RAPID ANTIMICROBIAL SUSCEPTIBILITY TESTING THROUGH IMPEDANCE SPECTROSCOPY USING LOW CONDUCTIVITY ZWITTERIONIC BUFFERS

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by

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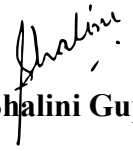
CERTIFICATE

This is to certify that the thesis entitled "**Rapid antimicrobial susceptibility testing through impedance spectroscopy using low conductivity zwitterionic buffers**" being submitted by **Pragya Swami** for the award of the degree of **Doctor of Philosophy** in Chemical Engineering is a record of bonafied work carried out by her under my guidance and supervision at the Indian Institute of Technology Delhi.

The results contained in the thesis have not been submitted elsewhere, either in part or in full, to any other University or Institute for the award of any degree or diploma.

I certify that she has pursued the prescribed course of research.



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Abstract

Established antibiotic susceptibility testing (AST) techniques based on cell culture, gene amplification and mass spectroscopy are laborious, expensive and time consuming. They are inherently limited by the doubling rate of bacteria or bacterial DNA. Impedance spectroscopy, on the other hand, is a rapidly emerging tool for bacterial AST as it is simple, label-free, cost-effective, high-throughput and real-time. The current research in impedance biosensors mostly focuses on electrode design and cell enrichment strategies to enhance impedance signal, whereas the role of buffers in impedance spectroscopy is relatively unexplored. In my thesis, we have for the first time, investigated the use of Good's zwitterionic buffers (*e.g.*, HEPES) for impedance spectroscopy and illustrated their application for bacterial cell viability analysis and rapid AST. Our results show that zwitterionic electrolytes perform significantly better than standard electrolytes (*e.g.*, PBS) because of their large molecular size, lower concentrations at which they are used, high dissociation constants, and most importantly, higher ion-ion interactions (compared to PBS) which leads to the lowering of their conductivity and hence, increase in impedance signal. At the same time, zwitterionic buffers maintain cell vitality despite having low osmolality. With respect to cell viability in the presence of antibiotics, zwitterionic buffers play a crucial role as they allow measuring ionic changes due to bacterial death/growth with much greater sensitivity, reducing the overall detection time. We used two AST approaches in my thesis. In the first, ionic release due to antibiotic-triggered cell death was measured in HEPES buffer with or without a dielectrophoretic cell capture step depending on the electrode size. As a result, resistant and susceptible strains were readily distinguished within 30 min of cell-drug interaction. In the second approach, ionic release due to cell growth was measured in the presence of antibiotic in a low conductivity growth buffer that was pre-optimized with respect to HEPES buffer and Lb broth composition. The results were

obtained in 60 min (approximately 2 to 3 doubling cycles) which is 8 to 9 times faster than the conventional approaches. The various cell-drug combinations used included clinically susceptible and resistant strains of *Salmonella typhi*, *Staphylococcus aureus*, *Klebsiella pneumonia*, *Enterococcus faecalis* and *Escherichia coli* and nine different classes of antibiotics, selected based on their action mechanisms, cellular targets, and resistance patterns. Both systems were found to be highly specific and were further used for rapid MIC/MBC determination under 80 min paving way for efficient and rapid bacterial diagnosis and management.

सार

सेल कल्चर, जीन एम्प्लीफिकेशन और मास स्पेक्ट्रोस्कोपी पर आधारित स्थापित एंटीबायोटिक संवेदनशीलता परीक्षण (एएसटी) तकनीक श्रमसाध्य, महंगी और समय लेने वाली हैं। वे स्वाभाविक रूप से बैक्टीरिया या जीवाणु डीएनए की दोहरीकरण दर से सीमित हैं। दूसरी ओर, प्रतिबाधा स्पेक्ट्रोस्कोपी, बैक्टीरियल एएसटी के लिए एक तेजी से उभरता हुआ उपकरण है क्योंकि यह सरल, लेबल-मुक्त, लागत प्रभावी, उच्च-थ्रूपुट और वास्तविक समय है। प्रतिबाधा बायोसेंसर में वर्तमान शोध ज्यादातर प्रतिबाधा संकेत को बढ़ाने के लिए इलेक्ट्रोड डिजाइन और सेल संवर्धन रणनीतियों पर केंद्रित है, जबकि प्रतिबाधा स्पेक्ट्रोस्कोपी में बफ़र्स की भूमिका अपेक्षाकृत अस्पष्ट है। मेरी थीसिस में, हमने पहली बार प्रतिबाधा स्पेक्ट्रोस्कोपी के लिए गुड्स ज़्विटरियोनिक बफ़र्स (जैसे, हेपेस) के उपयोग की जांच की और बैक्टीरियल सेल व्यवहार्यता विश्लेषण और तेजी से एएसटी के लिए उनके आवेदन को चित्रित किया। हमारे परिणाम बताते हैं कि ज़्विटरियोनिक इलेक्ट्रोलाइट्स अपने बड़े आणविक आकार, कम सांद्रता जिस पर उनका उपयोग किया जाता है, उच्च पृथक्करण स्थिरांक, और सबसे महत्वपूर्ण, उच्च आयन-आयन इंटरैक्शन (पीबीएस की तुलना में) के कारण मानक इलेक्ट्रोलाइट्स (जैसे, पीबीएस) की तुलना में काफी बेहतर प्रदर्शन करते हैं। जिससे उनकी चालकता कम हो जाती है और इसलिए, प्रतिबाधा संकेत में वृद्धि होती है। उसी समय, ज़्विटरियोनिक बफ़र्स कम ऑस्मोलैलिटी होने के बावजूद सेल जीवन शक्ति बनाए रखते हैं।

एंटीबायोटिक दवाओं की उपस्थिति में सेल व्यवहार्यता के संबंध में, ज़िवटरियोनिक बफ़र्स एक महत्वपूर्ण भूमिका निभाते हैं क्योंकि वे बैक्टीरिया की मृत्य/वृद्धि के कारण अधिक संवेदनशीलता के साथ आयनिक परिवर्तनों को मापने की अनुमति देते हैं, जिससे समग्र पहचान समय कम हो जाता है। हमने अपनी थीसिस में दो एएसटी दृष्टिकोणों का इस्तेमाल किया। पहले में, एंटीबायोटिक-ट्रिगर कोशिका मृत्यु के कारण आयनिक रिलीज को इलेक्ट्रोड आकार के आधार पर एक डाइइलेक्ट्रोफोरेटिक सेल कैप्चर चरण के साथ या बिना हेपेस बफर में मापा गया था। नतीजतन, सेल-ड्रग इंटरैक्शन के 30 मिनट के भीतर प्रतिरोधी और अतिसंवेदनशील उपभेदों को आसानी से पहचाना गया। दूसरे दृष्टिकोण में, सेल की वृद्धि के कारण आयनिक रिलीज को कम चालकता वृद्धि बफर में एंटीबायोटिक की उपस्थिति में मापा गया था जो हेपेस बफर और एलबी ब्रॉथ संयोजन के संबंध में पूर्व-अनुकूलित था। परिणाम 60 मिनट (लगभग 2 से 3 दोहरीकरण चक्र) में प्राप्त किए गए जो पारंपरिक तरीकों की तुलना में 8 से 9 गुना तेज है। प्रयोग किए गए विभिन्न सेल-दवा संयोजनों में साल्मोनेला टाइफी, स्टैफिलोकोकस ऑरियस, क्लेबसिएला निमोनिया, एंटरोकोकस फ़ेकलिस और एस्चेरिचिया कोलाई के नैदानिक रूप से अतिसंवेदनशील और प्रतिरोधी उपभेदों और उनके क्रिया तंत्र, सेलुलर लक्ष्यों और प्रतिरोध पैटर्न के आधार पर चयनित एंटीबायोटिक दवाओं के नौ अलग-अलग वर्ग शामिल थे। दोनों प्रणालियों को अत्यधिक विशिष्ट

पाया गया और कुशल और तेजी से जीवाणु निदान और प्रबंधन के लिए 80 मिनट के मार्ग के तहत तेजी से एमआईसी/एमबीसी निर्धारण के लिए उपयोग किया गया।

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DEFINITIONS OF TERMS AND SYMBOLS USED IN THE TEXT

Symbol	Notes	Units
I	Absolute impedance	Ohm (Ω)
$\tilde{I}$	Complex impedance	Ω
I'	Real part of impedance	Ω
I''	Imaginary part of impedance	Ω
$\widetilde{K}$	Complex conductivity	Siemens meter^{-1} (S/m)
$K'\ or\ \kappa$	Real part of conductivity/DC conductivity	S/m
K''	Imaginary part of conductivity	S/m
C_c	Geometrical cell constant	meter^{-1} (m^{-1})
ω	Angular frequency	Hertz (Hz)
$\check{\varepsilon}$	Complex permittivity	Farad meter^{-1} (F.m^{-1})
ε'	Real part of permittivity	F.m^{-1}
ε''	Imaginary part of permittivity	F.m^{-1}
θ	Phase angle	Degree (˚)
C_{dl}	Double layer capacitance	F.m^{-2}
R_{sol}	Solution resistance	Ω
C_{sol}	Solution capacitance/Bulk capacitance	F.m^{-2}
f_{cm}	The Clausius–Mossotti factor	Unit less
$Re(f_{cm})$	Real part of the Clausius–Mossotti factor	Unit less
$\phi/v_1/v_2$	Volume fraction	Unit less
$\check{\varepsilon}_{cell}$	Complex permittivity of cell	F.m^{-1}
$\check{\varepsilon}_{sol}$	Complex permittivity solution	F.m^{-1}
$\check{\varepsilon}_{mix}$	Complex permittivity cell and solution	F.m^{-1}
$\check{\varepsilon}_{cyto}$	Complex permittivity cell cytoplasm	F.m^{-1}
$\check{\varepsilon}_{mem}$	Complex permittivity cell membrane	F.m^{-1}
$\check{\varepsilon}_{wall}$	Complex permittivity cell wall	F.m^{-1}
$\check{\varepsilon}_{cyto-mem}$	Complex permittivity cell cytoplasm and membrane	F.m^{-1}
$\check{\varepsilon}_{cyto-mem-wall}$	Complex permittivity cell cytoplasm, membrane and wall	F.m^{-1}
$\check{\varepsilon}_{eff}$	Complex permittivity of inner sphere of the two phase dielectric spheres	F.m^{-1}
$\check{\varepsilon}_1$	Complex permittivity of outer layer of the two phase dielectric spheres	F.m^{-1}
$\check{\varepsilon}_2$	Complex permittivity of the two phase dielectric spheres	F.m^{-1}
v_1 and v_2	Volume Fraction	Unit less
C_{mem}	Capacitance of cell membrane	F.m^{-2}
C_{wall}	Capacitance of cell wall	F.m^{-2}

$C_{mem-wall}$	Capacitance of cell membrane and cell wall	$F.m^{-2}$
R_{mem}	Resistance of cell membrane	Ohm $meter^2$ ($\Omega.m^2$)
R_{wall}	Resistance of cell wall	$\Omega.m^2$
R_{cyto}	Resistance of cell cytoplasm	$\Omega.m^2$
A	Electrode surface area	$Meter^2$
ε_{dl}	Permittivity of double layer	$F.m^{-1}$
D	Distance between two electrodes	Meter
κ_+	DC conductivity of cations	S/m
κ_-	DC conductivity of anions	S/m
λ_+	Molar conductivity of the cation	Siemens $meter^2$ $mole^{-1}$ ($S.m^2.mol^{-1}$)
λ_-	Molar conductivity of the anion	$S.m^2.mol^{-1}$
C_+	Cation molar concentration	$Mole.meter^{-3}$ ($mol.m^{-3}$)
C_-	Anion molar concentration	$mol.m^{-3}$
C_{el}	Electrolyte concentration	$mol.m^{-3}$
x	Number of cation atom	-
y	Number of anion atom	-
A^{y+}	Dissociate equivalent moles of cation	Mole
B^{x-}	Dissociate equivalent moles of cation	Mole
Λ	Molar conductivity	Siemens $meter^2$ $mole^{-1}$ ($S.m^2.mol^{-1}$)
Λ^0	Limiting molar conductivity	$S.m^2.mol^{-1}$
$\Lambda_{theo.}$	Theoretical molar conductivity	$S.m^2.mol^{-1}$
λ_+^0	Limiting molar conductivity of the cation	$S.m^2.mol^{-1}$
λ_-^0	Limiting molar conductivity of the anion	$S.m^2.mol^{-1}$
C_{actual}	Actual electrolyte concentration	$Mole.meter^{-3}$ ($mol.m^{-3}$)
ε_r	Relative permittivity	Unit less
ε_w	Relative permittivity of pure water	Unit less
ε_{sol}	Relative permittivity of the electrolyte solution	Unit less
ε_{cyto}	Relative permittivity of cell cytoplasm	Unit less
ε_{mem}	Relative permittivity of cell membrane	Unit less
ε_{wall}	Relative permittivity of cell wall	Unit less
δ	Phenomenological constant	$Meter^3.mole^{-1}$ ($m^3.mol^{-1}$)
f	Frequency	Hz
κ_{ideal}	DC Conductivity at infinite dilution	S/m
$\kappa_{expt.}$	Experimental DC Conductivity	S/m
S_s	Constant	Siemens $mole^{-0.5}$ $meter^{0.5}$ ($S.mol^{-0.5}.m^{0.5}$)
u	Dipole moment	Debye, D
ΔP_0	Turgor pressure	Atm
ΔP	Osmotic pressure difference between the cell and the medium	Atm
P_e	Peclet number	Dimensionless number
a_c	Bacterial cell radius	Micrometer (μm)

ρ	Density	Gram/centimeter^3 (g/cm^3)
t	Time	Second (s)
T	Temperature	Kelvin (K)
K_b	Boltzmann constant	m^2 kg s^{-2} K^{-1}
x	Distance travelled by ions	Milimeter (mm)
D_s	Diffusivity/Diffusion Coefficient	$\mu m^2/s$
A	Radius of bacterial cytoplasm	Micrometer (µm)
a_{cyto}/a	Thickness of the bacterial cell cytoplasm	µm
a_{mem}	Thickness of the bacterial cell membrane	µm
a_{wall}	Thickness of the bacterial cell wall	µm
σ_{sol}	Medium conductivity	S/m
σ_{cyto}	Cell cytoplam conductivity	S/m
σ_{mem}	Cell membrane conductivity	S/m
σ_{wall}	Cell wall conductivity	S/m
F_1/F_2	Cut-off frequency	Hertz (Hz)
Σ	Standard deviation	-
C_{LOD}	Limit of detection of impedance sensor	Milimolar (mM)
$S_{impedance}$	Sensitivity of impedance sensor	Ohms/mM
λ	Debye length	Nanometers, nm
π	A dimensionless quantity written as pi with value equal to 3.14	-
I_s	Ionic Strength	Molar (M)
N_b	Total number of cells in 20 microliter volume	-
D_{PDMS}	Radius of the PDMS chamber	µm
A_{PDMS}	Total surface area of the PDMS chamber	μm^2
A_{FoV}	Area per field of view	μm^2
N_b	Total number of cells	-
N	Total number of cells captured on the entire electrode surface	-
N_c	Total number of cells captured per field of view on electrode surface	-
n_{FOV}	Total number of field of view	-
N	Number of doubling cycles of bacteria	-
K	Intercept of zone of inhibition and antibiotic disk concentration plot	mm^2
m	Slope of zone of inhibition and antibiotic disk concentration plot	$mm^2/\mu g$
V	Molar volume of the solute	cm^3/gmol
J	Diffusion flux	Mol.m^{-2}.s^{-1}
B_1 and B_0	Number of bacterial colonies formed at 160 and 0 min	-

ABBREVIATIONS

COVID-19 – Coronavirus disease 2019

WHO – World Health Organization

CDC – Centre for Disease Control and Prevention

AMR – Antimicrobial resistance

MS – Mass spectrophotometry

PCR – Polymerase chain reaction

ELISA – Enzyme-linked immunosorbent assay

FISH – Fluorescence in situ hybridization

AST– Antibiotic susceptibility testing

MIC – Minimum inhibitory concentration

CLSI – Clinical and Laboratory Standards Institute

R – Resistant strain/bacterium

S – Susceptible strain/bacterium

EUCAST – European Committee on AST

ZOI – Zone of inhibition

FDA – Food and Drug Administration

EF – Electric field

NPs – Nanoparticles

QCM – Quartz crystal microbalance

SPR – Surface plasmon resonance

POC – Point-of-care

PBS – Phosphate buffer saline

DI –Deionized

MBC – Minimum bactericidal concentration

LCGB – low conductivity growth buffer

LB – Lysogeny broth

DEP – Dielectrophoresis

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

EPPS – 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid

PIPES – 1,4-Piperazinediethanesulfonic acid

POPSO – Piperazine-1,4-bis (2-hydroxy-propanesulfonic acid) dihydrate

IDEs – Interdigitated electrodes

PDMS – Polydimethylsiloxane

DFT – Density functional theory

GGA – Hybrid generalized-gradient approximation

CM5 – Charge model 5

C-PCM – Conductor-like polarizable continuum model

MS – Mechanosensitive

DC – Direct current

AC – Alternative current

EDL – Electrical double layer

EP – Electrode polarization

DL – Double layer

CP – Counterion polarization

DLC – Double layer capacitance

MMT – Maxwell's mixture theory

ECM – Equivalent circuit modeling

FEM – Finite element method

ITO – Indium tin oxide

LOD – Limit of detection

AMPs – Antimicrobial peptides

Conc. – Concentration

nL – Nano-liters

pL – Pico-liters

mV – Milivolts

S/N – Signal to noise ratio

EC – *E. coli*

SA – *S. aureus*

KP – *K. pneumonia*

pDEP – Positive DEP

nDEP – Negative DEP

TSB – Tryptic soy broth

MHB – Mueller-Hinton broth

CM – Clausius–Mossotti factor

O.D. – Optical density

PMB – Polymyxin B sulfate

Sushi – Sushi S3 peptide

Bac – Bacitracin

Levo – Levofloxacin

MET – Methicillin

VA – Vancomycin

CMS – Colistin methanesulfonate

CF – Cefoxitin

AMP – Ampicillin

IMP – Imipenem

RIF – Rifampicin

GEN – Gentamicin

TR – Trimethoprim

CIP – Ciprofloxacin

MBC_{100}/ BC_{100} – Maximal bactericidal concentration

MBC_{50}/BC_{50} – Half-maximal bactericidal concentration

MBC_{lysis}/BC_{lysis} – Lysis bactericidal concentration

DEPIS – Dielectrophoresis with impedance spectroscopy

LPS – Lipopolysaccharide molecules

OM – Outer membrane

FOV – Field of view

MSSA – Methicillin-susceptible *S. aureus*

MRSA – Methicillin-resistant *S. aureus*

EFS – Levo-susceptible *E. faecalis*

EFR – Levo-resistant *E. faecalis*

PBP – Penicillin-binding proteins

PI – Propidium iodide