PREPARATION AND CHARACTERIZATION OF CHOLECYSTIC EXTRACELLULAR-MATRIX POWDER

DISSERTATION BY

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BIOMEDICAL TECHNOLOGY WING

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2015

DECLARATION

I hereby declare that the dissertation entitled "**Preparation and Characterization of Cholecystic Extracellular-matrix Powder**" submitted to Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, in partial fulfillment for the degree of Master of Philosophy in Biomedical Technology is a record of original work carried out by me under the guidance of Dr. T.V. Anilkumar, Scientist F, in the Division of Experimental Pathology, Biomedical Technology Wing, SCTIMST, Kerala, during the period of January 2015 to June 2015.

I further declare that the results of the work have not been previously submitted for any other degree or fellowships.

Place: Thiruvananthapuram
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CERTIFICATE

This is to certify that Ms. Reshmi Raj, in the Experimental Pathology Laboratory of this institute has fulfilled the requirements prescribed for the M. Phil. degree of the Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram. The dissertation entitled, "**Preparation and Characterization of Cholecystic Extracellular-matrix Powder**" was carried out under my direct supervision. No part of the thesis was submitted for the award of any degree or diploma prior to this date.

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ABBREVIATIONS

µg	Microgram
bFGF	Basic Fibroblast Growth Factor
˚C	Degree Celsius
C-ECM	Cholecystic Extracellular Matrix
Cp	Centipoise
DLS	Dynamic Light Scattering
DMSO	Dimethyl Sulfoxide
DMEM	Dulbecco's Minimum Essential Medium
DNA	Deoxy Ribonucleic Acid
ECM	Extracellular - Matrix
ESEM	Environmental Scanning Electron Microscopy
FBS	Foetal Bovine Serum
FTIR	Fourier Transform Infrared Spectroscopy
GAG	Glycosaminoglycan
IR	Infrared
L-929	Adherent fibroblast cells from adipose tissue of mouse
MTT	Methyl thiazolyl tetrazolium
n	Sample size
nm	Nanometer
NaCl	Sodium Chloride
NC-ECM	Sodium Chloride treated Cholecystic Extracellular Matrix
OD	Optical Density
PDI	Polydispersity Index
SD	Standard Deviation
SIS	Small Intestinal Mucosa
TGF-β	Transforming Growth factor Beta

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ABSTRACT

Biological scaffold materials derived from extracellular matrices (ECM) of intact mammalian organs and tissues have been extensively used in various clinical applications as xenografts or implants. Cholecystic ECM (C-ECM) is an acellular natural biodegradable matrix rich in collagen and other macromolecules useful in tissue remodeling. In most cases of C-ECM based tissue engineering applications, single or multilayered two dimensional forms of these materials have been used, which accompanies inherent limitations with regard to their shape, three dimensional form and clinical utility. Powder form of ECM provides a great deal more flexibility in terms of delivery of the biomaterial to the target sites. The objective of the present study was to prepare a particulate form of porcine cholecystic ECM. The study investigated the conventional grinding and salt precipitation methods for the preparation of particulate ECM. The ECM powder formulations prepared by these two methods were characterized by Dynamic Light Scattering, Environmental Scanning Electron Microscopy, Fourier Transform Infrared Spectroscopy and X-ray Diffraction. The salt precipitation technique yielded more uniform particle size (344.5 nm) and low polydispersity index value (0.5) when compared to freeze milling method (1805 nm). The content of collagen and glycosaminoglycan (GAG) remained similar in both forms of ECM. The results supported the notion that that ECM characteristic remained unaltered after salt precipitation treatment. The current study showed that a particulate form of C-ECM with uniform size and morphology can be prepared through salt precipitation method.

1. Introduction

Until the mid 1960s the cell and its intracellular contents, rather than the extracellular matrix (ECM), was the focus of attention for most cell biologists, molecular biologists, developmental biologists and other life scientists. However, with the discovery that the ECM plays a role in the conversion of myoblasts to myotubes [1] and that structural proteins such as collagen and glyocosaminoglycans are important in salivary gland morphogenesis [2] it became obvious that the ECM is much more than a passive bystander in events of tissue and organ development and in host response to injury. The ECM is the non-cellular component present within all tissues and organs, and provides not only essential physical scaffolding for the cells but also initiates crucial biochemical and biomechanical cues that are required for tissue morphogenesis, differentiation and homeostasis [3]. The major components of extracellular matrices include collagens, elastins, proteoglycans, and a variety of cell adhesion proteins, such as fibronectin, and laminin. The primary structural framework for ECM is provided by collagen [4]. The presence of elastin adds to the elasticity of ECM [4]. Proteoglycans consist of protein and glycosaminoglycans and serve as an amorphous packing material that fills the extracellular space [4]. These proteins perform diverse functions which include the provision of structural support and tensile strength, attachment sites for cell surface receptors, and as a reservoir for signaling factors that modulate diverse host processes such as angiogenesis, vasculogenesis, cell migration, cell proliferation, inflammation, and wound healing [5]. The concept of ‘dynamic reciprocity’ between the ECM and intracellular cytoskeletal and nuclear elements has become widely accepted [6]. The translation of this phenomenon to therapeutic use of the ECM as a scaffold for tissue engineering applications has recently been attempted [5].

ECM is well known as nature's ideal scaffold material [5]. ECM based materials when appropriately prepared and processed, can be used as inductive templates for tissue reconstruction [3]. Hence in regenerative medicine, extracellular matrices isolated from mammalian organs and tissues are usually used as xenografts. These xenografts are generally fabricated out of ECM of variable purity and can be called as scaffolds because of its ability to act as substrate for three dimensional growth of cells, *in vitro* and *in vivo*. The xenogeneic sources for ECM explored so far include dermis of the skin [7], small intestinal submucosa [8], urinary bladder mucosa [9], stroma of decellularized liver [10], decellularized tendon [11] and amniotic membrane [12]. Porcine small intestinal submucosa is probably the most favourite scaffold of mammalian origin and it has been clinically used in at least a million human patients worldwide [5]. Porcine small intestinal submucosa based products were marketed for different clinical applications such as soft tissue repair (Cuffpatch, Surgisis®) and dura mater repair (Durasis®). A lyophilized sheet form of porcine small intestinal submucosa(Oasis®) was available for the treatment for the treatment of partial and full thickness wounds.

Cholecystic extracellular matrix (C-ECM) has been best described as an acellular natural biodegradable matrix rich in collagen and other macromolecules useful in tissue remodeling [4]. The importance of C-ECM from porcine origin as a potential biological scaffold material was already reported by the host laboratory, revealing the existence of another promising candidate in the field of tissue engineering scaffolds [13]. Naturally occurring biodegradable materials has the ability to induce the host tissue to replace the graft with native tissue [14]. C-ECM elicits a regenerative response [15] in healing of skin excision wounds [16].

C-ECM proved its usefulness as a potential biomaterial in tissue engineering. It was a reliable natural collagen scaffold for bladder augmentation [17] and it was shown to act as

reinforcing buttressing staples for gastrointestinal resection [18]. It possess a micro-architecture similar to heart valves, supports proliferation of valvular endothelial and interstitial cells [19] and also a potential skin graft for treating full thickness skin wounds [16]. Cholecystic ECM elicits a very less immunogenic reaction compared to other commercially available xenogenic scaffolds [15]. Certain studies shown that C-ECM has the ability to withstand large strain [20][21] and its properties can be functionalized and modified using chemicals [22][23]. Most studies related to C-ECM conclude that it is a promising animal derived xenograft for biomedical applications.

ECM powders have been shown to have great potential for meeting clinical challenges in regenerative medicine [24]. Lyophilized sheets of ECM can be comminuted into particulate form of ECM, which allows for the delivery of the ECM as a suspension via minimally invasive techniques to the site of interest [25][5]. It will also aid the manufacture of three dimensional scaffolds by compaction methods [5]. The particulate form of ECM retains the ultrastructural and three dimensional properties of the parent scaffold. The size and shape of the particulate ECM may affect the host response *in vivo*. The size distribution and shape of the particles may differ depending upon the source and composition of the ECM and also upon the method by which the powder is formed [26][25]. A powdered form of ECM provides a great deal more flexibility in terms of delivery of the material to the target sites. Extracellular matrix suspensions could be administered locally, whereas this would not be possible with the sheet forms. However, in most cases of C-ECM based tissue engineering applications, single or multilayered two dimensional forms of these materials have been used, which also accompanies inherent limitations with regard to their shape, three dimensional form and clinical utility. Therefore, the utility of an ECM based biologic scaffold for many clinical applications is typically restricted to topical

administration or to invasive surgical procedures that can accommodate variations of the two-dimensional sheet forms [27]. Therefore, in view of wider use of the material, a particulate form of C-ECM may be of interest, for fabricating injectable ECM based materials.

For an injectable ECM suspension to be a reality, uniform particle size is required so that the particles do not obstruct the syringe needle lumen [25]. An ideal pharmaceutically acceptable parenteral suspension should have particle size less than 5µm [28].

Against the above background, this study aims to compare two methods proposed for particulate ECM formulations, suitable for making injectable C-ECM suspension for tissue engineering applications. In the first method, lyophilized sheet form of C-ECM was snap frozen and then comminuted in a freezer mill. The second method utilized a technique of salt precipitation, where the material was saturated in sodium chloride (NaCl) solution prior to snap freezing and grinding.

2. Review of literature

2.1. Extracellular matrix

Extracellular matrix, a complex blend of structural and functional proteins, glycoproteins, and proteoglycans arranged in a unique, tissue specific three-dimensional ultrastructure [29]. The ECM acts more than an inert packing material or nonspecific glue that holds cells together; it often plays a key regulatory role in determining the shape and activities of the cell. One of the best defined extracellular matrices is the basement membrane (or basal lamina), a continuous sheet, 50 to 200 nm thick that surrounds nerve fibers, muscles, and fat cells, underlies the basal surface of epithelial tissues, such as the epidermis of the skin or the lining of the digestive and respiratory tracts, and underlies the inner endothelial lining of blood vessels. Basement membranes provide mechanical support for the attached cells, generate signals that maintain cell survival, serve as a substratum for cell migration, separate adjacent tissues within an organ, and act as a barrier to the passage of macromolecules. The composition and ultrastructure of the ECM will vary depending on the factors that influence the phenotype of the cells residing in a tissue, which includes biochemical milieu, mechanical forces and oxygen requirements [29]. Even though the ECM may take diverse forms in different tissues and organisms, it tends to be composed of similar macromolecules. Unlike most proteins present inside of cells, which are compact, globular molecules, those of the extracellular space are typically extended, fibrous species. These proteins are secreted into the extracellular space where they are capable of self-assembling into an interconnected three-dimensional network, depicted in Figure 1. The major components of extracellular matrices include collagens, proteoglycans, and a variety of cell adhesive proteins, such as fibronectin, vitronectin and laminin. It also serves as a rich source of different growth factors depending on the type of source organ. Collagens are highly abundant,

fibrous proteins that give the ECM the capability to resist pulling forces. Proteoglycans consist of protein and glycosaminoglycans and serve as an amorphous packing material that fills the extracellular space. The ECM is in a state of dynamic reciprocity and will change in response to environmental cues such as hypoxia and mechanical loading. In the context of tissue engineering applications therefore, the use of the ECM as a scaffold indeed provides structural support, but more importantly provides a favourable environment for constructive remodeling of tissue and organs. Xenogeneic and allogeneic ECM has been used as a bioscaffold for the reconstruction of many different tissue types in both pre-clinical and human clinical studies [29]. Common features of ECM-associated tissue remodeling include extensive angiogenesis, recruitment of circulating progenitor cells, rapid scaffold degradation and constructive remodeling of damaged or missing tissues. The ECM-induced remodeling response is a distinctly different phenomenon from that of scar tissue formation.

2.2. Extracellular matrix as therapeutic scaffolds

The ECM, typically from a xenogeneic source, has been used as a scaffold for the replacement or reconstruction of many different tissues. These studies have utilized ECM that is derived from organs such as small intestine [30][31], urinary bladder [32], skin, cartilage [33], omentum [34] and many other organs too. The choice of organ or tissue for the isolation of ECM depends on the location of ECM within the organ or tissue, age of the host and physiologic requirements of the particular tissue. Organs rich in parenchymal cells, such as the kidney, have relatively little ECM. In contrast, tissues such as tendons and ligaments with primarily structural functions have large amounts of ECM relative to their cellular component [29]. An allogeneic or xenogeneic source of ECM has usually been used, thus providing the possibility of an off-the-shelf product for clinical application. There were differing opinions as to whether the ECM

should be seeded with site-specific cells prior to utilization as a repair scaffold or should be used in an acellular form. The effect of adding cells to an ECM scaffold upon the biologic remodeling response varies depending upon the intended application and the manner of preparation of the composite scaffold. A cellular component requires an autologous cell source and a controlled environment to assure cell viability during the translocation of the cell-scaffold composite to the recipient tissue site.

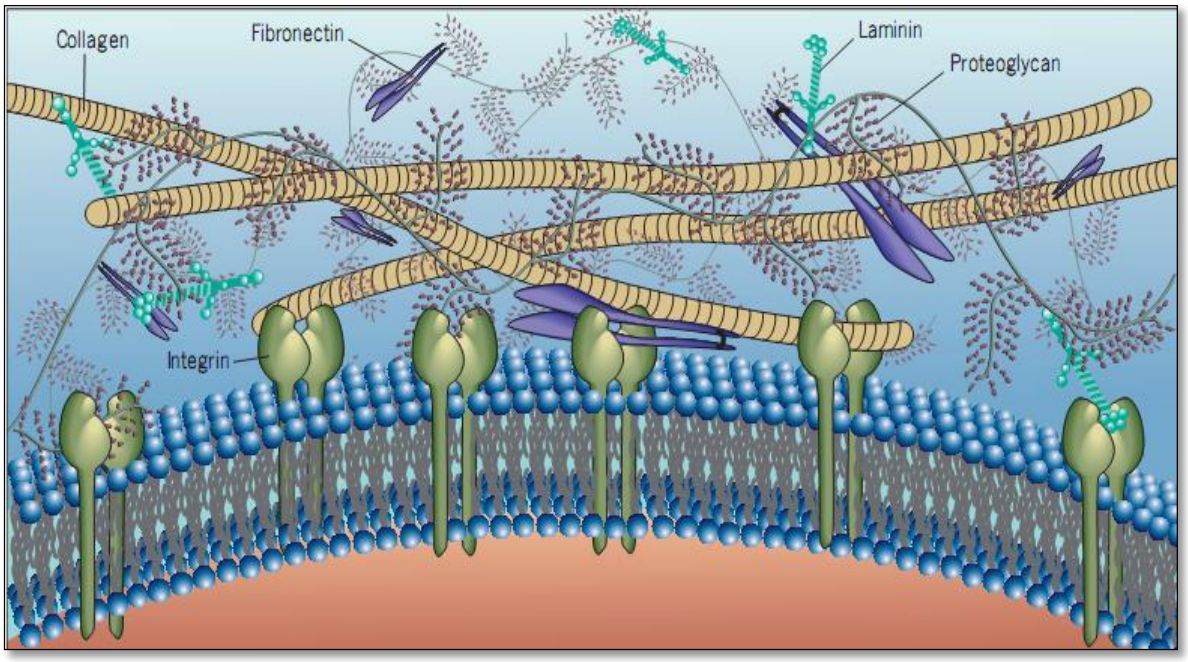

Fig. 1: An overview of the macromolecular organization of the extracellular matrix (adapted from *Cell and Molecular Biology Concepts and Experiments* (7th Edition) - Gerald Karp.pdf.)

Both cellular and acellular forms of ECM scaffolds have been used for tissue engineering applications [35]. The use of intact (i.e. not chemically modified) ECM as a tissue scaffold provides for a different host tissue response than purified components of the ECM such as collagen I [29] . However, if ECM was chemically cross-linked by such agents as glutaraldehyde, carbodiimide or photo-oxidizing agents, the constructive remodeling that was seen as part of the host response will be replaced by a typical biomaterial foreign body reaction

which includes fibrous encapsulation, slowed or completely inhibited scaffold degradation and chronic inflammation. Stated differently, although the ECM has been viewed favorably as a scaffold for tissue repair, its method of preparation can dramatically alter the host remodeling response [35].

2.2.1. Use of ECM scaffolds in vascular tissue engineering

ECM harvested from the porcine small intestinal submucosa (SIS) has been studied extensively for its utility as a vascular graft. SIS has been used as a large diameter vascular graft in dogs with results that showed excellent long-term patency and extensive remodeling of the blood vessel wall structures [36]. The remodeled scaffolds showed complete degradation and resorption of the originally implanted ECM material and replacement by host tissues that form an intimal structure, partially organized bundles of smooth muscle in the vascular wall, and a complete endothelial lining. In another study related to vascular tissue engineering, utilized a non-vascular extracellular material obtained by decellularization of porcine urinary bladder and examined for its potential as scaffold for vascular tissue engineering [37]. The study proved that vascular cell seeded-urinary bladder matrix constructs provide a suitable and affordable *in vitro* model for cell-physiology and drug development studies, which might elucidate to the mechanisms of vascular disease formation, prevention and its treatment.

2.2.2. ECM scaffolds in dental pulp tissue engineering

The current treatment for necrotic dental pulp tissue arising from dental caries is root canal therapy. This treatment results in loss of tooth sensitivity and vitality making it prone for secondary infections. Over the past decade, several tissue-engineering approaches have attempted regeneration of the dental pulp tissue. Although several studies have highlighted the potential of dental stem cells, none have transitioned into a clinical setting owing to limited

availability of dental stem cells and the need for growth factor delivery systems. Recent researches in dental pulp tissue engineering have demonstrated that it is possible to overcome these hurdles by using biomimetically engineered dental pulp ECM incorporated scaffolds[38]. These scaffolds possess the ability to trigger lineage specific differentiation of several mesenchymal stem cell types *in vitro* and *in vivo*. Therefore, dental stem cells can be avoided if appropriate sources are not available. On the other hand experiments also showed that these scaffolds contain an array of lineage specific proteins that includes both structural and functional proteins in physiologically relevant quantities. Therefore, the use of these scaffolds will overcome the need for using complex growth factor delivery systems [38] [39]. Although the results were promising, several factors need to be standardized before this technology can be ready for clinical translation. To achieve mass-production and minimal batch variability, it was a necessity to produce ECM incorporated scaffolds using dental stem cell lines. Standardized tests need to be developed to assess the quality of the scaffolds and to quantitatively evaluate the levels of different growth factors and ECM proteins within the scaffolds. These tests will ensure product reliability and minimum performance thresholds [38].

2.2.3. ECM scaffolds in skin tissue engineering

The use of decellularized human dermis (Alloderm®) and porcine SIS (OaSIS™) has been shown to positively affect the healing of chronic ulcers and traumatic skin wounds. These acellular materials serve not only to provide bulk as a dermal substitute but also to promote wound coverage by serving as an excellent substrate for growth of autologous keratinocytes [40] [41]. Depending upon the form of ECM used, there is typically a vascular ingrowth from the wound bed (angiogenesis), dense cellular infiltrate from surrounding dermis and subcutaneous tissue and rapid migration of keratinocytes from the wound edges. Contraction of the wounds

can be improved (i.e. minimized) with the use of ECM scaffolds but reconstitution of adnexal structures such as hair follicles and sweat glands has not been shown. Tissue engineered skin substitutes such as Dermagraft™ and ApliGraf® consists of composite grafts including neonatal fibroblasts and secreted ECM, added growth factors and keratinocytes. These composite grafts represent excellent scaffolds for wound repair but the specific advantages offered by the cell component vs. ECM alone are unclear. Without compelling evidence that these composites provide significantly superior results compared to the use of ECM scaffolds alone, the practical limitations such as shelf life, cell sourcing and regulatory approval limit the utility of these devices in human patients [35].

2.2.4. ECM scaffolds in cardiac tissue engineering

There have been numerous studies investigating the use of ECM scaffolds as a myocardial patch and heart valve substitute [42]. Another recent study investigated the *in vitro* properties and *in vivo* potential of an injectable myocardial matrix designed to mimic the natural myocardial extracellular environment. Porcine myocardial tissue was decellularized and processed to form a myocardial matrix with the ability to gel *in vitro* at 37˚C and *in vivo* upon injection into rat myocardium. The resulting myocardial matrix maintained a complex composition, including glycosaminoglycan content, and was able to self-assemble to form a nanofibrous structure. Endothelial cells and smooth muscle cells were shown to migrate towards the myocardial matrix both *in vitro* and *in vivo*, with a significant increase in arteriole formation at 11 days post-injection. The matrix was also successfully pushed through a clinically used catheter, demonstrating its potential for minimally invasive therapy. Thus the study demonstrated the initial feasibility and potential of a naturally derived myocardial matrix as an injectable scaffold for cardiac tissue engineering [43]. Recently, an injectable hydrogel derived from

ventricular ECM was shown promising for treating myocardial infarction. This was considered as the first *in situ* gelling material to be delivered via transendocardial injection in a large animal model, a critical step towards the translation of injectable materials for treating myocardial infarction in humans [44].

2.3. Effect of processing upon structure and function of ECM based scaffold materials

The processing steps involved in isolation of the matrix will affect the biomaterial grade properties of the scaffold. The preparation of an ECM scaffold material from intact mammalian tissue requires several processing steps that can markedly affect both the structure and the type of host response that these materials elicit when utilized as a template for tissue reconstruction. The native tissue from which an ECM scaffold is prepared must be mechanically or physically separated from unwanted tissue structures, decellularized, often lyophilized and terminally sterilized. Each of these processing steps can alter the integrity and architecture of the matrix as and, in turn, influences the mechanical and material properties of the ECM.

2.3.1. Decellularization

Chemical decellularization is regarded as an important step in isolation of ECM. It is the effective removal of antigenic epitopes associated with cell membranes and intracellular components of tissues and organs, necessary to minimize or avoid adverse immune response by allogeneic and xenogeneic recipients of ECM scaffold material. Chemical decellularization is usually carried out with enzymes and detergents and the procedure may have deleterious effects on the biomaterial quality of the scaffold [45] [20]. The tissues from which the ECM is harvested, the species of origin, the decellularization method and the methods by which the material is sterilized can vary widely. Xenogeneic and allogeneic cellular antigens are

recognized as foreign by the host and result in an adverse inflammatory response [45]. Commonly used methods of decellularization include a combination of physical and chemical treatments. Sonication, agitation, freezing and thawing are commonly used methods to disrupt cell membranes, release cell contents and facilitate the subsequent rinsing and removal of cell remnants from the ECM. The commonly used decellularization methods appear to be insufficient to achieve complete decellularization, as most, ECM scaffold materials retain some DNA [46]. Although it seems logical that the decellularization process will by definition affect the structure and composition of the ECM, the intent of the process is the preservation of as much of the native mechanical properties and biological properties of the original ECM as possible. Some detergents used to facilitate decellularization have been shown to disrupt collagen of certain tissues, thereby decreasing the mechanical strength of the tissue, while the same detergent may have no effect on the collagen in another tissue [47] [48]. Thus the decellularization method requires optimization for each tissue to remove cellular material without compromising the mechanical properties of the tissue.

2.3.2. ECM recovery without the use of chemical decellularization

Preparation of an optimal biomaterial grade scaffold is a multistage process. Traditional procedures involve: collection of organ or tissue of choice from slaughter houses and transfer to laboratory with minimal microbial contamination and maximum cell viability. Decellularization was then achieved by a combination of physical methods (e.g., freezing, direct pressure, sonication, and agitation) and chemical treatment. Incubation with enzymes like nucleases is done for promoting degradation of nucleic acid. Decellularization process aims at removing cells and debris while preserving the three dimensional organization and ultra structure of ECM [49].

Subsequent stabilization of biomolecules in the decellularized scaffold is an essential step in most scaffold isolation procedures. This is achieved in several ways. The use of protease inhibitors or treatment with cross linking agents like glutaraldehyde was widely preferred. Sterilization of the scaffold is also necessary for its use in clinics and is achieved by ethylene oxide treatment or radiation. Thus there is a wide range application of chemicals and reagents for extraction of ECM. These chemicals used for the processing of scaffold can alter the biophysical quality of the resulting scaffold and also these chemical residues have the potential to mount an adverse reaction when it comes to clinical applications [50] [51]. In 2013 Anilkumar, *et al* patented a new method for isolation of cholecystic ECM via a non enzymatic and non detergent method which improved the biomaterial grade properties of the existing scaffold material [13]. In contrast to the conventional scheme of activities they used a reverse procedure for isolation of scaffolds from cholecyst. The procedure started with collection of the organ or tissue in a stabilizing agent like formaldehyde that caused pre isolation ex situ stabilization of the scaffold molecules and this protocol proceeds by truncating the traditional steps drastically. The entire procedure omitted the use of chemicals conventionally used for preservation, transportation, and decellularization. Formaldehyde, well known for facilitating cross linking of protein molecules, early cross linking of biomolecules with formaldehyde caused quick preservation of biomolecules and from subsequent autolysis of the tissue components. This procedure was suitable for isolating scaffold from other hollow organs like urinary bladder and jejunum [13].

2.4. Clinical applications of particulate ECM

Lyophilized sheets of ECM can be comminuted into particulate form of ECM. A particulate form allows for the delivery of the ECM as a suspension via minimally invasive techniques to the site of interest or for the manufacture of three dimensional scaffolds by

compaction methods. The particles present in particulate ECM retain the ultrastructural and three dimensional surface characteristics of the parent ECM sheet [25]. Particle sizes ranging from 50 to 200 μm in diameter can be reproducibly manufactured. Suspensions made from a particulate form of lyophilized urinary bladder matrix have been successfully used as an injectable scaffold for the treatment of urinary incontinence in preclinical studies [52], and the results suggested that endoscopically guided injection of particulate ECM into the internal urethral sphincter may be useful for the treatment of acquired urinary incontinence in female dogs. Carriers such as glycerin are typically required to increase the viscosity of a suspension of particles intended for clinical use [5]. Acellular human dermal matrix has been investigated as a micronized form for injection into laryngeal tissue, but tissue augmentation was not possible due to its rapid *in vivo* degradation [53]. Choi, *et al* 2009 investigated the use of extracellular matrix powders derived from human adipose tissue as injectable cell delivery carriers for adipose tissue engineering [24]. The study proved that, the human ECM powders were highly effective for promotion of cell attachment and proliferation for three-dimensional cell culture. Moreover, their findings demonstrated that human ECM powders could act as efficient injectable cell delivery carriers and that long-term stable adipose tissue can be engineered *in vivo* by simple injection with cell-seeded human ECM powders, rather than surgical operation. Powdered forms of ECM scaffolds may also be used for topical delivery or may be combined with synthetic polymers to create hybrid scaffolds. Moreover a particulate form of ECM is a necessary step prior to ECM based hydrogel preparations. Since a particulate form of scaffold materials are not expected to serve load-bearing functions, the physical properties of the ECM, such as particle size, surface area and type of liquid carrier, are the relevant variables that affect the ease and convenience with which the material can be delivered to the intended sites.

2.5. Injectable ECM hydrogels

Hydrogels are water-swollen polymeric networks, usually consisting of crosslinked hydrophilic polymers that can swell but do not dissolve in water. This ability to swell under biological conditions makes them an ideal class of materials for biomedical applications, such as drug delivery and tissue engineering [54] [55]. Hydrogels possess a three dimensional network structure, crosslinked together either physically or chemically. This insoluble cross-linked structure allows effective immobilization and release of active agents and biomolecules. Owing to their high water content, hydrogels resemble natural soft tissue more than any other type of polymeric biomaterials. Hydrogel materials generally exhibit good biocompatibility and high permeability for oxygen, nutrients and other water-soluble metabolites, making them attractive scaffolds for use in cell encapsulation [56].

ECM based hydrogels are promising scaffolds for tissue engineering applications owing to their biocompatibility, inherent biodegradability and critical biological functions. ECM hydrogels proved its usefulness as a potential biomaterial through variable applications. ECM derived from mammalian tissues has been utilized to repair damaged or missing tissue and improve healing outcomes. More recently, processing of ECM into hydrogels has expanded the use of these materials to include platforms for three dimensional cell culture as well as injectable therapeutics that can be delivered by minimally invasive techniques and fill irregularly shaped cavities. At the cellular level, ECM hydrogels initiated a multifaceted host response that includes recruitment of endogenous stem/progenitor cells, regional angiogenesis, and modulation of the innate immune response [27]. A gel form can also serve as a cell delivery vehicle when appropriate. The rheological properties of the gel can be designed to be similar to those of the tissue that is being repaired. Ideally, the gel processing methods would minimize or avoid

purification steps that could remove or destroy the active growth factors and low molecular weight peptides present in the native ECM, and the gel form would retain the native bioactivity of the parent ECM scaffold.

Recent findings concluded that both soluble and structural components of ECM hydrogel contributes to the host response via different mechanisms [57]. It had been demonstrated that decellularized porcine corneal ECM hydrogels can be used for engineering corneal tissue [58]. The porcine corneal ECM hydrogels were highly transparent and able to maintain stromal cell viability. When compared to standard collagen hydrogels, the ECM hydrogels retained corneal biomolecules and appeared to enhance the native keratocyte phenotype. ECM composites for three dimensional culture of multipotent and pluripotent stem cells were developed recently by entrapping whole molecule ECM proteins in polyethlene glycol hydrogels using the chemistry of native chemical ligation [59]. Another study showed that urinary bladder matrix can be successfully solubilized using pepsin and can be formed into a gel *in vitro* that can be used as an injectable scaffold for clinical applications [27]. The above study also showed that matrix gels support the adherence and growth of rat aortic smooth muscle cells [27]. Porcine myocardial tissue was decellularized and processed to form a myocardial matrix with the ability to gel *in vitro* at 37˚ C and *in vivo* upon injection into rat myocardium. The resulting myocardial matrix maintained a complex composition, including glycosaminoglycan content, and was able to self-assemble to form a nanofibrous structure. Endothelial cells and smooth muscle cells were shown to migrate towards the myocardial matrix both *in vitro* and *in vivo*, with a significant increase in arteriole formation at 11 days post-injection. The matrix was also successfully pushed through a clinically used catheter, demonstrating its potential for minimally invasive therapy. Hence demonstrated the initial feasibility and potential of a naturally derived myocardial matrix as an

injectable scaffold for cardiac tissue engineering [43]. Later the same group investigated the safety and efficacy of the above mentioned injectable hydrogel derived from porcine myocardial ECM as a scaffold for cardiac repair through a pre clinical approach [60].

2.6. Cholecystic extracellular matrix (C-ECM)

Due to tremendous research advancements in ECM based scaffolds, porcine derived ECM isolated from sub mucosa of hollow organs like urinary bladder and small intestine has found extensive clinical applications, mainly in regenerative medicine and tissue engineering. Several clinical products made of biomaterial grade ECM scaffold are now available in the market[5]. Apart from small intestine and urinary bladder, cholecyst (gall bladder) is also a potential source of ECM scaffold, but its importance as a source of biomaterial grade ECM was recently explored. Herein, the diverse and versatile nature and properties of cholecystic ECM as a potential biomaterial grade scaffold has been reviewed to provide a rationale for the importance of C- ECM in tissue engineering.

ECM scaffolds contains structural and functional molecules secreted by the resident cells of each tissue and organ from which they are prepared, which means that the specific composition and distribution of the ECM constituents will vary depending upon the tissue source [5]. The complexity of native ECM structure, particularly the biological architecture of each tissue derived ECM determines whether it should be conducive for cell infiltration or not in tissue engineering applications. The conduciveness of natural ECM based scaffolds must be appreciated because it is a necessary factor in some cases of tissue engineering, especially in applications like grafts for skin, blood vessels, heart vessels, dura mater, peripheral nerves, heart valve and body wall. The more complex and compact nature of ECM makes them more

impermeable to cellular infiltration and upon implantation into the body, their resorption occurs through surface erosion. That means scaffolds are not conducive to host tissue infiltration, the implants are effectively contained by the surrounding host fibrous tissue and even a simple shear stress can disturb the implant tissue interface ultimately leading to implant failure. Because of this and many other defects there is a widespread need for tissue engineering scaffolds that are conducive for cellular infiltration. These scaffolds which are conducive to cellular infiltration would allow host tissue deposition, and the deposited ECM would interpenetrate with the implants bulk, promoting true biointegration of the scaffold with the host tissue. The C-ECM, had been initially identified from the perimuscular sub serosal connective tissue of porcine cholecyst which is a well known source for intact ECM [4]. The serosal layer of cholecyst has got a fibroporous structure which makes the C-ECM conducive for cellular infiltration thus promoting biointegration [23] [4].

2.6.1. Biomaterial grade applications of cholecystic ECM

Krishna Burugapally and his co workers in 2007 first reported the evidence of an intact ECM with a mesh like architecture, derived from sub serosal connective tissue of cholecyst [4]. This particular work laid the foundation stone for the current development and ongoing researches in C-ECM. The subserosal layer was isolated by mechanical delamination and followed by decellularization with a treatment with peracetic acid and ethanol solution in water to obtain the final matrix. This study assessed the qualitative and quantitative macromolecular compositions of the matrix and revealed that collagen is the primary structural component of the matrix. It is this collagen fiber architecture which plays a critical role in determining biomechanical behavior of the matrix scaffold. In addition to collagen, elastin components are also present. The studies proved that C- ECM is an ideal supporting structure for the attachment

and proliferation of murine fibroblasts. The important determinants for the quality of a biomedical grade biomaterial are its chemical composition and biomolecular content. Various biomedical applications of C-ECM scaffold had already revealed by the scientific community. It can be considered as a reliable natural collagen scaffold for bladder augmentation [17] and also it was shown to act as reinforcing buttressing staples for gastrointestinal resection [18] and anastomotic procedures. It possess a micro-architecture similar to heart valves, supports proliferation of valvular endothelial and interstitial cells [19] and also it is proved as a potential skin graft for treating full thickness skin wounds [16]. It also elicits a very less immunogenic potential when compared to other commercially available xenogenic scaffolds [15]. Certain studies shown that C-ECM possess the ability to withstand large strain suggesting its importance in load bearing applications [21]. No serious adverse inflammatory response has not been reported following deep tissue grafting studies in animal models [20]. Research activities were also carried out to improve the biomaterial properties of C-ECM scaffold via surface modification and functionalization with cross linkers which prolonged the rate of *in vivo* degradation and removal of C- ECM [61] [22].

Cholecystic ECM is a relatively novel scaffold material whose properties are in par with the existing scaffolds from other organs like urinary bladder and jejunum. Recent studies confirm the potential of C- ECM scaffold as satisfactorily good skin graft for wound healing applications. The scaffold is abundant in type I collagen [13]. The isolation procedure for this scaffold do not appreciate the traditional chemical decellularization methods much, the new procedure involved mechanical recovery of ECM scaffold after *ex situ* incubation of the organ with a stabilizing agent [13]. The successful utilization of C-ECM as a therapeutic device will depend the ability to further understand the native structure/ function relationship of the biological scaffold material.

A better understanding of the chemical composition and biomolecular content is required to improve the clinical outcome associated with the use of C- ECM based scaffold material.

2.7. Sodium chloride as a porogen

Tissue engineering aims to create functional tissue using cells seeded onto three dimensional (3-D) scaffolds, providing an alternative to traditional transplants. Tissue engineering techniques in general require the use of porous scaffolds, which serve as a 3-D template for initial cell attachment and growth leading to tissue formation. The architecture of the scaffold used defines the ultimate shape of the newly grown tissue. An ideal tissue scaffold must meet several requirements: the scaffold must comprise an interconnected, open pore network allowing nutrients and cells to pass into the scaffold while ensuring waste products can get out. Also the scaffold must have mechanical properties closely matching those of the tissues at the site of implantation. For example, the human heart is always beating, the muscle of the heart is constantly stretching and contracting so any tissue scaffold used in the heart must be able to withstand this constant motion (a brittle material would crack while a soft material might deform). In addition to structure and mechanics, the scaffold chemistry will be very important. The scaffold must have a surface chemistry suitable for cell attachment and growth. To be successful, a scaffold material must be easily processed into a variety of shapes and sizes, and its production must be inexpensive and reproducible on a large scale. A wide variety of techniques can be used to design and fabricate porous tissue scaffolds. One such technique is called salt leaching where salt crystals such as NaCl are put into a mold and polymer is poured over the salt, penetrating into all the small spaces left between the salt crystals. The polymer is hardened and then the salt is removed by dissolving it in a solvent (such as water or alcohol) which washes/leaches the salt out. Upon removal of the salt crystals all that remains is the hardened

polymer with open holes/pores where the salt once was. salt leaching is a widely used facile, convenient, cost-effective and simple technique for the creation of porous scaffolds in tissue engineering [62] [63]. Limitations include wide variations of pore sizes, lack of interconnectivity, irregular pore geometry, and the inability to produce submicron and nanoscale pore sizes. In most studies, the technique of salt leaching was used for synthetic polymers. A variant of salt leaching method was used to create uniform and porous ECM in a recent study, called salt precipitation [25]. The study confirmed that the production of a comminuted form of ECM is possible through the technique of salt precipitation and that the uniformity of particle size and shape are dependent upon the manufacturing methodology.

2.8. Gaps identified

The C-ECM is now well known for its ideal biomaterial properties and applications in tissue reconstruction. Future studies on C-ECM may include scaffold aided drug delivery, incorporation of other clinically relevant biomolecules within the scaffold, conditioning the scaffold within a bioreactor, followed by *in vivo* trials. The seeding of stem cells on the scaffold may help to regenerate the entire tissue. There are also possibilities for the development of hybrid scaffolds by combining a biodegradable polymer and C-ECM to meet complex tissue engineering needs. This combination will allow the generation of a more biocompatible scaffolds with advanced mechanical properties, flexural rigidity, suture retention strength and also prolonged *in vivo* biodegradation rate. C-ECM may be suitable for other graft applications also like vascular graft, hernia repair, dural repair etc. The C-ECM scaffold can be processed to various forms for different clinical applications. Each form will have relevant properties based on a suitable application. A hydrated sheet like form can be used as a scaffold material directly; it can be processed to lyophilized sheets. The lyophilized sheets can be comminuted to make a

particulate form of the material. The comminuted material can be enzymatically digested to make a liquid form of the material, which can be repolymerized into a gel or mixed with a synthetic polymer to make a hybrid scaffold [5]. Commercialization of the scaffold for biomaterial grade clinical applications is also an upcoming option.

3. Hypothesis and objectives

3.1. Hypothesis

A particulate form of C-ECM with uniform size distribution and morphology can be generated by salt precipitation for fabrication of injectable ECM based materials.

3.2. Objectives

- To prepare powder formulations of porcine cholecystic extracellular matrix by employing two methods: grinding and salt precipitation.

- To compare physicochemical and biochemical properties of the above two particulate forms for their biomaterial properties.

- To compare the cytocombatibility of the above two particulate forms using cell viability assay.

4. Materials and methods

4.1. Materials

Disodium hydrogen phosphate (Na_2HPO_4), Dimethyl sulfoxide (DMSO), Formalin, Glacial acetic acid, Hydrogen chloride (HCl), Methanol, Potassium chloride (KCl), Pottasium dihydrogen phosphate (KH_2PO_4), Sodium chloride (NaCl), Sodium dihydrogen phosphate (NaH_2PO_4.7H2O), and Sodium hydroxide (NaOH) were purchased from Merck chemicals (India). Acrylamide, Ammonium per sulphate (APS), Bromophenol blue, Beta mercaptoethanol, Coomassie brilliant blue-R250, Glycerol, Glycine, *N*, *N*, *N′*, *N′*-Tetramethylethane-1, 2-diamine (TEMED) N, N-methylene bis-acrylamide, Sodium dodecyl sulphate (SDS), and Tris were purchased from GE Healthcare Life Sciences (Sweden). Fetal bovine serum (FBS) and Pepsin were purchased from Himedia Pvt. Ltd India. Sircol collagen assay kit and BlyScan sulphated GAG assay kit were purchased from Biocolor Life science assays (UK). Rat tail collagen type I (Enzo Life Sciences), 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) (Invitrogen, US), TrypLE (Gibco life technologies, India), Dulbecco's modified Eagle's medium (DMEM) (Sigma Aldrich, US), Protein marker (Amersham Biosciences, US). All other chemicals and solvents used were of analytical grade unless or otherwise specified.

4.2. Preparation of porcine C-ECM powders

ECM for powder preparation was isolated from porcine gall bladder by a previously reported procedure, [13] that involved mechanical recovery of lamina propria after ex situ treatment with a stabilizing agent which resulted in controlled pre isolation *in situ* cross linking of biomolecules in the ECM scaffold [16].The isolated sheets of cholecystic ECM was washed thoroughly with running water for at least 4 hours and the samples were freezed by storing at

-80˚C. It was later lyophilized and sterilized by ethylene oxide (EtO) treatment. The sterilized porcine cholecyst ECM sheets were used for particulate ECM generation.

Two particulate forms of C-ECM were prepared. The first method utilized the conventional grinding technique, which involved lyophilizing the EtO sterilized ECM sheet followed by comminution and snap freezing, so that the particles were small enough to be placed in a freezer mill (Spex SamplePrep, 6770, USA). The second method was based on NaCl precipitation technique [25], as modified from a previously published protocol for bladder matrix. The method involved an initial soaking of the sterilized ECM sheets in a 30% (w/v) NaCl solution for 5 min. The material was then snap freezed to precipitate salt crystals, and lyophilized to remove residual water. This material was then milled as described in the first method. Finally, the particles were suspended in deionized water and centrifuged for 5min at 1000 rpm three times to remove the excess NaCl. The suspension was snap freezed and lyophilized again, followed by milling to disaggregate the individual particles. In both cases, six sets of milling cycles were conducted and generated fine powders, which is desirable for the purpose of the study. Finally, the obtained NaCl treated and non NaCl treated C-ECM powders were sieved through a strainer of 100µm and separated the particles based on their size. The particles of size below 100 µm were used for all the studies.

4.3. Physicochemical characterization of ECM powder

4.3.1. Moisture content analysis

The initial dry weight of NaCl treated ECM powder (NC-ECM) and C-ECM powder were noted. The samples (n = 4) were taken out, dried in an oven at 40°C for 24 h and the dried

weight was noted down. Percentage of moisture content was calculated as [(initial weight − dry weight) × 100]/initial weight.

4.3.2. Environmental scanning electron microscopy (ESEM)

The surface morphology of C-ECM and NC-ECM powders were observed using environmental scanning electron microscopy (FEI, Quanta 200, USA). Analysis was performed on gold coated lyophilized samples and visualized under low vacuum conditions. The ECM ultrastructure was observed at an accelerating voltage of 10 kV.

4.3.3. Energy-dispersive spectroscopy (EDS)

Energy dispersive X-ray spectroscopy (EDS) was performed with oxford detector of the above ESEM for elemental composition analysis.

4.3.4. Particle size analysis

The average particle size (hydrodynamic diameter, Z average) and polydispersity index (PDI) analyses of the prepared C-ECM and NC-ECM powders were determined by using Zetasizer NanoZS (Malvern, UK) by the principle of dynamic light scattering (DLS). The particle size of ECM particles in cell culture media was also analyzed.

4.3.5. Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR-spectra of lyophilized ECMs (C-ECM sheet, NC-ECM, C-ECM powders) were recorded in the infra-red range of 4000–400 cm^{-1} using Fourier transform Infrared spectrometer (JASCO, 4200 FTIR Spectrophotometer, Easton, USA) with attenuated total transmittance mode. Lyophilized samples were sandwiched between KBr plates and IR spectrum

was recorded at room temperature. Typically, 32 scans were signal-averaged for a single spectrum at a resolution of 64cm^{-1} using a KBr crystal at an incident angle of 45˚.

4.3.6. X ray diffraction (XRD)

X ray diffraction pattern of NC-ECM and C-ECM powders were measured using X-ray diffractometer (XPERT, Philips, Eindhoven, Netherlands) with nickel-filtered Cu-Kα radiation (= 0.154 nm) at a voltage of 40 kV and a current of 30 mA.

4.3.7. Determination of viscosity

Viscosity of solubilized NC-ECM and C-ECM suspensions at 37˚C was measured using a rotational viscometer (RVA-StarchMaster 2, Newport Scientific) at 200 rpm as per manufacturer's instructions. The solubilized ECM formulations were evaluated for injectability using viscosity measurements [64]. 25 ml of solubilized NC-ECM and C-ECM suspensions were directly subjected to constant stirring for 20 min using a rotational viscometer.

4.4. Biochemical characterization of ECM powders

4.4.1. ECM digestion and solubilization

NC-ECM and C-ECM powders were solubilized, as modified from a previously published protocol for bladder matrix [27]. Pepsin was used to solubilize the particulate ECM. Briefly, pepsin was dissolved in 0.1M HCl such that pepsin is at a concentration of 1mg/ml. When pepsin was fully dissolved, the solution was added to the scintillation vials containing NC-ECM and C-ECM powders and so that pepsin: matrix ratio was 1:5. Both the particulate ECMs were allowed to digest for 48h under constant stirring.

4.4.2. pH adjustment of the solubilized matrix

When completely solubilized, as indicated by the lack of particles in solution, both NC-ECM and C-ECM solubilized suspensions were brought to physiological pH 7.4 through the addition of sodium hydroxide (NaOH) and 10 X PBS , and diluted to 1 mg/mL with 1X PBS. Final solubilized matrix was kept in 4˚C until it was used for further studies.

4.4.3. Total protein estimation

Total protein concentration of C-ECM and NC-ECM powder was determined by the Bradford method [65] and the absorbance was read using microplate reader (Biotek Synergy 4, US) at 595 nm.

4.4.4. Content of selected biomolecules

Solubilized forms of NC-ECM and C-ECM powders were analyzed by SDS-PAGE and compared with Rat tail collagen type I. Solutions were run on a Tris–HCl, 8% polyacrylamide gel in Tris/Glycine/SDS buffer, with 100 mM reducing agent beta-mercaptoethanol. Gel electrophoresis was performed in a mini VE Vertical Electrophoresis System (GE Healthcare Life Sciences), compared to a broad range standard, and stained with Coomassie brilliant blue.

The chemical makeup of the particulate scaffolds were further characterized for collagen, and sulphated GAGs using Sircol collagen assay kit, and Blyscan sulphated-GAGs assay kit (Bicolor Life Science Assays, UK), respectively, in both solubilized NC-ECM and C-ECM powders using manufacturer's protocol.

4.5. Assesment of Cytotoxicity

4.5.1. Standardization of cell culture and maintenance of L-929 cell line

L-929 cell lines (adherent fibroblast cells from adipose tissue of mouse) were obtained from Tissue Culture Laboratory which was originally purchased from NCCS, Pune, India. The confluent cells in cell culture flask ($25cm^2$) were treated with 1X TrypLE solution in Phosphate buffered saline (PBS) and placed at 37˚C in CO_2 incubator for 1-2 minutes allowing the cells to detach from the surface. Once the cells were detached, the TrypLE present in the flask was inactivated using DMEM containing 10% FBS in the presence of antibiotics (10,000U Penicillin, 10mg Streptomycin and 25µg Amphotericin per 0.9 ml normal saline). The cells were then centrifuged at 2000 rpm for 2 min and the pellet formed was resuspended in 10% FBS supplemented medium and plated at a concentration of $1x10^5$ cells in culture flasks (for the purpose of maintenance) and seeded at a concentration of $1x10^4$ cells in 96-well plates for the experiments to be carried out. The cells were incubated at 37°C with 5% CO_2 in air and 99% humidity. Media change in cell culture flasks were carried out every 2-3days. Cryopreservation was carried out using 10% DMSO in whole serum.

4.5.2. Morphological Analysis

Cells were passaged and subcultured to 80% confluence before the experiments. Fresh suspensions of NC-ECM and C-ECM powders were prepared and checked to ensure consistency of physical and chemical properties of ECM. Experimental group consist of (1) control cells (2) cells treated with solubilized C-ECM powder (2 mg/ml) and (3) cells treated with solubilized NC-ECM powder (2 mg/ml) unless or otherwise stated. Observations were made after 24 h of incubation with test materials in all groups.

4.5.3. MTT assay

Procedure

Cells in exponential growth phase were plated at 1×10^4 cells per well in 96-well plate. After, cells were exposed to various concentrations (12.5µg, 25 µg, 50 µg, 100 µg, and 200 µg) of solubilized NC-ECM and C-ECM powders for 24 hr, they were subjected to MTT analysis. For this 10µL of MTT solution (5 mg/mL) was added to each well and incubated for 3 h at 37°C. The formazan crystals thus formed were dissolved in DMSO. Then the plates were read after 45 min in a microplate reader (Biotek Synergy 4, USA) at 570 nm. The percentage viability was calculated using the formula,

$$\text{Viability in \%} = (\text{OD of test}/\ \text{OD of control}) \times 100$$

80% or above was considered as viable.

4.6. Statistical analysis

Each experiment was conducted in triplicate. Results were expressed as mean and standard deviation. Data were subjected to paired Student's t test for calculation of the statistically significant difference among different groups and significance was accepted at $p \leq 0.05$.

5. Results and discussion

5.1. Preparation of C-ECM and NC-ECM powders

Non-NaCl treated and NaCl treated C-ECM powder (Fig.1 B-C) was prepared from cholecystic ECM sheet (Fig. 2 A) using simple physical treatments without the addition of potent chemicals or enzymatic factors (Fig. 2 C-D). There were no significant differences in the gross appearance of C-ECM and NC-ECM powder.

Fig. 2 A: C-ECM sheet, B: C-ECM powder, C: NC-ECM powder

5.2. Physicochemical characterization of ECM powders

5.2.1. Moisture content

Moisture contents of NC-ECM and C-ECM powder were similar (Fig. 3). Adequate moisture is required for satisfactory biological activity of growth factors and proteolytic enzymes. In addition, moisture is known to enhance fibroblast/endothelial cell proliferation immune function and wound healing [66]. Epithelial cells need a moist environment for migration to make the re-epithelization process faster [67]. Since both the ECM materials were showing similar percentage moisture content rates, it was inferred that the salt precipitation technique was not affecting the physical hydration parameters of the ECM.

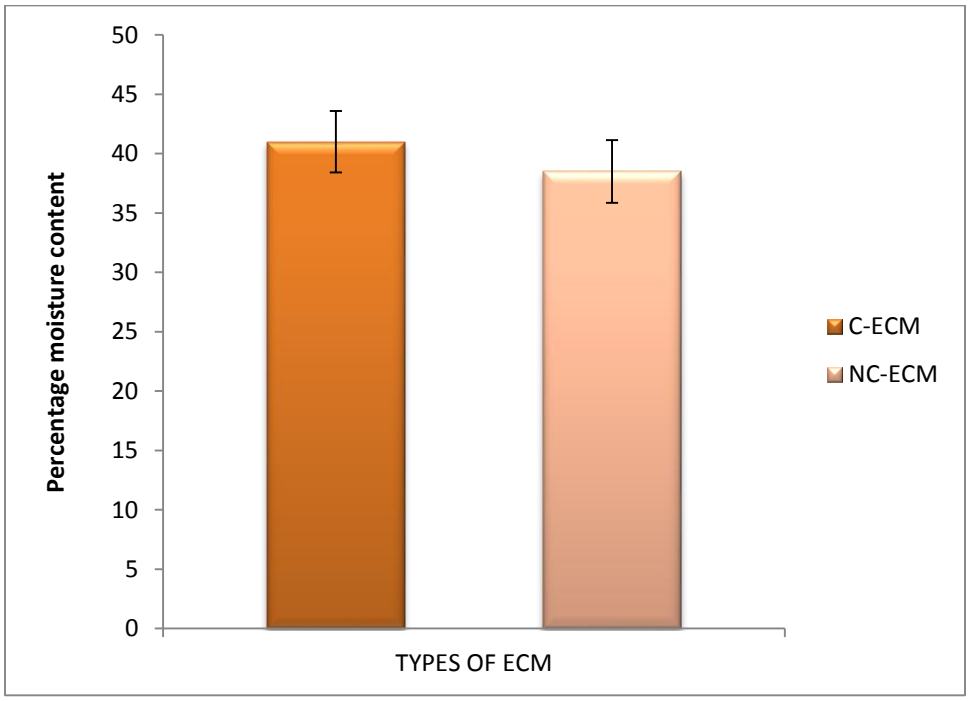

Fig. 3: Moisture content analysis of C-ECM powder and NC-ECM powder (Values represent mean ± SD, n=4)

5.2.2. Environmental scanning electron microscopy (ESEM)

The particles formed after NaCl precipitation had a different morphology than those formed by the grinding method as shown with ESEM (Fig. 6). The NC-ECM population was characterized by both larger and smaller particles which are rather porous in appearance, tends to agglomerate with each other and themselves. On the other hand, the electron microscopy image of C-ECM powder was indicated by irregular shape of the particles (Fig. 5). The irregular shape and size of the particles was likely due to the fibrous nature of this largely collagenous material [25]. The grinding process might have sheared the material between adjacent collagen fibers than to cut a fiber along its length. The ESEM image of cholecystic ECM sheet is shown in Fig. 4. The ECM sheet showed relatively gel like architecture characteristic of cholecystic ECM [13].

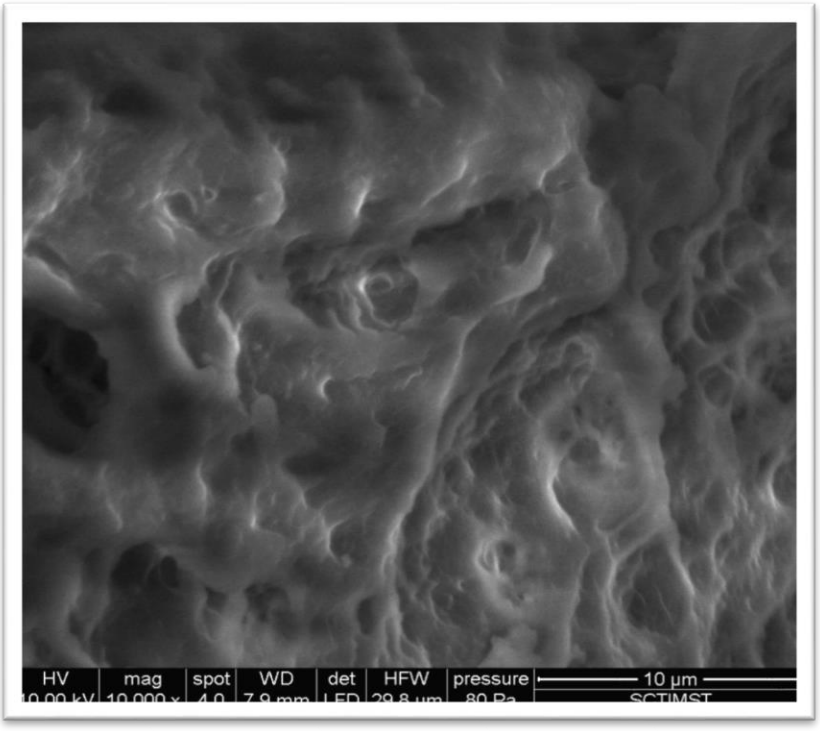

Fig. 4: ESEM image of C-ECM sheet

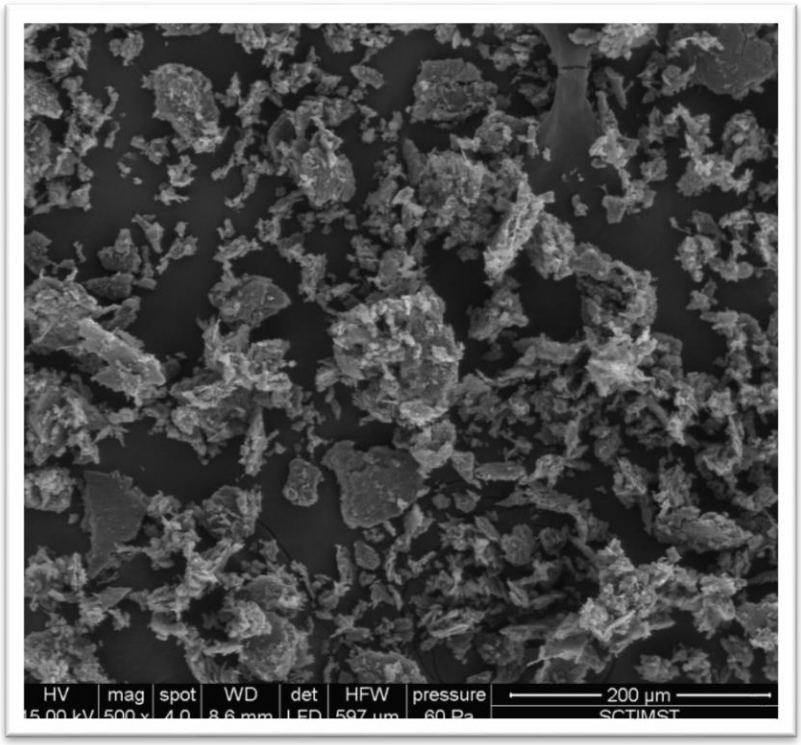

Fig. 5: ESEM image of C-ECM powder

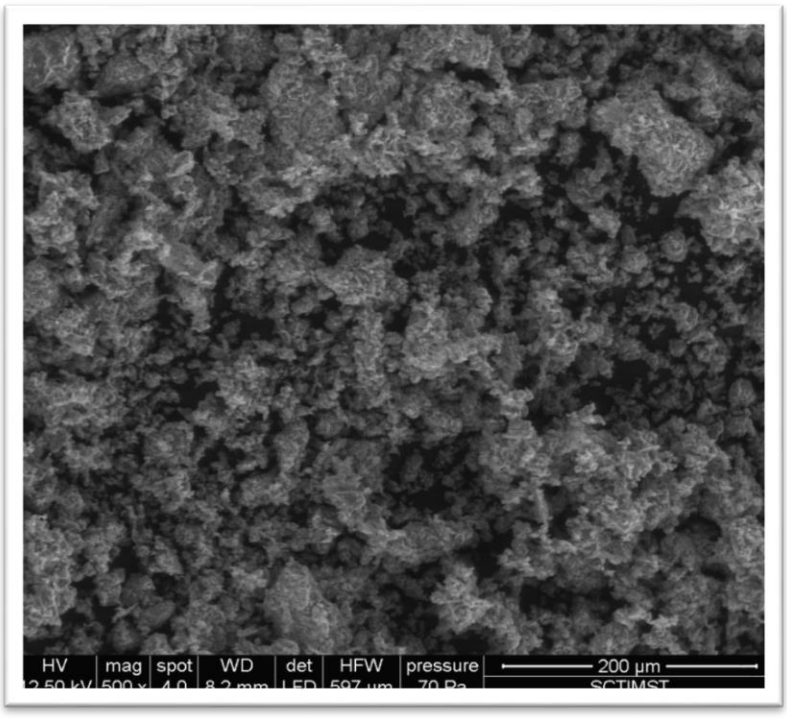

Fig. 6: ESEM image of NC-ECM powder

5.2.3. Energy-dispersive X ray spectroscopy (EDS)

Energy-dispersive X ray spectroscopy provided elemental analysis on areas as small as nanometers in diameter. In EDS, all the ECM materials showed common peaks of protein-rich organic elements (Fig. 7, 8 & 9).The EDS data of NC-ECM powder showed peaks of sodium and chlorine, indicating that traces of NaCl were retained within the ECM after the salt precipitation (Fig. 8). However, the NC-ECM powder retained the original biological composition of the parent ECM as confirmed by the presence of carbon, nitrogen and oxygen. Apart from the presence of sodium and chlorine in NC-ECM powder, the elemental composition between the three ECM materials was preserved.

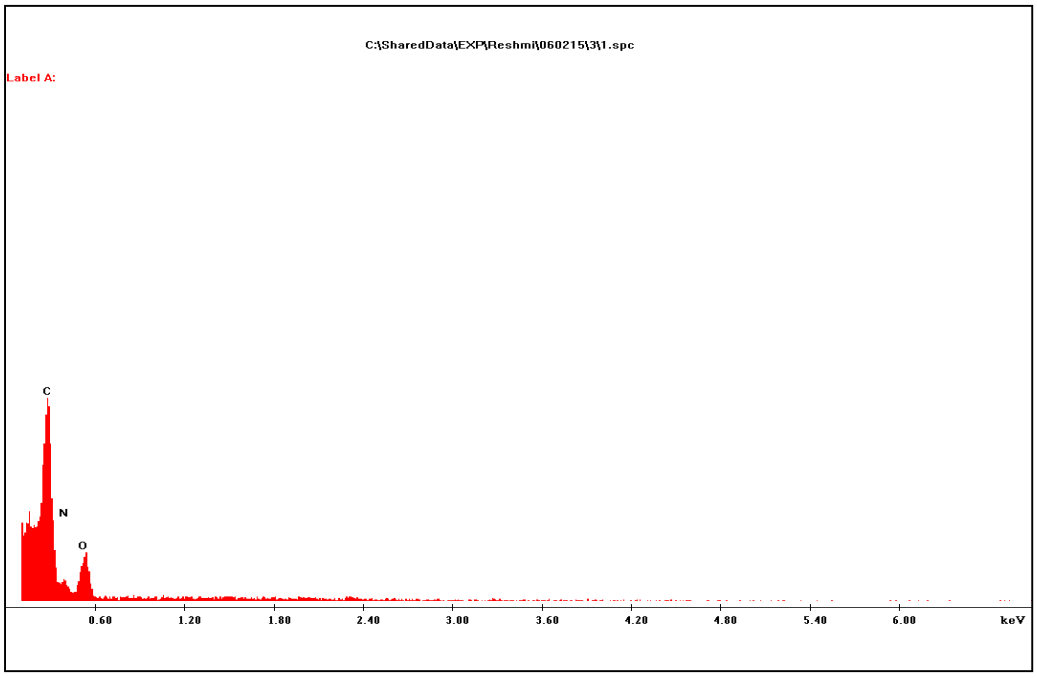

Fig. 7: EDX image of C-ECM sheet

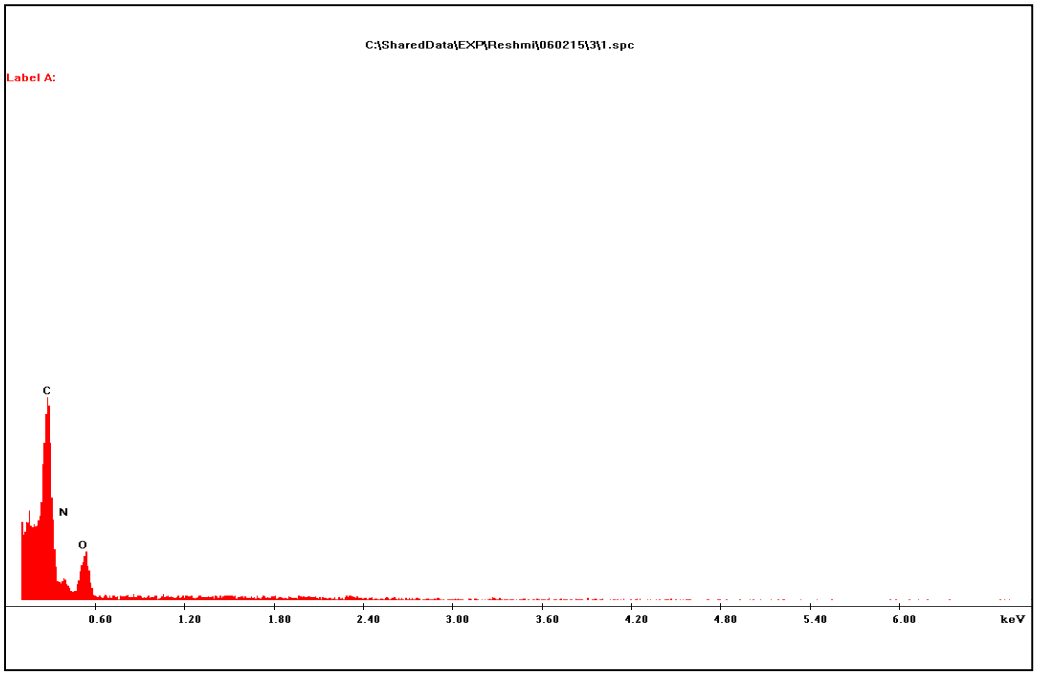

Fig. 8: EDX image of C-ECM powder

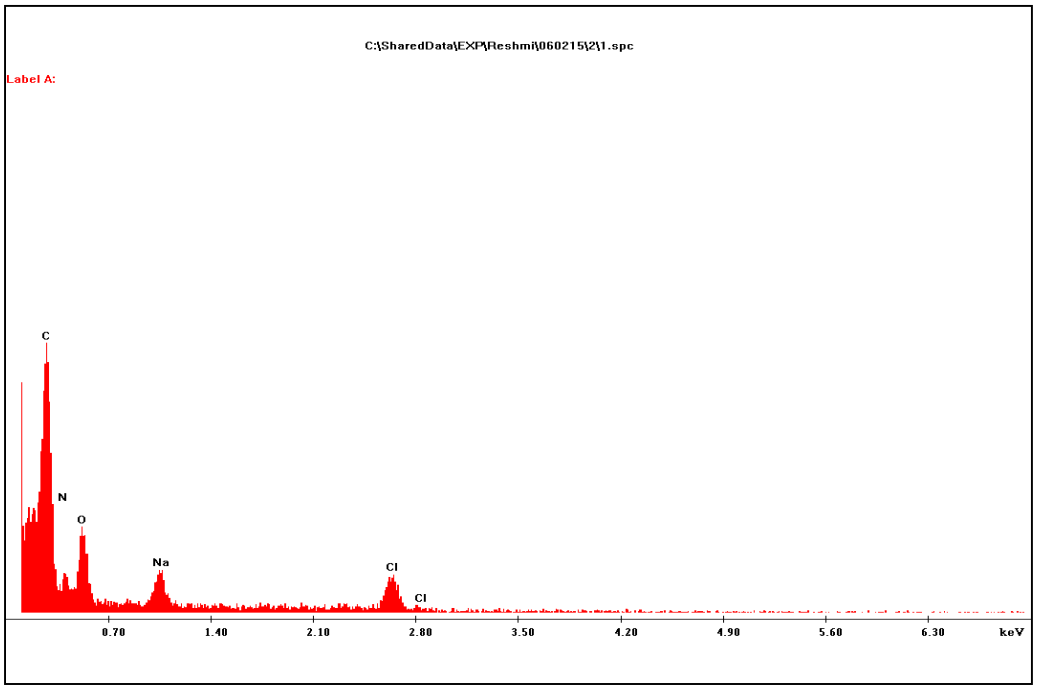

Fig. 9: EDX image of NC-ECM powder

5.2.4. Particle size analysis

The particle sizes of prepared NC-ECM and C-ECM powders were analyzed by the principle of DLS (Fig. 10). The mean particle size of NC-ECM powder was found to be 344.5 nm with a polydispersity index (PDI) of 0.5, confirming the formation of polydisperse ECM particles while the mean particle size of C-ECM powder was found to be 1805 nm with a PDI of 0.8, indicating that the sample has a very broad size distribution with an increased mean particle size (Table. 1). The DLS measurements of polydisperse nanoparticles was expected in the range of 100 – 300 nm with a PDI of 0.4 or below generally [68]. The mean particle size of C-ECM powder prepared by NaCl precipitation is also in the range of polydisperse nanoparticles, a slight increase in PDI and average particle size was due to agglomeration behavior of ECM components. A multimodal peak for NC-ECM powder indicates that, ECM components in the particulate level behave as polydisperse particle solutions. A low value of PDI corresponded to more homogeneous particle size in the case of NC-ECM powder when compared to the C-ECM powder as per DLS data. Hence NaCl precipitation technique has brought about reduction in particle size compared to conventional grinding technique of ECM powder preparation. An additional sieving procedure by sonic sifting may facilitate preparation of a powder formulation with uniform particle size. The host laboratory did not have facility for sieving hence could not prepare a powder formulation with uniform size.

5.2.5. Fourier transform infrared spectroscopy (FTIR)

The FTIR spectra (Fig. 11) indicated the presence of organic molecules of similar type in each of the ECM formulations examined. Characteristic bands found in the infrared spectra of proteins and polypeptides included the Amide I and Amide II. These might have arisen from the amide bonds that link the amino acids. It is known that the absorption associated with the Amide

I band leads to stretching vibrations of the C=O bond of the amide, absorption associated with the Amide II band leads primarily to bending vibrations of the N—H bond [69]. Characteristic collagen peaks at 1628 (amide I), 1402 (amide II), 2370, 3158, 3160, 3172, 1058 and 1063 cm^{-1} were observed for ECM materials indicating [69] that the biochemical composition of the C-ECM was not altered by the techniques utilized for the powder preparation.

Table. 1: Particle size and polydispersity index of NC-ECM and C-ECM powder

Sample code	Average particle size(nm)	Polydispersity index(pdi)
NC-ECM POWDER	344.5	0.5
C-ECM POWDER	1805	0.8

5.2.6. X ray diffraction

The purpose of XRD characterization was to analyze, whether the excess traces of NaCl content was interfering with the biochemical composition of ECM or not. The C -ECM powder showed a broad peak in the 2 theta range of 15–35 which was typical for pure collagen, [70] while NC-ECM powder was showing crystalline XRD pattern of NaCl along with the peak of collagen (Fig. 12). NC -ECM powder retained NaCl traces even after leaching, most probably in the vacant interstitial spaces of ECM without altering the original biochemical content of the ECM. Both NC-ECM and C-ECM powder had an amorphous behavior. In particulate ECM preparation, it was very important to control the particle size, shape and morphology. The prepared ECM powders were characterized by XRD as it is an important analysis tool in biomaterial science.

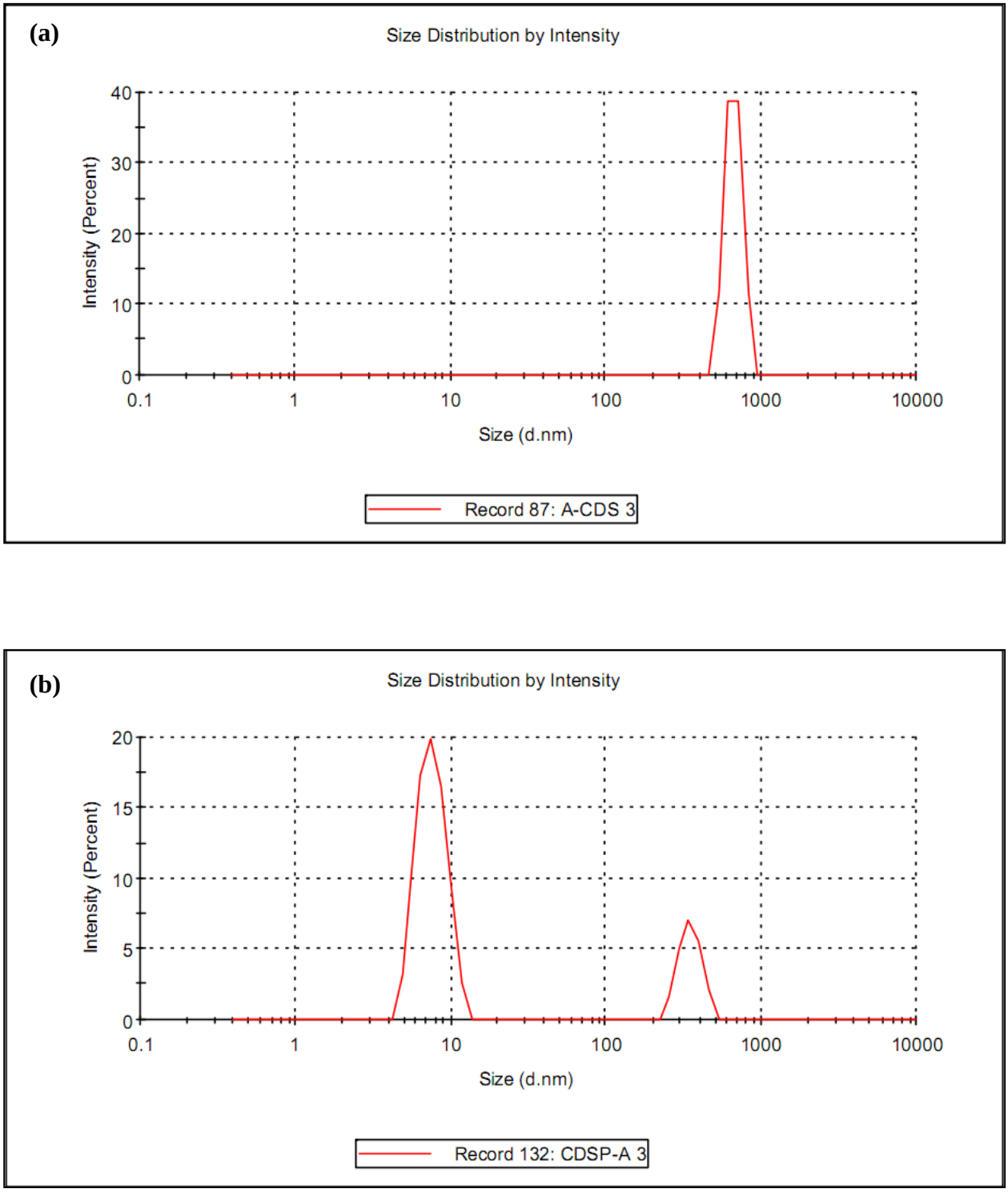

Fig. 10: Particle size distribution of (a) C-ECM powder and (b) NC-ECM powder

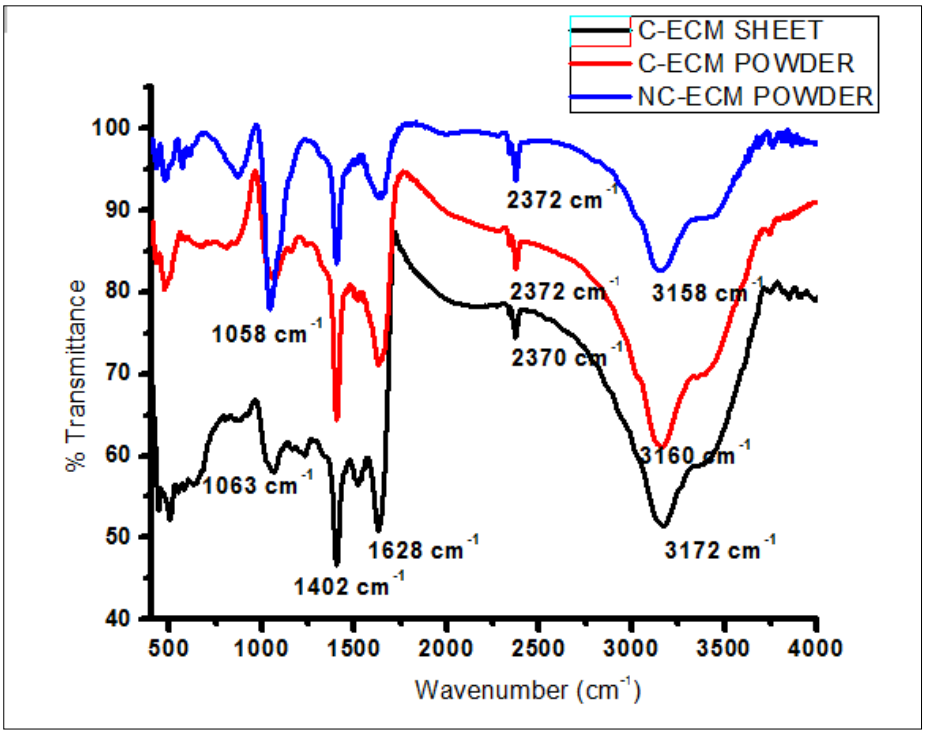

Fig. 11: FTIR spectra of C-ECM sheet, C-ECM powder and NC-ECM powder

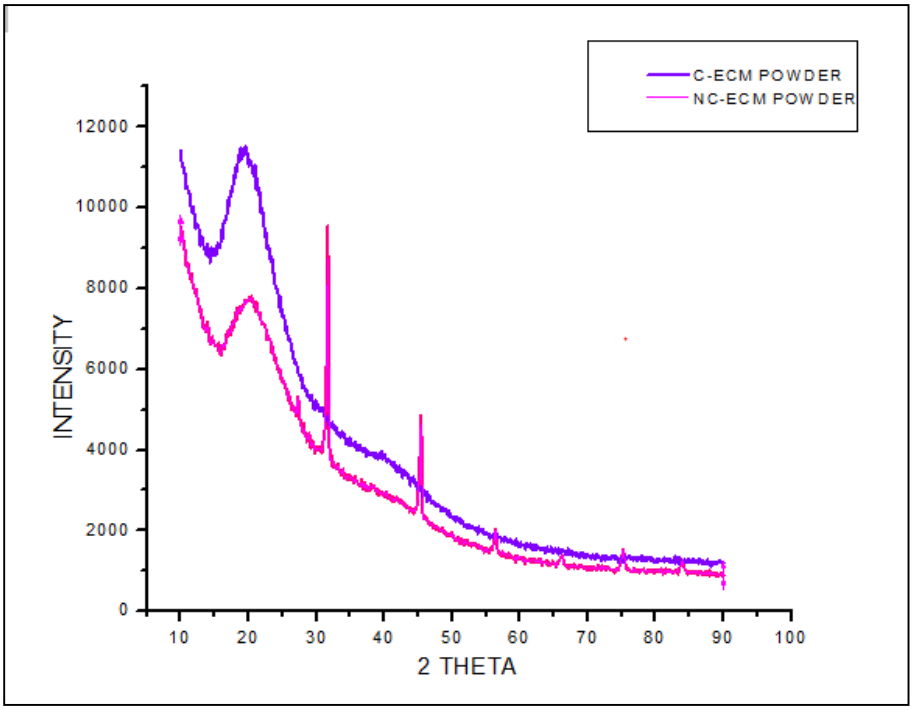

Fig. 12: XRD pattern of C-ECM powder and NC-ECM powder

5.2.7. Viscosity measurements

The viscosity of the solubilized ECM formulations in water at 37˚C was determined to evaluate their applicability as an injectable hydrogel *in vivo*. The data showed viscosity value of 16 centipoise (Cp) (Table 2) for ECM formulations, C-ECM and NC-ECM suspensions. Evidently these values fall well below the U.S. FDA approved permissible limit of approximately 50 cP for subcutaneous injectable systems [71], which clearly demonstrated that these ECM formulations had great potential as injectable preparations for biomedical applications.

Table 2. Viscosity measurement of C-ECM and NC-ECM powder

Sample	Viscosity (Cp)
C-ECM powder	16
NC-ECM powder	16

5.3. Biochemical characterization of ECM powders

The NC-ECM and C-ECM powders were solubilized using pepsin, and after visual confirmation of the lack of particles in solution, solubilized cholecystic matrix suspensions were brought to physiological pH .

5.3.2. Total protein estimation

Total protein content of pepsin solubilized suspensions of NC-ECM and C-ECM powder was analyzed by Bradford assay (Fig. 13). NC-ECM showed protein content of 321.5µg/ml

while C-ECM showed 294.8µg/ml. The total protein content appeared to be almost similar in both the suspensions. Since both the particulate ECM materials are dissolved in pepsin, it was a necessity to check the effect of pepsin on relative protein content of ECM materials, therefore pepsin was used as a control. Pepsin suspension dissolved in 0.1 M HCl showed protein content of 33.2µg/ml, markedly less than that of ECM. The observation deduced that the ECM was rich in proteins and also the salt precipitation technique has not altered the proteome of the original C-ECM.

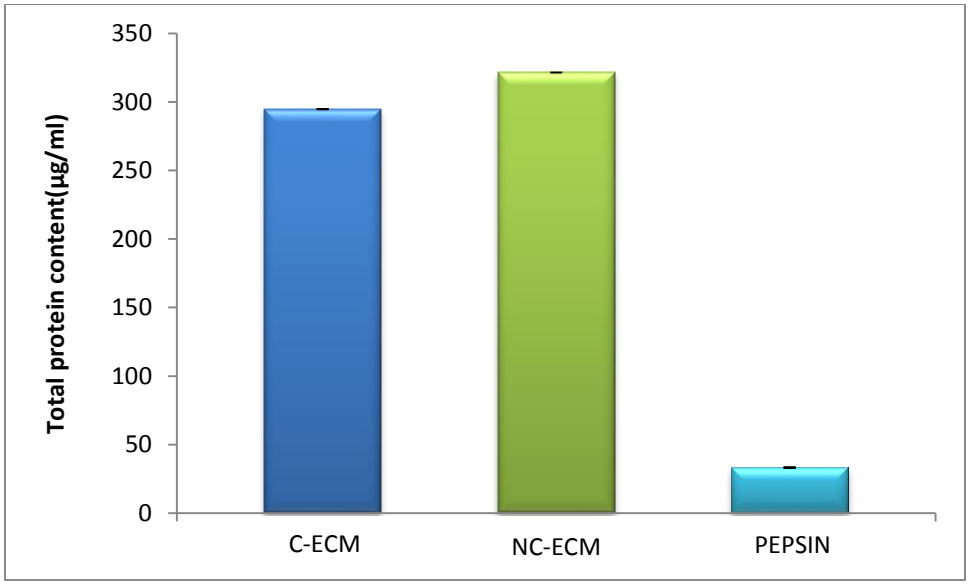

Fig. 13: Total protein content of C-ECM and NC-ECM powder (Values represent mean ± SD, n=3)

5.3.3. Content of Collagen and Glycosaminoglycan

Gel electrophoresis of solubilized NC-ECM and C-ECM powder demonstrated corresponding bands to collagen, as well as the presence of lower molecular weight bands. Thus, gel electrophoresis revealed additional peptides or ECM fragments, demonstrating the complexity of the cholecystic matrix as compared to strictly collagen type I (Fig. 14). Similar

banding pattern was observed for both C-ECM and NC-ECM powder revealing that salt precipitation technique did not alter the biological composition of cholecystic ECM.

Moreover, using Sircol collagen assay kit, the collagen contents of both the solubilized ECM materials were determined and it was found to be similar for both NC-ECM and C-ECM powder (Fig. 15). Content of sulphated GAG was also analyzed in the two ECM powder preparations and the content was found to be similar (Fig. 16). Thus it was confirmed that salt precipitation technique preserved the natural biochemical content of the ECM.

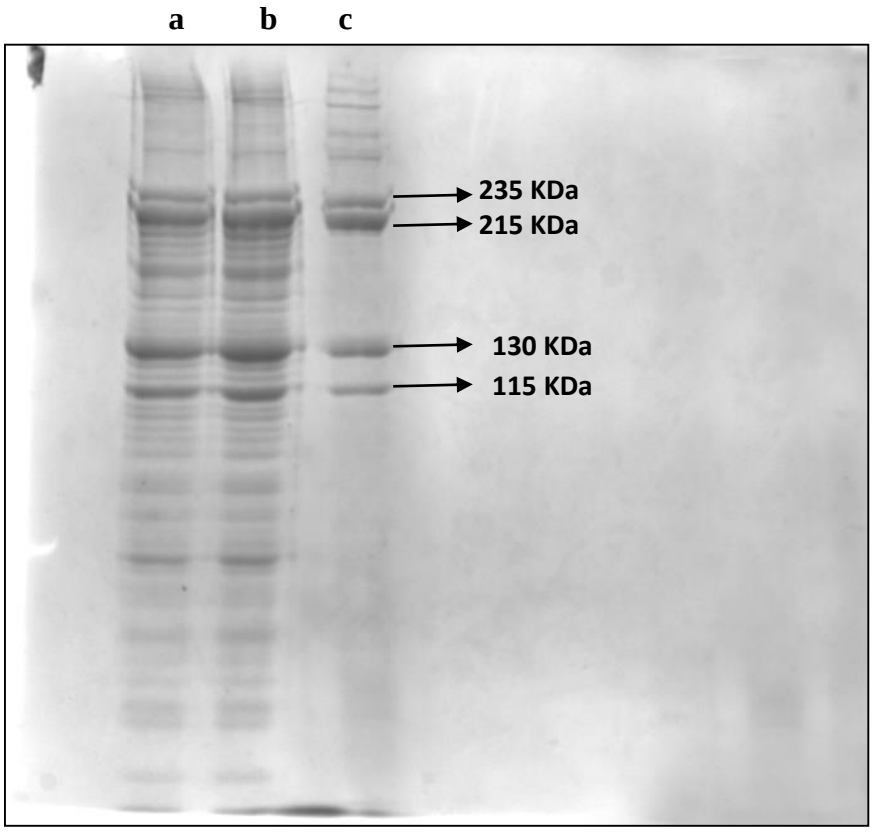

Fig. 14: SDS PAGE of solubilized (a) NC-ECM powder, (b) C-ECM powder, (c) Collagen

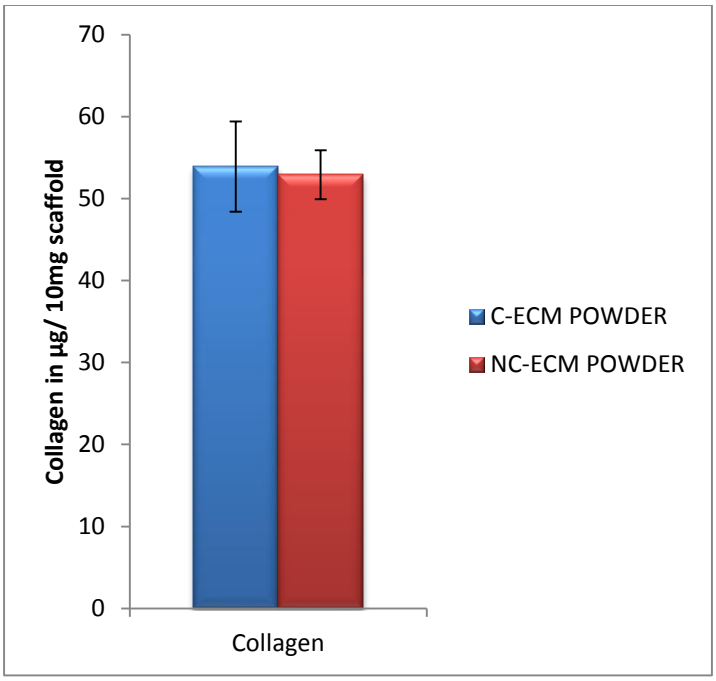

Fig. 15: Content of collagen in C-ECM and NC-ECM powder (Values represent mean ± SD, n=4)

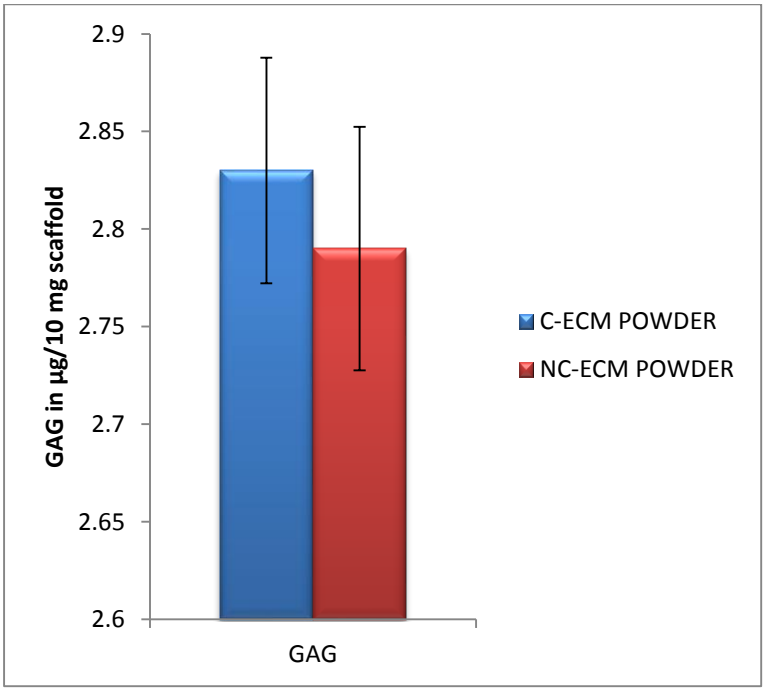

Fig. 16: Content of sulphated glycosaminoglycan in C-ECM and NC-ECM powder (Values represent mean ± SD, n=4)

5.3.4. Behavior of ECM particles in cell culture media

The particle size of C-ECM and NC-ECM powder in cell culture medium with serum was analyzed (Fig.17a and 17b). The size of the NC-ECM powder particles in cell culture medium with serum was found to be 74.2nm with a PDI value of 0.1. The particle size observed in DLS was considerably decreased from the previous results due to the enhanced repulsive interactions especially with proteins in serum containing medium [72]. The multimodal peak obtained for NC-ECM powder in cell culture media indicated a broad distribution of particle size in agreement with the primary DLS results. The observed PDI value of 0.1 indicated probably that NC-ECM powder possessed the ability to remain as primary particles in cell culture media suggesting that NC-ECM was relatively non toxic in solution and the particles remained homogenous in nature. Meanwhile the size of C-ECM particles in cell culture medium with serum was found to be 3353 nm with a PDI value of 1. The result suggested high degree of agglomeration of C-ECM with cell culture medium and a high PDI value indicated its interaction with serum components. The agglomeration of ECM particles in liquid medium has great importance in development of injectable suspensions. The size of the particles influences the agglomeration properties of the materials which may result in instability of the material in biologically relevant fluids.

5.4. Cytotoxicity studies

5.4.1. Cell morphology

The morphological examination of cells under a bright field microscope showed that the cells were capable of maintaining their normal morphology, upon treatment with NC-ECM powder up to 200 μg for 24 h (Fig. 18b–f). On the other hand, cells treated with C-ECM powder were also

able to maintain a healthy morphology, (Fig. 18g–k) but clumps of ECM suspension was clearly visible at higher concentrations. This indicates that the size of the C-ECM particle was too high and the particles were agglomerating with the cell culture media. However, the agglomeration behavior of C-ECM particles was not interfering with the cell morphology.

5.4.2. MTT assay

MTT assay gives an account of viability of cells. The percentage cell viability at different concentrations of NC-ECM and C-ECM powder treated for 24h (Fig. 18) was analyzed. The results of MTT assay showed above 100% cell viability upon treatment with NC-ECM powder for 24h in all the test concentrations. The observed percentage cell viability in NC-ECM powder was higher than that of C-ECM treated cells for all the concentrations. The overall results indicated that the NC-ECM powder prepared through salt precipitation technique was non-toxic to cells. The observations substantiated the claim that the powder formulation is a promising candidate for biomedical applications.

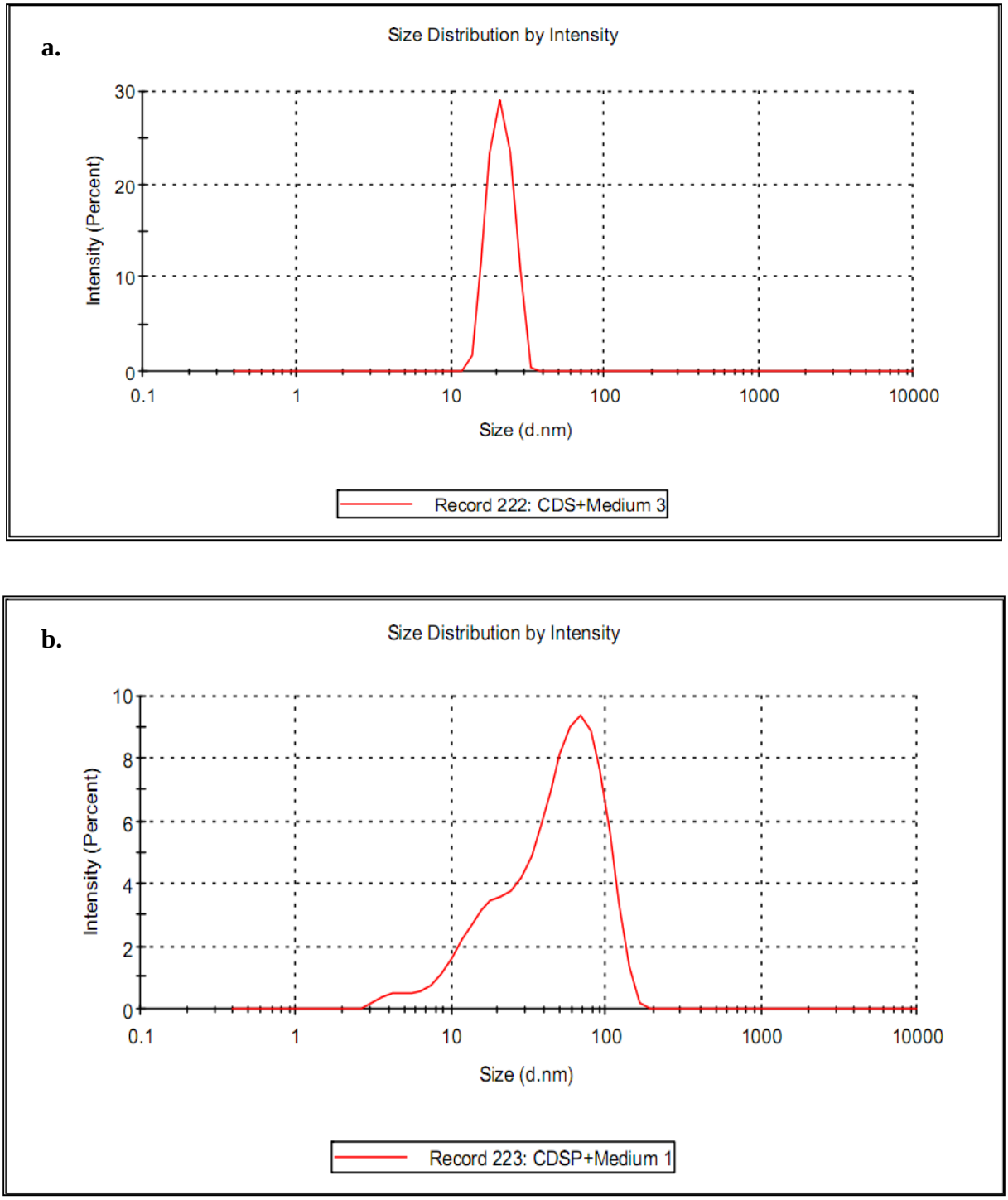

Fig. 17: Particle size distribution for (a) C-ECM powder and (b) NC-ECM powder in serum containing medium

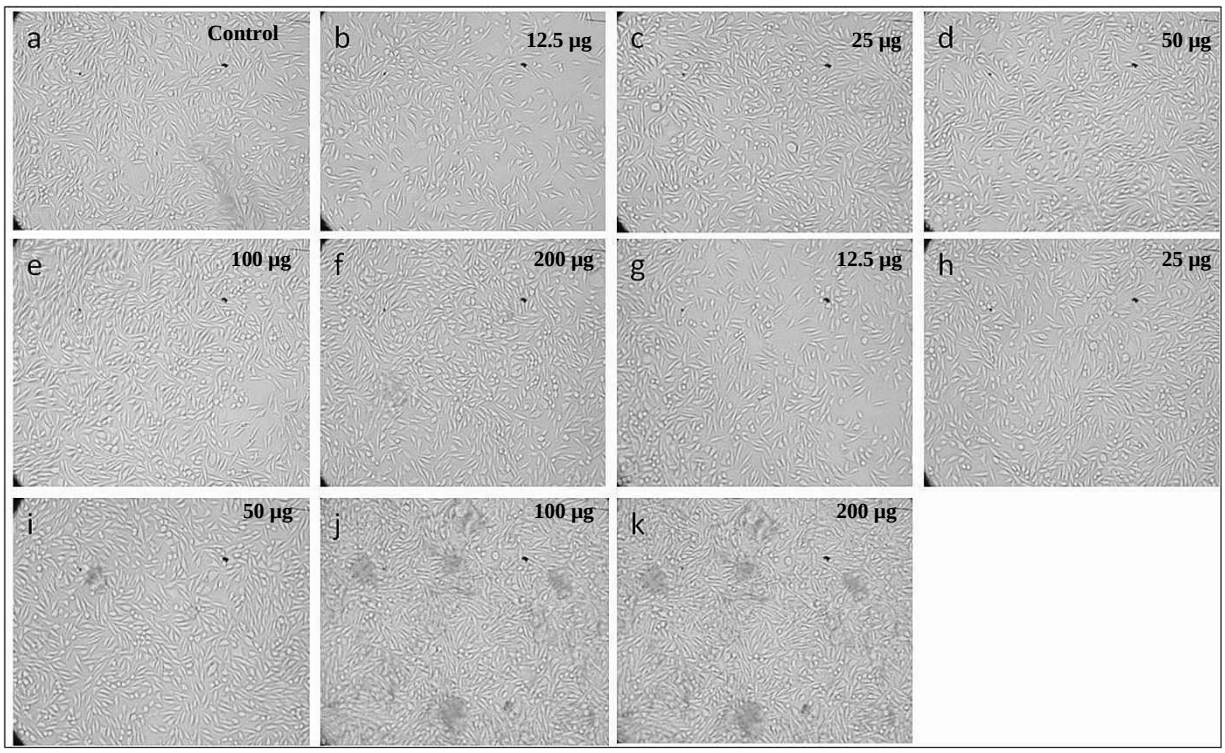

Fig. 18: Bright field images of ECM powder treated L-929 cells for 24 h: (a) control; (b-f) NC-ECM treated L-929 cells for 24 h; (g-k) C-ECM treated L-929 cells for 24 h

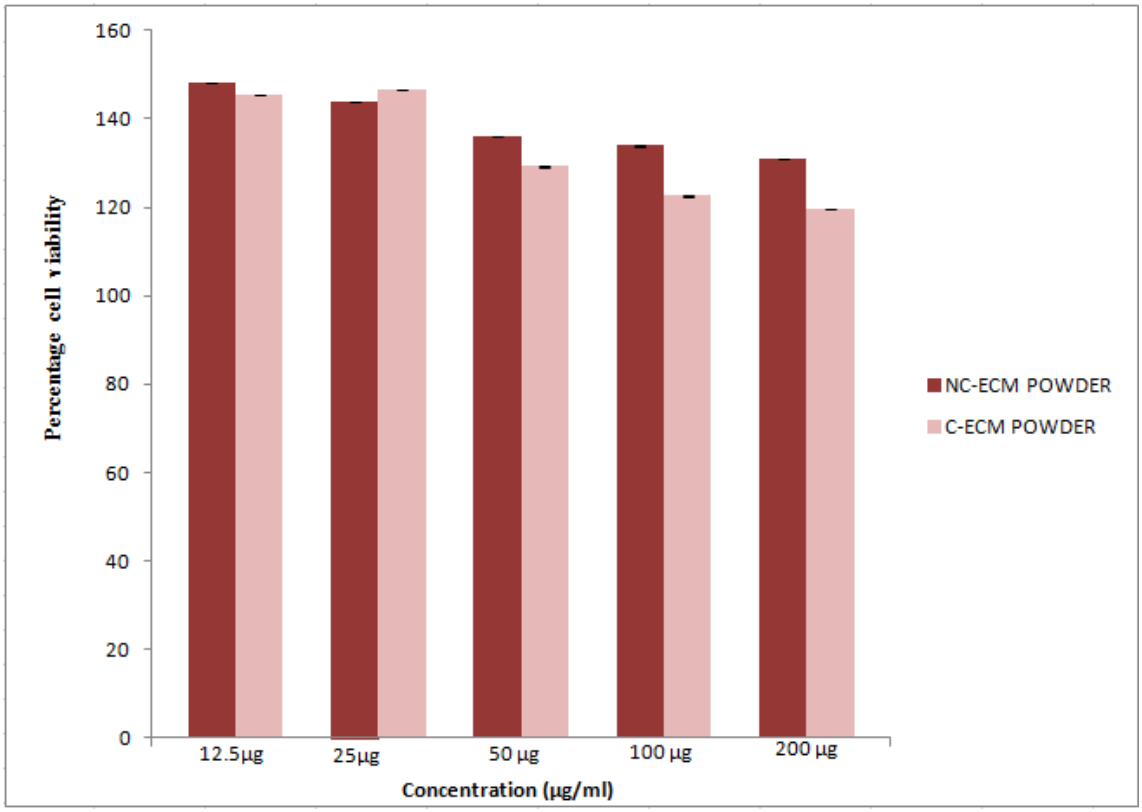

Fig. 19: MTT assay of C-ECM and NC-ECM powder treated for 24 h in L-929 cells (Values represent mean ± SD, n=6)

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6. Summary and conclusion

Extracellular matrices derived from mammalian organs and tissues have wide range of applications in regenerative medicine. This study investigated two methods for the preparation of particulate form of cholecystic ECM with the objective of evaluating the effect of method of production on ECM characteristics. The technique of conventional grinding and salt precipitation were used to produce C-ECM powder. The physical and biochemical properties between the two ECM materials were evaluated. Dynamic light scattering and Environmental scanning electron microscopy were used to evaluate the particle size distribution and ultrastructure of the two particulate forms. Upon comparing the two powdering techniques, the salt precipitation method produced smaller particles that were more uniformly distributed than those obtained by the conventional grinding method as per the results. The irregular shape and size of the particles seen with conventional grinding method was likely due to the fibrous nature of this largely collagenous material. The grinding process might have sheared the material between adjacent collagen fibers than to cut a fiber along its length. The particle size of prepared NC-ECM and C-ECM powders were analyzed by the principle of DLS. The mean particle size of NC-ECM powder was found to be 344.5 nm with a PDI of 0.5, confirming the formation of polydisperse ECM particles while the mean particle size of C-ECM powder was found to be 1805 nm with a PDI of 0.8, indicating that the sample has a very broad size distribution with an increased mean particle size. Hence it was clear that NaCl precipitation caused reduction in particle size compared to conventional grinding technique of ECM powder preparation. The difference in size and morphology of NC-ECM could have an effect on how cells interact with the matrix.

On an application point of view, one of the advantages of C-ECM was its rich load of natural biomolecules that promotes tissue remodeling reactions rather than pro-inflammatory

reactions. Therefore, any formulation procedure should not cause loss of these natural biomolecules. To know whether the salt precipitation technique is causing significant alteration with the biochemical composition of the C-ECM, both NC-ECM and C-ECM powders were evaluated for their functional groups, content of selected biomolecules, and total protein. The results showed that the salt precipitation method was not affecting the biochemical composition of the C-ECM. Moreover, cytotoxicity studies revealed that NC-ECM was non toxic to cells.

This study compared the physical properties of the particles in the two powder formulations. The results revealed that, a particulate form of C-ECM with uniform size distribution and morphology can be prepared by the method of salt precipitation. The ECM particles produced by conventional grinding method were irregularly shaped, and retained the ultrastructure observed on the sheet form of the material. With salt precipitation, the particles were uniformly smaller in size when compared to particulate ECM prepared through freeze milling method. However, this study did not attempt to determine whether, these particulate formulations, when suspended in a liquid medium, would meet the criteria of an injectable scaffold for tissue engineering applications. Certainly more studies are required to assess the potential of NC-ECM powder to form a gel *in situ* under physiological conditions. There is also a need to further refine the production techniques that result optimum particulate size suitable for clinical use. The effect of different concentrations of sodium chloride on C-ECM should be evaluated. The mechanical and degradation properties of NC-ECM powder remains to be elucidated. It should be noted that cross linking techniques can be explored to strengthen the mechanical properties of the material, as well control degradation mechanisms and rates. Despite all these deficiencies, the data collected as part of the study strongly indicates that the powder formulations of cholecystic ECM are promising biomaterials for tissue engineering applications.

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