

Structural, Functional and Morphological Modifications of Graphene Oxide-based Nanocarriers for Anticancer Drug Delivery



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**Structural, Functional and Morphological
Modifications of Graphene Oxide-based Nanocarriers
For Anticancer Drug Delivery**

*Thesis submitted in partial fulfillment of the requirements
for the degree of*

DOCTOR OF PHILOSOPHY

by

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*Dedicated to my Parents, sister,
brothers and friends for their
faith, love, understanding
and support*



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CERTIFICATE

It is certified that the work reported in the thesis entitled “**Structural, Functional and Morphological Modifications of Graphene Oxide-based Nanocarriers for Anticancer Drug Delivery**” by **Ankush Dasharath Sontakke**, a Ph.D. student of the Department of Chemical Engineering, Indian Institute of Technology Guwahati, has been carried out under my supervision. The work documented in this thesis has not been submitted to any other University or Institute for the award of any degree or diploma.

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Abstract

Chemotherapy has continued to be the most frequently utilized therapy for cancer treatment. Despite the abundance of chemical chemotherapeutic agents and anticancer medications, their unregulated administration and inability to distinguish between rapidly proliferating healthy cells and cancer cells lead to adverse impacts on cancer therapeutics. Moreover, due to the diversity of cancer cells, the emergence of drug resistance, and the undesirable effects induced by high and/or repetitive drug doses, the administration of a solitary chemotherapeutic agent frequently fails to accomplish a comprehensive cancer regression. The major challenges related to effective and sustainable consumption of chemotherapeutic agents can be subsided with targeted drug delivery systems (DDS) directed by the advanced nanomaterials (nanocarriers), natural chemotherapeutic agents such as gallic acid (GA), caffeic acid (CA), targeting ligands such as folic acid (FA) and employing co-delivery systems for chemotherapeutic agents. Previous studies related to anticancer DDS have demonstrated the therapeutic potential of graphene-based nanocarriers due to their distinct surface characteristics.

Graphene oxide (GO), a 2D hydrophilic derivative of graphene, is enriched with oxygenated functional groups and delivers significant advantages for improved drug loading and functionalization with targeting ligands and biocompatible biopolymers. Despite the several properties and applications of GO, it owns some disadvantages, like agglomeration or overlapping of GO sheets, larger lateral dimensions, less porous structure and the lower stability of GO suspension in an organic solvent. These limitations collectively affect the loading and release characteristics of GO-based nanocarriers along with their toxicological aspects. In line with the limitations of GO for drug delivery-related applications, this thesis work is focused on the modifications of surface characteristics of GO-based nanocarriers for anticancer DDS. These nanocarriers were structurally, functionally, and morphologically

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modified to improve their colloidal stability, targetability, cytocompatibility, loading, and release characteristics for conventional as well as natural chemotherapeutics agents.

Initially, this work elucidates a facile method for the structural and morphological modification of GO nanosheets into spirally wounded 1D graphene oxide nanoscrolls (GONS) of tunable dimensions by low frequency (20 kHz) ultrasonication. The GO was synthesized by modified Hummers' method using low-cost synthetic graphite as a base material. Later, GO suspension (0.05 gL^{-1}) was made using methanol as solvent and subjected to different ultrasonication conditions. GO sheets were found to curl themselves into nanoscrolls by overcoming the energy barrier for scrolling with the help of bubble cavitation energy provided by ultrasonication. The effect of ultrasonication power (100 - 150 W) for irradiation time (0.5 - 3 h) over the GONS dimensions was investigated. The spiral wounded GONS structure was visualized through electron microscopy. Thin-film X-ray diffraction, Raman, X-ray photoelectron, and FTIR spectroscopic analysis were also conducted to endorse GONS formation. Factors affecting GONS formation, such as sonication power and solvent selection, were studied as scrolling of GO sheets is strongly dependent on sonication parameters and solvent characteristics. It was found that the average length of obtained GONS decreases from 1311 nm to 1011 nm and 1165 nm to 516 nm for 100 W and 150 W sonication power, respectively, with an increase in irradiation time from 0.5 h to 3 h during ultrasonication. It was expected that the GONS with improved surface characteristics and morphology might support its applications in anticancer DDS.

Later, the obtained spirally wounded GONS are employed as nanocarriers for their applications in anticancer DDS. GONS nanocarriers were loaded with GA at different compositions, temperatures and pH of the media. In addition, an *in-vitro* study was conducted on drug loading and release behavior at physiological pH conditions to understand the GA

loading mechanism, thermodynamics, and release kinetics. The presented structural and morphological modification of GO into 1D GONS was favorable for a significant improvement in the loading and release behavior of GA for cancer therapeutics with a maximum loading efficiency of $29.82 \pm 0.8\%$ at a 1.25 weight ratio of GA and $73.78 \pm 2.21\%$ GA release in 5 days. The cytotoxicity studies of GONS-GA nanocomposites demonstrated higher toxicity for lung cancer cell lines (A549) without showing perceptible cytotoxicity for normal cell lines (HEK293), which advocates GONS as a suitable nanocarrier for the DDS.

Furthermore, in line with the targeted co-delivery principle for chemotherapeutic agents, the fabricated nanoscrolls were firstly functionalized with chitosan (CS) biopolymer and FA to further enhance their biocompatibility and target-specific delivery of anticancer drugs. The resultant GONS-CS-FA (GCF) nanocarriers were loaded with DOX and CA at different weight proportions with respect to nanocarrier and drug composition. The optimum loading efficiency of $51.14 \pm 1.47\%$ (DOX) and $49.70 \pm 1.19\%$ (CA) was observed for a drug: GCF ratio of 2.5 with drug composition of 1:1. It was observed that with an increase in drug composition DOX: CA, in contrast to CA the loading efficiency of DOX was enhanced. Additionally, to explore the release behavior of the GCF nanocarrier, an *in-vitro* release study was performed for GCF-DOX-CA nanocomposite under pH conditions mimicking both physiological and cancer micro-environments. At pH 5, ~ 83 and 75% DOX and CA were released from the GCF-DOX-CA nanocomposite, respectively, whereas ~ 71 and 61% release was observed at pH 7.4 over the course of 7 days, suggesting a higher and targeted drug release in the cancer microenvironment. The *in-vitro* cytotoxicity evaluations of GCF-DOX-CA nanocomposite demonstrated significant cytocompatibility for normal cells (HEK293) and selective apoptosis for cancer cell lines (A549).

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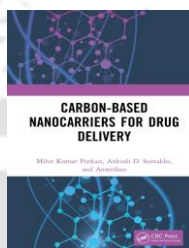
In addition, a sustainable, facile, and one-pot synthesis approach is adapted for the preparation of GO-impregnated Fe-based metal-organic frameworks [(GO/MIL-100(Fe)) (Gw-MF)] as doxorubicin (DOX) nanocarriers via the HF free route. The DOX loading efficiency of these nanocarriers was studied for Gw-MF at a fixed drug concentration and with varying DOX with respect to the carrier (w/w). Amongst various Gw-MF nanocarriers, G0.5-MF has shown a superior loading efficiency of 29.91 ± 1.49 % for a 1 w/w ratio of DOX. The DOX-released pattern of the G0.5-MF nanocarrier was more controlled in nature, which may improve the therapeutic efficacy. The release behavior of G0.5-MF was further studied using kinetic modeling. *In-vitro* cytotoxicity studies of G0.5-MF showed higher toxicity to cancer cells (A549) without showing noticeable toxicity for normal cells (HEK293). A similar approach is adopted for synthesizing CA nanocarriers using β -cyclodextrin conjugated graphene oxide (β -CD-GO)-functionalized Fe-based metal-organic frameworks [(β -CD-GO@MIL-100(Fe)) (BGw-MF)]. The structural, functional, and morphological attributes of obtained nanocarriers were inspected through respective characterization methods. Furthermore, the CA loading efficiency (LE) was assessed for various BGw-MF nanocarriers, with fixed drug concentration and variable CA masses (w/w) with respect to the carrier and pH of the media. The BG0.5-MF nanocarrier presented the highest LE for CA, reaching $56.44 \pm 2.25\%$ for a 2 w/w ratio of CA in a basic environment (pH 9). Moreover, the CA release pattern of BG0.5-MF-CA was controlled. The release characteristics of BG0.5-MF were subsequently investigated using a kinetic study. *In-vitro* cytotoxicity tests of BG0.5-MF-CA revealed significant toxicity to lung cancer cells (A549) deprived of inducing evident toxicity to normal cells (HEK293). Overall, a room temperature and sustainable synthesis approach of GO@MOF-based nanocarriers has emphasized their potential for higher drug loading capacity, improved release characteristics, suitable colloidal stability, and cytotoxicity performance towards cancer cells in the field of DDS.

Research Publications

1. Publications related to the thesis

1.1. Book

- 1) **Carbon-Based Nanocarriers for Drug Delivery** authored by Mihir Kumar Purkait, Ankush D. Sontakke, Anweshan: Imprint: **CRC Press** (Taylor & Francis), *eBook ISBN: 9781003358114*.



1.2. Journal articles

- 1) **A.D. Sontakke**, M.K. Purkait, A brief review on graphene oxide nanoscrolls: Structure, Synthesis, characterization and scope of applications, **Chem. Eng. J.** 420 (2021) 129914. <https://doi.org/10.1016/j.cej.2021.129914>.
- 2) **A.D. Sontakke**, M.K. Purkait, Fabrication of ultrasound-mediated tunable graphene oxide nanoscrolls, **Ultrason. Sonochem.** 63 (2020) 104976. <https://doi.org/10.1016/j.ultsonch.2020.104976>.
- 3) **A.D. Sontakke**, R. Fopase, L.M. Pandey, M.K. Purkait, Development of graphene oxide nanoscrolls imparted nano-delivery system for the sustained release of gallic acid, **Appl. Nanosci.** (2022). <https://doi.org/10.1007/s13204-022-02582-8>.
- 4) **A.D. Sontakke**, A. Bhattacharjee, R. Fopase, L.M. Pandey, M.K. Purkait, One-pot, sustainable and room temperature synthesis of graphene oxide-impregnated iron-based metal-organic framework (GO/MIL-100(Fe)) nanocarriers for anticancer drug delivery systems, **J. Mater. Sci.** (2022) <https://doi.org/10.1007/s10853-022-07773-w>.
- 5) **A.D. Sontakke**, S. Tiwari, M.K. Purkait, A comprehensive review on graphene oxide-based nanocarriers: Synthesis, functionalization and biomedical applications, **FlatChem.** 38 (2023) 100484. <https://doi.org/10.1016/j.flatc.2023.100484>.
- 6) **A.D. Sontakke**, S. Tiwari, P. Gupta, S.K. Banerjee, M.K. Purkait, Room temperature synthesis of β -cyclodextrin functionalized graphene oxide decorated MIL-100 (Fe): A sustainable drug cargo for anticancer drug delivery, **Materials Today Communications**, 38, (2024), 108560. <https://doi.org/10.1016/j.mtcomm.2024.108560>

- 7) **A.D. Sontakke**, P. Gupta, S. K. Banerjee, M. K. Purkait, Chitosan-grafted folic acid decorated one-dimensional graphene oxide nanoscrolls: a biocompatible drug cargo for targeted co-delivery of anticancer drugs. (*Under review - International Journal of Biological Macromolecules*)

1.3. Book chapters

- 1) **A.D. Sontakke**, Deepti, N.S. Samanta, M.K. Purkait, Smart nanomaterials in the medical industry, in: A. Husen, K. Siddiqi (Eds.), *Adv. Smart Nanomater. Their Appl.*, Elsevier, 2023: pp. 23–50. <https://doi.org/10.1016/B978-0-323-99546-7.00025-2>
- 2) **A.D. Sontakke**, P. Mondal, M.K. Purkait, Graphene oxide-based advanced nanomaterials for environmental remediation applications, in: S.J. Ikhmayies (Ed.), *Adv. Mater. Res. Technol.*, Springer International Publishing, Cham, 2022: pp. 155–190. https://doi.org/10.1007/978-3-031-11996-5_6.
- 3) **A.D. Sontakke**, S. Tiwari, M.K. Purkait, Surface Modifications of Graphene Oxide Nanomaterials for Analytical Applications in: K. Suresh (Ed.) *Analytical Applications of Graphene Oxide*, Elsevier (*In-Press*).
- 4) **A.D. Sontakke**, S. Tiwari, M.K. Purkait, Magnetic graphene oxide in analytical science in: K. Suresh (Ed.) *Analytical Applications of Graphene Oxide*, Elsevier (*In-Press*).

2. Publications from other work

2.1. Journal articles

- 1) **A.D. Sontakke**, P.P. Das, P. Mondal, M.K. Purkait, Thin-film composite nanofiltration hollow fiber membranes toward textile industry effluent treatment and environmental remediation applications: review, **Emergent Mater.** (2021). doi:[10.1007/s42247-021-00261-y](https://doi.org/10.1007/s42247-021-00261-y).
- 2) S. Tiwari, **A.D. Sontakke**, K. Baruah, M.K. Purkait, Development of graphene oxide-based nano-delivery system for natural chemotherapeutic agent (Caffeic Acid), **Mater. Today Proc.** (2022). <https://doi.org/10.1016/j.matpr.2022.11.373>.
- 3) M. Sharma, P. Mondal, **A.D. Sontakke**, A. Chakraborty, M.K. Purkait, High-performance graphene-oxide doped cellulose acetate based ion exchange membrane for environmental remediation applications, **Int. J. Environ. Anal. Chem.** 00 (2021) 1–22. doi:[10.1080/03067319.2021.1975276](https://doi.org/10.1080/03067319.2021.1975276).

- 4) S. Dhara, **A.D. Sontakke**, N.S. Samanta, R.V.S. Uppaluri and M.K. Purkait, Biowaste-alkaline lignin and GO integrated polysulfone ultrafiltration membrane fabrication for Pb^{2+} and Eosin Y dye removal, *Separation and Purification Technology* (2024), p.126894. <https://doi.org/10.1016/j.seppur.2024.126894>.

2.2. Book chapters

- 1) **A.D. Sontakke**, S. Tiwari, A. Bhattacharjee, P. Mondal, M.K. Purkait, Recent Advances in Biochar Production and Its Applications toward Textile Industry Effluent Treatment, in: Des. Biochar Assist. Bioremediation Ind. Effluents, **CRC Press**, Boca Raton, 2022: pp. 1–21. <https://doi.org/10.1201/9781003203438>.
- 2) **A.D. Sontakke**, P. Mondal, M.K. Purkait, Green Synthesis of Metallic Nanoparticles for Biofuel Production, in M. Srivastava, M.A. Malik, P.K. Mishra (Eds.), **Springer Nature Singapore**, Singapore, 2022: pp. 51–77. https://doi.org/10.1007/978-981-16-9356-4_3.
- 3) **A.D. Sontakke**, A. Tiwari, M.K. Purkait, Fabrication of Borate Cross-Linked Graphene Oxide Framework (GOF)-Laminated UF Membrane for Heavy Metal Removal, in: D. Deka, S.K. Majumder, M.K. Purkait (Eds.), Sustain. Environ., **Springer Nature Singapore**, Singapore, 2023: pp. 135–149. https://doi.org/10.1007/978-981-19-8464-8_8.
- 4) P.P. Das, **A.D. Sontakke**, M.K. Purkait, Rice Straw for Biofuel Production, in: M.P. Shah (Ed.), Green Approach to Altern. Fuel a Sustain. Futur., **Elsevier**, 2023: pp. 153–166. <https://doi.org/10.1016/B978-0-12-824318-3.00034-5>.
- 5) P.P. Das, **A.D. Sontakke**, N.S. Samanta, M.K. Purkait, Emerging Contaminants in Wastewater: Eco-Toxicity and Sustainability Assessment, in: M.P. Shah (Ed.), Ind. Wastewater Reuse, **Springer Nature Singapore**, Singapore, 2023: pp. 63–87. https://doi.org/10.1007/978-981-99-2489-9_4.

3. Conferences/ Seminars/ Workshops attended

- 1) **Ankush D. Sontakke** and Mihir K. Purkait, " Fabrication of ultrasound-mediated tunable graphene oxide nanoscrolls " at National Conference on Issues and Challenges in Water Treatment and Allied Research for Sustainable Environment (**WATER-2020**), 23-25 January 2020.

- 2) **Ankush D. Sontakke**, and Mihir K. Purkait, " Graphene Oxide Framework (GOF) Based UF Membrane for Removal of Heavy Metals" at International Conference on Advances in Chemical, Biological and Environmental **Engineering (ICACBEE-2021)**, 23-24 April 2021.
- 3) **Ankush D. Sontakke**, Ankit Tiwari, Mihir K. Purkait, Fabrication of Borate Cross-Linked Graphene Oxide Framework (GOF)-Laminated UF Membrane for Heavy Metal Removal, at North East Research Conclave (**NERC-2022**), 20-22 May 2022.
- 4) International Symposium on "Nanotechnology for Healthcare: Clinical and Industrial Perspectives" Conducted by Nuffield Department of Women's & Reproductive Health (**NDWRH**), University of Oxford, 10 June 2022.
- 5) National workshop on "Density Functional Theory for Heterogeneous catalysis" Conducted under the Global Initiative of Academic Networks (**GIAN**), by IIT Guwahati: Sponsored by "Ministry of Human Resource Development Government of India," 6-11 August 2018.

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Nomenclature

Notations

λ	Wavelength (nm)
$\lambda_{\max}$	Wavelength with the highest absorbance (nm)
ζ	Zeta potential (mV)
d-spacing	Interlayer spacing (nm)
d	Average scroll diameter (nm)
L	Average length of the scrolls (nm)
D	Debye
q_e	Drug loading capacity (mg g^{-1})
C_e	Equilibrium concentration (mg L^{-1})
m	Concentration of nanocarrier (g L^{-1})
T	Temperature (K)
ΔH°	Enthalpy (J mol^{-1})
ΔS°	Entropy ($\text{J mol}^{-1} \text{K}^{-1}$)
ΔG°	Gibbs free energy (J mol^{-1})
R	Gas constant (J mol^{-1})
M_t	Amount of drug released from the nanocomposite at time t (mg)
M_e	Amount of drug released from the nanocomposite at equilibrium (mg)
M_∞	Amount of drug released from the nanocomposite at infinite time (mg)
M_D	Amount of drug dissolved as a function of time (mg)
M_R	Total amount of drug released (mg)
W_o	Initial amount of drug (mg)
W_t	Remaining amount of drug at time t (mg)
k	Rate constants

Nomenclature

t	Time (h)
n	Release exponent
IC ₅₀	Half-maximal inhibitory concentration ($\mu\text{g mL}^{-1}$)

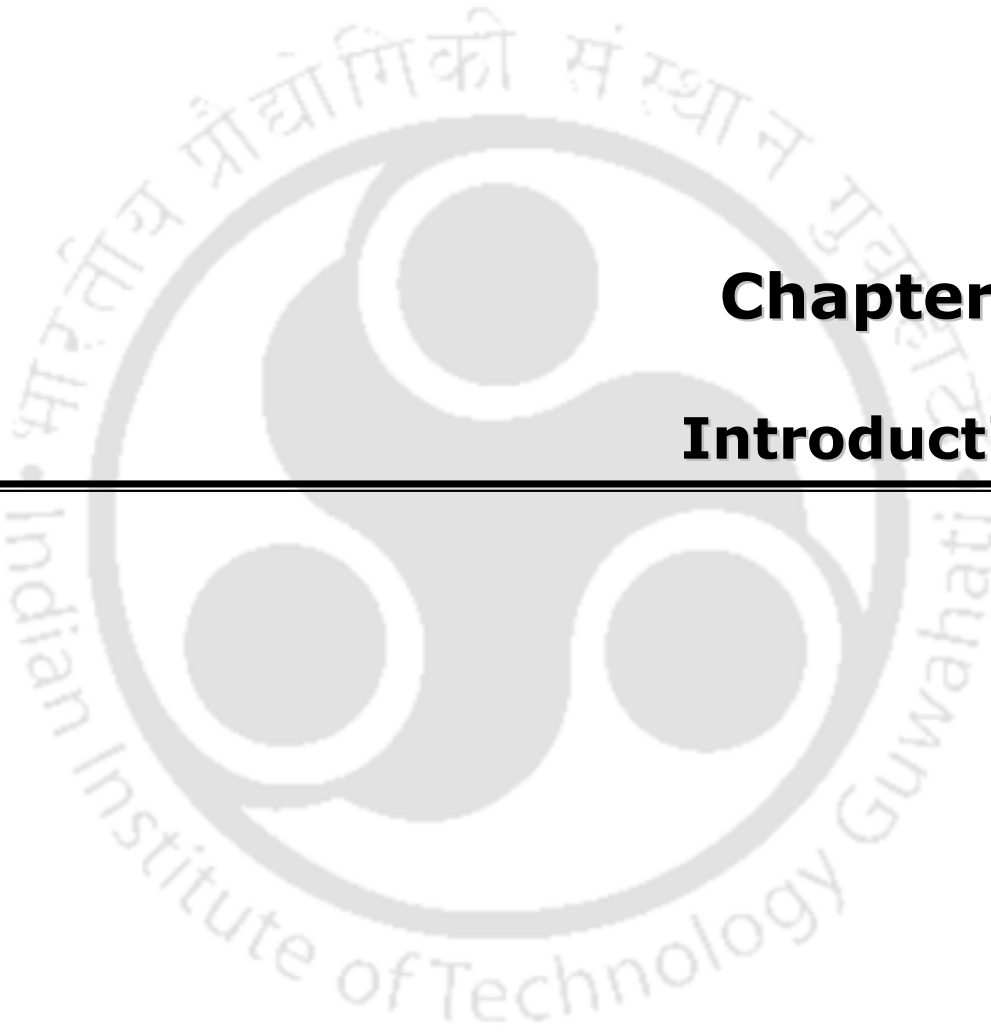
Abbreviations

1D	One-dimensional
A549	Human lung epithelial carcinoma cells
AFM	Atomic force microscopy
AT	Ambient temperature
CA	Caffeic acid
CMC	Carboxymethylcellulose
CNS	Carbon nanoscrolls
CNT	Carbon nanotubes
CPT	Camptothecin
CR	Controlled release
CS	Chitosan
DCC	N, N-dicyclohexylcarbodiimide
DDS	Drug delivery systems
DI	Deionized
DMSO	Dimethyl sulfoxide
DOX	Doxorubicin
DPPC	Dipalmitoylphosphatidylcholine
EDC	1-ethyl-3 (3dimethylaminopropyl)-carbodiimide
EE	Entrapment efficiency
FA	Folic acid

FESEM	Field emission scanning electron microscopy
FETEM	Field emission transmission electron microscopy
FTIR	Fourier transform infrared spectroscopy
FWHM	Full-width half maximum
GA	Gallic acid
GO	Graphene oxide
GOF	Graphene oxide flakes
GONS	Graphene oxide nanoscrolls
HACPN	Hyaluronic acid-chitosan-g-poly(N-isopropyl acrylamide)
HCl	Hydrochloric acid
HEK293	Human embryonic kidney cells
HF	Hydrofluoric acid
L-DOX	Liposomal doxorubicin
LE	Loading efficiency
LF	Low-frequency
MDR	Multidrug resistance
MEC	Minimal effective concentration
MF	MIL-100 (Fe)
MOF	Metal-organic framework
MTC	Minimum toxic concentration
MTT assay	3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide
MTX	Methotrexate
NHS	N-hydroxy succinimide
NPs	Nanoparticles
PAA	Polyacrylic acid

Nomenclature

PEG	Polyethylene glycol
P-gp	P-glycoprotein
PT	Physiological temperature
PTX	Paclitaxel
PVA	Polyvinyl alcohol
rGO	Reduced graphene oxide
ROS	Reactive oxygen species
RT	Room temperature
SA	Surface area
SR	Sustained release
SRL	Sisco research laboratories
TEM	Transmission electron microscopy
Tris-NTA	Tris- [nitrilotris (acetic acid)]
VFD	Vortex fluidic device
XPS	X-ray photoelectron spectroscopy
XRD	X-ray diffraction
β -CD	β -Cyclodextrin
γ -PGA	γ -polyglutamic acid AAP



Chapter 1:

Introduction

Chapter 1

Introduction

This chapter delivers a brief overview of the fundamentals involved in drug delivery systems. It summarizes the state-of-the-art in the structurally, functionally and morphologically modified forms of graphene oxide-based nanocarriers, their application in anticancer drug delivery systems (DDS) and the background of the problems undertaken in this work, i.e., the problems associated with the drug loading capacity, controlled release behavior, aqueous stability and cytocompatibility. It discusses the significance of graphene oxide (GO)-based nanocarriers in anticancer DDS, their functionalization strategies, and morphological modification into one-dimensional nano-morphology. Furthermore, this chapter enlists the recent developments in GO-based DDS through the detailed literature review on their application in anticancer DDS while addressing the allied limitations. Finally, the methods for the fabrication or synthesis of structurally, functionally and morphologically modified forms of GO-based nanocarriers are comprehensively reviewed as well as the objectives of the present work are highlighted.

1.1. Background

1.1.1. Introduction to anticancer drug delivery systems

Cancer is one of the life-threatening diseases which is certainly a leading cause of death worldwide [1]. It begins with the abnormal growth of cells and spreads uncontrollably within the human body [2]. In 2020, around 19.3 million additional cancer cases were diagnosed, and about 10 million deaths were noticed globally [3]. Traditional anticancer drugs or treatments such as chemotherapy used for cancer therapeutics function poorly in controlled delivery and

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Chapter 1

can induce toxicity with their doses [4,5]. In addition, most of these anticancer medicines are unable to distinguish between rapidly proliferating normal cells and cancer cells. Cells in the digestive system, as well as bone marrow and hair glands, may be harmed by such deficiency. The basis for side effects, including hair loss, nausea, exhaustion, and vomiting, had been the mutilation of healthy normal cells [6,7]. To address this problem, various DDS have been investigated in order to improve medication delivery efficiency and limit the adverse effects of chemotherapy.

Drug delivery systems, also known as DDSs, are pharmacological formulations that assist in the targeted distribution and controlled release of therapeutics in the body. After being administered, the DDSs will release the drug's active components that will reach the point of action after crossing several biological barriers. The fundamental aim of a DDS is to extend, contain, and target the therapeutic drug at the site of disease in a safe manner. DDSs are necessitated since it is well-known that certain drugs are known to exert unpleasant side effects on parts of the body that fall out beyond their intended targets. These side effects occur from the formulation of the medicine, route of administration, the reaction of the body, or often resulting from the accumulation of high blood plasma drug concentration with traditional drug administration [8,9]. It is preferable to employ a DDS to deliver drugs in the human body to obtain a controlled or sustained release rate, completely discard any side effects, and achieve a significant therapeutic effect.

The frequency of dose, drug clearance rates, the method of administration, and the DDS used all affect how long a drug remains in the therapeutic range. The therapeutic range must lie between the minimal effective concentration (MEC) and the minimum toxic concentration (MTC). **Fig. 1.1** [8] illustrates the concept of drug delivery through the variation in the concentration of a drug with respect to time. Subsequently, anticancer drugs have a range of

optimal doses where the most significant benefits are obtained; quantities lower or higher than this range can be harmful or have no therapeutic effect. Another effective method for simulating a drug delivery is pulsed delivery, which can control the concentration profile by permitting drug discharge from the drug nanocarriers only when prompted by an external trigger, such as temperature and pH conditions [8,10].

The dosing frequency of anticancer drugs can be decreased by lowering the drug release rate over a longer period of time with the nanocarrier-based matrix. Controlled release (CR) DDSs, on the other hand, are intended to forecast consistent plasma drug concentrations irrespective of the biological milieu at the site of application.

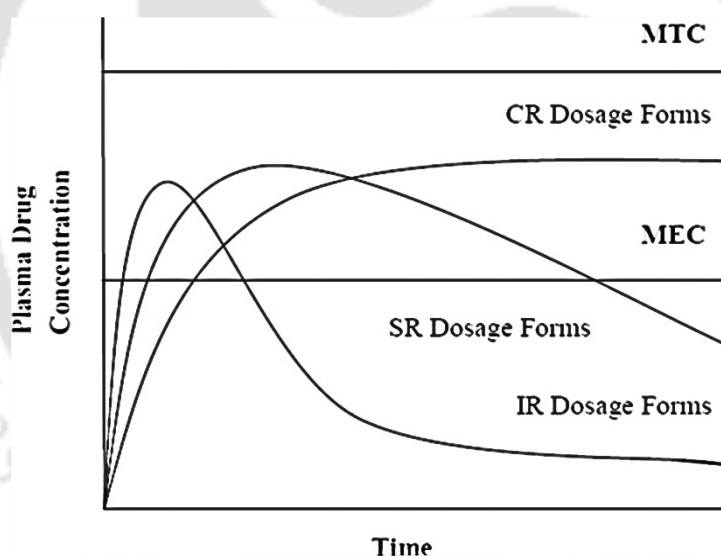


Fig. 1.1: The drug plasma levels following a single oral dose of a drug in the relevant immediate release (IR), sustained release (SR) and controlled release (CR) forms [8].

From burst release to prolonged release dosage forms, the resultant plasma concentration versus time curves flatten down more, showing that the medication is maintained in the therapeutic range for a longer period of time. To overcome the problem of fluctuating drug levels associated with conventional dose forms, controlled DDSs have indeed been developed [8]. The drug is not always delivered to the target organ via CR methods. In order to manage the bioavailability of drugs, target-specific drug delivery devices must be developed. As a

result, a number of original ideas have been developed to specifically address the requirements of the desirable DDS. Thus, nanocarriers-based novel DDS has come to light, with researchers focussing on increasing bioavailability and patient compliance while lowering toxicity and adverse effects to overcome the major deficiencies of conventional drug delivery methods [11].

The novel DDS has systematically subsided the shortcomings of conventional DDS through employing nanocarriers to selectively and effectively transport drug molecules to the diseased sites within the body. These nanocarriers are considered an essential element of the DDS. Several nanocarriers have been demonstrated for pharmaceutical and medical applications using a range of delivery methods. Since they are able to transport large amounts of drugs and do not require a high-dose carrier to attain the therapeutic concentrations due to the higher surface area of the nanocarriers [12]. Previously, extensive investigations have been conducted on nanocarrier-based DDS, including solid lipid nanoparticles, liposomes, dendrimers, and carbon-based nanomaterials [13–15]. Such nanosized drug delivery methods were invented to strengthen the drug's solubility and dissolution rate, which frequently results in enhanced pharmacokinetic/pharmacodynamic characteristics and better *in-vivo* effectiveness [16]. The bioavailability and safety of nanocarriers can be further increased by functionalizing them or incorporating targeted functionalities [17]. These nanomaterials exhibit certain advantages for their utilization in DDS, such as i) better stability and solubility of drugs through encapsulation and ii) selective and controlled release of the encapsulated drugs [18].

1.1.2. Graphene oxide-An overview

Carbon-based nanomaterials were extensively used in a variety of engineering fields owing to their large surface area and affordable production cost. In the family of graphene-derived materials, the oxidative and hydrophilic derivatives of graphene (GO and rGO) are enriched in hydroxyl, carboxyl, and epoxy functional entities, which provides them better stability and

dispersibility in aqueous media along with visible/infrared fluorescence [19,20]. Graphene, along with its derivative like GO, are currently being extensively researched, not only because of its fundamental physicochemical characteristics but also by its agitating potential of applications in varied arenas of biomedical engineering such as drug delivery, biosensing, cancer therapeutics, and tissue engineering [21]. The modulation of defects in graphene-based nanomaterials is of direct relevance for a variety of applications. GO is a 2D single atomic thick honeycomb-like structure that is indeed a hydrophilic derivative of graphene [22]. Other characteristics of GO, such as tunable morphology, functionalization ability, higher surface area [18] and colloidal stability in an aqueous environment, make it suitable for biomedical applications. As illustrated in **Fig. 1.2**, the oxygenated functional entities are located at the trailing edge and basal planes of the GO nanosheets. In addition to the attributes listed above, GO may be incorporated with polymers and other additives to make a variety of composites. Those additives can be polyacrylic acid (PAA), polyethylene glycol (PEG), folic acid and chitosan to improve their biocompatibility, loading capacity, targetability as well as structural properties of GO [23].

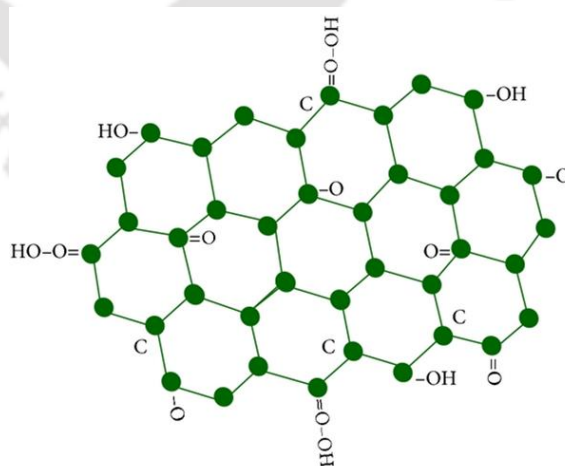


Fig. 1.2: Structure of graphene oxide [Replicated with permission from Song et al. (2014)][24].

However, GO has an aggregation tendency in the physiological solutions with proteins and salt due to the non-specific binding and electrostatic interactions, which produces a hindrance to

the development of biological probes [25,26]. The precise functionalization of GO boosts its solubility under physiological circumstances and optimizes it for biological applications.

1.1.3. Graphene oxide as a nanocarrier for anticancer DDS

Owing to the distinct physicochemical and surface characteristics of GO and rGO were extensively investigated as a nanocarrier for numerous biomedical applications, including cancer therapeutics [19], neurodegenerative diseases [27,28], gene delivery, and tissue engineering [29]. Recently, Liu et al. (2021) highlighted the recent advances in GO-based nanocarriers for cancer therapeutics [21]. Bellier et al. (2022) illustrated the recent progress in the application of GO/rGO-based nanocomposites for biomedical applications without describing their toxicological aspects [30]. Patil et al. (2021) listed the wound healing, cancer therapeutic and drug delivery applications of GO-based stimuli-responsive nanoplateforms [31]. Similarly, Jafari et al. (2022) reported the stimuli-responsive drug delivery mechanism of graphene-based nanocomposites [32]. Jain et al. (2021) demonstrated the nanographene oxide functionalization strategies and their application in cancer therapeutics, gene delivery and bioimaging [33].

Some of the favorable characteristics demonstrated by GO as a nanocarrier for anticancer DDS are; high surface area, amphiphilicity, pH sensitivity, and photo-thermal properties [34]. The amphiphilic characteristics of GO allow drugs to be stacked on the hydrophobic region via π - π staking and electrostatic interactions, while the hydrophilic region allows surface functionalization [35]; for instance, the ionizable carboxylic acid group on edge allows drug molecules to interact with electrostatically [36]. Through covalent and non-covalent modifications, GO can bind with functionalization agents that upgrade stability and provide multi-modal diagnostics [37]. Also, GO shows maximum stability at pH 7 or 8, and since the tissues at cancer sites have low pH, it triggers the drug release due to hydrogen bonds between

GO and drug molecules getting weakened [35]. The targetability of the GO-based nanocarriers can be enhanced by their functionalization with targeting ligands such as FA, aptamers, and lactoferrin. As the cancer cells are overexpressed to folate receptors, the FA functionalized nanocarriers loaded drug conjugates accumulate over the tumor cell, henceforth increasing their local concentration and enhancing the therapeutic efficiency in chemotherapy.

Overall, it was envisaged that the unique characteristics of GO had encouraged the world community for their application in various scientific and technological sectors, including the biomedical field. Specifically, due to its oxygen-containing functional entities, larger surface area, as well as functionalization capabilities, GO is a standout candidate for biological application where the interaction/combination of molecules with the surface of nanocarriers is obligatory. In the initial stages, GO-based nanocarriers suffered from low solubility and aqueous stability issues, limiting their use as drug-delivery vehicles for biomedical applications. However, covalent and non-covalent functionalization approaches alongside morphological modifications can resolve these issues, fortifying GO as an effective nanocarrier for the DDS [38].

1.2. Preparation of graphene oxide

GO is described as a graphene sheet or layer adorned by oxygenated functionalities. During the oxidation of graphite, the oxidizing agent such as KMnO_4 reacts with the carbon skeleton of graphite and further weakens the van der Waals force within the stacked sheets of graphite while increasing the interlayer spacings, subsequently [39]. GO is typically produced using two methods, namely, dry media and wet medium. In the case of the dry media production process, the graphene sheets are oxidized in a vacuum chamber using atomic oxygen. This type of production is costly since it demands graphene sheets as a carbon precursor, ultra-high vacuum environment and molecular oxygen [40,41]. Conversely, the wet medium technology is a less

expensive alternative since it uses natural and freely accessible synthetic graphite as a precursor of graphene/carbon and does not require rigorous experimentation. **Fig. 1.3** depicts the primary chemical pathways for the production of GO. In the first approach, graphite is utilized for exfoliation to yield graphene sheets, which are then succeeded by the oxidation process. Furthermore, in the next approach, the oxidation process is preceded by acoustic exfoliation in an aqueous phase [42,43]. Currently, the final and third approaches represent a well-known and widely used technique for manufacturing GO. In this case, the graphite is oxidized by employing strong oxidizing reagents while also being exfoliated in an acidic medium.

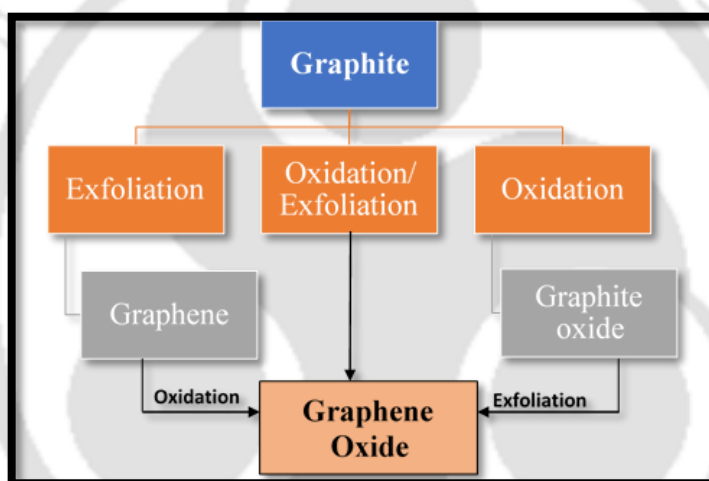


Fig. 1.3: Outlines for the synthesis of graphene oxide (GO).

Numerous strategies for synthesizing GO have indeed been explored over the past century. Brodie [44], Staudenmaier [40], and Hummers' [45] are indeed the key three approaches; each of these methodologies constitutes the successors of the formerly mentioned method. Currently, Hummers' approach is primarily used for GO synthesis; however, Sun et al. (2013) and Peng et al. (2015) modified it using free water oxidation methods to increase the quality and yield of GO. Tour et al. (2010) created a customized form of all of these fundamental GO synthesis techniques. **Table 1.1** illustrates the specifics of process conditions and attributes of various techniques for GO synthesis [40,44–50].

Table 1.1: Specifics of process conditions and attributes of methods for GO synthesis.

Method	Reagents	Parameters	Characteristics	C/O Atomic ratio	Reference
Brodie (1859)	KClO ₃ , HNO ₃	60 °C, 3-4 h	The very first methodology to GO synthesis Evolution of toxic gas, ClO ₂	1.63	[44]
Staudenmaier (1898)	KClO ₃ , HNO ₃ , H ₂ SO ₄	Room Temperature, 96 h	Successfully enhanced the graphite oxidation Evolution of Toxic gas ClO ₂	2.89	[40]
Hummers' (1958)	H ₂ SO ₄ , NaNO ₃ , KMnO ₄	35°C, 4 h	Safe reaction conditions A better degree of oxidation Reduced reaction time Time overwhelming separation process	2.25	[45]
Tour (2010)	H ₃ PO ₄ , NaNO ₃ , KMnO ₄	35-40 °C, 12h	Restraining the discharge of toxic gases like NO ₂ , N ₂ O ₄ or ClO ₂ A higher dose of KMnO ₄ Claimed higher degree of oxidation and reaction yield	-	[46]
Sun (2013)	H ₂ SO ₄ , KMnO ₄	Room Temperature, 2 h	Free water oxidation method Additional intercalation among GO sheets Safe and efficient in scalable applications The higher degree of oxidation	2.5	[47]
Peng (2015)	H ₂ SO ₄ , K ₂ FeO ₄	Room Temperature, 1 h	Free water oxidation A sustainable and adaptable strategy is proposed A better degree of oxidation	13.4	[48]

1.3. Functionalization of graphene oxide-based nanocarriers

The surface chemistry of the material is the most integral factor that influences its drug-loading capacity, biocompatibility and controlled-release rate. Though GO is soluble in water, it aggregates in salts and protein-rich solutions, owing to the charge screening effect [51]. Such

inadequacy limits the aqueous stability of GO, which certainly affects its bioavailability and biocompatibility for biomedical applications. These challenges can be subsided via functionalization or modification of GO using more compatible polymers and biomolecules. The resultant GO-based nanocarriers deliver significant advantages for biomedical applications, especially in drug delivery. The oxygen-containing functional sites, high interfacial area, hydrophilic nature, and surface modification potential of GO provide it with a substantial edge in real-world applications. Meanwhile, the morphological and surface modification of GO to improve its physiochemical characteristics is a crucial component of the applications-driven studies, which is another great and rapidly expanding field of research. GO may be functionalized by incorporating new moiety or sites into GO, which may provide distinctive and advanced properties to the parent GO without compromising its fundamental attributes [38,52]. The functionalization of GO results in a minor reduction in the respective oxygenated site and produces rGO, despite the fact that the various oxygenated functional moieties in GO have distinct properties.

The GO can be functionalized via various inorganic or organic compounds, natural or synthetic polymers, biomolecules and other building blocks such as hydrogels, nanoparticles and metal-organic frameworks (MOFs) to improve its solubility, biocompatibility, drug loading capacity, release behavior and adaptability underneath physiological conditions for biomedical and drug delivery-related applications. The functionalization of GO can be primarily established via employing covalent and non-covalent conjugation. The subsequent sections describe the specifics of each form of functionalization.

1.3.1. Covalent functionalization

The GO is enriched with oxygenated functional groups like carboxy, hydroxy and epoxy functional entities. Covalent bonding in GO refers to the process of integrating GO moiety with

newly introduced ligands via covalent bonding. The primary goal of covalent functionalization is to enhance surface properties, biocompatibility, loading efficiency and release behavior of GO-based DDS for biomedical applications [19]. GO is frequently triggered by providing immediate electrophilic functionalities on the surface prior to covalent interaction with biomolecules. The functional groups in GO can be employed in typical processes, including acetylation, isocyanation, diazotization, and others. There are several methods for covalently functionalizing GO, including hydroxy group, aromatic ring and carboxyl group functionalization. Such functionalization approaches also involve the attachment of polymers of admirable solubility via esterification, click chemistry, amidation, nitrene chemistry, and radical addition [38].

Due to its abundance and strong reactivity, the carboxyl functional group has been widely employed to functionalize GO, which can be observed in enormous quantities near the edges of GO nanosheets. The most common kind of carboxyl functionalization consists of reaction initiation, followed by the dehydration of hydroxyl and amino groups to produce ester or amide linkages. Numerous reagents, notably hexafluorophosphate, thionyl chloride (SOCl_2), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and N, N-dicyclohexylcarbodiimide (DCC) are used to carry out the activation. Recently, Liu et al. (2019) have developed highly biocompatible and stimuli-responsive nanoparticle-assisted anticancer DDS. The nanocarrier was obtained via covalent crosslinking of carboxyl functional groups of GO with chitosan oligosaccharide (CO), which was further ornamented by γ -polyglutamic acid (γ -PGA). The GO-CO- γ -PGA composites were obtained by an amidation coupling reaction, and the activation of carboxylic groups of γ -PGA and GO was done with the help of NHS and EDC. *In-vitro* analysis of the synthesized nanocomposite revealed easier transfer of nanocarriers within the Hela cells with good compatibility with the normal cells and higher anticancer activity for the tumor cells [53]. In the other study, Sousa et al. (2018) developed a carboxyl-

activated and functionalized nanocarrier based on the folic acid (FA) and GO of potential and targeted drug delivery of Camptothecin (CPT) for anticancer activity [54].

A substantial proportion of hydroxyl and carboxyl functional groups were located on the surface of GO nanosheets. The presence of the hydroxyl group not just to gives GO substantial hydrophilicity as well as better surface characteristics, but it also serves as a spacer for the covalent bonding of other ligands at the perimeter of GO nanosheets. Recently, Wang et al. (2018) have employed covalent crosslinking to synthesize octa arginine (R8) and antiHER2 antibody-functionalized GO as a new gene delivery vehicle for tumor treatment. Furthermore, R8 was conjugated onto GO surfaces as a cell-penetrating peptide to boost the effect of siRNA delivery. Moreover, as per the *in-vivo* and *in-vitro* studies, the GO-R8/anti-HER2/survivin-siRNA has displayed great potential as a gene silencing carrier for siRNA delivery in cancer therapy [55]. For cellular transport of oligohistidine and biotin-tagged biomolecules, Jana et al. (2013) have reported a flexible technique for dual functionalization of carboxyl groups of GO with Tris- [nitrilotris (acetic acid)] (Tris-NTA) and biotin via covalent interactions [56].

1.3.2. Non-covalent functionalization

Non-covalent functionalization is the best way to retain the unique characteristics and bulk structure of GO while improving its aqueous stability and dispersibility in regard to hydrogen bonding and electrostatic interactions among GO and its functional entities. The major attribute of the non-covalent approach is its moderate working conditions, which certainly preserve the parental properties of GO. These forms of interactions comprise direct sonication, followed by a washing step to remove unbound molecules. Various research outputs have claimed significant improvements in the drug loading efficiency, controlled and sustainable release behavior with better biocompatibility and anticancer activity of the nanocarriers synthesized via non-covalent bonding of functional entities to the GO.

One of the most adaptable methodologies for the functionalization of GO and rGO in the non-covalent surface modification of graphene is the hydrogen-bonding. Hu et al. (2016) demonstrated a novel one-pot synthesis of Dex-coated rGO nanocomposites for the targeted photo-chemotherapy, where rGO was synthesized using Dex as a reducing agent. During the reduction process, the Dex was directly coupled with the rGO nanoparticles via hydrogen bonding to form a self-assembled rGO/Dex nanocomposite. In this study, the Dex conjugation improved the DOX loading capacity (10.85%) and the aqueous dispersibility and biocompatibility of the rGO/Dex nanocomposite. The DOX-loaded rGO/Dex nanocomposite displayed efficient anticancer activity for B16F10 cells and achieved the therapeutic level in a short time with a lower concentration of DOX. [57]. Another approach for the functionalization of GO is the electrostatic interactions between the negatively charged surface of GO and other nanomaterials or functional entities with a positive surface charge, such as chitosan, liposomes and metal nanoparticles. Recently, Prasad et al. (2019) developed a novel graphene oxide flakes fortified liposomal (GOF-Lipo) nanohybrids for the red emissive nano delivery system. The nanohybrid was functionalized with the tumor-targeting ligand, folic acid (FA). The functionalization was established via electrostatic interaction amongst the positively charged dipalmitoylphosphatidylcholine (DPPC) lipid and negatively charged GOF (**Fig. 1.4a**). The nanohybrids have shown superior aqueous dispersibility, quick photo-thermal response with 90% cell viability and hemocompatibility [58].

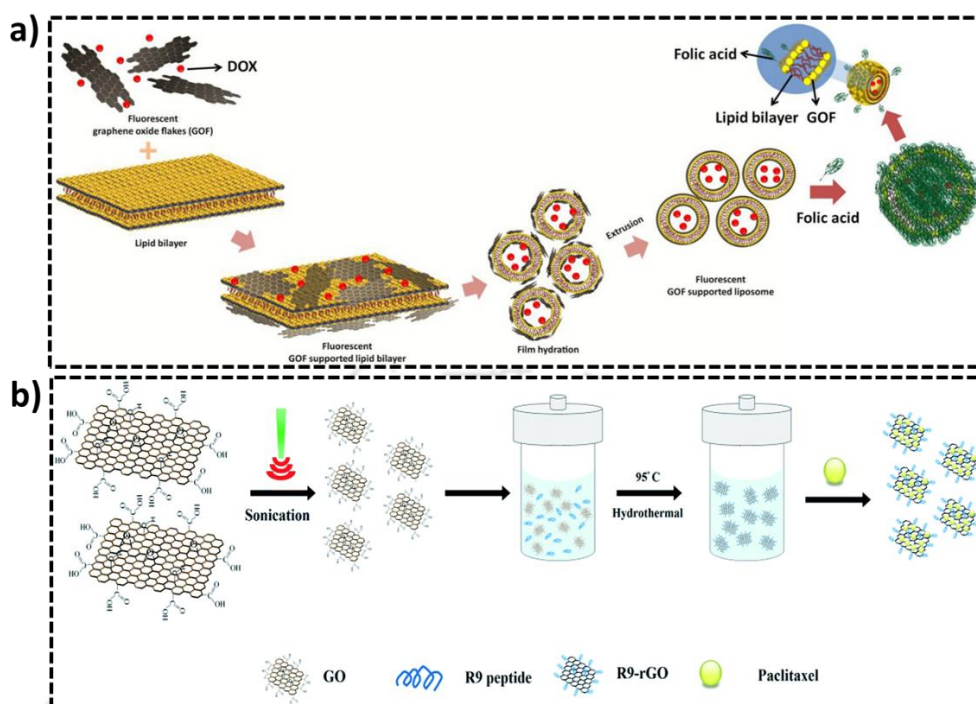


Fig. 1.4: Schematic illustration for the preparation of (a) DOX-loaded GOF-Lipo-FA conjugates (Reproduced with permission from Prasad et al. (2019) © ACS publication) [58], and (b) R9 peptide-rGO nanocarrier loaded with Paclitaxel (Reproduced with permission from Hashemi et al. (2016) © RSC) [59].

Regarding the smart delivery of hydrophobic agents, Hashemi et al. (2016) developed a unique and targeted graphene-based DDS via functionalizing rGO with the R9 peptide using a hydrothermal-assisted approach (**Fig. 1.4b**). The formation of the R9 peptide-rGO nanocarrier was accompanied via non-covalent interactions, which were further loaded with the paclitaxel by hydrophobic interactions. According to *in-vitro* investigations, the R9-rGO-Paclitaxel nanocomposite was readily uptake by Hela cancer cell lines and lowered the cell viability by > 90% after 72 h [59].

In most studies, the hydrophobic interaction and π - π bond functionalization are considered a mechanism behind the attachment of drug molecules to the hydrophilic surface of GO. There is no direct evidence of the functionalization of GO by certain additives via π - π bond functionalization for drug delivery-related applications [60,61]. In cancer therapeutics studies, the electrostatic interaction phenomenon was extensively used for material functionalization.

However, the drug loading capacity and release characteristics of the functionalized materials were not much reasonable, which can be improved via the introduction of micro-mesoporous materials like MOFs.

1.3.3. GO-based Metal-Organic Frameworks

Metal-Organic Frameworks (MOFs) are porous crystalline materials containing clusters of metal ions or interconnected organic linkers. MOFs are the emerging class of materials for smart drug delivery systems [62]. The drug molecules can be efficiently loaded into MOFs due to their large surface area, pore volume, controllable pore size, and ordered structure. However, the open framework of MOFs has the disadvantage of not providing stronger adsorption forces to bind small molecules [63]; hence, combining it with a substrate may improve its properties significantly. The properties of MOFs can be considerably improved by modifying them with functional groups/ligands to increase their drug-loading capacity. Developing MOF-based composites, such as MOF/GO, where to GO with its oxygenated functional groups and polycrystalline structure delivers improved drug loading capacity and aqueous stability; in addition, MOFs with a potential of controlled drug release characteristics leads to the suitable alternative for novel DDS [61]. In a MOF/GO hybrid, the MOF can load the drug on its surface by electrostatic interaction, while GO can absorb the drug through hydrogen bonding and π - π interactions [64]. These complexes can be employed in DDS owing to their characteristics like improved drug loading capacity, controlled release rates, colloidal stability, biocompatibility, and reduced toxicity [64]. Recently, Javanbakht et al.(2019) synthesized CMC/MOF-5/GO through a solvothermal technique to enhance the drug-loading capacity of GO [64]. GO was modified with carboxymethylcellulose (CMC) and a Zinc-based metal-organic framework (MOF-5) via non-covalent interactions. It was found that the solubility, colloidal stability and loading capacity of GO were drastically increased with its functionalization. The DOX-loaded

CMC/MOF-5/GO nanocomposite has displayed significant toxicity toward the K562 cell lines. It was illustrated that the MOF-based GO composites were mostly fabricated via non-covalent interactions and have shown great potential for their application in the DDS. Meanwhile, the cytocompatibility/ biocompatibility of these composites must be explored further.

1.3.4. Biocompatible GO-based nanocarriers

The biocompatibility of a material is an important researched characteristic for assuring its safe biological and biomedical application. The factors influencing the cytotoxicity levels of any nanocarriers must be investigated and studied for the scope of improvement. The factors that contribute to the biocompatibility and bioavailability of graphene hybrids are their structure, size and oxidative state. GO has a two-dimensional structure and contains oxygen-bearing functional groups, which make it less cytotoxic than CNTs [38]. The biocompatibility of nanocarriers is also depends on their colloidal stability and solubility. It was examined that the GO exhibits dose-dependent biocompatibility [65], which can be further improved by adapting various functionalization approaches. The covalent and non-covalent functionalization approaches entail binding polymers and biomolecules with excellent solubility and biocompatibility [66]. Polyethylene glycol (PEG) is the most common and established polymer that has been utilized for the functionalization of GO. Alongside, poly (vinyl alcohol) (PVA), polyacrylic acid (PAA), β -Cyclodextrin (β -CD), chitosan, and dextran are some of the other polymers that have been extensively explored for GO functionalization in-line with drug delivery [19,38].

Despite the suitable colloidal stability and presence of oxygenated functional entities, GO undergoes major drawbacks related to its higher lateral dimensions. Several *in-vivo* testing has signified that the higher length of GO reduced the survival rate, altered heart rate, and accumulated in the eyes, heart, yolk sac, and blood vessels. Such infirmities can be subsided

via morphological modification of GO by extended exfoliation or scrolling GO nanosheets in graphene oxide nanoscrolls (GONS) [38].

1.4. Morphological modifications of GO

In a family of materials based on carbon, such as carbon nanotubes (CNT), graphene and its derivatives (GO and rGO) have demonstrated remarkable prospective in several real-world applications due to their mechanical strength, functionalization ability and chemical activity with easy accessibility [67,68]. Graphene being hydrophobic in nature, impermeable to water, and zero band-gap nanomaterial limits its utilization in drug delivery, membrane separation, adsorption, and photocatalyst applications without further modifications [69,70]. However, GO, being the hydrophilic derivative of graphene, may work as a potential alternative to graphene for these applications [70,71].

As stated, GO single-layer nanosheets consist of hydroxyl, carboxyl and epoxy groups, resulted in a hydrophilic nature. GO is mostly synthesized by the well-known 'Hummers' method' and their modifications as reported in previous studies [45,70]. Despite the distinct characteristics and potential applications, GO owns some disadvantages, like agglomeration or overlapping of GO sheets. Agglomeration resulted in the reduction of active surface area and sites for adsorption, drug delivery, and filtration membrane applications [70,72]. Also, the stability of GO suspension in an organic solvent and physiological solutions is a significant issue as it directly affects the bioavailability and biocompatibility of GO-based nanocarriers in DDS. However, the thin and flat structure of GO provides flexibility in structural and morphological modifications, which delivers an effective way to overcome these disadvantages [22,73]. Furthermore, the oxygen functional groups in GO serve as both functionalization sites and spacers for molecular absorption, which assist the conversion of GO in various nanocomposites and nano-morphologies [73,74]. Structural modification and functionalization

via covalent and noncovalent bonding interaction could be helpful in the application of GO in DDS. The GO sheets are perhaps be folded, stretched, wrinkled, and scrolled by altering the processing parameters of different fabrication methods such as ultrasonication, microwave-assisted, vortex fluidic device (VFD), and lyophilization. Recent research studies on GO direct a significant potential for the nano-manufacturing of diverse nano morphologies without altering the fundamental characteristics of GO with an economical, cost-effective technique [70].

Previously, Amadei et al. (2016) and Fan et al. (2015), inspired from the structure of carbon nanoscrolls (CNS) and nanotubes (CNTs), had developed one of the potential nano-morphology of GO via rolling up single atomic thin GO nanosheets into the spirally wounded structure [73,75]. The nano morphology was termed to be graphene oxide nanoscrolls (GONS). The GONS have identical 1D morphology to CNTs, with adjustable interlayer distance and a more accessible inter-wall area. GONS exhibit similar properties to GO along with open-end morphology, porous structure and adjustable interlayer distance/spacing between scrolled layers [73,75]. In brief, identical properties to GO with morphological and structural modification make GONS more feasible for practical applications compared to GO and CNT. As discussed before, the shortcomings of the use of GO in DDS are a) stability in aqueous media, b) agglomeration of GO nanosheets, c) less porous structure, and d) lower BET surface area. Also, it was found that the size of GO nanosheets is a critical factor in their toxicity. On the contrary, GONS with enhanced structural morphology, de-agglomeration tendency, adjustable interlayer distance and dimensions may overcome the limitations of GO.

1.4.1. Structure, morphology and advantages of GONS

The GONS are termed to be the structural nano-morphology of GO, which imparts several advantages over GO and may be utilized for different engineering applications. The 1D nano-

morphology of GONS is formed by bending followed by scrolling of single-layer GO nanosheets. The nanoscrolls may exist in length from hundreds of nanometres to several micrometers of different diameters [76,77]. The topography of nanoscrolls suggests that it is formed by scrolling of GO nanosheet in several continuous Archimedean spiral shapes. Therefore, these spirals were described by the polar equation to determine the point's r belongs to the scroll with a defined core radius (r_0), as shown in **Fig. 1.5a**.

$$r = r_0 + \frac{h}{2\pi} \phi \quad (1.1)$$

Where h is the interlayer spacing and ϕ varies from 0 to $2\pi N$ (N - Number of turns made by nanoscroll). However, the type of nanoscrolls is defined by an angle Θ such as $\Theta = 0^\circ$ for zigzag scroll configuration, $\Theta = 90^\circ$ for armchair, and $0^\circ < \Theta < 90^\circ$ represents chiral scroll configuration [78,79]. The structure of nanoscrolls is strongly dependent on their synthesis process and composition. The parent material and its synthesis process significantly affect the size distribution, overall structure, and morphology of nanoscrolls [78–80]. Therefore, it was suggested that multiple sheets of layered material be considered for a better understanding of the scrolling process. However, in the case of GONS, the exfoliation of GO layers is inevitable for intercalation solvent within the sheets for twisting, bending, and scrolling subsequently.

The scrolling process of nanoscrolls can be self-driven once the energy barrier is overcome. The minimum requirement for self-driven scrolling of nanoscrolls is related to CNS and boron nitride nanoscrolls [78,80]. The postulations are as follows: nanoscroll formation is controlled or manipulated by two opposite energy contributions. Firstly, the increase in elastic strain or energy by bending of nanosheets, which ultimately decreases the stability and enhances the scrolling process. Secondly, there is a reduction in free energy due to van der Waals interaction in overlapping nanosheets. The postulations are also reconsidered by Wallace et al. [81] and Tang et al. [82] for the formation of carbon and GO nanoscrolls, respectively. GONS formation

is also based on a similar scrolling principle and exhibits identical energy requirements. However, the scrolling process is not initiating and self-propagating unless it overcomes the activation energy. The starting energy is obligatory for the initiation of scrolling, which can be provided through some external source of energy such as sonication, mechanical strain, and microwave irradiation [78,79,82].

The open-ended morphology (**Fig. 1.5c**) and porous structure of GONS offer additional accessible inner wall area to nanoscrolls. The more accessible area leads to improved performance for drug delivery and adsorption application, which substantiates the advantages over CNT. Several studies have revealed the significance of CNT similar to GO in the applications related to desalination, water treatment, drug delivery and adsorption [70,83]. However, the performance of CNT in reported studies does not reach the expected standards. The reason behind this could be a less porous structure, low stability in an aqueous medium, and, most importantly, a close structure of CNTs at its end, resulting in a long path of ion diffusion. In the case of GO, as discussed before, the agglomeration or overlapping of GO sheets may retards the active surface area and sites. The lower surface area and stability of GO in aqueous media may affect the performance of GO for its application in adsorption, drug delivery, photocatalysis, and filtration membrane [70,72]. Fortunately, GONS exhibits similar excellent properties to GO, along with added open-end morphology and a higher surface-to-volume ratio. Besides, GONS provides a porous structure as of GO, higher defect concentration and adjustable interlayer distance/spacing between scrolled layers with a more inner accessible area than CNT. Therefore, GONS is becoming an interesting nano- morphology distinct from GO sheets and CNT by offering numerous advantages and possibilities for its application in photocatalysis, adsorption, filtration membrane and drug delivery [70,73].

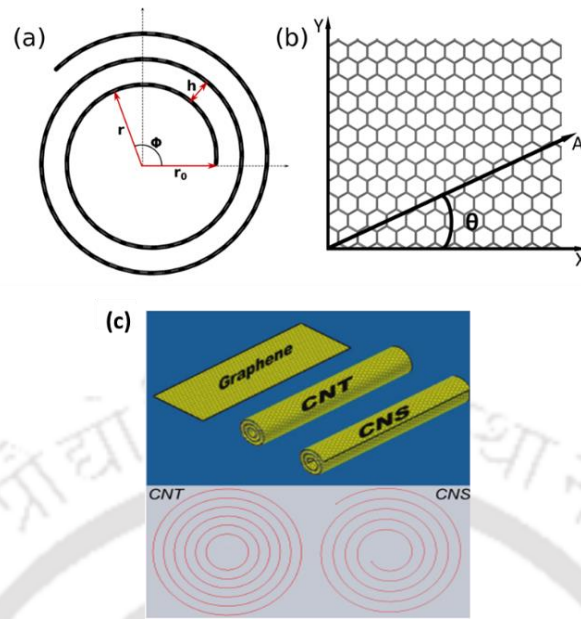


Fig. 1.5: (a) Scheme representing a cross-section of GONS relationship between r , r_o , h , and ϕ . (b) Scheme for rolling up GO nanosheets along with axis A and angle Θ . Reproduced with permission [78], (c) Structural comparison of MWCNT and CNS or GONS. Reproduced with permission [84].

1.4.2. Fabrication of GONS: limitations and suggestive measure

GONS can be fabricated via common nanomaterial synthesis techniques like ultrasonication lyophilization, molecular combing, vortex fluidic device (VFD) and microwave irradiation [22,70]. A starting material for the fabrication of GONS is the GO nanosheets, which is primarily being synthesized by the well-known Hummers' method and their modifications as described previously. The scrolling activation energy is a major parameter that affects the scrolling tendency of nanosheets. Therefore, to initiate the scrolling or curling of the GO nanosheets, this activation energy needs to be achieved during a process. Further, the scrolling will be self-propagative. The van der Waal and π - π bond interaction in GO assists the self-propagative scrolling of GO nanosheets for the formation of tubular or cylindrical GONS [22,73]. The barrier may conceivably be overcome by external stimuli such as ultrasonication and microwave sparks. Out of the stated fabrication techniques, ultrasonication is suggested to be the adequate method for GONS fabrication with its efficient utilization of ultrasound energy

through bubble cavitation. In addition, this approach delivers potential for tuning the dimensions of nanoscrolls via altering the sonication parameters. Herein, the subsequent section discusses in detail about the ultrasonication methods for the fabrication of GONS and factors affecting the GONS formation. Furthermore, the limitations of the ultrasonication method are described along with the suggestive measures for overcoming the difficulties during GONS formation subsequently.

1.4.2.1. Ultrasonication

Ultrasonication is a conventional method utilized for the synthesis of nanomaterial and nanocomposites or nanostructures [85]. In addition, high-intensity ultrasonic waves with bubble cavitation energy were extensively used in recent studies linked to nanomaterials synthesis and diverse applications [73,85]. Fundamentally, the synthesis of nanocomposites via ultrasonication is a top-down approach where the physical effects of cavitation are utilized [22,86]. Besides, ultrasonication provides the ability to de-agglomerate nanoparticles along with efficient energy consumption as an advantage [87].

It was proposed that the GONS is also fabricated using ultrasonic energy, where scrolling activation energy can be overcome by bubble cavitation [73]. The ultrasonication could be a useful tool for the formation of nanoscrolls with tunable dimensions in a scalable manner as per the application. Also, the efficiency in the utilization of energy along with de-agglomeration ability makes ultrasonication the best suitable tool for nanomaterial synthesis. However, the yield of GONS formed by ultrasonication was limited due to a low concentration of GO suspension used for sonication treatment. The reason behind the use of suspension with low concentration could be the sufficient cavitation energy distribution within the GO sheets to overcome the activation energy barrier. It was also discovered that controlling the particle size and scroll diameters was crucial during ultrasonication [70]. Meanwhile, the yield of

GONS and control over scroll dimensions may be enhanced by optimizing the process parameters such as frequency, irradiation time, power density and a higher concentration of GO suspension, which can still overcome the energy barrier for scrolling. The study related to the optimization of process parameters for GONS fabrication via ultrasonication is still in progress.

Furthermore, the 1D nanomorphology of GONS can be obtained via molecular combing, nanoparticle-assisted, and solvent-induced self-assembly approaches. The comparative analysis of these methods is presented in **Table 1.2** through describing their advantages, disadvantages and challenges.

Table 1.2: Typical methods for the fabrication of GONS; Advantages and disadvantages. (Reproduced with permission from Sontakke and Purkait (2021)) [70].

Methods	Advantages	Disadvantages/ Challenges
Ultrasonication	Versatile, Cheap, medium quality, and limited yield	Partial size control
Lyophilization	Relatively cheap and has no inclusion	Limited yield and difficult to reproduce
Self-assembly approach	Easy, cheap, higher yield, and better quality	Partial size control
Nanoparticle assisted	Reversible, easier process, better yield	Relatively expensive, difficult to reproduce, and limited de-agglomeration ability.
VFD	Higher yield, continuous process and easy to scale up	Energy-intensive and limited delivery volume.
Molecular combing	Versatile, better reproducibility, and provides reliable statistical analysis.	Inadequate for small GO sheets, lower yield, and difficult for stress manipulation.

1.5. Applications of GO-based nanocarriers in anticancer DDS

Graphene oxide, with its derivatives and functionalized forms, was effectively employed for a variety of biomedical applications, including biosensors, biodevices, drug delivery, gene therapy, bioimaging, nanomedicine, and tissue engineering (**Fig. 1.6**) [38,88]. The oxygen-rich functional groups on GO surfaces play important and critical roles in their characteristics and

bio-application. Alongside this, the controlled oxidation of GO allows for tuning its optical, electrical, and mechanical properties.

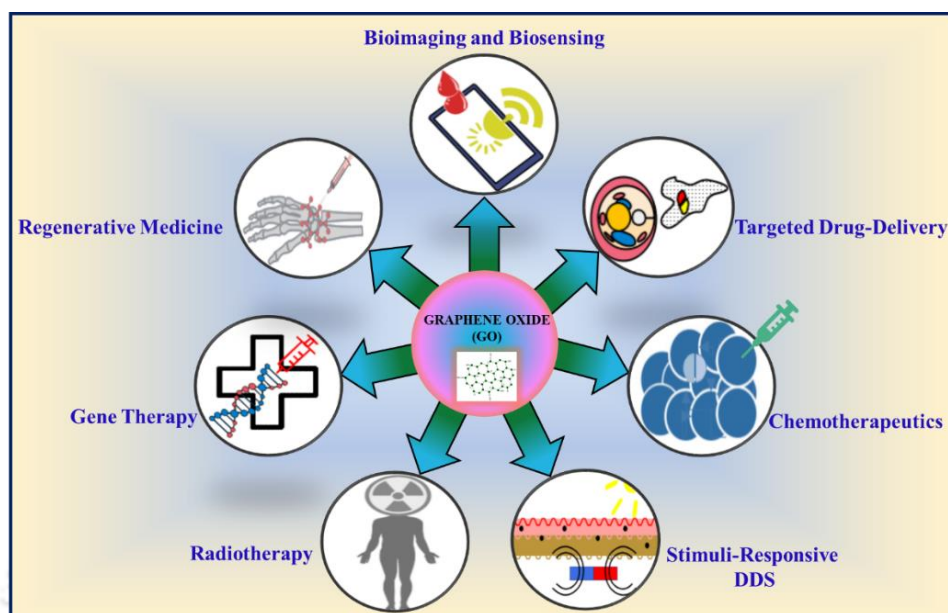


Fig. 1.6: Biomedical applications of graphene oxide-based nanocarriers [38].

Such a flexible characteristic of GO provided several ways to bind a variety of molecules on nanosheets, making it a more adaptable precursor for comprehensive applications. Some of the most recent breakthroughs in the field of GO-based nanomaterials for the delivery of conventional and natural chemotherapeutic agents are provided in the subsequent section.

1.5.1. Conventional chemotherapeutic agents

Chemotherapy is the most widely employed treatment method for cancer. It involves administering low-molecular-weight cytotoxic drugs to target tumor cells and prevent their growth [89]. However, chemotherapeutics target frequently dividing cells, including cancerous and healthy cells. The side effects associated with chemotherapy include gastrointestinal toxicity, neurotoxicity, hair loss, anaphylaxis, nephrotoxicity, bone marrow suppression, cardiac toxicity, and damaged lung tissues [90]. Apart from their non-specific drug release, the other disadvantages include water insolubility, a short blood circulation period, and rapid

elimination [37]. Thus, the anticancer drugs are conjugated with nanocarriers to reduce their adverse effects and improve bioavailability. Here is a brief discussion on different anticancer drug and their release mechanism with nanocarriers.

Doxorubicin (DOX) or Adriamycin ($C_{27}H_{29}NO_{11}$) is an antibiotic that is a member of the anthracycline group of chemotherapeutic drugs. It is derived from the *Streptomyces peucetius* bacterium [91]. DOX is among the most potent anticancer drug and is most commonly used for treating breast cancer, Hodgkin's disease, lymphoblastic leukemia, soft tissue sarcomas, and solid childhood tumors [92,93]. The working mechanism of DOX is that it intercalates with DNA and curbs the synthesis of RNA and DNA [101]. It further disrupts topoisomerase II-mediated DNA repair, which leads to DNA damage [91]. Apart from this, DOX can cause the generation of reactive oxygen species (ROS) along with the reduction of antioxidants. This leads to increased oxidative stress and damages cellular material [93]. DOX also attaches to the inner mitochondrial membrane due to its strong attraction to negatively charged membranes and causes lipid peroxidation [93]. Despite its anticancer nature, the major side effect of DOX is cardiovascular complications that are dose-dependent and lethal to the patients [92]. Smart DDS helps reduce the adverse side effects of chemotherapeutics. Conjugating DOX with GO and its derivatives increases its efficiency and decreases the side effects. DOX is non-covalently loaded on GO via π - π interactions and shows a pH-sensitive release mechanism [95].

Vovusha et al. (2018) used density functional theory calculations as well as fluorescence and X-ray photoelectron spectroscopies to investigate DOX adsorption on GO. It was established that DOX binds with GO by π - π interactions, and sp^2 regions of GO offer stronger interactions [96]. Quagliarini et al. (2020) studied the anticancer mechanism of GO-DOX on MCF-7 and MDA-MB 231 (breast cancer cells) and compared its performance with liposomal doxorubicin

(L-DOX), another commonly used drug for breast cancer. They reported that the GO-DOX complex had higher efficiency than L-DOX. Encapsulating drugs with nanocarriers increases their bioavailability and cellular uptake. GO provides higher cell internalization by binding with integrins at the plasma membrane of cells, which creates a pathway for conjugated drugs. Thus, GO-DOX exhibits superior performance as it delivers DOX by attaching it to the cell membrane and destroying the DNA [97].

Paclitaxel ($C_{47}H_{51}NO_{14}$) (PTX) is one of the most commonly administered chemotherapeutics for treating cancers of several kinds, including breast, lung, and ovarian cancer. It is a tricyclic diterpenoid, and its complex structure consists of a taxane and an amide function. PTX is often combined with other chemotherapy agents to enhance its efficiency, most frequently with doxorubicin for treating breast cancer and with cisplatin and carboplatin for treating lung cancer [98]. Guo et al. (2021) established a drug delivery system with PEG-modified and oxidized sodium alginate functionalized GO nanosheets to deliver PTX and treat gastric cancer. PTX@GO-PEG-OSA was designed to overwhelm the drug resistance faced by PTX in gastric cancer cells due to P-glycoprotein (P-gp). When exposed to NIR radiations, the PTX@GO-PEG-OSA NSs exhibit a photo-thermal effect that generates ROS, limits mitochondrial respiratory chain complex enzyme activity and diminishes ATP supplement to P-gp. Thus, this nanocarrier system provides better efficiency than free PTX [99].

Meanwhile, traditional drug delivery methods have some drawbacks, such as drug release before reaching the targeted site, low efficiency, low drug retention, poor solubility in the physiological medium, and side effects on healthy cells [20]. The advantages of smart drug systems, as summarized by Shah et al. (2021), are improved utilization of the drug and lowered administration frequency, drug protection from degradation, and reduced side effects [100]. The advances in nanotechnology have led to the advancement in nano-carriers-based targeted

DDS. The DDSs are target-specific, leading to the appropriate distribution of the drug in the patient's body as the nanostructures have the required size and display excellent drug loading and release behavior [100]. The amphiphilic characteristics of GO allow drugs to be stacked on the hydrophobic region via π - π staking and electrostatic interactions, while the hydrophilic region allows surface functionalization [35]; for instance, the ionizable carboxylic acid group on edge allows drug molecules to interact with electrostatically [36]. Through covalent and non-covalent modifications, GO can bind with functionalization agents that upgrade stability and provide multi-modal diagnostics [37]. Also, GO shows maximum stability at pH 7 or 8, and since the tissues at cancer sites have low pH, it triggers the drug release due to hydrogen bonds between GO and drug molecules get weakened [35].

Pourjavadi et al. (2020) designed a GO-based DDS for the loading and controlled release of both hydrophobic and hydrophilic anticancer drugs [101]. The hydrophobic drug (curcumin) was loaded by π - π interaction and hydrophilic (DOX) by covalent bonding. Also, GO was modified with oxygen-rich polymers to enhance its aqueous solubility. The system displayed pH-triggered release behavior, and dual-loading resulted in better drug internalization. Fong et al. (2017) recently developed a folic acid (FA) conjugated GO nanocarrier (GO-FA) for the targeted delivery of DOX, as shown in **Fig. 1.7**. For intratumoral drug administration, the drug-loaded nanocomposite (GO-FA-DOX) was encapsulated with the injectable and thermosensitive hyaluronic acid-chitosan-g-poly(N-isopropyl acrylamide) (HACPN) hydrogel. The breakdown duration of HACPN hydrogel may be modulated, potentially resulting in a controlled distribution of DOX from the GO-FA nanocarrier. Furthermore, biopsies of major organs and blood analysis indicated no adverse effects of the medication, confirming its safety [102].

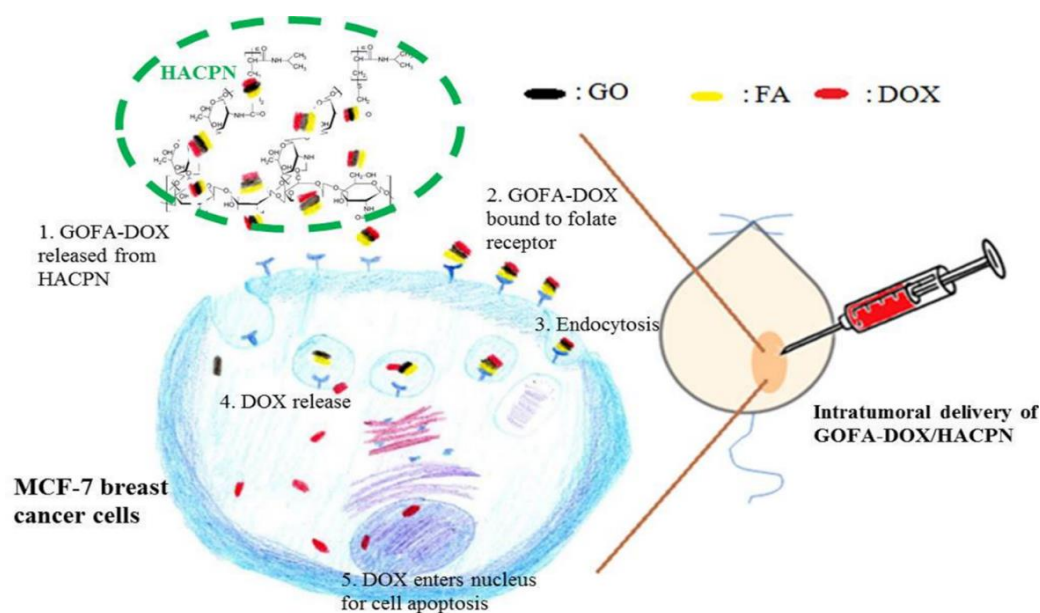


Fig. 1.7: Schematic representation of targeted delivery of DOX to tumor cells from the GO-FA-DOX- HACPN hydrogel [102].

1.5.2. Natural chemotherapeutic agents

Natural compounds that display anticancer behavior are abundant in dietary substances, plants, and fruits. They are generally present as phenolic compounds. Some of the natural chemotherapy agents are discussed below:

Gallic acid (3,4,5-trihydroxybenzoic acid) is a natural phenolic compound produced from plants and is reported to have the potential to inhibit the development of cancer. It is found in several plants, fruits, and food [103]. Gallic acid contains anti-inflammatory, antibacterial, antimelanogenic, antiviral, antimutagenic, and anticancer properties [104]. Gu et al. (2018) reported that GA exerts an anticancer effect by inhibiting mitochondrial respiration in cancer cells [105]. Zhang et al. (2019) studied the effect of GA on NSCLC A549 cells with and without cisplatin, another chemotherapeutic. The findings showed that GA controlled the proliferation and persuaded apoptosis of cancerous cells. Also, GA enhanced the effect of cisplatin [103]. Dorniani et al. (2016) loaded GA on GO nanocarriers. The application of the GO/GA system was studied on normal fibroblast and liver cancer cells at different concentrations. It was

conveyed that the complex hindered the growth of cancer cells and had no side effects on normal cells [104].

Another natural chemotherapeutic is chlorogenic acid, which is abundantly present in green coffee beans. Chlorogenic acid is a polyphenol, an ester of cinnamic acid and quinic acid [106]. Chlorogenic acid has been recognized to be beneficial in the treatment of colon, brain, lung, and cancer, and its consumption can prevent other chronic diseases [107]. Barahuie et al. (2017) designed a GO-based DDS for the controlled release of chlorogenic acid. The GO/CA complex was characterized, and it was revealed that chlorogenic acid was loaded on GO by hydrogen bonding and π - π interactions [108].

Caffeic acid (3,4-dihydroxy cinnamic acid) is a natural phenolic compound present in fruits, vegetables, olive oil, and coffee [109]. CA has been shown to have antioxidant, antithrombotic, antihypertensive, antifibrosis, antiviral, and antitumor effects [110]. Espindola et al. (2019) stated that CA showed antitumor activity against hepatocarcinoma cells by reducing the overproduction of ROS and helping in tumor cell apoptosis through DNA oxidation, as well as angiogenesis by inhibiting VEGF-induced vascularization and suppressing MMP-2 and MMP expression [111]. Min et al. (2018) combined caffeic acid with paclitaxel and tested their toxicity on H1299 cells. The results reported that the combined effect of the two drugs promoted cell apoptosis and curbed the growth of cancerous cells while having no adverse effects on normal cells [112].

1.6. State of the art

With a brief overview of contemporary research, this section outlines the research outcome of various literature so as to identify a few promising areas of research that needs to be addressed

in this thesis. The state of the art has been presented for the structural, functional and morphological modification of graphene oxide-based nanocarriers for anticancer DDS.

1.6.1. Fabrication of ultrasound-mediated tunable graphene oxide nanoscrolls

Literature survey

Graphene oxide, a hydrophilic derivative of graphene, exhibited significant physicochemical characteristics. Despite several properties and applications, GO owns some disadvantages, like agglomeration or overlapping of GO sheets and larger lateral dimensions. Also, the stability of GO suspension in an organic solvent is a significant issue [82,113]. Agglomeration leads to the reduction in active surface area and sites for applications such as adsorption, drug delivery and membrane filtration [72,73]. However, thin and flat GO structures provide flexibility in structural and morphological modifications, which delivers an effective way to overcome these disadvantages. The oxygen functional groups in the tail end of GO sheets act as both functional sites as well as spacers, which facilitates GO conversion by structural and functional means into nano-morphologies [73]. The structural and morphological modification of GO into nano-morphologies like GONS and quantum dots may subside the shortcoming of GO nanosheets, which could be vital for real-world applications such as drug delivery, filtration membranes, electrochemical sensors, and super-capacitors [72,114].

Graphene oxide nanoscrolls (GONS) are one of the potential nano-morphology formed by rolling up single atomic thin GO sheets into spiral wound structures. GONS has a similar 1D morphology to CNT but with a more accessible inter-wall area. Various studies explored the applications of CNT for drug delivery [38,83]. Since GONS exhibits similar properties of GO along with open-end morphology, porous structure, tunable dimensions, and adjustable interlayer distance/spacing between scrolled layers with a more inner accessible area than CNT could be a suitable alternative for DDS [26,70].

In previous studies, GONS were fabricated by ultrasonication [73], Lyophilization [115], Langmuir-Blodgett method [116] and using Vortex Fluidic Device (VFD) [117]. In recent studies, among the reported approaches for the synthesis of nanostructured materials for various applications, the utilization of high-intensity ultrasound waves and bubble cavitation energy has been applied extensively [118,119]. Cavitation includes the formation, growth and collapse of bubbles in a liquid. The bubble collapse leads to high pressure ($\sim 1000\text{ atm}$), intense local heating ($\sim 5000\text{ K}$) at the interphase, heating and cooling rate ($> 10^9\text{ K sec}^{-1}$) and liquid jet stream (400 km h^{-1}). However, classical heating conditions have limitations related to heating and cooling rates, higher energy consumption and longer processing time [87]. Also, ultrasonication processes can be used for the synthesis of nano-morphologies because of the de-agglomeration ability and efficient utilization of energy. The synthesis of nanoparticles is a top-down approach, where the size of particles is reduced by utilizing the physical effect of cavitation, which includes the breaking of agglomerates and aggregates [87]. Previously, Amadai et al. (2016) studied the fabrication of GONS with tunable dimensions via low-frequency (LF) and high-frequency (H.F.) ultrasonication. They have investigated the effect of ultrasonic frequency, power density and irradiation time on GONS production, morphology, dimensions and surface chemistry [73]. However, one of the important factors, such as solvent selection affecting the formation and structural characteristics of GONS, has not been reported. Furthermore, the detailed functional and structural characterization of GONS was lacking. Various literature related to the fabrication of GONS and a brief comparison among them is provided in **Table 1.3**.

Table 1.3: Literature related to the fabrication of GONS.

	Fan et al. (2015)[75]	Amadai et al. (2016) [73]	Alharbi et al. (2018)[117]	Tang et al. (2018)[113]
Base material	High-grade natural graphite	High-grade natural graphite	High-grade natural graphite	High-grade natural graphite
Method	Lyophilization	LF-H.F Ultrasonication	Vortex Fluidic Device (VFD	solvent-induced self-assembly
Solvent	Water	Ethanol	Water and 2-Propanol	N, N-Dimethylformamide (DMF)
Factor affecting GONS dimension	-	Ultrasonic frequency and power density	-	-
Characterization	FESEM, TEM, Raman, XRD, TGA, and Capacitance.	FESEM, TEM, Raman, XPS, and AFM.	FESEM, TEM, Raman, XRD, TGA, and XPS.	FESEM, TEM-SAED, Raman, XRD, XPS, and AFM.
GONS dimensions	-	Length -3-5 μm Dia.-0.193-1.6 μm .	-	Length-0.537-1.870 μm . Dia.-0.063-0.242 μm .
Applications	Super-capacitor	-	-	Surface-relief-gratings (SRG)

Possible scope for further research

As discussed before, the shortcomings for the use of GO in drug delivery are a) stability in aqueous media, b) agglomeration of GO nanosheets, c) less porous structure, and d) lower surface area, which together affect the loading and release characteristics of nanocarriers along with their toxicological aspects. On the contrary, GONS with enhanced structural morphology may overcome the limitations of GO with its de-agglomeration tendency, suitable aqueous stability, adjustable interlayer distance, tailored dimensions, and higher porous structure. In addition, the open-end morphology of GONS and similar properties to GO, along with the structurally and morphologically modified form, make it more feasible for DDS and other real-world applications compared to GO and CNT.

Although a few studies on the synthesis of GONS have been reported, most of the studies have employed high-grade graphite as a base material and other relatively harsh, expensive chemicals for the limited yield of GONS. To the best of our knowledge, no work has been done on the synthesis of GONS using synthetic graphite as a base material, which is comparatively inexpensive to high-grade graphite. Again, the influence of sonication parameters and solvent characteristics on the formation and stability of GONS have not been studied in detail. In the present work, an attempt was made to synthesize GONS by the LF ultrasonication (20 kHz) method using cheaper synthetic-grade graphite and methanol as solvent. The selection of methanol as a solvent was made due to its lower dipole movement, negative (–ve) zeta (ζ) potential and cost. These modifications are proposed to be favorable for the facile preparation of nanoscrolls. The primary objectives of the present study are a) Formation of tunable dimension GONS from low-grade synthetic graphite as a base material, b) The study of solvent characteristics for GONS formation, and c) The investigation of the effect of sonication parameters over GONS structure and morphology.

1.6.2. Development of graphene oxide nanoscrolls imparted nano-delivery system for the sustained release of gallic acid

Literature survey

The fabrication and design of novel drug and gene delivery systems, along with higher loading efficiency and controlled and sustainable release rates, are much imperative in order to enhance the therapeutic efficiency of diseases [113]. Previously, it was observed that the advanced nanocarriers, a part of novel DDS, are proficient in transporting the active drug ingredient to the targeted sites. The nanocarriers, a nanosized material, have a unique accumulation ability around the tumor side due to their smaller size, leading to a higher concentration of drugs in cancer cells [51,120]. In recent studies for DDS, various nanomaterials like nanoparticles, carbon nanotubes (CNTs), and polymers have been used extensively for controlled DDS via

conquering the release rate and drug half-life [121–123]. Recently, GO, a two-dimension derivative of graphene, has been used in applications related to pharmaceuticals and biomedicines [124,125]. Despite the significant physicochemical characteristics of GO, its application in DDS is limited due to the agglomeration of GO nanosheets, lower aqueous stability, higher lateral dimensions, and lower bioavailability and biocompatibility [38]. However, a flat and thin structure of GO provides an opportunity for its modifications in the 1D nanomorphology of GONS, which could deliver an operative way to overwhelm these shortcomings [73,126]. Spiral wounded GONS exhibit a similar one-dimensional structure to CNTs, with an additional and accessible inner wall area. However, CNTs showed lower performance during their application in drug delivery due to a longer diffusion path resulting from the closed-end morphology, less porous structure and aqueous instability [70,72]. In addition, CNTs as nanocarrier exhibits major biosafety and cytotoxicity-related issues in DDS due to their surface chemistry, colloidal instability, higher lengths, agglomeration tendency, and hydrophobicity [127].

Furthermore, to overcome the disadvantages of conventional chemotherapy, a novel DDS plays an important role along with natural chemotherapeutic agents such as gallic acid, caffeic acid and methyl caffeate [128]. Gallic acid is proven as a natural antioxidant and anticancer agent against various cancer cells; its low cost and lower toxicity also appeal to substantial attention as an alternative to synthetic anticancer compounds [125]. Gu et al. (2018) reported that GA exerts an anticancer effect by inhibiting mitochondrial respiration in cancer cells [105]. Zhang (2019) studied the effect of GA on A549 cells with and without cisplatin, another chemotherapeutic. The findings showed that GA controlled the proliferation and persuaded apoptosis of cancerous cells. Also, GA enhanced the effect of cisplatin [103]. Dorniani et al. (2016) used GO nanocarriers to load GA. In normal fibroblasts and liver cancer cells, the use of the GO/GA system was investigated at various doses. It was reported that the compound had

prevented the proliferation of cancer cells [104]. However, the GO-based nanocarriers demonstrated lower loading capacity and burst release of GA due to the weaker interaction with drug molecules.

Possible scope for further research

It is acclaimed that GO, with its agglomeration tendency and lower stability in aqueous media, affects the drug loading capacity, release rate, and drug concentration over the cancer cells. However, GONS with open-end morphology, higher surface area, reduced nature, and better aqueous stability may provide additional benefits over its utilization in the DDS. Also, the more accessible-inner wall area and modifiable interlayer distance between scrolled layers of GONS make it suitable for improving the drug loading capacity. The primary objective of this part of the thesis was to build up GONS based nano-drug delivery system for sustainable transport of GA to human cancer cells. Till then, no study had explored the use of one-dimensional morphologies like GONS in DDS for cancer treatment via a natural chemotherapeutic agent, gallic acid. The stated structural and morphological modification of GO is proposed to be favorable for a significant improvement in the loading capacity and release behavior of gallic acid.

In this part of the thesis, an endeavor has been made to synthesize GONS-GA nanocomposite of different compositions at different temperatures and pH of the media. The thermodynamics study, along with the kinetic study on the loading and release characteristics of GA, was expected to describe the mechanism related to higher loading capacity and improved release behavior, respectively. To the best of our knowledge, no study had been undertaken on the application of GONS in DDS for the treatment of cancer.

1.6.3. Chitosan-grafted folic acid decorated one-dimensional graphene oxide nanoscrolls: a biocompatible drug cargo for targeted co-delivery of anticancer drugs

Literature survey

Despite substantial advances in medical research and biomedical engineering, the therapeutic evolution of anticancer therapies is frequently hampered by drug administration issues [129]. Moreover, due to the diversity of cancer cells, the emergence of drug resistance, and the undesirable effects induced by high and/or repetitive drug doses, the administration of a solitary chemotherapeutic agent frequently fails to accomplish a comprehensive cancer regression. To address these concerns, oncologists have embraced combination chemotherapy as the predominant cancer treatment strategy, which includes multiple chemotherapeutic agents. It has been observed that employing numerous drugs which target various cellular pathways might boost the genetic barriers for cancer cell mutations, delaying the cancer adaption process [130]. Cancer is a broad term that encompasses a wide range of disorders. The majority of malignancies have the trait of not responding to existing chemotherapies because of the emergence of multidrug resistance (MDR). Cancer cells can become highly resistant to a variety of functionally and structurally unconnected substances due to a phenomenon known as MDR. Several mechanisms can cause this phenomenon, but the best-explained one is the overexpression of drug-efflux proteins like P-glycoprotein (P-gp). In 90% of patients, this results in cancer recurrence and death [131].

The primary objective of most anti-tumor medications is to eradicate tumors by activating signaling pathways that lead to apoptosis. It is possible for cancer cells to avoid apoptosis and develop greater chemoresistance. For instance, paclitaxel (PTX), an anticancer drug, does not necessarily cause tumor cells to undergo apoptosis ultimately. Conversely, PTX-induced apoptosis causes tumor cells to survive and increase their treatment resistance.

Chemosensitizers and resistance modulators, which are able to limit Pgp's ability to efflux drugs, can be used to resolve such concerns by enabling larger concentrations of medications to reach their cellular target sites [132,133].

The advancements in P-gp inhibitors have been made as cancer MDR reversal drugs and have been discovered through time, which are based on prior failure experiences [19]. Quinidine, verapamil, tariquidar, and zosuquidar were only a few of the chemical P-gp inhibitors formerly used to illustrate the MDR reversal effect; however, most of them are severely toxic and require higher dosages. As a result of the extreme toxicity and interaction of the aforementioned chemical reagents, researchers have shifted their focus to natural resources, intending to identify low-toxic and formidable compounds from plants, fungi, or marine animals. In line with this, phytochemicals such as catechin, quercetin, caffeic acid, and ellagic acid have received much interest due to their numerous pharmacological properties, notably antioxidant and anticancer activity [134]. Caffeic acid (CA), a phenolic compound found in many plants, fruits, and tea extracts, is well-known for its antioxidant properties [135]. Moreover, it has been shown that caffeic acid possesses anti-inflammatory, antibacterial, and antiviral properties. The distinguishing characteristics of CA encourage its utilization as a chemosensitizer in combination with chemotherapy to inhibit an MDR effect and improve the drug's therapeutic effect correspondingly.

Meanwhile, the clinical result of combination chemotherapy is greatly reliant on the drug ratio, which defines the degree of synergistic effects or antagonism. The coordination of the pharmacokinetics and cellular absorption of several medicines is another difficulty in combination chemotherapy. Importantly, combinatorial nanoparticles (NPs) as nanocarriers offer an excellent platform for addressing these obstacles due to their synchronized delivery of the precise ratio of drugs to cellular components, synergistic therapeutic potential, and

inhibition of drug resistance [130]. As drug carriers, a variety of polymers, dendrimers, and carbon-based nanomaterials, including graphene oxide (GO), have been widely used up to this point [38]. Unfortunately, the majority of carriers lack the ability to differentiate between healthy and malignant cells. As a result, for such systems, the anticancer drugs non-selectively accumulate in healthy tissues and cause serious toxicities. The commonly studied method for precise drug delivery to the tumor location is targeted drug delivery as a consequence of modification of the drug carriers with specific ligands like folic acid (FA). The majority of tumor-targeting drug delivery systems incorporate a tumor recognition moiety and a drug-loaded vesicle [136].

Recently, GO, with its distinct physicochemical properties and functionalization ability has attracted significant attention from researchers working in anticancer DDS. However, the main disadvantages of utilizing GO nanosheets in the delivery of anticancer drugs are size, aqueous stability, and sharp edges, which frequently cause non-cancerous cells to be disrupted. It has also been demonstrated to have dose-dependent hemolytic activity [137]. GO is often functionalized with other polymers, such as polyethylene glycol (PEG), chitosan (CS), polyvinyl alcohol (PVA), etc., to reduce the hazard involved in employing GO. The biocompatibility and targetability of GO-based nanocarriers for cancer therapies are greatly improved by functionalizing them with natural polymers, including CS, cyclodextrin, and folic acid (FA), as a targeting ligand for cancer cells [38]. Sousa et al. (2017) demonstrated the GO-FA-PEG nano-delivery system in the controlled release of Camptothecin (CPT). The results of the preclinical screening tests showed that HepG2 cells and J774 cells both internalized GO-FA-PEG effectively and attached to its surface robustly. Although the GO-FA-PEG + CPT nanocarrier increased tumor cell death through apoptosis, the early and late stages of apoptosis/necrosis depended on the cell type [54]. Pooresmaeil et al. (2022) derived a targeted dual DDS by D-mannose functionalized MgAl-LDH/Fe-MOF nanocomposite for a sustainable

delivery of DOX and methotrexate (MTX). The cytotoxicity studies of nanocarrier and drug-loaded nanocarriers were conducted for breast cancer cells (MDA-MB 231). The IC_{50} value for drug-loaded MTX@DOX@MgAl-LDH/Fe-MOF/D-Man nanocomposite was found to be $0.117 \mu\text{g mL}^{-1}$ [136].

Possible scope for further research

It is envisaged from the above literature that the co-delivery of chemotherapeutic agents assisted with novel nanocarriers based on targeted DDS may counter the issues related to the MDR and henceforth improve their therapeutic efficacy. With its distinct physicochemical characteristics, the GO provides a scope for its functionalization with targeting ligands such as FA and natural chemotherapeutic agents like gallic acid, caffeic acid (CA) and chlorogenic acid. These chemotherapeutic agents can be employed as chemosensitizer, which may further enhance the therapeutic efficacy of synthetic chemotherapy drugs such as DOX, MTX and PTX.

Meanwhile, as stated previously, the utilization of GO in DDS suffers from the agglomeration tendency, poor aqueous stability, higher lateral dimensions, and lower surface area, which inevitably affects its bioavailability, biocompatibility, drug loading capacity and release characteristics. Fortunately, GONS, a morphologically modified form of GO with identical functional characteristics and 1D open-end structure of tunable dimensions, may eventually address these issues. In this part of the thesis, an attempt has been made to develop a GONS-based biocompatible drug cargo for a targeted co-delivery of chemotherapeutic agents such as DOX and CA. The nanoscrolls were fabricated via LF ultrasonication technique and functionalized with chitosan (CS) biopolymer and FA to further enhance their biocapacity and target-specific delivery of anticancer drugs, respectively. The resultant GONS-CS-FA (GCF) nanocarriers were loaded with DOX and CA at different weight proportions. To the best of our

knowledge, it is a first-ever attempt for the development of GONS-based 1D, biocompatible and targeted drug cargo for the co-delivery of anticancer agents.

1.6.4. One-pot, sustainable and room temperature synthesis of graphene oxide-impregnated iron-based metal-organic framework (GO/MIL-100(Fe)) nanocarriers for anticancer drug delivery systems

Literature survey

Metal-Organic Frameworks (MOFs), a versatile porous material, comprise organic linkers and metal ions that are bonded together in a coordinated manner. MOFs are known for having exceptional characteristics like large surface area, porosity, functionality, crystalline structures, and thermal and chemical properties [138]. MOFs as drug carriers have recently received much interest among researchers due to their suitable loading capacity, controlled release patterns, and biocompatibility [139,140]. Among them, "Fe" metal-rich MIL-100 (Fe) is mostly preferred as a carrier due to higher uptake and minimal cytotoxicity [141]. MIL-100 (Fe) can be made via a traditional hydrothermal process at high temperatures, with or without HF (hydrofluoric) acid as a crystallizing agent [142]. The conventional hydrothermal methods involve environmentally harmful chemicals, and high-temperature conditions make it a more costly and unsustainable method. However, economic, environmental and chemical sustainability must be governed to discover the versatility of MIL-100 (Fe) in the present industrial framework. The sustainable approach for the synthesis of MOFs as well as their composite can be established via room temperature conditions, utilization of cheap and harmless solvents such as water, metal salts, and organic salts as linkers, avoiding tedious post-synthesis treatments, absence of any harmful by-products and corrosive inorganic acids such as HNO₃, and HF. Recently, Guesh et al.(2017) demonstrated an HF-free MIL-100 (Fe) production procedure at ambient temperature for photocatalytic degradation of dyes [143]. Yuan et al. (2019) have synthesized MIL-100 (Fe) similarly in room temperature conditions

using an oxidizing radical-promoted approach for the separation of light hydrocarbons via the adsorption process [144]. Similar approaches can be revised further for drug delivery-related applications. The literature related to the application of MOF-based nanocarriers in anticancer DDS is provided below.

Table 1.4: Application of MOF-based nanocarriers in anticancer DDS.

Nanocarrier	Synthesis method	DOX loading efficiency (%)	Cell lines	References
MIL-100 (Fe)	Hydrothermal route with and without HF	10.7, 23.40	-	[142]
Fe ₃ O ₄ @MIL-100 (Fe)	Hydrothermal route	15.70	BT-474, MCF-7	[145]
Fe ₃ O ₄ @MIL-100 (Fe)	Hydrothermal route	19.40	-	[140]
CeO ₂ -MIL-100 (Fe)	Hydrothermal route without HF	13.30	-	[146]
MIL-101-N ₃ (Fe)	Solvothermal	13.40	HeLa (human adenocarcinoma) cells	[147]
UPNC@MIL-100 (Fe)	RT	17.20	HEK-293, MCF-7	[148]
ZnO-MIL-100(Fe)	Hydrothermal route	23.50		[149]
ZIF-8	RT	20.00	MCF-7, HTB-26, HTB-132	[150]
NH ₂ -MIL-88B	Low-temperature synthesis (40 °C)	14.4	4T1	[151]
MgAl-LDH/Fe-MOF	Low-temperature synthesis (40 °C)	17.8	HTB-26	[136]
CMC/MOF-5/GO	Solvothermal route	6.45	K562	[64]

Possible scope for further research

The above literature envisaged that the GO and MIL-100 (Fe), with their higher porosity and surface area, have higher uptakes for an anticancer drug such as Doxorubicin (DOX). In addition, the composites of nanomaterials with MIL-100 (Fe) provided higher DOX uptake capacity than pristine MOFs [149]. Therefore, incorporating GO into the MIL-100 might further increase the loading capacity of the resultant composites. Also, sustainable synthesis methods can be revised for the preparation of GO/MIL-100 (Fe) nanocarriers to improve their

adaptability in real-world applications. However, such a sustainable approach had not been reported for GO/MIL-100 (Fe) nanocomposites or utilized for drug delivery-related applications. Besides, the control and sustainable release behavior of GO-impregnated nanocarriers must be established along with their toxicological aspects for mammalian cancer as well as normal cells, which may be favorable to reduce the dosing frequency and side effects of chemotherapy.

This work has adopted a sustainable approach to synthesizing HF-free GO/MIL-100 (Fe) nanocarriers with different amounts of GO at room temperature conditions. The obtained nanocarriers, including pristine GO, MIL-100 (Fe) (MF) and their nanocomposite, Gw-MF, were characterized to examine the surface morphology, structure and surface chemistry of nanocarriers and subjected to DOX loading subsequently. To the greatest of our knowledge, no research had been conducted on the sustainable, one-pot and in-situ preparation of GO-impregnated MIL-100 (Fe) nanocomposites at room temperature conditions as well as their application in DDS for cancer therapeutics. The presented synthesis approach for GO-impregnated MIL-100 (Fe) nanocomposites is proposed to be favorable for higher drug loading capacity and controlled and sustainable drug release with significant improvement in its cytotoxicity towards cancer cells.

1.6.5. Room temperature synthesis of β -cyclodextrin functionalized graphene oxide decorated MIL-100 (Fe): a sustainable drug cargo for controlled delivery of caffeic acid

Literature survey

Compared to traditional treatment methods, nanocarriers used in DDSs have demonstrated significant advantages such as enhanced biodistribution, increased plasma half-life, better cellular uptake, stability and maximized drug solubility via their encapsulation and, most importantly, the precise administration of the drug to the cancer sites [38]. As stated previously,

GO and MOFs (MIL-100(Fe)), with their substantial surface and functional characteristics, have been successfully utilized for the novel DDS with enhanced drug loading capacity, controlled release behavior, suitable colloidal stability, and cytocompatibility. However, the biocompatibility of the GO and MOF-based nanocomposites was hampered by their poor aqueous stability in organic solvents and physiological solutions. Furthermore, more efforts are obligatory to improve the pH-sensitive release of active drug components to the targeted sites. All these aspects can be overcome through employing functional or pristine biopolymers like oligosaccharides and polysaccharides [152,153].

β -Cyclodextrin (β -CD) is a natural oligosaccharide assembled by seven d-glucopyranose units connected with α -1,4-glycosidic bonds such that its structure has a hydrophilic exterior and hydrophobic core [154]. The toroidal shape of β -CD with its hydrophobic core allows easy incorporation of other molecules, and its high absorption capacity has been exploited for use in several domains such as wastewater treatment, remediation of environmental pollutants and drug delivery [155–157]. The conjugation of β -CD on two-dimensional surfaces can greatly improve their absorption capacity, solubility, and biocompatibility. Furthermore, because β -CD complexes are pH-sensitive, drug release can be regulated by altering the pH [152,154]. As a result, by optimizing each component's capability for chemotherapeutic drug delivery, GO may be conjugated with β -CD to integrate the advantages of both components.

Also, in order to address the shortcomings of conventional chemotherapy, a modern DDS, in conjunction with nature-found chemotherapeutics such as methyl caffeate, gallic acid, chlorogenic acid and caffeic acid, plays a vital role. The therapeutic nature of phenolic compounds usually found in plants is being extensively researched as their antioxidative nature can help to avert ailments such as cancer that arise due to oxidative stress. One of the most common classes of phenolic is hydroxycinnamic acids, and a typically found hydroxycinnamic

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acid is caffeic acid (CA) [158]. CA is present in various dietary items, fruits, vegetables, coffee and tea and is known for its medicinal properties like anti-inflammatory, antiviral, antioxidant, and anticancer nature. It exerts a therapeutic effect by reducing oxidative stress and lessening the free-radicals-induced DNA damage [159]. Previously, the anti-cancerogenic nature of plant-based chemotherapeutics such as gallic acid and caffeic acid has been explored for DDS [26,110].

Possible scope for further research

The literature described previously suggested that the pristine GO, β -CD-GO and MIL-100 (Fe) would have increased uptakes for an anticancer agent due to their excellent physicochemical characteristics, including higher porosity, surface area, aqueous stability, higher drug solubility and loading capacity, controlled release and increased biocompatibility, respectively. Additionally, the drug absorption capability of nanomaterial composites containing MIL-100 (Fe) was greater than that of virgin MIL-100 (Fe) [149]. Therefore, β -CD-GO, when added to the MIL-100 (Fe), can raise the resulting composites' loading efficiency even further. Additionally, in order to make β -CD-GO/MIL-100 (Fe) nanocarriers more adaptable for use in practical applications, sustainable production strategies can be altered. However, till now, no such sustainable strategy for β -CD-GO /MIL-100 (Fe) nanocomposites-based controlled delivery of natural chemotherapeutic agents has been documented.

The current study outlines a sustainable approach for producing HF-free β -CD-GO/MIL-100(Fe) nanocarriers comprising varied β -CD-GO contents at room temperature. The obtained nanocarriers subsequently were loaded with a natural chemotherapeutic agent, caffeic acid (CA). Loading characteristic CA on the developed nanocarriers has been investigated for various β -CD-GO concentrations in nanocarriers, pH of the media, and CA to nanocarrier mass

ratios. In addition, *in-vitro* cytotoxicity assessments of pure β -CD-GO, MF, and the optimized Gw-MF nanocarrier were done on normal cells (HEK293) and cancer cell lines (A549).

To the best of our knowledge, no research has been conducted on the room-temperature, one-pot synthesis of β -CD-GO impregnated MIL-100 (Fe) nanocomposites and their implementation for natural chemotherapeutics-based DDS. The β -CD-GO modified MIL-100 (Fe) nanocomposites synthesized in this part of the thesis are expected to have a better drug loading capacity, the loaded drug release in a sustainable and regulated manner, and improved cytotoxicity toward cancer cells. It is envisaged that this work will benefit the scientific community to further explore the sustainable synthesis processes of nanocarriers and their possible applications in DDS or biological science.

1.7. Objectives of thesis work

In line with the limitations of GO for drug delivery-related applications provided in the preceding sections, this thesis is focused on the modifications of surface characteristics of GO-based nanocarriers for anticancer DDS. As per the research gap based on the structural, functional and morphological modifications of GO-based nanocarriers, the possible scope of work was framed out, and the following objectives are outlined:

- Fabricating ultrasound-mediated tunable graphene oxide nanoscrolls (GONS)
- To develop a GONS-imparted nano-delivery system for the sustained release of gallic acid
- Synthesizing a chitosan-grafted and folic acid decorated one-dimensional GONS as a biocompatible drug cargo for targeted co-delivery of anticancer drugs
- To synthesize GO-impregnated metal-organic framework (GO/MIL-100(Fe)) nanocarriers for anticancer DDS by adopting a one-pot, sustainable and room temperature synthesis approach

- ☛ To develop a sustainable and biocompatible drug cargo for controlled delivery of caffeic acid by synthesizing a β -cyclodextrin functionalized graphene oxide decorated MIL-100 (Fe)

1.8. Organization of the thesis

This thesis work is divided into 7 chapters. **Chapter 1** discusses the background of GO and its morphologically as well as functionally modified forms, such as GONS, β -CD-GO, and GO-MOF as nanocarriers with their applicability for anticancer DDS. This chapter also discusses various synthesis and functionalization aspects of GO to enhance its surface attributes, drug loading and release characteristics, as well as targetability and biocompatibility. The problems undertaken in this work, i.e., drug loading capacity, burst release of chemotherapeutic agents, lower surface area, uncontrolled release behavior, lower aqueous stability, higher lateral dimensions, and lower biocompatibility of GO, have been critically reviewed in this chapter. The objectives of the present work are outlined and presented in this chapter based on the research gap and possible scope of work. **Chapter 2** describes the detailed fabrication process for the morphologically modified GONS with tunable dimensions using the low-frequency ultrasonication method. The effect of ultrasonication power (100–150 W) and irradiation time (0.5–3 h) on the GONS dimensions is investigated in this chapter; in addition, the detailed characterization of spirally wound 1D GONS is also provided. Furthermore, the application of fabricated GONS towards anticancer DDS is elucidated by loading gallic acid (GA) as a natural chemotherapeutic agent on the GONS nanocarriers in **Chapter 3**. The GA loading experiments are conducted for varied amounts of the drug, pH of the media and loading temperatures. The *in-vitro* release study, release kinetics, and cytotoxicity studies are also conducted in this chapter to investigate the efficiency of GONS as an anticancer drug cargo. **Chapter 4** deals with the targeted co-delivery of dual anticancer drugs: Doxorubicin (DOX)

and Caffeic acid (CA). Herein, a co-delivery system is developed via chitosan-grafted and folic acid decorated 1D GONS (GONS-CS-FA) using CA as a chemosensitizer to address the issues of biocompatibility, targetability, and efficiency of a chemotherapeutic agent (DOX), respectively. Furthermore, **Chapter 5** elucidates the fabrication and characterization of sustainable nanocarriers obtained via impregnation of parent material GO with MIL-100 (Fe) at room temperature. In this chapter, the nanocarriers are synthesized at variable wt.% of GO within the GO/MIL-100 (Fe) (Gw-MF) nanocomposites and utilized suitably for drug loading (DOX). Herein, *in-vitro* studies have been conducted on the drug loading and release characteristics of nanocarriers to understand the DOX loading mechanism, thermodynamics, and release kinetics. **Chapter 6** deals with the synthesis of β -cyclodextrin functionalized GO and its impregnation with MIL-100 (Fe) for a sustainable, biocompatible drug cargo and controlled delivery of CA, the natural chemotherapeutic agent. The cytotoxicity studies are also conducted for all the nanocarriers and drug-loaded nanocomposites, including GONS, GONS-GA, GONS-CS-FA, GONS-CS-FA-DOX-CA, G0.5-MF, G0.5-MF-DOX, BG0.5-MF, BG0.5-MF-CA on normal cell lines (HEK293) and lung cancer cell lines (A549), respectively. **Chapter 7** summarizes the inferences drawn from this work and provides suggestions towards future research.

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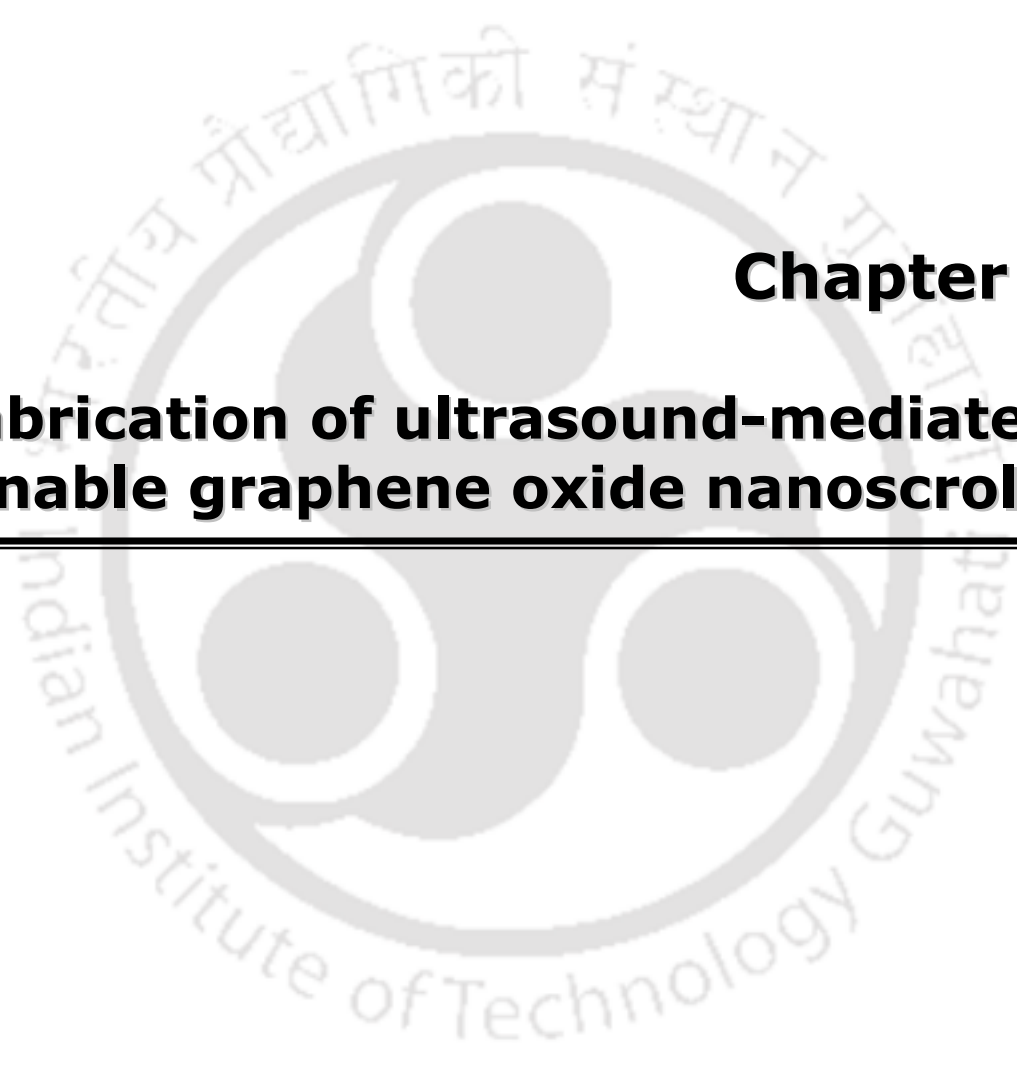
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Chapter 2:

Fabrication of ultrasound-mediated tunable graphene oxide nanoscrolls

Chapter 2

Fabrication of ultrasound-mediated tunable graphene oxide nanoscrolls

This chapter illustrates the fabrication approach for graphene oxide nanoscrolls (GONS) by low-frequency (LF) ultrasonication method using exfoliated graphene oxide (GO) nanosheets as a parent material and methanol as a solvent. The GO was synthesized via a modified Hummers' method by employing the oxidation of relatively cheaper synthetic-grade graphite. For GONS fabrication, methanol was selected as a solvent due to its lower dipole moment, negative (–ve) zeta (ζ) potential, and cost. Herein, GONS of tunable dimensions were obtained via altering the sonication parameters. The structure, morphology and functional characteristics of obtained GONS were investigated through FETEM, FESEM, FTIR, X-ray photoelectron spectroscopy (XPS), Raman spectroscopy and thin-film-X-ray diffraction techniques. Also, the effects of ultrasonication power (100–150 W), irradiation time (0.5–3 h) and solvent characteristics on the GONS dimensions were investigated. The structural and morphological modification of GO nanosheets into the 1D scrolled nano-morphology is proposed to be favorable for its further application in DDS.

2.1. Experimental

2.1.1. Materials

Synthetic-grade graphite powder (325 mesh) was obtained from Sisco Research Laboratories (Taloja, India). Chemicals such as Sodium nitrate (NaNO_3) (99%), Potassium permanganate (KMnO_4) (98.5%), Hydrogen peroxide (H_2O_2) (30%), Hydrochloric acid (HCl) (37%), Sulphuric acid (H_2SO_4) (98%) and Methanol (CH_3OH) (99%) were purchased from Merck

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Life Science Private Limited (Mumbai, India). Deionized water was obtained from Elix-3 M/s Millipore (USA) Milli-Q water purification system.

2.1.2. Preparation of GO sheets

Graphene oxide nanosheets were synthesized using synthetic grade graphite powder as starting material by the well-known Hummers' method with certain modifications [1,2]. The synthetic graphite used here for the synthesis of GO is comparatively inexpensive to that of natural graphite. The surface area of synthetic graphite used for GO synthesis was found to be higher than that of natural graphite. In a typical process for the synthesis of GO, 2 g of synthetic graphite was mixed with 1 g of sodium nitrate (NaNO_3) in a conical flask kept in an ice bath. Further, 50 mL of 98% sulphuric acid (H_2SO_4) was added slowly into the mixture and stirred continuously with a magnetic stirrer for 15 min. to obtain a homogenized mixture. The temperature of the mixture was kept within 5 °C by using an ice bath. After obtaining a homogenized mixture, the stirring rate was reduced, and 6 g of potassium permanganate (KMnO_4) was added slowly to avoid expelling out the mixture due to its exothermic nature. The temperature was monitored again and did not exceed 20 °C. Then, the mixture was removed from the ice bath and allowed to rise up to 35 °C. It was maintained for 45 min. until the mixture started thickening and became a paste of dark brown color. Later, deionized (DI) water was slowly added to the mixture while stirring at the same time. The violent effervescence and rapid increase in temperature were observed due to this exothermic step. Now, the temperature was monitored carefully using a hot plate and was kept between 98 - 100 °C for another 45 min. The mixture was further diluted with 200 mL of DI water, and 10 mL hydrogen peroxide (30%) (H_2O_2) was added gradually for the reduction of the residual potassium permanganate (KMnO_4), manganese dioxide (MnO_2) and manganese heptoxide (Mn_2O_7) to obtain soluble manganese sulfate (MnSO_4) salts. The resulting suspension was

centrifuged at around 6000 rpm for 30 min. The supernatant was cast off until the supernatant had a pH of around 6-7, and a viscous brown layer of pure GO appeared on top of the oxidation by-products. The layer was then separated carefully using warm water. This step should be done carefully to avoid remixing of the layer with the sediment. The obtained GO was freeze-dried for 12 h and placed in a vacuum desiccator for another 24 h in order to remove any remaining humidity.

2.1.3. Fabrication of GO nanoscrolls

The GO powder, produced after centrifugation and drying, was dispersed in methanol to obtain GO suspension of 0.05 g L^{-1} concentration in a glass beaker. The solvent (Methanol) was chosen based on the lowest value of dipole movement, -ve zeta (ζ) potential, which provides lower stability in suspension. The details are provided in the subsequent section. After that, 150 mL of homogenized GO suspension was subjected to ultrasonication treatment using a 20 kHz LF tip-sonicator (LABMAN Pro-650) (12mm tip geometry) with variable amplitude (15 - 30%) for different irradiation times. Then, the sonicated solution was taken out, and obtained GONS were vacuum filtered, followed by drying. The temperature of the suspension was measured before and after ultrasonication treatment to calculate the actual power dissipated by the sonicator. The study related to parameters such as irradiation time (0.5 - 3 h), power (100 - 150 W), and solvent characteristics were also investigated to understand the scrolling mechanism, morphological alterations, and yield of fabricated GONS. The sonicator parameters are selected based on the literature [3,4]. The schematic for the formation of GONS is shown in **Fig. 2.1**.

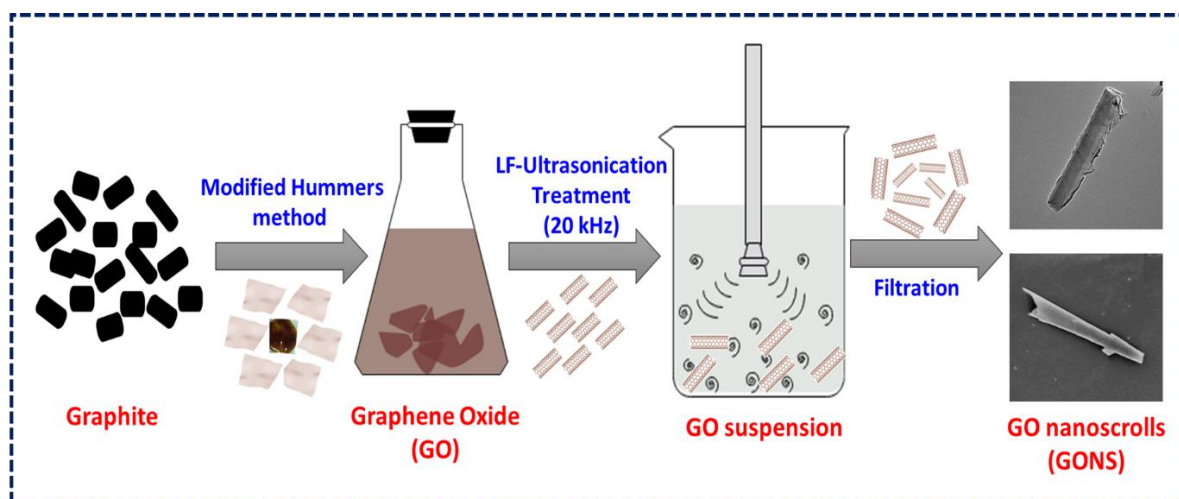


Fig. 2.1: Schematic representation of the fabrication of graphene oxide nanoscrolls (GONS).

2.1.4. Characterization techniques

The obtained GO nanosheets and GONS were characterized by Field emission transmission electron microscopy (FETEM), Field emission scanning electron microscopy (FESEM), X-ray diffraction (XRD), BET surface area, Particle size analysis, Raman spectroscopy, and Fourier transform infrared spectroscopy (FTIR). A high-resolution TEM (JEOL, 2100F) with an electron beam of 100 kV was used to observe a single-layer GO sheet and scrolling of GO sheets to GONS obtained after sonication. TEM samples were prepared by dispersing GO and GONS in methanol. X-ray diffraction spectra for synthetic grade graphite, GO, and GONS were recorded on Bruker (Netherlands) D8 advance diffractometer with Cu K α radiation (1.5406 Å) at 40 kV. The active BET surface area of samples was determined using Micromeritics, Tristar II model. The average particle size analysis of GO nanosheets was carried out by using the DLS method in Delsa Nano (Delsa nano C, Beckman Coulter). Raman spectra were recorded using the Horiba Jobin Vyon LabRam HR model with an excitation wavelength of 633 nm at room temperature. Field emission scanning electron (FESEM) microscopy images of obtained GO and GONS were captured by Zeiss, model Sigma 300, with a 2-3 kV voltage and working distance of 6-8 mm. The elemental

compositions of GO and GONS were acquired via XPS analysis. The XPS analysis was performed using ULVAC-PHI, Inc., Japan. The functional groups present in graphite, GO, and GONS were investigated using FTIR analysis (Shimadzu, Japan, IR Affinity model).

2.2. Results and discussion

2.2.1. Transmission electron microscopy

The morphological studies of obtained GO and GONS were done using TEM analysis. **Fig. 2.2** shows TEM images of GO and GONS samples obtained after dispersing in methanol for drop casting over the copper grid (325 mesh) and drying for overnight. The well-exfoliated GO nanosheets were obtained after centrifuging and freeze-drying. It has been found challenging to obtain GO nanosheets by ordinary drying compared to freeze drying due to tight stacking between sheets [4]. The freeze-dried GO sheets achieve wrinkles in their periphery, as shown in **Fig. 2.2 (a, b)**.

GONS were fabricated via low-frequency (LF) ultrasonication (20 kHz) of GO suspension in methanol. **Fig. 2.2c** signifies the initiation of nanoscroll formation at one end of the GO nanosheet. The concentration of GO suspension was chosen based on the Hansen solubility parameter, dipole movement of solvent, and ζ -potential. Initially, 0.1 g L^{-1} concentration of GO suspension in methanol was chosen for ultrasonication treatment; the resultant scrolling behavior is presented in **Fig. 2.2c**. The partial scrolling was observed as presented in the illustration. This was due to low sonication energy exposure at a higher concentration of GO suspension. However, this hurdle was overcome by preparing 0.05 g L^{-1} GO suspension for GONS formation (**Fig. 2.2d-i**). Also, 0.05 g L^{-1} GO suspension exhibits lower negative ζ -potential favoring GONS formation.

The TEM images (**Fig. 2.2**) demonstrated sequential growth in the scrolling process and structural morphology with variable lengths and diameters of GONS. The structural characteristics of formed GO scrolls were of spindle and cylindrical (tubular) hollow geometries. However, TEM images (**Fig. 2.2d-i**) reveal typical completely scrolled morphology formed after layer-by-layer rolling of flat GO sheets with open ends and more interlayer separation. This specific modification provides a more inner accessible area to atoms or molecules than the close-end structure of carbon nanotubes [3].

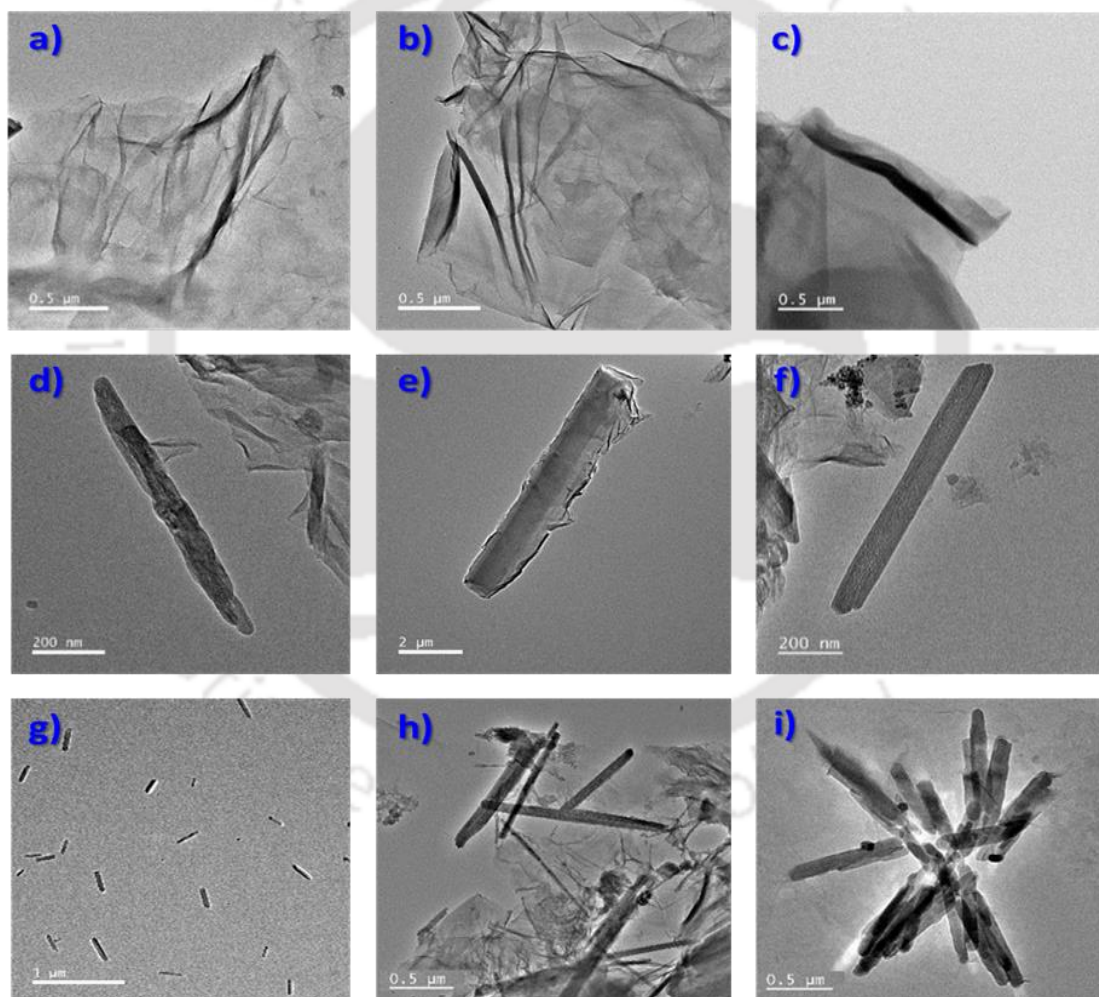


Fig. 2.2: TEM images for a stepwise transition of GO to GONS (a) GO sheets before sonication used for GONS preparation, (b) wrinkling of GO sheet indicates incomplete scrolling, (c) initiation of nanoscroll formation at one end of GO sheet (0.1 g L^{-1} suspensions), (d) fully scrolled GO sheets (GONS) with translucent surface signifies hollow nature between the layers (0.05 g L^{-1} suspension), (e, f) completely formed scrolls, (g, h, i) bunch of scrolls of different dimensions.

The active BET surface area of GONS obtained was around three times that of GO, as reported in **Table 2.1**. Also, it was observed that unlike GO nanosheets and carbon nanoscrolls, the scrolled GO (GONS) does not tend to aggregate/overlap. The confrontation of aggregation by GONS provides a significant advantage for applications like drug delivery, membrane fabrication, photocatalyst synthesis, and adsorption due to enhancement in an active surface area or sites [3,5–7]. The obtained GONS exhibit concentric geometries with several distinct outer walls, as shown in **Fig. 2.2(e, i)**.

Table 2.1: BET surface area of synthetic graphite, GO, and GONS.

Sample	BET surface area ($\text{m}^2 \text{g}^{-1}$)
Synthetic graphite	03.06
GO	39.78
GONS	118.36

2.2.2. FESEM analysis

The structural morphology of synthesized GO and GONS were studied via FESEM (**Fig. 2.3**). **Fig. 2.3a** shows FESEM images of exfoliated GO sheets synthesized from synthetic graphite prior to ultrasonication treatment for GONS fabrication. Here, the overlapping of GO nanosheets was observed, corresponding to the reduction in active surface area by hiding the inner and outer sides of overlying and underlying sheets, respectively, which is absent in the case of GONS (**Fig. 2.3b-f**). The FESEM images of GONS obtained at different sonication conditions are shown in (**Fig. 2.3b-f**). Herein, a spindle (tubular) shape open-ends morphology of GONS was observed, which was developed by sequential scrolling of GO nanosheets. As stated before, such an open-end morphology results in more accessibility of the inner wall area for numerous applications such as drug delivery and adsorption.

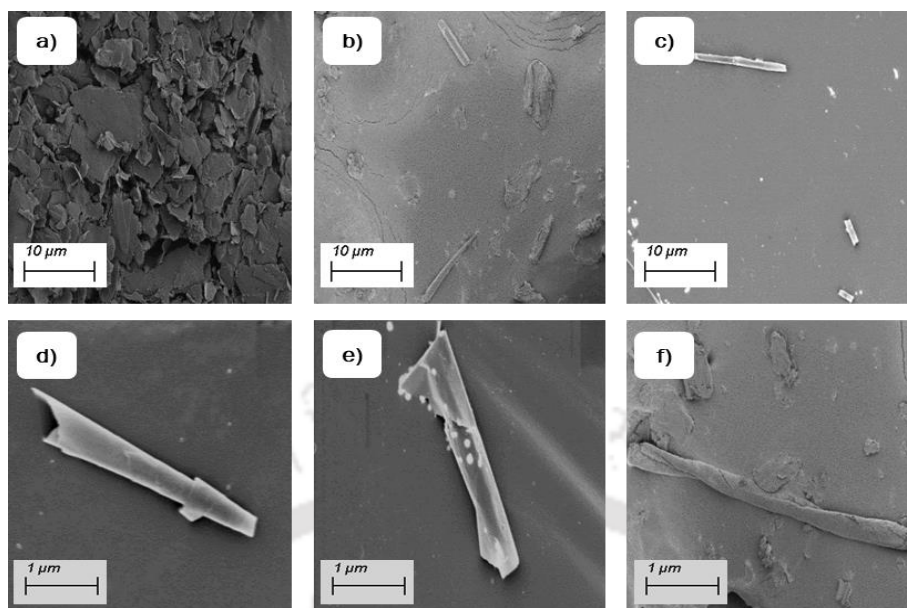


Fig. 2.3: FESEM image (a) GO synthesized by modified Hummers' method and GONS fabricated by the ultrasonication method at (b) 100W-0.5h, (c) 100W-1h (d) 150W-0.5h, (e-f) 150W-1h.

2.2.3. FTIR spectra

The surface chemistry related to functional groups present in obtained GO and GONS samples was studied by FTIR analysis using the KBr pellets method. **Fig. 2.4** demonstrates FTIR spectra for synthetic graphite, GO, and GONS. A reasonably broad and intense peak at 3430 cm^{-1} corresponds to a hydroxyl group of stretching vibrations, which may be formed due to absorbing water molecules or phenolic OH from carboxylic groups [8]. The minor peak at 2927 cm^{-1} indicates hydrogen-bonded OH of dimeric carboxylic acid (COOH) groups, also a smaller peak near 2857 cm^{-1} attributed to intramolecular bonded O-H stretching vibration of alcohols [2,8].

Three familiar peaks were observed at 1730 cm^{-1} and 1630 cm^{-1} to 1650 cm^{-1} before and after the oxidation of graphite, indicating C=O stretching of the carboxyl-carbonyl group and unchanged C=C bonding [8,9]. The peak near 1052 cm^{-1} attributed to the C-O-C bond indicates the epoxy group, and the presence of an oxide functional group with lower transmittance % signifies a high population of bonds in GO and GONS to that of synthetic

graphite [2,9]. As shown in **Fig. 2.4**, the formation of GONS does not affect any of the epoxy, carboxyl, and hydroxyl groups present in GO. Therefore, it can be stated that fabricated GONS exhibit similar chemical properties alike GO, and sonication does not affect functional groups present in GO. These findings advocate the application of GONS in place of GO in specific applications such as drug delivery, desalination, and adsorption.

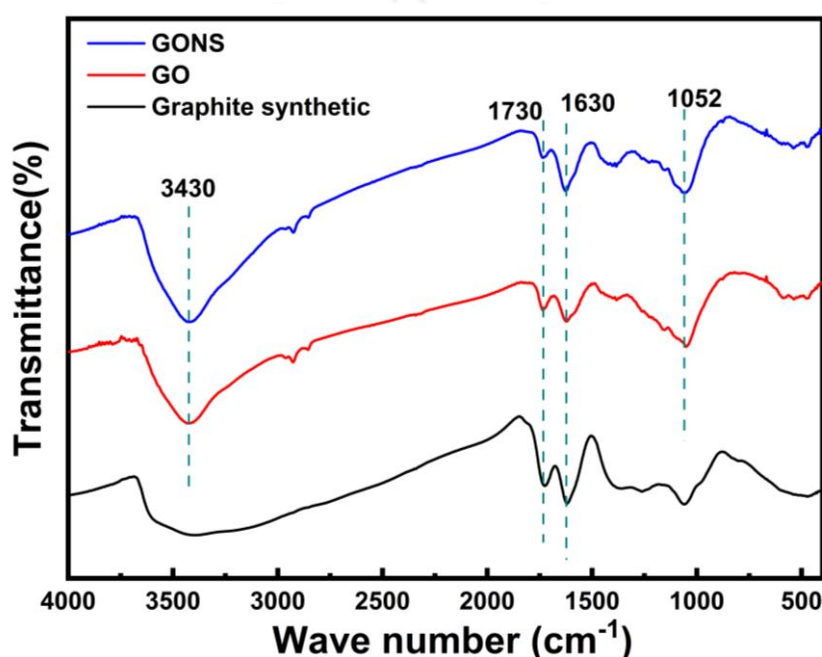


Fig. 2.4: FTIR spectra of graphite, GO, and GONS.

2.2.4. X-ray photoelectron spectroscopy (XPS)

The oxidation of graphite tends to the reduction in π - π bond interaction, which acts as a driving force for the exfoliation of graphite [10]. Oxidation of synthetic graphite was confirmed by FTIR and XPS analysis. **Fig. 2.5a** depicts the survey spectra of GO and GONS. The GO obtained by the modified Hummers' method resulted in a C/O atomic ratio of 1.98. It was found that during ultrasonication of GO suspension for GONS fabrication, some of the oxygen functional sites were removed, which caused a reduction in GO due to an upswing in the temperature of a suspension [11]. XPS analysis confirms the reduction in GO, where the C/O atomic ratio was increased from 1.98 to 2.25, which abets findings related to the lower oxygen content in GONS than GO.

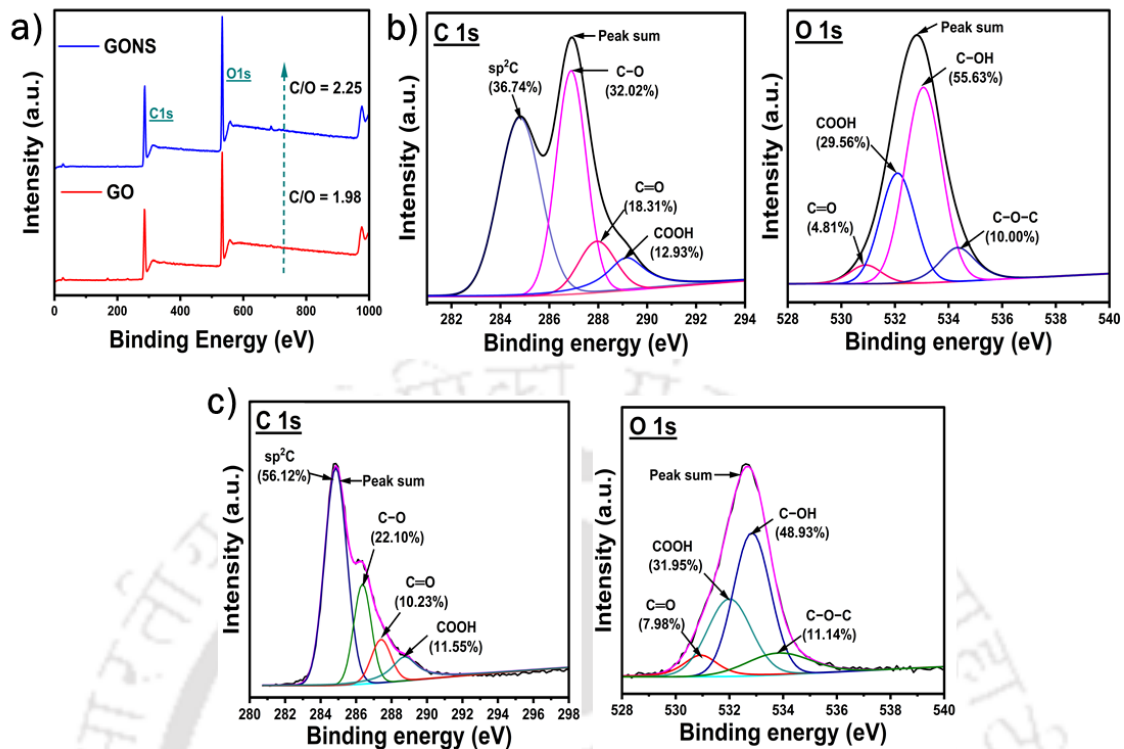


Fig. 2.5: (a) XPS survey spectra GO and GONS, High-resolution deconvoluted C1s and O1s spectra of (b) GO, and (c) GONS.

The deconvoluted C1s spectra of GO (**Fig. 2.5b**) lead to four major peaks at 284.8 eV, 286.6 eV, 287.6 eV and 288.9 eV, which are attributed to the sp^2 graphitic carbon, epoxy or hydroxyl groups (C-O/ C-OH), carbonyl group (C=O) in addition to the carboxyl (–COOH) functional entities present in GO, respectively [12,13]. In addition, the deconvoluted O1s spectra resulted in peaks centralized at 530.9 eV, 532.0 eV, 533.1 eV, and 534.4 eV are correspondingly ascribed to carbonyl (C=O), carboxylic (–COOH), hydroxyl (C–OH) and epoxide (C–O–C) functional groups in GO [13,14]. **Fig. 2.5c** illustrates a high-resolution deconvoluted spectrum of the C1s and O1s states of GONS, which have depicted identical functional groups to that of GO with a minor shift in the binding energies (0.1-0.2 eV), which may be due to the sonication treatment. Additionally, after GONS formation through ultrasonication treatment, the peak intensities of hydroxyl groups (C–OH), carbonyl group (C=O), and carboxyl groups (–COOH) in C1s spectra were significantly decreased, which

has been accompanied by increment in the peak intensity of sp^2 carbon. Such alterations in the peak area and intensity of GONS with respect to parent GO reveal the reduction of a large number of oxygenated groups while restoring the sp^2 graphitic network within GONS [15]. The FTIR study additionally confirmed the occurrence of listed oxygen-containing functional groups in GONS.

2.2.5. Atomic Force Microscopy (AFM)

The AFM analysis was carried out to investigate the GO sheet's depth and lateral dimensions. The produced GO sample was diluted to 1 ppm dispersion prior to AFM analysis. The suspension was drop cast over a silicon wafer and oven-dried at 60 °C overnight. The AFM image with an area of $2 \times 2 \mu m$ is shown in **Fig. 2.6a**. The height profile was obtained using WSxM 5.0 image analysis software (**Fig. 2.6b**). A profile defines the depth of a single GO flake in the range of 1.5-2 nm. As the single-layer GO sheets are just over 1 nm tall, so by measuring the depth of the flake, the number of layers stacked over one another was determined approximately [16]. Herein, **Fig. 2.6b** implies that the GO flake is of two GO sheets tall and subjected to further dilution of GO suspension. 3D imaging is a useful tool for topographical studies such as roughness parameters and height profiles. The figure depicted the height profile of a GO nanosheet. The average and RMS roughness parameters of graphene oxide were 0.4558 nm and 0.794 nm, respectively. The roughness parameters are the measure of the hydrophobic behavior of materials. Also, it was observed that most of the GO sheets were less than $2 \mu m$ wider. Also, the TEM image (**Fig. 2.2a**) confirms that GO samples were of single-layer nanosheets of 1-2 μm lateral size. The average particle size of graphene oxide nanosheets was found to be 450 nm; similar results are provided in the literature [17].

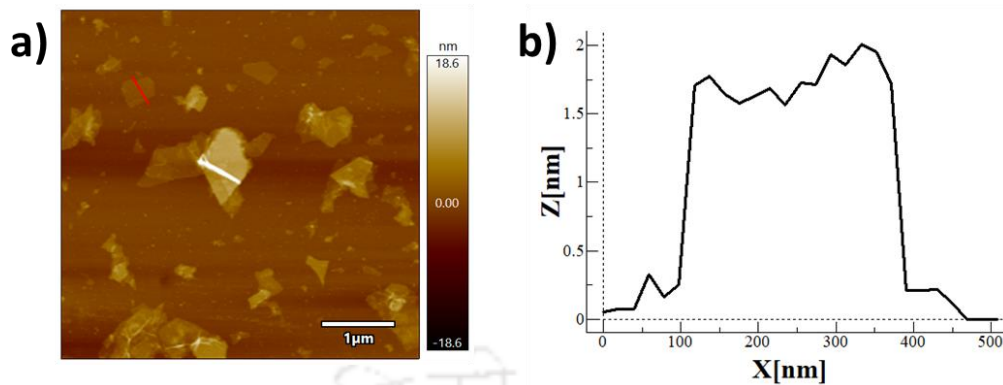


Fig. 2.6: AFM characterization of GO sheet (a) 2D AFM image, (b) Height profile.

2.2.6. Raman spectra

The best tool to analyze carbon material is Raman spectroscopy. The base material synthetic graphite, obtained GO and GONS samples were analyzed with an excitation wavelength of 633 nm for Raman analysis (**Fig. 2.7**). In the case of GO, two peaks were observed at 1589 cm^{-1} and 1335 cm^{-1} corresponds to G-band and D-band, respectively. G-band is termed as a graphitic hexagonal-pinch mode, which denotes sp^2 hybridized carbon atoms. D-band is a defect-related mode, which appears when defects exist and is attributed to the edges of the plane. Also, the 2D band was observed for graphite at 2672 cm^{-1} . In some cases, it is referred to as an overtone of the D-band. The shape, Raman shift, and intensity of the 2D band define the graphitic chemical structure as well as the number of graphite layers in the graphite nanostructure [18]. The oxidations of graphene sheets to obtain GO impart defects, which can be estimated by the I_D/I_G ratio. The intensity ratio for synthetic graphite was found to be 0.45 and enhanced to 1.14 in the case of GO. As provided in the literature, such increment is inversely proportional to the average size of the sp^2 hybridized GO [17,19]. The increase in the intensity of the D-band may cause by stress annealing of graphite, with an increase in 'unorganized' carbon in a sample, which reduces the average size of the sp^2 hybridized GO and increases in defect concentration of graphitic lattice structure [19]. The increase in defect intensity results in an increment in the intensity ratio.

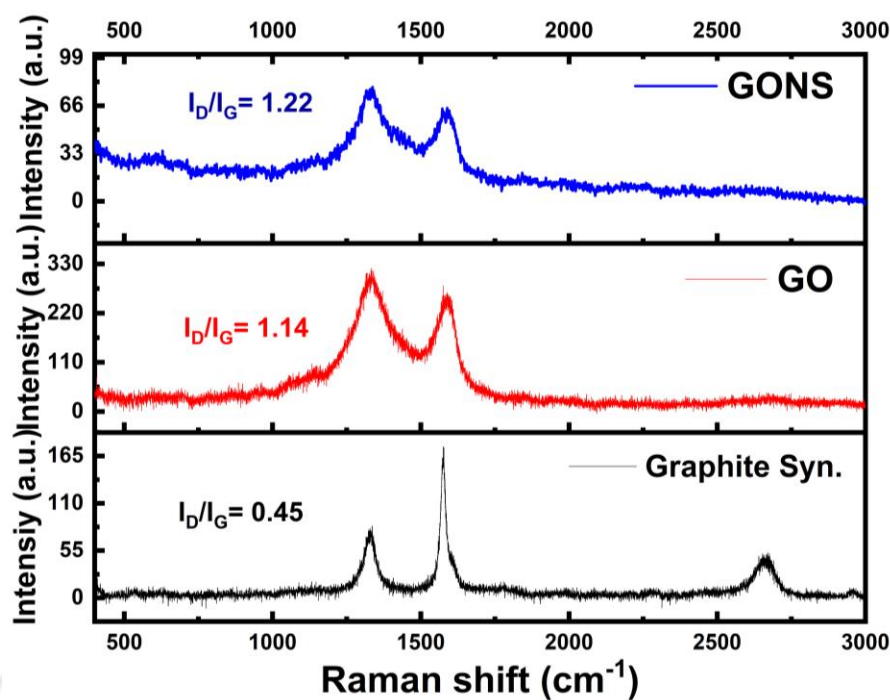


Fig. 2.7: Raman spectra of graphite (synthetic), GO, and GONS formed in methanol suspension.

In the case of GONS, the peak position of the D-band (1335 cm^{-1}) was found to be similar to GO with lower intensity. While the peak position of the G-band (1578 cm^{-1}) was shifted $\sim 11\text{ cm}^{-1}$ lower, suggesting an inferior degree of hybridization. The intensity ratio observation provides vital information for GO and GONS. The I_D/I_G ratio for GONS was 1.22, which is significantly higher than GO (1.14) and represents more defect formation over the surface of GONS. The difference in I_D/I_G advocated a partial reduction in GO due to the removal of a few oxygen functional groups by sonication, resulting in more π - π bond interaction [20].

In order to quantify the relative defect density of GONS and GO, obtained Raman spectra were fit using Gaussian distribution ($R^2 > 0.96$) shown in **Fig. 2.8**. The fits for D-band show that the Full-width half maximum (FWHM) remains nearly constant for both GO and GONS with $228 \pm 8\text{ cm}^{-1}$ and $227 \pm 6\text{ cm}^{-1}$, respectively. Whereas for G-band, the FWHM of GONS has increased from 99 ± 2 to $109 \pm 3\text{ cm}^{-1}$ to that for GO. Also, the area ratio A_D/A_G for GO ~ 1.81 has decreased to 1.68 after GONS formation.

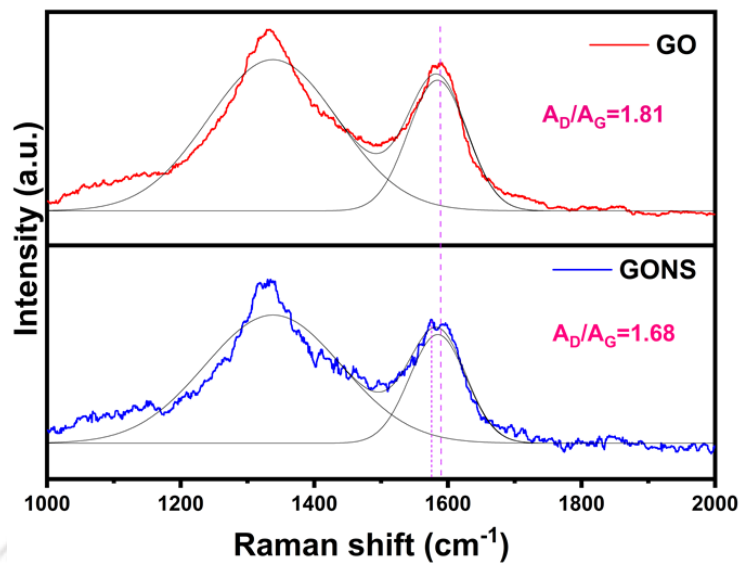


Fig. 2.8: Raman spectra (Gaussian fitted) of GO and GONS.

The above verdicts related to FWHM and area ratio suggest that GO facilitates the GONS formation with fewer oxygen groups, which is consistent with a reduced disorder with graphitic materials, resulting in the broadening of the G-band. [3,20]. The reduction was previously confirmed by XPS analysis. The decrement in the A_D/A_G ratio is observed due to the broader G-band, while the broader G-band in GONS appears because of an increase in elastic strain by the multilayer morphology of GONS. The increment in elastic strain was resulted from the sonication effect on its basal plane [21].

2.2.7. XRD analysis

X-ray diffraction technique plays an essential role in investigating crystal structure, phase identification, and qualitative analysis of both crystalline -amorphous materials. A qualitative- analysis of GO synthesized from synthetic graphite and GONS was done using the powder XRD technique. **Fig. 2.9** shows the XRD spectra of synthetic graphite, GO obtained by modified Hummers' method, and GONS fabricated by ultrasonication of GO suspension. In **Fig. 2.9**, a clear diffraction peak at 26.6° was observed, which corresponds to graphitic nature synthetic graphite with stronger van der Waals interaction. The oxidation of

graphite by Hummers' method weakens these interactions and forms fully exfoliated GO sheets.

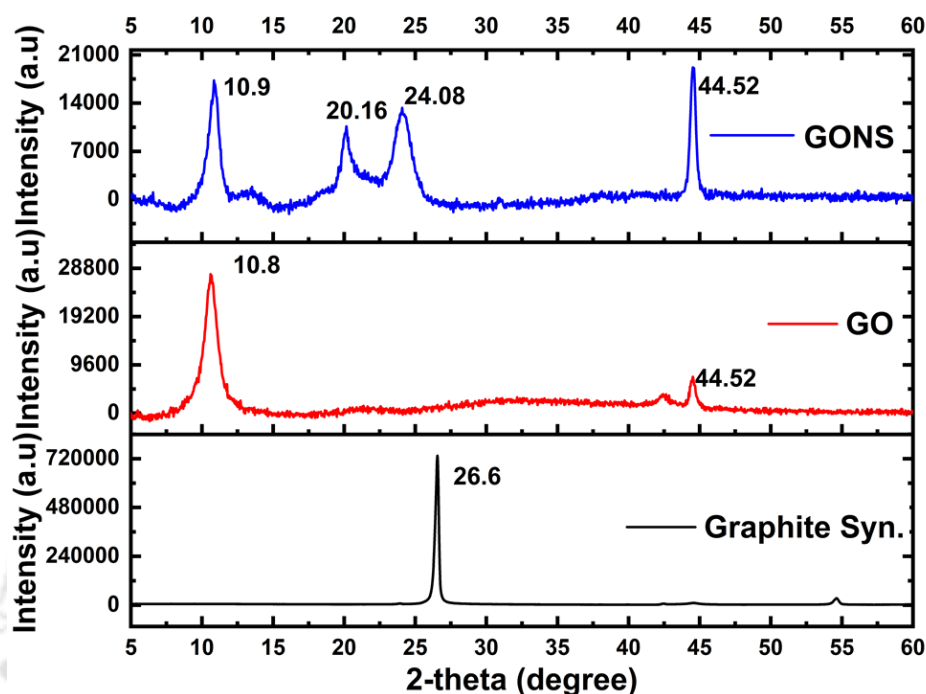


Fig. 2.9: XRD spectra of graphite, GO, and GONS.

After the oxidation of graphite, it can be observed that the graphitic peak at 26.6° has completely vanished, and a new peak formed at 10.8° , which corresponds to GO. A single diffraction peak at 10.8° (2θ) attributed to the (0 0 1) plane confirms the complete exfoliation of graphite with a larger interlayer spacing of 0.8254 nm; similar kinds of results were provided in the literature [22,23]. The increase in the d-spacing of GO was observed may be due to the inclusion of hydrophilic functional groups and other structural defects. The average crystal size of GO was found to be 8.63 nm. However, a GONS spectrum shows several other break peaks, including the GO peak at 10.9° . The peak position at 20.16° with a d-spacing of 0.4397 nm attributed to the (0 0 2) plane signifies an inevitable reduction in GO during GONS formation, as confirmed before by XPS analysis. Other broader peak at 24.08° with higher intensities, d-spacing 0.3694 nm and 44.52° with d-spacing 0.2050 nm indicates much closer packing of GONS, which helps to differentiate between GO and GONS for future

reference [3]. An increase in the intensity of the peak at 44.52° indicates more order, crystallization, and arrangement in the lattice of GONS. The average crystal size for GO and GONS is found to be 8.63 nm and 8.11 nm, respectively.

Overall, the above observations and characterizations have indicated the successful fabrication of quality GONS using inexpensive synthetic-grade graphite. The synthesized GONS exhibited similar characteristics as of GO with the more inner accessible area.

2.2.8. Factors affecting GONS formation

In order to curl/scroll GO sheets, the minimum energy barrier (scrolling activation energy) should be overcome. The scrolling activation energy for GNS formation was reported as 100–1000s J mol⁻¹ [3]. The ultrasonic pressure wave in ultrasonication imparts unsteady bubble cavitation, including nucleation, growth, and collapsation of microscopic bubbles. The energy provided by bubble cavitation has substantially been utilized by GO sheets to circumvent scrolling activation energy for the fabrication of GONS. It was also found that sonication parameters and solvent characteristics affect the scrolling process. Those are discussed in subsequent sections.

2.2.8.1. Effect of sonication power on GONS dimensions

Herein, the effect of ultrasonication power delivered by a sonicator (150, 100 W) on the time-dependent length and diameter of GONS was investigated. **Fig. 2.10** shows the effect of sonication power on the length of GONS with sonication time. It was observed that the average length of obtained GONS decreased with sonication time for both 150 W and 100 W ultrasonication power. These findings allow us for GONS fabrication with tunable dimensions as per the requirements. Herein, the length of obtained GONS decreases from 1311 nm to 1011 nm and 1165 nm to 516 nm for 100 W–150 W sonication power, respectively, with an increase in irradiation time from 0.5 h to 3 h during ultrasonication. The

dimensions of GONS were reported as the average length of scrolls and calculated using ImageJ software [24].

In this study, ultrasound energy has been utilized to overcome the energy barrier (activation energy) for the scrolling of GO nanosheets. The reduction of length and formation of GONS depends on the power and duration of sonication. It is because of the cleavage of oxygen defect sites and cavitation-mediated scission [22,23]. Also, the LF ultrasonication is harsher as the cavitation energy released during transient adiabatic bubble collapse is proportional to R^3_{max} . Where, R_{max} is the maximum bubble radius, which is inversely proportional to the frequency of ultrasonication that is responsible for excess energy dissipation [3]. The length and diameter of GONS for a typical condition (0.05 g L^{-1} , 150 W , 1 h) are shown in **Fig. 2.11** for 8 scrolls. It may be seen from the figure that the lengths and diameters of scrolls vary from $390\text{--}1607 \text{ nm}$ and $31\text{--}134 \text{ nm}$, respectively. It was also found that the effect of sonication parameters over the diameter of nanoscrolls was negligible and did not follow any specific trend.

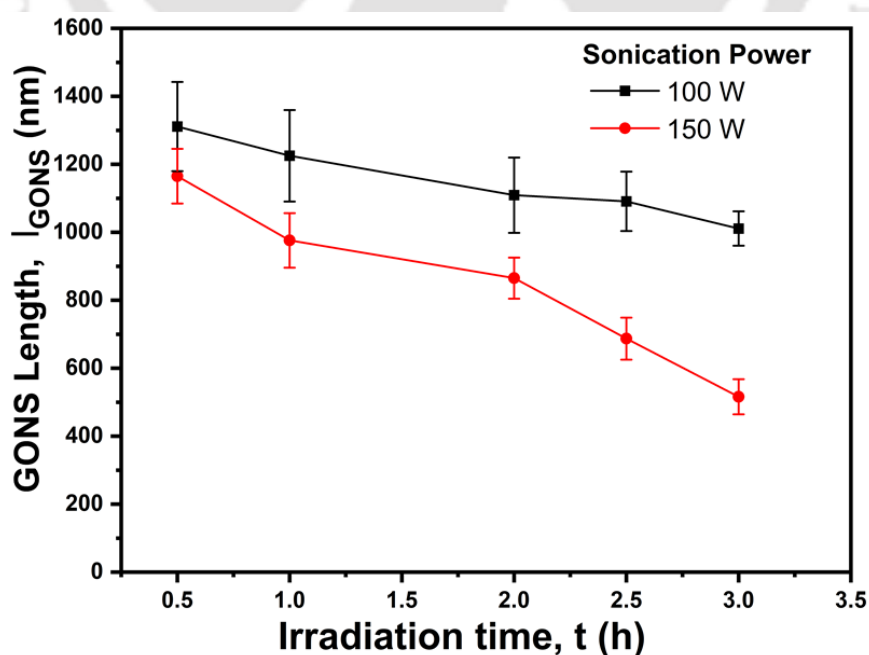


Fig. 2.10: Effect of sonication power on GONS length as a function of irradiation time (GO suspension concentration in methanol: 0.05 g L^{-1}) [The results are represented as mean $\pm$ SD (N=3)].

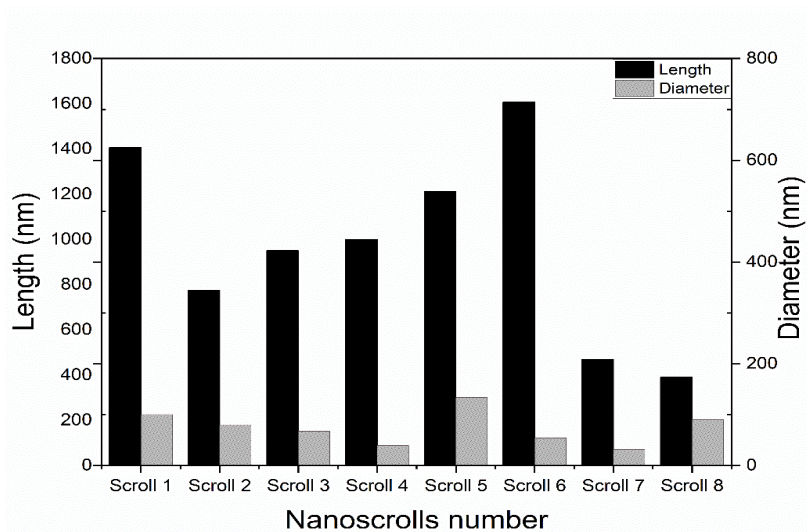


Fig. 2.11: Length and diameter of GONS obtained by ultrasonication (0.05 g L^{-1} , 150 W, 1h).

2.2.8.2. Solvent selection for GONS formation

The formation of GONS is closely related to the interaction of solvent with GO nanosheets and its suspension stability. The suspension stability of the solvent is associated with the Hansen solubility parameter, ζ -potential, and dipole movement [4]. Hence the selection of solvent becomes an essential factor during GONS fabrication. It has been observed that UV-vis absorbance of suspension is proportional to 2-D GO sheets because of the higher interfacial area exposed to solvent and lower density. The sedimentation rates are lower for stable suspension of GO in different aprotic and protic polar solvents like methanol, ethanol, IPA, pyridine, NMP, and DMF [4,25,26]. The solvent characteristics are listed in **Table 2.2**.

Table 2.2: Characteristics of organic solvent.

Solvent	Dipole movement (D) [4]	ζ potential (mV)	Hansen solubility parameter ($\text{MPa}^{0.5}$) [4]
Methanol	1.67	-20.90	34.6
Isopropyl alcohol	1.68	-29.30	22.5
Ethanol	1.68	-36.20	28.2
Ethylene glycol	2.20	-48.60	37.0
Pyridine	2.23	-60.00	14.7
DMF	3.82	-41.50	25.0

Previous studies show that protic polar solvents with Hansen solubility parameter >32 MPa^{0.5}, dipole movement > 1.8 D (Debye, $D= 1 \times 10^{-18}$ coulomb-meter) and aprotic polar solvents with dipole movement >3.82 D possesses more dispersion stability with lower suspension rate. Also, it was found that well-suspended GO nanosheets were unable to curl up to form nanoscrolls, and with an increase in dipole movement with the Hansen solubility parameter, the average length and yield of GONS declined, respectively [4]. Although the polar protic solvent has a similar dipole movement, the average lengths of GONS formed have dissimilarity, which depends on the size of GO sheets. Here, it was found that ζ potential of solvent plays a vital role. The increase in the absolute value of ζ potential increases the electric interaction of solvent with GO nanosheets. The electric interactions correspondingly electrophoretic mobility of GO affect the scrolling tendency by increasing van der Waals interaction [27,28]. Therefore, only relatively small-sized GO nanosheets can form GONS as the scrolling energy barrier is lower [4]. The GO suspensions of 0.05 g L^{-1} were made in different solvents for ζ -potential analysis; the results are reported in **Table 2.2**. The lower value of ζ -potential causes instability in suspension, which favors the scrolling process. Methanol as a protic solvent has Hansen solubility parameter $34.6 \text{ MPa}^{0.5}$, dipole movement 1.67 D , and potential -20.9 mV . From the listed solvents, methanol exhibits the lowest dipole movement and lowest $-ve$ ζ -potential, resulting in lower stability in suspension. As discussed above, the mentioned aspects and their cost and availability advocate methanol as the most suitable solvent for GONS fabrication. The comparison of work related to GONS preparation with the present study is reported in **Table 2.3**.

Table 2.3. Comparison of prepared GONS with the reported literature.

	Amadai et al. [3]	Fan et al. [29]	Alharbi et al.[20]	Present work
Base material	High-grade natural graphite	High-grade natural graphite	High-grade natural graphite	Synthetic graphite
Method	LF-HF Ultrasonication	Lyophilization	Vortex Fluidic Device (VFD)	LF- Ultrasonication
Solvent	Ethanol	Water	Water and 2-Propanol	Methanol
Factor affecting GONS dimension	Ultrasonic frequency and power density	-	-	Ultrasonic power density and solvent selection study
Characterization	FESEM, TEM, Raman, XPS, and AFM.	FESEM, TEM, Raman, XRD, TGA and Capacitance	FESEM, TEM, Raman, XRD, TGA and XPS	FESEM, XPS, TEM, Raman, XRD, BET surface area, FTIR, AFM, and particle size analysis for GO.
GONS dimensions	Length -3-5 μm Dia.-0.193-1.6 μm .	-	-	Length-0.516-1.311 μm . Dia.-0.044-0.155 μm .
Applications	-	Super-capacitor	-	Drug delivery

Summary

The fabrication of 1D GONS of tunable dimension was achieved from GO using the LF-ultrasonication technique. The structure and morphology of obtained GO and GONS were further verified using electron microscopy, Raman spectroscopy, and XRD analysis. In addition, the functional groups present in GO and GONS were investigated through FTIR and XPS analysis. It was found that the size of the nanoscrolls can be controlled by selecting suitable ultrasonication parameters, as with an increase in the sonication power and time, the length of the scrolls decreases. GONS exhibited identical functional groups to that of GO with enhanced surface morphology and characteristics suggesting its potential utilization in drug delivery-related applications.

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Chapter 3:

Development of graphene oxide nanoscrolls imparted nano-delivery system for the sustained release of gallic acid

Chapter 3

Development of graphene oxide nanoscrolls imparted nano-delivery system for the sustained release of gallic acid

This chapter elucidates the application of 1D GONS fabricated via ultrasonication treatment for sustained delivery of a natural chemotherapeutic agent, gallic acid (GA). The GA was loaded over the GONS to obtain a GONS-GA nanocomposite of different compositions at variable temperatures and pH of the media. The structural and functional characteristics of obtained GONS and GONS-GA nanocomposite were investigated via FTIR, XRD, Raman spectroscopy, XPS, and zeta-potential analysis. Also, the morphology of GONS and GONS-GA nanocomposites was analyzed using FETEM and FESEM. The GA loading behavior of GONS at different pH conditions has been studied using entrapment efficiency and loading capacity determination. Furthermore, a thermodynamics approach to the loading behavior of GONS nanocarriers was established, along with the kinetic study on the release behavior of GA. In addition, the storage stability and in-vitro cytotoxicity assessments of the GONS-GA nanocomposite were performed. At last, a detailed comparison of GA-based DDS with the reported literature is provided.

3.1. Experimental

3.1.1. Materials

The details of preparatory materials for the synthesis of GO and GONS are provided in **section 2.1.1**. Sodium hydroxide pellets (NaOH) (98%) and Hydrochloric acid (HCl) (37%) were supplied by Merck Life Science Private Limited, India. Gallic acid pure ($C_7H_6O_5H_2O$) (98%) was purchased from SRL Private Limited. Deionized (DI) and Milli-Q water was taken from Elix-3 M/s Millipore (USA) Milli-Q water purification system.

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3.1.2. Fabrication of graphene oxide nanoscrolls (GONS)

The GO was produced using the modified Hummers' method via oxidation of synthetic graphite and employed as a starting material for GONS fabrication; the detailed procedure is reported in **section 2.1.2** [1]. Furthermore, an identical procedure was adopted for GONS fabrication via ultrasonication with GO suspension (0.025 gL^{-1}) and fixed sonication parameters (**Fig. 3.1**); the details are provided in **section 2.1.3**. Later, the sonicated suspension was vacuum-filtered and dried in the oven at 50°C . Now, the obtained GONS was further stored in an air-tight vial and subjected to characterizations and GA loading experimentation.

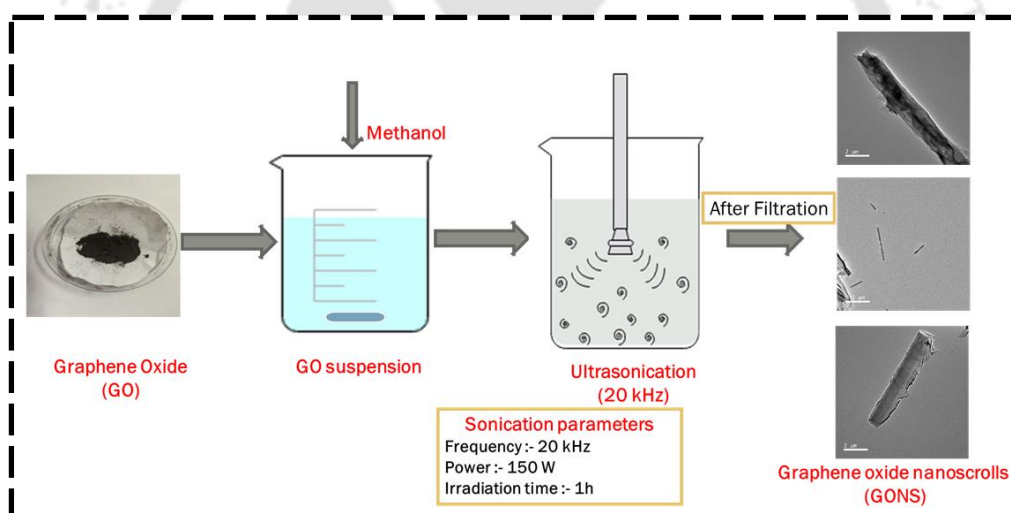


Fig. 3.1: Ultrasound-mediated fabrication of graphene oxide nanoscrolls (GONS).

3.1.3. Preparation of GONS-GA nanocomposites

A 50 mL aqueous dispersion of pure gallic acid (GA) and GONS was prepared using Milli-Q water. The amount of GONS and GA were taken in the diverse mass ratio of GONS:GA (1:0.25, 1:0.5, 1:0.75, 1:1, 1:1.25, and 1:1.50). The dispersions were well mixed by bath sonication for 10 min. Thereafter, the pH of the solution was attuned for different values, such as 4.7, 7, and 9, using 0.5 M NaOH solution to investigate the effect of the pH of the media on the drug loading efficiencies. Further, the dispersions were stirred (200 rpm) for 16

h at three different steady temperatures of 25 °C, 30 °C, and 35 °C under dark conditions in an incubator shaker (Daihan Labtech, LSI 3016R model). The slurry was taken out of the shaker, subjected to high-speed centrifugation (10000 rpm, 20 min.), and washed away with Milli-Q water. After centrifugation, the obtained GONS-GA nanocomposite was dried overnight in a vacuum oven at 40 °C. The schematic presentation of GA loading in GONS is shown in **Fig. 3.2**.

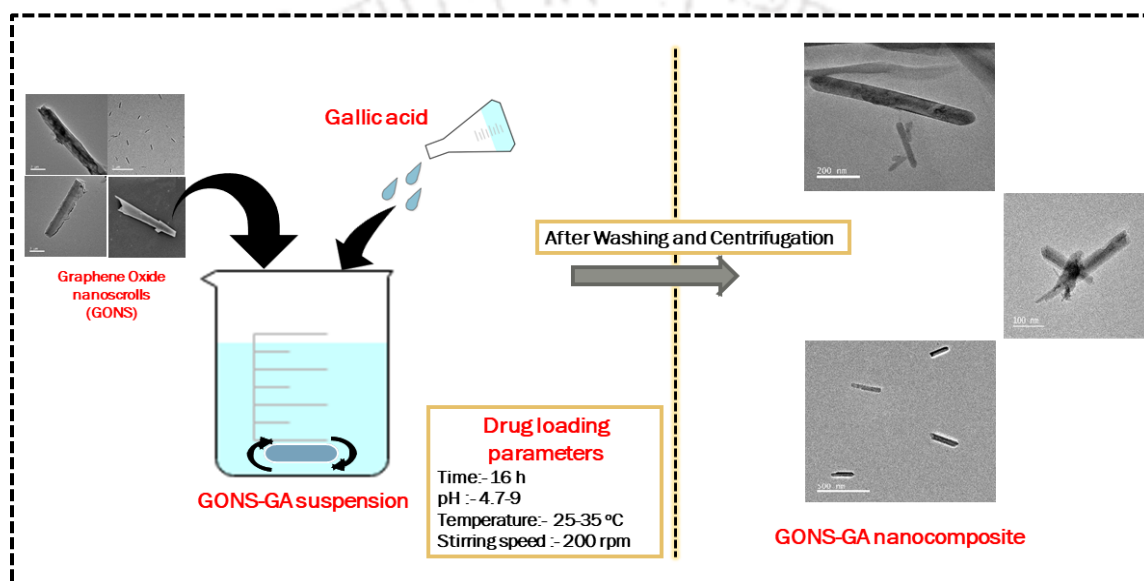


Fig. 3.2: Schematic illustration of GA loading on GONS.

3.1.4. Evaluation of entrapment efficiency and loading efficiency

The concentration calibration curve of GA has been plotted through UV-Vis spectroscopy at $\lambda_{\max} = 265$ nm. A concentration of GA supernatant remaining after loading of GA on GONS was evaluated via UV absorbance. Now, the entrapment efficiency (% EE) and drug loading capacity (% LE) of GONS were calculated using the following equations [2].

$$\text{Entrapment Efficiency (\%EE)} = \frac{W_1 - W_2}{W_1} \times 100 \quad (3.1)$$

$$\text{Loading Efficiency (\%LE)} = \frac{W_1 - W_2}{W_3} \times 100 \quad (3.2)$$

Where W_1 is the initial weight of GA; W_2 is the weight of unloaded GA in the supernatant; W_3 is the total weight of GONS-GA.

3.1.5. *in-vitro* drug release process

The *in-vitro* drug release behavior of the obtained GONS-GA nanocomposite was done using a phosphate buffer solution (PBS) of pH 7.4. Several studies have reported that the anions HPO_4^{2-} and H_2PO_4^- influence the drug release rate [2–4]. The sustainable release behavior of GA from GONS-GA nanocomposite was determined by adding 70 mg nanocomposite to 50 mL of PBS solution. The prepared PBS nanocomposite solution was further placed in an incubator shaker at 37 °C for 5 days. The amount of GA released out of GONS-GA nanocomposite was determined by collecting the aliquots at a regular interval (15 min.) and subsequent centrifugation for 15 min. at 10000 rpm and measuring the absorbance of the supernatant.

3.1.6. *in-vitro* cytotoxicity study

The cytocompatibility analysis of the synthesized nanocarriers was performed using the MTT assay (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) against human embryonic cells HEK293 and epithelial carcinoma cells A549 as normal and cancer cell lines, respectively [5]. The cell lines were cultured in Dulbecco's modified eagle medium (high glucose) supplemented with 10% fetal bovine serum and 1% antibiotics solution and incubated in a CO₂ incubator at 37 °C and 85% moisture. During the experiments, 1×10^4 cells were seeded in the 96 well plates along with the different concentrations of the free gallic acid (GA), unloaded nanocarrier GONS, and the gallic acid loaded GONS-GA nanocomposite. The concentrations of the samples varied in the range of 10 to 200 $\mu\text{g mL}^{-1}$. After the incubation period of 48 h, the wells were added with fresh media and 20 μL of MTT reagent (5 mg mL^{-1}), followed by further incubation for 4 h in a CO₂ incubator. Then, the media was carefully removed, followed by the dissolution of the precipitated formazan crystals using dimethyl sulfoxide. The absorbance of the plates was recorded using the

multiplate reader (Infinite 200 Pro, Tecan) at 570 nm. The cells incubated without materials were considered as the positive control, and cell viability was calculated using the following equation [6].

$$\text{Cell viability}(\%) = \frac{\text{Sample absorbance}}{\text{Control absorbance}} \times 100 \quad (3.3)$$

3.1.7. Characterization techniques

The structural and morphological characterizations of starting materials, such as GA, along with synthesized GO, GONS, and GONS-GA nanocomposites, were done by FETEM, FESEM, FTIR, XPS, and XRD). A FETEM (JEOL, 2100F) of the electron beam 100 kV was used to examine the single-layer GO nanosheet, along with the scrolled nanoscrolls and GONS-GA nanocomposite. FTIR (Shimadzu, Japan, IR Affinity model) and XPS (ULVAC-PHI, Inc.) analysis were conducted to confirm the existence of functional groups within GO, GONS, and GONS-GA nanocomposite, respectively. The XRD diffraction spectra of specified materials were established via Bruker (Netherlands) D8 advance diffractometer with Cu K α radiation (1.5406 Å) at 40 kV. The FESEM images of GONS and GONS-GA nanocomposite were obtained by Zeiss, model Sigma 300. Also, the XPS analysis (ULVAC-PHI, Inc., Japan) was performed to investigate the elemental composition of obtained GO, GONS, and GONS-GA nanocomposite. UV-Vis spectrophotometer was used for concentration calibration of gallic acid (GA) at 265 nm (Thermofisher Scientific Orion Aquamate 8000). The Raman spectrums were recorded to determine the oxidation state and reduction behavior of GONS with the help of Horiba Jobin Vyon LabRam HR model with an excitation wavelength of 488 nm at room temperature.

3.2. Results and discussion

3.2.1. Surface morphology of GONS and GONS-GA nanocomposite

The surface morphologies of GO, GONS, and GONS-GA nanocomposites obtained after loading natural chemotherapeutic agent GA were investigated using TEM and FESEM analysis. The TEM images of GO, GONS, and GONS-GA nanocomposites are shown in **Fig. 3.3A**. The morphological attributes of GO and GONS are described in **section 2.2.1**. The fabricated GONS have shown a great de-agglomeration tendency, as shown in **Fig. 3.3A (iii)**, which overcomes the conflict of GO applications as the agglomeration tends to reduce in active surface area for mass transfer. Previously, it was observed that the sonication parameters rightly affect the GONS dimensions (length) [1]. Therefore, in the present work, the sonication parameters were kept constant throughout the GONS fabrication experiments, as mentioned in the previous section. The fabricated GONS were subjected to GA loading. These GA-imparted nanoscrolls can be visualized in TEM images. The obtained GONS-GA nanoscrolls have multilayer geometry, as well as the hollow and porous structure that can be observed in **Fig. 3.3A (iv-vi)**. The resultant GONS-GA nanoscrolls depicted a de-agglomeration tendency similar to that of GONS. Herein, the average length of the scrolls provided in **Fig. 3.3A (iii-iv)** is $\sim 250 \pm 14.1$ nm (Estimated by ImageJ software) for both the pristine and GA-loaded nanocarrier.

The FESEM images of GO, GONS, and GONS-GA nanocomposite are shown in **Fig. 3.3B**. Here, exfoliated GO nanosheets synthesized via the modified Hummers' method can be observed before sonication treatment. Previously, such agglomeration behavior was observed for GO nanosheets, which is absent in the case of GONS [1,7]; a similar de-agglomeration trend was observed for GONS and GONS-GA nanocomposites.

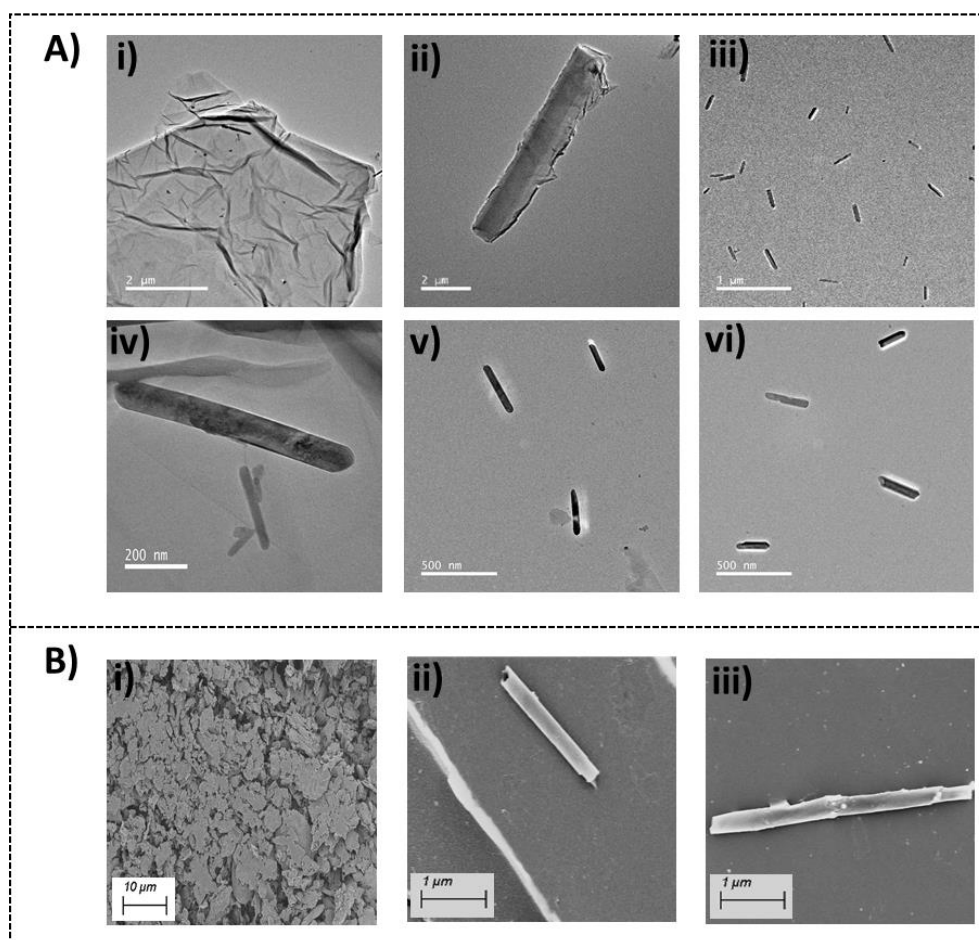


Fig. 3.3: (A) TEM images of (i) GO nanosheets, (ii) fabricated GONS via sonication treatment, (iii) a bunch of de-agglomerated GONS, (iv, v, vi) GONS-GA nanocomposites. The average length of the scrolls = 246.96 ± 3.3 nm. (B) FESEM images of (i) GO nanosheets, (ii) GONS, and (iii) GONS-GA nanocomposite.

3.2.2. Chemical interaction study

3.2.2.1. FTIR spectroscopy

The chemical interaction study of the obtained GONS-GA nanocomposite was investigated by FTIR spectroscopic analysis. Also, the evidence related to the reduction behavior of the GONS and nanocomposite was perceived with XPS analysis. The FTIR spectrum shown in **Fig. 3.4** provides the concluding remarks on the successful doping of GA over GONS, as the obtained nanocomposites include the functional groups of both GA and GONS. The discussion over functional groups of GO and GONS was explored in the previous chapter (**section 2.2.3**) related to the fabrication of ultrasound-mediated tunable GONS.

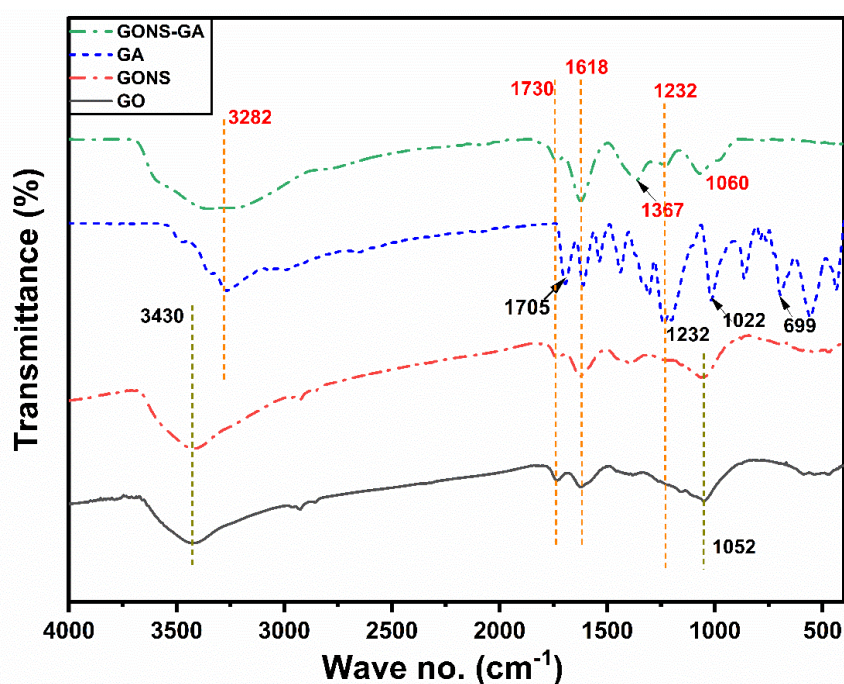


Fig. 3.4: FTIR spectrum of GO, GONS, GA and GONS-GA nanocomposite.

Here, in **Fig. 3.4**, the common peak position at 1618 cm^{-1} is designated as stretching vibrations of C=C bonds. For pure GA, the peak at 3282 cm^{-1} is attributed to acidic O-H stretching, and 1705 cm^{-1} confirmed the existence of the phenolic groups. The peak position at 1232 , 1022 , and 699 cm^{-1} confirms the existence of carboxylic, carbonyl, and benzene rings functional groups, respectively. In the case of GONS-GA nanocomposite, the peak at 3282 and 1232 cm^{-1} of the O-H functional group from the phenolic, carboxylic group of GA, along with the peak position at 1730 and 1618 cm^{-1} from GONS, still exist after composite formation. However, a slight deviation in the peak position of epoxy groups of GONS was observed in the nanocomposite, which suggests the efficient impregnation of GA [2,3]. Herein, the IR spectrum of GONS-GA nanocomposite represents the characteristic peaks for both GONS and GA that suggest successful nanocomposite formation.

3.2.2.2. XPS spectroscopy

Figure 3.5 illustrates the XPS spectra for pristine and GA-loaded GONS nanocarrier. **Fig. 3.5a** depicted the existence of C and O elements within the GONS and GONS-GA

nanocarrier. **Fig. 3.5b** describes a high-resolution deconvoluted spectrum of the C1s and O1s states of GONS. The deconvoluted C1s spectra of GONS bring in four significant peaks of 284.8 eV, 286.4 eV, 287.6 eV, and 288.8 eV, indicating the sp^2 or graphitic carbon, C–O mount in the hydroxyl or epoxy groups, carbonyl units (C=O), together with to the carboxyl units (–COOH) exist in the GONS, correspondingly. Also, four substantial peaks around 530.8 eV, 531.9 eV, 532.8 eV, and 533.9 eV are acquired by the O1s spectrum of GONS. The stated distinctive peaks correspond to the various oxygenated units like –COOH (carboxylic)/C=O (carbonyl) and the C–OH (hydroxyl)/C–O (epoxy) present in the GONS [8,9]. The FTIR study additionally confirmed the occurrence of listed oxygen-containing functional groups in GONS. In the case of GONS-GA nanocomposite (**Fig. 3.5c**), identical functional groups were observed to that of GONS with a minor positive shift in the binding energies (0.2-0.3 eV), which may be due to the stronger interaction among GONS and GA.

Additionally, after the loading of GA to obtain GONS-GA nanocomposite, the peak areas and intensities of epoxy groups (C–O) and carboxyl groups (–COOH) in C1s spectra were significantly decreased, which has been accompanied by increment in the peak area and intensity of sp^2 carbon. Such alterations in the peak area and intensity of the nanocomposite reveal the reduction of a large number of oxygenated groups while restoring the sp^2 graphitic network within GONS-GA [10]. The FTIR study additionally confirmed the occurrence of listed oxygen-containing functional groups in the GONS and GONS-GA nanocomposite. Furthermore, the XPS analysis confirms a slight reduction in the GO during the formation of GONS via sonication, as the C/O atomic ratio was enhanced from 1.98 to 2.25 and supports the findings related to the lower oxygen content in the GONS than GO. Meanwhile, an impregnation of antioxidative GA over GONS during nanocomposite formation also further improves the C/O atomic ratio by reducing in the oxygenated sites of GONS. It was found

that the C/O ratio of GONS was augmented from 2.25 to 3.19 for the GONS-GA nanocomposite. Here, the elemental analysis of nanocomposite advocates the antioxidant behavior of GA and the successful deposition of GA over the GONS. The elemental composition of GO, GONS, and GONS-GA nanocomposite is reported in **Table 3.1**. Further, the reduction of GONS during the nanocomposite formation is also validated via Raman spectroscopy and X-Ray Diffraction (XRD) analysis.

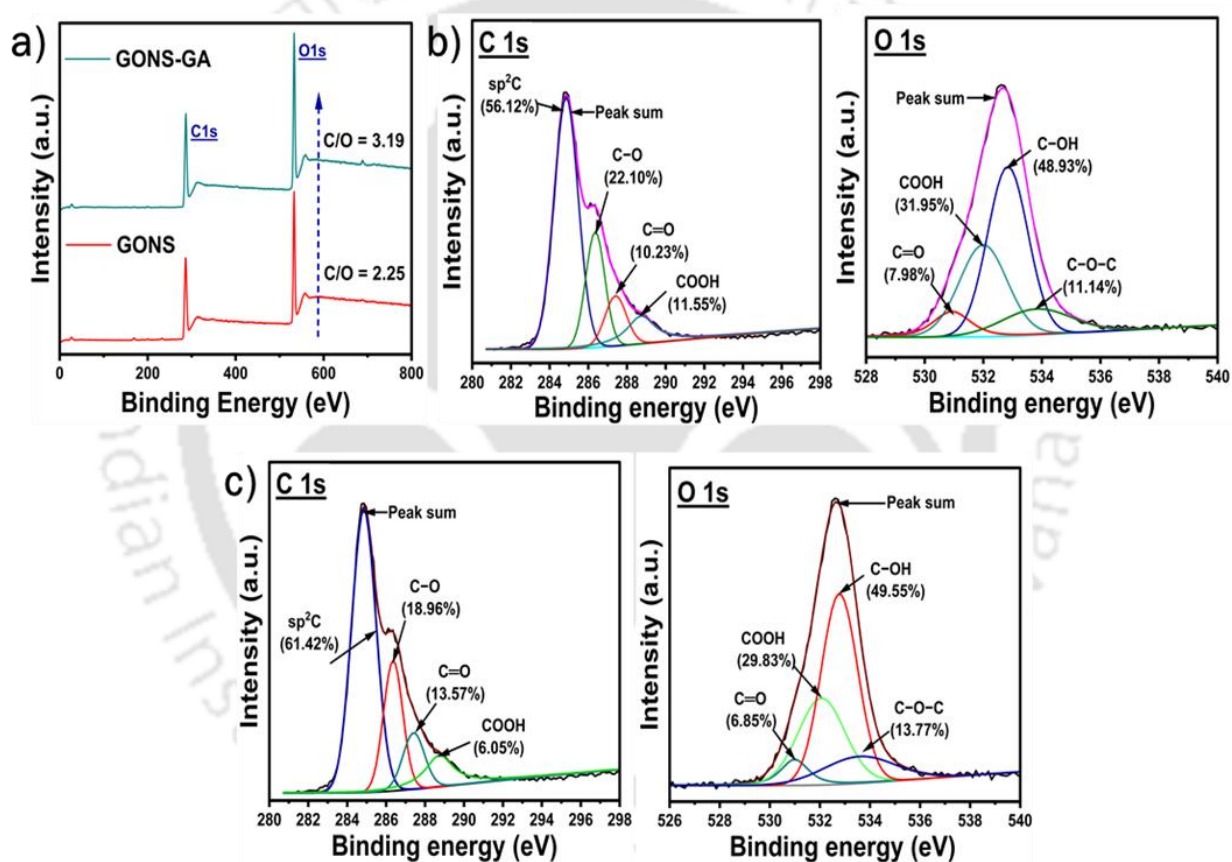


Fig. 3.5: (a) XPS survey spectra GONS and GONS-GA, High-resolution deconvoluted C1s and O1s spectra of (b) GONS and (c) GONS-GA.

Table 3.1: Elemental compositions of GO, GONS, and GONS-GA nanocomposites.

Sample name	C (At. %)	O (At. %)	C/O atomic ratio
GO	66.45	33.55	1.98
GONS	69.31	30.69	2.25
GONS-GA	76.14	23.86	3.19

3.2.3. Raman Spectra

Raman spectroscopy is considered as the best tool for analyzing carbon and related materials. The parent material, GO, along with the fabricated GONS and GONS-GA nanocomposite, are characterized using Raman spectroscopy for investigating the reducing behavior, degree of oxidation, and hybridization states. The Raman spectra of GO, GONS, and GONS-GA nanocomposite at 488 nm excitation wavelength are shown in **Fig. 3.6**. In the case of GO, two different peaks of G-band and D-band were detected at 1599 cm^{-1} and 1358 cm^{-1} , respectively. The intensity ratio (I_D/I_G) of GO was observed to be 0.99, which was a bit higher than that of parent synthetic graphite (0.45) used for oxidation and further synthesis of GO via the modified Hummers' method [1]. It was observed that the oxidation of graphite imparts defects within GO, which leads to an increment in the intensity of the D-band without changing the Raman shift. The increment in the intensity ratio suggests a lesser average size of sp^2 hybridized GO [8]. In the case of GONS fabrication after sonication of treatment, a slight change in the G-band (1586 cm^{-1}) with a blue shift of $\sim 13\text{ cm}^{-1}$ was observed, irrespective of the D-band. The change in the Raman shift of GONS advocates a lower degree of hybridization. The peak position of the D-band was observed to be identical to that of GO with inferior intensity. The intensity ratio for GONS was noted as 1.08, which was considerably greater than GO and represents higher defect formations during sonication treatments. The change in the I_D/I_G ratio also suggests a reduction of GO via eliminating some of the oxygenated functional groups during sonication, which leads to rapid π - π bond interaction [1,9].

However, for GONS-GA, nanocomposite blue shift for the D-band and redshift for the G-band were observed, which suggests a slight reduction and lower crystallinity than that of GONS during nanocomposite formation [2]. Also, it was found that the intensity ratio of

GONS-GA (1.05) decreased compared to parent GONS during the formation of nanocomposites.

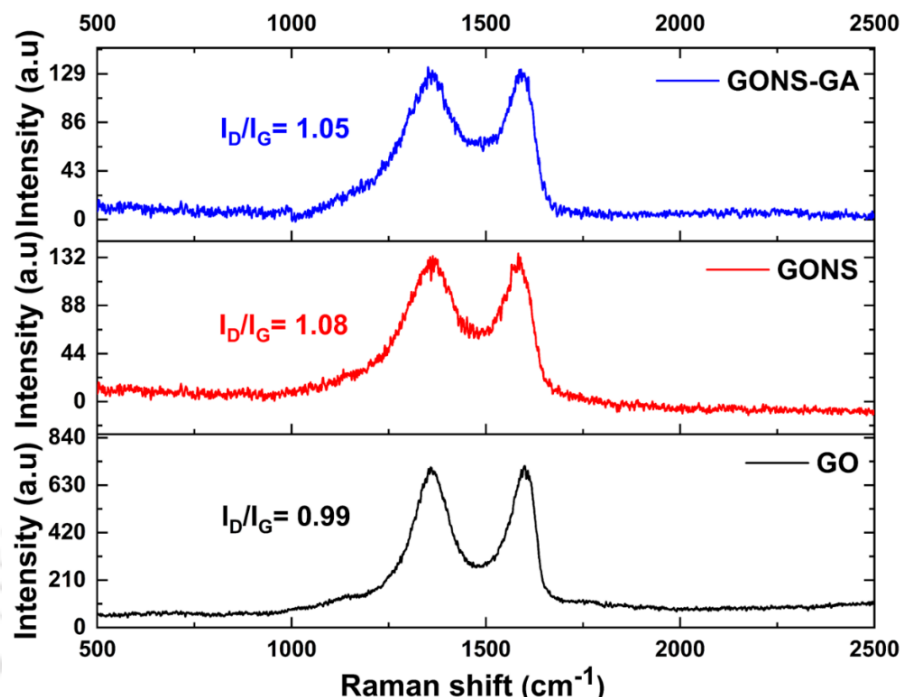


Fig. 3.6: Raman spectrum of GO, GONS, and GONS-GA nanocomposite.

The observation suggests that during GONS reduction, the aromatic structure of GONS was recovered by the defects and decreased the intensity ratio. The obtained Raman shift peak positions of the G-band and D-band, along with the intensity ratios of GO, GONS, and GONS-GA nanocomposites, are also reported in **Table 3.2**. The presented investigation may offer an opportunity for the restoration of the sp^2 network in graphene-related materials. In recent, it was reported that the GA, with its antioxidant molecules, provides a significant ability to preserve the 2D structure of phosphorene [2]. Similarly, the presented observations reinforce the potential of GA as a natural antioxidant for reducing structural defects in 1D materials such as GONS.

Table 3.2: Raman shifts and intensity ratio of D-band and G-band of GO, GONS, and GONS-GA nanocomposite.

Sample name	D-band peak (cm^{-1})	G-band peak (cm^{-1})	I_D/I_G ratio
GO	1358	1599	0.99
GONS	1358	1586	1.08
GONS-GA	1352	1591	1.05

3.2.4. X-Ray diffraction

The GO synthesized from synthetic graphite as well as GONS, GA, and GONS-GA nanocomposite were quantitatively analyzed using powder XRD, as shown in **Fig. 3.7**. The graphite with stronger van der Waals interactions possesses a strong diffraction peak around 26.6° , which corresponds to its graphitic nature [11]. However, the graphitic plane completely vanished after the oxidation of graphite during the synthesis of GO. The oxidation process weakens van der Waals interactions between the graphitic plane and forms thoroughly exfoliated GO nanosheets, resulting in a new peak at 10.68° as shown in **Fig. 3.7**. Further, XRD spectra of GONS obtained after the sonication treatment resulted to several other peaks at 20.16° , 24.08° and 44.52° , including the slightly reduced GO peak (10.84°). The peak position at 20.16° is ascribed to the (0 0 2) plane with a d-spacing of 0.44 nm. It indicates a foreseeable reduction in oxygenated functional sites of GO through the sonication treatment during GONS formation, as established in XPS analysis. However, additional broader and higher intensity peaks at 24.08° and 44.52° show d-spacing of 0.37 and 0.20 nm, respectively. Also, these peaks with lower interlayer distance signify more compact packing of GONS structure, which aids in the differentiation of GONS from GO. In the case of pure gallic acid (GA), sharp crystalline peaks were observed at 16.22° , 19.08° and 32.60° representing the characteristics of the organic molecules.

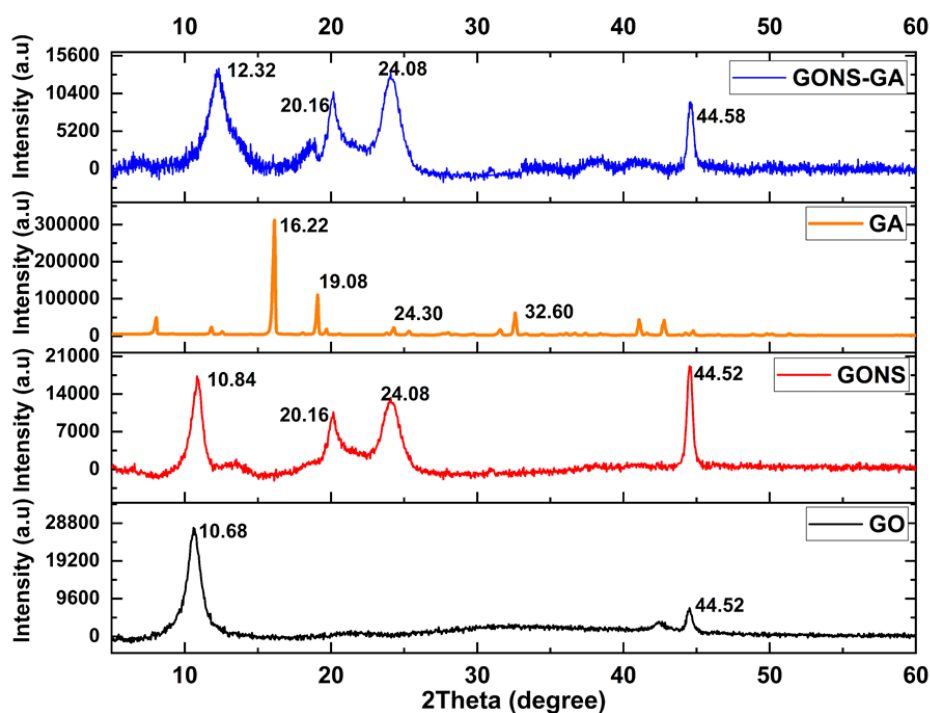


Fig. 3.7: X-ray diffractogram of GO, GONS, GA, and GONS-GA nanocarrier.

However, the GONS-GA nanocomposite obtained after loading of GA over the GONS exhibits a more amorphous structure. It was observed that the crystalline peaks of pure GA disappeared entirely from the XRD pattern of the GONS-GA nanocomposite, which demonstrated the successful loading of GA to the GONS nanocarrier, as confirmed by FTIR, XPS, and Raman spectroscopy [3]. Alongside, nearly identical diffractograms of GONS and GONS-GA nanocomposites had established that the impregnation of GA and the loading procedure do not affect the amorphous phase of the GONS nanocarriers.

3.2.5. Surface charge analysis

The properties of the nanoformulations, such as nanocomposites or nanocarriers, can be critically analyzed by investigating the surface charge of the nanocarriers to determine their aqueous stability and colloidal dispersion characteristics. Better stability of the nanocarriers can improve their bioavailability [12,13]. Therefore, the surface charges of parent GO, GONS, and GONS-GA nanocarrier suspensions of concentration 0.05 g L^{-1} in DI water were investigated by zeta potential (ζ) (Delsa nano C, Beckman Coulter). The surface charge of

GO was highly negative with a ζ potential value of -35.6 ± 1.6 mV. However, the negative charge of GO was decreased after the GONS formation via ultrasonication, with evidence from the ζ potential value of GONS was obtained as -24.2 ± 1.5 mV. The decrement in the negative surface charge of GONS may be attributed to the slight reduction in oxygenated groups of GO during the sonication treatment.

In contrast, the negative surface charge of the GONS-GA nanocomposite was enhanced to a ζ value of -37.1 ± 2.1 mV with more aqueous stability. The increment in negative ζ potential of the GONS-GA nanocomposite may be ascribed to the rich hydroxyl functional groups of GA. Consequently, enhancement in a negative ζ potential of the GONS-GA nanocarrier also elucidates better colloidal stability and the effective loading of GA on the pristine GONS [14].

3.2.6. Loading characteristics of GA

The drug molecules of gallic acid (GA) were loaded on the 1D porous and hollow structure of graphene oxide nanoscrolls (GONS). The loading was carried out by taking advantage of van der Waals interactions, intermolecular hydrogen bonding, and electrostatic interactions offered by GONS. The impact of various loading factors, such as pH, temperature, and the ratio of the GA vs. GONS, are investigated to enhance the effectiveness of loading and entrapment.

3.2.6.1. Effect of pH

The pH of the solutions acts as a substantial element in regulating the drug loading and entrapment efficiencies of the nanocarriers. Therefore, the impact of the pH of the solution on the loading efficacy of GONS nanocarriers (**Fig. 3.8A**) is investigated for pH 4.7, 7, and 9, representing an acidic, neutral, and basic nature of the solution, respectively. It was observed that the drug loading efficiency of GA was higher under acidic conditions (pH 4.7) than that

of neutral and basic environments. The higher drug loading efficiency of GA may be attributed to the hydroxyl (O^-) and carboxyl (COO^-) functional groups of gallic acid and GONS, which are expected to get protonated in an acidic medium [3]. The protonation further weakens the electrostatic interactions between GA and GONS while improving the other non-covalent interactions. In this case, the π - π stacking and intermolecular hydrogen bonding are the dominating forces that enhance the overall loading efficiency of the GONS nanocarriers. However, the basic environment ($\text{pH} \geq 7$) deprotonates the hydroxyl and carboxyl functional groups of GA and GONS. It produces negatively charged hydroxyl and carboxyl species, which causes electrostatic repulsion and intramolecular dehydration [2]. Such an environment further restricts the formation of hydrogen bonds and reduces the overall loading efficiency of the nanocarriers for both neutral and basic environments, as shown in **Fig. 3.8A**.

3.2.6.2. Effect of GA mass ratio

Figure 3.8 C-D shows the influence of the GA vs. GONS weight ratio over the drug loading efficiency as well as entrapment efficiency at the optimum pH of 4.7, respectively. It was observed that, with an increase in the amount of drug concerning the GONS nanocarriers, the drug loading efficiency was increased along with the entrapment efficiency up to 1.25 w/w ratio of GA:GONS. As stated earlier, the non-covalent interactions, including intermolecular H-bonding between hydroxyl and carboxyl functional groups, van der Waals interactions, and π - π stacking interactions between the aromatic rings of GONS and GA, plays a significant role in the efficient loading of GA on GONS. The maximum drug loading efficiency and entrapment efficiency of GA were found to be $29.82 \pm 0.8\%$ and $34 \pm 0.75\%$, respectively, at a 1.25 weight ratio of GA and GONS. However, a further increase in drug amount tends to a reduction in the loading and entrapment efficiency, which may be due to the lack of

availability of surface moieties and subsequent interactions between GA and GONS nanocarrier. The obtained loading efficiency of GONS nanocarriers was much higher than the pristine GO nanocarriers ($23.08 \pm 0.47\%$).

Overall, the acidic pH (4.7) during loading and the 1.25 w/w ratio of GA were optimum parameters for higher loading and entrapment efficiency of GA over the GONS. As the drug loading efficiency subjected to the amount of drug present in the end product, the proposed investigation demonstrated substantial drug loading capacity of GONS with every gram of GONS-GA nanocomposite comprising nearly 300 mg of GA. Previously, the maximum loading efficiency of GA for the GO nanocarrier was reported as 12.8% [2] and 12% [3], which suggests a loading capacity of nearly 130 mg g⁻¹ of GOGA nanocomposite. However, the structural and morphological modification of GO into the 1D spiral wounded structure of GONS has provided significant improvement in the loading capacity for GA under identical conditions.

3.2.6.3. Effect of loading temperature- a thermodynamic approach

The Effect of loading temperature on loading efficiency was investigated via performing the set of experiments for 250 mg L⁻¹ GA solution at three different temperatures (25, 30, and 35 °C). The experiments were conducted at 200 rpm for 16 h with a GONS solution of 1 g L⁻¹. It has been observed that the loading efficiency of GONS was increased with the rise in loading temperature, as shown in **Fig. 3.8B**. The thermodynamic parameters such as Gibbs free energy (ΔG^0), enthalpy (ΔH^0), and entropy (ΔS^0) for the loading of GA have been calculated with the help of the following thermodynamic equations [15].

$$\Delta G^0 = \Delta H^0 - T\Delta S^0 \quad (3.4)$$

$$\log\left(\frac{q_e m}{C_e}\right) = \frac{\Delta S^\circ}{2.303R} + \frac{-\Delta H^\circ}{2.303RT} \quad (3.5)$$

Where q_e is the amount of GA loaded per unit mass of GONS (mg g^{-1}), C_e is an equilibrium concentration (mg L^{-1}), m is the concentration of GONS (g L^{-1}), and T is a temperature in K. Here, $m = 1 \text{ g L}^{-1}$ therefore, Eq. 3.5 becomes:

$$\log\left(\frac{q_e}{C_e}\right) = \frac{\Delta S^\circ}{2.303R} + \frac{-\Delta H^\circ}{2.303RT} \quad (3.6)$$

The values of ΔG° have been determined from Eq. 3.4 via calculating the ΔH° and ΔS° . However, ΔH° and ΔS° have been determined from Eq. 3.6 by plotting the graph between $\log(q_e/C_e)$ vs. $1/T$. The obtained values of ΔG° , ΔH° , and ΔS° are reported in **Table 3.3**.

The loading of GA on GONS is termed to be an endothermic process since the value of ΔH° obtained was positive. Alongside this, an endothermic nature of the loading process was also confirmed via an increase in loading efficiency with temperature (**Fig. 3.8B**). In this case, as the loading process of GA on GONS was executed via the adsorption phenomenon, the higher heat of adsorption during the process indicates chemisorption rather than physisorption, which is a predominant mechanism as signified by the enthalpy of adsorption [15,16]. Furthermore, the negative values of Gibbs free energy (ΔG°) obtained for three different temperatures reveal the spontaneous nature of the loading process. At last, the positive value of entropy (ΔS°) suggests that the loading of GA on GONS is organized in a more random manner, which indicates increased randomness at the surface interfaces of GONS and GA during the loading process.

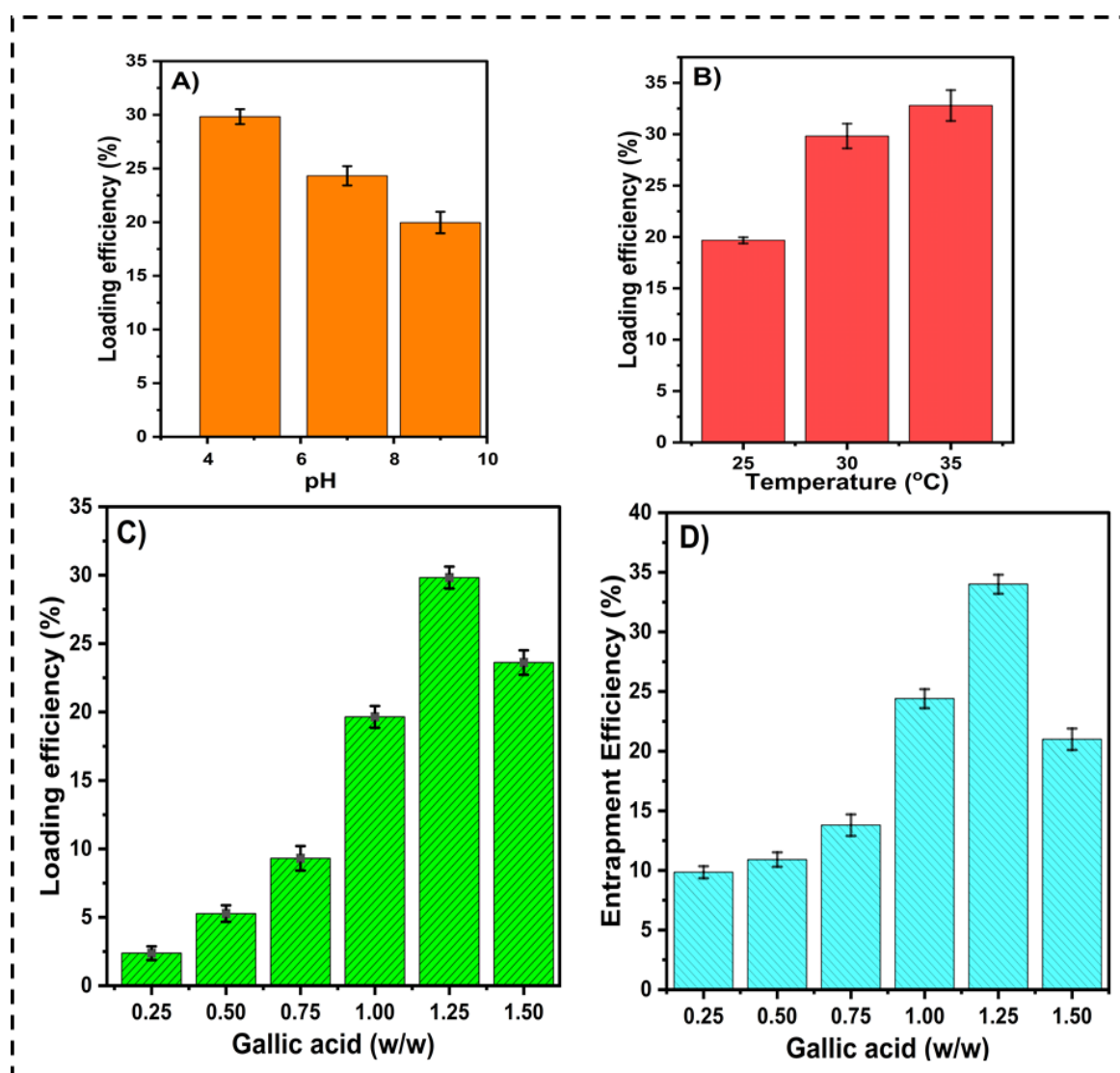


Fig. 3.8: Loading characteristics of GA on GONS. (A) effect of pH on loading efficiency, (B) effect of the loading temperature on loading efficiency, (C) influence of GA: GONS weight ratio on loading efficiency, (D) influence of GA: GONS weight ratio on entrapment efficiency. The results are shown as mean $\pm$ SD.

Table 3.3: Thermodynamic parameters for GA loading on GONS at different temperatures. (Results are shown as mean $\pm$ SD).

Drug concentration (mg L ⁻¹)	ΔH° (kJ mol ⁻¹)	ΔS° (J mol ⁻¹ K ⁻¹)	$-\Delta G^\circ$ (kJ mol ⁻¹)		
			T = 298 K	T = 303 K	T = 308 K
250	15.623 $\pm$ 0.467	54.622 $\pm$ 1.624	0.654 $\pm$ 0.017	0.928 $\pm$ 0.027	1.206 $\pm$ 0.035

3.2.7. Stability study of GONS-GA nanocomposite

The long-term stability or dispersibility of the nanocomposite/nanoformulation is a vital parameter for the efficient preparation of the colloidal pharmaceutical nanomedicine. The non-agglomerating tendency of the nanocomposites in aqueous media provides them with constant colloidal stability and improves their biocompatibility. In colloidal systems, the stability of nanocomposites is primarily governed by their surface charge, non-covalent interactions, and physical interactions between particles [2]. Herein, the storage stability and colloidal dispersibility of the freshly prepared GONS-GA nanocomposite were investigated for both the ambient (25 ± 1 °C) (AT) and physiological (37 ± 1 °C) temperatures (PT) for 21 days. The nanocomposites were observed for various physical instabilities such as agglomeration, crystallization, turbidity, precipitation, and change in color with respect to time and temperature.

The prepared GONS-GA nanocomposites have displayed long-term storage stability and colloidal dispersibility at both temperatures for 21 days. As shown in **Fig. 3.9A**, the nanocomposites have not displayed any other physical instability, such as agglomeration, crystallization, precipitation, or turbidity. However, a slight change in the color towards the darker side was observed for the nanocomposite at both temperatures, which might be due to the further reduction of oxygenated functional groups of GONS by GA. It was found that the GA not only acts as a reducing agent but also improves the colloidal dispersibility and stability of the GONS nanocarrier in aqueous media. The improvement in the colloidal stability of GONS by incorporation of GA was advocated by an increment in the magnitude of zeta potential from -24.2 ± 1.5 mV to -37.1 ± 2.1 mV, as discussed earlier, which signifies more electrostatic repulsion between the particles.

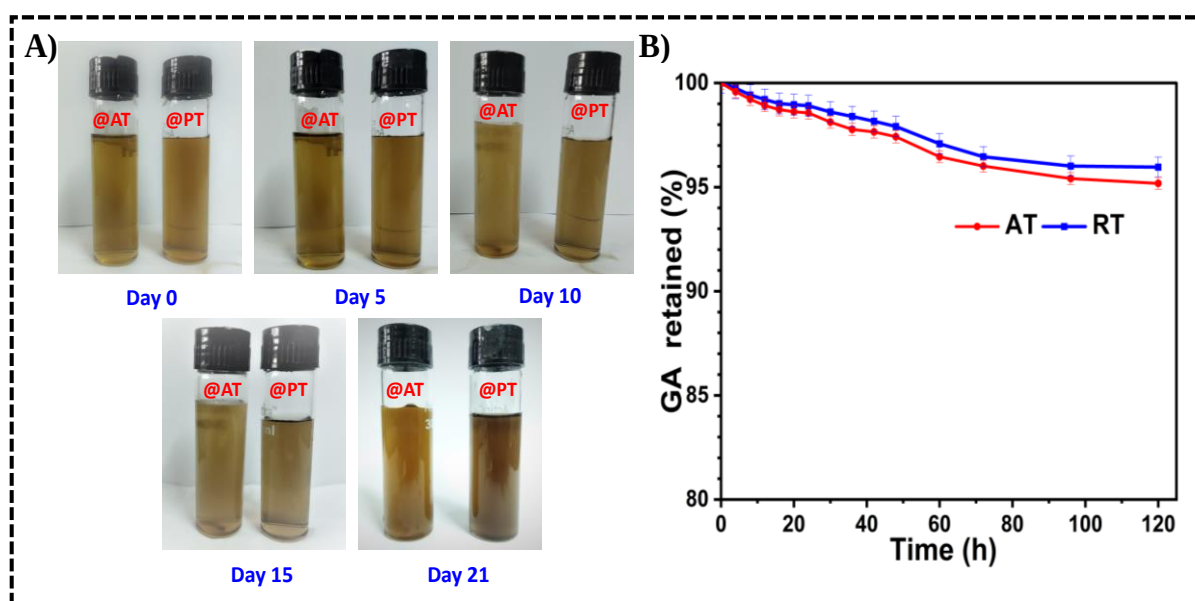


Fig. 3.9: (A) Storage stability and colloidal dispersibility of GONS-GA nanocomposite @AT ($25 \pm 1^\circ\text{C}$) and PT ($37 \pm 1^\circ\text{C}$), (B) % GA retained in GONS-GA nanocomposite @AT ($25 \pm 1^\circ\text{C}$) and RT ($4 \pm 1^\circ\text{C}$) (Results are shown as mean $\pm$ SD).

Furthermore, the degradation behavior of GA from GONS-GA was studied for ambient ($25 \pm 1^\circ\text{C}$) (AT) and refrigeration ($4 \pm 1^\circ\text{C}$) temperatures (RT). To determine the amount of GA retained in the GONS-GA nanocomposite, newly prepared GONS-GA nanocomposites were dispersed in the Milli-Q water and subjected to different temperatures (AT and RT) for 120 h (5 days). The aliquots were collected at a specific period of time, and the concentration of GA retained was determined via UV-Vis spectroscopy. The degradation behavior of GA from GONS-GA nanocomposite for both AT and RT is presented in **Fig. 3.9B**. It was found that the nanocomposites were stable up to 120 h, and only $\sim 4\text{-}5\%$ GA was degraded or leached out from the dispersed GONS-GA nanocomposite. The retention of GA was more at RT ($95.96 \pm 0.48\%$) than in AT conditions ($95.18 \pm 0.28\%$), suggesting that the GONS-GA should be stored in RT conditions. The obtained GONS-GA nanocomposite with long-term storage stability, better colloidal dispersibility, and lower degradation tendency of GA have presented its potential for drug delivery and cancer therapeutics.

3.2.8. *in-vitro* release behavior and kinetics study of GA from GONS-GA nanocomposite

The *in-vitro* release profile of GA from the GONS-GA nanocomposite into the phosphate buffer solution (PBS) at pH 7.4 for a period of 7200 min. (5 days) is presented in **Fig. 3.10**. In the first 1.5 h, a burst release of $31.74 \pm 0.94\%$ was observed, which may be due to the surface characteristics of the GONS nanocarrier. After that, a sustained release of GA was observed, which was apparently exhibited by the anion-exchange phenomenon between GA and GONS nanocarrier. However, over the period of 7200 min., only $73.78 \pm 2.21\%$ of GA was released from the GONS-GA nanocomposite.

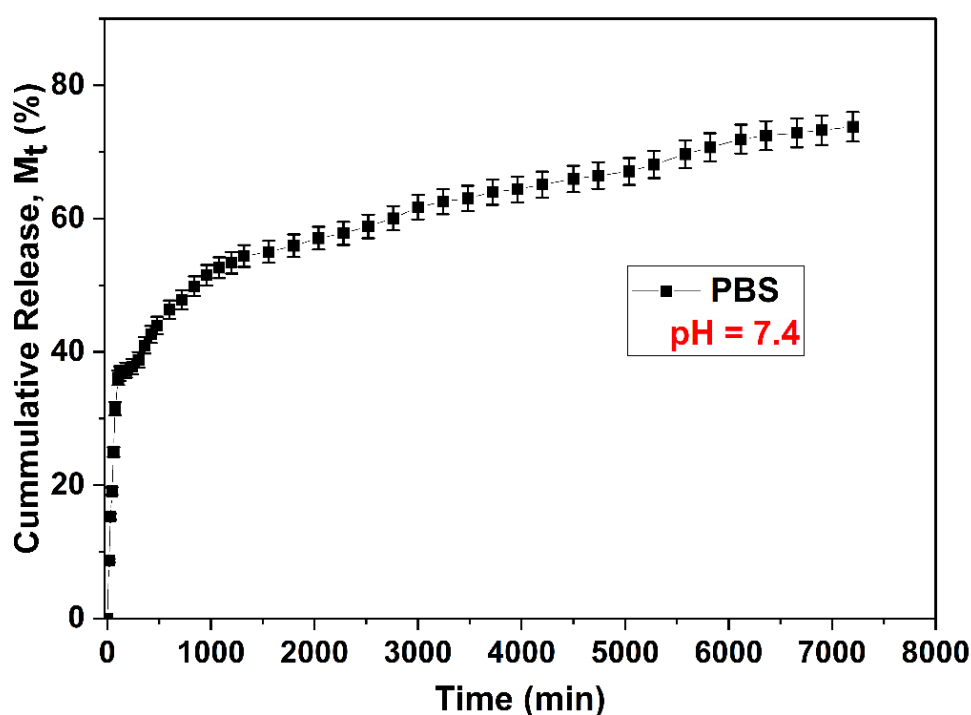


Fig. 3. 10: *in-vitro* release profile of GA from the GONS-GA nanocomposite at pH 7.4. (Results are expressed as mean $\pm$ SD).

The obtained results demonstrated that the releases of GA from nanocomposite were accompanied by an early burst phase and a sustained release phase subsequently over a longer duration. Previously, Dorniani et al. demonstrated a similar kind of release behavior for GO nanocarriers with 59% release of GA in an initial 1h followed by the sustainable

release of 81% in 7200 min. On the other hand, the loading efficiency of GO was only 12% [3]. From the cancer therapeutics' point of view, the initial burst release phase may be much more helpful for achieving the desired drug therapeutic level of GA. The successive sustained release may also contribute to upholding the overall dose of the drug during therapeutics for a longer timeframe. It was reported that the lower mass of GA is estimated to be leached out at physiological pH (pH 7.4) than the acidic pH, resulting in a slow-release tendency of GA from the nanocomposite. Such characteristics of nanocomposite are much more beneficial as less quantity of GA will be leaching out within blood (physiological pH). However, cancer tumor sites are acidic in nature, where the fast release of GA was observed [2].

Herein, eight different kinetic drug-release models were employed over the GA release pattern in PBS. The kinetic models such as zero-order (Eq. 3.7) [17], pseudo-first-order (Eq. 3.8), pseudo-second-order (Eq. 3.9), Higuchi model (Eq. 3.10), Korsmeyer-Peppas (Eq. 3.11) Hixson-Crowell model (Eq. 3.12), Baker and Lonsdale model (Eq. 3.13), and Weibull model (Eq. 3.14) [3,17–20] were applied to estimating the release kinetics of GA from the GONS-GA nanocomposite.

$$\frac{M_t}{M_e} = k_o t \quad (3.7)$$

$$\ln(M_e - M_t) = \ln M_e - kt \quad (3.8)$$

$$\frac{t}{M_t} = \frac{1}{kM_e^2} + \frac{t}{M_e} \quad (3.9)$$

$$M_t = k_h t^{1/2} \quad (3.10)$$

$$\frac{M_t}{M_\infty} = k_p t^n \quad (3.11)$$

$$W_0^{1/3} - W_t^{1/3} = k_{HC} t \quad (3.12)$$

$$\frac{3}{2} \left[1 - \left(1 - \frac{M_t}{M_\infty} \right)^{2/3} \right] - \frac{M_t}{M_\infty} = k_{BL} t \quad (3.13)$$

$$M_D = M_R (1 - e^{-kt}) \quad (3.14)$$

Where k is the corresponding rate constant of the respective kinetic model, M_t , M_e , and M_∞ are the amount of GA released from the GONS-GA nanocomposite at time t , at equilibrium,

and infinite time, respectively. n is the release or diffusional exponent. M_D and M_R are the amount of GA dissolved as a function of time and the total amount of GA released. Also, the W_0 and W_t are the initial amount and remaining amount of GA at time t , respectively.

In line with the applicability of these kinetic models, the zero-order kinetic model implies that the drug release rate is sustained throughout time and unaffected by the drug's concentration. It may be appropriate if drug/GA release from the drug-loaded/GONS-GA nanocomposite occurs in a constant, regulated manner that is unaffected by drug/GA concentrations or drug-nanocarrier interactions. This model is more appropriate in the case of oral delivery systems, matrix tablets with low water-soluble drugs in coated forms, osmotic systems, and, to some extent, transdermal systems for wound healing. At the same time, the pseudo-first-order kinetics implies that the drug release rate is precisely proportional to the amount of drug that remains in the system. It might be effective if the escape of drug/GA from the nanocomposite follows first-order kinetics, in which the rate of release declines exponentially with time. This model is primarily applicable in the case where the water-soluble active pharmaceutical agents are incorporated in a porous matrix [20–22]. The pseudo-second-order kinetics depict that the drug release rate corresponds to the square of the residual drug concentration in the composite matrix. This paradigm is frequently appropriate for systems where the drug release is regulated by chemical interactions between the drug and the matrix of molecules. If the release of GA exhibits pseudo-second-order behavior, it indicates a chemical adsorption/desorption mechanism. It may be more relevant if GA release follows a more complicated trend beyond first-order kinetics, potentially caused by drug-nanocomposite interactions. As described before in the thermodynamic study, the drug loading mechanism is primarily driven via the chemisorption process with the positive value of entropy (ΔS°), suggesting that the loading of GA on GONS is organized through rapid

interactions among its surfaces [20–22]. The Higuchi model is suggestive of diffusion-controlled release and represents the diffusion of drugs from a matrix as the square root of a time-dependent phenomenon. It may be appropriate if the release of GA from the nanocomposite is predominantly determined by diffusion across the matrix. This model can be employed to designate drug dissolution from numerous types of adapted release pharmaceutical dosage systems, similar to the case of some transdermal systems and matrix tablets with water-soluble drugs. This model describes the release of active drugs from solid and semi-solid matrices, including those that are less and more soluble. Korsmeyer-Peppas model is frequently employed to characterize drug release from polymeric compounds like hydrogels and may represent a variety of release processes, including Fickian diffusion, anomalous transport, and case II (relaxational) transport. It may be effective if the release of GA from the nanocomposite includes complicated pathways that cannot be characterized using basic first- or zero-order kinetics. Herein, GA released out of GONS-GA nanocomposite is illustrated via Fickian diffusion[20–22].

Furthermore, the Hixson-Crowell model is frequently employed to illustrate drug release in systems with changing surface area or shape over time, such as dissolving or eroding composites. It may be appropriate if the release of GA from the GONS-GA nanocomposite is followed by a change in the structural or morphological attributes of the GONS-GA. This statement is applicable to pharmaceutical dose forms like tablets, where the initial geometrical form remains constant throughout time, and the dissolving proceeds along planes parallel to the drug surface if the tablet dimensions decrease proportionately. The fitted Baker and Lonsdale model describes a non-homogeneous matrix with capillaries capable of enhancing the release of GA from the GONS-GA nanocomposite [20]. This model may be used to linearize the release data of numerous microparticle compositions (microcapsules and

microspheres). At last, the Weibull model is an essential methodology for investigating a wide range of release profiles, and it may reveal multiple release processes based on the shape parameter. It may be appropriate if the release of GA from the nanocomposite follows a non-linear or complicated pattern that simpler models do not represent well. [20–22].

The mentioned kinetic models are plotted as shown in **Fig. 3.11**, and the respective correlations coefficient (R^2), as well as rate constants (k), are listed in **Table 3.4**. The correlation coefficient, as the selection criteria of the best kinetic model for describing the release of GA from the GONS-GA nanocomposite, is also displayed in **Fig. 3. 11**. In this case, amongst all the mentioned kinetic models, the pseudo-second-order and Baker and Lonsdale model (**Fig. 3. 11 C, G**) was more acceptable, with a best fit R^2 value of 0.99. In accordance with the pseudo-second-order model, the GA release amount corresponded to the square of the residual GA concentration in the GONS-GA nanocomposite matrix; also, the model demonstrated that the GA release is regulated by significant chemical interactions between the GA and the GONS matrices. The fitted Baker and Lonsdale model describes a non-homogeneous matrix with capillaries capable of enhancing the release of GA from the GONS-GA nanocomposite [20]. Moreover, the release exponent was found to be 0.25 (< 0.5) for the Korsmeyer-Peppas model (**Fig. 3. 11E**) with an R^2 value of 0.97, signifying that the release of GA from GONS-GA nanocomposite is driven by the Fickian diffusion model [2]. The findings were also supported by the Higuchi model (**Fig. 3. 11D**), as the drug release counts on the square root of time [3].

As nanocarriers with higher surface area, porous structure, and better colloidal stability provide significant advantages for their use in biomedical applications such as drug delivery [12,23]. Previously, numerous nanocarriers like GO [3], Fe_3O_4 [24], BPBA- rGONC [2], and chitosan [25,26] have been extensively explored for the loading of GA towards their

application in drug delivery for cancer therapeutics. However, most of these nanocarriers exhibited lower loading capacity, colloidal stability and unsustainable delivery of GA. The obtained GONS nanocarrier with its porous/spongy structure, higher surface area (~3 fold higher than GO) [1], adjustable interlayer distance, and the additional inner accessible area has exhibited a loading efficiency of $29.82 \pm 0.8\%$ for GA, which was highest among the other nanocarriers (**Table 3.5**). In addition, the nanocarrier exhibited suitable colloidal stability in aqueous media with a zeta potential of -24.2 ± 1.5 mV, which was further increased after the loading of GA (-37.1 ± 2.1 mV). In contrast to the study done by Gupta et al. (2020) with BPBA@GA-rGONC nanoformulation, the increment in the magnitude of zeta potential signifies improved dispersibility of GONS-GA in aqueous media and hence the biocompatibility [2].

Table 3.4: Rate constants (k), correlation coefficients(R^2), and release exponent (n) for kinetic models over the release of GA.

Aqueous solution	Saturated release (%)	Kinetic model	Correlation coefficient (R^2)	Rate constant (k)	Confidence limits (Upper, Lower)
PBS Buffer pH=7.4	73.78	Zero-order	0.69	$k_0 = 6.78 \times 10^{-5} \text{ min.}^{-1}$	$6.96 \times 10^{-5}, 6.61 \times 10^{-5}$
		Pseudo first-order	0.87	$k = 1.38 \times 10^{-4} \text{ min.}^{-1}$	$1.41 \times 10^{-4}, 1.36 \times 10^{-4}$
		Pseudo second-order	0.99	$k = 4.92 \times 10^{-4} \text{ mg min.}^{-1}$	$2.07 \times 10^{-3}, -1.09 \times 10^{-3}$
		Higuchi	0.88	$k_h = 4.87 \times 10^{-2} \text{ min.}^{-0.5}$	$4.95 \times 10^{-2}, 4.78 \times 10^{-2}$
		Korsmeyer–Peppas	0.97	$k_p = 8.59 \times 10^{-2} \text{ min.}^{-n}$ $n = 0.25$	$8.69 \times 10^{-2}, 8.50 \times 10^{-2}$
		Hixson-Crowell	0.97	$k_{HC} = 7.22 \times 10^{-5} \text{ min.}^{-1}$	$7.38 \times 10^{-5}, 7.05 \times 10^{-5}$
		Baker and Lonsdale	0.99	$k_{BL} = 1.83 \times 10^{-5} \text{ min.}^{-1}$	$1.85 \times 10^{-5}, 1.81 \times 10^{-5}$
		Weibull	0.97	$k = 1.36 \times 10^{-4} \text{ min.}^{-1}$	$1.39 \times 10^{-4}, 1.34 \times 10^{-4}$

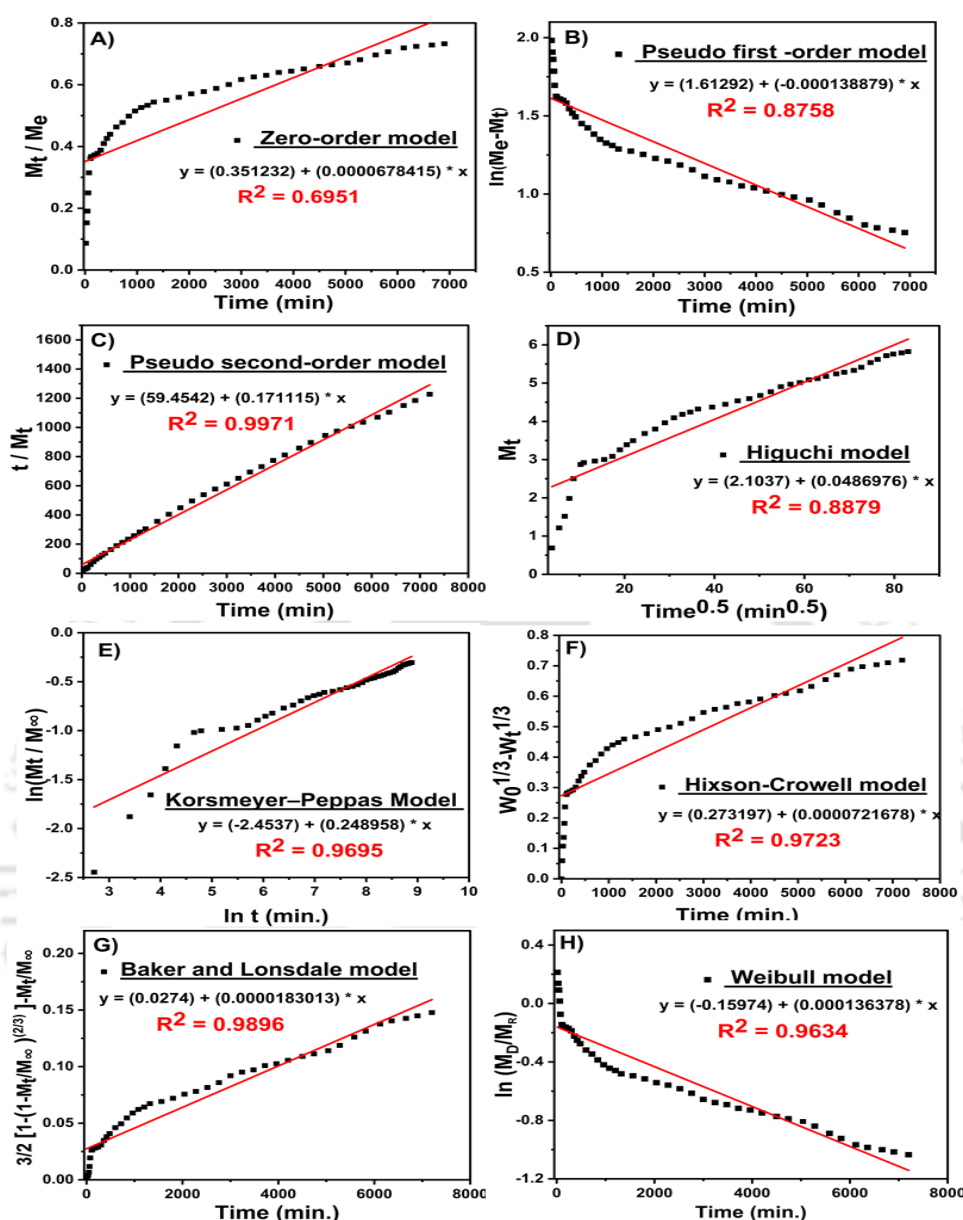


Fig. 3. 11: Kinetic model data fitting of GA release from GONS-GA nanocomposite (A) Zero-order model (B) pseudo-first-order model, (C) pseudo-second-order model, (D) Higuchi model, (E) Korsmeyer-Peppas model, (F) Hixson-Crowell model, (G) Baker and Lonsdale model, and (H) Weibull model.

In the case of the release of GA from the nanoformulations at physiological pH 7.4, GONS-GA displayed an initial burst effect, which may be due to the porous structure, nano capillaries between scrolled layers, and other surface characteristics of GONS alongside its interaction with the drug molecules. The burst effect was further followed by the sustainable release of GA. As stated earlier, the initial burst release stage may be beneficial in obtaining

the required therapeutic level of GA in cancer treatments. The sequential sustained release may also assist in maintaining the drug's total dosage during the treatment for a more extended period of time [2]. In contrast to the PLGA, Fe₃O₄-PVA, and chitosan nanoparticles, the GONS nanocarrier exhibited a slower and sustainable release of GA with a maximum release of ~74% in 120 h. A detailed comparison of loading efficiency and release behavior of the GA-based nanoformulation is provided in **Table 3.5**.

Table 3.5: Comparison of loading efficiency, release behavior, and zeta potential of GA carriers.

Carrier	Loading efficiency (%)	Maximum GA release (%)	Zeta potential (mV)	Reference
Fe ₃ O ₄ -PEG	7	60.9, 115 h	-	[24]
Fe ₃ O ₄ -PVA	5	80.9, 110 h	-	[24]
PLGA nanoparticles	-	98.24, 48 h	-	[27]
Chitosan nanoparticles	-	85.32 ± 3.76, 24 h	46.50 ± 2.09	[28]
Chitosan nanoparticles	3.10 ± 1.44	77.56, 24 h	28.1	[26]
Carboxymethyl chitosan (CMC)			-24	[25]
GO	12	81, 120 h	-	[3]
BPBA- rGONC	12.8 ± 1.15	52.5 ± 1.22, 48 h	-24.90 ± 1.03	[2]
GONS	29.82 ± 0.8	54.38 ± 1.63, 24 h 60.08 ± 1.80, 48 h 73.78 ± 2.21, 120 h	-37.1 ± 2.1	Present study

Overall, the major advantages over the use of GONS as a GA nanocarrier were observed as its porous structure, the improved surface-to-volume ratio as compared to parent GO, sustainable delivery of chemotherapeutic agents, highest drug loading capacity, reasonable

storage and colloidal stability in aqueous media, lower toxicity, and suitable biocompatibility. Such characteristics of GONS have advocated its application in DDS.

3.2.9. Cytotoxicity study of GONS, GA, and GONS-GA nanocomposite

The drug carriers are meant to deliver the drug to the desired site without causing any side effects to the surroundings and loss of the drug activity. Biocompatibility of the drug-carrying assembly plays a significant role in deciding its biological applications [6,29,30]. The in-vitro cytocompatibility of the synthesized GONS nanocarrier was performed on a normal cell line (HEK293) and a cancer cell line (A549). The cell viability in the presence of samples as compared to control is shown in **Fig. 3.12**. The GONS nanocarrier showed nearly a similar cytocompatibility behavior for both the cell lines, HEK293 and A549, which indicated the non-anticancer properties of the GONS nanocarrier. The cell viability up to a GONS concentration of $10 \mu\text{g mL}^{-1}$ was observed to be about 80%, i.e., above the recommended value of 70% [31]. However, further increases in concentrations resulted in a declination of cell viability for both cell lines. The IC_{50} values of the nanocarrier for both the cell lines were observed to be $\sim 99 \pm 2 \mu\text{g mL}^{-1}$, as provided in **Table 3.6**.

There are several intrinsic and external factors, such as structure, length, surface charge, colloidal stability, agglomeration tendency, surface chemistry, and functionalization, which may influence the toxicity of nanocarriers [32]. The carbon-based nanocarriers such as CNTs and GO have been explored previously for their cytocompatibility. In the case of pristine CNTs, the IC_{50} values were observed to be $42.10 \mu\text{g mL}^{-1}$ [33] and $87.58 \mu\text{g mL}^{-1}$ [34] towards HEK293 cells for 48 h, which was lower in comparison to GONS due to their shape, length, and surface chemistry. Furthermore, GO reportedly exhibited higher cytocompatibility towards HEK293 cells until the tested concentration of $125 \mu\text{g mL}^{-1}$ for 48 h, possibly due to more oxygenated functional groups than GONS [1,35]. Meanwhile, GONS

offers similar surface properties as of GO, along with an open-end 1D nanomorphology, porous structure, higher surface area, suitable colloidal stability, and adjustable interlayer spacing between scrolled layers, as well as an additional inner accessible area than CNTs [1,7]. Such characteristics of GONS, with its higher loading capacity for GA and suitable cytocompatibility ($IC_{50} \sim 99 \pm 2 \mu\text{g mL}^{-1}$) towards normal cell lines, make them favorable over GO and CNT.

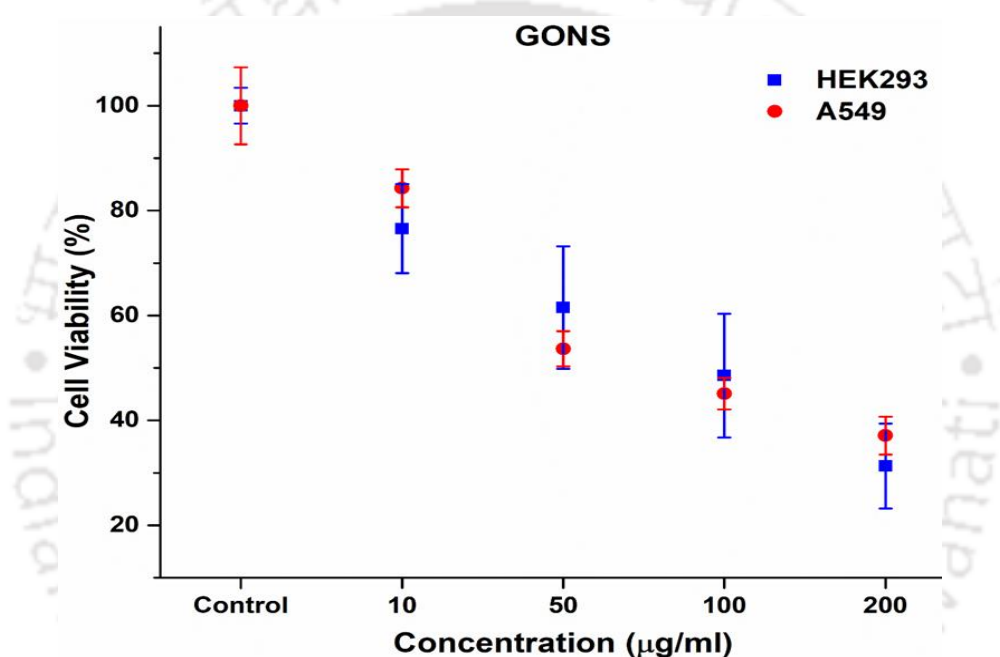


Fig. 3.12: *in-vitro* cytocompatibility of the GONS nanocarrier on the normal cell line HEK293 and cancer cell line A549. (The results are stated as mean $\pm$ SD).

Further, the cytotoxicity of a model drug GA in free form and loaded in the fabricated GONS nanocarrier (GONS-GA) was investigated. The tested drug GA is reported for anticancer activity with the cell viability data, as shown in **Fig. 3.13**. The cell viability of the cancer cells A549 decreased with an increasing GA concentration. For the GA concentration of 10 and $50 \mu\text{g mL}^{-1}$, the cell viabilities of 73.43% and 33.65% were observed, respectively. The reduced cell viability of cancer cells confirms the anticancer properties of the GA. Subramanian et al. highlighted the mechanisms of anticancer activity of GA as dose-dependent apoptosis due to DNA fragmentation and change in the cell morphology of the

lung cancer cells [36]. Kahkeshani et al. have also reported similar mechanisms of anticancer activity of GA involving cell cycle arrest, apoptosis induction, and reactive oxygen species (ROS) generation in the tumors [37]. The IC_{50} value of the GA for cancer cell line A549 was estimated to be $28.91 \pm 1 \mu\text{g mL}^{-1}$, i.e., $170 \mu\text{M}$ (**Table 3.6**), which was in accordance with the previously reported study [38]. GA acid has been reported to be compatible with normal cell lines and is widely used in healthcare products [39]. Similarly, in this study, GA did not show harmful effects on the human normal cell line HEK293 up to the concentration of $50 \mu\text{g mL}^{-1}$ with cell viability $>70\%$. The IC_{50} value of the GA for HEK293 cells was calculated as $91.98 \pm 1 \mu\text{g mL}^{-1}$ (**Table 3.6**), which is threefold higher than that for the cancer cells A549. These observations indicated the cytocompatibility as well as the anticancer properties of GA.

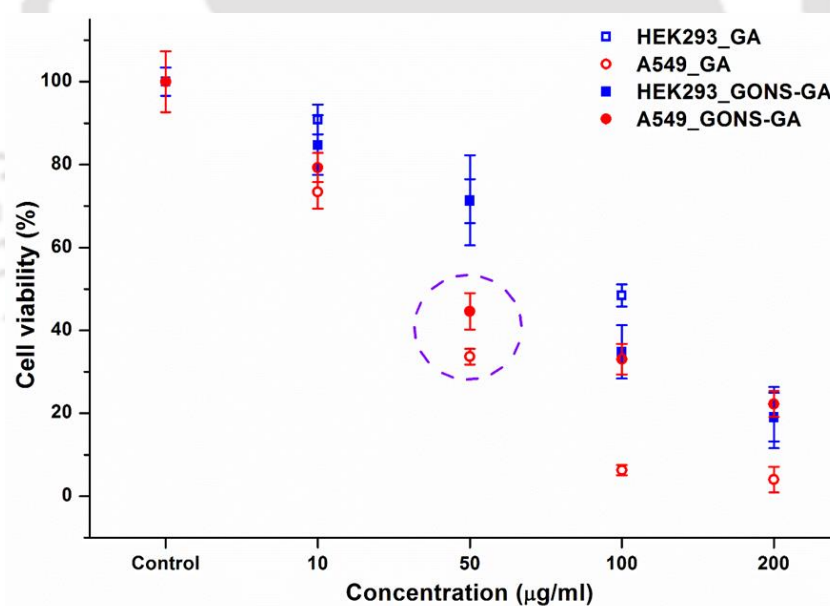


Fig. 3.13: Effect of GA and GONS-GA nanocomposite on the normal cell line HEK293 and cancer cell line A549 (The results are stated as mean $\pm$ SD).

The GA-loaded nanocarrier GONS-GA has also shown good cytocompatibility with HEK293 cells, with 84.73% cell viability at the concentration of $10 \mu\text{g mL}^{-1}$ and 71.4% at $50 \mu\text{g mL}^{-1}$. The IC_{50} value of GONS-GA for the HEK293 cell line was found to be $83.63 \pm 5 \mu\text{g mL}^{-1}$ (**Table 3.6**).

Table 3.6: IC₅₀ values ($\mu\text{g mL}^{-1}$) of the tested samples against the mammalian cells (normal HEK293 cells and cancer A549 cells).

Cell Type	GONS	GA	GONS-GA
HEK293	98.82	91.98	83.63
A549	98.86	28.92	60.7

However, the GONS-GA nanocomposite had displayed decent anticancer properties due to GA loading. The cell viability of cancer cells A549 was reduced to 44.8% at $50 \mu\text{g mL}^{-1}$ of GONS-GA, while the same was 71.4% for the normal HEK293 cells. The IC₅₀ value of the GONS-GA nanocomposite was estimated to be $60.7 \pm 5 \mu\text{g mL}^{-1}$ (**Table 3.6**), which is an intermediate value of that of GA and GONS values. The decreased cell viability of cancer cells due to the GA-loaded GONS nanocarrier has proven its efficiency towards drug delivery applications.

Summary

In the present study, 1D GONS nanocarriers were successfully fabricated via ultrasonication treatment for the anticancer DDS. The electron microscopic images demonstrated flat and exfoliated GO nanosheets along with the spirally wounded, tabular geometry of 1D GONS and GONS-GA. Also, the fabricated GONS have shown a great de-agglomeration tendency which is overcoming the conflicts related to the applications of GO. IR spectroscopy and XPS analysis confirmed the successful loading of GA on GONS. Raman spectroscopic observations have reinforced the potential of GA as a natural antioxidant with reducing structural defects in 1D material such as GONS. XRD analysis authenticates the quality of GO, GONS, and GONS-GA nanocomposites. Alongside the GONS nanocarrier, free drug GA and GA-loaded GONS-GA nanocomposite were found to be highly cytocompatible for normal cells HEK293. However, the obtained GONS-GA nanocomposite showed suitable anticancer activity against the lung cancer cells A549 (IC₅₀ = $60.7 \mu\text{g mL}^{-1}$).

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Chapter 4:

Chitosan-grafted folic acid decorated one-dimensional graphene oxide nanoscrolls: a biocompatible drug cargo for targeted co-delivery of anticancer drugs

Chapter 4

Chitosan-grafted folic acid decorated one-dimensional graphene oxide nanoscrolls: a biocompatible drug cargo for targeted co-delivery of anticancer drugs

The present chapter emphasizes the development of a GONS-based biocompatible drug cargo for a targeted co-delivery of chemotherapeutic agents such as DOX and CA. Herein, GONS were produced through a low-frequency (LF)-ultrasonication technique followed by functionalization with chitosan (CS) biopolymer and FA to further enhance their biocompatibility and target-specific delivery of anticancer drugs, respectively. The structural, functional and morphological attributes of GONS and GONS-CS-FA nanocarriers have been investigated through various characterization techniques. The GONS-CS-FA (GCF) nanocarriers were loaded with DOX and CA at different weight proportions and compositions. Furthermore, to investigate the release characteristics of the GCF nanocarrier, an in-vitro release study was conducted for GONS-CS-FA-DOX-CA (GCF-DOX-CA) nanocomposite at physiological and cancer environment pH conditions, followed by its release kinetics. In addition, in-vitro cytotoxicity assessments of pristine GCF and drug-loaded GCF-DOX-CA nanocomposite were done on cancer cell line (A549) and normal cells (HEK293).

4.1. Experimental

4.1.1. Materials

The details of preparatory materials for the synthesis of GO and GONS are provided in sections 2.1.1 and 3.1.1, respectively. The chitosan (deacetylated degree = 90%) was purchased from SRL Pvt. Ltd. (Taloja, India). Folic acid ($C_{19}H_{19}N_7O_6$, 97%) Sodium hydroxide (NaOH, 98%), N-Hydroxy succinimide (NHS) ($C_4H_5NO_3$, 98%), (1-Ethyl-3-(3-dimethylammoniumpropyl) carbodiimide cross linker (EDC) ($C_{12}H_{22}N_4O_2$, 98%) were purchased from Sigma-Aldrich. The content of this chapter has been submitted for publication as below:

Dimethylaminopropyl (Carbodiimide) hydrochloride (EDC-HCl) ($C_8H_{17}N_3\text{-HCl}$, 99%), and caffeic acid ($C_9H_8O_4$, 98%) were obtained from Merck Life Science Pvt. Ltd. (Mumbai, India) Doxorubicin (DOX) was acquired from Fresenius Kabi oncology limited. Solan, India.

4.1.2. Fabrication of chitosan-grafted GO nanoscrolls (GONS-CS)

The starting material GO and GONS were synthesized by employing the modified Hummers' and LF-ultrasonication techniques; their details are provided in **sections 2.1.2 and 3.1.2** of the previous chapters, respectively.

The obtained GONS were further functionalized with CS and FA. The CS was attached to the surface of GONS through an amidation reaction in the vicinity of NHS and EDC. During this process, an amide bond is created once the carboxyl ($-\text{COOH}$) group of GONS interacts with the amino groups of CS. For instance, an aqueous suspension of GONS (120 mL ; 1 mg mL^{-1}) was prepared via sonication for 15 min. Further, to activate the carboxyl functional entities within GONS, 1200 mg NHS and 600 mg EDC were gradually inserted into the GONS suspension and stirred continuously for the next 4 h. Later, an aqueous solution (120 mL) of chitosan (0.5 mg mL^{-1}) prepared in 1% acetic acid solution was gradually inserted into the GONS suspension and mixed for next 24 h. The obtained GONS-CS suspension was centrifuged, washed, and dried for FA functionalization.

4.1.3. Preparation of folic acid functionalized GONS-CS-FA nanocomposite

The obtained GONS-CS suspension was further subjected to functionalization with targeting ligand folic acid. In typical, 100 mg of GONS-CS powder was dispersed in a PBS buffer solution of pH 7.4 to obtain a 1 mg mL^{-1} suspension. Later, 75 mL of FA solution of 1 mg mL^{-1} concentration was prepared in PBS solution and gradually added to the GONS-CS suspension. The resultant reaction mass was allowed to be mixed under continuous stirring

for the next 24 h. At last, the FA functionalized GONS-CS suspension was collected, washed and filtered with DI water to remove free FA. The obtained GONS-CS-FA (GCF) nanoconjugate was vacuum dried at 50 °C overnight and stored for further characterization and drug loading experimentations. The schematics of GONS functionalization with CS and FA is presented in **Fig. 4.1**.

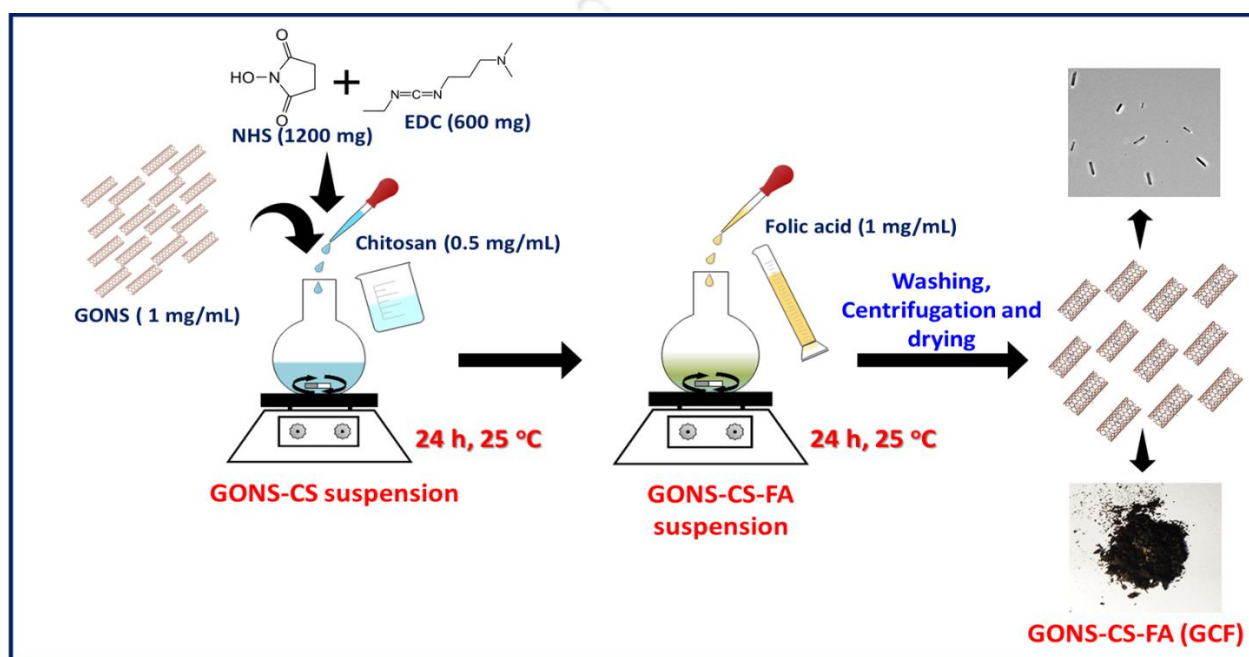


Fig. 4.1: Schematic description of GONS functionalization with CS and FA.

4.1.4. Dual drug loading process

The chemical and natural chemotherapeutic agents DOX and CA were co-loaded on the GONS-CS-FA nanoconjugate as per the previously reported protocols [1,2]. The loading experiments were conducted at different amount of chemotherapeutic agents (drugs) with respect to GCF nanoconjugate (nanocarrier) and drug compositions. Initially, the stock solutions of DOX and CA of concentration 1 mg mL⁻¹ were prepared in PBS buffer. In a typical drug loading process, 10 mg of GCF nanocarrier was added to the 5 mL drug solution containing an equal amount of DOX and CA with drug composition 1:1 to result in GCF-DOX-CA suspension of mass ratio of 1:0.5 for GCF nanocarrier: drug. Later, this suspension

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was positioned in an incubator shaker in dark conditions at 200 rpm for 24 h at 25 °C. The resultant GCF-DOX-CA suspension was exposed to centrifugation at 10000 rpm for 20 min. The supernatant was collected and diluted to determine the concentration of free DOX and CA using UV-Vis spectroscopy at $\lambda_{\text{max}} = 480$ and 325 nm, respectively. The GCF-DOX-CA suspension obtained after centrifugation was retrieved and vacuum dried at 40 °C for subsequent analysis. The concentration of DOX and CA in the supernatant was used to evaluate the amount of drugs unloaded and calculate the drug loading efficiency (LE%) of the GCF nanocarrier for individual drugs by the following equation:

$$\text{Loading Efficiency (\%LE)} = \frac{W_1 - W_2}{W_3} \times 100 \quad (4.1)$$

W_1 , W_2 , and W_3 denote different weights of drugs at the initial condition, free drugs in the supernatant, and drug-loaded nanocomposites (GCF-DOX-CA), respectively. Later, to inspect the effect of a variable quantity of drugs on the loading efficiency of the GCF nanocarrier, an identical procedure was conducted with 1:1 drug composition and varying nanocarrier: drug mass ratios as 1:1, 1:1.5, 1:2, 1:2.5, and 1:3.

Furthermore, the nanocarrier: drug mass ratio with maximum loading efficiency was employed to investigate the impact of drug composition on the loading efficiency of the GCF nanocarrier. Herein, the drug composition was varied as DOX: CA ration of 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, and 3. All loading investigations were done in triplicate, and the numerical findings were described with the mean and standard deviation (SD). After being loaded with DOX and CA, the GCF-DOX-CA nanocomposite was vacuum dried at 40 °C and subjected to further characterization.

4.1.5. *in-vitro* release of DOX and CA

Two distinct pH conditions, pH 7.4, representing physiological surroundings, and pH 5, signifying the cancer microenvironment, were employed to investigate drug release

characteristics of drug-loaded nanocomposite using phosphate-buffer solution (PBS) and sodium acetate buffer (SAB), respectively. The GCF-DOX-CA nanocomposite (20 mg) with the highest loading efficiency for DOX and CA was added to the 20 mL PBS and SAB buffer solutions and exposed to an incubator at 37 °C, 200 rpm for a duration of 7 days. The release samples were assembled from these solutions at a pre-set time interval of 15 min. For the next 15 min., these solutions were centrifuged at 10000 rpm, and later, the concentration of DOX and CA released was determined to set up the drug release profile for both the buffer solutions. The release experiments were carried out twice, and the mean $\pm$ standard deviation is represented for the numerical data.

4.1.6. Cytotoxicity study

The cell viability of HEK293 (human embryonic kidney) and A549 (human lung epithelial carcinoma cells) is determined by MTT assay (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) to check the compatibility of synthesized nanocarriers. The complete DMEM medium was used to culture the cell lines, which were then incubated in a CO₂ incubator at 37 °C. In a 96-well culture plate, 2×10^4 cells were seeded per well during experiments. Once the cells reach 70-80% confluency, the old media is removed and replaced with fresh DMEM media. Additionally, different concentrations of pure nanocarrier GCF and GCF-DOX-CA nanocomposite obtained after co-loading of DOX and CA were subjected to the cells for 48 h (ranging from 0, 100, 200, 300, and 400 $\mu\text{g mL}^{-1}$). After removing the media, 500 $\mu\text{g mL}^{-1}$ of MTT reagent was added to each well and incubated for 4 h. Following that, the MTT solution was separated from the plate, and DMSO was inserted into each well to dissolve the formazan crystals. Incubate the plate for 10-15 min. and measure the absorbance at 570 nm using a microplate reader (Epoch microplate spectrophotometer, Biotek) to determine the % cell viability [3,4].

4.1.7. Characterization techniques

The structural, functional, and morphological characteristics of prepared GONS and GONS-CS-FA (GCF) nanocarriers and GCF-DOX-CA nanocomposite were analyzed by FETEM, XPS, FTIR, FESEM, X-ray diffraction, and Raman spectroscopy; the instrumentation details are provided in **section 2.1.4** and **3.1.7**. DOX and CA concentrations were calibrated at 480 and 325 nm using a UV-Vis spectrophotometer (Thermofisher Scientific Orion Aquamate 8000), respectively. To examine the nanocarriers' oxidation status and reduction properties, Raman spectra were achieved at 633 nm excitation wavelength (Horiba Jobin Vyon LabRam HR model).

4.2. Results and discussion

4.2.1. Surface morphology of nanocarriers

TEM and FESEM investigations were used to evaluate the morphological attributes of GO, GONS, and GONS-CS-FA (GCF) nanocarriers and co-loaded GCF-DOX-CA nanocomposite. **Figure 4.2** shows TEM images of GO, GONS, GCF, and GCF-DOX-CA. A modified Hummers' technique was used to create exfoliated single-layer GO nanosheets (**Fig. 4.2a**), which had a few wrinkles around the edges. Additionally, **Fig. 4.2b** shows the GONS produced by LF-ultrasonication with completely scrolled morphology formed by successively scrolled thin and flat GO nanosheets. Contrary to CNTs, the GONS developed from scrolled GO nanosheets have open ends, making the inner wall region more readily available for drug molecules to transfer [5,6].

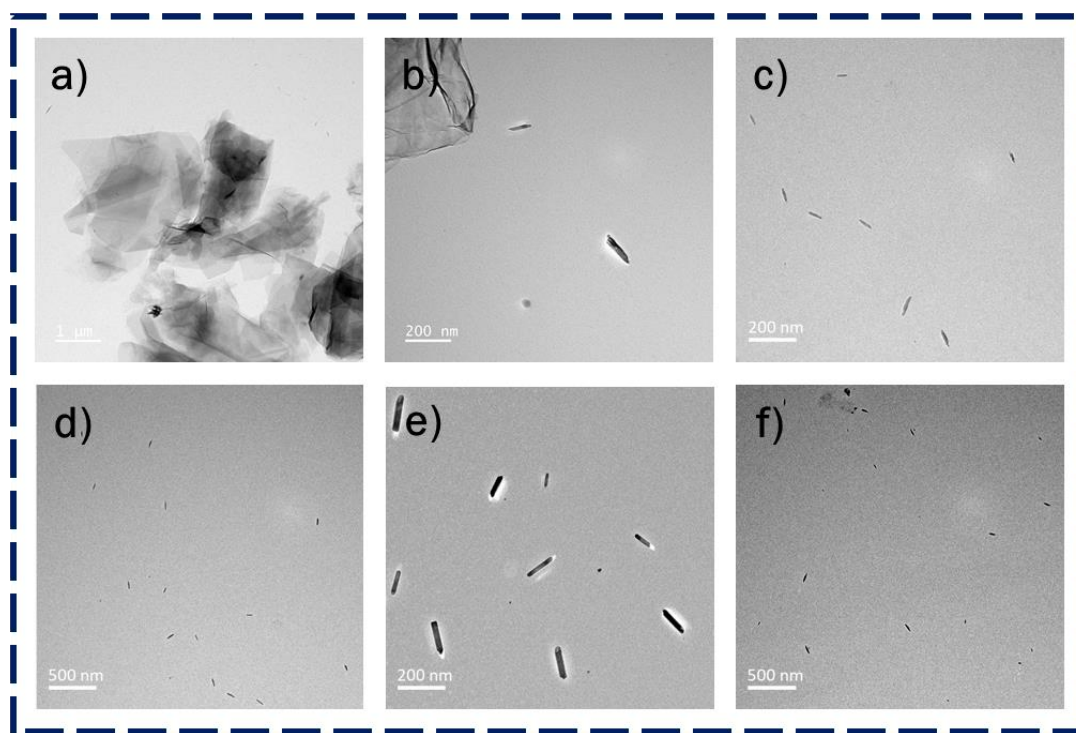


Fig. 4.2: FETEM illustrations of (a) GO, (b-c) GONS, (d) GCF nanocarrier, and (e-f) GCF-DOX-CA nanocomposite.

Additionally, as demonstrated in **Fig. 4.2c**, the produced GONS have a strong de-agglomeration ability, which resolves the diverge of GO deployments in numerous real-world applications since aggregation of nanosheets leads to reducing the active surface sites for mass transfer. Owing to an upsurge in the active surface area, these distinctive properties of GONS may offer considerable benefits for their application in DDS [7]. Previous investigations revealed that the sonication configurations appropriately influence the GONS dimensions (length) [6,7]. Therefore, as defined in the previous chapter, the sonication configurations in the current study were maintained constant across the GONS production processes. Furthermore, the obtained nanoscrolls were simultaneously functionalized via CS and FA targeting ligands to synthesize GCF nanocarrier, which was further co-loaded with natural and conventional chemotherapeutic agents CA and DOX. The identical morphological attributes were observed for the GCF nanocarrier (**Fig. 4.2d**) and drug-loaded GCF-DOX-CA nanocomposite (**Fig. 4.2e-f**) along with the de-agglomeration tendency and

smaller size of scrolls. Herein, the average length of GONS obtained via sonication treatment provided in **Fig. 4.2c** is ~ 190 nm, whereas GCF-DOX-CA nanocomposite exhibited an average length of ~ 175 nm (**Fig. 4.2 e-f**) (Assessed via ImageJ software).

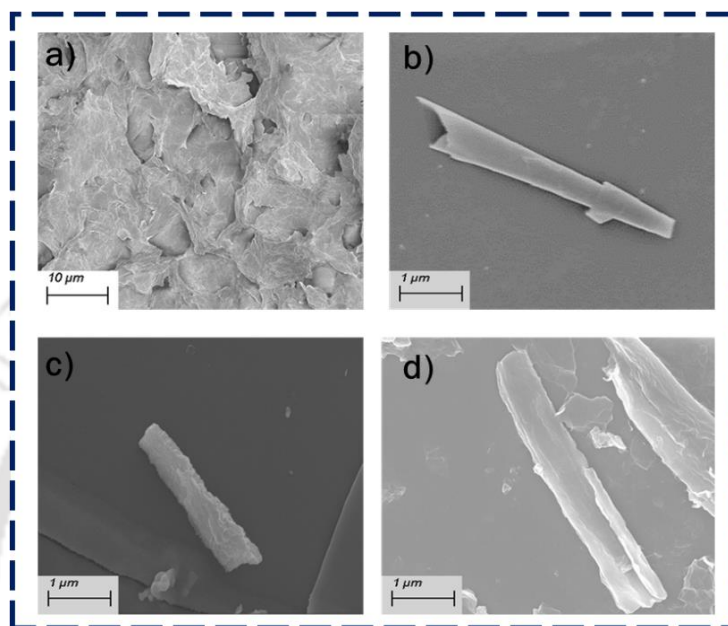


Fig. 4.3: FESEM illustrations of (a) GO, (b) GONS, (c) GCF nanocarrier, and (d) GCF-DOX-CA nanocomposite.

The FESEM descriptions of GO, GONS, GCF, and GCF-DOX-CA nanocomposite are presented in **Fig. 4.3**. In this instance, exfoliating GO nanosheets created using the modified Hummers' technique before being subjected to sonication treatment are apparent. The nanosheets were piled on top of one another, which caused the outer and inner surfaces of the underneath and overlaying nanosheets to end up in lower active surface sites. Such agglomerative characteristics were absent for GONS, GCF, and GCF-DOX-CA nanocomposites.

4.2.2. Chemical interaction study

4.2.2.1. FTIR spectroscopy

The existence of respective functional groups in GONS, GCF, and GCF-DOX-CA nanocomposites was investigated through a chemical interaction study. Additionally, XPS

analysis was conducted to interpret the data pertaining to the reduction attributes of the GONS and GCF nanocarrier. **Figure 4.4** illustrates the FTIR spectra of pristine GONS, GONS-CS, GCF, and co-loaded GCF-DOX-CA nanocomposite. The existence of hydroxyl, epoxy, carbonyl, and carboxyl oxygenated functional units within GONS were illustrated by characteristics peaks at 3430 cm^{-1} , 1052 cm^{-1} , and $1628\text{--}1730\text{ cm}^{-1}$, correspondingly [6,8]. In the case of the GONS-CS nanocomposite, the C=O stretching of the carboxyl-carbonyl units in GONS observed at 1730 cm^{-1} vanished due to the amide linkage amongst GONS and CS [9]. Furthermore, the emergence of peaks at 1069 cm^{-1} , 1152 cm^{-1} , and 1550 cm^{-1} demonstrates the C-N, N-H bending from NH_2 along with the augmentation in the transmittance of other characteristics peaks of GONS signifies interactions among oxygenated functional units of GONS and CS [9,10]. After functionalization with FA, the resultant GONS-CS-FA (GCF) nanocarrier exhibited undistinguishable functional units to that of GONS-CS with a slight budge in the bands and the appearance of the distinctive peaks of FA at 1677 cm^{-1} , 1384 cm^{-1} , 1069 cm^{-1} , 850 cm^{-1} correspond to the carboxylic acid group, C-H, C-N and C-H bending vibrations, respectively [10,11]. Also, the wide absorption peaks around 3550 cm^{-1} and 3102 cm^{-1} correspond to stretching in the O-H and C-H bonds of amine groups of FA [12].

Moreover, after loading the DOX and CA, the obtained GCF-DOX-CA nanocomposite illustrated decreased in the transmittance around $3100\text{--}3500\text{ cm}^{-1}$ due to an increase in the concentration of hydroxyl (-OH) functional within the drug-loaded nanocomposite. The reduction in the transmittance implies that hydroxyl functional groups from DOX and CA are conjugated to the structure of the GCF nanocarrier and establish the effective formation of the GCF-DOX-CA nanocomposite. Also, the existence of a specific peak position at 810 cm^{-1} linked to the DOX C-O-CH₃ group stretching vibrations was observed along with the

characteristic peaks at 1377 cm^{-1} representing the out-of-plane bending vibrations of carbonyl groups of CA [2,13].

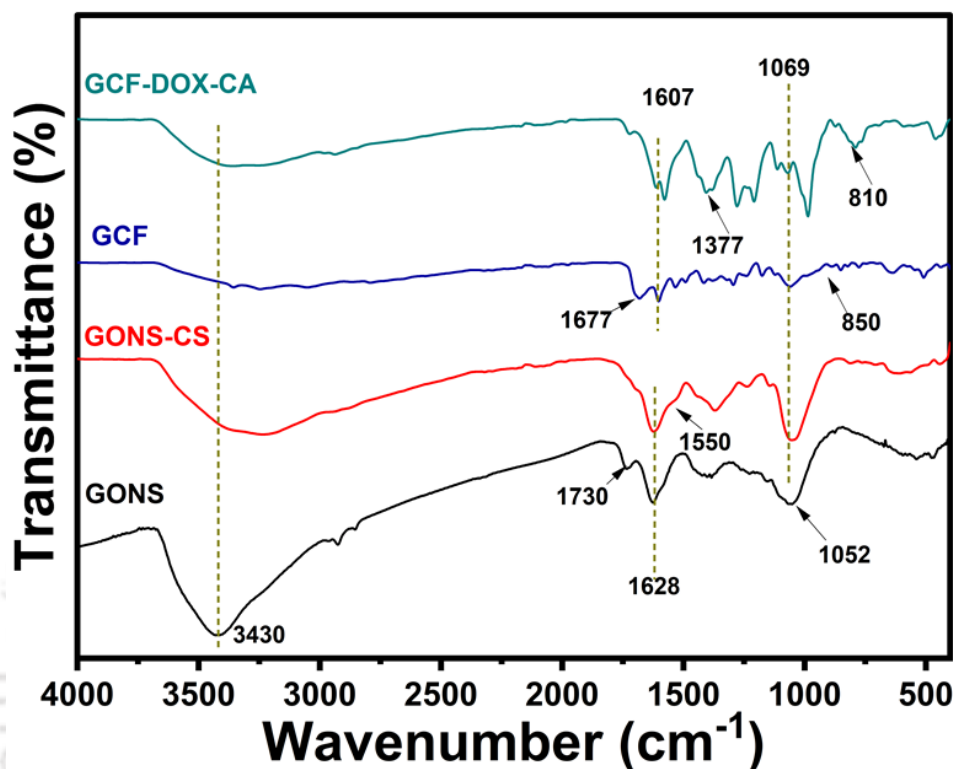


Fig. 4.4: FTIR spectrums of GONS, GONS-CS, GCF nanocarrier and GCF-DOX-CA nanocomposite.

4.2.2.2. X-ray photoelectron spectroscopy (XPS)

The investigation of corresponding functional groups and elements present in the interior of GONS, GCF nanocarrier, and GCF-DOX-CA nanocomposite was done via XPS analysis (**Fig. 4.5**). The elemental survey spectrums of pristine GONS, GCF, and GCF-DOX-CA nanocomposite is provided in **Fig. 4.5A**, which demonstrated the existence of O, C, and N elements in nanocarrier and nanocomposite. A deconvoluted high-resolution C1s and O1s spectrums of GONS are discussed in **section 3.2.2.2** of the previous chapter. An elemental survey spectrum of the GCF nanocarrier discloses the existence of O, C, and N elements (**Fig. 4.5A**). The deconvoluted C1s, O1s, and N1s XPS spectra of GCF are depicted in **Fig. 4.5C**. The C1s spectra produced four distinctive peaks at binding energies (BE) of 284.7 eV, 286.2

eV, 287.4 eV, and 288.5 eV, implying a negative shift in the BE (0.2-0.3 eV) of functional units of GONS, which may be due to the functionalization and ultimate interaction with CS and FA molecules. Likewise, the O1s spectrum of GCF nanocarrier exhibited identical functional units to GONS; however, the intensity of these function entities had been impeded by the reduction in the oxygenated functional groups and positive shift in BE. The reduction behavior was further endorsed via the elemental analysis listed in **Table 4.1**. A deconvoluted N1s spectrum of GCF nanocarrier further advocated the successful functionalization of GONS by CS and FA with the occurrence of a peak at 399.5 eV and 401.5 eV illustrating C-N/C=N and N-H/NC=O, separately [14–16].

The XPS survey spectrum for GCF-DOX-CA nanocomposite (with DOX and CA co-loaded on the GCF nanocarrier) is provided in **Fig. 4.5A**, demonstrating the existence of C, N, and O elements. In the case of GCF-DOX-CA nanocomposite (**Fig. 4.5D**), deconvoluted C1s spectra acceded into four primary peak positions at 284.7 eV, 285.8 eV, 286.4 eV, and 287.8 eV, illustrating identical functional entities to that of GCF nanocarrier with a peak at 285.8 eV representing the C-N functional groups of DOX. However, a positive swing (0.2-0.4 eV) in the BE of other functional groups was observed, which may be due to the partial reduction of GCF nanocarrier via DOX and CA by means of their antioxidant nature. Similarly, the O1s spectra yielded into the identical functional groups of GCF; however, it was observed that the intensity of hydroxyl (C-OH) groups was increased drastically, which may be due to the co-loading of DOX and CA, which are rich in hydroxyl groups. These findings were also endorsed by FTIR analysis. Furthermore, the N1s spectra advocated an interaction amongst DOX and CA molecules with GCF nanocarrier through a slightly negative shift in the binding energies. This shift could be ascribed to an electron energy transfer from nitrogen to the GCF surface through non-covalent interactions among GCF with CA and DOX molecules [17,18].

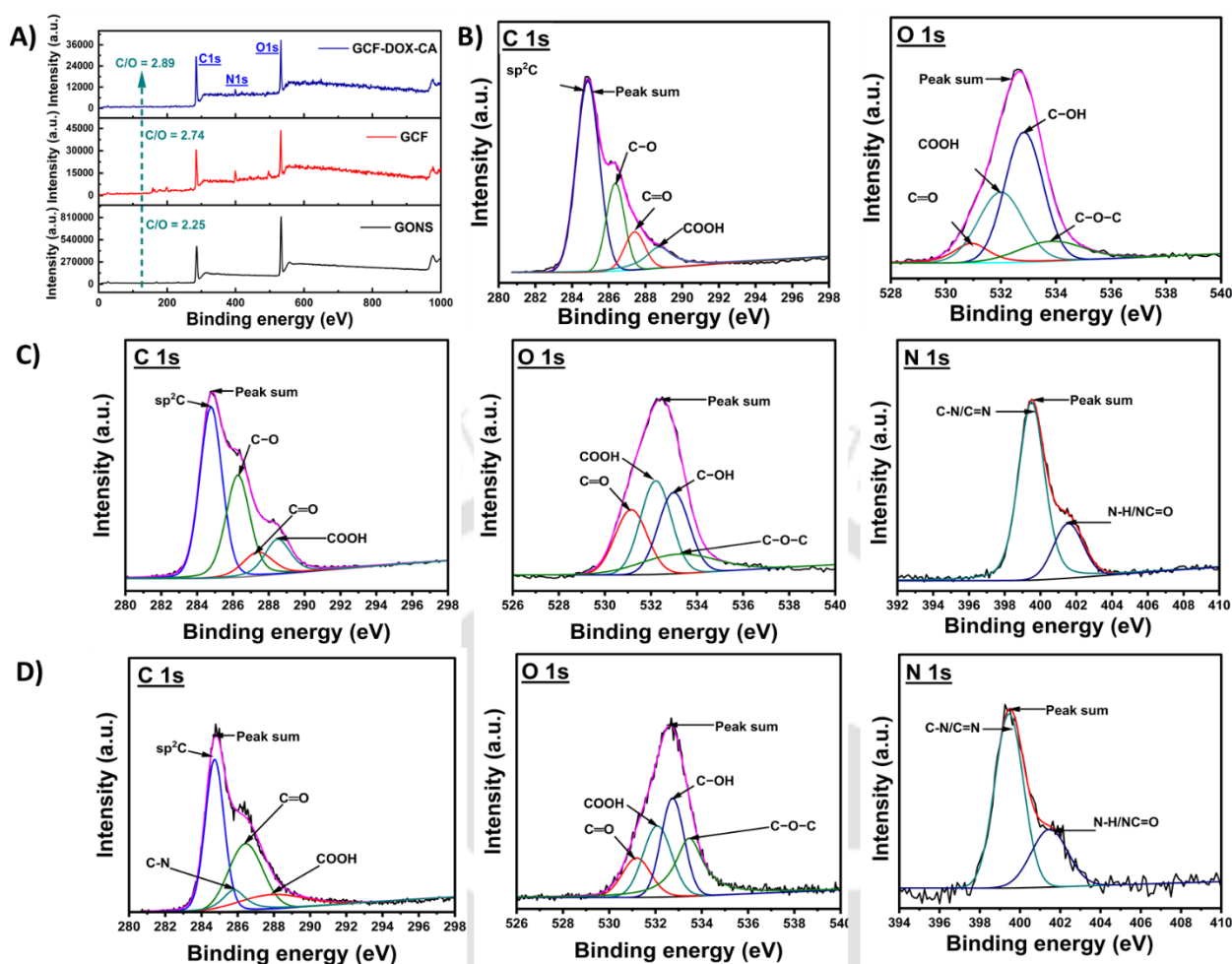


Fig. 4.5: XPS spectrums (A) Survey spectra, Deconvoluted spectrums of (B) GONS, (C) GCF nanocarrier and (D) GCF-DOX-CA nanocomposite.

Later, elemental analysis was conducted using XPS analysis and reported in **Table 4.1** to endorse the fabrication of GONS, GCF nanocarrier, and GCF-DOX-CA nanocomposite. The GONS fabricated via the LF-ultrasonication method resulted in lower oxygenated functional groups to that of GO due to reduction caused via ultrasonication treatment [6,7]. The 1D GONS exhibited C/O at. ratio of 2.25. Further, the CS and FA molecules were conjugated on GONS, which caused into the subsequent reduction in GONS to produce GCF nanocarrier, giving a 2.74 C/O atomic ratio. After loading with dual drugs, namely, DOX and CA, the ratio was increased to 2.89, owing to their antioxidant characteristics [2,19]. This increase in the atomic ratio owing to the partial reduction of GONS also resulted in a decrement in the d-

spacing, as validated by XRD analysis. The provided findings of the XPS study further validate the successful formation of GCF nanocarrier and GCF-DOX-CA nanocomposite.

Table 4.1: Elemental analysis of nanocarriers.

Samples	Elemental composition (at. %)			
	C	O	N	C/O
GONS	69.31	30.69	-	2.25
GCF	71.21	25.95	2.84	2.74
GCF-DOX-CA	71.04	24.66	4.30	2.89

4.2.3. X-Ray diffraction

Figure 4.6 elucidates the XRD spectrums of GO, GONS, GCF, and the drug-loaded GCF-DOX-CA nanocomposite. A strong peak diffraction pattern at around 26.6° is present in the graphite with greater van der Waals interactions, which is consistent with its graphitic origin [6,20]. However, once graphite was oxidized with the production of GO, the graphitic plane was utterly disappeared; the details are provided in **section 2.2.7**. As indicated in the newly formed peak at 10.68° , the oxidation progression diminishes van der Waals interaction within the graphitic planes and creates truly exfoliating GO nanosheets. Additionally, the XRD spectrum of GONS produced following the sonication treatment revealed a number of additional peaks at 20.16° , 24.08° , and 44.52° , as well as a significantly reduced GO peak (10.84°) with a d-spacing of 0.82 nm. A 0.44 nm d-spacing and the (0 0 2) plane are assigned to the peak location at 20.16° . According to XPS analysis, there is a likely decrease in the oxygenated functional regions of GO due to the sonication treatment throughout the synthesis of GONS. The d-spacing is 0.37 nm for the wider peak at 24.08° and 0.20 nm for the one with a greater intensity peak at 44.52° . Additionally, the increasingly compact stacking of the GONS structure shown by such peaks with reduced interlayer spacing assists in differentiating GONS from GO [6].

In the case of the GCF nanocarrier, a sharp shift in the crystalline plane of GONS from 10.84° to 8.98° was observed with an interplanar spacing of 0.98 nm, which may be attributed to the cross-linking of GONS with CS and FA [9,21]. Similarly, the contraction in other peaks of GONS was observed with improved crystallinity, suggesting the successful impregnation of folate functional entities, as illustrated in FTIR and XPS analysis.

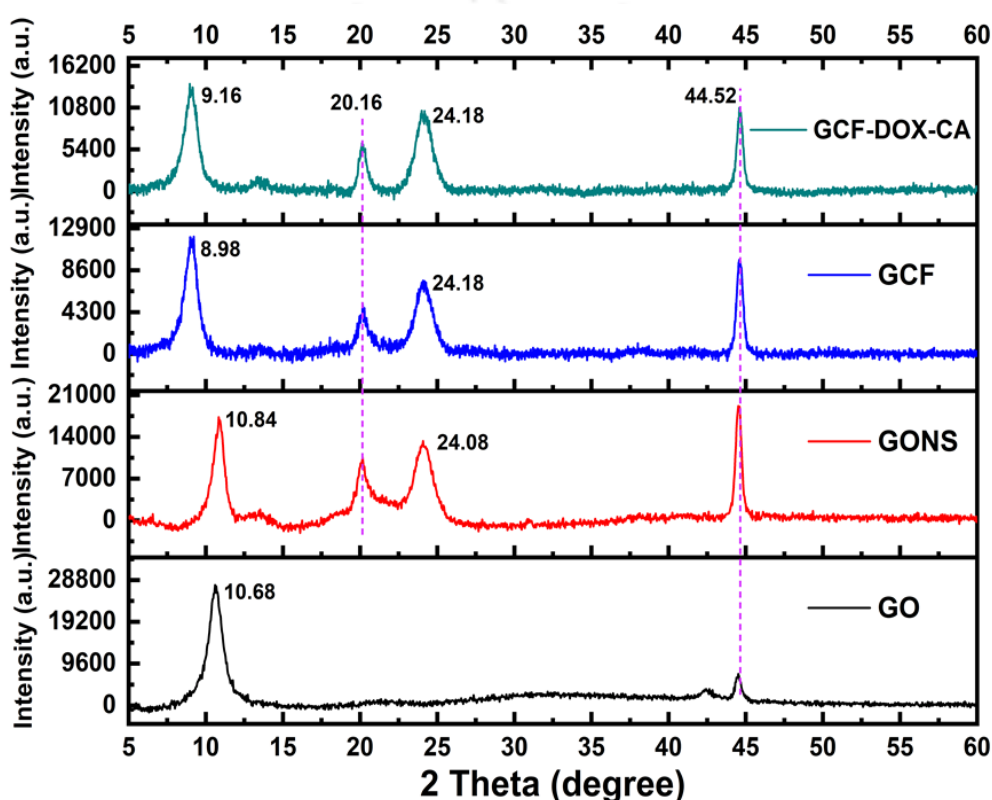


Fig. 4.6: X-ray diffraction spectrums of GO, GONS, GCF nanocarrier and GCF-DOX-CA nanocomposite.

Again, a major shift in the crystalline plane of GCF was observed from 8.98° to 9.16° with an interplanar distance of 0.97 nm, which may be due to the partial reduction in the oxygenated functional units of GCF nanocarrier. Such increment in the 2θ and the intensity of other peaks of graphitic planes signifies the successful impregnation of DOX and CA. Also, the partial reduction of GCF nanocarrier can be attributed to the antioxidant behavior of DOX as well as CA [22,23]. It was observed that the amorphous phase of GCF had been rightly

preserved during the loading process, suggesting the structural stability of the GCF-DOX-CA nanocomposite.

4.2.4. Raman Spectra

Raman spectroscopy, being a non-invasive characterization technique, offers significant findings regarding carbon-based nanomaterials. **Fig. 4.7** displays typical Raman spectra for GONS, GCF nanocarrier, and GCF-DOX-CA nanocomposite acquired at an excitation wavelength of 633 nm. For GONS, two distinctive peaks of the D-band at 1335 cm^{-1} and the G-band at 1588 cm^{-1} were discovered. Earlier, it was reported that the characteristic band peaks differ with excitation wavelength values ranging from $1320\text{--}1370\text{ cm}^{-1}$ and $1550\text{--}1630\text{ cm}^{-1}$ for D and G-band, separately [8,24]. The G-band depicts sp^2 hybridized in graphitic structure in the hexagonal-pinch form, whereas the D-band symbolizes the defects [25]. In contrast to GONS, the D-band in the GCF nanocarrier was slightly broader, which suggests the covalent conjugation of CS and FA on the GONS carbon skeleton.

The intensity (I_D/I_G) ratio for GONS was observed as 1.19, which was reduced to 1.05 after the conjugation of CS and FA. This retardation in the intensity ratio demonstrates fractional renewal of the sp^2 domains of GONS during the interaction of CS and FA macromolecule on the surface of GONS [7,26]. Furthermore, a Raman spectrum of GCF-DOX-CA nanocomposite reveals an identical D-band and blue shift in the G-band ($\sim 5\text{ cm}^{-1}$) with retardation in I_D/I_G ratio to 1.03, suggesting a stronger interaction among GCF nanocarrier with DOX and CA. Overall, the findings of the elucidated characterization studies validate the successful formation of GONS, GCF nanocarrier, and GCF-DOX-CA nanocomposite.

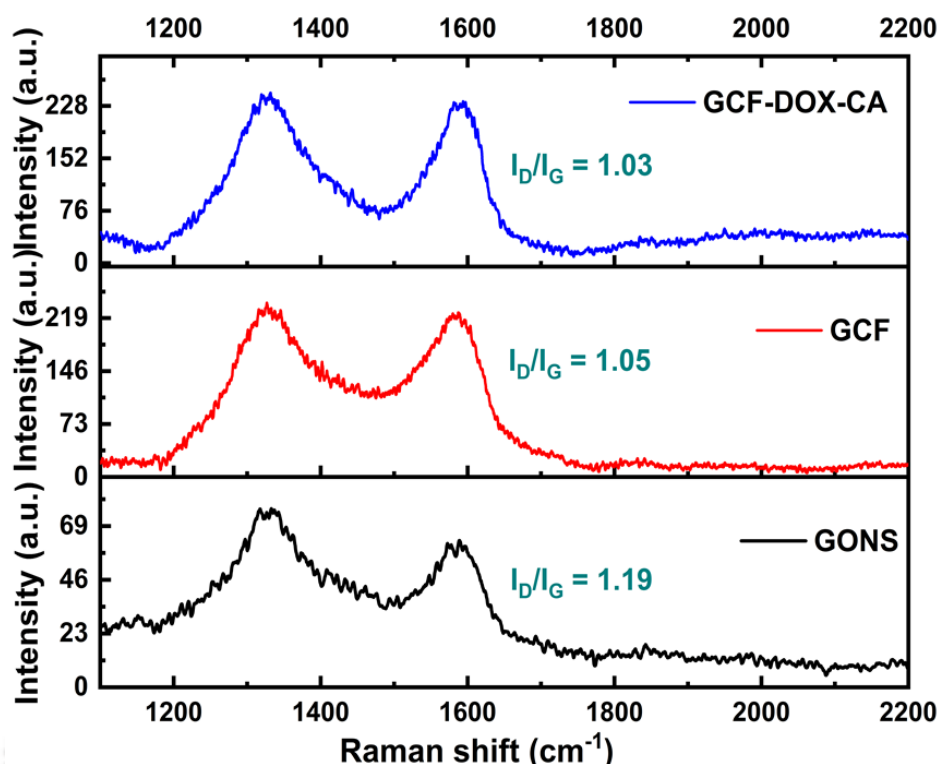


Fig. 4.7: Raman spectrums of GONS, GCF nanocarrier, and GCF-DOX-CA nanocomposite.

4.2.5. Loading characteristics

The drug molecules of the commercial (DOX) and natural chemosensitizer (CA) were successfully impregnated over the GCF nanocarriers. The loading experiments were conducted as per the protocol illustrated previously by varying the amount of drugs and compositions. The DOX and CA were co-loaded by intermolecular interactions in conjunction with the van der Waals, strong electrostatic, and π - π interactions provided by the GCF nanocarrier.

4.2.5.1 Effect of amount of drug

Figure 4.8A demonstrates the influence of drug vs. nanocarrier mass proportion on dual-drug loading efficiency (LE). The co-loading experiments were conducted under the identical parameters of drug composition 1:1, 25 °C, 200 rpm, and a 24 h loading period. The drug LE was observed to be increased with an upsurge in the drug quantity in regard to GCF, and it was highest for the 2.5 w/w ratios with LE of $51.14 \pm 1.47\%$ for DOX and $49.70 \pm 1.19\%$ for

CA. As previously mentioned, the loading of DOX and CA depends on non-covalent interactions amongst the oxygenated functional groups of drugs and the GCF nanocarrier [27,28]. Contrarily, a rise in drug concentration (>2.5 w/w) causes a decline in drug loading, which may be brought on by the absence of additional surface-active sites and associated interactions between the drug and GCF nanocarrier.

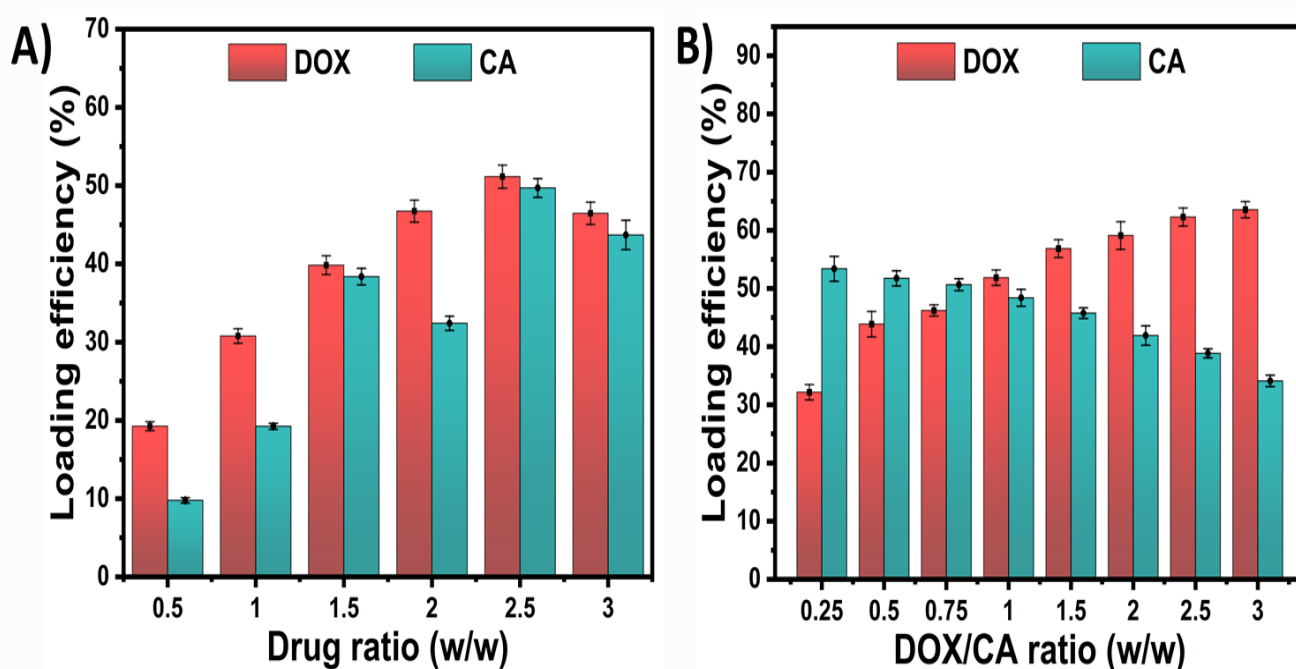


Fig. 4.8: DOX and CA loading characteristics of GCF nanocarrier for the variable (A) drug amount and (B) drug composition. The results are quantified as mean $\pm$ SD (N=3).

4.2.5.2 Effect of drug composition

The drug: nanocarrier ratio of 2.5 with the highest LE was further used to investigate the effect of drug composition. The drug composition, i.e., DOX:CA ratio, was varied from 0.25-3 (**Fig. 4.8B**). It was perceived that with an upsurge in drug composition, in contrast to CA, the LE of DOX was increased, which may be due to the augmentation in the amount of DOX and less accessibility of surface moieties for CA. The trend had been continued for DOX:CA ratio of 3 w/w. The optimum drug LE was considered as $51.14 \pm 1.47\%$ for DOX and $49.70 \pm 1.19\%$ for CA with 1 w/w drug composition; as a further increase in drug composition, a sudden decrease in the encapsulation efficiency of DOX as well as CA was detected. The

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maximum encapsulation efficiency of $93.91 \pm 1.88\%$ for DOX and $74.99 \pm 1.39\%$ for CA was observed for a DOX: CA drug composition of 1 w/w, drug: nanocarrier ratio of 2.5 w/w. The GCF-DOX-CA nanocomposite produced after co-loading was vacuum dried at $40\text{ }^{\circ}\text{C}$ overnight before being used to investigate the *in-vitro* release behavior and cytotoxicity. **Table 4.2** illustrates a detailed comparison of the drug loading efficiency of GCF nanocarriers with other nanocarriers.

Table 4.2: Comparative study of loading efficiency for DOX and natural chemotherapeutic agents.

Nanocarrier	Synthesis method	Chemotherapeutic agent	Drug loading efficiency (%)	References
MIL-100 (Fe)	Hydrothermal route without HF	DOX	23.40	[29]
GO@ MIL-100 (Fe)(GMF)	RT	DOX	29.91	[19]
MgAl-LDH/Fe-MOF	Low-temperature preparation ($40\text{ }^{\circ}\text{C}$)	DOX and methotrexate (MTX)	21.0 and 4.2	[30]
UPNC@MIL-100 (Fe)	RT	DOX	17.20	[31]
β -CD conjugated sialic acid	-	DOX	1.13	[32]
GO-CD/AD-COOH	-	DOX, TPT	15.2, and 16.2	[33]
β -CD-MGO	-	DOX, MTX	37.4, and 23.4	[34]
GONS	Ultrasonication	Gallic Acid (GA)	29.82 ± 0.8	[7]
GO	Improved Hummers' method	GA	12	[35]
GO	Improved Hummers' method	Chlorogenic acid	13.1	[36]
GONS	Ultrasonication	DOX and Caffeic acid (CA)	38.52 ± 1.24, and 33.36 ± 1.04	Present study
GONS-CS-FA	RT	DOX and CA	$51.14 \pm 1.47\%$ and $49.70 \pm 1.19\%$	Present study

4.2.6. Surface charge analysis

For the prospective utilization of nanomaterials in biomedical applications like drug delivery, their colloidal stability within physiological configurations is a crucial factor. The

bioavailability of nanocarriers may be increased with higher colloidal stability [37]. The zeta (ζ)-potential of nanocarriers, which represents the strength of electrostatic attraction/repulsion among nearby molecules with surface charge, may be used to assess the dispersion attributes of nanocarriers to stability [38]. In order to comprehend the drug loading and release process, a surface charge derived from the ζ -potential remains essential [39]. Herein, the ζ -potential (Delsa nano C, Beckman Coulter) analysis was conducted to determine the surface charges of the pristine GONS, GCF nanocarrier, and GCF-DOX-CA nanocomposite by preparing and analyzing their 50 mg L⁻¹ solutions at pH 7.4.

The fabricated GONS depicted a negatively (-ve) charged surface with a ζ -potential of -24.2 ± 1.5 mV. After functionalization with the FA and CA, the surface charge of the GCF nanocarrier was decreased to -18.5 ± 1.5 mV, which may be ascribed to the positively charged CS and caused as a result of the presence of amino groups. The GCF nanocarrier with a lower magnitude of ζ -potential displayed relatively lower stability than that of GONS. Meanwhile, the magnitude was improved to -21.3 ± 2.9 mV after loading with DOX and CA. DOX and CA, being a chemotherapeutic agent and chemosensitizer, illustrated the potential of 4.2 ± 1.5 mV and -2.7 ± 1.2 mV, respectively. The positive and lower negative surface charge of these drug molecules offers a driving force for their co-loading over GCF nanocarrier via electrostatic interactions. Overall, it can be predicted that the loading of DOX and CA on the GCF nanocarrier was primarily accomplished via non-covalent interactions. After loading, the GCF-DOX-CA nanocomposite exhibited a negative surface charge with a ζ -potential of -21.3 ± 2.9 mV, suggesting suitable colloidal stability for their application in DDS.

4.2.7. Release characteristics and kinetics study

The *in-vitro* release characteristics of DOX and CA out of the GCF-DOX-CA nanocomposite into PBS and SAB buffer solutions (for mimicking the physiological and tumor microenvironment at pH 7.4 and 5, individually) were investigated at 37°C (normal human body temperature) for 7 days. The release contour of DOX and CA in SAB and PBS solutions are illustrated in **Fig. 4.9**. In the human body, pH has a substantial biological stimulus; extra-cellular pH in normal tissue and tumor tissue is 7.4 and 4.5-6.5, respectively [40]. The drug release from a nanocarrier can be studied from a DDS constructed using these pH variations. Herein, an initial burst release with $15.96 \pm 0.60\%$ and $19.60 \pm 0.49\%$ of DOX and CA were observed within the initial 2 h for PBS solution. Whereas in SAB solutions, $20.13 \pm 0.62\%$ and $18.98 \pm 0.48\%$ of DOX and CA were released out of the GCF-DOX-CA nanocomposite. Such initial burst release, based on the nanocarrier's surface characteristic, could be essential in reaching the initial therapeutic level with an appropriate dosage of DOX at tumor sites. Furthermore, around 47% and 43% of DOX, 44%, and 52% of CA were released in PBS and SAB buffer solutions after a period of 24 h. After that, a sustained release profile was observed for DOX and CA release from the GCF-DOX-CA nanocomposite. In the initial 48 h, $50.98 \pm 1.19\%$ and $58.72 \pm 1.43\%$ of DOX and $48.52 \pm 1.23\%$ and $62.19 \pm 1.32\%$ CA release were observed, which was followed by the sustained release for 7 days (168 h) with $72.15 \pm 1.80\%$ and $82.44 \pm 2.47\%$ DOX and $60.59 \pm 1.63\%$ and $74.20 \pm 0.83\%$ CA release through the GCF-DOX-CA nanocomposite at pH 7.4 (PBS) and 5 (SAB), respectively.

Under acidic pH, the higher DOX and CA were released from the GCF-DOX-CA nanocomposite, which occurs as the hydrogen bonds between the drug molecules and nanocarriers become weak. Under basic conditions, an opposite trend is followed. It also

suggests that the drug will be distributed selectively at tumor sites as they are acidic in nature, which will help avoid the undesirable effects of chemotherapy [41,42]. The drug release was supplemented by an early burst release phase accompanied by a sustained release segment over a longer period of time. Such findings have also demonstrated the pH-dependent release of DOX and CA from GCF nanocarriers. Moreover, such environmentally sensitive release behavior may minimize drug loss during delivery while increasing the drug release once the nanocarrier arrives at the tumor site [43].

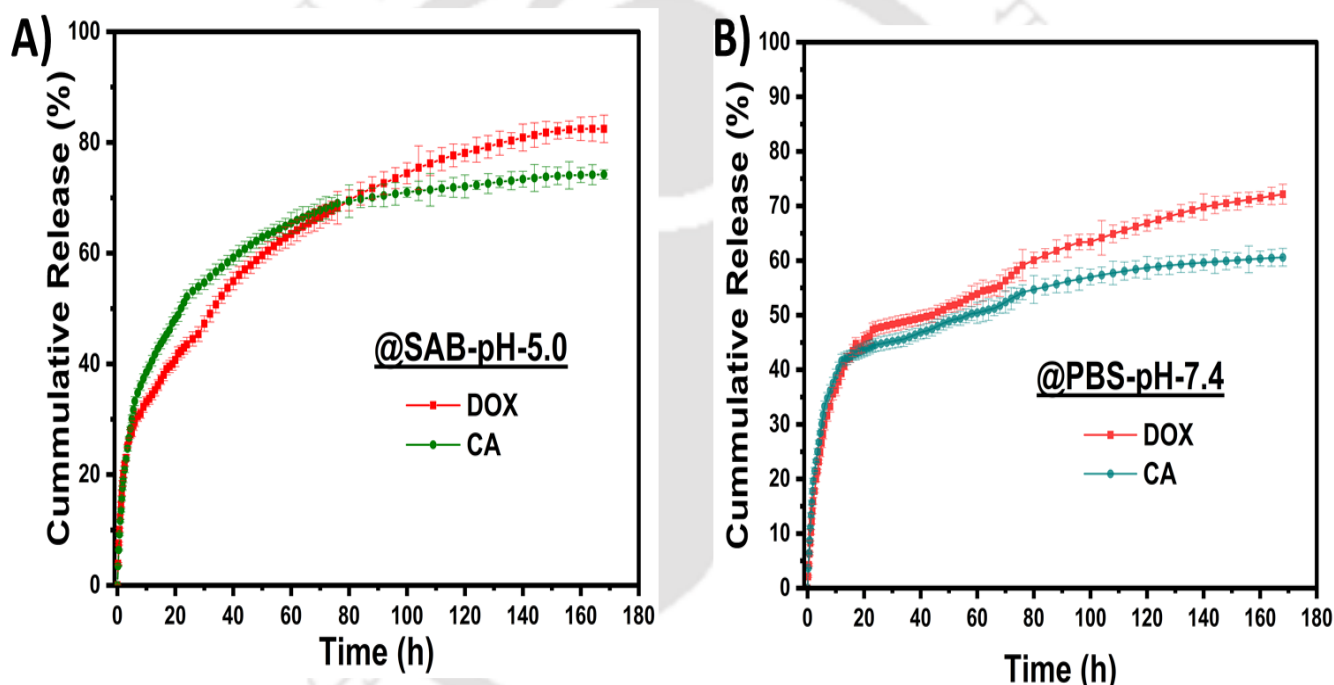


Fig. 4.9: Release behavior of DOX and CA in (A) SAB (pH = 5) (B) PBS (pH = 7.4). The results are quantified as mean $\pm$ SD (N=2).

Six distinctive drug-release models were exercised to study the release kinetics of DOX and CA from the GCF-DOX-CA nanocomposite in SAB at pH 5 [35,44–47], as given in **Table 4.3**. Where k is the corresponding rate constant of the respective kinetic model, M_t , M_e , and M_∞ are the respective amount of DOX and CA released from the co-loaded GCF-DOX-CA nanocomposite at time t , at equilibrium, and infinite time, individually. n is the diffusional or release exponent. Likewise, the W_0 and W_t are the original and residual amount of DOX and CA at time t , separately.

Table 4.3: Correlation coefficients (R^2), Rate constants (k), and diffusional exponent (n) of kinetic models for the release of DOX and CA from GCF-DOX -CA nanocomposite.

Release media	Drug Release (%)	Kinetic models	R^2	Rate constants (k)	Confidence limits (Upper, Lower)
SAB Buffer pH= 5	DOX = 82.44 ± 2.47 CA = 74.20 ± 0.83	Zero-order	0.85	$k_0 = 7.07 \times 10^{-3} \text{ min.}^{-1}$	$7.11 \times 10^{-3}, 7.03 \times 10^{-3}$
		$\frac{M_t}{M_e} = k_0 t$	0.78	$k_0 = 6.81 \times 10^{-3} \text{ min.}^{-1}$	$6.87 \times 10^{-3}, 6.75 \times 10^{-3}$
		Pseudo-first-order	0.96	$k = 1.01 \times 10^{-2} \text{ min.}^{-1}$	$1.01 \times 10^{-2}, 1.01 \times 10^{-2}$
		$\ln(M_e - M_t) = \ln M_e - kt$	0.81	$k = 7.36 \times 10^{-3} \text{ min.}^{-1}$	$7.44 \times 10^{-3}, 7.28 \times 10^{-3}$
		Pseudo-second-order	0.98	$k = 8.49 \times 10^{-3} \text{ mg. min.}^{-1}$	$8.97 \times 10^{-3}, 8.01 \times 10^{-3}$
		$\frac{t}{M_t} = \frac{1}{kM_e^2} + \frac{t}{M_e}$	0.99	$k = 2.19 \times 10^{-2} \text{ mg. min.}^{-1}$	$2.21 \times 10^{-2}, 2.17 \times 10^{-2}$
		Higuchi	0.97	$k_h = 0.46 \text{ min.}^{-0.5}$	$4.67 \times 10^{-1}, 4.63 \times 10^{-1}$
		$M_t = k_h t^{1/2}$	0.87	$k_h = 0.31 \text{ min.}^{-0.5}$	$3.17 \times 10^{-1}, 3.12 \times 10^{-1}$
		Korsmeyer-Peppas	0.97	$k_p = 1.31 \times 10^{-1} \text{ min.}^{-n}$ $n = 0.38$	$1.33 \times 10^{-1}, 1.29 \times 10^{-1}$
		$\frac{M_t}{M_\infty} = k_p t^n$	0.97	$k_p = 1.39 \times 10^{-1} \text{ min.}^{-n}$ $n = 0.37$	$1.40 \times 10^{-1}, 1.38 \times 10^{-1}$
		Hixson-Crowell	0.93	$k_{hc} = 4.86 \times 10^{-3} \text{ min.}^{-1}$	$4.89 \times 10^{-3}, 4.83 \times 10^{-3}$
		$W_0^{1/3} - W_t^{1/3} = k_{HC} t$	0.96	$k_{hc} = 3.41 \times 10^{-3} \text{ min.}^{-1}$	

The kinetic models for the release of DOX and CA in the SAB buffer are depicted in **Fig. 4.10** and **Fig. 4.11**, respectively, and the resultant rate constants (k) and correlation coefficients (R^2) are summarized in **Table 4.3**. The pseudo-second-order (**Fig. 4.10 C** and **Fig. 4.11 C**) was found to be more appropriate for this instance, with a best fit R^2 value of 0.98 and 0.99 for the release of DOX and CA, respectively. Additionally, the diffusional exponent for the Korsmeyer-Peppas model (**Fig. 4.10 E** and **Fig. 4.11 E**) was found to be 0.37 (< 0.5) with an R^2 value of 0.97, signifying Fickian diffusion-driven release of DOX and CA out of GCF-DOX-CA nanocomposite [43]. The Higuchi model (**Fig. 4.10 d** and **Fig. 4.11 D**) also corroborated the observations, as drug release is proportionate to the square root of time [35].

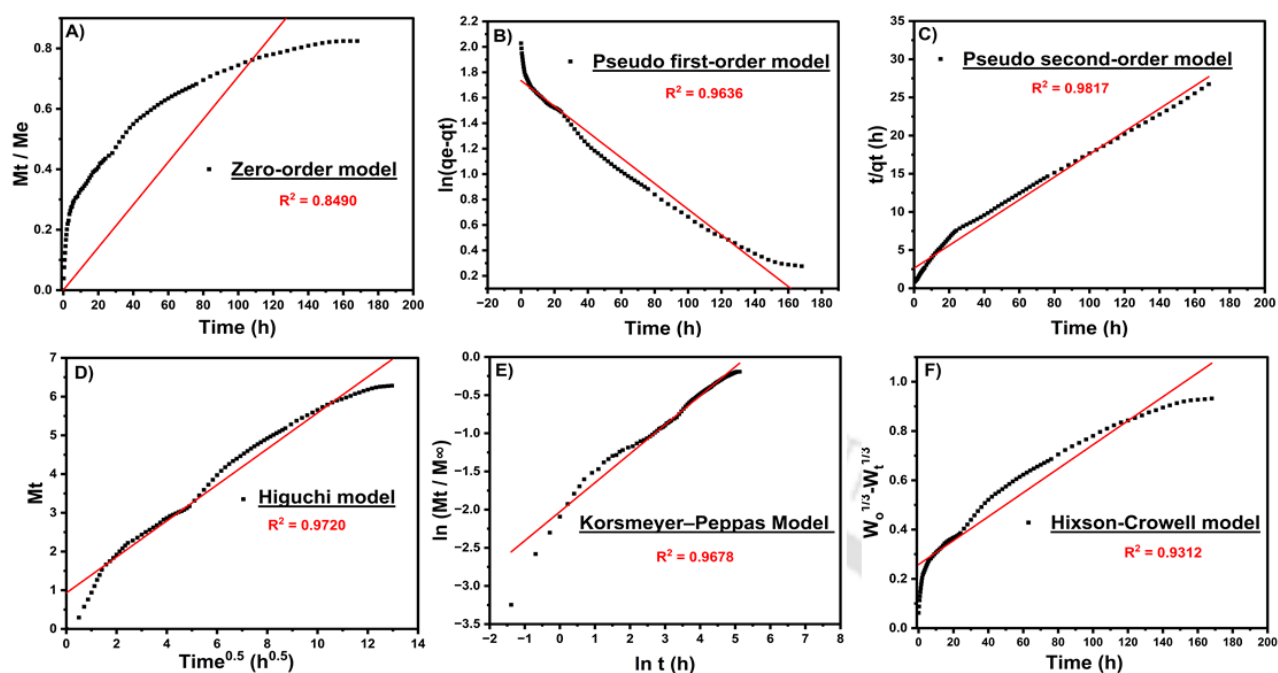


Fig 4.10: Kinetic model contour fit for DOX release out of the GCF-DOX-CA nanocomposite (A) zero-order (B) pseudo-first-order, (C) pseudo-second-order, (D) Higuchi, (E) Korsmeyer-Peppas and (F) Hixson-Crowell model.

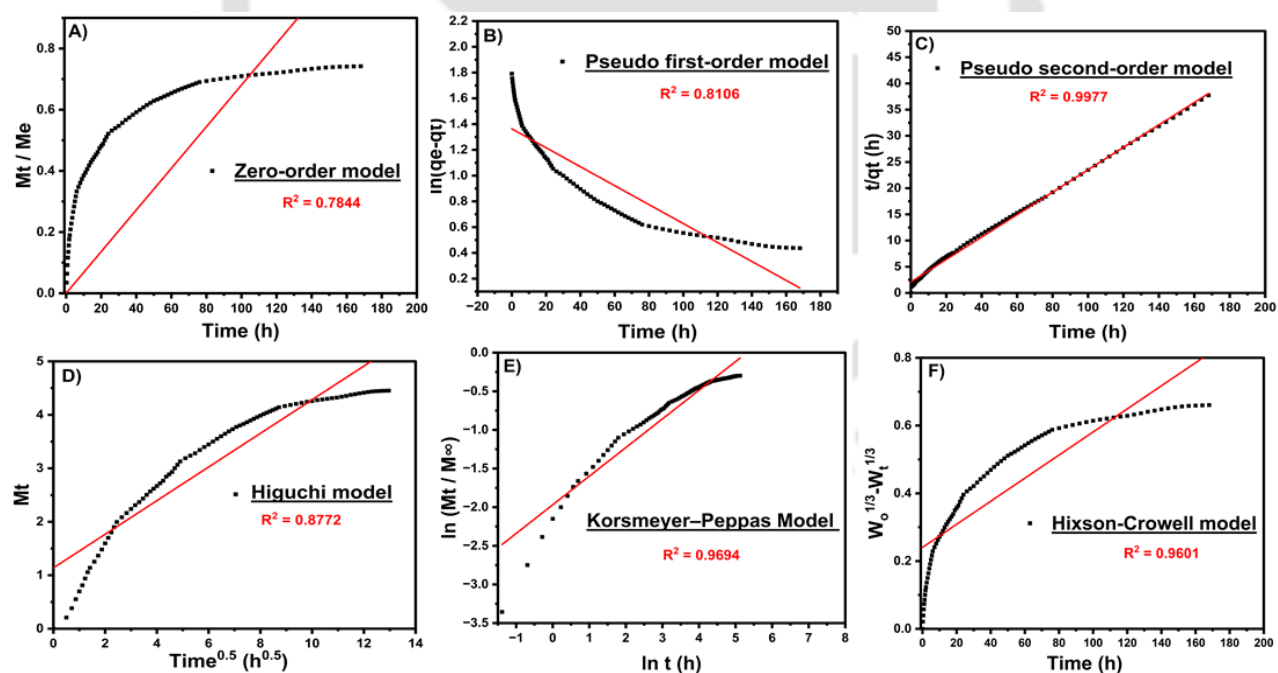


Fig 4.11: Kinetic model contour fit for CA release out of the GCF-DOX-CA nanocomposite (A) zero-order (B) pseudo-first-order, (C) pseudo-second-order, (D) Higuchi, (E) Korsmeyer-Peppas and (F) Hixson-Crowell model.

4.2.8. Cytotoxicity evaluation of GCF and GCF-DOX-CA nanocomposite

Nanocarriers are used to transport drug molecules to targeted sites without showing toxic effects and also enhance drug safety and drug efficacy. The cytocompatibility of synthesized GCF nanocarriers and drug-loaded GCF-DOX-CA nanocomposite was evaluated by performing an *in-vitro* cell viability assay using HEK293 as a normal cell line and A549 as a lung cancer cell line.

In the case of HEK293, the cell viability was varied with an increase in the concentration from 0-400 $\mu\text{g mL}^{-1}$ for the synthesized GCF nanocarrier and drug-loaded GCF-DOX-CA nanocomposite. However, with the cell viability of $\sim 81\%$ and $\sim 76\%$ for GCF and GCF-DOX-CA, respectively, both were found to be cytocompatible for a higher dose of 400 $\mu\text{g mL}^{-1}$ (Fig. 4.12).

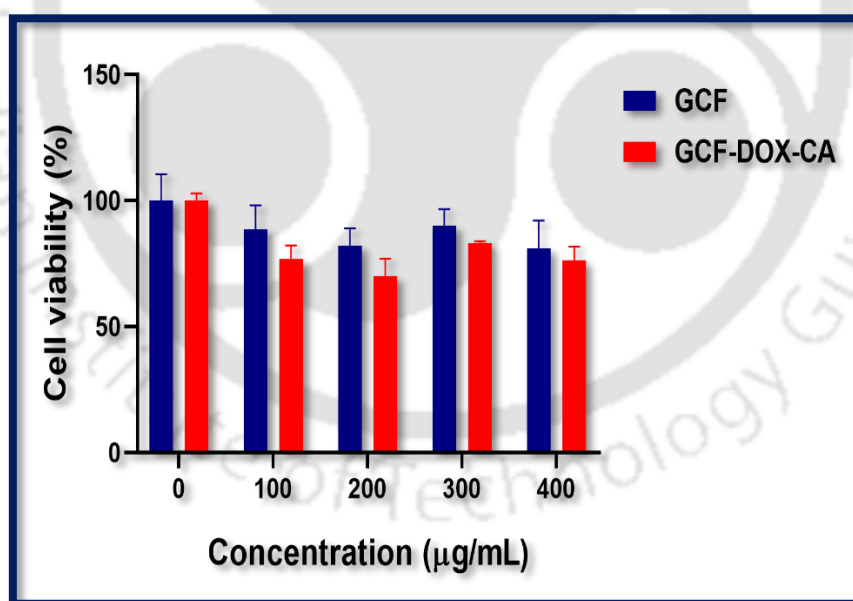


Fig. 4.12: *in-vitro* cytotoxicity assessment of GCF and GCF-DOX-CA nanocomposite against HEK 293. The results are stated as mean $\pm$ SD (N=4).

Furthermore, the cytotoxicity assessments were conducted for lung cancer cell lines A549. The GCF nanocarrier demonstrated a cell viability of $\sim 70\%$ against A549, suggesting its cytocompatibility for normal as well as for cancer cell lines. Whereas, in the case of GCF-

DOX-CA nanocomposite, the cell viability was reduced to $\sim 57\%$ at a concentration of $400 \mu\text{g mL}^{-1}$ (**Fig. 4.13**), suggesting the targeted apoptosis for cancer cells accompanied by the anticancer activity of DOX and CA. Overall, the present study revealed that the drug-loaded GCF-DOX-CA nanocomposite displayed high sensitivity toward A549 compared to the HEK293, which advocated its application in targeted DDS.

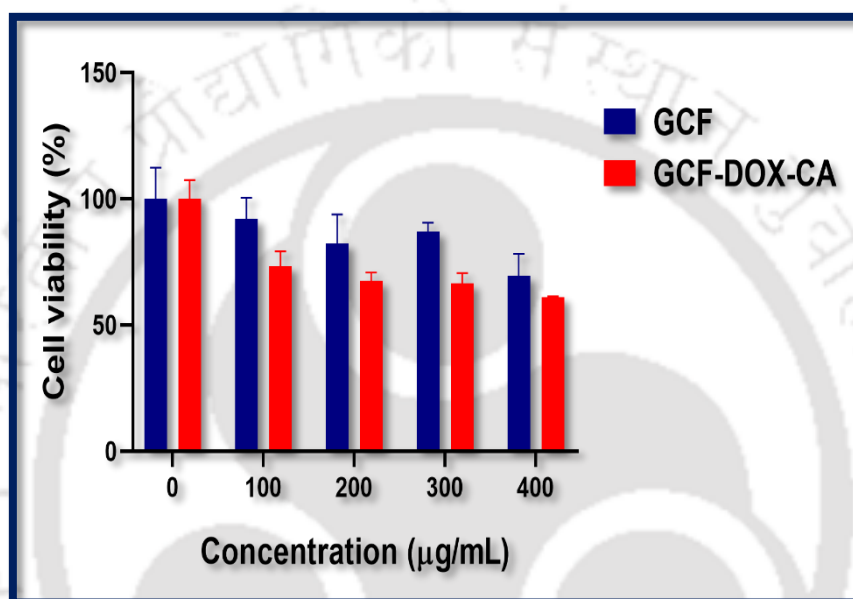


Fig. 4.13: *in-vitro* cytotoxicity assessment of GCF and GCF-DOX-CA nanocomposite against A549. The results are quantified as mean $\pm$ SD (N=4).

Summary

The 1D GONS were successfully fabricated and functionalized with CS and FA as a biopolymer and targeting ligand for the targeted co-delivery of DOX and CA for anticancer DDS. The electron microscopic images depict a tabular-shaped spirally wound 1D morphology of GONS, GCF, and co-loaded GCF-DOX-CA nanocomposite. The chemical interaction study elucidated a successful formation of GONS, GCF nanocarrier, and GCF-DOX-CA nanocomposite by illustrating the presence of respective functional groups. The reduction behavior of GONS and GCF nanocarrier was advocated by XRD, elemental analysis, and Raman spectroscopy. The obtained GCF nanocarrier displayed enhanced

loading efficiency to that presented in the literature for both the DOX and CA. The co-loading was primarily ascribed to non-covalent interactions, like hydrophobic and electrostatic interactions. An *in-vitro* release contour of the developed GCF nanocarriers demonstrated regulated and sustainable release of DOX and CA. The *in-vitro* cytotoxicity evaluations of GCF-DOX-CA nanocomposite demonstrated significant cytocompatibility for normal cells (HEK293) and selective apoptosis for cancer cell lines (A549).

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Chapter 5:

One-pot, sustainable and room temperature synthesis of graphene oxide-impregnated iron-based metal-organic framework (GO/MIL-100(Fe)) nanocarriers for anticancer drug delivery systems

Chapter 5

One-pot, sustainable and room temperature synthesis of graphene oxide-impregnated iron-based metal-organic framework (GO/MIL-100(Fe)) nanocarriers for anticancer drug delivery systems

This chapter presents a sustainable approach for synthesizing HF-free GO/MIL-100 (Fe) nanocarriers with different amount of GO at room temperature. The obtained nanocarriers, including pristine GO, MIL-100 (Fe) (MF), and their nanocomposite, Gw-MF, were characterized to investigate the surface morphology, structure, and surface chemistry of nanocarriers and subjected to DOX loading subsequently. The DOX loading behavior of obtained nanocarriers has been evaluated for the different amount of GO in the nanocarrier and DOX vs. nanocarrier weight ratio. The thermodynamic and kinetic study of the loading behavior at different temperatures and the release characteristics of DOX at physiological pH conditions were done, respectively. Alongside the in-vitro release study of DOX from the drug-loaded nanocarriers and cytotoxicity analysis of the optimized Gw-MF nanocarriers over normal cells (HEK293) and cancer cell lines (A549) was performed. It is the first-ever study commenced on the sustainable synthesis of GO-impregnated MOFs and their application in cancer therapeutics. Finally, a detailed comparative study was conducted with the application of MOF-based nanocarriers in anticancer DDS.

5.1. Experimental

5.1.1. Materials

The details of preparatory materials for the synthesis of GO are provided in **section 2.1.1**. For the synthesis of MIL-100 (Fe), Iron (II) sulfate heptahydrate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 99%), Benzene-

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1,3,5-tricarboxylic acid ($\text{C}_6\text{H}_3(\text{CO}_2\text{H})_3$) (Trimesic acid), 95%), Sodium hydroxide pellets (NaOH , 98%) were obtained from Merck Life Science Private Limited (Mumbai, India). The chemotherapeutic drug, i.e., Doxorubicin (DOX) (2 mg mL^{-1}), was produced by Fresenius Kabi Oncology Ltd, Solan, India. The materials and the drug acquired from commercial sources were used without additional purification for experimental purposes. Deionized and Milli-Q water was produced from Elix-3 M/s Millipore (USA) Milli-Q water purification system.

5.1.2. Synthesis of graphene oxide (GO)

The GO was synthesized via the renowned Hummers' and Offerman's method with some modifications; the details are provided elsewhere [1,2]. Initially, a mixture of 4 g of synthetic grade graphite, 6 g of P_2O_5 , and 6 g of $\text{K}_2\text{S}_2\text{O}_8$ was made, followed by adding 30 mL of H_2SO_4 in a conical flask consecutively. The mixture was subjected to react at 80°C for 5 h. Later, the reacted mixture was separated and washed using DI water and subjected to drying at 60°C overnight. Subsequently, a dried product (4 g) was employed for the synthesis of GO nanosheets as per the protocol described in **section 2.1.2**. The resultant GO suspension is subjected to purification and filtration steps followed by freeze-drying.

5.1.3. Synthesis of MIL-100 (Fe) and GO/MIL-100 (Fe) nanocarriers

The pristine MIL-100 (Fe) and GO-impregnated MIL-100 (Fe) nanocarriers were produced based on the previously reported sustainable approaches with certain modifications [3]. The sustainable strategy for the synthesis of MOF, as well as their composite, was accomplished via using water as a solvent, room temperature (RT) conditions, metal salts, organic salts as an anionic linker, non-formation of toxic by-products, adapting non-aggressive post-synthesis washing and absence of corrosive acids (HF or HNO_3). Initially, a pristine MIL-100 (Fe) was prepared; the schematic of the preparation process is shown in **Fig. 5.1**.

In a typical approach, two different solutions were made, namely solution 1 and solution 2. Solution 1 consists of an aqueous solution of iron (II) sulfate heptahydrate prepared by adding 3.16 g of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and 98 mL of H_2O . Whereas solution 2 was prepared by mixing trimesic acid (1.679 g) as an organic linker, NaOH (1.192 g) as a deprotonating agent, and H_2O (24 mL). Thereafter, solution 1 was placed in a round bottom flask and subjected to continuous stirring, followed by the dropwise addition of solution 2 in solution 1. The reaction mass was allowed to react at RT (25 °C) with continual stirring at 500 rpm for 24 h. During the development of MIL-100 (Fe), the color of the reaction mass changes from olive green to brownish-yellow. After maintaining the reaction for 24 h, the reaction mass was allowed to settle for the decantation of liquid. Now, the obtained MIL-100 (Fe) was washed with DI water (300 mL) 3 times, followed by ethanol washing (3 times). The solid MIL-100 (Fe) was later recovered via centrifugation at 5000 rpm for 20 min. and subjected to vacuum drying at 50 °C for 12 h. The obtained MIL-100 (Fe), hereafter denoted as MF, was weighed and stored for drug-loading experiments.

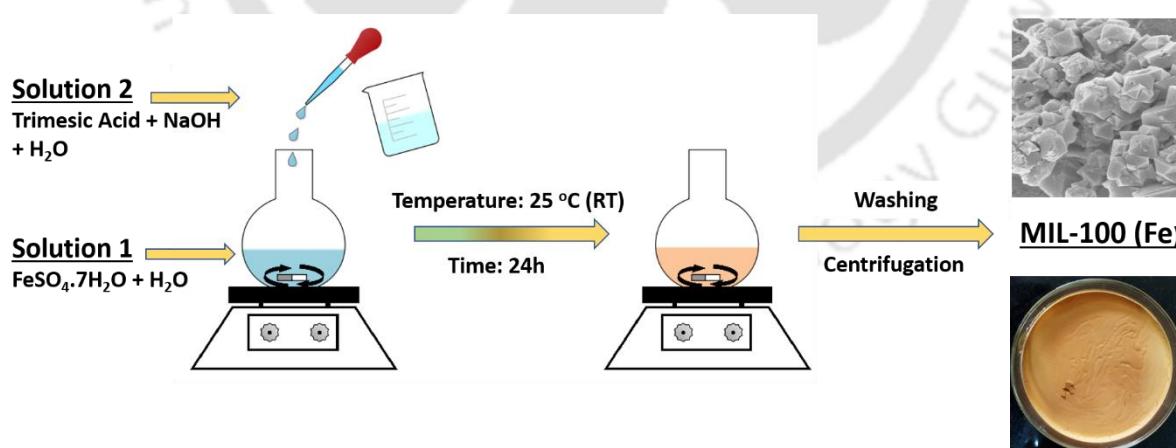


Fig. 5.1: Schematic representation for one-pot room temperature synthesis of MIL-100 (Fe).

Meanwhile, for the synthesis of GO-impregnated MIL-100 (Fe) nanocarriers (G-MF), an identical procedure was adapted with the introduction of GO powder in solution 1. The amount of GO (0.1, 0.2, 0.5, 0.7, and 1.0 wt.%) was taken with respect to the amount of

pristine MF obtained previously. The products obtained are hereafter referred to as Gw-MF, where $w = 0.1, 0.2, 0.5, 0.7$, and 1.0 represent the wt.% of GO was used to synthesize GO-impregnated MIL-100 (Fe) composites. All the samples were dried and stored for drug loading.

5.1.4. Loading of DOX on GO, MIL-100(Fe) and their nanocarriers

The DOX loading experimentations on parent materials such as GO, MIL-100(Fe) (MF), and their nanocomposites were accomplished via protocols provided in the literature [4]. For instance, 10 mg of MF was mixed with a purchased 2.5 mL DOX solution of concentration 2 mg mL^{-1} to obtain a mass ratio of 1:0.5 for MF: DOX. Further, the mixture was maintained in a shaker incubator (Daihan Labtech, LSI 3016R model) under a dark environment. The loading of DOX was performed at a steady temperature of 25°C with the help of an automated chiller assembly provided with the incubator shaker at 200 rpm for 24h. After that, the DOX-loaded MF was subjected to high-speed centrifugation at 10000 rpm for 20 min. The supernatant was diluted using Milli-Q ultrapure water to evaluate the concentration of DOX with UV-Vis spectroscopy at $\lambda_{\text{max}} = 480 \text{ nm}$.

In the meantime, MF loaded with DOX has been recovered after centrifugation and vacuum dried at 40°C for further characterization studies. Identical loading conditions were adapted for all other nanocarriers, namely, GO, G0.1-MF, G0.2-MF, G0.5-MF, G0.7-MF, and G1-MF. To determine the loading efficiency of nanocarriers, the concentration DOX calibration curve was depicted using a UV-Vis spectrophotometer at $\lambda_{\text{max}} 480 \text{ nm}$. UV absorbance was used to measure the concentration of DOX supernatant that was left over after DOX was loaded onto nanocarriers. The drug loading efficiency of nanocarriers was determined using the following equation.

$$\text{Loading Efficiency (\%LE)} = \frac{M_1 - M_2}{M_{ads}} \times 100 \quad (5.1)$$

M_1 is the original amount of DOX, M_2 is the weight of unloaded DOX present in the filtrate, and M_{ads} is the amount of dried nanocarrier.

Later, the effect of nanocarrier mass ratio with respect to DOX (Nanocarrier: DOX) was studied by performing loading experiments at different mass ratios such as 1:0.1, 1:0.2, 1:0.33, 1:0.5, 1:0.75, 1:1 and 1:1.25. The nanocarrier with higher loading efficiency was chosen to investigate the variation in the loading capacity of nanocarriers with a mass ratio of DOX. To understand the thermodynamics of the DOX loading process on nanocarrier with maximum loading efficiency, a similar loading process was conducted at different temperatures (25 °C, 30 °C, 35 °C). All the drug loading experiments were performed in a triplicate manner, and the numerical results are represented as the mean $\pm$ standard deviation (SD). The composites obtained after loading of DOX, namely GO-DOX, MF-DOX, and Gw-MF-DOX, were vacuum dried at 40 °C for overnight and stored for subsequent characterizations.

5.1.5. *in-vitro* release of DOX

The *in-vitro* DOX release behavior for obtained GO-DOX, MF-DOX, and the Gw-MF-DOX nanocomposite of maximum loading efficiency was investigated at physiological pH (7.4) environment by dissolving it into the phosphate buffer solution (PBS). Numerous reports have conveyed that the anions HPO_4^{2-} and H_2PO_4^- impact the rate of release of drugs [5,6]. In order to test the controlled release characteristic of DOX from Gw-MF-DOX nanocomposite, 70 mg of the nanocomposite was added to 50 mL of PBS solution. The nanocomposite- PBS mixture was then put into a shaker incubator at 37 °C for 25 days. The quantity of DOX released from DOX-loaded nanocomposites was evaluated by gathering the aliquots at regular intervals (30 min), centrifuging the mixture for 15 min. at 10,000 rpm, and then

analyzing the supernatant's absorbance. The release experiments were performed in a duplicate manner, and the numerical results are depicted as the mean $\pm$ SD.

5.1.6. Cytotoxicity study

The cytocompatibility and anticancer properties of prepared nanocarriers were inspected using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay [7]. Human embryonic kidney cells HEK293 and epithelial carcinoma cells A549 were used as normal and cancer cells, respectively. Both the cell lines were cultivated in Dulbecco's Modified Eagle Medium (high glucose) accompanied by the fetal bovine serum and antibiotics in the CO₂ incubator at 37°C. The experiments were performed in two parts. Firstly, 5×10^3 cells were seeded in each well of the 96-well plate with different concentrations of free DOX, unloaded nanocarrier G0.5-MF, and DOX-loaded nanocarrier G0.5-MF-DOX. First, the cytocompatibility of the prepared nanocarrier (G0.5-MF) was tested. For this, 5×10^3 cells were seeded in each well of a 96-well plate with different concentrations of the unloaded nanocarrier G0.5-MF in the range of 50 to 1000 $\mu\text{g mL}^{-1}$. Next, the anticancer properties of the DOX-loaded G0.5-MF nanocarrier were analyzed. The concentrations of free DOX varied in the range of 12.5 to 250 $\mu\text{g mL}^{-1}$, and the concentration of DOX-loaded nanocarrier G0.5-MF-DOX varied from 50 to 1000 $\mu\text{g mL}^{-1}$ considering 25% of drug loading. After the completion of periodic incubation of 01, 04, and 07 days, the wells were replaced by the fresh medium, and 20 μL of MTT solution was added and further incubated for 4 h incubation in a CO₂ incubator. Then, the medium was carefully removed, and the precipitated formazan crystals were dissolved in 100 μL dimethyl sulfoxide (DMSO). The absorbances were recorded at 570 nm using a multi-plate reader (Infinite 200 Pro, Tecan). The cell viability for the samples was calculated using the absorbance of the control

wells [8]. The cytotoxicity experimentations were done in triplicate; the respective numeric results are shown as the mean $\pm$ SD.

5.1.7. Characterization of nanocarriers

The structural and morphological characterizations of synthesized GO, MF, and Gw-MF nanocarriers were done by TEM, Brunauer-Emmett-Teller (BET), XRD, and Field emission scanning electron microscopy-electron dispersive x-ray analysis (FESEM-EDX); the instrumentation details are provided in **section 2.1.4 and 3.1.7**. Also, Atomic force microscopy (AFM) imaging was accomplished to investigate the depth and the lateral dimensions of the nanocarriers by a commercial AFM instrument, Oxford, Model Cypher. The BET and BJH-desorption methods were utilized to investigate the surface areas and to estimate the average pore sizes, total volumes, and structure of all the nanocarriers via N₂ adsorption-desorption isotherms (Micrometrics, TriStar II Plus). In addition, the XPS (Thermo Fisher Scientific Pvt. Ltd., ESCALAB Xi⁺) analysis was done to validate the existence of functional groups within the nanocarriers as well as drug-loaded nanocarrier. Also, to inspect the elemental composition of obtained nanocarriers, an EDX analysis was executed using Aztec V2.1 Oxford Instruments, assisted with FESEM. Doxorubicin Hydrochloride (DOX) concentration calibration was performed at 480 nm using a UV-Vis spectrophotometer (Thermofisher Scientific Orion Aquamate 8000). The Raman spectra of the nanocarriers were obtained at 488 nm excitation wavelength to investigate their oxidation state and reduction characteristics (Horiba Jobin Vyon LabRam HR model).

5.2. Results and discussion

5.2.1. Surface morphology of GO, MIL-100(Fe) and GO/MIL-100(Fe) nanocarriers

The surface morphology of GO nanosheets, MIL-100 (Fe), and the GO-impregnated MIL-100 (Fe) nanocarriers have been investigated using TEM and FESEM analysis, as shown in **Fig. 5.2**. A well-exfoliated GO sheet was recovered after centrifugation, followed by freeze-drying. The wrinkled surface of exfoliated GO nanosheets can be observed in **Fig. 5.2a**. The polycrystalline nature of GO was detected via a selected area electron diffraction (SAED) pattern, and a strong diffraction pattern of hexagonal lattice structure with six-fold spots was distinguished, as shown in the inset. The pristine MIL-100 (Fe) synthesized at RT is presented in **Fig 5.2b**. A sharp-edged octahedral morphology of MIL-100 (Fe) can be observed without any visible diffraction pattern, as shown in the SAED pattern. Previously, a similar pattern was observed for the MIL-100 (Fe) synthesized by the conventional hydrothermal method [4]. However, for the GO/MIL-100 (Fe) composite, a typical ring-like structure was observed, which indicates the minor polycrystalline nature of the nanocarrier due to the presence of GO. Here, in **Fig. 5.2c**, comparatively lighter areas with lower electron density represent multilayer GO nanosheets, whereas a darker area denotes octahedral MIL-100 (Fe).

Furthermore, a high-resolution transmission electron microscopic (HRTEM) investigation was implemented to estimate the lattice spacing of the nanocarriers. The HRTEM images of the pristine GO, MIL-100 (Fe), and GO-impregnated MIL-100 (Fe) are presented in **Fig. 5.2**. Herein, a single layer GO nanosheet exhibited slightly blurred lattice fringes with a spacing of 0.72 nm (**Fig. 5.2d**). In the case of pristine MIL-100 (Fe), much clear parallel lattice fringes were observed due to its crystalline structure with an interlayer spacing of 0.35 nm (**Fig. 5.2e**). In **Fig. 5.2f**, a well-distinct GO nanosheet and MIL-100 (Fe) of comparatively

lighter and darker areas and lattice fringes can be observed for the GO/MIL-100 (Fe) (G0.5-MF) nanocarrier. The lattice spacing of the individual nanocarrier in the composite is provided in **Fig. 5.2f** (inset). Herein, the lattice spacing of GO was decreased to 0.59 nm, which may be due to its reduction during composite formation. Reduction in the lattice spacing of GO further advocates stronger interaction between GO and MIL-100 (Fe) [2,9].

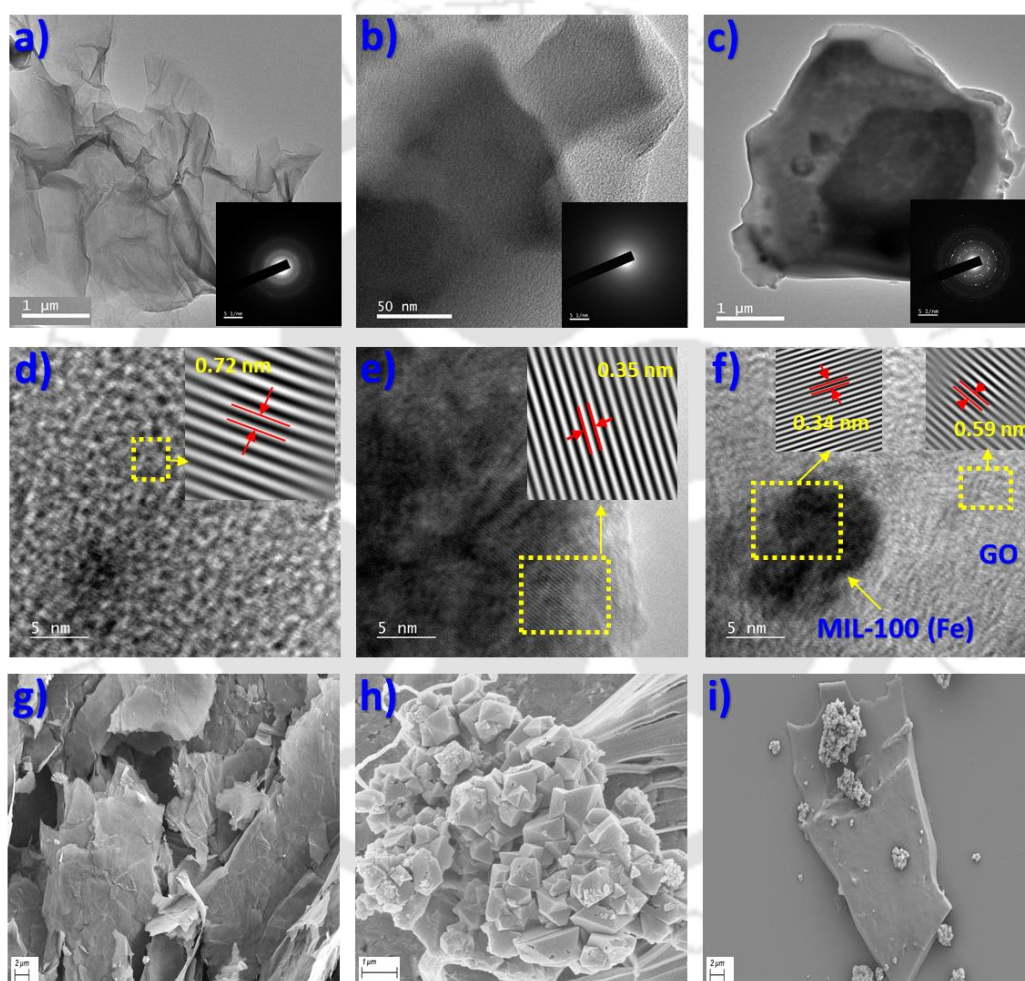


Fig. 5.2: FETEM, HRTEM, and FESEM images of (a, d, g) GO, (b, e, h) MIL-100 (Fe) and (c, f, i) GO/ MIL-100 (Fe) nanocarriers, respectively accompanied by the corresponding SAED patterns and interlayer spacings in the inset.

Similarly, the surface morphology of pristine GO, MIL-100(Fe), and nanocarriers have been investigated by FESEM analysis. **Fig. 5.2g** corresponds to the rumpled layer structure of GO with the accumulation of multiple single-atomic thin nanosheets. As illustrated in **Fig. 5.2h**, a distinct polyhedral crystal structure of MIL-100 (Fe) can be observed here, which is

accumulated with some irregular and smaller particles. In the case of the GO/MIL-100(Fe) nanocarrier, the MIL-100 (Fe) particles remained randomly attached in a cluster form over the surface of GO, as shown in **Fig. 5.2i**. However, in some cases, the MIL-100 (Fe) crystals act as a filler and lie between the stacked layers of GO; as presented in **Fig 5.2c**, an analogous observation was reported previously by Lou et al. (2018) [9]. The specified surface morphological observations by TEM, HRTEM, and FESEM convey preliminary shreds of evidence related to the successful formation of the GO/MIL-100(Fe) nanocomposite.

Besides, the lateral dimensions and the depth of the GO, MIL-100 (Fe) (MF), and GO/MIL-100(Fe) (G0.5-MF) nanocarrier were investigated through AFM analysis. Prior to analysis, the obtained nanocarriers were diluted by DI water to 1 ppm concentration, followed by sonication, drop casting over the silicon wafer, and drying at 60 °C. **Fig. 5.3** illustrates the AFM images and height profile of the nanocarriers generated using WSxM 5.0 image analysis software. In the case of GO, well-exfoliated, flat, and slightly stacked nanosheets of 0.4-1 μm lateral size can be observed in **Fig. 5.3a**. **Fig. 5.3d** further describes the respective height profile of a single nanosheet of 1.5-2 nm thickness. Since the solitary sheets are slightly taller than 1 nm, the number of layers stacked over one another can be roughly estimated by determining the flake's depth [2,10]. The AFM image and the height profile of octahedral MF are presented in **Fig. 5.3b** and **Fig. 5.3e**, respectively. A more microscopic and concrete structure of the pristine MF exhibited the highest thickness in a 78-85 nm range. In the case of GO-impregnated MF (G0.5-MF), the surface structure of GO nanosheets was retained after the modification with MF (**Fig. 5.3c**). The height of the MF crystals was recorded as ~80-85 nm, as displayed in **Fig. 5.3f**. Similar to the TEM, HRTEM, and FESEM, the existence of octahedral MF over the spongy GO nanosheet can be visualized in **Fig. 5.3c**, which further validates the successful impregnation of GO nanosheets with the MF of fairly identical thickness. It is quite possible that the pristine GO and MF are comprised of

abundant layered geometry and π -conjugation systems that further result in a dense layer stacking within themselves and allow GO nanosheets to preserve their flat structure [11].

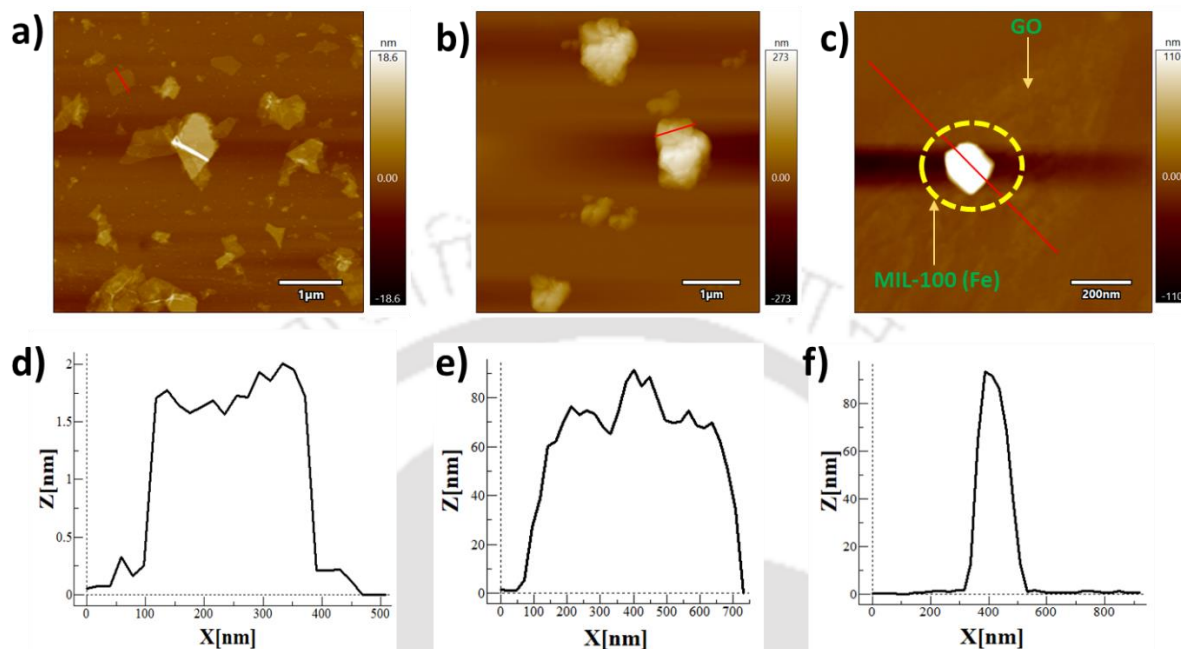


Fig. 5.3: AFM images and height profiles of (a, d) GO, (b, e) MIL-100 (Fe) (MF), and (c, f) G0.5-MF, respectively.

5.2.2. X-Ray diffraction

The X-ray diffractograms of synthesized GO nanosheets, pristine MF, and Gw-MF nanocarriers are presented in **Fig. 5.4**. In **Fig. 5.4A**, a solitary sharp crystalline peak of GO nanosheet was observed at 10.44° ascribed for (0 0 1) crystalline plane with d-spacing of 0.84 nm; similar type of outcomes were reported in the literature [12]. The GO nanosheets obtained via modified Hummers' describe their amorphous nature and enhancement in the interlayer spacing because of the presence of oxygenated hydrophilic functional entities and supplementary structural defects caused via oxidation of graphite [2]. Whereas the well-crystallized structure of MF, i.e., MIL-100 (Fe) synthesized at room temperature, can be observed further with the major characteristic peaks at 6.26° , 10.22° , 11° , and 20.04° , which are well in line with the previously reported studies [13,14]. Furthermore, identical XRD spectrums to that of pristine MF were obtained for G 0.1-MF, G 0.2-MF, G0.5-MF, G 0.7-

MF, and G1-MF nanocarriers signifying the well-conserved crystal structure of pristine MF within the nanocarriers. However, a significant peak of GO was not detected, possibly due to the overlapping of crystalline peaks around $10\text{--}10.5^\circ$ or the lower amount of GO was introduced during the synthesis of nanocarriers [15]. Also, it was observed that with an increase in the mass fraction of GO in Gw-MF nanocarriers, the intensity of diffraction peaks was lowered to some extent due to a decrement in the amount of MF present within the Gw-MF nanocarriers. **Fig. 5.4B** provides a magnified view of MF and G0.5-MF nanocarriers. The diffractogram findings demonstrate that the crystal structure of MF produced at room temperature is comprehensible to the literature, and Gw-MF nanocarriers have also persisted with similar reliability.

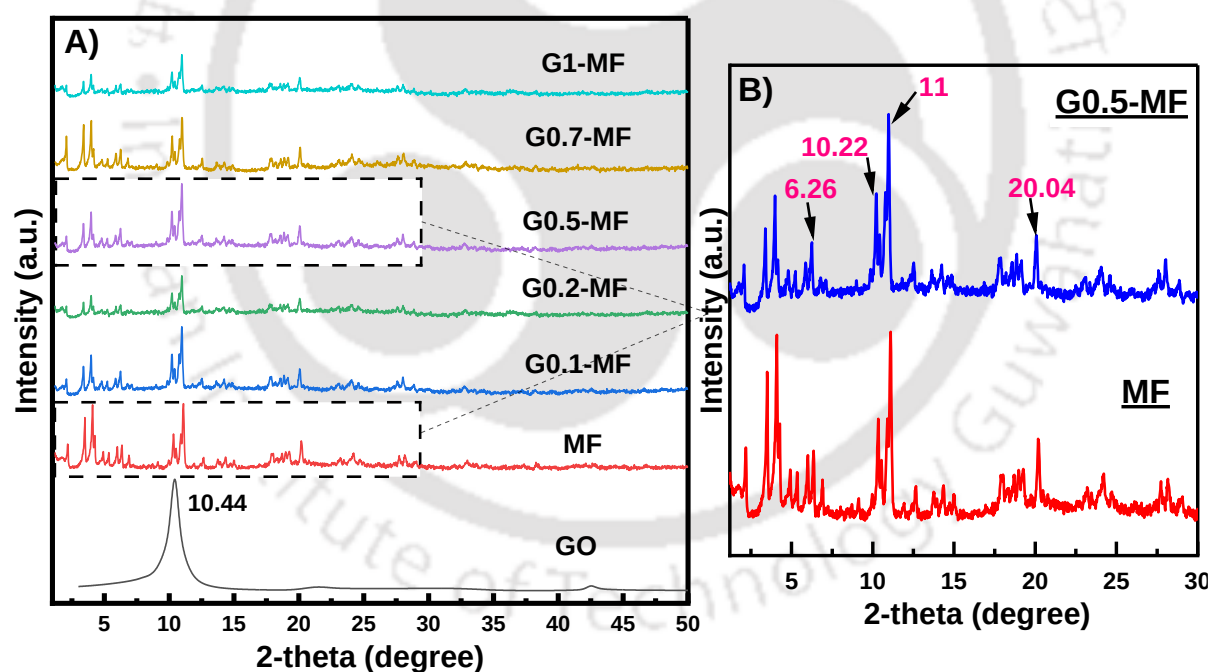


Fig. 5.4: X-ray diffractograms of (A) GO, MF, and Gw-MF nanocarriers and (B) MF with G0.5-MF nanocarrier (magnified view).

5.2.3. N₂ adsorption-desorption isotherms

The textural properties of as-prepared GO, MF, and GO/MIL-100 (Fe) (Gw-MF) nanocarriers were inspected using N₂ adsorption-desorption isotherms at 77 K (**Fig. 5.5**). The specific surface area of room temperature synthesized pristine MF was found to be $2203.26 \text{ m}^2\text{g}^{-1}$

with a total pore volume of $1.125 \text{ cm}^3 \text{ g}^{-1}$. As illustrated in **Fig. 5.5**, the MF nanocarrier follows type I N_2 adsorption-desorption isotherm per IUPAC classification through a minor hysteresis loop. The MF carrier exhibits an average pore size of 2.04 nm, indicating its microporous nature [14]. However, the GO synthesized via the modified Hummers' method follows type IV N_2 adsorption-desorption isotherm and manifests a lower surface area of $30.47 \text{ m}^2 \text{ g}^{-1}$ compared to pure MF. Meanwhile, in the case of Gw-MF composites nanocarriers, the pore volume and specific surface area declined with the increase in GO content, as reported in **Table 5.1**.

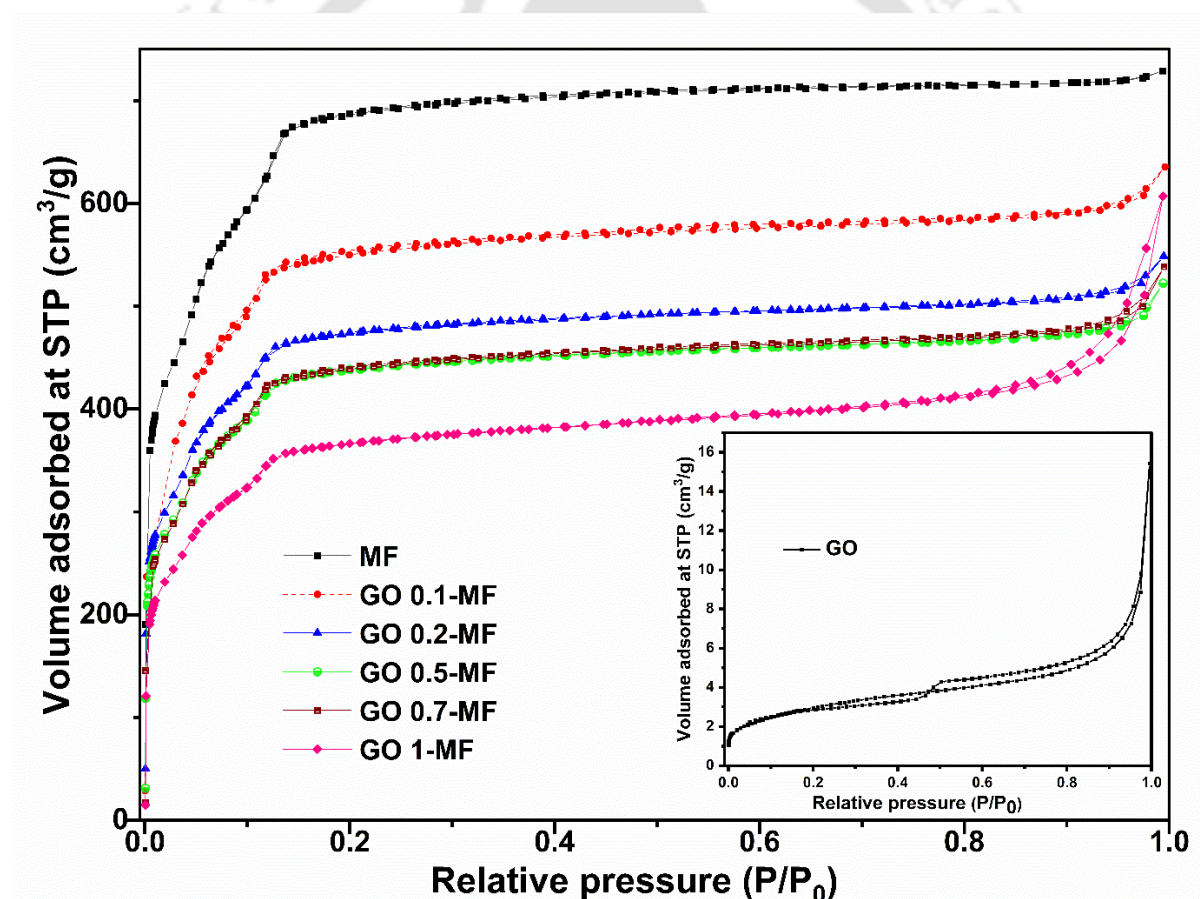


Fig. 5.5: N_2 adsorption-desorption isotherms of GO, MF, and Gw-MF nanocarriers.

The retardation in the surface area of all the Gw-MF nanocomposites, namely G0.1-MF, G0.2-MF, G0.5-MF, G0.7-MF, G1-MF, was due to the presence of relatively non-porous and covering structure GO [9]. The Gw-MF nanocarriers have shown an identical trend alongside an intermediate manner amongst type I and type IV N_2 adsorption-desorption isotherms, with

an increase in the hysteresis loop and higher GO content for the G1-MF nanocarrier. Such an intermediate mode of type I and IV correspondingly allied to the existence of microporous and mesoporous cavities in the nanocarriers [16]. Such porous structure and higher surface area of the nanocarriers may provide abundant active sites for drug adsorption and significantly improve drug loading efficiency. The textural characteristics, such as surface area, total pore volumes, and average pore size for all the nanocarriers, are provided in **Table 5.1**.

Table 5.1: Textural characteristics of nanocarriers.

Sample	Amount of GO (wt.%)	BET surface area ($\text{m}^2 \text{g}^{-1}$)	Average pore size (nm)	Total pore volume ($\text{cm}^3 \text{g}^{-1}$)
GO	100	30.47	7.95	0.097
MF	0	2203.26	2.04	1.125
GO 0.1-MF	0.1	1745.75	2.22	0.971
GO 0.2-MF	0.2	1503.75	2.23	0.840
GO 0.5-MF	0.5	1396.50	2.29	0.819
GO 0.7-MF	0.7	1393.71	2.34	0.798
GO 1-MF	1	1172.19	3.09	0.707

5.2.4. Chemical interaction study

5.2.4.1. FTIR and EDX analysis

The qualitative analysis of synthesized GO, MF, and Gw-MF nanocarriers was done using FTIR and elemental analysis to confirm the existence of major functional groups and elements within the nanocarriers, respectively. **Fig. 5.6** represents the FTIR spectrum of GO obtained after the oxidation of graphite, MF, and G0.5-MF nanocarriers synthesized at room temperature along with the G0.5-MF-DOX nanocomposite after loading of the anticancer drug, i.e., Doxorubicin. In the case of GO, a realistically broader peak with lower

transmittance can be observed at 3430 cm^{-1} , indicating -OH groups with stretching vibrations. A dimeric COOH group of hydrogen-bonded functionalities were observed at 2927 cm^{-1} alongside a minor peak at 2857 cm^{-1} , corresponding to the intramolecular bonded O-H stretching of alcohol. The familiar peaks of C=O stretched carbonyl-carboxyl groups and unaffected C=C bonding at 1730 cm^{-1} and $1630\text{--}1650\text{ cm}^{-1}$ were also detected in the IR spectra of GO [2,17]. However, the epoxy-functional groups in GO can be confirmed via peak position at 1052 cm^{-1} [2]. The existence of such oxygenated functional groups with lower transmittance signifies a good quality of GO [18].

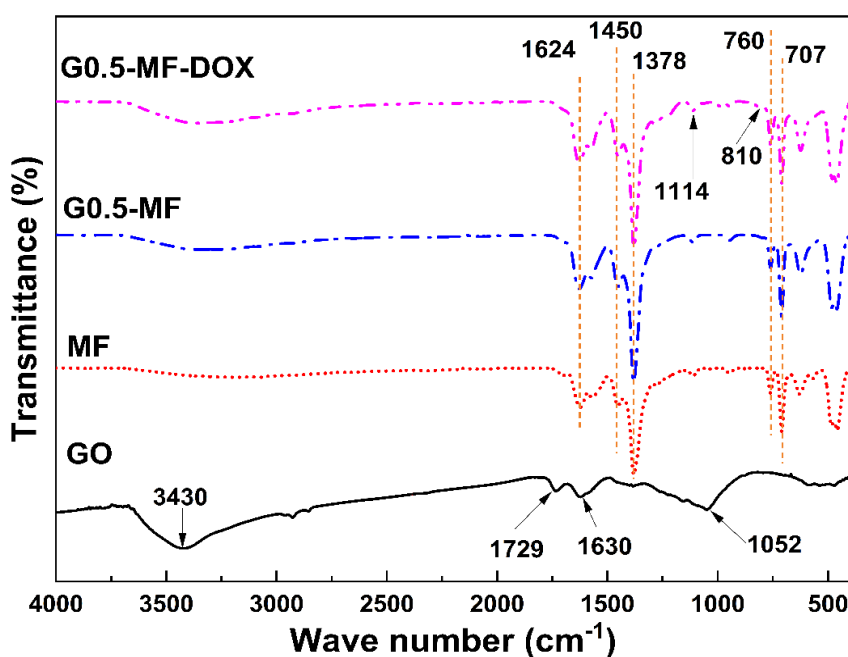


Fig. 5.6: FTIR spectrum of GO, MF, G0.5-MF nanocarriers, and DOX-loaded G0.5-MF-DOX nanocomposite.

Further, the FTIR spectrum for MF was provided in **Fig. 5.6**, where the clear transmittance peaks can be observed at 1624 cm^{-1} , 1450 cm^{-1} , 1378 cm^{-1} , 760 cm^{-1} , and 707 cm^{-1} . The peak position at 1624 cm^{-1} represents carboxylate groups, whereas the symmetric and characteristic asymmetric bands of O-C-O functional entities were detected at 1378 cm^{-1} and 1450 cm^{-1} , correspondingly. Here, the characteristic peak for trimesic acid at 1725 cm^{-1} representing the carboxyl functional group was not visible due to the low peak intensity as

the residual H_3BTC was removed during the washing process for the synthesis of MF, i.e., MIL-100 (Fe). Sharp peaks at 760 cm^{-1} and 707 cm^{-1} referred to the benzene structure with C-H bending vibrations[16]. Here, FTIR spectra with certain functional groups confirm the formation of MF and are well in line with the previous literature [14]. An identical IR spectrum was obtained for the G0.5-MF nanocarrier, which signifies that even after the introduction of GO nanosheets, the surface chemistry of MF remains nearly unaffected, and the structure was inevitably preserved. However, the transmittance of the spectra was decreased, which may be due to the exposure of MF within GO nanosheets. In the case of the G0.5-MF-DOX nanocomposite, the unchanged characteristics peaks of G0.5-MF nanocarriers were detected. In addition, the characteristic peak positions at 1114 cm^{-1} and 810 cm^{-1} attributed to the stretching vibrations of C-O-CH₃ groups in DOX were observed in the IR spectra of G0.5-MF-DOX nanocomposite with lower transmittance, which further confirms the successful loading of DOX [19].

Further, to confirm the successful synthesis of GO, MF, and G0.5-MF nanocarriers, the elemental analysis of these nanocarriers was done and reported in **Table 5.2**. The GO synthesized via graphite oxidation using the modified Hummers' method leads to the introduction of oxygenated functional groups. The oxidation of graphite was confirmed via elemental analysis, as reported in **Table 5.2**. The C/O elemental wt. ratio for GO nanosheets was found to be 1.82. However, a room temperature synthesized MF involves C, O, and Fe as elements with a composition of 55.33 ± 0.72 , 23.60 ± 0.36 , and 21.07 ± 0.2 wt.%, respectively; similar findings were observed in literature with lower Fe content [16]. In MF, the C/O wt. ratio was found to be 2.34, whereas it was decreased for the G0.5-MF nanocarrier to 1.96 due to an increment in oxygen wt.% caused by the introduction of GO, which suggests the successful impregnation of GO in MF.

Table 5.2: Elemental composition of nanocarriers. (Results are expressed as mean $\pm$ SD).

Nanocarriers	Elemental composition (wt.%)			
	C	O	Fe	C/O
GO	64.55 $\pm$ 0.92	35.45 $\pm$ 0.61	-	1.82
MF	55.33 $\pm$ 0.72	23.60 $\pm$ 0.36	21.07 $\pm$ 0.2	2.34
G0.5-MF	50.16 $\pm$ 1.04	25.51 $\pm$ 0.45	24.33 $\pm$ 0.31	1.96

5.2.4.2. XPS spectra

The X-ray photoelectron spectroscopy analysis of synthesized GO, MF, and G0.5-MF nanocarriers, as well as drug-loaded G0.5-MF-DOX nanocomposite, was conducted in order to validate the presence of their respective functional groups. **Fig. 5.7a** represents the surface survey spectrums of GO, MF, and G0.5-MF nanocarriers within a binding energy range of 0-1000 eV. The high-resolution characteristic and deconvoluted peaks in the C1s and O1s region of GO are presented in **Fig. 5.7b**. Deconvolution of C1s spectra resulted in the four major peaks at 284.8 eV, 286.8 eV, 287.9 eV, and 289.1 eV ascribed to the graphitic or sp^2 carbon, C–O present in the epoxy or the hydroxyl groups, C=O in a carbonyl group, alongside to the –COOH in carboxyl functional groups presents in the GO, respectively [20,21]. Besides, four significant peaks at 530.9 eV, 532.0 eV, 533.1 eV, and 534.4 eV were obtained by deconvoluting the O1s spectrum. These peaks were corresponding ascribed to the different oxygenated functional groups such as C=O (carbonyl), –COOH (carboxylic), along with the C–OH (hydroxyl) and C–O–C (epoxide) present in the GO [20,22]. Additionally, the FTIR analysis also validated the presence of these oxygenated functional entities within GO.

In the case of a pristine MF nanocarrier, a full-range survey spectrum indicates the existence of C, O, and Fe elements (**Fig. 5.7a**). **Fig. 5.7c** describes the high-resolution deconvoluted C1s, O1s, and Fe2p XPS spectrums, respectively. Herein, C1s spectra were deconvoluted,

which yielded three characteristic peaks at 284.8 eV, 285.1 eV, and 288.9 eV. The peaks at 284.8 eV and 288.9 eV are designated to the phenyl and carboxylic groups present in the structural cage of MF, whereas the existence of carbon skeleton on the surface of MF can be indorsed by a peak at 285.1 eV. Similarly, in a deconvoluted O1s spectrum, the presence of Fe–O–C species was advocated by the peak at 531.9 eV.

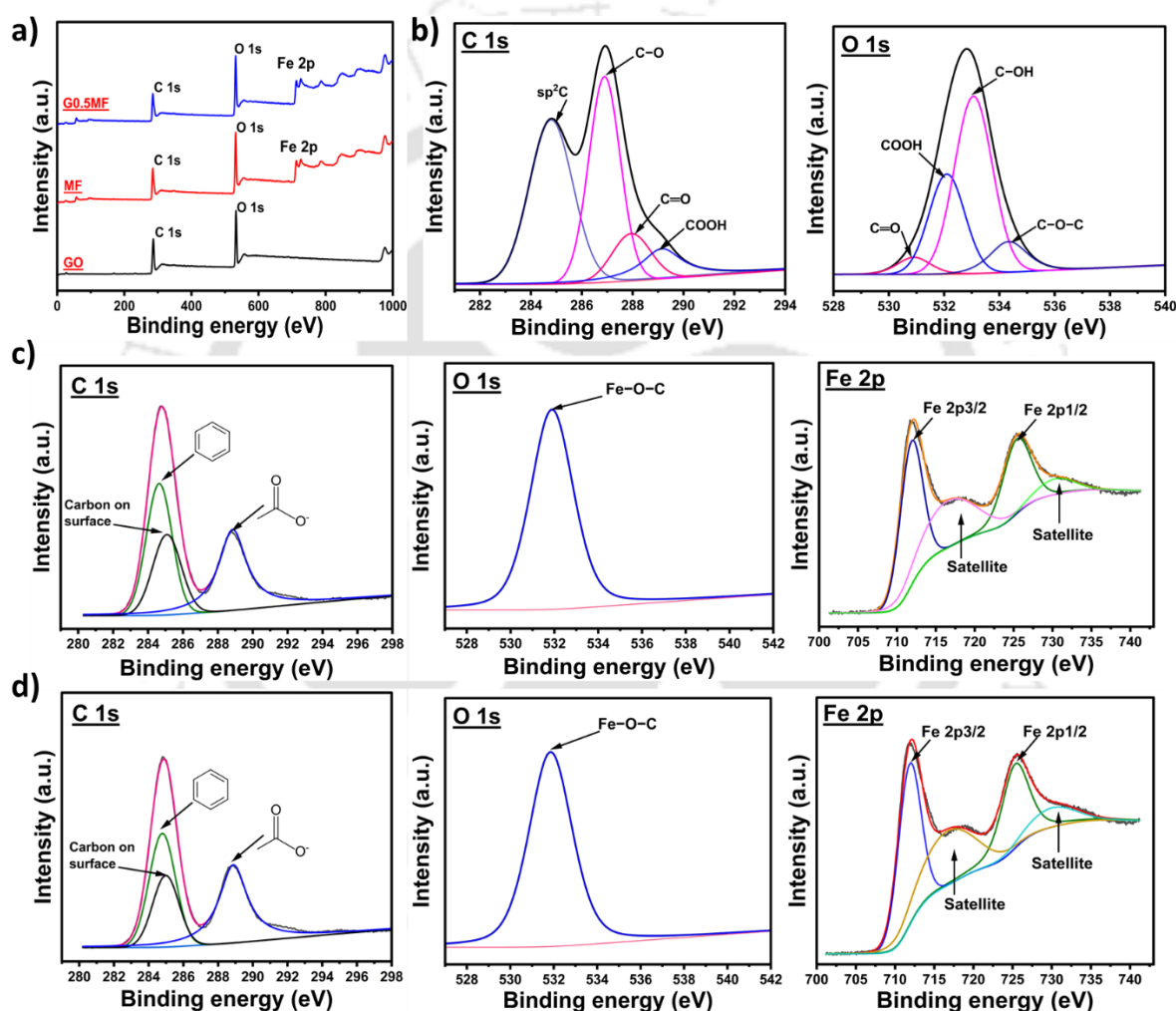


Fig. 5.7: XPS spectra of nanocarriers (a) Survey spectrum of GO, MF, G0.5-MF nanocarriers, (b) Deconvoluted C1s and O1s spectrum of synthesized GO (c, d) High-resolution C1s, O1s and Fe2p XPS spectra: deconvoluted peaks of synthesized pristine MF and G0.5-MF, respectively.

Further, high-resolution Fe2p XPS spectra were deconvoluted into four major peaks centered at 712.1 eV, 725.8 eV, 717.7, and 731.2 eV. Out of these, the characteristics peaks at 712.1 eV and 725.8 eV were designated to the Fe 2p_{3/2} and Fe 2p_{1/2} region, whereas peaks at 717.7

eV and 731.2 eV are attributed to the satellite peaks of $\text{Fe}_{3/2}$, and Fe $2p_{1/2}$ for the MF nanocarrier, respectively [23,24]. For the GO/MIL-100 (Fe) (G0.5-MF) nanocarrier, identical XPS survey spectra were observed, describing the presence of C, O, and Fe elements (**Fig. 5.7a**). In addition, identical peaks were detected after the deconvolution of C1s, O1s, and Fe 2p XPS spectrums for the G0.5-MF nanocarrier. The existence of identical peaks further advocates the presence of functional entities of MF within G0.5-MF. Moreover, a minor positive shift in binding energies (0.2 eV) was identified for G0.5-MF with respect to MF, as displayed in **Fig. 5.7d**. The positive shift in the binding energies of these XPS spectrums confirms that the interaction between GO and MF during the formation of G0.5-MF may be due to the non-covalent interactions [25–27].

The synthesized G0.5-MF nanocarrier was loaded with an anticancer chemotherapeutic agent, DOX. The obtained G0.5-MF-DOX nanocomposite was analyzed by XPS (**Fig. 5.8**), a survey spectrum elucidated the presence of C, O, N, and Fe elements corresponding to the G0.5-MF nanocarrier and DOX, as described in **Fig. 5.8a**. The high-resolution C1s, O1s, N1s, and Fe2p XPS spectrums have been deconvoluted and presented in **Fig. 5.8**. As shown in **Fig. 5.8b**, a deconvoluted C1s spectrum yielded into four peaks at 284.8 eV, 285.8 eV, 286.4 eV, and 288.5 eV, which are attributed to the characteristic peaks of C–C, C–N, C–O and C=O, respectively. A slight shift in the binding energy of epoxide/hydroxyl functional entities while preserving the binding energies of graphitic carbon as well as phenyl groups describes a strong interaction between the DOX and G0.5-MF nanocarrier caused may be due to the cation- π , non-covalent interactions [28,29]. In addition, the existence of C-N functional groups represents the amination of the carbon skeleton and advocates the successful loading of DOX on the G0.5-MF nanocarrier as described by FTIR spectroscopy (**Fig. 5.6**) [28,29]. Regarding the O1s spectra (**Fig. 5.8c**), a peak positioned at 532.3 eV signifying the Fe–O–C species of G0.5-MF or O=C– bonds of doxorubicin or absorbed water with a positive shift

(0.4 eV) in binding energy due to strong interaction between the nanocarrier and drug [25,29].

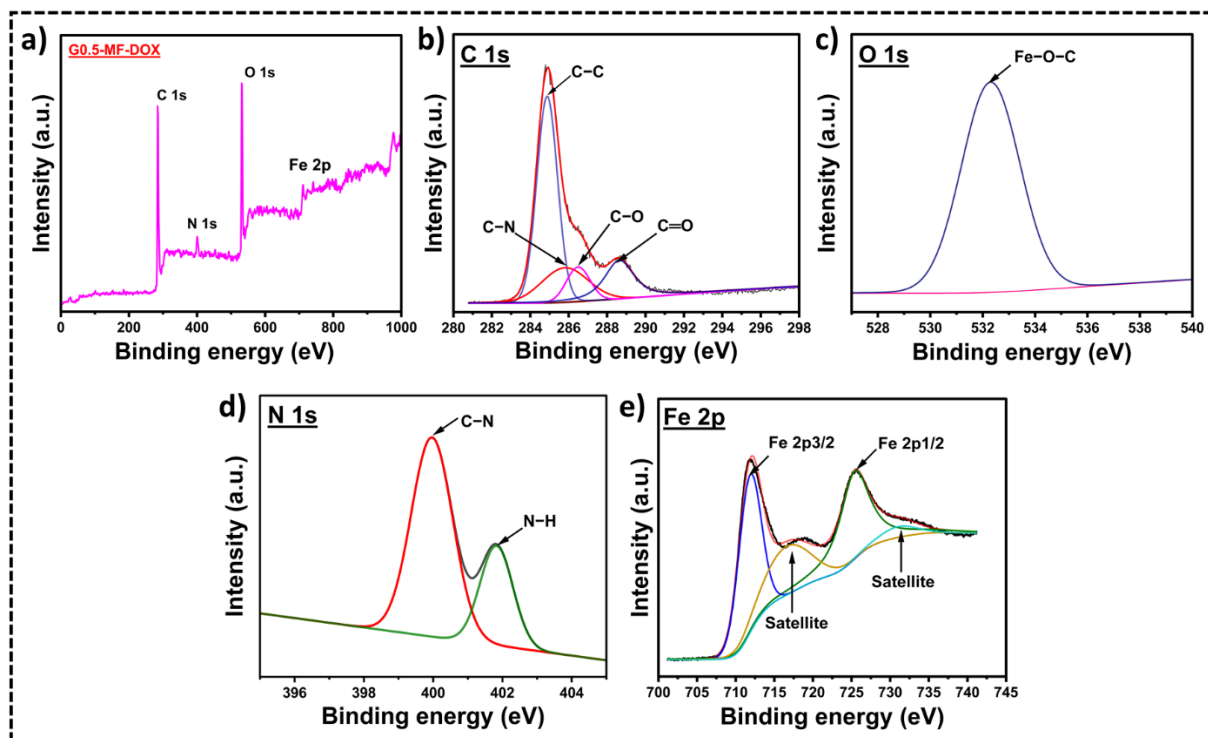


Fig. 5.8: XPS spectra of DOX loaded G0.5-MF nanocarrier (a) Survey spectrum of G0.5-MF-DOX nanocomposite, Deconvoluted (b) C1s spectrum, (c) O1s spectrum, (d) N1s spectrum, and (e) Fe2p spectrum.

Furthermore, as illustrated in **Fig. 5.8d**, the N1s spectra were alienated in two distinct peaks at 399.8 eV and 401.7 eV. A peak at 399.8 eV described C–N bonds of DOX with an expectation of decrement in the binding energy owing to its interaction with a metallic surface of G0.5-MF via electron density transfer from nitrogen groups to metals; similar observations were reported previously [29,30]. At the same time, a free amine group of DOX was attributed to the deconvoluted peak at 401.7 eV, which confirmed the loading of DOX on the G0.5-MF nanocarrier [28,30]. Finally, the high-resolution Fe2p spectra of the G0.5-MF-DOX were deconvoluted, as shown in **Fig. 5.8e**. Herein, major negative shifts in binding energies were observed for characteristics peaks of Fe 2p_{3/2} (711.9 eV) and Fe 2p_{1/2} (725.4 eV) region along with the satellite peaks of Fe_{3/2} (717.3 eV), and Fe 2p_{1/2} (731.0 eV) as compared to the MF/G0.5-MF nanocarriers. As stated earlier, the reduction in binding energy

could be contributed by the transfer of electron density from nitrogen and metallic surface as well as the non-covalent interactions between DOX and G0.5-MF [29,30].

5.2.5. Raman Spectra

Raman spectroscopy is a non-destructive characterization method that delivers valuable strips of evidence specifically for carbonaceous materials. The typical Raman spectra of GO, MF, and G0.5-MF nanocarriers obtained at an excitation wavelength of 488 nm are provided in **Fig. 5.9**. For GO, two characteristic peaks, namely, G-band and D-band, were identified at 1598 cm^{-1} and 1356 cm^{-1} , correspondingly. Previously, it was conveyed that G-band and D-band peaks vary within $1550\text{--}1630\text{ cm}^{-1}$ and $1320\text{--}1370\text{ cm}^{-1}$ based on the excitation wavelength [31,32]. Here, the G-band represents carbon atoms in an sp^2 hybridization state with the hexagonal-pinch mode of graphitic structure, and the D-band is attributed to the defect mode [33].

Further, a distinctive Raman spectrum of MF synthesized at room temperature conditions demonstrates specific characteristic peaks of MIL-100 (Fe) in the $400\text{--}2000\text{ cm}^{-1}$ region. In MF, the lattice vibrations mode and network binding modes can be observed in a region from 450 cm^{-1} to 600 cm^{-1} . However, the major peaks in Raman spectra of MF linked to the fingerprints of H_3BTC , i.e., trimesate linker and its aromaticity, were perceived at 800 cm^{-1} and 1002 cm^{-1} , respectively, as presented by peaks B and C. Furthermore, C–O–Fe stretching vibrations of Fe-trimesate were observed at around 1200 cm^{-1} with lower intensity in addition to the bonding vibrations of H–OH obtained in region D ($1400\text{--}1600\text{ cm}^{-1}$) due to the existence of coordinated water molecules in a network of Fe-trimesate linker [14]. The Raman spectra of the G0.5-MF nanocarrier follow the functional entities of both MF and predominantly of GO. It was observed that the principal characteristic peaks of MF in regions A, B, and C are sustained within the G0.5-MF nanocarrier with lower intensity. However, the

intensity of region 1200 cm^{-1} - 1600 cm^{-1} was increased for G0.5-MF compared to the MF nanocarrier, particularly for region D, which indicates the increase in defect concentration of G0.5-MF due to the presence of oxygenated GO nanosheets. The existence of GO in MF results in an upsurge in the oxygen content of G0.5-MF, as reported in **Table 5.2**, which may be responsible for the increase in defect concentration of G0.5-MF nanocarrier, similar to the oxidation of graphite into GO. The provided findings from the above characterization techniques confirm the successful synthesis of GO, MF, and Gw-MF nanocarriers.

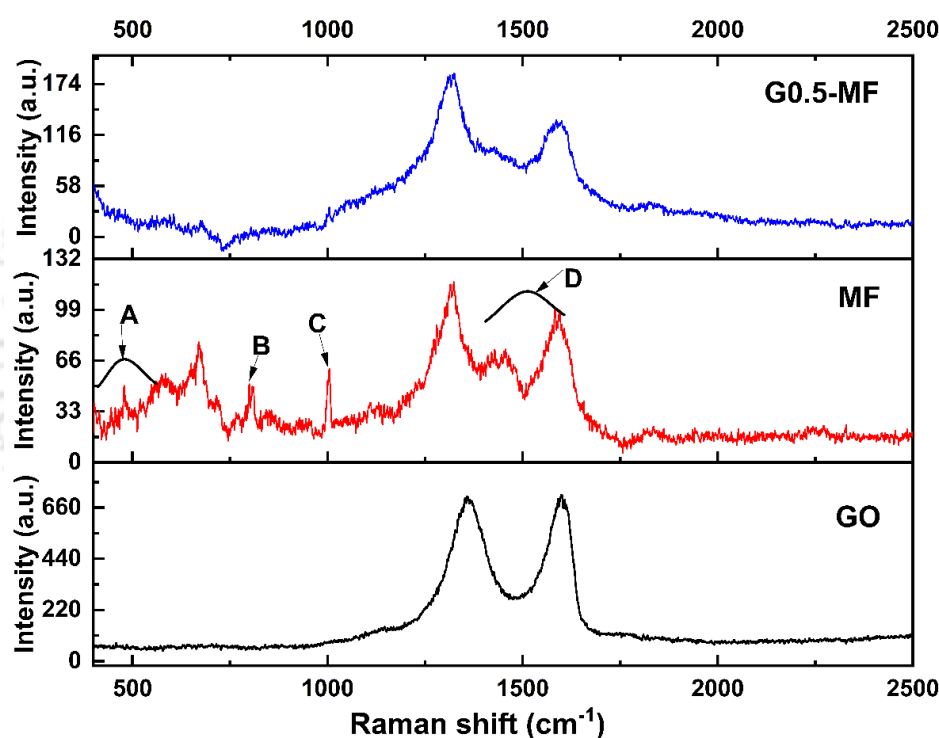


Fig. 5.9: Raman spectrum of GO, MF, G0.5-MF nanocarriers.

5.2.6. Loading characteristics of DOX

The DOX drug molecules were loaded on the pristine GO, MF, and Gw-MF nanocarriers, where $w = 0.1, 0.2, 0.5, 0.7$, and 1.0 represent the wt. % of GO within Gw-MF nanocarriers. The loading was accomplished via intermolecular hydrogen bonding along with the strong electrostatic, van der Waals, and π - π bonding interactions offered by GO, MF, and their composite nanocarriers. To improve the loading efficacy of a nanocarrier, the influence of loading parameters such as the amount of GO in nanocarriers and the weight ratio of the

DOX with nanocarrier was examined using a nanocarrier of maximum loading efficiency (G0.5-MF).

5.2.6.1 Effect of amount of GO in the nanocarriers

The DOX drug molecules were successfully loaded on the pristine GO, MF, and Gw-MF nanocarriers. The respective loading capacities of all the nanocarriers are shown in **Fig. 5.10A**. The maximum loading capacity of $37.32 \pm 1.86\%$ was observed for GO due to significant non-covalent interactions, encompassing intermolecular hydrogen bonding between hydroxyl and carboxyl groups, π - π stacking and van der Waals interfaces between the aromatic rings of GO and DOX. Whereas, in the case of pristine MF, the loading capacity was a minimum of $16.64 \pm 0.82\%$ due to the comparatively weaker surface interactions with DOX molecules. However, with an increase in the amount of GO in MF for Gw-MF nanocarriers, a significant increment in loading capacity was observed till the G0.5-MF nanocarrier may be due to adequate π - π stacking and van der Waals interactions within the nanocarriers and DOX molecules. It was observed that with a further increase in the amount of GO within Gw-MF nanocarriers, the DOX loading capacity was reduced, which may be due to partial hindering of MF surface by the GO nanosheets, lack of surface moieties and active sites for the adsorption process [9,34]. The maximum loading capacity of $23.53 \pm 1.18\%$ was observed for the G0.5-MF nanocarrier. Therefore, the G0.5-MF nanocarrier was used further to investigate the influence of the DOX weight ratio on loading capacity, subsequently.

5.2.6.2 Influence of DOX weight ratio

Figure 5.10B represents the effect of DOX vs. nanocarrier weight ratio on the drug loading efficiency. The loading was done in an identical condition of 25 °C, at 200 rpm for 24 h loading duration aimed for all the nanocarriers and the weight ratios. It was observed that,

with an increase in the amount of DOX with respect to the amount of G0.5-MF nanocarrier, the drug loading capacity was increased to $29.91 \pm 1.49\%$ for a 1 w/w ratio.

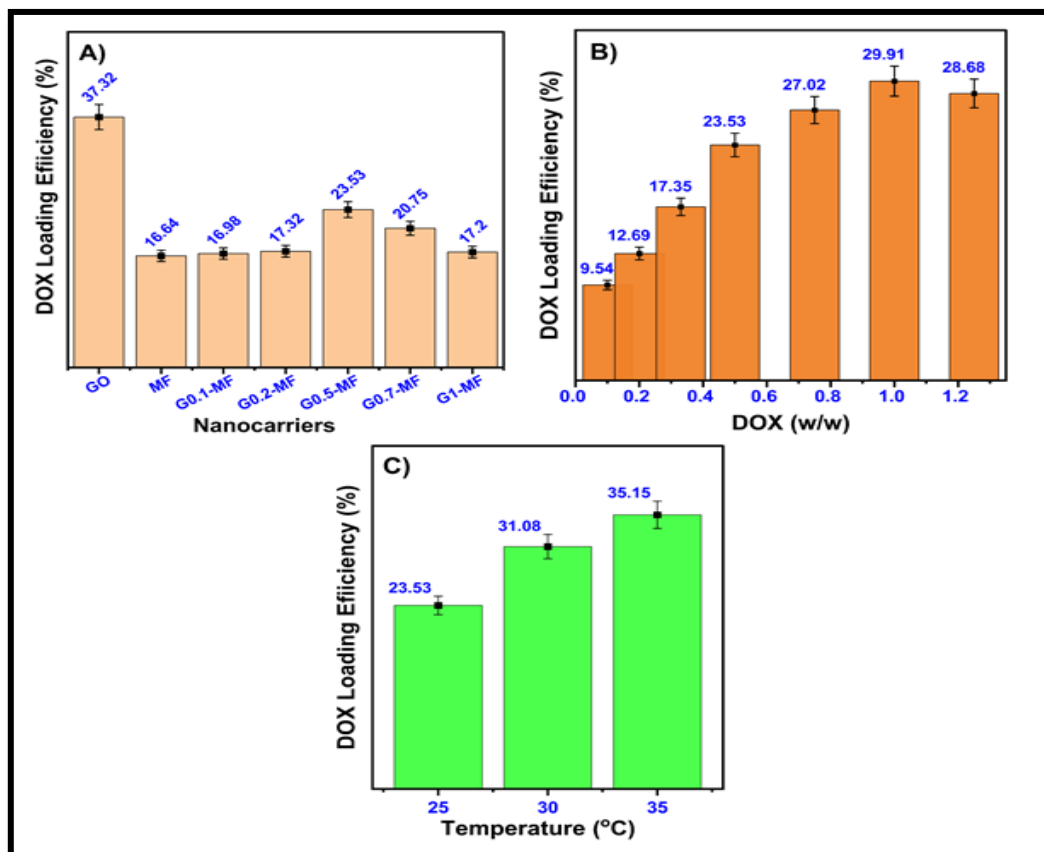


Fig. 5.10: Loading characteristics of DOX on GO, MF, and Gw-MF nanocarriers. (A) effect of the amount of GO on DOX loading efficiency, (B) influence of DOX: nanocarrier (G0.5-MF) weight ratio on drug loading efficiency, (C) influence of the loading temperature on DOX loading efficiency for G0.5-MF nanocarrier for 1:0.5 wt. ratio of G0.5-MF: DOX. The results are presented as mean $\pm$ SD.

Nevertheless, an additional increase in a drug quantity (>1 w/w) directs to the reduction in drug loading, which might be owing to a paucity of surface moieties and associated interactions across DOX and Gw-MF nanocarrier. The composites obtained after loading of DOX, namely, GO-DOX, MF-DOX, and G0.5-MF-DOX, were vacuum dried at 40°C for overnight and further used to study the *in-vitro* release behavior.

Table 5.3: Comparison of DOX loading efficiency of MOF-based nanocarriers.

Nanocarrier	Synthesis method	Loading conditions	Drug loading efficiency (%)	References
MIL-100 (Fe)	Hydrothermal route without HF	200 rpm, 25 °C, 24 h	23.40	[35]
Fe ₃ O ₄ @MIL-100 (Fe)	Hydrothermal route	RT, 30 rpm, 24 h	15.70	[36]
Fe ₃ O ₄ @MIL-100 (Fe)	Hydrothermal route	200 rpm, 25 °C, 24 h	19.40	[37]
CeO ₂ -MIL-100 (Fe)	Hydrothermal route Without HF	200 rpm, 25 °C, 24 h	13.30	[38]
MIL-101-N ₃ (Fe)	Solvothermal	RT, 24 h	13.40	[39]
UPNC@MIL-100 (Fe)	RT	RT, 24 h	17.20	[40]
ZnO-MIL-100(Fe)	Hydrothermal route	200 rpm, 25 °C, 24 h	23.50	[4]
ZIF-8	RT	-	20.00	[41]
NH ₂ -MIL-88B	Low-temperature synthesis (40 °C)	-	14.4	[42]
MgAl-LDH/Fe-MOF	Low-temperature synthesis (40 °C)	24 h pH 7.4	17.8	[43]
CMC/MOF-5/GO	Solvothermal route	RT, 72 h	6.45	[44]
MIL-100 (Fe)	RT	200 rpm, 25 °C, 24 h	16.64 ± 0.82	Present study
GO/MIL-100 (Fe)	RT	200 rpm, 25 °C, 24 h	29.91 ± 1.49	Present study

From the previous observations, G0.5-MF was considered to be an optimum nanocarrier with a GO of composition 0.5 wt.% and a maximum loading capacity of $29.91 \pm 1.49\%$ for a 1 w/w ratio of DOX. Therefore, it was further utilized to study the controlled release behavior of nanocarriers along with the pristine GO and MF. As the drug loading efficacy is related to the amount of drug present in the end product, the presented study indicated a significant drug loading capacity of G0.5-MF nanocarrier, with each gram of G0.5-MF-DOX nanocomposite containing roughly 300 mg of DOX. The DOX loading efficiency of the G0.5-MF nanocarrier is compared with the other MOF-based nanocarriers in **Table 5.3**. As reported in **Table 5.3**, the GO/MIL-100 (Fe) nanocarrier exhibits higher drug loading

efficiency than the virgin MIL-100 (Fe) prepared via conventional hydrothermal treatment, as well as the proposed sustainable approach.

5.2.7. Surface charge and colloidal stability of nanocarriers

The colloidal stability of the materials under physiological conditions is an important parameter for their potential application in biomedical fields such as drug delivery. Better stability can improve the bioavailability of the nanocarriers [45]. The colloidal dispersion characteristics of nanocarriers in terms of stability can be investigated by determining their zeta (ζ)-potential, representing the degree of electrostatic attraction/repulsion between adjacent particles and surface charge [46]. The surface charge defined from ζ -potential also plays a critical role in understanding the drug loading and release mechanism [47]. Herein, the surface charge of parent GO, MF, G0.5-MF nanocarriers, and drug-loaded G0.5-MF-DOX composites suspensions of concentration 0.1 mg mL^{-1} at physiological conditions (pH-7.4) were inspected by zeta (ζ)-potential analysis (Delsa nano C, Beckman Coulter) and the results are communicated as mean $\pm$ SD. Among all the nanocarriers, GO displayed better colloidal aqueous stability with a highly negative surface charge ($-35.6 \pm 1.6 \text{ mV}$). The pristine MF nanocarrier has a ζ -potential of $-24.6 \pm 0.8 \text{ mV}$, representing lower colloidal dispersion stability at physiological pH conditions. However, the negative surface charge was increased for the G0.5-MF nanocarrier to $-28.7 \pm 1.4 \text{ mV}$ due to the impregnation of GO, which illustrates better colloidal stability than the pristine MF.

Meanwhile, an anticancer chemotherapeutic agent, DOX, is a positively charged amphoteric drug ($6 \pm 1.5 \text{ mV}$) consisting of protonable amino and deprotonable phenolic groups [48]. A cationic surface of DOX provides a significant advantage for the effective loading of a drug over negatively charged nanocarriers such as GO, MF, and G0.5-MF. The loading was predominantly established in the interface between the drug and the carrier by non-covalent

bondings such as electrostatic and hydrophobic interactions [64], further decreasing the negative surface charge of DOX-loaded G0.5-MF nanocarriers to -19.4 ± 1.7 mV. The decrement in the zeta potential of the G0.5-MF-DOX nanocomposite further validates the successful loading of DOX with lower colloidal stability than the drug-free G0.5-MF nanocarrier under physiological conditions.

5.2.8. Thermodynamic study

The influence of loading temperature on the DOX loading efficiency was examined by implementing experiments for 250 mg L⁻¹ DOX solution at three distinct temperatures (25, 30, and 35 °C). The loading experiments were carried out with a G0.5-MF nanocarrier at 200 rpm for 24h and a solution of concentration 1 g L⁻¹. As per the observations, the loading efficiency of G0.5-MF was enhanced when the loading temperature was increased (**Fig. 5.10C**). The following thermodynamic equations have been used to estimate the thermodynamic parameters for the loading of DOX, including Gibbs free energy (ΔG°), entropy (ΔS°), and enthalpy (ΔH°) [49].

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ \quad (5.2)$$

$$\log\left(\frac{q_e m}{C_e}\right) = \frac{\Delta S^\circ}{2.303R} + \frac{-\Delta H^\circ}{2.303RT} \quad (5.3)$$

Where q_e is an amount of DOX laden per unit amount of G0.5-MF (mg g⁻¹), C_e is an equilibrium concentration (mg L⁻¹), m is a G0.5-MF nanocarrier concentration (g L⁻¹), and T is a temperature in K. Herein, $m = 1$ g L⁻¹ thus, Eq. 5.3 can be revised as follows:

$$\log\left(\frac{q_e}{C_e}\right) = \frac{\Delta S^\circ}{2.303R} + \frac{-\Delta H^\circ}{2.303RT} \quad (5.4)$$

The values of ΔG° have been defined from Eq. 5.2 via calculating the ΔH° and ΔS° . On the other hand, ΔH° and ΔS° have been evaluated from Eq. 5.4 by outlining the graph amongst $\log(q_e/C_e)$ vs. $1/T$. The calculated values of ΔG° , ΔH° , and ΔS° are stated in **Table 5.4**.

The loading of DOX on the G0.5-MF nanocarrier is termed to be an endothermic process as ΔH° obtained is positive [34,50]. Alongside this, the endothermic behavior of the loading process is additionally endorsed by the enhancement in loading efficacy by means of temperature (**Fig. 5.10C**). Herein, as the loading of DOX on the G0.5-MF nanocarrier was affected through the adsorption process, the elevated heat of adsorption throughout the loading process directs physisorption moderately [34,49]. Furthermore, the negative Gibbs free energy (ΔG°) attained for three diverse temperatures reveals that the loading process is spontaneous in nature. Moreover, the enhanced randomness at the surface interfaces of G0.5-MF and DOX throughout the loading process is demonstrated by the positive value of entropy (S°), which implies that the loading of DOX on G0.5-MF is arranged much more randomly [34,49].

Table 5.4: Thermodynamic parameters for the loading of DOX over G0.5-MF nanocarrier at different temperatures (Results are shown as mean $\pm$ SD).

Drug concentration (mg L ⁻¹)	ΔH° (kJ mol ⁻¹)	ΔS° (J mol ⁻¹ K ⁻¹)	$-\Delta G^\circ$ (kJ mol ⁻¹)		
			T = 298 K	T = 303 K	T = 308 K
250	6.914 $\pm$ 0.201	24.975 $\pm$ 0.749	0.529 $\pm$ 0.013	0.643 $\pm$ 0.019	0.778 $\pm$ 0.031

5.2.9. *in-vitro* release behavior of DOX and release kinetics

The *in-vitro* release contour of DOX from the GO-DOX, MF-DOX, and G0.5-MF-DOX nanocomposite into the PBS solution of pH 7.4 over a period of 600 h (25 days) is illustrated in **Fig. 5.11**. At an initial 5 h, the immediate burst release of around 20% was observed from GO-DOX nanocomposite, which may be due to the surface characteristics of the GO nanocarrier. However, a slower and controlled release of DOX was observed after the burst effect, which was deceptively revealed by the ion exchange phenomenon amongst DOX and GO nanocarrier. Meanwhile, around 50% of DOX was released within 24 h, and the release

trend was further extended for 25 days with a maximum release of 89%, as shown in **Fig. 5.11**. The obtained results have established that the releases of DOX out of GO-DOX nanocomposite were attended by an initial burst phase followed by a prolonged release phase. However, the conjugation of nanomaterials with GO may result in a slower release of DOX. Recently, Pooresmaeil and Namazi have investigated the DOX release behavior from β -CD grafted magnetic GO nanocarrier at pH 7.4. It was observed that nearly 8% of DOX was released in the first 5 h, followed by a sustained release of about 14 percent in 24 h [51]. In the case of pristine MF nanocarrier, burst release was not found, and $11.22 \pm 0.33\%$ of DOX was released in the initial 24h. Furthermore, around $20.41 \pm 0.60\%$ release was observed till the 5th day, resulting in sustained release of DOX from the MF-DOX composite and continued until 25 days with a maximum release of $34.39 \pm 1.03\%$, as shown in **Fig. 5.11**. A similar trend with a 30% release of DOX was observed for the MF nanocarrier synthesized via the conventional hydrothermal method [35]. However, the pristine MF nanocarrier synthesized by the proposed sustainable route has outperformed the hydrothermal route in DOX loading efficiency.

The G0.5-MF nanocarrier obtained from an in-situ process at room temperature had shown superior DOX loading capacity to that of pristine MF and another Gw-MF nanocarrier. However, in the release process, an initial burst effect was observed but to a lower extent than that of GO. At an initial 5h, $10.28 \pm 0.30\%$ of DOX was released, which signifies a reduction in the burst effect to nearly half of the GO nanocarrier; the comparison can be seen in **Fig. 5.11 (inset)**. In the first 24 h, $27.38 \pm 0.81\%$ of DOX was released, which was higher than the MF nanocarrier. After that, a well-controlled and sustained DOX release from the G0.5-MF-DOX nanocomposite was observed with $45.24 \pm 1.35\%$ release in 5 days and a maximum release of $70.96 \pm 2.10\%$ in 25 days. The resultant drug release from the DOX-loaded G0.5-MF nanocarrier was complemented by the initial low burst segment followed by a sustained

release phase over the longer duration. These observations indicated the synergetic effect over the impregnation of GO within the G0.5-MF nanocarrier due to strong interaction with DOX molecules and subsequent slower release.

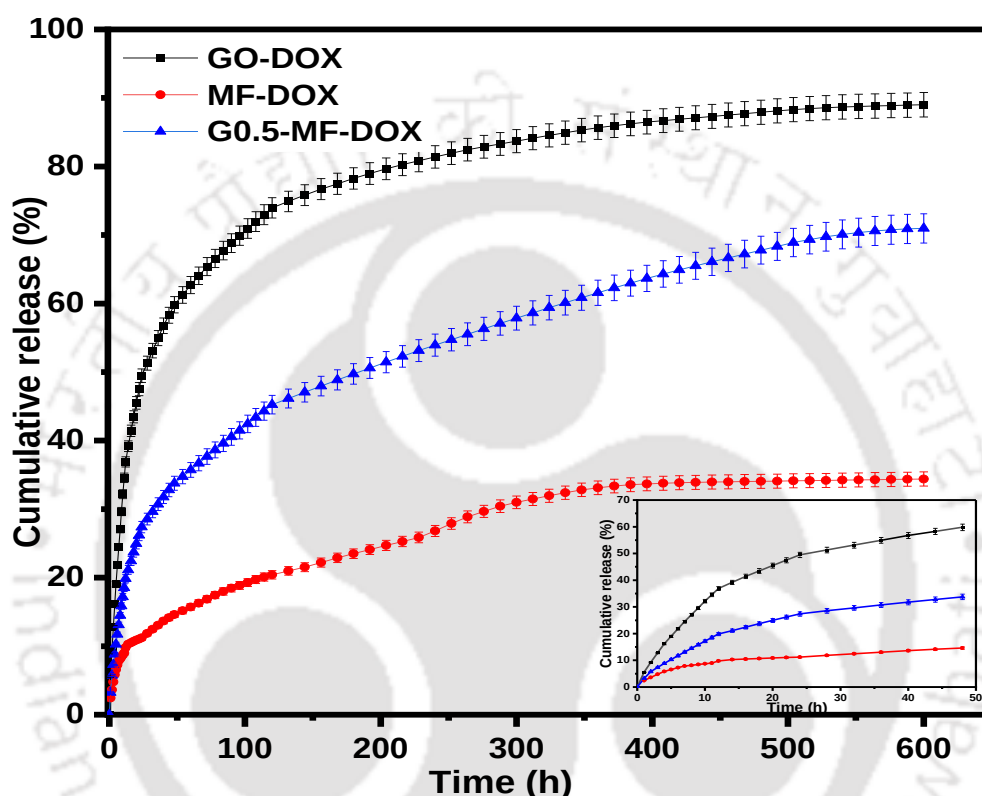


Fig. 5.11: *In-vitro* release contour of DOX from the GO-DOX, MF-DOX, and G0.5-MF-DOX nanocomposite at pH 7.4. (The results are represented as mean $\pm$ SD).

The first burst release phase may be very beneficial from the perspective of cancer therapies for obtaining the required drug therapeutic dose of DOX [12]. However, the higher initial burst release may result in rapid damage to normal cells due to overdosing; therefore, it is necessary to regulate the initial burst and overall release of the drug. In addition, the ensuing prolonged release potentially helps to maintain the cumulative dosage during the treatment for a longer period of time. The release profile of the G0.5-MF nanocarrier showed substantial tactics of controlled and sustainable drug release with higher drug loading capacity, which ultimately may result in the reduction in dosing frequency and the side effects of chemotherapy.

In the present study, we have employed five distinct kinetic drug-release models over the DOX release contour from the G0.5-MF nanocarrier at pH 7.4 in PBS. The kinetic models like zero-order [52], pseudo-first-order, pseudo-second-order, Higuchi model, and Korsmeyer-Peppas model [5,53,54] were employed to investigate the release kinetics of DOX from the G0.5-MF-DOX nanocomposite as reported in **Table 5.5**.

Table 5.5: Rate constants (k), Release exponent (n), and correlation coefficients (R^2) of kinetic models related to the release of DOX from G0.5-MF-DOX nanocomposite.

Release media	Saturated Release (%)	Kinetic model	(R^2)	Rate constant (k)	Confidence limits (Upper, Lower)
PBS Buffer pH=7.4	70.96	Zero-order $\frac{M_t}{M_e} = k_o t$	0.88	$k_o = 1.65 \times 10^{-3} \text{ min.}^{-1}$	$1.67 \times 10^{-3}, 1.63 \times 10^{-3}$
		Pseudo-first-order $\ln(M_e - M_t) = \ln M_e - kt$	0.94	$k = 1.84 \times 10^{-3} \text{ min.}^{-1}$	$1.86 \times 10^{-3}, 1.83 \times 10^{-3}$
		Pseudo-second-order $\frac{t}{M_t} = \frac{1}{kM_e^2} + \frac{t}{M_e}$	0.99	$k = 3.14 \times 10^{-3} \text{ mg. min.}^{-1}$	$3.69 \times 10^{-3}, 2.60 \times 10^{-3}$
		Higuchi $M_t = k_h t^{1/2}$	0.96	$k_h = 0.20 \text{ min.}^{-0.5}$	$2.09 \times 10^{-1}, 2.06 \times 10^{-1}$
		Korsmeyer-Peppas $\frac{M_t}{M_\infty} = k_p t^n$	0.96	$k_p = 6.29 \times 10^{-2} \text{ min.}^{-n}$ $n = 0.39$	$6.51 \times 10^{-2}, 6.07 \times 10^{-2}$

Where k is the respective rate constant of the kinetic models, M_t and M_e are the masses of DOX released out of G0.5-MF-DOX nanocomposite at time t, and equilibrium, correspondingly, n is diffusional or release exponent. The stated kinetic models are illustrated in **Fig. 5.12**, and the corresponding correlation coefficients (R^2) and rate constants (k) are provided in **Table 5.5**. As a criterion for choosing the most effective kinetic model to represent DOX release from the G0.5-MF-DOX nanocomposite, the correlation coefficients are also presented in **Fig. 5.12**.

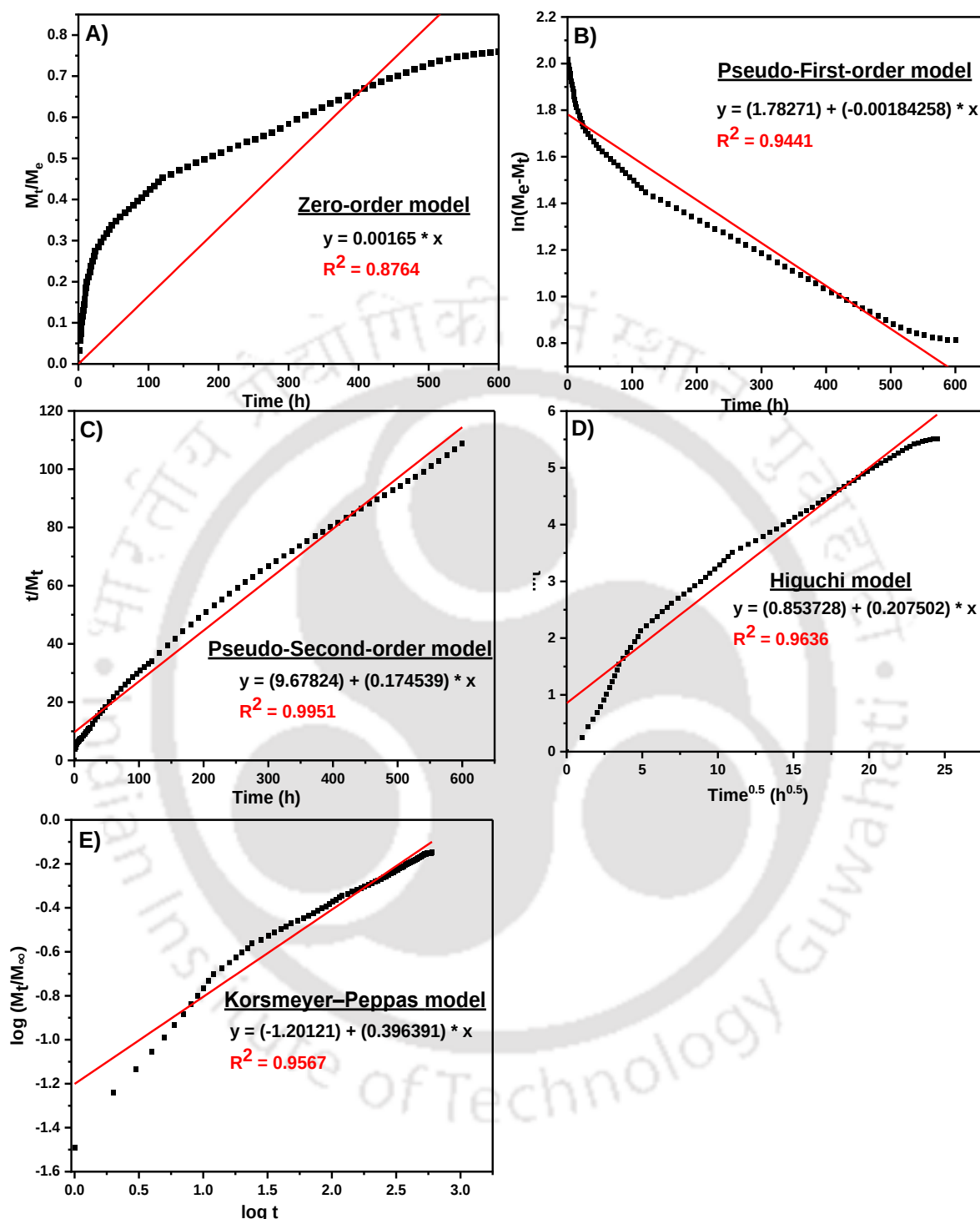


Fig. 5.12: Kinetic model data fit for DOX release out of the G0.5-MF-DOX nanocomposite (A) Zero-order (B) pseudo-first-order, (C) pseudo-second-order, (D) Higuchi, (E) Korsmeyer-Peppas.

Herein, amongst all the stated kinetic models, the pseudo-second-order kinetic model (**Fig. 5.12C**) was more suitable, with an R^2 of 0.99. Moreover, the release exponent was found to be around 0.39 (< 0.5) for the Korsmeyer-Peppas model (**Fig. 5.12E**) with $R^2 = 0.96$,

suggesting that the DOX release out of G0.5-MF-DOX nanocomposite is guided by the Fickian diffusion [6]. The Higuchi model (**Fig. 5.12D**), which relies on the time square root for drug release, again validated the observations [5].

5.2.10. Cytotoxicity study

The key objective of any drug carrier is to deliver the drug in its active form to the target site without instigating any undesired consequences on the environment. Cytocompatibility plays a significant role in the selection of nanocarriers for biomedical applications [8]. In this direction, the cytocompatibility of the pristine G0.5-MF nanocarrier and DOX-loaded G0.5-MF nanocomposite was analyzed by the MTT assay using cancer cell line A549 and normal cell line HEK293. As shown in **Fig. 5.13**, the nanocarrier G0.5-MF was observed to be highly cytocompatible toward normal and cancer cell lines for the tested incubation period of up to 07 days.

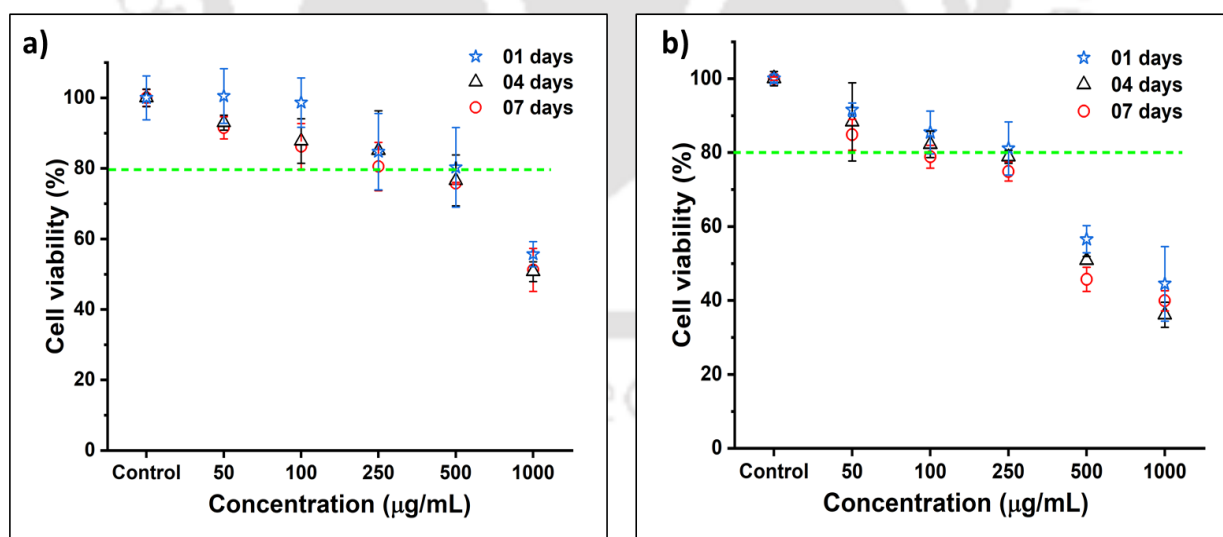


Fig. 5.13: Cell viability of G0.5-MF nanocarrier toward (a) Normal cell lines (HEK293) and (b) Cancer cell lines (A549) (Results are expressed as mean $\pm$ SD).

For the HEK293 cells, nanocarrier concentrations up to 500 $\mu\text{g mL}^{-1}$ showed above 80% cell viability for 01 and 04 days, which was slightly decreased to 75% for the 07 days of incubation. Such high cell viability confirmed its suitability as a biomaterial in accordance

with ISO 10993-5:2009 (Biological evaluation of medical devices – Part 5: Tests for in vitro cytotoxicity) [55].

The highest tested concentration of G0.5-MF showed decreased cytocompatibility with around $55 \pm 5\%$ cell viability. The IC_{50} values of G0.5-MF for HEK293 cells were calculated as $1314 \pm 27.84 \mu\text{g mL}^{-1}$, $1057 \pm 31.71 \mu\text{g mL}^{-1}$ and $982 \pm 39.28 \mu\text{g mL}^{-1}$ for the 01, 04 and 07 days, respectively. For A549 cells, the nanocarrier concentrations up to $250 \mu\text{g mL}^{-1}$ showed above safety criteria for cytocompatibility till the 07 days of incubation. The IC_{50} values for A549 cells were observed as $732 \pm 15.42 \mu\text{g mL}^{-1}$, $585.2 \pm 17.55 \mu\text{g mL}^{-1}$, and $576 \pm 17.28 \mu\text{g mL}^{-1}$ for the 01, 04 and 07 days, respectively. These observations indicated the excellent cytocompatibility of the prepared nanocarrier up to a concentration of $250 \mu\text{g mL}^{-1}$. The effects of a model anticancer drug DOX in free and nanocarrier-loaded forms on the normal and cancer cell lines were analyzed as shown in **Fig. 5.14 (a, b)**. The anticancer drug DOX was found to be toxic for both normal and cancer cells [56]. DOX concentration of $25 \mu\text{g mL}^{-1}$ showed above 80% cell viability for 01 and 04 days and slightly decreased 78% cell viability for 07 days incubation for HEK293 cells. In the case of A549 cells, the cell viability decreased to around 60% for the $25 \mu\text{g mL}^{-1}$ DOX, as expected. The IC_{50} values of DOX for HEK293 cells were observed as $105 \pm 4.30 \mu\text{g mL}^{-1}$, $83 \pm 1.66 \mu\text{g mL}^{-1}$, and $75.9 \pm 1.56 \mu\text{g mL}^{-1}$ for the 01, 04 and 07 days, respectively. While for the A549 cells, the IC_{50} values were $54 \pm 1.62 \mu\text{g mL}^{-1}$, $36 \pm 1.80 \mu\text{g mL}^{-1}$, and $28 \pm 0.77 \mu\text{g mL}^{-1}$ for the 01, 04 and 07 days, respectively. The reduction in the IC_{50} values for different incubation periods may be attributed to the improved uptake of the DOX by the cells with the incubation duration via endocytosis. The observed IC_{50} value of DOX against HEK293 cells was found to be comparable with the reported literature [57].

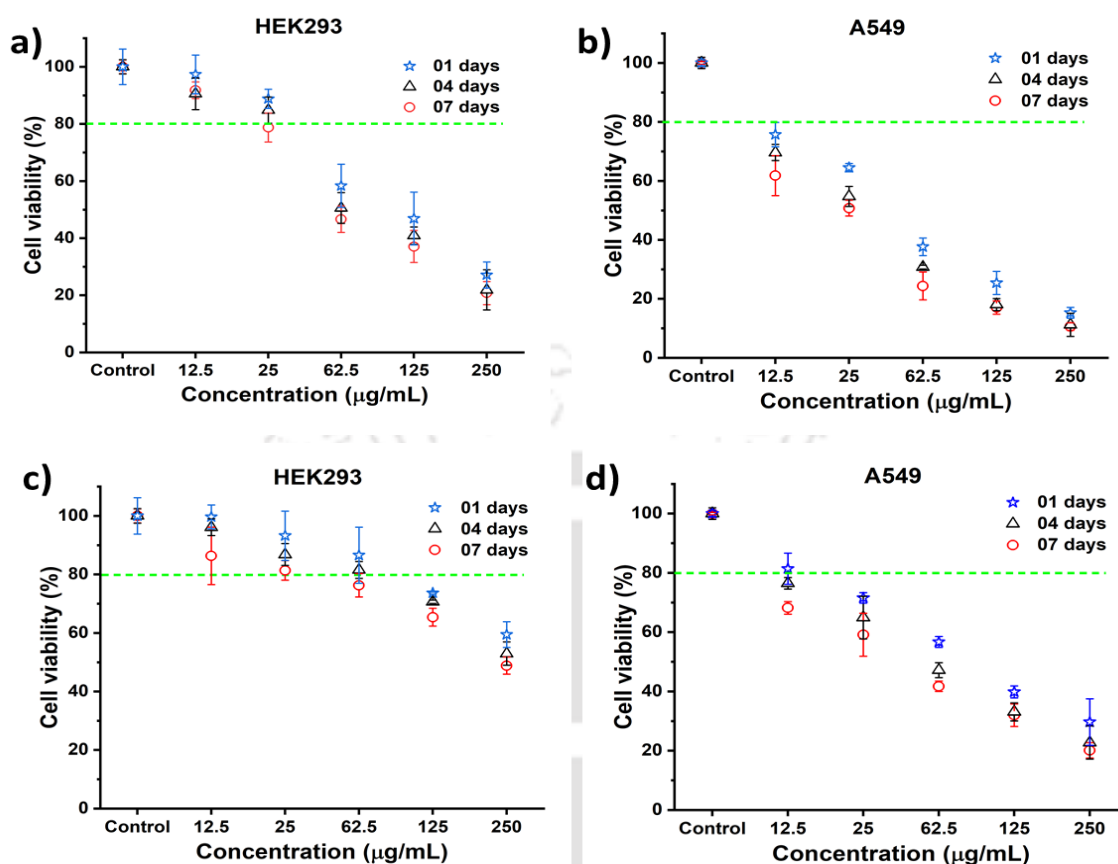


Fig. 5.14: Effects of concentrations of free DOX (a and b) and DOX in G0.5-MF-DOX (c and d) on the cell viability of HEK293 and A549 cell lines. (Results are communicated as mean $\pm$ SD).

Based on the cytocompatibility of nanocarrier and anticancer properties of DOX on HEK293 and A549 cells, G0.5-MF loaded with DOX (G0.5-MF-DOX) (~25% loading efficiency considering the degradation of DOX during experimentation) was evaluated for its potential application as drug nanocarrier. As shown in **Fig. 5.14 c**, HEK293 cells were observed to be $80 \pm 5\%$ viable till the G0.5-MF-DOX concentration of $250 \mu\text{g mL}^{-1}$, i.e., $62.5 \mu\text{g mL}^{-1}$ of DOX for the tested periods of incubation. The observed results were higher than that of free DOX ($25 \mu\text{g mL}^{-1}$), attributed to the excellent cytocompatibility of the prepared nanocarrier and the slower release of loaded DOX from the nanocarrier. The IC_{50} values for HEK293 were observed as $313 \pm 9.39 \mu\text{g mL}^{-1}$, $239.1 \pm 5.10 \mu\text{g mL}^{-1}$, and $194.4 \pm 4.85 \mu\text{g mL}^{-1}$ for the 01, 04 and 07 days, respectively. In the case of A549 cells (**Fig. 5.14 d**), the cytocompatibility was observed to decrease significantly up to $65 \pm 5\%$ for $25 \mu\text{g mL}^{-1}$ of

DOX for the incubation time of up to 07 days. The further increased concentration of $62.5 \mu\text{g mL}^{-1}$ resulted in the reduction in half of the viable cells. For the same concentration, HEK293 cells showed above 80% viability for 01, 03, and 07 days of incubation. The IC_{50} values of loaded DOX for A549 cells were found to be $101.7 \pm 2.45 \mu\text{g mL}^{-1}$, $75.2 \pm 1.67 \mu\text{g mL}^{-1}$, and $52 \pm 1.29 \mu\text{g mL}^{-1}$ for the 01, 04 and 07 days, respectively, as listed in **Table 5.6**.

Table 5.6: IC_{50} values ($\mu\text{g mL}^{-1}$) of the tested samples against the mammalian cells. (Results are communicated as mean $\pm$ SD).

Sample	HEK293			A549		
	Day 01	Day 04	Day 07	Day 01	Day 04	Day 07
DOX	105 ± 4.30	83 ± 1.66	75.9 ± 1.56	54 ± 1.62	36 ± 1.80	28 ± 0.77
G0.5-MF	1314 ± 27.84	1057 ± 31.71	982 ± 39.28	732 ± 15.42	585.2 ± 17.55	576 ± 17.28
G0.5-MF-DOX	313 ± 9.39	239.1 ± 5.10	194.4 ± 4.85	101.7 ± 2.45	75.2 ± 1.67	52 ± 1.29

Overall, the prepared nanocarrier G0.5-MF has shown excellent cytocompatibility towards normal as well as cancer cells, confirming its suitability for biological applications. The DOX-loaded nanocarrier has not shown any significant decrease in viability of normal cells up to $62.5 \mu\text{g mL}^{-1}$ of DOX concentration; however, the same was reduced to $50 \pm 5\%$ in the case of cancer cells. The observed anticancer properties of the loaded DOX were also comparable with the free DOX, suggesting the adequacy of DOX-loaded G0.5-MF nanocarrier in cancer therapy.

Summary

A novel one-pot, more accessible, and sustainable approach was adopted for the HF-free synthesis of GO/MIL-100 (Fe) nanocarriers of different GO weight fractions at room temperature. The electron and atomic force microscopic images of Gw-MF have demonstrated the effective formation of sharp-edged octahedral MIL-100 (Fe) crystals dispersed on flat and exfoliated GO nanosheets. The FTIR and XPS analysis have advocated

a successful synthesis of pristine GO, MF, and G0.5-MF nanocarriers, as well as DOX-loaded G0.5-MF composite. The synthesized Gw-MF nanocarriers have shown better loading capacity for DOX than pristine MIL-100(Fe) (MF). Among all the Gw-MF nanocarriers, the maximum loading efficiency of $29.91 \pm 1.49\%$ was observed for G0.5-MF nanocarriers. The *in-vitro* release profile of obtained MF and G0.5-MF nanocarriers showed a controlled and sustainable DOX release compared to an initial burst effect for pristine GO nanocarrier. As per the *in-vitro* cytotoxicity studies conducted for a period of 7 days, the G0.5MF-DOX nanocomposite presented improved cytotoxicity towards A549 without showing noticeable cytotoxicity towards HEK293.

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Chapter 6:

Room temperature synthesis of β -cyclodextrin functionalized graphene oxide decorated MIL-100 (Fe): a sustainable drug cargo for controlled delivery of caffeic acid

Chapter 6

Room temperature synthesis of β -cyclodextrin functionalized graphene oxide decorated MIL-100 (Fe): a sustainable drug cargo for controlled delivery of caffeic acid

The current chapter outlines a self-assembled and sustainable methodology for producing HF-free β -CD-GO/MIL-100(Fe) nanocarriers comprising varied β -CD-GO amount at room temperature. Detailed investigations were conducted on the surface morphology, chemistry, and structure of the produced GO, β -CD-GO, MIL-100 (Fe) (MF), and BGw-MF nanocarriers. The obtained nanocarriers were subsequently loaded with a natural chemotherapeutic agent, caffeic acid (CA). Loading characteristics of CA on the developed nanocarriers have been investigated for variable β -CD-GO amount in the nanocarriers, pH of the media, and CA to nanocarrier mass ratios. For analyzing the controlled release behavior of the nanocarrier, the optimized CA-loaded BGw-MF nanocomposite was subjected to the release of CA at physiological and cancer environment pH conditions, followed by its release kinetics. In addition, an in-vitro cytotoxicity assessment of the optimized BGw-MF nanocarrier was done on normal cells (HEK293) and cancer cell lines (A549).

6.1. Experimental

6.1.1. Materials

The details of preparatory materials for the synthesis of GO and MIL-100 (Fe) are provided in **sections 2.1.1 and 5.1.1**, respectively. The β -Cyclodextrin ($C_{42}H_{70}O_{35}$, 99%) and caffeic acid ($C_9H_8O_4$, 98%) were obtained from Merck Life Science Pvt. Ltd. (Mumbai,

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India). Cultured mammalian cells, SDS-HCl (10%), MTT reagent, and tissue culture medium were acquired from Gibco.

6.1.2. Synthesis of β -Cyclodextrin-graphene oxide (β -CD-GO) nanoconjugate

The β -CD-GO nanoconjugate was synthesized using a well-homogenized 2 mg mL⁻¹ aqueous solution of pristine β -CD and GO, respectively. The GO was synthesized by employing the Hummers' approach with certain modifications using synthetic graphite as a parent material; the process details are described in **section 5.1.2** of the preceding chapter.

For the preparation of nanoconjugate, the aqueous solutions of β -CD and GO were initially mixed in a beaker and sonicated for 15 min. Later, the pH of this solution was adjusted to 10 via dropwise addition of NH₃ solution, followed by continuous stirring and heating at 60 °C for 5 h. A black color solution of β -CD-GO was collected after the conjugation reaction, which was further washed and centrifuged till the pH of the supernatant reached that of DI water. The obtained β -CD-GO suspension was collected and vacuum-dried for further application.

6.1.3. Preparation of MIL-100(Fe) and β -CD-GO/MIL-100(Fe) nanocarriers

The pristine MIL-100 (Fe) (MF) and β -CD-GO/MIL-100 (Fe) (BGw-MF) nanocarriers were synthesized by adopting a self-assembled and sustainable approach described in **section 5.1.3** of the previous chapter. Herein, to investigate the impact of the quantity of β -CD-GO in MF-based nanocarriers on drug loading efficiency, different BGw-MF nanocarriers were synthesized by altering the weight of β -CD-GO in solution 1 as $w = 0.05, 0.1, 0.25, 0.5, 0.75$, and 1 wt.% with respect to pristine MF. **Figure 6.1** illustrates the methodology adopted for the preparation of MF and BGw-MF-based nanocarriers. The obtained BGw-MF nanocarriers

were dried and desiccated for subsequent characterizations and drug-loading experimentations.

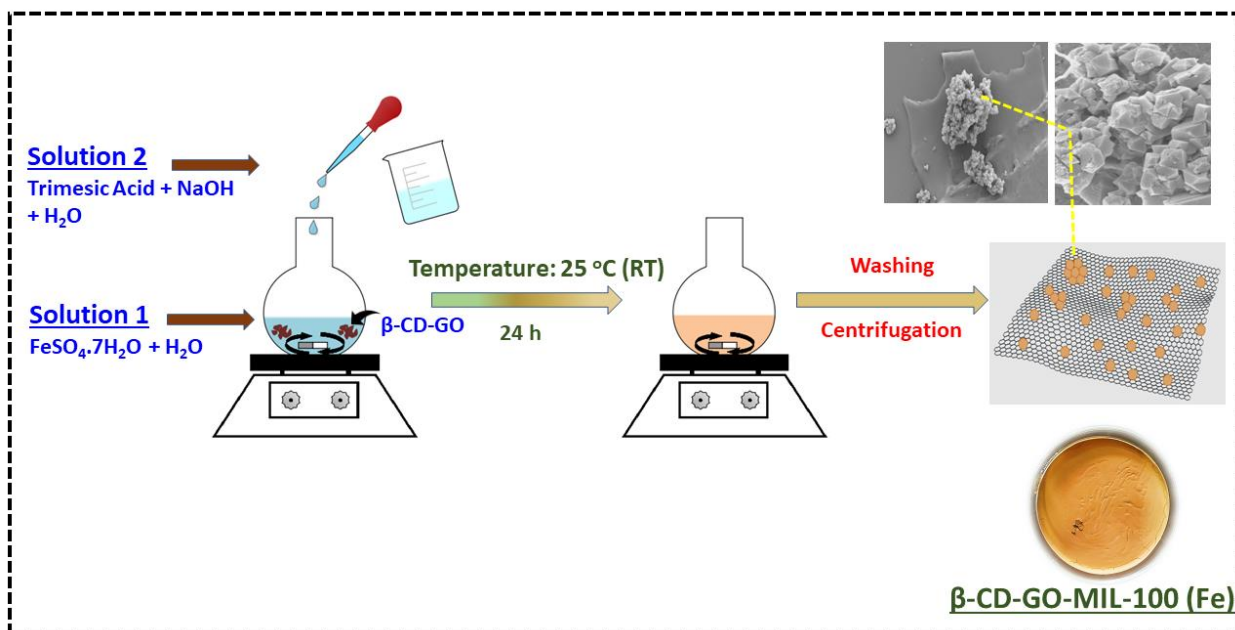


Fig. 6.1: Schematic representation for the sustainable synthesis of β -CD-GO/MIL-100 (Fe) nanocarriers.

6.1.4. CA loading process

The CA loading experiments for β -CD-GO (BG), MF, and BGw-MF nanocarriers were undertaken as per the previously reported protocols [1,2]. Typically, 10 mg CA was dispersed in 25 mL Milli-Q water using bath sonication. Subsequently, 10 mg MF was added to the CA solution to get a mass ratio of 1:1 for MF:CA nanocomposite. Through 0.1 M NaOH solution, the pH of MF-CA suspension was brought to 5. Later, this suspension was positioned in an incubator shaker in dark conditions at 200 rpm for 16 h at 25 °C. The resultant MF-CA was exposed to centrifugation at 10000 rpm for 20 min. The supernatant was collected and diluted to determine the concentration of free CA using UV-Vis spectroscopy at $\lambda_{\text{max}} = 325$ nm. The MF-CA suspension acquired after centrifugation was retrieved and vacuum dried at 40 °C for subsequent analysis. All other BGw-MF nanocarriers, including BG, BG0.05-MF, BG0.1-

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MF, BG0.25-MF, BG0.5-MF, BG0.75-MF, and BG1-MF, underwent the identical loading procedures.

The concentration of CA in the supernatant was used to evaluate the amount of CA unloaded and calculate the drug loading efficiency (LE%) and entrapment efficiency (EE%) of the nanocarrier with the formula:

$$\text{Loading Efficiency (\%LE)} = \frac{W_1 - W_2}{W_3} \times 100 \quad (6.1)$$

$$\text{Entrapment Efficiency (\%EE)} = \frac{W_1 - W_2}{W_1} \times 100 \quad (6.2)$$

W_1 , W_2 , and W_3 denote different weights of CA at the initial condition, free CA in the supernatant, and the CA-loaded nanocomposite, respectively.

Later, the BGw-MF nanocarrier with the highest loading efficiency was further used to inspect the effect of a variable quantity of CA on the LE of the nanocarrier by varying nanocarrier: CA mass ratios as 1:0.5, 1:1, 1:1.5, 1:2, and 1:2.5 under the same loading conditions. Furthermore, the influence of the pH of the media on the LE of the nanocarrier was also investigated by altering the pH from 5 to 7 and 9. All loading investigations were done in triplicate, and the numerical findings were described with the mean and standard deviation (SD). After being loaded with CA, the nanocomposites BG-CA, MF-CA, and BGw-MF-CA were vacuum-dried at 40 °C and subjected to further characterization.

6.1.5. *in-vitro* release of CA

Two distinct pH conditions, pH 7.4, mimicking physiological conditions, and pH 5, representing the cancer microenvironment, were employed to investigate drug release characteristics of drug-loaded nanocomposite using phosphate-buffer solution (PBS) and sodium acetate buffer (SAB), respectively. The BGw-MF-CA nanocomposite (50 mg) with

the highest loading efficiency for CA was added to the 50 mL PBS and SAB buffer solutions and subjected to an incubator at 37 °C, 200 rpm for a duration of 8 days. The release samples were assembled from these solutions at a pre-set time interval of 15 min. For the next 15 min., these solutions were centrifuged at 10000 rpm, and later, the concentration of CA released was determined to set up the CA release profile. The release experiments were carried out twice, and the mean $\pm$ standard deviation is represented for the numerical data.

6.1.6. Cell viability assay

Cell lines (HEK293 and A549) were purchased from ATCC and cultured according to protocol. In order to conduct cell viability experiments, cells were implanted into 96-well flat-bottom microtiter plates (CytoOne, Eppendorf) at a concentration of 2×10^4 cells per well. The cells were incubated with BG0.5-MF and BG0.5-MF-CA in HEK293 and A549 cell lines at variable concentrations (0, 100, 200, 300, and 400 $\mu\text{g mL}^{-1}$) for 48 h. Furthermore, the cells were treated for 3 to 4 hours using 10 μL of 2 mg mL^{-1} MTT solution (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (Merck Millipore)) prepared in PBS. After the MTT solution extraction from the plate, 100 μL of DMSO was injected into each well. After 30 min. on a rocking shaker, the plates were removed, and absorbance was measured at 570 nm using a spectrophotometer (Biotek) to determine the % cell viability [3,4]. The MTT test uses mitochondrial reductase to convert the water-soluble yellow dye MTT into the insoluble purple formazan. The cell viability was determined as follows:

$$\text{Viability}(\%) = \frac{\text{Sample absorbance}_{570}}{\text{Control absorbance}_{570}} \times 100 \quad (6.3)$$

6.1.7. Characterization

The structural, functional, and morphological characteristics of prepared BG, MF, and BGw-MF nanocarriers were analyzed by FETEM, N_2 adsorption-desorption isotherms, FTIR, XPS,

FESEM, XRD, and Raman spectroscopy; the instrumentation details are provided in **section 2.1.4 and 3.1.7**.

Furthermore, the materials' chemical functionalities were determined via X-ray photoelectron spectroscopy (XPS) analysis (ULVAC-PHI, Inc., Japan). The elemental analyses of nanocarriers were also inspected via XPS analysis. Caffeic acid concentration was calibrated at 325 nm by means of a UV-Vis spectrophotometer (Thermofisher Scientific, Aquamate 8000). To examine the nanocarriers' oxidation status and reduction properties, Raman spectra were achieved at 514 nm excitation wavelength (Horiba Jobin Vyon LabRam HR model).

6.2. Results and discussion

6.2.1. Surface morphology of nanocarriers

The TEM and FESEM analysis was employed to investigate the surface characteristics of β -CD-GO (BG), MF, and β -CD-GO decorated MF nanocarriers. The β -CD-GO nanocarrier recovered after vacuum drying displays a well-exfoliated and crumpled paper-like shape, as seen in **Fig. 6.2a**. A polycrystalline nature can also be observed in the selected area electron diffraction (SAED) pattern of β -CD-GO nanosheets, comparable to that of GO nanosheets with a distinguished diffraction pattern representing its hexagonal lattice structure and six-fold spots. A sharp-edged octahedral structured crystalline MF produced at ambient temperature is illustrated in **Fig. 6.2b**. As displayed in the SAED pattern, MF exhibits no discernible diffraction pattern. A similar trend was previously spotted for the MF produced using the traditional hydrothermal technique [1]. Conversely, a typical ring-like structure was discovered in the β -CD-GO-MF nanocarrier (**Fig. 6.2c-e**), indicating the slight polycrystalline structure of the nanocarrier by following the enclosure of β -CD-GO. The multilayer β -CD-GO nanosheets are represented with relatively lighter regions, as shown in **Fig. 6.2c-e**, with lower electron densities, while a darker region indicates octahedral MF.

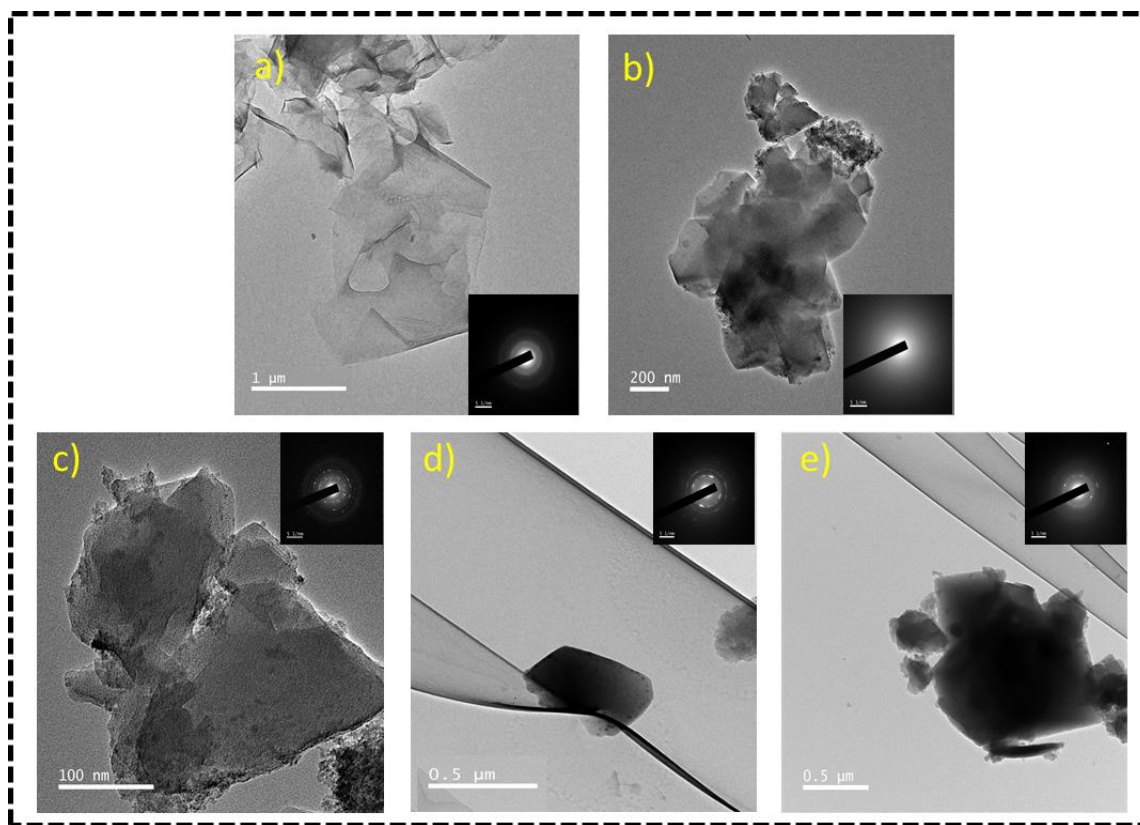


Fig. 6.2: FETEM illustrations of (a) β -CD-GO, (b) MF, and (c, d, e) β -CD-GO-MF nanocarriers, respectively, supplemented through the SAED patterns.

Furthermore, FESEM analysis of β -CD-GO, MF, and β -CD-GO-MF nanocarriers was conducted. A crumpled paper-like structure of β -CD-GO sheets, as observed in TEM images with the slight agglomeration of manifold nanosheets, is represented in **Fig. 6.3a**. As demonstrated in **Fig. 6.3b**, MF has a distinctive polyhedral crystalline morphology, aggregated with a few lumpy and tiny crystals. For the β -CD-GO-MF nanocarrier, as illustrated in **Fig. 6.3c, e**, the MF crystals were remained haphazardly connected in a cluster configuration over the surface of β -CD-GO. However, in other instances, as seen in **Fig. 6.2 d-e**, the MF crystals serve as a filler and are found in the space present through layers of β -CD-GO; our previous study [5] and Lou et al. (2018) [6] have made identical observations previously. The highlighted surface morphological findings made through TEM and FESEM provide early proof for successfully synthesizing the β -CD-GO, MF, and β -CD-GO-MF nanocarriers.

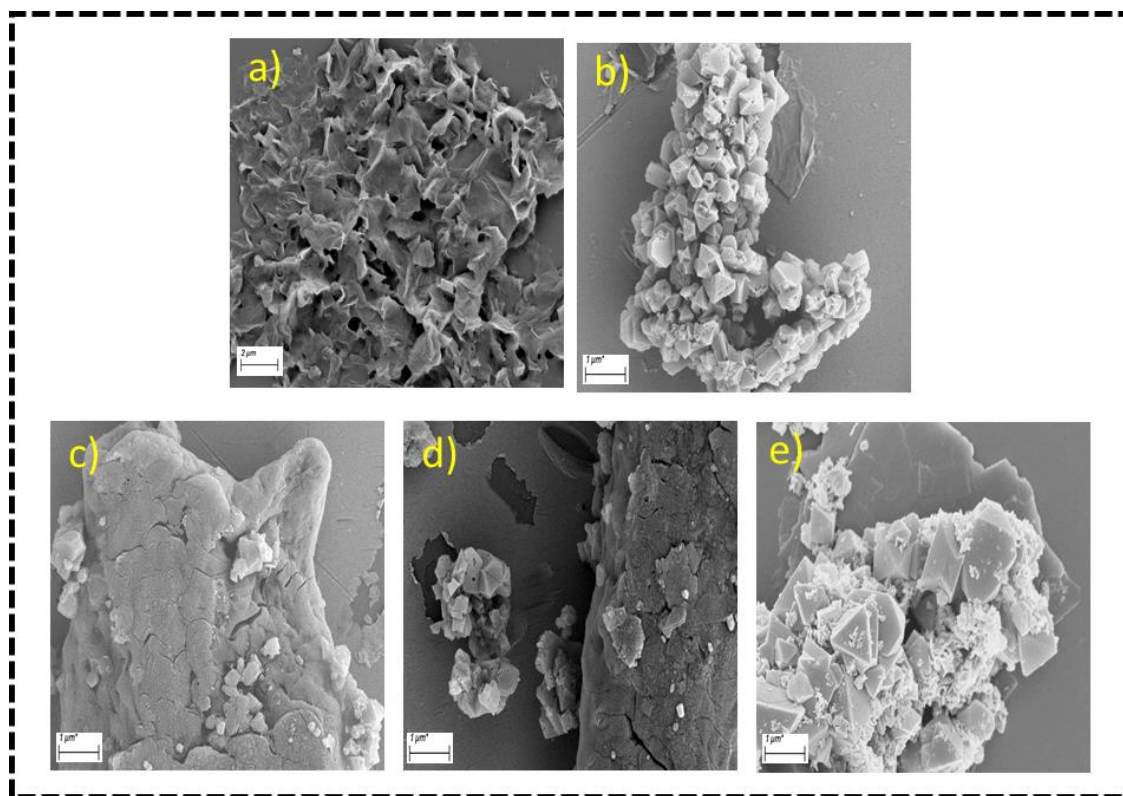


Fig. 6.3: FESEM illustrations of (a) β -CD-GO, (b) MIL-100 (Fe) (MF), and (c, d, e) β -CD-GO-MF nanocarriers.

6.2.2. X-Ray diffraction

X-ray diffraction analysis of pristine GO, β -CD-GO, MF, and β -CD-GO decorated MF nanocarriers with different proportions of β -CD-GO are illustrated in **Fig. 6.4**. A strong crystalline peak at $2\theta = 10.44^\circ$, corresponding giving a d-spacing 0.846 nm, attributing to the (0 0 1) crystalline plane of GO is shown in **Fig. 6.4A**. The graphite oxidation into GO resulted in the incorporation of oxygen-containing functional groups, which directs an increment in interlayer spacing within the GO, as also discussed in previous studies [7,8]. The resultant GO was further conjugated with β -CD, which resulted in a decrease in the d-spacing to 0.826 nm with $2\theta = 10.7^\circ$ for β -CD-GO nanoconjugate (**Fig. 6.4A**). Such reduction in interlayer spacing reflects a reduction of oxygenated functional groups of GO owing to β -CD conjugation [2]. **Fig. 6.4B** represents the XRD spectra for the pristine MF and β -CD-GO/MF nanocarriers obtained at room temperature. A well-crystallized structure was

observed for pristine MF with prominent distinctive peaks at 6.28° , 10.26° , 11° , and 20.08° , which are in accordance with the literature [5,9,10].

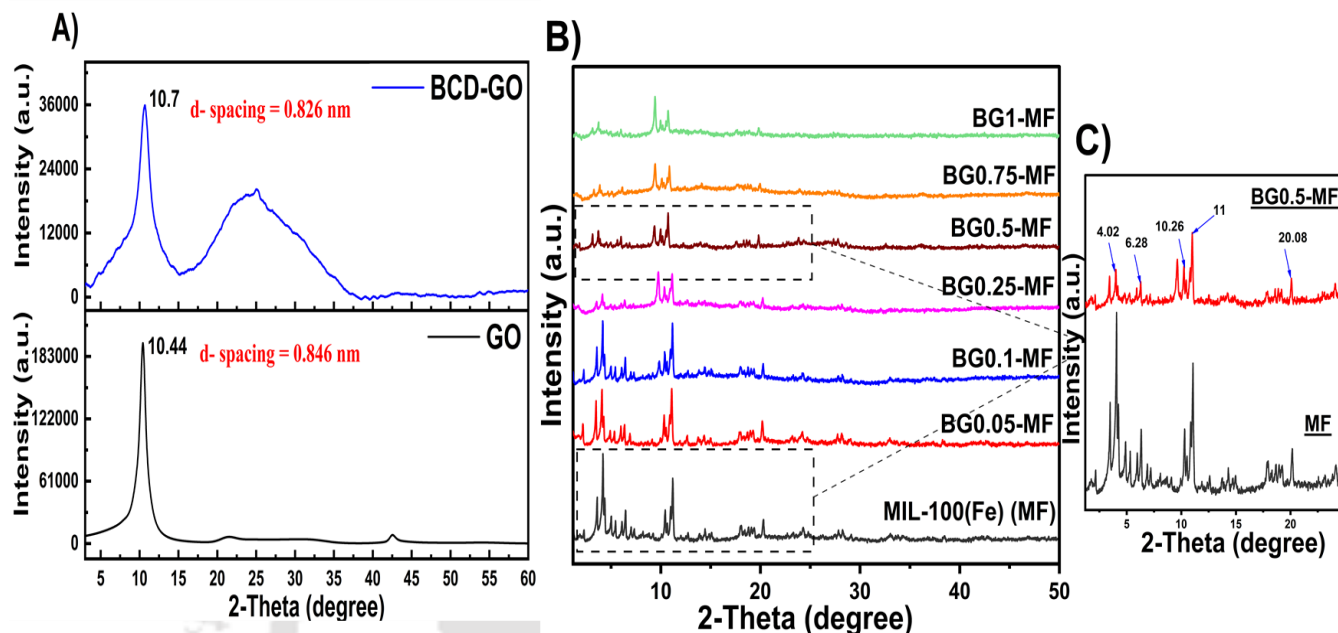


Fig. 6.4: X-ray diffraction spectra of (A) GO and β -CD-GO, (B) MF and BGw-MF nanocarriers, and (C) Magnified view of MF and BG0.5-MF.

Interestingly, equivalent XRD spectrums were obtained for BGw-MF nanocarriers, including BG0.05-MF, BG0.1-MF, BG0.25-MF, BG0.5-MF, BG0.75-MF, and BG1-MF, indicating well-preserved parent crystal structure of MF. Nevertheless, no discernible peak of β -CD-GO was witnessed, perhaps due to overlapping peak positions or the incorporation of a lower amount of β -CD-GO for the nanocarrier synthesis [11]. However, it was found that peak intensity was slightly reduced when the weight % of β -CD-GO in BGw-MF nanocarriers increased, which may be related to the corresponding decrement in MF concentration. A closer look at the MF and BG0.5-MF nanocarriers is depicted in **Fig. 6.4C**. The diffraction pattern results suggest that the configuration of MF synthesized in ambient temperature conditions is apparent throughout the literature. Additionally, BGw-MF has endured the findings with reasonable consistency.

6.2.3. N₂ adsorption-desorption isotherms

N₂ adsorption-desorption isotherms at 77 K investigated the textural characteristics of GO, β -CD-GO, MF, and β -CD-GO/MF (BGw-MF) nanocarriers (**Fig. 6.5**). **Fig. 6.5A** represents the isotherms for pristine GO and β -CD-GO nanocarriers. The modified Hummers' approach yielded GO having a $30.47 \text{ m}^2 \text{ g}^{-1}$ surface area, followed by a type IV isotherm, corresponding to the average pore size of 7.950 nm. After conjugating GO with β -CD, the specific surface area (SA) of GO was reduced to $11.13 \text{ m}^2 \text{ g}^{-1}$ while increasing the pore size to 8.822 nm and conserving the type IV isotherm along with mesoporous structure. The intercalation of β -CD moieties across the GO nanosheets most probably drove the expansion in the pore size from 7.950 to 8.822 nm. The β -CD-GO nanoconjugate's larger average pore size emphasizes its potential for use in drug adsorption [12]. Reviewing **Fig. 6.5B**, it was discovered that the produced MF exhibited a specific SA and total pore volume of $2203.26 \text{ m}^2 \text{ g}^{-1}$ and $1.125 \text{ cm}^3 \text{ g}^{-1}$, correspondingly. As given in **Fig. 6.5B**, the type I N₂ adsorption-desorption is followed by the MF nanocarrier, which follows the isotherm through a small hysteresis loop. The average pore size of the MF carrier is 2.044 nm, demonstrating its microporous structure [10]. In contrast, as illustrated in **Table 6.1**, as the proportion of β -CD-GO rose, the BGw-MF nanocarrier's specific surface area and pore volume were dropped.

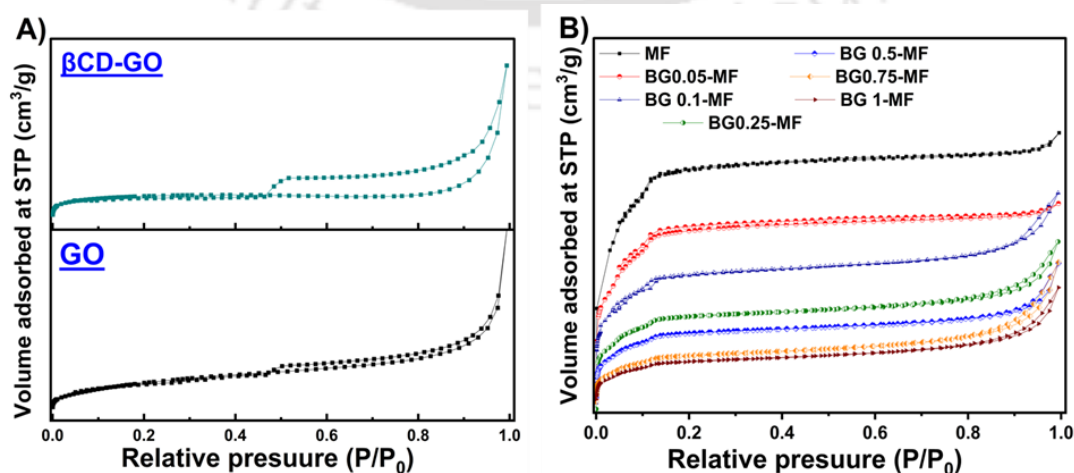


Fig. 6.5: N₂ adsorption-desorption isotherms of (A) GO and β -CD-GO, (B) MF and BGw-MF nanocarriers.

Table 6.1: Textural attributes of GO, β -CD-GO, and β -CD-GO/MF nanocarriers.

Nanocarrier	Amount of β -CD-GO (wt.%)	Surface area ($\text{m}^2 \text{g}^{-1}$)	Average pore size (nm)	Micro-pore volume ($\text{cm}^3 \text{g}^{-1}$)	Total-pore volume ($\text{cm}^3 \text{g}^{-1}$)
GO	0	30.47	7.950	0.00129	0.097
β -CD-GO	100	11.13	8.822	0.00342	0.024
MF	0	2203.26	2.044	0.50331	1.125
BG0.05-MF	0.05	1314.48	2.222	0.35597	0.730
BG0.1-MF	0.1	1001.39	3.386	0.25504	0.772
BG0.25-MF	0.25	698.48	3.152	0.17886	0.585
BG0.5-MF	0.5	579.43	3.071	0.14051	0.502
BG0.75-MF	0.75	473.09	3.056	0.11413	0.363
BG1-MF	1	421.55	2.786	0.09215	0.304

Introducing the relatively non-porous β -CD-GO resulted in the decreased surface area of BGw-MF nanocarriers, namely BG0.05-MF, BG0.1-MF, BG0.25-MF, BG0.5-MF, BG0.75-MF, and BG1-MF [6]. The BGw-MF nanocarriers exhibited the analogous behavioral patterns as type I and type IV isotherms, giving an elevation of the hysteresis loop with increased β -CD-GO amount in BG1-MF. These mode type I and IV are due to microporous and mesoporous cavities in nanocarriers [13]. The nanocarrier comprising high porosity and surface area could offer abundant active sites to the drug molecules, resulting in a high loading capacity. The textural attributes of nanocarriers are specified in **Table 6.1**.

6.2.4. Chemical interaction study

6.2.4.1. FTIR spectroscopy

The FTIR spectroscopic technique was utilized to validate important functional groups and qualitatively evaluate the produced GO, β -CD-GO, MF, and BGw-MF nanocarriers. **Fig. 6.6** depicts the FTIR spectrum of GO produced after oxidizing graphite, β -CD-GO obtained via conjugation of β -CD to GO, MF, and BG0.5-MF nanocarriers, natural chemotherapeutic agent caffeic acid (CA) as well as BG0.5-MF-CA nanocomposite following the loading of

CA. In the FTIR spectra of GO, the distinctive peaks for hydroxyl groups of stretching vibrations, C=O stretched carboxyl-carbonyl entities, unaltered C=C bonding, and epoxy-functional groups were present at 3400 cm^{-1} , 1725 cm^{-1} , 1620 cm^{-1} and 1052 cm^{-1} , correspondingly [8,14]. These functional groups that include oxygen and have a low transmittance signify superior GO quality [15]. For β -CD-GO, the transmittance of the C=O peak (1725 cm^{-1}) substantially increased with the near disappearance of the peak, suggesting that β -CD is coupled to GO via the ester bond between COO- of GO and OH- of β -CD [12,16]. Also, the lowering in the transmittance of peaks in the region $3500\text{--}3100\text{ cm}^{-1}$ compared to GO indicates a higher concentration of hydroxyl functional groups within GO after the β -CD-GO nanoconjugate is formed [2].

In the spectra of the MF nanocarrier, distinct transmittance peaks were present at 1624, 1450, 1378, 760, and 707 cm^{-1} representing carboxylate groups, symmetric-asymmetric bands of O-C-O functional groups, and benzene assembly with C-H bending vibrations, respectively. These acquired spectra with the designated functional groups are in line with previously reported literature and demonstrate the successful formation of MF [5,10]. For the BG0.5-MF nanocarrier, an analogous FTIR spectrum was realized, which indicated that even after the addition of β -CD-GO nanoconjugate, the structure of MF was conserved with no change in the surface chemistry. Moreover, the transmittance of the spectrum was reduced, possibly due to MF exposure within the β -CD-GO nanoconjugate.

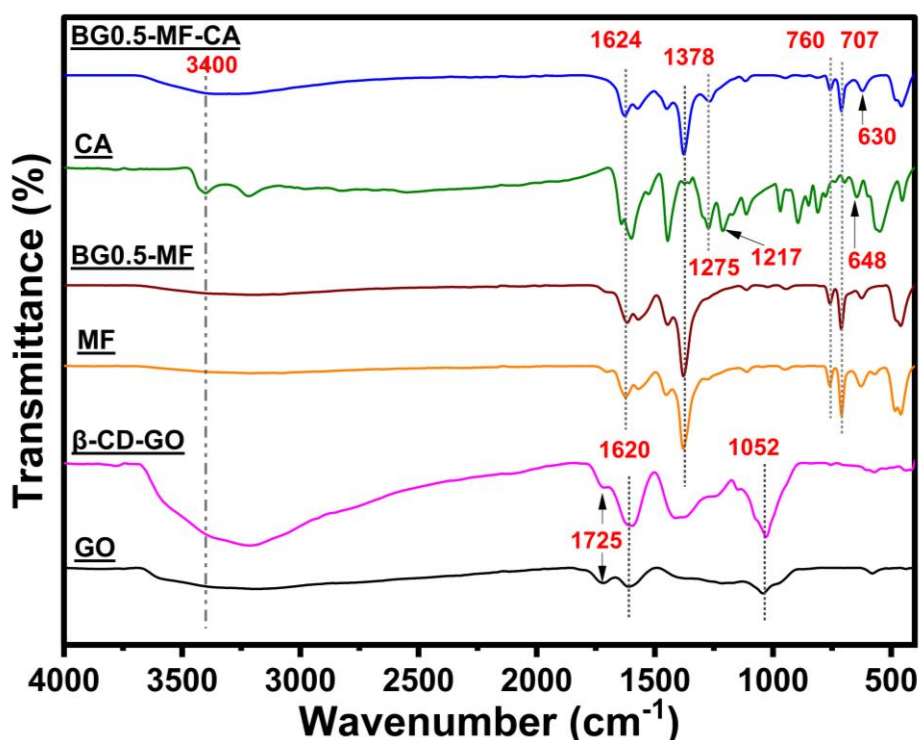


Fig. 6.6: FTIR spectra of GO, β -CD-GO nanoconjugate, MF, BG0.5-MF nanocarrier, CA and CA-loaded BG0.5-MF-CA nanocomposite.

The presence of the hydroxyl group is indicated by the appearance of prominent peaks in the FTIR spectra of CA between 3700 and 2500 cm^{-1} , which, to a certain extent, overlaid the feeble -CH stretching structures of the benzene molecule and acyclic chain [17,18]. The distinguishing peaks of CA at 1624 cm^{-1} and 1450 cm^{-1} are designated the aromatic and C-C stretching. Furthermore, an in-plane bending of olefinic groups, aromatic C-H bonds, and carbonyl groups was detected at 1275 cm^{-1} , 1217 cm^{-1} , and 648 cm^{-1} , exclusively [17].

For the BG0.5-MF-CA nanocomposite, the carboxylate groups, symmetric-asymmetric strips of O-C-O functional groups, and benzene structure were detected with identical peak positions of the BG0.5-MF nanocarrier. Also, the peaks at 1275 cm^{-1} verify that CA has been successfully loaded on the nanocarrier [17]. Furthermore, when particularly in comparison to BG0.5-MF, the transmittance of the hydroxyl functional groups peak (at 3400 cm^{-1}) is substantially decreased in BG0.5-MF-CA, implying that hydroxyl functional groups from CA

are conjugated to the structure of the nanocarrier and establishing the effective formation of the nanocomposite.

6.2.4.2. XPS spectra

The investigation of corresponding functional groups and elements present in the interior of β -CD-GO nanoconjugate, BG0.5-MF nanocarrier, and BG0.5-MF-CA nanocomposite was done via XPS analysis (**Fig. 6.7**). The elemental survey spectrums of pristine β -CD-GO, BG0.5-MF, and BG0.5-MF-CA nanocomposite is provided in **Fig. 6.7A**, which demonstrated the occurrence of O, C, and Fe elements in nanocarriers and nanocomposites. **Fig. 6.7B** depicts a high-resolution deconvoluted spectrum of the C1s and O1s states of β -CD-GO nanoconjugate. The deconvoluted C1s spectra of β -CD-GO bring in three significant peaks of 284.8 eV, 286.5 eV, and 288.9 eV, representing the graphitic or sp^2 carbon, C–O mount in the hydroxyl or epoxy groups, C=O in a carbonyl group, together with to the –COOH in carboxyl functional units exist in the β -CD-GO, correspondingly [19]. Also, two significant peaks, at 532.0 eV and 533.6 eV, are acquired by deconvoluting the O1s spectrum of β -CD-GO. These distinctive peaks correspond to the various oxygenated units like –COOH (carboxylic)/C=O (carbonyl) and the C–OH (hydroxyl) present in the β -CD-GO [19,20]. The FTIR study additionally confirmed the occurrence of listed oxygen-containing functional groups in β -CD-GO.

An elemental survey spectrum of the BG0.5-MF nanocarrier reveals the presence of O, C, and Fe elements (**Fig. 6.7A**). The deconvoluted C1s, O1s, and Fe2p XPS spectra of BG0.5-MF are depicted in **Fig. 6.7C**. The C1s spectra produced three distinctive peaks at energies of 284.8 eV, 285.0 eV, and 288.8 eV. Out of which, 284.8 eV and 288.8 eV are ascribed to the phenyl and carboxylic groups existing in the structural cage of MF, respectively, while 285.0

eV peak indicates a carbon backbone on the BG0.5-MF structure. Likewise, the 531.8 eV peak in a deconvoluted O1s spectra supported the existence of Fe–O–C groups.

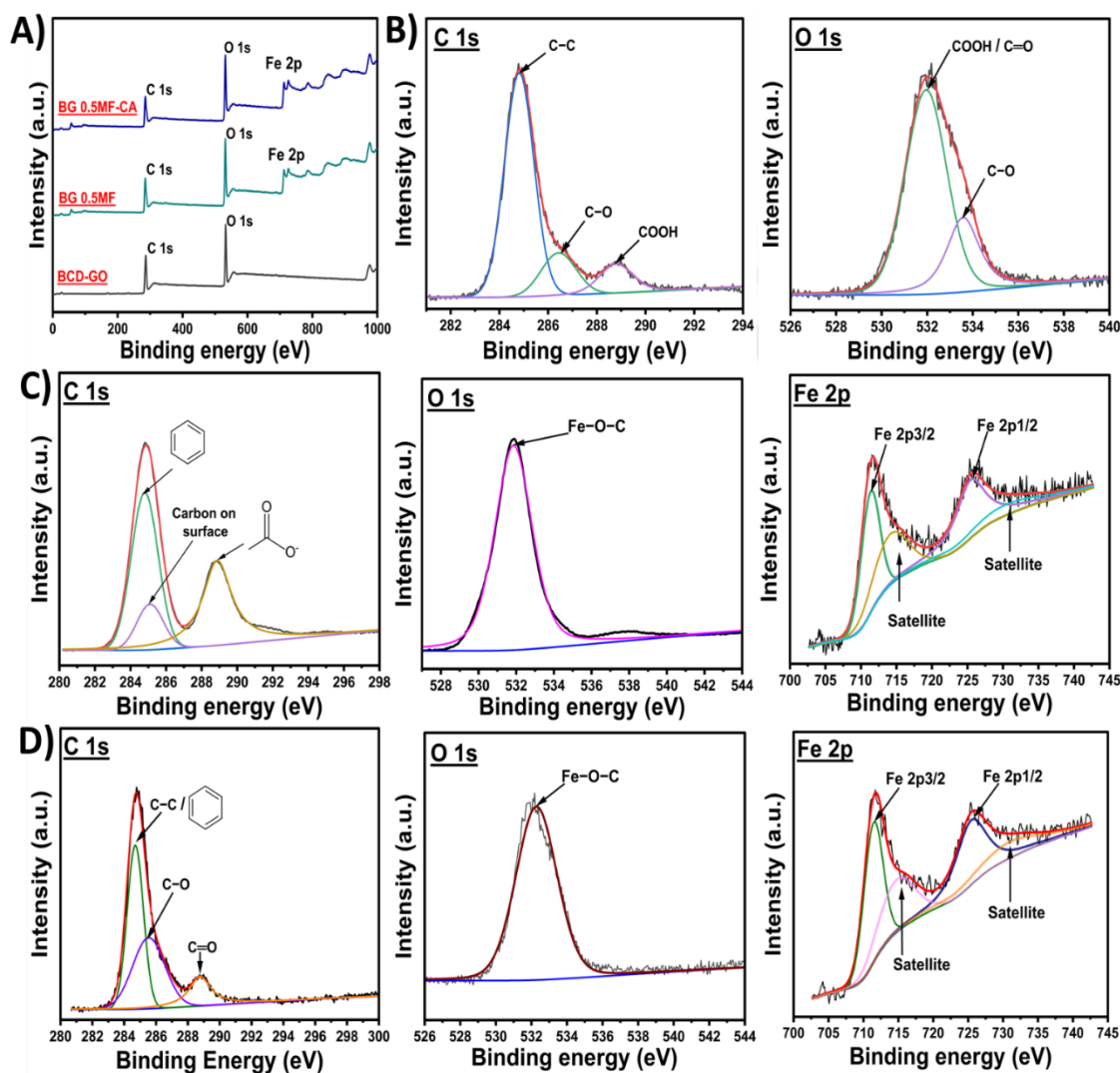


Fig. 6.7: XPS spectra of nanocarriers (a) Elemental survey spectra of β -CD-GO nanoconjugate, BG0.5MF nanocarrier, and CA-loaded BG0.5-MF-CA nanocomposite (b) C1s and O1s spectra of obtained β -CD-GO nanoconjugate (c, d) C1s, O1s, and Fe2p XPS spectra: BG0.5-MF and BG0.5-MF-CA nanocomposite, respectively.

In addition, the Fe2p XPS spectrum was deconvoluted into four primary peaks at 711.5 eV, 725.6 eV, 714.8 eV, and 730.9 eV. The Fe 2p_{3/2} and Fe 2p_{1/2} regions were distinctively assigned at 711.5 eV and 725.6 eV, correspondingly. Furthermore, peaks at 714.8 eV and 730.9 eV are ascribed to the satellite peaks of Fe_{3/2} and Fe 2p_{1/2} for the BG0.5-MF nanocarrier [21,22]. The XPS survey spectrum for BG0.5-MF-CA nanocomposite (with CA

loaded on the nanocarriers) is provided in **Fig. 6.7A**, demonstrating the occurrence of C, O, and Fe. In the nanocomposite, nearly indistinguishable spectra were observed after the deconvolution of the C1s, O1s, and Fe2p XPS spectra. The appearance of comparable peaks supports the existence of BG0.5-MF functionalities within the BG0.5-MF-CA nanocomposite. Furthermore, a positive shift in binding energies (BE) (0.2-0.5 eV) was observed for BG0.5-MF-CA in comparison to BG0.5-MF, as shown in **Fig. 6.7D**, demonstrating stronger interactions amongst CA and BG0.5-MF during the development of the BG0.5-MF-CA nanocomposite [23–25].

Further, to endorse the preparation of GO, β -CD-GO, BG0.5-MF nanocarriers, and BG0.5-MF-CA nanocomposite, the elemental analysis was conducted using XPS analysis and reported in **Table 6.2**. The modified Hummers' technique for synthesizing GO via graphite oxidation resulted in incorporating oxygenated functional groups. Elements analysis corroborates the graphite oxidation, as illustrated in **Table 6.2**. For GO nanosheets, the C/O elemental atomic ratio was discovered as 1.60. Further, the β -CD macromolecules were conjugated on GO sheets, which caused the fractional reduction of GO to produce β -CD-GO nanoconjugate, giving a 1.83 C/O atomic ratio. This increase in the atomic ratio due to the partial reduction of GO also resulted in a decrement in the d-spacing, as validated by XRD analysis.

A room temperature synthesized MF, on the other hand, has C, O, and Fe elements with compositions 55.78, 35.40, and 8.82 at. %, respectively; similar findings have been reported in the literature with very less content of Fe [13]. MF had a C/O at. the ratio of 1.58, which, however, was reduced to 1.10 for the BG0.5-MF nanocarrier due to an increase in oxygen at. % induced by the inclusion of β -CD-GO, indicating effective decoration of β -CD-GO in MF. For the BG0.5-MF-CA nanocomposite, this ratio was increased to 2.02 due to the reduction

of oxygenated sites at BG0.5-MF by the loading of CA, owing to CA's antioxidant nature. The provided findings of the XPS study further validate the successful formation of β -CD-GO, BG0.5-MF nanocarriers, and BG0.5-MF-CA nanocomposite.

Table 6.2: Elemental analysis of nanocarriers.

Nanocarriers	Elemental composition (at. %)			
	C	O	Fe	C/O
GO	61.58	38.42	-	1.60
β -CD-GO	64.61	35.39	-	1.83
MF	55.78	35.40	8.82	1.58
BG0.5-MF	50.60	46.04	3.36	1.10
BG0.5-MF-CA	64.87	31.98	3.15	2.02

6.2.5. Raman Spectra

Raman spectroscopy, being a non-invasive characterization technique, offers significant findings regarding carbon-based nanomaterials. **Fig. 6.8** displays typical Raman spectra for GO, β -CD-GO, MF, and BG0.5-MF nanocarriers acquired at an excitation wavelength of 514 nm. For GO, two distinctive peaks of the D-band at 1357 cm^{-1} and the G-band at 1599 cm^{-1} were discovered. Earlier, it was reported that the characteristic band peaks differ with excitation wavelength values ranging from $1320\text{--}1370\text{ cm}^{-1}$ and $1550\text{--}1630\text{ cm}^{-1}$ for D and G-band, separately [26,27]. The G-band depicts sp^2 hybridized in graphitic structure in the hexagonal-pinch form, whereas the D-band symbolizes the defects [28]. In contrast to GO, the D-band in β -CD-GO nanoconjugate was slightly broader, which suggests the covalent conjugation of β -CD on the GO carbon skeleton, additionally advocated with the blue shift in D-band ($\sim 10\text{ cm}^{-1}$) and G-band ($\sim 3\text{ cm}^{-1}$) characteristic peaks, compared with GO. The I_D/I_G ratio for GO was observed as 1.01, which was reduced to 0.92 after the conjugation of β -CD. This retardation in the intensity ratio demonstrates fractional renewal of the sp^2 domains of GO during the interaction of β -CD macromolecule on the surface of GO nanosheets [12,29].

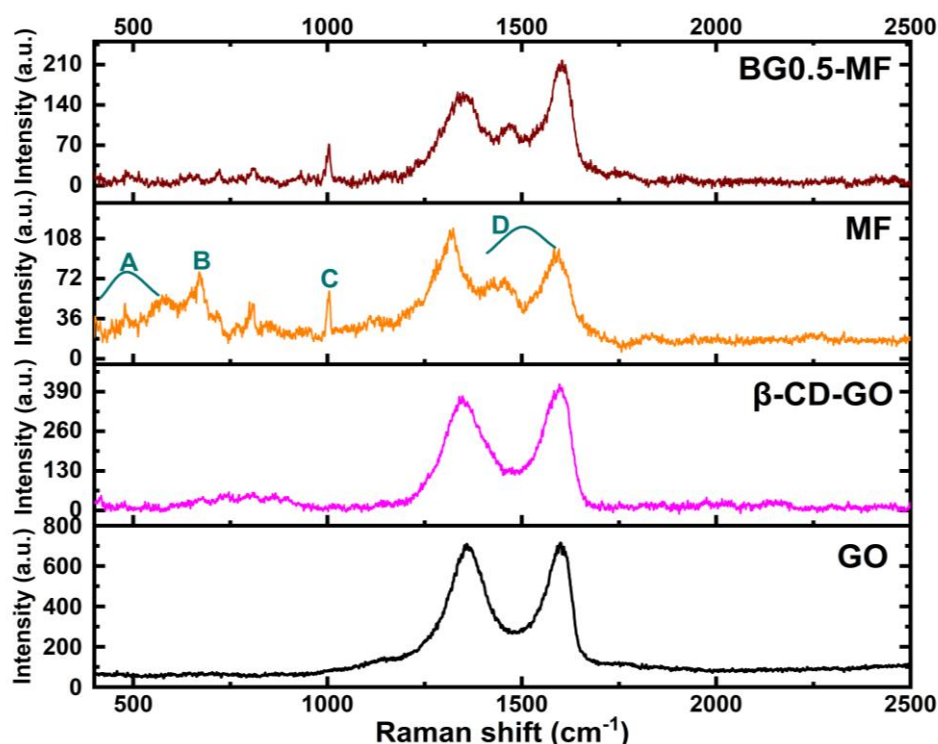


Fig. 6.8: Raman spectrum of GO, β -CD-GO nanoconjugate, MF, and BG0.5-MF nanocarriers.

Furthermore, a Raman spectrum of MF reveals distinctive MIL-100 (Fe) peaks in the assortment of 400-2000 cm^{-1} . Herein, between 450 cm^{-1} to 600 cm^{-1} , the lattice vibration and network binding modes were detected. At 800 cm^{-1} and 1001 cm^{-1} , indicated by peaks B and C, the prominent peaks of the H_3BTC footprints, namely the trimester linker and its aromaticity, were detected. Moreover, with the coordinated water molecules in the network of Fe-trimesate linkers, C-O-Fe stretching vibrations of 1200 cm^{-1} of lesser magnitude were detected, in complement with H-OH bonding vibrations recorded in region D (1400 - 1600 cm^{-1}) [10]. Raman spectra of the BGw-MF nanocarriers showed the functional elements of both MF and, more broadly, β -CD-GO. In the case of BG0.5-MF, the distinctive peaks of MF in areas A, B, and C were preserved with reduced intensity. In contrast to the MF nanocarrier, the intensity was relatively enhanced for BG0.5-MF, notably for region D, in the region 1200 cm^{-1} - 1600 cm^{-1} , demonstrating the presence of oxygenated β -CD-GO nanoconjugate raising the defect density of BG0.5-MF. The incorporation of β -CD-GO leads to increased oxygen

concentration, as illustrated in **Table 6.2**, which accounts for the up surging of defect density in BG0.5-MF nanocarrier, analogous to the formation of GO via graphite oxidation.

Overall, the successful formation of GO, β -CD-GO nanoconjugate, MF, and BG0.5-MF nanocarriers is validated by the findings of the elucidated characterization studies.

6.2.6. Loading characteristics

The drug molecules of the natural chemotherapeutic agent, caffeic acid, were successfully impregnated over the β -CD-GO nanoconjugate (BG), MF, and BGw-MF nanocarriers, where $w = 0.05, 0.1, 0.25, 0.5, 0.75$, and 1 are attributed to the wt.% of BG nanoconjugate within the BGw-MF nanocarriers. The CA was loaded by intermolecular interactions in conjunction with the van der Waals, strong electrostatic, and π - π interactions provided by BG, MF, and BGw-MF nanocarriers. The effect of loading factors like the quantity (wt.%) of BG in BGw-MF nanocarriers, the mass fraction of nanocarrier with respect to CA, and the influence of pH of the media were investigated. The nanocarrier with the highest loading efficiency (BG0.5-MF) was considered for further experimentations and characterizations.

6.2.6.1 Effect of amount of β -CD-GO in the nanocarriers

The pristine BG, MF, and BGw-MF nanocarriers were effectively loaded with the CA drug molecules. **Fig. 6.9A-B** displays the distinct loading (LE) and encapsulation/entrapment efficiency (EE) of each nanocarrier, respectively. Owing to substantial non-covalent interactions amongst the aromatic rings of BG and CA, the highest LE of $45.05 \pm 0.90\%$ and EE of $65.60 \pm 1.64\%$ were found for BG.

Conversely, in the case of intact MF, the LE was the lowest at $25.33 \pm 0.51\%$ with $33.92 \pm 0.83\%$ EE. The lower LE of the MF nanocarrier may be ascribed to the weaker surface interactions of MF with CA molecules. Nevertheless, a substantial improvement in loading

and entrapment efficiencies was noticed up to the BG0.5-MF nanocarrier when β -CD-GO content was increased in the MF-based nanocarriers. This improvement may result from adequate interactions among the nanocarriers and CA molecules [5]. It has been discovered that the CA loading capacity and entrapment efficiency decreased as the quantity of β -CD-GO in BGw-MF nanocarriers was increased further, which could be owing to the partial hindrance of MF surface by the β -CD-GO and availability of fewer active surface moieties for the encapsulation [6,30]. The maximum LE of $38.84 \pm 0.78\%$ with EE of $63.08 \pm 1.58\%$ was obtained for the BG0.5-MF nanocarrier. As a result, it was employed for further study on the impact of drug weight ratio on loading characteristics.

6.2.6.2 Effect of CA weight ratio

Fig. 6.9C demonstrates the influence of CA vs. nanocarrier weight ratio on drug LE. The loading experiments were conducted under the identical parameters of 25 °C, 200 rpm, and a 16 h loading period. The drug LE was observed to be increased with an upsurge in the CA quantity in regard to BG0.5-MF, and it was highest for the 2 w/w ratios with $48.37 \pm 1.45\%$ LE. As previously mentioned, the loading of CA depends on non-covalent interactions amongst the oxygenated functional groups of CA and the BG0.5-MF nanocarrier [30,31]. Contrarily, a rise in drug concentration (>2 w/w) causes a decline in drug loading, which may be brought on by the absence of surface-active sites and associated interactions between the CA and BG0.5-MF nanocarrier. The BG0.5-MF-CA nanocomposite produced after CA loading was vacuum dried at 40 °C overnight before being used to investigate the *in-vitro* release behavior.

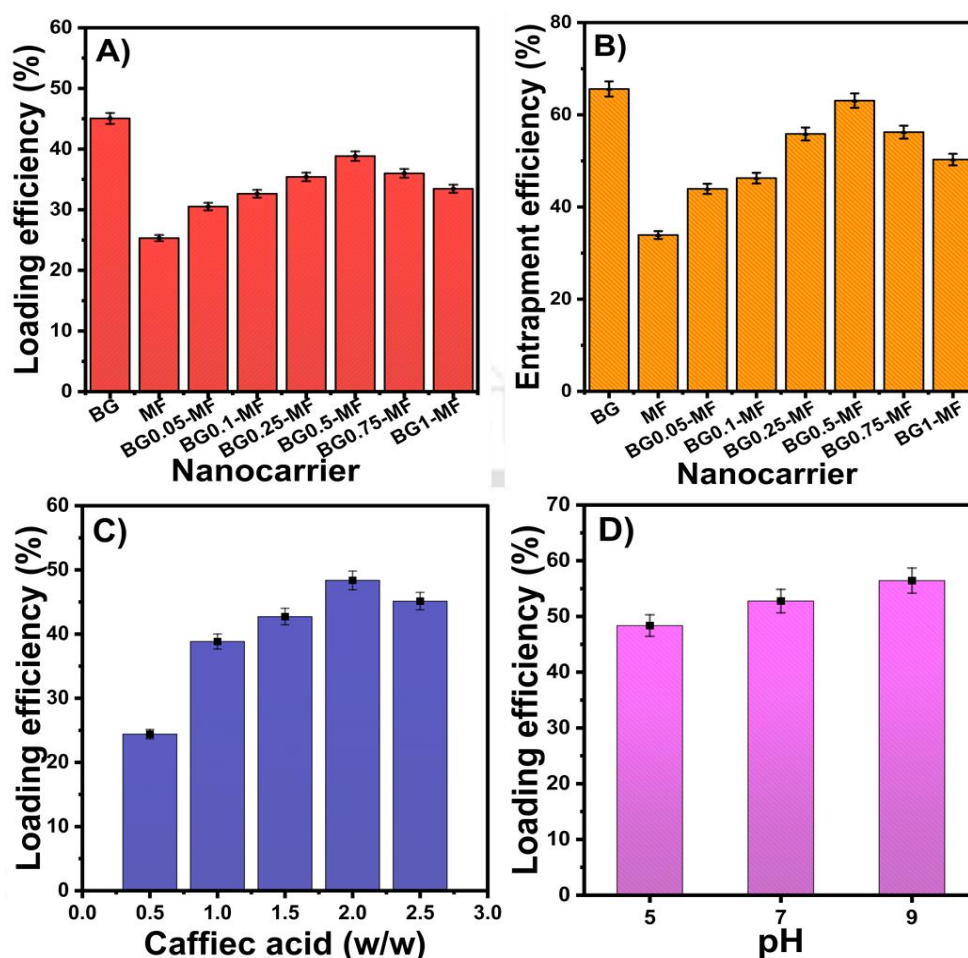


Fig. 6.9: Loading features of CA on β -CD-GO nanoconjugate (BG), MF, and BGw-MF nanocarriers. (A, B) effect of the amount of β -CD-GO in the nanocarrier on CA loading and entrapment efficiencies, respectively, (C) the effect of CA: nanocarrier (BG0.5-MF) mass ratio on drug LE, (D) the impact of pH of the media on the CA loading efficiency for BG0.5-MF nanocarrier. The outcomes are exposed as mean $\pm$ SD.

6.2.6.3 Effect of pH

The pH of the systems can greatly influence the drug LE and EE of the nanocarriers. As a result, the effect of the pH of the media on the LE of BG0.5-MF nanocarrier (**Fig. 6.9D**) was explored for pH 5, 7, and 9, signifying the acidic, neutral, and basic environments correspondingly. The drug-loading effectiveness of CA was observed to be highest under a basic environment ($56.44 \pm 2.25\%$) than in neutral and acidic environments, which may be due to the existence of hydroxyl (O-) and carboxyl (COO-) functional groups within BG0.5-MF and CA. These functional entities are anticipated to get deprotonate in a basic medium

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and may be accountable for the increased drug-loading efficiency of CA [32]. The deprotonation strengthens the electrostatic interactions between CA and BG0.5-MF as well as the loading efficiency. These findings have been advocated by surface charge evaluation of CA and BG0.5-MF.

Table 6.3: Comparison of loading efficiency of MIL-100 (Fe), β -CD, and GO-based nanocarriers for chemotherapeutic agents.

Nanocarrier	Synthesis method	Chemotherapeutic agent	Drug loading efficiency (%)	References
MIL-100 (Fe)	Hydrothermal route without HF	DOX	23.40	[33]
CeO ₂ -MIL-100 (Fe)	Hydrothermal route Without HF	DOX	13.30	[34]
Fe ₃ O ₄ @MIL-100 (Fe)	Microwave-assisted	DOX	15.70	[35]
GO@ MIL-100 (Fe)	RT	DOX	29.91	[5]
MgAl-LDH/Fe-MOF	Low-temperature preparation (40 °C)	DOX and methotrexate (MTX)	21.0 and 4.2	[36]
UPNC@MIL-100 (Fe)	RT	DOX	17.20	[37]
β -CD conjugated sialic acid	-	DOX	1.13	[38]
β -CD modified MGO (β -CD@MGO)	-	DOX, EPI	90.9 and 78.1	[39]
GO	Improved Hummers' method	Gallic Acid	12	[32]
GO	Improved Hummers' method	Chlorogenic acid	13.1	[40]
β-CD-GO	-	Caffeic acid (CA)	45.05 $\pm$ 0.90	Present study
MIL-100 (Fe)	RT	CA	25.33 $\pm$ 0.51	Present study
β-CD-GO/MIL-100 (Fe)	RT	CA	56.44 $\pm$ 2.25	Present study

Based on preceding findings, BG0.5-MF was deemed to be the ideal nanocarrier, with a 0.5 wt.% composition of β -CD-GO, which further delivered the highest loading capacity of 56.44 $\pm$ 2.25% corresponding to a 2 w/w ratio of CA at a basic environment (pH 9). As a result, this

system has been further employed to investigate the release characteristics in physiological and cancer environments. The presented BG0.5-MF nanocarrier system has demonstrated superior drug loading capacity for CA, as each gram of BG0.5-MF-CA nanocomposite contributes nearly 560 mg of CA. The LE of the nanocarriers is contrasted with that of the different MOF and GO-based carriers in **Table 6.3**. According to **Table 6.3**, the pristine MF nanocarrier prepared by both the suggested sustainable method and conventional hydrothermal treatment had lower drug loading efficiency than the GO and β -CD-GO decorated MIL-100 (Fe) nanocarrier.

6.2.7. Surface charge analysis

For the prospective utilization of nanomaterials in biomedical applications like drug delivery, their colloidal stability within physiological configurations is a crucial factor. The bioavailability of nanocarriers may be increased with higher colloidal stability [41]. The zeta (ζ)-potential of nanocarriers, which represents the strength of electrostatic attraction/repulsion among nearby molecules with surface charge, may be used to assess the dispersion attributes of nanocarriers to stability [42]. In order to comprehend the drug loading and release process, the surface charge derived from the ζ -potential remains essential [43]. Herein, the ζ -potential (Delsa nano C, Beckman Coulter) analysis was conducted to determine the surface charges of the pristine β -CD-GO, BG0.5-MF nanocarriers, CA, and BG0.5-MF-CA nanocomposite by preparing and analyzing their 50 mg L⁻¹ solutions at pH 5, 7, and 9, respectively. The outcomes of the surface charge evaluation are presented in **Table 6.4**.

The β -CD-GO nanocarrier depicted a negatively charged surface. However, the amplitude of the zeta potential changes with the pH of the medium. It was discovered that in the case of the β -CD-GO nanocarrier, the value of zeta potential increases with a rise in pH, which may be ascribed to the deprotonation of hydroxyl and carboxyl functional units present in the

nanocarrier. Similar observations were made for the BG0.5-MF nanocarrier. Whereas, in the case of CA, the magnitude of ζ potential decreases with a surge in pH, which further advocates the enhancement of its antioxidant nature in a basic medium [2,5].

Table 6.4: Surface charge analysis of nanocarriers and nanocomposite.

Material	@pH = 5	@pH = 7	@pH = 9
β -CD-GO	-24.2 ± 1.5 mV	-29.4 ± 1.8 mV	-46.8 ± 1.3 mV
BG0.5-MF	-24.9 ± 1.1 mV	-36.6 ± 1.1 mV	-42.1 ± 1.9 mV
CA	-4.3 ± 1.8 mV	-0.6 ± 0.4 mV	0.0 ± 0.3 mV
BG0.5-MF-CA	-31.2 ± 1.5 mV	-41.0 ± 3.8 mV	-50.5 ± 4.3 mV

Compared to the highly negatively charged nanocarriers like β -CD-GO and BG0.5-MF, the less negative surface of CA offers a substantial advantage for the efficient loading of CA via providing the driving force for an electrostatic interaction among the nanocarriers and CA. As this driving force amplifies with an upsurge in pH via the deprotonation principle, the highest loading capacity was observed for the basic media. Furthermore, a substantially -ve surface charge was observed for the BG0.5-MF-CA nanocomposites demonstrating their suitable aqueous stability. Also, it was depicted that the nanocomposite exhibited higher negative zeta potential in basic media than the acidic ones, which may be attributed to the ample hydroxyl functional units in CA [29]. Based on this result, it can be predicted that the BG0.5-MF-CA nanocomposite exhibited better colloidal stability in physiological conditions than in tumor cell conditions.

6.2.8. Release characteristics and kinetics study

The *in-vitro* release characteristics of CA out of the BG0.5-MF-CA nanocomposite into PBS and SAB buffer solutions (for mimicking the physiological and tumor microenvironment at pH 7.4 and 5, individually) were investigated at 37 °C (normal human body temperature) for 8 days. The release contour of CA is illustrated in **Fig. 6.10**. In the human body, pH has a substantial biological stimulus; extra-cellular pH in normal tissue and tumor tissue is 7.4 and

4.5-6.5, respectively [44]. The drug release from a nanocarrier can be studied from a drug delivery system constructed using these pH variations. Herein, an initial burst release with $13.18 \pm 0.39\%$ and $16.44 \pm 0.58\%$ of CA was observed within the initial 5 h for PBS and SAB solutions, respectively. Such initial burst release, based on the nanocarrier's surface characteristic, could be essential in reaching the initial therapeutic level with an appropriate dosage of CA at tumor sites. After that, a sustained release profile was observed for CA release from the BG0.5-MF-CA nanocomposite. In the initial 24 h, $26.21 \pm 0.79\%$ and $31.36 \pm 1.09\%$ of CA release were observed, which was followed by the sustained release for 4 days (96 h) with $40.05 \pm 1.20\%$ and $49.88 \pm 1.74\%$ CA release through the BG0.5-MF-CA nanocomposite at pH 7.4 and 5, respectively.

At the end of the 8th day (192 hours), the maximum release of $57.07 \pm 2.01\%$ at pH 5 and $48.04 \pm 1.44\%$ at pH 7.4 was obtained (**Fig. 6.10**). Under acidic pH, higher CA was released from the BG0.5-MF-CA nanocomposite, which occurs as the hydrogen bonds between that drug molecules and nanocarriers become weak. Under basic conditions, an opposite trend is followed. It also suggests that the drug will be distributed selectively at tumor sites as they are acidic in nature, which will help avoid the undesirable effects of chemotherapy [40,45,46]. The drug release was supplemented by a lower burst release phase accompanied by a sustained release segment over a longer period of time. Such findings have also demonstrated the pH-dependent release of CA from BG0.5-MF nanocarriers. Moreover, such environmentally sensitive release behavior may minimize drug loss during delivery during increasing drug release once the nanocarrier arrives at the tumor site [47].

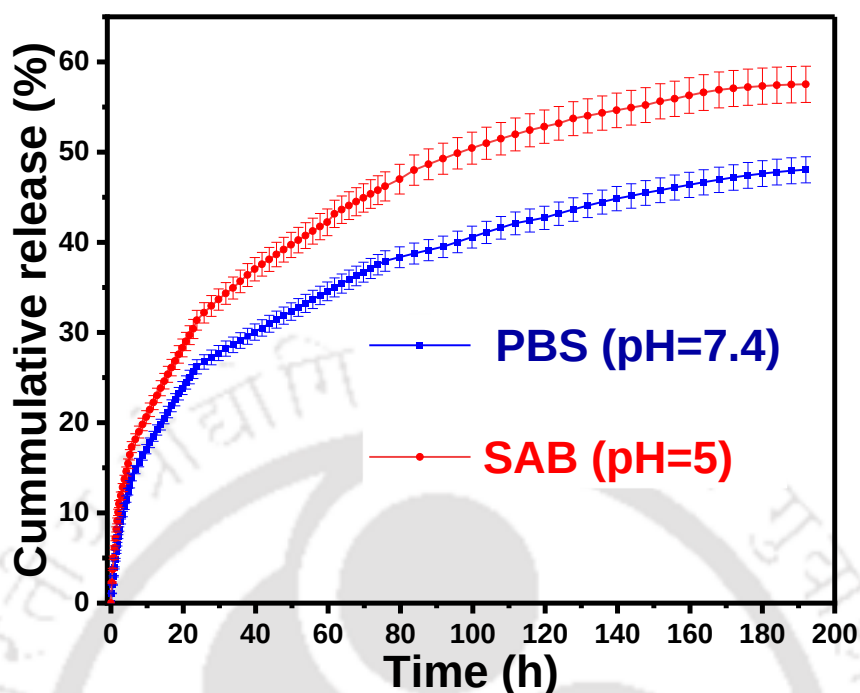


Fig.6.10: *in-vitro* release profile of CA out of BG0.5-MF-CA nanocomposite at pH 7.4 and 5. The results are exposed as mean $\pm$ SD.

Eight distinctive drug-release models were exercised to study the release kinetics of CA from the BG0.5-MF-CA nanocomposite in SAB at pH 5 [32,48–51], as given in **Table 6.5**. Where k is the corresponding rate constant of the respective kinetic model, M_t , M_e , and M_∞ are the quantity of CA released from the BG0.5-MF-CA nanocomposite at time t , at equilibrium, and infinite time, individually. n is the diffusional or release exponent. M_D and M_R are the quantity of CA suspended/dissolved at time t and the total sum of CA released. Likewise, the W_0 and W_t are the original and residual amount of CA at time t , separately.

The kinetic models are depicted in **Fig. 6.11**, and the resultant rate constants (k) and correlation coefficients (R^2) are summarized in **Table 6.5**. The Baker and Lonsdale models and pseudo-second-order (**Fig. 6.11 G, C**) were found to be more appropriate for this instance, with a best fit R^2 value of 0.99. The Baker and Lonsdale fitted model describes a non-uniform matrix with nano capillaries effective for boosting CA released from the BG0.5-

MF-CA nanocomposite [51]. Additionally, the diffusional exponent for the Korsmeyer-Peppas model (**Fig. 6.11 E**) was found to be 0.46 (< 0.5) with an R^2 value of 0.95, signifying Fickian diffusion-driven release of CA out of BG0.5-MF-CA nanocomposite [47]. The Higuchi model (**Fig. 6.11 D**) also corroborated the observations, as drug release is proportionate to the square root of time [32].

Table 6.5: Correlation coefficients (R^2), Rate constants (k), and diffusional exponent (n) of kinetic models for the release of CA from BG0.5-MF-CA.

Release media	CA Release (%)	Kinetic model	R^2	Rate constant (k)	Confidence limits (Upper, Lower)
SAB Buffer pH= 5	57.52	Zero-order $\frac{M_t}{M_e} = k_o t$	0.85	$k_o = 3.51 \times 10^{-3} \text{ min.}^{-1}$	$3.53 \times 10^{-3}, 3.49 \times 10^{-3}$
		Pseudo-first-order $\ln(M_e - M_t) = \ln M_e - kt$	0.88	$k = 3.07 \times 10^{-3} \text{ min.}^{-1}$	$3.10 \times 10^{-3}, 3.04 \times 10^{-3}$
		Pseudo-second-order $\frac{t}{M_t} = \frac{1}{kM_e^2} + \frac{t}{M_e}$	0.99	$k = 3.77 \times 10^{-3} \text{ mg min.}^{-1}$	$3.96 \times 10^{-3}, 3.58 \times 10^{-3}$
		Higuchi $M_t = k_h t^{1/2}$	0.98	$k_h = 4.05 \text{ min.}^{-0.5}$	4.01, 4.03
		Korsmeyer-Peppas $\frac{M_t}{M_\infty} = k_p t^n$	0.95	$k_p = 5.03 \times 10^{-2} \text{ min.}^{-n}$ $n = 0.46$	$6.07 \times 10^{-2}, 3.97 \times 10^{-2}$
		Hixson-Crowell $W_0^{1/3} - W_t^{1/3} = k_{HC} t$	0.97	$k_{HC} = 2.68 \times 10^{-3} \text{ min.}^{-1}$	$2.70 \times 10^{-3}, 2.66 \times 10^{-3}$
		Baker and Lonsdale $\frac{3}{2} \left[1 - \left(1 - \frac{M_t}{M_\infty} \right)^{2/3} \right] - \frac{M_t}{M_\infty} = k_{BL} t$	0.99	$k_{BL} = 2.74 \times 10^{-4} \text{ min.}^{-1}$	$2.75 \times 10^{-4}, 2.73 \times 10^{-4}$
		Weibull $M_D = M_R(1 - e^{-kt})$	0.97	$k_W = 3.07 \times 10^{-3} \text{ min.}^{-1}$	$3.09 \times 10^{-3}, 3.05 \times 10^{-3}$

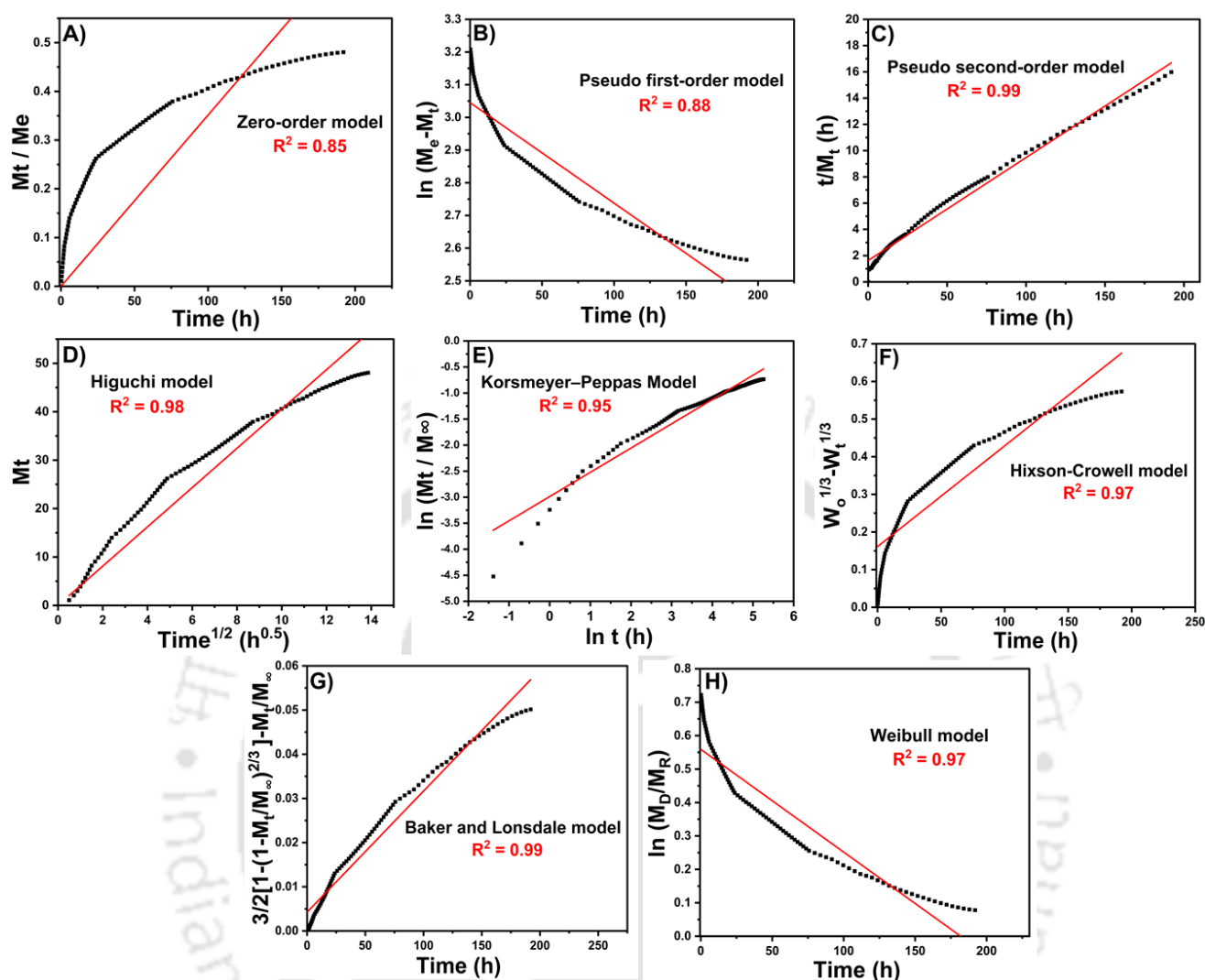


Fig. 6.11 Kinetic model fitting of CA release from BG0.5-MF-CA (A) Zero-order model (B) pseudo-first-order model, (C) pseudo-second-order model, (D) Higuchi model, (E) Korsmeyer-Peppas model. (F) Hixson-Crowell model, (G) Baker and Lonsdale model, and (H) Weibull model.

6.2.9. Cytotoxicity study of BG0.5-MF and BG0.5-MF-CA nanocomposite

Synthesized nanocarrier BG0.5-MF was used to load the natural chemotherapeutic agent, caffeic acid (CA). The BG0.5-MF nanocarrier was tested in HEK293 and A549 cell lines to investigate its cytocompatibility in the existence and absence of CA. In the HEK293 cell line, a cell viability of $80 \pm 5\%$ was observed after being treated with BG0.5-MF nanocarrier at a $100 \mu\text{g mL}^{-1}$ concentration for 48 h (**Fig. 6.12**). However, with an increase in the concentration of BG0.5-MF ($200\text{--}400 \mu\text{g mL}^{-1}$), an increase in cell death was observed.

Furthermore, the cytotoxicity of the BG0.5-MF nanocarrier was evaluated after loading the model drug CA. The drug-loaded BG0.5-MF-CA nanocomposite demonstrated significant improvement in cell viability in comparison to the pristine nanocarrier.

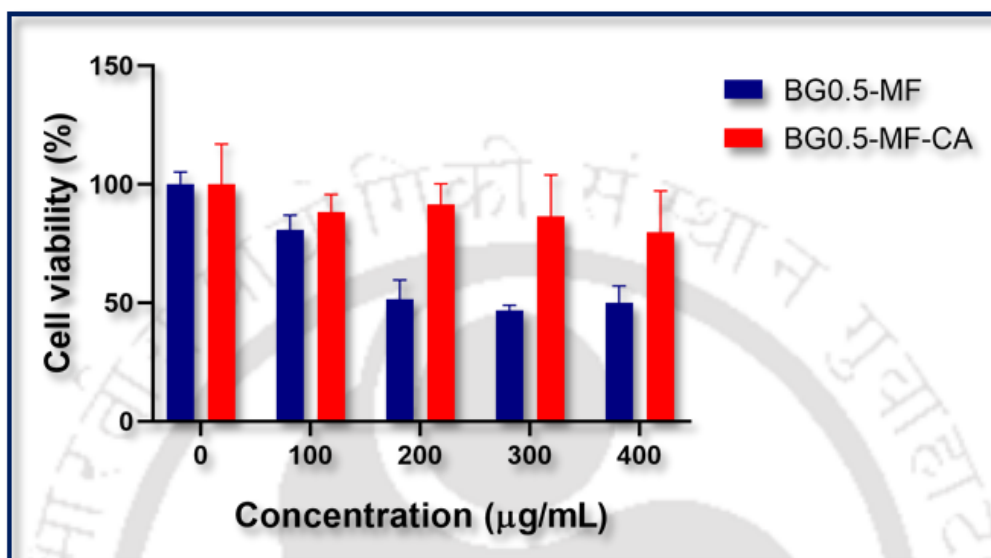


Fig. 6.12: Cell viability of pristine BG0.5-MF and CA-loaded BG0.5-MF-CA nanocomposite against HEK293. The results are stated as mean $\pm$ SD (N=4).

While in the case of the A549 cancer cell line, the unloaded nanocarrier is much more compatible with cell viability of $\sim 90\%$ until $400 \mu\text{g mL}^{-1}$ concentration (**Fig. 6.13**). However, the anticancer activity was detected once the cancer cells (A549) were treated with a drug-loaded BG0.5-MF-CA nanocomposite. A gradual decrease in cell viability was observed with an increment in the dose of BG0.5-MF-CA nanocomposite from 100 to $400 \mu\text{g mL}^{-1}$. The cell viability of BG0.5-MF-CA nanocomposite was $68 \pm 2\%$ for $400 \mu\text{g mL}^{-1}$ dose.

Overall, this assay elucidates that the loading of caffeic acid (CA) on the synthesized BG0.5-MF nanocarrier did not show any toxicity/cell death in the normal cell line while enhancing cell death for the lung cancer cell line. In line with this, the present study demonstrates a specific cytotoxic effect of CA-loaded BG0.5-MF-CA nanocomposite on cancer cell lines.

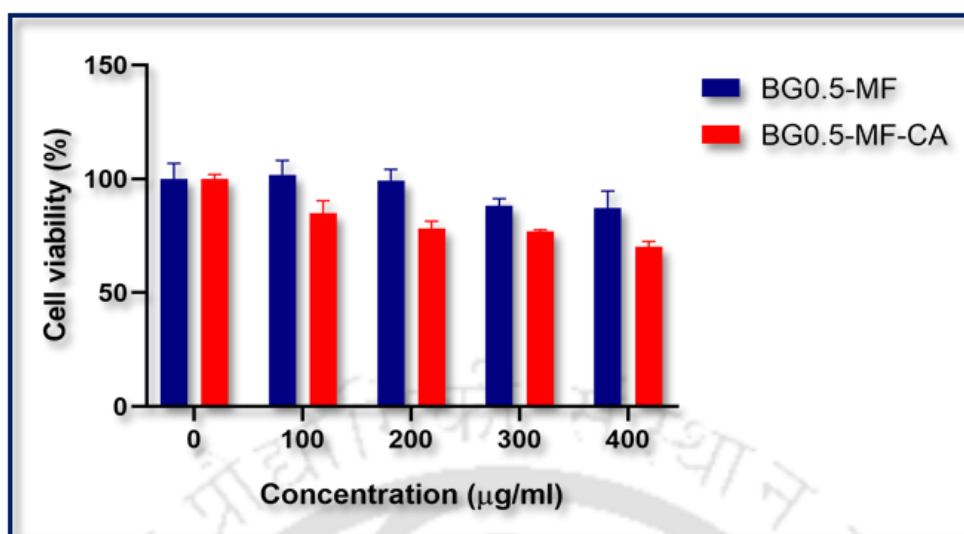


Fig. 6.13: Cell viability of pristine BG0.5-MF and CA-loaded BG0.5-MF-CA nanocomposite against A549. The results are stated as mean $\pm$ SD (N=4).

Summary

Herein, the β -CD-GO decorated MF nanocarriers of various β -CD-GO mass proportions were successfully manufactured using a self-assembled, sustainable, and facile technique. Before loading with the natural chemotherapeutic agent, CA, the nanocarriers were analyzed using several characterization methods. The electron microscopic images of BGw-MF show that sharp-edged octahedral MF crystals are effectively formed on the exfoliated β -CD-GO nanoconjugate. The polycrystalline SAED blueprints of β -CD-GO substantiated the effective production of β -CD-GO adorned MF nanocarriers. By using elemental analysis (XPS) and Raman spectroscopy, it was validated that the synthesis of β -CD-GO/MF nanocarrier caused a rise in defect concentration owing to the increment in the oxygen composition of nanocarriers. The synthesized BGw-MF nanocarriers demonstrated superior CA loading capacity than pristine MF. Furthermore, FTIR spectroscopy confirmed the effective loading of CA onto the BG0.5-MF. For the normal cell HEK293, the CA-loaded BG0.5-MF nanocarrier proved remarkable cytocompatibility. Meanwhile, the BG0.5-MF-CA nanocomposite revealed adequate anticancer efficacy against lung cancer cell A549.

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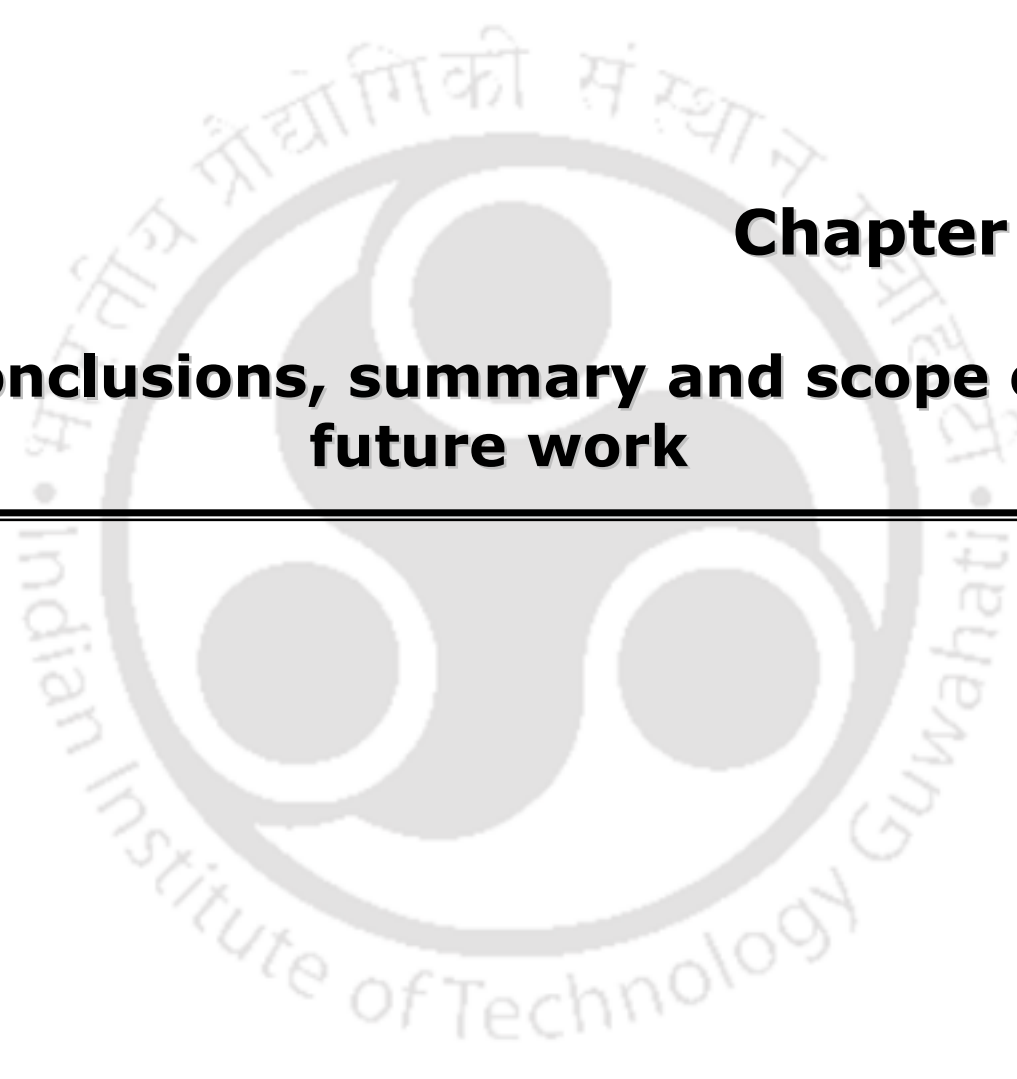
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Chapter 7:

Conclusions, summary and scope of future work

Chapter 7

Conclusion, summary and scope of future work

This chapter comprises three parts; the first part illustrates the major conclusions, which include the summary and results drawn from several works related to modifications of GO-based nanocarriers for anticancer DDS presented in this thesis. The second part demonstrates a comparative analysis of the synthesized GO-based nanocarrier in line with their characteristics and performance in anticancer DDS. The third and last part of this chapter discusses the ideas for future work.

7.1. Conclusions

The thesis focuses on the structural, functional, and morphological modifications of GO-based nanocarriers for their application in anticancer DDS. The state-of-the-art for modified forms of GO-based nanocarriers, their application in anticancer DDS, and the background of the problems undertaken in this work are illustrated in Chapter 1. Chapter 2 described the fabrication approach for 1D nano-morphology of GO, i.e., GONS of tunable dimensions using ultrasonication treatment. Chapters 3 and 4 outlined the application of GONS in sustainable and targeted co-delivery of natural as well as commercial chemotherapeutic agents. Chapters 5 and 6 highlighted the sustainable approaches for the synthesis of MOFs (MIL-100 (Fe)) and their composites obtained via impregnation of GO and β -CD-GO nanoconjugate at room temperature for controlled delivery of DOX and CA, respectively. The major conclusions from the different studies are presented below:

Fabrication of ultrasound-mediated tunable graphene oxide nanoscrolls (GONS) (Refer Chapter 2):

The 1D GONS of tunable dimensions were successfully fabricated from GO using low-frequency ultrasonication treatment, and the surface characteristics were investigated through respective characterization techniques. The conclusions of this study are drawn as follows:

- ❖ Electron microscopic images illustrated the spirally wounded, hollow, and tubular-shaped GONS and flat GO nanosheets (Refer Fig. 2.2 and 2.3).
- ❖ FTIR and XPS analysis indicated the oxidation of graphite to form GO and subsequently GONS with identical functional groups as of GO (Refer Fig. 2.4 and Fig. 2.5).
- ❖ Raman analysis overlays the rapid oxygen-defect site cleavage mechanism (Refer pages 59-61).
- ❖ A solvent with a relatively large Hansen solubility parameter but lower dipole movement and less –ve value of ζ potential supports GONS formation (Refer pages 65-66).
- ❖ The length of obtained GONS decreases from ~1311 nm to 1011 nm and ~1165 nm to 516 nm for 100 W and 150 W sonication power, respectively, with an increase in irradiation time from 0.5 h to 3 h during ultrasonication (Refer Fig. 2.10).
- ❖ The average length of the nanoscrolls can be tailored by selecting suitable ultrasonication parameters and solvents.

Development of graphene oxide nanoscrolls imparted nano-delivery system for the sustained release of gallic acid (Refer Chapter 3):

Fabricated 1D GONS was employed for anticancer DDS using GA as a natural chemotherapeutic agent. The GA loading and release characteristics were investigated at

variable GA amount, pH of the media, loading temperature, and physiological pH (7.4) to understand the loading mechanism and release behavior of GA. The following inferences were made:

- ❖ Electron microscopic images illustrated the unaffected tubular-shaped morphology of the GONS-GA nanocomposite (Refer to Fig. 3.3).
- ❖ FTIR and XPS analysis confirmed the successful loading of GA on GONS (Refer Fig. 3.4 and Fig. 3.5). Raman spectroscopic observations have reinforced the potential of GA as a natural antioxidant with reducing the structural defects in 1D material such as GONS (Refer Fig. 3.6).
- ❖ The synthesized GONS with a maximum loading efficiency of $29.83 \pm 0.8\%$ have shown better loading capacity for GA. Loading characteristics of GA revealed that with an increase in GA mass ratio, the loading efficiency was enhanced till 1.25 w/w, and it was maximum at the acidic pH (4.7) of the media (Refer Fig. 3.8).
- ❖ The *in-vitro* release study at physiological pH (7.4) presented a sustainable release behavior for GA, with $\sim 74\%$ release over a period of 5 days (Refer Fig. 3.10).
- ❖ The kinetic study over the release of GA from GONS-GA nanocomposite at pH 7.4 followed a pseudo-second-order kinetic model and Baker and Lonsdale model with an R^2 value of ~ 0.99 (Refer Fig. 3.11).
- ❖ Alongside the GONS nanocarrier, free drug GA and GA-loaded GONS-GA nanocomposite were found to be highly cytocompatible for normal cells HEK293. However, the obtained GONS-GA nanocomposite showed suitable anticancer activity against the lung cancer cells, A549 ($IC_{50} = 60.7 \mu\text{g mL}^{-1}$) (Refer Fig. 3.13 and Table 3.6).

Chitosan-grafted folic acid decorated one-dimensional graphene oxide nanoscrolls: a biocompatible drug cargo for targeted co-delivery of anticancer drugs (Refer Chapter 4):

Herein, the fabricated 1D GONS were successfully functionalized with CS and FA to enhance the biocompatibility and targetability of the nanocarrier. The obtained GONS-CS-FA (GCF) nanocarriers were employed for targeted co-delivery of anticancer agents, namely, DOX and CA. The loading characteristics were investigated for variable drug amount and compositions. In-vitro release and cytotoxicity studies were also conducted to investigate the release characteristics and cytocompatibility of nanocarriers. The conclusions of this study are drawn as follows:

- ❖ FTIR and XPS analysis confirmed the successful formation of GONS, GCF, and GCF-DOX-CA nanocomposite (Refer Fig. 4.4 and Fig. 4.5). Also, the reduction behavior of GONS and GCF nanocarrier was advocated by XRD, elemental analysis and Raman spectroscopy (Refer Fig. 4.6, Fig. 4.7 and Table 4.1).
- ❖ The fabricated GONS and GCF nanocarrier exhibited superior co-loading characteristics for DOX and CA. The optimum loading capacity for GCF nanocarrier was found to be $51.14 \pm 1.47\%$ and $49.70 \pm 1.19\%$ for DOX and CA, respectively, for 2.5 (w/w) drug: nanocarrier ratio and drug composition of 1 (DOX: CA) (w/w) (Refer Fig. 4.8).
- ❖ An *in-vitro* release contour of the developed GCF nanocarriers expressed regulated and sustainable release of DOX and CA. The overall DOX and CA release out of the GCF-DOX-CA nanocomposite for a period of 7 days (168 h) was roughly recorded as $72.15 \pm 1.80\%$ and $82.44 \pm 2.47\%$ and $60.59 \pm 1.63\%$ and $74.20 \pm 0.83\%$ at pH 7.4 (PBS) and 5 (SAB), respectively (Refer Fig. 4.9).

- ❖ At pH 5, the kinetics investigation of DOX and CA release from GCF-DOX-CA nanocomposite followed a pseudo-second-order kinetic model with an R^2 value of 0.98 and 0.99, individually (Refer Fig. 4.10 and Fig. 4.11).
- ❖ For HEK293, a cell viability of $\sim 81\%$ and $\sim 76\%$ was observed for GCF and GCF-DOX-CA for a higher dose of $400 \mu\text{g mL}^{-1}$, respectively, suggesting suitable cytocompatibility for normal cell lines (Refer Fig. 4.12).
- ❖ In the case of A549, the GCF with a cell viability of $> 70\%$ was found to be cytocompatible. In contrast, GCF-DOX CA nanocomposite displayed an adequate anticancer efficacy with cell viability of $\sim 57\%$ for $400 \mu\text{g mL}^{-1}$, suggesting targeted anticancer activity against the lung cancer cells (Refer Fig. 4.13).
- ❖ Apparently, the smaller size, deglomeration tendency, higher drug loading efficiency, suitable colloidal stability, targeted delivery of anticancer agents, higher toxicity to cancer cell lines, and sustained drug-release characteristics advocated the significance of obtained GCF nanocarrier for anticancer DDS.

One-pot, sustainable, and room temperature synthesis of graphene oxide-impregnated iron-based metal-organic framework (GO/MIL-100(Fe)) nanocarriers for anticancer drug delivery systems (Refer Chapter 5):

The HF-free GO@MIL-100(Fe) nanocarriers of different GO weight fractions were successfully synthesized by adopting a sustainable approach. The resultant Gw-MF nanocarriers were investigated for DOX loading, and the following observations were found:

- ❖ The electron microscopic images have illustrated the effective formation of flat and exfoliated GO nanosheets, the sharp-edged octahedral surface morphology of MIL-100(Fe), along with GO/MIL-100 (Fe) nanocarriers (Refer Fig. 5.2).

- ❖ The X-ray diffractograms, FTIR, XPS, and Raman spectroscopy confirmed the successful synthesis of GO, MIL-100(Fe), and their nanocomposites (Refer Fig. 5.4, Fig. 5.6, Fig. 5.7, and Fig. 5.9).
- ❖ It was observed that the formation of GO/MIL-100 (Fe) composite leads to an increase in defect concentration due to an upsurge in oxygen content of the nanocarrier, which has been confirmed by EDX and Raman spectroscopic analysis (Refer Table 5.2 and Fig. 5.9).
- ❖ The synthesized Gw-MF nanocarriers have shown better loading capacity for DOX than pristine MIL-100(Fe); the maximum loading efficiency was found to be $23.53 \pm 1.18\%$ for G0.5-MF nanocarrier consisting of 0.5 wt.% GO. However, the effect of the DOX weight ratio has suggested that, with an increase in the drug amount, the loading efficiency was improved to $29.91 \pm 1.49\%$ for a 1 w/w ratio (Refer Fig. 5.10).
- ❖ The thermodynamic study revealed that the DOX loading on the G0.5-MF nanocarrier was driven by the physisorption phenomenon, and the process is spontaneous in nature (Refer Table 5.4).
- ❖ An *in-vitro* release profile of obtained MF and G0.5-MF nanocarriers showed a controlled and sustainable DOX release compared to an initial burst effect for pristine GO nanocarrier. The cumulative release for the G0.5-MF nanocarrier was observed at around $70.96 \pm 2.10\%$ in 25 days (Refer Fig. 5.11).
- ❖ The kinetic study over DOX release from G0.5-MF-DOX nanocomposite at pH 7.4 follows a pseudo-second-order kinetic model with an R^2 value of 0.99 (Refer Fig. 5.12).
- ❖ As per the *in-vitro* cytotoxicity studies of DOX-loaded G0.5-MF conducted for a period of 7 days, the cell viability for A549 and HEK293 were $50 \pm 5\%$ and $80 \pm 5\%$ for the dose of $62.5 \mu\text{g mL}^{-1}$ of DOX, respectively (Refer Fig. 5.14).

Room temperature synthesis of β -cyclodextrin functionalized graphene oxide decorated MIL-100 (Fe): a sustainable drug cargo for controlled delivery of caffeic acid (Refer Chapter 6):

A facile, self-assembled, and sustainable technique was demonstrated to successfully synthesize β -CD-GO decorated MIL-100 (Fe) nanocarriers of various β -CD-GO weight fractions at room temperature. The nanocarriers were investigated for CA loading and release at pH = 5 and 7.4. The conclusions of this study are drawn as follows:

- ❖ The X-ray diffractograms, electron microscopic, FTIR, XPS, and Raman spectroscopic investigations confirmed the successful synthesis of GO, β -CD-GO, MIL-100(Fe), and their nanocomposites (Refer Fig. 6.3, Fig. 6.4, Fig. 6.6, Fig. 6.7, and Fig. 6.8).
- ❖ The synthesized BGw-MF nanocarriers displayed superior CA loading capacity than pristine MF. The highest loading capacity was found for BG0.5-MF with $56.44 \pm 2.25\%$ LE for a 2 w/w ratio of CA at pH 9 among all the BGw-MF nanocarriers (Refer Fig. 6.9).
- ❖ The overall CA release from the BG0.5-MF-CA nanocomposite was recorded as $57.07 \pm 2.01\%$ at pH= 5 and $48.04 \pm 1.44\%$ at pH= 7.4 in 8 days (Refer Fig. 6.10).
- ❖ The cytotoxicity assay elucidates that the loading of caffeic acid (CA) on the synthesized BG0.5-MF nanocarrier did not show any toxicity/cell death in the normal cell line ($80 \pm 5\%$) while enhancing cell death for the lung cancer cell line ($68 \pm 2\%$) for $400 \mu\text{g mL}^{-1}$ dose (Refer Fig. 6.12 and Fig. 6.13).
- ❖ Overall, the MF-based nanocarriers with enhanced surface characteristics have displayed improved colloidal stability, higher drug loading capacity, controlled release behavior, and suitable toxicity towards the cancer cells, which collectively advocates their applicability in anticancer DDS.

7.2. Comparative analysis

The comparative analysis is conducted on the synthesized nanocarriers and obtained nanocomposites, namely, GO, GONS, GONS-GA, GCF, GCF-DOX-CA, MF, G0.5-MF, G0.5-MF-DOX, BG, BG0.5-MF, and BG0.5-MF-CA. A comparative study is performed by considering the surface characteristics and performance of the listed nanocarriers and nanocomposites in anticancer DDS; the resultant observations are tabulated in **Table 7.1**. It was observed that, despite the higher drug loading efficiencies of GO nanosheets, their lateral dimensions have restricted their application in the anticancer DDS, with significant toxicity to normal cell lines. Meanwhile, GONS with identical function groups to that of GO and more accessible inner-wall area have resulted in higher drug-loading efficiencies, lower and adjustable scroll dimensions and lower toxicity for both the normal as well as cancer cell lines with suitable colloidal stability. The further modification of GONS with CS and FA has the lowest scroll dimensions and greatly improved the cell viabilities while illustrating a targeting approach for tumor cells through FA. The MOF-based nanocarriers such as MF, G0.5-MF, and BG0.5-MF demonstrated suitable loading capacities with delayed release of chemotherapeutic agents like DOX and CA. Out of which, the G0.5-MF nanocarrier exhibited the highest cytocompatibility for normal cells and BG0.5-MF for cancer cells. The DOX-loaded G0.5-MF nanocomposites demonstrated significant toxicity to cancer cells. Furthermore, BG0.5-MF nanocarriers illustrated the highest loading efficiency (LE) with improved colloidal stability to that of G0.5-MF nanocarriers.

Overall, it was depicted that the GONS-based nanocarriers (GONS, GCF) demonstrated higher toxicity for cancer cells with initial burst release of chemotherapeutic agents, and MOF-based nanocarriers (MF, G0.5-MF, BG0.5-MF) with improved colloidal stability and release characteristics illustrated lower toxicity for both normal and cancer cell lines. Although these

nanocarriers have distinguishable characteristics and performance in cytotoxicity analysis, the GCF nanocarrier is recommended for improved compatibility, higher LE, lower size, and suitable stability, whereas the G0.5-MF nanocarrier is recommended for controlled release characteristics, significant LE, and improved stability.

7.3. Recommendations for future work

This section highlights some of the new areas of research that can be carried out to further improve the adaptability of GONS and MOF-based nanocarriers in drug delivery and other biomedical applications. Some of the important areas of recommended research are suggested as an extension of the present study:

- ❖ Future fundamental work can include the optimization of the GONS fabrication process for tunable and lower dimensions with its cost analysis.
- ❖ Other than functionalities, GONS exhibits improved conductivity due to its reduced nature; therefore, it can be utilized for supercapacitors, sensing, and other environmental remediation applications.
- ❖ A sustainable synthesis approach can be explored for synthesizing other MOF-based nanocarriers, e.g., bismuth or copper-based MOFs.
- ❖ To enhance their release characteristics, a targeted co-delivery of chemotherapeutic agents can be employed for MIL-100 (Fe) nanocarriers.
- ❖ Based on the results of cytotoxicity studies, *in-vivo* and *in-silico* studies are strongly recommended to investigate the bioavailability of obtained nanocarriers.
- ❖ Pharmacological and pharmacokinetics studies are recommended to evaluate the efficacy of drug-loaded nanocomposite formulations.

Chapter 7

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Table 7.1: Comparative study of synthesized nanocarriers and obtained nanocomposites.

Nanocarriers/ Nanocomposites	Synthesis/ method	Structure/ morphology	BET surface area (m ² /g)	I _D /I _G ratio	Loading efficiency, LE (%)			Average particle size (nm)	ζ(mV)	Cytotoxicity, IC ₅₀ (µg/mL)		Remarks
					GA	DOX	CA			HEK293	A549	
GO	Modified Hummers method	Well-exfoliated 2D nanosheets	30-39	0.99-1.14	38.14 ± 1.1	37.32 ± 1.8	40.01 ± 2.1	450-2340	-35.6 ± 1.6	68.74 ± 3.2	74.89 ± 4.7	Higher LE, higher lateral dimensions, burst release, higher toxicity to normal cells
GONS	LF-Ultrasonication	1D scrolled tubular and open-end morphology	118.36	1.08-1.19	29.82 ± 0.8	38.52 ± 1.24	33.36 ± 1.04	190-400	-24.2 ± 1.5	98.82 ± 6.7	98.86 ± 4.4	Higher LE, lower size, adjustable sizes, higher surface area, suitable stability, lower toxicity to normal and cancer cells
GONS-GA	<i>in-vitro</i> drug loading	1D scrolled tubular and open-end morphology	-	1.05	-	-	-	~250	-37.1 ± 2.1	83.63 ± 3.6	60.7 ± 1.2	Suitable stability, hence bioavailability, lower size, sustained drug release, highest toxicity to cancer cells
GONS-CS-FA (GCF)	LF-Ultrasonication, Amidation reaction	1D scrolled tubular and open-end morphology	-	1.05	-	51.14 ± 1.47	49.70 ± 1.19	190 ± 20	-18.5 ± 1.5	> 400	> 300	Higher LE, lower size, adjustable sizes, lower toxicity to normal cells
GCF-DOX-CA	<i>in-vitro</i> drug loading	1D scrolled tubular and open-end morphology	-	1.03	-	-	-	170 ± 8	-21.3 ± 2.9	> 400	>250	Tumor targeting approach, smallest size, sustained drug release, higher toxicity to cancer cells, improved viability for normal cells.
MIL-100 (Fe) (MF)	Sustainable RT approach	sharp-edged octahedral morphology	2203.26	-	-	16.64 ± 0.82		391.2 ± 31.9	-24.6 ± 0.8	> 400	> 400	Lower LE, delayed drug release, suitable cytocompatibility, lower stability, higher surface area and particle size

Table 7.1: Comparative study of synthesized nanocarriers and obtained nanocomposites (Continued....)

G0.5-MF	Sustainable RT approach	Combination of 2D nanosheets and sharp-edged octahedral MF crystals	1396.50	-		29.91 $\pm$ 1.49	25.33 $\pm$ 0.51	1281.7 $\pm$ 201.8	-28.7 $\pm$ 1.4	982 $\pm$ 39.3	576 $\pm$ 17.2	Improved LE, highest cytocompatibility, suitable stability, higher surface area and particle size
G0.5-MF-DOX	<i>in-vitro</i> drug loading	Combination of 2D nanosheets and sharp-edged octahedral MF crystals	-	-				879.87 $\pm$ 87.9	-19.4 $\pm$ 1.7	75.2 $\pm$ 1.7	52 $\pm$ 1.3	Controlled and delayed drug release, significant anticancer behavior
β -CD-GO (BG)	Conjugation reaction	Well-exfoliated 2D nanosheets	11.13	0.92	-	41.35 $\pm$ 0.44	59.05 $\pm$ 0.90	1876.4 $\pm$ 406.5	-29.4 $\pm$ 1.8	> 400	> 400	Highest LE, higher lateral dimension, improved stability, suitable compatibility for both the normal and cancer cells
BG0.5-MF	Sustainable RT approach	Combination of 2D nanosheets and sharp-edged octahedral MF crystals	579.43	-	-	34.15 $\pm$ 0.71	56.44 $\pm$ 2.25	1481.2 $\pm$ 381.9	-36.6 $\pm$ 1.1 mV	350-400	> 400	Higher LE, higher lateral dimension, decent stability, suitable compatibility for both the normal and cancer cells
BG0.5-MF-CA	<i>in-vitro</i> drug loading	Combination of 2D nanosheets and sharp-edged octahedral MF crystals	-	-	-	-	-	1216.5 $\pm$ 136.84	-41.0 $\pm$ 3.8 mV	> 400	400-500	Controlled and delayed drug release, superior stability, higher lateral dimension, insignificant anticancer behavior

Appendix

Appendix

A. Calibration curves for UV-Vis spectroscopy

GA, CA, and DOX concentrations in different media (water, PBS, and SAB) are determined by first preparing a calibration curve using a UV-Visible spectrophotometer. Calibration curves are prepared for various concentrations of GA, CA, and DOX in water, as well as PBS and SAB solution at the specified wavelengths.

Figures A1, A2, and A3 present the obtained concentration calibration curve for GA, CA, and DOX loading as well as release experiments, respectively. It can be observed that the absorbance varied linearly with variation in GA, CA, and DOX concentration in water, PBS, and SAB solutions.

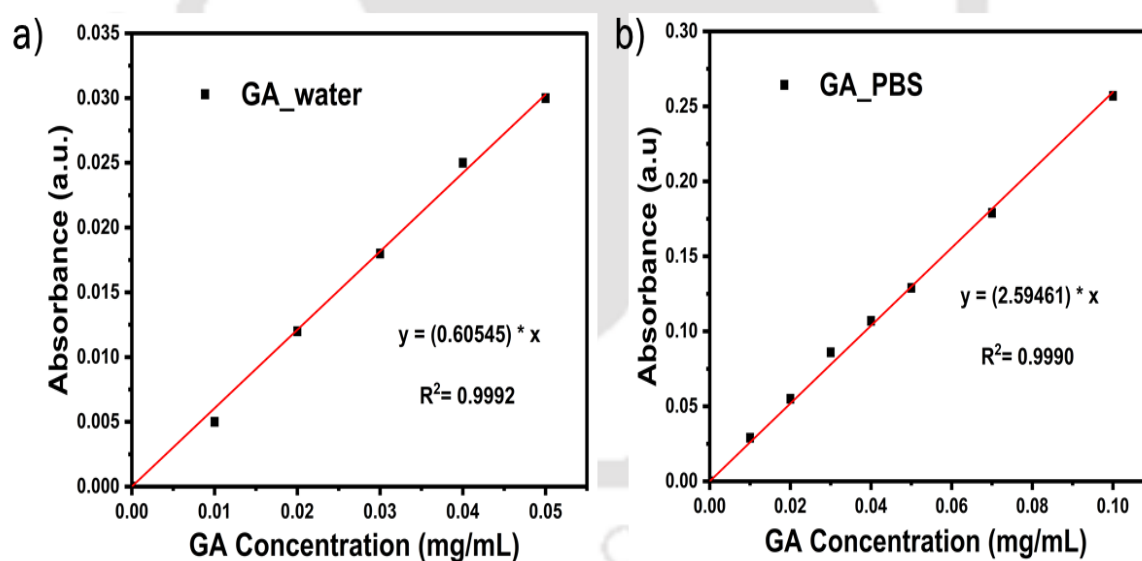


Fig. A1: Calibration curve for the evaluation of GA concentration in (a) water and (b) PBS solution for loading and release experiments.

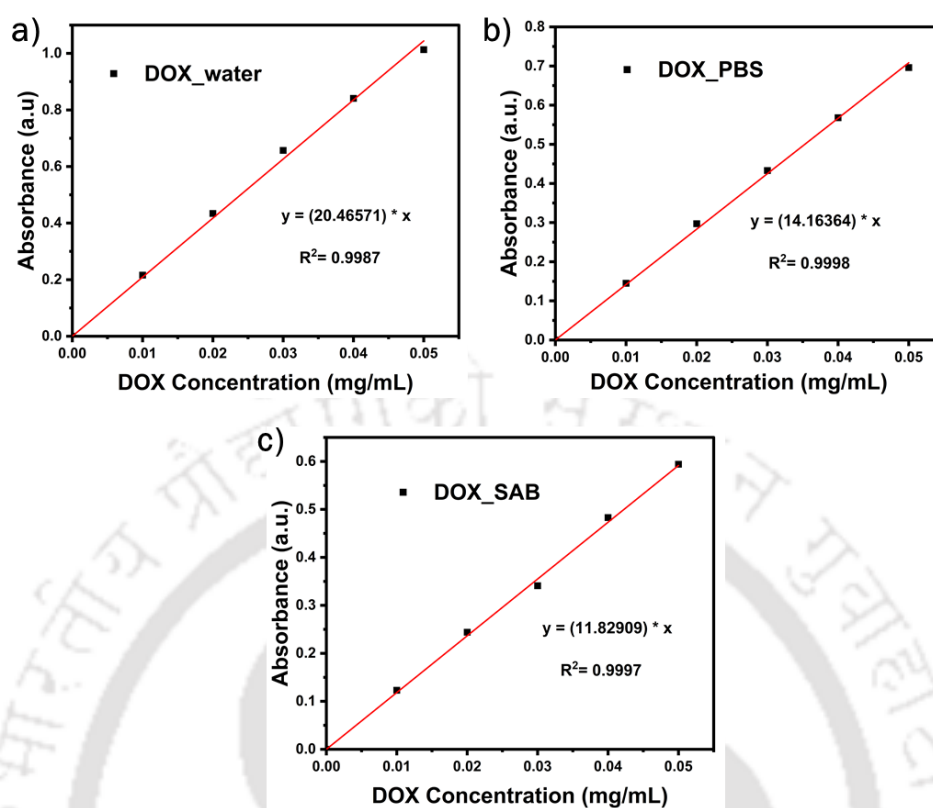


Fig. A2: Calibration curve for the evaluation of DOX concentration in (a) water, (b) PBS, and (c) SAB buffer solutions for loading and release experiments.

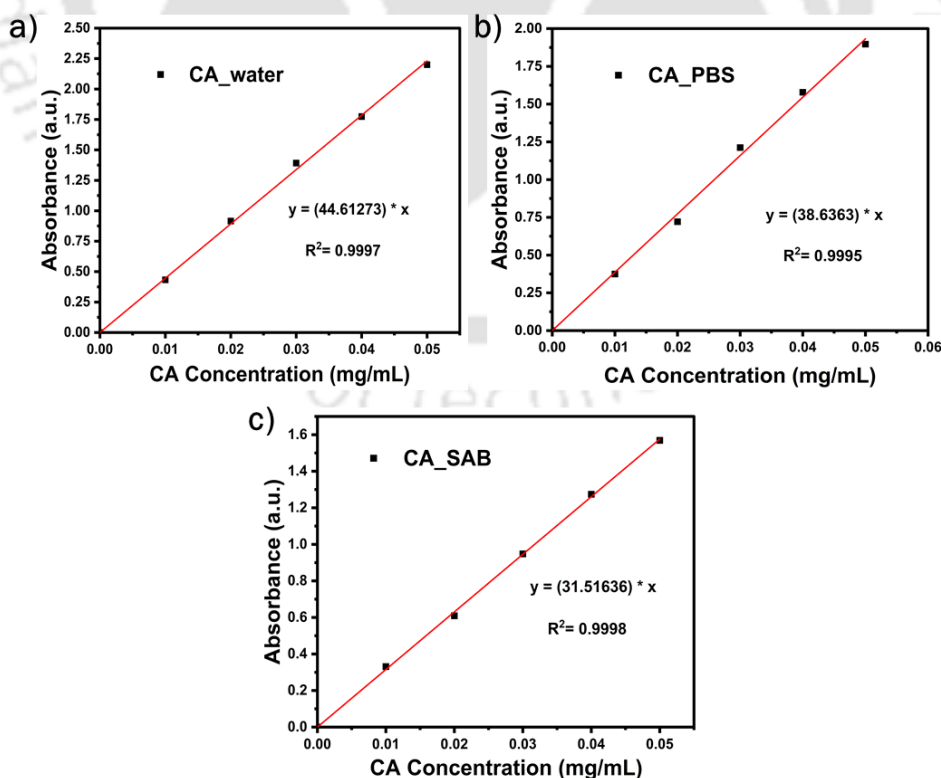


Fig. A3: Calibration curve for the evaluation of CA concentration in (a) water, (b) PBS, and (c) SAB buffer solutions for loading and release experiments.

B. Error analysis

The errors in experimentally measured quantities and in parameters calculated from those measurements are important in that they determine the accuracy of calculation and predictions using those quantities. There are two types of errors, viz., systematic error and random error. Systematic errors are the results of faulty assumptions or improper experimental measuring techniques. In this work, care was taken to eliminate systematic errors by appropriately designing the experiments and adopting qualified methods for the analysis of the data. On the other hand, random errors result from variations in the precision of measuring parameters and the slight variations that occur in successive measurements made by the same observer under nearly identical conditions. Random errors cannot be eliminated. The error analysis presented in this section focuses on the random errors.

In most of the experiments performed in this work, the quantities that are measured directly are the concentrations of drugs, namely, GA, CA, and DOX, which are used to determine the amount of drug loaded on the nanocarriers and released out of nanocomposites. In this section, statistical analysis (standard deviation) is used to estimate errors associated with the values of drug-loaded and released. Also, the error related to the measurement of cell viability for HEK293 and A549 cell lines is assessed through the mean standard deviation (SD) method, which is generally considered to be one of the best methods to estimate the error/uncertainty which is based on the following method:

If $x_1, x_2, \dots, x_N$ are the N results of the measurements of a particular quantity x , then the mean value of x (i.e. μ), is defined by

$$\mu = \frac{x_1 + x_2 + \dots + x_N}{N} = \frac{1}{N} \sum_{i=1}^N x_i \quad (\text{B.1})$$

Appendix

Standard deviation (σ) is defined by

$$\sigma = \sqrt{\frac{(x_1 - \mu)^2 + (x_2 - \mu)^2 + \dots + (x_N - \mu)^2}{N}} \quad (\text{B.2})$$

B.1. Error measurements for loading and release experiments

The amount of drug loaded and released was estimated by calculating the drug concentrations, which were determined by measuring the absorbance value at a specific wavelength in the UV-Vis spectrophotometer. Later, the error calculations were reported as mean $\pm$ SD.

- Error calculations were done for the GA, DOX, and CA concentrations in each loading experiment. An error of $\pm 2\text{-}3\%$ was taken into consideration for each data.
- Error calculations were also done for the GA, DOX, and CA concentrations in each release experiment. An error of $\pm 3\text{-}5\%$ was taken into consideration for each data.

B.2. Error measurements for cytotoxicity experiments

The cell viability of HEK293 and A549 was determined by measuring the absorbance value at 570 nm in the UV-Vis spectrophotometer. The error calculations were done for cytotoxicity experiments. An error of up to $\pm 8\%$ was taken into consideration for each data.

C. Cost estimation

Table C1. Cost estimation for the synthesis of GO.

Sr. No.	Raw material	Amount used	Price (INR)	Instrument used	Unit	Electric charge/Unit (INR)	Price (INR)
1.	Synthetic Graphite	53.45 g	40.41	Hot plate magnetic stirrer	1.846	5.96	11.01
2.	NaNO ₃	26.72 g	19.24	Centrifuge	0.272		1.62
3.	KMnO ₄	160.35 g	234.43	Fridge dryer	18		107.28
4.	H ₂ SO ₄ (98%)	1336.25 mL	1007.53				
5.	H ₂ O ₂ (30%)	267.25g	302.53				
6.	DI Water	26 L	101.40				
7.	HCl (35%)	20 mL	15.56				
(A)Total=			1721.10			(B) Total =	119.91
Overall cost for GO synthesis= (A+B) = 1841.01							
Overhead charges = 10% of Overall cost= 184.10						Batch Size =	100 g
Net cost for GO synthesis= (Overall cost+ Overhead charges) = 1841.01+184.10= 2025.11 INR/100 g							

Table C2. Cost estimation for the synthesis of GONS nanocarrier.

Sr. No.	Raw material	Amount used	Price (INR)	Instrument used	Unit	Electric charge/Unit (INR)	Price (INR)
1.	GO	1.78	36.05	Vacuum pump	0.088	5.96	0.52
2.	Methanol	30	1050.00	Sonicator	0.195		1.16
				Dryer	9		53.64
(A)Total=			1086.05			(B) Total=	55.33
Overall cost for GONS synthesis= (A+B) = 1141.37							
Overhead charges = 10% of Overall cost= 114.1						Batch size =	1 g
Net cost for GONS synthesis= (Overall cost+ Overhead charges) = 1141.37+114.1 = 1255.51 INR/g							

Appendix

Table C3. Cost estimation for the synthesis of GCF nanocarrier.

Sr. No.	Raw material	Amount used	Price (INR)	Instrument used	Unit	Electric charge/Unit (INR)	Price (INR)
1.	GONS	0.12	150.54	Hot plate magnetic stirrer	35.460	5.96	211.34
2.	CS	0.06	1.46	Centrifuge	0.272		1.62
3.	NHS	1.2	139.20	Fridge dryer	9		53.64
4.	EDC	0.6	94.01				
5.	PBS	0.1	368.00				
6.	FA	0.075	43.32				
7.	DI Water	26	101.40				
8.	AA Solution	0.12	175.68				
(A)Total=			1073.61	(B) Total=			266.60
Overall cost for GCF synthesis= (A+B) = 1340.21							
Overhead charges = 10% of Overall cost= 134.02						Batch size =	300 mg
Net cost for GCF synthesis= (Overall cost+ Overhead charges) = 1340.21+134.02= 1474.23 INR/300 mg							

Table C4. Cost estimation for the synthesis of MF nanocarrier.

Sr. No.	Raw material	Amount used	Price (INR)	Instrument used	Unit	Electric charge/Unit (INR)	Price (INR)
1.	FeSO ₄ .7H ₂ O	3.16	29.66	Hot plate magnetic stirrer	35.46	5.96	211.34
2.	C ₆ H ₃ (CO ₂ H) ₃ (Trimesic acid)	1.679	57.72	Centrifuge	0.13		0.81
3.	NaOH	1.192	0.79	Dryer	9		53.64
4.	ethanol	0.3	96.00				
5.	DI Water	1.5	5.85				
		(A)Total=	190.02			(B) Total=	265.79
Overall cost for GO synthesis= (A+B) = 455.82							
Overhead charges = 10% of Overall cost= 45.58						Batch size =	2 g
Net cost for GO synthesis= (Overall cost+ Overhead charges) = 455.82+45.58= 501.40 INR/2 g							

Table C5. Cost estimation for the synthesis of G0.5-MF nanocarrier.

Sr. No.	Raw material	Amount used	Price (INR)	Instrument used	Unit	Electric charge/Unit (INR)	Price (INR)
1.	FeSO ₄ .7H ₂ O	3.16	29.66	Hot plate magnetic stirrer	35.46	5.96	211.34
2.	C ₆ H ₃ (CO ₂ H) ₃ (Trimesic acid),	1.679	57.72	Centrifuge	0.13		0.81
3.	NaOH	1.192	0.79	Dryer	9		53.64
4.	ethanol	0.4	128				
5.	DI Water	2	7.80				
6.	GO	0.01	0.20				
(A)Total=			224.18			(B) Total=	265.79
Overall cost for GO synthesis= (A+B) = 489.97							
Overhead charges = 10% of Overall cost= 49.00						Batch size =	1.92 g
Net cost for GO synthesis= (Overall cost+ Overhead charges) = 489.97+49.00= 538.97 INR/1.92 g							

Table C6. Cost estimation for the synthesis of BG0.5-MFnanocarrier.

Sr. No.	Raw material	Amount used	Price (INR)	Instrument used	Unit	Electric charge/Unit (INR)	Price (INR)
1.	FeSO ₄ .7H ₂ O	3.16	29.66	Hot plate magnetic stirrer	35.46	5.96	211.34
2.	C ₆ H ₃ (CO ₂ H) ₃ (Trimesic acid),	1.679	57.72	Centrifuge	0.13		0.81
3.	NaOH	1.192	0.79	Dryer	9		53.64
4.	ethanol	0.4	128				
5.	DI Water	2	7.80				
6.	BGO	0.01	16.27				
(A)Total=			240.25			(B) Total=	265.79
Overall cost for GO synthesis= (A+B) = 506.04							
Overhead charges = 10% of Overall cost= 50.60						Batch size =	1. 85 g
Net cost for GO synthesis= (Overall cost+ Overhead charges) = 506.04 + 50.60= 556.64 INR/1.85 g							