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ABSTRACT

WET-SPINNING AND THERMAL ANALYSIS OF COLLAGEN FIBERS INCORPORATING HYDROXYAPATITE IN MIXED AND COATED FORM

by
Sandesh Prakash

The composition of natural bone comprises of collagen (Cg) as organic phase which acts as a matrix and inorganic calcium phosphate, particularly hydroxyapatite (HA), which is used as reinforcement. This study involved the wet-spinning of collagen fibers incorporating HA in mixed and coated form. The weight percent ratio of collagen and HA in the composite fibers was 95 to 5 respectively. HA coating on pure collagen fibers was achieved by immersing them in HA solution formed by mixing HA in water. With Thermomechanical analysis, the highest breaking point temperature was obtained for the composite fibers and the least with pure collagen fibers. The HA coated collagen fibers had breaking point temperature between these two fibers. However, tensile modulus was seen to be highest for pure collagen fibers and the least for the composite fibers. Thermogravimetric analysis was performed on the fibers to monitor the weight reduction with respect to temperature. The highest reduction was obtained with pure collagen fibers and the least with the composite fibers. HA coating on the collagen fibers was evident from X-ray diffraction analysis and EP-DIC images. This study showed a new method of coating collagen fibers with HA crystals. Cg/HA composite fibers were spun for the first time using Wet-Spinning apparatus. With thermal analysis it was evident that HA has low load bearing property and high melting point temperature. Osteoconduction and Osteoinduction are needed to be done on these fibers as a future work.

WET-SPINNING AND THERMAL ANALYSIS OF COLLAGEN FIBERS INCORPORATING HYDROXYAPATITE IN MIXED AND COATED FORM

by
Sandesh Nathaniel Prakash

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WET-SPINNING AND THERMAL ANALYSIS OF COLLAGEN FIBERS INCORPORATING HYDROXYAPATITE IN MIXED AND COATED FORM

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To my mummy, papa, Vaibhav and Saras for
their heartfelt love and support in my good and bad times
and to the Lord Almighty for his grace and blessings

The Lord is my shepherd, I shall not want.....Psalms 23:1

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CHAPTER 1

INTRODUCTION

1.1 General Information

Collagen is the principal structural protein inside the body and constitutes one third of the total body protein. It comprises most of the organic matter of the skin, tendons, bones and teeth and occurs as fibrous inclusions in most other body structures. Some of the properties of collagen are its high tensile strength, its ion exchanging ability, due in part to the binding of electrolytes, metabolites and drugs; its low antigenicity, due to masking of potential antigenic determinants by the helical structure, and its low extensibility, semipermeability, and solubility. Furthermore collagen is a natural substance for cell adhesion.

In human bone, collagen represents an organic matrix in which minerals consisting of calcium, phosphate, hydroxyl, fluoride and carbonate ions are embedded. The similarity to human tissue affords an important advantage by comparison with alternative materials [1, 2]. This advantage is utilized for producing prostheses and biomaterials. The composition of hard tissue, such as natural bone, comprises collagen and inorganic calcium phosphate, particularly biological apatite. Bone contains about 60% to 75% by weight of biological apatite, with tooth having more than 98% by weight of biological apatite. Biological apatite is a naturally occurring calcium apatite-type material which is formed in the body by precipitation from body fluids at body conditions. This biological apatite has a structure which is similar to pure hydroxyapatite,

but contains some substitute ions for the calcium, phosphate and hydroxyl ions. Strictly speaking, synthetically produced precipitated hydroxyapatite is more similar to biological apatite. Synthetically produced precipitated hydroxyapatite is a very fine powder [3].

It is one of the few materials that are grouped as bioactive, which means that it will support bone ingrowths and Osteointegration. The chemical nature of the hydroxyapatite makes it a non-stoichiometric material. The most common substitutions involve carbonate, fluoride and chloride ions for hydroxyl groups, while defects can also exist resulting in a deficient hydroxyapatite. Hydroxyapatite is a thermally unstable compound, decomposing at temperature from about 800°C -1200°C depending on its stoichiometry. However, hydroxyapatite does not have the mechanical strength to enable it to succeed in long term load bearing applications.

1.2 Objective

The objective of this work is to spin collagen/hydroxyapatite composite fibers and to coat pure collagen fibers with hydroxyapatite crystals. The collagen fibers will be spun with 2.5% dispersion of collagen in water with 2ml lactic acid. These fibers will be tested for their tensile modulus, breaking point temperature and percentage weight retention using thermal analysis techniques. The coating on the collagen fibers will be analyzed by X-ray diffraction for crystal deposition of hydroxyapatite. Also EP-DIC images will be taken for the fibers. The fibers will be spun by means of the wet-spinning apparatus installed in the Medical Device Concept Laboratory.

CHAPTER 2

BACKGROUND

2.1 Collagen

Collagen is a family of proteins that are major components of vertebrate tissue. The common structural feature found in all collagens is a triple helix that consists of three left handed helixes that are wound into a right handed triple helix. Individual alpha chains, which are the basic units of collagen, contain one or more polypeptide sequences (GLY-X-Y) that form the triple helix with one or more non-triple helical modules. These alpha chains vary in size. They range from six hundred to three thousand amino acids and have been classified into fibrillar, non-fibrillar and novel collagens. The fibrillar collagen includes type I, II, III, V and XI which form cross striated fibrils, and all share triple helical region containing about one thousand amino acids per chain. They have a length of about 300nm. Non-fibrillar collagens contain triple helical segments of varying lengths interrupted by sequences containing larger segments of non collagenous sequences found in von Willebrand factor, collagen type IV and fibronectin. The novel collagens are similar to the non-fibril collagens because they consist of a triple helical region separated by a non-triple helical region [4].

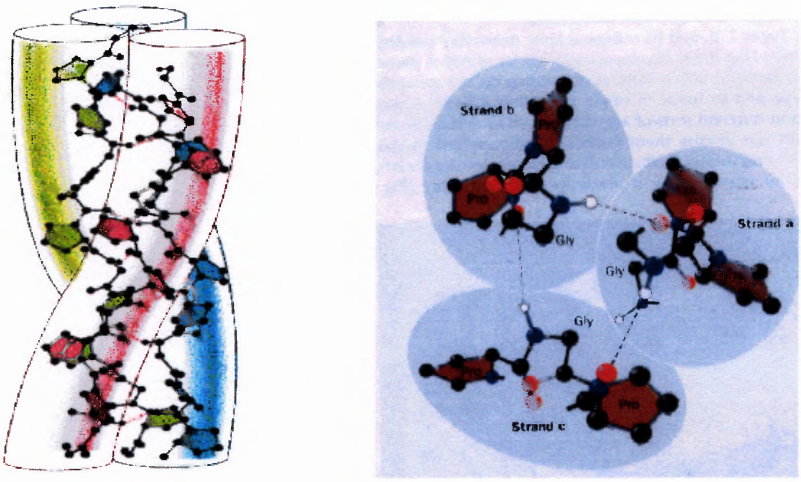

Figure 2.1 Triple Helix Structure of Collagen [5].

2.2 Collagen Type I

Collagen is the principal structural protein inside the body and constitutes one third of the total body protein. The most abundant of all the collagens is the type I collagen whose structure, function and synthesis have been thoroughly investigated. Collagen is characterized by a unique triple helix formation extending over a large portion of the molecule. Every third amino acid is glycine (the smallest amino acid with a hydrogen side group in every third position in order for the polypeptide chains to pack together close enough to form the collagen triple helix) and about 25% of amino acid residues are proline and hydroxyproline. Also, the high content of proline and hydroxyproline in the x and y positions respectively contribute for forcing each collagen subunit into a helical structure. The three subunits are then arranged into a form of a triple helical procollagen molecule similar to a triple stranded rope, which is then released into the extra cellular space. This procollagen is transformed into the extra cellular space by specific peptidases to form the collagen monomer [4, 5].

Glycine (Gly) has three different unique capabilities. Main chains approach each other very closely, when Gly molecules are present. Secondly, Gly can assume conformations normally restricted by close contacts of the beta-carbon and finally it is more flexible than any other residues, thus contributing to parts of the backbone that need to move or hinge.

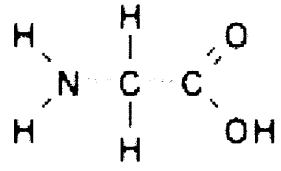

Figure 2.2 Glycine [5].

Proline ($C_5H_9NO_2$) has stronger stereochemical constraints than any other residue with only one instead of two variable backbone angles and it lacks the normal backbone NH for hydrogen bonding. It is both disruptive to regular secondary structure and also good at forming turns in the polypeptide chain. In spite of its hydrophobicity, it is usually found at the edge of the protein.

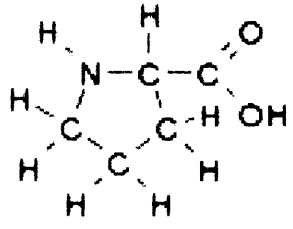

Figure 2.3 Proline [5].

Hydroxyproline ($C_5H_9O_3N$) is an uncommon amino acid produced by hydroxylation of proline. It is hydroxylated after protein synthesis. Along with proline, it is one of two cyclic amino acid found in proteins. It helps provide stability to the triple-

helical structure of collagen by forming hydrogen bonds. Proline hydroxylation requires ascorbic acid. Most effects of absence of ascorbic acid in humans come from the resulting defect in hydroxylation of proline residues of collagen with reduced stability of the collagen molecule [4, 5].

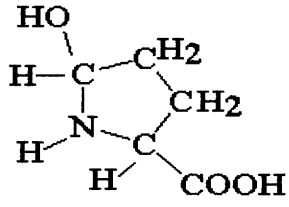

Figure 2.4 Hydroxyproline [5].

In addition to the helical portion of the molecule, the terminal amino acid sequence at each end of the molecule is comprised of short (less than 5% of the total) non-helical domains called telopeptides which are involved in the polymerization by non covalent binding in the sites on adjacent helixes.

Collagen fibers form strong, organized fibrils consisting of staggered arrays of tropocollagen molecules. Each triple helix is 300nm long and is stacked head to tail with 40nm gaps in between. These gaps called "hole regions" repeat every five strands and can be seen by electron microscopy. The holes are thought to be the site of hydroxyapatite, a mineral constituent of bone.

Hydroxylysine residues are covalently linked to sugar molecules in the hole region. Sugars may function in organizing fibril assembly. Lysine residues are also post translationally modified to aldehyde derivatives by a copper-dependent enzyme called lysyl oxidase. Two lysines can then form a very stable covalent intramolecular crosslink between two strands in tropocollagen via an aldol condensation and dehydration.

2.3 Levels of Structure Description in a Protein

The task of describing protein structure is approached at four hierarchical levels of complexity:

- Primary Structure
- Secondary Structure
- Quaternary Structure
- Tertiary Structure

Primary structure describes the proteins in terms of its monomeric subunits and the bonds that link them to one another. Considering only their primary structure, proteins differ from one another by the specific sequence of amino acids. Secondary structure describes the proteins in terms of recurring structural patterns, stable arrangements of amino acids. While the secondary structure describes protein structure locally, the tertiary structure describes the whole three-dimensional folding of a polypeptide. Quaternary structure is the structure of protein consisting of more than one polypeptide.

The primary structure of a protein is the amino-acid sequence of the polypeptide chains, without regard to spatial arrangement. The commonly occurring amino acids are of 20 different kinds which contain the same dipolar ion group $H_3N^+.CH.COO^-$. These amino acids have a central carbon atom to which are attached a hydrogen atom, an amino group (NH_2) and a carboxyl group (COOH).

Secondary structure describes the "local" ordered structure brought about via hydrogen bonding mainly within the peptide backbone. The spatial arrangement of amino

acids in a protein is known as a conformation. These conformations are generated by rotations around the bond length and angles which are invariant around the protein structures. Among these conformations, protein has a unique or nearly unique three-dimensional structure also known as a native conformation. This native conformation is the surviving conformation under specific biological conditions. The critical forces stabilizing conformations are noncovalent interactions and are responsible for common structural patterns. The local structure of proteins is characterized by specific backbone torsion angles and specific main chain hydrogen bond pairings. Therefore, the key to protein folding lies in the torsion angles (also known as dihedrals) of the backbone.

Tertiary structure describes folding of the total chain, a combination of the elements of secondary structure linked by turns and loops. Tertiary structures may contain only α helices, only β sheets, or both, or even other less common secondary structures. Stability of the tertiary structure is determined by non-bonding interactions and disulfide bonds. The tertiary structure is a tightly folded structure with polar groups on the surface and non-polar groups in the bulk.

Quaternary structures are foldings of more than one polypeptide chains. They describe the spatial arrangement of amino acids in proteins. The forces that stabilize a quaternary structure are much the same as those that stabilize the secondary and tertiary structure (non-covalent interactions). The hydrophobic groups combine together so as to exclude water.

2.4 Denaturing a Protein

The shape and the conformation of a protein can be altered by changing the environment of the protein. This is done by putting it in an environment which will change the ability of the Van Der Waals, hydrogen, ionic and covalent bonds to hold the protein molecules together in a particular conformation. Unfolding of the molecules is possible by breaking these bonds, which is achieved by changing the pH or heating it. When a protein is caused to be unfolded in this way, it is denatured. The denaturation process is mostly irreversible. However, it can also be reversed if the changes in conditions are not drastic. The primary structure dictates the kind of secondary, tertiary and quaternary structure. Given the time and the proper conditions, a protein which has retained its primary structure can regenerate its secondary and tertiary structure and thus its conformation making it a viable working protein. Changes in environmental conditions (temperature and pH) should have the same effect on collagen protein.

2.5 Hydroxyapatite Structure and Properties

Hydroxyapatite (HA) is the most well known bioactive ceramic material used in medicine [6]. The inorganic constituent of bone is made up of biological apatites, which provide strength to the skeleton and act as a storehouse for calcium, phosphorus, sodium, and magnesium. These biological apatites are structurally similar to the mineral apatites including hydroxyapatite (HA, $Ca_{10}(PO_4)_6(OH)_2$) and brushite (B, $CaHPO_4 2H_2O$). In physiological environment, two forms of calcium phosphates are stable. At pH < 4.2, the stable calcium phosphate phase is brushite. At pH>4.2, the stable phase is hydroxyapatite.

These apatites, along with fluorapatite (FAp, $Ca_5(PO_4)_3F$), monetite (M, $CaHPO_4$), tricalcium phosphate (TCP, $Ca_3(PO_4)_2$), tetracalcium phosphate (TTCP, $Ca_4(PO_4)_2$), and octacalcium phosphate (OCP, $Ca_8H_2(PO_4)_{6.5}H_2O$) belong to a family of minerals known as apatites. These materials demonstrate similar structures and possess the structural formula, $X_3Y_2(TO_4)Z$. This structure allows for easy substitution. In nature, X and Y include Ca, Sr, Ba, Re, Pb, U, or Mn (rarely Na, K, Y, Cu). T includes P, As, V, Si, S, or C (as CO_3)and Z includes F, Cl, OH, or O. In medicine, apatites of interest possess X=Y=Ca, T=P, and Z=F or OH. For example, the apatite is called hydroxyapatite (HA) when T=P and Z=OH. Hydroxyapatite forms crystals that are best described as hexagonal rhombic prisms. The lattice parameters for hydroxyapatite are a=9.432 Å and c=6.881 Å.

Hydroxyl ions (OH-) occur at the corners of the basal plane. These ions are positioned at every 3.44 Å (one half the unit cell), parallel to the c-axis and perpendicular to the basal plane. Thus, 60% of calcium ions in the unit cell are associated with the hydroxyl ions. The density of this material is 3.219 cm^3.

In addition to its similarity to natural bone, mineral HA is an osteoconductive material that provides a temporary scaffold for bone growth. The osteoconductive nature of HA coatings results in the formation of strong bonds with bone. The direct contact between living bone and the implant material is known as osseointegration. Brånemark et al. originally described the phenomenon in 1977 and later Albrektsson et al. defined it in the literature in 1981 [8]. In that paper, Albrektsson et al. proposed six factors that are crucial for successful osteointegration [8]. The osteointegration of a biomaterial depends

not only on the properties of the implant material but also on the surface conditions, status of the bone, the surgical technique and the implant loading conditions. Osteointegration has been shown to enhance the reduction of mechanical stresses and micro motions at the bone-implant interface.

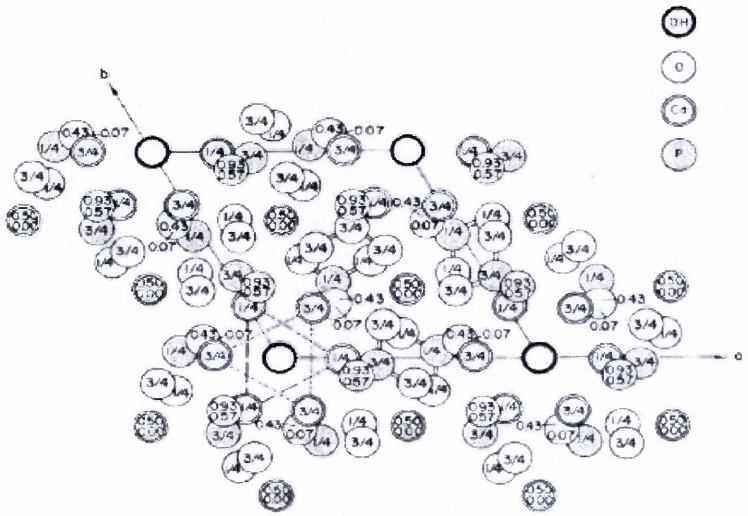

Figure 2.5 Hydroxyapatite Structure Projected down C Axis onto Basal Plane [7].

2.6 Composition of Bone

Bone is a natural composite material primarily consisting of an organic phase (mostly Type I collagen) as a matrix and a mineral phase (hydroxyapatite crystal, which is brittle in nature) as reinforcement. The mineral phase imparts the strength and stiffness to bone, whereas the organic phase the toughness and viscoelasticity. Mineral phase plays a vital role in withstanding the mechanical force applied to bone. It has been shown that decreasing bone mineral content is one of the major factors contributing to increased osteoporosis fracture risk with age. Type I collagen constitutes over 90% of the organic phase where collagen molecules cross-link with neighboring collagen molecules to make a three-dimensional lattice-like network. Additionally, collagen fibrils are strengthened

by mineral deposits, and vice versa. Although the movement of collagen fibrils is constrained by its mineral constituents, type I collagen may play an important role in the viscoelastic properties of bone due to its polymeric molecular structure [9, 10]. The other main phase is water for which the exact function and location in the bone is not fully understood. Water may exist in various forms and locations in bone. It contributes towards stabilizing the collagen triple helix by means of hydrogen bonding. Water is bonded to crystal surfaces for ion exchange and it is also bonded to extrafibrilar noncollagenous proteins. Water exists as bulk water in pores, both within and on the surface of bone. Water may play a notable role in determining the mechanical properties of bone. The degree of hydration of bone greatly influences its biomechanical properties in vitro. Moreover, the viscoelastic creep behavior of bone during mechanical tests is considered to be determined by both water and collagen [11, 12, 13].

2.7 Osteoconduction

For a successful cell implantation into bone defects, an ideal carrier is necessary to act as a scaffold for cell proliferation and differentiation at the transplantation sites. Studies demonstrated that type I collagen exhibits favorable effects on attachment, proliferation and differentiation of human osteoblastic cell lines.

Osteoblasts cultured in collagen sponge express highly differentiated osteoblastic phenotypes compared to the osteoblasts cultured on the plastic surfaces of tissue culture plates. Calcium deposition verifies the different mineralizing potentials of osteoblasts and gingival fibroblasts when cultured in collagen sponges [14]. 2–4 mM concentration of Ca^{2+} is suitable for proliferation and survival of osteoblasts, whereas slightly higher

concentrations (6–8 mM) favor osteoblast differentiation and matrix mineralization in both two and three dimensional cultures. Higher concentrations (>10mM) are cytotoxic [15]. Purely from the perspective of calcium deposition, higher concentrations lead to increased accumulation of Ca^{2+}. Collagen matrices appear to enhance the osteoblastic phenotypes of cells and exhibit accelerated mineralization in a manner similar to osteoblasts from other sources.

Osteoconduction is the replacement of a mineralized tissue with host bone and is sometimes described as creeping substitution whereby the osteoconductive agent serving as a passive scaffold is gradually resorbed and replaced by new bone growing in from the margins [14]. In simple words, osteoconduction is denoted to a material that will support bone formation. Numerous investigations confirm the osteoconductive and bioactive properties of HA. However, poor load bearing capacity of HA by itself and insufficient bending strength limits its usage. The composite of HA/collagen can be an alternative for bone replacement as bone itself is an organic-ceramic material.

Natural bone has a complex hierarchical structure. On the ultra structural level, an intimate association exists between collagen fibrils and apatite crystals. The type I collagen matrix has a characteristic quarter staggered arrangement of tropocollagen molecules assembled and aligned with an axial period of approximately 67nm. The matrix serves as a template for periodic deposition of mineral platelets. The thickness of bone crystals are usually uniform ranging from 2nm to 5nm. The length and width of bone crystals are variable and crystal growth in the c-axis direction can exceed collagen hole zones and periods. The crystallographic c-axes of mineral platelets are generally oriented parallel to one another and directed along the collagen long axes. Under light

microscope, the bone consists of consecutive lamellae and the collagen fibrils assume a parallel arrangement with the lamella. The preferential orientation of the bone minerals has a significant effect on the mechanical property of bone.

Living bone constantly undergoes a coupled resorptive-formative process known as bone remodeling. The process involves simultaneous bone removal and replacement through the respective activities of osteoclasts and osteoblasts together with the accompanying vascular supply and network of canaliculi and lacunae. In the case of cancellous bone, the cells are not very far away from the blood vessels and the process can take place on the surface of trabeculae, a phenomenon often referred to as creeping substitution. The osteoclasts ream out a resorption canal in compact bone, followed by blood vessels and accompanying cells that differentiate into osteoblasts and deposit the lamellar bone of the new osteon.

2.8 Osteoinduction

Osteoinduction can be defined as the induction of undifferentiated inducible osteoprogenitor cells that are not yet committed to the ostogenic lineage to form the osteoprogenitor cells. In simple form, it is the term given to the material which induces the formation of bone. One of the first evidence of osteoinduction was given by Urist in 1965, after implanting demineralized bone matrix (DBM) in soft tissue of rabbits, rats, mice and guinea pigs. Later studies suggested that, DBM contained morphogenetic factors are capable of inducing the differentiation of resident extraskeletal mesenchymal cells firstly into chondrocytes and then into osteoblasts. At present, highly purified native bone morphogenic proteins (BMP's) are used for osteoinduction. It was considered that

BMP's were always necessary for the bone induction. However, recently Winter et al. suggested the importance of calcification for the process of osteoinduction [16, 17]. An interesting finding of the Winter's and Simpson's study was that the implanted sponge polymer showed in vivo calcification prior to the process of bone formation, suggesting the significance of calcium phosphates (CaPs) in the process of osteoinduction [17]. Since the mechanism of osteoinduction by biomaterials is not completely understood, it is unknown whether it is the biomaterial or possibly an interaction between the biomaterial and the relevant proteins present in body that is responsible for the process of bone induction. Since most implants do not induce bone, specific material properties are apparently needed for starting the process of bone induction.

To start the differentiation of the undifferentiated inducible osteoprogenitor cells into bone-forming cells, it has been suggested that not only the chemistry, but also geometry of the biomaterial in contact with these cells are critical factors. In other words, the microenvironment around the cells is crucial [16]. Some of the properties affecting the osteoinduction are stated by Huipin Huan et al. as microporosity, sintering temperature, mild dissolution of the materials, three dimensional environment and material specificity [18]. Sintering temperature of a calcium phosphate ceramic has a consequence on its microstructure and crystal size, i.e. its specific surface area. The presence of macropores or concavities is shown to be a prerequisite for osteoinduction by biomaterials. The presence of a well-interconnected macroporous structure is important for a good supply of the body fluids with nutrients throughout the implant. Accompanied with this supply, the release of calcium and phosphate ions is believed to be the origin of the bioactivity of calcium phosphate (CaP) biomaterials followed by the precipitation of a

biological apatite layer. The precipitation of this apatite layer takes place when the concentration of calcium and phosphate ions had reached the supersaturation level in the material vicinity. This explains the fact that bone induction always takes place in the pores in the center of the implant and not on the implant periphery. The diffusion of the released calcium and phosphate ions might occur too fast at the implant periphery, and therefore not allow for ion concentration increase, required for the biological apatite formation.

It is expected that a material with a higher dissolution rate will release calcium and phosphate ions faster, followed by a faster formation of the biological apatite layer. One way to influence the in vivo dissolution rate of a material is by changing its specific surface area. Materials with a specific surface area below the optimum will degrade slower and will finally induce less bone. Materials with a specific surface area above the optimum might degrade too fast, losing thereby the shape and stability that is necessary to facilitate bone formation. Finally, there is a minimum threshold in the amount of micropores and specific surface area for the bone to be induced.

Osteoinduction couldn't be found in ceramics other than calcium phosphates. For example bone formation was not observed in titanium oxide ceramics, although they had micropores on the macropore surface, indicating the material specificity for osteoinduction [16, 18].

2.9 Solubility of Hydroxyapatite

The following reaction occurs when hydroxyapatite is in contact with water:

Precipitation $\rightleftharpoons$ dissolution

$Ca_{10}(PO_4)_6(OH)_2 \rightleftharpoons 10Ca^{2+} + 6PO_4^{3-} + 2OH^-$

Solid $\rightleftharpoons$ Solution.

A small amount of hydroxyapatite dissolves and releases calcium, phosphate and hydroxyl ions. This process continues until the water is saturated with respect to hydroxyapatite. At that point, the rate of the forward reaction (mineral dissolution) is equal to the rate of the backward reaction (mineral precipitation).

The solubility of hydroxyapatite can be split into separate ions and can be characterized by its solubility product (Ksp). It is the product of the concentrations (mol/L) of the component ions raised to the appropriate power in a saturated solution. Formations of precipitates are chemical equilibria phenomena. This heterogeneous equilibrium is given by the equilibrium constant called as the solubility product (Ksp).

For a solution saturated with respect to HA, the Ksp is $[Ca]^{10}[PO_4]^6[OH]^2$. The bracketed values represent the activities (effective concentrations) of the component ions rather than their actual concentrations. Hydroxyapatite is a highly insoluble mineral and since activities of the three component ions are expressed in the large units of mole/liters, the value of Ksp is very small for hydroxyapatite (10^{-117}). Although the Ksp is a constant, the concentrations of each of the 3 component ions in a saturated solution can vary, provided that their product remains equal to the Ksp. Thus, in a more acidic solution, in which the hydroxyl concentration is reduced, the concentrations of the calcium or phosphate ions (or both) would have to increase to maintain the saturation. If the solution

is not saturated, no precipitate will form and in this case it is given by the Ion Product (Ip). Ion Product is the product of the concentrations of the ions in a solution at any moment of time. The dissociated ions are present in the solvent phase in the same proportion as they are found in the solid phase. If Ip=Ksp, then the solution is just saturated with respect to hydroxyapatite. If Ip<Ksp, the solution is unsaturated and when Ip>Ksp, the solution is supersaturated. Solubility of hydroxyapatite in water is approximately 30mg/l. The Ip for HA in distilled water is zero as water contains only hydroxyl ions. It contains no calcium or phosphate ions. Since Ip<Ksp, the water is unsaturated and hydroxyapatite will dissolve in it till Ip=Ksp.

Value of critical pH for hydroxyapatite varies over a long range and it is determined by the concentration of calcium and phosphate in the solution. The critical pH is the pH at which a solution is just saturated with respect to a particular mineral such as hydroxyapatite. If the pH of the solution is above the critical pH, then the solution is supersaturated with respect to the mineral and more mineral will tend to precipitate out. Conversely, if the pH of the solution is less than the critical pH, the solution is unsaturated and the mineral will tend to dissolve until the solution becomes saturated.

2.10 Collagen-Hydroxyapatite-Water Interactions

Collagen macromolecule spontaneously forms triple helix fibrils of great tensile strength and thermostability with holes within the 3D-structure, where tiny hydroxyapatite (HA) nanocrystals can grow. This is called the mineralization of collagen. Water molecules (also called 'biological water' due to its specific structure near bio molecule surfaces) have a specific influence on the collagen-hydroxyapatite interactions. Two factors can be

formulated to explain this phenomenon. The first is the critical influence of the hydration shell on the triple helix collagen structure and its stability and the second is that P–O and Ca^{2+} groups of HA molecules are hydrophilic. When collagen and collagen-hydroxyapatite composite is treated with relative humidity ranging from 0% to 90%, the following hydration isotherm is obtained.

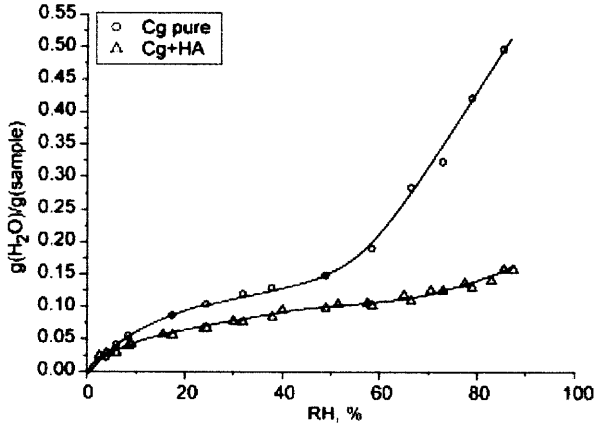

Figure 2.6 Hydration Isotherms for Collagen and Cg/HA composite [20].

Water molecules stabilize the triple helix structure of collagen molecule. From the behavior of the obtained isotherms, it follows that the level of sorption of water molecules for the pure collagen is rather higher than that for the collagen-hydroxyapatite system. It means that the HA molecules have a higher tendency to close some hydration sites within the collagen structure.

Figure 2.7 shows two collagen layers with a number of the cylindrically shaped molecules. Two types of hydration water around collagen peptide are seen. The first type corresponds to the so-called first cylinder layer of hydration, whereas the second type corresponds to the water shell that is localized in the gap area, probably with stronger interactions. These sites of hydration in the case of the mineralized collagen molecules are not available. Hence, it is seen that mineralization of the collagen fibrils by the hydroxyapatite crystals located within the gap area dramatically decreases a number of water molecules present in the initial hydration shell. The process causes some structural changes within the Collagen structure. However, this structural change does not denature the original collagen molecule.

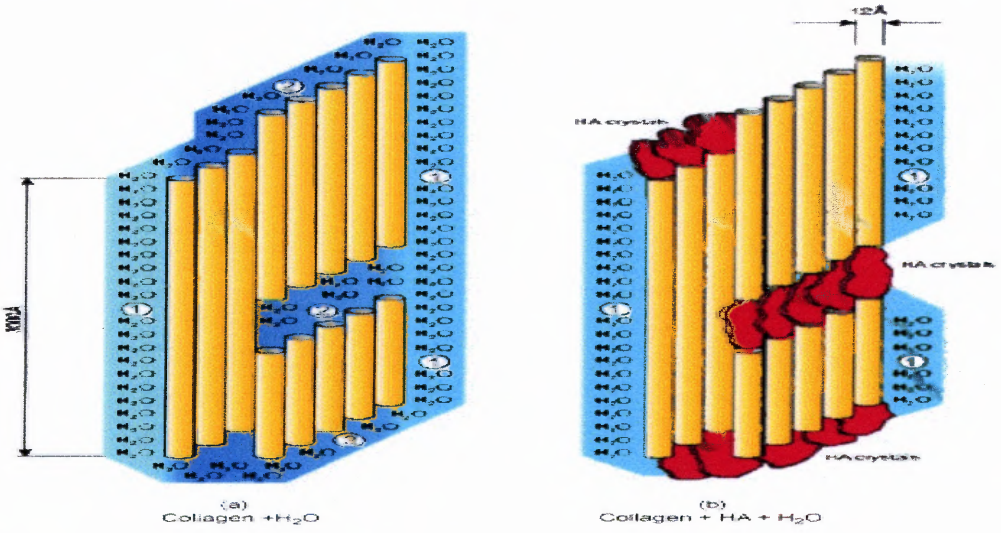

Figure 2.7 Early Mineralization of Collagen in Water Environment [20].

2.11 Simulated Body Fluid Environment

Increasing efforts have been made to produce biomaterials that will mimic bone, teeth and shell materials. Collagen/Hydroxyapatite (Cg/HA) is of special interest because it can mimic the composition of natural bone and the collagen has been shown to effect accelerated bone healing. Osteointegration could be greatly improved by the use of collagen–hydroxyapatite mixture. For these composites the key step is the growth of calcium phosphate on collagen matrix in an aqueous media.

In the Cg/HA composites, the collagen has a function of regulating the distribution of HA by preventing the aggregation of small HA particles. The negatively charged groups of some amino acids in collagen molecules probably play an important role in regulating this process. They have a good affinity for calcium ions in calcium solution and thus cause a uniform distribution of small HA particles on the collagen surface. Methods used for the formation of HA on collagen have some disadvantages such as altering the orientation, phase state and morphology of crystals on the surface of organic matrices. HA can be grown on collagen matrix by using 1.5X simulated body fluid (SBF). The collagen membranes were immersed in a 1.5 SBF solution and kept undisturbed at a temperature of 37.0±0.5 °C.

Collagen membranes were also immersed in distilled water as a reference condition. Collagen membrane is immersed in 1.5 SBF for 0, 3 and 7 days. As the soaking time increases, many small HA crystal grains can be seen to aggregate into spheroidal particles and the spherulites were grown quickly on the surface of collagen membrane. Critical incubation period of 24 h is observed before crystal growth occurs.

The incubation period of the HA crystal growth on the collagen membrane may be longer than that on the collagen gel. Only an extensive broadening peak in the 2θ range of 12–30° is observed in the pure collagen membrane. Several diffraction peaks appear in the Cg/HA, where positions of the most intense peaks and the not-resolved intensity bumps correspond well to the expected Bragg peaks for hydroxyapatite. This indicates that the HA crystal is formed on the surface of collagen membrane [21].

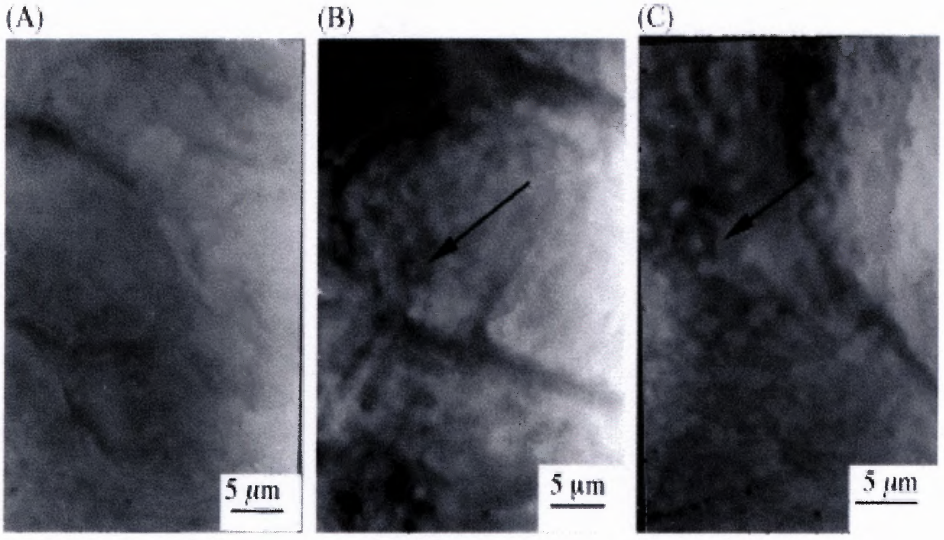

Figure 2.8 Collagen immersed in the 1.5 SBF, (A) 0 day, (B) 3 days, (C) 7 days [21].

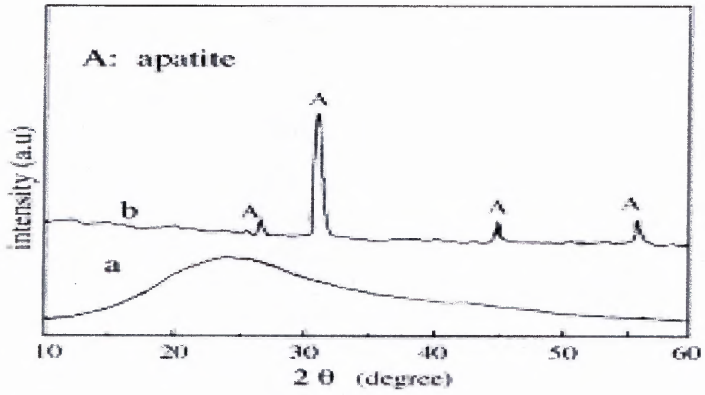

Figure 2.9 (a) Collagen in water for 7 days. (b) Collagen in 1.5 SBF for 7 days [21].

Table 2.1 Advantages and Disadvantages of Coating Techniques

Technique	Thickness	Advantages	disadvantages
Dip coating	0.05mm -.5mm	Inexpensive Coatings applied quickly Can coat complex substrates	Requires high sintering temperatures Thermal expansion mismatch
Sputter coating	0.02μm -1μm	Uniform coating thickness on flat substrates	Expensive Time consuming Cannot coat complex substrates Produces amorphous coatings
Pulsed laser deposition	0.05μm - 5μm	As for sputter coating	As for sputter coating
Electroporetic deposition	0.1 mm -2mm	Uniform coating Rapid deposition rates	Difficult to produce crack-free coatings high sintering temperatures
Plasma spraying	30μm -200μm	High deposition rates	High temperatures induce decomposition Rapid cooling produces amorphous coatings
Hot isostatic pressing	0.2 mm -2mm	Produces dense coatings	High temperature required. Thermal expansion mismatch Elastic property differences
Sol-gel	<1μm	Can coat complex shapes Low processing temperatures Relatively cheap as coatings are very thin	Some processes require controlled atmosphere processing Expensive raw materials

CHAPTER 3

MATERIALS AND METHODS

Collagen dispersion is prepared by a process developed with the help of Dr. Nels Lauritzen and Dr. J Nichols of Prodex Science Inc, located in Princeton, New Jersey. The dispersion of 2.5% collagen is obtained by mixing 25grams of dry collagen obtained from the bovine flexor tendon and mixing it in 1liter of water along with 2ml of lactic acid.

The technique used for the spinning of collagen was setup in the Medical Device Concept Laboratory at NJIT. Collagen dispersion is extruded out of a hollow needle and passed through a coagulation bath to form a continuous fiber. The bath consists of acetone and ammonium hydroxide in water. This bath is called a dehydrating bath since it removes the water from the dispersion coming out of the needle and forms a single collagen monofilament. The filament obtained is then air dried at room temperature.

3.1 Wet Spinning

Wet spinning apparatus basically consists of the following parts:

1. Syringe pump
2. Syringe and needle
3. Coagulation bath
4. Polystyrene belt
5. Take up rollers
6. Take up motor

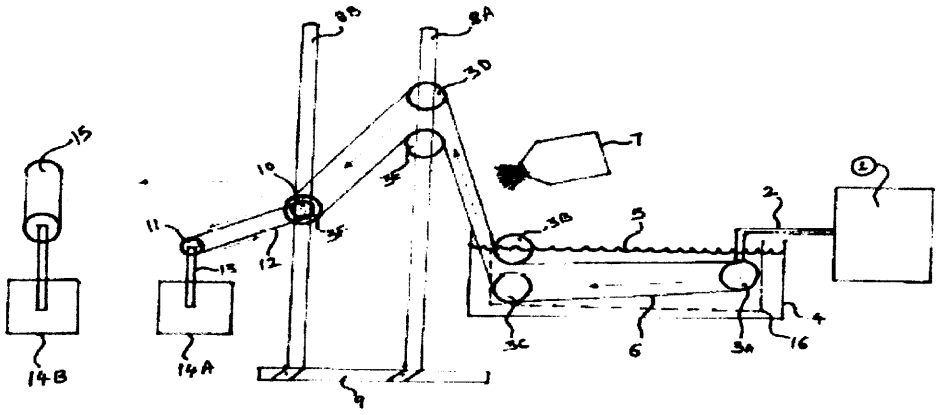

Figure 3.1 Schematic Diagram of Wet Spinning Apparatus installed at MDCL.

The coagulation bath consists of acetone, ammonium hydroxide and water. Acetone is used as a dehydrating agent and ammonium hydroxide as a neutralizing agent. Ammonium hydroxide can produce dual effect. If the quantity is more, the collagen filaments will be brittle and if it is less, the filaments will be soft. Water has the opposite effect on the fibers. Large quantity of water will make the filament soft and low quantity will make it brittle. The pH of the bath is maintained between 8 and 9. Two liters of HPCL grade acetone is used along with 60 grams of water and approximately 3 ml of ammonium hydroxide. The pH of the bath is constantly measured by a pH meter. According to the pH of the coagulation bath, the quantity of the ingredients of the bath can be varied.

The apparatus is used to make thin monofilaments of collagen fibers. Monofilament means a single thread of oriented collagen fibril extruded through a single orifice spinneret. This apparatus is used to spin collagen fibers from 2.5% dispersion of collagen in water and 2ml lactic acid. 25grams of dry collagen will require 1000ml of water and 2ml of lactic acid to produce the required 2.5% dispersion.

The syringe pump can host syringes from 10cc to 50cc. The syringe is filled up with the required amount of collagen dispersion to be spun. An 18 gauge needle is attached to this syringe which has an inner diameter of 0.033inch. The dispersion can be spun at variable speed of the syringe pump according to the quantity needed for dispersion and also according to the required diameter of the fibers. The infusion rate (throughput rate) of the dispersion is highest when the pump is set at 1 (10.6 ml/minute) and lowest, when it is set at 12 (0.00206 ml/min). The rate used in the spinning of fibers was 0.206 ml/min.

The needle is bent at its center such that it just touches the coagulation bath. The polystyrene belt has speed ranging from 3inches/min to 12inches/min. The speed for the spinning was kept at 6inches/min. The speed can be varied as per the diameter requirements of the monofilament. The dispersion comes out of the needle in a form of a filament which is allowed to pass twice through the coagulation bath. This is done to remove the water from the filament or to dehydrate it. The filament is picked up from the belt and allowed to air dry at room temperature.

There are two disadvantages for this apparatus. First, the dispersion has air bubbles in it and hence the monofilament formed by the wet spinning is not continuous and has certain weak points. This can be solved by centrifuging the dispersion at 5000 rpm which will remove most of the air bubbles from the dispersion. The other option is to let the dispersion sit in the needle for sometime until most of the air bubbles disappear. Secondly, the apparatus does not have a crosslinking bath and a separate bath is required for this process.

3.2 Collagen/Hydroxyapatite Composite

Collagen and hydroxyapatite composite is prepared by mixing 2.5% collagen dispersion and hydroxyapatite powder in the ratio of 95 and 5 by weight. It is difficult to obtain a uniform dispersion of hydroxyapatite in a solution. Solubility of hydroxyapatite increases from 30mg/liter to 30g/liter with the change in pH from 4 to 7. As you decrease the pH, the solvent becomes acidic. Acid in contact with collagen (protein) denatures it. Therefore it is not possible to mix the acidic solution with hydroxyapatite dissolved in it with collagen dispersion. Hence we are not able to disperse hydroxyapatite uniformly in the collagen dispersion.

Collagen and hydroxyapatite are mixed together by adding powdered hydroxyapatite while vigorously stirring the collagen dispersion. With this we can attain a certain level of uniform dispersion. Once hydroxyapatite is mixed with collagen, the collagen dispersion loses its transparent appearance and becomes white in color. The collagen/hydroxyapatite solution has higher viscosity than the collagen dispersion by itself and so it becomes difficult to spin fibers with the composite solution. The fibers obtained have their diameter ranging from 250 micrometer- 330 micrometer. The fibers lack the original strength of the collagen fibers.

3.3 Coating Hydroxyapatite on Collagen

Collagen has a function of regulating the distribution of hydroxyapatite by preventing the aggregation of small hydroxyapatite particles. The negatively charged groups of some amino acids in collagen molecules probably play an important role in regulating this process. They have a good affinity for calcium ions in calcium solution and thus cause a

uniform distribution of small hydroxyapatite particles on the collagen surface. Different methods for coating of hydroxyapatite can be used on metal substrates. These include thermal plasma spraying, sputtering, chemical vapor deposition, physical vapor deposition, surface induced mineralization, simulated body fluid environment etc. According to a Japanese patent, a method for forming a coated film was by suspending fine particles of apatite in water and coating the aqueous substance onto the surface of a substrate.

Bone consists of collagen, biological apaptite and water. In accordance with this relation, hydroxyapatite particles were suspended in water by continuous stirring. 2 grams of hydroxyapatite was introduced in 50ml of water and was stirred for 5 minutes. Some amount of hydroxyapatite dissolved in this process. However, the amount is negligible since hydroxyapatite has a very low solubility product (10^{-117}). 30mg of hydroxyapatite dissolves in 1liter of water at pH 7. Collagen fibers made from 2.5% dispersion of collagen are introduced into the solution having suspended hydroxyapatite crystals and kept for at least 2 days. After observing the fibers under optical microscope we see a uniform coating of hydroxyapatite particles. The size of hydroxyapatite particles ranges from 50micrometer-90 micrometer.

CHAPTER 4

EXPERIMENTS

4.1 Thermogravimetric Analysis

Thermal Gravimetric analysis is carried out with the Q50 Thermogravimetric Analyzer installed at MDCL. Thermogravimetric Analysis (TGA) is a thermal analysis technique used to measure changes in the weight (mass) of a sample as a function of temperature and/or time. TGA is commonly used to determine polymer degradation temperatures, residual solvent levels, absorbed moisture content and the amount of inorganic (noncombustible) filler in polymer or composite material compositions.

The heating of the samples is carried out in the range of 0 deg C to 350 deg C. The heating is carried out at the rate of 10 deg C per minute for each sample. The nitrogen flow rate taken is 60 cm^3 per minute and the size of the sample is approximately 2mg. A sample is placed into a tared TGA sample pan which is attached to a sensitive microbalance assembly. The sample holder portion of the TGA balance assembly is subsequently placed into a high temperature furnace. The balance assembly measures the initial sample weight at room temperature and then continuously monitors changes in sample weight (losses or gains) as heat is applied to the sample. The experiments are performed at non isothermal mode. The samples are cut into small pieces to fit into the pan. The samples analyzed are hydroxyapatite powder, pure collagen fiber, hydroxyapatite coated collagen fiber and collagen/hydroxyapatite composite fiber.

4.2 Thermomechanical Analysis

Dense hydroxyapatite does not have the mechanical strength to enable it to succeed in long term load bearing applications. The melting temperature of hydroxyapatite varies from 800 deg C to 1200 deg C depending on its stoichiometry.

Collagen to hydroxyapatite weight ratio in composite fibers is 95 to 5 respectively. Hydroxyapatite crystals occupy some gaps between collagen molecules which are supposed to be occupied by water molecules. By doing Thermomechanical Analysis, we can find out the temperature at which these fibers break. The analysis is done by raising the temperature from 35 deg C to 300 deg C. This is done under a constant force of 1N. The test is conducted by using the TMA 2940 made by TA instruments. The fibers are heated at the rate of 10 deg C per minute. Since an internal cooling system is not available, it is cooled by introducing liquid nitrogen in the furnace.

4.3 Tensile Modulus

This test is conducted by using the TMA 2940 Thermomechnical Analyzer made by TA Instruments installed at MDCL. This is used to find the Young's modulus of collagen fibers at isothermal temperature.

The equipment software plots the changes in the length of the collagen fibers with respect to the increase in force applied. The maximum amount of force applied is 1N. Collagen fiber is cut using the scissors and placed between the clamps used to mount the sample. The sample is then mounted onto the sample fixture. The initial length of the fiber is measured by the instrument. The test is conducted at isothermal temperature. The temperature is kept constant at 37 deg C.

4.4 X-ray Diffraction

Diffraction occurs as waves interact with a regular structure whose repeat distance is about the same as the wavelength. X-rays have wavelength in the order of a few angstroms, the same as typical interatomic distances in crystalline solids. Therefore X-rays can be diffracted from minerals which are crystalline and have regularly repeating atomic structures. Hence, X-ray diffraction (XRD) is an important tool in mineralogy for identifying, quantifying and characterizing minerals in complex mineral assemblages.

X-ray diffraction equipment was used to determine the crystalline coating nature of the hydroxyapatite coated collagen fibers. This equipment is installed in the materials and characterization laboratory at NJIT. X-ray diffraction was carried out on pure hydroxyapatite powder which was used as a reference. Further, both pure collagen and hydroxyapatite coated collagen fibers were studied. X-pert data collector software was used to collect the data from the instrument. Instrument settings are cited below.

Instrument Settings

1. Diffractometer

Positions

2theta = 100.00 deg

Offset = 0 deg

Omega = 50 deg

Phi = 0 deg (disabled)

Goniometer **PW3050/10 (Theta/Theta)**

Resolution = normal (0.001 deg)

Sample Stage **MPSS (Vertical System)**

Stage Accessory **quartz crystal for fibers and powder sample holder for HAp**

X-ray Generator **PW3040 (mppc)**

Status = on

Tension = 40 KV

Current = 20 mA

X-ray Tube **PW3373/00 Cu LFFDK 147380**

Shutter

Status = closed

Focus = line focus

Port = 1

Incident Beam Path

Radius = 200 mm

Take-off angle = 6 deg

Divergence Slit **Slit Fixed ½ deg**

Distance Sample = 100

Mask **Inc. Mask Fixed 15 mm (MPD/MRD)**

Distance Sample = not specified

Anti Scatter Slit **Slit Fixed ½ deg**

Soller Slit **Soller 0.04 rad**

2. Active Beam Path

Diffracted Beam Path 1

Optic number = 1

Radius = 200 mm

Offset = 0 deg

Used wavelength = K-alpha 1

Anti Scatter Slit **Slit Fixed ½ deg**

Receiving Slit **Prog. Rec. Slit**

Soller Slit **Soller 0.04 rad**

Detector **PW3011 (Miniprop small window)**

PHD lower level = 35 %

PHD upper level = 80 %

Bragg recognized a predictable relationship for the distance between similar atomic planes in a mineral (the interatomic spacing) called the d-spacing (measured in angstroms), the angle of diffraction theta (measured in degrees) and the wavelength of the incident X- ray radiation. For practical reasons, the diffractometer measures an angle twice that of the theta angle.

CHAPTER 5

RESULTS AND DISCUSSION

Fibers were characterized in terms of their mechanical behavior, temperature dependent dimension changes and temperature dependent weight reduction. This was done by Thermomechanical Analyzer and Thermogravimetric Analyzer. Hydroxyapatite coated collagen fibers were analyzed with X-ray diffraction and the spectrum was compared with the hydroxyapatite spectrum as reference. Surface structure was observed from the EPI-DIC images, taken for hydroxyapatite powder and the fibers.

5.1 Thermogravimetric Analysis

Thermogravimetric Analyzer was used to see the temperature dependent weight changes in the fibers. Samples analyzed were hydroxyapatite powder, pure collagen fiber, hydroxyapatite coated collagen fiber and hydroxyapatite mixed collagen fiber.

The melting point for hydroxyapatite is in the range of 800 deg C to 1200 deg C depending on its stoichiometry. The samples were heated from 0 deg C to 350 deg C at the rate of 10 deg C/min. The water in the samples gets evaporated at 100 deg C. Any further reduction in the weight is due to the sample itself. Hydroxyapatite mixed collagen fibers have dense hydroxyapatite and the content is more compared to the hydroxyapatite coated collagen fibers which have hydroxyapatite crystals only on the surface and the content of hydroxyapatite is low. The table shows the weight retention of the samples at 350 deg C. The weight percentage retention was seen highest with hydroxyapatite powder (Figure A.1) and least for pure collagen fiber (Figure A.2). The weight retention with the hydroxyapatite coated collagen fiber and collagen/hydroxyapatite composite fiber lies in between the two (Figures A.3 and A.4).

Table 5.1 Weight Retention as a function of Temperature

Samples	Weight retention at 350 deg C
Hydroxyapatite powder	96.66%
Collagen fiber	46.21%
Hydroxyapatite coated collagen fiber	60.94%
collagen/hydroxyapatite fiber	80.17%

5.2 Thermomechanical Analysis

Thermomechanical Analysis was done on the samples. The samples were heated at a constant rate of 10 deg C per minute. The fibers were under a constant force of 1N. The heating was carried out to obtain the breaking temperature for the fibers.

The results showed the collagen/hydroxyapatite composite fibers had the highest breaking temperature (Figure B.3). This can be related to the fact that hydroxyapatite has a melting temperature in the range of 800 deg C to 1200 deg C. Some of the water sites in the collagen fibers were occupied with hydroxyapatite molecules which would have led to the increase in breaking temperature for the collagen/hydroxyapatite composite fibers. Since hydroxyapatite coated collagen fibers have crystals on the surface, the temperature at which it breaks is lower than the collagen/hydroxyapatite composite fibers (Figure B.2). As the heating increases, there is a contraction in the helical structure of the collagen molecule. This contraction is caused by the rotation of the structural units in the protein. Since, some of the hydration sites in collagen are occupied by the hydroxyapatite molecules, these fibers denature at a higher temperature. The breaking point temperature for the pure collagen fiber can be seen in the Figure B.1.

Table 5.2 Breaking Temperature at a Constant Load

Samples	Breaking Temperature (deg C) at 1 Newton
Collagen fiber	252.78
Hydroxyapatite coated collagen fiber	257.09
Collagen/hydroxyapatite fiber	267.41

5.3 Tensile Modulus

With ageing of collagen, the tensile strength and moduli of the collagen fibril increases. This is associated with the crosslinking of the collagen fibrils. At maturity, the crosslinking process is reversed. This results in decreased tensile strength and decreased failure strength. Hydroxyapatite has a poor mechanical loading property. The results prove the poor mechanical loading property of hydroxyapatite. Collagen/hydroxyapatite mixed fibers showed the least tensile strength (Figure C.3). These fibers had hydroxyapatite and collagen in the weight ratio 5 to 95. Hydroxyapatite molecules occupied the sites between the collagen fibrils which decreased their crosslinking ability. The highest tensile strength was shown by pure collagen fibers (Figure C.1). Hydroxyapatite coated collagen showed the tensile strength between these two fibers (Figure C.2).

Table 5.3 Breaking Point for the Fibers

Samples	Breaking Point
Collagen fiber	N/A
Hydroxyapatite coated collagen fiber	.98 Newton
collagen/hydroxyapatite fiber	.25 Newton

Table 5.4 Tensile Modulus for the Fibers

Samples	Initial Slope in mm/Newton	Modulus Newton/meter^2
Collagen fiber	.4330	$1141.8 * 10^6$
HA coated collagen fiber	.8176	$490.5 * 10^6$
Cg/HA fiber	1.031	$198.2 * 10^6$

5.4 X-ray Diffraction

X-ray diffraction was performed to check the coating on the collagen fiber. For this reason, pure hydroxyapatite powder was analyzed as a reference. The spectrum of hydroxyapatite powder (Figure D.1) showed a prominent peak at 33 deg (2theta). The hydroxyapatite coated collagen fibers (Figure D.2) also showed a prominent peak at 33 deg (2theta). This showed that the coating technique used was a good method to deposit hydroxyapatite crystals on the collagen fibers.

5.5 Differential Interference Contrast Microscopy

Differential Interference Contrast (DIC) microscopy is a beam-shearing interference system in which the reference beam is sheared by a minuscule amount. It is an excellent mechanism for rendering contrast in transparent specimens. The technique produces a monochromatic shadow-cast image that effectively displays the gradient of optical paths for both high and low spatial frequencies present in the specimen. Those regions of the specimen where the optical paths increase along a reference direction appear brighter (or darker), while regions where the path differences decrease appear in reverse contrast. As the gradient of optical path difference grows steeper, image contrast is dramatically increased. Figure D.3 shows the crystalline hydroxyapatite powder. The hydroxyapatite crystals can be clearly seen on the collagen fiber in Figure D.5. The crystalline nature of hydroxyapatite was not observed on the surface of the composite fiber (Figure D.6). The surface of the pure collagen fiber can be seen in Figure D.4.

CHAPTER 6

CONCLUSION AND FUTURE SUGGESTIONS

This study was based on the wet-spinning of collagen. Thermal analysis proved that hydroxyapatite had a poor mechanical loading property. However, since hydroxyapatite has high melting temperature, the fibers having hydroxyapatite in them could withstand higher temperature at a constant load of 1N before breaking and had high weight retention at the said temperature of 350 deg C. Values of percentage weight retention, tensile strength and breaking point temperature for the coated fibers were between those of the composite fibers and pure collagen fibers. These results indicate that the hydroxyapatite deposited on these fibers was less than the hydroxyapatite in the composite fibers. This study showed a new technique for coating hydroxyapatite crystals on collagen fibers. Wet-spinning for the composite fibers was employed for the first time.

Metals coated with hydroxyapatite have been used as orthopedic implants. A good experiment will be to test the osteoconduction and osteoinduction properties on these fibers. Also, it is necessary to increase the strength of the fibers by crosslinking them with different agents without decreasing the percentage of hydroxyapatite in the fibers. Coated fibers with biomimetic methods such as simulated body fluid and the coated fibers obtained from the present study need to be compared. Methods for the uniform dispersion of hydroxyapatite in collagen fibers are to be investigated. As for the pure collagen fibers, comparing the properties of fibers spun with freshly prepared dispersion and preserved dispersion need to be compared.

APPENDIX A

TGA PLOTS FOR WEIGHT RETENTION OF THE FIBERS

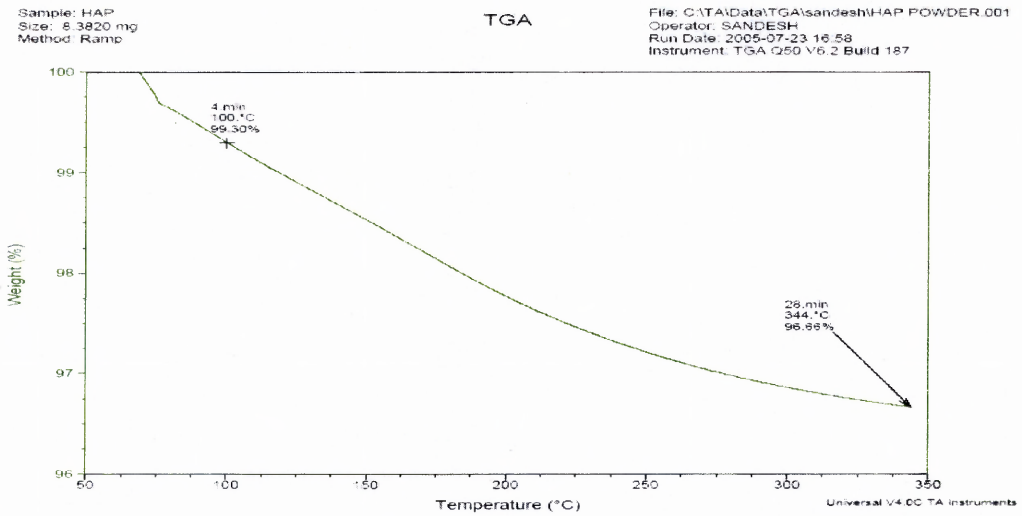

Figure A.1 Percentage Weight Retention for Hydroxyapatite Powder.

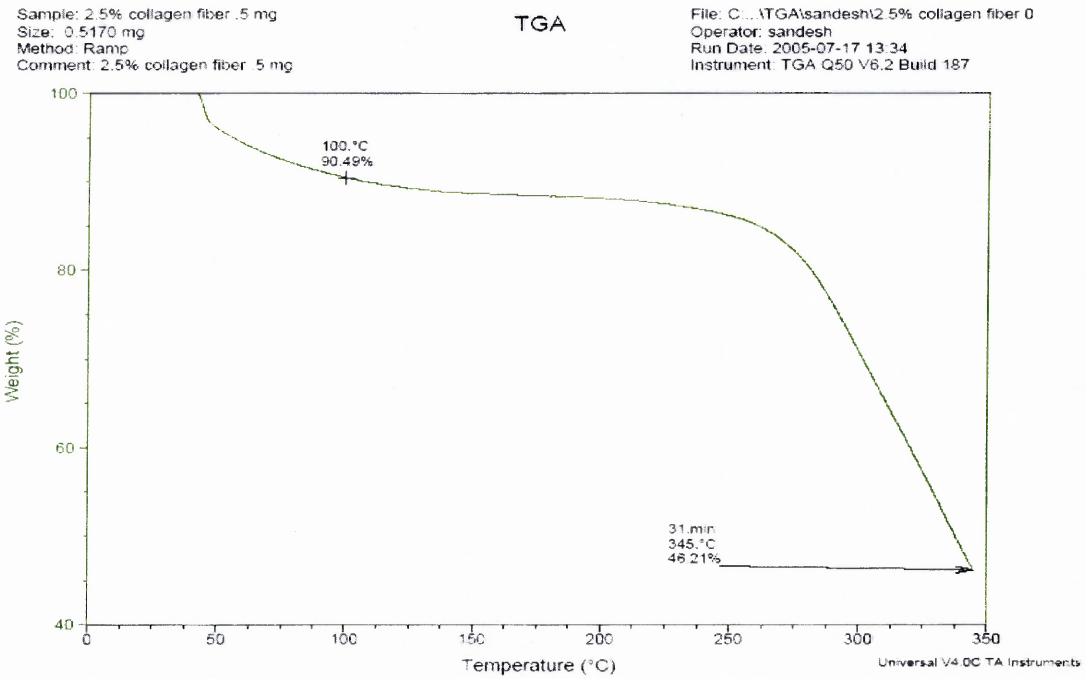

Figure A.2 Percentage Weight Retention for Pure Collagen Fiber.

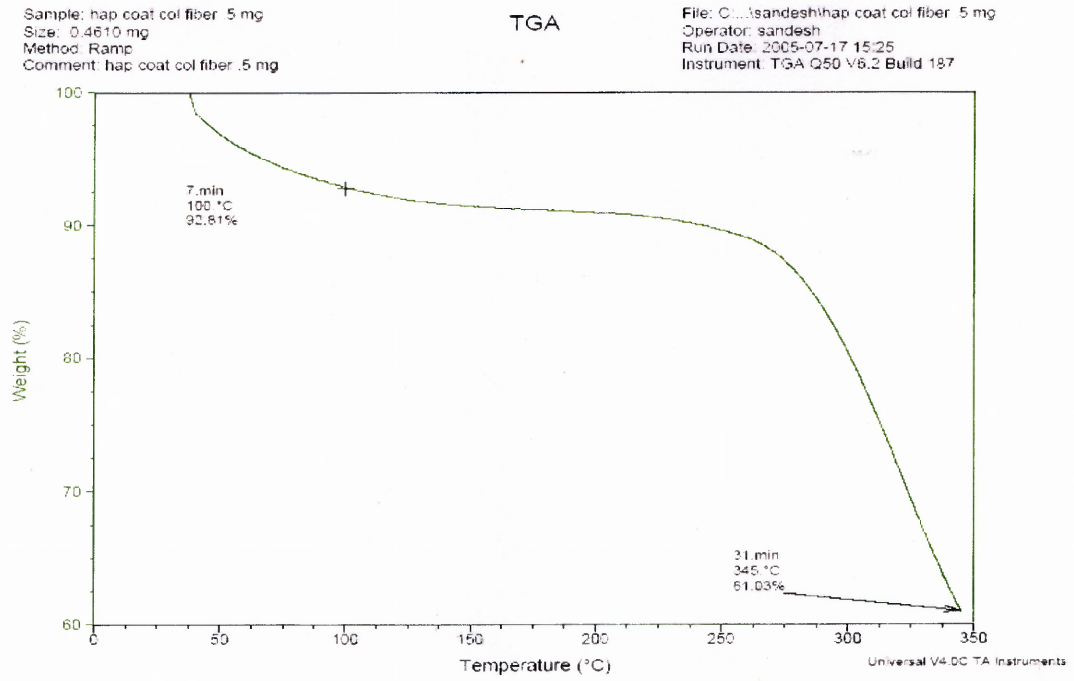

Figure A.3 Percentage Weight Retention in HA coated Collagen Fiber.

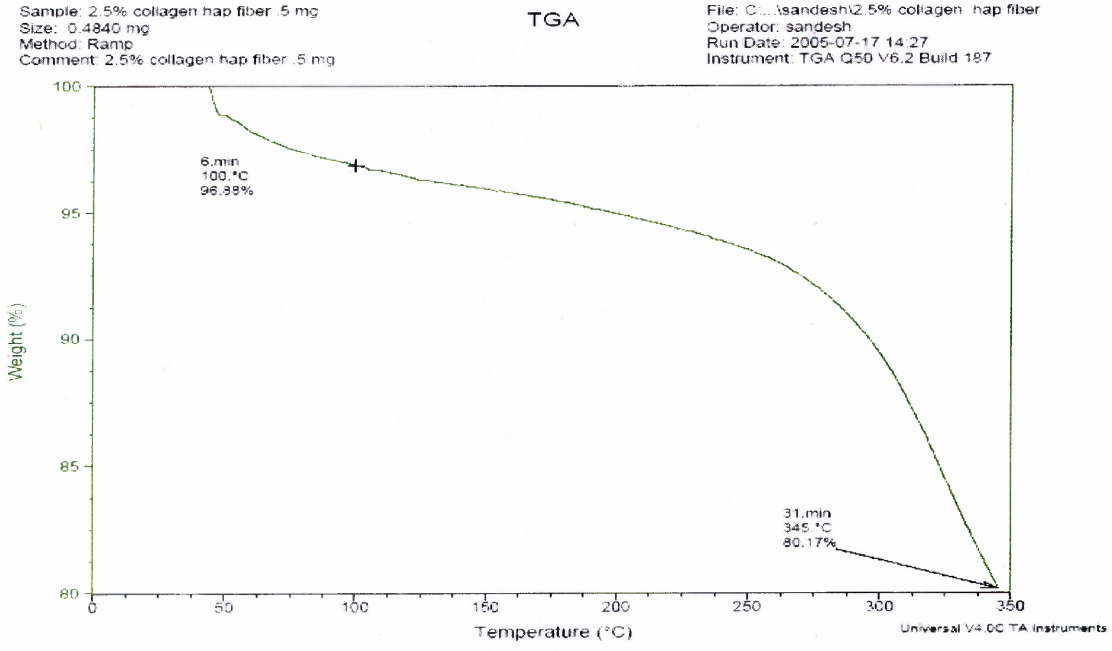

Figure A.4 Percentage Weight Retention in Collagen/Hydroxyapatite Composite Fiber.

APPENDIX B

PLOTS FOR BREAKING POINT TEMPERATURE OF THE FIBERS

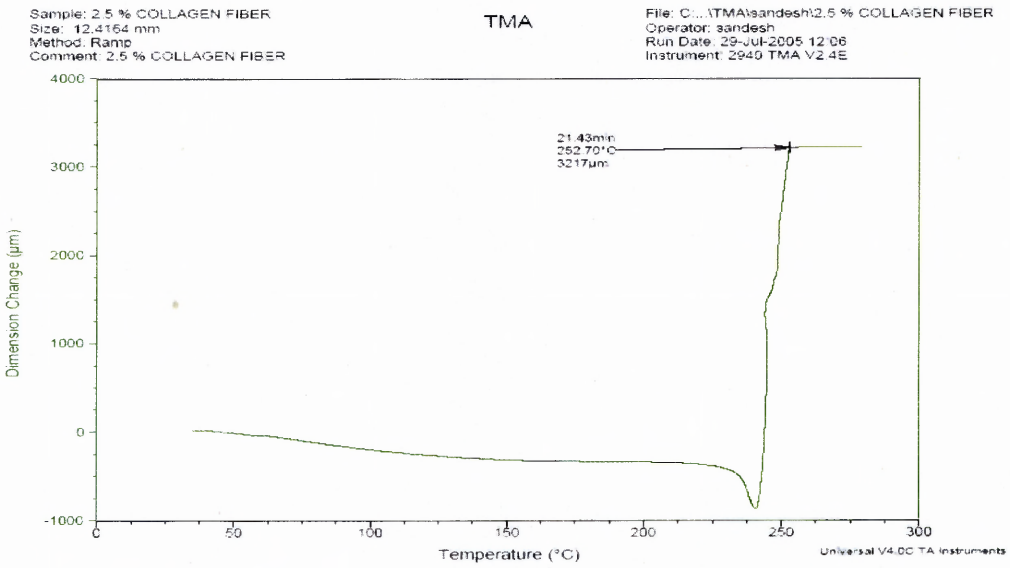

Figure B.1 Breaking Point Temperature for Pure Collagen Fiber.

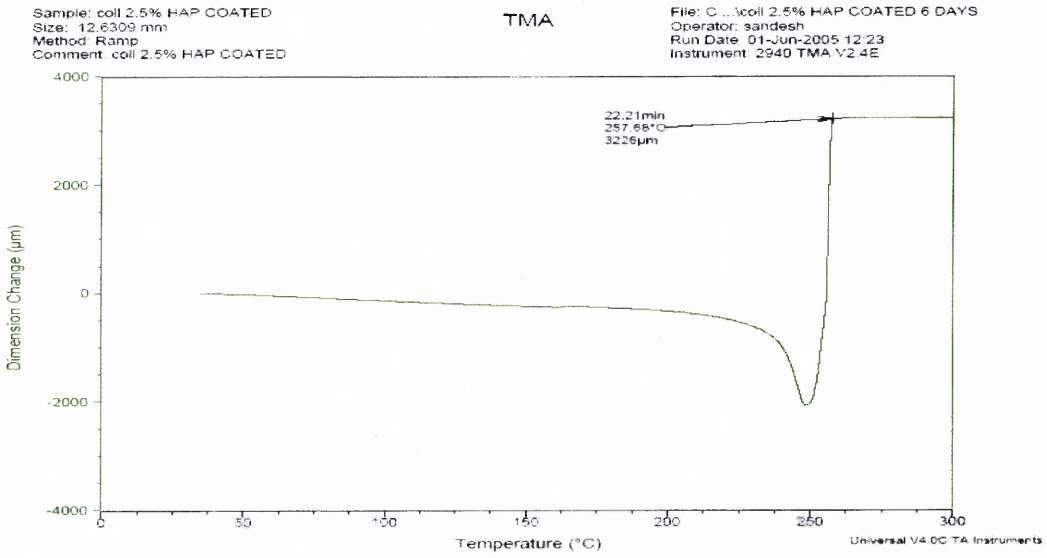

Figure B.2 Breaking Point Temperature for HA Coated Collagen Fiber.

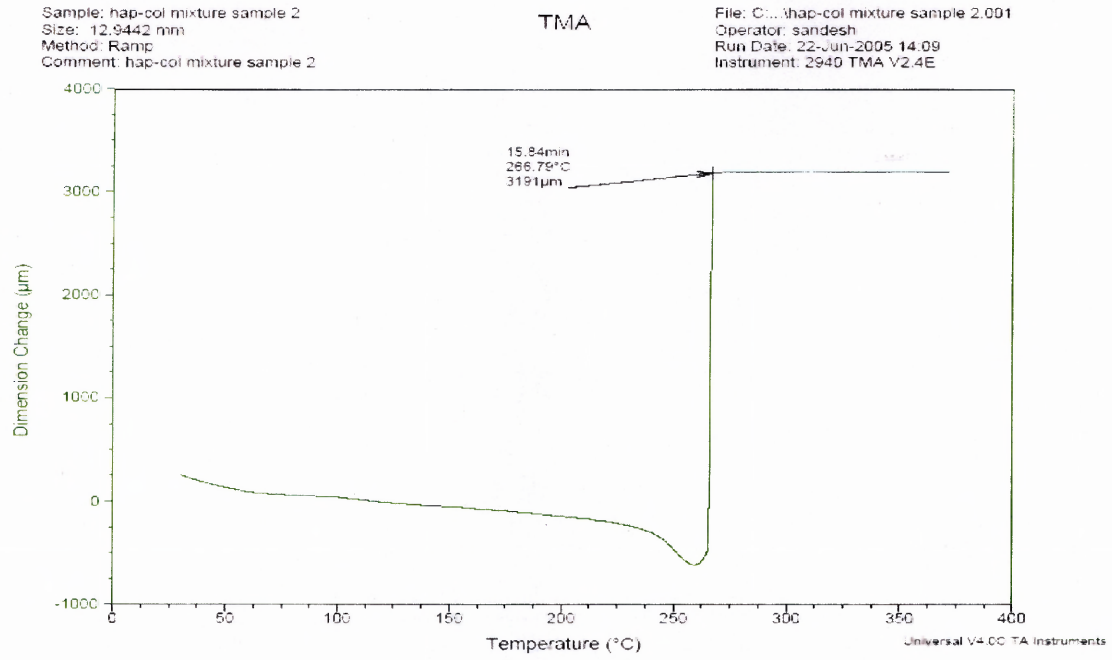

Figure B.3 Breaking Point Temperature for Collagen/Hydroxyapatite Composite Fiber.

APPENDIX C

PLOTS FOR THE TENSILE MODULUS OF THE FIBERS

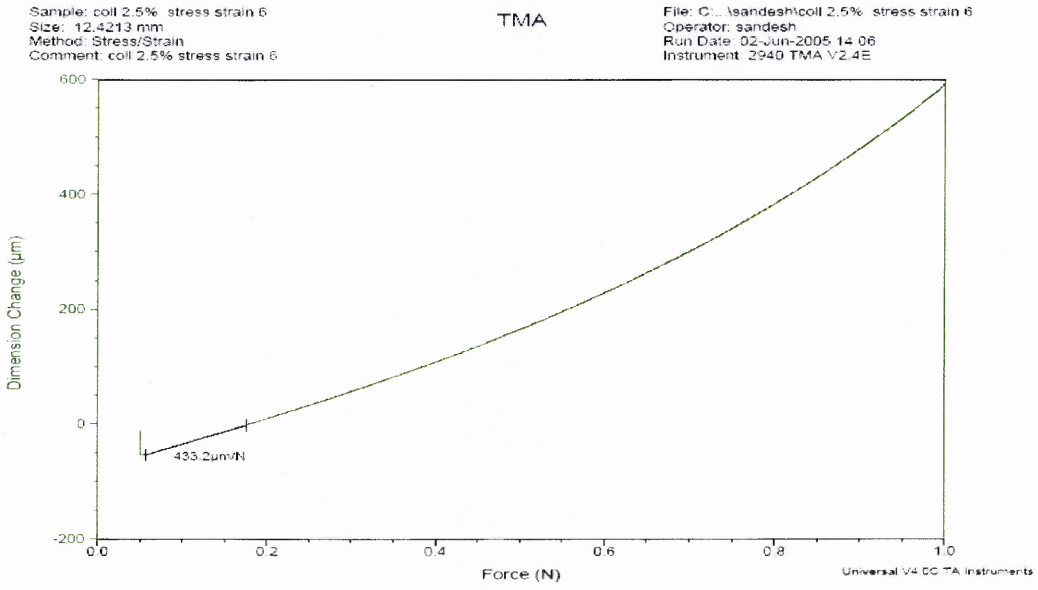

Figure C.1 Initial Tensile Modulus for Pure Collagen Fiber.

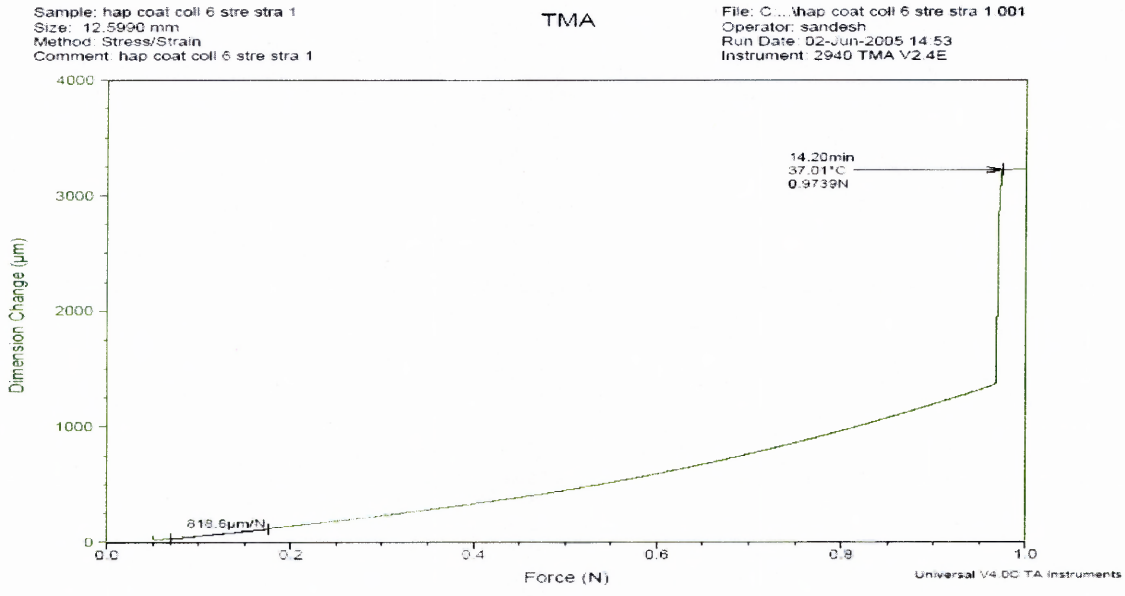

Figure C.2 Initial Tensile Modulus for HA coated Collagen Fiber.

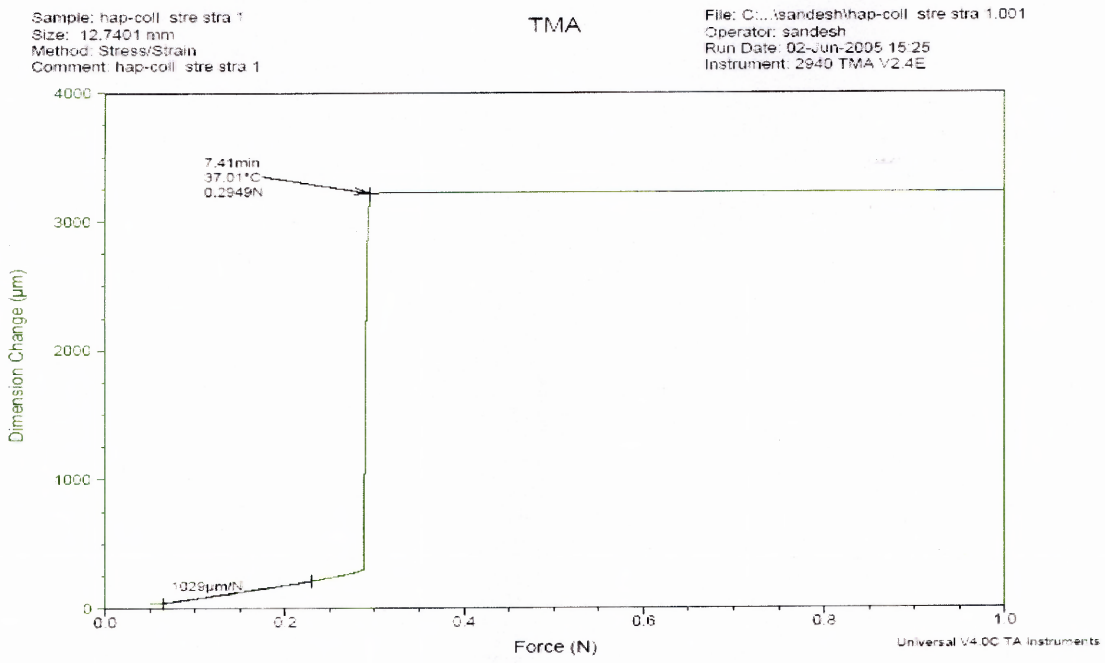

Figure C.3 Initial Tensile Modulus for Collagen/Hydroxyapatite Composite Fiber.

APPENDIX D

X-RAY DIFFRACTION SPECTRUM AND DIC IMAGES

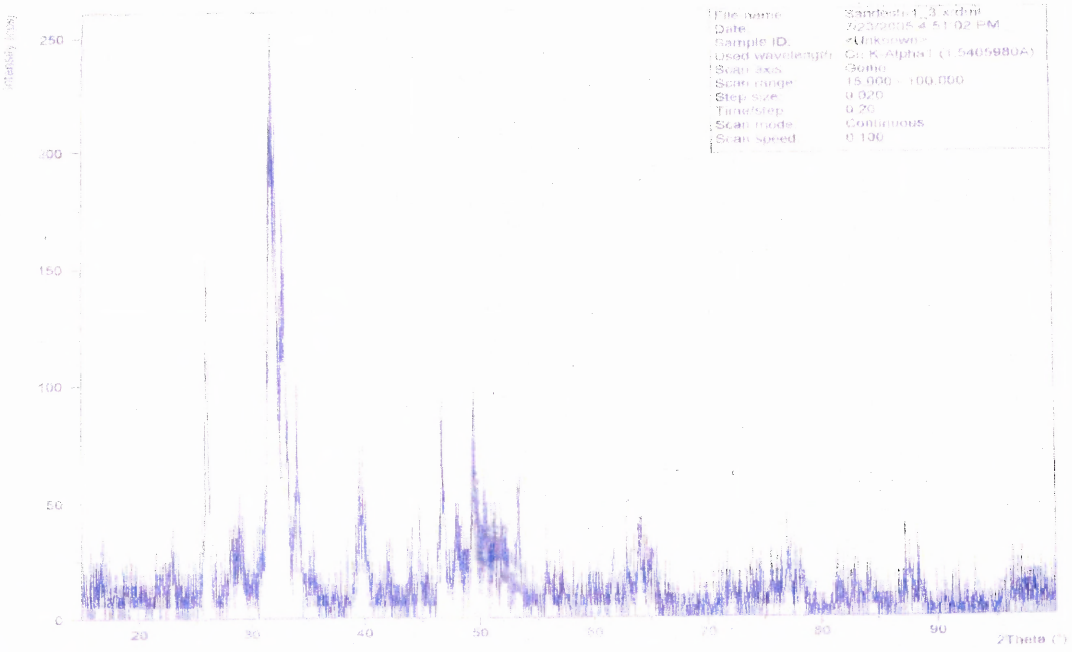

Figure D.1 XRD Spectra for Hydroxyapatite Powder.

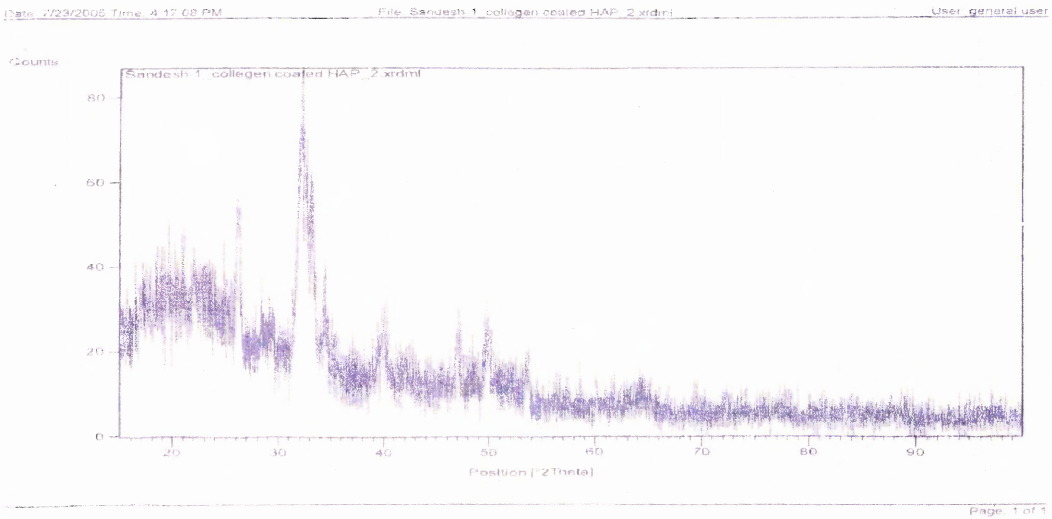

Figure D.2 XRD Spectra for HA coated Collagen Fiber.

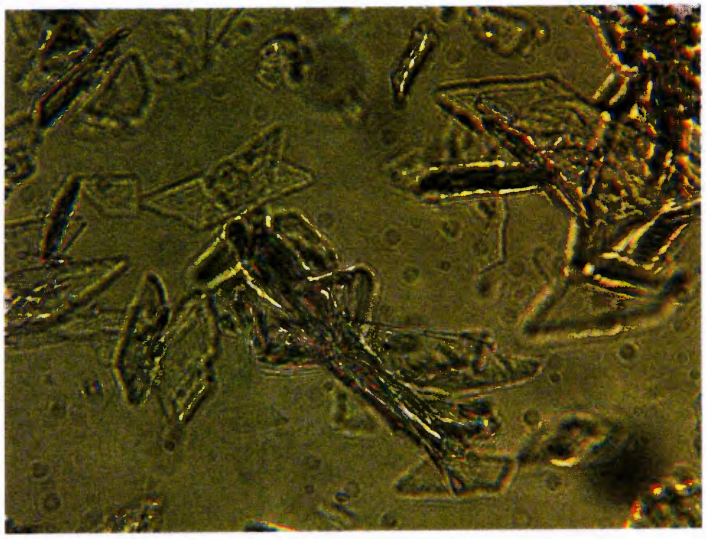

Figure D.3 Hydroxyapatite Crystals.

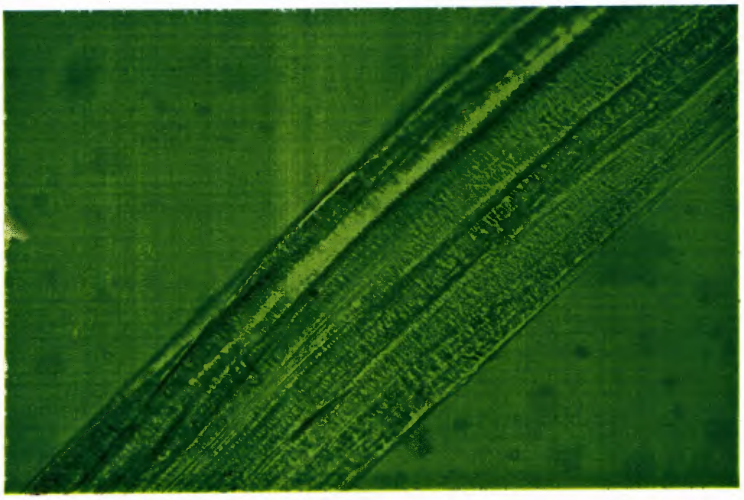

Figure D.4 Pure Collagen Fiber.

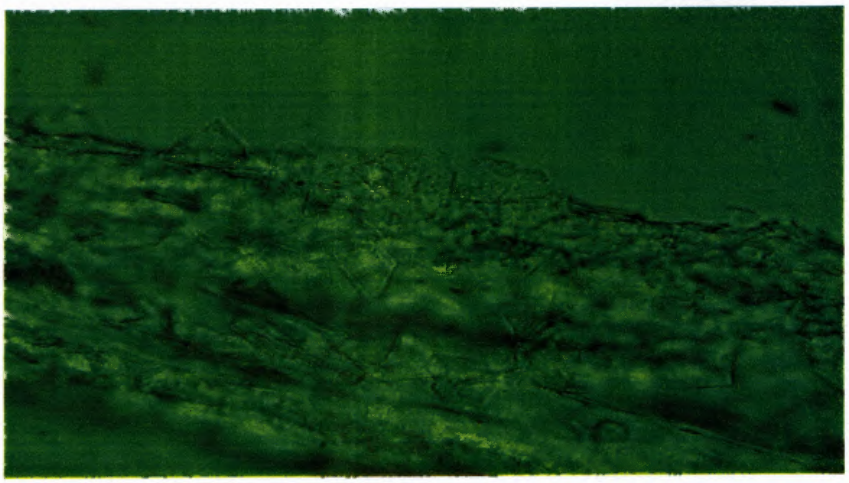

Figure D.5 Hydroxyapatite coated Collagen Fiber.

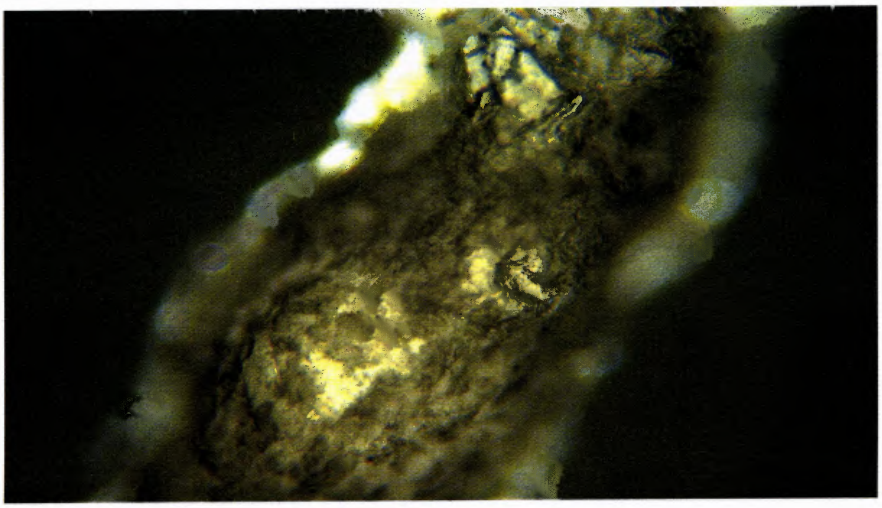

Figure D.6 Hydroxyapatite/Collagen Composite Fiber.

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