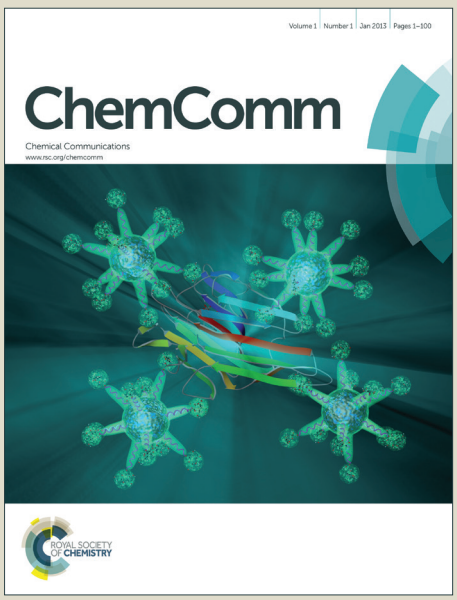
ChemComm

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/chemcomm

Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

ARTICLE TYPE

Tailored Lysozyme-ZnO Nanoparticle Conjugates as Nanoantibiotic†

Nirmalya Tripathy,[a] Rafiq Ahmad, [c] Seung Hyuck Bang, [b] Jiho Min, [b, c] Yoon-Bong Hahn [a, c,*]

Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX
DOI: 10.1039/b000000x

Covalently attached lysozyme-ZnO nanoparticles (L-ZNPs) conjugates were synthesized by low temperature solution route. Tailored L-ZNPs conjugates present pronounced antibacterial features against *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*).

Nanoantibiotics development has become imperative owing to rapid burgeoning of multiple antibiotics resistance by several pathogenic microorganisms, invalidating major clinical drugs currently in use.[1] Recently, use of antimicrobial enzymes targeting different bacterial cellular components and biofilm formation, has emerged as nature-inspired antibiotic-free strategy to treat several infectious diseases.[2] Lysozyme, a well-known antimicrobial enzyme, is widely used in food industry and healthcare products for effective bacteria killing. Lysozyme mostly exhibits more effective antibacterial action toward Gram-positive (Gram+) than Gram-negative (Gram-) bacteria by hydrolyzing β-1, 4-glycoside linkage of peptidoglycan in the bacterial cell wall. However, its high industrial production cost has limited their biomedical applications. Addressing this challenge, various enzymes-antimicrobial agent conjugates have been developed to inhibit/destroy existing microbial growth by acting in a complementary fashion to enhance its therapeutic efficacy and reduce overall cost.[3]

Particularly, inorganic antimicrobial nanoparticles offer several distinctive benefits in overcoming resistance, reducing dosage, improved patient-compliance, minimized side effects, and lowering overall cost compared to conventional antibiotics.[4] Synthesis of antimicrobial nanoparticles is cost-effective compared with antibiotics synthesis, as well stable enough for long-term storage with a prolonged shelf-life.[5] On the other hand, enzyme-nanoparticle conjugates have attracted considerable interest for biological usages due to its enhanced stability, high degree of mono-dispersity, high enzyme loadings and possibility of targeted delivery. Attempts have been made to improve lysozyme efficacy by immobilizing on silver, TiO_2 nanoparticles *etc.*, albeit the results were not immensely impressive.[6] Recently, Satish *et al.* have explored that the lysozyme covalently bound to positively charged polymer nanoparticles exhibits higher antimicrobial activity compared to free lysozyme and lysozyme attached to negatively charged polymer nanoparticles.[7] This breakthrough discovery has prompted us to design lysozyme conjugated positively charged inorganic nanoparticle with unique merits to enhance therapeutic efficacy.

ZnO is well-recognized as multifunctional material bestowed with exceptional optical, semiconducting and piezoelectric characteristics. It possesses the most diversified family of nanostructures, used in many avenues such as sunscreen products, sensors, industrial coatings, and antibacterial agents.[8] Zn^{2+} is an indispensable trace element for adults; 9.5 and 7.0 mg of Zn^{2+}/ day are needed for adult men and women, respectively. The intrinsic property of ZnO such as presence of surface -OH groups facilitates additional benefits of functionalization with various surface-decorating molecules.[9] Especially, ZnO nanoparticles (ZNPs) possess a wide range of antibacterial activities towards both Gram+ and Gram- bacteria, including major foodborne pathogen, attributed to the generation of reactive oxygen species (ROS) on these oxide surfaces. Also, they featured with remarkable photocatalytic and photo-oxidizing ability against chemical/biological species that is predicted to increase the ROS levels.[10] Herein, we demonstrate an efficient strategy for synthesizing covalently attached lysozyme to ZNPs (L-ZNPs conjugates) in order to achieve enhanced enzyme antimicrobial activity, through low temperature solution route. Furthermore, the effect of this modification was demonstrated by investigating the antibacterial effectiveness of L-ZNPs conjugates against *E. coli* and *S. aureus.*

Fig. 1 represents the synthetic procedure of L-ZNPs conjugates (for experimental details see ESI†). TEM image and XRD pattern (Fig. S1a, ESI†) displays uniform nanoparticle size of 30 ±10 nm along with good wurtzite crystallinity. To achieve L-ZNPs conjugates, aminated-ZNPs were prepared using 3-aminopropyltriethoxysilane/3-aminopropyltriethoxysilane. The TEM and HRTEM image of aminated-ZNPs showed displays a thin film on the particles surface with an uniform covering layer thickness measured to be ~2 nm (see ESI†, Fig. S1b insert), consistent with the reported aminated-ZnO nanostructures.[11] Further, the precise assessment of pristine ZNPs and aminated-ZNPs were studied using FTIR spectra and zeta potential measurements (see Fig S1c,d, ESI†).

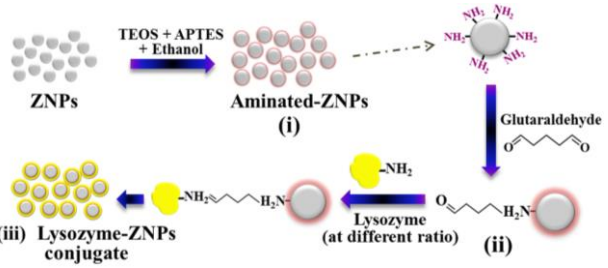


Fig.1 Schematic procedure for synthesis of L-ZNPs conjugates.

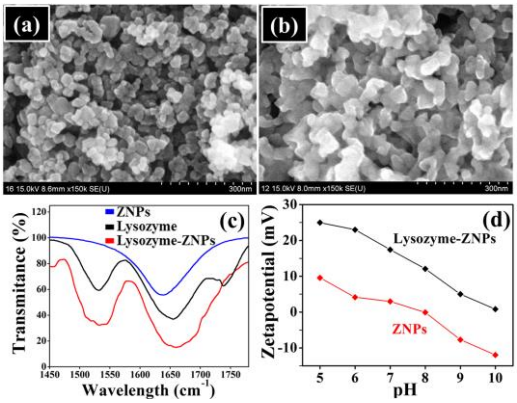


Fig.2 FESEM images of (a) pristine ZNPs, (b) L-ZNPs conjugates, (c) Zeta potential curves, and (d) FTIR spectra.

Fig. 2 shows FESEM images of pristine ZNPs (a) and L-ZNPs conjugates (b). FESEM image captured after lysozyme loading demonstrate a slight increase in particle size which indicates lysozyme immobilization. Moreover, FTIR measurements were performed to study the secondary structural changes in lysozyme induced by nanoparticle conjugation (Fig. 2c). Spectrum of the conjugates displays the presence of lysozyme native bands in L-ZNPs conjugates spectrum, confirming a well-maintained secondary structure of the enzyme with negligible changes after the conjugates formation.

The electrostatic properties of L-ZNPs colloidal suspension were characterized by Zeta potential measurements (Fig. 2d). A gradual increase in the isoelectric point (IP) of L-ZNPs was evident with increment in binding lysozyme yield. The initial step of covalent attachment of lysozyme to ZNPs involves its physical adsorption. However, it can be predicted that more positively charged lysozyme molecules (IP = 10.6) could be adsorbed to a negatively charged surface, than to a positively charged surface. To achieve higher binding lysozyme yield, a crosslinker (glutaraldehyde) was used to immobilize lysozyme molecules on modified-ZNPs surfaces. The glutaraldehyde consisting of two aldehyde groups (-COOH) can efficiently forms bond separately to the amino groups of lysozyme and aminated-ZNPs.

Primarily, L-ZNPs conjugates samples were prepared with different enzyme-to-nanoparticle ratio, followed by centrifugation and subjected to characterizations. Fig. 3a shows the binding kinetics curves of conjugated lysozyme to aminated-ZNPs measured by UV-visible absorbance peak. The loaded lysozyme concentration was calculated using Beer-Lambert equation: A (absorbance) = ε c l; where, ε = extinction coefficient (38,940 cm^{-1} M^{-1} for lysozyme), c = molar concentration and l = optical path length (1 cm). With different lysozyme:ZNPs ratio of 1:1, 10:1, 20:1, 30:1, 40:1 and 50:1, the loaded lysozyme amount was evaluated as 2.38, 7.87, 15.24, 19.1, 31.18 and 39.45 mg/g (Fig. 3b); and lysozyme conjugation degree was calculated as 0.2, 0.07, 0.08, 0.06, 0.08, and 0.08, respectively. Thereafter, TGA analysis was performed to evaluate the mass ratio between ZNPs and lysozyme (Fig. 3b). The ZNPs mass loss profile exhibits a single decrease in mass over ~220-280 °C, attributed to desorption of physically adsorbed water from the ZNPs surface. For L-ZNPs conjugates, a three- step mass loss was observed: first mass loss (~13%) below 300 °C due to trapped solvent evaporation; other two-weight loss at ~300-450 °C (42%) and

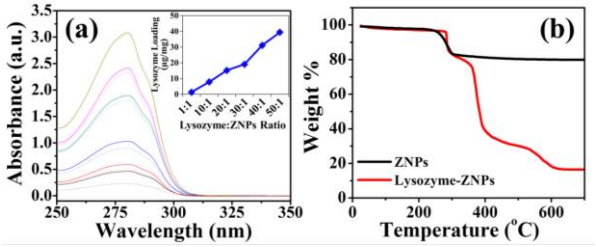


Fig.3 (a) Binding kinetics curves of conjugated lysozyme to aminated-ZNPs examined by UV-visible spectra (different lysozyme:ZNPs ratio i.e. 1:1, 10:1, 20:1, 30:1, 40:1 and 50:1). The initial and unbound lysozyme concentrations were shown by lines and dotted lines, respectively. Percentage of adsorbed lysozyme onto ZNP surfaces is shown in insert. (b) TGA curve.

450-600 °C (17%) were assigned to the decomposition and complete carbonization of loaded lysozyme. Thus, the total enzyme weight loss above 300 °C was calculated to be ~59 wt% of the overall conjugates (after reducing first mass loss %) and the remaining wt% of ZNPs is approximately 28%. These results are in good agreement with the UV-visible results, indicating high lysozyme binding yield.

Fig. 4 shows the antibacterial efficacy of native pristine ZNPs, lysozyme, and L-ZNPs conjugate (20:1 ratio) evaluated against *S. aureus* (Gram+) and *E. coli* (Gram-). The MIC of lysozyme against *S. aureus* and *E. coli* was found to be 191 and 236μg/mL, respectively, indicating higher toxicity for *S. aureus*. The MIC of ZNPs against *S. aureus* and *E. coli* were observed to be 82 and 48 μg/mL, respectively, which suggests a much stronger bacterial toxicity for Gram+ bacteria, in accordance with previous findings.[12] In sharp contrast, MIC value for L-ZNPs conjugated towards *S. aureus* and *E. coli* was evaluated as 18 and 12 μg/mL, respectively. The conjugates display superior antibacterial (Gram+ and Gram-) property compared to that of lysozyme and ZNPs. Interestingly, the conjugates presents higher toxicity towards Gram-bacteria, ascribed to the net charge difference of bacterial cell wall. Fig. 5a displays the CLSM micrographs of *E. coli* and *S. aureus* treated with native lysozyme, pristine ZNPs, and L-ZNPs conjugates followed by FITC and PI staining. Majority of untreated cells shows green fluorescence due to viable cells indicating intact bacterial cell wall. Compared to lysozyme and ZNPs, approx. 100% of bacteria exposed to the conjugates displayed strong red fluorescence, thus suggests cell death and/or enhanced bactericidal effect.

Keeping in view the storage stability and durability of L-ZNPs conjugates, antibacterial activity profiles were monitored after 30 days storage in 0.1 M PBS (pH = 6). Under identical conditions, the L-ZNPs conjugates maintained approx. 90% of their initial activity, however the activity of native lysozyme

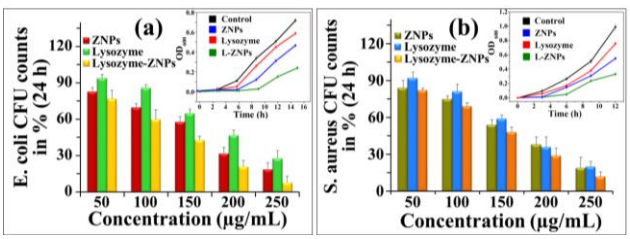


Fig.4 Bacterial growth curve plots of (a) *E. coli* and (b) *S. aureus* treated with ZNPs, lysozyme, and L-ZNPs conjugates on solid agar and on LB media (50 μg/mL of each sample shown in the inserts).

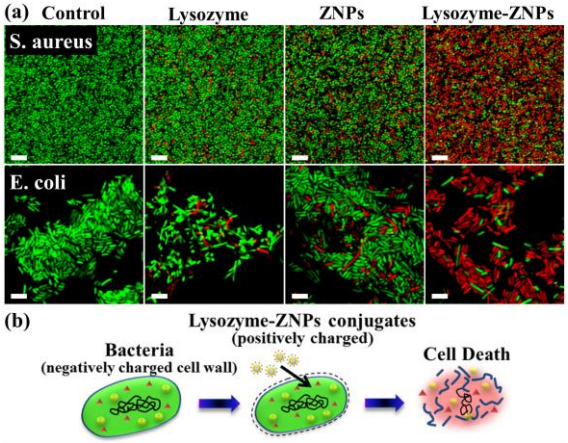


Fig.5 (a) CLSM images of *S. aureus* and *E. coli* followed by treatments and stained with FITC and PI dyes. The green or red fluorescence is defined as live or dead cells, respectively. Scale bar = 5 μm. (b) Antibacterial action of L-ZNPs conjugates.

significantly decreased after 24 h indicating good durability of the conjugates. Furthermore, the toxicity profile of L-ZNPs conjugates were assessed against HEK cells by MTT assay to demonstrate an effective implication of these therapeutic (see Fig S3, ESI†). Although, the L-ZNPs conjugates showed dose-dependent toxicity, however approximately 80% cell viability was obtained at a dosage of 200 mg/mL after 24 h. The high cell viability is may be due to lysozyme conjugation and ZNPs preferential toxic nature towards cancerous cells.[13]

Studies have reported that nanomaterial charge can be smartly utilized to tailor lysozyme's antibacterial activity, which is consistent with our results.[7] Compared to previous reports,[7,14] our results demonstrate pronounced antibacterial activity of L-ZNPs conjugates due to combined effects of active lysozyme and ZNPs, which is achieved through smart material organization and packaging. Vecitis *et al.* have proposed a three-step cytotoxicity mechanism: bacterial adhesion onto nanostructure; membrane disruptive interaction with bacteria, inducing membrane stress; and final step involves disrupting a specific microbial process by disturbing/oxidizing a vital cellular component.[15] We hypothesize that this three-step toxicity mechanism is also applicable to L-ZNPs conjugates and their antibacterial mechanism is likely to be the synergy of membrane stress and oxidative stress. Primarily, direct bacteria-L-ZNPs conjugates contact was established due to difference in electrostatic interactions (explained below), and incorporating these conjugates into the outer and/or inner layer surrounding the bacteria. The lysozyme enzymes then cause cell wall damage resulting in membrane stress.[16] On the other hand, ZNPs also induces ROS mediated oxidative stress which may be attributed to the released ionic Zn^{2+} or labile zinc complexes.[17]

Notably, the conjugates were more effective in inhibiting *E. coli* growth, attributed to the net charge difference of bacterial cell wall. In general, bacterial cell walls are negatively charged owing to teichoic, lipoteichoic, and teichuronic acids in their cell membrane, however *E. coli* is more negatively charged than *S. aureus* because of their cell wall chemical differences.[18] Since, electrostatic interactions are known to play a prominent role in contact establishment between the conjugates and bacteria, thus the observed antibacterial differences can be explained based on electrostatic contribution and zeta potential results. At pH = 7.4, the zeta potential of ZNPs is negative due to acetate ions on its surfaces, whereas aminated-ZNPs possess a positive value owing to $-NH_2$ groups. Further, covalent binding of lysozyme to aminated-ZNPs through glutaraldehyde crosslinking, results in higher positive zeta potential values of L-ZNPs conjugates. Consequently, these positively charged conjugates target towards negatively charged bacteria mediated by electrostatic interactions (Fig. 5b). In case of *E. coli*, the net negative charge is higher leading to higher electrostatic interactions, thus greatly attracts the positively charged conjugates and *vice versa* for *S. aureus*. Owing to the proximity between the *E. coli* and L-ZNPs conjugates, higher L-ZNPs conjugates were taken up by the cells facilitating cell death.

Conclusions

An enhanced antibacterial feature of lysozyme was achieved though covalent binding of positively charged lysozyme to modified-ZNPs against two bio-medically relevant bacteria strains (*S. aureus* and *E. coli*). Growth kinetics studies showed that L-ZNPs conjugate possess superior antibacterial property towards Gram+ and Gram- bacteria owing to the synergetic effects of lysozyme and ZNPs. A much stronger antimicrobial efficiency of the conjugates was observed for *E. coli*, assigned to the electrostatic interactions between the negative bacterial cell wall and positively charged conjugates. Collectively, these results were envisioned to provide useful insights for development of antibiotic-free enzyme-metal oxide conjugates for biomedical applications.

This research was supported by the National Leading Research Laboratory Program (2013064831) for financial support through National Research Foundation of Korea funded by the Ministry of Science, ICT & Future Planning.

Notes and references

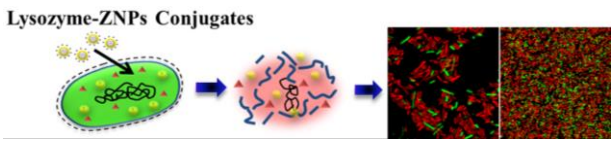
[a]*Dept. of BIN Fusion Technology,* [b]*Dept. of Bioprocess Engineering,* [c]*School of Semiconductor and Chemical Engineering and Nanomaterials Processing Research Center, Chonbuk National University, 567 Baekje-daero, Deokjin-gu, Jeonju 561-756, Republic of Korea.*
** Corresponding author. E-mail: ybhahn@chonbuk.ac.kr.*

† Electronic Supplementary Information (ESI) available: Experimental details for synthesis, characterization, antibacterial tests. See DOI: 10.1039/b000000x/

1 (a) C. Walsh, *Nature*, 2000, **406**, 775; (b) A.J. Huh and Y.J. Kwon, *J. Controlled Release*, 2011, **156**, 128.
2 D.J. Stickler, *Nature Clin. Pract.*, 2008, **5**, 598.
3 (a) B. Craigen, A. Dashiff and D.E. Kadouri, *Open Microbiol. J.*, 2011, **5**, 21; (b) Z. Alahdad, R. Ramezani, M. Aminlari and M. Majzoobi, *J. Agric. Food Chem.*, 2009, **57**, 6449.
4 (a) E. Weir, A. Lawlor, A. Whelan and F. Regan, *Analyst*, 2008, **133**, 835; (b) J. Song, H. Kong and J. Jang, *Chem. Commum.*, 2009, 5418; (c) L.C. Gerber, N. Moser, N.A. Luechinger, W.J. Stark and R.N. Grass, *Chem. Commum.*, 2012, **48**, 3869.
5 M.L. Cohen, *Nature*, 2000, **406**, 762.
6 (a) C.K. Bower, R.J. Avena-Bustillos, C.W. Olsen, T. H. McHugh and P.J. Bechtel, *J. Food Sci.*, 2006, **71**, 141; (b) S.H. Bang, A. Jang, J. Yoon, P. Kim, J.S. Kim, Y.H. Kim and J. Min, *Enzyme Microb. Technol.*, 2011, **49**, 260.
7 R.S. kumar and A. Vertegel, *Biotechn. Bioengineering*, 2008, **100**, 403.
8 (a) Y.-B. Hahn, R. Ahmad and N. Tripathy, *Chem. Commun.*, 2012, **48**, 10369; (b) R. Ahmad, N. Tripathy, D.-U.-J. Jung and Y.-B. Hahn, *Chem. Commun.*, 2014, **50**, 1890.

9 H. Hong, J. Shi, Y. Yang, Y. Zhang, J.W. Engle, R.J. Nickles, X. Wang and W. Cai, *Nano Lett.*, 2011, **11**, 3744.
10 (a) N. Jones, B. Ray, K.T. Ranjit and A.C. Manna, *FEMS Microbiol. Lett.*, 2008, **279**, 71; (b) A. Sasidharan, P. Chandran, D. Menon, S. Raman, S. Nair and M. Koyakutty, *Nanoscale*, 2011, **3**, 3657.
11 Y. Zhang, H. Wu, X. Huang, J. Zhang and S. Guo, *Nanoscale Research Lett.*, 2011, **6**, 450.
12 N. Padmavathy and R. Vijayaraghavan, *Sci. Technol. Adv. Mater.*, 2008, **9**, 035004.
13 C. Hanley, J. Layne, A. Punnoose, K. M. Reddy, I. Coombs, A. Coombs, K. Feris and D. Wingett, *Nanotechnology*, 2008, **19**, 295103
14 V. Ernest, S. Gajalakshmi, A. Mukherjee and N. Chandrasekaran, *Artif. Cells. Nanomed. Biotechnol.*, 2013, doi: 10.3109/21691401.2013.818010
15 (a) C. D. Vecitis, K. R. Zodrow, S. Kang and M. Elimelech, ACS Nano, 2010, 4, 5471; (b) S. Liu, T. H. Zeng, M. Hofmann, E. Burcombe, J. Wei, R. Jiang, J. Kong, and Y. Chen, ACS Nano, 2011, 5, 6971.
16 V. L. Hughey and E. A. Johnson, Appl. Environ. Microbiol., 1987, 53, 2165.
17 M. Li, L. Zhu and D. Lin, *Environ. Sci. Technol.*, 2011, **45**, 1977.
18 (a) A.T. Poortinga, R. Bos and H.J. Busscher, *Colloids Surf. B Biointerfaces*, 2001, **20**, 105; (b) R. Sonohara, N. Muramatsu, H. Ohshima, and T. Kondo, *Biophys. Chem.*, 1995, **55**, 273.

TOC



Covalently attached lysozyme-ZnO nanoparticles conjugates present pronounced antibacterial features against *Escherichia coli* and *Staphylococcus aureus.*