AZURIN PROTEIN FOR THE DEVELOPMENT OF BIONANODEVICES

NEETI KALYANI

DEPARTMENT OF BIOCHEMICAL ENGINEERING AND BIOTECHNOLOGY

INDIAN INSTITUTE OF TECHNOLOGY DELHI

JULY 2018

AZURIN PROTEIN FOR THE DEVELOPMENT OF BIONANODEVICES

by

NEETI KALYANI
Department of Biochemical Engineering and Biotechnology

Submitted

in fulfillment of the requirements of the degree of Doctor of Philosophy

to the

INDIAN INSTITUTE OF TECHNOLOGY DELHI

JULY 2018

Dedicated to

My dad, Sh. Ganga Ram

and

My dear husband, Parvesh Kumar

“The purpose of morality is to teach you, not to suffer and die,

but to enjoy yourself and live.”

- Ayn Rand

CERTIFICATE

This is to certify that the thesis entitled **"Azurin Protein for the Development of Bionanodevices"** being submitted by **Ms. Neeti Kalyani** 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 her under my supervision and guidance. She has fulfilled all the requirements for submission of this thesis, which to the best of my knowledge has reached the required standard. The results contained 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.

Prashant Mishra, PhD

Professor

Department of Biochemical Engineering and Biotechnology

Indian Institute of Technology Delhi

Hauz Khas – 110016,

New Delhi, India

ACKNOWLEDGEMENTS

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. Gopal P. Agarwal**, **Dr. Bishwajit Kundu** 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. Samaresh 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 thank to **Prof. Vikram Kumar and Prof. Neeraj Khare** for letting me be a part of Nanoscale research facility (NRF), IIT Delhi. I utilized the sophisticated characterization and clean room 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.

I want to give special thanks to all my friends without whom this journey couldn't have been so exciting and memorable especially **Sumit Murab**, **Sanjay Singh**, **Amandeep Jindal**, **Saundarya Singh**, **Nikhil Patidar**, **Chandel Angad, Swati Jain**, **Shilpa Patyal**, **Sandeep Raghav**, **Nitesh Jain**, **Shubham Gupta**, **Khushboo Rastogi**, **Rahul Agarwal**, **Vibhooti Kayastha**, **Divyansh Raj** and all others who never let me be alone. Their presence never let me sink into any dejection however grave the situation be.

It was a great pleasure to work in protein engineering laboratory. I sincerely acknowledge the help and assistance provided by **Dr. Bhawna Madan** during my initial Ph.D. days and for teaching me how to tackle problematic situations with patience and diplomacy. I would like to express special appreciation to all my lab-mates especially **Garima Goyal**, **Sanjay Singh**, **Gaurav Sinsinbar**, **Dr. Sujata Sinha, Dr. Surbhi Goel**, **Dr. Swati Jaiswal**, **Dr. Prabha Arya**, **Apoorva Verma**, **Ritu Bhardwaj** and **Tushar Dash** for their constant help, encouragement and support during this period. I would also like to thank **Akshay Moudgil** and **Samir Kumar** for their incredible help and support in research. I would like to acknowledge the staff of biochemical engineering and biotechnology department for their innumerable help especially **Dr Sumeet Kapoor** and **Mrs. Renu Sethi** for their valuable support and expertise.

When it comes to personal support and strength, I am extremely happy to express my sincere appreciation to my husband, **Parvesh Kumar** for his love, patience and understanding nature that allowed me to nurture my dream and keep looking forward to greater achievements in future. He has always been so helpful and kind to me. Without his support, it would not have been possible for me to complete my research work in a timely manner. I wholeheartedly thank my siblings, **Anshu**, **Sanju**, **Ankur** and **Reshav**. Their unflinching support, patience and love have helped me pursue my dreams. They helped me at various stages of my personal life and longed to see this achievement come true.

I express my deep sense of gratitude to my beloved parents **Mr. Ganga Ram** & **Mrs. Vishesh Kumari** whose support and inspiration have helped me realize this dream. Their support, blessings and constant love have sustained me throughout my life. My father, being a principal himself, has always understood me, believed in me and persistently encouraged me at every step. It was the dream of my first teacher, my father and his diligent and patient efforts made my journey up to here possible. He pushed me all the time that helped me achieve what I could in my research today. I thank him for inculcating in me the scientific temper and making me dream big.

No study is complete without adequate funding support from the funding agency. I gratefully acknowledge Council of Scientific and Industrial Research (CSIR), Ministry of Electronics and Information Technology (MeitY) and Department of Science and Technology (DST), Government of India, for providing the funding support for this study.

At last, I owe it all to Almighty God for granting me the wisdom, health and strength to undertake this research task and enabling me to its completion.

(Neeti Kalyani)

ABSTRACT

Pseudomonas aeruginosa azurin, a small copper containing redox metalloprotein was cloned in pET28a vector in fusion with an affinity tag (6 X histidine) for single step purification using Ni-NTA (Nickel-nitrilotriacetic acid). The protein was overexpressed and purified and the yield was 50 times higher for recombinant azurin than reported values for wild type azurin from *P. aeruginosa*. Recombinant azurin was characterized by UV-visible spectroscopy and the characteristic peak at 620 nm was observed with purity ratio (Abs_{620}/Abs_{280}) of 0.174. Azurin was also characterized by fluorescence, and CD spectroscopy. The electrochemical behavior of protein was studied by cyclic voltammetry and conductive atomic force microscopy. In cyclic voltammetry azurin protein showed oxidation and reduction peaks at 280 mV and 178 mV, respectively. Azurin variants (Cu-azurin, Fe-azurin and Ni-azurin) were reconstituted by modifying the metal ion center of the protein. They were characterized by UV-visible absorbance and fluorescence spectroscopy. All the three azurin variants showed good redox active characteristics observed by the presence of oxidation and reduction peaks. Azurin variants were prudently immobilized on three different devices which acted as a channel (200 nm) between the source and drain electrodes and the devices realized in highly scalable and reproducible manner to act as a field effect transistor (FET). Electrical measurements illustrated p-type FET behavior for all three variants. The devices possess low subthreshold swing (200 mV/decade) and high on-off current ratio (10^5). The FET devices showed consistent behavior in highly stable mode as measured for a span of 8 weeks. These FETs having high throughput with long term stability and exhibited commendable robustness. The Al/Azurin/ITO/PET (Aluminium/Azurin/Indium tin oxide/Polyethylene terephthalate) structure based on

recombinant azurin was used for the flexible memory applications. Highly stable LRS (low resistance state) and HRS (high resistance state) were demonstrated for 500 cycles. The flexible device has also exhibited stable current and both switching states upon bending test for multiple cycles. Azurin metal ion variants based memory devices were also studied. Stable HRS and LRS were observed in all three devices for 10 cycles. The Cu-azurin based device showed best memory behavior with larger memory window. Also, all three devices exhibited good repeatability and endurance. Recombinant azurin has shown good conducting behavior and may be used for the development of electronic biosensors. These devices also fill the technological gap to integrate protein based memory with other biocompatible and bio-integrated electronic device, which is extremely desirable.

सार

स्यूडोमोनास एरुजिनोसा ऐज़ूरीन, जो कि एक तांबा आयन युक्त छोटा सा अपचयोपचय धातु प्रोटीन है, को निकेल-एन. टी. ऐ. (नाइट्रिलोट्रायएसिटिक एसिड) द्वारा एकल चरण शुद्धिकरण के लिए एक एफ़िनिटी टैग (6xहिस्टिडाइन) के साथ pET28a वेक्टर में क्लोन किया गया। ऐज़ूरीन को ओवरएक्सप्रेस और शुद्ध किया गया और वाइल्ड टाइप स्यूडोमोनास एरुजिनोसा ऐज़ूरीन की तुलना में रिकॉम्बिनेंट ऐज़ूरीन 50 गुना ज़्यादा प्राप्त हुआ। रिकॉम्बिनेंट ऐज़ूरीन को पराबैंगनी-दृश्यमान स्पेक्ट्रोस्कोपी द्वारा चिन्हित किया गया और शीर्ष 620 nm पर 0.174 के शुद्धता अनुपात (Abs620 / Abs280) के साथ पाया गया। ऐज़ूरीन, फ्लोरोसेंस और सी. डी. स्पेक्ट्रोस्कोपी द्वारा भी चिन्हित किया गया। प्रोटीन के विद्युत् रासायनिक व्यवहार का अध्ययन चक्रीय वोल्टामेट्री और प्रवाहकीय परमाणु बल माइक्रोस्कोपी (कंडक्टिव एटॉमिक फोर्स माइक्रोस्कोपी) द्वारा किया गया। ऐज़ूरीन प्रोटीन में चक्रीय वोल्टामेट्री द्वारा ऑक्सीकरण और अपचयन शीर्ष क्रमशः 280 mV और 178 mV पर प्राप्त हुए। ऐज़ूरीन वेरिएंट्स (Cu-ऐज़ूरीन, Fe-ऐज़ूरीन और Ni-ऐज़ूरीन) प्रोटीन के धातु-आयन केंद्र को संशोधित करके पुनर्निर्मित किए गए। वे पराबैंगनी-दृश्यमान अवशोषण और फ्लोरोसेंस स्पेक्ट्रोस्कोपी द्वारा भी चिन्हित किये गए। सभी तीन अज़ुरिन वेरिएंट्स की अपचयोपचय क्रिया ऑक्सीकरण और अपचयन शीर्ष द्वारा देखी गई। एज़ुरिन वेरिएंट्स तीन अलग-अलग उपकरणों पर इम्मोबिलाईज़ किये गए जिन्होंने स्रोत और निकास इलेक्ट्रोड के बीच एक माध्यम (200 nm) के रूप में कार्य किया और तीनों उपकरण अत्याधिक स्केलेबल और पुनरुत्पादित फील्ड इफ़ेक्ट ट्रांजिस्टर की तरह काम करने के लिए अनुकूल पाए गए। विद्युत मापन द्वारा सभी तीन उपकरणों में p-प्रकार के फील्ड इफ़ेक्ट ट्रांजिस्टर का व्यवहार देखा गया। उपकरणों में कम सबथ्रेशोल्ड स्विंग (200 mV/ डिकेड) और उच्च ऑन-ऑफ विद्युत प्रवाह अनुपात (10^5) मिला। फील्ड इफ़ेक्ट ट्रांजिस्टर उपकरणों के व्यवहार

को आठ सप्ताह की अवधि के लिए मापा गया और अत्याधिक स्थिर व्यवहार प्राप्त हुआ। इन फील्ड इफ़ेक्ट ट्रांज़िस्टरों में दीर्घकालिक स्थिरता के साथ उच्च थ्रूपुट दिखाया गया और सराहनीय सुदृढ़ता प्रदर्शित हुई। लचीली मेमोरी अनुप्रयोगों के लिए रिकॉम्बिनेंट ऐज़ूरीन पर आधारित एलुमीनियम / ऐज़ूरीन / आई. टी. ओ. / पी. ई. टी. (इंडियम टिन ऑक्साइड / पोलीएथिलिन टेरिपथेलेट) संरचना का उपयोग किया गया। 500 चक्रों के लिए अत्याधिक स्थिर एल. आर. एस. (मंद प्रतिरोधी अवस्था) और एच्. आर. एस. (उच्च प्रतिरोधी अवस्था) पायी गयी। लचीले यन्त्र को कई बार मोड़ने के बाद भी स्थिर प्रवाह और दोनों स्विचिंग अवस्थाएं पायी गयी। एज़ुरिन धातु आयन वेरिएंट्स आधारित मेमोरी उपकरणों का भी अध्ययन किया गया। 10 चक्रों के लिए सभी तीन उपकरणों में स्थिर एल. आर. एस. और एच्. आर. एस. प्राप्त हुए। Cu-एज़ुरिन आधारित यन्त्र ने बड़ी मेमोरी विंडो के साथ सबसे अच्छा मेमोरी व्यवहार दिखाया। इसके अलावा, सभी तीन उपकरणों ने अच्छा दोहराव और स्थिरता प्रदर्शित की। रिकॉम्बिनेंट एज़ुरिन द्वारा अच्छा संचालन व्यवहार दिखाया गया है और इसे विद्युतीय संवेदको के विकास के लिए इस्तेमाल किया जा सकता है। ये उपकरण प्रोटीन आधारित मेमोरी को अन्य जैव-संगत और जैव-एकीकृत विद्युतीय यन्त्र के साथ एकीकृत करने के लिए तकनीकी कमी को पूरा करते हैं, जो की बेहद वांछनीय है।

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

℃	Degree Celsius
11-MUA	11-mercapto-undecanoic acid
Abs_{280nm}	Absorption at 280 nm
Abs_{620nm}	Absorption at 620 nm
Abs_{max}	Absorption maxima
AFM	Atomic force microscopy
APS	Ammonium persulfate
APTS	3-aminopropyl triethoxysilane
Az	Azurin
BSA	Bovine serum albumin
cAFM	Conductive atomic force microscopy
CD	Circular dichroism
CHAPS	3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate
cm	Centimeter
CV	Cyclic voltammetry
cytC	Cytochrome c

DNA	Deoxyribonucleic acid
DTSSP	3,3-dithiobis(sulphosuccinimidyl propionate)
E^0	Standard reduction potential
EBL	Electron beam lithography
ECSTM	Electrochemical scanning tunneling microscopy
ET	Electron transfer
ETp	Electron transport
FET	Field-effect transistor
FTIR	Fourier transform infrared spectroscopy
h	Hour
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HOMO	Highest occupied molecular orbital
HRS	High resistance state
I_D	Drain current
IPTG	Isopropyl-β-D-thiogalactopyranoside
ITO	Indium tin oxide
I-V	Current-voltage

kDa	Kilodaltons
k_{ET}	Electron transfer rate constant
$kVcm^{-1}$	Kilo volt per centimeter
LB	Luria Broth
LRS	Low resistance state
LUMO	Lowest unoccupied molecular orbital
$M^{-1}cm^{-1}$	Per molar per centimeter
mg	Milligram
mg/mL	Milligram per milliliter
mg/L	Milligrams per liter
mm	Millimeter
mM	Millimolar
$n\Omega \cdot m$	Nanoohm-meter
MPTS	3-mercaptopropyl trimethoxysilane
mV	Millivolt
$mVsec^{-1}$	Millivolt per second
MWCO	Molecular weight cut-off

nA	Nanoampere
NHE	Normal hydrogen electrode
Ni-NTA	Nickel-nitrilotriacetic acid
nm	Nanometer
OLED	Organic light emitting diode
pA	Picoampere
PCR	Polymerase chain reaction
PET	Polyethylene terephthalate
PMMA	Poly(methyl methacrylate)
POC	Point of care
rpm	Revolutions per minute
s	Second
SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscopy
STM	Scanning tunneling microscopy
SS	Subthreshold swing
TB	Terrific Broth

TC	Transfer characteristics
TEMED	N,N,N′,N′-tetramethylethylene diamine
T_m	Melting temperature
Tris	Tris(hydroxymethyl)aminomethane
µM	Micromolar
UV	Ultraviolet
V	Volt
V_{DS}	Drain-source voltage
V_{GS}	Gate-source voltage
v/v	Volume by volume
V_{reset}	Reset voltage
V_{set}	Set voltage
V_{TH}	Threshold voltage
w/v	Weight by volume

LIST OF SYMBOLS

α	Alpha
β	Beta
cm	Centimeter
ε	Molar absorption coefficient
g	Relative centrifugal force
μ	Micro
λ	Wavelength