Development of Polymer/nano Hydroxyapatite Composite Membrane

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BY

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ABSTRACT

The main purpose of this project is to prepare a biodegradable, resorb-able composite membrane which assists in bone healing. This composite is developed by adding hydroxyapatite in a polymer matrix in varying weight percentages. Effects of hydroxyapatite in dispersion, decomposition temperature and improvement in mechanical properties are studied.

Hydroxyapatite is synthesized by wet precipitation method which involves the reaction of phosphate and calcium precursors and results in nano-HA particles. Solutions of both polymer and HA are prepared in their respective solvents. Then both solutions are mixed together and rigorously stirred to achieve maximum dispersion. Membranes are prepared by solvent casting method. The solution is poured in the petri dish and allowed to be air dried. Finally the membranes are vacuum dried for maximum solvent evaporation.

After the samples are prepared they are characterized and mechanical properties are evaluated. Water absorption studies are performed to see the effect of hydrophobicity of HA on composite membrane. As HA content increases, the composite membrane absorbs less water. Tensile testing is done to evaluate the increase in strength due to filler content. As HA weight percentage increases the tensile strength also increases. SEM is performed to observe the morphology of the membranes. It shows even distribution of HA in polymer matrix.

As the amount of HA increases, the properties of the membrane also improve. Tensile strength increases and it absorbs less water content which makes it more suitable to be used as a barrier membrane in the orthopedics and dentistry. But after certain amount of HA, the properties decline.

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ABREVEATION LIST

FTIR	Fourier Transformation Infra-Red Spectroscopy
SEM	Scanning Electron Microscopy
TG/DTA	Thermo Gravimetric/ Differential Thermal Analysis
XRD	X-Ray Diffraction
DSC	Differential Scanning Calorimetry
T_g	Glass Transition Temperature
T_d	Degradation Temperature
HA	Hydroxyapatite
CS	Chitosan
CTAB	Cetyl Trimethyl Ammonium Bromide
GTR	Guided Tissue Regeneration

Chapter 1

Introduction

Hard tissues of the human body are of vital importance. The skeleton provides support and shape to the human body. It also offers a network where all soft tissues can attach. One of the most usual problems faced by people is bone fracture which could be caused by any accident or carelessness. Bones are hard tissues and require special attention if fractured. Over the time researchers have developed strong knowledge in the field of tissue engineering. This development promises to solve many hard tissue problems. Although they come with inherent issue of insufficient mechanical properties which can be resolved [1].

Biomaterials are the materials which interact with human body. They can be natural or of synthetic origin. Chief criteria which a biomaterial must be fulfilled are biocompatibility and bio-functionality. Institute of Materials London published a report in 1995. They estimated world market for biomaterials which is around $12 billion per year [2]. This gives a clue of the size of the economy about this area. Apart from economical aspect they own a vital importance for human life in many cases.

Potential candidates are ceramics, polymers, metals and composite materials which are modified to solve bone problems. Every material has its own advantages and disadvantages. Polymers lack mechanical strength, metals possess superior mechanical strength but they are very corrosive. Ceramics although possess preferred properties for instance wear resistance and hardness but the main drawback is that they are brittle and have low fracture toughness. In order to obtain a bone substitute material a combination of these properties is required which is possible only through composite materials.

Composition of bone composite is shown in Figure 1. HA and collagen form a complex hierarchical microstructure which is responsible for the superior mechanical properties of the bone. There has been extensive amount of research done for bone substitute materials comprising HA and polymer. HA possesses good properties such as

bioactivity, biocompatibility, non-toxicity and osteo-conductivity [3]. There are also some problems linked with HA. For instance it is brittle, shows poor formability and tends to wander from the implanted sites. In order to avoid these issues, HA composites with polymers are preferred.

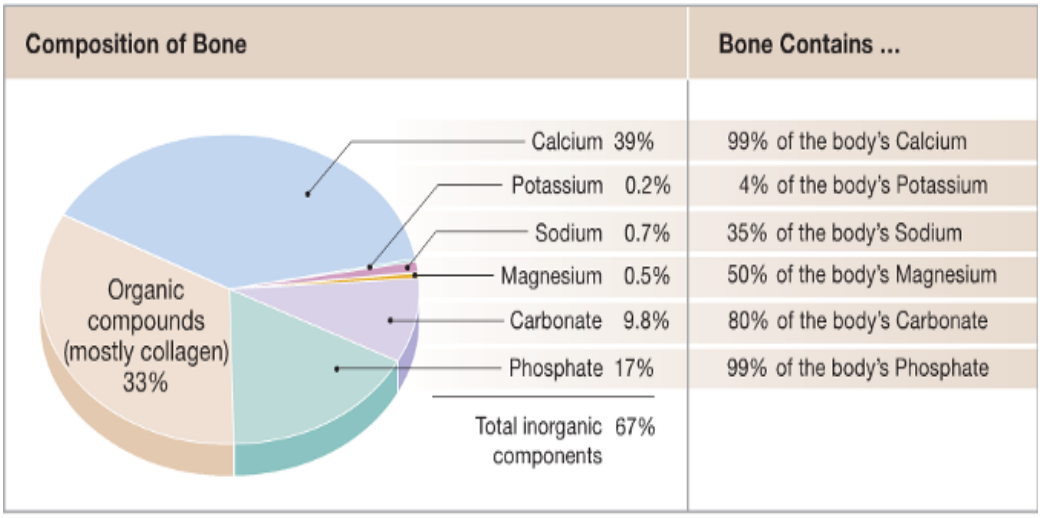


Figure 1 Composition of Bone

Collagen is the first choice when a polymer matrix is considered. A major shortcoming of using collagen is its high cost that confines its medical application in treating bone defects because of its indifferent formability and flexibility. Chitosan is promising candidate to serve as a matrix in composite membranes. It is a natural polymer found in immense amounts in crustaceans. It is deacetylated form of chitin. It is considered a significant polymeric matrix for HA reinforced composite membrane because of its noticeable features like biocompatibility, biodegradability, flexibility, adhesiveness and anti-infectivity [4]. Its degradation rate is higher than bio-ceramics. Addition of HA in a chitosan matrix causes increased osteoconductivity and biodegradability with substantial improvement of mechanical strength. New bone inter grows around HA particles as the polymer matrix degrades.

In this project biodegradable composite membranes comprising chitosan as polymer matrix and hydroxyapatite as reinforcement are prepared and analyzed. The effects of varying weight percentages of HA in polymer matrix on mechanical, microstructural and physical properties have been studied.

Chapter 2

Literature Review

2.1 Bio-Resorbable Films

A barrier membrane is a device used in oral surgery and periodontal surgery for the prevention of epithelium, which regenerates quickly, and grows in another area where, relatively slow tissue growing type is desired e.g: a bone. A method used for preventing epithelial migration into another specific area is termed as guided tissue regeneration (GTR). The membrane serves as a medium to prohibit cells penetration, which are primarily epithelial, through its configuration i.e. structure. [5]

The first films developed were non-resorbable and require a second surgery for membrane removal after certain period of time. The additional requirement for a second surgery makes it difficult to utilize original barrier membranes, setting a bench mark to develop resorbable films. A huge range of resorbable films is commercially obtainable and is now in clinical use as well. Composites based on polymers are classified as shown in Figure 2.

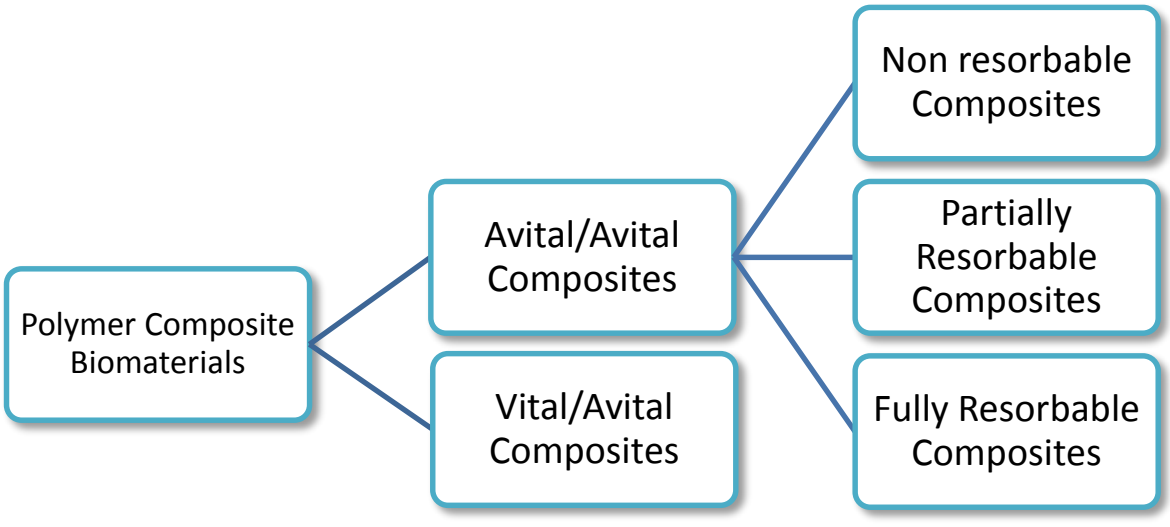


Figure 2 Types of Polymer Based Bio-composites

Many important components are involved in the concept of bio-resorbability. The films must undergo in vivo macromolecular degradation and resorption. In currently used resorbable films, in-vivo degradation and resorption is done by both hydrolysis and direct enzymatic degradation by enzymes such as acid phosphatase and chitosanase. It also demands complete elimination of the degradation products from the body without any residual side effects. A film is known to be bio-resorbable only when there is confirm elimination [6].

The ideal bioresorbable films for Guided Bone Regeneration should have the following characteristics:

- Biocompatibility
- No inflammatory reaction
- Totally resorb-able, degradable, and get eliminated
- Easy to handle, cut, contour, and adapt
- Maintains geometry (configuration and shape)
- Resistant to bacterial attack and hinder their growth
- An expected resorption time comparable with bone growth

At present bio-resorbable membranes, based on polymers, are divided into natural and synthetic polymers. Natural polymers are derived from living origin. Resulting products can be strengthened by filler addition and cross linked with aldehyde solutions to increase resistance against enzymatic degradation. Membranes with synthetic polymers used as matrix have several noticeable advantages. Such as production in large quantities, and availability of numerous variety of materials allow the development of variety of membranes in broad spectrum range covering mechanical, physical and chemical properties.

Material properties are greatly varied when the composition and the ratio of these polymers is changed. Such properties are difficult to calculate before their preparation and testing. Modification in properties is not dependent on added polymers into the matrix, which makes it difficult to predict the change of properties due to specific polymer being used in formulation [7].

Addition of reinforcement into polymer matrix yields a bio-resorbable membrane. An ideal polymer/ceramic composite intended for guided tissue regeneration should have following characteristics:

- Biocompatibility
- Macro porosity
- Mechanically stable and importantly easy to handle
- Structural integrity during initial stages
- Osteo-conductivity
- Carrier for growth factors

2.2 Polymers as Biomaterials

For composite membranes matrix can be metal, polymer or ceramic. But mostly polymers are preferred over other options. Polymers have assumed an important role in medical applications. In most of the applications polymers have little or no competition from other types of materials, because of their outstanding properties compared below:

Materials	Advantages	Disadvantages	Examples
Metals Titanium Co-Cr alloys Stainless steel	High Strength Tough Ductile	Dense Corrosive	Joint Replacement Bone plates and screws Dental root implant
Ceramics Alumina Carbon Hydroxyapatite	Wear resistant Biocompatible Bioactive Strong in compression Bio-inert	Brittle Difficult fabrication High modulus Non resilient	Dentistry Hip socket

Polymers Nylon Silicones	Resilient Easy fabrication Bio-resorbable Bio-inert	Insufficient mechanical properties Deform with time May degrade	Sutures Hip socket Blood vessels Ear, Nose
Composites	High Strength, Controllable microstructure and mechanical properties	Difficult fabrication	Joint implants Heart valves

Table 1 Comparison of Different Materials as Biomaterials

2.3 Chitosan

Chitosan is a non-toxic biopolymer. It plays a vital role in targeted action because of its ability to bind and activate living cells. It degrades within the human body and produces harmless and innocuous compounds which are easily eliminated. A wide range of biomaterials are employed in guided tissue engineering. Chitosan has attracted much attention because it is inexpensive, easily available in large quantity, biocompatible and shows resistance against microbial activity. Chitosan is proposed as a second best polymer for orthopedic applications. Because of its biocompatibility and inherent wound healing characteristics, it provides short-term mechanical strength for the regeneration of bone cells [8].

Chitosan is not soluble in distilled water, alkali or organic solvents but dissolves in a range of dilute aqueous organic acids. Mostly acetic acid and formic acid are used as solvent. After dissolution it forms a cationic polymer which has a high positive charge density and hence forms polyelectrolyte complexes with an extensive variety of anionic components [4].

2.3.1 Structure

Chitosan comprises of beta (1-4) 2-amino-2-deoxy-D-glucopyranon unit which repeats [9]. Figure 3 shows the structure of chitosan. The primary repeating unit for chitosan is 2-amino-2-deoxy-Dglucose with beta, 1-4 glucosidic linkages [10]. Chitosan

is a natural polysaccharide derived from chitin by its deacetylation. Chitosan and collagen show a number of physical and structural similarities which provides a foundation for the preparation of n-HA/chitosan composites for use in bone tissue engineering [11]. Chitosan consists of three types of reactive functional groups, primary NH_2 and primary and secondary OH groups. These functional groups help in the modification of chitosan for instance graft copolymerization. Modified chitosan is very useful for making scaffolds for tissue engineering applications [4].

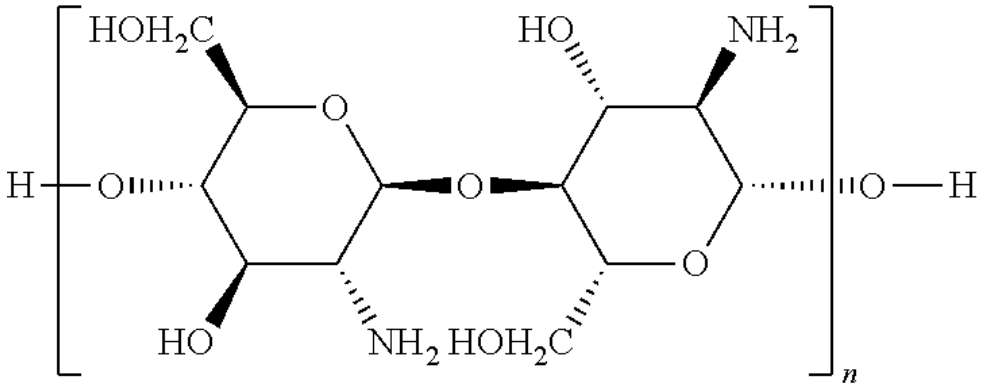


Figure 3 Structure of Chitosan

2.3.2 Properties

2.3.2.1 Degree of Deacetylation

Degree of Deacetylation alters the physical properties of chitosan film and is not affected during ultrasonic degradation of chitosan. Degree of deacetylation determines the molecular weight of chitosan prepared. More is the degree of deacetylation large is the molecular weight. The process is shown in Figure 4.

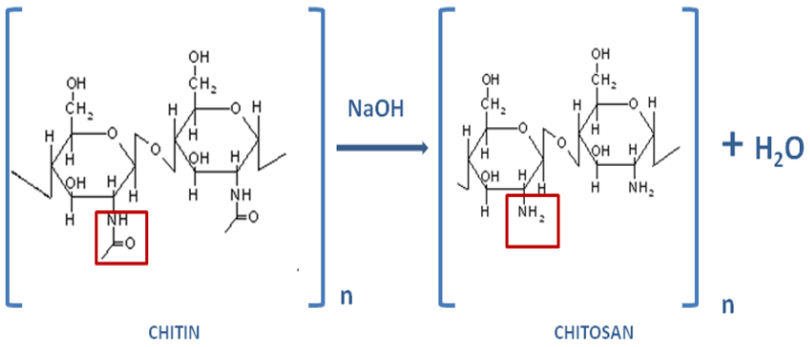


Figure 4 Deacetylation of Chitin into Chitosan

Chitosan is flexible and has a high resistance upon heating due to the intra-molecular hydrogen bonds formed between hydroxyl and amino groups. The degradation rate is inversely related to the degree of crystallinity which is controlled mainly by the degree of deacetylation (DD). Highly deacetylated forms (85 %) exhibit relatively a low degradation rate and can take a number of months in vivo, compared with the forms having lower DD which degrade more rapidly. The degradation rates also integrally affect both the mechanical and solubility properties [8].

Intra-molecular hydrogen bonds between OH and NH_2 groups provides resistance to chitosan during heating. It becomes very easy to handle. It is inherently a flexible biopolymer having a wide range of forming properties like film, fiber, and micro/nano-particles. It has unique features of bio-compatibility, non-toxicity and biodegradability. Deacetylation of chitin with a degree of deacetylation more than 50 % gives chitosan, which is soluble in aqueous solutions of organic acids like acetic or formic acid, and is used more abundantly than chitin as films, membranes, fibres and particles [4, 8].

2.3.2.2 Mechanical Properties

Chitosan allows several possibilities for covalent and ionic modifications that leads to extensive modification of mechanical and biological properties. At temperature slightly higher than normal room temperature, the chitosan film is highly crystalline and its particles are much smaller in size, which ultimately results in improved mechanical properties. High temperature causes a change in chitosan properties and the chitosan film losses the crystal structure at this point, which eventually decreases the mechanical properties. Chitosan is flexible and has a high resistance upon heating due to the intra-molecular hydrogen bonds formed between hydroxyl and amino groups. A bio-composite film of HA and chitosan is anticipated to display an increased osteoconductivity and biodegradation along with a sufficient tensile strength for orthopedic use [12].

2.3.2.3 Degradation in Body

In order to have a long term performance of tissue-engineered material, the rate of degradation of a scaffold plays a critical role. The scaffolds disturb several cellular processes inside the body like cellular growth, tissue regeneration, and response of the

host. A biomaterial composite being used for tissue engineering of skeletal system should degrade slowly, because it must sustain the mechanical strength until the regeneration of the specific part is complete. Chitosan has shown degradation in in-vivo, mainly because of enzymatic hydrolysis. Lysozyme, chitosanase, and papain are capable of degrading chitosan in vitro.

An enzyme known as Lysozyme targets the acetylated residues which leads to in vivo degradation of chitosan. In vivo hydrolysis of chitosan and its derivatives by lysozyme gives rise to oligomers that activate the macrophages. The degradation leads to the formation of N-acetyl-glucosamine which is a major component of dermal tissues and its presence is essential for scar-less tissue repair. Hence, chitosan could possibly act as a wound healing accelerator as well [9].

If degree of acetylation is high and polymer is highly crystalline than the rate of degradation of chitosan becomes small. Therefore highly deacetylated form of polymer exhibits the slowest degradation and may last a number of months in vivo.

2.3.3 Applications

Currently the research aims to estimate the capability of chitosan as a scaffolding material for engineering various tissues including cartilage, skin and bone [13].

- It is soluble in weak aqueous acids which is used to fabricate various structures and forms, such as gels, nano-fibers, nano-spheres, micro-spheres and combined with its pH sensitivity, excellent biocompatibility and biodegradability, makes chitosan a promising candidate for developing drug delivery devices and as scaffolds for tissue engineering.
- It interacts with the cell membrane due to its property to act as a permeation enhancer. It helps to reorganize the structure of tight-junction associated proteins. Furthermore its muco-adhesive property, makes it an appropriate candidate for use in both oral and nasal vaccination formulations. As such, several solution and microsphere vaccine formulations based on chitosan have been developed.
- It is employed to prepare injectable temperature-sensitive carrier devices for biomedical applications.

- Chitosan is used in the form of hydrogels. In local therapy it is used as a device which provides growth factors and delivers small molecular weight drugs.
- It is employed in targeted drug delivery and access to deliver drugs in inflammatory and tumor areas.
- Medical devices and artificial organs implants due to its inert nature are prepared. These implants are used in dentistry and bone healing processes.

2.4 Hydroxyapatite

Hydroxyapatite is very similar to the carbonated apatite, present in human bones and teeth, both in crystallography and in chemical nature. That's why it is extensively used in musculoskeletal surgeries [14]. HA is easy to machine and used in pre-fabricated forms, several formulations (Figure 5) of calcium phosphate cements can be molded as pastes and harden in situ. HA is the key component of teeth and bones in vertebrates. It is opted as an implant material for bones and teeth because it shows superior biocompatibility and at the same time good mechanical properties [15].

Figure 5 Different Forms of Hydroxyapaptite

For the last two decades HA is considered an excellent candidate for bone healing and regeneration. n-HA particles show instability when they come in contact with the blood of a patient. These particles tend to migrate from the implanted site into surrounding tissues and cause harm to healthy tissues [16]. HA particles are very hard and brittle so it is very difficult to achieve a specific shaped material for bone

substitution. As a result, a composite of HA and organic polymer has become of great concern to compensate the weak mechanical properties of HA [17].

2.4.1 Structure

Pure HA, $Ca_{10}(PO_4)_6(OH)_2$, has the theoretical composition of: 39.68 wt% Ca, 18.45 wt% P, Ca/P ratio, 2.151 and Ca/P molar ratio of 1.67 [18]. The term apatite describes a family of compounds which have similar structures but not necessarily same composition. HA is named as calcium hydroxyapatite which has a definite composition and a crystallographic structure. HA falls into the hexagonal system, with a space group, P63/m. This space group is characterized by a six-fold c-axis perpendicular to three equivalent a-axes (a1, a2, a3) at angles 120^o to each other. Unit cell consists of Ca, PO_4, and OH groups closely packed together [17, 18]. Figure 6 represents the crystal structure of HA.

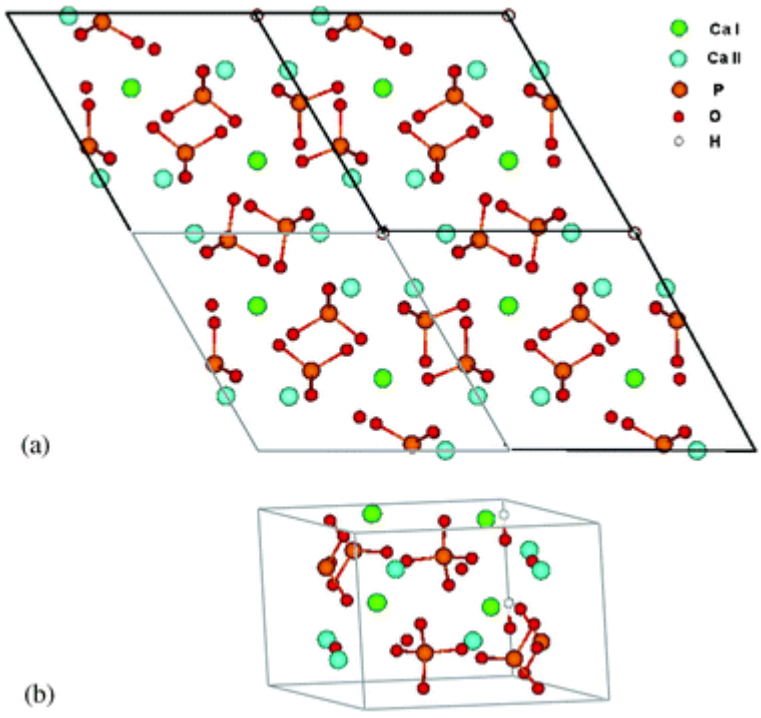


Figure 6 Structure of Hydroxyapatite

2.4.2 Properties

Hydroxyapatite, a major inorganic component of bone, has been used widely for biomedical implants and bone regeneration due to its bioactive, biodegradable and osteoconductive properties [19]. HA is accessible commercially in several forms like solids blocks, micro-porous blocks and granules [20]. n-HA is famous for its biological effectiveness. n-HA precipitates may have higher solubility and therefore affect the biological responses. It is able to promote the attachment and growth of human osteoblast-like cells [21]. Clinical trials confirmed biocompatibility of HA cement and its immunity to infection. It also shows improvement in success rate of implant if coated with HA. HA supports mesenchymal stem cell attachment, cell proliferation, and differentiation [22]. n-HA possess exceptional properties such as high surface area to volume ratio and ultra-fine structure identical to that of biological apatite. It poses great effect on cell-biomaterial interaction. It is being used to treat bone defect as it has the ability to bond with the living bone in implanted area [23].

HAp has an excellent ion-exchange performance, which exchanges calcium ion site with cation and phosphate group or hydroxide ion site with anion respectively. Indeed, in bones and teeth though it is fine, calcium ion is exchanged with iron ion, magnesium ion or strontium ion, and also hydroxide ion is exchanged with fluorine ion or carbonate ion. For example, cavity prevention using fluorine in dental field is a practical application by applying medicinal substance containing fluorine ion to teeth for the ion-exchange of fluorine ion and hydroxyl giving acid resistant to the teeth surface as a result [24]. This is shown in Figure 7.

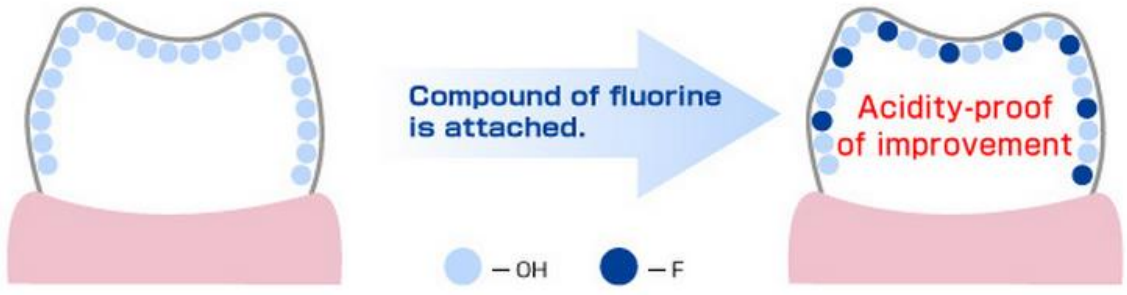


Figure 7 Ion Exchange Property of HA

2.4.3 Synthesis

There are many methods to prepare nano sized HA like precipitation method, hydrothermal synthesis and sol-gel method [25, 26, 27and 28]. Each method gives a different form, size and surface area of the powdered hydroxyapatite. Table 2 compares these methods on the basis of their advantages and disadvantages.

Method	Advantages	Disadvantages
Wet Precipitation Method	Simple Cost Effective Non-Polluting Nature	H_3PO_4 dropping rate and temperature affects the size, shape and surface area of the HA particles
Hydrothermal Method	Creates crystalline phases unstable at T_m. Materials having a high vapor pressure at T_m can be grown Large good-quality crystals with controlled composition.	Need of costly autoclaves Impossibility of observing the crystal as it grows
Sol-gel Method	Controlled composition which is not possible with conventional methods Low temperature synthesis Pure homogeneous product	Expensive raw materials Products contain high carbon content when organic reagents are used which inhibits densification during sintering. Several steps require close monitoring of the process

Table 2 Comparison of Synthesis methodologies for Hydroxyapatite

2.4.3.1 Wet Precipitation Method:

In view of the simplicity and ease of process, wet precipitation method is employed to prepare HA [25]. Wet method comprises of a neutralization reaction and the reaction between calcium and phosphate salts. In neutralization reaction, H_3PO_4

solution is slowly dropped into a $Ca(OH)_2$ aqueous solution. The chemical reaction is stated below:

$$\mathbf{10\ Ca\ (OH)_2 + 6H_3PO_4 + H_2O\ \rightarrow\ Ca_2(PO)_4)6(OH)_2 + 18H_2O}$$

The reaction between the calcium and the phosphate precursors produce hydroxyapatite as shown below:

$$\mathbf{6Ca\ (NO_3)_2 + 6(NH_4)_3PO_4 + H_2O \rightarrow Ca_{10}\ (PO_4)_6(OH)_2 + 18NH_3 + 20HNO_3}$$

2.4.4 Applications

Bone imperfections are commonly caused by tumor resection, trauma, and congenital abnormality. These defects are medically treated by the implantation of bio-ceramics and by either autogenously or allogeneic bone grafts. Even though auto grafting is a widespread technique in reconstructive surgery, it comprises a number of drawbacks, such as deficiency of donor supply, a persistent pain, the nerve damage, fracture, and cosmetic disability at the donor site. In contrast, for all types of grafting, donor site does not cause any problem, but they do involve some clinical risks including disease transmission and immunological reactions [29].

Hydroxyapatite has been integrated into a wide variety of biomedical devices for instance dental implants, HA coatings on Ti based hip implants, biodegradable scaffolds, and other kinds of orthopedic implants [30]. HA, is a bioactive material that is chemically identical to biological apatite HA, induces bioactivity in the composite films or scaffolds, as a coating on metal implants, and as a granular filler for direct integration into defected tissue site [31]. This is shown in Figure 8.

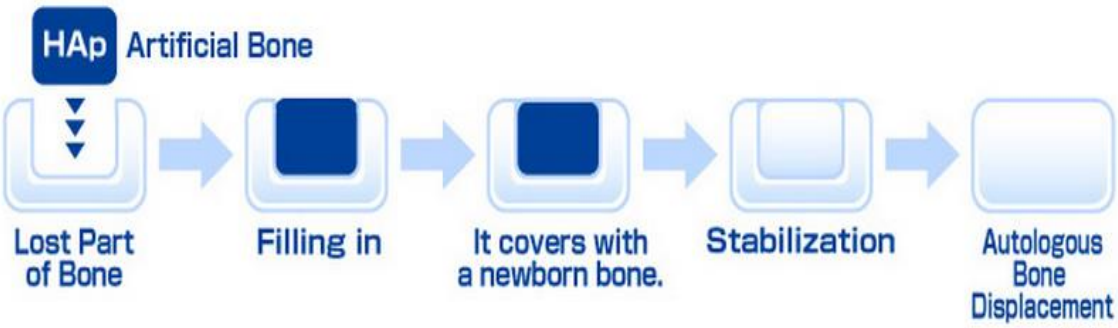


Figure 8 Application of HA

2.5 Hydroxyapatite/Chitosan Bio-composites

Composites with polymer matrix and bioactive ceramic fillers are being fabricated for bone tissue engineering. These composites attain the mechanical strength required for their function as either skeleton or teeth of organisms. Among these composites, HA/polymer composites have gained much attention since such composites may have osteo-conductivity due to the presence of HA, which has a similar chemical composition and structure to the mineral phase of human bones and hard tissues. A self-organized n-HA-collagen composite was prepared by a biomimetic co-precipitation method. It had similar microstructure to native bone and showed osteoclastic resorption and good osteoconductivity [32].

Polymers such as chitosan degrade in vivo or in vitro. Addition of HA into a chitosan matrix has shown to increase osteo-conductivity and biodegradability with significant improvement in mechanical strength [33]. Chitosan's chief attractive features include its biocompatibility, biodegradability, flexibility, adhesiveness and anti-infectivity, which make it as a feasible wound healing agent and an ideal polymeric matrix for HA ceramic [34].

2.5.1 Structure

In chitosan a free amino group is protonated to $C\text{-}NH_3^+$, when chitosan solution is prepared in aqueous acetic acid and then further mixed with HA slurry. The reaction is shown below:

$$C\text{-}NH_2 + HAc \longrightarrow C\text{-}NH_3^+ + Ac \qquad pH = 4.2$$

Dissolution of chitosan creates calcium and phosphate ions in the solutions. It produces HA\CS composites through electrostatic interactions between $C\text{-}NH_3^+$ and Ca^{2+} or PO_4^{3-} ions to form C-Ca and $C\text{-}PO_4$ complexes. Hydrogen bond is also present between OH of chitosan and OH of HA [35].

2.5.2 Properties

Incorporation of HA in chitosan can enhance the bioactivity and the bone bonding ability of the CS/HA composites [36]. Chitosan acts as an adhesive which prevents the movement of HA particles from the implanted site. It is anticipated that the nanostructure of HA/chitosan composite will have the best biomedical properties for the biomaterials applications [37].

It has been established that CS/n-HA composites prompt osteo-conductivity in osseous defects and can act as a drug delivery agent. It is necessary to fabricate composites with short life time and controlled action anti-inflammatory for the reduction or elimination of detrimental inflammatory processes [38]. The fabricated composites are expected to have an optimal mechanical performance and a controllable degradation rate as well as eminent bioactivity. These characteristics are of huge importance for bone remodeling and growth [39]. The tensile strength of the CS/HA composite increases with an increased concentration of HA [40].

In composite film a chemical and mechanical interlocking exists between n-HA and chitosan which accounts for the efficient stress- transfer. In addition, the physical interactions such as hydrogen bonding and chelation between two phases, also improves the mechanical properties of CS/n-HA composite [39]. The simulated physiological environment has a very damaging effect on mechanical properties of the composite films. It significantly decreases the values for strength and modulus. The CS/n-HA composite films containing 10 wt% HA are considered an optimum composition when mechanical strength and degradation rate is considered [41].

2.5.3 Preparation

Powder ceramic is an intelligent option for filling small and irregular defects but it causes loss of the therapeutic effect because particles migrate from the defect site. Block type ceramics are challenging to handle and cannot be kept densely for appropriate preparation and application [42]. A binder can be added that overcomes these problems. Several preparation methods for HA/CS composites have been stated, for instance addition of HA particles in a chitosan solution, coating of HA particles onto

a chitosan sheet, coating of HA crystals onto a tendon chitosan and a co-precipitation method [43]. Currently, the most common methodologies to acquire composite films are either mechanical mixing or co-precipitation methods.

Co-precipitation is a single step method. In this method CS solution is prepared by dropping aqueous H_3PO_4 solution into aqueous $Ca(OH)_2$ solution. HA is precipitated and at the same time mixed in the polymer solution [2]. Other methodologies include either bio-mineralization of chitosan in the solid form of a membrane in simulated body fluids (SBF) [44] or addition of a polymer solution with different $Ca_3(PO_4)_2$ fillers and later its precipitation as hydrogel composite. These methods help in the addition of inorganic fillers are embedded into the structure of composites, as nano or micro-sized particles [45]. The traditional process to develop CS/n-HA composite film is solution casting. HA slurry is added in chitosan solution, prepared in 2% aqueous CH_3COOH. The resulting mixture is poured in the mold and air drying is performed to obtain the composite film [46].

2.5.4 Applications

2.5.4.1 Barrier Membrane

HA nano-particles tend to migrate from the implanted site. In order to keep them at a certain place HA particles are integrated into chitosan matrices to make composite films. These composites are highly bioactive which helps in regeneration of hard tissues. Hence, a composite film of CS/n-HA is anticipated to be a good degradable barrier membrane which keeps the deposited HA at the implanted site [47]. Figure 9 shows the use of HA/Polymer composite film as a barrier membrane.

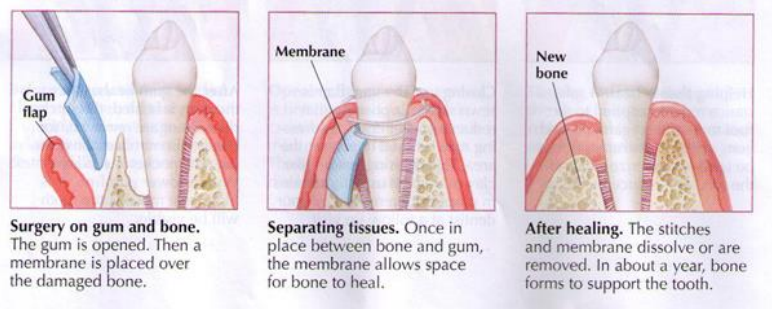


Figure 9 Application of Composite Barrier Membrane

2.5.4.2 Ridge Preservation

When a teeth is removed ridge tends to elevate in response of the absence of external stress. Sometimes the ridge gets absorbed and a bone graft is needed to enhance the ridge height or width. If the jaw ridge gets too thin it is very difficult to place conventional implants. In order to restore lost bone dimension, the ridge of the jaw is stretched by mechanical means. Bone grafts like CS/n-HA composite films are placed and matured for 2-3 months prior placing the implant [48]. Figure 10 represents different steps involved in ridge preservation.

Figure 10 Ridge Prevention By HA/Polymer Composite Membranes

2.5.4.3 Bone Grafts

Bone grafting is the process of replacing missing bone to repair bone fractures and bone loss. There are a lot of bone grafts that may be done all over the body. In case

of loss of teeth through gum disease or any kind of trauma, bone grafts are employed in order to keep bone in its current form. Bone grafts are employed in order to replace damaged vertebrae [49]. Figure 11 represents bone graft for vertebral column.

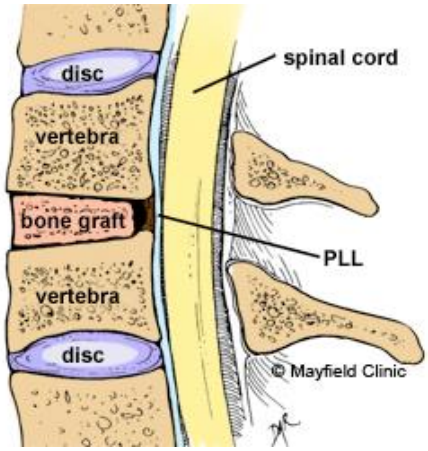


Figure 11 Bone Grafts

2.5.4.4 Bone Healing and Repair

Implants made of HA and polymer composite is employed in bone fractures and healing. In case of fracture metal rods are inserted and two step surgeries are required. But in case of HA implants only one step surgery is required because the implants become an integral part of the body. As they provide strength and due to their close composition tissue rejection is not an issue. So there is no need for the implants to be removed or replaced [50]. Figure 12 represents different types of bone fractures.

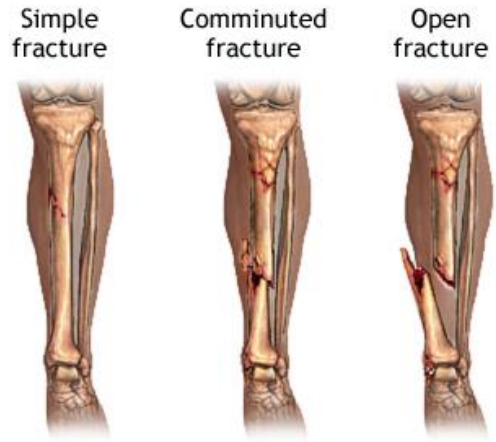


Figure 12 Bone Fractures

Chapter 3

Materials and Treatments

3.1 Synthesis of Hydroxyapatite

The simplest method to synthesize hydroxyapatite is wet precipitation method. This procedure requires two precursors of calcium and phosphate. $Ca(NO_3)_2$ (Calcium nitrate tetra hydrate) is purchased from Sigma Aldrich with 99% purity and used as a calcium precursor. $(NH_4)_2HPO_4$ (Diammonium hydrogen phosphate) is purchased from Merck Germany with 99% purity and used as a phosphate precursor. In order to maintain pH NH_4OH solution is used which is purchased from Honeywell, Burdick and Jackson and is 33% ammonia extra pure solution. This process includes following steps:

1) **0.2M** $(NH_4)_2HPO_4$ and **0.5M** $Ca(NO_3)_2.4H_2O$ solutions are prepared in distilled water. Solutions are stirred for 1 hour till they become clear and NH_4OH solution is added to adjust the pH up to the value of 10.
2) Phosphate solution is added drop wise to the calcium solution. It takes around 2 hours to complete the mixing of both solutions. In order to maintain pH solution is kept undisturbed for 5-6 hours. The reaction between the precursors is as followed [51]:

   $10Ca(NO_3)_2.4H_2O + 6(NH_4)_2HPO_4 + 8NH_4OH \rightarrow Ca_{10}(PO_4)_6(OH)_2 + 20NH_4NO_3 + 46H_2O$

   The structure of hydroxyapatite is shown in Figure 13.

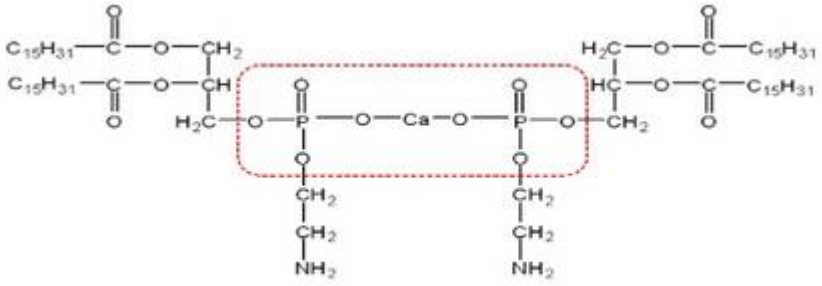


Figure 13 Structure of Hydroxyapatite

3) Solution is put on stirring for 24 hours. After vigorous stirring it is allowed to be aged for 12 hours.
4) The solution is filtered with the help of filter paper. The filtrate is washed with distilled water to maintain a neutral pH.
5) Powder is dried in oven at $80^{O}C$ for 12 hours.
6) Finally the powder is calcinated at $500^{O}C$ for 3 hours which yields high purity HA.

Flowchart for HA synthesis is shown in Figure 14.

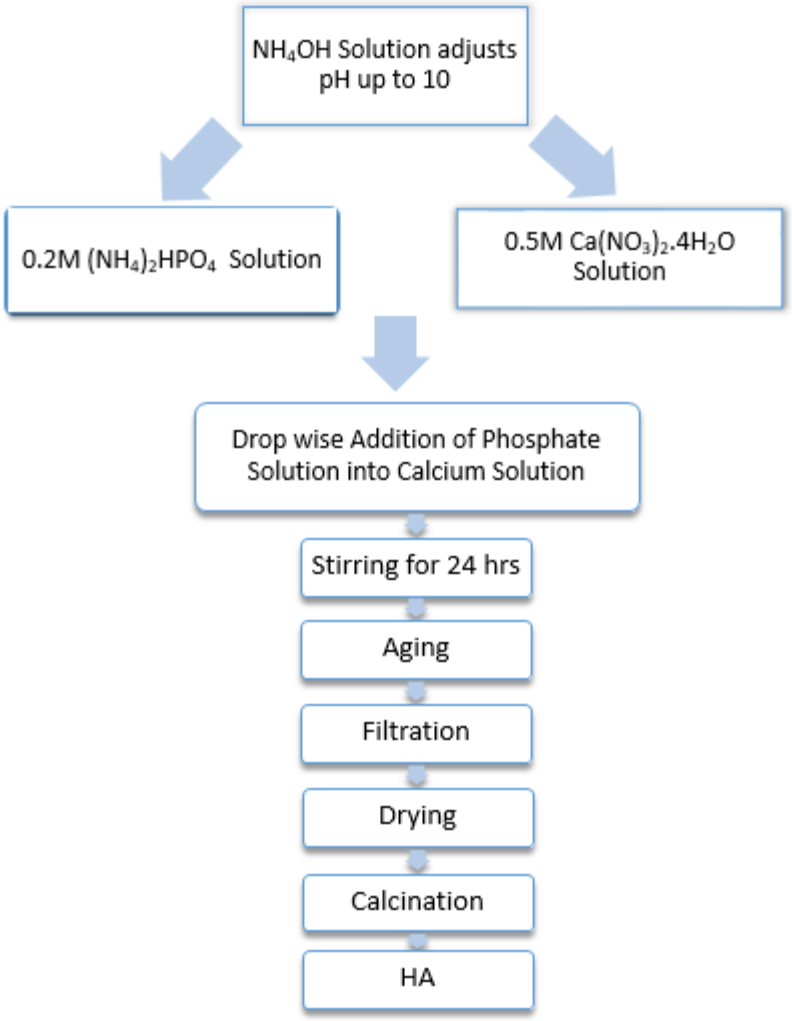


Figure 14 Synthesis of Hydroxyapatite

3.2 Composite Film Preparation

Composite films are prepared by varying weight percentages of hydroxyapatite in the polymer matrix. Due to high viscosity of polymer solution, HA tends to form agglomerates. In order to avoid this problem another set of samples is also prepared containing an additional component CTAB (Cetyl trimethylammonium bromide). CTAB, an antiseptic agent, is also used as a biocompatible surfactant. It lowers the surface tension of the solution and helps to make a better dispersion. So the effect of a surfactant can also be studied and results can be compared with samples without CTAB. Prepared samples are classified in Table 3.

SAMPLES		Wt% Chitosan	Wt% HA
Without CTAB	**CTAB=0.037g**		
A		100	0
B	**B'**	99.5	0.5
C	**C'**	99	1
D	**D'**	95	5
E	**E'**	90	10
F	**F'**	80	20
G	**G'**	70	30

Table 3 Sample Classification

3.3 Procedure

Composite films are prepared by solution casting method. This procedure is preferred over many other processes because it is a very simple, easy and inexpensive process. The drawback is that it consumes a lot of time when vacuum drying is involved. [52]

To prepare composite film, chitosan is dissolved in 2% aqueous acetic acid, purchased from Sigma Aldrich and 99% purity, and the solution is stirred until the entire polymer is dissolved. Then the prepared HA is also dispersed in deionized water. In order to obtain an elastic membrane glycerol is added to the polymer solution. It is purchased from Sigma Aldrich and 99% purity. Glycerol is added 1.5% by volume

which will help in easy removal of membranes from the mold and also protects the shrinkage of membranes. Glycerol is biocompatible so its presence will not cause any damage. But for safety, maximum of glycerol is removed during final drying step. Then in polymer solution, solution of hydroxyapatite is added and the mixture is put on stirring for 12 hours because it is the optimum time to achieve maximum dispersion.

The stirring of solution and its pouring in the mold are shown in Figure 15.

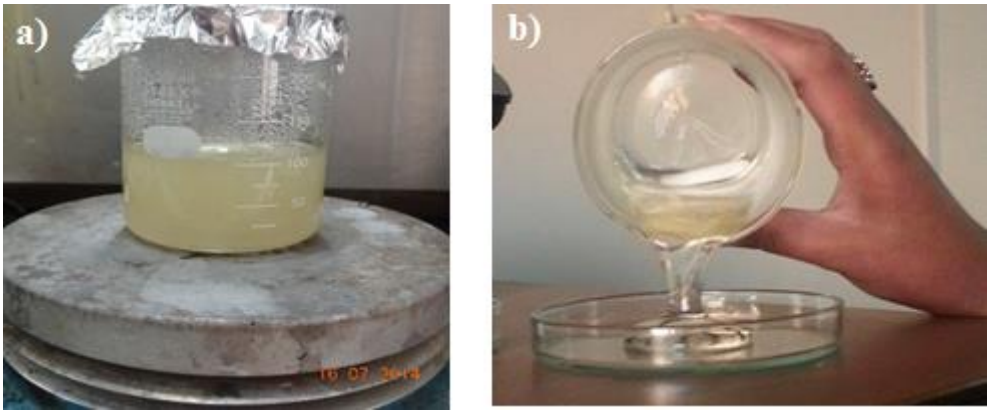


Figure 15 Preparation of Composite Film a) Stirring of Solution of Chitosan and HA b) Pouring of Solution in the Mold

After stirring the solution is sonicated for 25-30 minutes. After giving enough setting time, the solution is poured in glass petri dish and placed under fan for 24 hours and allowed to dry in air. Membranes when dried under forced air will cause maximum removal of acetic acid from the membrane which is not possible if samples are dried in normal air. That's why samples are dried under forced air.

Completely dried membranes can be easily removed from petri dishes using surgical blades. For maximum drying of solvent the samples are placed in vacuum oven at $60^{O}C$ for overnight. The final shape of composite film is shown in Figure 16.

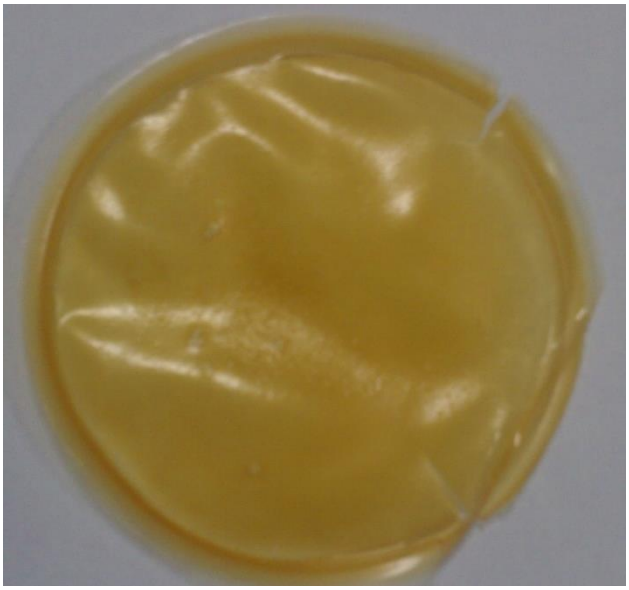


Figure 16 Prepared Chitosan/HA Composite Film

The solution casting process for the preparation of composite CS/HA film is summarized in Figure 17.

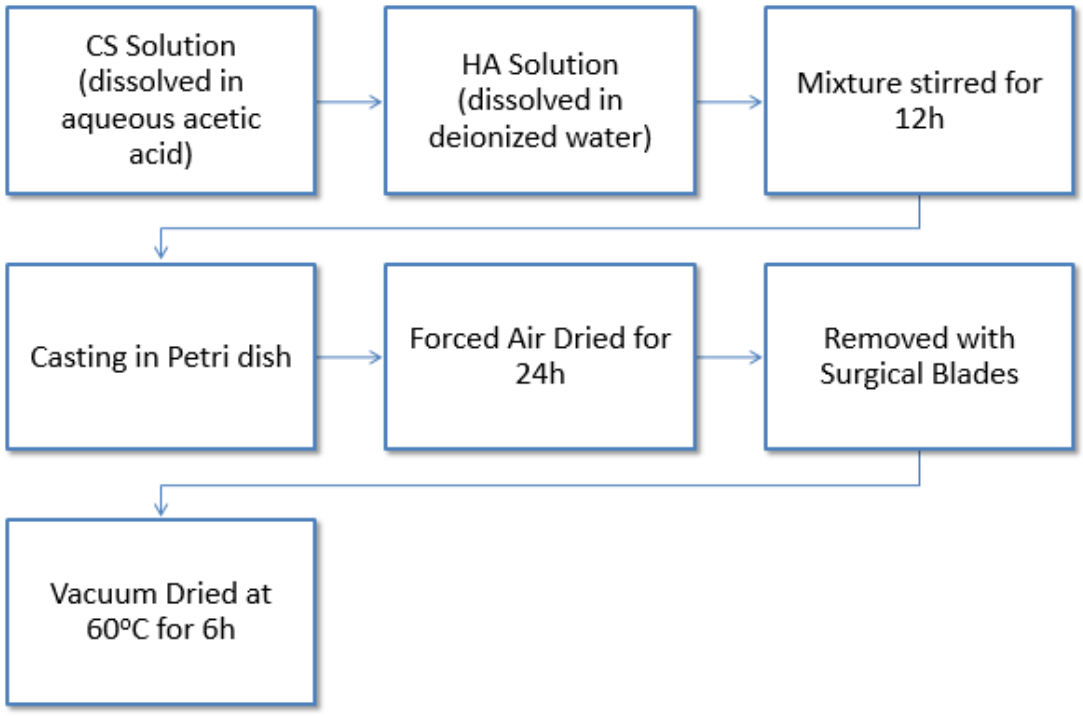


Figure 17 Flowchart for the Synthesis of Composite Film

Chapter 4

Results and Discussion

4.1 Criterion for Barrier Membrane

A biomaterial is defined as a nonviable material used in medical device, intended to interact with biological systems [53]. Any device introduced into the body to address a particular need has to fulfill two major requirements, safety and efficacy. Safety is addressed through a wide selection of in vitro and in vivo assays, designed to address specific aspects of biocompatibility. A design criteria for guided tissue regeneration (GTR) membranes includes biocompatibility, cell exclusion, space maintenance, tissue integration and ease of use [54]. An additional characteristic, biological activity is also being considered for future regeneration devices.

4.1.1 Bio-compatibility

Biocompatibility is defined as the ability of a material to perform with an appropriate host response in a specific situation [55], which means that neither the material adversely and significantly affects the body nor the physiological tissue environment adversely and significantly affects the material. Barrier membrane should be composed of material which should not elicit an immune response, sensitization or chronic inflammation which may interfere with healing and present a hazard to the patient.

4.1.2 Cell-Exclusion

The composite film should behave as a barrier which prohibits detrimental cell types from entering the isolated space next to the root surface. Cell exclusion requires the membrane to separate gingival flap from the maturing fibrin clot in the wound space.

4.1.3 Tissue Integration

A barrier membrane should integrate tissues by preventing rapid epithelial growth on the outer surface of the material or encapsulation of the material. It should also provide stability to the overlying flap. Tissue integration helps in the incorporation of structural elements in the membrane that promote tissue ingrowth which simultaneously achieve cell exclusion.

4.1.4 Space-making

Barrier material is capable of creating and maintaining a space adjacent to the root surface. This will allow the ingrowth of tissue from the periodontal ligament. Space maintenance for regeneration requires mechanical properties and/or structural features allow membrane to withstand the force of flap (tissue tension) or occlusion and prevent collapse of soft tissue and elimination or reduction of wound space.

4.1.5 Clinical Manageability

A composite film should be easy to use. Operator should be able to use this membrane without any difficulty. Is should be available in structures which are convenient to trim and place.

After preparation, the samples are characterized using different techniques to ensure that required properties are achieved. Following techniques are employed and the subsequent results are as followed:

4.2 X-ray Diffraction

It is an ultimate method for the evaluation of structure and lattice parameters of crystalline materials. X-rays of defined wavelength are projected at the sample at a certain angle of incidence. Diffraction occurs from atomic layers of the material, the out of phase waves cancel each other out and in phase interfere constructively. It relies on the distance between the parallel planes of atoms in the material which varies for every material due to variation in atomic sizes forming the crystal structure [56].

Constructive interference produces an intense signal which is detected by detector and the information is obtained by Bragg's Law as:

$$2dsin\theta = n\,\lambda$$

As,

d = inter planar space

θ = angle of diffraction

λ = x-ray wavelength

n = order of diffraction

4.2.1 Blank CS Film

Chitosan exhibits a large hump in the range of 15^{O}-25^{O} and a comparatively weak peak at an angle of 28^{O}. As shown in Figure 18, the XRD pattern of blank chitosan membrane shows a noticeable peaks at about 18^{O} and 28^{O}, respectively.

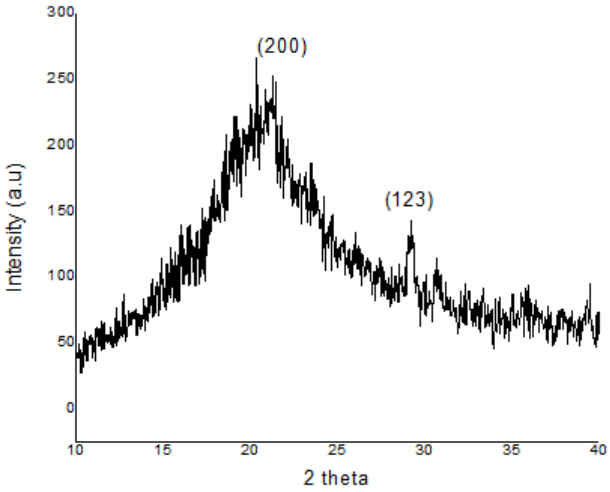


Figure 18 XRD Pattern of Blank CS Film

The reference pattern for chitosan is shown in Figure 19. As the chitosan used is amorphous in nature so only one peak matches while the rest of the peaks are not shown in the XRD pattern.

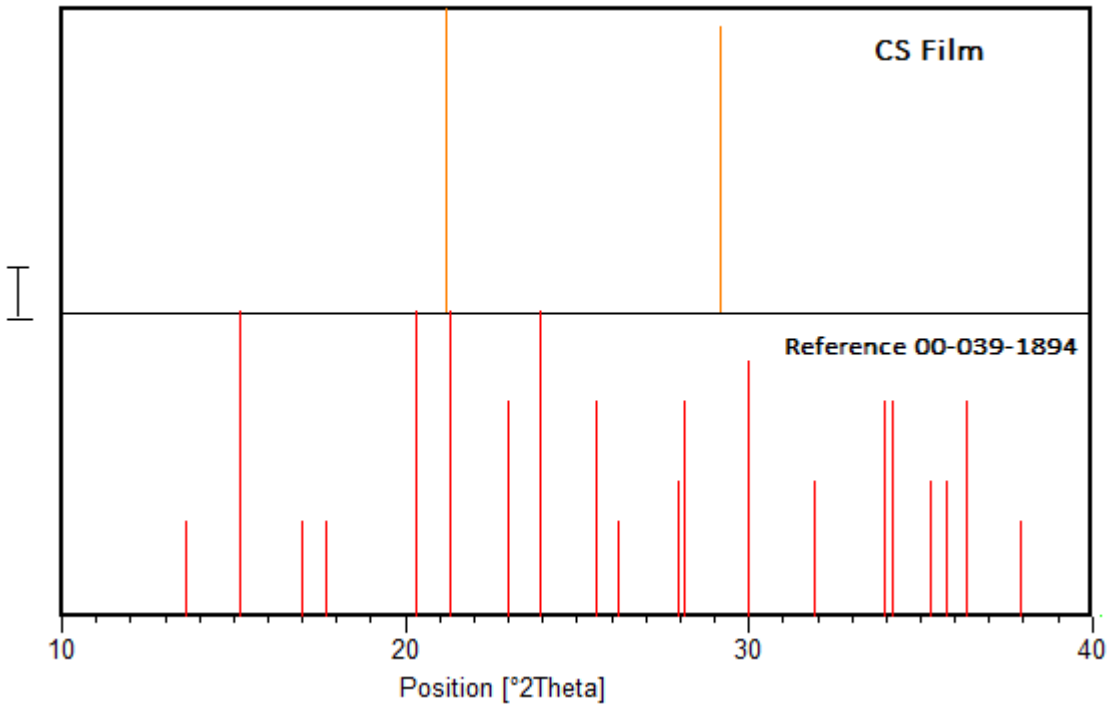


Figure 19 Reference Pattern for CS Film

4.2.2 Pure HA

The XRD pattern of the synthesized n-HA is shown in Figure 20. It shows sharp peaks that indicate better crystallinity at low sintering temperature. The characteristic peaks of crystalline n-HA are observed at an angle of 25.8^{o}, 32.2^{o}, 33^{o}, 34.1^{o}, 35.5^{o} and 40^{o}, which resembles to the n-HA found in natural bone. The XRD pattern of hydroxyapatite is compared with a reference pattern of HA (00-001-1008) in Figure 21. The peaks are in agreement with the peaks found in reference patterns.

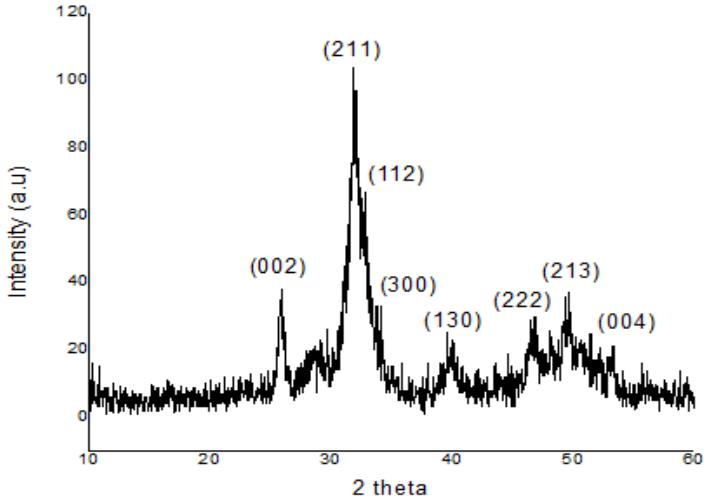


Figure 20 XRD of HA

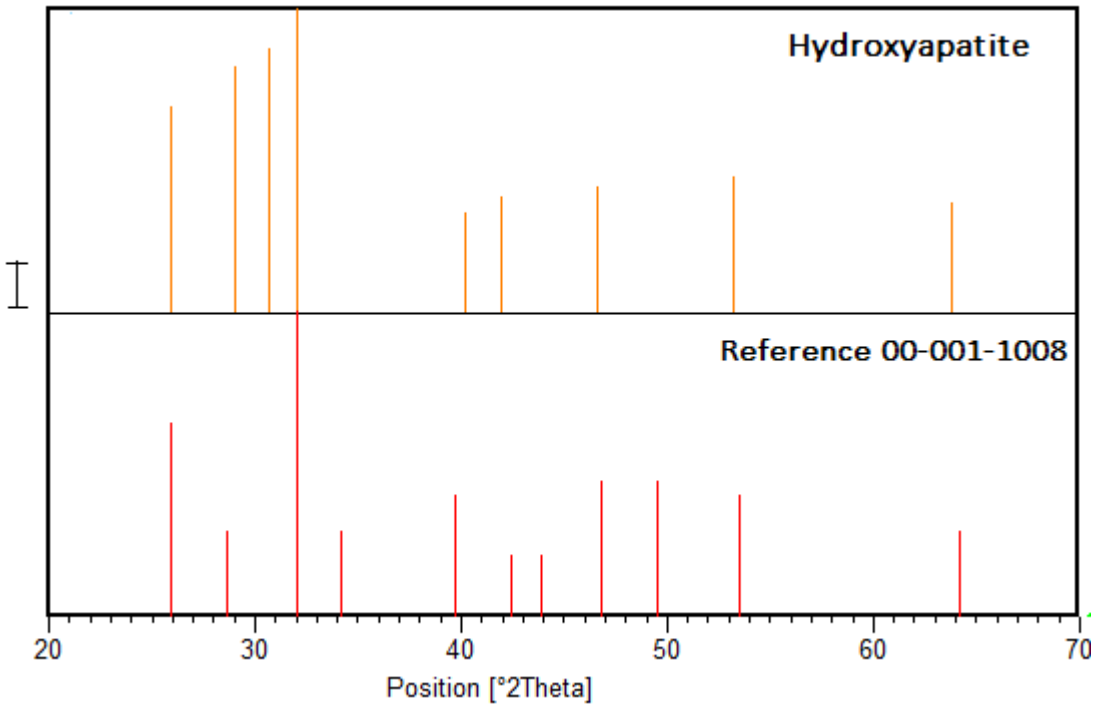


Figure 21 Stick Pattern for Reference Hydroxyapatite

4.2.3 Comparison

In composite films the XRD patterns of samples with and without CTAB remain the same. This is also evident from Figure 22.

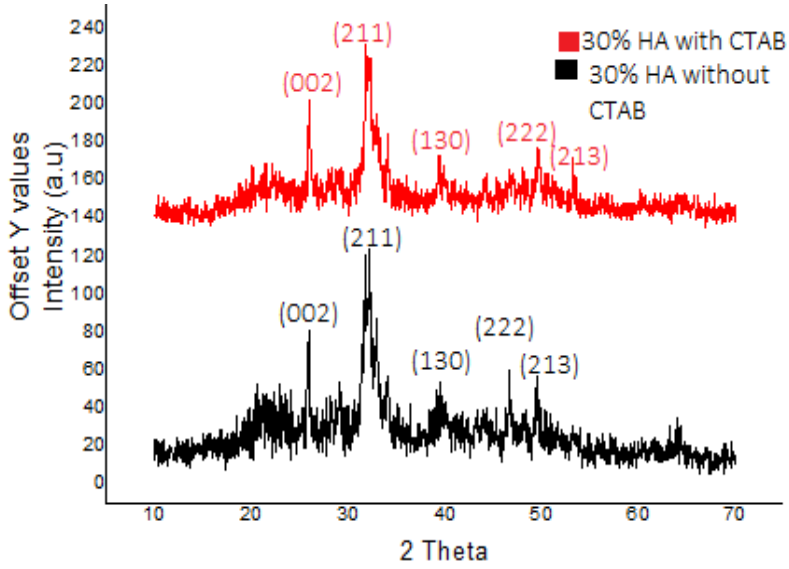


Figure 22 Comparison of Samples with and without CTAB

In composite membrane peaks of chitosan at 10^{O} and 28^{O} become weaken because of the addition of HA into CS film which affects the amount of crystallinity in chitosan. It results in distinct XRD spectra. At low HA content the hump is reduced in intensity but HA peaks are not much visible may be due to the amorphous structure of the composite film. As the HA content reaches 30%, the hump disappears and the HA peaks are clearly seen. It shows that addition of HA has altered the structure of composite film and reduced amorphous nature. Furthermore main characteristic peaks of HA are evidently detected in the composite film which imply that there is not transformation of HA into other Ca-P phase during the synthesis.

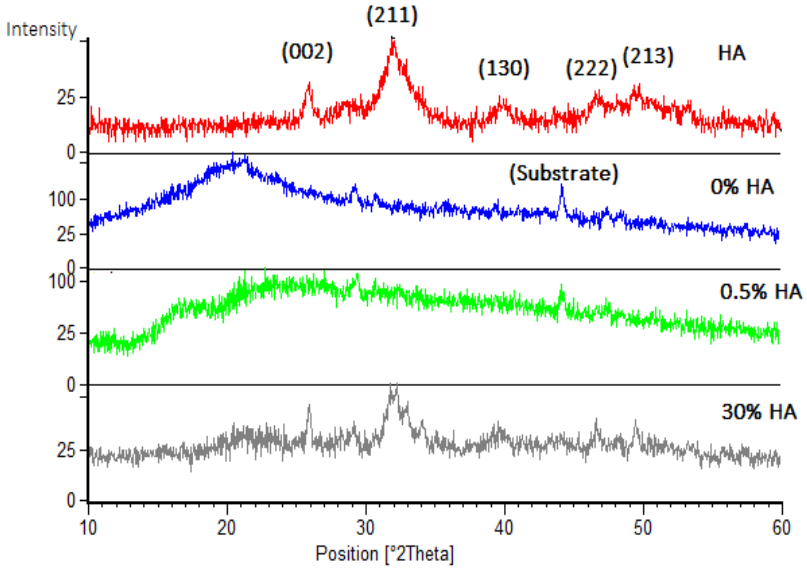


Figure 23 Comparison of XRD Pattern of Samples with varying HA Content

4.3 FTIR

The bond between the atom within a molecule and strong atomic interactions among the molecules are sensitive to the Infra-Red radiations. The bond strength affects the response of different linkages to vary and they response to specific wavelengths. FTIR uses this property of molecules to analyze the possible linkages in a material. This property is mainly applied in the semiconductor industry to confirm the presence of impurity atoms. This nondestructive method is quantitative in case of identification of impurity type and qualitative when concentration is determined.

Nakamoto257 and Griffiths and de Haseth worked on FTIR theory [57]. FTIR analysis in this work was performed by forming pallets of sample solutions in KBr. Testing was performed using Perkin-Elmer Precise spectrum 100, for the range 400 − 4000 cm^{-1}.

4.3.1 Pure CS Film

The FTIR spectra of pure chitosan film is shown in Figure 24. Chitosan consists of three reactive functional groups, primary NH_2 group and primary and secondary OH groups. It shows a very broad absorption band at 3336.04 cm^{-1} because of stretching

vibration of OH bond. This occurs because of the hydrogen bonding present in the chitosan structure. There are characteristic absorption bands which specifically indicate the presence of chitosan. It corresponds to a band at 1047.05 cm^{-1} which indicates the existence of C-O-C bonds while a band at 1559.30 cm^{-1} indicates the existence of NH_2 bond. No extra absorption bands are found which shows that chitosan film is free from foreign particles.

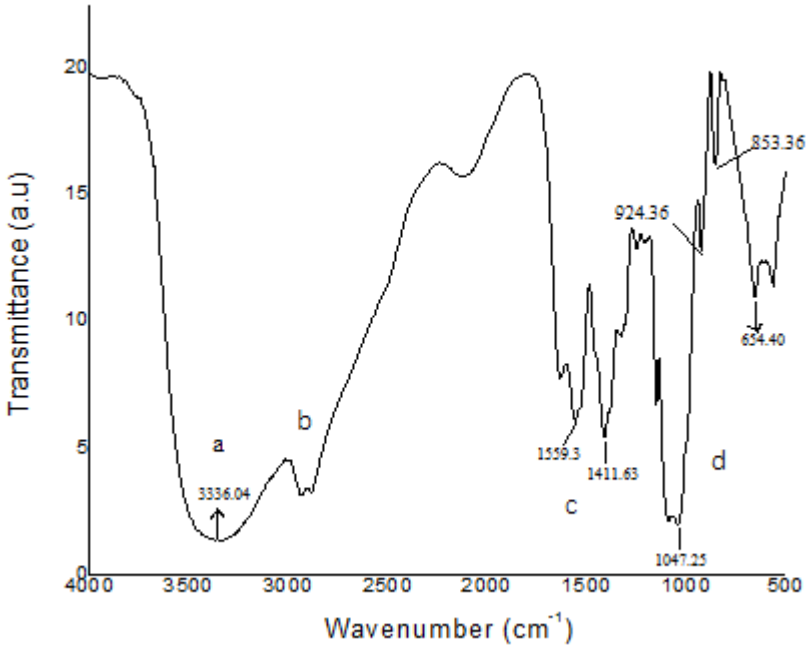


Figure 24 FTIR of Pure CS Film showing characteristic peaks at a) OH groups at 3336.04 cm^{-1} b) C-O-C bonds at 1047.05 cm^{-1} c) NH_2 groups at 1559.3 cm^{-1}

4.3.2 Pure HA

Functional groups associated with hydroxyapatite were identified by FTIR spectroscopy and shown in Figure 25. The ion stretching vibration around 3416.05 cm^{-1} confirms the presence of a hydroxyl group. Phosphate ions comprises of four vibrational modes $\nu 1$, $\nu 2$, $\nu 3$ and $\nu 4$. In the hydroxyapatite FTIR pattern, the $\nu 3$ band exists at three different sites, which are seen in the range of 1096-1056 cm^{-1}. Phosphate $\nu 1$ band is present at 961-2 cm^{-1} and can be observed in the FTIR pattern of hydroxyapatite Phosphate $\nu 4$ band is present in the range of 660-520 cm^{-1} and is a well-

defined and sharp band, observed in HA particles. The functional groups observed are listed in Table 4.

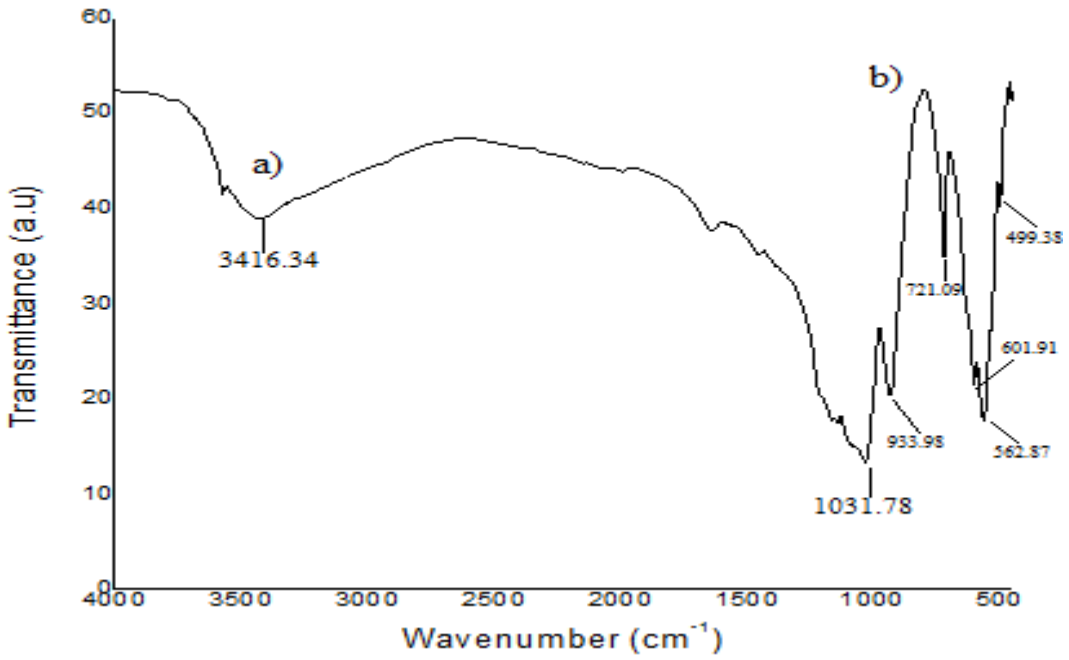


Figure 25 FTIR of Hydroxyapatite showing a) OH bonds at 3416.34 cm^{-1} b) Phosphate groups in the range of 1096-520 cm^{-1}

Wavenumber cm^{-1}	**Stretching Mode**	**Functional Group**
3568	Ion Stretching	OH^-
1461	Asymmetric stretching	CO_3^{2-}
1041	Asymmetric stretching	PO_4^{3-}
869	Out of plane bending mode	CO_3^{2-}
570	Asymmetric bending vibration	PO_4^{3-}

Table 4 Some Important Functional Groups of n-HA

4.3.3 Comparison

Surfactant CTAB is added to the composite films to ensure proper dispersion of HA particles in the chitosan matrix. Samples with and without surfactant are compared and there is no difference observed. CTAB was added in a very small amount which thus does not influences the composition of composite film. Figure 26 shows the comparison.

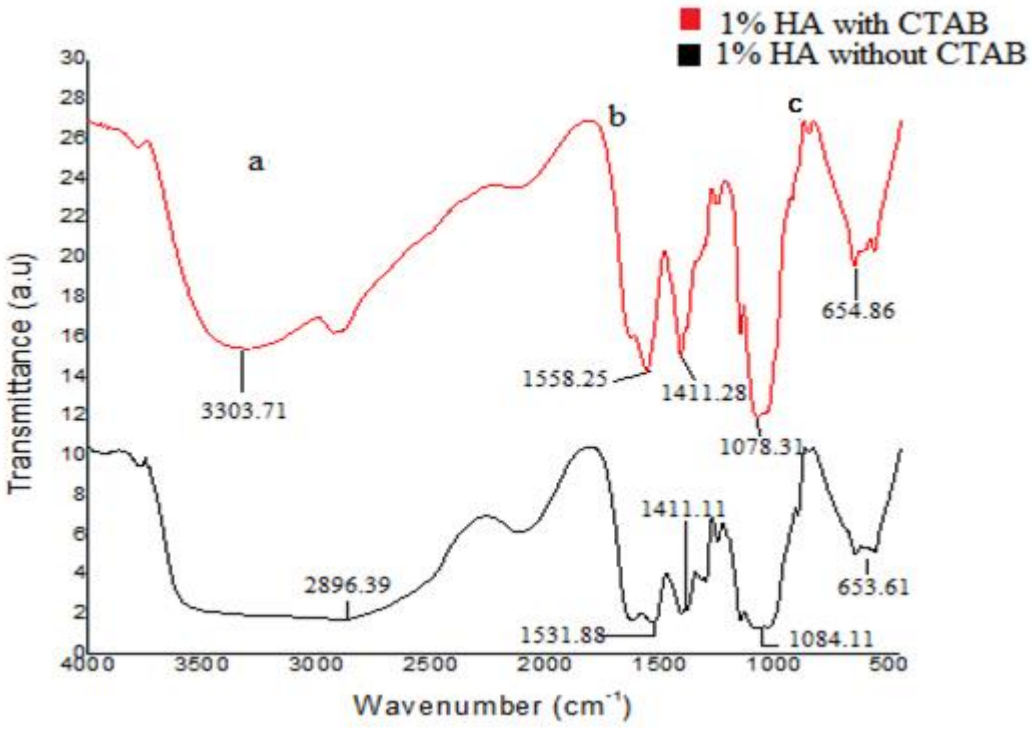


Figure 26 Comparison of Composite films with and Without Surfactant showing a) OH band b) NH_2 groups c) Phosphate groups

From the spectrum of n-HA/CS composite film, it is observed that a characteristic peak of NH_2 group present in chitosan at 1631 cm^{-1} disappears and amide I band at 1548 cm^{-1} becomes less intense, which indicates the possibility of physical interaction (electronic and hydrogen bonding) between the PO_4^{3-} of n-HA and NH_3^+ of chitosan. Conversely, the characteristic band of HA in HA/CS composite film becomes low in intensity and shifts to lesser wavenumbers (formly 1110 cm^{-1} to 1026 cm^{-1}) when compared with the peak present in pure n-HA.

From FTIR spectra (Figure 27) the presence of hydrogen bond and electronic interaction is confirmed in the composite film. There is no sign of chemical bond between n-HA and chitosan. Characteristic peaks of HA are observed in composite films both with and without CTAB which implies that HA retained its phase and is not changed into any other Ca-P phase during the whole process.

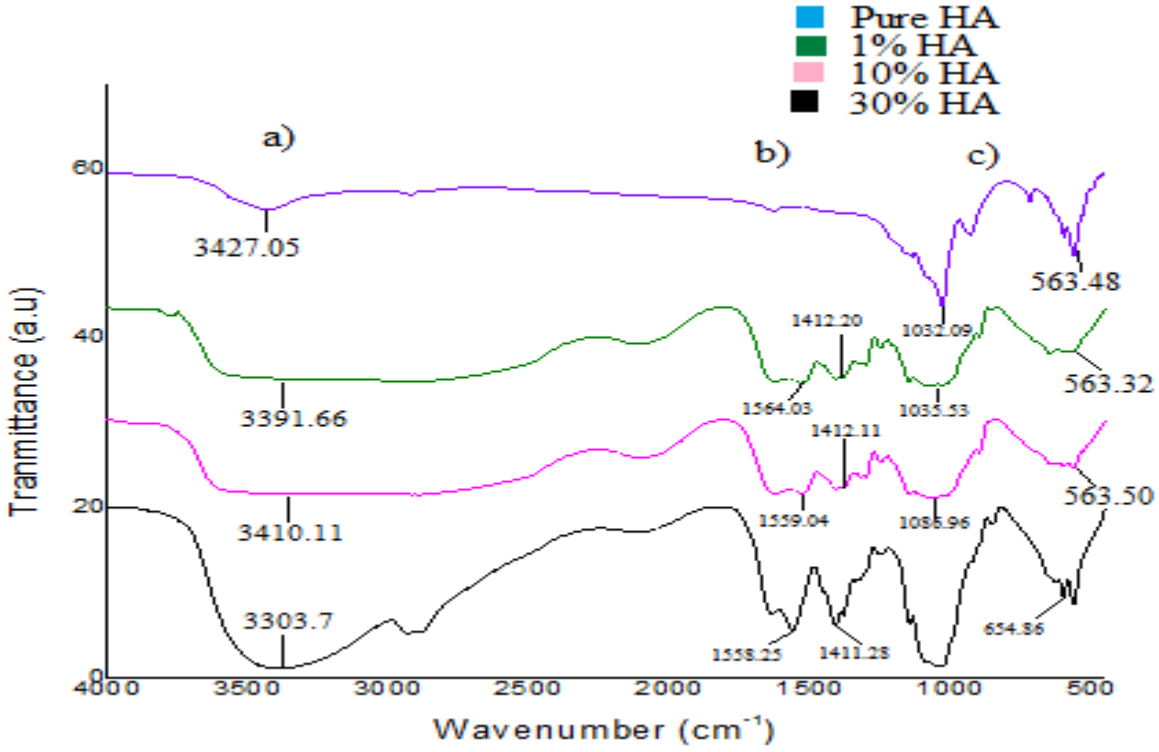


Figure 27 Comparison of FTIR Patterns a) shows OH stretching bonds b) shows amide bonds c) shows characteristic HA bands

4.4 SEM

Scanning electron microscopy is performed by bringing a sample specimen in contact with an electron beam [58]. An electron gun, powered by thermionic emmission or field emision, generates a strong beam of electrons. This beam is focused by magnetic condensers and a rectangular area of the specimen is scanned. Electron beam generates different types of signals comprising secondary electrons, backscattered electrons, photons etc, depending on the type of interaction and sample material. Each type of signal can be detected by specialized detectors. Amplified signals produce a

magnified gray scale image of the surface which is extremely high in resolution (nm range).

SEM was conducted on JEOL scanning electron microscope. Operating Voltage of 5-20 kV was used with a maintained working distance of 10mm. The secondary electron imaging mode was used to produce the higher and lower magnification images.

4.4.1 Pure Chitosan Membrane

The surface morphology of chitosan film is shown in Figure 28. It indicates the formation of a dense membrane with plain texture. No evidence of porosity is found. Surface of the chitosan film is smooth and uneven. Surface characteristics of the membrane plays an important role in allowing water uptake. Morphology of pure CS films suggests that it has the ability to uptake large quantity of water. On the other hand CS/HA composite film shows rougher and uneven structure compared with pure CS film, which prohibits water molecules to be adsorbed. SEM micrographs does not show any structure in neither pure CS film nor CS/HA composite film because of the amorphous nature of chitosan.

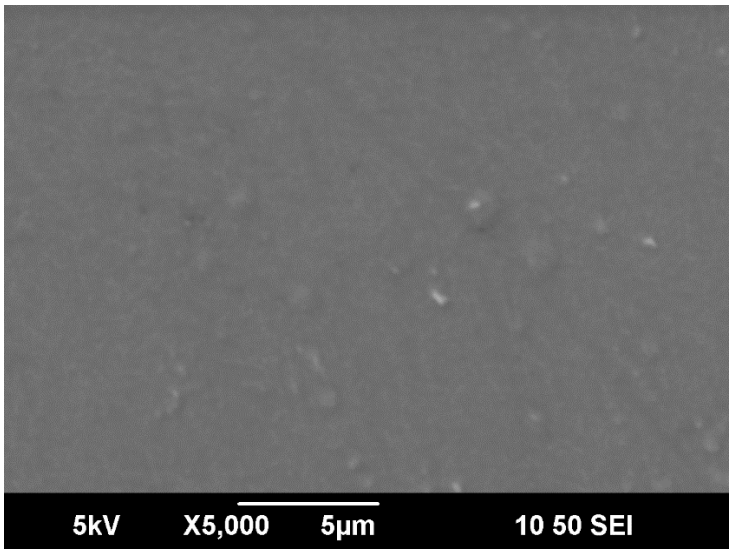


Figure 28 SEM Image of Chitosan Film

The chemical formula for chitosan is $C_6H_{11}NO_4$. The mass percentage of elements is obtained through EDX and is compared with the mass percentage calculated from chemical formula. Both values are almost very close to the calculated ones which

shows that pure chitosan film has been formed without any foreign particle and without any influence of the solvent or plasticizer added.

Elements	Mass Percentage in SEM	Calculated Mass Percentage
C	50.99%	48%
N	9.54%	9.33%
O	39.47%	42.67%

Table 5 Compositional Analysis of Pure Chitosan Film

4.4.2 Pure Hydroxyapatite

The SEM image of HA was obtained by dispersing HA in deionized water. The water was then evaporated and dispersed HA particles on a glass slide are observed. The micrograph shows HA particles, mostly in the form of agglomerates. It is shown in Figure 29.

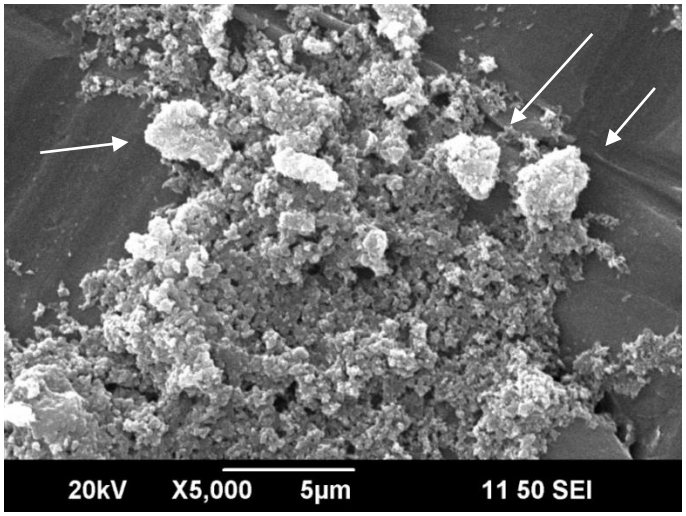


Figure 29 SEM Image of Hydroxyapatite showing large sized agglomerates

The average particle size of HA particles is estimated with the help of scale present in the scanning electron microscope. With the help of that scale the length between the initial and final point of a particle is measured which roughly gives the size of the particle. Average value is calculated from random 10 readings of particle sizes in the same micrograph. The size of prepared HA particles is roughly estimated to be 40nm.

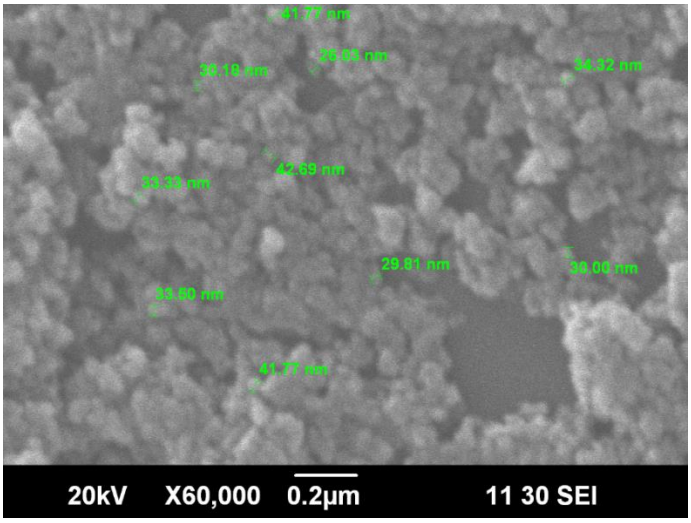


Figure 30 SEM Image of HA Particles for Estimating Particle Size

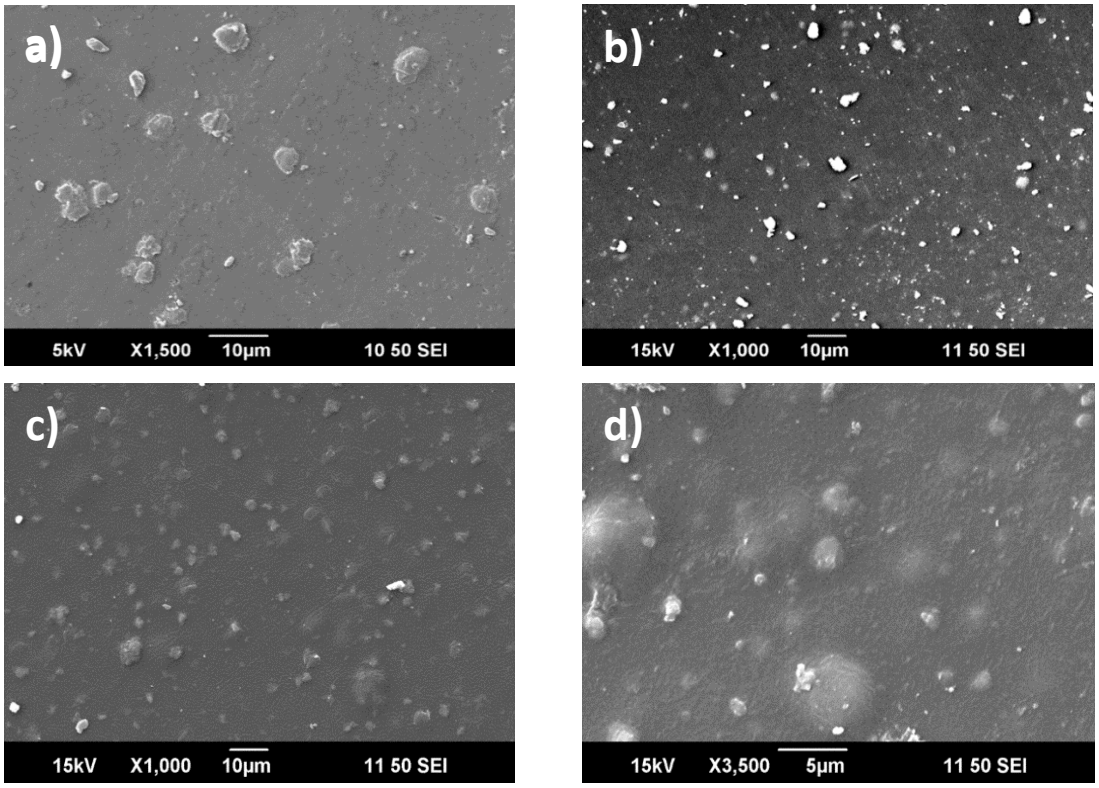
The chemical formula for hydroxyapatite is $Ca_{10}(PO_4)_6(OH)_2$. Mass percentage of elements is calculated from chemical formula and compared with the mass percentage obtained through SEM analysis. The values are very close to the calculated one. Composition has a large effect on its transformation to any other Ca-P phase. So this close composition will ensure that HA particles will not transform into any other Ca-P phase. The comparison is shown in Table 6. The theoretical value for Ca/P is 1.67 and the calculated value for the prepared HA particles is 2.68. The huge difference in Ca/P ratio is due to the sintering temperature. Theoretical value is achieved at high sintering temperatures. Since the temperature employed for composite films is low i.e., $500^{O}C$ so the ratio increases.

Elements	Mass Percentage in SEM	Calculated Mass Percentage
Ca	44.28%	40%
P	16.5%	18.5%
O	39.21	41.5%

Table 6 Compositional Analysis of Hydroxyapatite Particles

4.4.3 Composite Films without Surfactant

Surface morphology of pure chitosan film is rough and uneven. The surface roughness of composite films, compared with the pure chitosan film, gradually increases when HA content is added in a very low amount. HA particles that are dispersed all over the composite film are held responsible for the increase in roughness. As the HA content becomes higher i.e,. 20% and 30% by weight, the surface becomes rougher because HA particles precipitate during the fabrication of composite films. HA particles are dispersed throughout the polymer matrix but mainly in the form of agglomerates or clusters. Clusters are formed due to the anti-plasticizing effect of glycerol. That's why in high HA content membranes, HA is found mostly in the form of agglomerates. Figure 31 shows SEM micrographs of composite membranes.



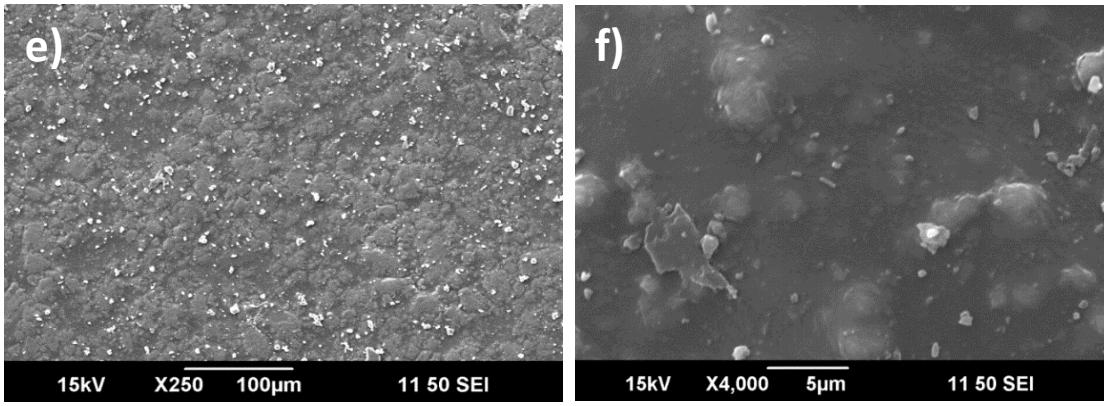
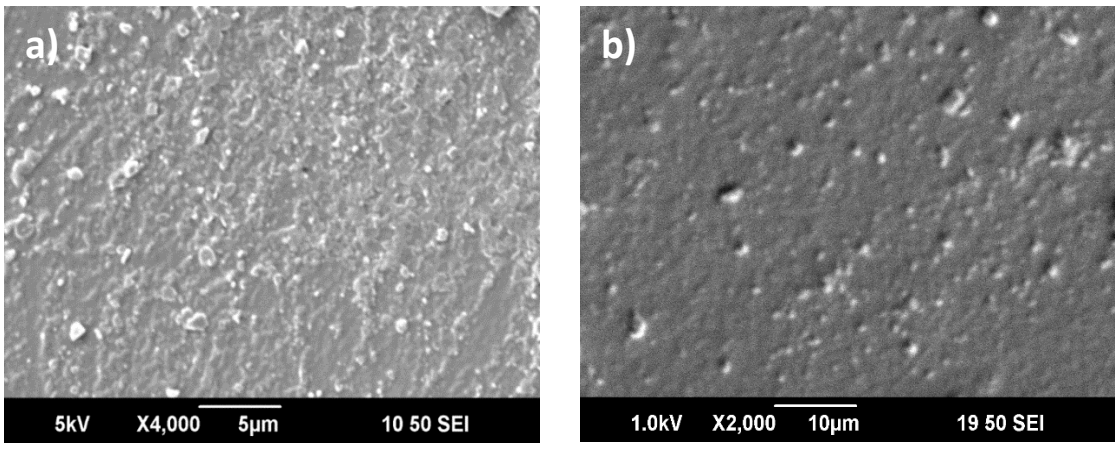


Figure 31 SEM Images of CS/HA Composite Films having dispersed HA content a) 0.5% HA b) 1% HA c) 5% HA d) 10% HA e) 20% HA f) 30% HA

4.4.4 Composite Films with Surfactant

CTAB is a biocompatible surfactant which is added to lower the surface tension of the solvent. In the composite films it is added to enhance the dispersion of HA. With the addition of CTAB the dispersion is improved slightly. The surface roughness of composite films increases as the HA content is increased. The roughness on lower side of the composite film is more compared with the upper side. Roughness is attributed to the addition of HA particles. HA, despite of the addition of CTAB, is dispersed mostly in the form of clusters throughout the polymer matrix. But the cluster size is lowered. The prepared films are dense and no sign of porosity is found in any of the composite film. The images are shown in Figure 32.



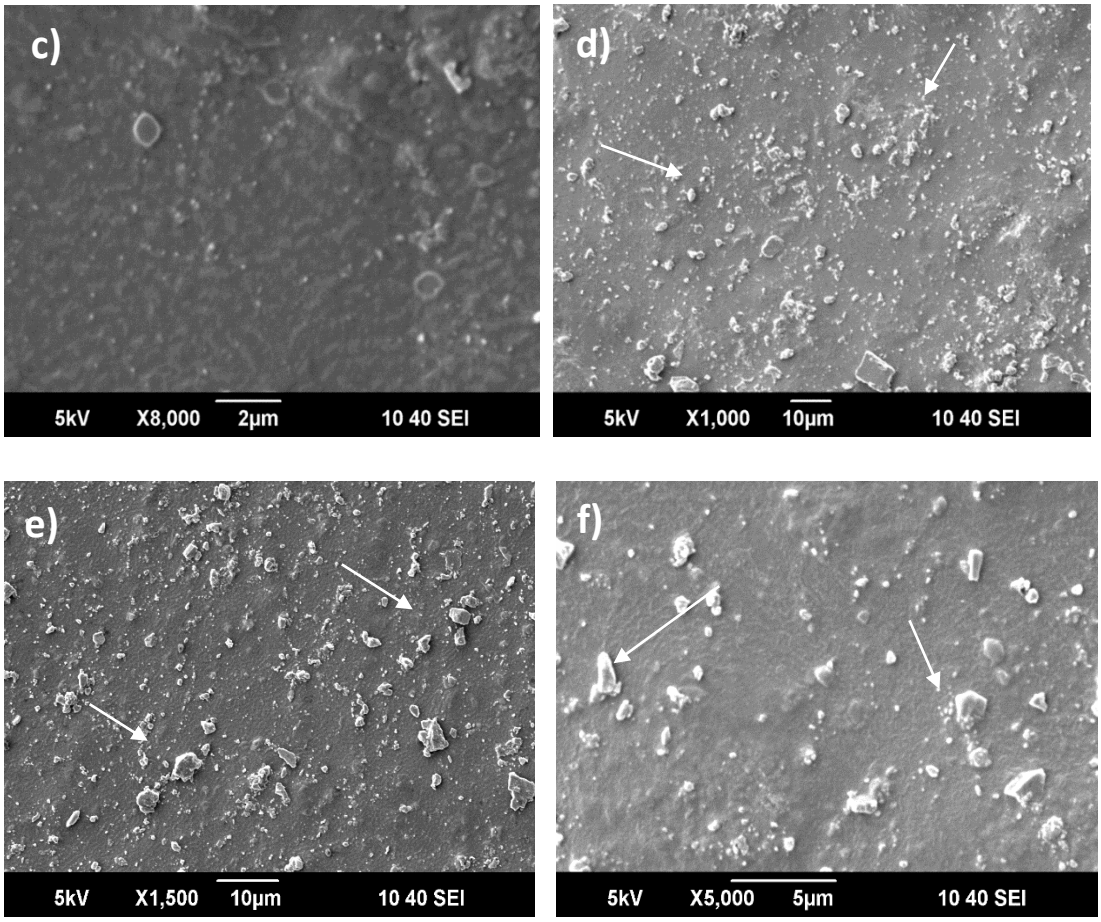


Figure 32 SEM Images of Composite Films with Surfactant a) b) c) d) e) 20% HA shows porosity and uniform dispersion of HA content f) mostly shows agglomerates of 30 wt% HA

4.4.5 Fracture Analysis

After performing tensile test the cross-section of fractured samples is examined through scanning electron microscope. The surface texture is very rough and uneven and there is no sign of porosity. A very good affinity of nano HA particles with the chitosan matrix is observed. Due to fracture particles are also broken along with the matrix which indicates adequate load distribution. Mechanical strength of chitosan film alone is very low. HA particles are added which will bear the maximum load and the chitosan matrix will distribute the load throughout the structure. This will enhance the mechanical properties of composite films. This is also verified by SEM micrographs of composite films with varying HA content, as shown in Figure 33.

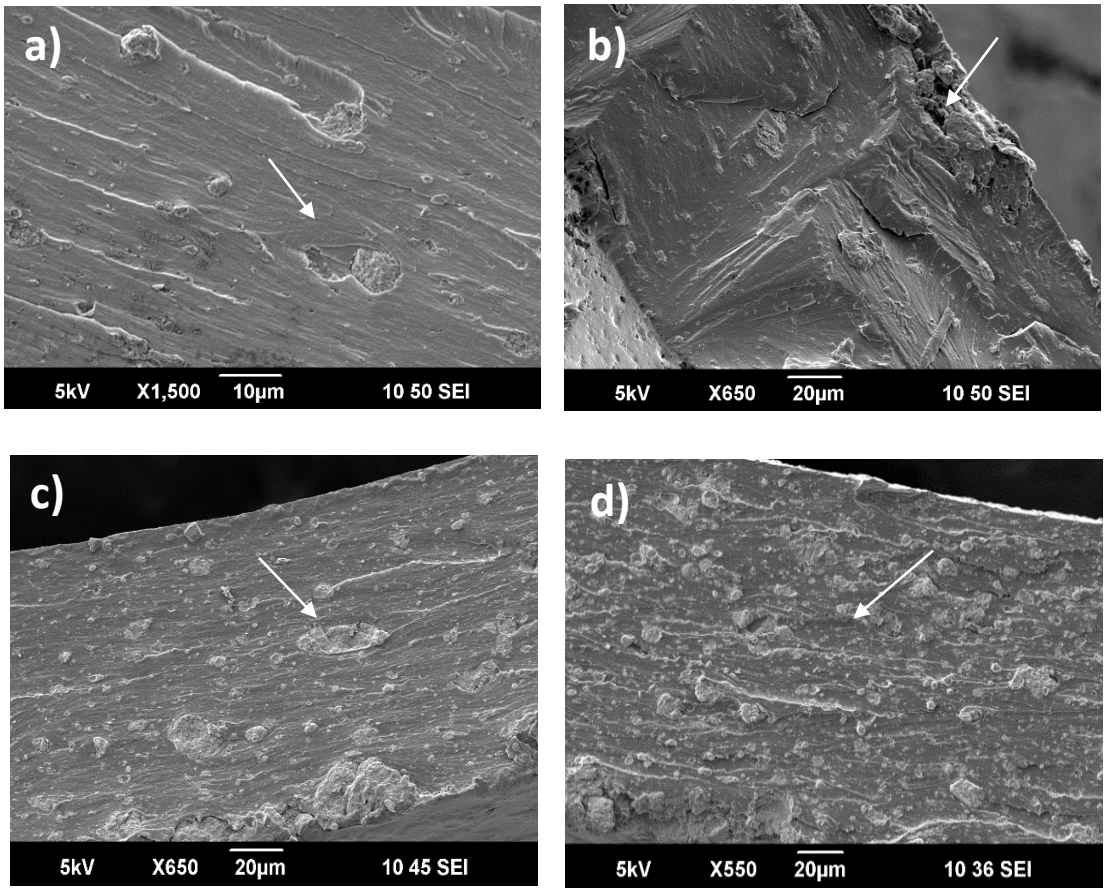


Figure 33 Fracture Analysis of Composite films in which arrows indicate particle separation along with the matrix in the samples containing HA weight percentage of a) 5% HA b) 10% HA c) 20% HA d) 30% HA

4.5 Thermo Gravimetric Analysis

TGA determines change in weight when the temperature is varied. It depends on precise measurements of weight, temperature, and the rate at which temperature changes. DTA is a technique based on the thermodynamics of materials and is used to classify the regions where the variation of energy absorption and release takes place [59].

The two techniques performed simultaneously can be used to attain the following information:

- Melting Point/ Boiling Point
- Glass transitions and solid-solid transitions

- Volatile content
- Aging/lifetime breakdown,
- Thermal stability
- Phase changes

The testing was performed on the Perkin-Elmer Diamond TG/DTA at School of Chemical and Materials Engineering, NUST. The samples were kept under a stable nitrogen atmosphere in the temperature range of 25^{o}C – 450^{o}C. Heating was done at a constant rate of 10^{o}C/min.

4.5.1 Pure Chitosan Film

Decomposition behavior of pure CS film and HA/CS composite films at high temperature is analyzed by TGA. As shown in Figure 34 it is noticed that pure chitosan membrane shows three stage degradation behavior. In case of pure chitosan membrane the solvent is aqueous acetic acid which is removed during vacuum drying, but some of the water content still remains. When heating of membrane is started the initial weight loss is due to evaporation of water. This first event of mass loss, removes bond water in the membrane, occurs in the temperature range of 95-100^{o}C. Percentage mass loss is calculated to be 11.18%. The second event of weight loss occurs in the temperature range of 150-270^{o}C. Maximum mass loss occurs during this event which is calculated to be 47.32%. Chitosan film degrades during this temperature range and 265^{O}C is considered to be the temperature at which chitosan thermally degrades. Third event of loss in mass occurs in the temperature range of 270-450^{o}C. Percentage mass loss is calculated to be 7.66%. Mass loss during third stage is due to the removal of carbonaceous material from the chitosan membrane.

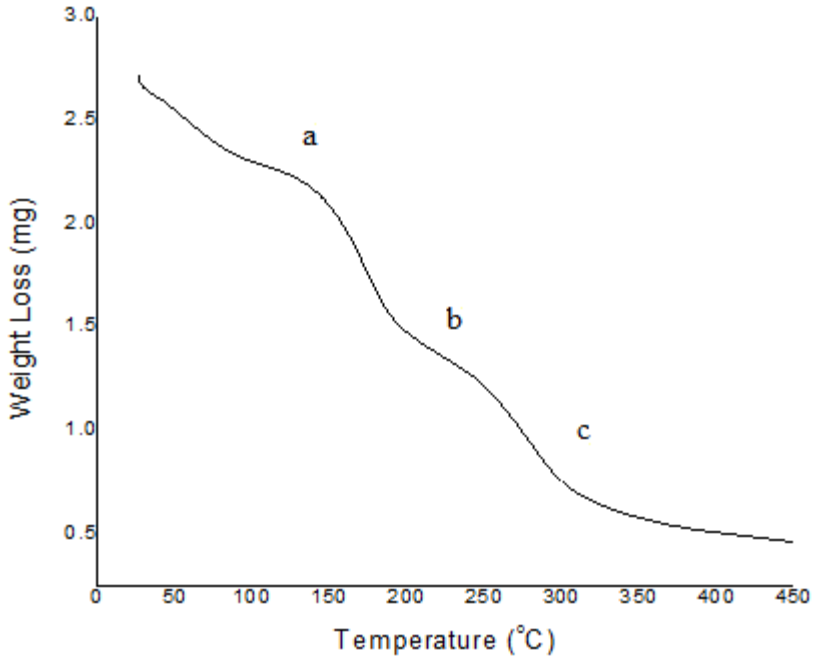


Figure 34 TGA of Pure CS Film showing three events of mass loss a) First in the range of 95-100^{O}C b) Second in the range of 150-270 ^{O}C c) third in the range of 270-450 ^{O}C

4.5.2 Low HA Content Films

HA/CS composite films are also prepared in aqueous acetic acid solvent. There is water bond to the composite film as well as to the hydroxyapatite. So first event of mass loss is simply the removal of water bond to the composite film. This happens in the temperature range of 34-72^{o}C with a percentage mass loss of 12.3%. Second event of mass loss take place in the temperature range of 150-280^{o}C with a percentage mass loss of 48.8%. The third event of mass loss occurs in the range of 280-450^{o}C with a percentage mass loss of 18.35%. The amount of HA has a large influence on the degradation pattern of composite membrane. It tends to stabilize the composite film. Degradation temperature (T_d) of HA/CS membrane is gradually increased from 265^{O}C to 281^{O}C as the HA weight percentage is increased from 0% to 10%. A physical interaction (hydrogen bond and electronic interaction) is present between the components which results in increased T_d. Blending of n-HA in the chitosan matrix could raise the T_d of chitosan due to the physical interaction between the components. TGA of composite films containing 1% HA content is shown in Figure 35.

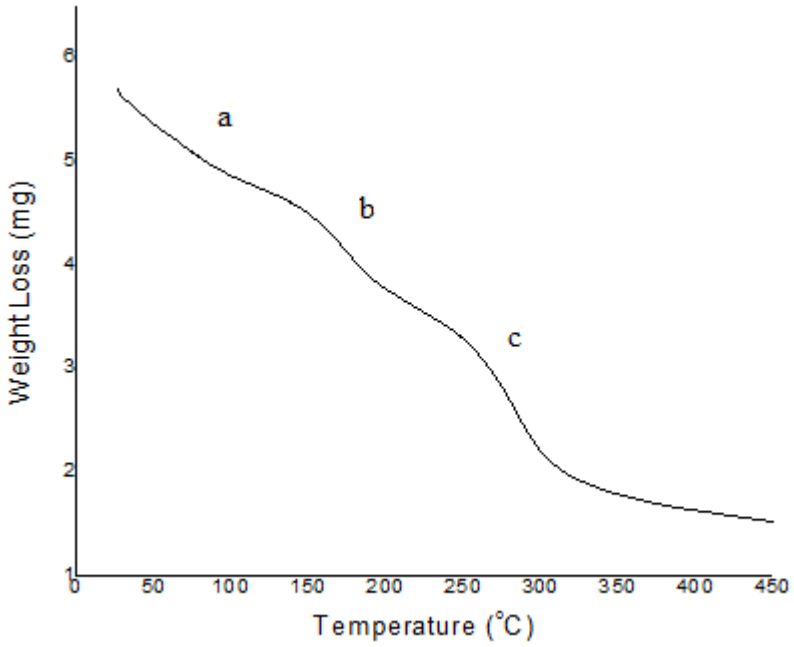


Figure 35 TGA of Composite Film Containing 1% HA showing three events of mass loss a) First in the range of 95-100^{O}C b) Second in the range of 150-280 ^{O}C c) third in the range of 280-450 ^{O}C

4.5.3 High HA Content Film

Decomposition behavior of composite films containing higher content of HA i.e., 30 wt% is shown in Figure 36. It again shows three events of mass loss. First happens in the temperature range of 34-72^{o}C with a percentage mass loss of 12.37%. Second event takes place in the temperature range of 110-280^{o}C with a percentage mass loss of 25%. The third event happens in the temperature range of 280-450^{o}C with a percentage mass loss of 19.4%. First and second events show water removal while third event shows the degradation of composite film.

An interesting phenomenon is observed when the HA content is increased till 30%. The decomposition temperature of n-HA/CS composite film decreases dramatically from 281^{O}C to 273^{O}C. Excessive amount of HA in the composite film is held responsible for destroying the structure of film as well as the bonding between the components. A relatively higher decomposition temperature of n-HA/CS composite film compared with pure chitosan film implies that there is existence of physical

bonding (hydrogen bond and electronic interaction) among the components of composite film, which is in consent with the observations of FTIR.

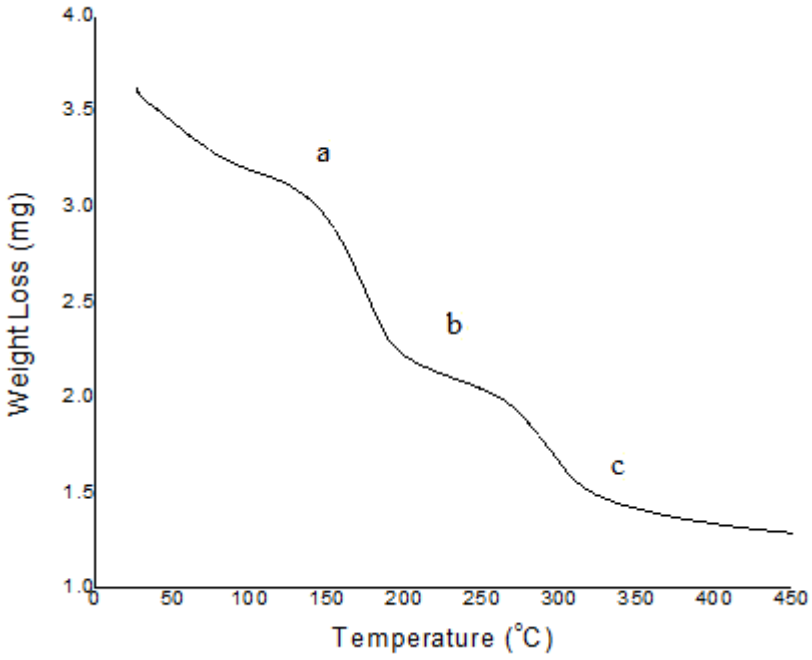


Figure 36 TGA of Composite Film Containing 30% HA showing three events of mass loss a) First in the range of 95-100^{O}C b) Second in the range of 150-280 ^{O}C c) third in the range of 280-450 ^{O}C

4.5.4 Variation in Decomposition Temperature

From the results of TGA it is indicated that HA has a huge influence on the decomposition temperature of the composite films. Addition of HA in low concentration shows a gradual increase in the decomposition temperature. But when HA content is increased up to 30% by weight the decomposition temperature decreases. Figure 37 shows the variations in decomposition temperature. Chitosan degrades in vivo. To be used as a barrier membrane, a prime requirement is that it should stay stable over a range of temperature. The results of TGA are suggestive that by controlling the HA content, the degradation temperature of the composite films can be enhanced. This will lead to a more stable composite film stable. Increase in HA weight percentage

beyond 10% decreases the stability of the composite film and decreases the degradation temperature.

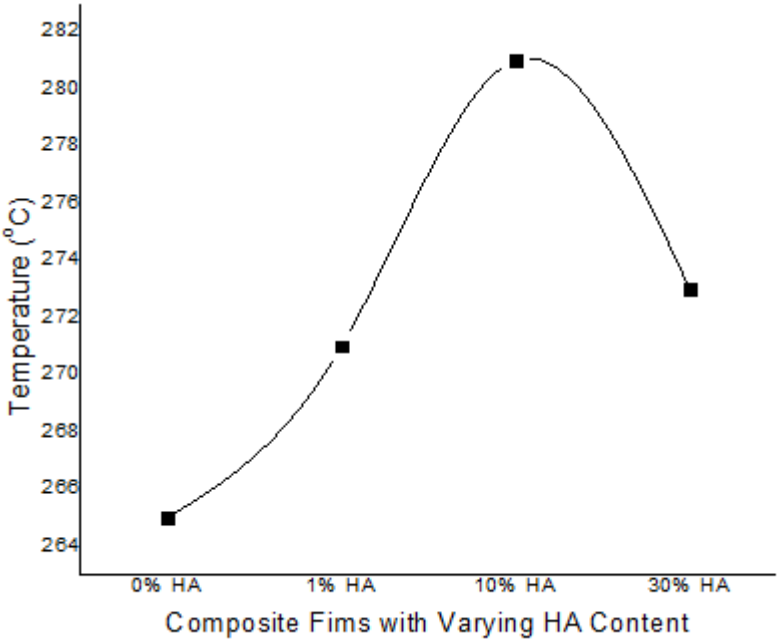


Figure 37 Variations in Decomposition Temperature

4.6 Differential Scanning Calorimetry

Differential Scanning Calorimetry is a thermo-analytical technique in which sample and reference are maintained at same temperature and heat required to maintain the temperature of a sample and reference is measured with respect to temperature. This technique is based on a simple principle which is that due to heat sample will show a physical transformation like a phase change. Depending on the type of event, exothermic or endothermic, more or less heat will flow through it than the reference to maintain both at the same temperature. By calculating the difference in heat flows between the sample and reference, DSC measures the amount of heat absorbed or released during such transitions.

DSC can be used to measure a number of characteristic properties of a sample. Using this technique it is possible to observe

- Glass transition temperature T_g
- Melting temperature T_m
- Crystallization temperature T_c
- Chemical reactions like oxidation etc.
- Curing of polymers

This test was performed on the DSC at Institute of Space Technology, Islamabad. The samples were kept under a stable nitrogen atmosphere in the temperature range of 25°C – 300°C. Heating was done at a constant rate of 10°C/min.

4.6.1 Pure CS Film

DSC curve for the pure chitosan film is exhibited in Figure 38. Chitosan is an amorphous polymer so the DSC curve does not show any peak for the crystallization. The CS film revealed an endothermic event at 96 °C. This event is supported by TGA which also shows a peak at this temperature. This occurs due to the loss of water associated with the hydrophilic groups. Next endothermic event takes place at 265°C which is again confirmed by TGA which shows maximum weight loss of 47.32% at this temperature. This is attributed to the thermal decomposition of amino groups of polymer and removal of carbonaceous groups. DSC curve shows glass transition temperature T_g at 128°C. Pure chitosan film will behave in a brittle manner when used in ambient temperature. When the temperature of the film rises above the T_g, it will become more rubber-like. Thus, knowledge of T_g is essential in the selection of materials for a specific application.

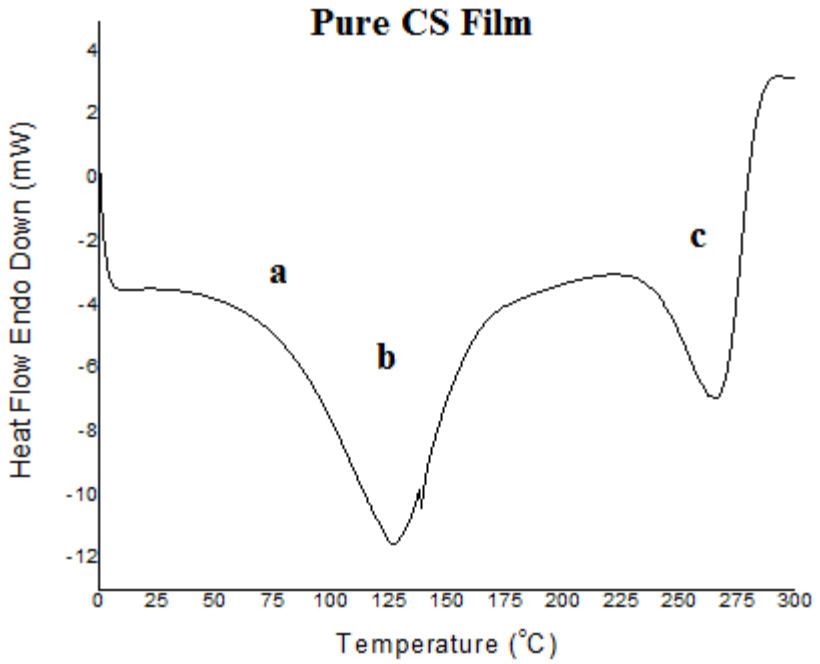


Figure 38 DCS Curve for Pure CS Film showing a) Water Loss at 96°C b) Glass Transition Temperature at 128°C c) Degradation Temperature at 265°C

4.6.2 Composite Films

DSC curve for CS/HA composite film with very low HA content, 1% by weight, is shown in Figure 39. Same results are obtained as were obtained from TGA. Initial water bond to the composite film is lost at 55°C. Second endothermic event occurs at 118 °C which shows glass transition temperature. With the addition of HA T_g has decreased significantly compared with the T_g value of the pure chitosan film. Third endothermic event occurs at 270 °C which is the degradation temperature of the film. Small amount of HA helps in increasing the degradation temperature.

DSC curve for CS/HA composite film containing 30% HA is shown in Figure 40. The results are in agreement with the TGA. Initial water bond to the film starts to evaporate at 55°C. Degradation temperature of the film decreases to 271°C and an endothermic curve verifies it. The glass transition temperature is increased to 251°C which shows that high HA content increases the glass transition temperature.

Overall DSC curves show small shifts and changes in intensity. This confirms the presence of physical interaction between HA particles and the chitosan matrix.

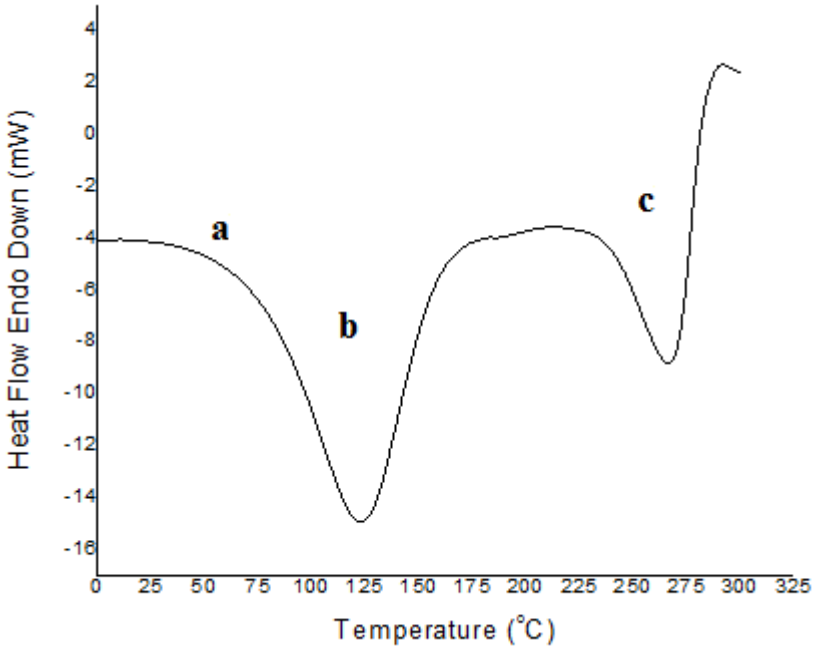


Figure 39 DSC Curve for Composite Film Containing 1% HA Content a) Water loss at 55°C b) Glass Transition Temperature at 118°C c) Degradation Temperature at 260°C

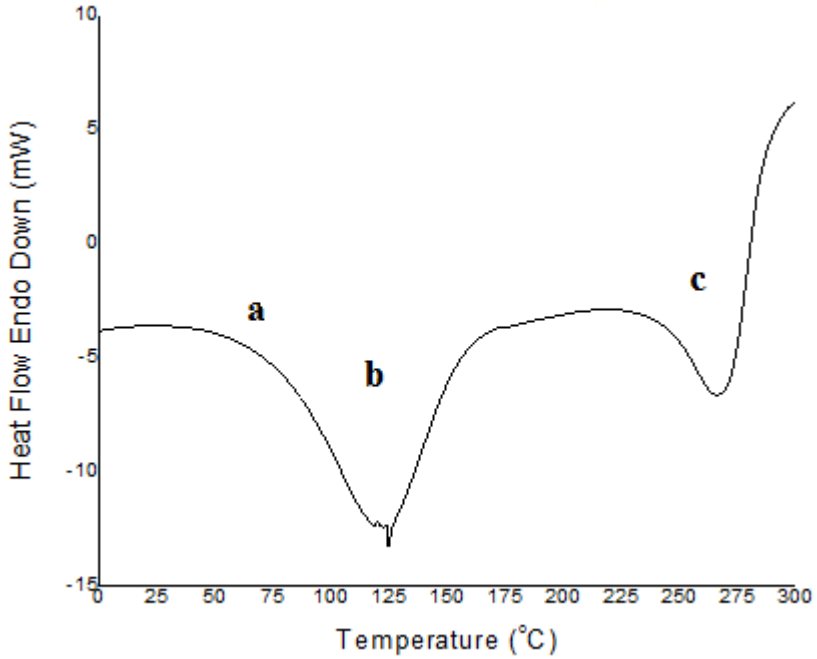


Figure 40 DSC Curve for Composite Film Containing 0.5% HA Content a) Water loss at 55°C b) Glass Transition Temperature at 125°C c) Degradation Temperature at 271°C

4.6.3 Effect on Glass Transition Temperature

A change in glass transition temperature is observed for the composite films. Pure chitosan films shows T_g at 128^{o}C. When HA is added in a very small amount i.e. 0.5% by weight the T_g decreases sharply to 118^{o}C but as the HA content is increased the T_g also increases and for composite film containing 30% HA by weight T_g comes very near to the value obtained for pure chitosan film.

T_g is greatly affected by the amount of plasticizer. Glycerol is added as a plasticizer in the composite film. Glycerol penetrates in the chitosan matrix which reduces cohesive forces between the chitosan chains and develops polar attractive forces between polymer chains and itself. This decreases Tg. So when small amount of HA is added, glycerol is enough in quantity to attach to polymer chains and thus decreases T_g significantly. But as the amount of HA is increased, a competition occurs between HA and glycerol for the attachment to the chitosan chains. Due to huge amount of HA glycerol gets less opportunity to attach and thus acts as an anti-plasticizer. That's why T_g increases when the amount of HA is increased.

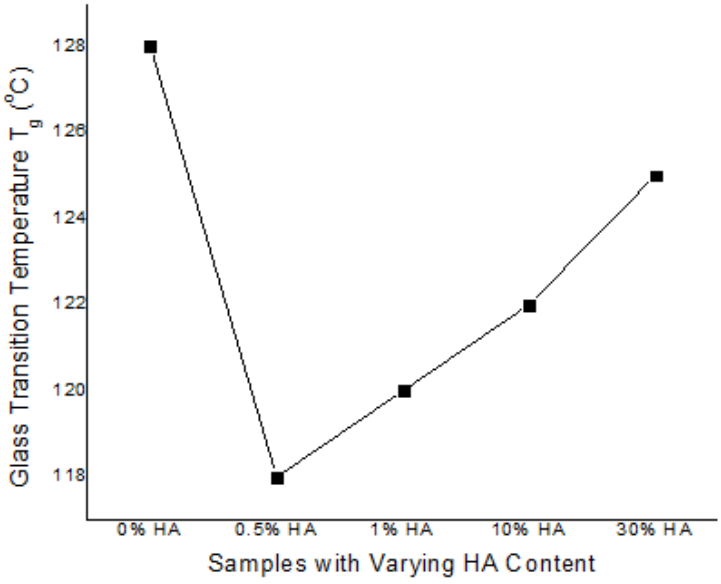


Figure 41 Effect of HA on Glass Transition Temperature of Composite Films

4.7 Mechanical Properties

Pure HA lacks mechanical properties. It is not suitable for load bearing applications. So it is always combined with another material to enhance its properties. Pure chitosan membrane is also not mechanically very strong but it is expected that the composite membranes will show good strength. In order to test this hypothesis samples for tensile test were prepared. DIN 53 504 – S3A standard is followed according to which the dimensions of samples are as followed:

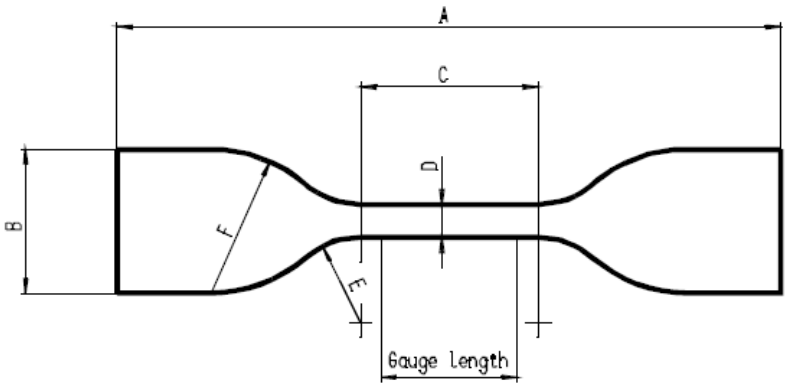


Figure 42 Tensile Test Specimen

Category	Dimensions (mm)						
	A	**B**	**C**	**D**	**E**	**F**	**Gauge**
III [c]	Mini 50	8.5 ± 1.0	16 ± 0.5	4 ± 0.05	7.5 ± 0.3	10 ± 0.3	10 ± 0.5

Table 6 Standard Dimensions for Tensile Test of Composite Films

For every sample the strain rate was set at 20N/min. Three representative samples were prepared for each composite films and following were the results obtained. The values obtained are average values of the repeated tests.

4.6.1 Tensile Strength

From the stress-strain curve, tensile strength for each specimen is calculated. As the HA particles are added they tend to bear more load which is at the same time distributed by the matrix. Therefore tensile strength shows a gradual increase with increasing HA content. The specimen with 10% HA shows maximum tensile strength which indicates a good dispersion of HA particles in the matrix, and efficient load bearing ability. But as the content of HA is increased beyond 10%, the HA particles tends to form agglomerates. Such clusters are very fatal to the strength of the composite. So at very high content of HA, agglomerates are held responsible for decreasing the tensile strength

Samples containing CTAB show increasing trend of tensile strength but the values at high HA content are higher than composite films without CTAB. It can be attributed to more uniform dispersion of HA in this sample which leads to good strength.

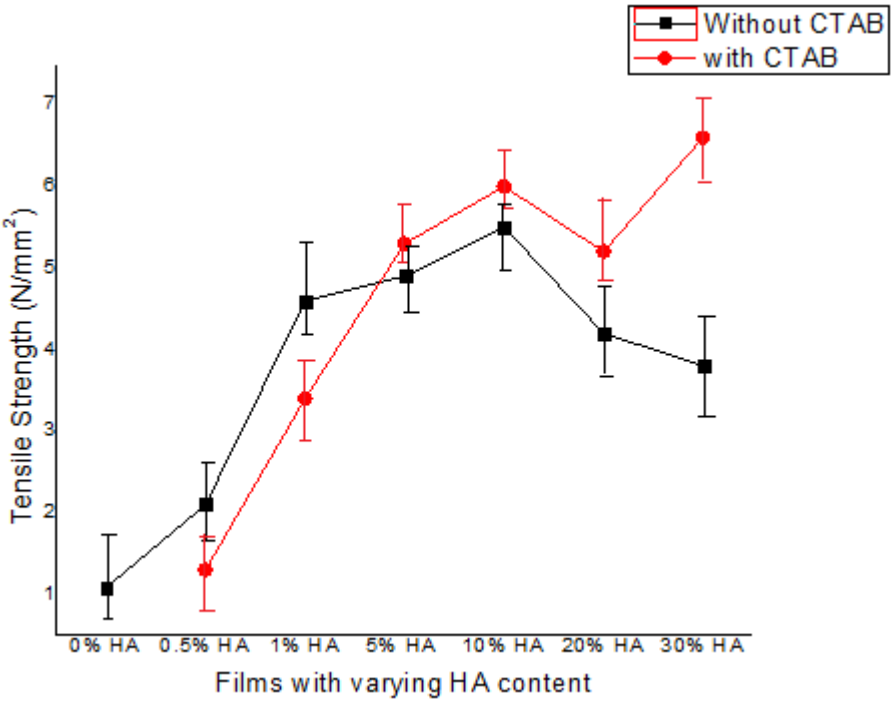


Figure 43 Comparison of Tensile Strength of Samples with Varying HA Content with and without CTAB

4.6.2 Young's Modulus

Young's modulus determines the resistance of a material to elastic deformation under load. The value for elastic modulus increases till 10 wt% HA and then the value decline.The addition of glycerol affects the stiffness of the composite film. At low HA content the stiffness increases which is attributed mainly due to the addition of HA. Afterwards as the polymer content decreases, glycerol starts to act as anti-plasticizer which tends to make HA clusters thus decreasing strength and stiffness of composite films containing high HA content. Same trend is shown by the composite films with CTAB only the values are much lower than the samples without CTAB. This indicates better dispersion of HA in the chitosan matrix which makes it flexible compared with the films without CTAB. Figure 44 shows a comparison of the change in elastic modulus of the composite films as the amount of HA varies.

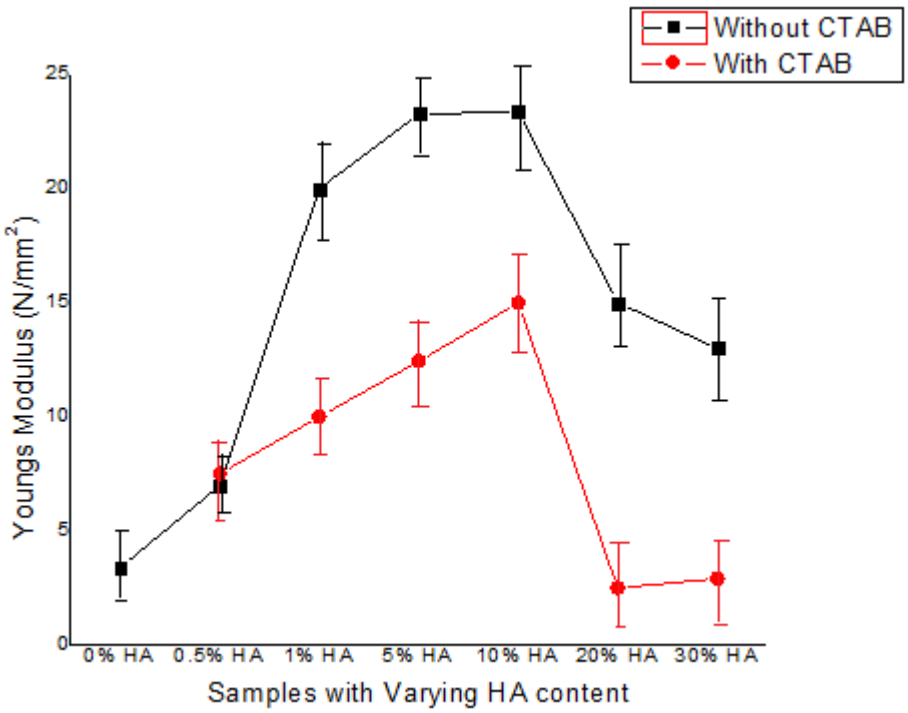


Figure 44 Comparison of Young's Modulus of Composite Films with Varying HA Content with and without CTAB

4.6.3 Percentage Elongation at Break

Inherently the film formed is very brittle. Glycerol is added in the n-HA/chitosan composite solution before casting the membrane. It introduces elasticity in the film. The film confirms elasticity by showing elongation after fracture. At high HA content anti-plasticizing effect of glycerol comes in effect which leads to a decrease in elongation.

Initially the elongation is small, becomes maximum for CS film with 10%HA and then decreases at high HA content. At low HA content the physical interactions between CS/HA particles are strong but as the HA content increases these interactions are weaken due to agglomeration and the elongation value also decreases.

CTAB is added to improve the dispersion of HA in the chitosan matrix. Samples containing surfactant show elongation values more than the films without surfactant. Initially the elongation value is very high which then continuously decreases. At high HA content the ceramic interface bears most of the load that is why the elongation decreases. The comparison is shown in Figure 45.

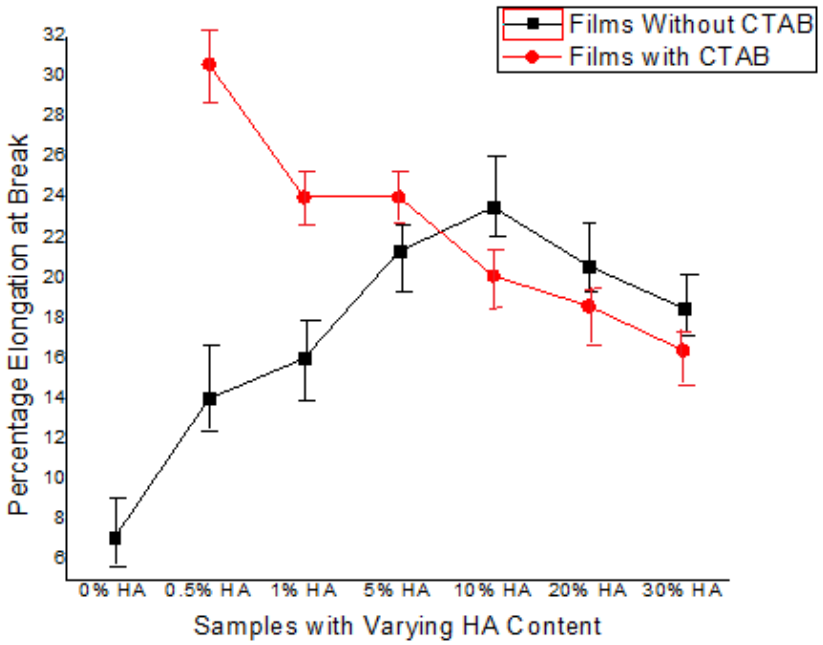


Figure 45 Percentage Elongation at Break of the Composite films with and without CTAB

4.8 Absorption Studies

In a polymer network addition of solvent swells it which leads to increase in volume. The degree of volume increase depends upon:

a) Degree of cross-linking of polymer

b) Affinity of polymer with solvent

Large number of cross links enhance the degree of cross-linking and the chain length between network junctions becomes small. Thus as volume increases, chains extend very little in amount which decreases the equilibrium degree of swelling.

4.8.1 Procedure

Samples with 1x1 cm dimensions are used as shown in Figure 46.

Figure 46 Samples for Water Absorption Studies

They are weighed in dry form and then dipped in distilled water in the time span of 5 minutes to 120 minutes as shown in Figure 47.

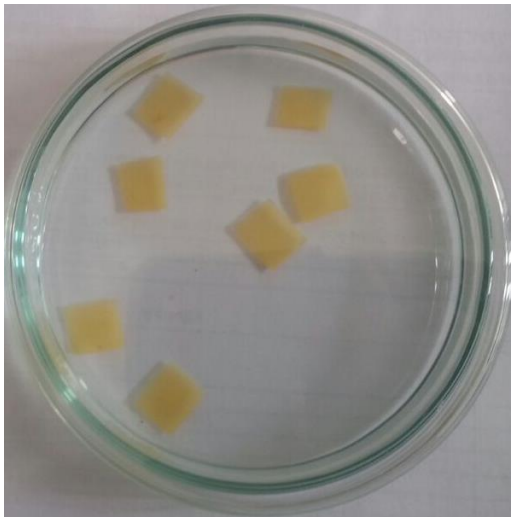


Figure 47 Samples Immersed in Distilled Water

After time completion the samples are gently pressed in a filter paper and weighed again. Then percentage mass swell ratio is calculated by the following formula:

$$\%\text{age mass swell ratio} = \frac{\text{final weight} - \text{initial weight}}{\text{initial weight}} \text{ x } 100$$

4.8.2 Results

As the percentage of HA varies the absorption of distilled water increases. The composite membranes swell maximum till 10% HA composition. Afterwards the absorption rate decreases. This can be implied that the polymer is not cross linked enough naturally. During fabrication of composite films no cross-linking agent is added so the percentage absorption becomes very high for each sample. Addition of any cross-linking agent will increase the cross linking of the polymer network which will decrease the value of percentage absorption. Figure 48 shows absorption behavior of composite films.

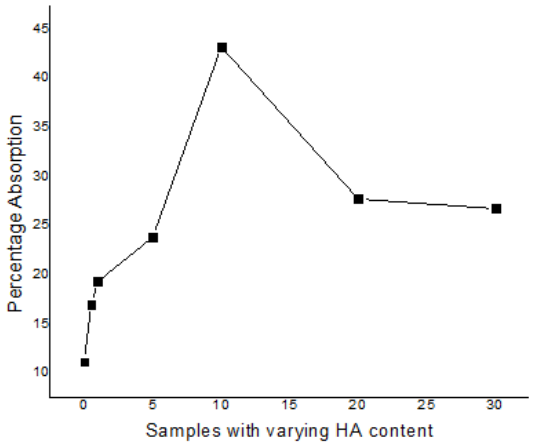


Figure 48 Percentage Water Absorption in Composite Films without Surfactant

Water absorption pattern of the composite films containing CTAB is also the same as for the samples without CTAB. Only the values differ. Composite films containing CTAB absorb more water initially. This can be attributed to the improved dispersion of the HA which shows its hydrophobicity only at high content and at low content allows more water absorption. This is shown in Figure 49.

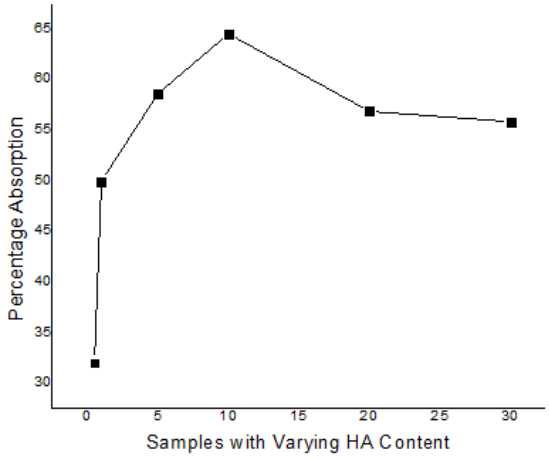


Figure 49 Percentage Water Absorption in Composite Films with Surfactant

4.8.3 Equilibrium Mass Swell Ratio

In a dry amorphous polymer chain stay in an entangled and relaxed state. When it comes in contact with a solvent the polymer tends to swell. At a time two forces come in action. First is the stress experienced by chains as a result of increased distance between network junctions. Second is the counter force which tends to keep polymer in relaxed state. Equilibrium is achieved when polymer stops to absorb solvent. It depends upon chain length and the affinity of polymer for the solvent. The time period of 90 minutes is selected as the time where equilibrium mass swell ratio is achieved. After 90 minutes the composite membrane will not absorb any more water and absorption decreases consequently. This is also shown in Table 6 and Table 7 shown below:

Time	Equilibrium Mass Swell Ratio						
	A	B	C	D	E	F	G
5	8.3	20.1	17	24.5	13.6	26.7	49.8
10	13.6	17.3	20.6	27.5	33.9	29.2	43.6
20	7.3	25.6	20.3	22.9	31.5	28.9	48.5
40	10.7	8	20.5	17.5	26.4	25.9	48.3
60	9.5	19.7	14.5	24.5	32.9	28.5	38.9
90	**14.6**	**16.9**	**29.7**	**27**	**29.2**	**37**	**36.8**
120	13.8	11	12.6	22.6	19.4	17.5	36.4

Table7 Equilibrium Mass Swell Ratio

Time	Equilibrium Mass Swell Ratio					
	B’	C’	D’	E’	F’	G’
5	36.1	59	57.2	59.6	63.9	55.2
10	28.4	52.5	55.3	66.5	63.6	86.1
20	32.3	50.3	60.9	69.6	62.1	52
40	30.9	39.2	58.3	66	47.3	45.1
60	32.5	57.9	60.7	63.9	57.7	52.3
90	**33.4**	**49.8**	**62**	**63.3**	**56**	**55.2**
120	30.1	40.7	55.3	62.4	47	44.2

Table 8 Equilibrium Mass Swell Ratio of Samples with CTAB

Chapter 5

Conclusion

During this project a composite of hydroxyapatite and biodegradable chitosan as polymer matrix is prepared and characterized.

The composite films are analyzed by FTIR and XRD. It appears that both components existed in the composite without changing into any other form. No peaks of any new substance was detected. In the composites crystallinity of HA was not effected by solvent and chitosan content. XRD patterns revealed that increasing HA content decreased chitosan crystallinity. However FTIR showed characteristic peaks of chitosan which implies that chitosan was not totally decomposed.

The SEM images revealed uniform dispersion of HA particles and proper embedding in the chitosan matrix. Due to the addition of HA the surface roughness of membranes was noted to increase. The fracture analysis also revealed a good bonding between HA particles and the matrix because at the time of fracture particles were broken along with the matrix. Chitosan is hydrophilic so the composite membrane absorbs water in noticeable amount. But HA is hydrophobic so when HA content is increased beyond 10% the water absorption decreases.

Composite film with 10 wt% HA is considered as an optimum candidate for the application. The test results from TGA and DSC show improved stability of the composite film and an increase in degradation temperature. Composite film with surfactant shows uniform dispersion of HA and shows minimum number of HA clusters. Due to this good dispersion it shows highest tensile strength compared with other composite films.

5.1 Future Work

5.1.1 Mechanical Properties

When the tensile strength of the composites is considered it is seen that they are suitable only for low load bearing applications and when the properties of the constituents are considered they make the composites suitable to be used as scaffold for bone growth.

To improve mechanical properties which would lead the usage for higher load bearing applications, the reinforcement-polymer interface may be strengthened and some ways to make chitosan regain its crystallinity may be observed.

The composite scaffolds can be studied in situ to investigate bone growth process and change in the microstructural, chemical and mechanical properties.

5.1.2 Porosity

The composite membranes prepared are dense and intended to be used as barrier membranes. In order to facilitate bone growth through the membranes porosity in needed. Pores can be generated by the addition of porogens or by changing the membrane casting method like solvent inversion or freeze drying.

5.1.3 Scaffolds

Solid porous scaffolds can also be prepared by dry pressing. This procedure will require usage of polymer and hydroxyapatite in solid form. By using a binder a solid form of any shape can be prepared. Heat treatment can increase the strength of the scaffold.

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