CHAPTER 7

A: Preparation of Glycolic Acid-g-Chitosan-Co$_3$O$_4$-Fe$_3$O$_4$ Hybrid Nanoparticles Based Nanohybrid Scaffolds for Drug-Delivery and Tissue Engineering
7.1 INTRODUCTION

In the field of nanotechnology, polymer matrix based nanocomposites have become a prominent area of current research and development. These materials exhibit unique optical\(^1\), thermal, electrical and mechanical properties due to the interaction of the polymer with the particle and state of dispersion\(^2\)-\(^4\). Transition metal nanoparticles are one of the most–studied system due to their quantum size effects\(^5\)-\(^7\), novel electronic\(^8\), optical\(^9\), magnetic\(^10\) and chemical properties. These metal nanoparticles play an important role in many different fields of science such as nano-electronics, catalysis\(^11\)-\(^13\) and recently, in biomedical application\(^14\)-\(^16\). Cobalt oxide and Fe\(_3\)O\(_4\) nanoparticles are currently attracting enormous interest owing to their unique size- and shape dependent properties and potential applications in the field of catalysis, sensors, electrochemistry, magnetism, energy storage, etc\(^17\). Here, we have demonstrated Co\(_3\)O\(_4\)-Fe\(_3\)O\(_4\) composite magnetic nanoparticles based materials can be use in the field of controlled drug release and cell proliferation systems, which is having major scientific application in the field of biomaterials\(^18\).

A wide range of materials have been employed as drug carriers such as lipids, surfactant, dendrimers and natural or synthetic polymers\(^19\)-\(^22\). Chitosan has prompted the continuous movement for the development of safe and effective drug delivery systems because of its unique physicochemical and biological characteristics. It is polycationic biopolymer\(^23\). Chitosan is hydrophilic and compatible with nanoparticle and has better processability due to the presence of amino group (pKa value is 6.2) in the chain. Chemical modification of chitosan is useful for the association of bioactive molecules to polymer and controlling the drug release profile. The grafting of side glycolic acid leads to marked changes in the chitosan structure\(^24\),\(^25\).
7.2 EXPERIMENTAL

7.2.1 Materials

Chitosan of low molecular weight (Mw = $1.5 \times 10^5$, degree of deacetylation was 85%), glycolic acid (99% pure), iron (0) pentacarbonyl (Fe(CO)$_5$), cobalt acetate (Co(OAc)$_2$), citric acid (C$_6$H$_8$O$_7$) was obtained from sigma Aldrich. Lithium chloride (LiCl), Tri phenyl phosphate (TPP), pyridine (Py), Sodium hydroxide (NaOH) and phenyl ether was obtained from M/s Sisco Research Laboratories, Mumbai. Deionised water was used throughout the work, which is prepared by Milli-Q-system.

7.2.2 Synthesis of Co$_3$O$_4$-Fe$_3$O$_4$ hybrid nanoparticles (CFNP)

Cobalt oxide nanoparticles (CoNP, synthesis is given in chapter 6 section 6.2.3), 1-octadecene, OAM and OA were heated to 120 $^\circ$C under argon atmosphere. At the temperature of 120 $^\circ$C, Fe(CO)$_5$ was injected to the reaction mixture. The reaction mixture was slowly heated to reflux (1 $^\circ$C min$^{-1}$) for 4.5 h. After completion of reaction, it is cooled to room temperature and stirred for 1 h, followed by precipitation with acetone. The precipitate was then dried in air (Scheme 7.1).

![Scheme 7.1 Schematic illustration of synthesis of Co$_3$O$_4$/Fe$_3$O$_4$ hybrid nanoparticles](image-url)
7.2.3 Preparation of nanohybrid scaffolds and drug loading

Glycolic acid grafted chitosan (1g) (Discussed in chapter 3B. section 3.2.2.3) was dispersed in deionised water (50 ml) and stirred for 1 h at room temperature. After 1 h, Co$_3$O$_4$-Fe$_3$O$_4$ composite magnetic nanoparticles (50 mg) was added to the solution and stirred overnight at room temperature. The resulting solution was heated up to 80 $^0$C with continuous degassing for 30 min. The resulting solution was cooled to room temperature after degassing. The drug (CPA) (10 mg) was added to the resulting solution and stirred for 5 h, so that drug completely mixes with the solution. The drug loaded solution was poured in tissue culture plates (20×20 mm diameter) and quenched in liquid nitrogen. The quenched sample was freeze dried by lyophilisation under -100 $^0$C temperature for 6 h. In lyophilisation water molecules were removed by freezing and sublimation of ice crystals, which lead to the formation of pores. The formulation is shown in the Table 7.1.

Table 7.1 Formulation of Cyclophosphamid (CPA)-loaded nanohybrid of chitosan-g-glycolic acid and Co$_3$O$_4$-Fe$_3$O$_4$ hybrid magnetic nanoparticles

<table>
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<tr>
<th>S.No</th>
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<th>Co$_3$O$_4$-Fe$_3$O$_4$ (mg)</th>
<th>CPA (%)</th>
<th>Drying Process</th>
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<td>CGCF-1</td>
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<tr>
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<td>CGCF-2</td>
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<tr>
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<td>1</td>
<td>50</td>
<td>_</td>
<td>Freeze</td>
<td>CGCF (S)</td>
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<tr>
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<td>1</td>
<td>50</td>
<td>10</td>
<td>Freeze</td>
<td>CGCF (D)</td>
</tr>
</tbody>
</table>
7.3 CHARACTERIZATIONS OF NANOHYBRID

7.3.1 Transmission Electron Microscopy (TEM)

High Resolution Transmission Electron Microscopy (HR-TEM model Technai TF30, 300KV FEG) was used to analyse the particle size, morphology and Selected Area Diffraction pattern (SAED) of Co$_3$O$_4$-Fe$_3$O$_4$ hybrid magnetic nanoparticles.

7.3.2 Physical property measuring system (PPMS)

The formation of Co$_3$O$_4$-Fe$_3$O$_4$ hybrid nanoparticle was confirmed by measuring hysteresis loops of the synthesised nanoparticles using a physical property measuring system (PPMS) (quantum design Inc. San Diego, USA) equipped with 7T superconducting magnet and a vibrating sample magnetometer.

7.3.3 Fourier transform infrared spectroscopy

Attenuated total reflectance Fourier transform infrared (ATR-FTIR) Nicolet Nexus 870 FTIR spectrometer equipped with a smart Endurance diamond accessory (64 scans, 4 cm$^{-1}$ resolution, wave number range 4000-550 cm$^{-1}$) was used to analyse fourier transform infrared spectra of neat chitosan (CTS), chitosan grafted glycolic acid (CGCF-1), nanohybrid scaffold (CGCF (D)) and drug (CPA).

7.3.4 X-ray photoelectron spectroscopy (XPS)

XRD patterns of the samples were recorded on X-ray Diffractometer (WAXRD – Rigaku (Japan)) with Cu-$\alpha$ radiation at a voltage of 50 KV. The scanning rate was 4$^0$/min and the scanning scope of 20 was from 2$^0$ to 80$^0$ at room temperature.

7.3.5 Scanning Electron Microscopy (SEM)

Scanning Electron Microscopy (SEM) (Model, JOEL Stereoscan 440, Cambridge) was used to investigate the surface morphology of the porous scaffolds. Prior to the observation, specimens were fixed on the copper grid.
7.3.6 Swelling behavior

The swelling behavior of porous scaffold was determined by exposing them to media of different pH, 1N HCl, 1N NaOH and simulated body fluid (SBF) (pH 7.4) solutions. The shape retention of porous scaffold was determined by measuring the change in its diameter of scaffold as a function of time in the media. The drug “CPA” content in the aliquot was investigated by UV-vis spectrophotometer (UV-NIR- PL Lamda 950) at 180 nm.

7.3.7 Cell viability study

In vitro cell culture was carried out using L929 cell. These cells are derived from an immortalized mouse fibroblast cell line, are internationally recognized cells that are routinely used in in-vitro cytotoxicity assessments. The scaffold was sterilised by putting it in 6 well tissue culture plate containing isopropanol (5 mL) and exposed to UV radiation for 4 h. L929 cells were further seeded on nanohybrid scaffold placed in 6-well plate at a density of 5×10³ cells/well and incubated at 37 °C, 5% CO₂ and 95% humidity incubation conditions. The tissue culture plate containing only cells were used as control. To study the cell proliferation on different substrates, cell proliferation was determined by the colorimetric MTT assay. MTT assay is based on the reduction of yellow 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) salt in MTT to form purple formazan by dehydrogenase enzymes secreted from the mitochondria of metabolically active cells. The amount of formazan formed is directly proportional to the number of viable cells. After 2 h, 4 h, 6 h, 24 h, 48 h, 72 h, the cells solution (100 µl ) was transferred to an ELISA micro-plate and optical density (OD) was measured at 540 nm using the spectroscopic method. The relative cell growth was compared to control cell, which exhibit cell culture medium without chitosan. It was calculated by using the given eq. (1)

\[
\% \text{Live cell} = 100 - \left[ \frac{(C - T)}{(C - B)} \times 100 \right]
\]
C = OD of control
T = OD of test sample
B = OD of blank
OD = Optical density

All the in vitro tests were done in triplicate and results were reported as an average value.

7.4 RESULTS AND DISCUSSION

**Scheme 7.2** Grafting of glycolic acid on chitosan, formation of Chitosan-g-glycolic acid and $\text{Co}_3\text{O}_4\text{-Fe}_3\text{O}_4$ hybrid nanoparticles based nanohybrid scaffold and interaction between chitosan-g-glycolic acid, drug and $\text{Co}_3\text{O}_4\text{-Fe}_3\text{O}_4$ hybrid nanoparticles.

7.4.1 TEM analysis

The TEM image of $\text{Co}_3\text{O}_4\text{-Fe}_3\text{O}_4$ composite magnetic nanoparticles (Figure 7.1 (a)) exhibit uniformly spherical morphology almost same overall size. Figure 7.1 (b) shows the high
resolution TEM image of these composite magnetic nanoparticles, which are crystalline as shown in selected area diffraction (SAED) pattern Figure 7.1 (c). The distance between the two adjacent lattice planes in Co$_3$O$_4$ domain is 2.31 Å, close with the reported value of 2.33 Å for (2 2 2) plane$^{28}$ and that in Fe$_3$O$_4$ domains 4.85 Å, close to the literature value of 4.88 Å for (1 1 1) plane. TEM-EDAX also confirms the formation of Co$_3$O$_4$-Fe$_3$O$_4$ hybrid nanoparticles (Figure 7.1 (d)).

Figure 7.1 (a) TEM image Co$_3$O$_4$-Fe$_3$O$_4$ hybrid nanoparticles; (b) HRTEM image of Co$_3$O$_4$-Fe$_3$O$_4$ hybrid nanoparticles (White line delineate distance between two lattice plane in Co$_3$O$_4$ domain and Fe$_3$O$_4$ domain); (c) SAED pattern of Co$_3$O$_4$-Fe$_3$O$_4$ hybrid nanoparticles; (d) TEM-EDAX of Co$_3$O$_4$-Fe$_3$O$_4$ hybrid nanoparticles.
7.4.2 Magnetization study

The magnetic properties of the hybrid nanoparticle were investigated to evaluate the influence of the diamagnetic Co$_3$O$_4$ on the Fe$_3$O$_4$ domains. Figure 7.2 shows magnetic hysteresis loops recorded at 300 k of Co$_3$O$_4$-Fe$_3$O$_4$ hybrid nanoparticle with Fe$_3$O$_4$ nanoparticle of size 5-10nm. Hybrid nanoparticles are super paramagnetic, however the saturation magnetization increases with Co$_3$O$_4$ particles. The decrease in the magnetization of Co$_3$O$_4$-Fe$_3$O$_4$ hybrid nanoparticle confirms the formation of Co$_3$O$_4$-Fe$_3$O$_4$ hybrid nanoparticles.

![Figure 7.2](image)

**Figure 7.2** Magnetic hysteresis curve recorded at 300 k of Co$_3$O$_4$-Fe$_3$O$_4$ hybrid nanoparticle (CoFNP) with Fe$_3$O$_4$ nanoparticles (FeONP).

7.4.3 X-ray photoelectron spectroscopic (XPS) analysis

Figure 7.3 shows the electron binding energy of Co$_{2p}$ measured from XPS for 10nm CoNP and 10-15 nm CoFNP. Co$^{3+}$ in CoNP exhibit binding energy at 779.8eV$^{25}$. Whereas, Co$_3$O$_4$-
Fe₃O₄ hybrid nanoparticles exhibit ~5.4 eV increase in binding energy. The change in binding energy is likely due to the transfer of electron from Fe₃O₄ to Co³⁺ of Co₃O₄ nanoparticle. The shift in the peak of binding energy indicates the formation of Co₃O₄-Fe₃O₄ hybrid nanoparticles.

![XPS spectra of 5nm Co₃O₄ nanoparticles and 5-10nm Co₃O₄-Fe₃O₄ hybrid nanoparticles.](image)

**Figure 7.3** XPS spectra of 5nm Co₃O₄ nanoparticles and 5-10nm Co₃O₄-Fe₃O₄ hybrid nanoparticles.

### 7.4.4 FTIR analysis

Fourier transform infrared (FT-IR) spectra reveals information about the structure of neat chitosan (CTS), chitosan grafted glycolic acid (CGCF-1), nanohybrid scaffold (CGCF (D)) and drug (CPA) (Figure 7.4). The characteristic peaks in the FTIR spectrum of CTS include 1633 cm⁻¹ (-NH stretching) and 3500cm⁻¹ (-OH stretching). The presence of extra peak at 1730 cm⁻¹ (-C=O stretching) and shifting of peak (-NH stretching) towards the lower
frequency region (1568 cm\(^{-1}\)) confirms the interaction of glycolic acid with NH\(_2\) group of chitosan. The grafting of glycolic acid on chitosan was confirmed by the formation of amide (–NH-C=O) linkage between amine (-NH\(_2\)) group of chitosan and –C=O group of glycolic acid. In the FTIR spectrum of CPA include peak at 1237 cm\(^{-1}\) (P=O stretching) and 1652 cm\(^{-1}\) (NH stretching). The FTIR spectrum of CGCF-(D) include shift in peak 1067 cm\(^{-1}\) (P=O stretching) and 3214 cm\(^{-1}\) (OH stretching) it may due to the interaction of Co\(_3\)O\(_4\)-Fe\(_3\)O\(_4\) hybrid nanoparticles with –P=O group of drug molecule and –OH group of chitosan via metallic bond. The peak at 1568 cm\(^{-1}\) in CGCF-(D) is attributed to shift in –C=O stretching towards lower frequency region, it may be due to the interaction of CPA with –C=O group of grafted glycolic acid via H- bonding (Scheme 7.1).

![Figure 7.4 FTIR spectra of neat chitosan (CTS), grafted chitosan (CGCF-1), grafted chitosan and Co\(_3\)O\(_4\)-Fe\(_3\)O\(_4\) hybrid nanoparticles based nanohybrid scaffold (CGCF (D)) and drug cyclophosphamide (CPA).](image)

*Figure 7.4 FTIR spectra of neat chitosan (CTS), grafted chitosan (CGCF-1), grafted chitosan and Co\(_3\)O\(_4\)-Fe\(_3\)O\(_4\) hybrid nanoparticles based nanohybrid scaffold (CGCF (D)) and drug cyclophosphamide (CPA).*
7.4.5 XRD analysis

Figure 7.5. (a) Illustrates the X-ray diffraction pattern of neat chitosan (CTS) and glycolic acid grafted chitosan (CGCF-1). It was observed that neat chitosan (CTS) shows the characteristic peak at 10.9° and 19.8°, which correspond to a hydrated crystalline structure and an amorphous structure of chitosan, respectively. Grafting of chitosan with glycolic acid (CGCF-1) resulted in a shift of peak from 10.9° to 10.1° and 19.8° to 20.6° which confirms the interaction of chitosan with glycolic acid. These peaks were shifted from 10.1° to 8.1° and 20.6° to 22.5°, showing the interaction of Co₃O₄-Fe₃O₄ hybrid nanoparticles with the grafted chitosan (CGCF-2) as shown in Figure 7.5. (b).
Figure 7.5 (a) X-ray diffraction spectra of neat chitosan and grafted chitosan. (b) X-ray diffraction spectra of CS-g-glycolic acid and Co3O4-Fe3O4 hybrid nanoparticles based nanohybrid scaffold.

7.4.6 Morphological study

The SEM image (Figure 7.6 (a, b)) reveals the morphology of nanohybrid scaffold before drug loading and after drug addition (Figure 7.6 (c, d)). It is observed that pore size of scaffold before drug addition was ranging from 30.10 µm to 40.10 µm, but upon addition of drug pore size decreases and lies in the range of 12.87 µm to 11.07 µm. The decrease in the pore size may be due to the incorporation of drug molecule in the pores of scaffold. The SEM-EDAX of scaffold confirms the incorporation of Co3O4-Fe3O4 hybrid nanoparticles in it (Figure 7.6 (e)).
Figure 7.6 (a, b) SEM image of grafted chitosan and Co$_3$O$_4$-Fe$_3$O$_4$ nanohybrid scaffold without drug; (c, d) SEM image of grafted chitosan and Co$_3$O$_4$-Fe$_3$O$_4$ nanohybrid scaffold with drug; (e) EDAX of nanohybrid scaffold (CGCF (D)).

7.4.6 In vitro drug release

Figure 7.7 shows UV-vis spectra of in vitro drug release study illustrating variation in the absorbance of the drug in the scaffold with respect to time. In vitro drug release was examined with SBF (pH 7.4) and release media was quantified by UV-visible spectral absorbance values. It is observed that initially the release of drug was high and it decreases with the time because the drug which is at the surface of scaffold is released much faster than the drug incorporated deeply into the pores of the scaffold. The effect of incorporation of
Designing of Chitosan and metal/metal oxide nanoparticle based nanocomposites

Co$_3$O$_4$-Fe$_3$O$_4$ hybrid nanoparticles can be significantly observed as reduced rate of release at initial stage of immersion (upto 200 min). Initially specimen is solvated, which facilitates the lateral diffusion of drug after 250 min$^{32}$. The rate of release of drug decrease over the time, it may be due to the interaction of Co$_3$O$_4$-Fe$_3$O$_4$ composite nanoparticles and grafted glycolic acid chains with the loaded drug$^{31}$.

![Graph showing drug release profile](image)

**Figure 7.7** Drug release profile from the prepared nanohybrid scaffold (CGCF (D)).

7.4.8 Shape retention study

In general, swelling of chitosan involves the protonation of amino/imine groups and the mechanical relaxation of coiled chitosan chain$^{33,34}$. Shape retention was studied by measuring the change in the diameter as a function of immersion time in the media$^{35}$. Swelling behavior of scaffold strongly depends upon the pH of the implantation site for their practical use in tissue engineering. It was investigated by exposing it to media at different pH, 1N HCl (pH
1.2), 1N NaOH (pH 14) and simulated body fluid (SBF) (pH 7.4) solutions for 24 h. The in vitro cell culture studies indicate that initial swelling is desirable\textsuperscript{36, 37}, but continuous swelling reduces the mechanical integrity and leads to the generation of compressive stress to the surrounding tissue. It is observed that scaffold CGCF (S) dissolve completely in the HCl solution within 2.5 h of immersion, whereas, rate of swelling is very low in NaOH and reached the plateau level around 3 h of immersion but increase in size of scaffold is observed within 6 h in SBF solution. In the case of scaffold CGCF (D), its complete dissolution was observed in HCl solution within 2.5 h of immersion, whereas slight swelling was observed in SBF within 3.5 h. These results showed that nanohybrid scaffold is stable towards the SBF and higher pH solution (Figure 7.8).

![Figure 7.8](image-url)

\textit{Figure 7.8} Shape retention of scaffolds prepared from grafted chitosan and Co\textsubscript{3}O\textsubscript{4}-Fe\textsubscript{3}O\textsubscript{4} nanohybrid.
7.4.9 Cell viability study

MTT assay was carried out to evaluate the proliferation of L929 on (CGCF-(D)). Growth of the cells cultured on the scaffold was higher during the first 2 h but slight decrease in the cell number was observed in next 4 h. It may be because during proliferation cells have occupied all the available spaces on the scaffold. Present study implies that the cell proliferation is not affected by the incorporation of Co$_3$O$_4$-Fe$_3$O$_4$ composite nanoparticles into glycolic acid grafted chitosan. This may be due to the enhanced interaction between Co$_3$O$_4$-Fe$_3$O$_4$ composite nanoparticles and growing cells on the biopolymer matrix. These results of improved cell proliferation and cell adherence on scaffold was mainly due to the presence of reactive groups on the polymer surface and improved hydrophilicity after hydrolysis, similar to those reported by other researchers. The Co$_3$O$_4$-Fe$_3$O$_4$ composite nanoparticles may develop London- van der Waals forces with cells. These Co$_3$O$_4$-Fe$_3$O$_4$ composite magnetic nanoparticles can act as adhesive between biopolymer and cells.

![Cell viability study done with MTT assay of cultured cells.](image-url)

**Figure 7.9.** Cell viability study done with MTT assay of cultured cells.
7.5 CONCLUSIONS

The present study examined the potential use of hybrids of chitosan-g-glycolic acid and Co$_3$O$_4$-Fe$_3$O$_4$ composite magnetic nanoparticles as biomaterial. The FTIR confirmed the interaction of cationic chitosan with Co$_3$O$_4$-Fe$_3$O$_4$ composite nanoparticles via metallic bond and linkage of drug with the polymer matrix via H-bond. The nanohybrid scaffolds are stable regardless of pH of the medium. The nanohybrid scaffold posses porous morphology. The porous nanohybrid scaffolds have shown faster and higher drug release. The incorporation of Co$_3$O$_4$-Fe$_3$O$_4$ composite nanoparticles was observed to control the initial release of drug. From the results we conclude that, the prepared nanohybrid scaffold is biocompatible and also Co$_3$O$_4$-Fe$_3$O$_4$ composite magnetic nanoparticles are viable additive for formulating sustained drug delivery systems and could be applied in the field of biomaterials.

7.6 REFERENCES


CHAPTER 7

B: Preparation and Characterization of Chitosan-g-Glycolic Acid-Co$_3$O$_4$-Fe$_3$O$_4$ Hybrid Nanoparticles Based Nanocomposite Film
7.2.1 INTRODUCTION

The nanotechnology field is one of the interesting areas for current research and development in all technical discipline. In this field the investigation cover a broad range of topics, this obviously includes polymer science and technology. Other areas include polymer based biomaterials, layer by layer self assembled polymer films, imprint lithography, electro-spun nanofiber, nanocomposites, fuel cell electrode polymer bound catalysts and nanoparticle drug delivery. The nanocomposites of inorganic materials in polymer matrices have attracted a great deal of attention because of their wide area of applications as micromechanical devices\(^1\), optical device\(^2\), catalytic membrane\(^3\) and biosensors\(^4\). The field of nanocomposites include composites rein-enforcement, electro-optical properties, barrier properties and cosmetic applications. Different approaches have been developed for the synthesis of nanocomposites such as incorporation of premade nanoparticles into the polymer matrix. This can be achieved with the use of a common blending solvent or by reduction of metal salt dispersed in polymer matrix using an external reducing agent\(^5\). Various methods are known for the embedment of nanoparticles in polymer matrices such as physical and chemical vapour deposition, ion implantation and sol-gel synthesis\(^6\)\(^-\)\(^8\). The Metallic and metal oxide nanoparticles have become an area of growing interest of fundamental studies and technological applications, due to their unique mechanical, electronic, chemical, magnetic and optical properties\(^9\)\(^-\)\(^{14}\). The metal nanoparticle embedded polymer film can show enhanced mechanical properties. Therefore, attempts have been made to synthesize metal nanoparticles embedded chitosan films.

Chitosan is used to prepare a variety of forms such as powders, hydrogels, membranes, fibers, porous scaffolds and films that have been tested in many medical and biological applications\(^15\)\(^-\)\(^18\).
7.2.2 EXPERIMENTAL

7.2.2.1 Materials

Chitosan of low molecular weight (Mv 1.5×10^5, degree of deacetylation was 85%), glycolic acid with (99% purity), iron (0) pentacarbonyl (Fe(CO)_5), oleylamine (OAM), oleic acid (OA), 1-octadecene, cobalt acetate (Co(OAc)_2) and citric acid (C_6H_8O_7) was obtained from sigma Aldrich. Sodium hydroxide and phenyl ether was obtained from Sisco Research Laboratories. Deionised water was used throughout, which is prepared by Milli-Q-system.

7.2.2.2 Preparation of nanocomposite film

Chitosan-g-glycolic acid and Co_3O_4-Fe_3O_4 nanoparticle (synthesis of nanoparticles has been given in chapter 7A, section 7.2.2) nanocomposite film was prepared by dispersing chitosan in deionised water for 1 h with constant stirring at room temperature. Glycolic acid was added to the solution after 1 h, which is allowed to stirred for 12 h. After 12 h, Co_3O_4-Fe_3O_4 hybrid nanoparticles were added to the resulting solution and stirred overnight at room temperature. Degassing of resulting solution was done at 80 °C for 25- 30 min. The solution was casted on a glass plate and dried at 60 °C for 8 h under vacuum to promote the dehydration of grafted chitosan copolymer with formation of the amide linkage. The thickness of the prepared film were measured and found to be 0.18 mm. The formulation of chitosan and nanoparticles are given in Table 7.2.1. The unreacted glycolic acid and the oligomers of glycolic acid were extracted with methanol in soxhlet apparatus for 48 h.
Scheme 7.2.1 Schematic illustration of interactions between glycolic acid grafted chitosan and Co$_3$O$_4$/Fe$_3$O$_4$ hybrid nanoparticles.

Table 7.2.1 The formulation of chitosan-g-glycolic acid and Co$_3$O$_4$-Fe$_3$O$_4$ nanoparticle

<table>
<thead>
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<th>S. No</th>
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<td>0</td>
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</tbody>
</table>

7.2.3 CHARACTERIZATION OF NANOCOMPOSITE FILM

7.2.3.1 Attenuated total reflectance Fourier transform infrared (ATR-FTIR)

The Nicolet Nexus 870 attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectrometer equipped with a smart Endurance diamond accessory (64 scans, 4 cm$^{-1}$ resolution, wave number range 4000-550 cm$^{-1}$) were used to analyse Fourier transform
infrared spectra (FT-IR) of neat chitosan (CS), chitosan grafted glycolic acid (CGCoF-1) and grafted chitosan nanocomposite with Co$_3$O$_4$-Fe$_3$O$_4$ hybrid nanoparticles (CGCoF-2).

**7.2.3.2 X-ray diffraction (XRD)**

XRD pattern of the samples were recorded by using X-ray Diffractometer (WAXRD – Rigaku, Japan) with Cu-κα radiation at a voltage of 50 KV. The scanning rate was 4°/min and the scanning scope of 2θ was from 2° to 80° at room temperature.

**7.2.3.3 Atomic force microscopy (AFM)**

The surface morphology of nanohybrid film were investigated by atomic force microscopy (AFM) (Model: Nanoscope IV) under contact mode.

**7.2.3.4 Water absorption measurement**

The water absorption measurement was investigated by ASTM D 570 method, according to which, the clean, dried film samples of known weights were immersed in distilled water at 25°C for 24h (1 Day). The films were removed, blotted quickly with absorbent paper and weighed. The absorption percentage of prepared samples was calculated using the Eq. (1):

\[
X\% = \frac{(W_1 - W_0)}{W_0}
\]

where $W_0$ and $W_1$ are the weight of dry and swollen samples, respectively.

**7.2.3.5 Dynamic mechanical analysis (DMA)**

The mechanical strength of prepared nanohybrid films were investigated with dynamic mechanical thermal analyser (DMTA RSA3, TA instrument) in tensile mode at a frequency of 1Hz with heating rate of 5°C/min in the temperature range from -10°C to 200°C.

**7.2.3.6 Tensile strength testing (TST)**

The tensile stress testing of the nanohybrid film was determined with Linkam TST 350. The break stress and strain was calculated with the associated software (Linkam). A dumb bell strip was cut from each membrane and strained to break at a constant crosshead speed of 10 mm/min.
7.2.3.7 Thermogravimetric analysis (TGA)

TGA Q5000 instrument were used to conduct the thermogravimetric analysis (TGA) of the sample. Temperature ranges from 50 °C to 900 °C with the heating rate of 10 °C/min under nitrogen with flow rate 20

7.2.4 RESULT AND DISCUSSION

7.2.4.1 FTIR analysis

The structural information about pure chitosan (CS), glycolic acid grafted chitosan (CGCoF-1) and its nanocomposite with Co$_3$O$_4$-Fe$_3$O$_4$ composite nanoparticles (CGCoF-2) were investigated by Fourier transform infrared (FT-IR) spectra (Figure 7.2.1). In pure chitosan spectrum, peak at 1634 cm$^{-1}$ is attributed to the --N-H bending vibration of amine (–NH$_2$) group in chitosan. In glycolic acid grafted chitosan (CGCoF-1) spectrum, the peak attributed to the --N-H bending vibration is shifted towards the lower frequency region confirming the interaction of glycolic acid onto NH$_3^+$ group. In CGCoF-1 a new peak appeared at 1734 cm$^{-1}$ corresponds to $\nu_{co}$ stretching. These two peaks confirms the conversion of amine (NH$_2$) to amide (–NH–C=O). The band at 3437 cm$^{-1}$ is corresponding to $\nu_{NH}$ stretching. The N-H bending of the molecule as well $\nu_{co}$ stretching band is observed to be shifted towards lower frequency region, indicating the interaction of Co$_3$O$_4$-Fe$_3$O$_4$ with N-H group of chitosan and C=O group of glycolic acid through metallic-bond, in FTIR spectrum of CGCoF-2 (Scheme 7.2.3).
**Designing of Chitosan and metal/metal oxide nanoparticle based nanocomposites**

**Figure 7.2.1** FTIR spectra of neat chitosan (CS), grafted chitosan (CGCoF-1) and grafted chitosan/Co$_3$O$_4$-Fe$_3$O$_4$ hybrid nanoparticle nanocomposite film (CGCoF-2).

### 7.2.4.2 XRD analysis

The XRD pattern of neat chitosan (CS), chitosan grafted with glycolic acid (CGCoF-1) and nanocomposite of grafted chitosan with Co$_3$O$_4$-Fe$_3$O$_4$ composite nanoparticles (CGCoF-2) was shown in **Figure 7.2.2** (a, b). The chitosan structure is strongly dependent on its processing treatment, such as dissolving, precipitation and drying, as well as its origin and characteristics, such as degree of deacetylation and molecular weight\(^{19}\). The neat chitosan film shows the peaks at \(\theta = 10.3^0\) and \(20.1^0\), corresponds to hydrated crystalline structure and an amorphous structure of chitosan, respectively\(^{20-21}\). The XRD peaks were shifted from \(10.3^0\) to \(9.6^0\) and \(20.1^0\) to \(19.6^0\) confirming the interaction of glycolic acid with chitosan in CGCoF-1 sample film. More shifts in CGCoF-2 nanocomposite film was observed that is, \(\theta = 9.6^0\) to \(9.1^0\) and \(19.6^0\) to \(21.5^0\). This is probably due to higher compatibility of the Co$_3$O$_4$-Fe$_3$O$_4$ nanoparticle with the grafted chitosan matrix.
Figure 7.2.2 (a) X-ray diffraction spectra of neat chitosan (CS) and grafted chitosan (CGCoF-1). (b) X-ray diffraction spectra of grafted chitosan/Co$_3$O$_4$-Fe$_3$O$_4$ hybrid nanoparticle nanocomposite films.
7.2.4.3 Morphological studies

Atomic Force Microscopy (AFM) illustrates the surface topography of pure chitosan, grafted chitosan and nanocomposite film. Figure 7.2.3 (a) shows the AFM image of neat chitosan film, which is observed to exhibit smooth surface. The image size was 5 μm × 5 μm. Grafting of chitosan with glycolic acid increases the roughness and height of the surface (Figure 7.2.3 (b)). The incorporation of Co₃O₄-Fe₃O₄ composite nanoparticle in the matrix of chitosan film is shown in Figure 7.2.3 (c, d).

![AFM images](image)

**Figure 7.2.3** (a) AFM image of pure chitosan film; (b) AFM image of grafted chitosan film. (c, d) AFM image of grafted chitosan/Co₃O₄-Fe₃O₄ hybrid nanoparticle nanocomposite films.

7.2.4.4 Water absorption Behavior

The water absorption property of the glycolic acid grafted chitosan is higher than that of the pure chitosan. The pure chitosan is hydrophilic but it does not absorb much water, probably due to many –OH and –NH groups in chitosan, which causes strong intermolecular
and intramolecular hydrogen bonds. The higher water absorption in grafted chitosan is probably because the molecular structure integrity is broken in the grafted chitosan, which can expose more functional groups for water absorption. This swelling extent will depend on the osmotic pressure and charge repulsion, the degree of ionization and grafting extent. In comparison of grafted chitosan, nanocomposite films show lower water absorption and decreases with the increasing content of nanoparticles. This is probably due to the formation of a barrier in the form of cross linking points, which prevents water permeation into chitosan. Since nanoparticle is hydrophobic, resulting nanocomposite were expected to be hydrophobic. It can be attributed to the interaction between nanoparticle and copolymer. Upon increasing content of nanoparticle reduces the exposure of more functional group towards the water, thus the hydrophobicity of the nanocomposite film increases. The formation of nanocomposite occurs through the metallic bond formation between Co₃O₄-Fe₃O₄ composite nanoparticle and copolymer, which decreases the water absorption (Table 7.2.2). The nanocomposite films were kept in water for 24 h till the equilibrium is reached. After complete swelling, film was dried under vaccume at 65 °C to evaluate the moisture retention capacity of the nanocomposite films. Grafted chitosan shows high water retention capacity. Nanoparticles act as a physical barrier for the moisture to exude out from the films this is because on increasing the nanoparticle content, the water absorption decreases.

Table 7.2.2 Sorption behavior of the nanocomposites

<table>
<thead>
<tr>
<th>Sample Code</th>
<th>Water Absorption (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS</td>
<td>54</td>
</tr>
<tr>
<td>CGCoF-1</td>
<td>70.7</td>
</tr>
<tr>
<td>CGCoF-2</td>
<td>52.98</td>
</tr>
<tr>
<td>CGCoF-3</td>
<td>33.2</td>
</tr>
</tbody>
</table>
7.2.4.5 Dynamic mechanical analysis

The change in viscoelastic property, stability and glass transition temperature of polymer determines the variation in dynamic mechanical thermal property of glycolic acid grafted \( \text{Co}_3\text{O}_4-\text{Fe}_3\text{O}_4 \) hybrid nanoparticle nanocomposites. The storage modulus \( [E'] \) of pure chitosan film at 77.92 °C is observed to be \( 1.766 \times 10^8 \) [Pa] (Figure 7.2.4 (a)). The storage modulus \( [E'] \) increases to \( 2.502 \times 10^8 \) [Pa] for grafted polymer film (Figure 7.2.4 (b)). Storage modulus \( [E'] \) of nanocomposite film increases with the increase in \( \text{Co}_3\text{O}_4-\text{Fe}_3\text{O}_4 \) hybrid nanoparticle content (Figure 7.2.4 (c, d)). The loss factor \((\tan \delta)\), which is the ratio of the loss modulus to the storage modulus decreases with the increase in glass transition temperature. The neat chitosan film exhibit \( T_g = 153.63 °C \). Decrease in \( T_g \) of grafted film is observed due to increase in the mobility of the polymer chains. The addition of \( \text{Co}_3\text{O}_4-\text{Fe}_3\text{O}_4 \) hybrid nanoparticle restricts the mobility of the chains, thus the storage modulus increases and improves the mechanical strength of polymer film Table 7.2.3.

**Table 7.2.3** Viscoelastic properties of grafted chitosan- \( \text{Co}_3\text{O}_4-\text{Fe}_3\text{O}_4 \) hybrid nanoparticle nanocomposites

<table>
<thead>
<tr>
<th>Sample Code</th>
<th>( \text{Co}_3\text{O}_4-\text{Fe}_3\text{O}_4 ) (Wt %)</th>
<th>Storage modulus (Pa) at 77.92 °C</th>
<th>( T_g ) (°C)</th>
<th>( \tan \delta )</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS</td>
<td>0</td>
<td>( 1.766 \times 10^8 )</td>
<td>153.86</td>
<td>0.43</td>
</tr>
<tr>
<td>CGCoF-1</td>
<td>0</td>
<td>( 2.507 \times 10^8 )</td>
<td>113.54</td>
<td>0.59</td>
</tr>
<tr>
<td>CGCoF-2</td>
<td>40</td>
<td>( 4.133 \times 10^6 )</td>
<td>24.62</td>
<td>0.55</td>
</tr>
<tr>
<td>CGCoF-3</td>
<td>80</td>
<td>( 2.306 \times 10^7 )</td>
<td>38.02</td>
<td>0.43</td>
</tr>
</tbody>
</table>
Designing of Chitosan and metal/metal oxide nanoparticle based nanocomposites

(a)

(b)
Figure 7.2.4 Temperature variation of tanδ, glass transition temperature, storage modulus $[E']$, and loss modulus $[E'']$ (a) Pure chitosan film (CS); (b) Grafted chitosan film (CGCoF-1); (c) Grafted chitosan/Co$_3$O$_4$-Fe$_3$O$_4$ hybrid nanoparticle nanocomposite films (CGCoF-2); (d) Grafted chitosan/Co$_3$O$_4$-Fe$_3$O$_4$ hybrid nanoparticle nanocomposite films (CGCoF-3).
7.2.4.6 Tensile stress testing

All the membranes had uniform thickness of 0.17mm and were semi-transparent. The mechanical properties of chitosan are inconsistent and lack clarity in the mode of analysis such as crosshead speed or molecular weight\textsuperscript{22-24}. Therefore, the tensile properties of chitosan were analysed first (Figure 7.2.5 (a)). The tensile properties varied significantly with the crosshead speed. Crosshead speed used while testing is 10mm/min at 27 °C. The neat chitosan film exhibits break strain as 25-26%. The elastic modulus of neat chitosan film was observed to be 0.9855 MPa. The grafted chitosan exhibits relatively decrease in elastic modulus. Addition of Co\textsubscript{3}O\textsubscript{4}-Fe\textsubscript{3}O\textsubscript{4} hybrid nanoparticle improves the tensile strength of the polymer (Figure 7.2.5 (b)).

![Tensile stress testing graph](image-url)
Figure 7.2.5  (a) Stress strain behavior of pure chitosan membranes (CS); (b) Effect of grafting and nanoparticle stress strain behavior of grafted and nanocomposite chitosan membranes.

Table 7.2.4  Tensile strength and testing of chitosan and nanocomposites

<table>
<thead>
<tr>
<th>Sample Code</th>
<th>Elastic modulus (MPa)</th>
<th>Stress (%)</th>
<th>Strain (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS</td>
<td>0.9855</td>
<td>47.97</td>
<td>25.83</td>
</tr>
<tr>
<td>CGCoF-1</td>
<td>0.9556</td>
<td>6.38</td>
<td>83.29</td>
</tr>
<tr>
<td>CGCoF-2</td>
<td>0.9829</td>
<td>9.98</td>
<td>113.4</td>
</tr>
<tr>
<td>CGCoF-3</td>
<td>0.9838</td>
<td>13.71</td>
<td>113.3</td>
</tr>
</tbody>
</table>
7.2.4.7 Thermogravimetric analysis

Figure 7.2.6 shows thermal degradation of neat chitosan, grafted chitosan and its nanocomposite with various ratio of Co$_3$O$_4$-Fe$_3$O$_4$ nanoparticles under nitrogen flow. Two steps of non-oxidative degradation were observed. The weight loss at 50-150 °C is attributed to the water absorbed in chitosan. Whereas, the weight loss in the temperature range of 200-350 °C corresponds to the degradation and deacetylation of chitosan$^{21,25}$. In TGA curve, three parameters were measured: temperature of thermal degradation at 20% weight loss, the temperature at 50% weight loss and the yield of charred residue under nitrogen flow. Upon grafting thermal degradation temperature of chitosan decreases by 20-30 °C. The thermal stability of chitosan is decreased. This is probably due to the poor heat barrier properties of nanoparticle for polymer matrix during the formation of chars$^{21}$. The grafted chitosan matrix have highest char residue ((27.7% at 900 °C). The char residue is increased upon increasing the nanoparticles wt %. However decrease of 75-80 °C was observed upon addition of Co$_3$O$_4$-Fe$_3$O$_4$ nanoparticles (Table 7.2.5). The amount of weight loss at this temperature range decreases with the increasing content of nanoparticles in samples. This implies that due the grafted chitosan-Co$_3$O$_4$-Fe$_3$O$_4$ nanoparticles bonding water absorbability, that is hydrophillicity of the films decreases. This was also confirmed in water swelling behavior section.

Table 7.2.5 TGA results for chitosan and its nanocomposites

<table>
<thead>
<tr>
<th>Sample Code</th>
<th>Temperature at 20% loss (°C)</th>
<th>Temperature at 50% loss (°C)</th>
<th>char at 900 °C (wt %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS</td>
<td>310</td>
<td>360</td>
<td>27.1</td>
</tr>
<tr>
<td>CGCoF-1</td>
<td>246</td>
<td>347</td>
<td>27.7</td>
</tr>
<tr>
<td>CGCoF-2</td>
<td>168</td>
<td>272</td>
<td>18.9</td>
</tr>
<tr>
<td>CGCoF-3</td>
<td>172</td>
<td>280</td>
<td>21.0</td>
</tr>
</tbody>
</table>
7.2.5 CONCLUSION

In summary, novel nanocomposite film of chitosan-g-glycolic acid and Co$_3$O$_4$-Fe$_3$O$_4$ hybrid nanoparticles was prepared. The interaction of cationic chitosan with Co$_3$O$_4$-Fe$_3$O$_4$ nanoparticles is through metallic bond, which results in enhancement in structural and functional properties. The chemical modification of chitosan with glycolic acid and nanoparticles increases its mechanical as well as tensile strength. The grafting of chitosan with glycolic acid imparts hydrophillicity to the film. The results showed that increasing content of nanoparticles reduces hydrophillicity of the nanocomposite film. The longer water retention and swelling behavior properties were discussed, which could be applied in the field of biomedical. AFM showed the morphological study of nanocomposite film.

Figure 7.2.6 Thermogravimetric curves of prepared nanocomposites
7.2.6 REFERENCES


