

**DEVELOPMENT OF NANOPROBES AND 3D
IN VITRO MODELS FOR DISEASE-SPECIFIC
BIOMARKER DETECTION**

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Development of Nanoprobes and 3D *In Vitro* Models for Disease-Specific Biomarker Detection

by

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Submitted

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



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Dedicated to Mom and Dad

Certificate

This is to certify that the thesis entitled '**Development of Nanoprobes and 3D *In Vitro* Models for Disease-Specific Biomarker Detection**' being submitted by **Ms. Simran Kaur Rainu** to the Indian Institute of Technology Delhi for the award of degree of **Doctor of Philosophy** is a record of bonafide research work carried out by her. **Ms. Simran Kaur Rainu** has worked under my guidance and supervision and has fulfilled the requirements for the submission of this thesis, which to our knowledge has reached the requisite standard.

The results contained in this thesis are original and 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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Simran Kaur Rainu

Abstract

Alteration in the physicochemical characteristics of the extracellular matrix (ECM) and tissue environment are commonly observed in various pathological conditions such as cancer, arthritis, cardiovascular diseases, inflammatory diseases, and liver diseases. These changes drive initiation and progression of diseases by influencing cell-matrix interactions. Dysregulated expression of matrix metalloproteinases (MMPs) and alterations in pH levels are the two key factors commonly associated with cancer and fatty liver disease progression. Early and accurate identification of these molecular and physical changes is essential for timely diagnosis and intervention in these diseases, thereby improving patient outcomes. Therefore, there is a critical need for probes capable of real-time monitoring and distinguishing diseased tissue from healthy one.

Chapter 1 of the thesis provides an introductory overview, discussing the role of ECM in disease progression. It introduces fluorescent nanoprobe and 3D *in vitro* platforms, outlining their importance in the field. Additionally, it identifies gaps in current research, laying the groundwork for further investigation in subsequent chapters. The goal of this thesis is to develop fluorescent nanoprobe for detecting multiple disease-specific parameters and to validate these nanoprobe in various 3D *in vitro* disease platforms that closely mimic *in vivo* conditions. To achieve this goal, in **Chapter 2**, a dual-sensitive nanoprobe using carbon nanoparticles as pH-sensing fluorescent components, decorated with MMP-9 sensitive peptide sequence attached to a fluorophore-quencher pair, was synthesised. To validate these nanoprobe, in **Chapter 3**, a classic cancer model was developed wherein low pH and elevated MMP expression is observed. Stiffness-tuneable dual-sensitive nanoprobe-laden 3D scaffolds were employed to understand the impact of mechanical cues on MMP-9 expression in cancerous cells and to visualise changes in pH in the tumor microenvironment. Additionally, a 3D core-shell model with stiff cancerous

core and soft non-cancerous shell, mimicking a tumor-margin condition was developed and these regions were distinguished visually using dual-sensitive nanoprobe. Furthermore, this platform was utilised to visually study the role of cytokines (TNF- α) secreted by macrophages in upregulating MMP-9 expression in cancerous cells.

Subsequently, the potential of these nanoprobe in distinguishing stages of non-alcoholic fatty liver disease (NAFLD) was explored. NAFLD is a heterogenous condition that encompass various liver diseases from simple hepatic steatosis to cirrhosis, requiring comprehensive analysis of the physicochemical and biological factors that drive its progression. The earliest stage of NAFLD is hepatic steatosis which is marked by triglyceride accumulation in the cytosol of hepatocytes. This fatty acid accumulation is usually accompanied by hepatic inflammation leading to tissue acidification and dysregulated protease expression. Moreover, this condition further progresses to fibrotic stage, which is accompanied with extensive ECM remodelling characterised by substantial deposition and crosslinking of ECM proteins particularly collagen, resulting in increased tissue stiffness and altered MMP expression profile.

Therefore, taking cues from biological parameters of the disease and to validate nanoprobe, in **Chapter 4**, a simplistic 3D *in vitro* liver disease model encapsulating triple-marker sensitive fluorescent nanoprobe (pH, MMP-3, MMP-9) was developed for real-time monitoring of these changes to better understand the disease condition and hence, enabling the distinction between healthy and diseased liver tissue (steatotic) based on pH changes and MMP expression. In the final chapter, **Chapter 5**, the utility of nanoprobe to understand the intricate dynamics of matrix composition, elasticity and diseased cell conditions in the progression of NAFLD was explored. 3D NAFLD models representing healthy, steatotic, and fibrotic liver matrices were prepared to examine how changes in MMP expression and collagen deposition drive the progression of NAFLD. A

comprehensive investigation of the combined effects of mechanical cues, collagen deposition, and fatty acid treatment from day 1 yielded valuable insights into the correlation between MMP-9 and matrix properties. Real-time monitoring using nanoprobe facilitated a critical understanding into the underlying mechanisms of liver disease, offering a promising NAFLD platform for further research and therapeutic development. The conclusion of the thesis is found in **Chapter 6** with discussion on future prospects.

In summary, the development of fluorescent nanoprobe has provided a crucial tool for real-time monitoring and understanding of disease progression, particularly in cancer and non-alcoholic fatty liver disease (NAFLD). Through innovative 3D *in vitro* models, this research offers insights into the interplay of biochemical and mechanical factors driving disease, facilitating early diagnosis and targeted interventions for improved patient outcomes. These findings lay the groundwork for further research and therapeutic advancements in combating these complex diseases.

सार

बाह्य कोशिकीय मैट्रिक्स (ईसीएम) और ऊतक वातावरण की भौतिक रासायनिक विशेषताओं में परिवर्तन आमतौर पर कैंसर, गठिया, हृदय रोग, सूजन संबंधी बीमारियों और यकृत रोगों जैसी विभिन्न रोग स्थितियों में देखा जाता है। ये परिवर्तन सेल-मैट्रिक्स इंटरैक्शन को प्रभावित करके रोगों की शुरुआत और प्रगति को प्रेरित करते हैं। मैट्रिक्स मेटालोप्रोटीनिस (एमएमपी) की अनियंत्रित अभिव्यक्ति और पीएच स्तर में परिवर्तन दो प्रमुख कारक हैं जो आमतौर पर कैंसर और फैटी लीवर रोग की प्रगति से जुड़े होते हैं। इन रोगों में समय पर निदान और हस्तक्षेप के लिए इन आणविक और शारीरिक परिवर्तनों की प्रारंभिक और सटीक पहचान आवश्यक है, जिससे रोगी के परिणामों में सुधार होता है। इसलिए, वास्तविक समय की निगरानी करने और रोगग्रस्त ऊतक को स्वस्थ ऊतक से अलग करने में सक्षम जांच की अत्यधिक आवश्यकता है।

थीसिस का **अध्याय 1** एक परिचयात्मक अवलोकन प्रदान करता है, जिसमें रोग की प्रगति में ईसीएम की भूमिका पर चर्चा की गई है। यह क्षेत्र में उनके महत्व को रेखांकित करते हुए फ्लोरोसेंट नैनोप्रोब और 3डी इन विट्रो प्लेटफॉर्म पेश करता है। इसके अतिरिक्त, यह वर्तमान शोध में कमियों की पहचान करता है, और बाद के अध्यायों में आगे की जांच के लिए आधार तैयार करता है। इस थीसिस का लक्ष्य कई रोग-विशिष्ट मापदंडों का पता लगाने के लिए फ्लोरोसेंट नैनोप्रोब विकसित करना है और इन नैनोप्रोब को विभिन्न 3डी इन विट्रो रोग प्लेटफॉर्मों में मान्य करना है जो विविध स्थितियों की बारीकी से नकल करते हैं। इस लक्ष्य को प्राप्त करने के लिए, **अध्याय 2** में, पीएच-सेंसिंग फ्लोरोसेंट घटकों के रूप में कार्बन नैनोकणों का उपयोग करते हुए एक दोहरे-संवेदनशील नैनोप्रोब को फ्लोरोफोर-क्वेंचर जोड़ी से जुड़े एमएमपी-9 संवेदनशील पेप्टाइड अनुक्रम से सजाया गया था, जिसे संश्लेषित किया गया था। इन नैनोप्रोबों को मान्य करने के लिए, **अध्याय 3** में, एक क्लासिक कैंसर मॉडल विकसित किया गया था जिसमें कम पीएच और ऊंचा एमएमपी अभिव्यक्ति देखी गई है। कैंसरग्रस्त कोशिकाओं में एमएमपी-9 अभिव्यक्ति पर यांत्रिक संकेतों के प्रभाव को समझने और ट्यूमर माइक्रोएन्वायरमेंट में पीएच में परिवर्तन की कल्पना करने के लिए कठोरता-ट्यून करने योग्य दोहरे-संवेदनशील नैनोप्रोब-युक्त 3डी मचानों को नियोजित किया गया था। इसके अतिरिक्त, ट्यूमर-मार्जिन स्थिति की नकल करते हुए कठोर कैंसरयुक्त कोर और मुलायम गैर-कैंसरयुक्त खोल वाला एक 3डी कोर-शेल मॉडल विकसित किया गया था और दोहरे-संवेदनशील नैनोप्रोब का उपयोग करके इन क्षेत्रों को दृष्टिगत रूप से अलग किया गया था। इसके अलावा, इस प्लेटफॉर्म का उपयोग

कैंसर कोशिकाओं में एमएमपी-9 अभिव्यक्ति को विनियमित करने में मैक्रोफेज द्वारा सावित साइटोकिन्स (टीएनएफ- α) की भूमिका का दृश्य अध्ययन करने के लिए किया गया था।

इसके बाद, गैर-अल्कोहल फैटी लीवर रोग (एनएएफएलडी) के विशिष्ट चरणों में इन नैनोप्रोब की क्षमता का पता लगाया गया। एनएएफएलडी एक विषम स्थिति है जो साधारण हेपेटिक स्टीटोसिस से लेकर सिरोसिस तक विभिन्न यकृत रोगों को शामिल करती है, जिसके लिए भौतिक-रासायनिक और जैविक कारकों के व्यापक विश्लेषण की आवश्यकता होती है जो इसकी प्रगति को बढ़ाते हैं। एनएएफएलडी का प्रारंभिक चरण हेपेटिक स्टीटोसिस है जो हेपेटोसाइट्स के साइटोसोल में ट्राइग्लिसराइड संचय द्वारा चिह्नित होता है। यह फैटी एसिड संचय आम तौर पर यकृत सूजन के साथ होता है जिससे ऊतक अम्लीकरण और अनियंत्रित प्रोटीज़ अभिव्यक्ति होती है। इसके अलावा, यह स्थिति आगे चलकर फ़ाइब्रोटिक चरण तक पहुंच जाती है, जिसमें व्यापक ईसीएम रीमॉडलिंग होती है, जिसमें ईसीएम प्रोटीन विशेष रूप से कोलेजन के पर्याप्त जमाव और क्रॉसलिंग की विशेषता होती है, जिसके परिणामस्वरूप ऊतक कठोरता में वृद्धि होती है और एमएमपी अभिव्यक्ति प्रोफ़ाइल बदल जाती है।

इसलिए, रोग के जैविक मापदंडों से संकेत लेते हुए और नैनोप्रोब को मान्य करने के लिए, **अध्याय 4** में, ट्रिपल-मार्कर संवेदनशील फ़्लोरोसेंट नैनोप्रोब (पीएच, एमएमपी -3, एमएमपी -9) को समाहित करने वाला एक सरल 3डी इन विट्रो लीवर रोग मॉडल वास्तविक रूप से विकसित किया गया था। रोग की स्थिति को बेहतर ढंग से समझने के लिए इन परिवर्तनों की समय पर निगरानी करना और पीएच परिवर्तन और एमएमपी अभिव्यक्ति के आधार पर स्वस्थ और रोगग्रस्त यकृत ऊतक (स्टीटोटिक) के बीच अंतर करना संभव बनाता है। अंतिम अध्याय, **अध्याय 5** में, एनएएफएलडी की प्रगति में मैट्रिक्स संरचना, लोच और रोगग्रस्त कोशिका स्थितियों की जटिल गतिशीलता को समझने के लिए नैनोप्रोब की उपयोगिता का पता लगाया गया था। स्वस्थ, स्टीटोटिक और फ़ाइब्रोटिक लीवर मैट्रिसेस का प्रतिनिधित्व करने वाले 3डी एनएएफएलडी मॉडल यह जांचने के लिए तैयार किए गए थे कि एमएमपी अभिव्यक्ति और कोलेजन जमाव में परिवर्तन एनएएफएलडी की प्रगति को कैसे बढ़ाते हैं। पहले दिन से यांत्रिक संकेतों, कोलेजन जमाव और फैटी एसिड उपचार के संयुक्त प्रभावों की व्यापक जांच से एमएमपी-9 और मैट्रिक्स गुणों के बीच संबंध में मूल्यवान अंतर्दृष्टि प्राप्त हुई। नैनोप्रोब का उपयोग करके वास्तविक समय की निगरानी ने यकृत रोग के अंतर्निहित तंत्र में एक महत्वपूर्ण समझ की सुविधा प्रदान की, जो आगे के अनुसंधान और चिकित्सीय विकास के लिए एक

आशाजनक एनएएफएलडी मंच की पेशकश करता है। थीसिस का निष्कर्ष **अध्याय 6** में भविष्य की संभावनाओं पर चर्चा के साथ मिलता है।

संक्षेप में, फ्लोरोसेंट नैनोप्रोब के विकास ने रोग की प्रगति की वास्तविक समय की निगरानी और समझ के लिए एक महत्वपूर्ण उपकरण प्रदान किया है, विशेष रूप से कैंसर और गैर-अल्कोहल फैटी लीवर रोग (एनएएफएलडी) में। नवोन्मेषी 3डी इन विट्रो मॉडल के माध्यम से, यह शोध रोग को प्रेरित करने वाले जैव रासायनिक और यांत्रिक कारकों की परस्पर क्रिया में अंतर्दृष्टि प्रदान करता है, जिससे रोगी के बेहतर परिणामों के लिए शीघ्र निदान और लक्षित हस्तक्षेप की सुविधा मिलती है। ये निष्कर्ष इन जटिल बीमारियों से निपटने के लिए आगे के शोध और चिकित्सीय प्रगति के लिए आधार तैयार करते हैं।

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List of Abbreviations

µg	Microgram
µL	Microliter
2D	2 Dimensional
3D	3 Dimensional
6'TAMRA	6-Carboxytetramethylrhodamine
BSA	Bovine serum albumin
Calcein AM	Calcein acetoxymethyl ester
CNP	Carbon Nanoparticle
D ₂ O	Deuterium Oxide
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
ECM	Extracellular Matrix
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
EDX	Energy Dispersive X-ray analysis
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal Bovine Serum
FRET	Fluorescence Resonance Energy Transfer
GelMA	Gelatin Methacryloyl
HBSS	Hank's Balanced Salt Solution
HRTEM	High Resolution Transmission Electron Microscopy
IL-6	Interleukin-6
kDa	Kilo Dalton
LPS	Lipopolysaccharide
M.A.	Methacrylic anhydride
MES	2-(N-morpholino)ethanesulfonic acid
mg	Milligram
mL	Milliliter
mM	Millimolar
mm	Millimeter
MMP	Matrix Metalloproteinase

MTT	3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide
NAFLD	Non-alcoholic fatty liver disease
ng	Nanogram
NHS	N-hydroxysuccinimide
nM	Nanomolar
NMR	Nuclear Magnetic Resonance
°C	Degrees Celsius
PBS	Phosphate Buffered Saline
PEG	Poly ethylene glycol
PFA	Paraformaldehyde
PI	Propidium Iodide
QY	Quantum Yield
ROS	Reactive Oxygen Species
rpm	Rotations per minute
RPMI	Roswell Park Memorial Institute
SEM	Scanning Electron Microscopy
TEM	Transmission Electron Microscopy
TNF α	Tumor necrosis factor alpha