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Extracellular Matrix Hydrogels from Decellularized Tissues for Biological and Biomedical Applications

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1.1 Introduction to Hydrogels

1.1.1 Definition and Use of Hydrogels in Biomedical Applications

A hydrogel is a hydrophilic three-dimensional (3D) network composed of polymer units that is capable of absorbing large amounts of water relative to the dry weight of the component polymers [1–3]. Interaction between polymer units and a gelation stimulus in a hydrated environment, which in the context of biomedical applications may include physiologic liquids rather than pure water, results in formation of the 3D network. Since pioneering work by Wichterle and Lim [4], hydrogels have been used in numerous biological and biomedical applications including tissue engineering, drug delivery, cell culture, and as biosensors, hemostatic agents, and self-healing materials [1, 3]. Their widespread and growing use is due to numerous advantageous characteristics, such as tunable mechanical properties, ability to add functional groups to polymers that may act as ligands or be responsive to external stimuli, cytocompatibility, and low toxicity [1].

Hydrogels have received particular attention in cell culture and tissue engineering applications. Specific advantages of hydrogels in this field include the ability to encapsulate cells, proven biocompatibility and cytocompatibility, the potential to mimic physiologic environments, and act as cell and drug delivery vehicles [3, 5, 6]. In the context of these applications, some practical problems related with the use of hydrogels remain: the hydrogels are often difficult to handle, relatively fragile, hard to sterilize once gelation has occurred, and many are not currently produced using Good Manufacturing Protocol (GMP) grade processes limiting their translation into human patients [3, 5].

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1.1.2 Classification and Properties of Hydrogels

There are numerous methods to classify and describe hydrogels, many of which are discussed in detail elsewhere in this book, and are summarized in Table 1.1 [1, 3, 5–7]. The variables most relevant when considering biomedical applications of hydrogels are the nature of the forces holding the network together (response), the source of the polymers, and the cross-linking stimulus. The network of a reversible hydrogel is held together by molecular entanglements and/or secondary forces (ionic, hydrogen, or hydrophobic bonding), which can be reversed by changes in physical conditions. By contrast, permanent hydrogels are held together by covalent cross-linking [3]. Polymers may be naturally occurring, synthetic, or a hybrid natural-synthetic copolymer and may be tunable, allowing variation of mechanical

Table 1.1 Classification of hydrogels.

Classification variable	Subtypes
Response	Physical/reversible *Ionotropic hydrogel* *Polyelectrolyte complexes* Permanent/chemical *Copolymerization of polymer and cross-linking molecule* *Cross-linking of water-soluble polymers*
Cross-linking stimulus	pH Temperature Light/laser Chemical and ionic Magnetic field
Source of polymers	Synthetic Natural Hybrid
Macromolecular structure of polymers	Linear polymers Block copolymers Graft copolymers
Mix of polymers	Homopolymeric Copolymeric Multipolymer interpenetrating networks (IPNs)
Degradability	Degrading Nondegrading

Source: Adapted from Chai et al. [1], Hoffman [3], Giobbe et al. [5], Holloway et al. [6], and Capeling et al. [7].

properties. The stimulus to initiate cross-linking may be a defined range or change in temperature, pH, light, magnetic field, or ion strength/chemical composition of solution, or the addition of a specific cross-linking molecule [1, 2].

Temperature is a useful trigger of the gelation in the context of biological applications. When polymer units and water (or physiologic liquid) are mixed, the polymer network will take on water to swell, followed by gelation in a definable temperature range for a given hydrogel [1, 2, 5]. The lower bound of this range is termed the lower critical solution temperature (LCST) – above the LCST phase separation and gelation occurs, while below the LCST the system is miscible in all proportions – with transition from gel to sol or sol to gel states related to changes in Gibbs free energy [1, 3]. This property can be extremely useful in biological applications. For example, a hydrogel may be in solution at room temperature but undergo gelation at 37 °C.

The amount of water in a hydrogel is termed total bound water, composed of primary bound water (the first water to enter the 3D network and hydrate the most polar groups) and secondary bound water (which is bound to hydrophobic groups exposed by initial swelling), and the proportion of water in a hydrogel is called the volume fraction of water. Osmotic force will draw water into the network toward infinite dilution until an equilibrium is reached with the opposing force of elastic retraction by the network (equilibrium swelling theory) [1, 3]. When equilibrium is reached, water exists in the spaces in the hydrogel between polymers (pores), which are usually inhomogeneous in size, distribution, and connectedness in a given gel. These variables, in combination with solute size, shape, polarity, and solute–matrix interactions, determine the permeation of a solute through a hydrogel [3].

Properties of hydrogels can be studied using a multitude of techniques including histology, immunostaining, electron microscopy, and proteomics by liquid chromatography–tandem mass spectrometry (LC–TMS). Additionally, turbidity (measured by spectrophotometry) is commonly used to determine gelation kinetics, while atomic force microscopy and rheometric techniques are used to study the mechanical properties of hydrogels, for example, storage (G') and loss (G'') moduli [3, 5, 8].

Finally, any hydrogel under consideration for use in biomedical or biological applications must be cytocompatible. Whenever a hydrogel is modified (polymer type, polymer concentration, change in functional groups, etc.), it must be proven to be cytocompatible or it cannot be used *in vitro* or in clinical applications.

1.1.2.1 Synthetic Hydrogels

Synthetic hydrogels are composed of polymers that have been chemically synthesized and are not found in nature. Examples include poly(ethylene glycol) (PEG), poly(lactic acid) (PLA), poly(*co*-glycolic acid) (PGA), and poly(vinyl alcohol) [2]. A brief summary of advantages and disadvantages of this broad range of hydrogels will be presented here but is more comprehensively discussed elsewhere in this book.

Potential advantages in the use of synthetic hydrogels for biomedical applications are several. They have well-defined molecular composition and architecture, can be manufactured with low batch-to-batch variability, and avoid the use of xenogeneic material in production process [1, 6]. The monomers are uniform and

modular, leading to a relatively predictable relationship between composition and physicochemical properties when polymerized [9]. Therefore, the concentration of monomers can be varied, or the monomers can have functional units added rationally to alter the mechanical and biological signally properties of the resulting hydrogel. These features allow synthetic hydrogels to be easily tuned for specific circumstances [6, 8, 9].

There are several important disadvantages of synthetic hydrogels in biological contexts. Many synthetic hydrogels are covalently cross-linked and resistant to enzymatic degradation (due to lack of proteolytic sensitive domains), such that these gels behave as linear elastic networks. This is of particular relevance in cell culture contexts as these networks can accumulate substantial compressive forces that are then transmitted to encapsulated cells [9, 10]. Furthermore, the breaking of covalent bonds is irreversible, meaning that permanent synthetic hydrogels cannot "self-heal" like reversible gels [9]. Despite the ability to tune synthetic gels, in most products, this occurs as a bulk property of the hydrogel; that is, there is minimal spatial control over variation in mechanical properties. However, recent innovation in the functionalizing of synthetic gels with photosensitive functional groups may allow for improved spatial control of mechanical properties, which could be used to study symmetry breaking and other aspects of stem cell and niche biology [9, 11, 12]. Perhaps the most important disadvantage of synthetic hydrogels in biological applications is the lack of biological ligands to interact directly with cell surface receptors, such as integrins [2, 9]. However, several groups have taken advantage of this fact to study the influence of microenvironment on cell behavior or have functionalized the hydrogel to overcome this barrier [8–10].

PEG is a commonly used synthetic hydrogel for biomedical applications because of its modular macrostructure (e.g. 4 or 8 arm PEG) and ability for functional groups to be added, as recently described by Urciuolo et al. [12]. Gjorevski et al. [10] used 8-arm PEG to study the effect of matrix stiffness on the culture of murine intestinal stem cells (mISCs). First, they demonstrated that functionalizing of PEG with 1 mM of cell adhesion peptide arginylglycylaspartic acid (RGD) on vinylsulfone facilitated mISC survival, expansion, and passaging with comparable expression to Matrigel® of intestinal stem cell (ISC) marker leucine-rich repeat-containing G-protein coupled receptor 5 (Lgr5). The stiffness of the PEG–RGD was then varied that resulted in the optimal expansion of Lgr5-expressing mISC at 1.3 kPa with concomitant nuclear translocation of yes-associated protein 1 (YAP). Furthermore, at lower stiffness (190 Pa), the mISC-containing epithelium-only organoids were shown to bud and differentiate. Human ISC and colorectal cancer organoids were also shown to be viable in PEG–RGD [10]. In a similar strategy, Cruz-Acuña et al. [8] used 4-arm PEG with a maleimide group on each terminus functionalized with 2 mM RGD and cross-linked with protease-degradable peptide GPQ-W to culture human embryonic stem cell (hESC) and induced pluripotent stem cell (hiPSC) derived intestinal organoids, which contain both mesenchymal cells and epithelial cells, with morphology and protein expression (immunofluorescence) and RNA expression (real-time polymerase chain reaction [PCR]) similar to culture in Matrigel. Amongst other findings, this hydrogel was used as a delivery vehicle

with *in situ* polymerization that facilitated engraftment of the organoids in a mouse model of colon epithelial injury [8].

1.1.2.2 Natural Hydrogels

Natural hydrogels are composed of polymer chains that are naturally occurring or where the subunits do not need to be chemically synthesized in laboratory or commercial settings. The polymer network is composed of peptides and/or polysaccharides and may be homopolymeric or contain a mixture of polymers [1]. However, recent work has utilized DNA to produce tunable hydrogels, opening another category of natural polymers for use [13]. Commonly used examples are presented in this book and include gelatin, collagen I, hyaluronic acid, chitosan, alginate, dextran, cellulose, and agarose [2]. Extracellular matrix-derived hydrogels (ECM), including Matrigel, are a subset of natural hydrogels [9].

Natural hydrogels are already extensively used in laboratory and clinical applications. Gelatin, Matrigel, and collagen I are routinely used in cell culture. Gelatin is produced from the partial hydrolysis of animal collagens, retaining the cell-binding motifs (including RGD) and matrix metalloprotease (MMP)-sensitive degradation sites of the original proteins [1]. This inherent cell-binding activity and protease-mediated degradability result in gelatin being commonly used as a substrate in 2D cell culture without further modification. However, modified gelatin has been used in more complex applications. For example, methacrylated gelatin has been used as an injectable vehicle for human endothelial cells into the subcutaneous space of immunodeficient mice, followed by *in situ* polymerization using ultraviolet (UV) light [14].

Similarly, Matrigel can be used as a substrate for 2D cell culture, and it is currently accepted as the gold standard for 3D cell and organoid culture to which other hydrogels are compared in this context. Matrigel is a laminin-rich mixture of ECM proteins secreted by the Engelbreth–Holm–Swarm mouse sarcoma cell line. It is a proprietary product known to contain key components of the basement membrane, including laminin-1, collagen IV, and nidogen-1, as well as heparan sulfate and numerous growth factors [8, 9]. Matrigel has been used in seminal papers published on epithelium-only [15] and mesenchyme-containing organoids [6], amongst innumerable other works.

While Matrigel is the current gold standard for most 3D cell culture, it is also an example of the limitations of natural hydrogels. There is significant batch-to-batch variability in protein and growth factor composition, it is not chemically defined, not easily tunable (beyond simple dilution), it is xenogeneic, and expensive [6, 7]. However, the most important limitation, in terms of clinical translation, is the fact that Matrigel is derived from a sarcoma cell line.

Homopolymeric natural hydrogels can support 3D cell culture. Mouse small intestinal epithelium-only organoids were encapsulated in collagen I hydrogel (0.75 mg/ml), allowed to gelate in a ring shape, then the ring detached and cultured floating in media [16]. This method allowed the organoids to fuse together into a tube with a continuous lumen and cause contraction of the gel ring, demonstrating the ability of a simple defined ECM-mimetic hydrogel to support structurally

complex cell culture [16]. Others have shown that growth factors and adhesive cues can be added to simple natural hydrogels with positive effects on organoid growth, while exhibiting minimal batch-to-batch variability [6].

However, not all natural hydrogels are inherently biologically active. For example, alginate, a polysaccharide derived from algae, is biologically inert and lacks inherent cell recognition molecules. The Spence lab has demonstrated that 1% alginate, cross-linked with calcium ions, can be used to differentiate hiPSC-derived intestinal organoids, albeit with yields <50% of that in Matrigel [7]. The mesenchymal cells of the hiPSC-derived organoids did not penetrate into the alginate and, importantly, epithelium-only intestinal organoids did not proliferate in the alginate and lost polarity, suggesting that the basement membrane secreted by the mesenchymal cells was crucial in the absence of such signals from the hydrogel itself. However, in contrast to Matrigel, alginate is cheap (US$0.44 vs. US$300 per 10 ml) and U.S. Food and Drug Administration (FDA) approved for clinical use in humans already (see Section 1.4), making it worthy of further study for translational purposes [7, 17].

DNA-based hydrogels have recently been investigated, aiming to take advantage of several fundamental features of nuclei acids that could be useful in hydrogels [13]. These features include the modular structure of DNA, ability to control the changes in the macromolecular structure of DNA (for example, strand displacement), and the existence of sequence-specific DNA-cleaving technologies. This potentially allows for rational predesign of the hydrogel matrix. Yue et al. [13] demonstrated reversible control of shape and stiffness in a DNA-based hydrogel using oligonucleotide sequences as triggers. Furthermore, they showed the ability for the hydrogels to be loaded with doxorubicin and control its release using the same triggers.

1.2 Key Features and Functions of the Extracellular Matrix in Homeostasis and Development

Considering both the broad categories and specific examples described above, some important themes emerge with regard to biomedical applications of hydrogels:

1. Mechanical properties of hydrogels are important but not sufficient to sustain cells in culture.
2. At least some of the signaling essential for successful cell culture is derived from cell–matrix interactions.
3. Mimicry of the native organ microenvironment is important for *in vitro* cell culture to recapitulate *in vivo* cell behavior.
4. GMP-grade processes need to be available for the manufacture of any hydrogel intended for translation to clinical applications in humans.

The ECM comprises “structural” ECM proteins, such as collagens, glycoproteins, and proteoglycans, and a vast array of ECM-associated proteins, including ECM-modifying enzymes, ECM-bound growth factors, and ligands for cell surface receptors [18]. The structural “matrisome” is estimated to comprise ~300 proteins,

not including ECM-associated proteins, and is highly conserved across mammalian species.

Collagens are essential to strength in almost all forms of ECM and are composed of the basic repeating unit "glycine-X-Y," where X is usually proline and Y is 4-hydroxyproline. Glycoproteins serve multiple functions in the ECM, including interactions that facilitate the assembly of structural proteins like collagen, as well as the display of motifs for cell signaling and adhesion [19]. Glycoproteins where a substantial proportion of the molecular mass is made from glycosaminoglycans (GAGs) are called proteoglycans. Proteoglycans and glycoproteins of particular relevance to this chapter include perlecan (also called heparan sulfate proteoglycan 2) and laminins, which are core components of basement membrane, and fibronectin, which binds integrins [5, 10, 19]. GAGs on proteoglycans and glycoproteins are highly polar, acting to sequester cations and water, and have many binding sites for growth factors and matrix-bound extracellular vesicles [20]. In fact, this binding allows the ECM to act as a reservoir for growth factor that can be released during ECM remodeling or be presented as ligands in their bound state [21]. This is perhaps one of the most important functional aspects of the ECM in development and homeostasis. The ECM can be remodeled by numerous classes of proteases, such as MMPs and elastases, which can restructure collagens, liberate growth factors and matrix-bound extracellular vesicles, and produce cryptic peptides that may be further sources of local signals to cells [20, 21]. Examples of common cell surface receptors for ECM proteins include integrins, which bind to RGD motifs and laminin, and dystroglycan, which binds laminin and perlecan. Cell surface receptors provide the links between the ECM and the cytoskeleton, transmitting physical signals for cell shape, polarity, and motility, and between the ECM and the nucleus, altering gene expression [18, 19]. An example of such signaling with importance to ECM hydrogels is the negative regulation of transforming growth factor-β (TGF-β) signaling following laminin binding by epithelial cells [18]. In fact, the signaling functions of ECM adhesion receptors are likely as complex as soluble growth factor signals but are yet to be studied in the same depth. The interested reader is referred to an excellent review by Hynes and Naba for further details [19].

Together, the ECM and associated proteins not only give physical structure to tissues and organs but also critical inputs to cells by controlling cell shape, survival, proliferation, differentiation, migration, and polarity. This is true during organogenesis and development, normal homeostasis, and is increasingly recognized as important in disease states [6, 18, 19]. That is, the ECM is a critical component of the cellular microenvironment, which, in addition to the above-mentioned functions, also includes paracrine, endocrine, and neural signals [18, 19]. However, it is clear that cells secrete ECM and this leads to the important theory of "dynamic reciprocity," which is the concept of tissue architecture as a cause of tissue specific functions but also a consequence to tissue-specific cell activity. Put another way, the ECM provides biophysical and soluble cues that influence cell morphology and phenotype, and in turn cells modify the secretion of ECM products in response to these cues [18, 22].

These fundamental roles for ECM in biology have spurred intense research into the derivation and use of ECM-based hydrogels from the decellularization of organs, including as a mechanism for increasing the biomimicry of *in vitro* cell culture with the aim of increasing complexity of cell culture models [5, 6]. In Sections 1.3 give an overview of methods of production and the published work using ECM hydrogels derived from several different organs.

1.3 Extracellular Matrix-Based Hydrogels Derived from Decellularization of Organs

1.3.1 Production of ECM Hydrogels

ECM hydrogels have been produced from several organs. While the detail of published protocols varies widely, the principal steps of most methods are [20] as follows:

1. Removal of cellular components and nucleic acids from the organ (decellularization)
2. Solubilization of protein components (digestion)
3. Restoration of physiologic pH and electrolyte concentration (neutralization)
4. Gelation under appropriate conditions

Approaches to decellularization can be broadly classified as perfusion or immersion techniques. Perfusion-based protocols take advantage of the vascular network and/or the lumen of the organ itself to deliver the decellularizing agents into an intact organ. The distribution of solution recapitulates blood flow in the tissue by utilizing the native vasculature. This technique has been shown to produce scaffolds that retain the macroscopic and ultrastructure of the original organ in esophagus [23, 24], intestine [25], heart [26], trachea [27], lung [28], liver [29], and kidney [30]. By contrast, immersion-based protocols bathe the tissue in the decellularization agent, which usually requires the tissue to be cut into volumes small enough for the agent to penetrate by diffusion. To aid this process, immersion-based protocols usually occur in agitation and with regular changes of decellularization solution. While this process does not preserve whole-organ level structure, it maintains ECM components in appropriate ratios and is capable of retaining the native ultrastructure in the fragments of tissue [5].

Whether by immersion or perfusion, decellularization is usually based on supplying a combination of hypotonic solutions (e.g. de-ionized water), detergents, and enzymes, collectively known as detergent-enzymatic treatment (DET) (Table 1.2). DET may be preceded by freezing and thawing cycles, which can be used to mechanically disrupt cells through the changes in volume of water between phases. DET protocols take advantage of the post-assembly modifications of ECM, for example disulfide bonding, transglutaminase-mediated cross-linking, and the actions of lysyl oxidases and hydroxylases, which render the ECM relatively insoluble in detergents such as deoxycholate [5, 25]. Established DET processes have been shown to preserve glycoproteins, proteoglycans, GAGs, and growth factors, as well as the structural

Table 1.2 Example reagents used in decellularization protocols.

Detergents	Sodium deoxycholic acid (SDC)
	Sodium dodecyl sulfate (SDS)
	Triton X-100
	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)
	Polyoxyethylene [20] sorbitan monolaurate (Tween 20)
Enzymes	DNase
	Trypsin
	Dispase
Other	Ethylenediaminetetraacetic acid (EDTA)
	Peracetic acid
	Chloroform
	Methanol
	Ethanol

proteins, retaining the biochemical complexity of the tissue of origin in appropriate stoichiometric ratios [5, 20, 25, 31].

It is important to remember that inherent in these protocols is a trade-off between complete removal of cellular proteins and nucleic acids vs. the retention of ECM proteins and ECM-associated proteins (including ECM-bound growth factors). The optimal balance between retention of the diversity of ECM components and complete removal of cellular components is not clear. There is evidence that incomplete removal of cellular components may elicit an adverse inflammatory response to decellularized scaffolds [22]. However, future work may demonstrate that preservation of matrix supersedes the complete removal of cellular components, or vice versa, and the protocol chosen will depend on the intended application of the decellularized material.

Following decellularization, the ECM is lyophilized and milled to allow solubilization by pepsin-mediated digestion. Two commonly used methods are the Freytes method (0.1 M hydrochloric acid + pepsin) and the Voytik–Harbin method (0.5 M acetic acid + pepsin). Utilizing pepsin-based digestion is particularly useful for xenogeneic ECM as it cleaves the telopeptide of collagen, which is the main antigenic component [32]. Changing the duration of digestion produces pre-gels that contain different profiles of cryptic peptides, theoretically allowing tailoring of the hydrogel at this stage of production to optimize it for a given application [20]. During digestion, there is the desired fragmentation of larger structural ECM proteins, but also some depletion of growth factors compared to intact decellularized scaffolds and native tissues. The extent to which this affects the bioactivity of the final ECM hydrogel is not known [20, 33]. Neutralization is simply performed with small volumes of high-molarity hydrochloric acid (HCl) or sodium hydroxide (NaOH) to restore

physiologic pH and the combination of the digested ECM with an appropriate volume of solution with physiologic salt concentration. Once neutralized, the hydrogel is ready for use with cells.

ECM hydrogel gelation is an entropy-driven self-assembly process, the kinetics of which are governed predominately by collagen [20]. However, ECM hydrogels from decellularized organs have been shown to have distinct matrix assembly kinetics and fiber morphologies, compared to purified collagen I hydrogels. These differences depend on proteoglycans (e.g. decorin), GAGs (e.g. heparan sulfates), fibronectin, and minor collagens, which are known to act as nucleation sites for collagen fibrillogenesis, again demonstrating the importance of their conservation during production of the hydrogel. Gelation occurs by reformation of bonds and entanglements at physiologic temperature [20, 34].

An example protocol from our group utilizes 4% sodium deoxycholate, purified and deionized water, DNase-I for decellularization of porcine small intestinal mucosa-submucosa (pSIS), digestion using the Freytes method (1 mg/ml pepsin in 0.1 M HCl), and neutralization with 10× Dulbecco's modified eagle medium (DMEM)/F12 and 10 M NaOH. The final hydrogel is used while working on ice to encapsulate cells before thermally induced gelation at 37 °C [5]. An alternative process involves homogenization by physical disruption in high salt buffer; use of dispase to cleave fibronectin, collagen IV, and collagen I; a urea extraction step for further solubilization; and centrifugation and removal of insoluble remnants. The resulting extract undergoes gelation at 37 °C or by lowering the pH to 4 with acetic acid. This is included for completeness, as it is based on the method used to produce Matrigel [20].

1.3.2 Characterization of ECM Hydrogels

As introduced in the opening Section 1.1.2 of the chapter, ECM hydrogels can be characterized in a multitude of ways, including ultrastructure, mechanical properties, and biochemical composition. The ultrastructure of ECM hydrogels is influenced by tissue of origin, protein and GAG concentration, and processing and gelation methods. Light, confocal, and electron microscopy is used to interrogate gel ultrastructure, including pore size and fiber architecture, with scanning electron microscopy usually demonstrating a loosely organized nanofibrous scaffold with interconnecting pores [35].

Rheometry and turbidimetric assays are the main methods to assess gelation and mechanical properties of ECM hydrogels, but other methods include atomic force microscopy, indentation, and compression testing. Turbidimetric assays can be used to describe gelation kinetics such as time to initiation of gelation (lag phase), time to 50% turbidity, and describe the curve of gelation (sigmoidal, exponential) [20]. Rheology gives more granular detail regarding pre-gel viscosity and storage and loss moduli of the final gel. Viscosity of ECM pre-gel, rate of gelation, and storage modulus of final gel tend to be positively correlated with protein concentration. The solid-like behavior of the final gel is confirmed on oscillatory rheology when the storage modulus is independent of frequency and is greater than the loss modulus

by an order of magnitude. Using these methods, it has been shown that ECM hydrogels, as other gels, exhibit viscoelastic behavior with similarity to embryonic tissues [7, 20].

The biochemical composition of ECM hydrogels can be evaluated in most detail using paired LC–TMS [5], while commercially available kits can be used to easily measure residual nucleic acid content or specific matrix components, such as collagens or GAGs. Proteomic profiling of decellularized ECM in this manner has been done for numerous organs and has shown that biochemical composition may vary by source organ, but only subtly between tissues of the same embryonic germ layer [5, 20].

As the examples below demonstrate the diversity of tissue of origin, processing, and preparation methods that allow ECM hydrogels to be tuned in a near-infinite way. However, any such changes require the resulting gel to be treated as a novel gel and tested accordingly prior to use. For example, it has been shown that hydrogels derived from dermis have ultrastructural differences depending on whether they undergo temperature-dependent or pH-dependent gelation [36]. Furthermore, when considering the subsequent examples and their biological applications, it is crucial to remember that cells (encapsulated in the gel or recruited from the recipient animal) interacting with the ECM hydrogel will remodel it and eventually replace it with *de novo* matrix. Therefore, it is likely that each application will require an "individualized" gel. For example, in one setting it may be the aim that the gel provides sustained support for the cells and is replaced slowly, while in another the ECM hydrogel may be tuned to allow ECM signaling molecules to be released from the gel and diffuse into the surrounding tissue.

1.3.3 Pancreatic ECM-Derived Hydrogels

Diseases of the endocrine and exocrine pancreas are major causes of mortality and morbidity worldwide. Pancreatic ECM-derived hydrogels may be useful in the study of islet cell biology and further development of islet cell transplantation as a human therapy, as 3D culture methods have shown promising effects on beta-cell survival and functionality, with basement membrane proteins in particular having a crucial role *in vitro* and *in vivo* [37, 38]. However, the pancreas presents two unique challenges to the production of a pancreas ECM hydrogel. Namely, the fat content and the presence of proteases in the native pancreas could inhibit the formation of a stable gel or further digest the ECM during decellularization and digestion phases, respectively.

Gaetani et al. [39] utilized readily available porcine pancreas in a study to optimize a protocol for the production of a pancreas ECM hydrogel by immersion–agitation DET and Freytes digestion. They examined a range of concentrations of sodium dodecyl sulfate (0.1%, 0.5%, 1% SDS) and Triton X-100 as the detergent, but also examined the effect of adding a synthetic serine protease inhibitor (0.1 mM Gabexate) to the protocol, aiming to mitigate the effect of pancreatic proteases on the gel. Interestingly, they demonstrated that successful gelation (no residual liquid phase) only occurred in 1% Triton X-100 groups, both with and without Gabexate, and that

laminins were not detectable at the conclusion of any of the SDS protocols. At a molecular level, addition of Gabexate improved the sulfated-GAG (sGAG), basement membrane, and matricellular protein content of the gel. The 6 mg/ml hydrogel produced by 1% Triton X-100 with Gabexate supported 3D cell culture of rat insulinoma cells to 10 days, which were able to secret insulin in response to glucose stimulation. However, the gel was not used to passage cells, culture human cells, or in any *in vivo* experiments [39].

In a major work, Sackett et al. [40] were the first to demonstrate the production of an ECM hydrogel from decellularization of human pancreas. Importantly, they showed that reproducibly successful gelation only occurred when utilizing a homogenization and centrifugation protocol to de-lipidise the pancreas prior to decellularization with sodium deoxycholic acid (SDC). Fat content decreased from 38% in native pancreas to 14% in the SDC only protocol, which was further reduced to 4% when the homogenization step was added. Furthermore, this study was the first to demonstrate preservation of laminins in decellularized pancreatic scaffold or hydrogel. The hydrogel was able to sustain 2D culture of rat insulinoma cells, human umbilical vein endothelial cells (HUVECs), and expansion culture of hiPSCs and H9 and H1 hESC lines. The hydrogel performed well in 3D culture of ESC-derived pancreatic progenitors and islet-like cell clusters, demonstrating similar numbers of PDX1, Ki-67, and Caspase-3 positive cells compared to standard suspension cultures. With a view to future clinical application, the hydrogel could be injected subcutaneously into humanized immunodeficient mice with successful *in situ* gelation and minimal host inflammatory response [40].

1.3.4 ECM Hydrogels Derived from Liver

The development of high-fidelity *in vitro* systems to study human liver development, disease, and function has proven challenging. Primary hepatocytes and sinusoidal endothelial cells lose maturity in plastic-adherent or pure collagen-based culture [41, 42], iPSC-derived hepatocytes show a relatively immature phenotype, and immortal cell lines are inappropriate for translational applications [22, 43]. As a result, many groups have developed strategies utilizing hepatic ECM in cell culture models attempting to overcome these issues. For example, it has been shown that a solution of porcine hepatic ECM contained >40 liver-derived growth factors and supplementation of culture media with this solution improved albumin gene expression and secretion in porcine iPSC-derived hepatocytes [33].

ECM hydrogels have been successfully produced from the livers of several species, including rat [44], pig [32, 33], sheep [43], and human [45, 46]. Depending on the protocol and species, lyophilized hepatic ECM has 75%–100% sGAG and 60% total collagen content of native liver [33, 43, 45], with 18 mg of powder produced per 1 g wet weight of porcine liver [32]. Gelation has been successful with protein concentrations varying from 3 to 10 mg/ml [32, 43]. Cytocompatibility of hepatic ECM hydrogels has been shown with a range of cell lines, including human hepatocellular carcinoma (HCC) and hepatoblastoma (HepG2) cells [43, 46], and porcine [33] and human [45] iPSC-derived hepatocytes.

Saheli et al. [43] cocultured human HCCs with HUVECs and mesenchymal stem cells (MSCs) to examine the effect of 3D culture and a sheep liver-derived ECM hydrogel on *in vitro* hepatocyte maturity. Three-dimensional liver organoids cultured in hepatic ECM hydrogel demonstrated the most mature cellular phenotype by RT-PCR and secretion of albumin, α_1 antitrypsin, and urea, compared to all other conditions (HCC only 2D culture, 3D organoid culture in collagen, 2D culture supplemented with solubilized ECM, hydrogel-free 2D culture). Alpha-fetoprotein, cytochromes 3A4 and 3A7 expression approximated native adult human liver, and while albumin and tyrosine aminotransferase did not reach native liver levels, their expression was greatest in 3D ECM hydrogel culture.

Most iPSC-hepatocyte differentiation protocols, including seminal work by Taniguchi's group [47], use laminin-rich Matrigel as the culture substrate. However, laminin is not a major component of liver ECM, and such protocols have generated human iPSC-hepatocytes with maturity similar to fetal hepatocytes [45, 47]. Jaramillo et al. [45] studied the effect of adult human liver-derived hydrogel on iPSC-hepatocyte differentiation. In comparison to Matrigel, iPSC differentiated entirely on ECM hydrogel showed upregulation of albumin, most major cytochromes (CYP3A4, CYP2B6, CYP2D6), and hepatic nuclear and transcription factors (forkhead box A [FOXA1], FOXA2, hematopoietically expressed homeobox [HHEX], hepatic leukemia factor [HLF], hepatocyte nuclear factor [HNF4α], luteinizing hormone receptor [LHR1], polysaccharide biosynthesis domain containing [PBDC1]). Furthermore, the expression of these markers was highest when iPSCs were differentiated on ECM from the induction of definitive endoderm, compared to switching to ECM hydrogel later in the protocol, and markers of hepatoblast differentiation (e.g. HNF6) appeared earlier and were upregulated in the ECM protocol compared to Matrigel. Functionally, ECM-differentiated iPSC-hepatocytes demonstrated intracellular glycogen storage, bile canaliculi formation, cytosolic albumin expression, and secretion of albumin into the media. Importantly, levels of albumin secretion and CYP3A4 activity were comparable to human neonatal hepatocytes, but still lower than human adult hepatocytes [45].

1.3.5 Lung ECM Hydrogels

Early studies into the decellularization of lung tissue focused on obtaining intact acellular lung scaffolds for repopulation with lung epithelial and vascular endothelial cells for whole organ tissue engineering [28, 48, 49]. While successful removal of DNA and cellular components was achieved, this was usually at the expense of complexity of the remaining ECM, with as little as 15% fibronectin, 40% elastin, and <10% sGAG content remaining (compared to native lung levels) at the conclusion of protocols using CHAPS [48, 49]. The result was somewhat improved with a change to SDC/Triton X-100 in a later study by the same group [48].

The first group to produce a lung ECM hydrogel was Pouliot et al. [50]. They decellularized porcine lung by perfusion DET via trachea and pulmonary artery using 0.1% Triton X-100 and 2% SDC. After 48 hours of digestion, the pre-gel elastin content was reduced by 60% compared to native lung levels, but collagen content

and sGAG content were reduced by 40% and 50%, respectively. The resulting hydrogel was shown to be cytocompatible with human bone marrow mesenchymal stem cells (BM-MSCs) *in vitro* and increased the engraftment of rat BM-MSC by 2.5× (compared to saline vehicle) when injected intratracheally in an elastase-induced rat model of emphysema [50].

Recently, a group from Groningen reported the production of ECM hydrogels from human lungs, including donor lungs unsuitable for transplantation and lungs affected by chronic obstructive pulmonary disease (COPD) and idiopathic pulmonary fibrosis (IPF) [51]. The protocol used was complicated – homogenization in a blender, 3 hours in 0.05% trypsin, then 24 hours each of 6 M NaCl, 70% ethanol, 1% SDS, 1% Triton X-100, 1% SDC, and finally DNase. While characterization of the hydrogels was very limited and they were not used with cells *in vitro* or *in vivo*, the stiffness of native lung and hydrogel from lungs affected by IPF was significantly greater than normal and COPD-affected lungs and their respective hydrogels [51]. This suggests the potential value of using hydrogels from diseased organs for modeling *in vitro*.

1.3.6 Hydrogels Derived from Decellularized Colon

Most studies of decellularized colon have focused on intact scaffolds in colorectal neoplasia [52, 53] or using other hydrogels to deliver cells into the lumen of the colon [54, 55]. Decellularized human colorectal carcinoma scaffolds have been demonstrated to polarize macrophages to an anti-inflammatory M2 phenotype and that this phenotype promotes invasion through basement membrane *in vitro*, while scaffolds from normal colon did not [53]. In mouse models of colonic mucosal injury, ECM-based hydrogels, fibrin glue, and gelatin have been used as delivery vehicles for organoids with successful engraftment in the recipient animal [54, 55].

Keane et al. [56] performed a detailed analysis of a novel hydrogel derived from decellularized porcine colon. Following delipidization 2 : 1 chloroform: methanol and graded ethanol baths, the decellularization protocol utilized 0.02% trypsin/0.05% EDTA and 4% SDC, and the lyophilized powder was then digested using the Freytes method. By immunostaining, laminin was largely absent and fibronectin signal was much reduced. sGAG concentration was reduced by ~1/3 and hyaluronic acid by about 75%. Interestingly, basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) were retained after decellularization, albeit at much reduced concentrations. Stable gels were formed at 4 and 8 mg/ml, with a positive correlation between concentration and maximum storage modulus. Intestinal epithelial cells were compatible with the hydrogel with minimal cell death seen at 24 hours. In further experiments, supplementation of media with soluble porcine colon ECM promoted M2-like phenotype in mouse macrophages *in vitro*, similar to most reports, but in contrast to Pinto et al., perhaps suggesting interspecies difference [53, 56]. The M2 macrophage phenotype was also seen after implantation of the colon hydrogel in a rat abdominal wall defect model [56].

Romero-López et al. [57] generated a hydrogel by decellularization of human colorectal cancer metastases to the liver. The hydrogel derived from metastases

had a threefold higher stiffness compared to hydrogel from normal colon and on LC–TMS contained higher amounts of collagen IV, VI, XIV, fibrillin, emilin, vitronectin, and laminin-γ1. Interestingly, fibronectin, versican, thrombospondin-2, periostin, tenascin, and several other laminin subunits were only detectable in tumor ECM hydrogel. However, it is important to note that the decellularization protocols differed significantly in the detergent used – 1% SDS for tumor and, 2% SDC for normal colon. In terms of disease modeling, SW620 colon tumor cells grew faster in tumor hydrogel and demonstrated an increase in glycolysis compared to normal colon hydrogel. Furthermore, when endothelial cells were cultured in the tumor hydrogel, they formed vessels mimicking tumor vasculature with high variability in vessel diameter and fractal dimension of the network [57].

1.3.7 ECM-Derived Hydrogels from Small Intestine

Acellular pSIS-based products have been in commercial use since the 1990s and have become an accepted and routine part of mechanical reconstruction in most surgical disciplines including cardiovascular surgery, otorhinolaryngology, and abdominal surgery [58]. The regulatory acceptance and established clinical use of pSIS products make it an attractive option to derive hydrogels for clinical translation.

The effect of a pSIS hydrogel on tissue regeneration was studied in a dextran sodium sulfate model of colitis [59]. When delivered to the rodents by enema, the acellular hydrogel adhered to injured colonic mucosa and resulted in a reduction in clinical and histologic severity of the disease, with corresponding improvement in colonic epithelial barrier function. This occurred in the setting of reduced numbers of pro-inflammatory macrophages in the colon following pSIS hydrogel treatment [59].

Initial investigations into small intestine submucosa (SIS) hydrogels suggested that collagen and sGAG content are similar to the decellularized scaffold and contain at least some intact growth factors [20, 34]. A recent study by our group reported a deep characterization of a pSIS hydrogel and its potential for human organoid culture and regenerative medicine [5]. The hydrogel was prepared by dicing pSIS, immersion-agitation decellularization using 4% SDC and DNase, lyophilization, milling, and digestion by the Freytes method, before neutralization with 10X DMEM-F12 and 10 M sodium hydroxide (Figure 1.1). This protocol allowed for effective removal of cellular material and nucleic acid from the tissue but preserved concentrations of elastin and GAGs and an (expected) increase in total collagen concentration, compared to native tissue. Successful gelation of the pSIS hydrogel was observed at 6, 8, and 10 mg/ml with storage and loss moduli by temperature ramping oscillatory rheology and elastic modulus by nanoindentation showing that 6 mg/ml pSIS hydrogel and Matrigel had similar mechanical profiles [5].

Deep characterization of the proteomic profile of the pSIS hydrogel was undertaken with LC-TMS. The most abundant proteins included several types of fibrillar (types 1, 2, 3, 5, 6), fibril-associated (types 12, 14, 21), and sheet-forming (type IV) collagens. Interestingly, on comparison to the publicly available map of the *human* proteome, the set of ECM proteins identified in the pSIS gel showed a high degree

Figure 1.1 Example process for ECM hydrogel preparation. The gelation preparation protocol consists of decellularization of the SI mucosa/submucosa, freeze-drying, milling into a fine powder, γ-irradiation for sterilization, Freytes method digestion for 72 hours, and neutralization to a physiological pH and salinity. The hydrogel undergoes temperature-dependent gelation at 37 °C. Source: Giobbe et al. [5]/Figure 1a/Springer Nature/Attribution 4.0 International (CC BY 4.0).

of similarity to the group of tissues of endodermal origin by principal component analysis (PCA) and hierarchical clustering analyses (Figure 1.2). Indeed, when the pSIS gel was used to culture organoids in 3D, multiple human endodermal organs were supported, including gastric, intestinal, ductal hepatic, fetal intestinal, fetal hepatic, and fetal pancreatic, with comparable performance to culture in Matrigel. For example, human small intestine (SI) organoids culture in pSIS hydrogel showed morphology and protein expression of stem, crypt, and villus markers similar to culture in Matrigel for up to four passages, and fetal SI organoids could be passaged to single cells in pSIS hydrogel with successful *de novo* organoid formation. Furthermore, human ductal and fetal hepatocytes showed similar levels of phenotype-specific markers whether cultured in basement membrane extract or pSIS hydrogel and produced similar levels of albumin [5].

Analysis of the transcriptome of human SI organoids by bulk RNA-Seq demonstrated upregulation of OLFM4, SMOC2, and lysozyme (LYZ) in pSIS hydrogel but similar levels of LGR5, BMI1, and LRIG1 (stem and transit amplifying [TA] cell markers). Villus domain markers Ezrin (EZR), VIL1, MUC12, MUC13, MUC17, and MUC20 were upregulated in Matrigel; FABP1, MUC1, MUC3A, MUC5B were similar between the two conditions; while chromogranin A (CHGA) was the only differentiated cell marker overexpressed in pSIS hydrogel-cultured organoids. Together, this suggests a higher fraction of crypt cells in human SI organoids cultured in pSIS hydrogel compared to Matrigel. Interestingly, ECM-specific proteins detected on proteomic analysis of the decellularized pSIS were found to be overexpressed in Matrigel-cultured organoids, leading us to speculate that human SI organoids may need to produce their own intestine-specific ECM to compensate when cultured in Matrigel [5].

From a regenerative medicine standpoint, we were able to use the pSIS hydrogel for successful *in vivo* transplant of human fetal pancreatic and mouse SI organoids with preserved morphology and marker expression at eight weeks. Importantly, all reagents used in generating the hydrogel are already commercially available at GMP-grade and we demonstrated that while the highly antigenic galactose-α1,3-galactose (α-Gal) was present in whole decellularized tissue but was absent in final gel. These points underscore the potential utility of this hydrogel to

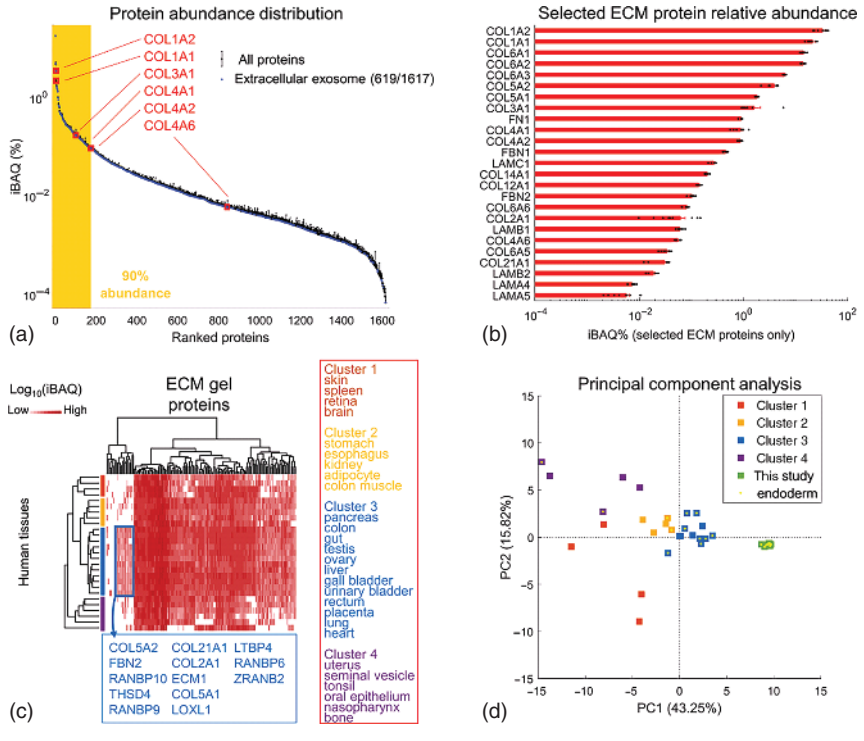

Figure 1.2 pSIS ECM proteomic analysis. (a) Protein abundance range, with 619 (on 1617 total) proteins mapped to GO-CC:0070062~extracellular exosomes highlighted. Yellow-shaded area represents the range covering 90% of total protein abundance. Example collagens also highlighted. (b) Relative abundance of selected ECM proteins. (c) Hierarchical clustering analysis of mass spectrometry native human tissue data from a draft map of the human proteome, conducted for proteins in our data mapped to GO-CC:0031012~ECM. Four main clusters are identified whose color-coded tissues are shown on the right. A small group of proteins especially expressed in cluster 3 is highlighted. (d) Principal component analysis (PCA) of data from native human tissue reported in (c) and from decellularized pSIS. Tissues and samples having endodermal origin are also highlighted. Source: Giobbe et al. [5].

deliver cell-based therapeutics in humans and suggests there may be less of a need for organ-specific cell–hydrogel combinations than first thought [5, 60].

1.3.8 Cellular Responses to ECM Hydrogels

As discussed, *in vitro* methods of evaluating cellular responses to ECM hydrogels include supplementing the media with solubilized ECM, 2D culture on ECM hydrogel substrate, and 3D culture with cells encapsulated in ECM hydrogel. Cellular response can be assessed in terms of survival, proliferation and differentiation capacity, and the preservation or *in vitro* rescue of tissue-specific functions. In these regards, ECM hydrogels have shown impressive abilities across numerous cell types and sources of ECM, including cross-species and interorgan compatibility, likely related to the conservation of the matrisome across mammals [5, 19, 20].

Current experience with *in vivo* applications of ECM scaffolds and hydrogels has shown several important beneficial components of the host response including [5, 20, 22, 56, 58, 59, 61]:

- Promotion of angiogenesis
- Modulation of the immune response, including M2-like macrophage activation and Th2-type T-cell response, which favor tissue regeneration
- Stem cell recruitment
- Promotion of innervation

While the mechanisms underlying these responses are beginning to be elucidated in the setting of decellularized ECM scaffolds, they have not been studied in depth for ECM-derived hydrogels. As discussed earlier, there is evidence that bulk mechanical properties of ECM hydrogels can influence cell behavior but questions regarding the effect of alterations to ECM proteins, matrix-bound nano-vesicles, and growth factors during solubilization and gelation are only beginning to be addressed [20, 22, 62]. For example, in our study of pSIS hydrogel, LC–TMS revealed 619 proteins in the decellularized material known to be from extracellular exosomes [5].

1.4 Commercially Available Products

Possible physical forms of hydrogels used in biomedical applications include solid/molded (e.g. contact lenses), pressed powders, microparticles, coatings, membranes and sheets, and encapsulated semi-solids [3].

Natural hydrogels have been in clinical use for decades with proven safety and efficacy. Commercially available alginate and gelatin-based dressings are particularly useful in burns and wound management, maintaining a favorable moist environment, having hemostatic effects, allowing diffusion of nutrients and gases, while also acting as a barrier to microorganisms [2, 17].

As mentioned earlier, pSIS-based products are in regular therapeutic use in humans. Most common commercially available pSIS products are based on laminated sheets fabricated into appropriate shapes for the clinical application of interest. Products include Surgisis®, Biodesign®, and Durasis® (Cook Medical). For example, CorMatrix® make a range of cardiovascular implants from pSIS, which have been used in congenital cardiac and vascular reconstruction, pericardial reconstruction, and valve replacement [58].

By contrast, ECM hydrogels from decellularized organs have yet to be introduced into regular clinical practice. A Phase I clinical trial was recently completed using porcine myocardial ECM hydrogel (Ventrigel®, Ventrix Inc.) injected into the infarcted area of patients with evidence of left ventricular remodeling following ST segment elevation myocardial infarction. The trial showed Ventrigel to be safe and well tolerated, and patients had some improvement in six-minute walk test and New York Heart Association symptom class [63]. The study was not designed to assess efficacy of the treatment but does pave the way for further clinical trials.

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