AZURIN AND ITS DERIVATIVES FOR PROSTATE CANCER DETECTION AND THERAPY

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Azurin and its Derivatives for Prostate Cancer Detection and Therapy

by

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Submitted

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

to the

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To my parents,

Whose endless love and support have sustained me.

My thanks and love go to my younger brother, Ashu for being my pillar of strength.

CERTIFICATE

This is to certify that the thesis entitled "**Azurin and its Derivatives for Prostate Cancer Detection and Therapy**" submitted by **Ms. Ritu Bhardwaj** 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 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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Ritu Bhardwaj

ABSTRACT

Despite diagnostic advancements in prostate cancer, early detection of aggressive forms remains challenging. Prostate specific antigen (PSA) screening, followed by biopsy, is the standard diagnostic method. However, this approach is hampered by over-detection and inability to differentiate between dormant and aggressive tumors by PSA, leading to unnecessary invasive procedures and overtreatment. Prostate specific membrane antigen (PSMA), a tissue-based biomarker linked to prostate cancer aggressiveness has shown promise, but heterogeneity in expression necessitates exploration of new biomarkers. Treatments like surgery, radiotherapy, hormonal therapy, chemotherapy and immunotherapy have lessened the burden of metastatic prostate cancer. Nonetheless, undesirable side effects like recurrence and development of insensitive and resistant tumors makes it imperative to search for alternative treatment approaches.

Protein-based nanomedicines have emerged as promising alternative in cancer detection and therapy by utilizing diverse functional properties of various proteins to overcome the limitations of conventional small molecules drugs. In contrast to conventional chemotherapy, protein-based nanomedicines offer precise targeting, reduced off-target effects and enhanced therapeutic efficacy. In the present work, novel composite protein engineered nanomedicines were designed while conjugating different class of proteins via genetic engineering for prostate cancer management.

Erythropoietin-producing hepatocellular (Eph) receptors a subfamily of receptor tyrosine kinases, play a pivotal role in cancer development and progression. Thus, Ephs, have emerged as prominent cancer biomarkers due to their aberrant expression with cancer progression. Azurin, a bacterial protein isolated from *Pseudomonas aeruginosa,* has demonstrated its potential in inhibiting tumor progression by disrupting Eph–ephrin signaling via its C-terminal domain as one of the mechanisms. In prostate cancer, overexpression of EphA6 receptors has been associated with prostate cancer metastasis. Thus, for the first-time cell surface receptor, EphA6 has been explored as a theranostic biomarker for detecting and simultaneously killing of metastatic prostate cancer in *in vitro* studies. The *in silico* binding studies, demonstrated that the fusion protein, his_6EGFP-azu (80–128) exhibited higher binding affinity for the EphA6 receptor compared to traditional ephrin A ligands via azu (80–128) peptide. Inspired by the *in*

silico binding studies, a novel recombinant protein his_6EGFP-azu (80-128) was designed, cloned, expressed, and purified. The his_6EGFP-azu (80–128) fusion protein, spontaneously self-assembled as nanoparticles, effectively targeted EphA6 receptors on prostate cancer cells (LNCaP) and demonstrated remarkable imaging potential, antiproliferative, apoptotic, antimigratory, and anti-invasive effects, while sparing EphA6-negative human normal lung cells (WI-38).

Further, to enhance the therapeutic efficacy of azurin via it's nanodelivery as cross-linked azurin-ELP micelles, a fusion protein composed of azurin and a thermally responsive structural cationic elastin-like protein (ELP), was designed, cloned, expressed, and purified. A simple method of inverse transition cycle (ITC) was employed to purify the fusion protein azurin-ELP diblock copolymer (d-bc). Further, it's self-assembly properties were investigated. Interestingly, the engineered azurin-ELP d-bc in response to increasing temperature shows a dual step phase separation into bio-functional nanostructures. Around physiological temperature azurin-ELP d-bc formed stable coacervates which was dependent on the concentration and time of incubation. These coacervates were formed below the lower critical solubility temperature (LCST) of the ELP block at physiological temperature. Above LCST micelles of size ranging from 25-30 nm are formed. The cytotoxicity of azurin-ELP d-bc depends on the size of coacervates formed and their cellular uptake at physiological temperature. Further, MTT assay of azurin-ELP d-bc in the cross-linked micelles prepared *ex situ* showed > six times higher killing of LNCaP cells than the unimeric form of azurin-ELP at 5 µM concentration. The flow cytometric results of these micelles at 20 µM concentration showed, ~97 % LNCaP cells in apoptotic phase. Thus, azurin-ELP cross-linked micelles enhanced potential for anticancer therapy due to their higher avidity.

Thus, in retrospect protein-engineered nanomedinces based on the recombinant proteins, azurin-ELP and his_6EGFP-azu (80–128) were designed by fusing, an anticancer bacterial protein, azurin with a thermoresponsive structural protein, ELP and azurin's peptide azu (80-128) with fluorescent protein EGFP, respectively. The unique functions of both the components in the fusion systems aided in the nanoscale self-assembly of the recombinant proteins to achieve enhanced therapeutic effect along with real time imaging of the prostate cancer cells. The work collectively represents innovative approaches for targeting prostate cancer aiming to overcome the limitations associated with conventional approach. These findings contribute

valuable insights to the evolving field of protein-based nanotherapeutics and nanotheranostics with significant potential for precision cancer therapy.

सार

प्रोस्टेट कैंसर में नैदानिक प्रगति के बावजूद, आक्रामक रूपों का शीघ्र पता लगाना चुनौतीपूर्ण बना हुआ है। प्रोस्टेट विशिष्ट एंटीजन (पीएसए) स्क्रीनिंग, उसके बाद बायोप्सी, मानक निदान पद्धति है। हालाँकि, पीएसए द्वारा अति-पहचान और निष्क्रिय और आक्रामक ट्यूमर के बीच अंतर करने में असमर्थता के कारण यह दृष्टिकोण बाधित होता है, जिससे अनावश्यक आक्रामक प्रक्रियाएं और अत्यधिक उपचार होता है। प्रोस्टेट विशिष्ट झिल्ली एंटीजन (पीएसएमए), प्रोस्टेट कैंसर की आक्रामकता से जुड़ा एक ऊतक आधारित बायोमार्कर ने वादा दिखाया है, लेकिन अभिव्यक्ति में विविधता के कारण नए बायोमार्कर की खोज की आवश्यकता होती है। सर्जरी, रेडियोथेरेपी, हार्मोनल थेरेपी, कीमोथेरेपी और इम्यूनोथेरेपी जैसे उपचारों ने मेटास्टैटिक प्रोस्टेट कैंसर के बोझ को कम कर दिया है। बहरहाल, असंवेदनशील और प्रतिरोधी ट्यूमर की पुनरावृत्ति और विकास जैसे अवांछनीय दुष्प्रभाव वैकल्पिक उपचार दृष्टिकोण की खोज करना अनिवार्य बनाते हैं।

पारंपरिक छोटे अणुओं वाली दवाओं की सीमाओं को दूर करने के लिए विभिन्न प्रोटीनों के विविध कार्यात्मक गुणों का उपयोग करके प्रोटीन-आधारित नैनोमेडिसिन कैंसर का पता लगाने और उपचार में एक आशाजनक विकल्प के रूप में उभरी है। पारंपरिक कीमोथेरेपी के विपरीत, प्रोटीन-आधारित नैनोमेडिसिन सटीक लक्ष्यीकरण, कम-लक्ष्य प्रभाव और बढ़ी हुई चिकित्सीय प्रभावकारिता प्रदान करती है। वर्तमान कार्य में, प्रोस्टेट कैंसर प्रबंधन के लिए आनुवंशिक इंजीनियरिंग के माध्यम से प्रोटीन के विभिन्न वर्ग को संयुग्मित करते हुए नवीन मिश्रित प्रोटीन इंजीनियर नैनोमेडिसिन डिजाइन किए गए थे।

एरिथ्रोपोइटिन-उत्पादक हेपैटोसेलुलर (ईएफ) रिसेप्टर्स, रिसेप्टर टायरोसिन किनेसेस का एक उपपरिवार, कैंसर के विकास और प्रगति में महत्वपूर्ण भूमिका निभाते हैं। इस प्रकार, कैंसर की प्रगति के साथ उनकी असामान्य अभिव्यक्ति के कारण इफ़्स प्रमुख कैंसर बायोमार्कर के रूप में उभरे हैं। स्यूडोमोनास एरुगिनोसा से अलग किया गया एक जीवाणु प्रोटीन, अज़ुरिन, ने एक तंत्र के रूप में अपने सी-टर्मिनल डोमेन के माध्यम से एफ़-एफ़्रिन सिग्नलिंग को बाधित करके ट्यूमर की प्रगति को रोकने में अपनी क्षमता का प्रदर्शन किया है। प्रोस्टेट कैंसर में, EphA6 रिसेप्टर्स की अधिक अभिव्यक्ति प्रोस्टेट कैंसर मेटास्टेसिस से जुड़ी हुई है। इस प्रकार, पहली बार कोशिका सतह रिसेप्टर, EphA6 को इन विट्रो अध्ययनों में मेटास्टैटिक प्रोस्टेट कैंसर का पता लगाने और साथ ही मारने के लिए एक चिकित्सीय बायोमार्कर के रूप में खोजा गया है। सिलिको बाइंडिंग अध्ययनों से पता चला है कि संलयन प्रोटीन, his_6EGFP-azu (80-128) ने azu (80-128) पेप्टाइड के माध्यम से पारंपरिक एफ्रिना लिगैंड की तुलना में EphA6 रिसेप्टर के

लिए उच्च बाध्यकारी संबंध प्रदर्शित किया है। सिलिको बाइंडिंग अध्ययनों से प्रेरित होकर, एक नवीन पुनः संयोजक प्रोटीन his_6EGFP-azu (80-128) को डिजाइन, क्लोन, व्यक्त और शुद्ध किया गया था। his_6EGFP-azu (80-128) संलयन प्रोटीन, नैनोकणों के रूप में स्वतः एकत्रित होकर, प्रोस्टेट कैंसर कोशिकाओं (LNCaP) पर EphA6 रिसेप्टर्स को प्रभावी ढंग से लक्षित करता है और EphA6-नकारात्मक मानव सामान्य फेफड़ों की कोशिकाओं (WI-38) को बख्शते हुए उल्लेखनीय इमेजिंग क्षमता, एंटीप्रोलिफेरेटिव, एपोप्टोटिक, एंटीमाइग्रेटरी और एंटी-इनवेसिव प्रभावों का प्रदर्शन करता है।

इसके अलावा, क्रॉस-लिंक्ड एज़ुरिन-ईएलपी मिसेल्स के रूप में नैनोडिलीवरी के माध्यम से अज़ुरिन की चिकित्सीय प्रभावकारिता को बढ़ाने के लिए, अज़ुरिन से बना एक संलयन प्रोटीन और एक थर्मल प्रतिक्रियाशील संरचनात्मक धनायनित इलास्टिन-जैसे प्रोटीन (ईएलपी) को डिजाइन, क्लोन, व्यक्त और शुद्ध किया गया था। संलयन प्रोटीन अज़ुरिन-ईएलपी डाइब्लॉक कॉपोलीमर (डी-बीसी) को शुद्ध करने के लिए व्युत्क्रम संक्रमण चक्र (आईटीसी) की एक सरल विधि को नियोजित किया गया था। इसके अलावा, इसकी सेल्फ-असेंबली संपत्तियों की जांच की गई। दिलचस्प बात यह है कि बढ़ते तापमान के जवाब में इंजीनियर्ड अज़ुरिन-ईएलपी डी-बीसी जैव-कार्यात्मक नैनोस्ट्रक्चर में दोहरे चरण चरण पृथक्करण को दर्शाता है। शारीरिक तापमान के आसपास अज़ुरिन-ईएलपी डी-बीसी ने स्थिर कोएसर्वेट्स का निर्माण किया जो ऊष्मायन की एकाग्रता और समय पर निर्भर था। ये कोएसर्वेट शारीरिक तापमान पर ईएलपी ब्लॉक के निचले महत्वपूर्ण घुलनशीलता तापमान (एलसीएसटी) से नीचे बने थे। एलसीएसटी के ऊपर 25-30 एनएम आकार के मिसेल बनते हैं। अज़ुरिन-ईएलपी डी-बीसी की साइटोटॉक्सिसिटी गठित कोएसर्वेट्स के आकार और शारीरिक तापमान पर उनके सेलुलर अवशोषण पर निर्भर करती है। इसके अलावा, क्रॉस-लिंक्ड मिसेलस तैयार पूर्व सीटू में अज़ुरिन-ईएलपी डी-बीसी के एमटीटी परख से पता चला कि 5 μM सांद्रता पर अज़ुरिन-ईएलपी के यूनिमेरिक रूप की तुलना में एलएनसीएपी कोशिकाओं की छह गुना अधिक हत्या हुई है। 20 μM सांद्रता पर इन मिसेलों के प्रवाह साइटोमेट्रिक परिणामों से पता चला, एपोप्टोटिक चरण में ~97 % LNCaP कोशिकाएं। इस प्रकार, अज़ुरिन-ईएलपी क्रॉस-लिंक्ड मिसेलस ने अपनी उच्च अम्लता के कारण कैंसर रोधी चिकित्सा की क्षमता बढ़ा दी।

इस प्रकार, पुनः संयोजक प्रोटीन पर आधारित पुनः संयोजक प्रोटीन इंजीनियर नैनोमेडिसिन में, एज़ुरिन-ईएलपी और उसके (80-128) को फ़्यूज़िंग द्वारा डिज़ाइन किया गया था, एक एंटीकैंसर बैक्टीरियल प्रोटीन, थर्मोरिस्पॉन्सिव संरचनात्मक प्रोटीन के साथ एज़ुरिन, ईएलपी और एज़ुरिन के पेप्टाइड azu (80 - 128) क्रमशः फ्लोरोसेंट प्रोटीन ईजीएफपी के साथ। संलयन प्रणालियों में दोनों घटकों के अनूठे कार्यों ने

प्रोस्टेट कैंसर कोशिकाओं की वास्तविक समय इमेजिंग के साथ-साथ उन्नत चिकित्सीय प्रभाव प्राप्त करने के लिए पुनः संयोजक प्रोटीन के नैनोस्केल स्व-संयोजन में सहायता की। यह कार्य पारंपरिक दृष्टिकोण से जुड़ी सीमाओं को दूर करने के उद्देश्य से प्रोस्टेट कैंसर को लक्षित करने के लिए सामूहिक रूप से नवीन दृष्टिकोण का प्रतिनिधित्व करता है। ये निष्कर्ष सटीक कैंसर थेरेपी के लिए महत्वपूर्ण क्षमता वाले प्रोटीन-आधारित नैनोथेराप्यूटिक्स और नैनोथेरानोस्टिक्स के विकसित क्षेत्र में मूल्यवान अंतर्दृष्टि प्रदान करते हैं।

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LIST OF SYMBOLS

α	alpha
β	beta
^{o}C	degree celsius
g	grams
h	hour
L	liter
μ	micro
M	molar
%	percentage
s	second
U	units
v/v	volume/volume
λ	wavelength

LIST OF ABBREVIATIONS

APS	ammonium persulfate
bp	base pairs
DNA	deoxyribonucleic acid
CD	circular dichroism
cm	centimeter
CMT	critical micellar temperature
d-bc	diblock copolymer
DSC	differential scanning calorimetry
DIC	differential interference contrast
EGFP	enhanced green fluorescent protein
ELP	elastin-like protein
Eph	erythropoietin-producing hepatocellular
FITC	fluorescein isothiocyanate
IPTG	isopropyl-β-D-thiogalactopyranoside
ITC	inverse transition cycling
kDa	kilodaltons
LB	luria bertani
LCST	lower critical solubility temperature
LNCaP	lymph node carcinoma of the prostate
µM	micromolar
µL	microlitre
mm	millimeter
mg	milligram
mL	millilitre

mins	minutes
nm	nanometer
MTT	3-4,5-dimethylthiazol-2-yl-2,5-diphenyl tetrazolium bromide
Ni-NTA	nickel-nitrilotriacetic acid
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
RPM	rotation per minute
TB	terrific broth
TEM	transmission electron microscopy
TEMED	N,N,N′,N′-tetramethylethylene diamine
UV	ultraviolet
w/v	weight/volume
w/w	weight/weight