Development of porcine omentum fat derived extracellular matrix based scaffold and microbeads for soft tissue engineering

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By

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CERTIFICATE

This is to certify that the summer project report entitled, **"Development of porcine omentum fat derived extracellular matrix based porous scaffold and microbeads for soft tissue engineering"** submitted by **Mr. Krishan Kumar** in partial fulfillment of the requirements for the award of the Masters of Technology in Biotechnology and Medical Engineering with specialization in "Biotechnology" 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 this thesis has not been submitted to any other University/ Institute for the award of any other Degree or Diploma.

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ABSTRACT

Trauma, tumor resection, change with age, burns, accidental injury and congenital abnormality causes soft tissue defects or voids within the dermis layer or subcutaneous layer of fat. These defects are due to the loss of fat that is difficult to self-repair. Tissue engineering can be viewed as the best methods for these defects by scaffold, biofilms and microbeads. In this study we prepared scaffold containing porcine adipose tissue derived extracellular matrix by freeze drying and composite microbeads of alginate and extracellular matrix by electrospraying respectively. Adipose tissue was decellularized by physical methods like homogenization, freeze thaw, centrifugation and chemical methods like acetic acid and SDS treatments. Then ECM scaffold was stained with Verhoeff's Van Gieson characterized by SEM, FTIR, DSC, and swelling ratio study. ECM scaffold shown hydrophilic porous structure and having antibacterial activity. The microbeads were characterized by SEM and FTIR. The beads are highly porous containing small microparticles of 7-8 μm.

Keywords: adipose tissue, decellularization, scaffold, microbeads

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INTRODUCTION

Soft tissue engineering is the study of the growth and reconstruction of the connective tissues and organs by the cells, scaffold and growth stimulating signals that will produce the fully functional tissue for the implants in the body. Soft tissue defects are common because of the tumor resections, trauma and burns, accidental injury and congenital abnormalities. Soft tissue defects or voids within the dermis layer or subcutaneous layer of fat causes impaired function and appearance (1). Furthermore, these defects which are mainly to loss of adipose tissue, is very difficult to self-repair (2-3). Researchers have found success in restoring the soft tissue defects (4) by using the synthetic injectable implants, autologous fat, adipose cells, stem cell and other synthetic scaffold (5-6).But all of the above mentioned soft tissue restoration methods have their own advantages and disadvantages.

The extracellular cellular matrix based tissue engineered scaffold is relatively new entry in soft tissue engineering field. The extracellular matrix plays an important role for the cell seeding, growth and maintenance. It provides the biological cues for the cell attachment and signaling of the cells. It also provides the structural support to the cells and gives the tissue its strength. It has the reservoir of growth factors and it has antibacterial activities also.

Due to the shortage of human tissues and organs for the extraction extracellular matrix, researchers have found the alternative of human tissues in animal tissues. Among the animals, pig is most commonly used for the extraction of extracellular matrix. Harvesting the ECM from pig has its own advantage. Pig is the domestic animal and multiplies very fast. It becomes sexually mature in six months and their biochemical profile is similar to the humans (7, 8). But the use of xenogeneic tissue cause hyper acute rejection due to xenogeneic antigen α-gal (9). These antigen can be

removed by the using of proper decellularized techniques. The proper decellularization technique removes all cellular, nuclear and lipid contents (10). There are several decellularization techniques for the decellularization of the porcine tissue (11-12). In our study porcine adipose tissue was used for the extraction of ECM because in addition of having all the advantages of using ECM in tissue engineering application, Adipose derived ECM stimulates the differentiation of stem cells towards adipocytes, the main constituents of soft tissues. The ECM of adipose tissue consists of collagen I-VI, laminin, fibronectin, elastin and glycosamino-glycan (13). There is one disadvantage of using these ECM for the soft tissue engineering. The degradation rate of the ECM scaffold is very high due to which the contour of the soft tissue defects is hard to maintain. There degradation rate should match with the remodeling speed of the soft tissues. To prevent the degradation rate the ECM was composited with alginate, a natural polysaccharide whose degradation rate is slower than that of ECM. For the small defects or voids injectable form of composite alginate-ECM microbeads were prepared.

In our studies first the adipose tissue was decellularised by physical treatment (homogenization, freeze thawing and centrifugation) to remove the fat content from tissues and chemical treatment (acetic acid and SDS) for complete decellularization. Then after final centrifugation, the ECM forms the white semisolid pellet. Now these ECM was used for two purposes one is for scaffold preparation and second is for microbeads preparation.

1. For the scaffold preparation the collected ECM was freeze thawed in the lyophillizer and then the dried ECM scaffold was characterized by light microscopy, Scanning electron microscopy, Differential scanning colorimetry, Fourier transform infra-red spectroscopy.

2. The microbeads were formed by the mixing the sodium alginate with ECM pellet solution and

then by electrospraying the solution at different voltage and flow rate. The microbeads formed were filtered and freeze dried. Then it was characterized by light microscopy, SEM, Fourier transform infra-red spectroscopy and differential scanning calorimetery.

REVIEW OF LITERATURE

2.1 Soft tissue

Soft tissue are the soft parts of the body that connect, support and surrounds other structure and organs of the body. Soft tissue includes the muscles that supports the bone, tendons that connects muscles to the bones, ligaments that connects bones to bones, synovial tissue that makes joint capsules, which gives upright integrity to the human form by surrounding the musculoskeletal components, and other examples are nerves, blood vessels and fat. Soft tissue contains different types of cells, fibers and ground substances

2.1.1 Types of cells in soft tissue

- ✓ **Mesenchymal cells:** it is the embryonic cells which persist in the adult and are capable of differentiation and proliferation during regeneration. Mesenchymal stem cells (MSCs) are mostly found in the bone marrow but it can also be isolated from other tissues such as cord blood, peripheral blood, fallopian tube, and fetal liver and lung
- ✓ **Fibroblast cells:** These are large, flat, and spindle shape cells. It is the most common cell of the connective tissue which play a role for the collagen synthesis and wound healing. It also synthesis the ground substances like proteoglycan.
- ✓ **Macrophage:** these cells are found in the abundance amount in the loose connective tissue. Initially they are in non-motile form but during inflammation they become phagocytic cells. These cells engulf blood cells, dead cells, bacteria and debris digesting this material with powerful enzymes.
- ✓ **Adipose cells:** adipose cell, adipocyte or fat cells are commonly found in the loose

connective tissue. These cells synthesize the fats and globules. These cells are of two types, white and brown adipose cells. White cells contains large droplet of fats, non-centrally located nuclei and small amount of cytoplasm, while brown adipose cells contains small fat droplets, large cytoplasm and centrally located nuclei.

- ✓ **Leukocytes:** it is also called the white blood cells that wander the tissue that surrounds the blood vessels. Eosinophiles are the types of leukocytes that found in the respiratory and digestive tract. Neutrophils are found commonly in the inflammation area. B lymphocytes are commonly found in the chronic inflammation areas.
- ✓ **Mast cell:** these cells are found in the soft tissue that is resemble similar to the basophiles cells. Like basophiles it also secretes histamine and heparin and binds to the vitamin E for the anticoagulant property.
- ✓ **Melanocytes:** these are the pigment cells that found in the connective tissues like skin and choroid coat of the eye. The melanin that is produced by these cells absorb ultra violet light.

2.1.2 Connective tissue fibers

Connective tissue fibers are the part of extracellular matrix found in the tissue. The numbers, types and alignment of fibers determines the property of the tissue.

Collagen: it is the most abundant protein found in the ECM and more than 20 types of collagen has identified.

Types of collagen

Collagen 1: it is more characterized and ubiquitous protein in all animal and plant kingdom (14). Due to the low antigenicity, collagen 1 is most commonly used protein for the scaffold preparation for tissue repair. Bovine type 1 collagen is most commonly used scaffold for therapeutic treatment due its abundant source.

Type II collagen: it is mostly found in the cartilage and articular cartilage. It helps in the entrapment of proteoglycan and gives the tensile strength to the tissue (15).

Type III collagen: it is mostly found in the tissue that has elastic properties, such as skin, blood vessel and various internal organs. It yields gelatin on boiling.

Type IV collagen: it is present in the basement membrane of all vascular structure and acts as a ligand for the endothelial cells (16).

Type V collagen: it is found in the tissue that contains type V collagens. Mutation in this protein cause Ehlers-Danlos syndrome (18).

Type VI collagen: it is mostly found in Skelton muscle (17).It acts as a connector between the functional proteins and GAG to the larger proteins such as collagen I.

Type VII collagen: it is the important component of the anchoring fibrils of the keratinocytes underlying the basement membrane of epidermis (19). Mutation of these proteins cause epidermolysis bullosa acquisita.

Fibronectin: it is the second most abundant protein after collagen. It is high molecular weight glycoprotein that binds to membrane spanning receptor protein and other component of extracellular matrix like collagen, fibrin and heparan sulphate proteoglycan. Vertebrates has two

types of fibronectin. Soluble fibronectin: it is found in blood plasma and it is produce in the liver by hepatocytes. Insoluble fibronectin: it is the major component of the extracellular matrix and it is produce by many cells primary by the fibroblast cells. It plays important role in cell adhesion, growth, migration and differentiation.

Laminin: these proteins helps the formation of vascular structure during repair or remodeling, especially in case of extracellular matrix laminin plays a vital role in the vascularization because it is friendly biomaterial for the endothelial cells.

Elastic fiber: It is also called yellow fibers, it is the bundles of elastin proteins secreted by the fibroblast cells in the connective tissue. These fibers can be stretched up to 1.5 times of the original length and back to its original position when it is released.

2.1.3 Ground substances

Ground substances are the part of the extracellular matrix that occupy the spaces between the fibers and cells in the tissue.

1. Hyaluronic acid – It is mostly found in cartilage, skin, blood vessels, and the umbilicus. Hyaluronic acid is the major component of vitreous humor and synovial fluid.

2. Chondroitin sulfate – it is mostly found in bone, cartilage, blood vessels, skin and the cornea.

3. Dermatan sulfate – it is found in skin, heart valves, blood vessels, tendons and connective tissues of the lung.

4. Keratan sulfate – it is mostly found in the cornea and in cartilage.

5. Heparin sulfate – it is mostly found in the liver, aorta and lung.

2.2 Adipose tissue

It is also called fat tissue, is a type of connective tissue that are specific, dynamic and multifunctional tissue that are distributed throughout the body(20, 21).the role of the adipose tissue are balancing body energy, maintain the body shape, it occurs with other tissue and provides thermal and mechanical insulation. It is also called endocrine glands because it secretes large amount of autocrine, endocrine and paracrine factor (22). In the lean man it consists of 9 to 18 % of the body weight while in the female it consist of 14 to 18 % of the body weight (23). In the male it mainly accumulate in the abdominal region and in the female it accumulate in the gluteofemoral region (24). The adipose tissue consists of differentiating cells that are called adipocytes or fat cells that are organized with other cells, cytokines and growth factors (25). Depending upon these they are differentiated in to different types of adipose cells. Adipose tissue mainly are divided in two types depending upon the cellular morphology and tissue property whit adipose tissue and brown adipose tissue (26).

2.2.1 White Adipose Tissue: these adipose tissue are found in the subcutaneous layer of the muscles and dermal layer. It is also found in the intraabdominal, as a visceral tissue and also surrounds heart, kidney and liver of the body. White adipose tissue consists of white adipocytes that has lipid in the form of triglycerol (27). These white adipocytes has single flattened nucleus and elongated mitochondria. These cells have large globules of lipid droplets. In these types of cells 90% of the cells weight are lipid or fat .due to the high lipid contents these tissue has white or yellow color appearance.

2.2.2 Brown adipose tissue: these tissue are present in the neonatal body, and it produce heat for adaption to the body temperature (28). The brownish color of these tissue is due to the high vascularization of the tissue. These cells are smaller than the white adipose cells. It consists of centrally located nucleus and rich in mitochondria (29). These consists of multiocular distribution of many small droplets of lipid content. With the age these tissue convert in to the white adipose tissue (30)

2.3 Soft tissue injury

A wound is a type of injury in which skin is torn, cut, or punctured (an *open* wound), or where blunt force trauma causes a contusion (a *closed* wound)

Wounds are of two types

Open wound

Open wounds can be classified according to the object that caused the wound. The types of open wound are:

- **Incisions or incised wounds**: caused by a clean, sharp-edged object such as a knife, razor, or glass splinter.
- **Lacerations**: irregular tear-like wounds caused by some blunt trauma. Lacerations and incisions may appear linear (regular) or stellate (irregular). The term *laceration* is commonly misused in reference to incisions.
- **Abrasions:** superficial wounds in which the topmost layer of the skin (the epidermis) is scraped off. Abrasions are often caused by a sliding fall onto a rough surface.

- **Puncture wounds**: caused by an object puncturing the skin, such as a nail or needle.
- **Penetration wounds**: caused by an object such as a knife entering and coming out from the skin.
- **Gunshot wounds:** caused by a bullet or similar projectile driving into or through the body. There may be two wounds, one at the site of entry and one at the site of exit, generally referred to as a "through-and-through."

Closed wound

Closed wounds have fewer categories, but are just as dangerous as open wounds. The types of closed wounds are:

- **Contusions**: more commonly known as bruises, caused by a blunt force trauma that damages tissue under the skin.
- **Hematomas**: also called a blood tumor, caused by damage to a blood vessel that in turn causes blood to collect under the skin.
- **Crush injury**: caused by a great or extreme amount of force applied over a long period of time.

2.4 Treatment methods for soft tissue defect

Injectable Implants for Soft-Tissue Reconstruction: This tissue implants offer a less invasive technique for the rebuilding of loss of soft tissue that may be due to trauma or diseases (31-34). They will be especially useful for facial tissue, which is difficult to reconstruct without scarring or loss of function. This implant is formed by the combination of naturally occurring hyaluronic acid and the synthetic polymer polyethylene glycol (PEG).

The implant is injected under the skin as a liquid, then message it in to shape. Then use the light emitting diode it will trigger a photo initiator. This causes link between the PEG molecules, creating a solid hydrogel that trap and entangles the particle of hyaluronic acid, fixing the implant in to place.

Skin Graf: It is a type of graft surgery involving skin for the transplantation. Skin grafting is mostly used for the treatment of

- Extensive wound healing or Trauma (35-36)
- Skin loss due to the infection such as necrotizing fasciitis or purpura fulminans
- removing of skin cancer

There are two types of skin graft,

Split-thickness: the most commonly used graft where the thin layer of the skin is removed from healthy donor like peeling the tomato (35, 36, 37, 38, and 39).

Full thickness: skin graft which involves the pitching and cutting of skin away from the donor. The graft is carefully spread over the bare area and fixed with the help of stitched and surgical staples.

Synthetic polymers scaffold: preparation of scaffold by using biodegradable polymers PLLA, PGA, PLGA[40], PCL [41], PDLLA, PEE and PBT [42]etc. for the formation of the porous scaffold that acts as the ECM in the body used for the restoration and wound healing purpose

Extra cellular matrix scaffold: The extra cellular matrix that are derived from the soft tissue like adipose tissue, SIS, skin will provide the natural scaffold for soft tissue engineering

Autologous fat: autologous fat is most commonly used for the soft tissue augmentation. These

is autologous fat so there will not be immune rejection. It is commonly used in the face for the aesthetic improvement (43)

2.5 Xenogeneic tissue for soft tissue engineering

The meaning of the xenogeneic is the different species. Now days the use of xenogeneic animal for the human beneficial is common. The most appropriate example of this is xenotransplantation. It is defined as the transplantation, implantation and infusion of animal cell, tissue, or organ in the human body.

These are of two types

Concordant xenografting: transplantation of tissue between closely related species. E.g. rat to hamster, baboon to man. Rejection of organ occurs within few days.

Discordant xenografting: transplantation of tissue between phylogenetic distant species like pig to human. Rejection of organ occurs within minutes or hours.

We are using porcine xenograft because the use of primates are restricted due to ethical and retroviral considerations, size constraints, and slow gestation periods (44, 45).
The domestic pig is identified as an identical xenogenic species because

- ✓ Pig is the domestic animal
- ✓ It has organs of the sufficient size, that multiply very fast
- ✓ It will become sexually mature in six months (46).

when we transplant tissue from pig to human (47) the hyper acute reaction and severe rejection occur due to the natural antibodies present in the human body which recognize the glycoprotein antigen present in the endothelial cell and platelets (48, 49). This hyper acute rejection occur due

to the presence of the carbohydrate galactose-alpha-(l, 3)-galactose (50-55). the galactose-alpha-(l, 3) linkage is catalyze by an enzyme that present in all mammalian cells, except humans, old world monkeys, and the great apes.

There are three possible methods of blocking HAR

I. **Depletion of pre-formed antibodies:** it is done by the use of anti-heavy chain monoclonal antibodies. These is very effective methods by using this monoclonal I_gm is reduced by 100 folds. The addition of plasmaphoresis, splenectomy, and even drugs inhibiting antibody formation but did not successfully block HAR (56-58).

II. **Reduction of target antigen expression:** this can be done by changing the antigens of the pig by human fucosyl transferases, which convert the alpha-galactose epitope into an antigen similar to that of human blood group antigens (59)

III. **Inhibition of complement activation:** the complement system is regulated by the complement control proteins which are present in the plasma. The activation of complement system cause huge damage of the transplant tissue. It can be inhibit by the C I-inhibitor, which will bind to C I to stop the regulation.

2.6 Importance of ECM as biomaterial for tissue engineering

All cells in the mammalian except blood cells are anchorage dependent cells residing in the matrix called extracellular matrix. There are different types of extracellular matrix with different types of components and tissue specific composition.

Function of extracellular matrix

i. It provides the structural support to the cells and help in attachment, grow and migrate of the cells.

ii. Extracellular matrix provide the structural support to the cells in the tissue, its means it is also giving the strength to the tissue. For examples high concentrated collagen I fibers gives the rigidity to the tissue as in the tendon, it is highly resistance to the tensile strength. On the other hand randomly distribution of collagen and elastin gives the elastic property to the tissue and less strength as compared to the high collagen content tissue.

iii. ECM provides the biochemical and bioactive cues to the cells for residing, cell migration and other cell activities (60, 61). For examples RGD sequence of the fibronectin has the binding property (62).

iv. Extracellular matrix provides the reservoir for the growth factors and potentiates their activities. For examples heparin sulphate helps in fibroblast growth factor demineralization (63).

v. The degraded product of the extracellular matrix provides the environment for the vascularization, pathological challenges, and in remodeling process during the homeostasis, morphology and in wound healing process.

2.7 Decellularization

The extracellular matrix for the scaffold preparation are derived from the variety of the tissue like heart valves [64-68], blood vessels [66–67], skin [68], nerves [69-70], skeletal muscle [71-72], tendons [73], ligaments [74], small intestinal submucosa (SIS) [75], urinary bladder [76-77] and liver [78]. Decellularisation is the technique to remove the cells from tissues or organs so that it

leave the complex mixture of structural and functional proteins that is called extracellular matrix. There are various methods to remove the cellular component of each tissue. Decellularization is considered important because it removes potential adverse immune response elicited by cell membrane epitopes, allogeneic or xenogeneic DNA, and damage associated molecular pattern (DAMP) molecules

There are various methods for the decellularisation.

2.7.1 *Physical methods*

Snap freezing: in this method tissue is subjected to very low temperature that will cause intracellular ice formation that will cause lyse of the cell. This methods has already applied for tendinous and ligament tissue (79-84) and nerve tissue (85). Freezing is a cell lyses method so it must be followed by the methods to remove cells materials from the tissue.

Merits: Rapid cell lyses method

Demerits: ECM can be disrupted or fractured during rapid freezing.

Mechanical force: In this methods a direct pressure is applied on the tissue and organs that will cause lyse of the cells. This method is applied on the tissue and organs that has not well densely packed ECM like lungs and liver (86).

Merits: easy and fast process of decellularisation.

Demerits: it can cause damage of ECM

Mechanical agitation: mechanical agitation can be used with the help of magnetic stirrer, orbital shaker or a low profile roller. This method is done simultaneously with the help of chemical treatment for cell lyses and removal of cell debris (92, 93).

Merit: cell lyses and removal of cellular debris occur simultaneously by exposure in chemical treatment.

Demerit: aggressive agitation can disrupt ECM as cellular material is removed.

2.7.2 *Chemical methods*

Alkaline and acid treatment: This methods is used for the decellularisation of tissue by solubilizing the extra cellular matrix and removes DNA and RNA content. . For example, acetic acid, peracetic acid (PAA), hydrochloric acid, sulfuric acid, and ammonium hydroxide (NH4OH) disrupt cell membranes and intracellular organelles [86, 88-91]. However, these chemicals also dissociate important molecules such as GAGs from collagenous tissues.

Merit: solubilize cytoplasmic component of cells and disrupt nucleic acid component.

Demerits: removes GAG

Non-ionic detergents: Non-ionic detergents are widely used chemical because of its mild effect upon the tissue structure. These detergent disrupts the lipid-lipid and lipid-protein interaction but leave protein –protein interaction intact so that protein should be left in the functional groups (16). Triton X-100 is most widely used detergent for different periods ranging from several hour to 14 days (85, 94-99).

Merit: it disrupt lipid-lipid and protein-lipid interaction and leave protein-protein interaction intact.

Demerits: it will give mixed result; efficiency depends upon types of tissue. It will also remove GAG

Ionic detergents: ionic detergents are effective in solubilizing in both cytoplasmic and nuclear membrane, but tends to disrupting protein-protein disruption (16). The most commonly used ionic detergents are sodium dodecyl sulphate, sodium deoxycholate, and triton X-200(85, 98, 100-104).

Merit: solubilize cytoplasmic and nuclear membrane. Remove nuclear remnant and cytoplasmic protein.

Demerit: it disrupt the native tissue structure, removes GAG and damage collagens.

Zwitterion detergents: it has the property of both non-ionic and ionic detergent and it has a great tendency to denature the protein than non-ionic detergents.

Examples: 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS): it has been studied for the decellularization of the blood vessels (93).

Sulphobetaine-10 and-16: it has been studied for the decellularisation of the nerves (102, 104).

Merit: efficient cell removal.

Demerit: disruption of cellular ECM.

Tri (n-butyl) phosphate: It is an organic solvent that disrupt protein-protein interaction. Recently this has been used for the decellularisation of tendons and ligaments.

Merit: complete removal of the nuclear remnant. Effect on mechanical property is minimum.

Demerits: loss of collagen (97-98).

Hypotonic and hypertonic solution: hypertonic or hypotonic solution creates osmotic shock that cause lyse of cells within the tissue or organs (93, 98,). A treatment in a hypotonic solution with hypertonic solution show better cell lyses.

Merit: it will not effect on the protein-protein interaction

Demerit: it will not effectively remove the cellular remnant.

Chelating agent: chelating agents such as EDTA and EGTA binds the divalent ions ($Ca^{2+}$and Mg2+) that is necessary for the binding of cells to the collagen and fibronectin at the Arg-Gly-Asp receptor (108-111).

Merit: it resist cells binding to the ECM

Demerit: there is no isolated exposure. It is typically used with chemical and enzymatic treatments.

2.7.3 *Enzymatic methods*

There are many enzymes that are used for the decellularisation.

Trypsin: it is a highly specific enzymes that cleaves the proteins at the c side of the arginine and lysine, if next residue is not proline (112). But if trypsin exposure is more then it will disrupt the ECM. Remove laminin, fibronectin, elastin and GAG.

Endonuclease: catalyze the hydrolysis of interior bonds of ribonucleotide and deoxyribonucleotide. Exonuclease: catalyze the hydrolysis of terminal bonds of ribonucleotide and deoxyribonucleotide (113).

Merit: it cleaves specifically protein site

Demerit: difficult to remove from the tissue that invoke the immune response.

2.8 Microsphere

Microspheres are the small spherical particles with the diameter range between 1 – 1000 μm. Now days various department of medicine like cancer, pulmonary, cardiology, radiology,

gynecology, and oncology etc. are using microsphere as a drug delivery system (114-116). These microspheres are used as a controlled drug delivery with the systematic release of a pharmaceutical agent to maintain a therapeutic level of the drug in the body for a sustained period of time. This may be achieved by incorporating the therapeutic agent or drugs into a degradable polymer vehicle that releasing the agent or drugs continuously as the polymer degrades. Microspheres are manufactured by various types of natural and synthetic materials.

Types of polymers for preparation of microspheres

Synthetic Polymers:

Non-biodegradable: Acrolein, Glycidyl methacrylate, Epoxy polymer, PMMA

Biodegradable: Polyanhydides, Lactides and glycolides and their copolymer, Polyalkyl cyno acrylates

Natural Materials:

Proteins: Albumins, Gelatins, Collagens

Carbohydrates: Chitosan, Carrageenan, Starch, Agarose

Chemically modified carbohydrate: Poly (acryl) dextran, Poly (acryl) starch, DEAE cellulose

2.8.1 *Methods for the Preparation of the Microspheres*

There are many methods for the preparation of the microspheres that depends upon the requirement likes particles size, route of administration,

Emulsion solvent evaporation technique

In this process the polymer is mixed in the water immiscible solvent and the drug was dissolved in this polymer solution. Then the resulting solution is added to the aqueous phase that contain emulsifying agent. Then this mixture is agitated at 500 rpm for the formation of the emulsion (121). The polymer and the drug is transformed in to small droplet that is converted in to solid

microsphere by the solvent evaporation and the microparticles is collected by the filtration and washed with the water and dry at room temperature.

Emulsion cross linking method

In this method the drug or protein is dissolved in the polymer solution that is previously dissolved in the aqueous solvent. Then this solution is added to the oil solvent and stir it at different rpm 200 to 1500 rpm according to our particle size requirement. The emulsifying agent is also added to the solution for the stable emulsion formation (122). After the droplet formation the crosslinking agent is added to the solution for the gelation of microbeads. Then filter it through cloth and then wash with tween 80.

Co-accervation method

In the co-accervation method the drug first dissolved in the polymer and then the polymer was dissolved in the aqueous solution. Then change the condition of the solution so that the polymer solubility decrease and the phase difference will occur.

Thermal co-accervation: ethylene cellulose was dissolved in the cyclohexane with vigorously stirring at 80^{o}C. Then drug was added to the solution with stirring then the phase separation occurs with the reducing the temperature. Then collect the microbeads and air dry.

Co-accervation by non-solvent addition: ethylene cellulose was mixed in the toluene solvent that contains propylisobutylene in a beaker with 6 hr. stirring. Then drug was added to the solution and

stirring it. The phase separation was occurred by adding of petroleum benzene and then wash the bead with n-hexane (118).

Spray drying technique

In this method the polymer was dissolved in the volatile solvent and then drug was added to the solution by homogenization. Then the polymer solution was spray in the hot air stream due to which the solvent was vaporized and lead the formation of microbeads (117).

Ionic gelation

Sodium alginate and chitosan microbeads are mostly formed by these methods. First the drug was dissolved in the polymer solution and then the polymer solution was added drop wise to the ionic solution. For sodium alginate the ionic solution is ca^2/al^3 and for chitosan the ionic solution is of acetic acid. Then keep the microbeads in the solution for 24 hr. for internal gelation.

Electrospraying method

In this method the sodium alginate solution was prepared and then put in to the syringe and then the syringe was fitted in to the electrospraying machine above the calcium chloride solution. Electric charge is supplied in to the solution through needle. The high voltage was applied to the needle at different flow rates. The microbeads will be form in the solution. It was filtered and keep in the water solution.

2.9 Related Works

Porcine adipose tissue was chopped and then homogenized and centrifuged for the deflating of the tissue. Decellularisation process was done by the chemical treatments (1.5M sodium

chloride and .5% SDS), enzymatic treatments (DNase, RNase) and at temperature 40^{o}C for prevent congeability. After decellularisation it was showing significant reduction in the immunogenic component such as nucleic acid and α- gal. It was also showing significant amount of collagen, elastin and glycosaminoglycan in the decellularized scaffold (123).

The DNA content and the fragment length of the commercial available scaffold was quantified by picogreen assay and electrophoresis on a 3% LMP agarose gel with Ethidium Bromide. It was found that mostly all commercial scaffold contain very less amount of nuclear remnant. But its quantity is so less that has not adverse effect on the remodeling process (124).

Decellularized porcine esophageal scaffold was cross-linked with genipin, glutaraldehyde, and carbodiimide and a untreated scaffold bovine pericardium as a positive control were implanted subcutaneously into Sprague-Dawley rats. The host response was quantified by the infiltration of fibroblasts, lymphocytes, macrophages and giant cells after 1, 9, and 30 days. The inflammatory response was recognized in all scaffolds but the genipin group was showing less infiltration. Its means the genipin is the most effective and tolerated cross linker in all other group (125).

Bovine pericardium was first decellularized by standard hypotonic solution and treated with DNAse and RNAse. After this the decellularized tissue was treated for 24 h by 0.1% sodium dodecyl sulphate (SDS), 0.5% sodium deoxycholate(SD), 0.5% triton X 100, α galactosidase (5U/ml) and phospholipase A2 (150U/ml). Standard decellularized tissue resulted in partial removal of cellularity, and α-gal. Adding SD resulted in acellularity but persistence of antigen. Adding SDS resulted in complete removal of cellularity and xenogenic antigen. Galactosidase help in selectively removal of α-gal (126).

The trachea was treated with 3 % triton, 0.1 peracetic acid, and 4% ethanol for 4 hrs. at 300 rpm. Then the decellularized scaffold was sterilized by gamma irradiation. Decellularized tracheal

scaffolds was used for the formation of site specific epithelium and have a good mechanical strength (127).

Porcine urinary bladder ECM and liver ECM scaffolds were degraded by using acid and high temperature and then fractioned by ammonium sulphate. Degraded ECM product was demonstrated to S. aureus and E.coli for see antimicrobial activity. This study showed that the low molecular peptides between 5 to 16 KD shows antibacterial activity (128).

The relationship was demonstrated between tissue remodeling and macrophage phenotype. The abdominal wall of rats were repaired by using autologous body wall, porcine urinary bladder and their ECM scaffold. The ECM scaffold showed M2 type macrophage and constructive remodeling while cellular tissue even autologous wall showed M1 type macrophage and result in scaring (129).

Intra-articular injection of UBM-ECM in the dog improve the lameness and motion of the dogs compared to the control dog (130).

Bovine pericardium was solubilized to two different solution, first in the hydrophilic solution of dithiothreitol and potassiumchloride and then in the lipophilic solution of amidosulfobetaine-14 (ASB-14). Lipophilic antigen removal step removes the two common barriers galactose-α (1, 3)-galactose and major histocompatibility complex I (131).

The ECM sheet of thickness 80 µm derived from either porcine small intestinal submucosa (SIS) or the porcine urinary bladder (UB) matrix was used for the repairing of ventricular anterior walls of pigs and dogs. There was complete replacement of acellular scaffold to the different types of tissues like adipose tissue, cartilage tissue, fibrous connective tissue and myocardial tissue (132).

Porcine heart was decellularized by using pulsatile retrograde aortic perfusion in less than 10 hrs. The perfusion was done by enzymatic, ionic and non-ionic detergent, hypertonic and hypotonic solution and acidic solution. The decellularized ECM retained intact collagen, elastin, and glycosaminoglycan and mechanical integrity (133).

The biological properties of porcine ECM derived from different tissue like water absorption, antimicrobial activity, histological structure, mechanical property, biodegradability and in vitro biocompatibility test was compared. Urinary bladder ECM was showing good biological properties as compared to other tissue ECM (134).

Porcine extracellular matrix was examined for the immune response in the mouse abdomen. The immune response produced was of Th2 types that related to remodeling of the ECM scaffold rather than rejection (135).

Alginate microbeads were formed by electrospraying method to achieve the protection. The polymer solution was fabricated in to the ionic solution of calcium chloride by applying different voltage and flow rate (136).

MATERIALS AND METHODS

Instruments

Blender: surmit, Magnetic stirrer (SPINT), Freeze (-20^{o}) [Elanpro], Centrifugation (REMI), Freeze-dryer (Operon Chemical free freeze dryer-120C) under -115^{0}C and 650-950mmtorr Electro spin, Vacuum drier (fisher scientific), Weighing balance (CONTECH), Micro oven (Samsung), Water bath

Glassware and plastic ware

Beaker (1l, 500ml, 100ml, 50ml, 25ml) [Qualigens and LOBA Life], Centrifuge tube (50ml) [fisher scientific], Measuring cylinder (1000ml, 100ml, 10ml) [Qualigens and Borosil), Syringe

(5ml), Needle (26G), Magnetic bead

Chemicals

All chemicals Acetic acid (HPLC grade, RAMKEM), Sodium dodecyl sulphate, Sodium alginate, Sodium hydroxide, Picric acid, Biebrich scarlet, Acid fuchsin, Formaldehyde, Hematoxylin, ferric chloride, hydrochloric acid, Phosphotungstic acid, Phosphomolybdic acid, Aniline blue, Lugol's iodine purchased from LOBA Chemie.

3.1 Collection of Porcine Adipose Tissue

The adipose tissue was obtained from slaughter house of freshly killed porcine in Mall Godam, Rourkela. Then the adipose tissue washed with water 3-4 times and kept in -20^{o}C Freeze until use.

3.2 Extraction of Extracellular Matrix form Porcine Adipose Tissue

Porcine adipose tissue was thawed at 37^{o}C if it was kept in freeze. Then removed skin and muscles form the adipose tissue and processed. This adipose tissue chopped in to small pieces and washed thoroughly with distilled water 3 to 4 times. Distilled water (200ml) was added to porcine adipose tissue (100gm), which was homogenized for 10 minutes by using the housewares blender with pulsation. The tissue suspension was centrifuged at 8000 rpm and the upper layer of the oil was discarded. Then it was kept in the -20^{o} freeze and thaw in water bath at 37^{o}C. This two steps

were repeated several times until the complete removal of oil component. For the decellularisation the extracellular matrix suspension was treated with .5 M acetic acid solution at 40^{o}C for 2 or 3 days. Then the ECM suspension was centrifuged for the collection of pellet and discard supernatant. After the decellularization the tissue was treated with .1% SDS for 24 h to remove the cellular component and α-gal. After the SDS treatment the decellularized tissue was washed for 24 h and then centrifuged. Then the ECM pellet was stored at -20^{o}C for the future use.

3.3 Freeze-Drying

Extracellular matrix pellet was filled in a small petri plate and kept in -20^{o}C freeze for minimum 6 hr. Then for the freeze drying the sample was transferred to the vacuum freeze dryer under -115^{0}C and 650-950mmtorr (Operon Chemical free freeze dryer -120^{o}C). The lyophilization process was done 24-36 hr. After lyophilization it will give the porous scaffold.

3.4 Microbeads Preparation

The microbeads were formed via the electrospraying machine. The 10% w/v of ECM pellet was dissolved in 5 % sodium hydroxide solution and then the solution was mixed with different concentration of sodium alginate by magnetic stirrer. Then the mixed solution was loaded in the 5 ml syringe that is fitted with 26G stainless steel nozzle. Then the syringe was fitted in the electrospraying machine above the collector that contain 5 % calcium chloride solution. The solutions were extruded through the nozzle at a constant rate of either 1ml or 4 mL/h using a

syringe pump. The tip-to-collector (TTC) distance was set to 15cm respectively and the High-voltage of 18KV was applied between the needle and collector. After electrospraying the microbeads were transferred to the glass vials.

3.5 Characterization of microbeads

3.5.1 Histological Staining

The decellularized ECM was held by the forceps and sliced in to thin section of 10µm with the help of razor blade. To visualize the cell nuclei and collagen fiber the decellularized scaffold was stained by Collagen - Masson's Trichrome stain (TRI) method and for the elastic fiber staining the decellularized scaffold was stained with Verhoeff'S Van Gieson methods.

3.5.2 Swelling ratio

The scaffold was immersed in PBS solution and then the water uptake amount of the ECM scaffold was calculated by the weight after dipping in the PBS solution. The swelling ratio was measured as the ratio of increase weight (W-W_O) to the initial weight (W_O).

3.5.3 Antimicrobial activity

The antimicrobial activity was tested for the intact ECM and degraded ECM against E coli and Bacillus Subtilis bacteria. The ECM was degraded by acetic acid (.5 M), and heating at 120^{o}C. Then the solution was kept at room temperature and then centrifuge it at 8000rpm for 10 min. the supernatant was discarded and the pellet was collected. Then the intact ECM and degraded ECM was kept on the nutrient agar plates that was streaked by E Coli and Bacillus bacteria. Then the

plates were kept in incubator at 37^{o}C for 24 h. Then the antibacterial activity was examined by the zone of inhibition by the ECM and degraded ECM.

3.5.4 Particle size measurement

Particle size and size distribution of different microsphere were measured by using an optical microscope, and the mean particle size was calculated

3.5.5 Shape and surface Morphology

The shape and surface morphology of the microbeads and the scaffold was determined by using scanning electron microscope (JEOL-JSM 6480, Japan) after coating with a thin layer of platinum (Pt). SEM images were used to measure the microbead diameters and pore size of the scaffold.

3.5.6 Fourier Transform Infra-Red Spectroscopy

Infrared spectroscopy of the samples was carried out by using ATR-FTIR instrument (Alpha-E by Brucker, USA) for the functional group identification. The alginate bead, composite alginate and gelatin bead, composite alginate and ECM beads and scaffold in the range of 3500 cm-1 to 500 cm-1 to understand the interactions amongst the components of the microbeads.

3.5.7 Thermal Analysis

Thermal properties of microbeads and ECM scaffold were studied using differential scanning Calorimetry (DSC 200 F3 Maia, NETZSCH).

RESULTS AND DISCUSSION

4.1 Extraction of extracellular matrix from adipose tissue

The extracellular matrix was extracted from the porcine adipose tissue by mechanical treatment like homogenization, centrifugation and freeze thawing and decellularized by chemical treatment in .5 M acetic acid solution. After centrifugation of decellularized ECM solution the white viscous fibrous pellet formed. These pellet represented near 8 % of the total original tissue. Freeze thaw and centrifugation process helps in the removal of the oil component from the adipose tissue. The extraction and Decellularization procedure were performed at 40^{o}C to prevent the congealability of lipids of the porcine adipose tissue. The role of decellularisation is the removal of xenogeneic cellular, nuclear material and damage associated molecular pattern (DAMP) antigens that may cause inflammation and immune mediated rejection by host. To see the decellularisation the ECM

was stained by Collagen – Masson's Trichrome Stain (TRI) and Verhoeff'S Van Gieson (EVG) methods. The result demonstrates the reduction of immunogenic component of tissue after decellularisation.

4.2 Scanning electron microscopy of Scaffold

This study demonstrate that the decellularized ECM scaffold has variablle pore size of average maximum diameter 182.5 µm and average minimum diameter 113.5 µm. these SEM result indicates that the scaffold material has good porus structure for the cell infilteration.

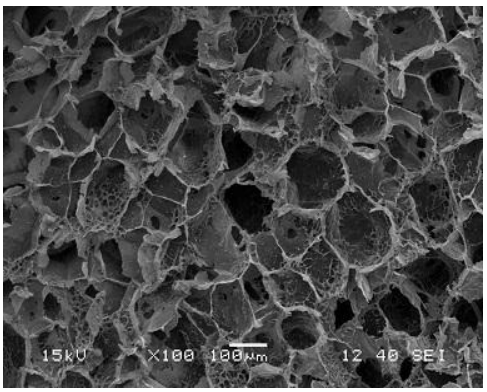


Fig: 1

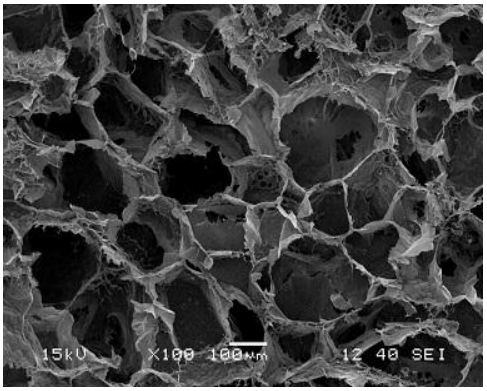


Fig: 2

Fig 1: SEM image of flat scaffold

Fig 2: SEM image of cross section of scaffold

4.3 Verhoeff's Van Gieson staining of ECM

The biochemical and histological images of ECM scaffold was shown in figure 3. After decellularization of tissue a large amount of collagen and elastic fibers was preserved as shown in figure 3 the black color is showing the presence of elastin and red color is for collagen.

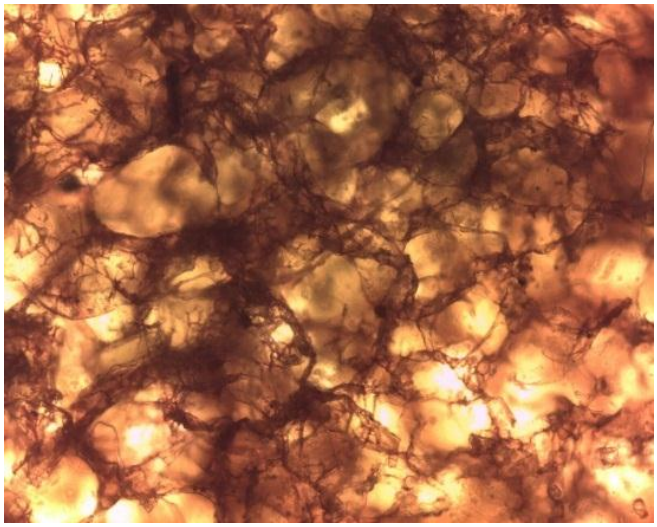


Fig: 3: Elastic tissue fibers- Verhoeff's Van Gieson (EVG)

4.4 Swelling Study of ECM Scaffold

In the early stage of wound healing cells required the high nutrients that are present in the tissue fluids surrounding the wound. So the scaffold should have water absorbance property for the uptake of nutrients. The swelling ratio of the scaffold depends upon the hydrophilicity and pores of the scaffold. The pore size and the swelling helps in the nutrient uptake in the interior of the scaffold and it also increase the area of the scaffold for the adhering of cells that is required for the tissue engineering. But swelling also affect the mechanical property of the scaffold so scaffolds should have controlled swelling. The absorbed water of the scaffold is 10.5 times of its initial weight in 3 hr. its means the scaffold has good water absorbable property.

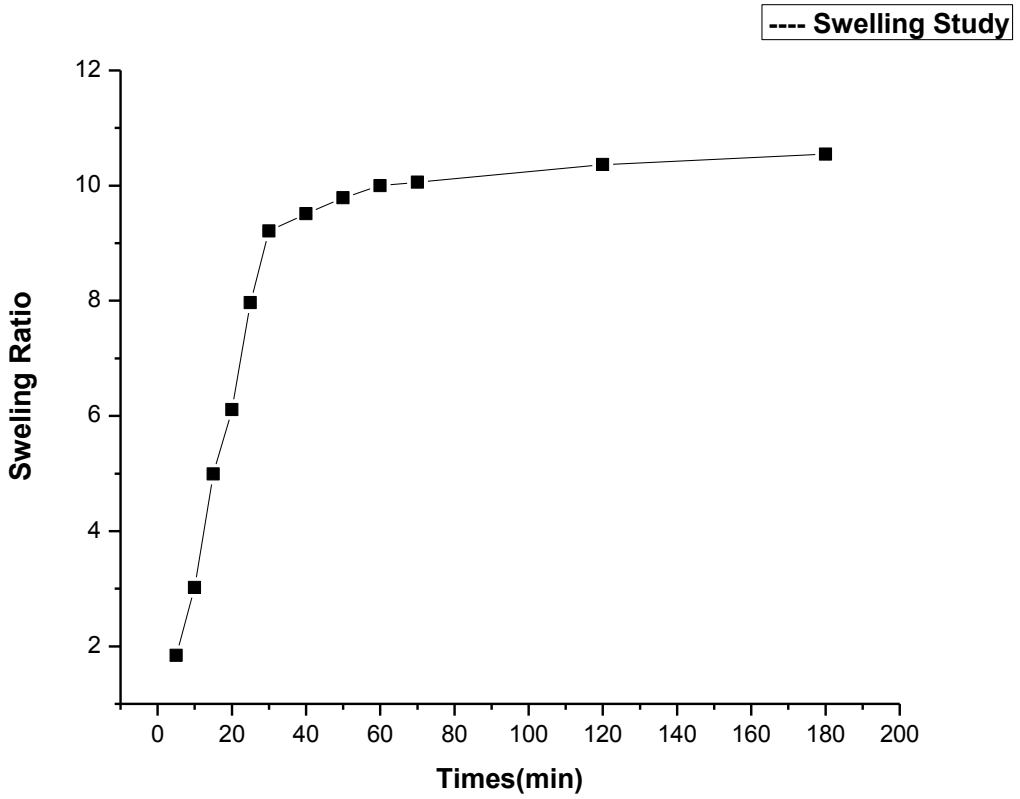


Fig: 4 swelling study of ECM

4.5 Antimicrobial Activity of ECM

The extracellular matrix that was degraded by acid and heat was showing antibacterial activity against both gram negative bacteria (E. Coli) and gram positive bacteria (Bacillus Subtilis) while the intact ECM has no effect on the bacterial population as shown in figure.

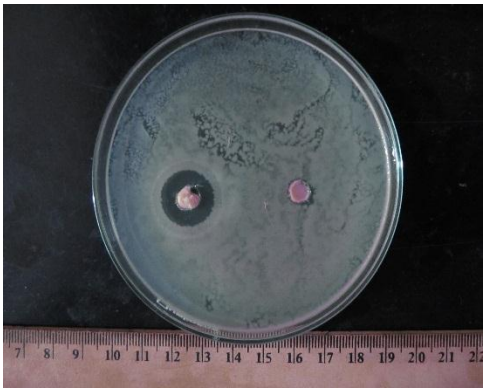


Fig 5: ECM Vs E Coli

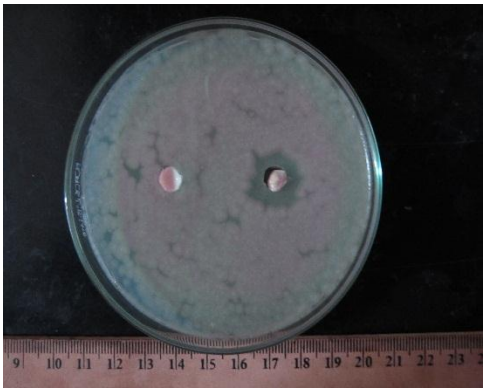


Fig 6: ECM Vs Bacillus

4.6 Microbeads Preparation

Sodium alginate polymer was selected for the preparation of microbeads because it has its own biodegradability and it polymerize easily by the calcium ion. ECM pellet was first dissolved in the 5 % sodium hydroxide solution then add different ratio of sodium alginate. The bead formed at different concentration of sodium alginate and at different flow rate by electrospraying method.

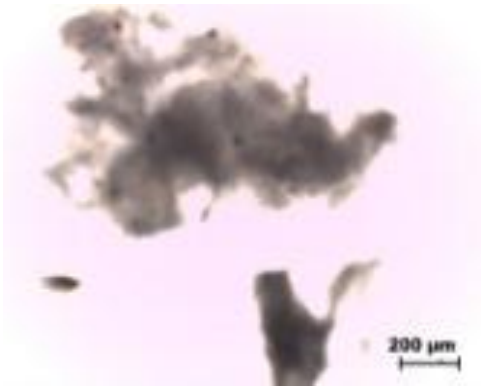
Particle size analysis of microbeads

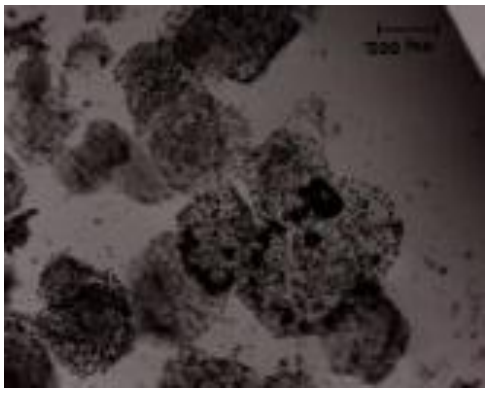
The particles size was seen by the optical microscope at 4X and the size was quantified by the ImageJ software. The microbeads were formed with the different flow rate 1ml/hr. and 4ml/hr. and with the different concentration of sodium alginate polymer. The result microbeads formed

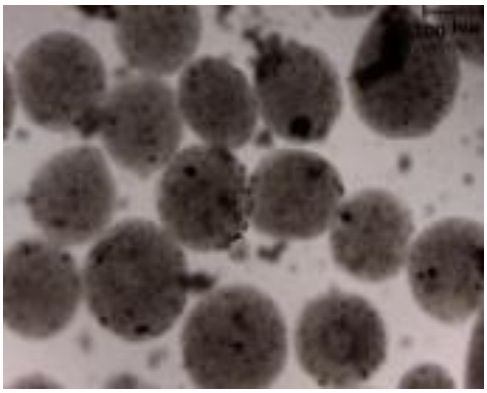
are shown in the table. 1.2 % Sodium alginate with 1ml/hr was showing good result with particle size less than 200 µm.

Table: 1: microbeads preparation parameters

Sample	Concentration	Flow rate (ml/hr)	Status
A	**.8%**	**1**	**----------**
B	**.8%**	**4**	**Bead form**
C	**1.2%**	**1**	**Bead form**
D	**1.2%**	**4**	**Bead form**
E	**1.6%**	**1**	**Ellipse form**
F	**1.6%**	**4**	**Ellipse form**
G	**2%**	**1**	**----------**
H	**2%**	**4**	**----------**

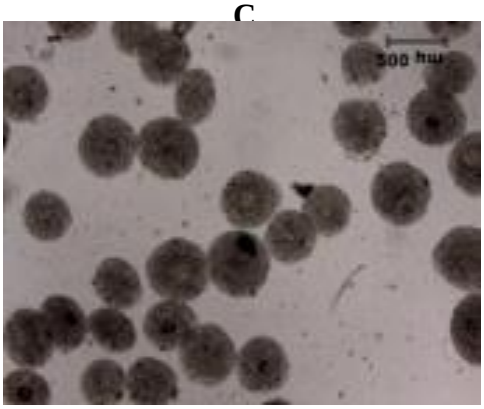


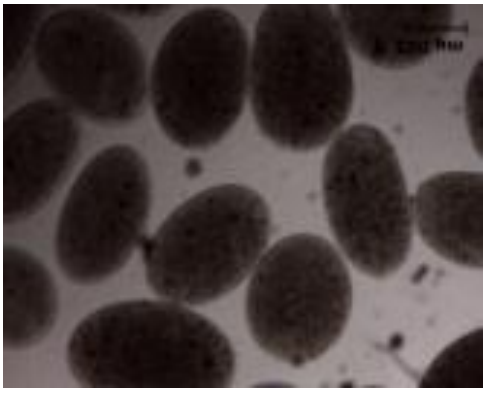


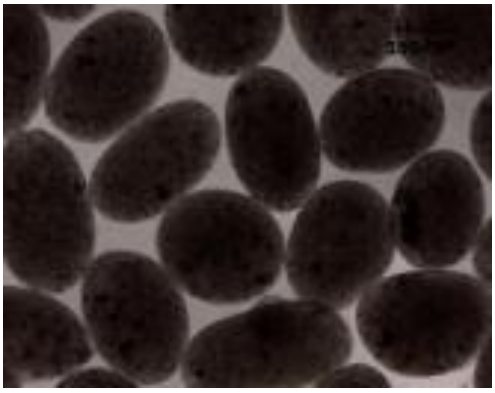


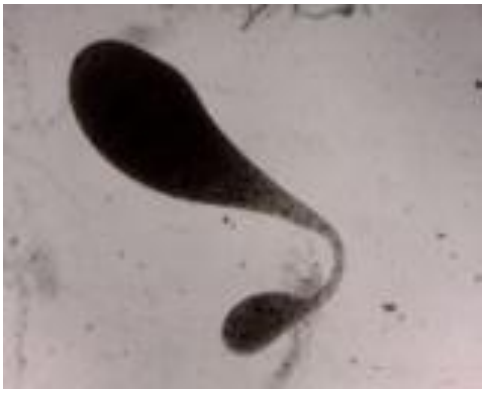
C

D









E **F**

G **H**

Fig: 7: Simple microscopy of microbeads

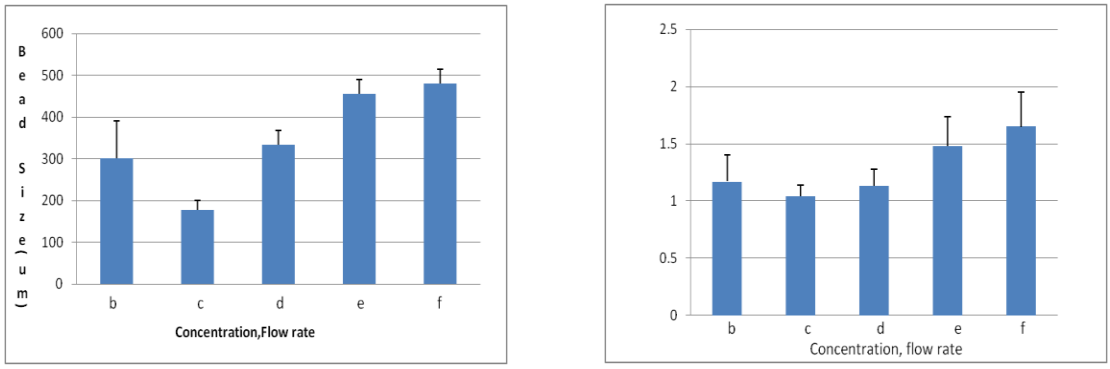


Fig: 8: Particle size analysis of microbeads

4.2 Scanning Electron Microscopy of Microbeads

The SEM image are showing that microsphere contains small microparticles of 7-8 µm size. These image indicates that these small microparticles are fairly smooth and spherical in shape with apparently homogenous surface. These microsphere will be more biodegradable due to porous structure and having more surface area because each microsphere made of many small microparticles.

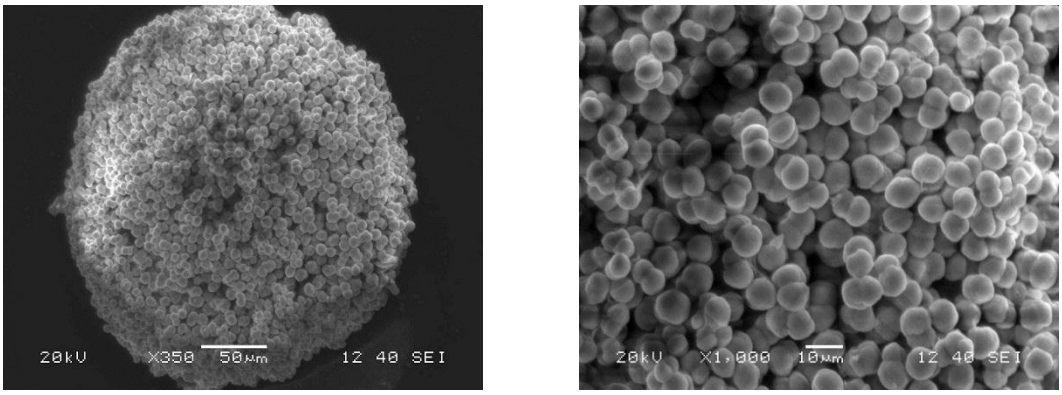


Fig: 9: SEM images of microbeads

4.3 FTIR of Scaffold and Microbeads

FTIR is used to see the organic constitute of the ECM scaffold and microbeads. FTIR are showing the peaks of amide I, amide II, amide III, CH, CH_2, CH_3, NH, C-O, COO^-, C-N, N-H, and C=O and cross linked C=O functional groups as shown in table that resembling the peaks of collagen protein and sodium alginate.

Table: 2: Wavenumber of different functional groups present in ECM

Sl No	**Functional Group**	**Wave number cm^{-1}**
1	Amide I region: mainly C=O , C-N bonds	1585-1720
2	Amide II region: mainly combination of C-N stretching and N- H bending vibrations	1500-1585

3	Amide III region: mainly C-N stretching, N-H bending, C-C stretching, and sulfate stretching vibrations	1200-1300
4	CH_2 bending vibrations, CH_3 asymmetric bending vibrations, COO^- stretching vibrations, and CH_2 side chains vibrations of collagen	1300- 1500
5	CH_2 side chains vibration	1340
6	Carbohydrate region: stretching vibrations of C-O and C-OH, as well C-C ring vibrations	985-1140
7	Free C=O group of alginate	1651
8	Cross-linked C=O group of alginate	1759

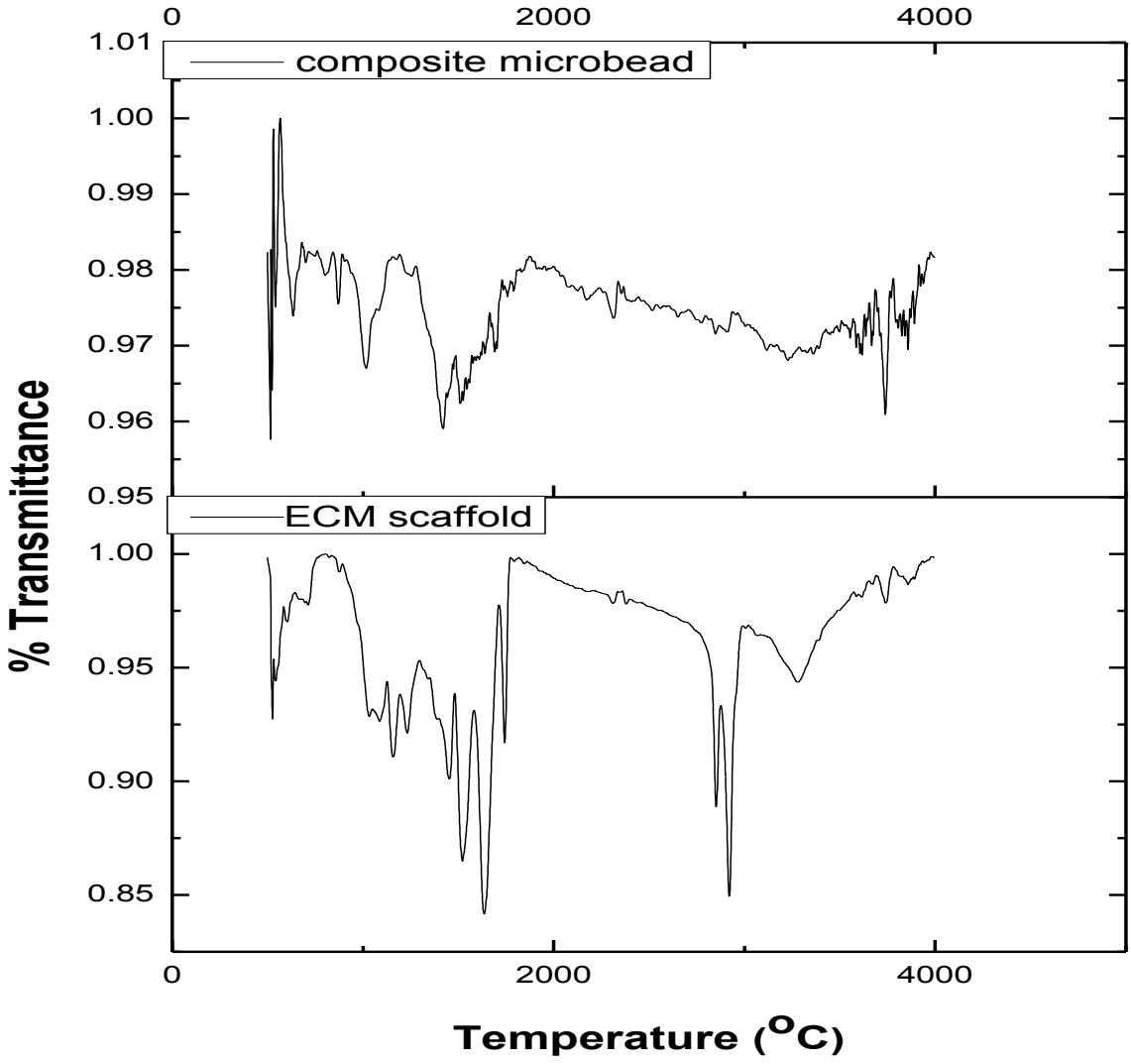


Fig: 10: FTIR of ECM scaffolds and microbeads

CONCLUSION AND FUTURE PLANS

Porcine adipose tissue can be successfully decellularized by homogenization, freeze thaw and SDS treatment. Freeze drying of adipose tissue gives highly porous structure having excellent hydrophilicity and antimicrobial activity. The decellularized ECM can be used for the preparation of microbeads which will be great contribution to the soft tissue engineering. Electrospraying of alginate and ECM mixture can give porous microbeads. The future plan of my work is further optimization of scaffold and microbeads, cell seeding in scaffold ,in vivo study of ECM scaffold and microbeads

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