

**ANTIBIOTIC FUNCTIONALIZED METALLIC
NANOPARTICLES FOR BACTERIAL DETECTION
AND ANTIMICROBIAL ACTIVITY**

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INDIAN INSTITUTE OF TECHNOLOGY DELHI**

August 2020

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AND ANTIMICROBIAL ACTIVITY**

by

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Submitted

in fulfilment of the requirements of the degree of Doctor of Philosophy

to the



INDIAN INSTITUTE OF TECHNOLOGY DELHI

August 2020

Dedicated to

My Parents

Shri. R. K. Singh & Smt. Munni Devi

CERTIFICATE

This is to certify that the thesis entitled “**Antibiotic Functionalized Metallic Nanoparticles for Bacterial Detection and Antimicrobial Activity**” submitted by **Mr. Sanjay Singh** to the Indian Institute of Technology Delhi for the award of the degree of **Doctor of Philosophy** in Biochemical Engineering and Biotechnology, is a record of the authentic research work carried out by him under my supervision and guidance. He has fulfilled all the requirements for submission of this thesis, which to the best of my knowledge has reached the required standards. The results presented in this thesis have not been submitted in part or full to any other university or institute for the award of any other degree or diploma.

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ACKNOWLEDGEMENTS

“No one who achieves success does so without the help of others. The wise and confident acknowledge this help with gratitude.”

– Alfred North Whitehead

I wish to acknowledge several key figures who have contributed significantly to my research endeavor. It is my pleasure to express my deep sense of gratitude to my guide and mentor **Prof. Prashant Mishra**, under whose valuable guidance, inspiration, keen interest and support, I had the opportunity to carry out this study. I sincerely thank him for his supervision, constant encouragement, healthy criticism and never-ending patience throughout the journey of my Ph.D. He provided me an ideal balance of freedom to pursue my own ideas and guided me in the right direction that allowed me to conceptualize my research work problem independently. His allegiance to perfection on furbishing the papers helped me to learn the art of paper writing in a precise and concise manner.

I am highly obliged to all the faculty members of Department of Biochemical Engineering and Biotechnology (DBEB), and my SRC Committee members, **Prof. Saroj Mishra**, **Prof. S. K. Khare** and **Dr. Preeti Srivastava** for their precise observations, continuous criticism and encouragements to enrich the quality of my doctoral work. I am grateful to **Dr. Samarsh Das** and **Prof J. P. Singh** for providing their valuable suggestions, advice and facility for research work. I also take the opportunity to express my thanks to **Prof. Vikram Kumar** and **Prof. Neeraj Khare** for letting me be a part of the Nanoscale research facility (NRF), IIT Delhi. I utilized the sophisticated characterization facilities at NRF, IITD that helped me in my research in a big way. I would also like to express my thanks to all the technical staff and office members of NRF for their constant help and co-operation. Next, I would like to thank the Ministry of Human Resource Development, Govt. of India for providing me with a scholarship to support the course of my Ph.D.

I want to give special thanks to all my friends without whom this journey couldn't have been so exciting and memorable especially **Neeti Kalyani**, **Sumit Murab**, **Shasanka Shekhar Sinha**, **Deepak Tiwari**, **Garima Goyal**, **Amanpreet Kaur Nagpal**, **Soumya Rajpal**, **Arushi Agarwal**, **Amandeep Jindal**, **Moolchand Sigar**, **Jitendra Singh Verma**, **Ashmita Pandey**, **Saurav**, **Manik**, **Jai**, **Nawab**, **Jyoti Sharma**, **Vibhooti Kayastha** and all others who never let

me be alone. Their presence never let me sink into any dejection however grave the situation be.

My heartfelt gratitude to my seniors and my mentors **Dr. Neeti Kalyani, Dr. Swati Jaiswal** and **Dr. Surbhi Goel** who have taught me about the lab culture, experiment planning and execution and stood by my side from the beginning till the end. I would like to express special appreciation to all my lab-mates especially **Soumya Rajpal, Amanpreet Kaur Nagpal, Garima Goyal, Gaurav Sinsinbar, Dr. Snehasish Bhakta, Dr. Senthilguru, Ritu Bhardwaj, Dr. Sujata Sinha, Dr. Garima, Dr. Sweta, Dr. Apoorva Verma and Dr. Tushar Dash** for their constant help, encouragement and support during this period. I owe a special thanks to **Akshay Moudgil, Samir Thakur** and **Nishant Mishra** for their invaluable help and making this work a success. I would like to acknowledge the staff of the Biochemical Engineering and Biotechnology department for their innumerable help especially **Mrs. Renu Sethi** and **Dr. Sumeet Kapoor** for their valuable support and expertise.

I express my deep sense of gratitude to my beloved parents **Mr. R. K. Singh** and **Mrs. Lal Munni** whose support and inspiration have helped me realize this dream. I would like to thank my uncle and my mentor **Mr. M. L. Singh** who always supported me and gave me the right direction, inspiration and motivation to achieve my dreams. I would also like to thank my sisters **Vidya** and **Vibha**, who have been my energy through this journey. Most importantly, thanks to the universal energy for helping me in this effort.

Sanjay Singh

ABSTRACT

Nowadays, one of the greatest health challenges worldwide is an infectious disease caused by pathogens. Antibiotics play a significant role in decreasing mortality and morbidity. However, current antibiotics therapies are not effective due to the rise of multiple drug-resistant (MDR) infections which is an increasing threat to the human race. To address this problem there is an urgent need to discover a novel antimicrobial agent for killing as well as early-stage screening methods for the detection of bacteria. Metal-antibiotic hybrid can play an important role in achieving these twin objectives. Based on the antibiotic-specific interaction with the cell wall of bacteria, detection of pathogens becomes highly specific. In this study, the metal-antibiotic hybrid has been employed for killing of bacteria as well as for detection of bacteria using electrochemical impedance spectroscopy (EIS) and surface-enhanced Raman spectroscopy (SERS) technique.

The impedance sensor was fabricated using gold interdigitated electrodes onto a tungsten oxide thin film. Tungsten oxide was functionalized with vancomycin, a glycopeptide antibiotic known to have a specific interaction with the peptidoglycan layer of Gram-positive bacteria. The functionalized tungsten oxide (metal-antibiotic hybrid) sensor was highly efficient in the capture of Gram-positive bacteria with a limit of detection of 10^2 colony forming units/mL. Label-free detection under physiological conditions was tested and achieved. The impedance measurement was also sensitive to differentiate between viable and non-viable Gram-positive bacteria.

For the detection of bacteria using SERS, vancomycin functionalized bimetallic magnetic/silver core-shell nanoparticles for specifically capturing and concentrating Gram-positive pathogen were used. The capture efficiency and calibration graphs of these van-coated Fe@Ag nanoparticles were examined over different bacterial concentrations of *S. aureus* (10^1 - 10^7 CFU/mL). These captured bacteria were detected at a low concentration of up to 10 CFU/mL using SERS. These nanoparticles were able to capture Gram-positive bacteria that were separated using an external magnetic field and were rapidly detected (~ 20 min).

For specific detection of Gram-negative bacteria, ampicillin was used and was similarly functionalized on the silver nanorods substrate (AgNRs) prepared using glancing angle deposition technique (GLAD). For this, ampicillin concentration was optimized initially to

allow specific capture of Gram-negative bacteria. Ampicillin coated AgNRs SERS substrates sensitively detected Gram-negative bacteria with a LOD of 10 CFU/mL. It was also able to differentiate viable cells from non-viable Gram-negative bacteria. Ampicillin coated AgNRs SERS substrates provide a rapid (~ 3 min) detection method for bacteria having a simple operating procedure and high sensitivity.

In addition, the metal-antibiotic hybrid has been fabricated for developing a new class of metal-antibiotic nanoformulation to kill bacteria. Antibiotic coated AgNPs with highly efficient antibacterial and antibiofilm activity were synthesized by the coating of AgNPs with bacitracin (BA) and allyl isothiocyanate. BA is a cyclic peptide that disrupts bacterial cell wall synthesis whereas isothiocyanate (ITC), a natural plant product is also known for its antibacterial effect. The ITC and BA were coated on the AgNPs surface and characterized by FTIR, UV-spectrometer, and TEM. ICP-MS results showed a very low silver content present in antibiotic coated AgNPs. The antibiotic coated silver nanoparticle efficiently inhibited the growth of both Gram-negative and Gram-positive bacteria. This fact was confirmed by MIC study, cell viability assay and time-kill curve. In addition, they also showed the activity of inhibition of biofilms, and selective cytotoxicity against bacterial cells over mammalian cells.

Keywords: Vancomycin, ampicillin, electrochemical impedance sensor, surface enhanced Raman spectroscopy, bacitracin, allyl isothiocyanate, antibiotic coated silver nanoparticles

सार

आज दुनिया में सबसे बड़ी स्वास्थ्य चुनौतियों में से एक रोगाणुओं के कारण होने वाली संक्रामक बीमारी है। एंटीबायोटिक दवाइयाँ मृत्यु दर और रुग्णता को कम करने में महत्वपूर्ण भूमिका निभाते हैं। हालांकि, वर्तमान समय में एंटीबायोटिक दवाइयाँ कई दवा-प्रतिरोधी (एम.डी.आर) संक्रमणों के बढ़ने के कारण प्रभावी नहीं हैं, जो मानव जाति के लिए बढ़ता खतरा है। इस समस्या के समाधान के लिए नवीन रोगाणुरोधी यौगिक की खोज के साथ-साथ प्रारंभिक अवस्था में जीवाणुओं का पता लगाने के लिए तत्काल-स्क्रीनिंग तरीकों की भी आवश्यकता है। इन दोनों उद्देश्यों को प्राप्त करने में धातु-एंटीबायोटिक संकर (हाइब्रिड) महत्वपूर्ण भूमिका निभा सकते हैं। जीवाणुओं की कोशिका भित्ति के साथ एंटीबायोटिक-विशिष्ट जुड़ाव के आधार पर, जीवाणुओं का पता लगाना अत्यधिक विशिष्ट हो जाता है। इस अध्ययन में, इलेक्ट्रोकेमिकल प्रतिबाधा स्पेक्ट्रोस्कोपी (ई.आई.एस) और सतह-संवर्धित रमन स्पेक्ट्रोस्कोपी (एस.ई.आर.एस) तकनीक का उपयोग करके बैक्टीरिया की रोकथाम के लिए नियोजित करने के लिए धातु-एंटीबायोटिक हाइब्रिड का उपयोग किया गया है।

टंगस्टन ऑक्साइड की पतली फिल्म पर सोने के अंतःसंबंधित इलेक्ट्रोड का उपयोग करके प्रतिबाधा सेंसर गढ़ा गया था। टंगस्टन ऑक्साइड को वैनकोमाइसिन के साथ क्रियाशील किया गया, जो की एक ग्लाइकोपेप्टाइड एंटीबायोटिक है, जिसे ग्राम-पॉजिटिव बैक्टीरिया की पेप्टिडोग्लाइकेन परत के साथ एक विशिष्ट जुड़ाव के लिए जाना जाता है। न्यूनतम प्रति मिलीलीटर 10^2 कॉलोनी बनाने वाली इकाइयों का पता लगाने की सीमा के साथ ग्राम-पॉजिटिव बैक्टीरिया को पकड़ने में कार्यात्मक टंगस्टन ऑक्साइड (धातु-एंटीबायोटिक हाइब्रिड) सेंसर अत्यधिक कुशल पाया गया। शारीरिक स्थितियों के तहत लेबल मुक्त पहचान का परीक्षण किया गया और परिणाम हासिल किया गया। प्रतिबाधा माप व्यवहार्य (जीवित) तथा गैर-व्यवहार्य (मृत) ग्राम पॉजिटिव बैक्टीरिया के बीच अंतर करने के लिए भी संवेदनशील था।

सतह-संवर्धित रमन स्पेक्ट्रोस्कोपी (एस.ई.आर.एस) के उपयोग से बैक्टीरिया का पता लगाने के लिए, विशेष रूप से ग्राम-पॉजिटिव रोगजनक बैक्टीरिया को पकड़ने और केंद्रित करने के लिए वैनकोमाइसिन फंक्शनलिज्ड द्विधात्विय चुंबकीय / चांदी कोर-शेल नैनोकणों का उपयोग किया गया था। इन वैनकोमाइसिन फंक्शनलिज्ड द्विधात्विय चुंबकीय / चांदी नैनोकणों की पकड़-दक्षता और अंशांकन रेखांकन को एस. ऑरियस ($10^1 - 10^7$ कॉलोनी बनाने वाली इकाइयां प्रति मिलीलीटर) के विभिन्न बैक्टीरियल सांद्रता पर जांच की गई थी। सतह-संवर्धित रमन स्पेक्ट्रोस्कोपी (एस.ई.आर.एस) का उपयोग करके इन पकड़े गए बैक्टीरिया को न्यूनतम 10 कॉलोनी बनाने वाली इकाइयां प्रति मिलीलीटर तक की

सांद्रता तक पता लगाया गया। यह नैनोकण उन ग्राम-पॉजिटिव बैक्टीरिया को पकड़ने तथा उनका पता लगाने में (~ 20 मिनट) सक्षम थे जिन्हें बाहरी चुंबकीय क्षेत्र का उपयोग करके अलग किया गया था।

ग्राम- नेगेटिव जीवाणुओं की विशिष्ट पहचान के लिए, एम्पीसिलीन का उपयोग किया गया था और इसी तरह चांदी के नैनोरोड्स सबस्ट्रेट पर फंक्शनलिज़्ड किया गया था, जिसे ग्लान्सिंग कोण निक्षेप तकनीक (जी.एल.ए.डी) का उपयोग करके तैयार किया गया था। इसके लिए, ग्राम- नेगेटिव बैक्टीरिया को विशिष्ट रूप से पकड़ने के लिए एम्पीसिलीन सांद्रता को प्रारम्भ में अनुकूलित किया गया था। एम्पीसिलीन लेपित चांदी के नैनोरोड्स सबस्ट्रेट (एजी.एन.आर) ने प्रति मिलीलीटर 10 कॉलोनी बनाने वाली इकाइयों का पता लगाने की सीमा के साथ ग्राम- नेगेटिव बैक्टीरिया का संवेदनशील रूप से पता लगाया। यह गैर-व्यवहार्य (मृत) ग्राम- नेगेटिव बैक्टीरिया से व्यवहार्य (जीवित) कोशिकाओं को अलग करने में भी सक्षम था। एम्पीसिलीन लेपित चांदी के नैनोरोड्स सबस्ट्रेट, एक सरल ऑपरेटिंग प्रक्रिया है और उच्च संवेदनशीलता द्वारा तीव्रता (~ 3 मिनट) के साथ बैक्टीरिया का पता लगाने की विधि प्रदान करता है।

इसके अलावा, बैक्टीरिया को मारने के लिए धातु-एंटीबायोटिक नैनोफॉर्मूलेशन का एक नया वर्ग विकसित करने के लिए धातु-एंटीबायोटिक संकर का निर्माण किया गया है। चांदी के नैनोकणों के साथ बैक्त्रासीन (बी.ए) और एलिल आइसोथियोसाइनेट (आई.टी.सी) की कोटिंग द्वारा संश्लेषित किए गए एंटीबायोटिक लेपित चांदी के नैनोकण अत्यधिक कुशल जीवाणुरोधी पाए गए जो बायोफिल्म को खत्म करने में भी सक्षम थे। बैक्त्रासीन (बी.ए) एक चक्रीय पेप्टाइड है जो बैक्टीरिया कोशिका भित्ति के संश्लेषण को बाधित करता है जबकि आइसोथियोसाइनेट (आई.टी.सी), एक पौधे का प्राकृतिक उत्पाद है जिसे जीवाणुरोधी प्रभाव के लिए भी जाना जाता है। आई.टी.सी और बी.ए को चांदी के नैनोकणों सतह पर लेपित किया गया और इनका फ.टी.आई.र, यू.वी-स्पेक्ट्रोमीटर, आई.सी.पी.-एम.एस और ट.इ.म विधि द्वारा परिक्षण किया गया। आई.सी.पी.-एम.एस के परिणामों के द्वारा यह पाया गया कि एंटीबायोटिक लेपित चांदी के नैनोकणों में चांदी की मात्रा बहुत कम थी। एंटीबायोटिक लेपित चांदी के नैनोकणों ने ग्राम-नेगेटिव और ग्राम-पॉजिटिव बैक्टीरिया दोनों के ग्रोथ को कुशलता से रोक दिया। इस तथ्य की पुष्टि एमआईसी अध्ययन, सेल व्यवहार्यता परख और टाइम किल कर्व द्वारा की गई थी। इसके अलावा, यह भी पाया गया कि ये बायोफिल्म को खत्म करने में भी सक्षम हैं और उन्होंने स्तनधारी जीव की कोशिकाओं से अधिक बैक्टीरिया कोशिकाओं के खिलाफ चयनात्मक साइटोटॉक्सिसिटी की गतिविधि को भी दिखाया।

प्रमुख शब्द: वैकोमाइसीन, एम्पीसिलीन, इलेक्ट्रोकेमिकल प्रतिबाधा सेंसर, सरफेस एनहांस्ड रमन स्पेक्ट्रोस्कोपी, बैसिट्रैसीन, एलिल आइसोथियोसाइनेट, एंटीबायोटिक लेपित चांदी के नैनोकण

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LIST OF ABBREVIATIONS

Ab	Antibiotic
AFM	Atomic force microscopy
AGM	Alternate gradient magnetometer
AgNP	Silver nanoparticles
AgNR	Silver nanorods
ITC	Allyl isothiocyanate
AO	Acridine orange
ATCC	American type culture collection
BA	Bacitracin
BIC	Biofilm inhibitory concentration
BPE	Trans-1, 2-bis (4-pyridyl) ethylene,
CFU	Colony forming units
CDC	Centers for disease control and prevention
CLSI	Clinical and laboratory standards institute
CPE	Constant phase element
CTAB	Cetyl trimethyl ammonium bromide
DLS	Dynamic light scattering
DMEM	Dulbecco's modified eagle's medium
DSM	German collection of microorganism

EtBr	Ethidium bromide
EIS	Electrochemical impedance spectroscopy
EDTA	Ethylene diamine tetraacetic acid
EDX	Energy-dispersive X-ray spectroscopy
FBS	Fetal bovine serum
FDA	Food and drug administration
FET	Field effect transistor
FESEM	Field emission scanning electron microscope
FI	Fluorescence intensity
FTIR	Fourier-transform infrared spectroscopy
GLAD	Glancing angle deposition
HaCaT	Immortalized human epidermal keratinocytes
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IC ₅₀	Concentration at which 50% inhibition takes place
IC ₉₀	Concentration at which 90% inhibition takes place
IDE	Interdigitated gold electrodes
IONP	Iron-oxide nanoparticles
ITO	Indium Tin Oxide
LAF	Laminar air flow

LB	Luria broth
LPS	Lipopolysaccharide
m	Milli
M	Molar
mM	Millimolar
mL	Millilitre
mg	Milligram
MBC	Minimum bactericidal concentration
MBEC	Mature biofilm eradication concentration
MHA	Mueller Hinton agar
MHB	Mueller Hinton broth
MIC	Minimum inhibitory concentration
MS	Magnetization saturation
MNP	Magnetic nanoparticles
MTT	3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
NCCS	National centre for cell science
NCIM	National collection of industrial microorganisms
nm	Nanometre
NP	Nanoparticle
NPs	Nanoparticles
NRRL	Agricultural research service culture collection

OD	Optical density
OH [•]	Hydroxyl radical
PAE	Post antibiotic effect
PBS	Phosphate buffer saline
PCA	Principal component analysis
PCR	Polymerase chain reaction
PDI	Polydispersity index
ROS	Reactive oxygen species
Rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute
SD	Standard deviation
SEM	Scanning electron microscope
SERS	Surface enhanced Raman spectroscopy
SPION	Superparamagnetic iron oxide nanoparticles
TEM	Transmission electron microscope
TEOS	Tetraethyl orthosilicate
TQ	Thymoquinone

UV-Vis	Ultraviolet-visible
WHO	World Health Organization
WO ₃	Tungsten oxide

LIST OF SYMBOLS

w/v	Weight per unit volume
v/v	Volume per unit volume
g	Relative centrifugal force
h	Hour
μ	Micro
$^{\circ}$	Degree
λ	Wavelength
θ	Angle of incidence
eV	Electron volt
I	Current
Z	Impedance
D_h	Hydrodynamic diameter
D_t	Translational diffusion coefficient
k_B	Boltzmann's constant
η	Dynamic viscosity
Ω	Ohm