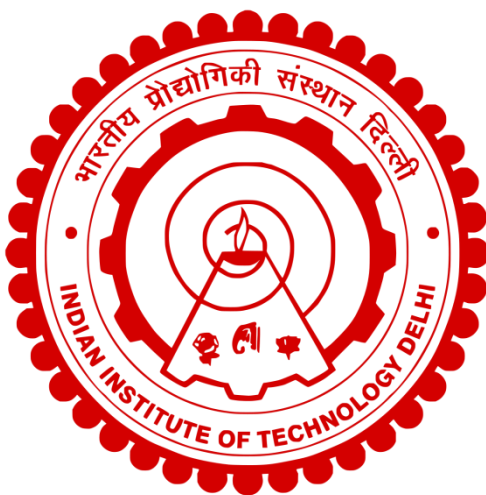


**SYNTHESIS AND CHARACTERIZATION OF DERMAL
NANO-BIOCOMPOSITES FOR WOUND HEALING AND
EVALUATION BY NMR METABOLOMICS**

ISHA GUPTA



**DEPARTMENT OF CHEMISTRY
INDIAN INSTITUTE OF TECHNOLOGY DELHI**

AUGUST 2024

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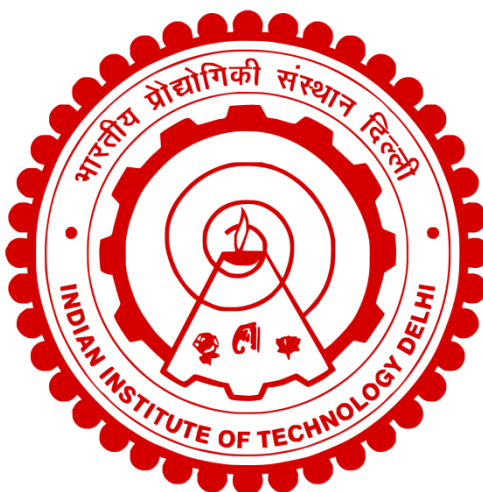
ISHA GUPTA

Department of Chemistry

Submitted

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

to the



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AUGUST 2024

*Dedicated to
My Parents
& Husband*

CERTIFICATE

This is to certify that the thesis entitled, “**SYNTHESIS AND CHARACTERIZATION OF DERMAL NANO-BIOCOMPOSITES FOR WOUND HEALING AND EVALUATION BY NMR METABOLOMICS**” being submitted by **Ms. ISHA GUPTA** to the **Indian Institute of Technology Delhi** for the award of the degree of **Doctor of Philosophy** in Chemistry, is a record of bonafide research work carried out by her. Ms. ISHA GUPTA has worked under our guidance and supervision. She has fulfilled the requirements for the submission of this thesis, which to our knowledge has reached the requisite standard.

Dr. Sonia Gandhi
Scientist ‘F’
Head, Functional MR & Spectroscopy Department
Institute of Nuclear Medicine and Allied Sciences
Defence Research and Development Organisation
Delhi - 110054

Prof. Sameer Sapra
Department of Chemistry
Indian Institute of Technology Delhi
New Delhi – 110016

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Isha Gupta

Abstract

Wound care is a clinical challenge due to the prevalence of antibiotic-resistant bacterial strains, degradation of living standards, extended hospital stays, and complex and lengthy treatments. The battlefield scenario also becomes uglier with increased wound complexity and haemorrhage. In India, 4.5 and 10.5 per 1000 people suffer from chronic and acute wounds, respectively. Thus, wound care management is expected to reach 16.36 billion USD globally by 2030, leading to demand for new bioactive hemostatic wound dressings to reduce this burden on the healthcare system. Recently, nanoparticle-based bioactive dressings (nano-biocomposites) have garnered the attention of researchers and caregivers. These are made of nanoparticles and biopolymers as fillers and matrices, respectively. The nano-biocomposites are better than the macro-biocomposites due to their unique properties, such as the large interfacial area between biopolymers and nanoparticles, mimicking the native tissue, and their antimicrobial nature. The nanoparticles (NPs) such as silver, zinc oxide, gold, magnetite, selenium and titanium oxide have complex antimicrobial actions, considerably reducing the development of drug resistance in bacteria. The size and surface of the NPs can be easily manipulated depending on the application and increasing popularity among scientists. Natural polymers (biopolymers) such as chitosan and gelatin accelerate wound healing. Sooner or later, human exposure to them is inevitable. Hence, toxicity studies should also be carried out meticulously. The current toxicity studies lack molecular information regarding the toxicity mechanism, which may even result in adverse reactions among the population. The present thesis is divided into developing a nanoparticle-reinforced biocomposite and then investigating the toxicity of nanoparticles and nano-biocomposite at the molecular level by metabolomics. In this regard, silver nanoparticles (Ag NPs) are synthesized through green synthesis and then used to develop biocomposites through solution

blending. Furthermore, their cytotoxicity and biocompatibility are evaluated against murine fibroblast (L929) cells through NMR spectroscopy.

Chapter 1 deals with the skin anatomy, classification of wounds, physiology of wound healing, factors responsible for impaired wound healing and commercially available hemostatic dressings. This chapter elaborately discusses the inorganic nanoparticle-reinforced biocomposites, their components and their potential in healing wounds. This chapter also highlights the synergism of cell culture and pharmacology-based metabolomics to evaluate the material's efficacy. The objective of the thesis is also covered in this chapter. **Chapter 2** includes the synthesis of nanoparticles, composites and their characterization techniques. It also briefly covers NMR spectroscopy as an analytical technique for Metabolomics. This chapter also discusses the different *in vitro* assays conducted to evaluate the material's efficiency. **Chapter 3** is devoted to the synthesis and characterization of silver nanoparticles (Ag NPs) and zinc oxide nanoparticles (ZnO NPs) from an eco-friendly route using *Ocimum sanctum* (tulsi). Ag NPs are found to be better than ZnO NPs because of their better dispersion in water, which is responsible for their sensitivity to the growth of bacteria. **Chapter 4** is dedicated to preparing and characterizing multifunctional biocomposite from chitosan and gelatin (ratio = 1:1) based on Ag NPs. Different concentrations of Ag NPs (0, 1, 2%, w/v) are used to prepare nano-biocomposite films (CG, CG₁ and CG₂) through the physical blending process followed by the solvent evaporation method. This chapter inspected the features of nano-biocomposites as a potential topical wound dressing. In **Chapter 5**, we employed NMR-based metabolomics to investigate the effects of Ag NPs and silver nitrate (AgNO₃) on murine fibroblast (L929) cells, as these cells are primarily used for *in vitro* studies of NPs and topical drugs. Based on the MTT assay, the L929 cells are incubated with 0, 1 and 5 µg/mL of green synthesis-mediated Ag NPs and AgNO₃ for 24 and 48 h. The treated cells are also morphologically observed with the help of Acridine orange

(AO) and Ethidium bromide (EB) staining under the fluorescence microscope. The cellular metabolome was analyzed with the help of high-resolution NMR spectroscopy, and the datasets were evaluated using multivariate analysis. NMR-based metabolomics showed that AgNO₃ exposure induced early metabolic alteration (membrane, energy metabolites and amino acids), while Ag NPs took another 24 hours to stimulate cell alterations at the same concentration. This study presented NMR-based metabolomics as a potential analytical tool to reveal early phenotype-dependent cellular events in L929 cells on exposure to green silver nanoparticles. In **Chapter 6**, we exploited the NMR spectroscopy technique on Ag NPs reinforced biocomposite exposed murine fibroblast (L929) cells to detect early metabolic alterations associated with cell-nanomaterial interaction. It demonstrated that cellular metabolic profiles reset towards control upon more exposure. **Chapter 7** summarizes the results of the present thesis, emphasizing the application of Ag NPs reinforced biocomposite as a potential candidate for topical application in wound healing and taking a closer look toward the future prospects of the work described in this chapter.

सार

एंटीबायोटिक-प्रतिरोधी जीवाणु उपभेदों की व्यापकता, जीवन स्तर में गिरावट, लंबे समय तक अस्पताल में रहने और जटिल और लंबे उपचार के कारण घाव की देखभाल एक नैदानिक चुनौती है। घाव की जटिलता और रक्तस्राव में वृद्धि के साथ युद्धक्षेत्र का परिदृश्य भी बदसूरत हो जाता है। भारत में, प्रति 1000 लोगों में क्रमशः 4.5 और 10.5 लोग जीर्ण और तीव्र घावों से पीड़ित हैं। इस प्रकार, 2030 तक वैश्विक स्तर पर घाव देखभाल प्रबंधन 16.36 बिलियन अमेरिकी डॉलर तक पहुंचने की उम्मीद है, जिससे स्वास्थ्य देखभाल प्रणाली पर इस बोझ को कम करने के लिए नए बायोएक्टिव हेमोस्टैटिक घाव ड्रेसिंग की मांग बढ़ेगी। हाल ही में, नैनोकण-आधारित बायोएक्टिव ड्रेसिंग (नैनो-बायोकंपोजिट्स) ने शोधकर्ताओं और देखभाल करने वालों का ध्यान आकर्षित किया है। ये क्रमशः फिलर्स और मैट्रिसेस के रूप में नैनोकणों और बायोपॉलिमर से बने होते हैं। नैनो-बायोकंपोजिट अपने अद्वितीय गुणों के कारण मैक्रो-बायोकंपोजिट से बेहतर हैं, जैसे कि बायोपॉलिमर और नैनोकणों के बीच बड़ा इंटरफेशियल क्षेत्र, मूल ऊतक की नकल करना और उनकी रोगाणुरोधी प्रकृति। सिल्वर, जिंक ऑक्साइड, गोल्ड, मैग्नेटाइट, सेलेनियम और टाइटेनियम ऑक्साइड जैसे नैनोकणों (NPs) में जटिल रोगाणुरोधी क्रियाएं होती हैं, जो बैक्टीरिया में दवा प्रतिरोध के विकास को काफी कम कर देती हैं। NPs के आकार और सतह को अनुप्रयोग और वैज्ञानिकों के बीच बढ़ती लोकप्रियता के आधार पर आसानी से हेरफेर किया जा सकता है। चिटोसिन और जिलेटिन जैसे प्राकृतिक पॉलिमर (बायोपॉलिमर) घाव भरने में तेजी लाते हैं। देर-सबेर, उनका मानव संपर्क अपरिहार्य है। इसलिए, विषाक्तता का अध्ययन भी सावधानीपूर्वक किया जाना चाहिए। वर्तमान विषाक्तता अध्ययनों में विषाक्तता तंत्र के संबंध में आणविक जानकारी का अभाव है, जिसके परिणामस्वरूप आबादी के बीच प्रतिकूल प्रतिक्रियाएं भी हो सकती हैं। वर्तमान थीसिस को नैनोकण-प्रबलित बायोकंपोजिट विकसित करने और मेटाबोलॉमिक्स द्वारा आणविक स्तर पर नैनोकणों और नैनो-बायोकंपोजिट की विषाक्तता की जांच करने में विभाजित किया गया है। इस संबंध में, सिल्वर नैनोकणों (Ag NPs) को हरित संश्लेषण के माध्यम से संश्लेषित किया गया है और फिर समाधान सम्मिश्रण के माध्यम से बायोकंपोजिट विकसित करने के लिए उपयोग किया गया है। इसके अलावा, एनएमआर स्पेक्ट्रोस्कोपी के माध्यम से म्यूरिन फाइब्रोब्लास्ट (L929) कोशिकाओं के खिलाफ उनकी साइटोटॉक्सिसिटी और बायोकम्पैटिबिलिटी का मूल्यांकन किया गया है।

अध्याय 1 त्वचा की शारीरिक रचना, घावों का वर्गीकरण, घाव भरने की फिजियोलॉजी, घाव भरने में बाधा के लिए जिम्मेदार कारक और व्यावसायिक रूप से उपलब्ध हेमोस्टैटिक ड्रेसिंग से संबंधित है। यह अध्याय अकार्बनिक नैनोकण-प्रबलित बायोकंपोजिट, उनके घटकों और घावों को ठीक करने की उनकी क्षमता पर विस्तृत रूप से चर्चा करता है। यह अध्याय सामग्री की प्रभावकारिता का मूल्यांकन करने के लिए सेल कल्चर और फार्माकोलॉजी-आधारित मेटाबोलॉमिक्स के तालमेल पर भी प्रकाश डालता है। थीसिस का उद्देश्य भी इस अध्याय में शामिल है। अध्याय 2 में नैनोकणों और कंपोजिट का संश्लेषण और उनकी लक्षण वर्णन तकनीकें शामिल हैं। इसमें मेटाबोलॉमिक्स के लिए एक विश्लेषणात्मक तकनीक के रूप में एनएमआर स्पेक्ट्रोस्कोपी को भी संक्षेप में शामिल किया गया है। यह अध्याय सामग्री की दक्षता का मूल्यांकन करने के लिए किए गए विभिन्न इन विट्रो परीक्षणों पर भी चर्चा करता है। अध्याय 3 ऑसिमम सैंकटम (तुलसी) का उपयोग करके पर्यावरण-अनुकूल मार्ग से सिल्वर नैनोकणों (Ag NPs) और जिंक ऑक्साइड नैनोकणों (ZnO NPs) के संश्लेषण और लक्षण वर्णन के लिए समर्पित है। Ag NPs पानी में बेहतर फैलाव और बैक्टीरिया के विकास के प्रति संवेदनशीलता के कारण ZnO NPs से बेहतर पाए गए हैं। अध्याय 4 Ag NPs के आधार पर चिटोसिन और जिलेटिन (अनुपात = 1:1) से बहुक्रियाशील बायोकंपोजिट तैयार करने और चिह्नित करने के लिए समर्पित है। Ag NPs (0, 1, 2%, w/v) की विभिन्न सांद्रता का उपयोग विलायक वाष्पीकरण विधि के बाद भौतिक मिश्रण प्रक्रिया के माध्यम से नैनो-बायोकंपोजिट फिल्म (CG, CG₁ and CG₂) तैयार किया है। इस अध्याय में संभावित सामयिक घाव ड्रेसिंग के रूप में नैनो-बायोकंपोजिट की विशेषताओं का निरीक्षण किया गया है। अध्याय 5 में, हमने म्यूरिन फ़ाइब्रोब्लास्ट (L929) कोशिकाओं पर Ag NPs और सिल्वर नाइट्रेट (AgNO₃) के प्रभावों की जांच के लिए NMR-आधारित मेटाबोलॉमिक्स को नियोजित किया, क्योंकि इन कोशिकाओं का उपयोग मुख्य रूप से NPs और सामयिक दवाओं के इन विट्रो अध्ययन के लिए किया गया है। MTT के आधार पर, L929 कोशिकाओं को 24 और 48 घंटों के लिए 0, 1 और 5 µg/mL हरित संश्लेषण-मध्यस्थता वाले Ag NPs और AgNO₃ के साथ इनक्यूबेट किया गया है। उपचारित कोशिकाओं को प्रतिदीप्ति माइक्रोस्कोप के तहत एक्रिडीन ऑरेंज (AO) और एथिडियम ब्रोमाइड (EB) धुंधला की मदद से रूपात्मक रूप से भी देखा गया है। उच्च-रिज़ॉल्यूशन NMR स्पेक्ट्रोस्कोपी की मदद से सेलुलर मेटाबोलोम का विश्लेषण किया गया है, और मल्टीवेरिएट विश्लेषण का उपयोग करके डेटासेट का मूल्यांकन किया गया है। NMR-आधारित मेटाबोलॉमिक्स ने दिखाया कि AgNO₃ एक्सपोजर ने प्रारंभिक चयापचय परिवर्तन (झिल्ली, ऊर्जा मेटाबोलाइट्स और अमीनो एसिड) को प्रेरित किया, जबकि Ag NPs को उसी एकाग्रता में सेल परिवर्तन को उत्तेजित करने में 24 घंटे लग गए। इस अध्ययन ने पर्यावरण के अनुकूल Ag NPs के संपर्क में आने पर L929 कोशिकाओं में प्रारंभिक फेनोटाइप-निर्भर सेलुलर घटनाओं को प्रकट करने के लिए NMR-आधारित

मेटाबोलॉमिक्स को एक संभावित विश्लेषणात्मक उपकरण के रूप में प्रस्तुत किया गया है । अध्याय 6 में, हमने सेल नैनोमटेरियल इंटरैक्शन से जुड़े प्रारंभिक चयापचय परिवर्तनों का पता लगाने के लिए Ag NPs प्रबलित बायोकंपोजिट एक्सपोज़्ड म्यूरिन फ़ाइब्रोब्लास्ट (L929) कोशिकाओं पर NMR स्पेक्ट्रोस्कोपी तकनीक का उपयोग किया। इससे पता चला कि सेलुलर मेटाबॉलिक प्रोफाइल अधिक एक्सपोज़र पर नियंत्रण की ओर रीसेट हो जाता है। अध्याय 7 वर्तमान थीसिस के परिणामों को सारांशित करता है, घाव भरने में सामयिक अनुप्रयोग के लिए संभावित उम्मीदवार के रूप में Ag NPs प्रबलित बायोकंपोजिट के अनुप्रयोग पर जोर देता है और इस अध्याय में वर्णित कार्य की भविष्य की संभावनाओं पर करीब से नज़र डालता है।

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GLOSSARY OF SYMBOLS AND ABBREVIATIONS

NPs	Nanoparticles
2D	two-dimensional
g	Gram
h	Hour
min	Minute
°C	degree centigrade
mM	Millimolar
mL	Millilitre
s	Second
μL	microlitre
mm	millimeter
nm	nanometer
Å	Angstrom
rpm	revolutions per minute
h	Hour
Hz	Hertz
w/v	weight/volume
v/v	volume/volume
kV	kilovolt
mA	milliAmpere
ECM	Extracellular matrix
PEC	Polyelectrolyte complex

DNA	Deoxyribonucleic acid
UV-Vis	Ultraviolet visible
PXRD	Powder X-Ray diffraction
TEM	Transmission electron microscopy
FTIR	Fourier-transform infrared (FTIR) spectroscopy.
FESEM	Field emission scanning electron microscopy
TGA	Thermogravimetric analysis
DLS	Dynamic light scattering
MS	Mass spectrometry
¹ H NMR	Proton Nuclear magnetic resonance
ROS	Reactive oxygen species
PBS	Phosphate buffered saline
L929	Murine fibroblast cells
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
DMEM	Dulbeco's Modified Eagle Medium
FBS	Fetal Bovine serum
CFU	colony forming units
TSP	Trimethylsilyl-2,2,3,3-tetra-deuteriopropionic acid
NOESYPR	Nuclear overhauser effect spectroscopy presaturation
JCPDS	Joint Committee on Powder Diffraction Standards
Calcein-AM	Calcein acetoxymethyl
AO	Acridine orange
EB	Ethidium bromide
PI	Propidium iodide
TXI	Triple resonance probe

FID	Free induction decay
HMDB	Human metabolome database
ANOVA	Analysis of variance
PCs	Principle components
PCA	Principle component analysis
PLS-DA	Partial least squares discriminant analysis
VIP	Variable importance in projection
Q ²	predictive ability
R ²	goodness of fit
SD	Standard deviation
TCA	Tricarboxylic acid
ADP	Adenine diphosphate
ATP	Adenosine triphosphate
NAD	Nicotinamide adenine dinucleotide
NADP ⁺	Nicotinamide adenine dinucleotide phosphate
GlcN	Glucosamine
UDP	Uridine diphosphate
UMP	Uridine 5'-monophosphate
UDP-GlcNAc	Uridine diphosphate-N-acetylglucosamine