

DECELLULARIZED LIVER SCAFFOLDS FOR INDUCED REGENERATION IN ADULT MAMMALIAN MODEL

JESNA P. V.

PhD THESIS

2024



**SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND
TECHNOLOGY, TRIVANDRUM**

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MAMMALIAN MODEL**

A THESIS SUBMITTED BY

JESNA P. V.

TO

SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND
TECHNOLOGY, TRIVANDRUM.

IN PARTIAL FULFILMENT OF THE REQUIREMENTS

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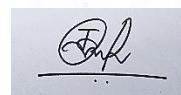
DOCTOR OF PHILOSOPHY

2024

DECLARATION BY THE STUDENT

CERTIFICATE

I, **Jesna P. V.**, hereby certify that I had personally carried out the work depicted in the thesis titled, **“Decellularized Liver Scaffolds for Induced Regeneration in Adult Mammalian Model”** under the guidance of Dr. Umashankar P. R., Scientist-G, Division of In-vivo Models and Testing, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum. No part of this thesis has been submitted for the award of any other degree or diploma prior to this date.



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*Clearance was obtained from the Institutional Animal Ethics Committee (IAEC) for carrying out the study as per the order SCT/IAEC-432/JANUARY/2022/112, dated 03/03/2022.

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The thesis entitled

**“Decellularized liver scaffolds for induced regeneration in
adult mammalian model”**

Submitted by

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Doctor of Philosophy

of

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LIST OF ABBREVIATIONS

S No	Abbreviation	Full Form
1	TERM	Tissue engineering and regenerative medicine
2	ECM	Extracellular matrix
3	DEM	Decellularized extracellular matrix
4	DAMP	Damage-associated molecular patterns
5	TLRs	Toll-like receptors
6	ADCC	Antibody-dependent cell-mediated cytotoxicity
7	RGD	Arg-gly-asp
8	H&E	Hematoxylin and Eosin
9	DAPI	4',6-Diaminidino-2-phenylindole hydrochloride
10	HGF	Hepatic growth factor
11	FGF	Fibroblast growth factor
12	TGF	Transforming growth factor β
13	PDGF	Platelet-derived growth factor
14	IGF	Insulin-like growth factors
15	CA	Citric acid
16	GRAS	Generally regarded as safe
17	SF	Silk fibroin
18	SFG	Silk fibroin/gelatin
19	CBB	Citrate-based biomaterial
20	ROS	Reactive oxygen species

21	FBGC	Foreign body giant cells
22	M	Macrophage
23	T _H	T- helper cells
24	TE	Tissue engineering
25	DFU	Diabetic foot ulcer
26	SDS	Sodium dodecyl sulphate
27	SDC	Sodium deoxycholate
28	TX100	Triton X-100
29	GAG	Glycosaminoglycans
30	GFs	Growth factors
31	MBVs	Membrane-bound vesicles
32	SIS	Small intestinal submucosa
33	GA	Glutaraldehyde
34	PAA	Peracetic acid
35	ETO	Ethylene oxide
36	PMNCs	Polymorphonuclear cells
37	PRRs	Pattern recognition receptors
38	IL	Interleukin
39	TNF	Tumour necrosis factor
40	IFN	Interferon
41	CTL	Cytotoxic T lymphocytes
42	DPL	Decellularized porcine liver
43	H&E	Hematoxylin and Eosin
44	DNA	Deoxyribo nucleic acid

45	AGE	Agarose gel electrophoresis
46	TAE	Tris base, acetic acid, EDTA
47	EDTA	Ethylene diamine tetra acetic acid
48	NaOH	Sodium hydroxide
49	Na ₂ CO ₃	Sodium carbonate
50	SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
51	MT	Masson's trichrome
52	AB	Alcian blue
53	DMMB	Dimethyl methylene blue
54	K ₂ HPO ₄	Dipotassium hydrogen phosphate
55	ELISA	Enzyme linked immunosorbent assay
56	TMB	3,3',5,5'-tetramethylbenzidine
57	PBS	Phosphate buffered saline
58	UTM	Uniaxial tensile testing machine
59	SEM	Scanning electron microscopy
60	L929	Murine fibroblast cell line
61	MEM	Minimum essential medium
62	UHMWPE	Ultra high molecular weight polyethylene
63	PVC	Polyvinyl chloride
64	DMEM	Dulbecco's modified eagle medium
65	RSF	Regenerated silk fibroin
66	LiBr	Lithium bromide
67	NHN	Ninhydrin

68	-NH ₂	Free amine group
69	FTIR	Fourier transform infrared spectroscopy
70	MTT	3-(4, 5-Dimethyl thiazol -2-yl)-2,5-diphenyltetrazolium bromide
71	HRP	Horse radish peroxidase
72	HGB	Hemoglobin
73	HCT	Hematocrit
74	RBC	Red blood cell
75	WBC	White blood cell
76	PLT	Platelet
77	DPX	Dibutylphthalate polystyrene xylene
78	DMSO	Dimethyl sulfoxide
79	SI	Stimulation index
80	PBMNCs	Peripheral blood mononuclear cells
81	RPMI	Roswell park memorial institute
82	BSA	Bovine serum albumen
83	IHC	Immuno histochemistry
84	CAXL	Citic acid cross linked
85	UnXL	Un crosslinked
86	SWA	Scratch wound assay
87	AWR	Abdominal wall regeneration

SYNOPSIS

Healing in adult mammalian organisms takes place either by regeneration or repair based on the regenerative potential of the organ or tissue. Imparting regenerative repair potential to non-regenerative organs by applying cells, scaffolds, signaling molecules, or a combination of these is termed induced regeneration, which replaces the normal structure and function of the injured or defective organ by site appropriate functional tissue regeneration (termed constructive remodeling). The scaffolds used for such constructive repair applications include both synthetic and biological ones. Compared to biological scaffolds, the disadvantage of synthetic scaffolds is that they induce foreign body reactions and elicit adverse inflammatory responses leading to fibrosis. Biological scaffolds composed of decellularized extracellular matrix form an important avenue in regenerative surgical grafting because of their potential to induce regeneration instead of scarring. Decellularization is the removal of cellular and nuclear components associated with tissue to remove the antigenic determinants associated with it. There are physical, chemical, enzymatic, and combinational decellularization strategies to bring about efficient cellular removal. They are advantageous over their synthetic counterparts because of their inherent bioactivity, acceptance by xenogeneic recipients, insignificant inflammatory/immune potential, and their ability to promote constructive remodeling leading to regeneration. The present research focuses on bioscaffold fabrication using decellularization with minimal damage to the resulting ECM and preserving biomolecules such as glycosaminoglycans (GAG) and growth factors (GFs) which are crucial in providing

regenerative efficacy to the scaffold. The ECM resulting from the process has structural and functional molecules such as collagen, elastin, laminin, fibronectin, GAG, GFs etc. The peculiarity of such a matrix is that the structural and functional components associated with ECM are conserved across species and are accepted even by xenogeneic recipients. However, the reduced mechanical behavior of biological scaffolds makes their application in clinical settings difficult. Decellularization renders the porcine liver tissue mechanically weak and easily degradable. An easily degrading matrix may fail structurally and functionally without leading to constructive remodeling. So, it is very important to augment the mechanical behaviour of such matrices by various methods. Among these methods, chemical crosslinking remains the most extensively used, but it is associated with adverse responses such as cytotoxicity, adverse immune responses, and fibrotic reactions. Such limitations created the need to identify a non-toxic and biocompatible method to improve the biomechanical properties of ECM without destroying the inherent biomolecules preserved within the matrix. The modified scaffolds should be cytocompatible, immunocompatible and with inherent bioactivity to promote constructive remodelling.

On this ground, the hypothesis formulated for the study was to “develop a decellularized and mechanically strengthened porcine liver scaffold that maximally preserves growth factors and to study its potential to induce constructive remodelling”.

The four objectives to prove the hypothesis are the following

Proposed Objectives:

The following objectives will be addressed with specific analyzing methodologies.

1. Development of decellularized porcine liver scaffold (membranous form) which maximally preserves the candidate growth factors and structural components and its mechanical, chemical and biological characterization.
2. Improving mechanical strength of the decellularized liver scaffold by impregnation with biocompatible natural polymers.
3. Evaluation of inflammatory and immunogenic potential of mechanical strength improved decellularized porcine liver scaffold by rat subcutaneous implantation
4. Studying the phenomenon of induced regeneration in adult mammalian models such as rat abdominal wall regeneration model

The whole research work was divided into 6 chapters which include an introduction, literature review, materials and methods, results, discussion, and summary & conclusion.

Chapter 1 give an **introduction** about biological scaffolds composed of decellularized extracellular matrices (ECM), various methods of fabrication of decellularized ECM scaffolds, and the advantages and disadvantages associated with it. The importance of preserving the biomolecules and their role in promoting regenerative healing was also described. The need to enhance the mechanical properties of such biological scaffolds using green crosslinking methods and incorporating natural polymers such as silk fibroin was described in detail with a brief description of the inflammatory and immune responses elicited by such materials, and the importance of immunocompatibility to promote constructive remodeling.

Chapter 2 presented the **literature review** on the current status of research in the area by citing available literature on various decellularization methods, criteria to be satisfied by a decellularization protocol, the importance of preserving the biomolecules, advanced methods of mechanical augmentation and the commercially available biological grafts for soft tissue regenerative healing.

Chapter 3 contains the **materials and methods** used to develop the decellularized extracellular matrix scaffolds from porcine liver followed by their characterization to confirm successful cellular removal, preservation of collagen architecture, retention of glycosaminoglycans and growth factors along with the in-vitro cytotoxicity evaluation. The proprietary procedure for augmenting the mechanical property of the ECM scaffold was also described and their successful characterization. This also includes animal implantation protocols and the evaluation of the inflammatory, immune, and regenerative potential.

Chapters 4 and 5 included the **results** followed by a **discussion** of the experiments carried out to prove the hypothesis in an objective-dependent manner. As part of the first objective, three ECM scaffolds were fabricated from the porcine liver. Three methods of detergent decellularization were used which included various ionic and non-ionic detergents. They were 0.1% sodium dodecyl sulfate (SDS), a combination of 0.05% SDS+0.05% sodium deoxycholate (SDC), and the final method involving enzymatic digestion and TX-100 treatment followed by 0.1% SDS. The acellular matrices developed were abbreviated as SDS DPL, SDS+SDC DPL, and TX100 DPL respectively. Characterization of acellular matrices proved the SDS+SDC DPL to be the best decellularization method which satisfied the essential criteria of

decellularization with preserved collagen integrity and highest concentration of GAG and the candidate growth factors HGF and bFGF. Therefore SDS+SDC DPL was chosen for mechanical augmentation followed by in-vitro and in-vivo evaluations.

The second objective of enhancing the mechanical strength of the selected ECM was carried out by impregnation of the fibrous natural polymer, silk fibroin (SF). SF was selected for the study because it is an FDA-approved biomaterial and is associated with high intrinsic mechanical strength, biocompatibility, bio-resorbability, water-based processing, and low immunogenicity. SF was isolated from *B. mori* silk cocoon. The modification procedure involved a green crosslinking strategy mediated by citric acid crosslinking. The underlying principle was alkaline-catalyzed low-temperature mediated crosslinking between free amine groups present within the ECM and SF. SF blended with gelatin (G) was also used to modify the pliability of the scaffolds and to incorporate bioactive RGD sequence motifs which further enhances its bioactivity. The code names of the modified scaffolds were SF100 DPL, SFG5050 DPL, and SFG3070 DPL. The incorporation of silk fibroin into the scaffold was proven, and subsequent strength and stability augmentation was demonstrated by significant improvement in tensile strength, Young's modulus, suture retention strength, and delayed in-vitro degradation compared to the unmodified control. The swelling behavior and surface wettability were also significantly reduced compared to the decellularized matrix. The in vitro cytotoxicity studies using L929 fibroblast cells by 'direct' and 'test on extract' methods based on ISO 10993-5 proved the scaffold to be non-cytotoxic. An in-vitro cell migration study using scratch wound assay confirmed the ability of material extracts to promote cell migration. SFG5050 DPL extract-treated L929 cells exhibited a comparatively higher potential to promote cell migration

followed by those treated with SF100 DPL. This ensures the suitability of the materials for animal implantation studies to prove their inflammatory, immunogenic, and regenerative potential.

In the third objective, the potential to modulate inflammatory and immune response was confirmed by rat sub-cutaneous implantation for 14, 30, and 90 days. Histomorphometric evaluation revealed a prominent reduction in acute inflammation at 30 days and 90 days compared to an increased inflammatory cell infiltration at 14 days. Lymphocyte proliferation test, in-vitro cytokine release study, and antibody response revealed the immune response exhibited by the material implanted animals showed an initial moderate immune stimulation followed by tolerance on 90 days.

In the final objective, a comparative study on the regenerative potential of the modified scaffolds in comparison to unmodified control was evaluated using the rat abdominal wall defect model. Regeneration by neo-muscle formation was confirmed by Masson's trichrome (MT) staining and immunohistochemistry (IHC). It demonstrated skeletal muscle island formation initially at the host-graft interface on 21 days while extending towards the mid-graft region as time progressed. Herovici's staining demonstrated new immature collagen type III deposition predominant at 21 days which was maturing to collagen type I at 90 days. Among the modified scaffolds, SFG5050DPL exhibited comparatively high regenerative potential followed by SF100DPL

The whole research findings are summarised in the **summary and conclusion** given in **Chapter 6** with potential limitations of the study. Briefly, a standardized liver decellularization protocol that satisfied the essential criteria of decellularization and preserving maximum GAG and GFs was developed. Its mechanical strength was

augmented successfully using a non-toxic and biocompatible method using silk fibroin impregnation. In-vivo implantation study in the subcutaneous implantation model has revealed the scaffold-implanted animals showed tolerable inflammation and immunogenicity. Biomaterial-induced immunogenicity was proven to be antibody-mediated through the activation of B-cells. The rat abdominal wall defect model has given evidence that the material well supported the defect site mechanically and promoted positive remodeling by neo-muscle formation. SF100DPL and SFG5050DPL possessed comparatively higher regenerative potential than SFG3070DPL.

1 INTRODUCTION

A major research focus in tissue engineering and regenerative medicine (TERM) constitutes finding innovative solutions for the reconstruction of disease-affected tissues. Soft tissues are frequently exposed to traumas, burns, tumors, and other types of tissue damage that need remedial interventions using scaffold-based therapies. The healing patches designed for such repair applications should provide structural support, promote natural healing, reduce post-operative complications, and improve regenerative outcomes. Biocompatible xenografts developed by tissue engineering, with minimized risk of adverse inflammatory and immune responses, integrate with the host tissue easily and promote healing by regeneration instead of repair (“Acellular matrices for the treatment of wounds – Wounds International,” n.d.; “Cardiovascular and Soft Tissue Repair Patches Market Size,” n.d.). Three cornerstones of tissue engineering include cells, scaffolds, and signaling molecules. A major area of regenerative medicine uses scaffolds as templates for triggering the body’s own regenerative mechanism. Scaffolds can be of natural or synthetic origins that act as supporting structures for the defect and provide an appropriate platform for the functional cells to grow and lay new extracellular matrix (ECM). The major considerations while developing a scaffold for regenerative applications are its biocompatibility and mechanical strength, allowing it to afford mechanical constraints during surgical procedures (Bertsch et al., 2023).

There is a multitude of emerging scaffold fabrication methodologies in tissue engineering that are used for regenerative medicine applications. They include both conventional methods that use autografts and modern techniques of applying artificial

scaffolds as supporting structures for injured or diseased tissues or organs with non-regenerative anatomical defects (Eltom et al., 2019). Decellularized extracellular matrix (DEM) scaffolds refer to those biomaterials developed from human or animal tissues/organs, by the elimination of immunogenic cellular and nuclear components. This extracellular matrix (ECM) can minimize or prevent exaggerated inflammatory and immune responses. ECM remains a complex network of proteins and polysaccharides such as collagen, elastin, fibronectin, laminin, glycosaminoglycans, etc., providing a dynamic regulatory environment for supporting cells and cell functions. It can also act as a repository of growth factors, cytokines, and other bioactive molecules, crucial in regulating cell functions and leading to tissue regeneration (Neishabouri et al., 2022; Xu et al., 2021). It is associated with various biophysical, biochemical, and biomechanical cues that facilitate cellular functions such as cell survival, intercellular communication, proliferation, and differentiation. Thus acellular extracellular matrices can function as ‘biological modulators’, a substance derived from biological sources that influence biological processes such as wound healing (“Acellular matrices for the treatment of wounds – Wounds International,” n.d.; Borrelli and Buckley, 2020; Brown et al., 2022; Hwang et al., 2020). Examples of commercially available xenografts from porcine or bovine origin are Dermagraft®, Alloderm®, and Matristem®. Acellular collagenous matrix combined with synthetic materials form composites, Integra® is an example of such bilayer composite wound dressing (“Acellular matrices for the treatment of wounds – Wounds International,” n.d.).

The scaffold fabrication technique followed in the present study was decellularization. The tissue taken for making the scaffold was porcine liver, because of its excellent

regenerative capability on account of its high growth factor content and, its inherent mechanical strength due to strong intercollagenous connective tissue compared to other mammals used for meat purposes. The removal of antigenic epitopes associated with the cell membranes and intracellular components is termed decellularization. The methods utilized to produce acellular ECM scaffolds mainly involve physical, chemical, enzymatic, or combinational approaches (Crapo et al., 2011a). Physical treatments involve applying temperature, freeze-thaw cycles, agitation, scraping, sonication, etc. for cell disruption and removal of its contents. The chemical way of decellularization makes use of various detergents and other chemical agents in particular acids and bases, alcohols, and hypertonic/hypotonic solutions. Detergents such as ionic, non-ionic, and zwitterionic detergents, disrupt cellular bonding, solubilizes cell membranes, dissociate DNA, and bring about effective cellular removal. Enzymatic treatments break cell-cell and cell-matrix interactions by their comparatively specific action and remove undesirable ECM constituents. Different decellularization agents used are enlisted in Figure 1.1. A decellularization procedure may involve a combination of these methods to balance the adverse effects of each in the resulting matrix and also for an optimized cellular removal (Zhang et al., 2022).

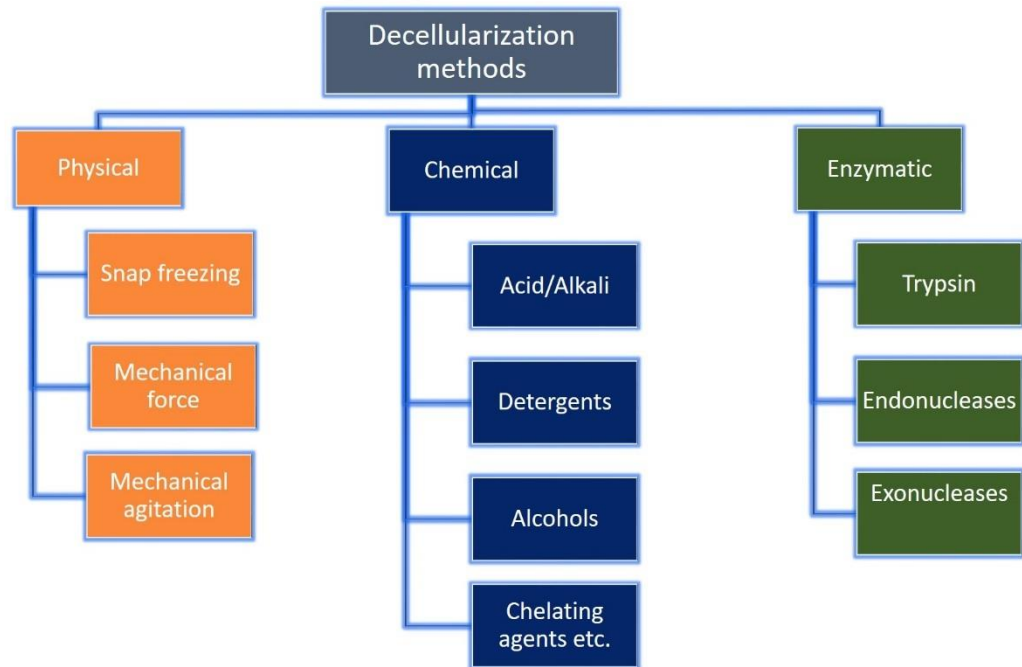


Figure 1.1. Various methods of decellularization: Physical, chemical, and enzymatic agents used for the decellularization procedure

The ultimate aim of a decellularization process is to remove all antigenic epitopes without compromising the structural integrity and eventual functionality. The more damaging the process is, the more adverse host reactions it will result (Cornwell et al., 2009). Inappropriate decellularization involving retention of nuclear and cellular remnants above certain threshold levels can act similarly as damage-associated molecular patterns (DAMP) involving toll-like receptors (TLRs) and activate various signaling cascades. This may lead to a prolonged inflammatory response (acute or chronic) or antibody-dependent cell-mediated cytotoxicity (ADCC), which may either lead to seroma formation, foreign body reaction, abscess formation, fibrous encapsulation, or complement-mediated rejection of the material at the host site (Brown et al., 2009; Faulk and Badylak, 2014a; Keane et al., 2012a; Neishabouri et

al., 2022). Insufficient removal of residual chemicals post-decellularization may render the scaffold cytotoxic. Residual genetic materials like DNA or RNA and antigenic peptides are the major determinants of immunocompatibility. Among these DNA forms an important one, based on which the three criteria of decellularization were formulated (Gilbert et al., 2009b). The criteria suggested by Crapo *et. al.* describe that the acellular matrix should possess no nuclei visible by H&E staining and DAPI, dsDNA content less than 50ng/mg of ECM, and the length of residual DNA fragment to be less than 200bps. When implanted in a compatible microenvironment, a scaffold satisfying these criteria can act as a biological matrix that promotes cellular infiltration without eliciting any adverse inflammatory or immune responses (Crapo et al., 2011a).

Appropriately manufactured extracellular matrix scaffold provides micro-environmental cues for promoting angiogenesis, recruits host-specific stem/progenitor cells, and prevents bacterial infection at the implant site (Badylak et al., 1994a; Badylak, 2002a; Daly et al., 2012; Faulk and Badylak, 2014b; Hillebrandt et al., 2019). They act as a skeleton to support and hold cells and offer a wide range of advantages over individual protein and polysaccharide scaffolds. ECM contains mainly the structural proteins (such as collagen and elastin); adhesive proteins (like laminin and fibronectin) and ground materials (proteoglycans and glycoproteins) which provide cell attachment domains such as RGD (Arg-Gly-Asp) for integrins (Frantz et al., 2010; Hynes and Naba, 2012). In contrast, glycosaminoglycans sequester growth factors, prevent unnecessary activation, and ensure its availability by controlled release during *in-vivo* scaffold degradation. ECM contains nanovesicles, within which the enzymes, miRNAs, growth factors, and other signaling factors are stored (Huleihel et al., 2016).

They are readily degradable by proteases releasing non-toxic, resorbable, or biologically active components such as matricryptins/matrikines (Mendibil et al., 2020). The biomechanical and bioinductive properties of the ECM are also highly influential in directing a reconstructive sequel. The biomechanical properties of ECM play a significant role. They are defined mainly by the collagen fibers' size, orientation, compactness, and alignment. In contrast, the bioinductive properties of ECM are defined by the growth factors and matricryptic peptides released during the degradation of the material. Growth factors, the most critical players in various events of regenerative remodeling like stem/progenitor cell recruitment, angiogenesis, mitogenesis, and activation of cells to deposit host-specific extracellular matrix, include hepatic growth factor (HGF), Fibroblast growth factor (FGF), Transforming growth factor β (TGF- β), Platelet-derived growth factor (PDGF), and Insulin-like growth factors (IGF) (Akbari Zahmati et al., 2017; He and Callanan, 2013). The xenogeneic compatibility of molecular components of ECM is because of the conservation of amino acid sequence and structure of these molecules throughout evolution during the divergence of species (Schroeter et al., 2017). Figure 1.2 explains the role of growth factors in bringing about different cellular and tissue responses.

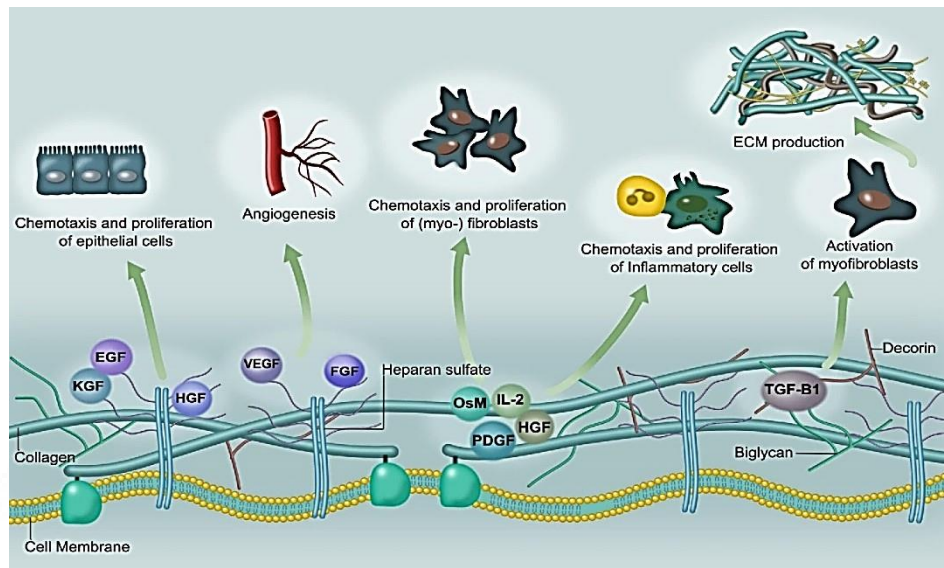


Figure 1.2. ECM structural and functional components: Role of growth factors in regulating various cellular and tissue responses. [Figure adapted from (Karsdal et al., 2015)]

After confirmation of decellularization and preservation of biochemical and mechanical properties, it can be modified and refined without compromising its ability to boost tissue regeneration. As decellularization renders the resulting extracellular matrix mechanically weak, the method adopted should improve mechanical strength of the acellular matrix to withstand the mechanical forces that the material may experience during surgical implantation. Also, the scaffold must resist faster degradation to promote a constructive remodeling outcome instead of early structural failure. An easily degradable matrix cannot be used as it may lead to structural failure due to its early degradation. Hence, the scaffold's degradation time should match the time required for neo-tissue formation (Xu et al., 2021). Major approaches to improve the mechanical properties remain crosslinking using various methods like physical, chemical, or enzymatic agents. Chemical crosslinking is a prominent method that

forms intermolecular and intramolecular crosslinks that render the material good mechanical properties, degradation resistance, and stability compared to other methods. The most widely used chemical cross-linking agents (such as glutaraldehyde, N-hydroxysulfosuccinimide, 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide, 4-aminoethanol-N-hydroxyethyl-1,8-naphthalimide, epichlorohydrin, sodium metaphosphate, epoxy compounds etc.) were already known to cause undesirable modifications to the matrices by causing functionality changes leading to cytotoxicity, chronic inflammation, calcification and fibrous encapsulation leading to its failure or occlusion. Physical crosslinking methods which crosslink through non-covalent crosslinks and biological crosslinking which make use of enzymes to form covalent crosslinks also have several drawbacks (GhavamiNejad et al., 2020; Reddy et al., 2015a). Physical methods mainly include the application of irradiation techniques that make undesirable changes to the biomaterial and sometimes compromise its mechanical properties and long-term stability. Enzymatic crosslinking also leads to residual enzymatic activity within the material and may cause cytotoxic results and reduce its stability (Hussein et al., 2017a). These crosslinking methods can cause inflammatory and immune reactions that can compromise the integration of the implants with the surrounding host tissue. These limitations created the need to identify alternative methods to impart desirable mechanical characteristics to the extracellular matrices with desirable compliance and without compromising its inherent biological activities (Salihu et al., 2021).

Improving the structural stability of ECM can be carried out by adding or incorporating synthetic or biological polymers to it. Thus stabilizing it with increased degradation

resistance and improved mechanical strength to effectively promote tissue repair or regeneration (Ijima et al., 2018). Biopolymers offer a wide range of advantages over synthetic polymers for modifying biological matrices. They include polysaccharides (cationic, anionic, or neutral), polypeptides, and polyphenols. They can be either plant-based or derived from animals that provide enhanced biocompatibility and bio-functional motifs (Mohan et al., 2016). Silk fibroin is a natural protein polymer obtained from *Bombyx mori* silk cocoon, containing two animal proteins, fibroin and sericin. Fibroin is characterized by low immunogenicity, biocompatibility, excellent mechanical strength, and controlled biodegradability (B et al., 2013; Jao et al., 2016). The biological activities of cells were not readily supported by the *B. mori* silk fibroin as it lacks inherent bioactive peptides in it. Blending with gelatin offers RGD sequence motifs that support cell adhesion, differentiation, and growth. Both the protein polymers, silk fibroin and gelatin are good candidates for blending as they carry opposite charges which helps them to bind each other by electrostatic forces (Taddei et al., 2013a). These two candidate polymers are being explored here, silk fibroin for augmenting the biomechanical stability and gelatin to modify the pliability and offer bioactive peptides (RGD-peptides).

For stabilizing the interaction between the ECM and the incorporated polymers, a green crosslinking strategy was also adopted. Citric acid (CA) is a known green covalent crosslinker that is listed in the generally regarded as safe (GRAS) category by the US FDA. CA crosslinking of protein and polysaccharides was proved to be biocompatible and provide necessary improvements in its properties. Crosslinking using CA having 3 carboxyl functional groups is one of the safer ways to modify

protein or polysaccharide-based polymers. Also, CA, a metabolic intermediate within the body, can be metabolized without producing toxic by-products. Carboxylic acids are advantageous over aldehyde-containing chemical crosslinkers in terms of cytotoxicity and providing desired tensile properties to the scaffolds. Conventionally such crosslinking is temperature-driven (150-175°C) and was carried out in the presence of phosphorus-containing catalysts (sodium hypophosphite) under low pH (3-4) (Xu et al., 2008). This way of crosslinking was not found suitable for biological scaffolds intended for regenerative applications, as the catalyst was reported to be cytotoxic and the high temperature may alter the properties of the material adversely (Jiang et al., 2010a). Therefore, an alkaline catalyzed low temperature mediated CA crosslinking between the ECM and silk fibroin followed by vacuum-assisted deposition was developed and characterized. The principle is been given in Figure 1.3.

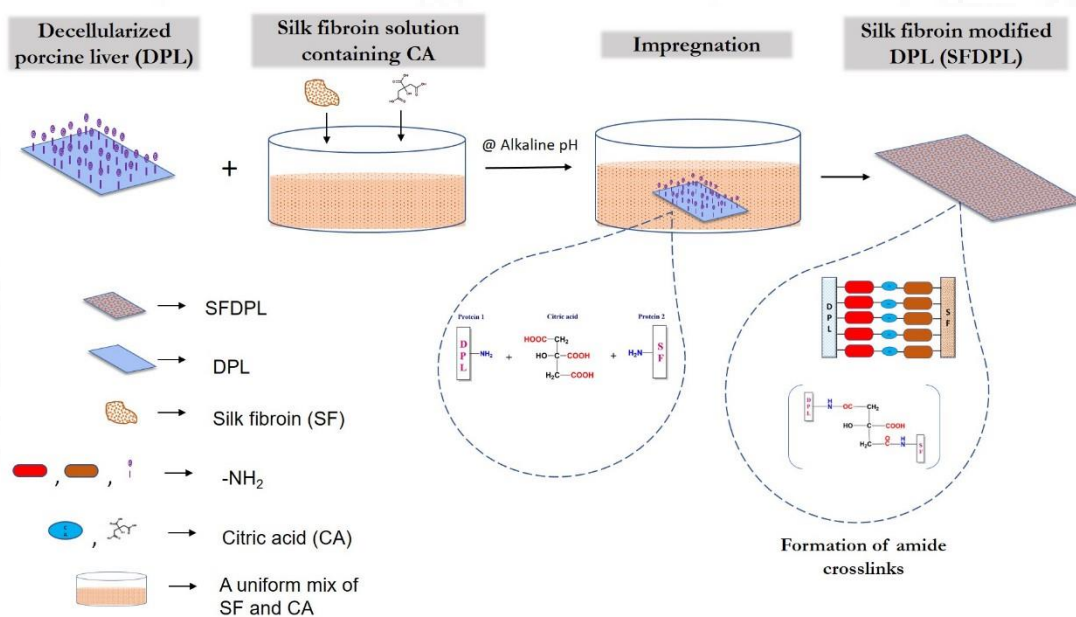


Figure 1.3. The principle and procedure for mechanical strengthening of the decellularized porcine liver matrix.

In this work, the biomechanical properties of ECM were improved by impregnation of citric acid crosslinked silk fibroin (SF) and blended silk fibroin/gelatin (SFG) in two different combinations. This can be included in the category of “citrate-based biomaterial (CBB)” which refers to any substance or material of biological origin (other than drugs) that has been chemically modified or amended with CA and is intended for use on biological systems to treat, augment or replace any tissue, organ or function of the system (Salihu et al., 2021)

Application of a suitable scaffold that provides cell attachment sites for anchorage-dependent cells is essential to promote repair or regeneration in defective tissues and organs (Griffith and Naughton, 2002; Kim et al., 2007a; Modulevsky et al., 2016a). A major hurdle in bringing a biological scaffold for a specific clinical application in medical settings is to understand its short and long-term immune reaction. The interaction of the biological graft material with the host microenvironment is influenced by various factors which is hard to study *in vitro*. Host immune response is necessary to some extent but should not lead to a catastrophic influx of immune cells (Ground et al., 2021). Immunomodulation plays a necessary role which leads to tissue homeostasis, site-directed remodeling, or repair. An effective strategy to evaluate innate and adaptive immune responses to bioscaffolds is the use of small animals, especially rats. The peculiarities of small animal studies include comparatively low cost, easiness to house them, and genetic similarity. Systemic inflammation at various time durations can be tracked by serum sampling and histopathological evaluations.

An insight into the acute or chronic phase of the host immune system towards an implanted graft can be very well understood by evaluating innate and adaptive immune responses by cell sensitization assays, cytokine release studies, and molecular biology techniques. The rat subcutaneous implantation model represents a well-accepted model to study the biocompatibility of intact matrices (Jeinsen et al., 2018). The most widely accepted definition for biocompatibility includes “the ability of a material to perform with an appropriate host response in a specific application” (*Definitions of Biomaterials for the Twenty-First Century*, 2019; Williams, 2008). The biological response associated with a scaffold is influenced not only by the antigenic determinants associated with it, but the host-material interaction and chemical changes that happen during the scaffold preparation (Prudente et al., 2016).

Both innate and adaptive arms of the host immune system are the key determinants of downstream remodeling outcomes, influenced by the physical and chemical properties of the biomaterial. The resolution of an initial inflammatory and immune reaction towards the material and deposition of site-appropriate tissue at the defect site is influenced by the proliferative potential of the infiltrated cells (Anderson, 2001; Londono and Badylak, 2015a). Briefly, the implantation of a foreign material follows a cascade of events populated by various immune cells and signaling molecules. They include acute inflammation which is characterized by a heavy influx of neutrophils within hours of implantation. After extravasation of neutrophils, cytokine release attracts monocytes. Neutrophils have short lifetimes of hours to days, but monocytes persist around the implant for longer periods and can differentiate into macrophages that become part of chronic inflammation and fibrosis. Neutrophils and monocytes

secrete various factors including a wide range of chemokines and pro-inflammatory cytokines, that promote immune cell migration, and reactive oxygen species (ROS) that greatly influence the behavior of monocytes and macrophages (Anderson and McNally, 2011; Tamassia et al., 2018). Chronic inflammation starts 4 to 7 days after implantation and is characterized by macrophages and lymphocytes, around the implant site (Anderson et al., 2008). Due to the presence of the implant and the prevailing strong acute pro-inflammatory phase, macrophages are generally activated towards a pro-inflammatory state, releasing more cytokines and ROS and attempting to phagocytose or break down the implanted biomaterial (Mariani et al., 2019). The frustrated inflammatory response promotes the fusion of macrophages to form foreign body giant cells (FBGCs) to degrade the biomaterial followed by encapsulating the material in a fibrous capsule (Sridharan et al., 2015). The degree of frustrated response determines the acceptance or integration of the biomaterial implant. In the context of biomaterial efficacy, the pro-inflammatory innate response should be minimized. The macrophage behavior during chronic inflammation dictates whether the biomaterial will undergo tissue integration or fibrous encapsulation. In the typical injury response cascade, macrophages tend to adapt a classically pro-inflammatory activation behavior and after several days shift to an alternatively pro-healing and reparative activation state. A balance between pro-inflammatory and reparative behaviors is needed for constructive tissue repair and remodeling (Davenport Huyer et al., 2020; Griffith and Naughton, 2002). Similar to the immune response following implantation of a foreign object into the body, decellularized ECM also is invaded by an abundant mononuclear cell population with neutrophil response as well. They start degrading the ECM, releasing matricryptins, exposing matricryptic epitopes, and releasing contents of

nanovesicles. Macrophages are the key players in regulating ECM degradation and remodeling. They determine the inflammatory or anti-inflammatory response based on its phenotype. The M₁ phenotype is responsible for pro-inflammatory and fibrotic responses, whereas the M₂ phenotype promotes an anti-inflammatory phase leading to repair and remodeling. There are hybrid macrophages also which exhibit the markers of both M₁ and M₂. T cells forms another important cell type in eliciting immune response to decellularized ECM (Huleihel et al., 2017). The cytokine profile of T-helper cells (T_H cells), i.e., T_{H1} response leads to cellular immunity and graft rejection, T_{H2} cells are responsible for immune tolerance against the implanted scaffold. A properly synthesized decellularized ECM scaffold promotes very little T_{H1} response and predominant T_{H2} response which can be demonstrated as an initial short-duration pro-inflammatory phase followed by organized, site-appropriate constructive remodeling (Allman et al., 2001). During two weeks post-implantation, the remodeling starts with a strong chemotactic response which stimulates the host cells to migrate within the ECM and deposits neo-ECM with stimuli to angiogenesis. Thus acellular ECM matrices induce an anti-inflammatory pro-remodeling response in-vivo with site-appropriate functional tissue formation at the anatomical defect site with reduced scar tissue formation (Sicari et al., 2014). Figure 1.4 explains these events schematically.

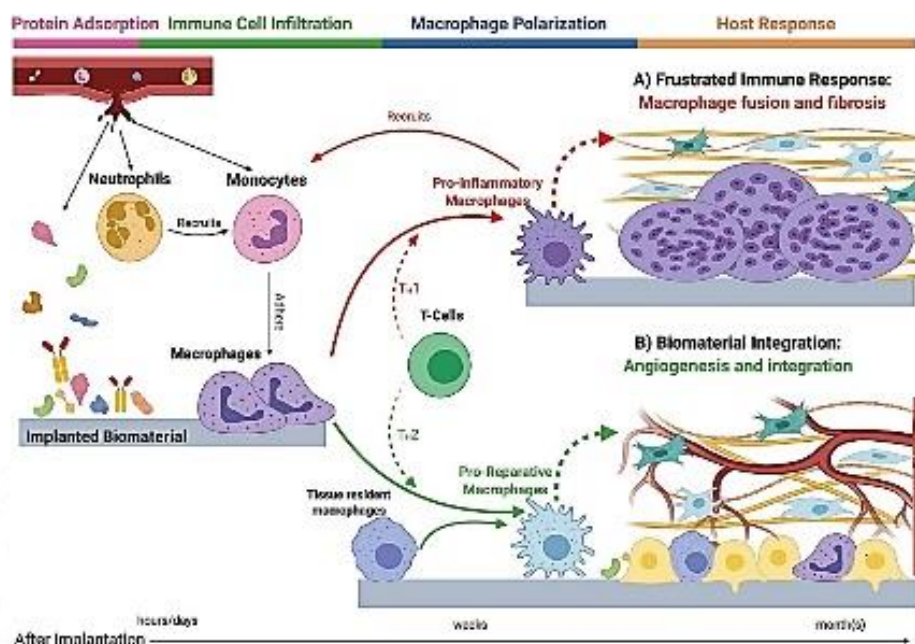


Figure 1.4. The immune-biomaterial microenvironment determining the fate of the implanted biomaterial: Schematic representation of the cascade of events following biomaterial implantation leading to either fibrosis or biomaterial integration (constructive remodeling). [Figure adapted from (Davenport Huyer et al., 2020)]

Adult rat abdominal wall defect model established in this laboratory is utilized to compare the regenerative potential of different scaffolds produced (Umashankar et al., 2013). Minor injury to muscle tissue get repaired by regeneration, but a critical-sized defect needs to be treated with effective regenerative therapy (Qu et al., 2023; Zhong et al., 2011). The composite scaffold developed in the present study was evaluated for its induced regenerative potential in abdominal wall defects created in Wistar rats. Induced regeneration refers to the recovery of the physiological structure and function of non-regenerative organs by applying appropriate cells, matrices, signaling molecules, or a combination of these (Yannas, 2015). In general, an optimal scaffold intended for tissue repair by inducing regeneration should possess features like defining a space for shaping the regenerating tissue, providing a temporal structural

function at the implantation site, stimulating progressive deposition of functional tissue and its rate of degradation should match with the rate of new tissue growth, without producing any toxic degradation products (Salerno and Netti, 2014). The regenerative effect of modified and unmodified scaffolds was studied to evaluate acute, and chronic responses by maintaining the animals implanted with the test materials for 21 days and 90 days.

The presence of an extended inflammatory response indicates poor tissue compatibility of the graft. In the present study, decellularized ECM was fabricated from porcine liver using three different detergent decellularization methods. Among the ECM scaffolds, the one that satisfied all the major criteria of decellularization, preserved ultrastructure, collagen integrity, GAG and GFs was selected. This basal ECM matrix was modified using a biocompatible modification procedure involving the incorporation of silk fibroin and fibroin-gelatin blends with citric acid-mediated crosslinking. Modified composite scaffolds were tested to prove its efficiency in modulating immune response towards healing by regeneration using in-vivo rat implantation models.

2 LITERATURE REVIEW

2.1. Bioscaffolds and regenerative medicine

Tissue engineering (TE) constitutes a division of regenerative medicine that merges biological sciences with engineering to develop functional constructs that restore, maintain, or improve damaged tissues and organs. Three cornerstones of TE are cells, scaffolds, and signaling molecules which act as triggering components to activate the inherent regenerative potential of tissues or organs. Regenerative medicine is a broad field that uses the body's systems to rebuild tissues and organs structurally and functionally with the help of foreign biological material. The scaffolds constituting tissue triads are crucial in tissue engineering, determining the efficiency to promote repair, regeneration, or remodeling. Among the natural, synthetic, and composite scaffolds, using those with biocompatibility and biodegradability for regenerative applications is appropriate (Eltom et al., 2019; Kumar and Jacob, 2022).

2.2. Decellularized extracellular matrix scaffolds

Decellularization is one of the tissue engineering strategies in regenerative medicine to manufacture biological substitutes for organ replacement therapy partially (as grafts) or completely (whole organs). Extracellular matrix (ECM) developed by decellularization forms a naturally occurring biological material that inherently possesses all the requirements such as structural and functional proteins, glycosaminoglycans, glycoproteins, membrane-bound vesicles, tissue-specific ultrastructure, mechanical properties, small bioactive molecules playing a crucial role in modulating cellular behavior in various stages of remodeling (*Comprehensive Biomaterials II*, 2017).

Decellularized ECM (DEM) is used for medical applications for more than two decades. More recently, DEM fabricated from different tissue sources such as small intestinal submucosa (SIS), urinary bladder, pericardium, cholederm, fish skin, etc. in the form of sheet, powder and hydrogels have shown great promise in promoting repair, remodeling, and regeneration (Esmaeili et al., 2023; Revi et al., 2016; Zhao et al., 2024). One of the pioneers in working on DEM to create scaffolds includes WFIRM (Wake Forest Institute for Regenerative Medicine), who constructed vaginas using decellularized intestinal submucosa to treat congenital vaginal agenesis. The engineered vaginas were able to develop into functional vaginal tissue similar to that of normal vagina (Raya-Rivera et al., 2014). Badylak's lab from the University of Pittsburgh revealed oesophageal repair and treatment of volumetric muscle loss by decellularized ECM scaffold placement at the defect site (J. Dziki et al., 2016). The role of xenogeneic ECM scaffolds in tissue reconstruction (Badylak, 2002b), host immune response towards the scaffold (Badylak and Gilbert, 2008), the detailed process of decellularization and recellularization in whole organ tissue engineering (Badylak et al., 2011), their resistance to infection (Badylak et al., 1994b) and resorbability (Badylak et al., 2001) were extensively studied and elucidated by Badylak's research group. In a comparatively recent approach, DEM from adipose tissue was 3D printed to develop a wound patch and had shown accelerated wound healing in a clinical trial to treat diabetic foot ulcer (DFU) (Kesavan et al., 2024).

As ECM scaffolds constitute an important avenue to tissue repair, addressing its major drawbacks and developing measures to improve them is necessary. Hence this review focuses mainly on various techniques to create such scaffolds, their advantages, disadvantages, and multiple approaches to modify the mechanical strength of DEM to

suit them to be used more appropriately for tissue engineering and regenerative applications.

Decellularization is the technique of natural scaffold development by which the cellular and nuclear antigens associated with a tissue or organ are removed. These antigens are responsible for rendering the material immunologically reactive by the host and eliciting adverse host responses upon implantation at host specific site. This method helps to develop an immunomodulatory natural matrix to treat various diseased or injury conditions. In short, decellularization reduces the material's immunological impact (Montoya and McFetridge, 2009). Synonym of decellularized matrices commonly found in the literature include acellular matrices (*Comprehensive Biomaterials II*, 2017). The peculiarity of using decellularized scaffolds over allogeneic or xenogeneic scaffolds is that the components associated with the ECM are conserved across species and are found to be accepted even by xenogeneic recipients upon implantation (Londono and Badylak, 2015b).

2.3. Various decellularization methods

To decellularize a tissue it is important to standardize a protocol based on the tissue taken. A decellularization procedure is a multistep cellular extraction process that involves a combination of physical, chemical, and enzymatic agents applied through appropriate methods such as perfusion, immersion & agitation, using pressure gradients, supercritical carbon dioxide, etc. (Crapo et al., 2011a) Tissues and organs differ based on their species of origin, cellular density, architecture, thickness, and overall structural organization. The decellularization procedure for each organ and tissue should be standardized considering the above features of the tissue. The critical structural and functional components should be preserved to achieve the application

for which the material is developed. The surface morphology and mechanical properties of the ECM scaffolds are greatly affected by the manufacturing process which has a significant influence on its efficiency in bringing about constructive remodeling (Crapo et al., 2011a) which is the site-appropriate functional tissue deposition at the defect site that is promoted by the degradation of the ECM scaffold..

2.3.1. Physical methods

For successful cellular removal, it is important to destroy the cellular connection with the matrix and its plasma membrane integrity. Physical methods are employed to meet this purpose and also it makes the entry of chemical cell lysing agents into the cell and its eventual removal from the tissue. Physical methods include the use of freeze-thaw cycles, applying mechanical force, mechanical agitation, delamination of tissue layers, (Freytes et al., 2004), sonication, use of pressure gradients, electroporation, and the use of dissolved supercritical fluids such as CO₂ (Guruswamy Damodaran and Vermette, 2018). Disrupting the integrity of the cell membrane causes the release of intracellular contents (Hussein et al., 2016). While applying physical methods, it is very important to choose the appropriate method and optimize it carefully, as the methods are equally disruptive to the extracellular matrix and damage to the matrix is capable of eliciting adverse responses when used for future applications (Keane et al., 2015a). Physical methods alone cannot be effective in accomplishing complete cellular removal, especially in thicker tissues, so to achieve optimum removal of cells, it should be conjugated with chemical and enzymatic methods.

2.3.2. Chemical methods

The use of chemical baths is the simplest method of decellularization. Usually, solid organs are sliced into smaller pieces, bathed, and agitated with various chemical agents

to accomplish decellularization. The major chemical decellularizing agents employed include detergents, comprising of ionic, non-ionic, and zwitterionic forms (akbari zahmati et al., 2017). Even though detergents exhibit higher decellularization efficiency compared to other chemical agents, retention of chemicals within the matrix after washing remains a major issue in bioscaffold development because these chemicals can be toxic to the cells, elicit a negative immune response, interfere with growth factor-induced bioactivity and compromises regenerative potential (Liu et al., 2022). Despite this fact, it is one of the predominant means of decellularization. Other chemical agents that are actively used for the purpose include acids, bases, specific hypotonic and hypertonic solutions, alcohols, chelating agents, and organic solvents such as tributyl phosphate (Gilbert et al., 2006a). SDS, which is the most commonly used ionic detergent for decellularization, affects live cells mainly by exerting physical and biochemical effects on the cell membrane (Singer and Tjeerdema, 1993). Another ionic detergent that is less damaging than SDS is sodium deoxycholate (SDC). There are reports that SDC is more effective in decellularizing when it is used in combination with other detergents (such as zwitterionic or non-ionic or ionic) and is easy to wash out as well (Keane et al., 2015a). Non-ionic detergents (such as Triton X-100) disrupts the lipid-lipid and lipid-protein interactions, was proven to cause complete loss of GAGs within the matrix (Gilbert et al., 2006a)

2.3.3. Enzymatic methods

Biological methods include the use of enzymes that have specificity in removing cellular and extracellular matrix elements. A few of the enzymes that are usually employed in decellularization comprise trypsin, nucleases, collagenase, lipases, dispases, thermolysin, and α -galactosidase (Liu et al., 2022). The peculiarity of

enzymatic methods is they specifically remove cells and cellular components without causing damage to other tissue components.

2.3.4. Combinational methods

Improving the penetration of decellularizing agents is important in accentuating the elimination of cellular and genetic materials. Each method described above has its own merits and demerits in removing cellular components and also harms the structure, composition, and properties of the resulting ECM. Previous research work by Rahman *et. al.*, produced human auricular cartilage ECM with a combination of SDS (chemical), DNase & trypsin (enzymatic), and freeze-thaw (physical) cycles. By merging these methods harsh impact on ECM can be minimized with maximized removal of cellular components [21, 22].

2.4. Advantages and disadvantages of extracellular matrix (ECM) scaffolds

Tissue-engineered matrices developed from various sources regulate cell functions variably, so there is a need to develop extracellular matrix mimicking scaffold (Hoshiba and Yamaoka, 2019). The removal of cells from the tissues mostly results in the formation of porous ECM with interconnected pores. These structures promote cellular adhesion to the scaffold, facilitate cellular metabolism, proper exchange of nutrients, removal of waste products generated, cellular differentiation, proliferation, penetration of blood vessels, etc. All these processes are genetically controlled which finally results in optimum regeneration. (Liao et al., 2020)

The ECM generated by decellularization constitutes, collagen, elastin, proteoglycans containing core proteins, glycosaminoglycans, and glycoproteins such as fibronectin and laminin. All these components play a very important role in contributing to matricryptins and matrikines which are crucial in providing guided regeneration

potential to the ECM scaffold. Collagen has the property to promote functional cell proliferation and angiogenesis, GAG is important in storing and releasing growth factors whenever required. Proteoglycans and fibronectin provide cell adhesion sites and help to establish stable cellular connections with the matrix. There are matrices engineered using individual ECM components and ECM-coated constructs, but native ECM is more potent in inducing cell functions compared to others (Liu et al., 2022). There are several advantages associated with DEMs which mainly include their unique structural property to provide cellular support, a pool of bioactive molecules residing within the matrix, reduced antigenic molecules to prevent severe immune rejection, ability to modulate immune reaction, undergo degradation releasing non-toxic byproducts and acts a promising organ replacement approach. Endogenous bioactive factors like growth factors, micro RNAs within the membrane-bound vesicles (MBVs), protein and lipid degradation products, etc. which are released during in vivo degradation of the DEM help the cells to physically interact with the matrix eliciting anti-inflammatory macrophage polarization and TH₂ response which leads to a pro-healing and finally a remodeling outcome (Cramer and Badylak, 2020). Major obstacles associated with DEM include the optimization of decellularization, uncontrolled degradability, insufficient mechanical strength to act as a supportive matrix for tissue regeneration, customizing scaffold properties to suit specific needs, and recellularization (McInnes et al., 2022)

2.5. Mechanical reinforcement of ECM scaffolds

The shortcomings associated with DEM scaffolds include batch-to-batch variability due to intraspecies differences and the physical and chemical properties of the tissue are difficult to control. The porosity, mechanical properties, and degradability also

vary in batches (Liao et al., 2020). Even though ECM has so many advantages, its easy degradability and reduced mechanical properties need to be addressed with biocompatible methods to effectively promote repair. The existing strategies to improve the stability of ECM include the following

2.6. Various strategies of mechanical reinforcement

Poor mechanical properties of decellularized ECM fail to provide cell attachment sites and affect the overall performance of the matrix by inhibiting structural and functional cell proliferation and differentiation. The biomechanical properties of ECM do not satisfy the criteria to be used for surgical applications. For DEM to be used for applications that need the material to be sutured, it should possess sufficient suture retention strength and tear resistance. The scaffold in general should also withstand the biomechanical load it may experience when implanted in specific in vivo environments.

Various methods adopted to improve the mechanical properties include multilamination, different crosslinking methods (physical, chemical, enzymatic), complexing with other natural or synthetic polymers, surface coating, etc. Multilamination which is stacking the sheet-like DEM in multiple layers is one of the methods to improve its mechanical properties to be used as biological patches in abdominal wall repair, musculotendinous repair, etc. This can be carried out by pressing the individual sheet-like DEMs such as small intestinal submucosa (SIS) to make it into multiple sheet forms to exhibit increased suture retention strength and tensile properties, making it suitable for tissue repair (Rajabi-Zeleti et al., 2014). Crosslinking refers to the formation of intermolecular and intramolecular crosslinks within and between extracellular matrix molecules such as collagen to prevent the

increased rate of degradation and overcome the poor mechanical strength, and also to give good structural stability to the DEM (Cao et al., 2019). Crosslinking reduces the immunogenicity of the DEM by masking various antigenic epitopes and also enhances its shelf life (Ma et al., 2014). Even though crosslinking improves the mechanical properties, most of them especially the chemical crosslinkers were found to cause undesirable changes to the ECM leading to cytotoxicity and reduced vascularization efficiency (Reddy et al., 2015b).

2.6.1. Physical crosslinking

Physical crosslinking involves the use of different irradiation techniques such as electron beam, ion beam, gamma ray, plasma, and ultraviolet rays. Such crosslinking methods offer simultaneous sterilization also without using any toxic chemicals. A safe and biocompatible physical crosslinking method is dehydrothermal treatment in which ECM scaffolds are heated under vacuum. It relies on water molecule depletion to exert crosslinking. It also brings about a reduction in immunogenicity and, an increase in mechanical integrity and pore size that make the scaffold suitable for cell attachment (Aslan et al., 2018). UV irradiation to crosslink ECM has inferior biocompatibility because of its ability to deplete growth factors and proteins (Walker et al., 2022).

2.6.2. Chemical crosslinking

Chemical crosslinking involves the use of synthetic or naturally derived agents that bridge the amino acid residues within the matrix proteins to be bonded together by covalent crosslinks. Some of the synthetic reagents used include glutaraldehyde (GA), N-hydroxysulfosuccinimide, 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide, 4-aminoethanol-N-hydroxyethyl-1,8-naphthalimide, epichlorohydrin, sodium

metaphosphate, epoxy compounds, etc. The principle of crosslinking by these agents has been described in detail by Hussein *et al.* (Hussein *et al.*, 2017b). The most renowned chemical crosslinker is GA, due to its water solubility, high crosslinking efficiency, and immunomodulatory properties. The resistance of ECM to chemical and enzymatic agents is significantly improved after GA crosslinking. They are associated with increased mechanical strength and stiffness, but hurdles such as immunogenicity, loss of glycoproteins, cytotoxicity, and calcification are associated with GA-processed DEM in bioprosthetic heart valves. (Schmidt and Baier, 2000). Natural crosslinkers such as genipin, quercetin, chondroitin sulfate, procynidins and enzymatic crosslinkers such as transglutaminases, and peroxidases are used as alternatives to overcome the GA related shortcomings. Dose-dependent cytotoxicity of such agents and adverse responses associated with remnant crosslinkers still need to be addressed (Gao *et al.*, 2019a)

2.6.2.1. Citric acid crosslinking

A cytocompatible way of improving the mechanical strength and stability of protein and polysaccharide-based biomaterials is by using carboxylic acids such as citric acid. This crosslinking allows the formation of ester bonds and amide bonds in polysaccharides and proteins respectively, leading to better haemocompatibility and also desired improvement in its properties. Conventional method of such crosslinking is carried out at very high temperatures (150-175°C) and involve cytotoxic mediators (such as sodium hypophosphate) (Yang *et al.*, 1997). Recent studies have proven alkaline catalyzed low temperature mediated crosslinking of CA in both wet and dry conditions (Reddy *et al.*, 2009a). The peculiarity of CA crosslinking over aldehyde-containing crosslinkers includes its non-toxicity and low cost. There are already

proven reports of crosslinking soy protein, gliadin (Reddy et al., 2009b), zein (Jiang et al., 2010b), starch (Reddy and Yang, 2010), chitosan (Jiang et al., 2023), etc., with CA that possessed promising results and advantages in tissue engineering

2.6.3. Enzymatic crosslinking

Crosslinking with enzymes is carried out using transglutaminase (TG), horse radish peroxidase (HRP), mushroom tyrosinase (MT), etc. Taddei *et al.* used TG and MT to crosslink silk fibroin/gelatin films (Taddei et al., 2013b). TG facilitates amide bonding between glutamine and lysine residues and promotes angiopoiesis and fibronectin deposition (Wang et al., 2013). HRP crosslinks by dityrosine links or isodityrosine linkages (Chirila et al., 2017). The advantages of enzymatic crosslinking compared to chemical crosslinkers is that the crosslinking needs only mild aqueous conditions and the reaction can be controlled by changing temperature, pH, or ionic strength (Johari et al., 2020).

Various crosslinking strategies

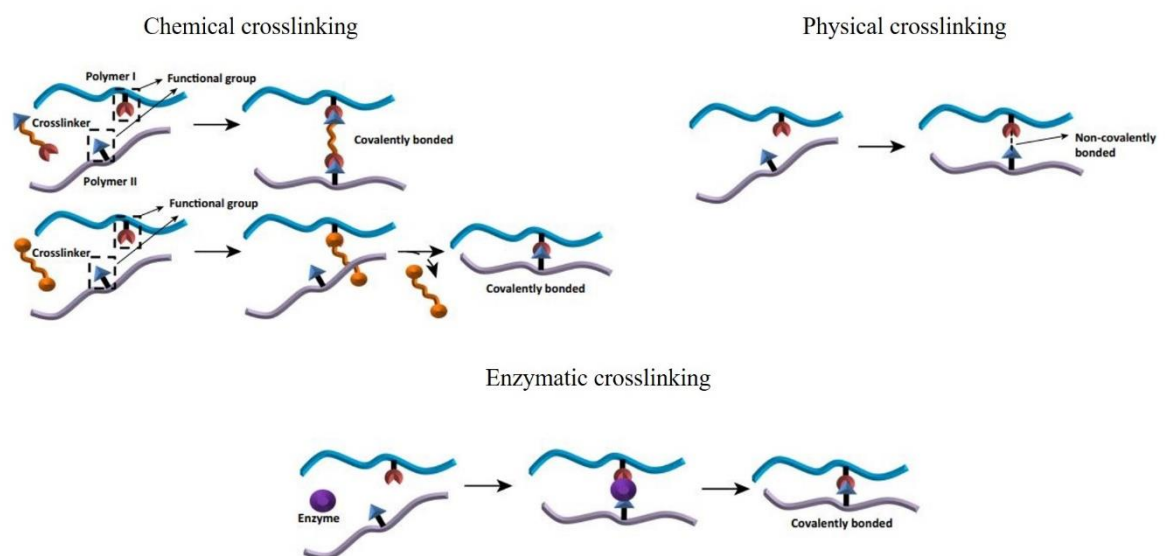


Figure 2.1: Schematic representation of the principle behind three methods of crosslinking. [Figure adapted from (Reddy et al., 2015b)]

2.6.4. Crosslinking mediated by natural crosslinking agents

Genipin (Wang et al., 2016), quercetin, procyanidins, nor-dihydroguaiarctic acid, and tannin acid are some of the natural crosslinking agents (Ma et al., 2014). Major advantages of using natural crosslinking agents are their extremely low cytotoxic effects, reduced enzymatic degradation by improving the mechanical properties and stability, no calcification, promotes cell migration as well as angiogenesis, better hemocompatibility, and anti-inflammatory potential (Wang et al., 2016). Genipin crosslinking of collagen/chitosan scaffolds has reduced its swelling behavior and increased the degradation resistance without affecting cell viability (Bi et al., 2011). Genipin is an aglycone synthesized from *Gardenia jasminoides*, Ellis plant. It promotes angiogenesis and cell migration leading to beneficial tissue repair. Procyanidins are abundantly present in fruits, leaves, bark, flowers, and vegetables of different plants. The biomaterials crosslinked with PC show less toxicity and absence of calcification (Hussein et al., 2017b)

2.6.5. Hybrid scaffolds

A rapidly degrading matrix does not allow tissue to grow properly and those degrading slowly may result in fibrosis. Combining ECM with natural or synthetic polymers is an important way to enhance the biomechanical properties, biocompatibility, sufficient stability, and bio-functionality of ECM scaffolds. This can be achieved by adding natural or synthetic polymers to it, making it into a composite scaffold that improves stability and degradation resistance. Poly 1,8 octane diol citrate (POC) integration with the ECM offers mechanical integrity (Jiang et al., 2015). Other biodegradable

polymers such as polycaprolactone (PCL) and poly lactic-co-glycolic acid (PLGA) showed suitable properties like enhanced stem cell migration and growth with increased biomechanics of ECM (Ghorbani et al., 2017). There are proven reports of modification of dECM with natural polymers /polysaccharides such as silk fibroin, gelatin, chitosan, etc., to produce biofunctional hybrid scaffolds with biocompatibility, enhanced biomechanical properties and antibacterial properties for skin wound healing (Dhasmana et al., 2019a; Xu et al., 2021)

2.6.5.1. Biopolymers

Biopolymers offer a wide range of advantages over synthetic polymers to combine with DEM scaffolds and develop composite scaffolds. Implant materials derived from organic polymers of natural origin are associated with immunogenicity and the absence of inherent bioactivity. Combining it with DEM mutually complements each other in such a way that it provides biofunctional motifs and signaling molecules such as growth factors, in turn, biopolymers offer improved mechanical strength and stability. Biopolymers are associated with high biocompatibility, biodegradability, and low toxicity. Natural polymers being incorporated with DEM to develop functional composite scaffolds include natural polysaccharides (alginate (Varaprasad et al., 2020), hyaluronic acid (Shen et al., 2023), chitosan (“(PDF) A Sponge-Like Double-Layer Wound Dressing with Chitosan and Decellularized Bovine Amniotic Membrane for Promoting Diabetic Wound Healing,” 2024)) and natural proteins (collagen (Hong et al., 2019), gelatin (Kara et al., 2022), silk fibroin (Dhasmana et al., 2019a), etc.). The present study employed silk fibroin and gelatin to impart increased biomechanical strength and degradation resistance to the ECM-based porcine liver scaffold. Silk fibroin isolated from *Bombyx mori* silk cocoon has enormous mechanical strength and

structurally it contains mainly two types of proteins, fibroin and sericin (Barbosa and Martins, 2017; Rockwood et al., 2011a). Fibroin has the long history of being used for biomedical applications and is an FDA approved biomaterial as well.

2.7. Sterilization

A biological scaffold has the potential to be infected by a wide range of microorganisms and their endotoxins increase the immunogenicity of the scaffold. Utilization of an appropriate sterilization method during the manufacture of DEM scaffolds is very important to avoid infection and immunological rejection. The most commonly employed agents to bring about efficient sterilization of DEM scaffolds include ethanol, antibiotics, antifungals, peracetic acid (PAA), ionizing radiations, ethylene oxide (ETO), propylene oxide, and supercritical CO₂ (de Sousa Iwamoto et al., 2022). Antibiotics such as penicillin, streptomycin, and antimycotics like amphotericin B are the most commonly used disinfectants in the decellularization process to preserve ECM. 70% ethanol brings about polypeptide denaturation but bacterial spore destruction cannot be done by this method. PAA has shown promise in the sterilization of ECM scaffolds and is cytocompatible too. The physical sterilizers like ionizing radiations induced denaturation of nucleic acids to clear out pathogens. However, their application is limited because it releases cytotoxic lipid-derived particles and may damage ECM ultrastructure as well. ETO sterilization was proved to be carcinogenic, toxic and releases immunogenic residues that compromise the biocompatibility of the scaffold. The sterilization method should be selected based on unique properties and the intended clinical application of the DEM scaffold (Tao et al., 2021).

2.8. Inflammatory and immune response to biologic scaffolds

An important aspect of developing new biomaterials in tissue engineering is to evaluate the inflammatory and immune potential resulting from the implantation of those materials. The material must be immune-interactive rather than immune-evasive to support its integration with the host tissue and promote healing by regeneration. Biological responses to biomaterials have been extensively evaluated by James Anderson (Anderson, 2001; Anderson et al., 2008). The inflammatory and immune response starts with the injury caused by its introduction. The response can be due to the injury and the presence of the foreign material. Acute inflammation following injury starts with the recruitment of polymorphonuclear cells (PMNCs), by complement activation and recognition of damage-associated molecular patterns (DAMP) by pattern recognition receptors (PRRs) of host cells that lead to the release of inflammatory cytokines such as IL-1 β , IL-6, and TNF- α . Monocytes infiltrated into the injury site start differentiating into macrophages. PMNCs and macrophages clear cellular debris and pathogens and release more and more inflammatory cytokines, exaggerating the inflammation. During this acute inflammatory phase, macrophages are differentiated towards an M₁ phenotype, the proinflammatory type which is responsible for amplifying the local inflammation and release of matrix metalloproteinases that are responsible for graft degradation. Based on the immunological trait of the implant, an anti-inflammatory response occurs when the T regulatory (Treg) cells modulate the shift in macrophage subtype to M₂. M₂-associated cytokines support immunoregulation and tissue remodeling. The interplay with the adaptive immune system takes place through the mediation of T_H-cells. Activation of complement cascades by DAMPs acts as chemoattractants to T_H cells. They migrate towards the graft, activate cytotoxic T lymphocytes (CTLs), and trigger a humoral

immune response by activation and maturation of B-cells. Most important cause of graft rejection in transplantation is mediated by antibodies (Davis and Cooper, 2017). Interaction between the antigens released from DEM and the antibodies produced further activates the complement cascade, leading to scaffold failure. The decellularization procedure aims to reduce or inhibit DAMP by preserving ECM structure, thus escaping the destructive responses from the host's innate and adaptive immune systems (Crupi et al., 2015; Kasravi et al., 2023; Vasconcelos et al., 2019; Zhong et al., 2023).

2.9. Tissue response to biologic scaffold,

Host tissue response to biological scaffolds composed of xenogenic ECM varies depending upon the variability in the preparation of the scaffold and post-processing methods adopted (such as crosslinking, sterilization, residual chemical removal, packaging, and storage conditions). Host response to biological scaffolds is influenced by various factors associated with it. That includes its source animal, composition, extent of decellularization, ligand landscape, alpha-Gal epitope (α Gal), DNA content, DAMP molecules, chemical crosslinking, and ECM proteins & their degradation products and the host micro-environment (Londono and Badylak, 2015b; Lopresti and Brown, 2015). Biologic scaffolds have shown constructive, functional tissue restoration of site-appropriate tissue, in a majority of pre-clinical animal studies and most of the human clinical trials. Based on the variations in manufacturing processes and source tissue, alternative outcomes associated with such scaffolds on in-vivo implantation include serous fluid accumulation, foreign body response, scar tissue formation, calcification, necrosis, fibrosis, etc. The bioactive ECM components exhibit high cross-species homology and, hence do not elicit adverse inflammatory

responses on xenotransplantation. The same decellularization can have different impacts on the decellularized scaffold from different source tissues (VeDepo et al., 2017). There are significant differences in host response elicited by scaffolds prepared from younger and older source animals. A constructive remodeling with predominant anti-inflammatory macrophages was shown as a response to young ECM. A stark contrast in remodeling with no evidence of structural tissue formation was observed with ECM developed from older animals (Sicari et al., 2012). Inadequate removal of cellular and nuclear components leads to intense inflammatory responses that cause poor remodeling outcomes (Keane et al., 2012a). Mineralization of the implanted material is one of the adverse outcomes of the most commonly used crosslinking agent, glutaraldehyde (Manji et al., 2006). It is the formation of nodular deposits of calcium-containing compounds within the scaffolds is termed mineralization or calcification (Schoen et al., 2020). The degradation products of ECM such as bioactive cryptic peptides, embedded growth factors, and storage products of MBVs (proteins, mRNA, and lipid cargo) act as chemoattractants to progenitor cells, recruit and activate pro-remodeling macrophage phenotype, promote angiogenesis and antimicrobial activity. Prevention of their degradation by crosslinking may lead to foreign body reactions. Commercially available ECM products which chemically crosslinked were invariably associated with chronic responses and fibrous encapsulation. Adhesion surface area and adhesion tenacity exhibited by non-crosslinked ECM scaffold was significantly lower compared to crosslinked ones in ventral hernia repair model (Kaufmann et al., 2019).

2.10. Induced regeneration

ECM scaffolds developed from natural tissues enhance endogenous tissue differentiation leading to regeneration. They have superior biocompatibility and promote favorable immune responses due to the inherent inductive niche present within the matrix (Zhu et al., 2019). An ideal ECM scaffold that is thoroughly decellularized, and sterile can promote a series of in-vivo events following implantation. This is termed constructive remodeling which is characterized by timely degradation and gradual replacement of site-appropriate functional tissue. Such an outcome involves the following events

- 1) Degradation of the scaffold to release bioactive molecules
- 2) Immunomodulation of host immune response to a pro-remodeling and regulatory type 2 phenotype
- 3) Recruitment and differentiation of endogenous host-specific stem/progenitor cells
- 4) Deposition of functional tissue (Cramer and Badylak, 2020; J. L. Dziki et al., 2016)

2.11. Present status of research in the area

The decellularized extracellular matrix has been an effective scaffold in many translational applications in various forms such as films, hydrogels, powders, microparticles, etc. It is an FDA-approved biomaterial also. ECM not only provides a scaffold for cellular attachment but host immune degradation releases many hidden bioactive molecules such as matricryptic peptides and matricryptins, growth factors, and release products of membrane-associated nanovesicles (MBVs) which in turn creates a pro-regenerative environment crucial for healing by regeneration (or constructive remodeling). There is an emerging need to fabricate bio-engineered

organs to replace the demand for functional organs. Even though there are various decellularization strategies, a method that preserves the ECM-associated biomolecules such as growth factors need to be developed and evaluated to retain the bioactivity of the scaffold. In the same way, the drawbacks associated with such scaffolds, such as their untimely degradation and weak mechanical strength also should be addressed without compromising their bioactivity.

2.12. Aim of the study

The present research work aims to develop a decellularized scaffold with maximum preservation of growth factors and minimum adverse impact on the ECM structure and biochemical and biomechanical properties. The ECM scaffold developed will be modified to augment its mechanical and degradative resistance in a biocompatible way. The modified scaffolds were aimed to characterize in-vitro and in-vivo for confirming their cytocompatibility, cell migration potential, inflammatory & immunocompatibility, and regenerative efficacy.

2.13. Hypothesis

Develop a decellularized and mechanically strengthened porcine liver scaffold that maximally preserves growth factors and to study its potential to induce constructive remodeling

2.14. Objectives

1. Development of decellularized porcine liver scaffold (membranous form) which maximally preserves the candidate growth factors and structural components and its mechanical, chemical, and biological characterization.
2. Improving mechanical strength of the decellularized liver scaffold by impregnation with biocompatible natural polymers.

3. Evaluation of inflammatory and immunogenic potential of mechanical strength improved decellularized porcine liver scaffold by rat subcutaneous implantation
4. Studying the phenomenon of induced regeneration in adult mammalian models such as rat abdominal wall regeneration

3 MATERIALS AND METHODS

The research problem was addressed sequentially under each objective. Thus, for achieving each objective formulated in the present study, the following methodologies were adopted and executed sequentially.

3.1. Porcine liver collection, decellularization, and its characterization

3.1.1. Materials

Ampicillin/Cloxacillin (Megapen^R, Himedia), Amphotericin B (A011, Himedia), Sodium dodecyl sulfate (GRM205, Himedia), sodium deoxycholate (GRM6216, Himedia), Triton X-100 (MB031, Himedia), Trypsin (TCL006, Himedia), Haematoxylin, Eosin (E4009, Sigma-Aldrich), DAPI, Agarose (MB080, Himedia), Ethidium bromide, HiPurA[®] Mammalian Genomic DNA Purification Kit (MB506, Himedia), Folin & Ciocalteu's phenol reagent (RM10822, Himedia), Coomassie Brilliant Blue G-250 (27815, Sigma-Aldrich), proteinase K (MB086, Himedia), chondroitin sulfate A sodium salt from the bovine trachea (C9819, Sigma). For estimating growth factors, Elisa kits were bought from MyBioSource, U.S.A., Biebrich scarlet (RM3204, Himedia), acid fuchsin (A3908, Sigma), methylene blue (M7766, Sigma), phosphotungstic acid (P4006, Sigma-Aldrich), phosphomolybdic acid (GRM629, Himedia), alcian blue (A3157, Sigma), dimethyl methylene blue (341088, Sigma-Aldrich), Elisa kits to estimate growth factors (MBS2510167 & MBS266673, MyBiosource).

3.1.2. Collection of porcine liver

The porcine liver with minimum bioburden was collected from adult pigs slaughtered for meat in a local slaughterhouse. Immediately after receiving the liver in clean rectangular glass dishes, it was washed in running water and sliced into desired-sized pieces with 5 mm thickness. The slices were then appropriately packed and immediately stored by plunging in cryocan containing liquid nitrogen (-196°C) until proceeding with the decellularization procedure. On the day of decellularization, the liver slices were taken out from liquid nitrogen and slowly thawed under refrigeration. This was followed by the decellularization procedure.

3.1.3. Preparation of decellularized porcine liver scaffold (DPL)

3.1.3.1. Method 1

The thawed liver segments were washed twice with cold PBS containing 500mg ampicillin, 500 mg cloxacillin, and 0.1% amphotericin B for 1hr each under vigorous agitation using a shaking incubator (at 200 to 250 rpm; MAXQ 4450 shaker, Thermo Scientific). To avoid microbial growth, all solutions contain ampicillin, cloxacillin, and 0.1% amphotericin B solutions. Detergent treatment with 0.1% sodium dodecyl sulfate (SDS) was done at room temperature (25-30°C) with at least 2-3 changes daily until complete cellular removal was achieved (Park et al., 2013a). Finally, samples were washed with PBS for 2 to 3 days, to remove residual detergents in the decellularized liver matrix. They were sterilized by keeping in 70% ethanol at 37°C for 14 days followed by storage at 4°C.

3.1.3.2. Method 2

In this method, the step-by-step procedure carried out for decellularization was the same as that of method 1. Here the detergent used for decellularization was a combination of 0.05% SDS and 0.05% sodium deoxycholate (SDC) followed by

washing to remove residual detergents, sterilization, and storage (as described in method 1).

3.1.3.3. Method 3

Fresh porcine liver tissues of slices of 0.5 cm thickness were continuously rinsed in ultrapure water for one hour. Then, undergone digestion with 0.025% trypsin for 30 minutes at 37°C, followed by treatment with 1% Triton X-100 solution for 24 h. Wash with ultrapure water for another 24 hours. 0.1% SDS was used to treat the washed liver slices for another 24 hours until the liver tissue slices became white and transparent. Finally, washing was followed by sterilization and storage (Mao et al., 2020).

3.1.4. Characterization of decellularization

3.1.4.1. Cellular removal

3.1.4.1.1. H&E staining

Completely decellularized and native samples were fixed in 10% neutral buffered formalin for a week, followed by short acetone processing and xylene treatment, which was then followed by embedding in paraffin wax. The embedded samples were cut into 5-micron thick sections in microtome (Model RM 2125, Leica). The sections mounted on glass slides were deparaffinized in xylene and hydrated by immersing in a graded series of alcohol (100% and 70%) followed by dH₂O and stained with Haematoxylin (Harris Haematoxylin) and Eosin. Finally, the stained sections were dehydrated, cleared in xylene, and mounted in DPX mountant. Microscopic images were acquired using the Olympus CX41 microscope and Progres CapturePro 2.10.0.1 software.

3.1.4.2. Removal of DNA

3.1.4.2.1. DAPI staining

To find the presence of any DNA remnants retained within the acellular matrices, DAPI staining was carried out. Dissolve 10mg DAPI (4',6-Diaminidino-2-phenylindole hydrochloride in 2 mL N, N-dimethylformamide to make a 14.3 mM stock solution. From the stock solution, 300µM intermediate stock was prepared in PBS. 1000 times dilution of intermediate stock made a working solution of DAPI, which was used to stain deparaffinized and hydrated sections in the dark at room temperature for 5 minutes. After removing excess staining by washing in PBS, the sections were mounted in glycerol and viewed under an inverted fluorescence microscope (Leica DMI3000B), and images were captured using LAS. V4.3 software.

3.1.4.2.2. DNA extraction

HiPurA® Mammalian Genomic DNA Purification Kit was used for DNA extraction from native and decellularized samples in triplicates. The extraction procedure was followed as per the directions given by the manufacturer. Briefly, 25 mg tissue was cut into smaller pieces, and lysis AL solution was added to it, followed by vortexing. Proteinase K was added, and after vortexing, the sample was incubated at 55°C until complete digestion. After complete digestion, the sample was incubated with RNase A solution and lysis C1. 100% ethanol was added, and the sample was loaded in a HiElute miniprep spin column and centrifuged and washed twice in diluted wash solution and centrifuged. Finally, the elution buffer was added and centrifuged, and the eluate was transferred to a fresh collection tube and stored at -20°C until further estimation was done.

3.1.4.2.3. DNA estimation by Nanodrop method

Total DNA in the extracted samples was quantified by measuring the absorbance using a Nanodrop spectrophotometer (Nanodrop ND1000, Thermoscientific). The absolute amount of DNA per mg of tissue was calculated.

3.1.4.2.4. Agarose gel electrophoresis (AGE)

Agarose gel electrophoresis was carried out to determine the fragment size, quality, and purity of the extracted DNA. DNA samples were added to each well of a 1% agarose gel along with sample loading buffer. The gel was immersed in 1X TAE buffer (Tris base, acetic acid, EDTA) and run by applying a voltage of 100 V for 30 minutes between the electrodes. The bands obtained were visualized using Gel Doc by making use of the staining agent ethidium bromide (0.05 µg/ml of agarose solution). DNA ladder of known fragment size was run as standard. The image of the gel was acquired using Gel Doc.

3.1.4.3. Removal of protein

3.1.4.3.1. Extraction of protein

Samples frozen at -80°C were thawed, and 100 mg each (N=3) of native and decellularized tissue was homogenized in ice-cold lysis buffer containing 0.05M Tris HCl and 0.002M EDTA in a tissue homogenizer along with protease inhibitor cocktail. Tissue homogenate was transferred to sterile prechilled microcentrifuge tubes for centrifugation (5000rpm, 10 minutes, 4°C), and the supernatant containing extracted protein from ECM was collected and stored at -20°C until used for further estimation.

3.1.4.3.2. Estimation of protein (Lowry's method)

The protein content was estimated using Lowry's method. Briefly, the procedure involved thawing the extracted protein by keeping it on ice. 2 mL of alkaline carbonate solution (containing 2% Na₂CO₃, 0.1N NaOH, 1.56% CuSO₄.5H₂O, 2.37% NaK

tartrate) was added to 50 µL of the protein sample and incubated for 30 minutes, followed by adding diluted Folin & Ciocalteu's phenol reagent. The absorbance was measured spectrophotometrically at 660 nm. The protein concentration was calculated using the standard graph prepared by using known concentrations of bovine serum albumen. Absorbance was measured spectrophotometrically at 660 nm and compared with the standard graph prepared using known concentrations of bovine serum albumen and the concentration of the unknown sample was calculated from the linear regression plot (Shen, 2023).

3.1.4.3.3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Done as per Biorad protocol for SDS PAGE. Briefly, 30% acrylamide was being used. The stacking and resolving gels prepared were 4% and 7% respectively to run protein samples in tris-glycine-SDS buffer at an electric current of 100 V. A standard protein marker (Takara, USA) was used to compare the molecular weight of intact protein bands obtained after electrophoresis, carried out in the Biorad electrophoresis unit. The gel stained using Coomassie Brilliant Blue G-250 was followed by de-staining in a solution containing methanol, acetic acid, and water followed by capturing its image using GelDoc.

3.1.4.4. Retention of collagenous architecture

3.1.4.4.1. Masson's trichrome staining

Completely decellularized and native samples were fixed in 10% neutral buffered formalin for two days, and short acetone processing and xylene treatment were followed by embedding in paraffin wax. The embedded samples were cut into 5-micron thick sections in microtome. The sections mounted on glass slides were

deparaffinized in xylene and hydrated by immersing in a graded series of alcohol (100%, 70%, and dH₂O) and then fixed in Bouin's fluid (picric acid, formalin, and glacial acetic acid) followed by staining with Haematoxylin. Staining was continued with Biebrich scarlet-acid fuchsin followed by incubation in combination of phosphotungstic acid-phosphomolybdic acid and in aniline blue solution continued by rigorous washing in 1% acetic acid to remove unbound dyes. Before coverslipped, the sections were dehydrated and cleared in xylene. The mountant used was DPX. Microscopic images were acquired using an Olympus CX41 microscope and Progres CapturePro 2.10.0.1 software.

3.1.4.5. Preservation of Glycosaminoglycans

3.1.4.5.1. Alcian blue staining

Alcian blue stains both sulfated and carboxylated acid mucopolysaccharides and sialomusins. Alcian blue staining was done following the manufacturer's instructions given in Alcian Blue 8GX. Shortly, acetic acid-alcian blue solution was prepared at a pH of 2.5. Formalin-fixed, paraffin-embedded tissue sections were deparaffinized, rehydrated conventionally, and progressed through alcian blue staining for 30 minutes. Nuclear fast red aluminium sulfate counterstaining was done following dehydration and clearing. Finally, sections were mounted in DPX mountant and observed under an Olympus CX41 microscope fitted with Progres CapturePro 2.10.0.1 software.

3.1.4.5.2. Dimethylmethylene blue (DMMB) assay

To quantify the GAG content in both native and decellularized samples, dimethyl methylene blue assay (DMMB assay) was carried out. Briefly, 30 mg dry tissue was digested in a 50 µg/mL proteinase K solution in 100 mM K₂HPO₄ pH 8.0 at 56°C overnight. Proteinase K was then inactivated by heating the preparation for 10 minutes

at 90°C. After centrifugation, digested tissue was filtered through an ultra-free filter to eliminate DNA and tissue debris from the extract. This preparation was used for sulfated GAG quantification (Barbosa et al., 2003). 20 µL sample taken in 5ml capped vials were made up to 1 mL using distilled water. DMMB solution was added and vortexed vigorously, and the absorbance was measured using a spectrophotometer (Shimadzu, UV-1700 Pharmaspec) at 525 nm immediately (Farndale et al., 1986). The absolute amount of GAG per mg of dry tissue was calculated from the standard plot prepared using chondroitin sulfate A sodium salt from the bovine trachea.

3.1.4.6. Growth factor preservation

3.1.4.6.1. Sandwich Elisa

The growth factors retained within native and decellularized tissue samples were evaluated using ELISA kits to specifically detect porcine HGF and bFGF following the manufacturer's instructions. Soluble proteins extracted from native and decellularized tissue were analyzed for GF content. Briefly, the tissue was minced into small pieces, rinsed in ice-cold PBS (0.01M, pH 7.4), and then homogenized in PBS (1 g: 9 mL PBS) with an electric homogenizer (Polytron) on ice. The tissue homogenates were centrifuged for 5 minutes at 5000-10000 rpm to get supernatant. The supernatant was used either for immediate testing or placed at -20°C/-80°C (1-3 months) for storage. Total protein content was estimated using Lowry's method. The kit utilizes the double antibody sandwich ELISA technique. The pre-coated antibody is an anti-porcine HGF/bFGF monoclonal antibody, while the detection antibody is a biotinylated polyclonal antibody. After incubation, samples and biotinylated antibodies are added into ELISA plate wells and washed out with PBS. Then, avidin-peroxidase conjugate was added to the wells. TMB (3,3',5,5'-Tetramethylbenzidine)

substrate reacts with peroxidase to form a blue-coloured product and finally turns yellow after the addition of the stop solution. The GF content was estimated by comparing the absorbance obtained for the respective test samples with the standard graph plotted with known concentrations of the target analyte (i.e., HGF/FGF).

3.1.4.7. Mechanical properties

3.1.4.7.1. Tensile testing

A comparison of the tensile properties of decellularized porcine liver slices developed by three detergent decellularization protocols was analyzed using a universal tensile testing machine, mainly for stress-strain behaviour. Decellularized and hydrated liver tissue strips of 50x10 mm size were prepared and subjected to uniaxial tensile testing using an Instron UTM machine. The testing conditions provided were 23°C and 60% humidity, a 5 mm/min crosshead speed, and a full-scale load range of 100 N.

3.1.4.8. Ultrastructure

The surface topography of the extracellular matrix scaffold produced by the three methods of decellularization, SDS DPL, SDS+SDC DPL, and TX100 DPL, was analyzed using scanning electron microscopy (SEM). The sample preparation involved fixation in 3% glutaraldehyde overnight by keeping it at 4°C, washing in PBS, and finally dehydration in ascending series of alcohol (30%, 50%, 70%, 90%, and 100%) for at least 15 minutes in each. Isoamyl acetate was added to the samples, followed by critical point drying (HCP-2 Critical point dryer, Hitachi) for 20 minutes. The dried samples were firmly fixed on aluminium stubs and coated with gold and finally viewed under SEM, and images were acquired (Cengiz et al., 2019a).

3.1.4.9. Cytotoxicity

Cell culture studies were performed using mouse fibroblast cell line (L-929, American Type Cell Cultures, and U.S.A.). Cells were cultured in Minimum Essential Medium containing 5% fetal bovine serum (South American Origin) and 100IU/mL penicillin and 100 µg/mL streptomycin in a CO₂ incubator (New Brunswick, Eppendorf India) set at 37°C, 5% CO₂ and 95% relative humidity. Confluent cells were harvested using trypsin (0.25%) –EDTA (0.02%), and 5×10⁴ cells were seeded to 96 well plates for experiments. Test samples immersed in ethanol was rinsed with serum-free culture media three times before the test.

3.1.4.9.1. Direct contact

An *in-vitro* cytotoxicity test using the direct contact method was performed based on ISO 10993-5. The culture medium from the L-929 monolayer was replaced with fresh medium. Test samples, negative and positive controls in triplicate were placed on the cells. After incubation at 37±1°C for 24 h, the cell monolayer was examined microscopically for the response around the test samples. The reactivity was graded as 0, 1, 2, 3, and 4 based on the zone of lysis, vacuolization, detachment, and membrane disintegration. The cytotoxic reactivity of test and control samples was evaluated under an inverted phase contrast microscope. UHMWPE was used as negative control and PVC disc reinforced with tin as positive control.

3.1.4.9.2. Test on extract

An *in-vitro* cytotoxicity test in the L929 fibroblast cell line, using a test on extract method was performed with test samples SDS DPL, SDS+SDC DPL, and TX100 DPL based on ISO 10993-5. The extract was prepared by incubating the test samples in DMEM containing FBS at 37±1°C for 24 h at 6 cm²/mL extraction ratio. Cells cultured in a typical standard medium were considered cell control. 100% extracts were

prepared by incubating the test material in serum-free media. 100% extract of test samples, positive control (UHMWPE), and negative control (phenol), in triplicate, were added to a subconfluent monolayer of L-929 cells. Cell culture was examined microscopically for cellular response after incubation of cells with extracts of the test sample and controls at $37\pm 1^{\circ}\text{C}$ for 24 h. Cells were examined microscopically using an inverted phase contrast microscope, and cellular responses were scored as 0, 1, 2, 3, and/or 4.

3.2. Mechanical reinforcement of decellularized porcine liver matrix (DPL)

The method involved the preparation of regenerated SF and gelatin solutions as detailed below.

3.2.1. Materials

B. mori cocoon (purchased from Sericulture Division, Tenkasi, Tamil Nadu, India), Citric acid (GRM1023, Himedia), Gelatin Type A from porcine skin (MB169, Himedia), Lithium bromide (746479, Sigma-Aldrich), dialysis tubing (MEMBRACEL, 3.5KDa MWCO, Serva), ethanol (MB228, Himedia), Haematoxylin, Biebrich scarlet (RM3204, Himedia), acid fuchsin (A3908, Sigma), methylene blue (M7766, Sigma), phosphotungstic acid (P4006, Sigma-Aldrich), phosphomolybdic acid (GRM629, Himedia), alcian blue (A3157, Sigma), dimethyl methylene blue (341088, Sigma-Aldrich), Ninhydrin (GRM 248, Himedia), Collagenase enzyme from *Clostridium histolyticum* (C6885, Sigma), Cell compatibility studies included fibroblast cell line (L-929, American Type Cell Cultures, and USA), Minimum Essential Medium (MEM: Gibco, USA), 5% fetal bovine serum (South American Origin, Himedia, India), penicillin and streptomycin (Gibco, USA), MTT (TC191, Himedia).

3.2.2. Preparation of silk fibroin (SF) and gelatin (G) solutions containing citric acid

Silk fibroin (7-8%): Silk fibroin (SF) was obtained by degumming *B. mori* silk cocoons (purchased from Sericulture Division, Tenkasi, Tamil Nadu, India) at 120°C for 30 minutes to remove sericin. Dried SF was dissolved in 9.3M LiBr for 4 h at 60°C to obtain silk fibroin (SF) solution. The solution was dialyzed against distilled water for 48 hours until the complete removal of salt. After dialysis, the solution obtained was centrifuged to remove any flocculants or particles to get a clear fibroin solution. Its w/v was determined and stored at 4°C until used (Rockwood et al., 2011b). 7% citric acid (w/w) was added to the corresponding w/v% of regenerated silk fibroin (RSF) solution obtained after dialysis. The solution was stirred to ensure complete dissolution. The solution may get coagulated as the stirring progresses. Sodium hydroxide (NaOH) was added and stirred well until it reached alkalinity (pH 9-11). Even after attaining the pH, stirring was continued to obtain a clear solution.

Gelatin (1%): Type A gelatin from porcine skin was used to prepare 1% (w/v) solution by dissolving 1 g of gelatin crystals in 100 mL distilled water under gentle heating and continuous stirring until complete dissolution (Taddei et al., 2013a). 7% citric acid (w/w) was added to 1% gelatin solution, and stirred to ensure complete dissolution. Concentrated NaOH was used to make the solution alkaline.

3.2.3. Preliminary study

A preliminary evaluation to prove the efficiency of CA crosslinking on the casted SF and SFG blend films was carried out as described below.

3.2.3.1. SF and SFG blend film preparation

The purified silk fibroin (7-8 w/v %) and 1% (w/v) Gelatin solutions were prepared similar to that mentioned above. While preparing the above solutions 7 w/w% of citric acid was also added and pH of the solutions was adjusted to alkalinity. Silk fibroin-Gelatin blend films were made by mixing regenerated silk fibroin (7-8% w/v) with Gelatin (1% w/v) at different blending ratios (0, 70:30, 50:50, 30:70), adjusting the pH of the final solutions to 10-11 followed by pre-crosslinking for 24 h. The aged solution was casted onto flat-bottomed polystyrene plates and dried overnight in under ambient conditions in a flow hood. The films obtained were post-crosslinked for 2-3 days at 24°C, 64% RH. These casted films were cut into similar-sized pieces to conduct hydrolytic degradation studies.

3.2.3.2. Hydrolytic degradation study on casted films

SF and SF-G blend films were weighed and immersed in PBS (pH 7.4) in the tissue culture plates to a concentration of 1 % (weight of the film/volume of PBS), and kept at 37°C O/N. Then the film was taken and dried, weighed, and compared with the initial mass of the films to obtain the residual mass (%) or percentage reduction in mass.

3.2.4. Modification of DPL

Using the above CA containing SF and gelatin solutions, SF100 (Silk fibroin 100%), SFG5050 (prepared by mixing silk fibroin and gelatin in a 1:1 ratio), and SFG3070 (prepared by mixing silk fibroin and gelatin in a 3:7 ratio) solutions were prepared and used for impregnation. SF and SF-G blend solutions were mixed and stirred well to get an appropriate uniform blend of the solutions. The final pH of the respective solutions was adjusted using NaOH. Three major steps were followed to modify the acellular porcine liver matrix with the above-mentioned polymer blends, including

pre-crosslinking, drying, and post-crosslinking. The decellularized matrices were immersed in 20 times higher volume of solution i.e., for every 1 g tissue weight under vacuum conditions (310 mbarr pressure and 29°C) for 48 h (pre-crosslinking). Pre-crosslinked samples were kept in Teflon-coated dishes and dried by keeping at 4°C (drying). After complete drying, the samples were post-crosslinked for 72 h in sealed petri dishes by keeping them at ambient conditions (post-crosslinking). Finally, the samples were hydrated and incubated with 70% ethanol for 7 days to stabilize the crosslinks formed. The samples were finally washed to remove any unbound polymer remnants and remaining CA. Sterilization of the scaffolds was done using 70% ethanol and storage at 4°C. Sample codes for the modified test groups were SF100DPL, SFG5050DPL, and SFG3070DPL for those impregnated in CA containing SF100 and two polymer blend solutions of SFG5050 and SFG3070 respectively. The unmodified control was named DPL.

3.2.4.1. Characterization of modified scaffolds (SF100DPL, SFG5050DPL and SFG3070DPL)

3.2.4.1.1. Masson's trichrome staining

The staining was carried out based on the standard staining procedure for trichrome staining, which involved Scarlet acid fuchsin, aniline blue, and Hematoxylin counterstaining followed by microscopic visualization (detailed in section 3.2.2.4.1).

3.2.4.1.2. In-vitro remodelability

An *in-vitro* enzymatic degradation test was carried out using a collagenase enzyme. 25 mg of decellularized control and impregnated test samples were suspended in 0.5 mL reaction buffer containing 0.1 M Tris, 0.05 M CaCl₂, 0.05 mg/mL Sodium azide (pH

7.4), and 500 U/mL collagenase from *Clostridium histolyticum* and incubated at 37°C for 1 day, 3 days and 7 days respectively (Umashankar et al., 2011a).

3.2.4.1.2.1. Percentage reduction in dry weight

After each period, the samples were centrifuged and the pellets were kept in a refrigerator at 4°C for 2 to 3 days until a constant weight is obtained and the percentage reduction in dry weight was calculated using the equation

$$\% \text{ Reduction in weight} = (\text{Difference in dry weight} / \text{Initial dry weight}) \times 100 \quad (1)$$

3.2.4.1.2.2. Ninhydrin assay (Reactive amine concentration)

Ninhydrin assay was performed in 24 h collagenase enzyme-degraded tissue supernatants to determine the percentage of free amine groups. To 100 µL samples, 1mL of ninhydrin solution (0.1% in 95% methanol) was added and heated for 20 minutes in the dark (De Clercq et al., 2016). Samples were cooled to room temperature. The optical absorbance of the solutions was measured at 570 nm using a spectrophotometer (Shimadzu, UV-1700 Pharmaspec). The amount of free amine groups in the sample, after heating with ninhydrin, is proportional to the optical absorbance of the solution. The concentration of free -NH₂ groups in the sample was determined from a standard curve obtained using various known concentrations of glycine. An increase in degradation resulted in more free amines in the solution and therefore higher absorbance (Sung et al., 2000; White et al., 1973).

3.2.4.1.2.3. Silk fibroin impregnated within the matrix (%)

From the collagenase degradation study with DPL, the percentage reduction in dry weight corresponds to the amount of collagen degraded from the sample and the

remaining part can be due to the presence of non-collagenous proteins such as elastin. Assuming the same amount of non-collagenous proteins to be left behind in each test group after the specified degradation period, the percentage of silk fibroin incorporated can be calculated using the formula.

$$\text{Amount of silk fibroin (\%)} = 100 - (\text{Collagen} + \text{non-collagenous proteins}) \quad (2)$$

Gelatin can be removed hydrolytically or being denatured collagen, it is susceptible to collagenase enzyme action also.

3.2.4.1.3. Gravimetry

For determining the approximate amount of silk fibroin and fibroin-gelatin blend incorporated within the extracellular matrix using the modification carried out, the difference in weight of the modified scaffolds with that of unmodified one was found and the percentage increase in weight corresponded to the amount of polymers incorporated.

3.2.4.1.4. Swellability

Air-dried slices of different groups ($n = 3$ for each) were weighed (W_0) and then immersed for 3 h in PBS at room temperature ($19\text{--}21^\circ\text{C}$). Excess water was then blotted with filter paper, the wet slices were weighed (W_1) again. The percentage swelling was calculated as the ratio of wet weight increase ($W_1 - W_0$) to the initial dry weight (W_0), multiplied by 100 (Saleh et al., 2018)

$$\text{Percentage swelling} = \frac{\text{Wet weight (} W_1 \text{)} - \text{Initial dry weight (} W_0 \text{)}}{\text{Initial dry weight (} W_0 \text{)}} \times 100 \quad (3).$$

3.2.4.1.5. Biomechanical properties

3.2.4.1.5.1. Tensile testing

Comparison of tensile properties of decellularized porcine liver and natural polymer-impregnated test samples were analyzed using a universal testing machine (Instron 3345). The materials were hydrated and rectangular strips of 50x10 mm size were made and subjected to uniaxial tensile testing using an Instron UTM machine. Triplicate samples were prepared, and thickness was measured using a thickness gauge. The testing conditions provided were 23°C and 60% humidity with a crosshead speed of 5mm/min and a full-scale load range of 100 N. The test results were obtained using the software Blue Hill -3.

3.2.4.1.5.2. Suture retention strength

Suture retention strength was estimated in decellularized and polymer-incorporated test samples in triplicates using a UTM machine (Instron 3345, UK). Rectangular strips of size 4 cm in length and 1cm width were taken and 3-0 prolene suture was inserted 5 mm from one end, looped, and secured. The sample was gripped using suitable jigs of the machine using a 100 N load cell and pulled at a rate of 5 mm per minute until the material failed (Chaparro et al., 2016). The maximum load applied was taken as suture retention strength (Kimitata et al., 2020).

3.2.4.1.6. Ultrastructure

The ultrastructure of the material surface and cross-sectional surfaces of the scaffolds were evaluated using SEM (FEI Quanta 200). The sample was fixed in 3% glutaraldehyde overnight by keeping at 4°C, washed in PBS, and dehydrated in ascending series of alcohol (30%, 50%, 70%, 90%, and 100%) for at least 15 minutes in each. To the sample, isoamylacetate was added followed by critical point drying (HCP-2 Critical point dryer, Hitachi) for 20 minutes. The dried sample coated with

gold was firmly fixed on an aluminium stub and finally viewed under SEM and images were acquired (Cengiz et al., 2019b).

3.2.4.1.7. Fourier Transform Infrared Spectroscopy (FTIR)

ATR-FTIR was carried out using a Nicolet iS50 FTIR analyzer (Thermo Nicolet Corporation, Waltham, MA, USA). The infrared absorption spectrum of the samples with a scanning step range of 400~4000 cm^{-1} based on ASTM E1252-98 (Re-approved 2021) and E573-01 (Re-approved 2021) (“Standard Practice for General Techniques for Obtaining Infrared Spectra for Qualitative Analysis,” n.d.; Zhao et al., 2021) was recorded and analyzed.

3.2.4.1.8. Surface wettability

The water-in-air contact angle of samples was measured at room temperature (23°C) using the sessile drop method by a video-based contact angle measuring device (Data Physics OCA15 plus, Germany) and imaging software (SCA20 software, Germany) within 10 seconds after inoculating water droplet of 4 μL . Six independent measurements at different sites on each sample were performed for the study. Smaller contact angles ($<90^\circ$) represent increased hydrophilicity and larger angles ($>90^\circ$) indicate improved hydrophobicity (Zhang et al., 2019).

3.2.4.1.9. GAG preservation

Preservation of GAG before and after mechanical modification was evaluated qualitatively by Alcian blue staining (detailed in section 3.2.2.5.1) and quantitative estimation through DMMB assay (as detailed in section 3.2.2.5.2).

3.2.4.1.10. In-vitro cytotoxicity

The cytotoxicity evaluation of the modified scaffolds was carried out using L929 cells based on ISO-10993 part 5. Direct contact test and test on extracts were done as

detailed in sections 3.2.2.9.1 and 3.2.2.9.2 respectively. In the test on extract method, MTT assay was also carried out in L929 cells treated with the material extracts as per the given protocol.

3.2.4.1.10.1. MTT assay

MTT assay was performed to measure the activity of cells to reduce yellow-coloured tetrazolium salt 3-(4, 5-Dimethyl thiazol -2-yl)-2,5-diphenyltetrazolium bromide to purple-coloured formazan. The test sample and control medium were replaced with 200 μ L MTT, prepared at a concentration of 1 mg/mL in a serum-free medium. The plates were incubated at 37°C for 2 h. The MTT medium was removed and 100 μ L of Isopropanol was added to all wells. The plates were kept in the dark for 5 minutes to solubilize the formazan product formed due to cell activity. The color developed was quantified by obtaining absorbance at 570 nm using a multi-well plate reader (Biotek, USA).

Percentage cell activity = (OD of the Test / OD of the Negative Control) X 100 **(4)**

3.2.4.1.11. In-vitro cell migration study (Scratch wound assay)

L929 cells were seeded on 4-well plates and allowed to grow until 90% confluency was reached. Using a sterile 200 μ L micropipette tip, a wound was scratched, and the cells were washed with sterile PBS to eliminate dead cells and debris. Extract prepared by incubating the test samples in culture medium containing serum at 37 $\pm$ 1°C for 24 h at an extraction ratio of 6 cm²/mL was added, and photographs were taken at different time intervals to determine the wound-healing effect of scaffolds on fibroblast cells. Cells cultured in a normal medium were considered cell control. Triplicate samples were used for the study. Cell migration rate was calculated as the distance traveled by

the cells, over time: just after the scratch (T_0), after 4 h (T_4), after 8 h (T_8), and after 24 h (T_{24}).

3.3. Inflammatory and Immune potential of acellular and modified scaffolds by rat subcutaneous implantation

3.3.1. Materials

Cytokine analysis was carried out with Elisa kits (Cat # 900-M73 and Cat # 900-M109, Peprotech, Israel), Ab response was evaluated using IgG+IgM+IgA antibody conjugated with HRP (ab102199, Abcam) and all staining solutions were also prepared using chemicals from Himedia, India, and Sigma Aldrich, USA.

3.3.2. Approval for animal study

All animal studies conducted were reviewed and approved by the Institutional Animal Ethics Committee (IAEC approval No: SCT/IAEC-432/JANUARY/2022/112, dated 3/3/2022).

3.3.3. Subcutaneous implantation

Three-week-old juvenile male Wistar rats (80-120 g body weight) were grouped into SF100DPL, SFG5050DPL, SFG3070DPL, and DPL control groups for study durations of 14 days, 30 days, and 90 days, with each group consisting of 3 animals/time point. Three animals per cage were housed in a temperature and humidity-controlled room with ad libitum feed and water. Animals housed at least for a week were taken for surgery. Animals were anesthetized using intraperitoneal injection of premixed xylazine (7 mg/kg body weight) and ketamine (70 mg/kg body weight). After preparing the dorsal area by shaving and sterilizing with iodopovidone 10% solution, three dorsal midline linear incisions of 0.5-1 cm long were created and two implants (size 0.5 cmX0.5 cm) of the same treatment group were placed at each

incision. After placing the materials, incisions were closed using a 3-0 silk interrupted suture. Thus, each animal received a total of six implants. Oxytetracycline (1mg/mL) was given orally by adding to the drinking water. Post-operative care was given for a duration of 7 days until the sutures were removed. At the end of each time point the animals were euthanized. Blood and spleen were collected to evaluate immunogenicity (Kim et al., 2007a).

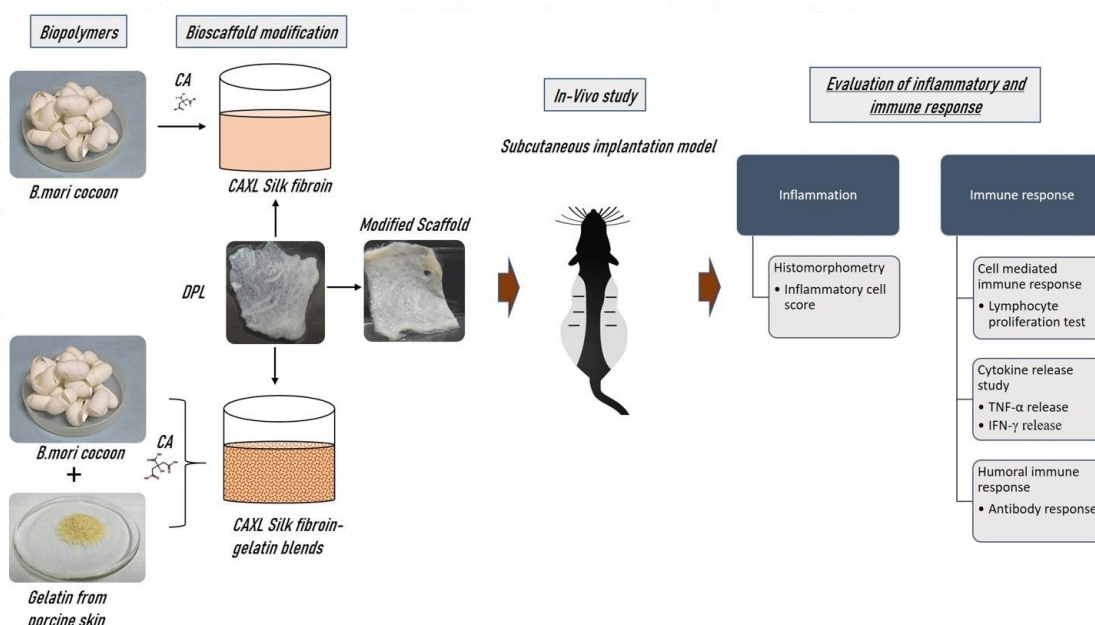


Figure 3.1. Subcutaneous implantation and evaluation: Shows the modification of porcine decellularized ECM scaffolds and its implantation in rat subcutaneous model and evaluation of its inflammatory and immunogenic potential.

3.3.3.1. Hematology and percentage weight gain of implanted animals

Fresh venous blood was collected from each animal in heparinized blood collection tubes and was subjected to hematology analysis. Hematology blood parameters, including hemoglobin (HGB) level, hematocrit (HCT), red blood cell (RBC), white

blood cell (WBC), and platelet (PLT) count, were analyzed using an automated hematology analyzer, (MEK 6450K, Nihon Kohden, Japan). A percentage increase in weight exhibited by the implanted animals was also noted.

3.3.3.2. Histological evaluation of inflammation (Inflammatory cell scoring)

Three explants from each animal was taken for evaluate the extent of inflammation within it by fixation in 10% neutral buffered formalin for a week and short acetone processing and xylene treatment was followed by embedding in paraffin wax. The embedded samples were cut into 5-micron thick sections in microtome (Model RM 2125, Leica). The sections mounted on glass slides were deparaffinized in xylene and hydrated by immersing in a graded series of alcohol (100% and 70%) followed by dH₂O and stained with Haematoxylin (Harris Haematoxylin) followed by bluing in Schott's tap water and staining with Eosin. Finally, the stained sections were dehydrated, cleared in xylene, and mounted in DPX mountant. Microscopic images were acquired using the Olympus CX41 microscope and Progres CapturePro 2.10.0.1 software. Mononuclear cell scoring was carried out using H&E-stained images to study inflammatory cell infiltration such as polymorphonuclear cells (PMNCs), lymphocytes, plasma cells, and macrophages within the implanted scaffolds. Necrosis, fatty infiltration, neoangiogenesis, and the presence of foreign body giant cells were also scored based on ISO10996-part 6 (14:00-17:00, n.d.). Five high power fields (hpf) from each tissue section, a total of 15 hpf for each test material was scored and the average response was found. The scoring criteria followed was given in the table 3.1.

Cell/Tissue	Score				
Response	0	1	2	3	4
PMNCs	0	1-5	5-10	Heavy infiltrate	Packed
Lymphocytes	0	1-5	5-10	Heavy infiltrate	Packed
Plasma cells	0	1-5	5-10	Heavy infiltrate	Packed
Macrophages	0	1-5	5-10	Heavy infiltrate	Packed
FBGCs	0	1-5	5-10	Heavy infiltrate	Packed
Explant cellularity	Cellular/acellular				
Peri-implant necrosis	Nil	Minimum	Mild	Moderate	Severe
Capsule description	Nil	Narrow	Moderately thick	Thick	Extensive
Neovascularisation	Nil	1-3	4-7	Broad	Extensive
Fatty infiltration	Nil	Minimal	Several layers	Broad	Extensive

Table 3.1: Histomorphological scoring criteria

3.3.3.3. Lymphocyte proliferation test (Cell-mediated immune response)

Presence of lymphocytes sensitized against the scaffold derived antigens were studied using lymphocyte proliferation test. For this test, sensitized lymphocytes were isolated from homogenized spleen. Approximately 10^6 cells/mL was used for seeding on to sterile 96-well microtiter plates. Exactly 100 μ L cell suspension was added to each well of the microtiter plate. Scaffold-derived extractable proteins prepared sterile at a concentration of 50 μ g/mL was used for stimulating lymphocytes. Cell control without any antigen was taken for comparison. After adding the antigens, the plates were incubated for 7 days at 37° C and 5% CO₂ concentration. Color changes to the medium were noted, regularly and fresh medium was introduced whenever required. At the end of the seventh day MTT assay was done in the cultures. The plates were wrapped in aluminum foil and incubated for 4 h at 37° C in a humidified atmosphere. The pellets were dissolved in DMSO and the color developed was read at 544nm immediately using a Chameleon microplate reader (Model no. 425-106, Hidex Finland). The proliferation of the lymphocytes was expressed as stimulation index (SI) calculated by dividing the absorbance obtained for antigen-activated lymphocytes to non-activated lymphocytes. A stimulation index greater than 2 was considered as an indication of significant proliferation (“ASTM F1906-98(2003) - Standard Practice for Evaluation of Immune Responses In Biocompatibility Testing Using ELISA Tests, Lymphocyte, Proliferation, and Cell Migration (Withdrawn 2011),” n.d.; Emery et al., 1988; Lambrecht et al., 2004).

$$\text{Stimulation index} = \frac{\text{Absorbance for antigen-activated lymphocytes}}{\text{Absorbance for non-activated lymphocytes}} \quad (5).$$

3.3.3.4. Evaluation of immunogenicity by IFN- γ release (T-cell activation) and TNF- α release (B-cell activation) study

This test was done to identify sensitized T and B cells in the non-concentrated peripheral blood mononuclear cells (PBMNCs) of scaffold-implanted animals. On completing the observation periods of 14, 30, and 90 days in sub-cutaneous implantation, animals were euthanized and whole blood was collected into sterile tubes containing sodium heparin. Within two hours of collection, it was diluted 1:5 in RPMI 1640 supplemented with 2 mM L-glutamine, 100 IU/mL penicillin, and 0.1 mg/mL streptomycin (Sigma–Aldrich, Kempton Park, South Africa), for preparing peripheral blood mononuclear cells (PBMNCs). Aliquots of the PBMNC suspension were incubated at 37° C and 5% CO₂ in triplicates in 96-well plates (200µL/well) with 10µg/mL of extracted protein, and medium alone control was also maintained. Following 5 days of incubation, supernatants from each treatment group were harvested, pooled, and stored at -20° C. Interferon-gamma and tumour necrosis factor-alpha concentrations (IFN-γ & TNF-α) of pooled samples were determined by enzyme-linked immunoassay (ELISA) in triplicates. Antigen-stimulated samples were assayed in duplicate. For each animal, the IFN-γ in the stimulated sample was compared with those of unstimulated samples to obtain IFN-γ response resulting from the implantation of the test materials (“Fact Sheets | Testing & Diagnosis | Fact Sheet - Recommendations for Human Immunodeficiency... Clinics | TB | CDC,” 2022; Litwin, 2007; Parsons et al., 2012).

3.3.3.5. Antibody (IgG+IgM+IgA) response

The collective antibody response (IgG+IgM+IgA) elicited by animals implanted with SF100DPL, SFG5050DPL, SFG3070DPL, and DPL control samples was assessed using Indirect ELISA. For this, extractable proteins from the test materials were coated in the 96-well ELISA immunoplate (96-well plate, Himedia) in antigen-diluting

bicarbonate/carbonate coating buffer (2 µg protein per well). Serum samples collected from animals implanted with the test materials at 1:25 dilution prepared in blocking buffer (1% BSA in PBS) formed the primary antibody. Serum from animals immunized with extractable protein from fresh decellularized porcine liver together with Freund's adjuvant was treated as positive control. The secondary antibody used was rabbit polyclonal to rat IgG+IgM+IgA conjugated with HRP (Abcam ab102199) and TMB being the HRP substrate. After the reaction, the ELISA plates were read with microplate reader Expert Plus (ASYS Hitech GmbH, Austria). OD readings of microwells without secondary antibodies were taken as a negative control (Umashankar et al., 2011b).

3.4. Induced regenerative potential of the scaffolds by rat abdominal wall regeneration study

3.4.1. Materials

New and improved Super Sensitive™ Polymer-HRP IHC Detection system (QD400-60KEN, BioGenex, Netherlands) was employed to carry out immunohistochemistry with primary antibody against myosin heavy chain (ab37484, Abcam). All staining solutions were also prepared using chemicals from Himedia, India, and Sigma Aldrich, USA.

3.4.2. Approval for animal study

All animal studies conducted were reviewed and approved by the Institutional Animal Ethics Committee (IAEC approval No: SCT/IAEC-432/JANUARY/2022/112, dated 3/3/2022).

3.4.3. Rat abdominal wall defect model

Adult male/female Wistar rats (Total=48) of weight range 350-410 g, were grouped into SF100DPL, SFG5050DPL, SFG3070DPL, and DPL control groups for surgically creating the abdominal wall defect. The rats were utilized for the study only after quarantine for a minimum of 1 week. Each group of 6 animals was kept for two durations of 21 days and 90 days post-surgery. Briefly, the procedure included, anesthetizing the animals by general anesthesia using xylazine (7 mg/kg body weight) and Ketamine (70 mg/kg body weight) after which the ventrolateral abdomen was prepared for aseptic surgery by shaving, scrubbing, and disinfecting, finally covering with sterile draping (Wang et al., 2014). A linear skin incision of about 3–4 cm is made; abdominal muscles are exposed. A full-thickness abdominal defect of about 1.5–2 cm in size consisting of all the abdominal wall layers such as external and internal oblique abdominus muscle, transverse abdominus muscle, and peritoneum is created by excising these structures. The test material in a circular shape, of about 1.5–2 cm in size, was first positioned at the defect site using 4-point anchoring and then implanted using 5/0 prolene continuous sutures, and the outer skin was closed with 3/0 silk interrupted sutures. After completing the procedure animals were returned to cage and observed for recovery and given free access to food and water. The animals were given postoperative antibiotics (oxytetracycline, 1 mg/mL), orally in drinking water for 7 days. Following the recovery of the animals, they were monitored until sacrificed at 21 days and 90 days (n=6/group/time point). The animals were euthanized, blood was collected and the graft with a 5mm border of adjacent host tissue was collected immediately followed by its fixation in 10% formaldehyde solution and used for further evaluation (Umashankar et al., 2013)

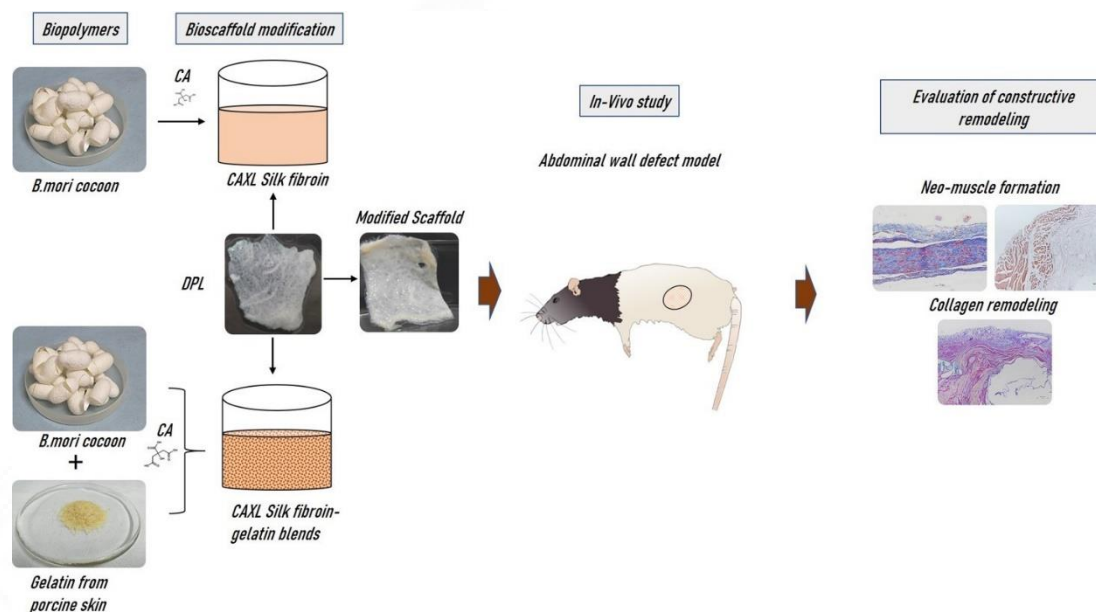


Figure 3.2. Abdominal wall defect study and its evaluation: Graphical image showing the modification of the DPL and implantation of modified matrices in rat abdominal wall defect model. Evaluation of explants for constructive remodelling by new muscle deposition and collagen maturation within the explants.

3.4.3.1. Hematology

The method of blood collection and analysis was given in section 3.4.2.1.

3.4.3.2. Inflammatory cell scoring

The method adopted is the same as that used to evaluate subcutaneous explants as detailed in section 3.4.2.2.

3.4.3.3. Evaluation of regeneration

3.4.3.3.1. Masson's trichrome staining

Neo collagen deposition and neo-muscle island formation within the recovered abdominal wall explants were evaluated using Masson's trichrome differential staining method as explained in section 3.2.2.4.1.

3.4.3.3.2. Immunohistochemistry

For the immunohistochemical study, 5 µm thick sections were taken in charged slides (StarFrost, Germany). After deparaffinization and hydration, heat-mediated antigen retrieval was performed. Myosin heavy chain specific primary antibody in 1:10000 dilution was applied followed by visualization using SS Polymer HRP detection system (BioGenex) as per manufacturer's instructions. Each step was accompanied by washing with TBS twice. Color development was done using the substrate for HRP, 3,3'-diamino-benzidine followed by counterstaining with hematoxylin. Then the sections were dehydrated in an ascending series of alcohols and finally mounted in DPX. Microscopic images were acquired using the Olympus CX41 microscope and Progress CapturePro 2.10.0.1 software.

3.4.3.3.3. Collagen remodeling (Herovici's polychrome staining)

Herovici's polychrome staining was performed following a modified protocol which was first described by Herovici (1963) (Herovici, 1963). The modification minimized staining variability previously described by others (Lillie et al., 1980; Fitzgerald et al., 1996) (Fitzgerald et al., 1996; Lillie et al., 1980). The stain was prepared as a mixture of 0.1 % w/v acid fuchsin in picric acid and 0.05 % w/v methyl blue in 1 % v/v acetic acid solutions at a 2:1 ratio. The tissue sections were incubated in this solution for 5 minutes after deparaffinization and hydration, then differentiated in 1% acetic acid for 2 minutes, followed by a 5-minute wash in 100% ethanol. The slides were then dehydrated through alcohol and xylene and mounted (Turner et al., 2013). Selected slides stained with Herovici staining were viewed using the Olympus CX41 microscope and images were captured using Progress CapturePro 2.10.0.1 software.

Analysis of the red and blue areas of the slide was completed using ImageJ and the Threshold Colour plugin. Images were loaded, and colors were isolated by using the hue histogram filter available in Threshold Colour. Images were subjected to a threshold such that each non-white pixel was turned black and each white pixel remained white. Then, the number of black pixels in each image was used to calculate the percentage of the image area that corresponded to a certain color. This process of filtration and threshold quantification was repeated for each color. Ratios of collagen III to collagen I were calculated by taking the ratio of the percentage area blue versus the percentage area red of each image. Subsequently, data was pooled for DPL, SF100DPL, SFG5050DPL and SFG3070DPL samples (Gilbert et al., 2009a).

3.5. Statistical analysis

All data were expressed as mean $\pm$ S.D., F-test, and Student's t-test for equal or unequal variances were done in Excel, and p-values less than 0.05 were considered to be statistically significant. Karl Pearson's coefficient of correlation was calculated for selected parameters to find a positive or negative correlation between them.

4 RESULTS

4.1. Decellularization of porcine liver slices and its characterization

4.1.1. Preparation of decellularized porcine liver scaffold (DPL)

Three decellularization methods were carried out and the step-by-step procedure adopted for its fabrication is given in Figure 4.1.

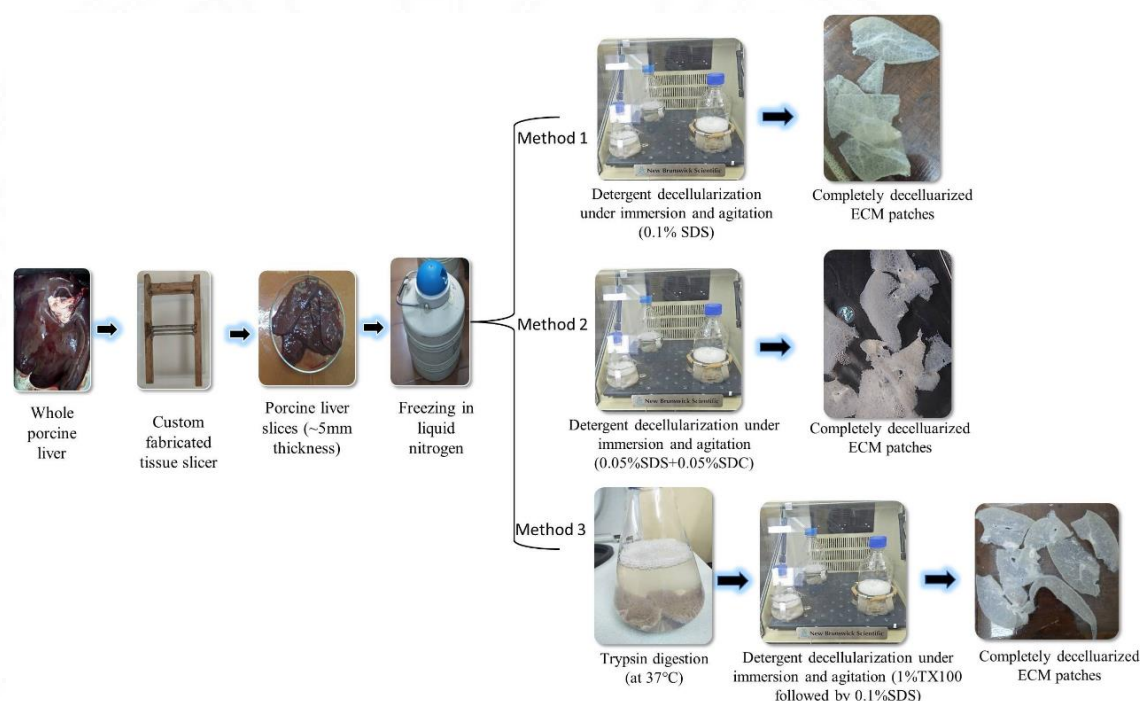


Figure 4.1. Decellularization procedure: Pictorial representation of different steps involved in the development of decellularized porcine liver scaffolds from native liver slices following three different detergent decellularization procedures

4.1.2. Evaluation of decellularization efficiency

4.1.2.1. Cellular removal

4.1.2.1.1. H&E staining

As seen in H&E-stained images (Fig. 4.2), the native porcine liver sections show intact nuclei, hepatocytes, inter-collagenous connective tissue sheath connecting the portal triads, blood sinusoids, central vein, and hepatic cords of the biliary system. Higher magnification images indicated the presence of the hepatic artery, portal vein, and hepatic duct constituting the portal triad (Fig 4.2. e). The decellularized tissue sections were observed to have complete cellular removal proven by the absence of nuclear staining in all three decellularization methods (Fig 4.2. f, g, h). The test groups SDS DPL represents the decellularized ECM developed using the ionic detergent sodium dodecyl sulfate, SDS+SDC DPL, using a combination of SDS and sodium deoxycholate and TX100 DPL, that developed using the detergent triton X-100 followed by SDS respectively.

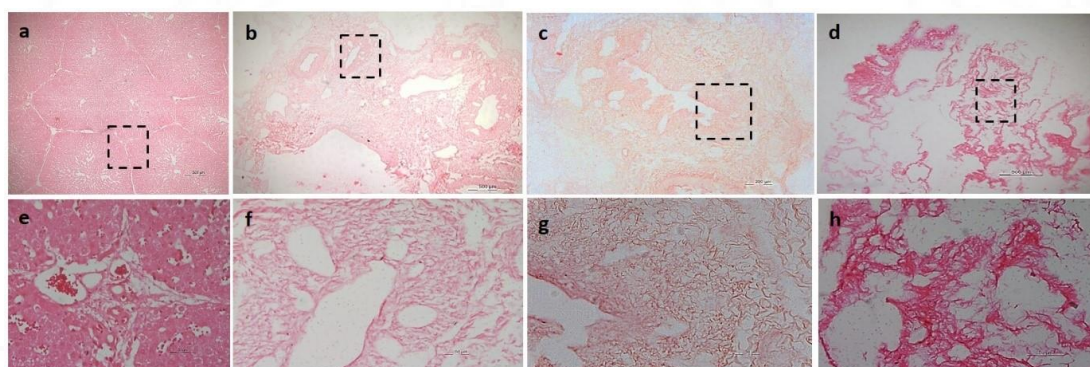


Figure 4.2. H&E staining: H&E-stained images of native and decellularized porcine liver. Images showing H&E stained images at low (4x) and high (40x) magnification showing native porcine liver (a, e), SDS DPL (b, f), SDS+SDC DPL (c, g), and Triton X-100 DPL (d, h) respectively. Scale: 500µm (Mag. 4x) and 50 µm (Mag. 40x)

4.1.2.2. Removal of DNA

4.1.2.2.1. DAPI (4',6-diamidino-2-phenylindole) staining

One of the major criteria of an efficient decellularization protocol is that it should eliminate DNA so that there shouldn't be DNA fragments in recognizable amounts to elicit adverse inflammatory and immune responses. Qualitative analysis of DNA removal by DAPI staining proved the decellularization protocols followed were quite successful in achieving excellent elimination of DNA. There were no DAPI stained regions detected in any of the decellularized tissue sections (Fig 4.3. b, c, d) while it was observed to be associated with the minor groove of DNA present in the nuclei of native liver cells, evidenced by the abundant intensely blue stained regions (Fig 4.3. a)

4.1.2.2.2. Nanodrop method

The concentration of DNA estimated in native and decellularized samples again confirms the efficient removal of DNA. A greater concentration of DNA was obtained in native samples (514.05 ± 93.5 ng/mg tissue). DNA detected in tissues after three methods of decellularization were 33.98 ± 9.88 ($P=0.012$), 11.32 ± 2.69 ($P=0.011$), and 120.97 ± 8.16 ($P=0.018$) ng/mg tissue in SDS DPL, SDS+SDC DPL and TX100 DPL respectively (Fig. 4.3f). Even though there is statistical significance in the reduction of DNA in all three methods of decellularized tissues compared to native porcine liver, the TX100 DPL was not found to satisfy the guideline of retaining less than 50 ng/mg ECM which proves its inefficiency in bringing out an effective decellularization.

4.1.2.2.3. Agarose Gel Electrophoresis (AGE)

In agarose gel electrophoresis (AGE), no intact visible DNA bands of specific fragment length were obtained in SDS DPL and SDS+SDC DPL, at the same time a faint and thin band of high molecular weight genomic DNA was feebly visible in the ECM obtained in TX100 DPL group. The DNA sample extracted from native tissue

showed a thick, distinct, and visible genomic DNA band with a size >3000 bps (Fig. 4.3g).

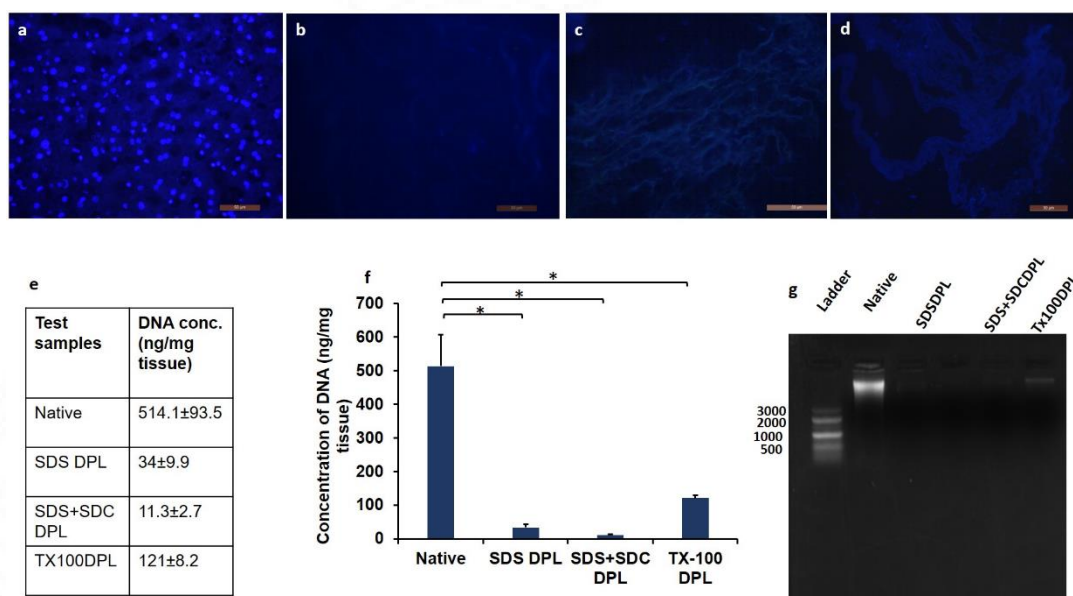


Figure 4.3. DNA content: DAPI stained images of native (a) tissue section, and absence of DAPI staining in SDS DPL (b), SDS+SDC DPL (c), and Triton X-100 DPL tissue (d). Table (e) shows the concentration of DNA estimated using Nanodrop, its graphical representation (f), and agarose gel image (g) containing DNA ladder, native genomic DNA band, and DNA samples from decellularized tissue samples. (Image magnification 40x; Scale 50µm). *p<0.05

4.1.2.3. Removal of protein

4.1.2.3.1. Lowry's method

Total protein content in decellularized tissue significantly declined in all three acellular samples compared to the native control. The concentration of protein estimated by Lowry's method in native, SDS DPL, SDS+SDC DPL, and TX100 DPL were 144.38±7.9, 18.88 ± 4.8, 18.71±1.8 and 17.88±5.5 µg/mg tissue respectively (Fig. 4.4a).

4.1.2.3.2. SDS-PAGE (Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis)

SDS PAGE done using the extracted protein showed numerous protein bands in the native protein sample loaded well. The lanes loaded with the proteins from acellular tissues do not have any intact bands (Fig. 4.4b).

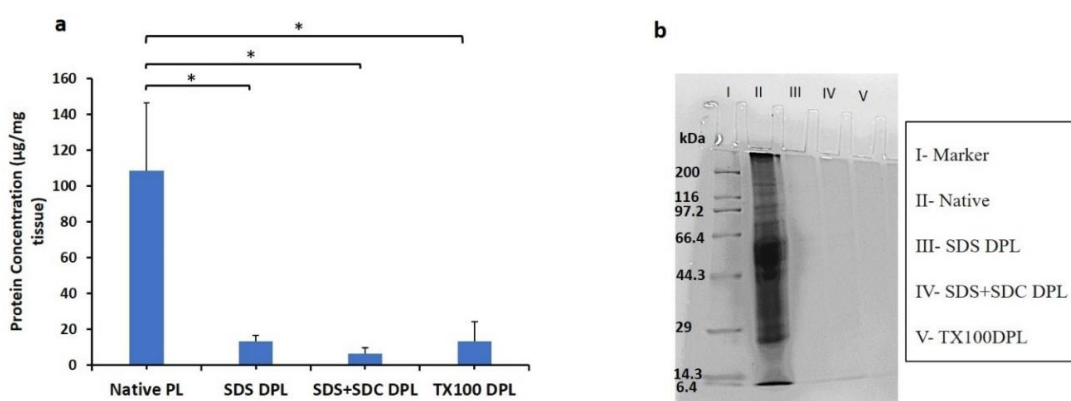


Figure 4.4. Protein content: Chart showing total extracted protein (a) estimated in native porcine liver and three methods of decellularized samples by Lowry's method and the gel image (b) obtained from SDS PAGE. *p<0.05

4.1.2.4. Retention of collagenous architecture

4.1.2.4.1. Masson's trichrome (MT) staining

Collagen distribution in the native porcine liver was mainly localized at the hexagonal-shaped regions which demarcate different lobules constituting the structural unit that explains the lobular concept of the liver (Fig. 4.5. a, e). In the decellularized matrices developed by SDS and SDS+SDC methods, the collagen was preserved with no cytoplasmic and nuclear remnants (Fig. 4.5. b, c, d, f, g, h). The collagen structure was heavily damaged in the decellularized matrix which was enzyme-digested before

decellularizing with Triton X-100 (Fig. 4.5. d, h) detergent.

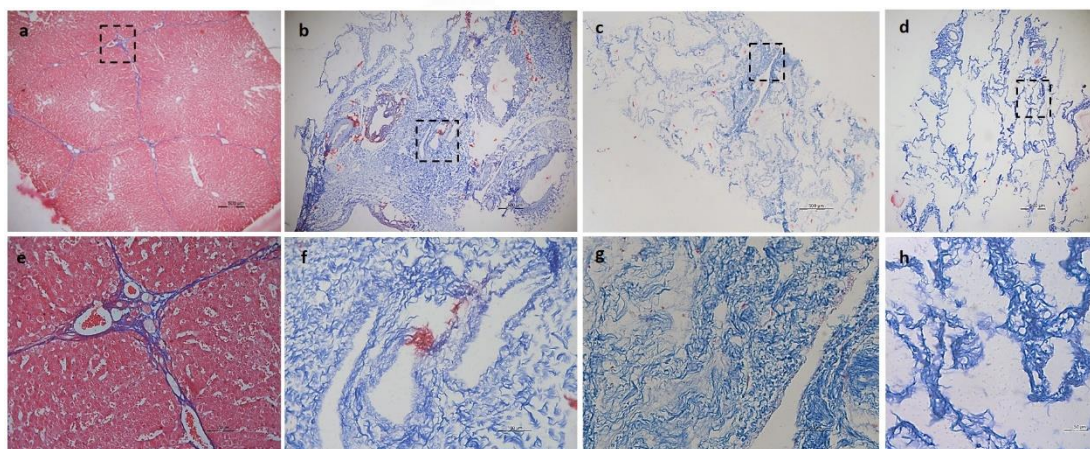


Figure 4.5. MT staining: Masson's Trichrome stained images of native and decellularized porcine liver, red staining indicates cells and blue represents the collagen. 4x and 40x images showing native porcine liver (a, e), SDS DPL (b, f), SDS+SDC DPL (c, g), and Triton X-100 DPL (d, h) respectively. Scale: a-d: 500 μ m (Mag. 4x) and e-h: 50 μ m (Mag. 40x)

4.1.2.5. Preservation of Glycosaminoglycans

4.1.2.5.1. Alcian blue (AB) staining

Decellularization using various detergents has a significant impact on the GAG content of the tissue. A qualitative study using Alcian blue staining has proved that GAG content was retained even after three methods of decellularization which was evident from the blue-stained regions (Fig 4.6 b-d). Fig 4.6a represents the GAG present in native porcine liver in low and high magnifications, and is mainly found to be associated with the intercollagenous connective tissue sheath.

4.1.2.5.2. Dimethylmethylene blue (DMMB) assay

The GAG content estimated in native porcine liver by dimethyl methylene blue assay was 4.57 ± 0.55 μ g/mg dry weight. Among the three acellular matrices, GAG

concentration was reduced with a concentration of 3.87 ± 0.88 and 1.46 ± 0.72 $\mu\text{g}/\text{mg}$ dry tissue in SDS DPL and TX100 DPL respectively with corresponding statistical significance of 0.3 and 0.004. However, in the combinatorial approach of decellularization, the GAG concentration was increased, which was significantly higher than the GAG content estimated in native porcine tissue i.e., 9.92 ± 2.1 $\mu\text{g}/\text{mg}$ dry tissue ($P = 0.05$) (Fig 4.6e).

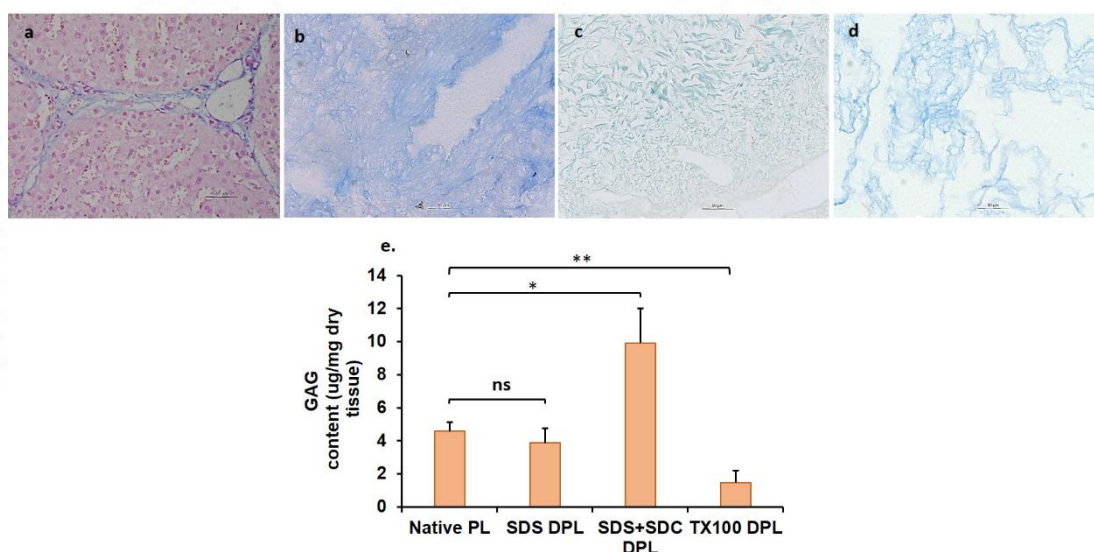


Figure 4.6. GAG retention: Alcian Blue stained images, light blue color indicating the presence of GAG in native (a) and decellularized porcine liver matrices such as SDS DPL (b), SDS+SDC DPL (c), and Triton X-100 DPL (d) respectively with 40x magnification and a scale of 50 μm . Graph showing sGAG estimated in native and the three acellular matrices estimated by dimethyl methylene blue (DMMB) assay (e). ns (non-significant), * $p < 0.05$, ** $p < 0.005$

4.1.2.6. Growth factor preservation

4.1.2.6.1. HGF and bFGF (Sandwich Elisa)

Among the two growth factors tested, both HGF and bFGF were retained in the SDS+SDC DPL in comparatively higher concentrations. Native porcine liver matrix

contained an HGF pool of 1638 ± 51 pg/mg dry tissue followed by SDS+SDC DPL with 458 ± 25 pg/mg dry tissue and the least amount in TX100 DPL that contained only 187 ± 12 pg/mg dry tissue. SDS DPL retained an intermediate level of HGF compared to the other two methods, which was 254 ± 8 pg/mg dry tissue of HGF (Fig. 4.7b). bFGF estimated was 104.5 ± 3.4 pg/mg dry tissue in native tissue and among the acellular matrices SDS+SDC DPL retained maximum bFGF content of 6.6 ± 1.4 pg/mg dry tissue and TX100 DPL did not contain any detectable amount of the bFGF (Fig. 4.7a). A correlation was observed between the preserved GAGs and the GFs within the decellularized matrices, from which it was inferred that the decellularization method that preserved highest GAG retained highest GF as well (Fig. 4.7c, 4.7d). The Karl Pearson's coefficient of correlation obtained between GAG preserved and HGF was 0.99 and that of GAG and bFGF was 0.92. So, GAG estimation can be used as an indirect way of estimating the GFs preserved within the matrix.

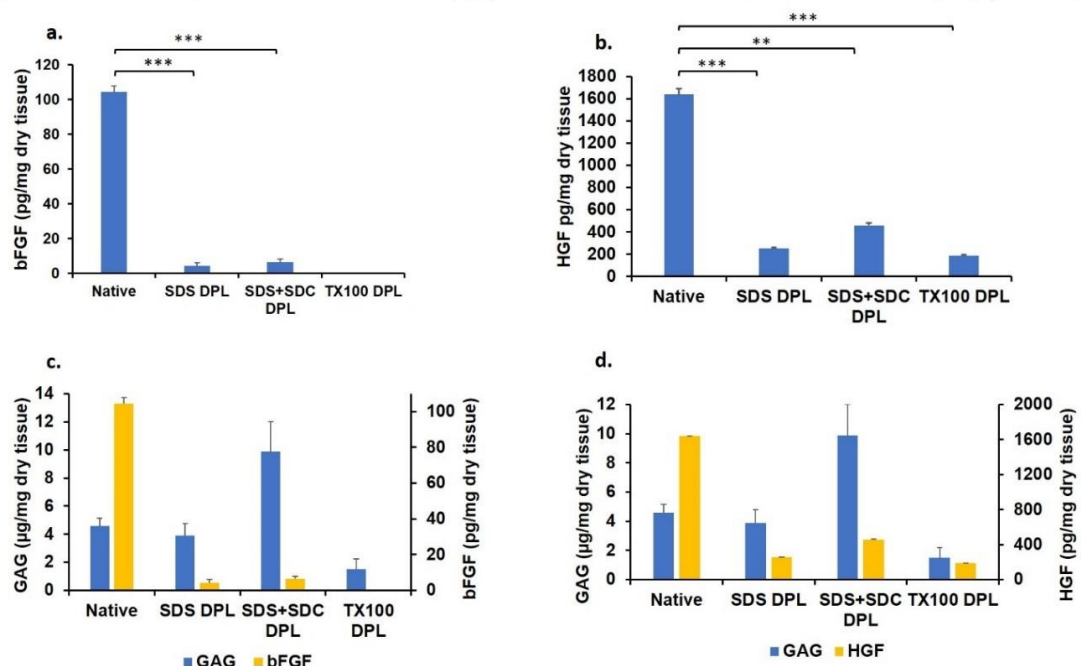


Figure 4.7. GFs retained and comparison with GAG: Graphs showing the growth factors bFGF (a), HGF (b), the relation between GAG & bFGF (c) and GAG&HGF (d) retained within the native and acellular matrices. ** $p < 0.005$, *** $p < 0.0005$

4.1.2.7. Mechanical properties

4.1.2.7.1. Tensile testing

SDS+SDC DPL exhibited a considerable reduction in tensile strength (5 ± 2.7 MPa) and Young's modulus (19.7 ± 13.03 MPa) compared to the other two methods (Fig. 4.8a, b). SDS DPL had the highest Young's modulus whereas the matrix produced using the TX100 method exhibited the highest load at break and maximum load (Fig. 4.8c, d).

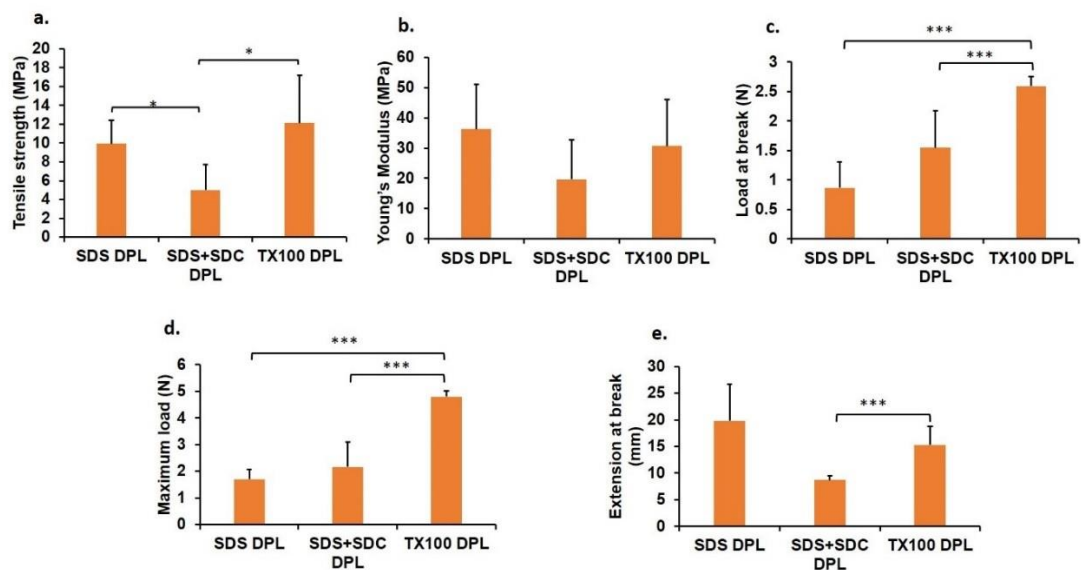


Figure 4.8. Mechanical properties: Charts showing the tensile strength (a), Young's modulus (b), Load at break (c), maximum load (d), and Extension at break (e) of extracellular matrix scaffolds developed by 3 methods of decellularization. * $p < 0.05$, *** $p < 0.0005$

4.1.2.8. Ultrastructure

The micro-architectural evaluation has revealed the high cellularity in the native liver (Fig. 4.9a) and the absence of cells in 3 decellularized matrices along with the fibrous nature of the extracellular matrix. The compact and bundle-like network of collagenous fibers was found to be evident associated with SDS DPL (Fig. 4.9b) and SDS+SDC DPL (Fig. 4.9c) in a well-preserved manner. The collagenous structure of the TX100DPL matrix has been observed to be completely distorted and deteriorated (Fig. 4.9d).

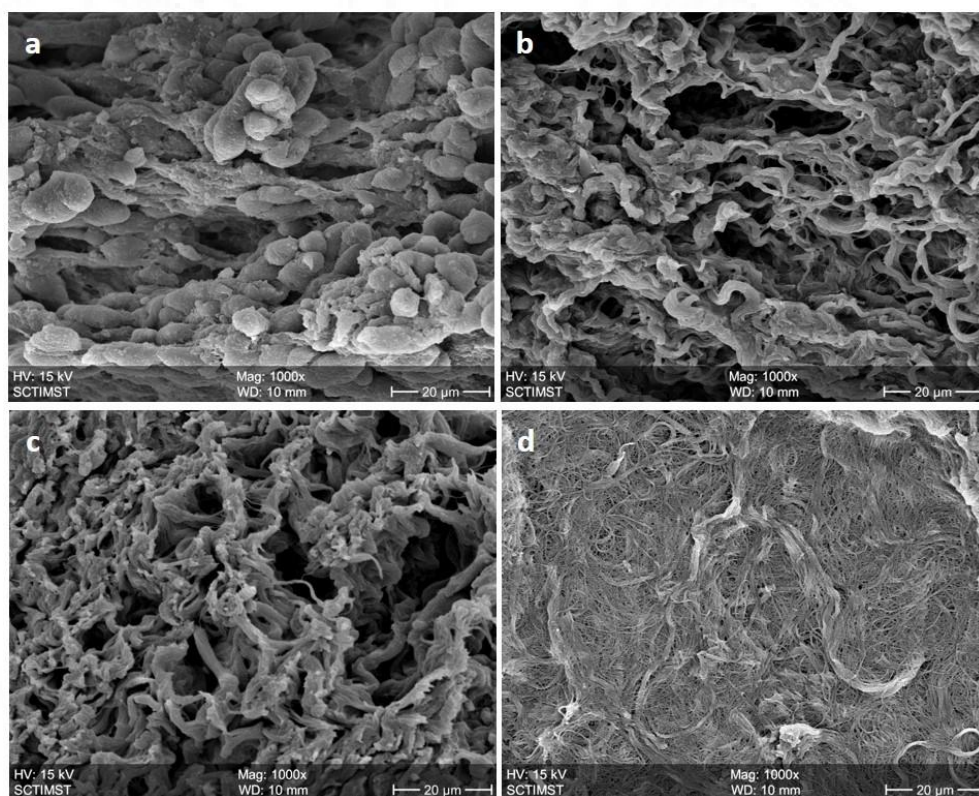


Figure 4.9. Ultrastructure: Scanning electron microscopic images of native porcine liver (a) and decellularized matrices SDS DPL (b), SDS+SDC DPL (c), TX100 DPL (d) respectively.

4.1.2.9. Cytotoxicity

4.1.2.9.1. Direct contact and Test on extract

All three acellular matrices developed were proved to be non-cytotoxic by direct contact and test on extract method of evaluation carried out based on ISO10993-part 5 (Fig. 4.10).

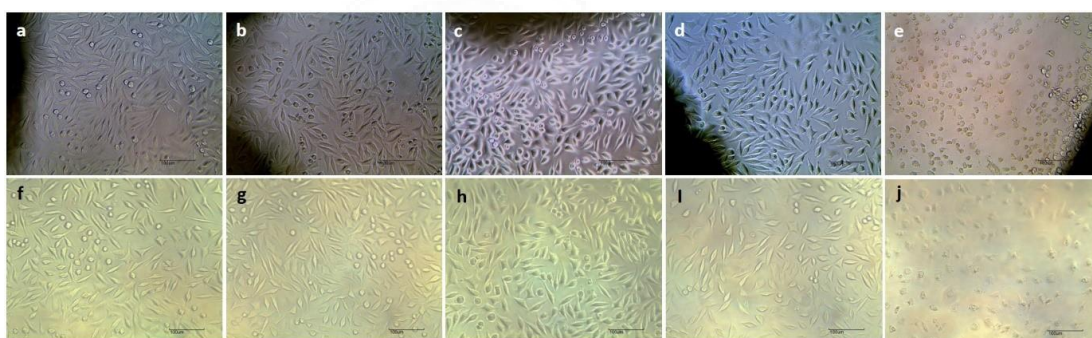


Figure 4.10. Cytotoxicity: Microscopic images showing non-cytotoxic cellular response exhibited by L929 cells with direct contact with the materials (a-e) and exposed to 100% material extracts (f-j). Test samples used include SDS DPL (a, f), SDS+SDC DPL (b, g), TX100 DPL (c, h), negative control UHMWPE (d, i), positive control of direct contact PVC disc reinforced with tin (e) and test on extract being diluted phenol (j).

4.2. Mechanical reinforcement of decellularized porcine liver matrix (DPL).

4.2.1. Preparation of silk fibroin (SF) and gelatin (G) solutions

The systematic procedure adopted for the extraction of silk fibroin from *B. mori* silk cocoon has been picturized in Fig.4.11.

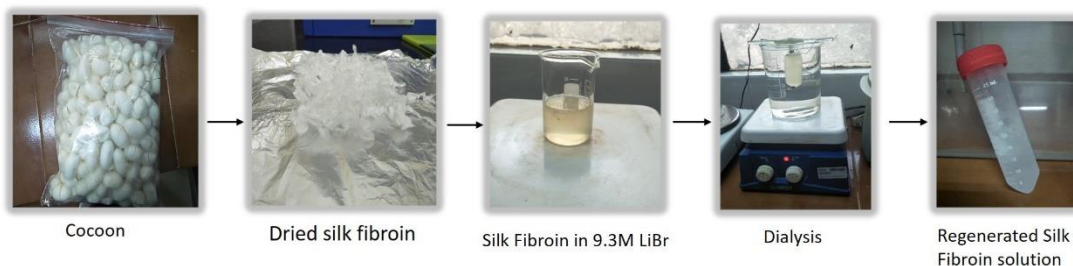


Figure 4.11: Silk fibroin extraction: Image showing various steps in silk fibroin extraction from *B. mori* cocoon.

4.2.2. Preliminary study

The films casted with and without CA (Fig. 4.12), exhibited a clearcut difference in the hydrolytic degradation study. There was a prominent degradation observed in the uncrosslinked films compared to the significantly reduced degradation of the CA crosslinked films. The statistical significance was more prominent for SF/G blend films than the SF alone casted one (Fig. 4.13). This proves the CA crosslinking provides increased stability to the solvent casted films.

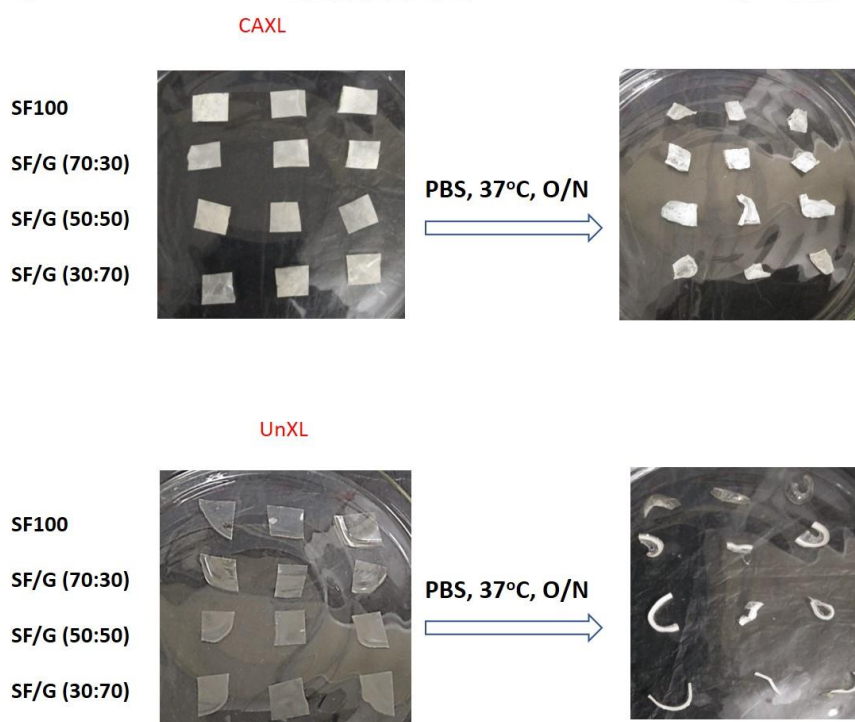


Figure 4.12: Hydrolytic degradation study of SF and SF-G blend films: Image showing both CA crosslinked and un-crosslinked films before and after incubation with PBS. SF100; film containing 100% silk fibroin, SF-G(70:30); film containing fibroin and

gelatin in 7:3 ratio, SF-G (50:50); film casted using fibroin and gelatin in 1:1 ratio, SF-G (30:70); film containing fibroin and gelatin in 3:7 ratio.

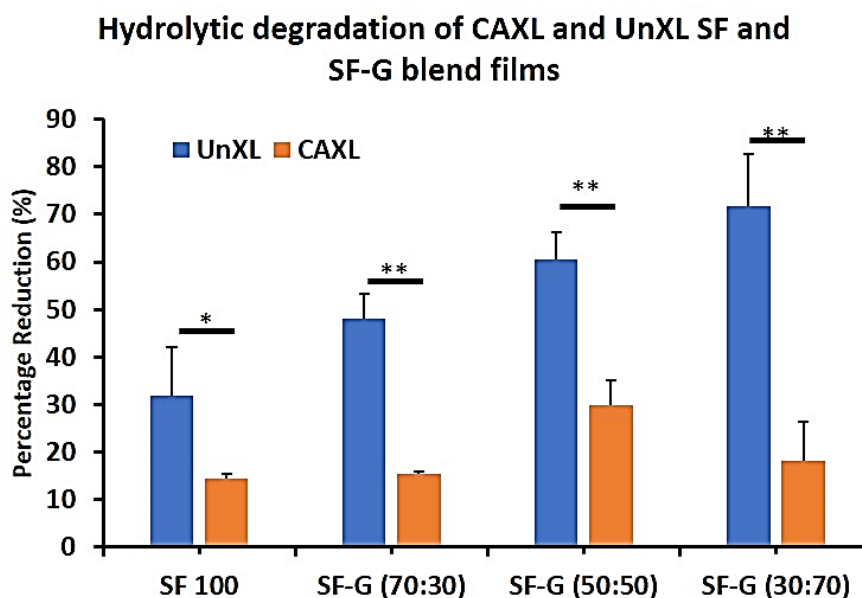


Figure 4.13: Percentage reduction in weight: Chart representing the percentage reduction in weight undergone by CA crosslinked and un-crosslinked SF and SF-G casted films.

4.2.3. Modification of DPL

Figure 4.14 illustrates the systematic procedure adopted for modifying the acellular porcine liver matrices through impregnation with selected natural polymers. Sample codes for the modified test groups were SF100DPL, SFG5050DPL and SFG3070DPL for those impregnated with CA containing SF100 and two polymer blend solutions of SFG5050 and SFG3070 respectively. The unmodified control was named DPL.

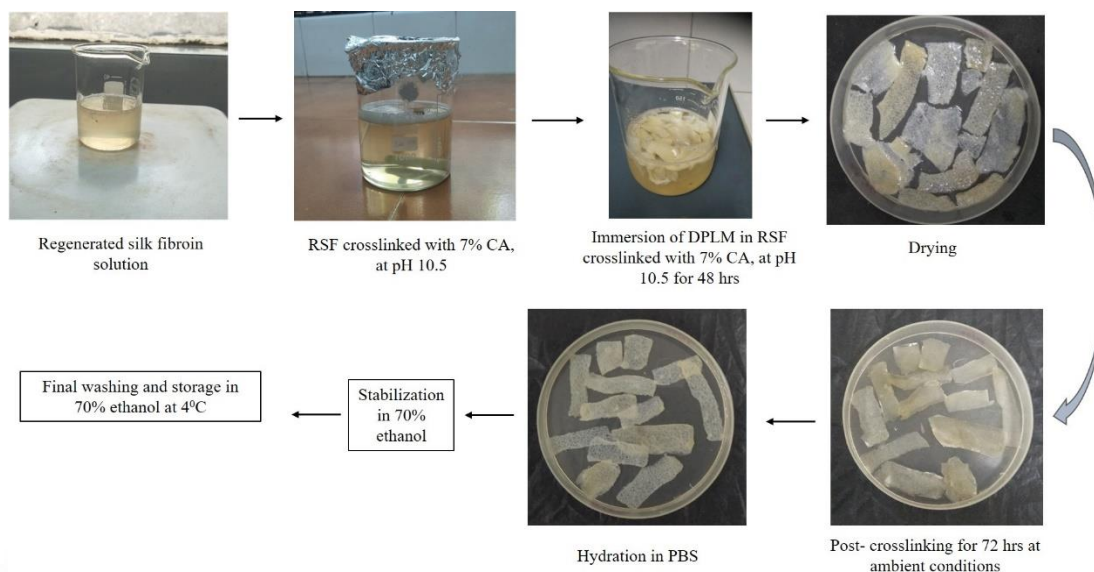


Figure 4.14: The impregnation procedure: Image representing various steps involved in the impregnation of CA-containing silk fibroin solution with DPL.

4.2.3.1. Characterization of modified scaffolds (SF100DPL, SFG5050DPL and SFG3070DPL)

4.2.3.1.1. Masson's trichrome staining

The experimental test groups developed were histologically evaluated using MT staining. The silk fibroin solution as well as the SFG blends incorporated within the test materials can be clearly distinguished in tissue sections. The blue color of aniline blue indicated the presence of collagen in the decellularized matrix whereas the unstained and whitish transparent region within the matrix indicated the polymers percolated inside the decellularized matrix (Fig. 4.15). SFG3070DPL showed the least incorporation of polymer blends, as blue regions are predominantly seen in the image, which appeared similar to that of decellularized control.

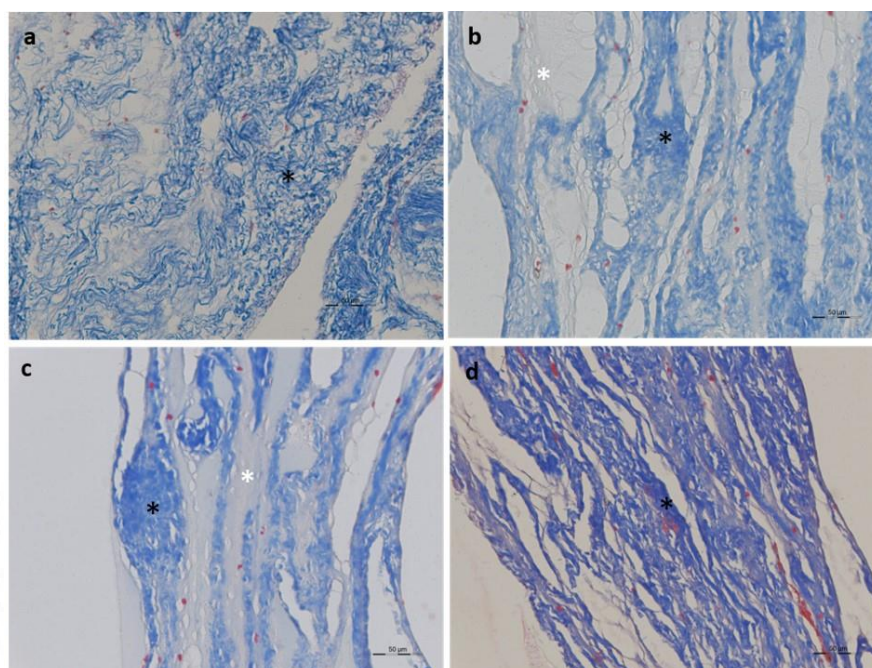


Figure 4.15: Histological assessment: Masson's trichrome images of DPL and impregnated samples. (a-e) Images showing the MT-stained images with a black star (dark blue color) representing the collagenous matrix part and a white star (greyish unstained region) representing the polymer-impregnated region within a) DPL, b) SF100DPL, c) SFG5050DPL and d) SFG3070DPL respectively. Magnification 400x; scale bar: 50 μ m

4.2.3.1.2. In-vitro remodelability

An *in-vitro* degradation study by collagenase susceptibility test has proved that the modified test materials show improved resistance to degradation compared to the unmodified control. DPL was found to be undergoing 78-82% reduction in weight in 1 to 7 days. The natural polymer-impregnated samples showed a significantly low weight reduction of 32-39% in 1 to 7 days, except for SFG3070DPL, which exhibited comparatively increased degradation of 68% at 7 days (Fig 4.16a). Determination of free amine concentration in the 24 h digested supernatants has proved that the

decellularized control exhibited the highest concentration of free amines (26.1 ± 1.6 $\mu\text{g}/\text{mg}$ tissue) and the least by SF100DPL (9.7 ± 1.2 $\mu\text{g}/\text{mg}$ tissue) as evident in Fig. 4.16b. The result proved that the impregnation of CA crosslinked natural polymer has been successful in reducing the free amines by effective crosslinking. From the same data, the degree of crosslinking was derived as a subsidiary information, by comparing the free amine concentration obtained for the impregnated samples with that of the non-impregnated (DPL) one. This has shown that among the impregnated test groups, SF100DPL and SFG5050DPL exhibited a crosslinking degree of 82.9 ± 6.1 and 79.5 ± 7.8 respectively while SFG3070DPL showed a decreased crosslinking degree of 42.07 ± 23.8 (Fig 4.16c).

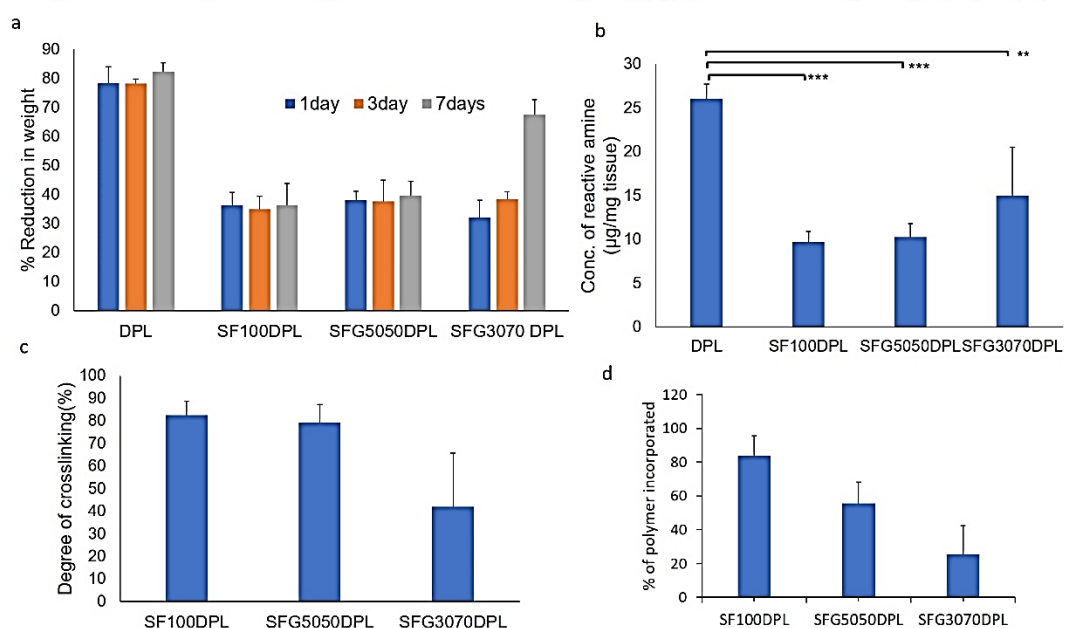


Figure 4.16: *In-vitro* degradation study: a) Percentage weight loss following incubation in collagenase enzyme for 1 day, 3 days, and 7 days. b) Free amine concentration determined by ninhydrin assay. c) The graph representing SF100DPL and SFG5050DPL having higher degree of crosslinking than SFG3070DPL. d) The

percentage of polymer incorporation achieved in the modified test materials derived from the gravimetric analysis. $p \leq 0.001$ (**) and $p \leq 0.0001$ (***)

4.2.3.1.3. Gravimetry

In gravimetry, a significant increase in the dry weight of modified scaffolds was increased compared to that of unmodified DPL. From the difference in their dry weights, the percentage of silk fibroin was estimated to be 83.8 ± 11.7 (in SF100DPL), 55.8 ± 12 (in SFG5050DPL), and 25.7 ± 16 (in SFG3070DPL) respectively (Fig. 4.16d).

4.2.3.1.4. Swellability

The water uptake capacity of the matrices expressed as percentage swelling is shown in Figure 19d. The data shows that the protein polymer impregnation into the decellularized matrix has reduced its swellability. Owing to the impregnation of DPL scaffolds with CA crosslinking protein, the porosity decreased and correspondingly the swelling percentage decreased. The percentage swelling of the decellularized control was 197 ± 49.1 and that of the test groups SF100DPL and SFG5050DPL was of 63 ± 9.9 and 70 ± 3.6 respectively. However, SFG3070DPL proved to have a percentage swelling of 104 ± 25 . All three modified scaffolds exhibited reduced swellability with a statistical significance of $p < 0.05$ compared to unmodified DPL (Fig. 21d).

4.2.3.1.5. Biomechanical properties

Mechanical properties evaluated for the decellularized control and all three impregnated test samples proved the impregnation method has significantly improved the tensile strength and Young's modulus. The tensile strength of DPL improved from 2.43 ± 0.4 MPa to 3.84 ± 0.66 MPa in SF100DPL with a statistical significance of $p < 0.05$. The tensile property exhibited by SFG5050DPL and SFG3070DPL was not

observed to be significant statistically (Fig 4.17a). The elastic modulus of the modified scaffolds experienced a prominent increase from 9.1 ± 1.7 to 20 ± 1.9 , 14 ± 1 and 15.2 ± 1.9 MPa respectively in the three test groups (Fig. 4.17b). Poor suturability was a major hurdle as far as the decellularized liver matrix was concerned, which had increased from 0.9 ± 0.08 in DPL to 2.25 ± 0.2 , 2.8 ± 1.2 and 2.6 ± 0.9 in the modified samples SF100DPL, SFG5050DPL, and SFG3070DPL respectively which is expressed as maximum load (N) (Fig 4.17c).

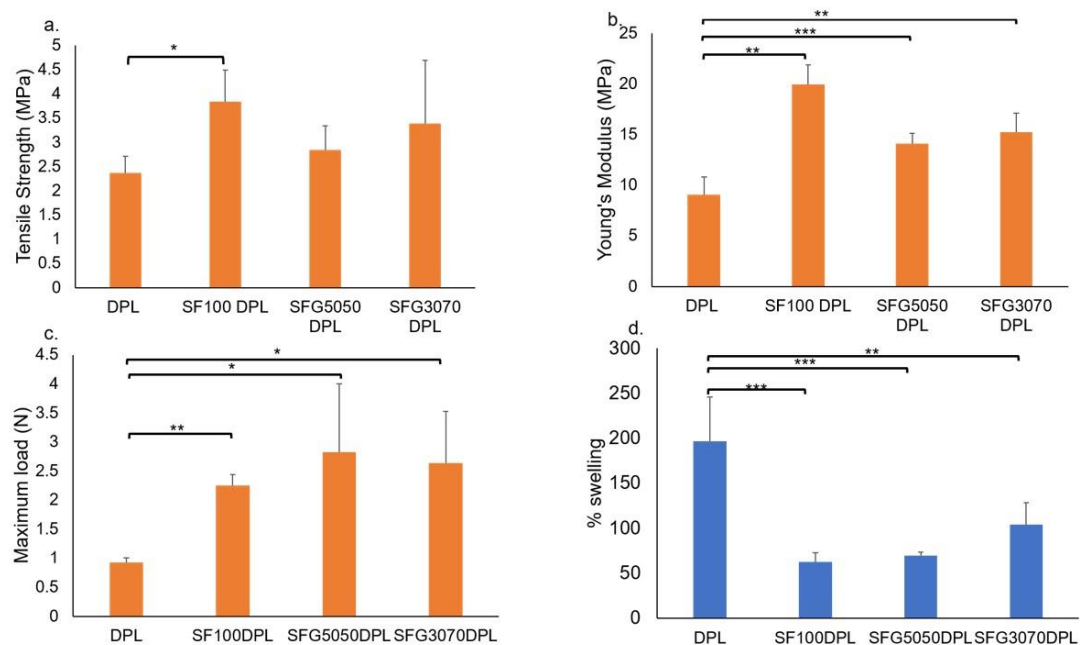


Figure 4.17: Biomechanical strength and swelling behavior: Graphs showing the improvement in a) Tensile strength b) Young's modulus, c) Suture retention strength, and d) reduction in swelling behavior exhibited by the modified test samples significantly reduced compared to the decellularized control. $p \leq 0.01$ (*), $p \leq 0.001$ (**) and $p \leq 0.0001$ (***)

4.2.3.1.6. Ultrastructure

The ultrastructural evaluation by SEM proved the DPL devoid of any cells, and the matrix developed is highly porous with collagen bundles being intact which confirmed the decellularization method not adversely influencing the matrix architecture. All three test groups appeared smooth compared to DPL, which further proved that the polymers employed had percolated within the matrix through the pores and had changed their surface characteristics (Fig. 4.18 a-d). SFG3070DPL shows a patchy appearance, which might be due to the removal of gelatin during the hydration of the crosslinked sample before sterilization. SEM carried out in cross sections (Fig. 4.18 e-h) of the test materials adds additional evidence that the natural polymers had successfully percolated within the bulk of the matrix.

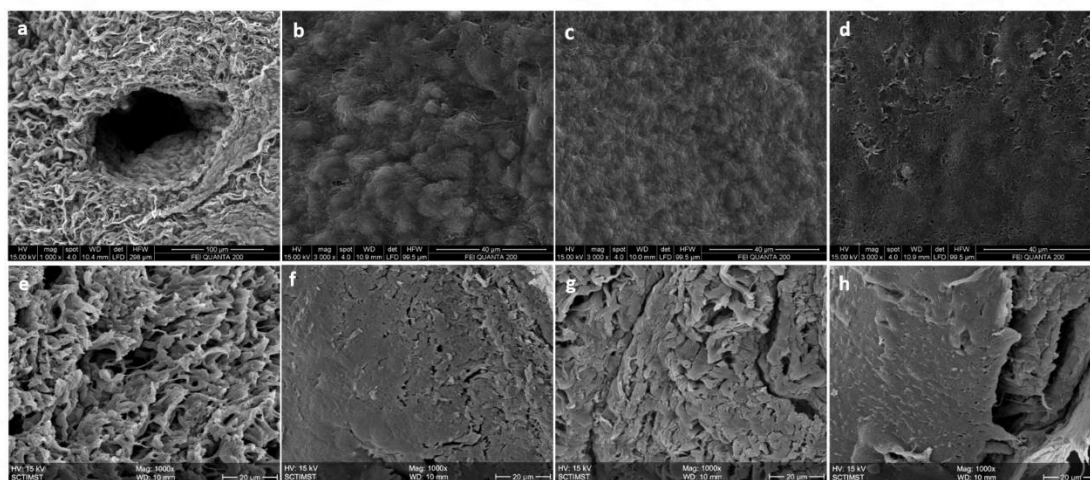


Figure 4.18: Ultrastructure: Images displaying ultrastructural surface view (a-e) and cross-sectional view (e-h) of (a, e) decellularized porcine liver (DPL) and modified acellular matrices (b, f) SF100DPL, (c, g) SFG5050DPL and (d, h) SFG3070DPL respectively. Magnification: 1000X

4.2.3.1.7. FTIR

A more or less similar spectral pattern was obtained for the modified and unmodified samples. SF100DPL exhibited only a very slight shift in the spectral peaks compared

to that of unmodified DPL. Very strong absorption bands at $1625\text{--}1630\text{ cm}^{-1}$, $1520\text{--}1530\text{ cm}^{-1}$ and $1260\text{--}1270\text{ cm}^{-1}$ correspond to amide bands I, II, and III, which represent mainly C=O stretching, N-H deformation and C-N stretching/ C=O bending respectively (Dhasmana et al., 2019b). Changes in the spectral pattern obtained for the modified scaffolds by the incorporation of the silk fibroin and its blend with gelatin are as follows. The sharpness of the peak at 1540 (amide II) observed in DPL has become less intense in SF100DPL (Fig. 4.19a), the sharpness and shift of the peak at 1396 cm^{-1} , 1079 cm^{-1} and 1031 cm^{-1} was significant in the modifications SF100DPL and SFG5050DPL, and the peak at 1164 cm^{-1} which was less pronounced in DPL became slightly prominent in SF100DPL and more prominence was observed for SFG3070DPL. In SF-G blend modified samples (SFG5050DPL & SFG3070DPL) the shift to lower wave number was found to be more prominent especially the peaks of amide I, II and III. The peak at 1396 cm^{-1} and 1031 cm^{-1} were absent in SFG3070DPL (Fig. 4.19b and 4.19c).

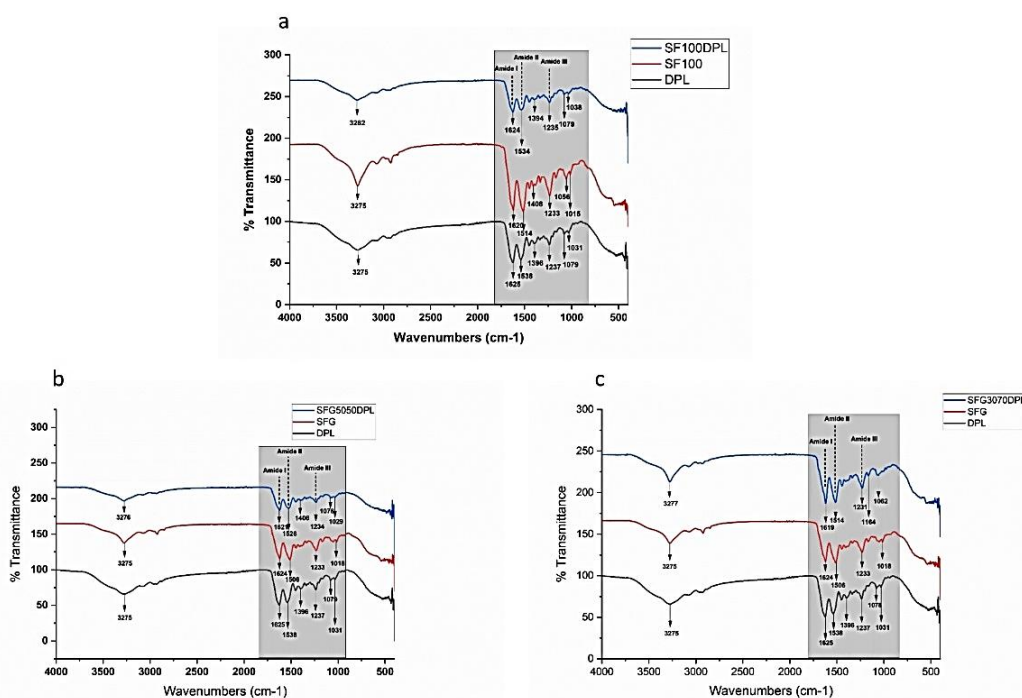


Figure 4.19: FTIR spectral analysis: Showing the spectral pattern and stacked spectra obtained for modified scaffolds in comparison with the unmodified control and the polymer alone casted films. (a) SF100DPL, (b) SFG5050DPL and (c) SFG3070DPL

4.2.3.1.8. Surface wettability

The surface characterization by static water contact angle measurement has proven that the hydrophilicity exhibited by DPL was significantly decreased towards more hydrophobicity in the modified bioscaffolds (figure 4.20 a-d). The contact angle obtained for unmodified DPL was 26 ± 3 , which indicates its hydrophilicity. The modified matrices exhibited a higher contact angle of 83 ± 7 in SF100DPL, 78 ± 3 in SFG5050DPL, and 88 ± 13 in SFG3070DPL respectively (Fig 4.20e).

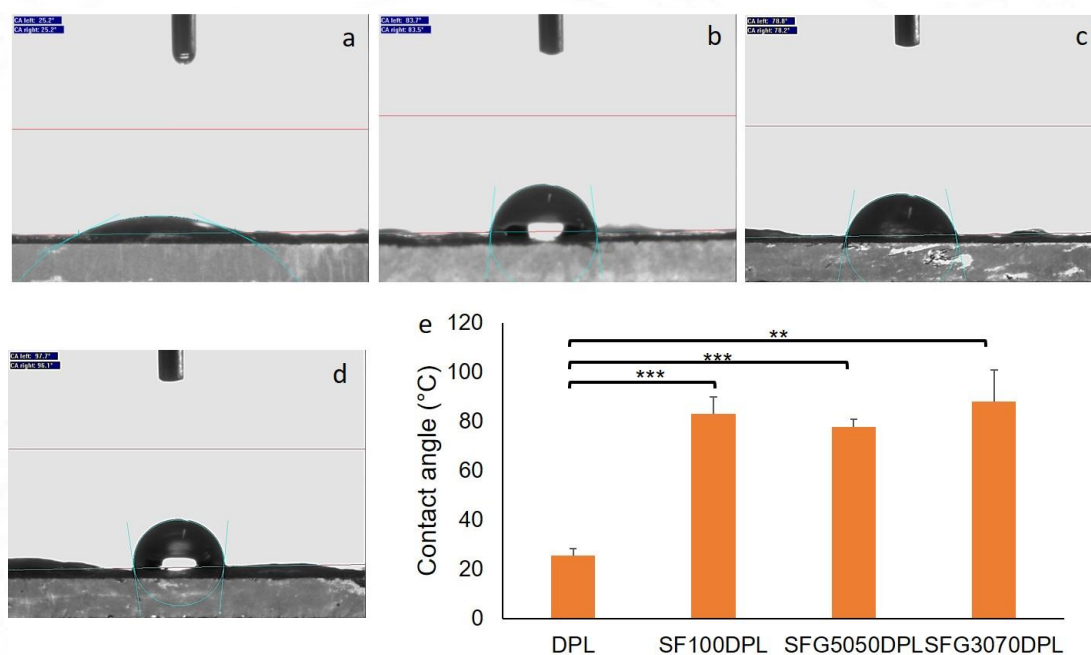


Figure 4.20: Surface contact angle: Images of the static water contact angle formed on the surfaces of a) DPL b) SF100DPL c) SFG5050DPL and d) SFG3070DPL along with e) the graphical representation of average contact angles with statistical significance marked. $p \leq 0.001$ (**) and $p \leq 0.0001$ (***)

4.2.3.1.9. GAG preservation

A qualitative study using Alcian blue staining has proved that GAG content was retained even after the mechanical augmentation procedure to strengthen the acellular porcine liver matrix, evidenced by the lightly blue-stained regions in SF100DPL (Fig. 4.21b), SFG5050DPL (Fig. 4.21c) and SFG3070DPL (Fig. 4.21d) and quantitatively confirmed by DMMB assay (Fig. 4.21e). The GAG content estimated in acellular porcine liver matrix by DMMB assay was 9.93 ± 2.08 $\mu\text{g}/\text{mg}$ dry weight. GAG concentration was reduced in the modified scaffolds in comparison to unmodified control, which was numerically estimated to be 3.2 ± 0.33 , 4.78 ± 0.28 and 4.94 ± 0.47 $\mu\text{g}/\text{mg}$ dry tissue respectively in SF100DPL, SFG5050DPL, and SFG3070DPL.

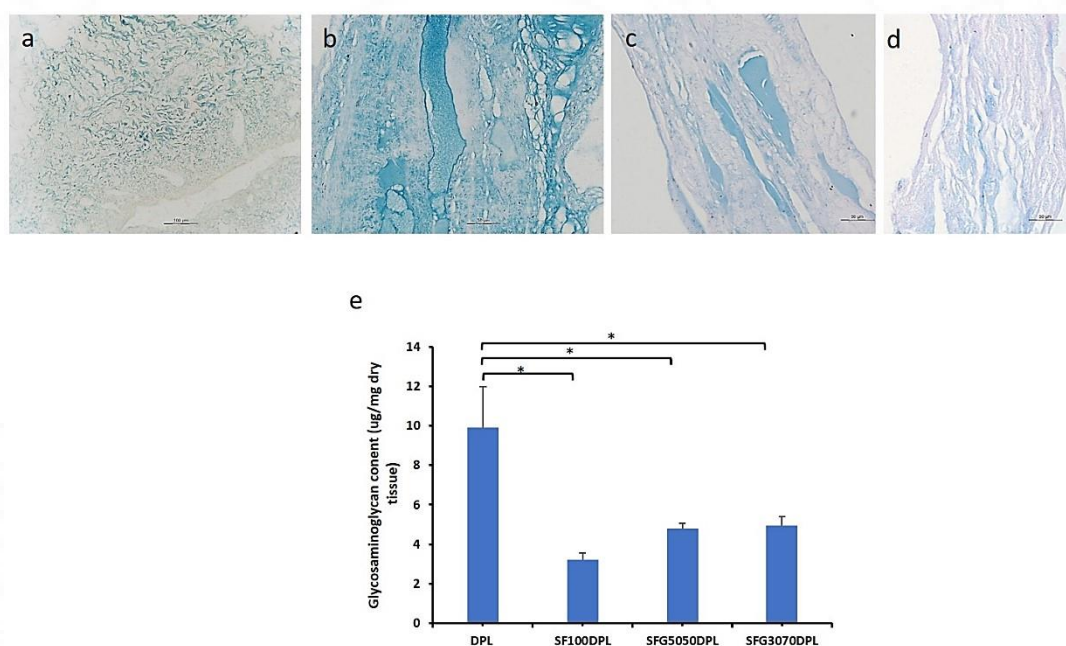


Figure 4.21: Glycosaminoglycan: Alcian Blue stained images of a) DPL, b) SF100DPL, c) SFG5050DPL and d) SFG3070DPL. e) Chart representing the quantity of sulfated GAG preserved within the unmodified and modified scaffolds using dimethyl methylene blue (DMMB) assay ($p < 0.05$). Scale bar- 40 μm

4.2.3.1.10. In-vitro cytotoxicity

The cytotoxic reactivity of test and control samples evaluated by direct contact test was given in Table 4.1. The evaluation was carried out using an inverted phase contrast microscope based on ISO10993-part5.

Sl. No.	Sample	Grade	Reactivity
1	Negative control (UHMWPE)	0	None
2	Positive control (Phenol)	4	Severe
3	DPL	0	None
4	SF100DPL	0	None
5	SFG5050DPL	0	None
6	SFG3070DPL	0	None

Table 4.1: Cytotoxicity grading: The cytotoxicity grades of the test samples and control materials proving their cytocompatibility.

The cell response around the test sample is shown in Figure 4.22. All three test groups including the control have obtained a cytotoxicity score of zero, which proves the materials tested to be non-cytotoxic. The cell morphology was comparable to negative control, without showing any zone of cell inhibition around the test materials. Negative control has no cytotoxic reactivity and positive control showed severe cytotoxic reactivity as expected. The cytotoxic reactivity of cells to the extracts of DPL, SF100DPL, SFG5050DPL, and SFG3070DPL also showed no cell lysis or reduction of cell growth confirming its non-cytotoxicity. Negative control (UHMWPE) gave no cytotoxic response and positive control (Phenol) gave severe cytotoxic reactivity as

expected. Cells incubated with extract of all test samples at 100%, 50%, and 25% dilutions showed more than 80% cell viability compared to the negative control evident by MTT assay. This further ensures the materials tested were non-cytotoxic (Fig. 4.22 e-h and Fig. 4.23).

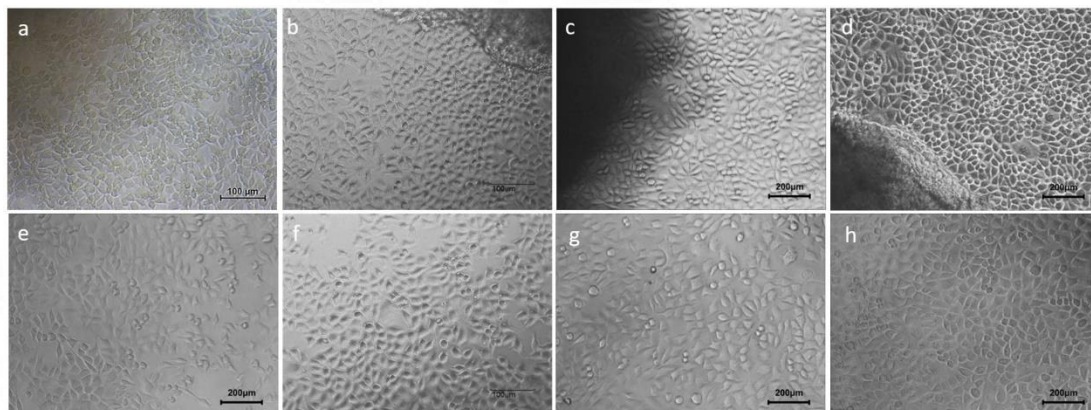


Figure 4.22: Cytotoxicity: Microscopic images showing non-cytotoxic cellular responses (normal cellular morphology and proliferation and no zone of cell lysis) of L929 cells with direct contact with the materials (a-d) and also when exposed to 100% material extracts (e-h). Test samples used include DPL (a & e), SF100DPL (b & f), SFG5050DPL (c & g) and SFG3070DPL (d & h)

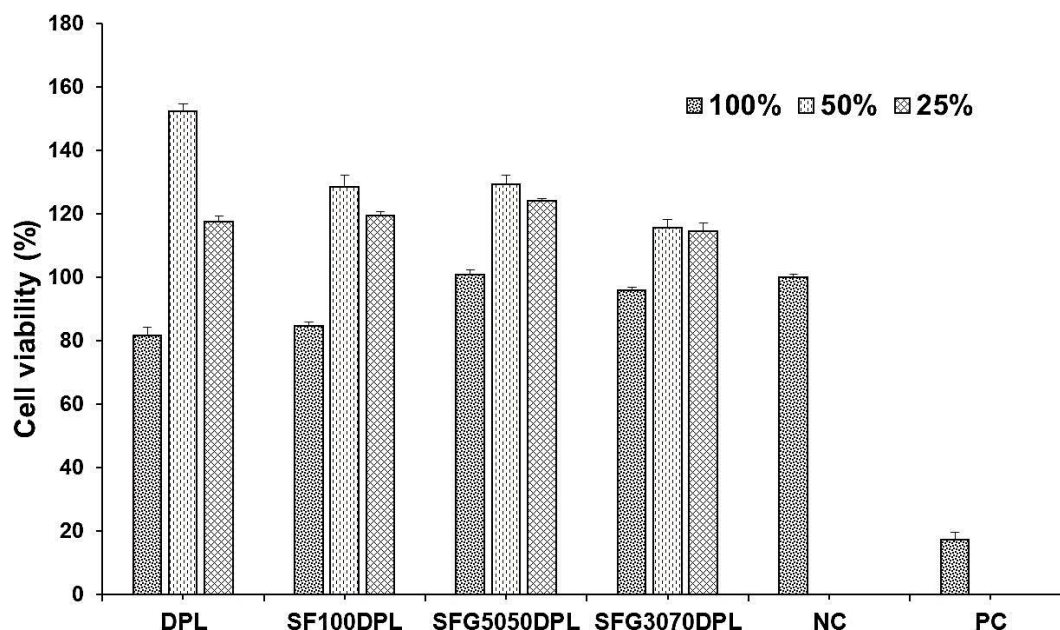


Figure 4.23: Cell viability: Percentage cell viability of L-929 cells exposed to extracts of DPL, SF100DPL, SFG5050DPL and SFG3070DPL. The values were normalized to negative control (NC UHMWPE). Positive control (PC Phenol) showed <20% activity indicating severe cytotoxicity.

4.2.3.1.11. In-vitro cell migration study (Scratch wound assay)

In-vitro wound healing study has proven the efficiency of the material extracts in promoting fibroblast cell proliferation and migration leading to wound closure. The cell-free area at different time points was semi-quantitatively analyzed using Image J software and expressed as a percentage of wound closure. The data demonstrated that at a 4 h time point, cells treated with SF100DPL extract migrated faster with a percentage cell migration of 26.8 ± 4.4 to the wound area. At 8 h and 24 h time points, DPL, SF100DPL and SFG5050DPL extract-given cells showed comparatively higher cell migration than the cell control which was estimated semi-quantitatively as 58.3 ± 7.9 , 51.4 ± 7.6 , 55.8 ± 10.5 at 8 h and 73.3 ± 5.9 , 69 ± 6.5 , 79.1 ± 3.3 at 24 h

respectively (Fig. 4.24B). The cells incubated with extracts prepared from SFG3070DPL exhibited significantly least migration at 8 h and 24 h compared to the media control

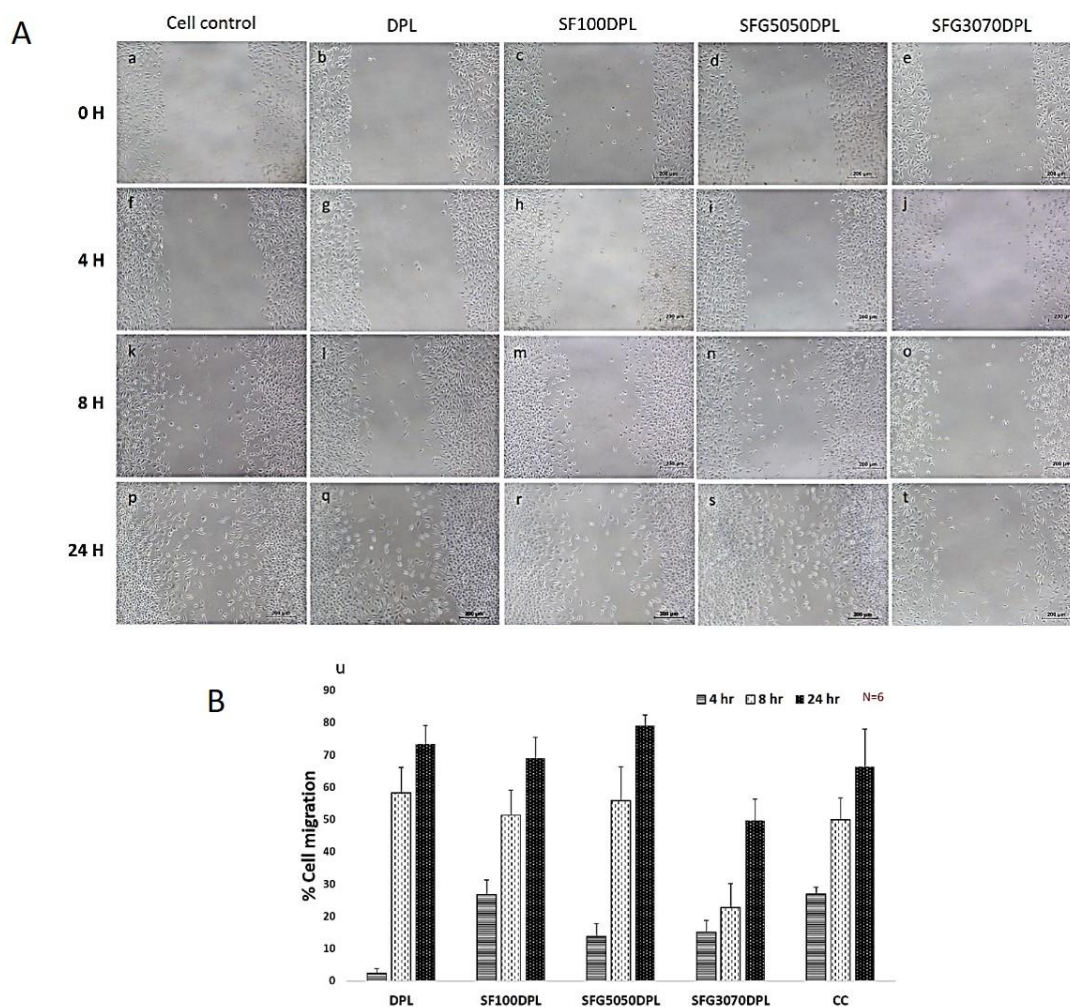


Figure 4.24: Scratch wound assay: Representative light microscopic images showing different rates of wound closure at 0H, 4H, 8H and 24H by L929 fibroblast cells treated with 100% extracts prepared from DPL (b, g, l, q), SF100DPL (c, h, m, r), SFG5050DPL (d, i, n, s) and SFG3070DPL (e, j, o, t) compared with cell control (a, f, k, p). Graphical representation of semiquantitative data of percentage wound closure

exhibited by L929 cells treated with respective material extracts done using Image J software. Scale bar- 200 μ m.

4.3. Inflammatory and Immune potential of acellular and modified scaffolds

4.3.1. Subcutaneous implantation

Every animal tolerated the implantation procedure and no healing-associated complications were observed in any of the study groups. All animals survived the entire observation period without showing any adverse responses during the study. The site of implantation was normal and didn't show any sign of redness, infection, or bulging. The unmodified DPL showed high degradation on 90 days, and only remnants of the material could recover (Fig. 4.25c). All modified materials were recoverable at the time of autopsy. The majority of them were found to be in the folded form (Fig. 4.25 d-f).

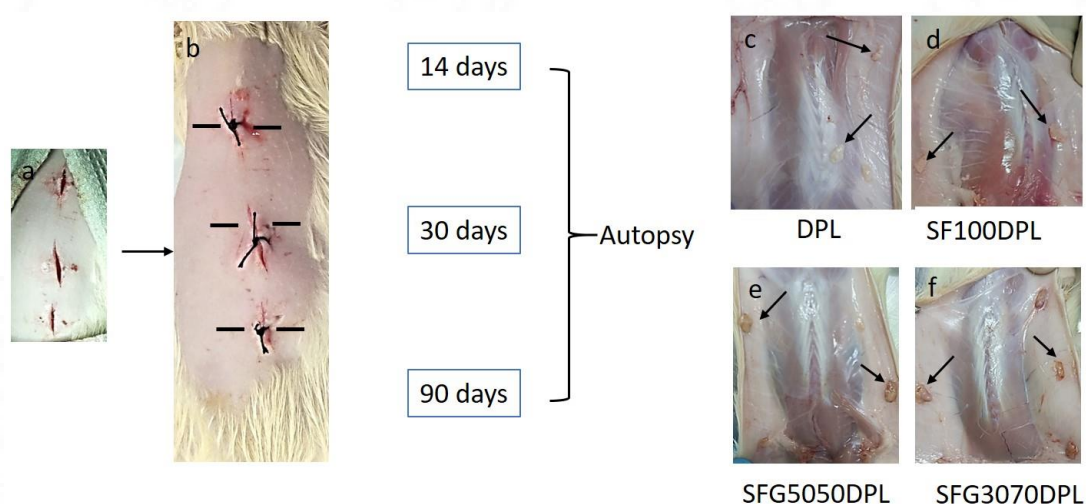


Figure 4.25: Subcutaneous implantation: Images showing the procedure of creation of dorsal incision (a) and subsequent insertion of test materials and its closure with 3-0 silk suture (b). Representative images showing the presence of explants after

completion of the respective observation period, (c) DPL, (d) SF100DPL, (e) SFG5050DPL, and (f) SFG3070DPL respectively.

4.3.1.1. Hematology

The basal hematological parameters and improvement in percentage body weight gain were observed to be within normal range, indicating the normal physiological characteristics exhibited by the material implanted animals (Fig. 4.26).

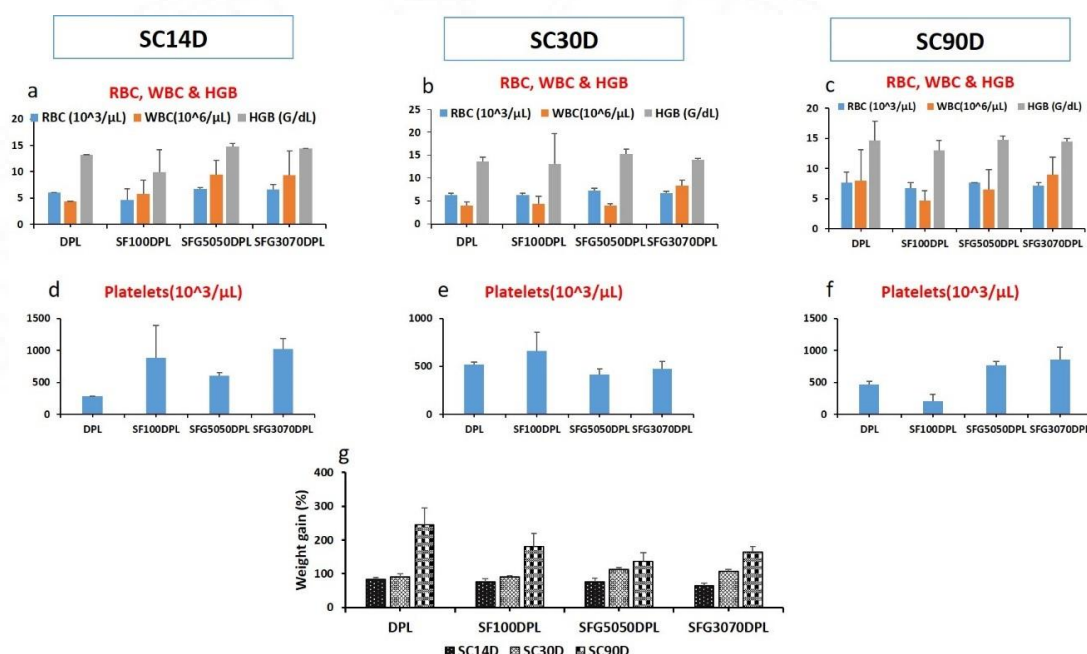


Figure 4.26. Hematology and body weight gain: The RBC, WBC, HGB, Platelets, and percentage body weight gain exhibited by the animals subcutaneously implanted with the test materials.

4.3.1.2. Histological evaluation of inflammation (Inflammatory cell scoring)

Histomorphological examination with H&E staining showed more pronounced cell infiltration in all four implanted test groups. Major inflammatory cells, which were taken into account included neutrophils (PMNCs- polymorphonuclear cells),

lymphocytes, macrophages, plasma cells, and foreign body giant cells (FBGCs). More robust cellular response was seen at the edges of the implant adjacent to the recipient tissue on 14 days and 30 days, which was considerably reduced at 90 days (Fig. 4.27). Lymphocytes, macrophages, and neovascularization were visible at all time points without much difference between the modified and unmodified materials. Inflammatory cell scoring based on ISO10996-part 6 (2016) and determination of reactivity score was also carried out. On 14 days of implantation, there was a predominance of PMNCs represented by mild to moderate response, irrespective of the material implanted. At 90 days, the PMNCs have significantly reduced to minimal. The reduction in PMNC score has considerably reduced from 14 days to 90 days from a score of 2 to 0.5 for DPL, 2.7 to 0.4 for SF100 DPL, 2.6 to 0.6 for SFG5050DPL and 3.2 to 1.1 for SFG3070DPL respectively (Fig. 4.28a). This marks a reduction in acute inflammation on 90 days in all the test materials irrespective of their modification. Lymphocytes and macrophages were predominantly present at all time points as it indicates an active immune reaction taking place within the materials by the host (Fig. 4.28b and 4.28d). Plasma cell response was initially very less but increased at 30 days and 90 days in DPL, but SF100DPL, SFG5050DPL, and SFG3070DPL exhibited a peaking at 30 days (Score of 1.6, 1.7, and 2) which downregulated on 90 days (score of 1.1, 1.1 and 1.5) (Fig 4.28c). The minimal giant cell response was observed in modified scaffolds, highest in SFG3070DPL at 90 days (score of 1.2) (Fig. 4.28e). Moderate neovascularization was evident at the beginning of 14 days, which became more abundant at 30 days. Neovasculature has diminished in 90 days. The above trend was observed in all the material explants (Fig. 4.28f).

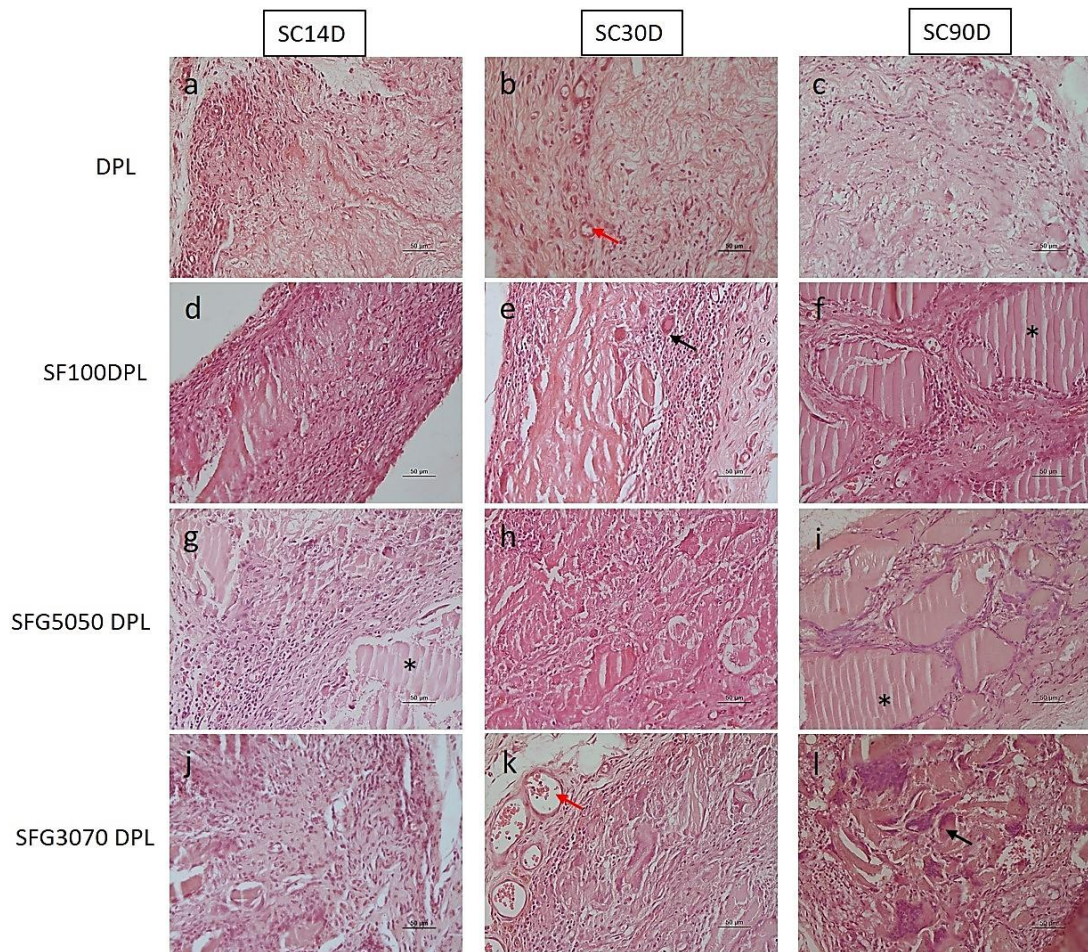


Figure 4.27: H&E staining: Representative images showing the extent of mononuclear infiltration in subcutaneous explants of DPL (a, b, c), SF100DPL (d, e, f), SFG5050DPL (g, h, i) and SFG3070DPL (j, k, l) of subcutaneous implantation at 14 days (a, d, g, j), 30 days (b, e, h, k) and 90 days (c, f, i, l) of time. Scale bar- 50 µm.

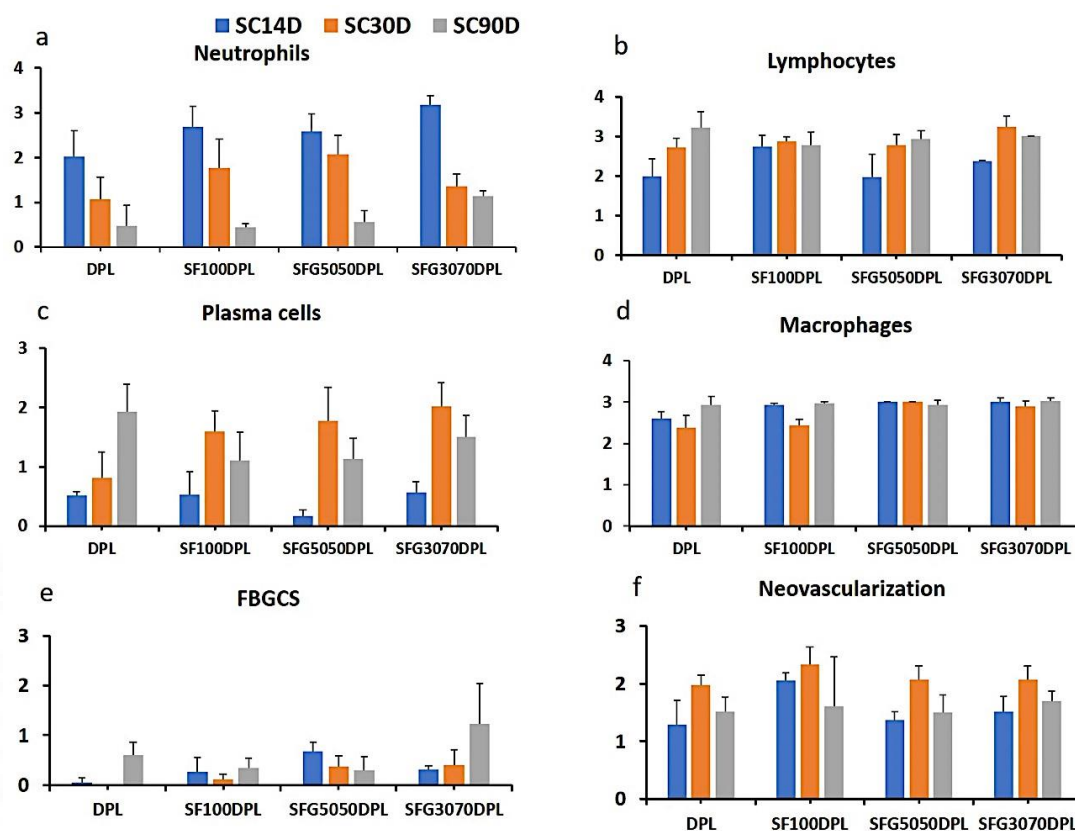


Figure 4.28: Histopathology scoring: Histological scores of the unmodified and modified scaffolds after explant recovery. (a) The average scores for the number of neutrophils; (b) lymphocytes; (c) plasma cells; (d) macrophages; (e) FBGCs; and (f) amount of neovascularization.

4.3.1.3. Lymphocyte proliferation test (Cell-mediated immune response)

The cell-mediated immune response elicited by the animals against the implanted material proteins was estimated by lymphocyte proliferation test to find out the presence of sensitized lymphocytes. The results obtained showed a higher standard deviation due to inter-animal variation. There was no significant lymphoproliferation observed except at the initial time point of 14 days. An insignificant proliferation which exhibited a decrease in trend on increasing the implantation time was evident in

DPL, SFG5050DPL, and SFG3070DPL. In SF100DPL, the SI was highest on 30 days which considerably reduced at 90 days. The modified scaffolds SF100DPL, SFG5050DPL, and SFG3070DPL did not result in an uncontrolled multiplication of splenic lymphocytes (Fig. 4.29), especially at 30 days and 90 days of implantation.

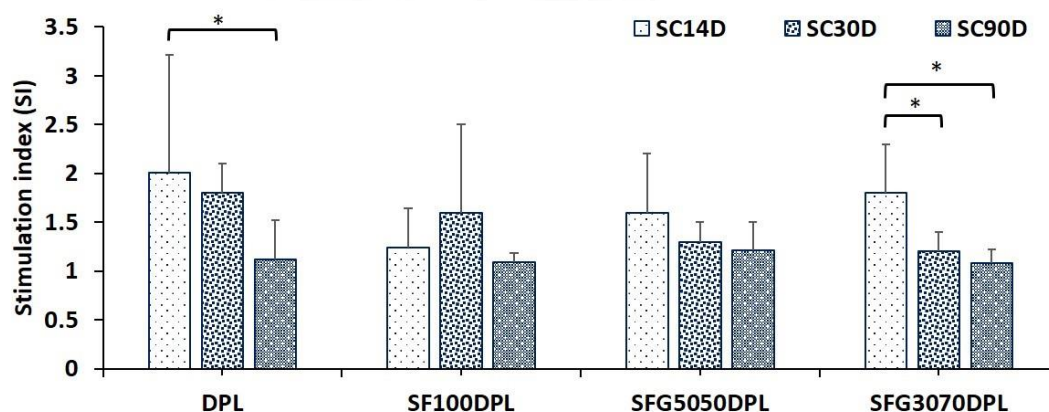


Figure 4.29. Lymphocyte proliferation: Chart showing cell-mediated immune response studied using lymphocyte proliferation test and the result expressed as stimulation index. * $p < 0.05$

4.3.1.4. IFN- γ release (T-cell activation) and TNF- α release(B-cell activation) assays

The implanted materials in any of the animals under study did not influence IFN- γ release. Its release was observed to be insignificant in comparison to the non-stimulated control (Fig. 4.30). TNF- α release had shown an initial activation by significantly increased production of TNF- α on 14 days that became insignificant on 30 days on exposure to unmodified DPL antigen. However, on 90 days, further activation of cytokine-producing B-cells was observed in the same group. Even though not stimulated initially on 14 days, PBMCs derived from SF100DPL implanted animals exhibited a biologically significant increase in production of TNF- α on 30

days that was masked on 90 days. SFG blend modified DPL implantation also had shown an initial non-stimulation followed by an apparent increase resulting from SFG5050DPL implantation on 30-day and 90-day periods. Such an increase was observed at 90 days upon exposure to SFG3070DPL (Fig. 4.31).

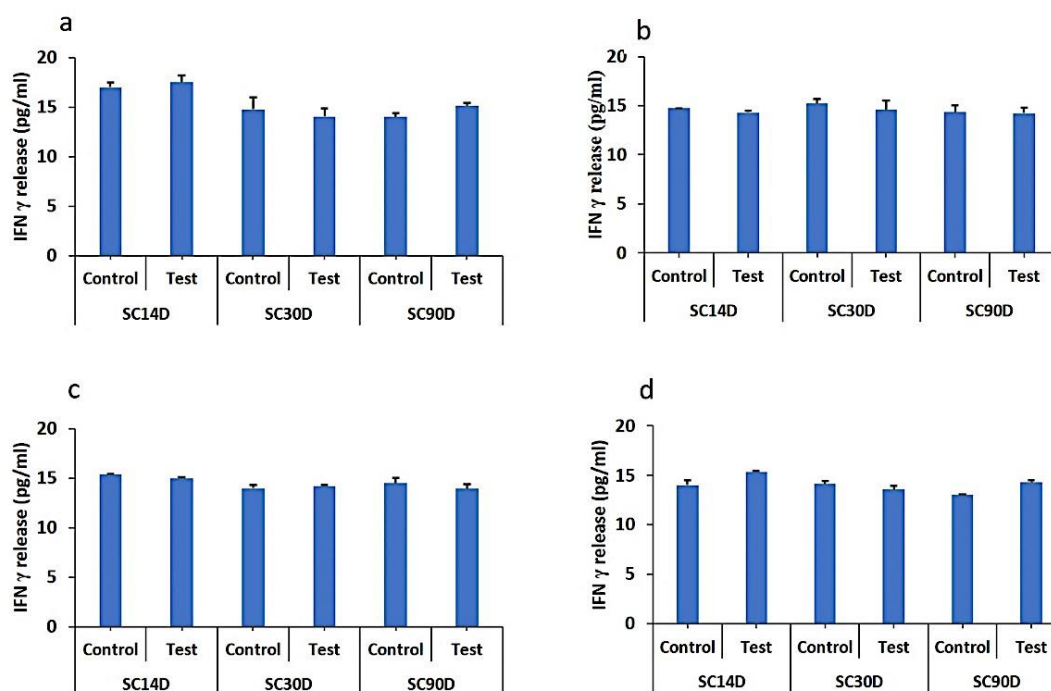


Figure 4.30: Sub-cutaneous implantation- IFN- γ release: Graphical images showing the inflammatory cytokine, IFN- γ release estimated in serum samples of subcutaneously implanted animals with DPL (a), SF100DPL (b), SFG5050DPL (c) and SFG3070DPL (d) compared to unstimulated control

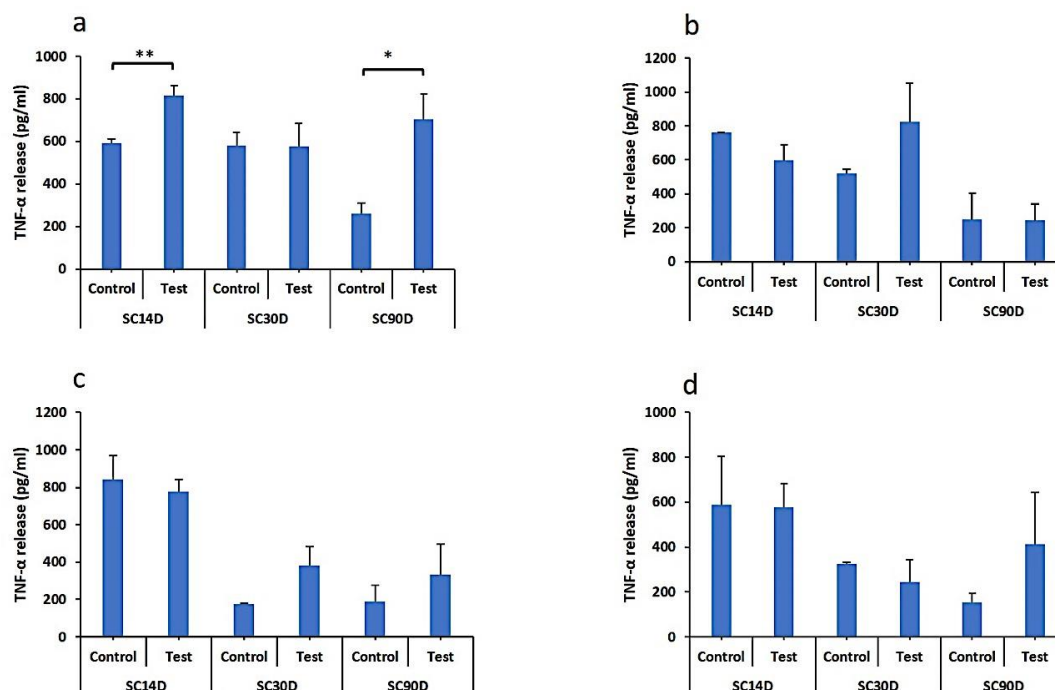


Figure 4.31: Subcutaneous implantation- TNF- α release: Graphical images showing the inflammatory cytokine, TNF- α release estimated in serum samples of subcutaneously implanted animals with DPL (a), SF100DPL (b), SFG5050DPL (c) and SFG3070DPL (d) compared to unstimulated control. $p \leq 0.05$ (*) and $p \leq 0.001$ (**).

4.3.1.5. Antibody (IgG+IgM+IgA) response

Differences in collective antibody response elicited by the animals against the materials implanted have been compared in Figure 4.32. The positive control used here was obtained from an immunization experiment carried out using test material extract at a concentration of 100 $\mu\text{g/mL}$. Antibody response exhibited a decreasing trend or increasing the duration of implantation for SFG5050DPL and SFG3070DPL implanted animals. Even though a significantly reduced antibody-mediated response was evident on 30 days ($p=0.01$) for DPL, it showed a significant hike on 90 days ($p=0.008$). From the results, it was evident that all three modified scaffolds were not triggering the activation of cell-mediated or humoral immune responses.

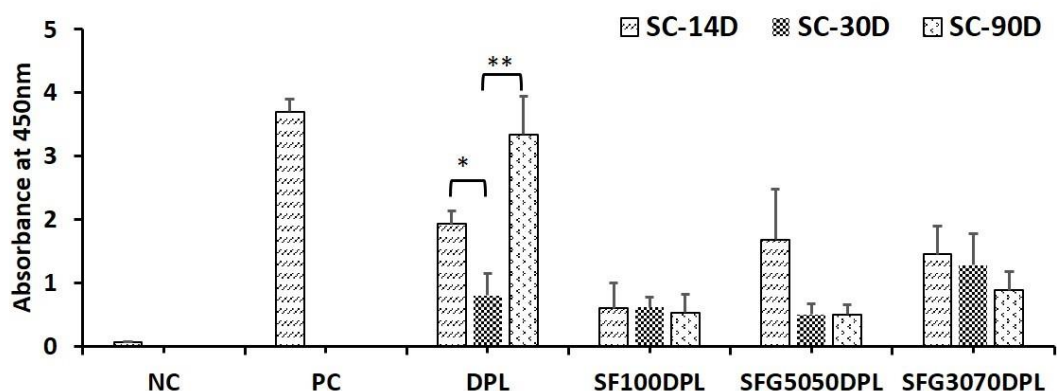


Figure 4.32. Antibody response: Chart representing the humoral immune response evaluated by indirect Elisa using IgG+IgM+IgA antibody and HRP detection system. * $p < 0.05$, ** $p < 0.01$

4.4. Induced regenerative potential of the scaffolds

4.4.1. Rat abdominal wall defect model

The surgical procedure carried out included the creation of the abdominal wall defect and closing the defect using the scaffolds as shown in Figure 4.33. The animals in which the abdominal wall regeneration (AWR) study was conducted survived for the entire study period of 21 days and 90 days. None of the animals exhibited any sign of infection, graft rupture or herniation, or graft rejection. The presence of the graft during euthanasia was identified by the presence of polypropylene suture. The grafted part in 21 days study, was visible and distinct from that of the host abdominal wall, but on reaching 90 days, the graft gradually integrated with the host abdominal wall and increased in thickness than at 21 days (Fig. 4.34). Abdominal wall adhesion was prominent at 21 days (Fig. 4.34 f & g) which markedly reduced and became absent in SF100DPL and SFG5050DPL at 90 days (Fig. 4.34 n & o).

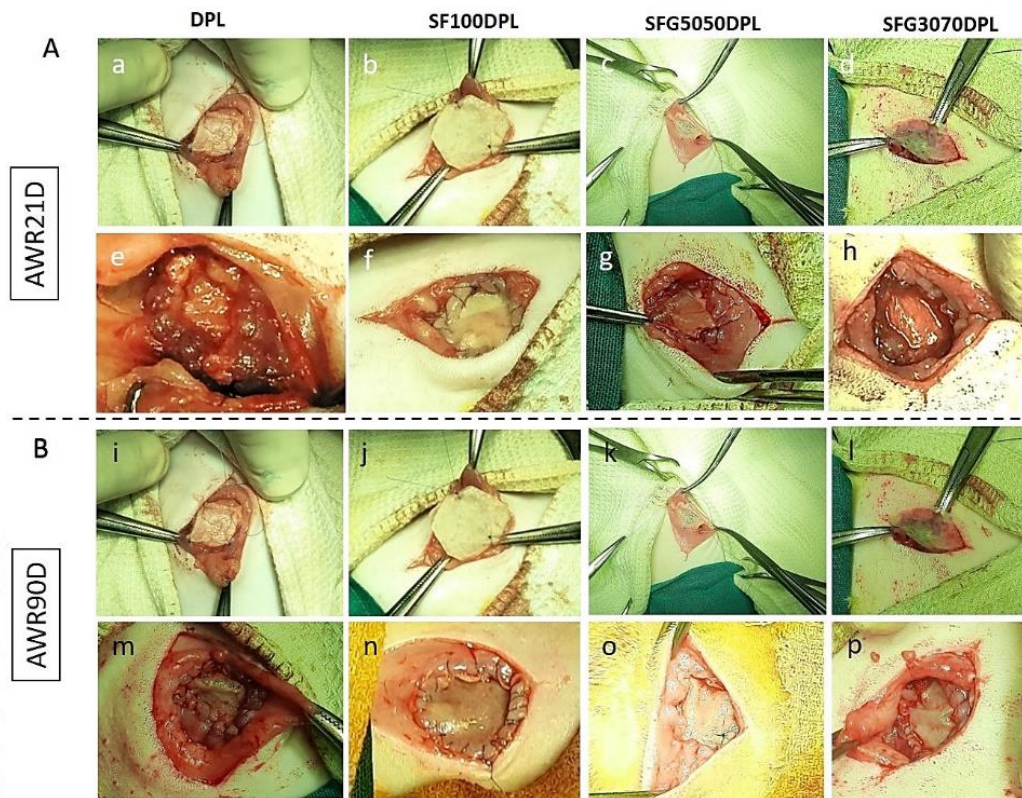


Figure 4.33: Rat abdominal wall defect creation and implantation- Gross images showing the surgical procedure of (a-e and i-l)) creation of the defect and placing the implants and positioning with anchoring sutures, (e-f and m-p)) appearance of the defect site immediately after suturing the implant.

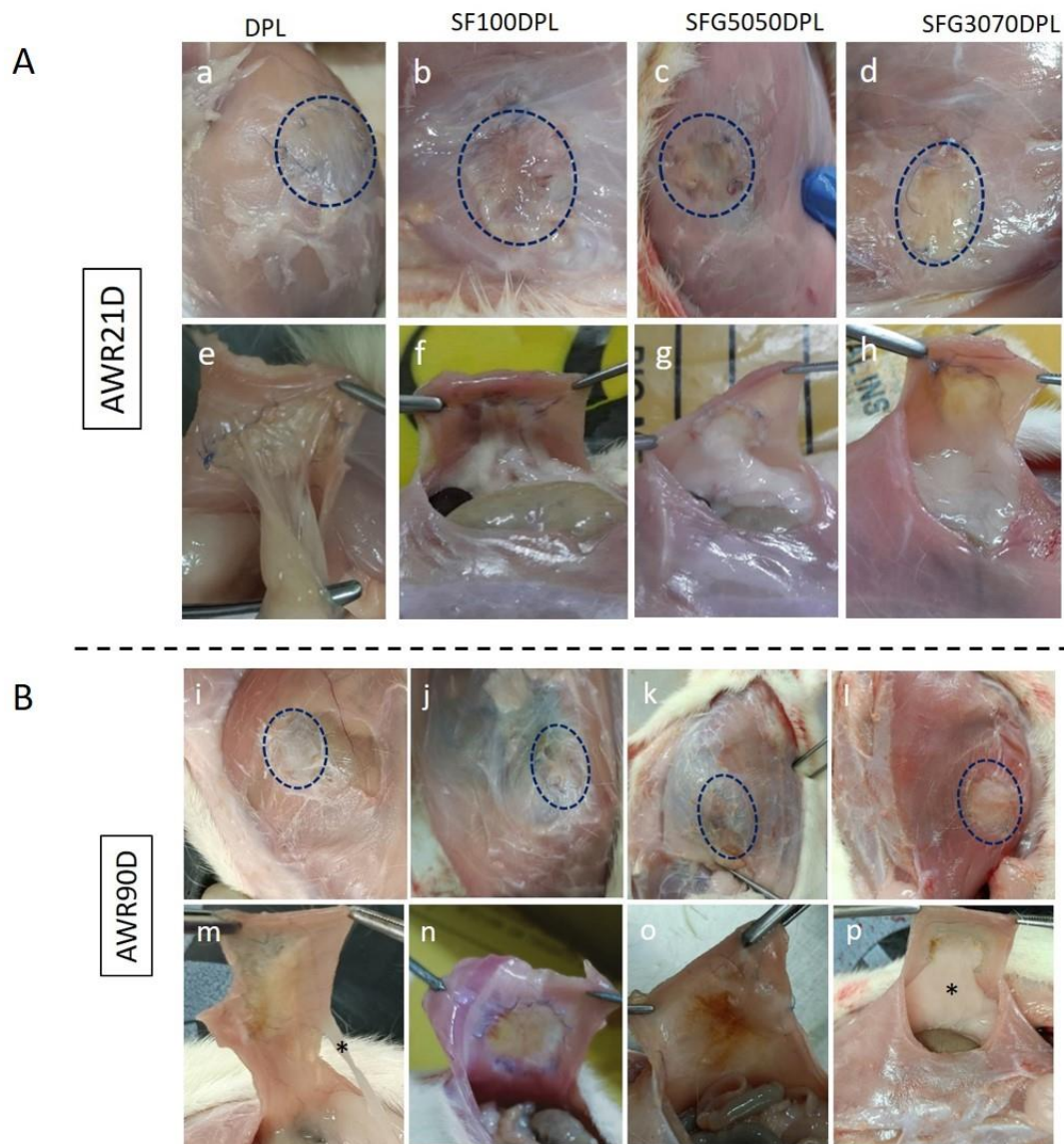


Figure 4.34: Gross observation on completion of observation period: Gross external appearance of the implant on completing 21 days (a-d) and 90 days (i-l). The internal side of the graft, with exhibiting fatty adhesion at 21 days (e-h) and 90 days (m-p). DPL and SFG3070DPL alone show fatty adhesion on 90 days.

4.4.1.1. Hematology

The basal hematological parameters such as RBC, WBC, HGB, and platelets were observed to be within normal range, indicating the normal physiological characteristics

exhibited by the material implanted animals (Fig. 4.35). The percentage increase in body weight was not significant for animals kept for 21-day study but those maintained for 90 days shown prominent increase in body weight indicating the materials implanted at the abdominal wall defect to be well accepted and healing might be taking place within the animals (Fig. 4.35e).

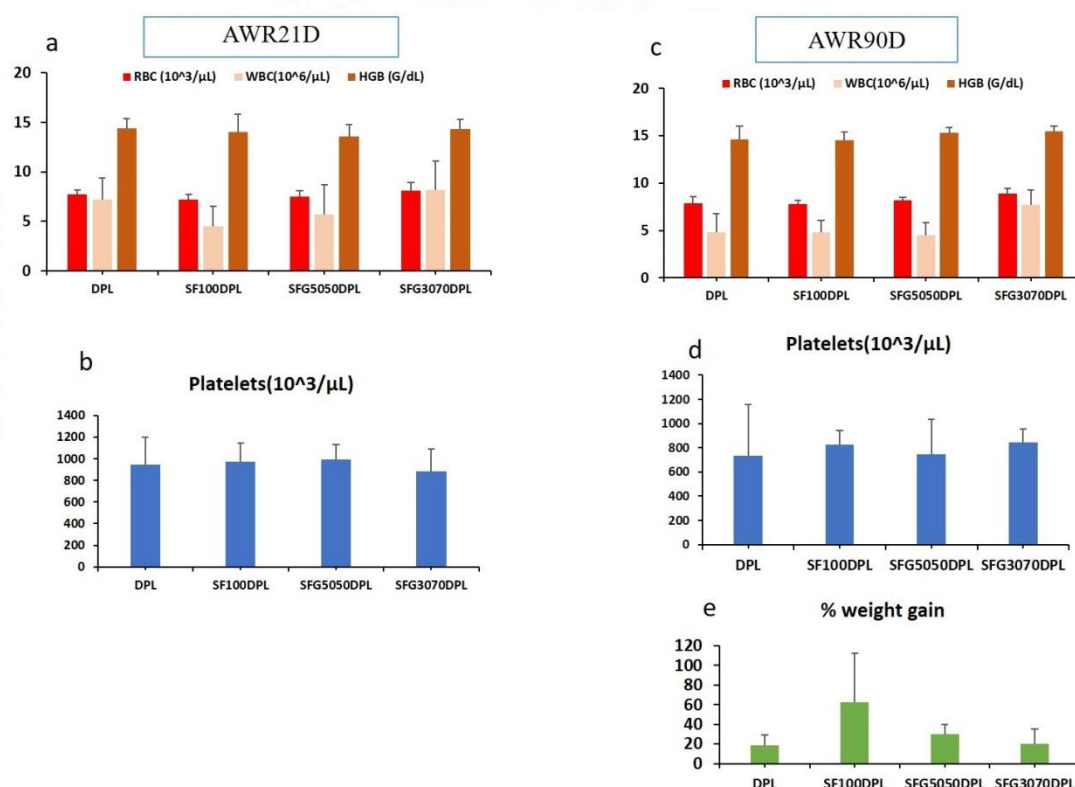


Figure 4.35: Hematology and body weight gain: The RBC, WBC, HGB, Platelets, and percentage body weight gain exhibited by the animals implanted with the test materials in the abdominal wall defect.

4.4.1.2. Inflammatory cell scoring

The histological examination followed by H&E staining in explants recovered on 21 days had significant inflammatory cell infiltration detected in all the test groups (Fig. 4.36). Among the modified test groups the polymer incorporated part was not infiltrated with these cells and more pronounced infiltration was observed at the host-graft

interface and the peripheral region of the explant compared to the center. Inflammatory cell infiltration was found to be subsiding significantly in 90 days which itself is a positive symptom leading to constructive remodeling. A considerable reduction in PMNC score from moderate to minimum was observed as the implantation time increased from 21 days to 90 days, and the score changed from 1.5 to 0.2 for DPL, 2.1 to 0.4 for SF100DPL, 2 to 0.4 in SFG5050DPL and 1 to 0.2 in SFG3070DPL respectively (Fig 4.37a). Lymphocyte and plasma cell response was also found to be decreasing (Fig 4.37 b&c). Macrophages were predominating at both the time points as it play a major role in remodeling as well (Fig. 4.37d). The extent of vascularization increased from 21 days to 90 days with broader vessels visible at 90 days (Fig. 4.37f).

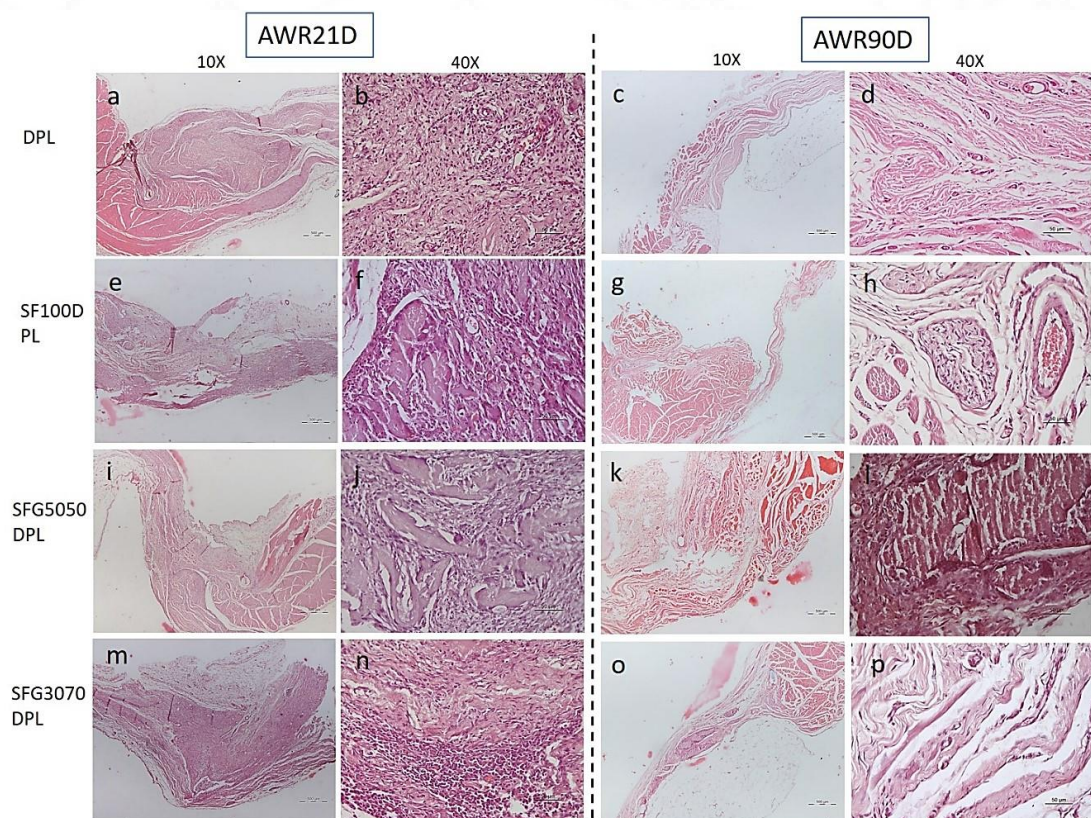


Figure 4.36: H&E staining: Explant evaluation using H&E staining showing heavy infiltration of mononuclear cells on 21 days proven by DPL (a, b), SF100DPL (e, f),

SFG5050DPL (i, j) and SFG3070DPL (m, n) at 10x and 40x magnification respectively. AWR 90-day explants showed a considerably decreased cellularisation by 90 days for DPL (c, d), SF100DPL (g, h), SFG5050DPL (k, l), and SFG3070DPL (o, p) respectively at 10x (scale bar- 200 μ m) and 40x (scale bar- 50 μ m) magnifications.

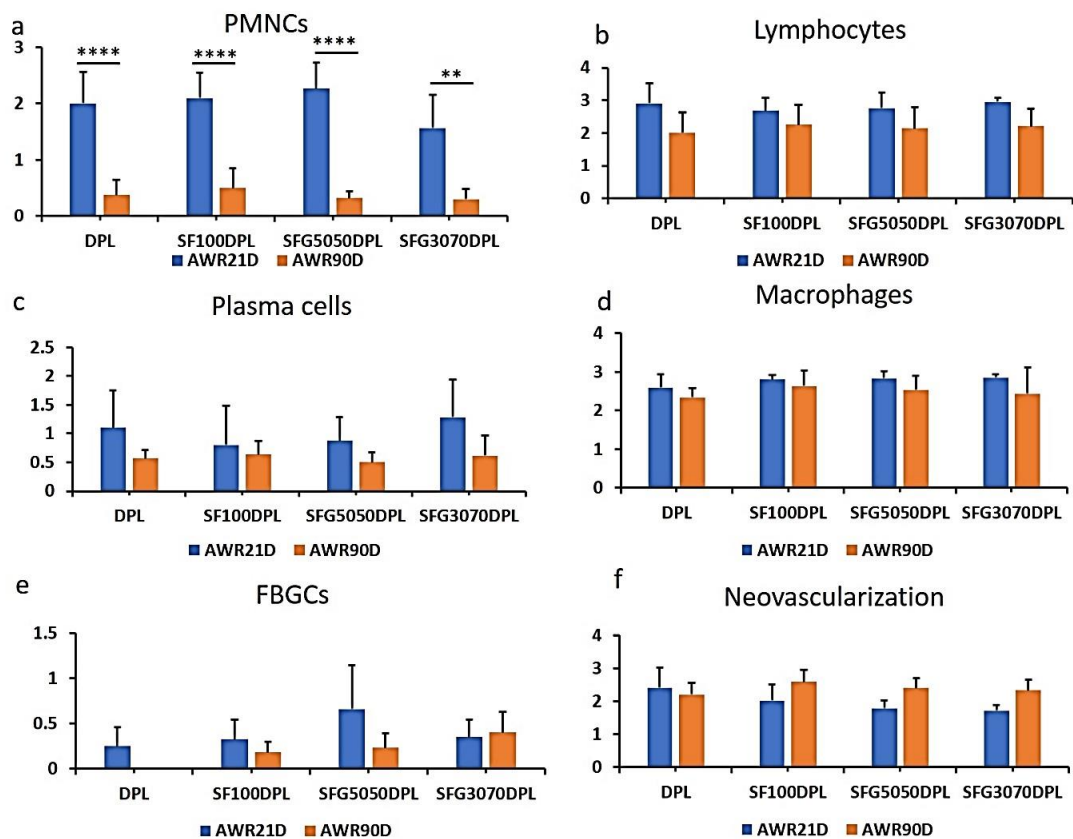


Figure 4.37: Histopathology scoring: Histological scores of the unmodified and modified scaffolds after explant recovery. (a) The average scores for the number of polymorphonuclear cells; (b) macrophages; (c) lymphocytes; (d) plasma cells; (e) FBGCs; and (f) amount of neovascularization. ** $p \leq 0.001$, **** $p \leq 0.0001$

4.4.1.3. Evaluation of regeneration

4.4.1.3.1. Neo-muscle formation (Masson's trichrome staining and Immunohistochemistry)

Structural regeneration evaluated using MT staining and IHC against myosin had shown the formation of neo-muscle islands at the host-graft interface in 21 days study and it has extended till the mid-graft region on increasing the duration of implantation study to 90 days. The percentage of muscle formation varied between the modified test groups compared to the unmodified DPL. Masson's trichrome staining of 21-day explants revealed more disoriented collagen fibers which were less intense in blue color, due to degradation of the graft-associated collagen and deposition of new collagen. New muscle formation was restricted only at the host-graft interface. The mid-graft region contained only loosely packed collagen with rich infiltration of mononuclear cells. All the study groups exhibited similar observations which were further confirmed by myosin staining by IHC (Fig. 4.38). But on 90 days post-implantation, the collagenous matrix appeared intensely blue, denser, and bundle-like in MT staining. In this group, the new muscle formation was not only restricted to the host-graft interface but extended to the mid-graft region (Fig. 4.39). New muscle island formation was visible by the intense red-colored regions in the mid-graft region in MT stained images whereas the same appeared brown pigmented areas in immunohistochemistry. While comparing regeneration by neo-muscle island formation in the mid-graft region among the modified scaffolds, the SFG3070DPL exhibited the least regeneration compared to SF100DPL and SFG5050DPL (Fig. 4.40).

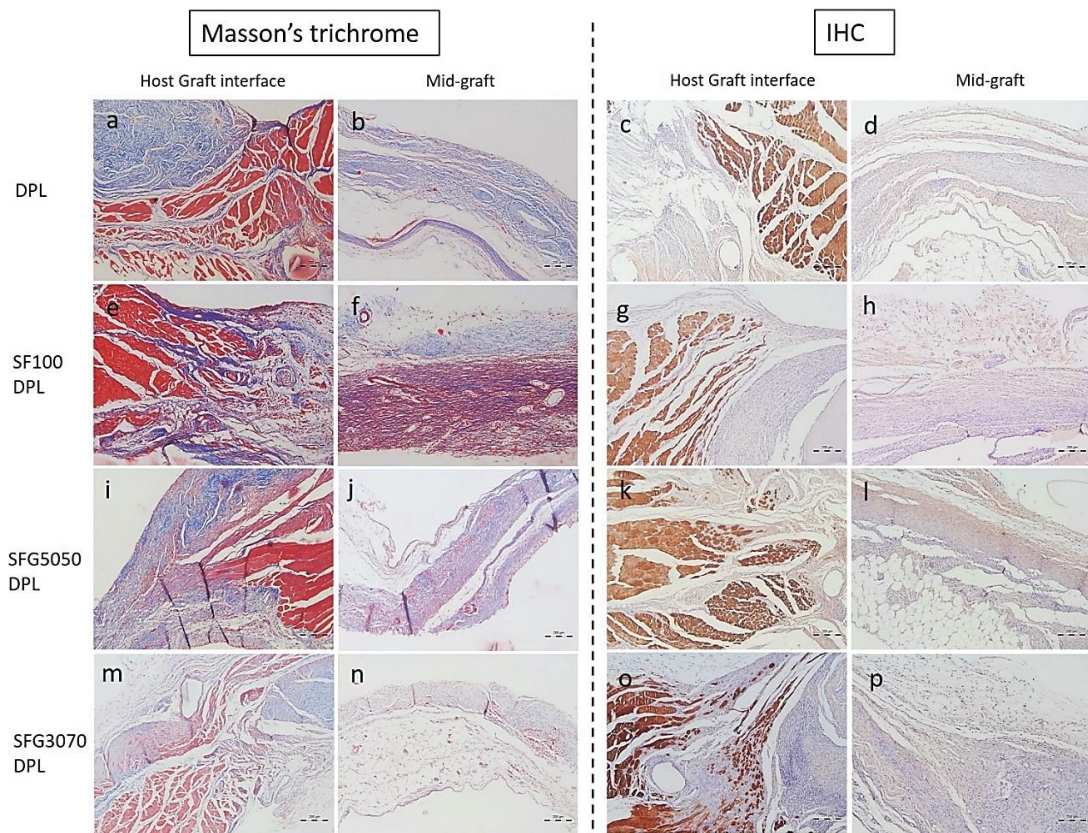


Figure 4.38: Neo-muscle regeneration on 21 days: Representative images (10x magnification, scale bar 200 μ m) of AWR21day explants showing MT stained and immunostained images of host-graft interface and mid-graft region.

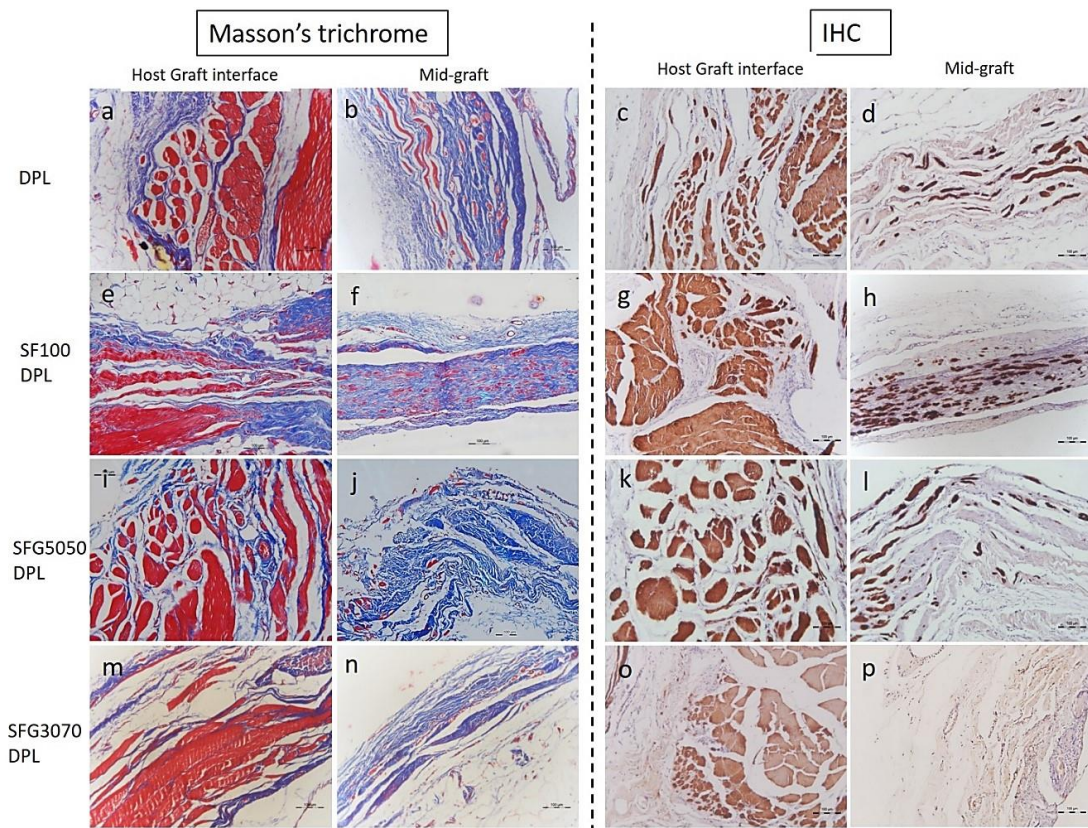


Figure 4.39: Neo-muscle regeneration on 90 days: Representative images (20x magnification, scale bar-100 μ m) of AWR90day explants showing MT stained and immunostained images of host-graft interface and mid-graft region.

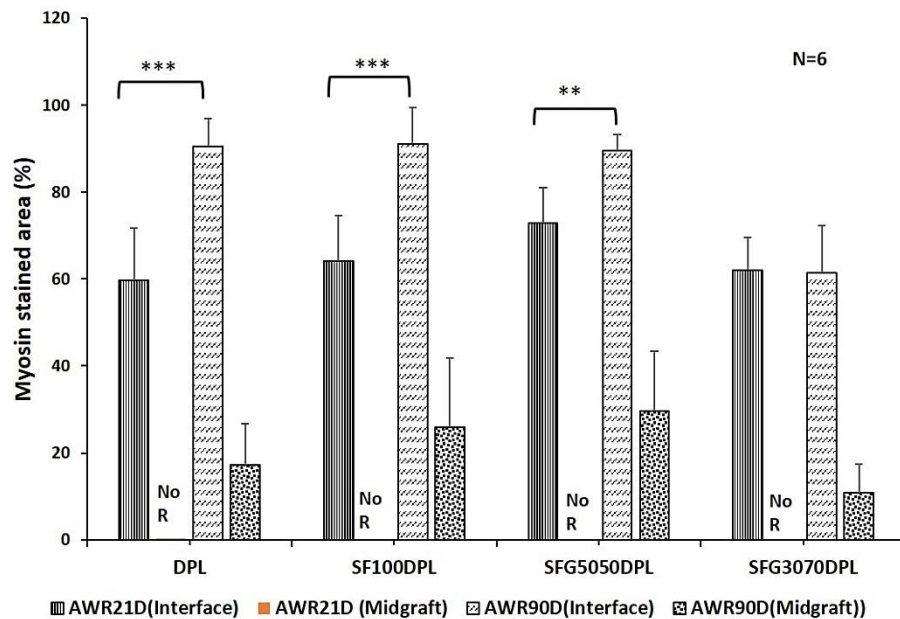


Figure 4.40: Extent of muscle regeneration: Graphical representation of semi-quantitative evaluation of new muscle formation using myosin stained IHC images by ImageJ software. Images of explants from AWR21day and AWR90day studies were evaluated at the regions of host-graft interface and mid-graft. No R. represents no regeneration

4.4.1.3.2. Collagen remodeling (Herovici's polychrome staining)

Herovici's polychrome staining is used to distinguish the type of collagen predominating in the specimen under study. In this staining purple staining corresponds to collagen type I and type III collagen appears in bright blue, and muscle appears as yellow. Quantification of collagen III: I ratio had proven a significant difference between the AWR21day and AWR90day explants under study. All the study groups exhibited statistically significant differences in collagen III: I ratio which indicates the transformation of newly laid immature collagen type III initially

deposited on 21 days, maturing to collagen type I on 90 days. While comparing the statistical significance in collagen remodeling by a change in the type of collagen, SF100DPL exhibited higher significance ($p < 0.0001$) than the rest of the two groups. The collagen III: I ratio showed a decreasing trend which included 4 ± 1 to 1.6 ± 0.6 ($p = 7.3E-05$) for DPL, 4.6 ± 0.9 to 1.8 ± 0.5 ($p = 0.0001$) for SF100DPL, 5.7 ± 1.9 to 1.5 ± 0.3 ($p = 0.003$) for SFG5050DPL and 8.2 ± 2.5 to 4 ± 1.8 ($p = 0.008$) in SFG3070DPL at AWR21day and AWR90day respectively (Fig. 4.41).

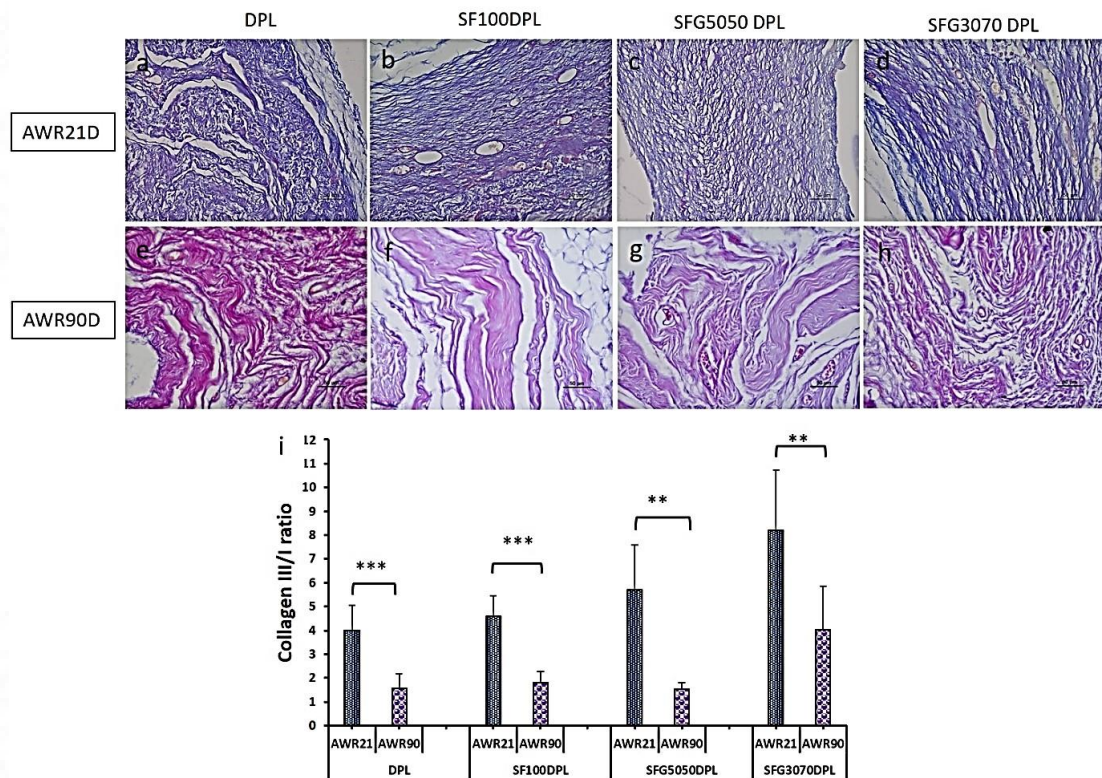


Figure 4.41: Collagen remodeling: Herovici's polychrome stained images of DPL, SF100DPL, SFG5050DPL, and SFG3070DPL explants recovered from AWR21day (a, b, c, d) and AWR90day (e, f, g, h) study. Graph showing the semiquantitative data on collagen III: I ratio (i) of the explants

5 DISCUSSION

Decellularization is an effective strategy for developing biological scaffolds to overcome the fibrotic and immunologic responses associated with the implantation of an allogeneic or xenogeneic cellular scaffold (Keane et al., 2012b). This study carried out three methods of decellularization of porcine liver slices and its comparison in removing antigenic determinants associated with the tissue such as nuclear, cytoplasmic, and membrane-bound, mainly DNA and proteins. Further, the evaluations were carried out to confirm its structural (including ultrastructure), biochemical, biomechanical, and cytotoxic properties. The glycosaminoglycan and growth factor preservation within the matrix was done to prove its bioactivity. Such decellularized extracellular matrix (DEM) scaffolds provide an inductive niche that influences inflammatory and immune responses in various stages of downstream tissue remodeling by regeneration. Host immunomodulation that precedes tissue remodeling by constructive/functional repair was characterized by inflammatory cell scoring and immunomodulation by the trend in cytokine release exhibited by the DEM-implanted host animal model. Antibody response also added evidence to the immunological acceptance of the bioscaffolds. Most importantly such immunomodulation progressed toward a significant reduction in inflammatory cytokine release and subsequent deposition of functional tissue was proven by in-vivo abdominal wall regeneration study.

The porcine liver was the organ of interest in the present study to develop decellularized extracellular matrix scaffolds. The liver is an organ with the highest regenerative potential. Compared to other mammalian models, the pig liver has strong

intercollagenous connective tissue, which may confer a minimum mechanical strength to the DEM scaffold. The close relatedness with humans also makes its selection advantageous. Decellularization was carried out by simple immersion and agitation in the chemical bath with an incubator shaker, which helps the tissue slices readily be exposed to the detergents in the solution to achieve efficient cellular removal (Baptista et al., 2009; Park et al., 2013b). The thickness of the tissue slices plays a vital role in determining the efficacy of decellularization because thicker regions exhibited compromised cellular removal compared to thinner regions and took a prolonged time to achieve complete decellularization, which may also render the tissue susceptible to microbial contamination. Another limitation is the release of proteases during cell lysis, which may degrade the extracellular matrix (Rabbani et al., 2021). Two of the detergent decellularization procedures (using SDS and SDS+SDC) followed physical means initially by freezing the porcine liver tissue slices in liquid nitrogen, which disrupts the cells by intracellular ice crystal formation. The freeze-thaw process is equally disruptive to ECM ultrastructure, but it readily accelerates decellularization (Wu et al., 2015). The TX100 method used enzymatic digestion followed by detergent exposure to the tissue slices. Vigorous agitation was adopted in all three methods because the liver is a heavily cell-laden tissue with organized trabeculae and blood sinusoids. It was observed that the degree of cellular removal was directly related to the aggressiveness of agitation (Keane et al., 2015b). The cellular removal in liver slices can be easily monitored visually by observing its change in color from dark reddish brown to white and transparent after complete decellularization (Geerts et al., 2016; Khan and Quigley, 2011).

All three methods lysed and removed most cells and nuclei from native liver tissue as confirmed by histological examination using H&E and DAPI staining. Preserved collagen structural integrity was evident in MT staining and ultrastructural evaluation. Among the three methods of decellularization followed, the detergents 0.1% SDS and 0.05% SDS + 0.05% SDC fulfilled the three standard criteria of decellularization qualitatively and quantitatively. The threshold level of DNA required to elicit an adverse inflammatory response, which hinders the scaffold's performance *in vivo* is >50ng/mg tissue, and a DNA fragment length of >200bps (Crapo et al., 2011b; Gilbert et al., 2009b; Keane et al., 2012b; Zheng et al., 2005). 0.1% SDS decellularization was proved to be very effective in removing xenogeneic antigens, as reported by Park *et al.* (Park et al., 2016, 2013b). DNA removal in the Triton X-100 decellularized matrix was ineffective because of the retention of a higher amount of DNA and structural deterioration. The combinatorial approach of decellularization was observed to be the most efficient, as evidenced by approximately 98% removal of DNA, whereas the removal of DNA by 0.1% SDS was around 93%, and by Triton X-100 method was only 73%. No evidence of DNA fragments was observed in SDS DPL and SDS+SDC DPL. A clear-cut band in the agarose gel at the high molecular weight genomic DNA region in TX100 DPL has proven its inferior efficacy in bringing about an effective decellularization (Faucher and Goddard, 1978). Residual DNA content with a fragment length of >200bp within the bioscaffold is capable of promoting pathological innate immune response within the host making it unsuitable for medical applications (Keane et al., 2012b; Sf and Tw, 2008).

Previous reports showed that the detergent penetration rate into the tissue under decellularization relies on its critical micelle concentration (CMC). Here, the

concentrations of detergents chosen for three methods of decellularization were 0.1% (for SDS/DPL), 0.05% (for SDS+SDC/DPL), and 1% (for TX100/DPL) respectively. The difference in decellularization efficiency among the three methods was due to the critical micelle concentration of the detergents under use. SDS is the most extensively used ionic detergent for developing DEM scaffolds because of its efficiency in solubilizing phospholipids and proteins. However, it has also been found to alter matrix-bound components such as GAG and collagen (Keane et al., 2015b). 0.1% SDS has been proven to have CMC that allows its easy diffusion into the liver cells, causing cell lysis. Compared to 0.1% SDS, the efficiency of the non-ionic detergent 1% Triton X-100 was found to be much less, which might be because of its poor penetration into the tissue, because of ten times higher concentration of Triton X-100. A combination of ionic detergents 0.05% SDS and 0.05% SDC, can form mixed micelle whose critical concentration allowed its' easy penetration into the tissue and brought about lytic removal of cells more effectively than 0.1% SDS alone. The SDS+SDC method was less damaging to collagen integrity mainly because SDC is reported to be less damaging than SDS. Furthermore, this method utilizes only 0.05% SDS which further reduces the deteriorating effect of SDS on extracellular matrix (ECM) (Gilbert et al., 2006b). As residual detergents can be cytotoxic, their complete removal after decellularization is crucial, which is one of the challenges faced in developing bioscaffolds. Here, the use of low concentrations of ionic detergents enabled its easier removal. Preliminary studies by another research group showed that 0.05% SDS alone is not sufficient for porcine liver decellularization (Park et al., 2013b). However, in this work, it is demonstrated that when used with 0.05% SDC, a lower concentration

of SDS (0.05%) can fulfill the decellularization criteria (Keane et al., 2012b; Park et al., 2016, 2013b), without imparting any deleterious effects by residual detergents.

Disruption of collagen integrity was prominent in H&E and MT-stained images of TX100 DPL. This observation was consistent with that reported by Nakayama *et al.*, (Nakayama et al., 2010a) in which residual nuclear debris retention was reported in 1% Triton X-100 decellularized kidneys of rhesus monkeys. Among three methods of decellularization carried out in the present study, TX100 treated tissue alone contained DNA of more than 100ng/mg tissue, and a clear band in agarose gel electrophoresis (AGE). Enzyme treatment using trypsin not only disrupts the cellular connection but also has a role in disrupting tissue architecture. It deteriorates its glycosaminoglycans (GAG) and growth factors (GFs), which further adds to the disruptive effect of the trypsin/Triton X-100 method on ECM (Nakayama et al., 2010b). The biomechanical strength exhibited by the SDS+SDC method was the least among the three methods, which needs to be augmented with biocompatible approaches to retain its bioactivity. GAG represents the functionally relevant family of negatively charged long-chain polysaccharides, which are found associated with protein core to form macromolecular assemblies called proteoglycans (Yeung et al., 2001). Usually, a reduction in GAG content was observed after detergent decellularization because of the destructive effect of detergents on ECM. Hence, a method that uses a minimal concentration of detergents for a shorter duration to achieve optimum decellularization is preferred (Akbari Zahmati et al., 2017; Ren et al., 2013). Among the three methods studied here, the GAG estimated in the SDS+SDC method was significantly increased compared to the native liver. This can be explained by the work by Coronado *et al.*, where the contributing effect of GAG-concentrated ECM and GAG-poor cells in native liver, is

demonstrated. It was observed that the decellularized dry tissue has ECM rich in GAG without cellular components, causing "compaction" of ECM, which is finally observed as high GAG content (Coronado et al., 2017) in SDS+SDC DPL compared to native liver tissue. At the same time, the rest of the two detergents were damaging the GAG associated with proteoglycans, resulting in reduced binding of the chromogen to GAG in DMMB assay.

Growth factors play a significant role in restoring injured tissue's typical structure and function by activating various signal transduction pathways essential for regeneration. An ECM scaffold with a detectable amount of GFs preserved in it remains a promising material that may aid in accelerated structural and functional restoration of damaged tissue or organs. HGF and bFGF are two major GFs essential for liver functions. Among the two, HGF is the most abundant GF present within liver ECM whereas bFGF was detected considerably in reduced amounts and even undetected in TX100 DPL. HGF is very much essential for the cells involved in tissue repair, such as epithelial and endothelial cells, to undergo proliferation, migration, and survival. bFGF can promote neoangiogenesis which is crucial for the survival of the newly infiltrating host-specific cells. The interplay between matrix components, various cell types that migrate towards the defect site, such as fibroblasts and endothelial cells, and the GFs preserved within the graft and secreted by the cellular infiltrate promote accelerated tissue remodeling by regeneration at the defect site (Koria, 2012). Therefore, the decellularization method, which maximally preserves the GFs and structural components, should be very effective in inducing regeneration in non-regenerative tissues (Ren et al., 2013). It was also noted in this study that the amount of GFs positively correlated with the amount of GAG estimated in the decellularised

tissues, thereby indicating that the estimated GAG alone can indirectly indicate the amount of GFs preserved. GAG estimation is much cheaper and simpler compared to GF estimation. Cellular removal makes the ECM scaffold porous, which is crucial for the infiltration of different types of cells into the matrix and for the diffusion of nutrients as well (Bonferoni et al., 2021). The cytocompatibility proven by *in vitro* studies again ensures the suitability of the matrices in regenerative scaffold-based therapy. Even though proven to be suitable for such applications, the reduced mechanical properties and untimely degradation hinder with its clinical application. It is always post-processed by crosslinking treatment to mitigate the adverse effects of decellularization on ECM (Zhang et al., 2021).

The current study envisages a green crosslinking strategy of incorporating CA-containing natural protein polymers into the decellularized matrix. Silk fibroin with excellent biological properties and enormous mechanical strength was selected to improve the compromised mechanical strength of the acellular porcine liver scaffold. The method involved CA crosslinked silk fibroin impregnation within the matrix using a low temperature driven alkaline catalyzed reaction. It involves pre-crosslinking, drying, and post-crosslinking without using any toxic chemical intermediates. Acellular matrix is susceptible to high temperatures, low pH, and toxic chemical catalysis, as it causes several adverse toxic and undesirable changes in its properties (Reddy et al., 2009c). Based on research done by Jiang *et. al.*, (Jiang et al., 2010a) the optimized CA concentration adopted in this study was 7 w/w%. It was experimentally proven that an increase in CA concentration beyond 7% did not improve the fiber strength in electrospun zein fibers (Jiang and Yang, 2011; Xu et al., 2015a). The pH of the solution was adjusted to alkalinity (Reddy et al., 2009c) using NaOH which

significantly reduced the viscosity of the solution and made it more translucent and easily penetrable into the matrix during pre-crosslinking at vacuum conditions. The reaction mechanism underlying crosslinking between CA and protein polymers has been explained by Xu *et. al.* (Xu et al., 2015a) The amide linkages can be intrachain linkages within the protein polymer or the extracellular matrix proteins and interchain linkages connecting the proteins of the matrix and the polymers. The pH is crucial here because amino groups cannot carry a partial positive charge at alkaline pH, so it could attack the positively charged carbonyl carbon to initiate the reaction forming an amide bond (Reddy et al., 2009c).

To modify the compliance of the resulting scaffold gelatin has been blended with silk fibroin. Silk fibroin and gelatin are good candidates for blending as they carry opposite charges which help them to bind each other by electrostatic forces (Taddei et al., 2013b). Both these proteins are water-soluble and gelatin offers bioactive RGD peptides that are crucial moieties for cell interaction involved in binding with integrin receptors on the cell surface promoting cell adhesion (Bellis, 2011). The viscosity of silk fibroin has decreased by adding gelatin, at the same time the weak mechanical property of gelatin can be supported by fibroin. They mutually complement each other (Su et al., 2014; Wang et al., 2019). Ethanol provides a dehydrated condition for stabilizing the intrachain and interchain crosslinks formed, which produces strong interaction between the protein-CA-protein. This in turn improves the structural stability of the incorporated polymers as well as the ECM (Kaewpirom and Boonsang, 2020; Puerta et al., 2020, 2019).

The spectral pattern obtained in FTIR for unmodified and modified scaffolds was more or less the same due to the proteinaceous nature of the extracellular matrix and the

incorporated polymers. The characteristic bands for the presence of citric acid at 1690 cm^{-1} and 1743 cm^{-1} were absent in all three test groups indicating the absence of C=O stretching of citric acid (Uranga et al., 2020, 2016). This confirms the absence of residual citric acid and the complete catalytic incorporation of citric acid in the crosslinking reaction to form amide linkages in the resultant modified matrices (Xu et al., 2015b). The porous architecture of decellularized porcine liver (DPL) changes to a compact structure by the percolation of polymer solutions into the matrix. The reduction in porosity decreases the extent of swelling of the modified scaffolds. The major role of a tissue-engineered scaffold is not only to provide specific microenvironmental cues but also to provide suitable mechanical support to facilitate cellular migration, differentiation, and function (Gao et al., 2019b). The biomechanical properties have significantly improved indicated by increased tensile strength, Young's modulus, and suture retention strength compared to the unmodified control. There is research work done using decellularized human amniotic membrane modified with electrospun silk fibroin that had shown significantly improved mechanical strength such as maximum load and suture retention strength compared to amniotic membrane alone. Maximum load has increased from $1.3\pm0.17\text{ N}$ to $1.9\pm0.18\text{ N}$ and suture retention strength from $512\pm63\text{ mN}$ to $692\pm31\text{ mN}$. Because of the increased mechanical properties and non-cytotoxicity of the 3-D scaffold, it has potential application in grafting for skin wound healing as per the study conducted by Gholipourmalekabadi M. *et. al.* (Gholipourmalekabadi et al., 2018) Similar studies with hybrid silk fibroin-acellular goat dermal matrix (SFAGDM) and coupling of DEM from placenta with SF also showed enhanced wound healing by remodeling when substituted as a skin graft (Dhasmana et al., 2019b; Rameshbabu et al., 2018).

Surface wettability plays a major role in protein adsorption and cell adhesion. Hydrophilic surfaces favor cell adhesion than hydrophobic surfaces. The contact angle obtained for the modified scaffold surfaces falls between 70° and 98°. Fibroblast cell adhesion was observed to be the highest between the contact angles 60° and 80°, closer to the contact angle obtained in the study (Cai et al., 2020).

The degradation properties of silk fibroin depend on various factors such as fibroin extraction, processing conditions, scaffold properties, etc. (Taddei et al., 2006) The other two test groups exhibited comparatively faster degradation as they also contained gelatin with silk fibroin. Gelatin even though CA crosslinked was not found to be stable as it dissolves out during hydration. It is also susceptible to collagenase enzyme because it is a collagen derivative. DPL proved to be more prone to degradation by the enzyme due to lack of strength and availability of more active sites for the enzymes, thus degrading faster and releasing more free amines in solution. The significantly reduced free amine groups in the modified scaffolds indicates its reduced antigenicity and further confirm its involvement in crosslinking reaction (Imamura et al., 1989; Mohan et al., 2016). The Scaffolds were non-cytotoxic proven by L929 cells in direct contact with the modified materials and more than 80% cell viability in cells treated with their extracts. All these results strongly support the scaffolds to be a good substrate for various soft tissue repair applications.

The subcutaneous implantation model revealed inflammatory and immune responses elicited by the animals on the implantation of the matrices at subcutaneous pouches. Macroscopically the explanted matrices of both unmodified and modified scaffolds showed size reduction. DPL exhibited the highest degree of reduction compared to modified ones. This reveals an early degradation of unmodified acellular matrix over

time compared to the modified scaffolds. The hematological parameters proved the animals that carried the subcutaneous implants exhibited normal physiological conditions. Semiquantitative histological analysis of the explants revealed a prominent accumulation of inflammatory cells within the implant, which decreased significantly as implantation duration increased. There was a significant reduction in acute inflammation on 30 days and 90 days after implantation and an earlier moderate to severe inflammation marked by heavy neutrophil and macrophage response on 14 days. An elevated level of lymphocytes, macrophages, and plasma cells was evident at 30 days in explants of SF100DPL, SFG5050DPL, and SFG3070DPL, among which the low levels of plasma cells at the 90-day time point revealed a reduction in immune response. The prevalence of macrophages at all three time points showed its role in inflammation initially and as the implantation time increased, macrophages had a beneficiary role in secreting growth factors, proangiogenic factors, and ECM-specific proteins that actively regulate fibro-proliferation and also in angiogenesis leading to tissue repair (Modulevsky et al., 2016b). A similar observation was reported by Kim *et. al.*, during the evaluation of host tissue response to PLGA or porcine SIS-based scaffolds (Kim et al., 2007b). Earlier studies by our research group have validated the difference in biological response exhibited by mildly and completely glutaraldehyde crosslinked bovine pericardium and its long-term healing response in vascular reconstruction. Improvement of decellularized scaffolds with glutaraldehyde compromises their regenerative efficacy and results in fibrous encapsulation. The method advocated here has proven to reduce acute inflammatory response marked by a significant reduction in neutrophil response in 90 days (Umashankar et al., 2017, 2011a).

For the activation and suppression of the immune system, the cytokine environment produced by the activated cells of the immune system plays a crucial role (Belardelli, 1995; Muñoz-Carrillo et al., 2018; Raetska et al., 2017). The cytokines TNF- α and IFN- γ have immunomodulatory effects are cell immunity inducers and play a central role in the development of chronic inflammation. IFN- γ secreted by T_H and T_c cells are the most potent activators of macrophages that are effective in the elimination of endogenous antigens. They mainly contribute to chronic inflammation (Royal, n.d.). The level of IFN- γ remained similar to that of control in all animals on three study durations, which proved the absence of T-cell activation in material-implanted animals. B-cells have an important role in transplantation either leading to graft destruction by antibody production or tolerance via immunomodulation. Activated B-cells produce antibodies against the antigens present within the ECM that further activated the complement cascade, finally resulting in graft failure. Reducing the antigenic determinants and preservation of ECM structure thus inhibiting DAMP release are key factors to overcome the destructive adaptive immune responses against the DEM scaffold (“Human Anti-Gal and Anti-Non-Gal Immune Response to Porcine Tissue Implants,” n.d.). TNF- α , a cytokine produced with proven pro-inflammatory role, stimulation of T and B cells, antibody production, etc., is released by regulatory B-cells (Bregs) and the mechanism of action is dependent on the receptor it binds (Poznansky et al., 2023). The material-induced TNF- α production was increased mainly in animals implanted with DPL due to their high degradability, thus more antigens being available for B-cell activation. Statistically insignificant change in TNF- α production by peripheral blood mononuclear cells (PBMNC), indicates abatement of immune response leading to tissue integration of the implants.

A necessary feature of tissue remodeling by ECM-based scaffolds is immune modulation. The adaptive immune response is triggered upon recognizing specific implant-derived antigens by T-cells or B-cells and undergoing its expansion to mitigate them. Lymphocyte proliferation remains a classical tool in measuring cell-mediated immunity upon antigen stimulation (Giambrone et al., 1980; Reynolds and Maraqa, 2000). All three durations of subcutaneous implantation of the modified scaffolds have revealed an average SI, of less than 2 which reveals there is no active lymphocyte proliferation taking place in the presence of implants upon antigen re-stimulation *in-vitro* (14:00-17:00, n.d.; Breed et al., 1999; Hotaling et al., 2015). Host immune reaction is necessary for defect repair by tissue regeneration, at the same time prolonged stimulation may lead to implant degradation and failure (Hotaling et al., 2015). B-cells have a proven role in immune response due to tissue injury. Immediately after injury, IgM and IgG antibodies are rapidly released into the blood resulting in acceleration of critical events in wound healing (Nishio et al., 2009; Ulndreaj et al., 2017). Here B-cell response to the biological scaffold implantation was investigated by evaluating the presence of collective IgG+IgM+IgA antibodies in sera collected from animals under subcutaneous implantation study. It has revealed a decreasing trend in collective antibody response against implant-derived antigens on secondary exposure *in vitro*. Thus revealing an immunosuppressive role.

Proving the materials to be immunotolerant by subcutaneous implantation study, its regenerative efficacy was proven by implanting them into rat abdominal wall defect model. In humans, abdominal wall defects can be formed due to various reasons such as hernias, trauma, abdominal wall tumor resection, congenital conditions, infections, abdominal surgeries, etc. (Meintjes et al., 2011). Tissue-derived biomaterials of animal

origin exhibit predominant advantages over synthetic meshes because of their proven non-immunogenicity, biocompatibility, degradability, remodelability, less chance of infection, and least long-term complications (Costa et al., 2019). As our modification focused on modifying acellular matrix without affecting the GAG preserved within the matrix (which is an inherent pool of bound growth factors), these bioactive molecules could contribute to cellular infiltration, proliferation, and differentiation. The Wistar rats that underwent abdominal wall defect study with the modified scaffolds, were maintained for 21 days and 90 days. Significant weight gain by the implanted animals over 90 days suggests better tolerability and acceptance of the material by the animals which was evident in the subcutaneous implantation study as well. The insignificant increase in animal body weight on 21 days post-implantation signifies that, during the initial days the animals will have post-surgical difficulties and high inflammation following implantation, also due to compromised food intake. The hematological parameters (RBC, WBC, platelets, and HGB) of all implanted animals have further confirmed their normal health.

Semi-quantitative histological analysis of the explants revealed a prominent accumulation of inflammatory cells highly infiltrated at the host-graft interphase and around and periphery of the graft which decreased significantly as implantation duration increased. There was a significant reduction in inflammation 90 days after implantation and an earlier moderate inflammation marked by the presence of neutrophils and macrophages on 21 days. An elevated level of lymphocytes and plasma cells, evident at 21 days in all modified and unmodified explants exhibited a visible reduction on the 90-day point revealing a regulation in the humoral immune response. The prevalence of macrophages at all the time points shows its active role in

inflammation initially and as the implantation time increased, they have a beneficiary role in secreting growth factors, proangiogenic factors, and secreting ECM-specific proteins that actively regulate fibro-proliferation and also in angiogenesis leading to tissue repair and regeneration (Modulevsky et al., 2016a). A similar observation was reported by Kim *et. al.*, during the evaluation of host tissue response to PLGA or porcine SIS-based scaffolds (Kim et al., 2007a). The inherent growth factors such as VEGF, FGF, HGF, etc. promote angiogenesis, migration of site-specific cells from the host tissue, and its differentiation. Early vascularization has a significant role to play in promoting repair without graft failure. Vascular supply provides sufficient oxygen and nutrient supply along with waste product removal that hastens the repair process progressing to repair by remodeling. The richly infiltrated blood vessels facilitate the proliferation of fibroblasts, vascular endothelial cells, and smooth muscle cells, those involved in the active repair of the defect (Song et al., 2023).

Structural regeneration was evaluated by new skeletal muscle formation and collagen remodeling. The neo-muscle formation was investigated at the host-graft interphase and the mid-graft region within the recovered explants. Host-graft interphase showed indications of sprouting small islands of new muscles during the 21-day study, which has become larger and with mature skeletal muscle islands in 90 days. The extensive growth of the new muscle tissue formation at the mid-graft region showed a difference in percentage between the groups. SFG5050DPL promoted the highest percentage deposition of new muscle fibres followed by SF100DPL and the unmodified DPL. The difference in regeneration by new muscle formation can be due to the difference in mechanical properties of the modified scaffolds because for regeneration to occur there should be a balance between the degradation rate of the scaffold and replacement of

new tissue which is very critical for abdominal wall reconstruction (Badylak et al., 2001; Shi et al., 2010). The collagen that constitutes the extracellular matrix of extensible tissues includes collagen type I along with type III. They are the most prevalent fibrillar collagens in which collagen I is composed of thick fibers with a low turnover rate whereas collagen III is thin and less durable with a high turnover. During wound healing as well as in ECM remodeling the ratio of collagen I and III is visibly altered. Collagen III is more prevalent during the healing phase but in a healed wound collagen I increases (Singh et al., 2023). In the case of the abdominal wall, they serve as structural support for the infiltrating muscle cells. The increase in collagen I density 90 days post-implantation was observed in all the test groups, among which DPL and SF100DPL exhibited high significance compared to SFG5050DPL and SFG3070DPL. Conversion of immature collagen III to mature collagen I can be an indication of healing by scarring as well as by regeneration which needs further clarification. The conversion was more prominent in DPL and SF100DPL compared to the rest of the two groups. Compared to the disoriented structure of predominant collagen III observed at 21 days has become more structured and well organized at a 90-day time point which ensures collagen remodeling within the graft.

Summarising, the method of CA crosslinked natural polymer impregnation strategy for developing a mechanical strength augmented decellularized porcine liver scaffold has been systematically characterized. The method successfully improved the mechanical strength, stiffness, and suturability, suiting the material to be used for surgical applications. Resistance to degradation has also been increased, as proven by enzymatic degradation study. Subcutaneous implantation study further confirms an immunomodulatory response by the modified scaffolds progressing towards a healing

phase not leading to an adverse immune response on extended implantation period. The evaluation of the induced regenerative potential of the modified scaffolds revealed the capability to be infiltrated by host-specific cells, promoting regeneration within the graft by new muscle deposition and its maturation and collagen remodeling as well. Among the three modified scaffolds, SF100DPL and SFG5050DPL exhibited promising regenerative potential than SFG3070DPL in comparison to the unmodified control

Overall, the research work has systematically developed and evaluated three methods of decellularization. The importance of using a combination of detergents to develop a DEM scaffold with minimal antigenic determinants and maximum GAG and GFs was proven. A major concern and drawback associated with the use of ECM scaffold in clinical application (reduced mechanical strength and stability) was addressed in the study and a completely non-toxic, biocompatible and immunologically acceptable way of improving the biomechanical strength of ECM scaffold was developed and characterized both in-vitro and in-vivo. The modified scaffolds were accepted immunologically, proven by rat subcutaneous implantation, and promoted repair by site-appropriate constructive remodelling, proven by rat abdominal wall regeneration model. Such scaffolds gives great promise in the field of soft tissue repair such as skin wound healing and as tissue expanders in mastectomy cases.

6 SUMMARY AND CONCLUSIONS

6.1. Summary

Biological scaffolds derived from natural sources form an important category of biomaterials intended for regenerative clinical applications. Among the naturally derived scaffolds, decellularized extracellular matrix (DEM) scaffolds created an ideal biomaterial with intrinsic bioactivity to promote host cellular penetration to promote reconstructive repair in various defective conditions. The method of fabrication of DEM scaffolds is decellularization, whose standardization poses a major challenge without any adverse effect on its structural and functional efficacy. The present work characterized three detergent-based decellularization methods to develop ECM scaffolds intended for soft tissue repair by scaffold-based therapy. Among the three methods, the combinatorial approach to decellularization of porcine liver slices readily preserved the ultrastructure and satisfied all the major criteria of decellularization. This also contained maximum glycosaminoglycan that can be used as an indirect estimate of the bound GFs retained within the ECM scaffold. The highest retention of HGF and bFGF further confirms the crucial bioactive molecules needed to impart regenerative potential. This acellular ECM scaffold can be a promising regenerative patch that can promote regenerative outcomes in soft tissue healing applications such as skin wound healing, burn wound repair, or as a tissue expander in mastectomy cases. ECM is a complex material whose intricate properties are difficult to replicate. Therefore, it is very important to address its drawbacks to explore its tremendous potential to promote regeneration. This work has also put forward a solution to improve the poor

mechanical strength of decellularized porcine liver ECM with the incorporation of a natural fibrous polymer, silk fibroin. This is the first attempt to augment the biomechanical strength of acellular porcine liver matrix by depositing silk fibroin within the porous ECM mediated by vacuum-assisted alkaline catalyzed low-temperature mediated CA crosslinking. The method has been proven non-toxic and biocompatible, significantly improving the mechanical property, suturability, and resistance to degradation. Subcutaneous implantation further proved a reduction in acute inflammation, absence of cytotoxic T-cell (T_c cell) activation, and an insignificant B-cell response (due to the influence of activated T_H cells and their release products) associated with modified scaffolds. All these positive outcomes support the present modification strategy to be highly promising to make use of the inherent strength and bioactivity of this ECM for regenerative surgical applications to treat full-thickness or partial-thickness wounds such as skin wounds or burn wounds. We herein successfully examined the immunomodulatory effect elicited by the modified acellular porcine liver scaffold *in vitro* as well as *in vivo*. The regenerative potential was further proved in the rat abdominal wall defect model. Among these, the SF100DPL and SFG5050DPL came out to be the best in promoting structural regeneration and brought about new muscle and collagen deposition within the graft followed by their maturation. This non-toxic and biocompatible method of strength improvement of ECM scaffolds was proven to be immunotolerant and regeneration-promoting. This can be explored with other biopolymers and extracellular matrices to ensure the broader applicability of this method to produce new functional bio-grafts.

6.2. Conclusion

Clinical translation of biomaterials for functional tissue repair is dependent on host immune response toward the material. They must be immunocompatible for a successful outcome. Acellular ECM scaffolds are associated with the absence of any adverse host immune responses because it is devoid of antigenic determinants and the conserved nature of ECM structural and functional components. Through our research a decellularization protocol to develop porcine acellular liver patch was developed without compromising the preserved biomolecules in it and without causing any damage to the ECM. An ECM scaffold modification strategy was also developed and evaluated. It was revealed to be non-cytotoxic and biocompatible which is advantageous over other existing chemical crosslinking strategies. Host inflammatory and immune responses towards the modified porcine liver scaffolds were also found to be within acceptable limits and significantly reduced on extending the implantation time. These results indicate a promising way of biomechanical augmentation of decellularized matrices that gave positive remodeling by collagen deposition and neo-muscle formation within the graft on rodent abdominal wall defect model.

6.3. Significance of the study

The study has given evidence that decellularization using a combination of detergents brings about maximum efficiency in cellular and nuclear material removal. They preserved the ECM structure, glycosaminoglycan, and growth factors as well which are important in reducing the DAMP release and providing inherent biofunctional and bioactive matricryptins or matricryptic peptides that play a major role in contributing to the resolution of immune response and regenerative potential of the ECM. The destructive effect of detergents in eliminating the GAG and growth factor content within the matrix was also proven here. A positive correlation between the GAG and

GFs preserved within the matrix was also revealed. The detergent which is less damaging to ECM, was found to preserve GAG and growth factor as well. A biocompatible and immunotolerant method of augmenting the mechanical properties and stability was successfully developed and proven in both in-vitro and in-vivo models.

6.4. Limitations and Future Perspectives

Even though the suture retention strength of the modified decellularized matrix showed significant improvement compared to unmodified DPL, this strength was insufficient for surgical applications such as hernia or vascular graft, which require higher strength. The average strength of modified scaffolds obtained in the present study was 2.5 N, which may not be enough for the above applications. However, this strength may be sufficient for skin grafting, which can be explored in future studies. In this study, as the candidate material, silk fibroin was preferably used because of several advantages over other polymers, concerning its easy availability, application, biocompatibility, immunogenicity, and bio-resorption. This can be modified in a better way to develop a multi-layered skin graft (containing epidermal, dermal, and hypodermal layers) by incorporating gradient concentrations of silk fibroin into the basal ECM matrix. The possibility of applying the crosslinking and impregnation strategy with other polysaccharides and other protein-based natural polymers needs to be explored to expand this concept further.

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ANNEXURES

Appendices

APPENDIX A – ETHICS COMMITTEE APPROVAL

Clearance was obtained from the Institutional Animal Ethics Committee (IAEC) for carrying out the study as per the order **SCT/IAEC-432/JANUARY/2022/112, dated 03/03/2022.**

APPENDIX B – SUPPLEMENTARY TABLES

APPENDIX C - PUBLICATIONS

List of publications from Thesis

- 1) Jesna Puthiya Veettil, Devika Sasikumar Lolitha, Rekha Mannemcherril Ramesan, Ramesh Parameswaran, and Umashankar Payanam Ramachandra. A Nontoxic and Biocompatible Method for Augmenting Mechanical Strength of Acellular Matrix by Silk Fibroin Impregnation, *ACS Applied Biomaterials*, 2024, 7(10): 6665-6681, DOI: 10.1021/acsabm.4c00843 (IF-4.7)
- 2) P. V. Jesna, S. L. Devika, and P. R. Umashankar. Combinatorial decellularization as a better approach to porcine liver extracellular matrix scaffold fabrication with preserved bioactivity: A comparative evaluation, *Xenotransplantation*, DOI: 10.1111/xen.70025 (IF-3.3)
- 3) P. V. Jesna, S. L. Devika, and P. R. Umashankar. In-vivo Immunocompatibility and induced regenerative potential of silk fibroin modified acellular porcine liver matrix scaffolds in rat subcutaneous and full-thickness abdominal wall defect models (Resubmitted to *International Journal of Biological Macromolecules* and is under review)

APPENDIX D – PLAGIARISM CHECK REPORT

DECELLULARIZED LIVER SCAFFOLDS FOR INDUCED REGENERATION IN ADULT MAMMALIAN MODEL

ORIGINALITY REPORT

3%

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