FABRICATION OF FIBROUS 3D COMPOSITE SCAFFOLD BY RAPID PROTOTYPING FOR TISSUE ENGINEERING APPLICATIONS

Thesis submitted in partial fulfilment of the requirements for the degree of

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In

Biomedical Engineering

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Under the guidance

Of

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National Institute of Technology Rourkela

CERTIFICATE

This is to certify that the thesis entitled ‘’**FABRICATION OF FIBROUS 3D COMPOSITE SCAFFOLD BY RAPID PROTOTYPING FOR TISSUE ENGINEERING APPLICATIONS**’’ submitted by **Amit Kumar Singh (213BM1017)** in partial fulfilment of the requirements for the award of **Master of Technology Degree** in **Biomedical Engineering** at National Institute of Technology, Rourkela is an authentic work carried out by him under my supervision and guidance.

To the best of my knowledge, the matter embodied in the thesis has not been submitted to any other University/Institute for the award of any Degree or Diploma.

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ABSTRCT

To develop the technique or finding the solution of unsolved medical problem, biological and medical practices are not enough to find the ways of treatment, that's why engineering is continuously involve in the treatment process and combination of biological, medical and engineering principle named as Tissue engineering. In modern medical era Tissue engineering revolutionize the ways of healing process to restore, enhance and replace diseased or damaged organ or tissue. The concept of tissue engineering reincarnate, the creation of an extra cellular matrix named as Scaffold that has the appropriate physical, chemical, and mechanical properties to enable cell penetration and tissue formation in three dimensions. There are numerous method for fabrication of 3D scaffold. Among them Rapid prototyping is most ideal technique to fabricate 3D extra cellular matrix because of its accuracy in designing of complex structure with control of pore size as well as structure.

Thing-O-Matic Replicator instrument is a Rapid Prototyping technique which is designed for the filament form of material. This work is focused on modification of instrument for scaffold fabrication with liquid phase of material. After modification, process parameters were optimized for the sodium alginate and gelatin composite such as concentration of individual solution, ratio of alginate and gelatin sodium, crosslinking agent, pressure, distance between nozzle and platform etc. Fabricated scaffolds were further characterized by SEM, XRD, FTIR, contact angle measurement, and tensile strength testing. Sodium alginate and gelatin composite scaffolds were produced by the modified setup with the range of diameter 150μm to 190μm and porosity with horizontal length 190μm to 300μm. The tensile strength measurement gives yield strength 60 kilopascal for prepared scaffold reinforced with β-TCP. Analysis of contact angle measurement data shows the high hydrophilic nature of scaffold after reinforcing with β-TCP.

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Chapter 1

Introduction

1. GENERAL INTRODUCTION

As the population increases number of disorder, loss, failure of organ or tissue is also increases due to disease, injuries, accident. The problem in tissue or organ as like failure or loss of tissue or organ is very difficult and devastating problem and the cure of such kind of problem is very costly. Stats said that in the US alone, every year approximate twenty million patients suffer with different types of organ and tissue related maladies such as tissue defects and diseases. Every year approximately eight million surgical treatment are performed to curing these cases and still over thousands of people are on waiting lists for transplantation, and an additional lakhs of patients die without even get chance of treatment through waiting list [1]. The cost for healing of these problem is so far away from the average people. To overcome these problem tissue engineering is developed which combines the biological principles and technology to healing of these problem. Tissue engineering is an application of engineering principles in biological and medical field to replace, regenerate and improve the condition of diseased organ and enhancement in the function of organ for the betterment of the human being [2, 3]. Strategy of tissue engineering has focused on provide the favorable cell environment by the modulation of cell– ECM (extracellular matrix) and cell–cell interactions. The ECM may influence cell behavior through it material properties, surface treatment, degree of porosity, and pore size. Controlling each of these environmental influences has been used to facilitate the development of functional tissue [4]. The selection of biomaterial for is very important parameter for designing of 3D ECM scaffold that is depends on the tissue that is to be regenerated. The size and geometry of pores to be designed within the scaffold is also important to the cell behavior and has always been one of the key parameters in the design of the ECM scaffold [5].

Scaffold used in Tissue engineering should have some specific properties like it should be biocompatible, large surface area, biodegradable, sufficient porosity with interconnected pores, non-toxic and it allow cells to attach on the surface and allow to grow on it and promotes neo-vascularization when being implanted in vivo and very important, it should be vital for cell regeneration. In present days as biomaterial hydrogels are in fashion. They are composed of hydrophilic polymer chains that could be either synthetic or natural. Examples of synthetic hydrogels include HEMA (2-hydroxyethyl methacrylate), poly (ethylene oxide) and its copolymers, and PVA (poly vinyl alcohol). Natural hydrogels, which are usually a biopolymer, include alginate, collagen, gelatin, fibrin, chitosan, agarose, and hyaluronate [6].

For fabrication of scaffold there are lot of methods but in the recent years Rapid Prototyping technique become very popular due to its specific and complex process at the fabrication level. Basically Rapid prototyping is a common and general method for 3D fabrication of prototype of any object. Present days this technique is very popular in field of Tissue engineering. Traditional method which was used in Tissue Engineering have number of limitation to overcome those limitations, the rapid prototyping (RP) has been explored by many scientists. RP technologies enable us to provide scaffolds with well-defined and controlled internal architecture. The RP technologies, including stereo lithography (SLA), selective laser sintering (SLS), fused deposition modeling (FDM), three-dimensional printing (TDP or 3DP) have been widely used in fabrication of scaffolds for tissue engineering [7]. Dimensional accuracy is limited in these processes by the nozzle size which is the main advantage of RP technique.

In RP technique the complex model firstly made into the software then after the structure is imported into the driving software and software process on the model and generate codes for the RP machine and according to the codes machine work.

Here the main frame of work is to modify the machine to develop the scaffold liquid form of biomaterial. Biomaterial in the form of filament is very costly so it is not possible to buy the filament biomaterial for every application then this is very important to find cheapest way to design the 3D fibrous scaffold using biomaterial. Normally biomaterial for the scaffold fabrication available in the powder form and it can be easily converted in liquid or gel form, in powder form biomaterial are cheap and easy in availability. Machine has been modified in this way that liquid biomaterial material can used to fabricate 3D scaffold. After modification the scaffold has been prepared and the characterization has been done for analysis of surface morphology, chemical composition, phase composition, strength, hydrophilic or hydrophobic nature. Then after to improve the characteristic of prepared scaffold, ceramic coating has been done and again characterization has been done to analyze enhance property.

1.1 Hydrogel of Sodium Alginate and Gelatin

Alginate is a natural polysaccharides which is derived from the different type of brown seaweeds of the phylum after that it is converted into sodium alginate. These are the composite of β-D-mannuronic acid (M units) and α-L-guluronic acid (G units) monomers along the polymer backbone. The ratio of these mannuronic acid units and guluronic acid units depends upon the source form which these are derived. M and G both have carboxylic groups which is capable in the formation of salt such as Sodium Alginate where the sodium monovalent ions are attached ionically to the carboxylic groups.

In this work $CaCl_2$ is used as crosslinking agent for the sodium alginate. When sodium alginate solution drop into $CaCl_2$ solution, sodium ions replaced by the calcium ions and one calcium ion bind the two of the polymer strands.

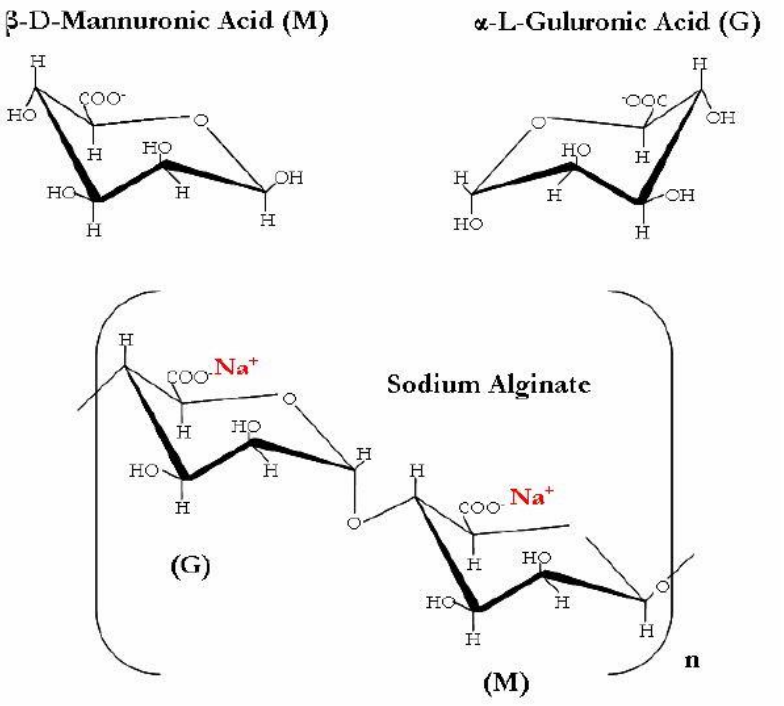

Figure 1: Diagram of β-D- mannuronic acid (M units) and α-Lguluronic acid (G units), and sodium alginate

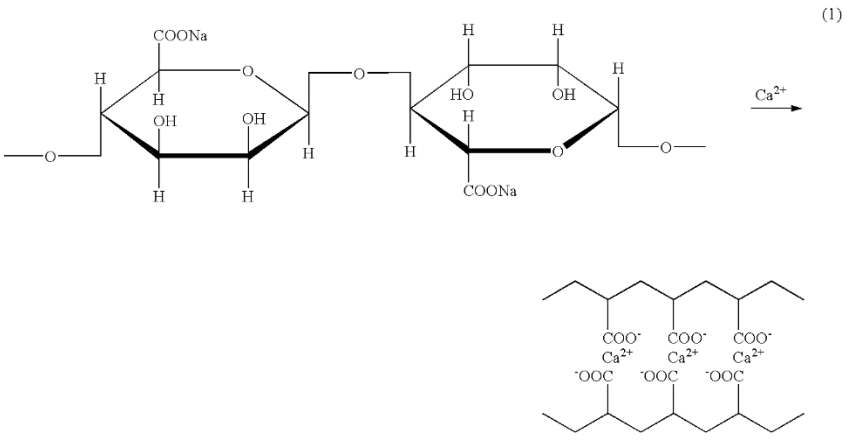

Fig 2: Crosslinking of Sodium alginate In Calcium Chloride solution

Gelatin is natural protein which is prepared by the thermal defacement of collagen, isolated from animal bones and skin, with highly dilute acid. It can also be extracted from fish skins. Gelatin contains glycine, proline and 4-hydroxyproline residue. Aldehyde reacts with the amine of the lysine residues of the gelatin chain

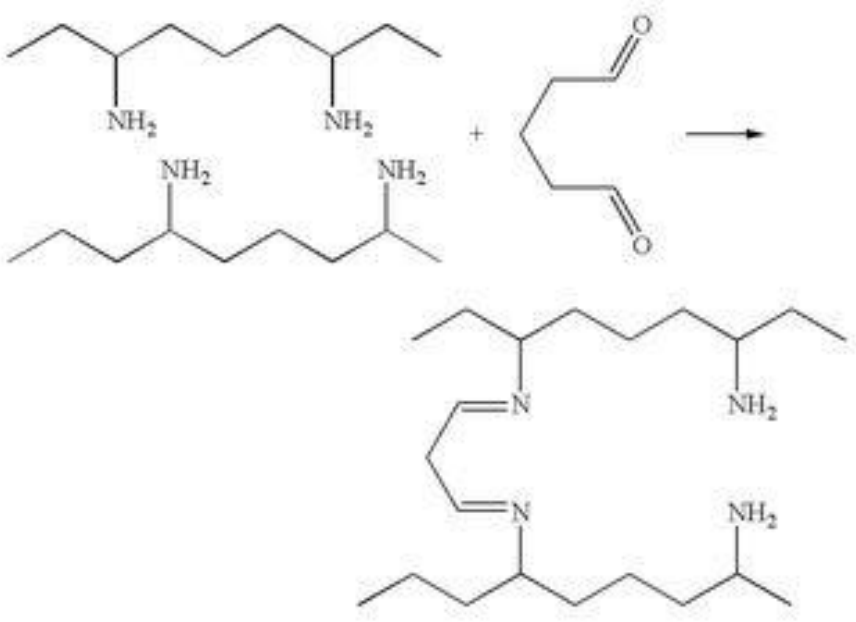

Fig 3 Crosslinking of Gelatin in diluted Glutraldehyde solution

1.2 Objective

1.2.1 Develop a viable technique for the fabrication of tissue substitute construct

1.2.2 Deposition feasibility study for biomaterial composites (sodium alginate + gelatin) solution

1.2.3 Study 3D bio-composite scaffold structural formation

1.2.4 Enhancement of surface property to improve cell viability

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Chapter 2

Literature Review

2. LITERATURE REVIEW

2.1.Tissue Engineering

Tissue engineering is an emergent multidisciplinary area of research that applies the principle of medicine, biology and engineering that is likely to discover the ways to improve the health and quality of life of people by maintaining, enhancing or restoring tissue and organ function.

It involves the assembly of tissue structure by combining cells and biomaterials and ultimately attempts to replace or restore physical functions lost in diseased or damage organ.

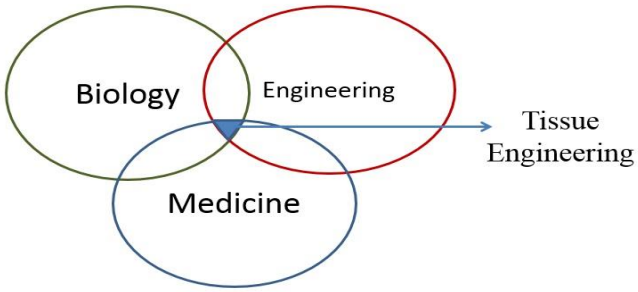

Fig 4: Tissue engineering Venn diagram

2.2 Scaffold

Scaffold is an artificial extracellular matrix (ECM) and is a 3D structure which is made up of biomaterial like natural and synthetic polymer, ceramic, metals and semiconductor etc. on which cells attach and differentiate on it and form the tissue. Scaffolds play very important role, such as

2.2.1 Allow cells to attach and migrate

2.2.2 Deliver & retain cells and biochemical factors

2.2.3 Enable diffusion of vital cell

2.2.4 Exert certain mechanical and biological influences to modify the behavior of the cell phase

2.2.1 Important properties of scaffold:

2.2.1.1 Biocompatible

The material chosen to fabricate scaffold for tissue engineering application must be compatible and should not interfere with biological system at the site of application.

2.2.1.2 Biodegradable

The scaffold material should degrade in a controlled way with the regeneration of tissue at site of implantation.

2.2.1.3 Should have sufficient mechanical strength

The mechanical property such as tensile strength, compressive strength and the elasticity of the scaffold should be in accordance to the tissue of application.

2.2.1.4 High porous

The scaffold should be highly porous (80-90%) to promote the cell penetration, proliferation uniformly throughout the scaffold. Porosity of the scaffold also help in proper distribution and availability of nutrient as well as removal of toxic material from the scaffold.

2.2.1.5 Non toxic

The scaffold material should not be toxic and help in cell attachment and proliferation.

2.2.1.6 Bioactive

Scaffold material should promote cell attachment, proliferation and differentiation of mesenchymal stem cells.

2.3 Scaffold fabrication technique

There are various technique available to fabricate scaffold, some of the techniques are described here:

2.3.1 Salt leaching method

Salt leaching developed by Mikos et al. in the year of 1994 [8], is relatively simple method used to develop porous scaffolds**.** This process involves the addition of soluble salt particles as porogen in polymer solution and casting into suitable molds [9-10]. After evaporation of the solvent, the salt particles are removed by leaching thereby porous scaffolds are formed. The major drawbacks of this method are poor interconnectivity, lower mechanical strength, difficulty in controlling porosity and usage of high toxic solvent [11]. However, by controlling the size of the salt particle, it is possible to tailor the properties of the resultant porous structure [12-13].

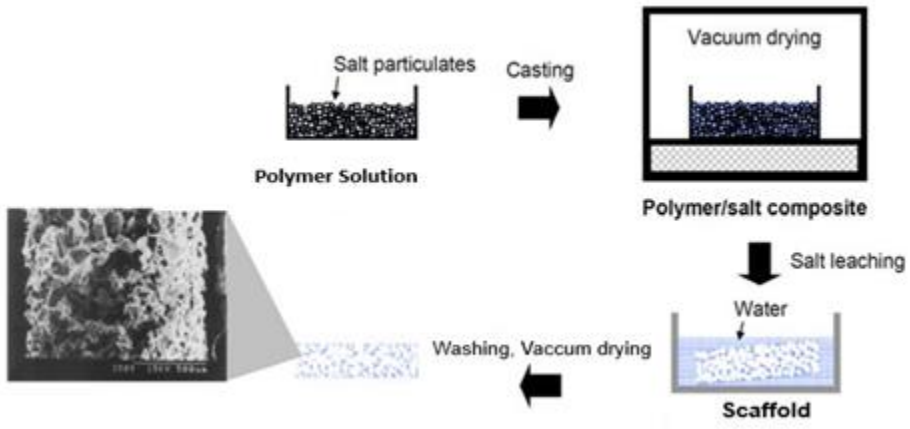

Fig 5: Salt leaching method for Scaffold fabrication

2.3.2 Gas forming

Gas foaming is a technique used to develop porous matrices. This technique can produce porous materials without the interference of any solvent [14]. Carbon dioxide is the most commonly

used gaseous agent for the formation of porous foam. Polymer disks at their solid state are subjected to CO_2 environment where gas bubbles are allowed to form in the polymer system, thereby creating porous sponges. The disadvantages of this method include need of specialized equipment to handle high pressure CO_2 and the process is limited to very few polymers [9].

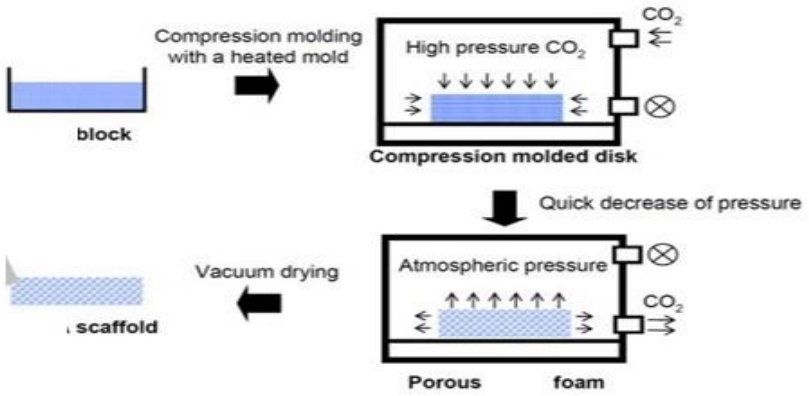

Fig 6: Gas forming technique for Scaffold fabrication

2.3.3 Freeze drying

Freeze-drying works on the principle of phase separation which is thermally induced. During this process, the phase-separated mixture is maintained at low temperatures and subjected to a high vacuum to sublime the solvent [15]. Pores are generated by the removal of ice crystals of solvent formed within the polymer solution. These ice crystals serve as porogen and size of these ice crystal can be controlled by adjusting freezing temperature and concentration of polymer solution [16]. The main drawbacks of freeze drying include scaffolds with low mechanical

strength, smaller pore size and difficulty in complete removal of the residual solvent [17].

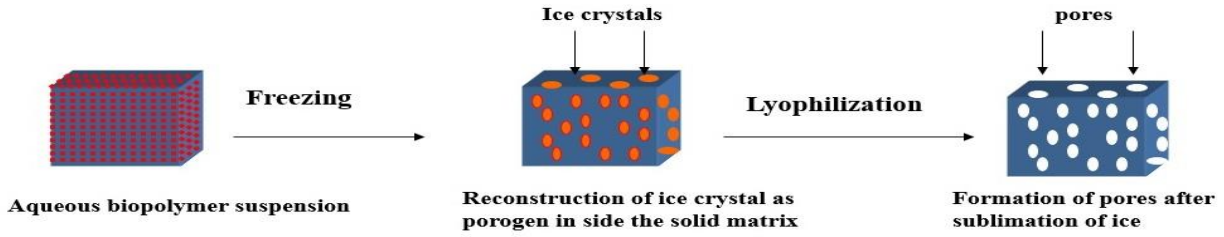

Fig 7: Freeze drying method for Scaffold fabrication

2.3.4 Freeze gelation

Freeze-gelation is a unique method of scaffold fabrication, which involves the principle of thermally induced phase separation. Problems encountered with the freeze drying process are destruction of pores in scaffold and formation of surface skin which are overcome by freeze-gelation method [18]. At the lower temperature, frozen polymer solution is immersed in a gelation environment and this temperature should be lower than freezing point of polymer solution [19]. In this method, since the polymer matrix becomes gel before the drying stage, the porous structure is retained without freeze-drying. The formation of ice crystals within the solution occurs at 273 K, but gelation does not occur until the temperature reaches a few degrees below freezing (~270 K). After freezing, the gel is warmed to melt the ice crystals and then dried. This causes a relatively high degree of continuous porosity with pores duplicating the morphology and dimension of the ice crystals formed during the freezing process. This method overcomes the limitations of freeze drying and sol-gel processing. It permits the formation of essentially zero-shrinkage, crack-free and low cost scaffolds [20]. Freeze-gelation has been used in wide areas of tissue engineering applications by using natural, synthetic polymers and their composites such as CS, silk, CS/β-TC, CS/PGA and CS/alginate/carboxy methyl cellulose.

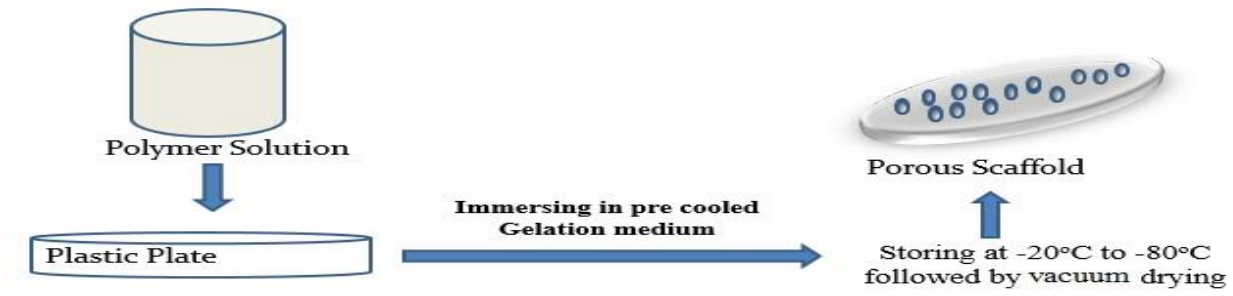

Fig 8: Freeze Gelation method for Scaffold fabrication

2.3.5 Electrospinning

Electrospinning is a method to generate nanofibers from various biomaterials. Electrospinning is a widely used technique that utilizes electric field to facilitate the formation of polymer deposits over a suitable collector [21-23]. The polymer solution is induced using a strong electric potential due to which it acquires an imbalanced charge. After a critical voltage is attained, surface tension of polymer solution is overcome by the charge imbalance leading to an electrically charged jet. This jet is focused towards a target which is grounded. After solvent evaporation, nanofibers are deposited over the collector. Process parameters that make the formation of ideal nanofibers include concentration of the polymer solution, tip to collector distance, applied voltage etc. [24]. Different natural and synthetic polymers were used to fabricate nanofibers for various applications such as wound healing [25], bone, skin, cartilage regeneration and drug delivery applications. The main disadvantages of Electrospinning include high energy requirement and high cost. Low pore size of the developed scaffolds further limit the cellular infiltration inside the fibers [26].

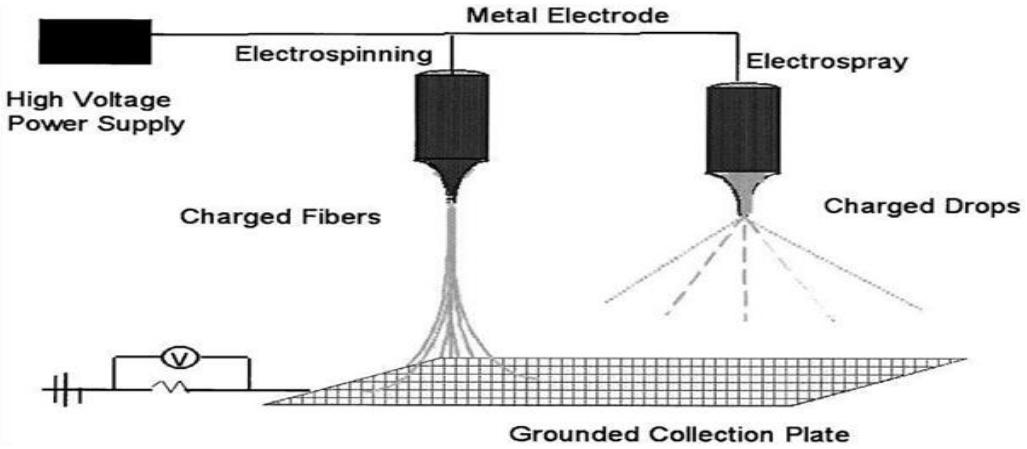

Fig 9: Electrospinning method for Scaffold fabrication

2.3.6 Rapid prototyping:

3D scaffolds are defined in three dimensions length, width and height. Scaffold fabrication in 2D is also very useful for healing for defected organ or tissue but at very complicated distortion of tissue or organ and we need a scaffold with specific shape and size for treatment then we need 3D form of scaffolds. Basically 3D scaffolds are designed for specific part of body with very specific purpose because according to the need 3D scaffold can be shaped into particular shape of organ or tissue. There are several method are presently used at the industrial level to develop 3D scaffold like freeze drying, solvent casting particulate leaching, freeze gelation, 3D printing, rapid prototyping.

2.4 Rapid prototyping Technique

Rapid prototyping is a technique to design 3D models of any object like parts of machine, machine tools, toys etc. at industrial level but now this method is also very popular in the field of biological regeneration of tissue or organ. Rapid prototyping is a technique which is used to design 3D scaffold into desired shape and size of prototype of any tissue or organ.

2.4.1 Robotics in the Rapid Prototyping

Rapid prototyping working principle is completely based upon robotics. In this machine there are one arduino circuit, three driver circuitry, one extruder circuit and two DC motor.

Arduino circuit is also known as controller circuit. From Arduino circuit all other component are connected: Mother board, driver circuit, nozzle heating setup and DC motor. It is basically used to command the all component of the device. It have microcontroller chip through which the component are interfaced and according to the command send by the computer it control the component. Extruder part is also connected to the Arduino which is used to push the filament into heating chamber through ***PTFE tube***.

Mother board is directly connected to the power supply and it guided the platform and nozzle to their maximum entropy of motion with using stop switch.

Driver circuitry is connected to the platform and nozzle part. Basically driver circuitry is used to move the platform into x-axis and y-axis and the nozzle part into z-axis. There are three stepper motors are connected to the driver circuitry among which two are used for x and y-axis motion and third one is used for z-axis motion.

End Stop switch is used to control the motion in all three direction. It is responsible for the maximum displacement of the platform in x-axis and y-axis and nozzle part into z-axis. At the time of motion at its maximum limit the moving part touch the switch and it makes switch open then it stop the movement and decide the limit of movement in one direction.

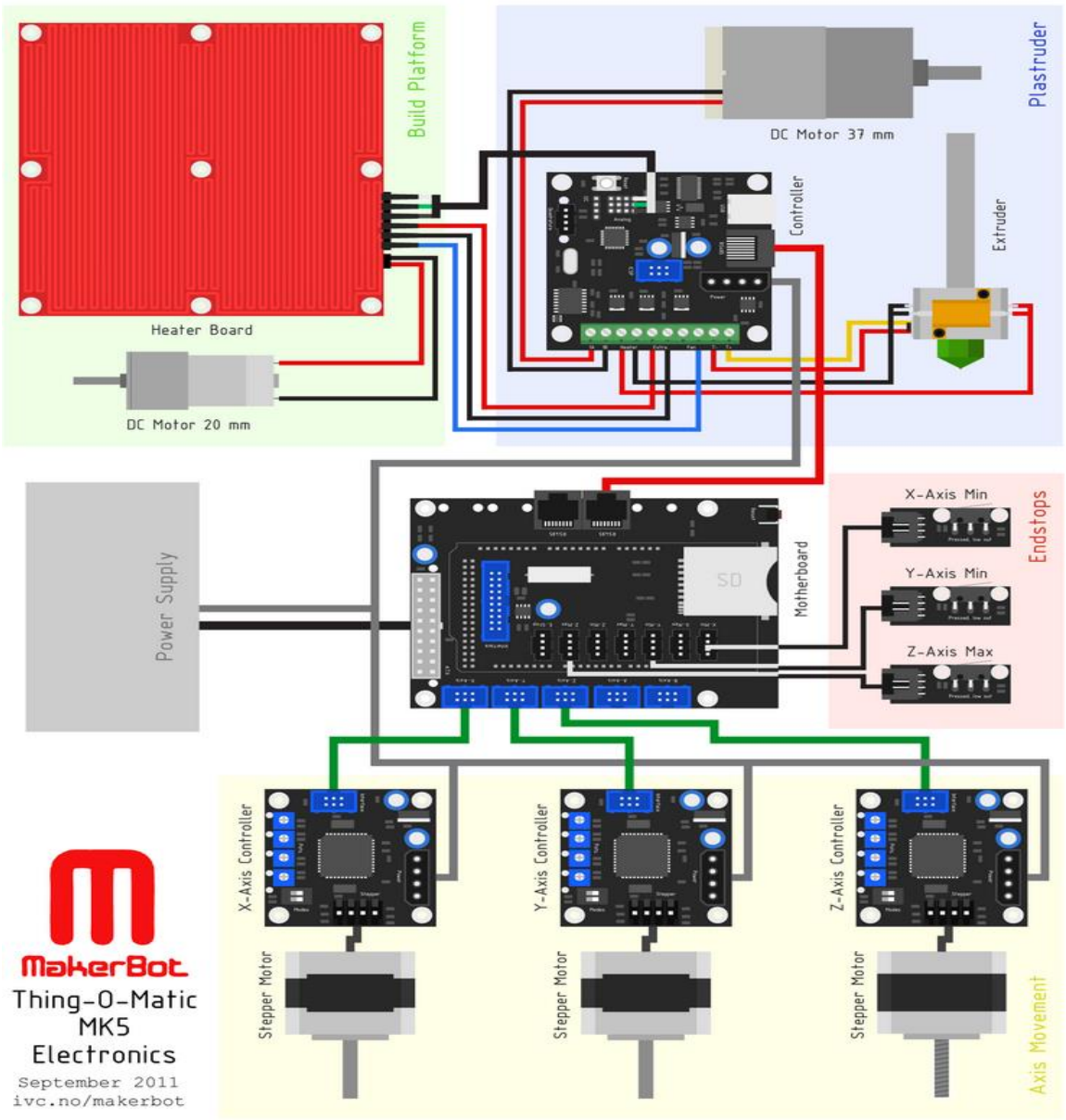

Fig 10: Circuit board of Thing-O-Matic MK5 Rapid Prototyping

2.5 Driving Software: Replicator G

Replicator G is an open source software which is used to drive the different type of 3D printing technique. In this project Replicator G software used as the drive control unit for the Rapid Prototyping Machine which perform very important role in the process of 3D scaffold

fabrication. This is very advance technology which is used to drive not only the Thing-O-Matic Rapid Prototyping Replicator G perform very important functions like:

2.5.1 Importing 3D models

2.5.2 Slicing 3D model into number of 2D layers

2.5.3 Generate driving codes for each 2D layer (called G Code)

2.5.4 Control the movement, speed and distance of the platform and the nozzle moving axis.

2.5.5 Upload the new instruction to Arduino circuit board

2.6 Different forms of Rapid prototyping

Rapid prototyping basically are two different forms depending upon the state of materials:

2.6.1 Material in Filament form

Filament type material based Rapid Prototyping machine is work on the principle of FDM (Fused Deposition Modelling). In this type of rapid prototyping firstly material fused at set temperature then comes out through the nozzle and deposit on the platform. In this method the material should be in the form of filament. For the melting purpose the heating chamber is there and nozzle is connected to the chamber. Filament insert into the chamber through the metal tube inside which there is a tube called Pitot tube which have very high melting point then material reaches in the heating chamber, material fused inside the chamber and comes out through nozzle. Important parameter of this method:

2.6.1.1 Material should be in the form of filament

2.6.1.2 Filament diameter: it should be less than the diameter of PTFE tube

2.6.1.3 Melting point of the material: it should be less than available temperature ($170^{O}\mathbf{C}$) in machine

2.6.1.4 Material should be solidify rapidly

2.6.1.5 Nozzle diameter

2.6.1.6 Distance between nozzle and platform

2.6.1.7 Speed of the movement of the platform and nozzle part

2.6.2 Material in liquid form

Liquid material based rapid prototyping is a fiber deposition technique. In this type of rapid prototyping machine the heating chamber and attached nozzle part is replaced by the dispensing unit which carry dispenser with uniform pressure device. Liquid material loaded into the dispenser and fix the dispenser at their place and apply very small uniform pressure. By this force liquid material comes out form the nozzle and the solidifier setup is placed with the dispenser but sometimes cross linker is used to solidify the liquid material. Important parameters in this method:

2.6.2.1 Concentration of the solution

2.6.2.2 Viscosity of the solution

2.6.2.3 Surface tension of the solution

2.6.2.4 Nozzle diameter

2.6.2.5 Working temperature

2.6.2.6 Applying pressure

2.6.2.7 Speed of the movement of the platform and nozzle part

2.7 Working principle of Rapid prototyping Thing-O-Matic

2.7.1 Filament material based Rapid prototyping

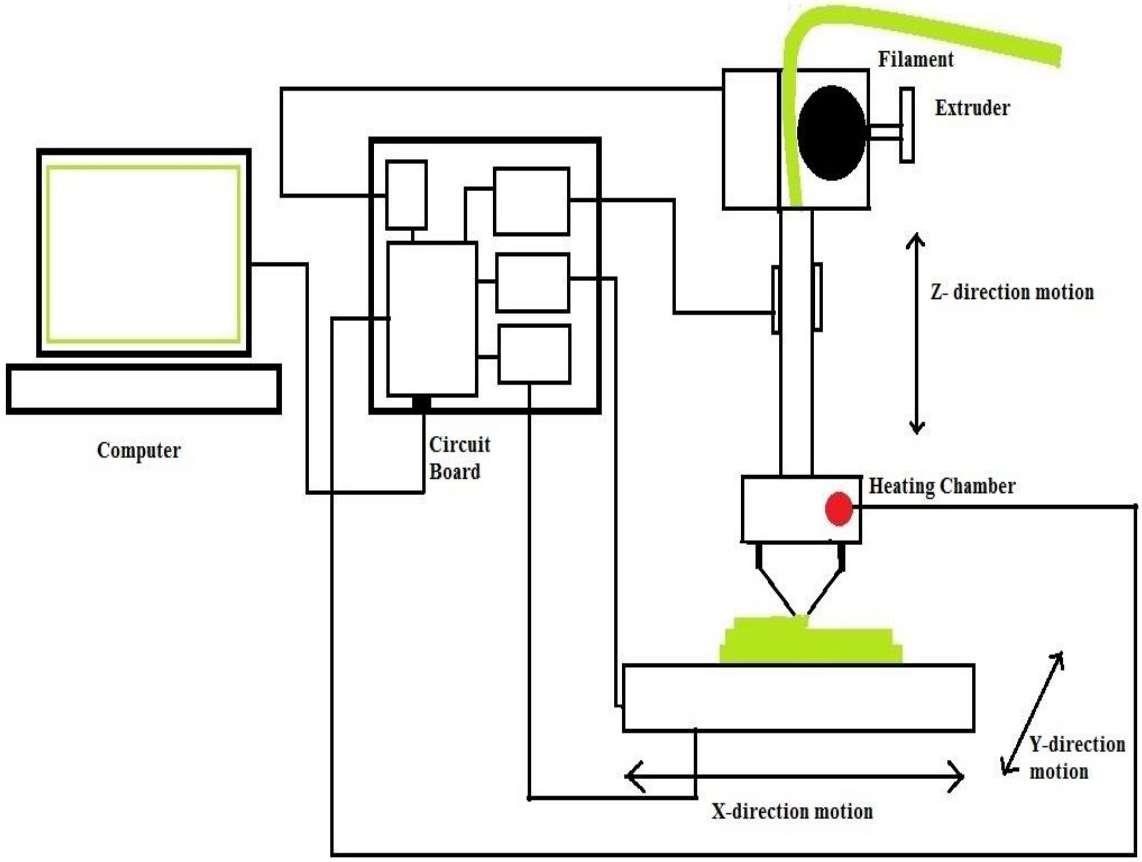

Fig 11: Working principle of Filament material based Rapid prototyping

Filament material based Rapid prototyping is work on the principle of Fused Deposition Modeling (FDM). Filament material based Rapid prototyping process involves the following 4 important steps-

2.7.1.1 Insertion of filament into the heating chamber

2.7.1.2 Melting of filament material

2.7.1.3 Movement of platform according to the 3D model

2.7.1.4 Deposition of material on the surface

2.7.1.5 Solidification of melted material

2.9.1.1 Insertion of filament into the heating chamber

Filament inserted in the heating chamber through the metal tube. In the metal tube a second tube arranged named as PTFE tube which have high melting temperature having material. The hollow cylindrical tube having diameter just equal to the filament diameter. Before entering into the tube filament came through the cylindrical cavity in which pulley attach to the extruder which is set by the naube. When the extruder run pulley rotating in anti-clock wise direction forasmuch filament is tightly trapped between the pulley and wall it goes inside the tube by pressing and at the time when pressing is not required extruder stop working.

2.9.1.2 Melting of filament material

The material in the form of filament. It inserted through metallic tube to the heating chamber. Metallic tube having PTFE tube fixed inside the tube. Actually filament inserted to the chamber through PTFE tube with force applying bye the extruder. The filament melted into the heating chamber by the application heating rod which provide heat to chamber up to 170^{o} C.

2.9.1.3 Movement of platform according to the 3D model

There are driver circuitry which is responsible for the movement of platform. Three stepper motor are used for this purpose, one for X-axis, 2^{nd} one for Y- axis and 3^{rd} one for Z-axis movement. According to the code generated by the Rpelicator G software for specific 3D model the platform move in x, y and z-axis direction.

2.9.1.4 Deposition of material on the surface

Material melted into the heating chamber and comes out through the nozzle. Extruder continuously press the filament inside the chamber due to which the material continuously comes

out through the nozzle. But after completion of one layer the nozzle tip again come at the starting point at this period the extruder has been stop while moving to the origin point then after again extruder motor starts.

2.9.1.5 Solidification of melted material

Material comes out through the nozzle and deposit on the platform in specific pattern. Normally melted filament material very fast solidified when it comes in air but in some advance machine the melted material is solidified by the application of laser. As the nozzle moves on the platform and layer is form, the laser beam moves through the layer and solidified it.

2.9.2 Liquid material based Rapid prototyping

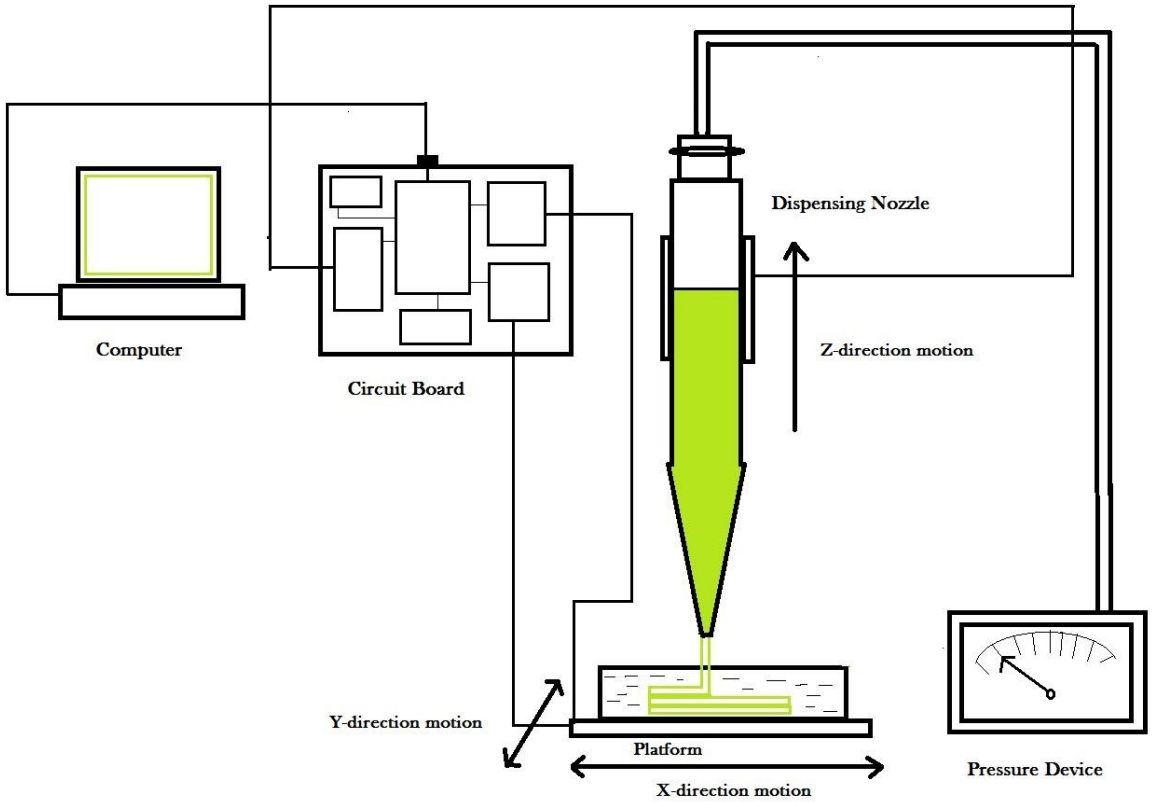

Fig 12: Working principle of Liquid material based Rapid prototyping

Liquid material based Rapid prototyping is a Fiber Deposition Technique. Liquid material based Rapid prototyping process involves the following 4 important steps-

2.7.1.6 Solution of desired material

2.7.1.7 Movement of platform according to the 3D model

2.7.1.8 Deposition of material on the surface

2.7.1.9 Solidification of liquid material

2.9.2.5 Applied pressure

2.9.2.1 Solution of desired material

Solution should be suitable for the process. In the formation of solution some parameters having very important concern:

2.9.2.1.1 Viscosity

2.9.2.1.2 Surface tension

2.9.2.1.3 Concentration

2.9.2.2 Movement of platform according to the 3D model

There are driver circuitry which is responsible for the movement of platform. Three stepper motor are used for this purpose, one for X-axis, 2nd one for Y- axis and 3rd one for Z-axis movement. According to the code generated by the Rpelicator G software for specific 3D model the platform move in x, y and z-axis direction.

2.9.2.3 Deposition of material on the surface

Liquid material filled into the dispenser and bye the application of pressure the material comes out through the nozzle continuously comes out through the nozzle. But after completion of one

layer the nozzle tip again come at the starting point at this period the extruder has been stop while moving to the origin point then after again extruder motor starts and pressure applied.

2.9.2.4 Solidification of liquid material

Liquid material comes out through nozzle and get into the cross-linker solution. As the material get into the crosslinking solution, it solidified. Normally for any material some specific cross-linkers are defined but here the solidification should very fast.

2.7.1.10 Applied pressure

It is very important parameter in this process because it decide the rate of liquid material flow through the nozzle to outside. Pressure is used to press the liquid material in the dispenser so that it comes out through the nozzle continuously. Pressure should be sufficient and uniform.

Chapter 3

Material and Method

3. MATERIALS AND METHODS

3.1 Material

3.1.2 Sodium Alginate

3.1.3 Gelatin

3.1.4 3.1.3 $CaCl_2$

3.1.5 Glutaraldehyde

3.1.6 β-TCP (Tri Calcium Phosphate)

3.2 Method

3.2.1 Modification of Rapid Prototyping set up

In this chapter the modification of the Rapid Prototyping machine has been describe. The main challenge was to develop a 3D model by use of liquid form of biomaterial from this machine.

The Rapid Prototyping set up model Thing-O-Matic bought from the Makerbot Industries. Basically this machine was designed for the material which should be in the form of filament. In this method the process was very easy and fast. Simply insert the filament into the heating chamber through PTFE tube and material melted and molten material came out through the nozzle and deposit on the platform. It was work on the principle of **Fused Deposition Modelling (FDM).** The main problem associated with this method is the material should be in the form of filament and every material is not available in the form of filament and those biomaterial which are available in the form of filament have very high cost.

Initially the plastic filament was used to create 3D model. This machine was developed to create prototype of any object in 3D for just general use such as toy design, dye design, screw etc. so the plastic material is suitable for this purpose but for tissue engineering application material should have biological properties and should be supportive nature in the biological environment.

3.2.1.1 Parts of Thing-O-Matic Replicator setup

In the above figure the part of the machine had been shown. There are different parts as given

A. Material

B. Metal tube and PTFE tube

C. Heating Chamber

D. Nozzle

E. Platform

F. Nozzle Part

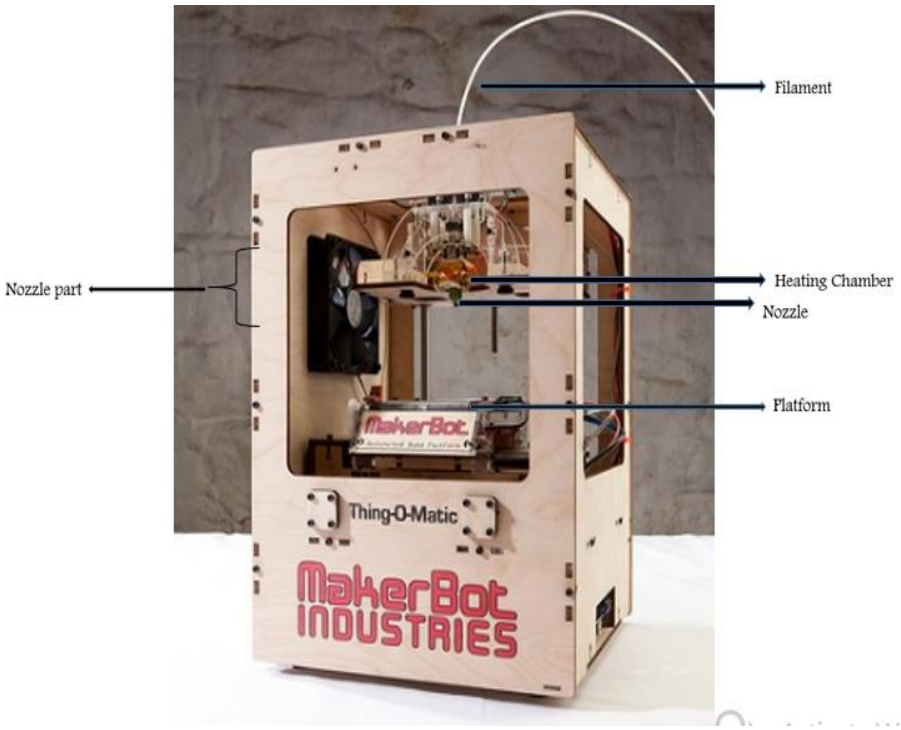

Fig 13: Thing-O-Matic Replicator setup which is used for the filament type material

A. Material

Material in the form of filament which feed to the heating chamber, it is directly inserted into the metal tube by the extruder. Extruder is a stepper motor which press the filament into the heating chamber through the tube.

B. Metal tube and PTFE tube

Filament inserted into Heating Chamber through the Metal tube inside which PTFE tube is present which provide the specific area to move filament. The area of filament and hollow area of the PTFE tube is just equal.

C. Heating Chamber

Heating chamber is used to melt the filament material. Basically it is a just a vacant box of metal in which a heating element has been inserted. When heat is applied then the element get heated and it transfer the heat to the chamber and it provide sufficient heat that is applicable to melt the filament.

D. Nozzle

A nozzle is associated with heating chamber. Filament material melted inside the heating chamber and melted material came out from the nozzle. The nozzle which are provide it this set up having tip diameter 1mm.

E. Platform

Just below nozzle the platform was situated on which material comes out through the nozzle tip deposited. Platform movable into X and Y direction due to which nozzle always static and platform move.

F. Nozzle Part

Nozzle Part include extruder set up, metal tube, PTFE tube, heating chamber and nozzle part.

3.2.1.2 Modification of Thing-O-Matic Replicator set up

The main challenge was to develop the machine for the biomaterial in liquid phase. To fulfill the objective modification was done. For this purpose the nozzle part in previous machine which was defined for the filament material was completely replaced by the dispensing unit which include nozzle tip, cylindrical volume, and valve and pressure device.

3.2.1.2.1 Formation of dispensing nozzle

Dispensing nozzle is formed by joining two parts: Centrifuge Tube and micropipette. Cut the centrifuge tube at its cone area and fit the micropipette by inserting it from its open area and made tight at the end. After that it fixed by pasting glue at the joining point of centrifuge tube and pipette.

This dispensing unit closed by the valve connecting at its mouth and fixed by the glue. This valve was fixed for the pressure pipe insertion and lock is associated with lock key which is used to lock the pressure pipe inside it. Holder had been made with card board for the holding of dispensing unit at the machine. Holder designed in this way that it could be fix at the machine with holding dispensing unit.

Fig 14: Dispensing Nozzle

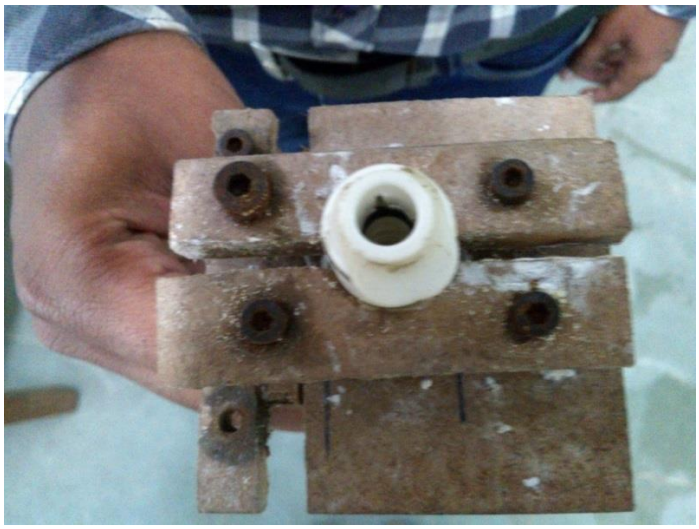

Fig 15: Dispensing Nozzle with the valve fixed by the glue and arranged with the holding setup for the machine

The dispensing unit was placed at the place of filament nozzle setup and fix it with the holding setup. That complete dispensing with holding setup fix at the place of extruder part of the machine.

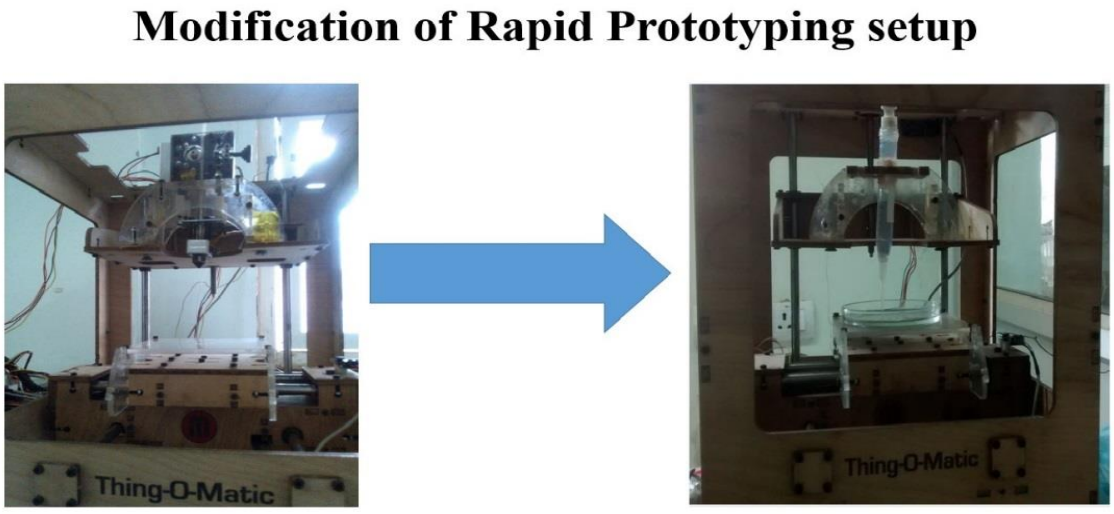

Fig 16: Modification of the Filament Based Rapid Prototyping Machine

3.2.2 Steps Involved for the preparation of the Solution

3.2.2.1 First of all a solution was prepared using distilled water in a beaker making it up to a final solution of 10 ml.

3.2.2.2 Sodium alginate was taken and the required amount in gram.

3.2.2.3 Gelatin was taken and the required amount in gram.

3.2.2.4 Calcium Chloride was taken and the required amount in gram.

3.2.2.5 Then all three solution is formed separately in beakers.

3.2.2.6 Sodium alginate aqueous solution was kept on a magnetic stirrer for more than 4 hours to dissolve uniformly to form a homogenous solution.

3.2.2.7 Gelatin aqueous solution was kept on a magnetic stirrer for more than 2 hours to dissolve uniformly to form a homogenous solution.

3.2.2.8 Calcium chloride aqueous solution was kept on a magnetic stirrer for more than 10 minutes to dissolve uniformly to form a homogenous solution

3.2.2.9 Glutraldehyde aqueous solution was kept on a magnetic stirrer for more than 10 minutes to dissolve uniformly to form a homogenous solution

3.2.3 Fabrication of 3D fibrous Scaffold

- To make the 3D fibrous scaffold with sodium alginate and gelatin bio composite these polymers had been taken in the ratio of 90:10 dissolved in distilled water. Then the 3D model was designed in Solid Works software, which is a modelling mechanical software to use to create models of any kind of 2D and 3D object. (Now in present days in tissue engineering this is very useful to create 3D construct for tissue engineering application such bone modelling, scaffold modelling, oral representation of diseased part of the body).

- Then 3D model saved in .STL format (STL (Stereo Lithography) is a file format for the stereo lithography CAD software developed by 3D Systems [27, 28]. STL is also known as Standard Tessellation Language. STL file format is supported by many other software packages; it is widely used for rapid prototyping and computer-aided manufacturing [29]. STL files describe only the surface geometry of a 3D object without any representation of texture, color or other common CAD model attributes. The STL format specifies both ASCII and binary representations. Binary files are more common, since they are more compact [30]).
- Then after import the saved file into the Replicator G software. Replicator G is an open source software which is basically use to drive the Rapid Prototyping machine. Apart from Makerbot replicator it is also used in some other machine CupCake CNC, RepRap machine, or generic CNC machine.
- After importing .STL file into Replicator G adjusted the model at the middle point of the platform into the software by using x, y and z –axis motion. After then G-code has been generated. These G-codes are basically responsible for the movement of platform in x, y and z axis. Replicator G software slices the three dimensional model into number of layers and each layer in defined into two dimensional and generate driving codes for 2D layers. Now set the default position of platform and dispensing nozzle. While generating G code the speed of motion has been adjusted and the number of layers has been specified.
- Then solution filled dispenser load on the machine and kept a $CaCl_2$ and gutraldehyde compound filled petry plate just below the nozzle and run the machine by clicking on the run option at Replicator G software. As the machine started running the pressure had been applied by the extruder circuit.

- As the pressure applied the compound solution came out through the nozzle and it was directly collect by the cross-linker solution filled in the petry-plate.
- According to the G codes generated by the software, platform moves in x and y direction and after completion of one layer the nozzle moved in z direction (upward) just equal to the thickness of first layer.
- In this way the specified number of layer formed one by one upon each. The number of layers is dependent upon the thickness of the model.
- Obtained construct was a fibrous structure. It had been washed by distilled water three times to remove extra cross-linker which was stuck on fibers.
- After wash it was freeze dried by freeze drying method and it was kept for 6 hours in the freeze drying chamber.

3.3 Coating on prepared tissue construct fibrous scaffold with β-TCP (Tri Calcium Phosphate)

To coat the scaffold Dip coating method was applied and it was done at different concentration of β-TCP. Nano level β-TCP in powder form was used for this purpose. Three different concentration of β-TCP in aqueous solution was made and tissue construct scaffold dip in those three solution for 2 hours and after that it was dried at 60^0 C for 1 hour.

3.4 Characterization of prepared Scaffold

- Surface morphology and microstructural observations of the prepared scaffold were carried out by Scanning Electron Microscopy (SEM)

- The phase composition was analyzed by using X-ray diffraction (XRD) of the prepared scaffold
- The presence of bond between different chemical content in the scaffold was analyzed by Fourier Transform Infrared Spectroscopy (FTIR)
- Tensile strength of the scaffold was analyzed by using Universal Tensile Strength Testing machine.
- The hydrophobic or hydrophilic nature of scaffold was analyzed by using Contact Angle Measurement Technique

Chapter 4

Results and Discussion

4.1 3D Scaffold fabrication

The 3D model designed in solid works was successfully fabricated by the Rapid Prototyping method with defined parameters. The circular structure was chosen to fabricate with 40 mm diameter of 20 layers with thickness of 8 mm and fabricated layers one by one. Thickness of the scaffold just after the fabrication was quite more than the scaffold after freeze drying process. The moisture was removed in the freeze drying process that's why the thickness of the scaffold was decreased.

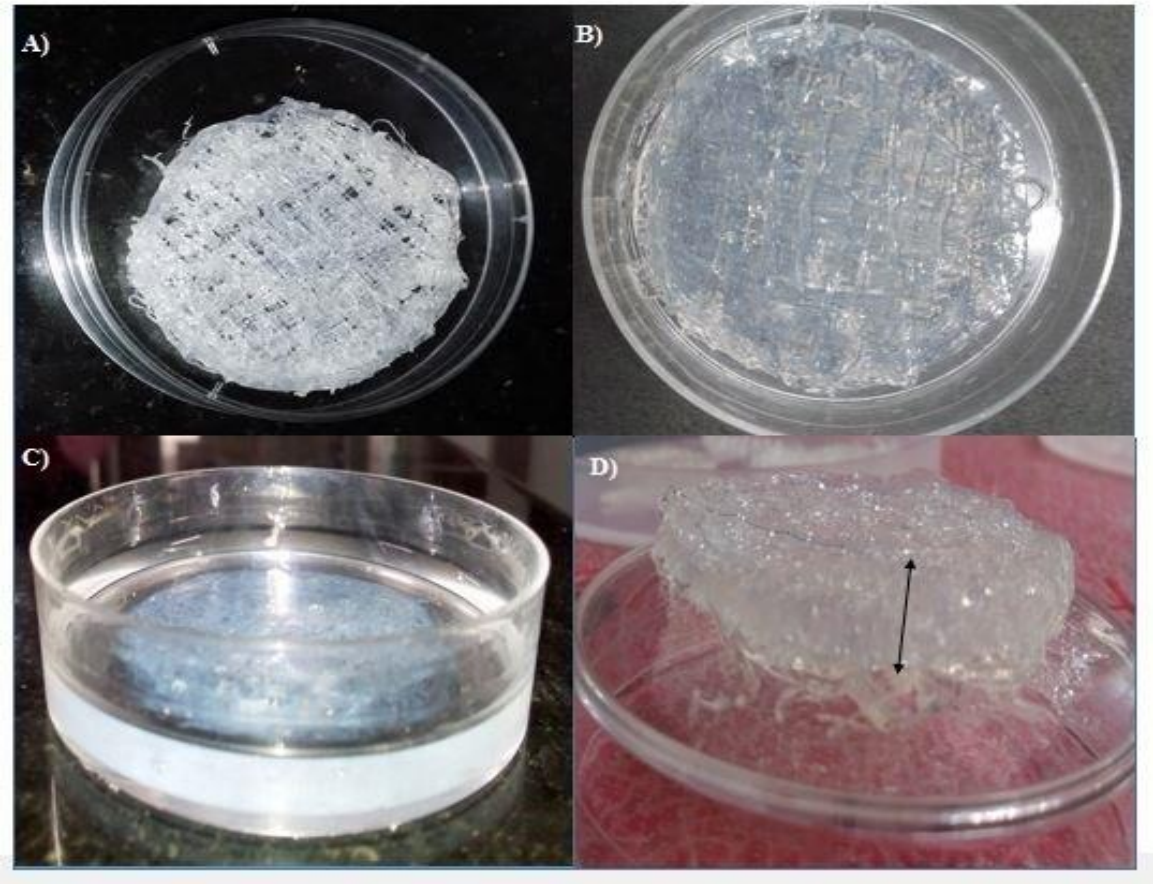

Fig 17: Images of prepared Scaffold. A), B), D) shows the just after fabrication. C) Image while washing of scaffold to remove the excessive amount of $CaCl_2$

Fig 18: E), F) Image of Freeze Dried Scaffold with showing the diameter of the scaffold.

4.2 Contact angle measurement

The measurement of contact angle of the surface of scaffold is very important aspect in respect of cellular response. It is decided by contact angle that scaffold surface is hydrophilic or hydrophobic in nature, if the contact angle is less than 90^o, it is hydrophilic (water attractant) and above the 90^o, it is hydrophobic (water repellent). Contact angle measurement was done with respect to water. The scaffold surface should be hydrophilic in nature. The contact angle of the prepared SA/GE scaffold surface observed 36.24^0 and SA/GE scaffold reinforced with β-TCP surface observed 28.06^0 which shows their hydrophilic characteristics. The considerable change found between the β-TCP coated surface and non-coated surface of the scaffold. By these result it has been concluded, SA/GE scaffold surface reinforced with β-TCP have better surface property which much favorable for tissue engineering application than SA/GE scaffold.

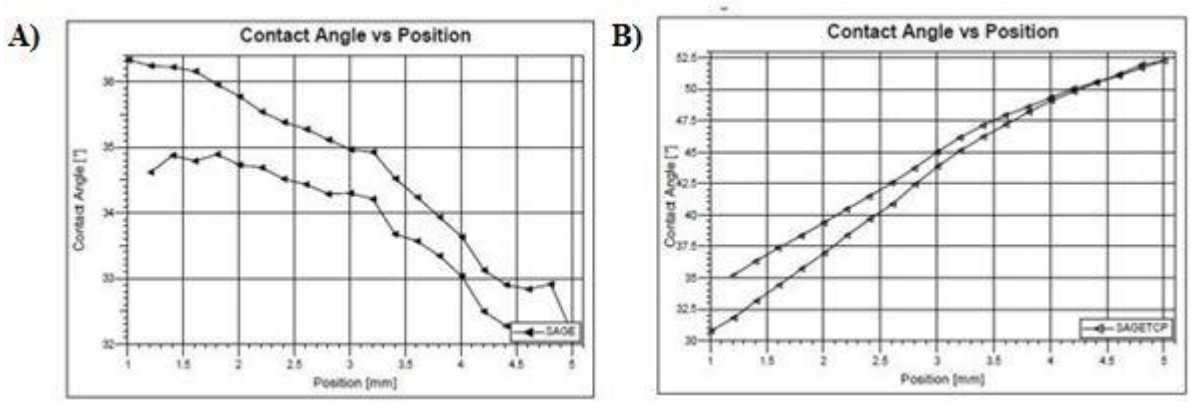

Fig 19: Contact angle A) SA/GE scaffold B) SA/GE scaffold coated with β-TCP

4.3 Morphological analysis by SEM

Morphological study of prepared scaffold was done by the NOVA NANOSEM 450. Before doing morphological study gold coating was done for 3 minutes. Four images was taken at 10kv at different magnification value A) at 1000X, B) and C) at 500X, D) and E) at 200X, F) at 100X. In image D the complete fibrous structure can be seen. Fibers are aligned one upon one. The pores between the fibers are clearly visible. In figure C and B the thickness of the fibers was shown i.e. 154µm and the pore size was varying from 194µm to 294 µm horizontally, means a varying pore structure was found in the scaffold which was considered as the range 200 µm-300 µm. FESEM images C), D), E) and F) the coating of β-TCP was found on the scaffold. It is clearly visible that the micro particles of β-TCP were attached to the fiber surface and distributed over the scaffold.

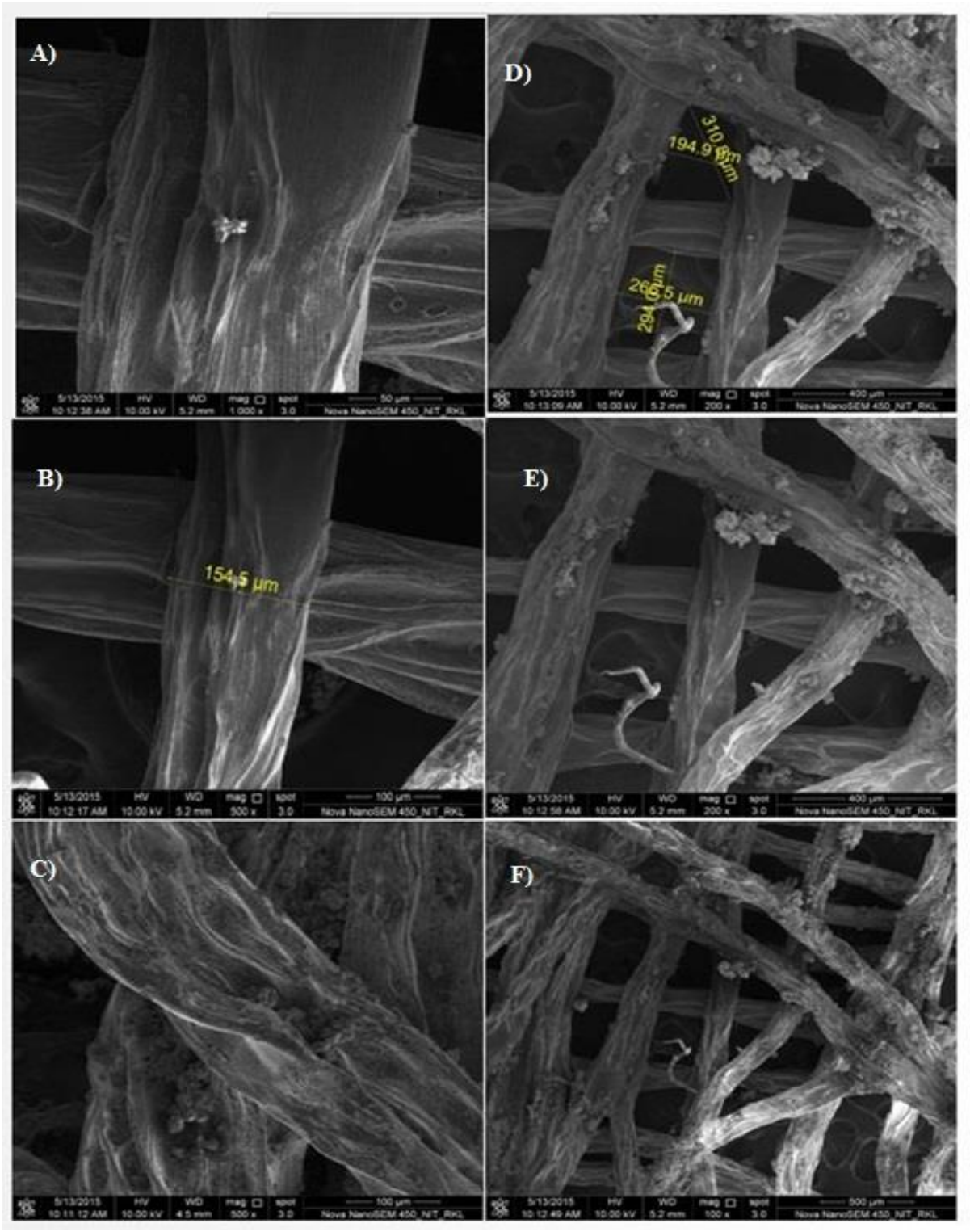

Fig 20: Scanning Electron Microscopy of SA/GE fibrous scaffold. A) And B) are SA/GE scaffold and C), D), E) and F) are the scaffold reinforced by β-TCP

4.4 Tensile strength measurement

Tensile strength measurement was performed on the prepared scaffold by UTM (Universal Tensile Testing Machine) provided by Super Duper Multi National Conglomerates R Us.

Reinforcing of β-TCP particles result to decrease the ultimate tensile strength but very small difference was found between the SA/GE and SA/GE β-TCP coated. The ultimate tensile strength of the SA/GE scaffold was 0.08MPa and SA/GE β-TCP coated scaffold was 0.06MPa.

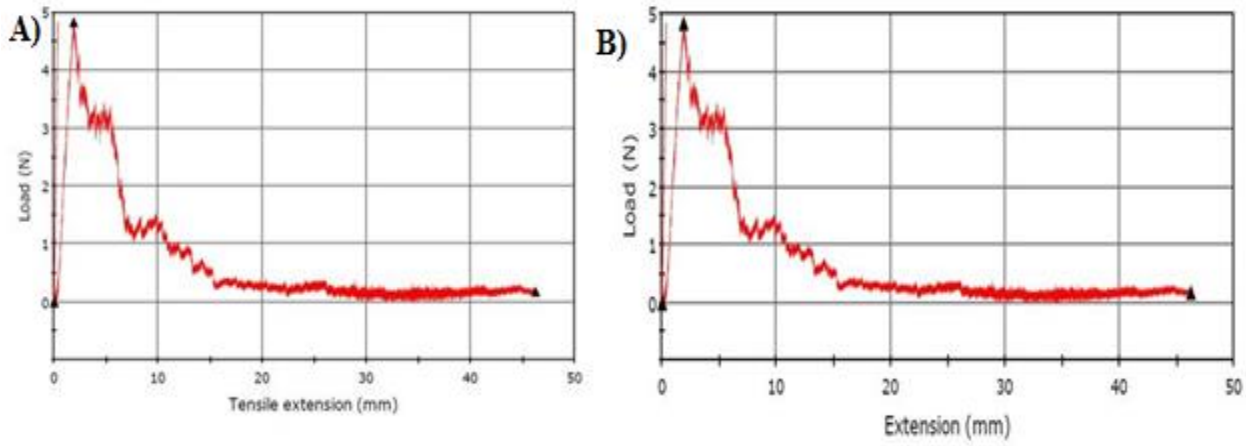

Fig 21: SA/GE scaffold. A) Load Vs Extension, B) Stress Vs Strain

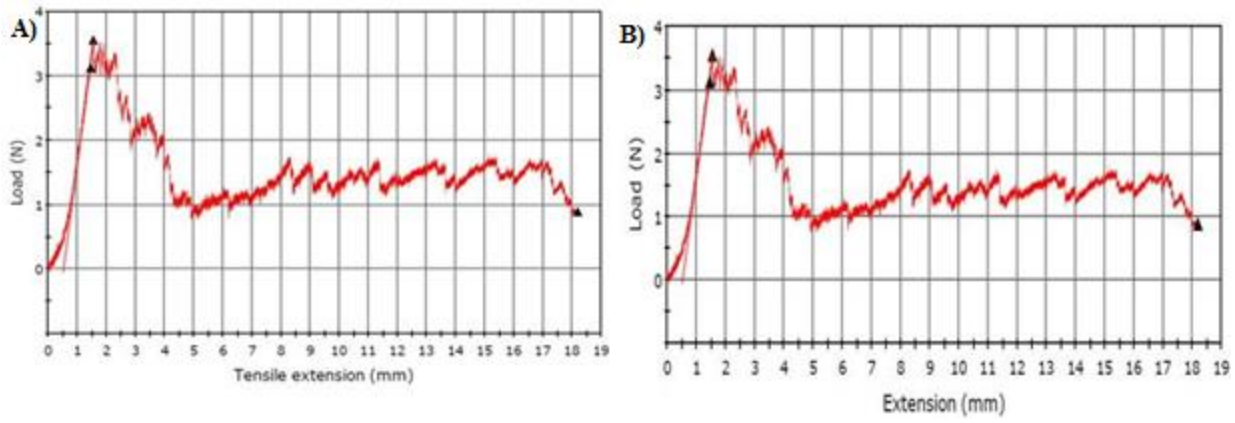

Fig 22: SA/GE β-TCP scaffold. A) Load Vs Extension, B) Stress Vs Strain

4.5 FTIR Analysis

FTIR spectra of Sodium alginate and gelatin composite scaffold reinforced with β-TCP has been showed in figure. From the peaks obtain in the graph from which can know the type of bond present. The wide adsorption band at around from 3250 to 3450 cm^{-1} was due to the stretching vibration of O–H. For alginate, absorption bands at 1639 cm^{-1} and 1423 cm^{-1} were attributed to the asymmetric and symmetric stretching vibration of COO– group, respectively [31]. For gelatin, absorption bands at 1630 cm^{-1} were attributed to C=O, stretching vibration [32]. Compared to 7gelatin/alginate and gelatin/ alginate/HAP, the adsorption bands of gelatin at 1637 cm-1 and 3450 cm^{-1} shifted to a lower wave number, suggesting intermolecular interactions between alginate and gelatin.

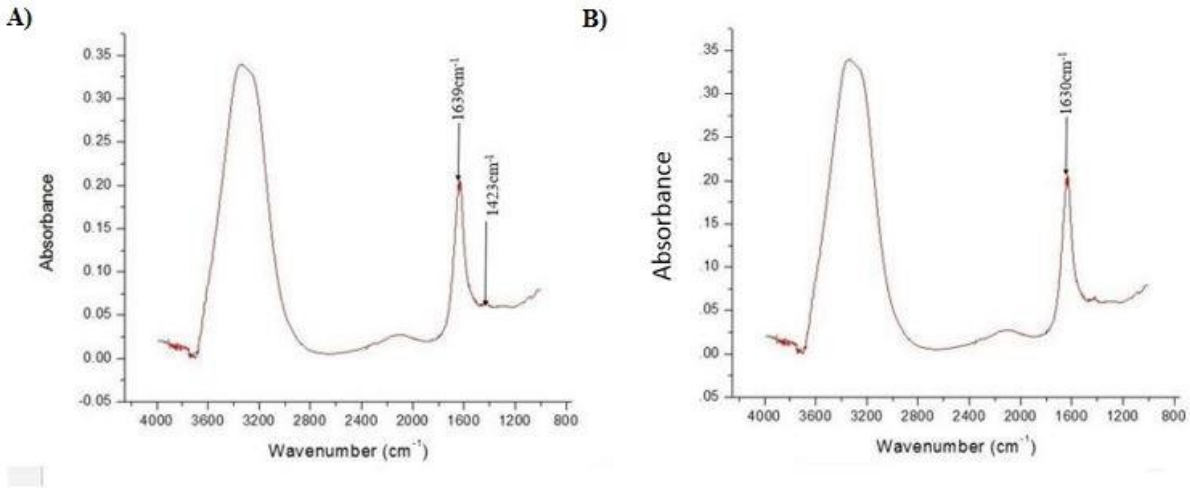

Fig 23: A) Showing the peaks for Sodium alginate, B) showing the peaks for gelatin

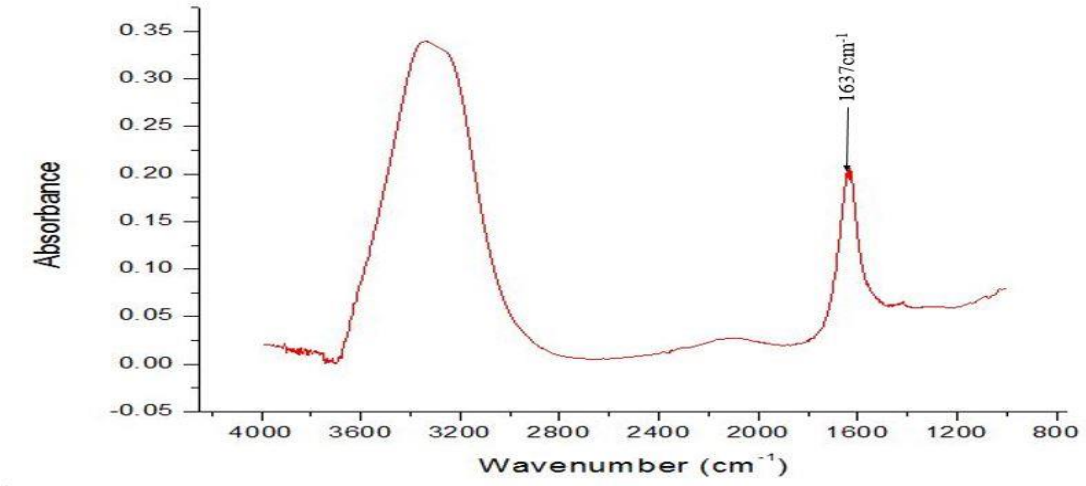

Fig 24: showing the peaks for TCP

4.6 XRD Analysis

Fig. (5.6.1) shows XRD diffractogram for sodium alginate, gelatin and SA/Gel/β-TCP composite scaffold. The characteristic peak for SA was observed at 13.7 whereas for gelatin it was observed at 21.8. The XRD diffractogram for SA/Gel composite shown in figure 5.6.1 (b) it may be seen that the diffraction peaks of alginate disappeared at 13.7◦ and intensified at 23.0◦, with the increasing of gelatin content. XRD analysis of Sodium alginate and gelatin reinforced by β-TCP revealed the presence of broad peaks between 10 and 30º, characteristic of amorphous materials. It may be seen, in Fig. 5.6.1, the X-ray diffraction patterns of gelatin and alginate. The diffracto gram of alginate consisted of two crystalline peaks at 2θ = 13.7◦ and 23.0◦ [33]. Gelatin only had a typically wide crystalline peak at 2θ = 21.8◦. The X-ray diffraction pattern of the β-TCP reinforced on the scaffold showed peaks at 2θ = 25.70°, 27.77°, 31.02°, 32.44° and 34.33°. B-TCP is more crystalline so that the peak at 31.02 very highly intensify [34].

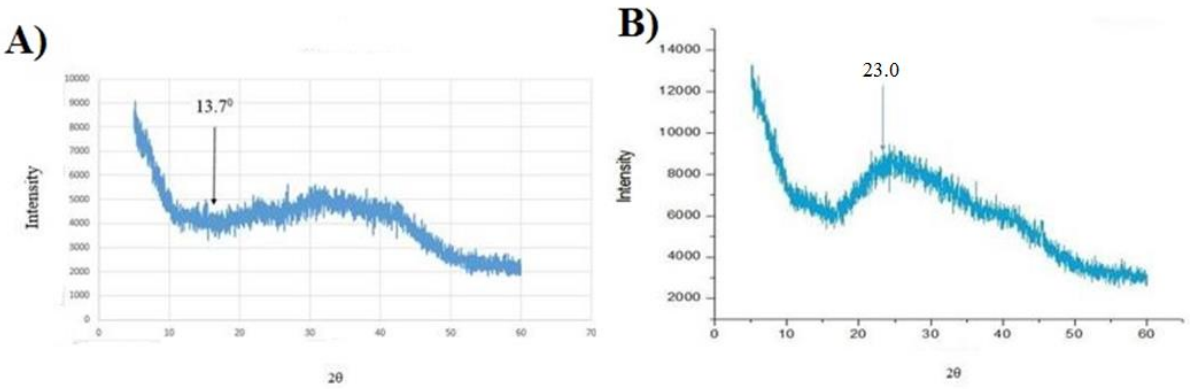

Fig 25: A) XRD of SA B) XRD of SA/GE

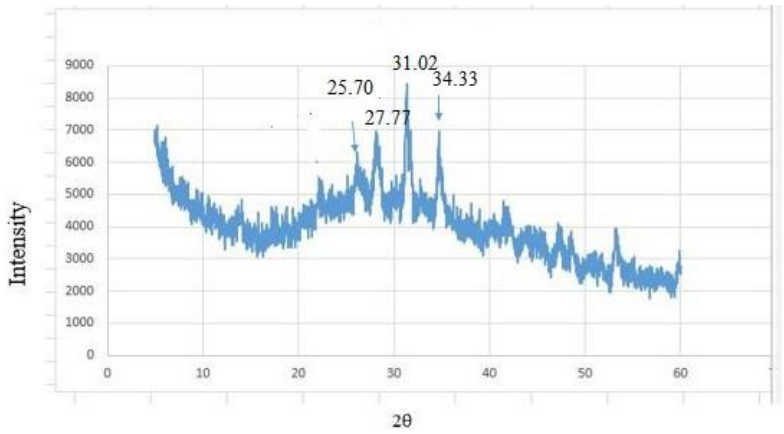

Fig 26: shows the XRD graph of SA/GE-β-TCP scaffold

Chapter 5

Conclusion

5. CONCLUSION

In this study Thing-O-Matic Replicator Mk5 was modified for the liquid phase of biomaterial and a 3D scaffold was fabricated by modified version of the instrument. The structure, chemical composition, hydrophilicity, crystallinity and tensile strength, were characterized by FESEM analysis, FTIR study, contact angle measurement, XRD analysis and Universal tensile strength testing. Furthermore, previous experiments were carried out to find the optimum parameters to achieve fibrous 3D scaffold by modified Rapid Prototyping technique.

The most interesting result of the present study is noted as follows –

1. Modification of Thing-O-Matic Replicator Mk5 Rapid Prototyping instrument successfully modified.
2. Process parameters for the process obtained as:

Pressure	0.3 bar
Nozzle diameter	650 micrometer (approx. value)
Distance between Nozzle tip and platform	3mm
Temperature	Room temperature

3. The best obtained fibrous scaffold with fiber diameter 150 micrometer.
4. The best obtained fibrous scaffold with pore size 195-300 micrometer.
5. Effect of other processing parameters can be varied to get optimum results for the formation of fibrous scaffold.

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