

BIOCOMPATIBILITY OF GRAPHENE OXIDE- INCORPORATED MAGNESIUM IMPLANTS FOR BONE REGENERATION

Submitted in partial fulfillment for the award of
Bachelor of Technology degree in Biotechnology

By

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SATHYABAMA INSTITUTE OF SCIENCE AND TECHNOLOGY

(DEEMED TO BE UNIVERSITY)

Accredited with Grade 'A' by NAAC

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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **Winnie Prahelika W (39230059)**, who carried out the project entitled "Biocompatibility of Graphene Oxide-incorporated Magnesium Implants for bone regeneration" under our supervision from September-2022 to April-2023.

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I, **Winnie Prahelika W (39230059)**, hereby declare that the Project Report entitled **"Biocompatibility of Graphene Oxide Incorporated Magnesium Implants for bone regeneration"** done by me under the guidance of **Dr. V. Ramesh Kumar** (Internal) and **Dr. T. Stalin Dhas** (External) at **Sathyabama Institute of Science and Technology, Chennai, Tamilnadu, India**, is submitted in partial fulfillment of the requirements for the award of **Bachelor of Technology degree in Biotechnology**.

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ABSTRACT

In the quest of finding suitable alternatives for Titanium implants due to their frequent failure, allergic reactions, and non-degradability, amidst some advantages such as increased durability and mechanical strength, Magnesium-based bone implants have gained popularity for reasons such as biodegradability, biocompatibility, ability to reduce stress-shielding, etc., Though Magnesium is an element that is naturally found in the body and has great osteopromotive and regenerative properties, it is also known to exhibit high corrosive properties in its purest form, and hence there is a need for it to be alloyed. Magnesium-based alloys are highly efficient as biodegradable materials owing to their ductile property and strong nature. The research problem addressed here is the lack of durability of Magnesium-based implants, which leads to the accumulation of Hydrogen locally, which ultimately leads to its degradation before the complete healing of the wounds. The current work is based on the hypothesis that fabricating Magnesium implants along with Graphene oxide, zinc, and Hydroxyapatite and coating the implant with pectin can enhance the durability, antibacterial properties, and hemocompatibility of such implants and hence can be tested for the same.

Keywords: Biocompatibility, Cytocompatibility, Cytotoxicity, Hemocompatibility, Bone implants, Pectin coating

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LIST OF ABBREVIATIONS

ACRONYM	EXPANSION
Ca	Calcium
Ca (NO₃)₂.4H₂O	Calcium Nitrate Tetrahydrate
Ca₁₀((PO₄)₆(OH)₂)	Calcium phosphate dihydrate
Co-Cr	Cobalt-Chromium
<i>E.coli</i>	<i>Escherichia coli</i>
EDS	Energy-dispersive X-ray spectroscopy
ELISA	Enzyme Linked Immunosorbent Assay
FESEM	Field emission scanning electron Microscopy
FTIR	Fourier Transform Infrared Spectroscopy
g/cm³	gram per centimeter cube
GO	Graphene Oxide
GPa	Giga pascal
H₂O₂	Hydrogen Peroxide
HAp	Hydroxyapatite
HBSS	Hank's Balanced salt solution
Hrs	Hours
HR-SEM	High Resolution-Scanning Electron Microscopy
HR-TEM	High Resolution-Transmission Electron Microscopy
K₂HPO₄	Dipotassium Hydrogen Phosphate
Mg	Magnesium
mm	millimetre
MPa.m^{1/2}	Mega pascal square root meter
NaNO₃	Sodium Nitrate
OH	Hydroxide
PO₄	Phosphate
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
UV-Vis	Ultraviolet-visible spectroscopy
X-RD	X-Ray Diffraction

CHAPTER-1

1. INTRODUCTION:

Bone is constantly remodeled by the osteoclasts which aid in bone resorption and osteoblasts which help in the formation of bone (Kokkonen *et al.*, 2007). Bone repair is contributed by various biomechanical, chemical, pathological, hormonal, and cellular factors (Zhi *et al.*, 2022). The natural healing process of the bone after a fracture includes four major steps namely, swelling (0 to 7 days after the occurrence of injury), formation of soft callus (2 to 14 days after the occurrence of injury), mineralization (14 to 40 days after the occurrence of injury), and repair (40 days to many years after the occurrence of injury). The process of bone fracture healing has been illustrated in Figure 1.1. (Holt *et al.*, 2017; Wright *et al.*, 2019).

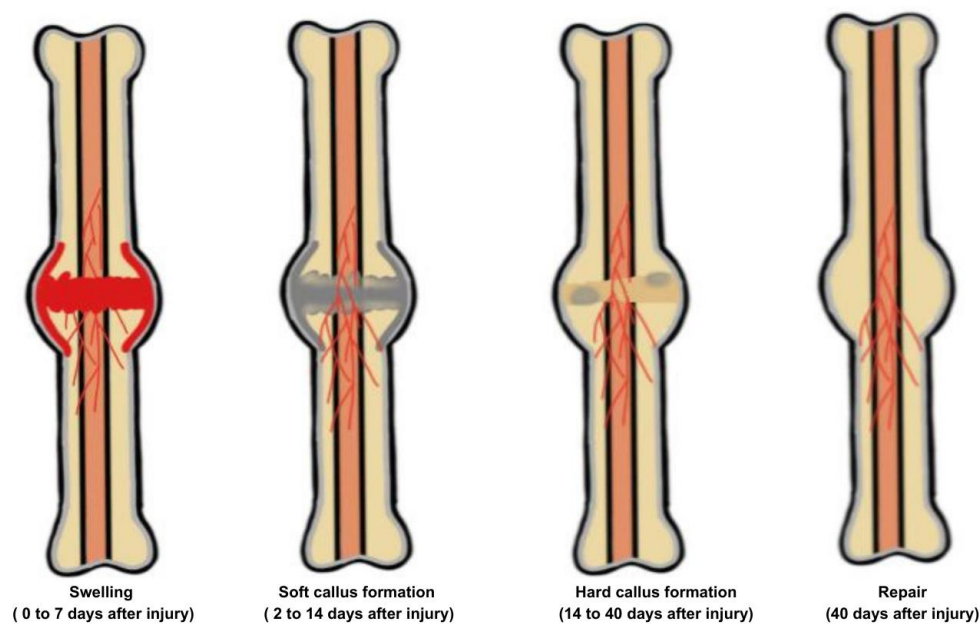


Figure 1.1: Healing Process of a Bone fracture (created using sketchbook and canva)

If the bone damage length goes above double the diameter of the fractured bone, the bone tissue loses its ability to self-heal, hence autografts, allografts or artificial substitutes for bones, come into play (Maia *et al.*, 2022), to heal the defects like tumour formations, traumatic fractures, infections and osteoporosis (Bahrami, Baheiraei and Shahrezaee, 2021). Autografts have higher failure rates, though they are being used widely (Li *et al.*, 2019). Implants made of metals may lead to an uncomfortable feeling at the site of injury, the need for repetitive surgeries, the occurrence of infections, etc., Implants that use Magnesium in the purest form,

degrade before the fracture completely heals itself (Shuai *et al.*, 2019), while Titanium is non-degradable (Bai *et al.*, 2019). The fracture healing process in Magnesium and Titanium-based implants is illustrated in Figure 1.2. (Gu *et al.*, 2009; Shuai *et al.*, 2019).

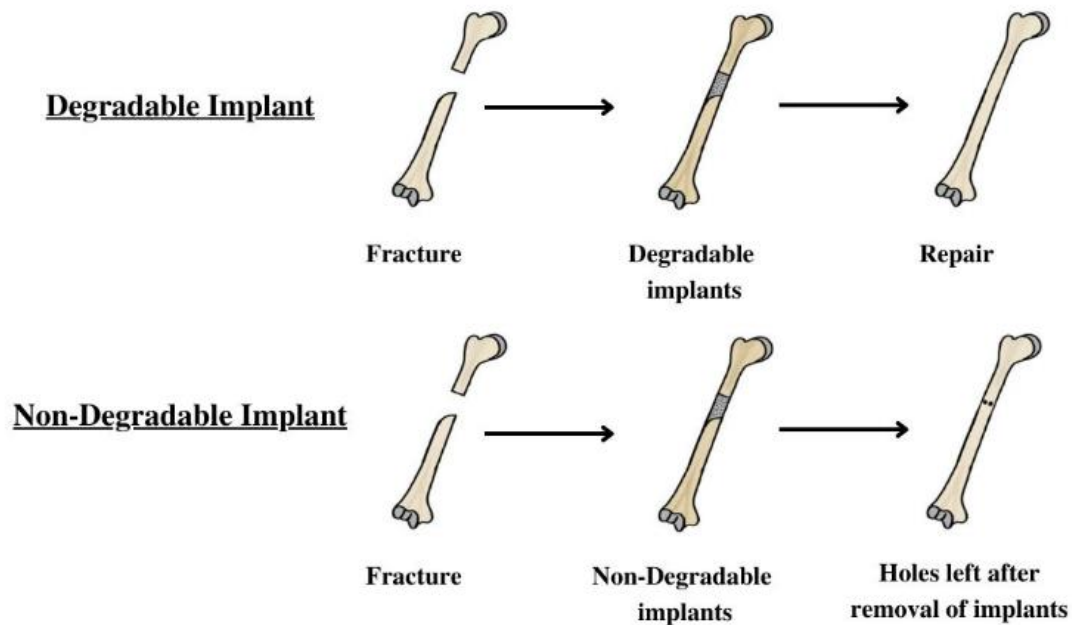


Figure 1.2: Healing process in the presence of degradable and non-degradable implants (created using sketchbook and canva)

Traditionally used metal implants are made up of Titanium and its alloys, Stainless steel, Gold, Co-Cr alloys, Magnesium, Silver, Palladium, Iron, Zinc, Tantalum and electrodes made of platinum. Polymers used are poly ethylenes, propylenes, methyl-methacrylate esters and amides and hydrogels. Ceramics utilized are Alumina, Zirconia, HAp, bioactive glasses, porcelains and phosphates where, the ceramics and polymers are used as coatings and the metals can be either alloyed or can be used as a whole implant material. A description of the properties of the materials used in conventional bone implants has been shown in Figure 1.3. The non-degradable nature of Titanium, Silver, Palladium, Gold and Tantalum make them less favourable for bone implants. Co-Cr alloys leave out toxic products of corrosion. Stainless steel is less preferred due to corrosions and cracks caused by stress and Iron can cause degeneration to neural cells. Zinc and Magnesium are favourable due to their

degradability (Bai *et al.*, 2019; Banerjee *et al.*, 2019; Korkusuz, 2015; Walley *et al.*, 2016; Xia *et al.*, 2021).

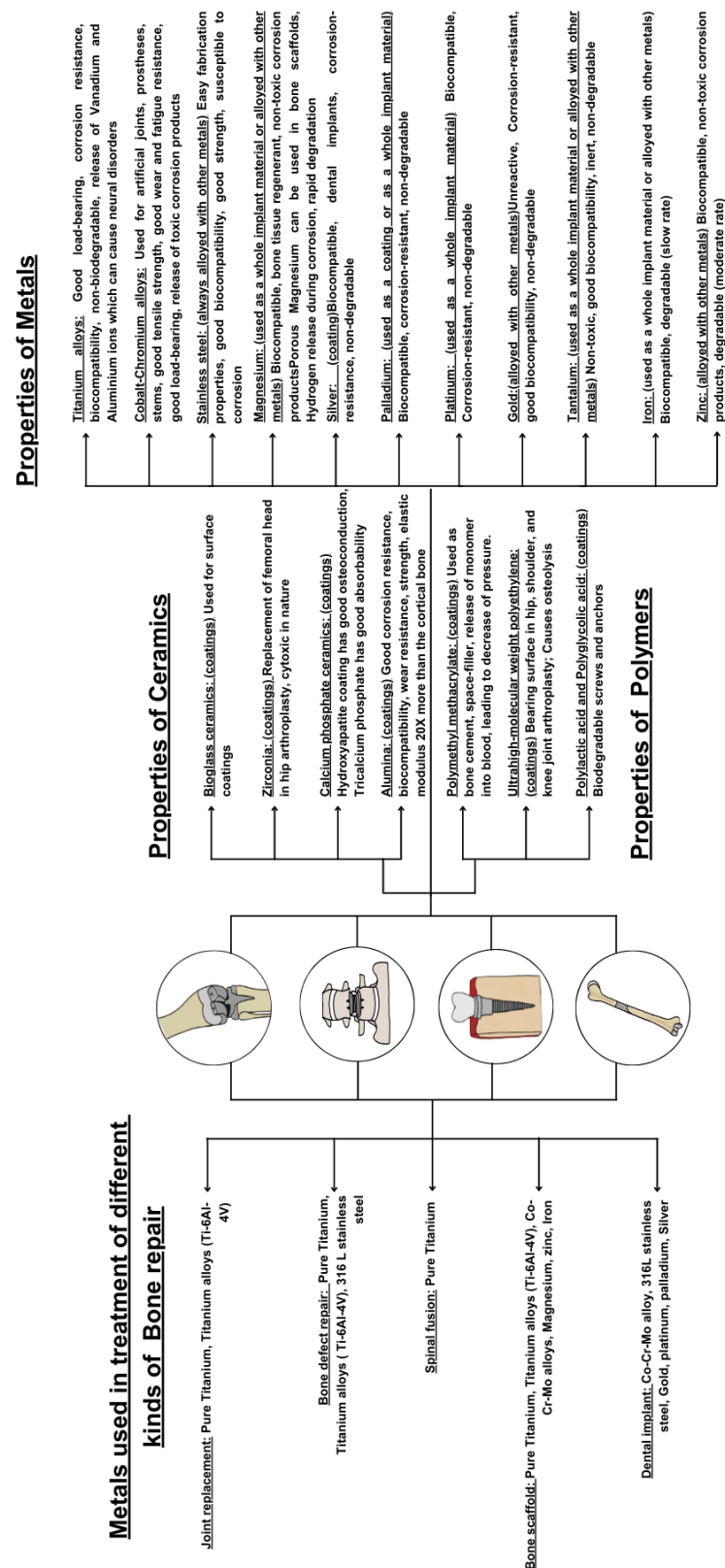


Figure 1.3: Common materials used in Bone implants and their properties (created using sketchbook and canva)

Though Titanium alloy and stainless-steel-based fracture fixation devices, help to achieve effective treatment, they may also lead to stress shielding effect which leads to bone resorption and loosening (Li J *et al.*, 2019; Shahin *et al.*, 2020). This problem develops mostly when the substantial modulus difference found between implant and the bone, which ultimately interferes with the healing of fractures (Li J *et al.*, 2019). An ideal bone implant is expected to possess a stronger and enduring osseointegrative property, a good *in vivo* timespan, an ability to promote osteoconduction, vascularization and osteoinduction, an ability to avoid the stress-shielding effect, a good storage-stability, etc., (Shahin *et al.*, 2020). High porous nature and favourable size of pores are necessary for the creation of a bone scaffold because pores support cell proliferation, infiltrate blood arteries, and aid in the creation of bone tissue, which is extremely favourable (Kim *et al.*, 2014). Implants that use Magnesium in the purest form, degrade before the fracture completely heals itself (Shuai *et al.*, 2019).

Graphene is the thinnest and strongest 2-D nanostructured allotrope of carbon, which is the basic component of graphene quantum dots, nanofibers, 3D graphite and many other allotropes of carbon. Graphene oxide, the Graphene in its highly oxidized state, is extracted using strong oxidizers (Jodati *et al.*, 2021). The graphene oxide in its reduced state can be obtained by reduction methods such as Microwave, thermal, chemical and Bacterial methods (Jodati *et al.*, 2021). Graphene oxide-based scaffolds have shown promising results in addressing the common research problem, which is inadequate bone regeneration (Shahin *et al.*, 2020). Graphene and Graphene oxide being two-dimensional, can reduce toxic effects caused by implants. Besides, they exhibit enhanced osteogenic performance and encourage osteogenesis, which is ideal (Holt *et al.*, 2017). They also have a high surface area, extraordinary mechanical strength, high osteoconductive efficiency and supreme versatility (Al-arjan *et al.* 2021). Pure Magnesium based implants are naturally degradable, biocompatible and possess advantageous mechanical properties, but it is disadvantageous in terms of wound healing due to the lack of durability, which becomes the primary cause of local hydrogen accumulation (Yang Y *et al.*, 2020). Magnesium is also known to exhibit poor antibacterial performance (Banerjee *et al.*, 2019). Magnesium alloys are reported to be advantageous due to their higher specific strength, young's modulus that is remotely similar to that of human bone, naturally degradable nature, biocompatible nature, osteopromotive property, plasticity, stiffness, etc., (Yang Y *et al.*, 2020).

Magnesium alloys get gradually dissolved, absorbed, ingested or eliminated and no further surgery is required. They exhibit no toxicity and side effects on the patient (Abazari *et al.*, 2021). Zinc can be used to overcome the major disadvantage of Magnesium possessing a low strength which is lesser than 350 MPa. Alloying Zinc with magnesium can improve its mechanical integrity and biocompatibility while enhancing osteogenesis and integration (Yang H *et al.*, 2020). Hydroxyapatite has great biocompatibility and bioavailability in the extracellular matrix. Additionally, it aids in the control of the bone's biomechanical characteristics (Al-arjan *et al.*, 2021). Pectin, with a chemical structure similar to alginate, is an innate polysaccharide obtained from the cell walls of plant cells, which serves as an extracellular matrix artificially, to immobilise cells for the regeneration of bone tissue, can aid in enhanced cell proliferation and adhesion within the microspheres. It has the ability to be degraded by the microflora in the colon and it can also remain intact throughout the gastrointestinal tract (Munarin *et al.*, 2011). Natural polymers lack mechanical strength hence graphene oxide can be added as a filler. The above-mentioned properties of these materials can widely aid in achieving the ideal bone implant's specifications.

Biocompatibility assessments play a significant part in confirming the capacity for bone implants to be used for clinical purposes. Biocompatibility means the efficiency of a material to perform a function without causing harmful effects on the body (Shuai *et al.*, 2019). The prepared bone implants were tested under the aspects of Antibacterial properties, Corrosion aspects and Hemocompatibility.

CHAPTER-2

2. REVIEW OF LITERATURE:

2.1 MAGNESIUM ALLOYS AND THEIR PROPERTIES:

Alloys of Magnesium are shown more interest in the designing process of bone implants as these alloys' young's modulus is strikingly similar to the Human bone's modulus. The young's modulus of human bone is around 15 to 30 GPa, while that of Magnesium alloys is 45 GPa. Their density is approximately 1.79 g/cm^3 , which is also very similar to human bone, whose density is 1.75 g/cm^3 . The limit of elasticity of Magnesium alloys is 100-200 MPa, while that of bone is 130-180 MPa and the fracture toughness is $3\text{-}6 \text{ MPa.m}^{0.5}$. The elastic modulus of bone is 3-20 GPa and that of magnesium is 41- 45 GPa, while the Titanium alloys have a higher elastic modulus (Abazari *et al.*, 2021; Guo *et al.*, 2021; Yang Y *et al.*, 2020). Due to electrochemical interactions with water that result in the release of hydrogen and magnesium oxide but do not shield the magnesium matrix from further corrosion, the deterioration of Magnesium-based alloys in acid or neutral solutions is more prone to occur. Magnesium implant's deterioration rate can be slowed through alloying, heating or surface treatment. Alloying can be used to modify the distribution of phases, size of grains and microstructure of alloys based on Magnesium. Additionally, it lessens flaws and galvanic corrosion (Shuai *et al.*, 2019). Alloying zinc with magnesium can improve yield stress and minimize the evolution of hydrogen gas during biological corrosion (Radha *et al.*, 2017). Haemolysis can result from corrosion in Magnesium alloys (Zhang E *et al.*, 2009; Zhou *et al.*, 2015).

2.2 ZINC-BASED ALLOYS:

Zinc-based alloys execute an anodic reaction when exposed to the solution and show cathodic reactions during the reduction of oxygen. The insoluble zinc hydroxide that is released, acts as a passive layer which becomes a salt that dissolves, when chlorine ions are present, which reduces the layer's protection causing the matrix to get exposed to the solution again and the electrochemical reactions restart. Degradation rate ultimately depends on the structure and composition of alloys. Compared to other alloys, Zinc based alloys have a moderate degradation rate. Zinc when alloyed; showed a greater reduction in corrosion rates (Shuai *et al.*, 2019). Zinc

addition to Magnesium can reduce the corrosion performance because of the grain refinement which leads to a uniform surface of corrosion (Hassan *et al.*, 2022).

2.3 CHARACTERISTICS OF REDUCED GRAPHENE OXIDE:

One of the most popular techniques for synthesizing Graphene Oxide is modified Hummer's method. Reduced graphene oxide, which has wide range of uses, is what we get at the end of the procedure. Daneshmandi.L. *et al.*, demonstrate that Graphene oxide doped Hydroxyapatite; when coated with Magnesium, greatly increased the hydrophilic and biomineralization capability of the implants, which paved the way for improvement in degradation rate. After adding graphene oxide in lesser concentrations, magnificent improvements in the mechanical properties of polymeric scaffolds have been seen (Forati *et al.*, 2014). Carbonaceous nanomaterials usually have a higher Young's moduli, a greater aspect ratio and higher thermal activity. The intrinsic strength is approximately equal to 130 GPa and the intrinsic strength is 1 TPa. The density is as low as 1 g/cm³. The precise surface area measures as much as 2630 m²/g. Natural degradation supported by oxygen is observed in biological reactions as the enzymatic oxidation reaction by peroxidase, naturally degrades Graphene nanoplatelets. Shuai *et al.*; confirmed that Graphene oxide could inhibit corrosion attacks and can promote apatite formation when coupled with Magnesium to form a composite (Abazari *et al.*, 2021). Graphene oxide can increase roughness and Flexural strength (Zapata *et al.*, 2019). When Graphene oxide with a smaller sheet size ranging from 0.01 to 0.65 micrometers, is used in bone implants, it provides higher antibacterial activity (Hatamie *et al.*, 2019).

2.4 PECTIN AND ITS PROPERTIES:

As it contains both carboxyl and hydroxyl groups, the polysaccharide pectin is hydrophilic, and hence has great biocompatibility, degradability and low toxicity. Esterification, transesterification, coordination and electrophilic addition are the reactions through which the hydrophilic groups promote pectin's physical and chemical properties. (Li *et al.*, 2021). Pectin can be helpful in modifying the surfaces of implants and other medical instruments (Fullana *et al.*, 2010). However, Pectin is also known to have unfavourable corrosive and swelling properties. Pectin is strongly advised for biomedical applications because it contains Rhamnogalacturonan II, which is an essential component to enhance biocompatibility (Li *et al.*, 2021). The polysaccharides

of pectin are recommended for biomedical uses as they lack toxicity, are affordable and possess anti-bacterial and anti-inflammatory properties (Clifford *et al.*, 2020). There is proof that when the concentration of Pectin is increased in a Gelatin-Pectin-based bone scaffold, the viability of cells increased to a greater extent (Nguyen *et al.*, 2015). Pectin can be subjected to degradation by microorganisms in the colon and hence can be used for delivering drugs to the colon. Mg and pectin-based composites proved to be less toxic to Huh7 cells (derived from hepatoma) (Mishra *et al.*, 2012). A nanocomposite containing Pectin and nanohydroxyapatite showed good support bearing when Graphene oxide was used in increased concentrations. (Al-arjan *et al.*, 2021).

2.5 HYDROXYAPATITE AND ITS PROPERTIES:

HAp is a natural phosphate-based compound. HAp's chemical composition and chemical formula ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) are quite close to human bone. It covers around 65% of the inorganic phase of native bone (Iviglia *et al.*, 2016). The density is around 3.22g/cm^3 . It is a crystalline compound made up of closely packed Ca, PO_4 and OH ions that form an apatite structure. The calcium-to-phosphate ratio of hydroxyapatite is 1.67, which is identical to human bone (Fathyunes *et al.*, 2018). They are highly stable due to the wide range of pH, which is 4 -12 and also the Calcium to Phosphorus molar ratio, which is 1.67. HAp nanoparticles are more stable than the bulk form as they have higher surface energy which leads to better mechanical properties, resorption potential along with great activity. HAp can help in corrosion control and reduces the chances of leaching, by retarding the metallic ions' rate of dissolution (Beig *et al.*, 2020). Hydroxyapatite is known to have high compressive strength, biocompatibility, immunotolerance, osteoconduction and integration. The disadvantages are that they have low tensile as well as fatigue strength (Shuai *et al.*, 2019). It is not an effective load-carrying material due to poor fracture toughness, wear resistance and tensile strength (Sobczak-Kupiec *et al.*, 2021). There is evidence that HAp nanocoatings on Magnesium implants play a crucial part in cell adherence and biocompatibility (Li *et al.*, 2017).

2.6 INVITRO BIOCOMPATIBILITY OF MAGNESIUM IMPLANTS INCORPORATED WITH GRAPHENE OXIDE:

2.6.1. ANTIBACTERIAL PROPERTIES:

Somayeh *et al.*, tested the antibacterial activity of ZM31-xRGO composite (where x represents various graphene oxide concentrations, ranging from 0 to 1.5 weight percent and ZM31 stands for Mg-3Zn-1Mn), using *Staphylococcus aureus* and *Escherichia coli* by agar diffusion method, where the controls were inoculated with 1×10^4 CFU/mL of both the bacteria. The inhibition zones formed around the composites placed in an agar medium inoculated with the bacteria in an incubator at room temperature for one day were checked with their respective controls. The results showed significant antibacterial activity after the encapsulation of reduced graphene oxide at a concentration of 1.5 wt% (Abazari, Shamsipur and Bakhsheshi-Rad, 2021). Safari N *et al.*; studied the antibacterial activity of Magnesium- 1 Aluminium- Copper based implant against bacteria like *Escherichia coli*, *Staphylococcus aureus*, etc., and found that addition of Graphene oxide at a weight% of 0.18 significantly increased the diameter of the inhibition zone (Safari *et al.*, 2020). *Staphylococcus aureus* has been reported as the most common bacteria in wounds that require surgical intervention and hence has been used in various studies (Zhang *et al.*, 2020). The other pathogens are *Escherichia coli* and *Pseudomonas aeruginosa*. Hydroxyapatite when doped with Zinc showed considerable antibacterial activity MSCs, MRC-5 and rat primary osteoprogenitor cell lines (Szurkowska *et al.*, 2018). Sinulingga *et al.*, have proven that 2.5 Mg-2.5 Ag-HAp and 2.5 Zn-2.5 Ag- HAp showed significant antimicrobial effectiveness against *Escherichia coli* (Sinulingga *et al.*, 2021). Gopi *et al.*, have reported improved antibacterial performance, when tested with strains of *S.aureus*, *C.albicans*, *E. coli*, etc., when pectin-doped HAp nanocomposite is used to implant bones (Gopi D *et al.*, 2015). Zhang C *et al.*, demonstrated that 2 Mg-0.5 Zn-Ca-based bone implant greatly reduced surface bacterial adhesion more efficiently than pure magnesium (Zhang C *et al.*, 2019). Qu X *et al.*, have established and proven that Zn-2Ag alloy can greatly minimize bacterial adhesion and the biofilm formation significantly (Qu *et al.*, 2021).

2.6.2. HEMOCOMPATIBILITY:

ASTM standards suggest that the samples with a hemolysis rate below 2 % are preferable and a Graphene oxide-Titanium-Silk fibroin-Hydroxyapatite-based nanocomposite was found to have a hemolysis rate ranging from 0 to 0.3% hence it is safe to use (Li M *et al.*, 2016). Yuan *et al.*, have reported that Zn-1Mg-based alloys can provide excellent hemocompatibility, where the rate of hemolysis is below 0.2% (Chen YW *et al.*, 2021). Escudero *et al.*, tested the hemocompatibility of Graphene oxide on a cobalt and chromium-based alloy using J774A (mouse macrophage) and found that graphene oxide that has been synthesized by electrochemical reduction can increase the biocompatibility of the alloy (Escudero *et al.*, 2020). Zinc in its purest form as well as in combination with Graphene-based nanosheets showed hemolysis less than 2 % and hence are biocompatible (Fan *et al.*, 2022).

2.6.3. IN VITRO LEACHING:

Slow degradation conserves the integrity in the structure of implants, while rapid degradation is detrimental to bone and tissue cells due to the non-uniform degradation and release of gases (Hassan *et al.*, 2022). The Simulated body fluids are prepared by the protocol proposed by Kokubo *et al.*, Chen K *et al.*, evaluated the degradation rates of 2Sr-Mg, 2Sr-Mg-Ca and 2Sr-Mg-Zn alloys and found that alloys with Magnesium offered great resistance to corrosion (Chen K *et al.*, 2020). Yuan *et al.*, have shown that Zn-1Mg alloy can increase the resistance to corrosion and leaching (Chen YW *et al.*, 2021). Bavya Devi K *et al.*, have tested the degradation of MgS *invitro* in an alloy of 0.5Zn-Mg in SBF and proved enhanced degradation capacity (Devi *et al.*, 2018). Bodhak S *et al.*, have established that when HAp is electrically polarized, the cell attachment process becomes rapid and tissue in-growth fastens up, hence proving its osteogenic nature (Bodhak *et al.*, 2011). Rhamnogalactouronan II in pectin can improve cell adhesion (Gurzawska *et al.*, 2014). Yuan *et al.*, have shown proof that when Zn-1Mg alloy is used, they can collectively improve adhesion and proliferation of cells (Yuan *et al.*, 2022). Chen.D *et al.*, have demonstrated that cells attach more easily to Magnesium and Zinc alloy than to Poly-L lactic acid-based implants when MC3T3-E1 cells were cultured on them (Chen D *et al.*, 2011). A 15% reduction in weight loss was observed in SBF after 30 days of immersion, when 5wt% HAp was incorporated along with Magnesium and Zinc. A 27%

reduction in weight loss was observed in SBF after 24 days of immersion, when 1 wt % HAp was incorporated along with 3 wt % Zinc and 0.5 wt % Zirconium. A 75 % reduction in weight loss was observed in PBS after 4 days of immersion, when 5wt % HAp was incorporated along with Magnesium (Jaiswal *et al.*, 2019).

CHAPTER-3

3. AIM AND OBJECTIVE:

- To increase the durability of Magnesium implants that are being used for bone regeneration.
- Utilizing Graphene Oxide, Zinc, Hydroxyapatite and Pectin in the fabrication process of implants.
- To study the Biocompatibility of the prepared implants under the aspects of Corrosion, Antibacterial properties and Hemocompatibility.

CHAPTER-4

4. MATERIALS AND METHODS:

4.1. MATERIALS AND PROCESSING:

Magnesium powder with a purity of $\geq 99\%$ (assay $\geq 99\%$, $\text{Fe} \leq 500\text{ mg/kg}$), supplied by Sigma-Aldrich, St.Louis, MO, USA, was used as the base material. Zinc, with a particle size of $< 45\text{ }\mu\text{m}$, obtained from Sigma-Aldrich, Canada, Oakville, Ontario is used as one of the components. Graphene Oxide nanosheets were synthesized by Modified Hummer's method, using Graphite (Assay $\geq 99.50\%$), obtained from HiMedia, Mumbai, India and, NaNO_3 (Assay $\geq 99\%$), obtained from Merck Lifescience Pvt.Ltd., Mumbai, India, as the precursors. Hydroxyapatite was synthesized using Low molecular weight Heparin method using K_2HPO_4 (assay $> 99.5\%$) obtained from Sisco Research Laboratories Pvt.Ltd., Mumbai, India and $\text{CaNO}_3.4\text{H}_2\text{O}$ (assay $> 99\%$, pH (5 % aqueous solution) – 5 to 7) as the precursors.

4.2. GRAPHENE OXIDE PREPARATION BY MODIFIED HUMMER'S METHOD:

In a beaker, 0.5 g of graphite and 0.25 g of NaNO_3 were taken and 11.5 mL of Sulphuric acid was added after placing the beaker in an icebath for continuous stirring for 1 hour. Temperature is increased to 35°C and maintained for 30 minutes. 23 mL of distilled water is added and stirring is stopped and the temperature is maintained at 70°C for 2 hrs. The synthesized Graphene Oxide nanosheets were cleaned by adding a solution of 70 mL of distilled water and 5 mL of 10 % v/v H_2O_2 followed by cleaning 5 times with dilute HCl and distilled water, before subjecting them to vacuum oven treatment for 24 hrs. The finished product is shown in Figure 4.1.

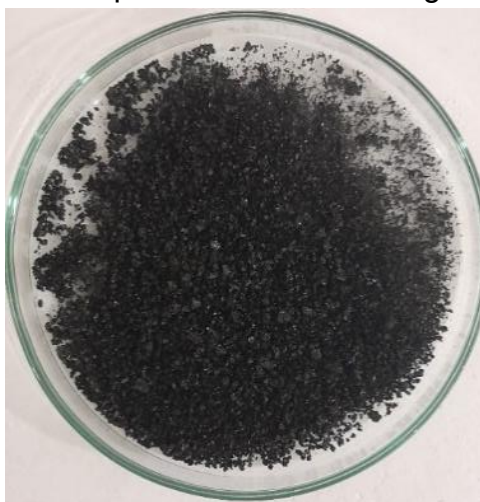


Figure 4.1: Synthesized Graphene Oxide Nanosheets

4.3. HYDROXYAPATITE PREPARATION BY LOW MOLECULAR WEIGHT HEPARIN METHOD:

In 100 mL of distilled H₂O, 1.67 g of CaNO₃.4H₂O and 10 g of K₂HPO₄ was added and stirred at room temperature for 6 hrs. About 69 mg of heparin was added to the mixture and stirred until it forms a gel. The solution is oven dried at 80 °C and the dried gel is placed in silica crucible and calcinated at 400 °C in a muffle furnace for 6 hrs. The finished product is shown in Figure 4.2.



Figure 4.2: Prepared Hydroxyapatite nanoparticles

4.4. FABRICATION BY POWDER METALLURGY METHOD:

Different concentrations of the synthesized Graphene oxide (0.1, 0.5 and 1 wt %), HAp, Zinc and Magnesium were ground together in a mortar and pestle and 0.05 g of the mixture is taken and pressed in a hydraulic press to create implant implants of 12 mm diameter, while 0.03125 g of the mixture was taken for preparing implant implants of 8 mm diameter. The prepared implants were then sintered at 350 °C for 3 hrs in a muffle furnace and then coated with 10 wt % pectin using dip coating method, where the implants were dipped in 10 wt % pectin for 1 hr, followed by drying in a vacuum oven for 2 hrs until completely dry. The fabricated implants have been shown in Figure 4.3.

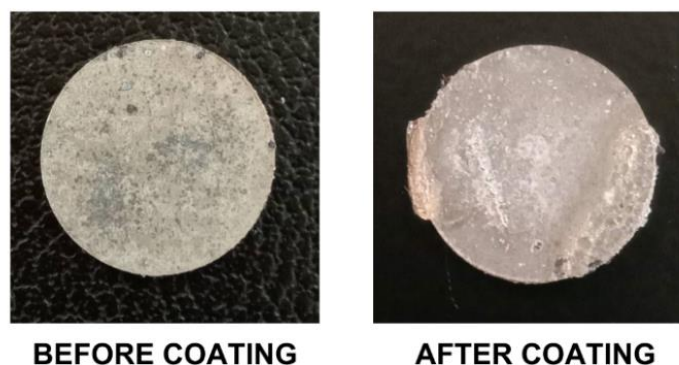


Figure 4.3: Fabricated Implants

4.5. CHARACTERIZATION

The UV absorbance of the samples were measured using UV-1800, Shimadzu Japan. The particle size analyzer used was Nanopartica PSA, HORIBA, Japan. An Agilent Cary 630-ATR FTIR instrument was used to identify the functional groups. The morphological characterizations were carried out using a Transmission Electron Microscope, obtained from JEOL-JAPAN. An automated Shimadzu LAB-XRD-6000 (Cu K; $14=1.54056\text{\AA}$) spectrometer with a scan speed of 2° min^{-1} was used to analyse X-ray diffraction patterns on flat, ground, and ultrasonically cleaned specimen. The samples were examined under the digital optical microscope with LES 4.0 software to investigate the grain distribution. JEOL JSM-5800 LV Scanning Electron Microscope (SEM, Kyoto, Japan) was used for studying the distribution of secondary phases and nanoparticle distribution. Contact angle measurements were done by using a $3 \mu\text{l}$ droplet of deionized water addition on the surface of the samples using Drop Shape Analyzer DSA25, Kruss GmbH, Germany. The fractured surfaces were investigated using Hitachi S-4300 FESEM (Tokyo, Japan) to understand the failure mechanisms.

4.6. BIOCOMPATIBILITY ASSESSMENTS

4.6.1. ANTIBACTERIAL ACTIVITY:

Biofilm formation is a process whereby microorganisms irreversibly attach to and grow on a surface and produce extracellular polymers that facilitate attachment and matrix formation, resulting in an alteration in the phenotype of the organisms with respect to growth rate and gene transcription. Broth cultures of *E. coli* and *S. aureus* was inoculated into fresh media, add discs washed with phosphate buffer saline and incubate for 24 hrs at 37°C in orbital shaker. The setup has been shown in Figure 4.4.

Optical density of adhered cells were observed using ELISA reader and antibacterial activity was evaluated.

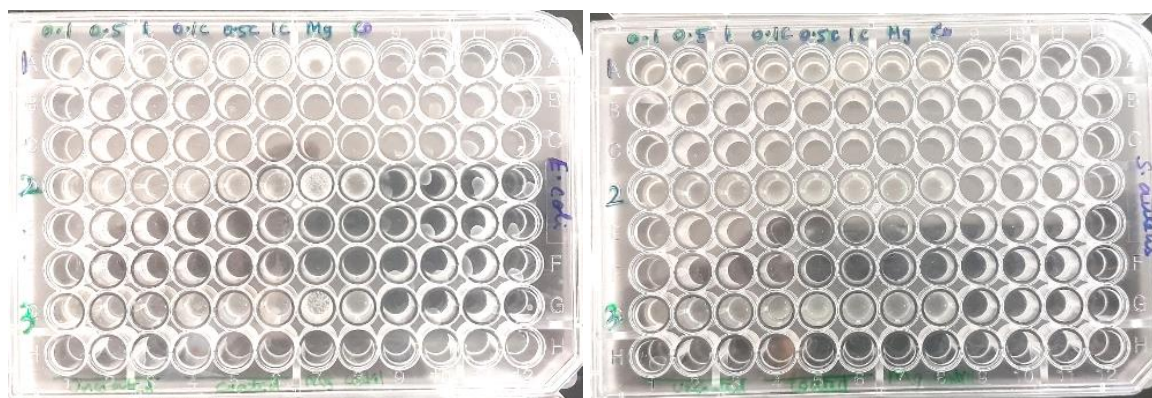


Figure 4.4: Gram positive and negative Bacteria suspended in 96 well plates for absorbance measurement

4.6.2. CORROSION STUDIES:

Cylindrical samples of (12 mm diameter) were immersed for 96 hrs in Hanks balanced salt solution (HBSS). The setup shown in figure 4.5, was maintained at 37 °C to simulate the temperature of the human body. The sample dimensions of 12 mm diameter were used. Solution to sample ratio was maintained at 20 mL-1 cm². Weight loss and absorbance measurements were measured after every 24 hrs. The uv-vis spectroscopy result was plotted in a line graph for each day and the differences in the spectrums were noted.

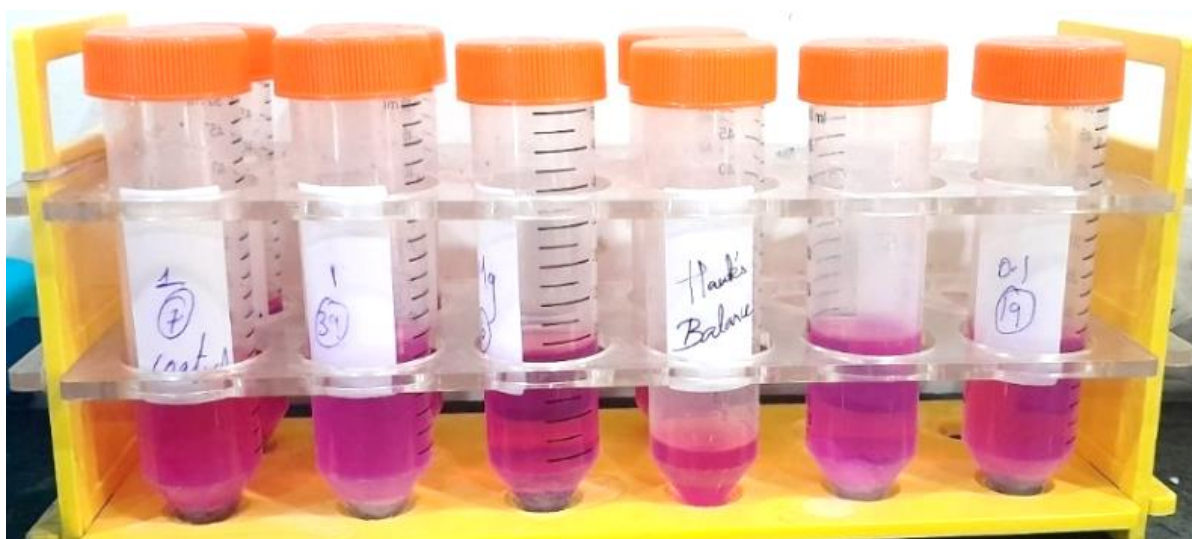


Figure 4.5: Implants immersed into Hank's balanced salt solution for corrosion testing

4.6.3. HEMOLYSIS ASSAY:

Add 5 % (v/v) sterile defibrinated blood that has been warmed to room temperature to sterile Blood Agar Base, which has been melted and chilled to 45 to 50 °C. To properly combine, swirl the flask while preventing the production of bubbles. Next, pour the mixture into sterile plates while continuing to avoid surface froth and bubbles. In this process, cooling the agar and reheating the blood are necessary procedures. Cold blood can lead the agar to solidify before being poured, while hot agar can harm red blood cells (Buxton, 2016).

100 mL of Blood agar was prepared and poured onto 4 sterilized plastic petridishes and each plate was partitioned into two sections. Each of the partitions were treated with 3 pectin coated implants with 3 different concentrations of Graphene oxide (0.1, 0.5 and 1 wt %) and 3 uncoated implants with 3 different concentrations of Graphene oxide (0.1, 0.5 and 1 wt%). The 4th plate was treated with Pure Magnesium in order to note the significance of the new creation. The analysis of hemolytic activity was performed macroscopically after the incubation time of 24 hrs at 37 °C (Lima *et al.*, 2022). The experimental setup is shown in figure 4.6.

Red blood cell lysis that is full or legitimate is known as beta hemolysis. The colony is surrounded by a clear zone that is similar in hue and transparency to the foundation medium. The oxidation of haemoglobin from red blood cells to methaemoglobin in the media around the sample is known as alpha hemolysis. The medium becomes stained green or brown as a result. It is possible to compare the hue to "bruising" the cells (Buxton, 2016).

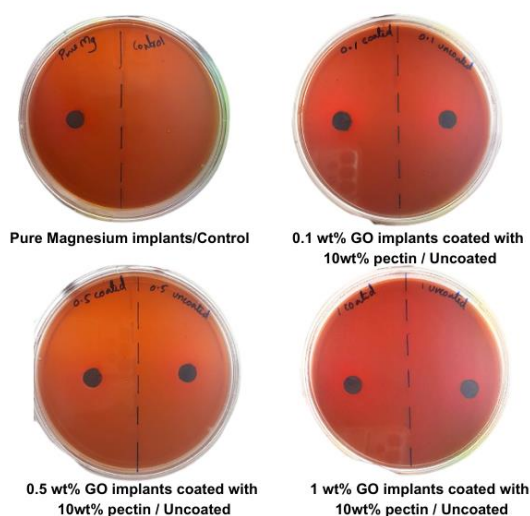


Figure 4.6: Implants suspended on Blood Agar for Hemolysis Assay

CHAPTER-5

5. RESULTS AND DISCUSSION:

5.1. MATERIAL CHARACTERIZATION:

5.1.1. GRAPHENE OXIDE:

UV-VISIBLE SPECTROSCOPY:

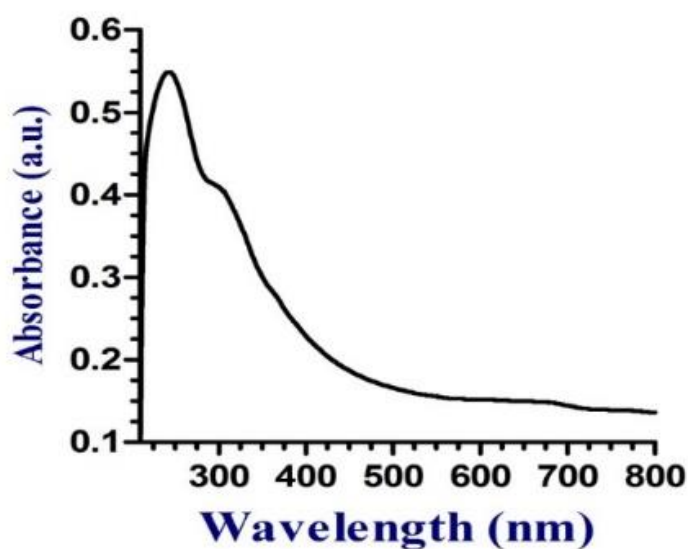


Figure 5.1: UV-visible spectroscopy results of Graphene Oxide

UV-Vis Spectroscopy (also known as Spectrophotometry) is a quantitative method for calculating how much light an individual molecule absorbs. This is accomplished by comparing the amount of light that passes through a sample to the amount of light that goes through a reference sample or a blank. It is a common practise to measure graphene oxide (GO) using its UV absorption, which commonly occurs at 230 nm (Jayavardhini *et al.*, 2022). The absorption peak was observed at 242 nm and a shoulder peak at 300 nm which are attributed to π - π^* transition of C=C bonds and n- π^* transition of C=O bonds, respectively as depicted in Figure 5.1. Both the absorption peaks confirmed the presence of GO nanoparticles.

RAMAN SPECTRA:

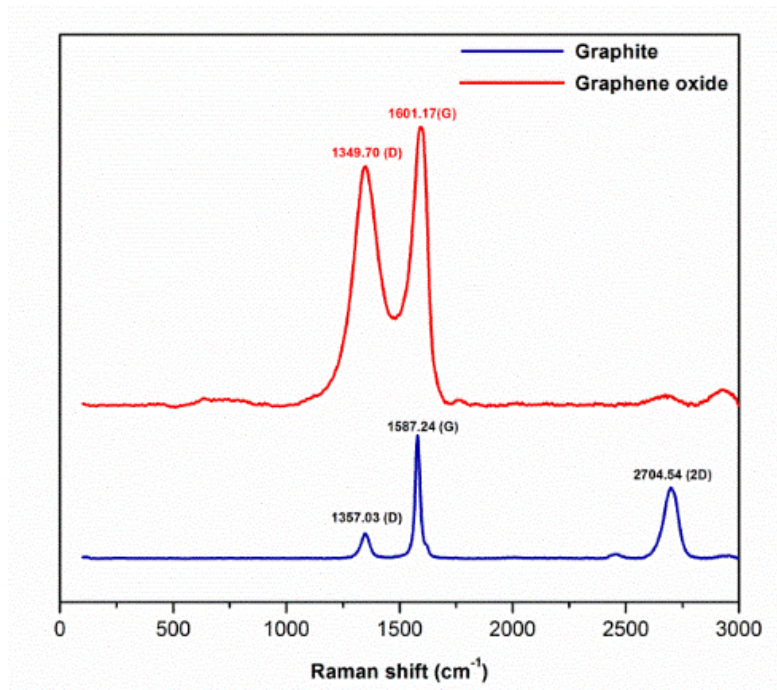


Figure 5.2: Raman spectroscopy results of Graphene Oxide

Raman spectroscopy is a non-destructive method of chemical examination that offers thorough details on crystallinity, chemical structure, phase and polymorphy, and molecular interactions (Sivashankari *et al.*, 2021). It is discovered that the Raman spectra of graphene oxide are solely associated to oxygen-free graphene-like areas, with Raman bands at 1130 and 3155 cm^{-1} that likely include C-H vibrations of rings and edges, while a band at 1700 cm^{-1} is ascribed to irregular rings resembling Stone Wales faults (Díez-Betriu *et al.*, 2013). The G band (strong band) at 1601 cm^{-1} is assigned to the first order scattering of the E_{2g} phonon from sp^2 carbon atoms and the D band (weak band) at 1349 cm^{-1} is attributed to a breathing mode of κ -point photons of A_{1g} symmetry as shown in Figure 5.2, which is related to the local sp^3 disorder bands formation through oxidation process especially the edges of graphite sheets.

PARTICLE SIZE:

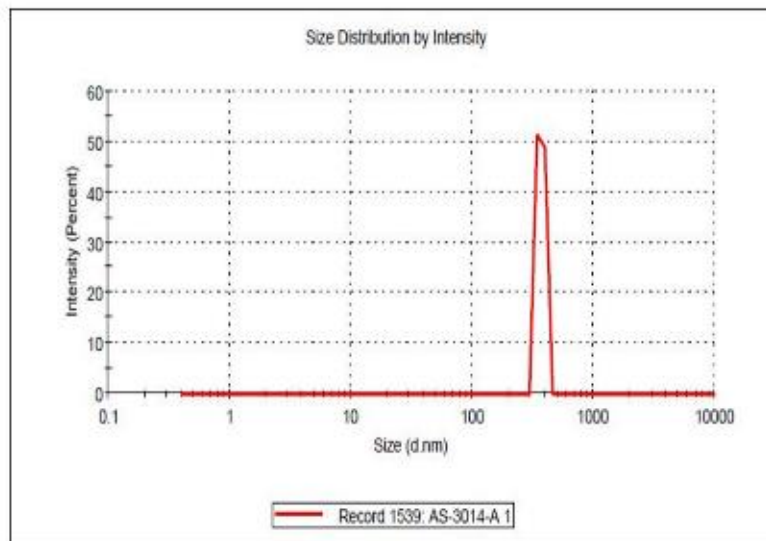


Figure 5.3: Particle size results of Graphene Oxide

The phrase particle size analysis refers to a variety of technical approaches used in laboratories to estimate the size distribution and/or average or mean size of the particles in a powder or liquid sample. The particle size analysis results are shown in Figure 5.3. The average size of the particle is found to be around 363.3 nm. The Graphene oxide nanosheets are oxidized during the synthesis process, which can introduce several functional groups such as carboxyl, hydroxyl and epoxy groups, which could have possibly resulted in the larger particle size.

ZETA POTENTIAL:

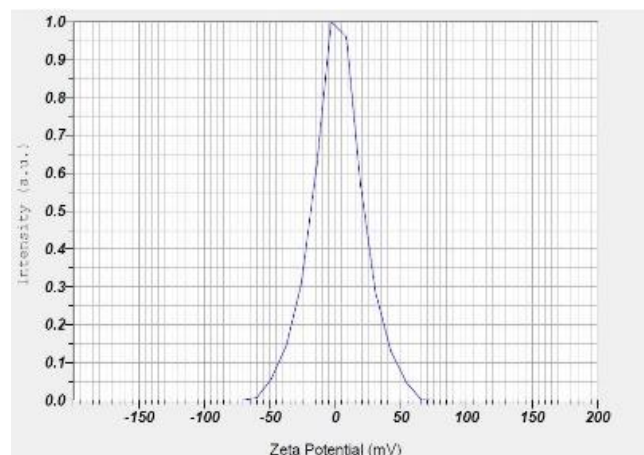


Figure 5.4: Zeta potential results of Graphene Oxide

The stability of nanoparticles in suspension depends on the zeta potential, which also plays a significant role in the initial adsorption of nanoparticles onto the cell membrane. The mobility is counted positive if the particles move toward lower potential and negative in the opposite case. (Rasmussen *et al.*, 2020). Zeta potential of graphene oxide is found to

be 1.8 mV, as shown in Figure 5.4, whereas the Electrophoretic mobility of the sample is $0.000014 \text{ cm}^2/\text{Vs}$.

XRD:

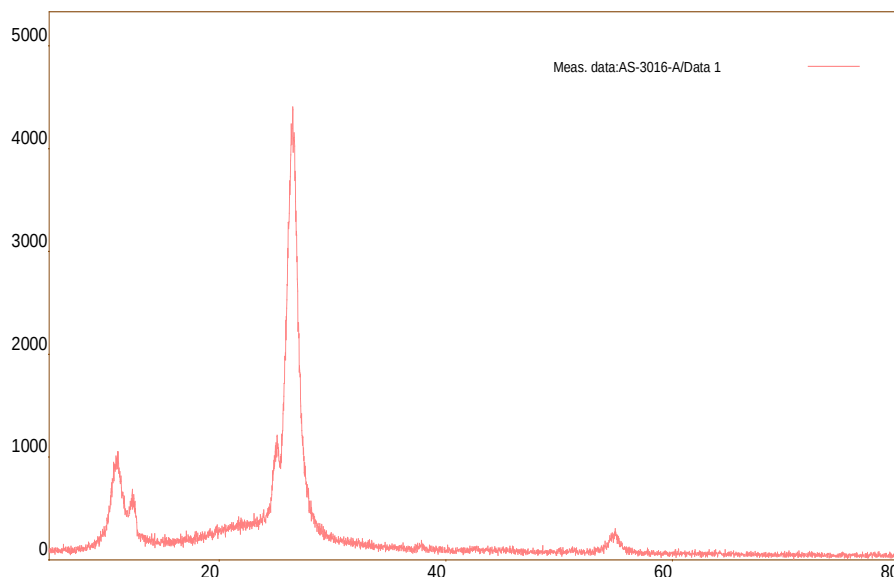


Figure 5.5: X-RD results of Graphene Oxide

The obtained $2\theta = 26.9$ degrees from the Figure 5.5, corresponds to the crystalline structure of the synthesized GO sheets. Increasing distance between fields in the graphene oxide is due to the presence of oxygen-functional groups and water molecules into the carbon layer structure (Fathyunes *et al.*, 2018).

HR-SEM:

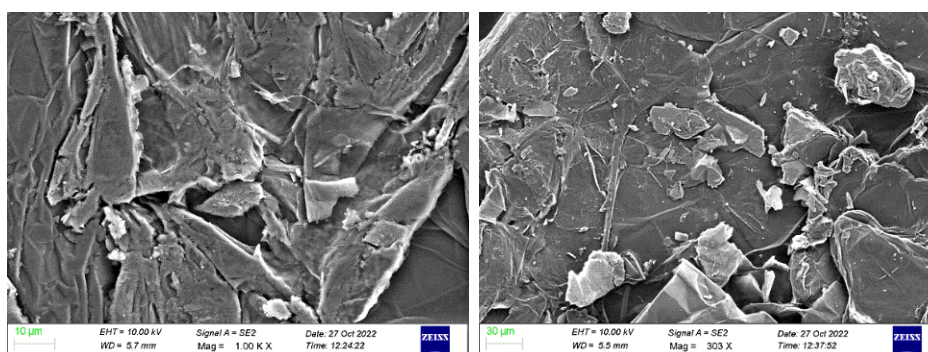


Figure 5.6: HR-SEM results of Graphene Oxide

The synthesized GO nanosheets were asymmetric patterns as observed in Figure 5.6. The GO nanosheets were observed to be in the form of sheets. The grain size and surface morphology were observed by the high resolution scanning electron microscope HR-SEM images of the GO have well defined and interlinked three-dimensional graphene sheets, forming a porous network.

HR-TEM:

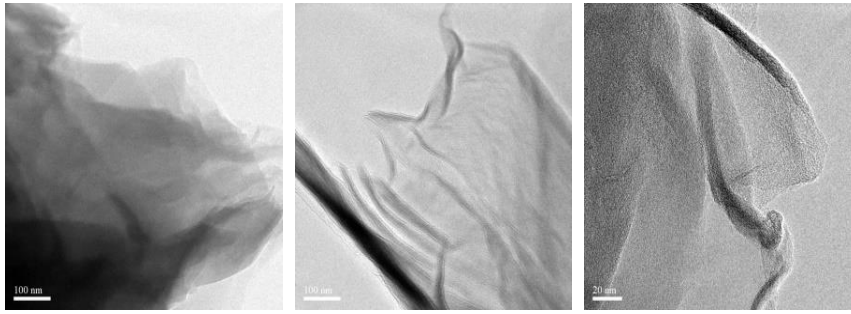


Figure 5.7: HR-TEM results of Graphene Oxide

The High-resolution Transmission electron microscopy enables the imaging of a sample's atomic-scale crystallographic structure. It is a useful instrument for researching the nanoscale characteristics of crystalline materials because of its great resolution. The results have been shown in Figure 5.7. The wrinkles in the HR-TEM suggest the formation of graphene oxide sheets.

5.1.2. HYDROXYAPATITE:

FTIR:

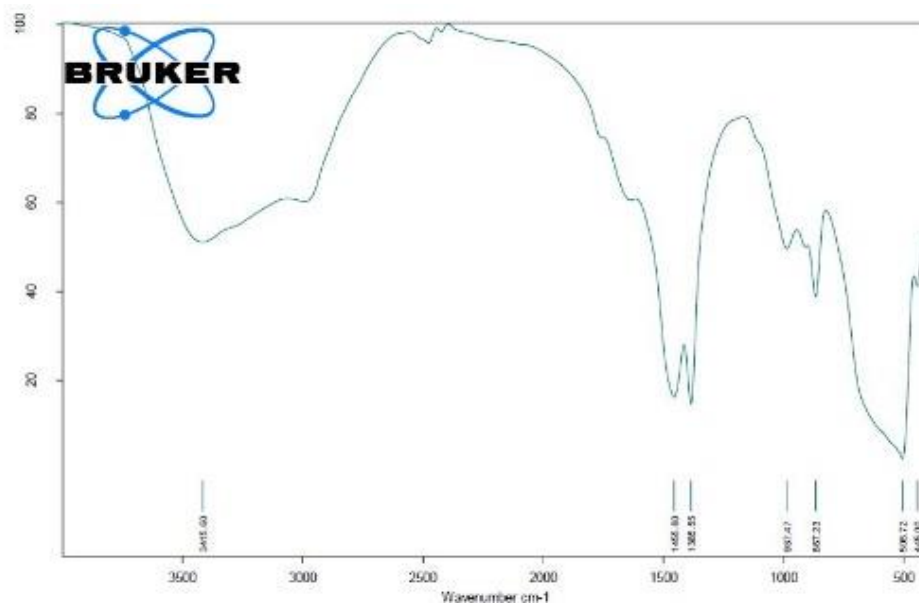


Figure 5.8: FTIR results of Hydroxyapatite

FTIR is used in the characterization of unknown materials. The band obtained at 560 cm⁻¹ C-I stretching (triply degenerated pending mode). The band at 951 cm⁻¹ (C=C) indicates the non-degenerated symmetric stretching vibration mode. The band at 1150 cm⁻¹ (C-O) triply degenerated asymmetric stretching vibration mode. On the other hand, OH⁻ of HAp has stretching vibration modes (3770 cm⁻¹) as seen in Figure 5.8.

RAMAN SPECTRA:

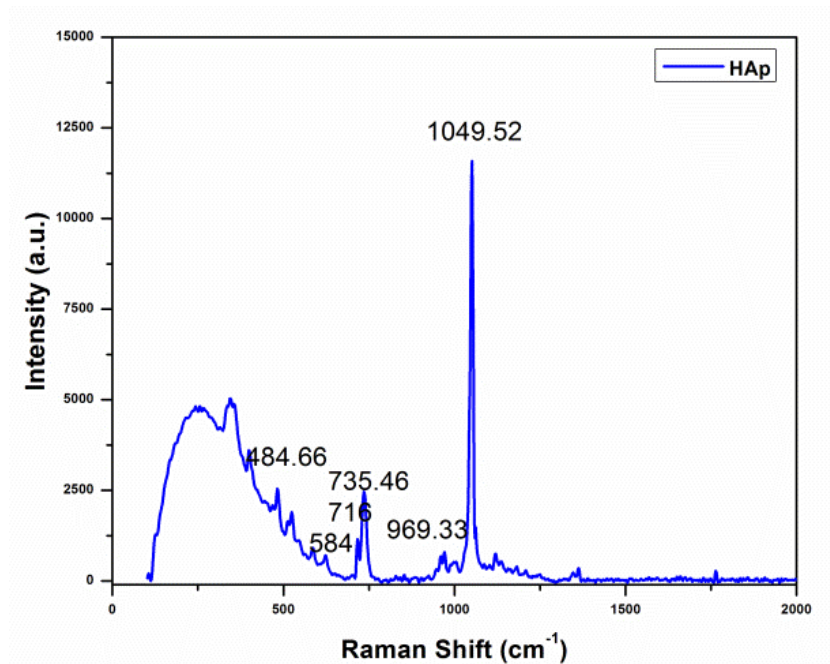


Figure 5.9: Raman Spectroscopy results of Hydroxyapatite

The Raman spectrum showed strong band at 484.66 cm^{-1} corresponding to O-P-O doubly degenerated bending mode ν_2 . The band at 584 cm^{-1} corresponds to the O-P-O triply degenerated bending mode, ν_4 . The peak observed at 969.33 cm^{-1} indicates the P-O non-degenerated symmetric stretching mode, ν_1 ; and the intense band at 1049.52 cm^{-1} is indicative of the P-O triply degenerated antisymmetric stretching mode, ν_3 as observed in Figure 5.9.

ZETA POTENTIAL:

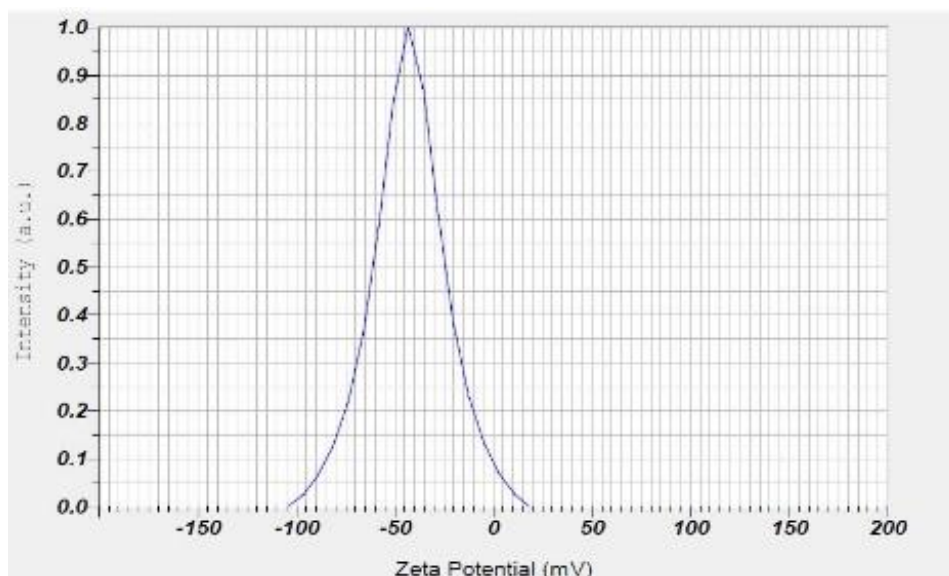


Figure 5.10: Zeta Potential results of Hydroxyapatite

The zeta potential of HAp as observed in Figure 5.10 was found to be -46.6mV, in which the negative sign is attributed to the negatively charged compounds in HAp that enhance the stability.

PARTICLE SIZE:

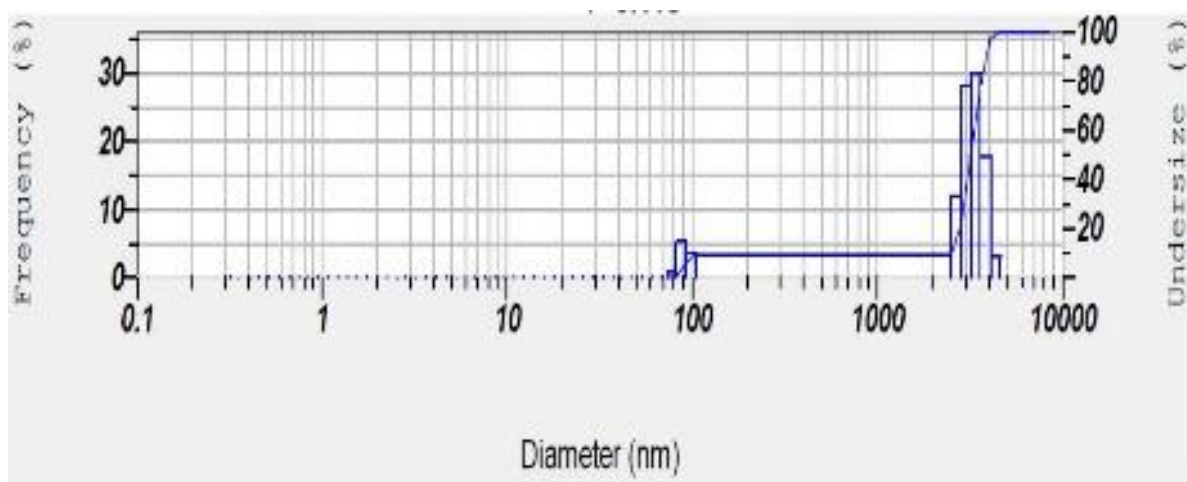


Figure 5.11: Particle size results of Hydroxyapatite

The HAp average size is 3547 nm as per Figure 5.11. The larger particle size can be justified by the usage of water as the dispersion medium, which might have led to the aggregation of the nanoparticles.

HR-SEM:

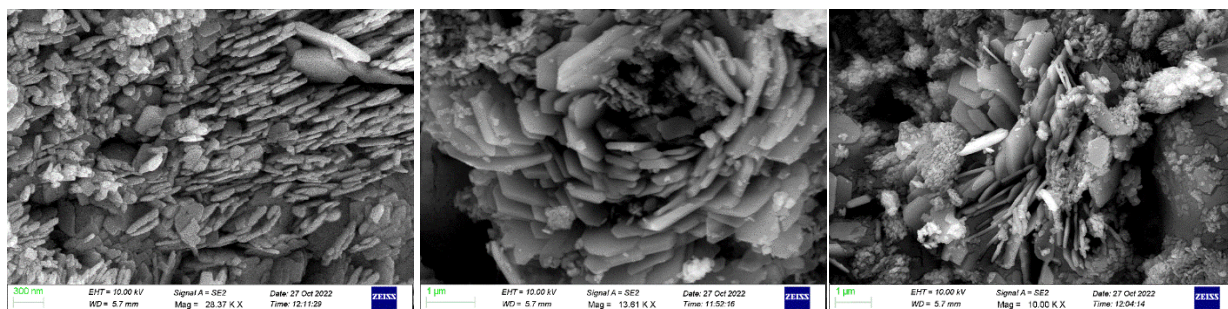


Figure 5.12: HR-SEM results of Hydroxyapatite

The particles with specific geometrics are observed in Figure 5.12, with rectangular and square shape being the most dominant. A good shape control has been achieved as uniform sized HAp are seen in the electron micrographs with the exception of a very few particles of anisotropic nature. Hexagonal particles are more commonly observed during the synthesis of HAp.

5.1.3. FABRICATED IMPLANTS:

5.1.3.1. UNCOATED IMPLANTS:

FESEM and ELEMENTAL MAPPING:

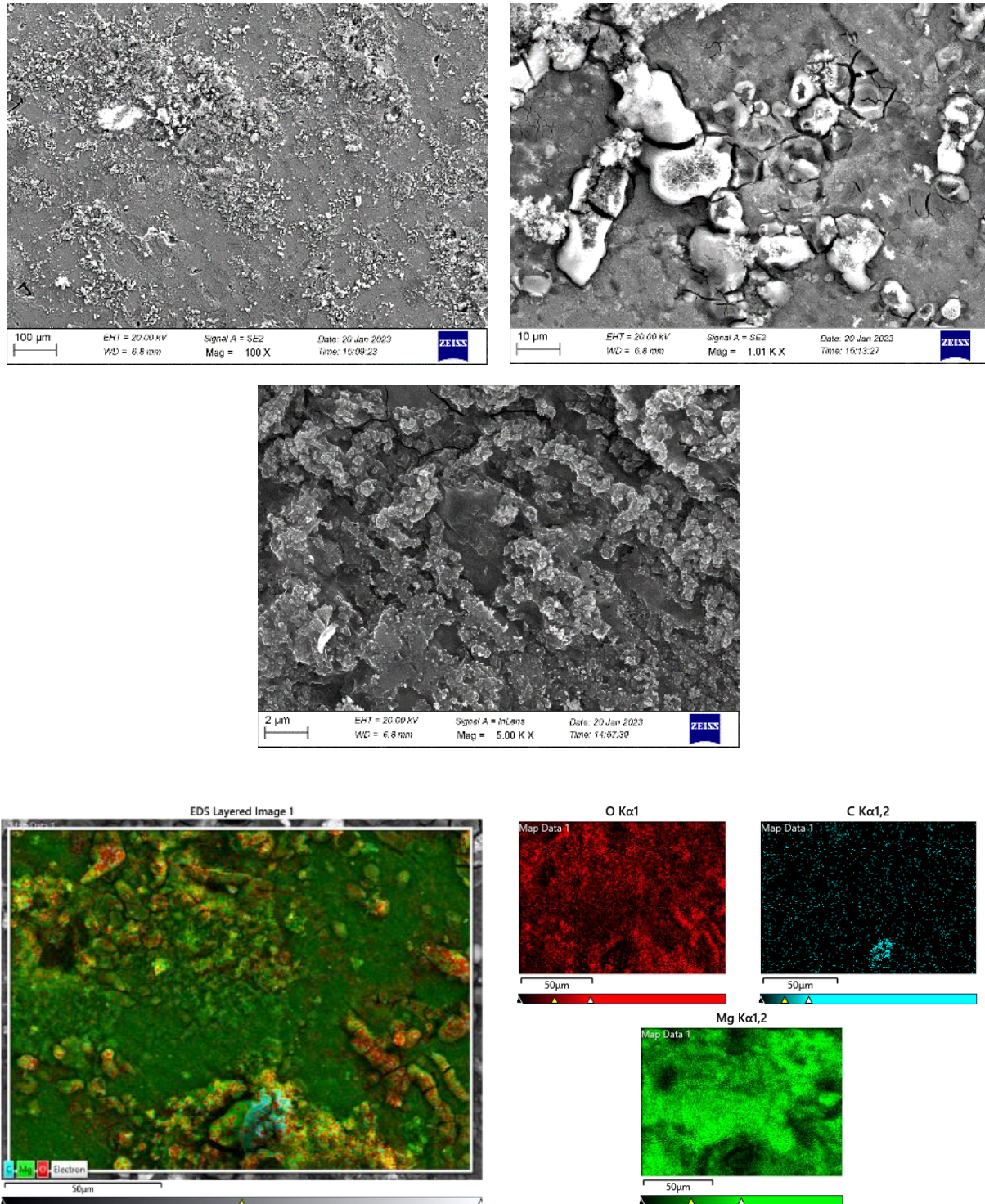


Figure 5.13: FESEM-ELEMENTAL MAPPING results of Uncoated implants

The foundation of elemental mapping is the collection of incredibly precise elemental composition data over a sample's surface. Usually, a SEM or TEM is used for this, along with EDS analysis (Newbury and Ritchie, 2013). Along with the EDS data, a high resolution picture of the region of interest is gathered, and the two are then correlated. A homogenous dispersion of all the incorporated components (Graphene oxide, Hydroxyapatite, Zinc, Magnesium) were observed as in Figure 5.13. A reasonable distribution is visible meaning that the sintering process has managed to break down the large particles and clusters. Due to surface alteration during the production of the material, the elemental mapping examination of the matrix revealed a mostly Mg phase with traces of Oxygen. The predominant Ca, O, and P peaks in the spectrum provide quantitative evidence that the grain boundary pinning mechanism responsible for grain refinement is supported by the fact that the mg composites have settled at the composites' grain boundaries. The EDS also verifies that there are no impurities or composites from the secondary phase.

XRD:

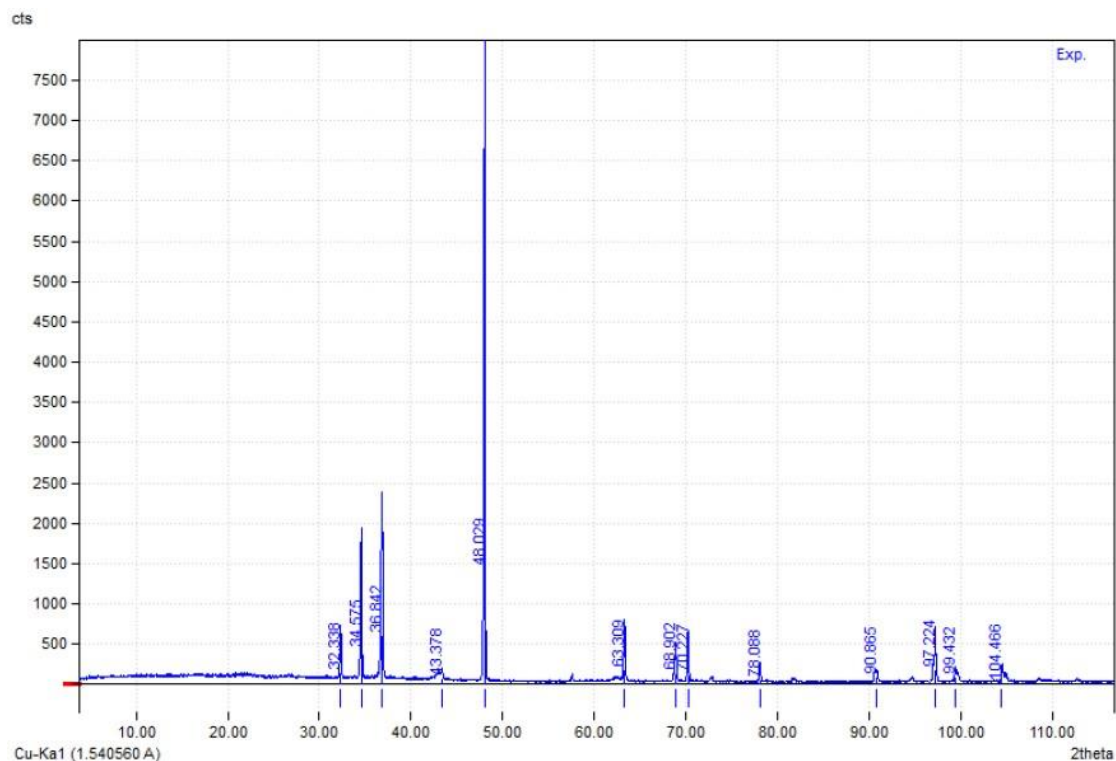


Figure 5.14: X-RD results of Uncoated implants

The obtained $2\theta = 48.029$ degrees from Figure 5.14, corresponds to the crystalline structure of the synthesized implants.

RAMAN SPECTRA:

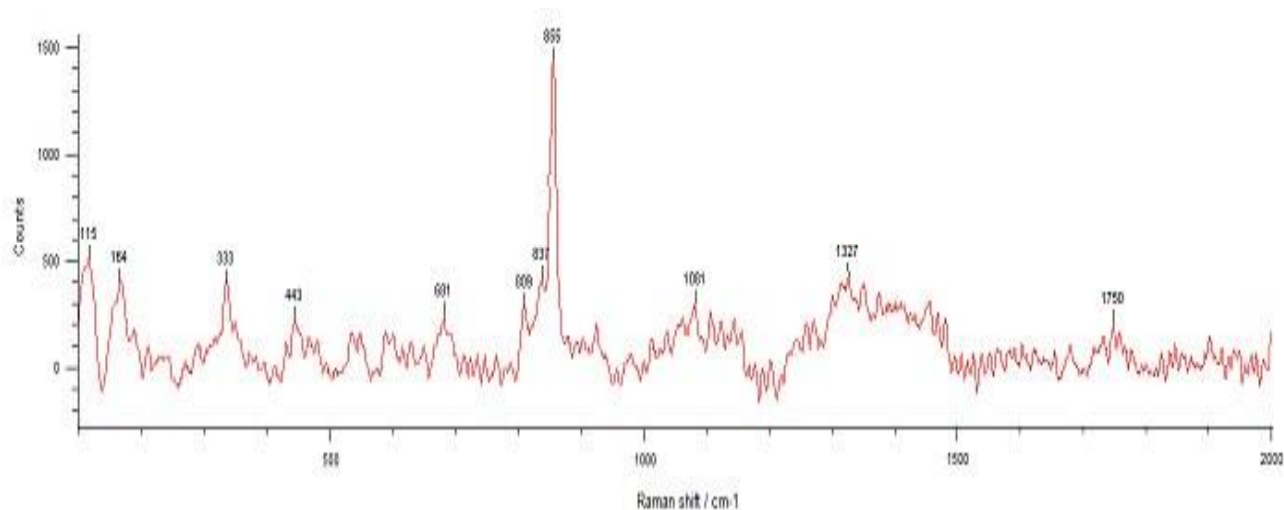


Figure 5.15: Raman Spectroscopy results of Uncoated implants

The peaks at 809cm^{-1} , 837cm^{-1} , 855cm^{-1} , and 1061cm^{-1} from the graph indicated in Figure 5.15, show the presence of carbon-carbon double bonds.

5.1.3.2. COATED IMPLANTS:

FESEM-ELEMENTAL MAPPING:

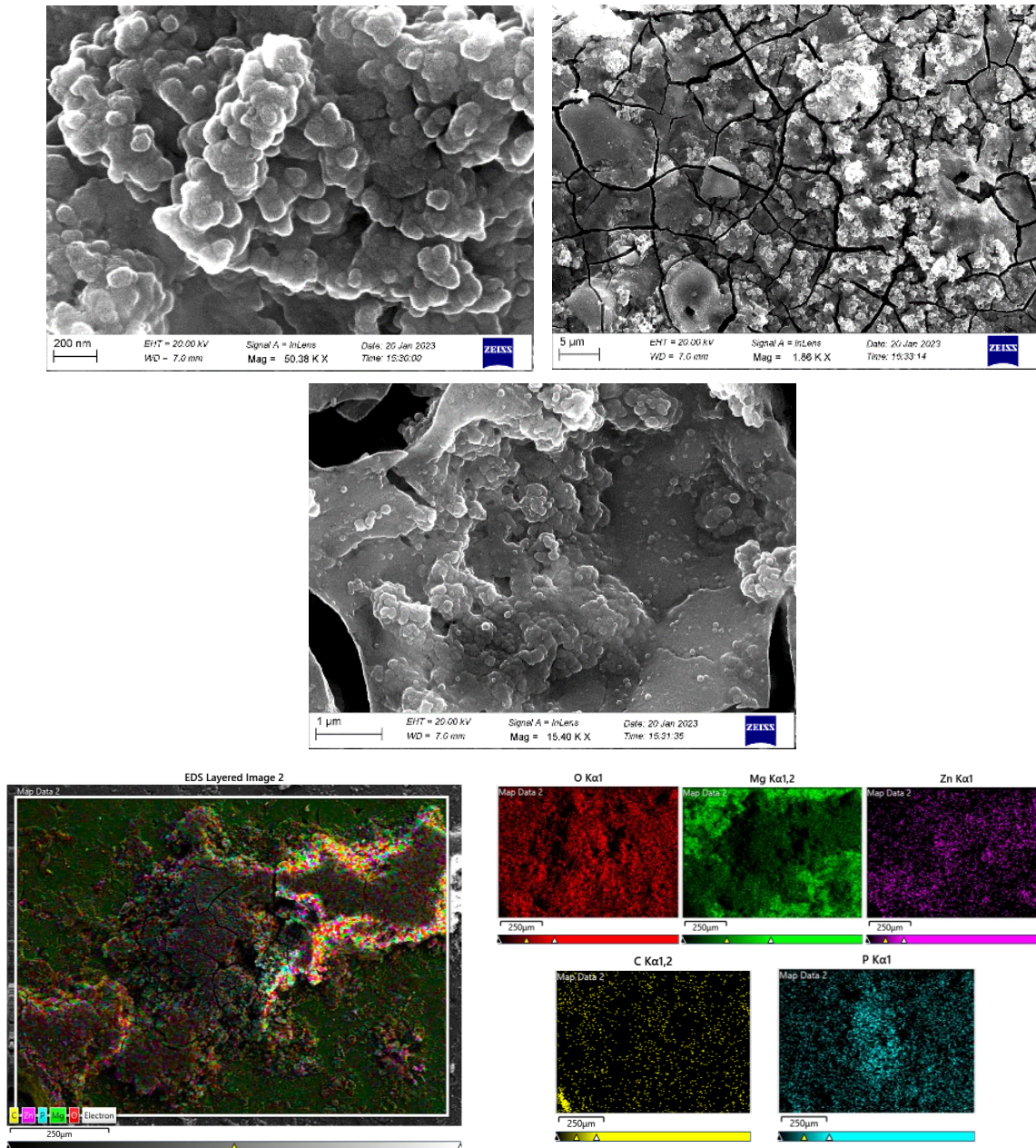


Figure 5.16: FESEM-ELEMENTAL MAPPING results of Coated implants

The SEM images (Figure 5.16) confirm that the pectin coating has been incorporated uniformly on the surfaces of the synthesized implants. The pectin covering on the magnesium implant and the underlying magnesium substrate are both confirmed by the elemental analysis to be present. While the presence of carbon indicates a good coating procedure, the presence of oxygen in both the pectin coating and the magnesium substrate reveals a robust adhesion between the two layers. The

study can aid in coating process optimisation and enhance the implant's functionality in biomedical applications.

XRD:

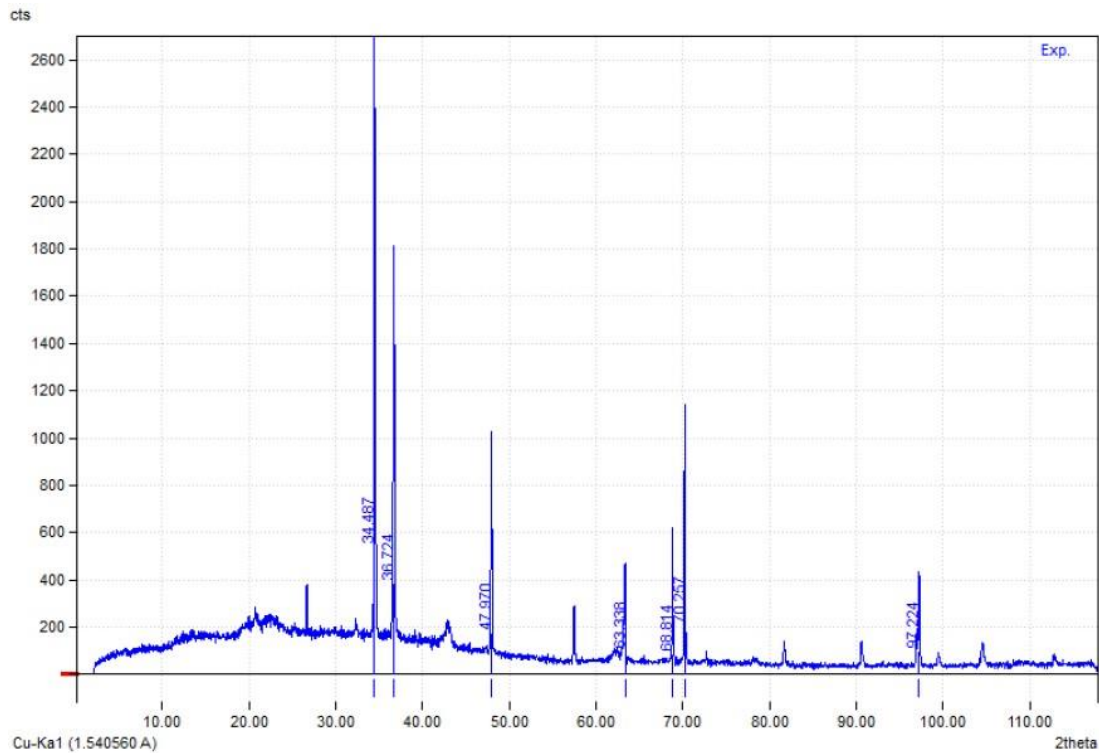


Figure 5.17: X-RD results of Coated implants

The obtained $2\theta = 34.487$ degrees (Figure 5.17) corresponds to the crystalline structure of the coated implants.

RAMAN-SPECTRA:

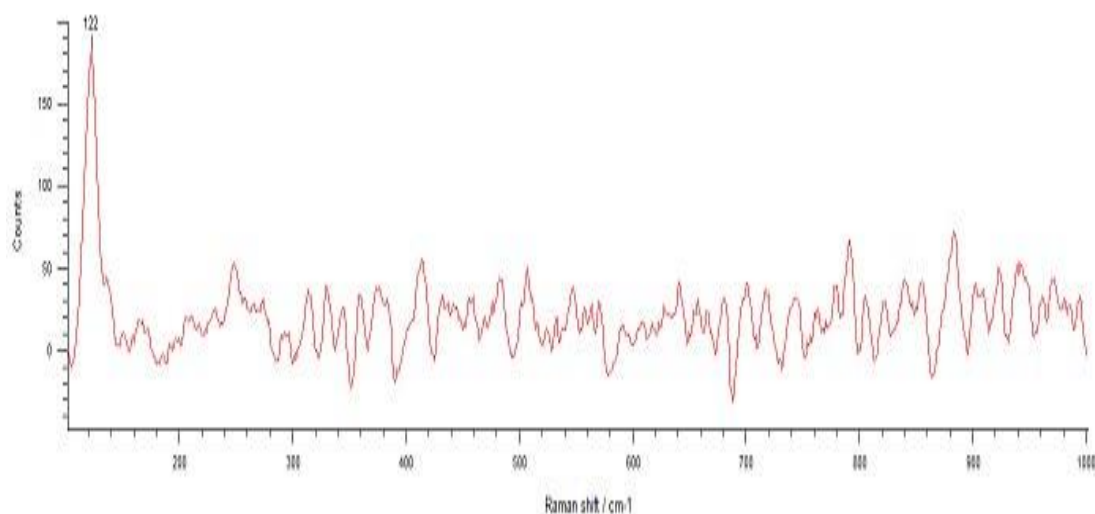


Figure 5.18: Raman spectroscopy results of Coated implants

Typically, a peak at 122 cm^{-1} represents the stretching mode of a C-N bond or the bending mode of a C-C bond. Hence the peak in Figure 5.18, indicates the presence of a polymer.

5.2. OPTICAL MICROSCOPIC IMAGES:

5.2.1. UNSINTERED IMPLANTS:

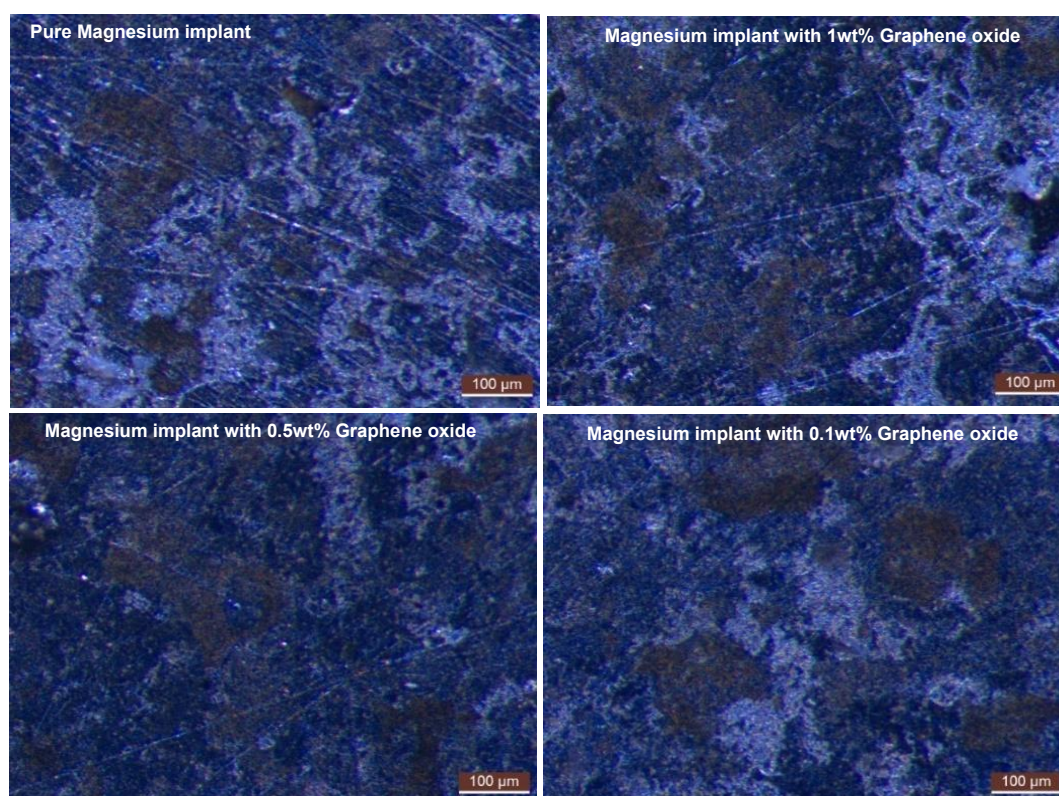


Figure 5.19: Optical microscopic images of unsintered implants

An optical microscope, commonly referred to as a light microscope, makes use of one or more lenses to magnify visible light pictures of tiny materials. The lenses are positioned between the sample and the viewer's eye to enlarge the picture and enable closer examination. The implants tend to appear irregularly shaped and there are a lot of prominently visible gaps as seen in Figure 5.19.

5.2.2. SINTERED IMPLANTS:

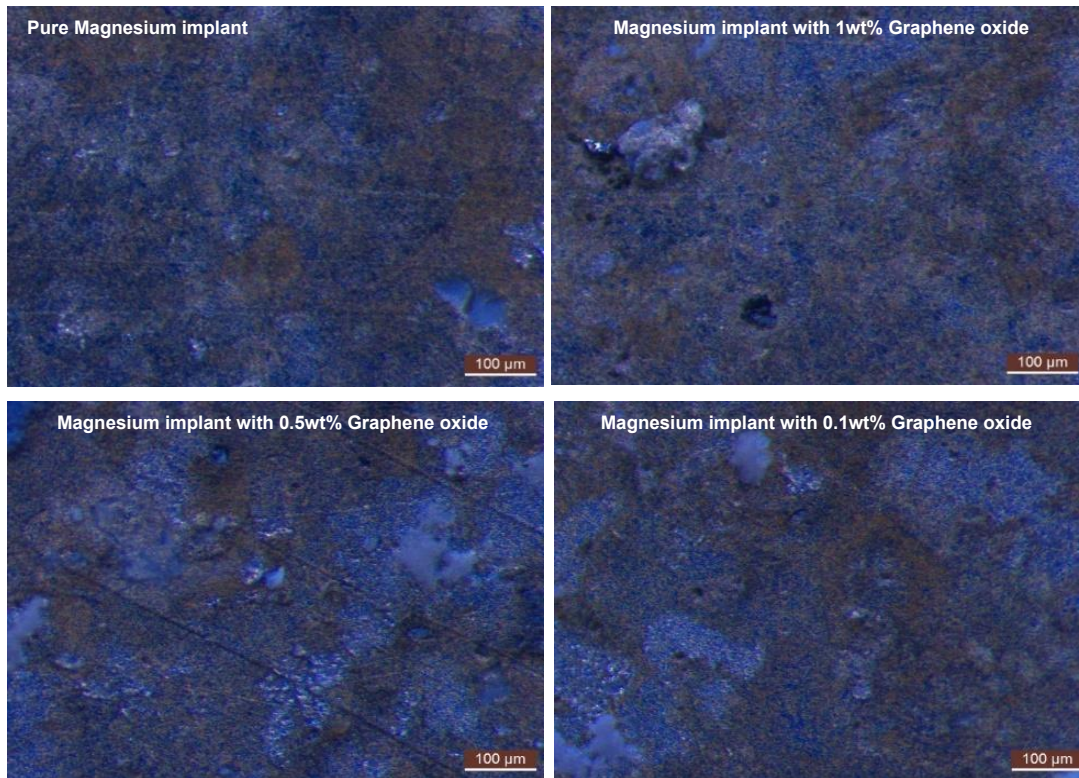


Figure 5.20: Optical microscopic images of sintered implants

The appearance of the fabricated implant after sintering drastically becomes smoother and the number of gaps significantly reduces and a uniform dispersion is visible as seen in Figure 5.20.

5.3. CONTACT ANGLE MEASUREMENT:

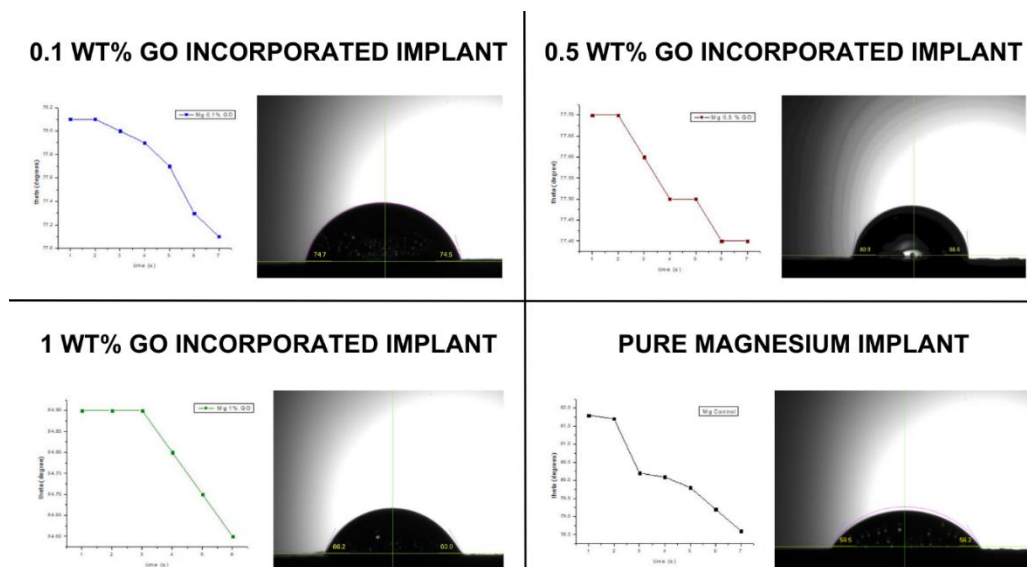


Figure: 5.21: Water contact angle measurement of Fabricated implants

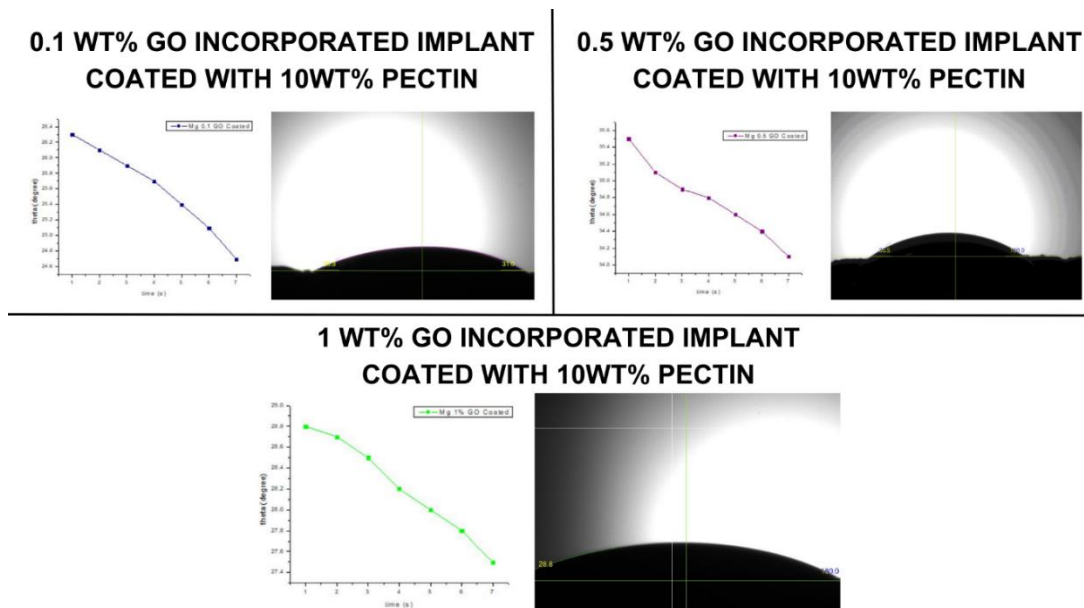


Figure: 5.22: Water contact angle measurement of pectin coated implants

The water contact angles (Figure 5.21, 5.22) of both the pectin coated and uncoated implants were measured. Any value greater than 90° is considered to be hydrophobic and a value below is considered to be hydrophilic (Latthe *et al.*, 2014). It was found that the implants made of 0.1wt% and 0.5wt% GO were slightly hydrophilic, and the implant made of 0.1 wt% GO was highly hydrophilic. On the other hand, the implant made of pure Magnesium showed the most hydrophilicity. All the pectin coated implants showed contact angles between the range of 25° to 35° , indicating the favourable property of high hydrophilicity, when compared to the uncoated implants.

5.4. LEACHING ASSAY:

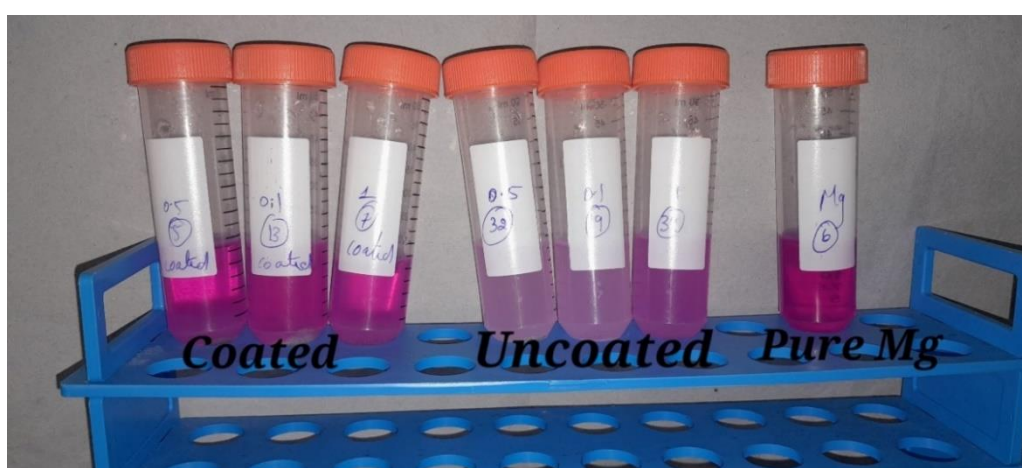


Figure 5.23: Leaching assay setup after 2 weeks

Visible change in the colour of solution containing coated and uncoated implants was noted after 2 weeks indicating the durability of implants coated with 10wt% pectin evidently seen in Figure 5.23.

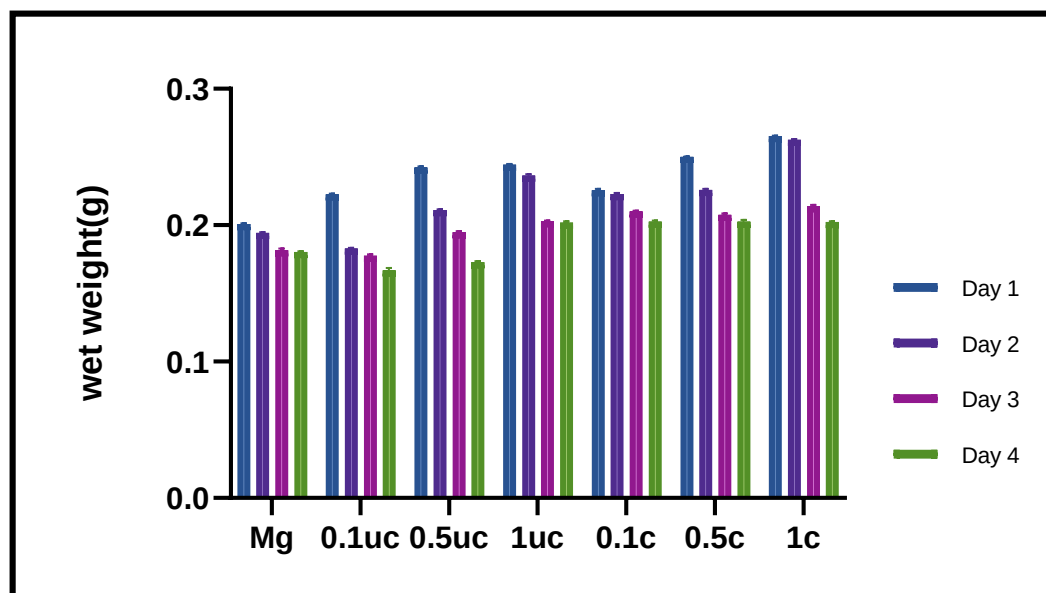


Figure 5.24: Leaching Assay (Plot between wet weights and days)

A plot between the days and wet weights (Figure 5.24) indicated that the leaching rates of implants with 1 wt % Graphene oxide without coating, 0.5wt% and 1wt% Graphene oxide, which were coated with 10wt% pectin, are considerably less compared to the implants made with pure Magnesium.

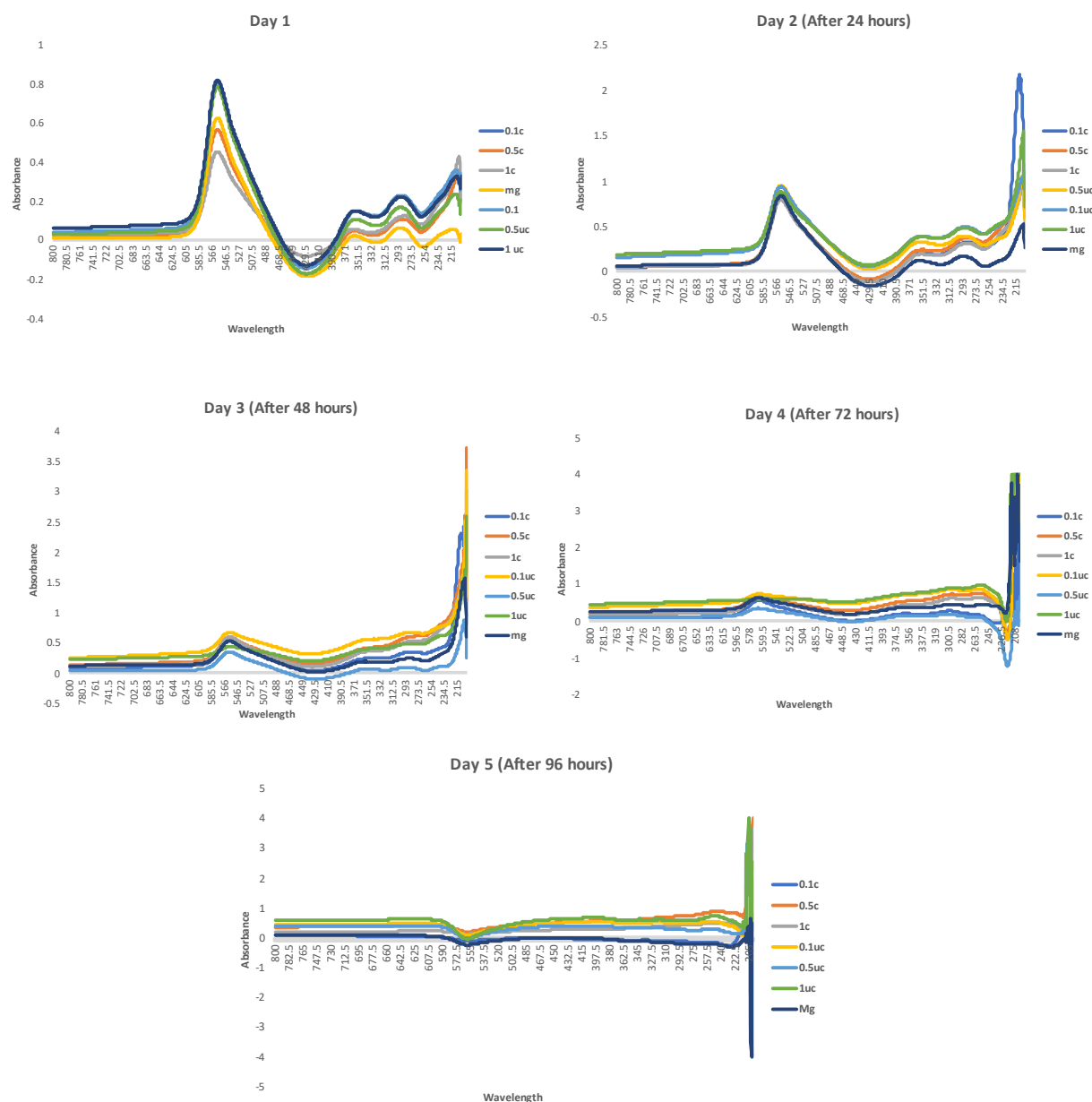


Figure 5.25: Leaching Assay (Plot between Absorbance and Wavelength)

The plot between the wavelength and absorbance (Figure 5.25) confirms the results obtained from the wet weight measurements.

5.5. ANTIBACTERIAL ACTIVITY

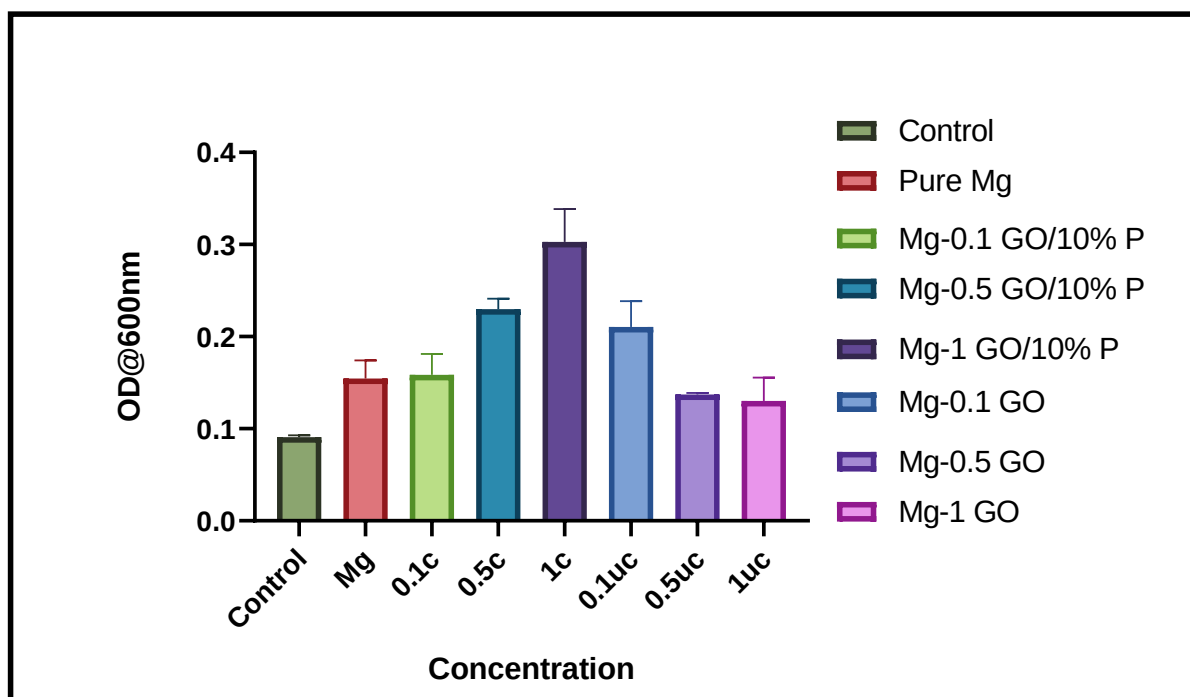


Figure 5.26: Antibacterial activity on *E. coli*

Gram negative bacteria: Implants made of 1 wt % Graphene oxide, showed a considerable amount of antibacterial activity (Figure 5.26), compared to other samples.

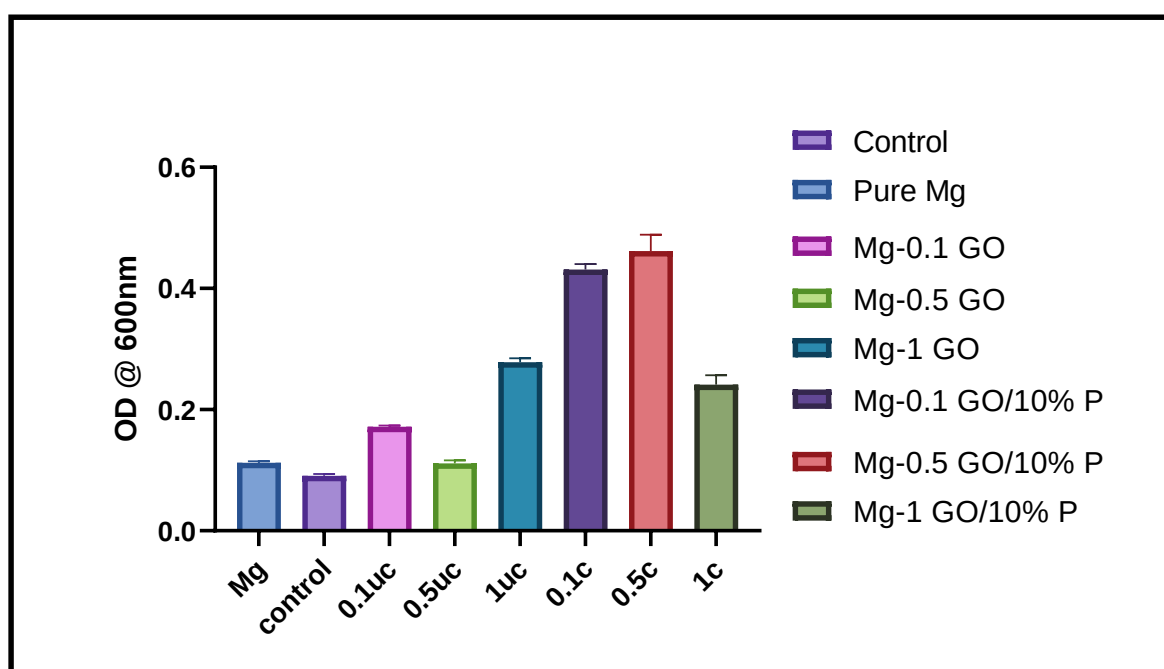


Figure 5.27: Antibacterial activity on *S. aureus*

Gram positive bacteria: Implants made of 0.5 wt % Graphene oxide, showed a considerable amount of antibacterial activity, compared to other samples as seen in Figure 5.27.

5.6. HEMOLYSIS ASSAY:

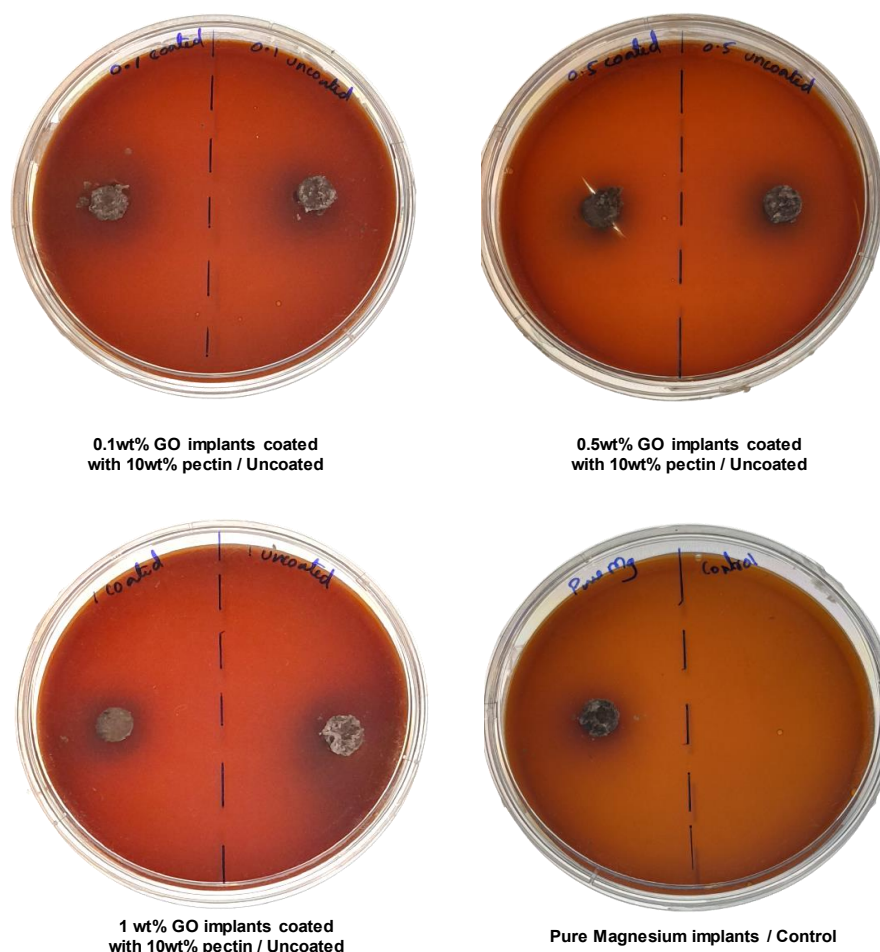


Figure 5.28: Hemolysis assay results

α hemolysis was observed in all the plates treated with implants made of three different concentrations of Graphene Oxide (0.1wt%, 0.5wt%, and 1wt%) coated with 10 wt% pectin, as observed in Figure 5.28, indicating the partial destruction of red blood cells, caused by the release of Hydrogen peroxide from the implants, thus oxidizing the Hemoglobin present in blood (Buxton, 2016). Gas bubbles may occur in the circulation as a result of this, which may result in embolisms or blood vessel blockages. H_2O_2 can also kill or harm red blood cells, which can result in hemolysis. Numerous symptoms, such as anaemia, jaundice, and weariness, may come from this. H_2O_2 may also harm other blood cells, including platelets and white

blood cells, which might reduce the body's capacity to clot and fight infections (Ghabach and Davarpanah, 2020). Oxidization was noted in all the implants except for implants made of 1wt% Graphene Oxide coated with 10 wt % pectin.

CHAPTER-6

6. CONCLUSION AND SUMMARY

In summary, *in vitro* biocompatibility investigations on the utilisation of graphene oxide integrated magnesium implants for bone regeneration have yielded encouraging findings. The mechanical and biological qualities of the magnesium implants have been discovered to be improved by the addition of graphene oxide, Zinc, HAp and Pectin coating, making them more appropriate for use in bone regeneration applications. Furthermore, the positive hemocompatibility, antimicrobial activity, and durability seen in *in vitro* investigations validated the biocompatibility of these implants. These results imply that the proposed novel implant has a good potential to replace conventional implants in bone regeneration applications. To validate these results and assess the long-term effectiveness and safety of these implants, more *in vivo* research is required.

7. REFERENCES:

1. Abazari, S., Shamsipur, A. and Bakhsheshi-Rad, H. R. (2021) 'Reduced graphene oxide (RGO) reinforced Mg biocomposites for use as orthopedic applications: Mechanical properties, cytocompatibility and antibacterial activity', *Journal of Magnesium and Alloys*. doi: 10.1016/j.jma.2021.09.016.
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