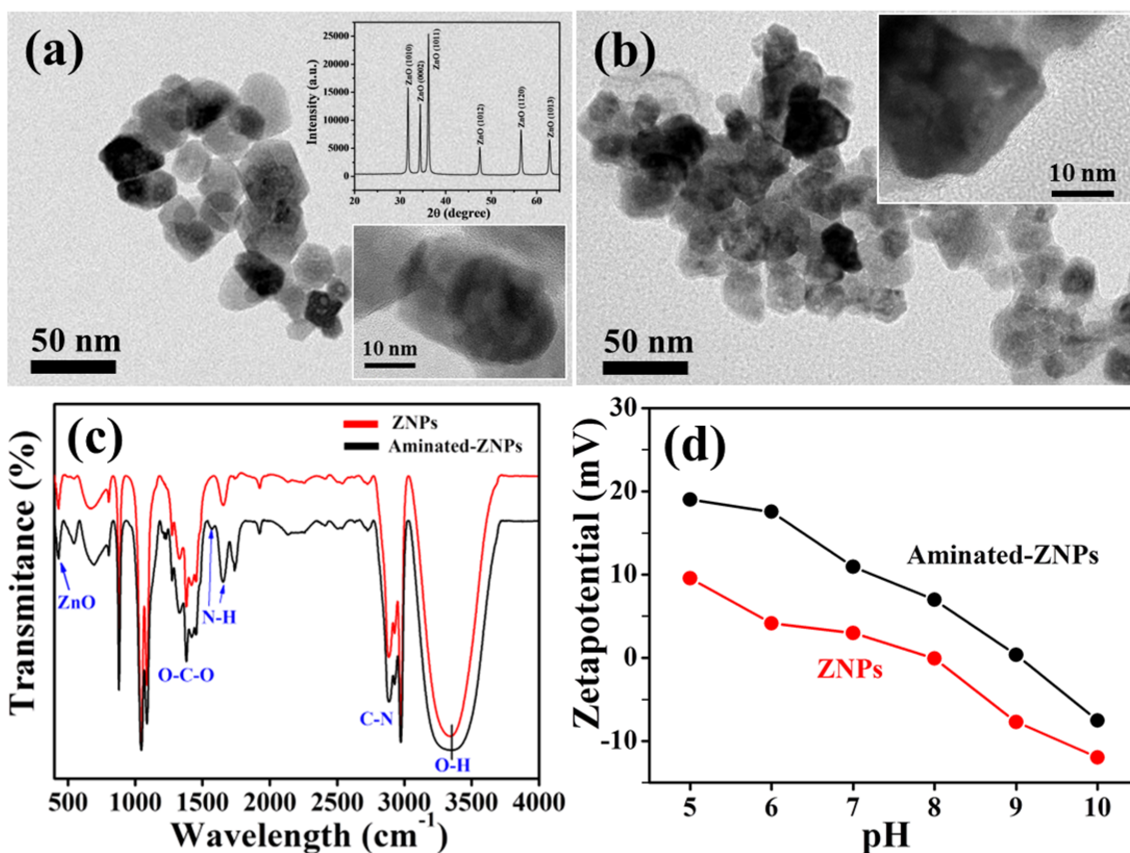

Fig. S1 Typical TEM images of (a) pristine ZNPs and (b) aminated-ZNPs. The insert of (a) shows the XRD pattern of ZNPs (top) and HRTEM image (bottom). The insert of (b) displays the respective HRTEM image. (c) Zeta potential curves. (d) FTIR spectra.

Furthermore, the Zn^{2+} concentration for ZNPs and L-ZNPs (each 200 µg/mL) was evaluated using ICP-MS analysis. At pH = 5, Zn^{2+} concentration for ZNPs and L-ZNPs were 46.2 and 29.9 mg/mL, respectively. Whereas, at pH = 6, the Zn^{2+} concentration for ZNPs and L-ZNPs were observed as 25.1 and 10.4 mg/mL, respectively.

2.2. Characterization of Lysozyme-ZNPs conjugates

Fig 2c shows the FTIR spectra of pristine ZNPs, native lysozyme and L-ZNPs conjugates. Infrared spectroscopy is a well-known method for protein analysis since it provides important information about its secondary structure. Among various bands of protein, the amide I C=O stretching mode (1600-1700 cm^{-1}) is used to follow the secondary structure of protein and the absorbance intensity of amide II C-N stretch coupled with N-H bending mode (1500-1600 cm^{-1}) has been reported to be proportional to the loaded protein quantity on the nanostructure surface.[6] The spectrum of native lysozyme (black color) demonstrates the amide I band (1658 cm^{-1}) is due to C=O stretching mode; the amide II band (1543 cm^{-1}) ascribed to the bending and stretching mode of N-H and C-N vibrations, respectively. The spectrum of L-ZNPs conjugates (red color) displays a small shift from 1530 to 1533.5 cm^{-1} corresponding to amide II band with low intensity, suggesting efficient conjugation of lysozyme on ZNP surfaces. Different regions of the amide I band are contributed by different secondary structural elements: 1620-1645 cm^{-1} by β-sheet, 1645-1652 cm^{-1} by random coil, 1652-1662 cm^{-1} by R-helix, and 1662-1690 cm^{-1} by turns.[7] Additionally, a shift from 1657.6 to 1656.8 cm^{-1} indicates small alterations in lysozyme structure owing to ZNP conjugation. The disappearance of native lysozyme peak (1649 cm^{-1}) after conjugates formation indicates loss in the non-regular structural region in the protein.[8] Hence, the presence of lysozyme native bands in L-ZNPs conjugates spectrum clearly reveals the well-maintained secondary structure of the protein with negligible changes after the conjugates formation process.

Fig S2 shows the EDX spectra of (a) pristine ZNPs and (b) L-ZNPs conjugates. The EDX spectrum demonstrates that the products are made of Zn and O only (a). And, the L-ZNPs EDX spectrum shows Zn, O and C, which confirms the presence of lysozyme on ZNPs (b).

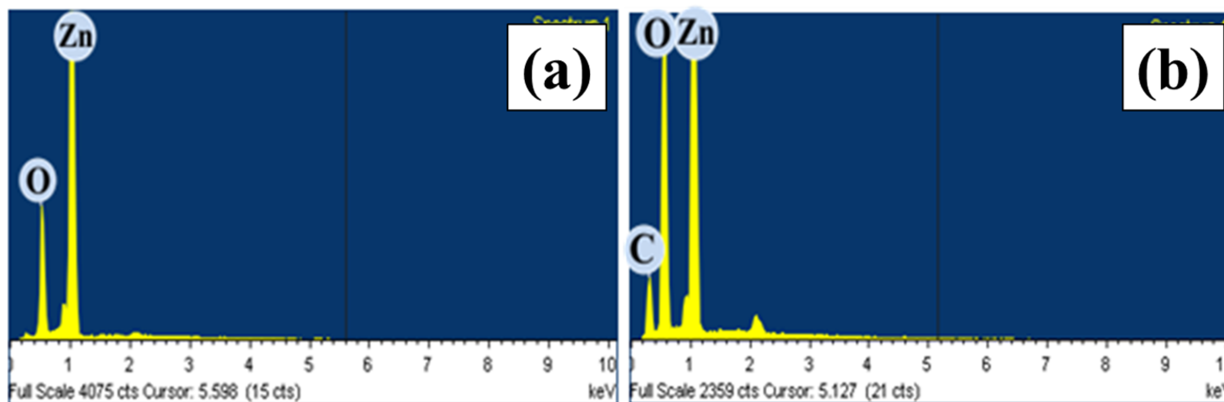

Fig. S2 EDX spectra of (a) pristine ZNPs and (b) L-ZNPs conjugates.

2.3. Cytotoxicity

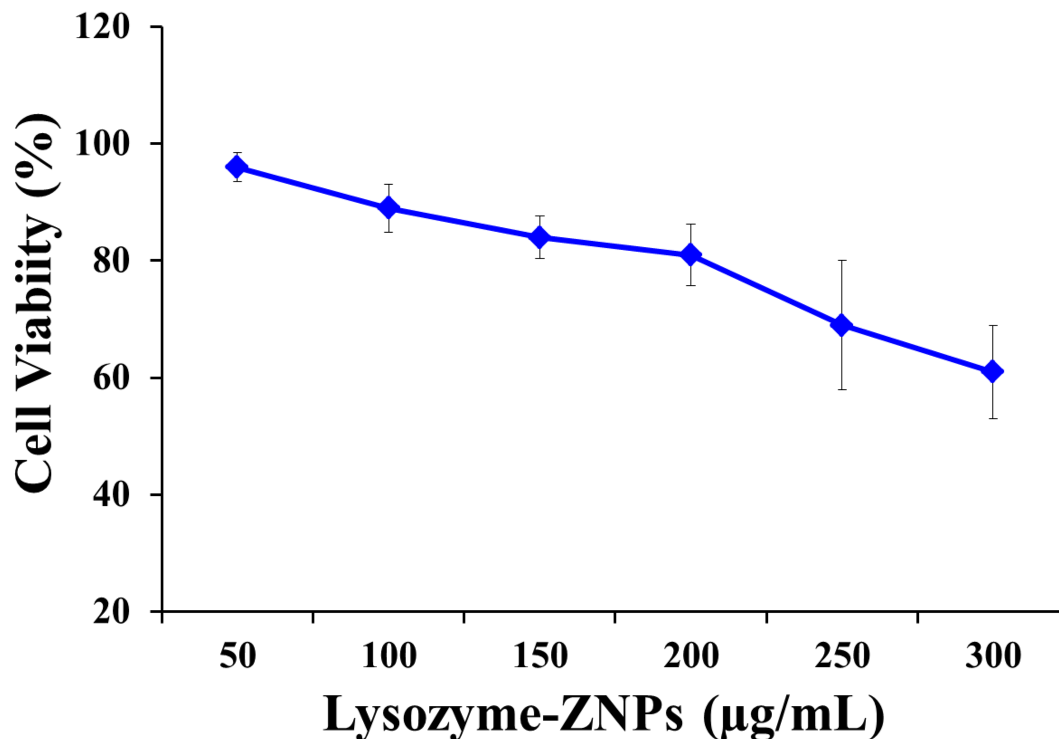

Fig. S3 *In vitro* HEK cell viability studied by MTT assay. Cells were treated with L-ZNPs conjugates for 24 h. Each treatment was performed three times independently.

4. Reference

1 A. Umar, M.M. Rahman, M. Vaseem, and Y.-B. Hahn, *Electrochem. Commun.*, 2009, **11**, 118.

2 Z. Huang, X. Zheng, D. Yan, G. Yin, X. Liao, Y. Kang, Y. Yao, D. Huang and B. Hao, *Langmuir*, 2008, **24**, 4140.

3 (a) R. Wahab, S.G. Ansari, Y.S. Kim, M.A. Dar and H.-S. Shin, *J. Alloy Compd.*, 2008, **461**, 66; (b) K. Yang, D.H. Lin, B.S. Xing, *Langmuir*, 2009, **25**, 3571.

4 Y. Guo, H. Wang, C. He, L. Qiu and X. Cao, *Langmuir*, 2008, **25**, 4678.

5 R. Satishkumar and A. Vertegel, *Biotechnology and Bioengineering*, 2008, **100**, 403.

6 W.K. Surewicz, H.H. Mantsch and D. Chapman, *Biochemistry*, 1993, **32**, 389.

7 J.O. Speare and T.S. Rush, *Biopolymers*, 2003, **72**, 193.

8 S. Chakraborti, S. Sarwar and P. Chakrabarti, *J. Phys. Chem. B*, 2013, **117**, 13397.