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The matrix reloaded: the evolution of regenerative hydrogels

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Cell-laden hydrogels can regenerate lost, damaged or malfunctioning tissues. Clinical success of such hydrogels is strongly dependent on the ability to tune their chemical, physico-mechanical, and biological properties to a specific application. In particular, mimicking the intricate arrangement of cell-interactive ligands of natural tissues is crucial to proper tissue function. Natural extracellular matrix elements represent a unique source for generating such interactions. A plethora of extracellular matrix-based approaches have been explored to augment the regenerative potential of hydrogels. These efforts include the development of matrix-like hydrogels, hydrogels containing matrix-like molecules, hydrogels containing decellularized matrix, hydrogels derived from decellularized matrix, and decellularized tissues as reimplantable matrix hydrogels. Here we review the evolution, strengths and weaknesses of these developments from the perspective of creating tissue regenerating hydrogels.

Introduction

Biological tissues often contain highly complex hydrogels [1,2]. They contain dynamic, heterogeneous and spatially defined mixtures of cell types, growth factors, nutrients, and intricate extracellular matrices (ECMs) [3]. Importantly, the matrices of natural tissues have complex structures that start with the defined arrangement of amino acids that compose ECM proteins at the nanoscale, to the formation of fibrils and fiber bundles at the microscale, and to the alignment of fibers in a specific direction and crosslinking of the fibers at the macroscale [4]. The hierarchical structure of the ECM not only controls the tissue's biochemical and physico-mechanical properties, but also the concentration, location and distribution of cells and growth factors, cytokines, and hormones within the tissue. The ECM thus acts as a key element in inducing, orchestrating and maintaining the multifaceted processes that govern tissue phenotype, function, and fate [5–8]. Naturally derived hydrogels are used in engineered constructs to support the growth and maturation of implanted cells, but lack the minimum stiffness required to resist soft tissue compression [9,10]. Conversely, synthetic hydrogels provide the required mechanical support but lack the intricate arrangement of ligands that regulate cell fate. Not surprisingly, much effort has been dedicated to recreate or incorporate ECM – or their derivatives or biomimetic counterparts – in hydrogels. Here, we review the evermore sophisticated approaches to integrate ECMs in hydrogels by orthogonal conjugation of cell-interactive ligands, copolymerization with functionalized ECM molecules, doping with decellularized ECM, or hybridization with digested

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tissue ECM, designed for the regeneration of complex multicellular tissues.

Hydrogels as extracellular matrices

Like natural ECMs, hydrogels consist of hydrophilic networks of nano- and microfibers that provide a mechanically robust shelter for cells, while retaining a large fraction of water in their structure [11]. Consequently, they allow for nearly free diffusion of oxygen, carbon dioxide, and nutrients and proteins to maintain the viability and function of encapsulated cells [12]. Hydrogels can be segregated in two broad categories: natural and synthetic hydrogels.

Natural hydrogels are as suggested by its name, isolated from biological sources that include amongst others collagen [13], gelatin [14], silk [15], alginate [16], hyaluronic acid [17] and dextran [18]. Hydrogels of natural sources typically support cell adhesion and proliferation, but are mechanically weak and provide little control over remodeling. Advanced modifications or processing strategies are therefore often required to match the biomaterial with the injured tissue. In addition, minor changes in sequence distribution of natural gels can dramatically affect the fate and function of the encapsulated cells in the matrix giving rise to batch-to-batch variation [19,20].

Synthetic hydrogels such as polyethylene glycol (PEG), polyvinyl alcohol (PVA), and polyvinyl pyrrolidone (PVP) have advantages that include well-defined composition and easily tunable physiochemical properties [21–23]. Synthetic hydrogels generate matrices with enormous range of physical, mechanical, and chemical properties for regeneration of complex multiphase tissues [3,24–27]. Reinforcement with fillers, nanofibers, nanotubes and optimization of network structure can improve the mechanical properties of hydrogels by several orders of magnitude [28–31]. Synthetic hydrogels are often characterized by slow degradation rates unless proteolytically degradable peptides are incorporated in the gel network [32]. However, additional modifications can remedy this challenge. For example, short hydroxy acid (HAc) segments can be polymerized to PEG chains to generate asymmetric HAc-chain extended PEG gels with tunable resorption times [33]. This enables a resorption time ranging from a few days for glycolic acid-chain extended gels, to a few weeks for lactic acid, to a few months for dioxanone, and to many months for caprolactone-chain extended gels (Fig. 1) [27]. Such modifications allow for the matching of hydrogel degradation with cellular invasion, vascularization, innervation and mineralization during tissue regeneration [33].

Extracellular matrix modified hydrogels

The regenerative potential of hydrogels heavily depends on our ability to develop man-made matrices that mimic the composition and microstructure of native tissues [34]. Although hydrogels resemble natural ECM on the abstract level, they do not incorporate the biological complexity derived from the vast variety of distinct ECM molecules [35]. To recapitulate this, hydrogels are commonly decorated with one or a few matrix molecule types [36–38]. These include amongst others hyaluronic acids [38], collagens [36,37], laminins [39], elastins [40], vitronectins and fibronectins [41]. It is well established that such modifications affect the function, proliferation and migration of cells. In addition, most of these ECM molecules can affect the biomaterials' porosity, swelling or degradation characteristic. In consequence, this often increases the difficulty of controlling the hydrogel's behavior [42]. As an alternative, numerous bioactive peptide sequences have been identified and conjugated to the polymer chains in the hydrogel network [28,43–45]. For example, cell-adhesive, vasculogenic and osteogenic hydrogels were generated by copolymerization of PEG macromonomers with acrylamide-terminated GRGD peptide (IP), propargyl acrylate and 4-pentenal (aldehyde moiety) monomers [46]. Aminooxy-functionalized vasculogenic SVVYGLRK peptide (VP) derived from osteopontin protein was conjugated to the PEG network by an aminooxy-aldehyde reaction whereas the azide-functionalized osteogenic KIPKA SSVPT ELSAI STLYL peptide (OP) derived from *recombinant human* bone morphogenetic protein-2 (rhBMP-2) was conjugated by a propargyl-azide reaction (Fig. 2) [46]. Functionalization of the hydrogels with IP, IP + OP, and IP + OP + VP significantly increased osteogenic

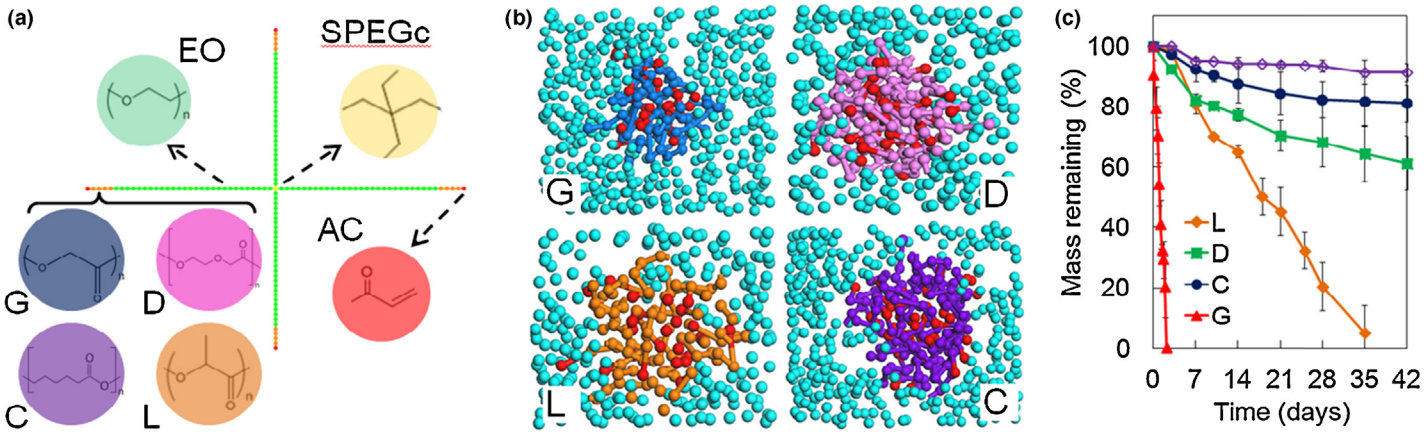

FIGURE 1

Representation of the SPEXA (X= L, G, C or D) macromonomer. Beads SPEGc (yellow), EO (green), G (blue), D (pink), L (orange), C (purple) and Ac (red) represent star PEG core, ethylene oxide repeat unit, glycolide, p-dioxanone, lactide, ε-caprolactone repeat unit, and acrylate functional group, respectively. (b) Simulation of the effect of degradable G (blue), L (orange), D (pink), and C (purple) monomers on the distribution of water beads around the micelles' core. Red and light blue beads in b are water and reactive acrylate beads, respectively. (c) Effect of monomer type G (red), L (orange), D (green), and C (blue) on the measured mass loss of SPEXA hydrogels with incubation time. The purple curve in C is the mass loss of PEGDA hydrogel without chain extension with a hydroxy acid. Reproduced with permission [27].

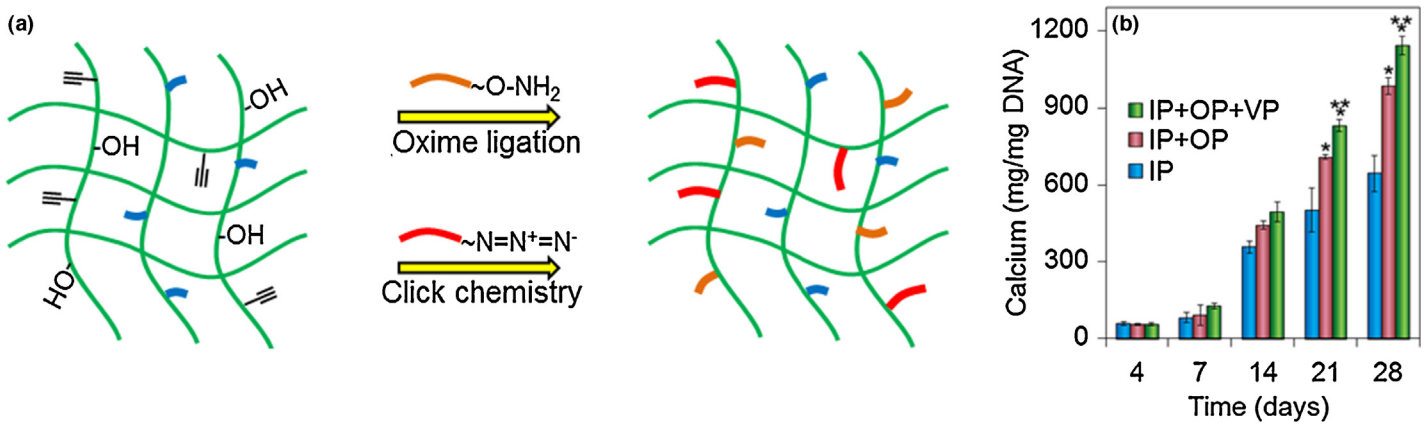

FIGURE 2

RGD (blue) is conjugated to a degradable hydrogel (green) by co-polymerization of PEG precursor macromonomer with acrylamide-terminated RGD. Propargyl acrylate and 4-pentenal are added to the copolymerization reaction to form a RGD conjugated gel with propargyl (triple bond) and aldehyde moieties, respectively. A vasculogenic peptide (orange) was grafted to the hydrogel by the aqueous reaction between the aminooxy on the peptide with the aldehyde moiety in the hydrogel. An osteogenic peptide (red) was grafted orthogonally to the hydrogel by the aqueous reaction between the azide moiety on the peptide with the propargyl moiety in the hydrogel. The orthogonal reactions led to the formation of a hydrogel with a cell-adhesive RGD peptide, a vasculogenic peptide and an osteogenic peptide. The extent of mineralization with incubation time by MSCs seeded in the hydrogel was highest when all three peptides were grafted to the hydrogel (green bars, IP + OP + VP) as compared to the cell adhesive peptide only (blue bars, IP) or cell adhesive plus osteogenic peptide (red bars, IP + OP). Reproduced with permission 46.

differentiation and mineralization of marrow stromal cells seeded in the hydrogels (Fig. 2) [46]. In addition, IP + OP + VP conjugation increased the expression of vasculogenic markers PECAM-1 and VE-cadherin by the seeded stromal cells whereas the IP + BP conjugation only increased the expression of α-SMA [46].

Although the ability to incorporate one or a few matrix molecule types into biomaterials represents a significant improvement, it still does not rival the sophisticated complexity that is present in natural ECMs. Specifically, it does not recapitulate the variety of cell-interactive ligands present in natural tissues and thus only supports part of its expected functions. A practical solution was found in processed decellularized mammalian tissues [47–53]. This process yields a purified matrix that subsequently can be digested and processed into a concentrated liquid or ground into a fine hydrophilic powder. The extracted ECM can then be combined with hydrogels to generate hybrids with tunable physical and biological properties (Fig. 3). For example, a soluble matrix derived from cartilage, meniscus, and tendon tissues by digestion with pepsin was successfully methacrylated by reaction with methacrylic anhydride [54,55]. The resulting methacrylated matrix could be covalently bound into a photocrosslinking natural hydrogel such as gelatin methacryloyl (GelMA) to produce hybrid hydrogels [54]. Moreover, the photocrosslinking approach allows the production of micropatterned scaffolds with controlled topog-

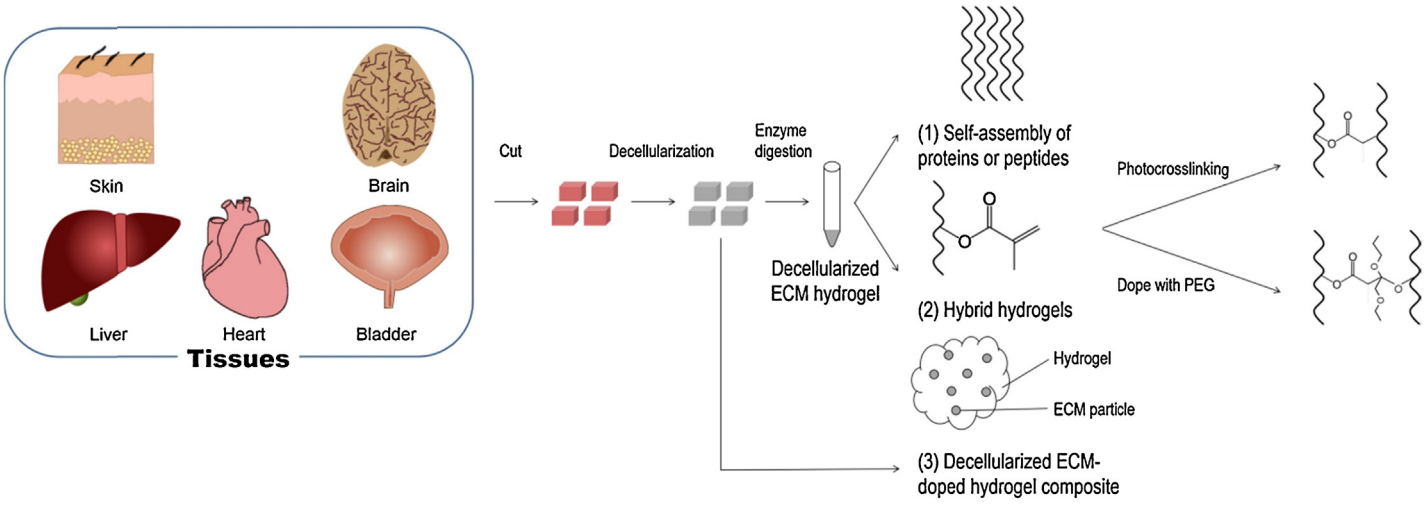

FIGURE 3

A typical process for the production of ECM derived hydrogels. The tissue is cut and minced into smaller particles and decellularized. The decellularized ECM is mixed with a hydrogel precursor solution to form a decellularized ECM-doped hydrogel composite (3). The decellularized ECM is enzymatically digested to produce a decellularized ECM solution. The decellularized ECM solution is physically crosslinked by the self-assembly of proteins and peptides to form a hydrogel (1). The decellularized ECM solution is functionalized with methacryloyl or acryloyl groups to produce a decellularized ECM hydrogel precursor solution (2). The precursor solution is mixed with PEG or crosslinked directly with UV to produce a hydrogel based on decellularized and digested ECM.

raphy and mechanical properties [56]. Synthetic polymers such as PEG [57] and PVA [58] have also received a plethora of distinct ECM modifications. For example, myocardial matrix–PEG hybrids were synthesized either by crosslinking the precursor matrix molecules with an amine-reactive PEG-star or photo-induced radical polymerization of multi-armed PEG-acrylates [57]. Both techniques allow for the conjugation of myocardial matrix to PEG [57]. Such approaches allow for increased control over both physicomechanical and biochemical properties.

Incorporated ECM additionally improves the hydrogel's tissue-like behavior by acting as a reservoir for delivery of functional biologics to induce the desired cell functions. In consequence, the choice of incorporated ECM also determines the panel of growth factors present in the hydrogel [59]. It is reported that hybrid hydrogels composed of native heart matrix induced cardiac differentiation of human embryonic stem cells without supplemented growth factors [60]. This hydrogel was prepared from decellularized ECM of porcine hearts by mixing ECM and collagen type I at varying ratios. The high ECM content hybrid gels promoted cardiac maturation and improved contractile function of cardiac cells. In addition to serving as a reservoir for the release of biologically active molecules, the decellularized ECM also provided an anchoring point for other biological molecules, for example heparin-binding growth factors could bind to the sulfated GAG in the decellularized ECM [61,62]. In that regard, hydrogels produced from pericardial matrix were utilized for the binding of growth factors such as basic fibroblast growth factor (bFGF) [63]. Binding of bFGF to pericardial matrix increased its retention both *in vitro* and *in vivo* in ischemic myocardium as compared to delivery of bFGF in a collagen matrix [63].

Despite the numerous advantages, these methods still require further optimization. Specifically, ground matrices typically consist of coarse micrometer sized particles and digestion physically cleaves the matrix, which can result in the partial loss of microstructure and function [64]. Another challenge lies in controlling the batch-to-batch variations, which otherwise could confound clinical outcomes. Although incorporation of ground or digested ECM effectively provides a natural variety of ECMs, it does so at matrix densities lower than those found in natural tissues. Thus the intensity of the ECM stimuli from these approaches will remain significantly lower than those present in natural tissues. This relatively low ECM concentration is an inherent and unavoidable characteristic of this approach as it requires a hydrogel-bulk to provide the implant with its mechanical stability.

Hydrogels derived from decellularized tissues

A recent approach that has been received with great interest is based on producing hydrogels purely of whole decellularized tissues. Such hydrogels are expected to display superior biocompatibility and bioactivity as compared to conventional hydrogels [9]. Hydrogels solely derived from decellularized ECM have several desirable characteristics for therapeutic applications. These include targeted delivery by minimally invasive techniques, ease of repeated delivery, ability to quickly fill an irregularly shaped space, polymerization to form a support structure suitable for host cell infiltration and remodeling, and the inherent bioactivity of native matrix [65,66]. Many tissues including skin [67], muscle [68], bladder [67], tendon [69], cartilage [70], heart [71], liver [72], bone [73], fat [74] and nucleus pulposus [75] have been processed into hydrogels. The driving force for hydrogel formation is protein and peptide self-assembly. In a typical process [71], the tissue is harvested, sliced into small pieces, decellularized using a detergent, lyophilized, milled and enzymatically digested. Importantly, the pH, temperature, ionic strength and solution's concentration of digested ECM affect gelation kinetics, mechanical properties and nanoscale architecture of the hydrogel [76].

The physical and biological properties of an ECM derived hydrogel can be controlled by the specific type and concentration of digested ECM in solution [67]. However, the tuning range of mechanical properties and degradation kinetics of these hydrogels is narrow [57]. Chemical crosslinking, for example with glutaraldehyde, can be used to increase the stiffness of the ECM hydrogels, while slowing the rate of degradation and cellular migration through the hydrogel [77]. ECM hydrogels remain injectable via a catheter following chemical crosslinking. Other chemical crosslinkers such as ethyldimethylaminopropyl carbodiimide and N-hydroxysuccinimide have also been used to generate chemically crosslinked hydrogels from ECM [75]. Despite the limited control over the hydrogel's physical characteristics, several studies report promising data that support the potential clinical translation of injectable ECM derived hydrogels [70,71,74,78]. For example, a myocardial-specific hydrogel precursor solution derived from decellularized ventricular ECM successfully gelled by self-assembly after delivery via trans-endocardial injection in a large animal model [78]. This increased the endogenous cardiomyocytes present in the infarct area without the induction of arrhythmias [78]. Regardless, although these methods allow the formation of pure ECM hydrogels, they do not provide any spatial organization. Instead, they generate a homogeneous construct without any zonal or organotypic structure, which are important to organ function.

Spatially organized ECM hydrogels

Natural tissues have a multilayered or organotypic organization with gradients in matrix stiffness, cell density, and growth factors [79–81]. These complex tissue architectures are imperative to their respective tissue functions [82]. For example, articular cartilage is composed of multiple zones including superficial, middle, deep, and calcified zones, which by acting together allow for the absorption of mechanical stress generated by movements [83]. Each zone is characterized by distinct cellular phenotypes, ECM compositions, and growth factors [84]. Moreover, each zone is mechanically unique and contains a different collagen fiber morphologies that ranges from thin to thick that run parallel, oblique or perpendicular from the superficial zone to the calcified zone, respectively [85]. The change in tissue composition and collagen fibers orientation substantially increases the compressive modulus from 80 kPa in the superficial zone to 320 MPa in the calcified zone [86]. Hydrogels entirely composed of digested cartilage ECM have lost this important instructive organization. In consequence, evermore attention is dedicated to the generation of zonal or organotypic structures [87,88]. For example, to recapitulate the microstructure and compressive properties of the calcified zone of articular cartilage, a suspension of gel-coated and aligned nanofiber microsheets, transforming growth factor-β1 (TGF-β1) and human mesenchymal stem cells (hMSCs) was crosslinked into a disk-shape matrix such that the aligned nanofibers were oriented

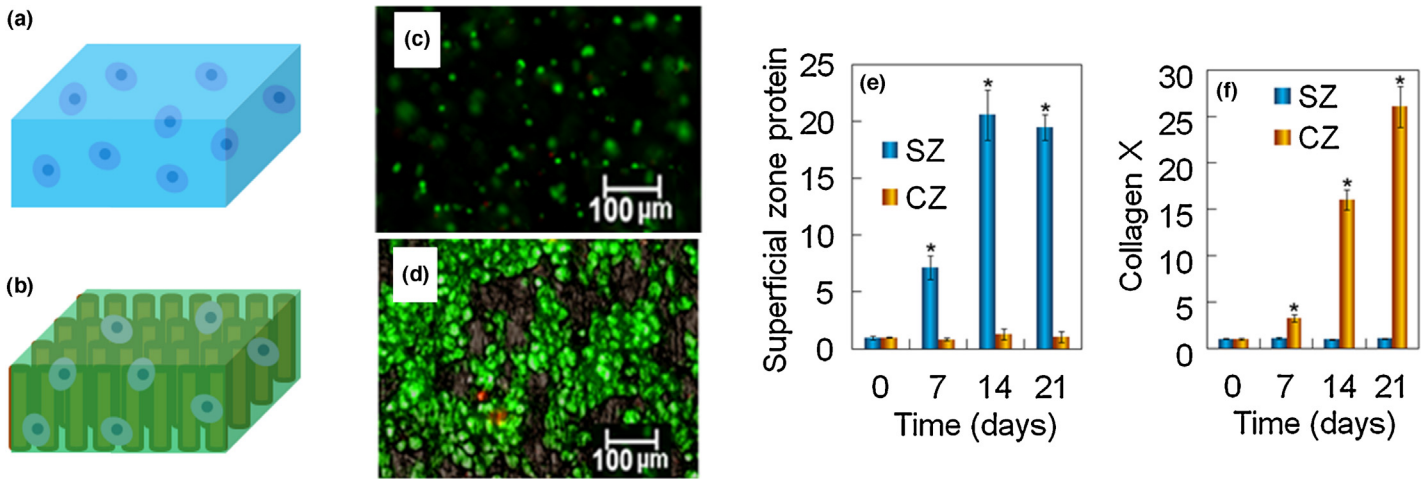

FIGURE 4

hMSCs are encapsulated in a soft gel with 80 kPa compressive modulus (a, light blue) to simulate the superficial zone (SZ) of articular cartilage or in a stiff gel with nanofibers aligned in the direction perpendicular to the plane of the gel layer with 320 MPa compressive modulus (b, green) to simulate the calcified zone (CZ). hMSCs in the soft gel are uniformly distributed in the gel (green dots in C) whereas hMSCs in the stiff gel clustered around the aligned nanofibers with a columnar morphology (green dots in d). hMSCs in the soft gel differentiated to the superficial zone chondrocytes and secreted a matrix rich in superficial zone protein (SZP, blue bars in b) whereas hMSCs in the stiff gel with aligned nanofibers differentiated to the calcified zone chondrocytes and secreted a matrix rich in collagen X (orange bars in f). Reproduced with permission [30].

perpendicular to the disk's surface (Fig. 4) [30]. hMSCs encapsulated in the fibrous hydrogel with 320 MPa compressive modulus expressed markers of hypertrophic chondrocytes found in the calcified zone (COLX and ALP) whereas compliant gels with a relatively low modulus of 80 kPa without nanofibers expressed early markers of chondrogenesis found in the superficial zone (SOX9 and SZP) of articular cartilage [30]. Remarkably, hMSCs encapsulated in the fibrous hydrogel were oriented along the direction of nanofibers [30]. Similarly, carbon nanotubes embedded in GelMA hydrogels not only controlled the hydrogel's viscoelasticity and electrical conductivity, but they also aligned myoblasts in a specified direction [89,90].

Another approach to recreate the natural organization of tissues can be found in the three-dimensional (3D) printing of decellularized ECM, which is recently pioneered [91]. Various tissues including heart, cartilage and fat were decellularized, processed, laden with stem cells and 3D printed [91]. As expected, stem cells within 3D printed tissue-specific ECM expressed significantly higher levels of biomarkers specific to the matrix of the chosen tissue [91]. The use of such biological ECM-based inks is predicted to enhance our capabilities and accelerate the development of man-made organ-like implants.

Together, these studies underline the importance of recreating the zonal and organotypic structures as found in natural tissues. Unfortunately, these techniques are unable to present the matrix molecules in their natural conformation. Natural matrices have a tightly orchestrated organization, which allows for presentation of the right motif at the right place at the micrometer level. Tissue digestion irrevocably removes this organization, even when the macro level is recapitulated in the form of zonal and organotypic structures.

Intact decellularized ECM as hydrogels

Decellularized tissues effectively are crosslinked ECM hydrogels with a highly controlled spatial organization [92–94]. Decellularization of a tissue can be achieved using physical, chemical or enzymatic approaches [95,96]. A well-known clinical example of this approach is the creation of acellular dermal matrices for the treatment of burn wounds and cosmetic surgery [97]. Decellularization is not only compatible with tissues, but also with whole organs. Uniquely, this yields implantable hydrogels that contain truly organotypic structures [98]. The resulting decellularized ECM constructs allow for reseeding with cells [92–94]. This can produce hybrid implants that are composed of allogeneic ECM and autologous cells, which could reduce the chance of organ rejection while improving implant function. In recent years, several studies have indeed reported on the functionality of this type of implant for amongst others lung, heart and trachea [99–101].

Despite its many advantages, decellularization of a tissue can alter properties of the matrix as it also can damage or remove part of the organ's matrix [102]. To counter this detrimental event, decellularized ECM has been combined with other types of hydrogels to once again form ECM/hydrogel composites [103,104]. In one report, the decellularized bladder matrix was seeded with bladder smooth muscle cells and a crosslinkable form of the non-sulfated glycosaminoglycan hyaluronan (HA) [103]. The HA-modified bladder cellular matrix displayed a notable increase in matrix contraction and triggered a higher level of cell-secreted gelatinase activity compared to the unmodified bladder acellular matrix [103]. In another report, human myocardium was first decellularized with retained biological elements of the ECM as well as the underlying mechanical properties [104]. The decellularized human myocardium was then sliced into sheets, coated with MSCs in a fibrin gel and laminated to form a fully biological composite scaffold for cardiovascular repair [104]. Implantation of the composite implant onto the myocardial infarct bed in a nude rat model enhanced the recovery of baseline levels of left ventricular systolic dimensions and contractility [104]. As such, these approaches partly reverse the traditional roles of biomaterials and ECMs in tissue engineering; the biomaterial supports the implant's

TABLE 1

Examples of regenerative matrices.

	Advantage	Disadvantage
Unmodified hydrogels	• Wide variety of available biomaterials • High degree of control over physicochemical properties • Able to conformally fill irregular tissue defects	• Provides little to no bioactive sites • Low level of control over bioactive sites, if at all present
Hydrogels modified with matrix-like molecules	• Contains tunable, biomimetic, and bioactive sites of choice • High degree of control over physicochemical properties • Able to conformally fill irregular tissue defects	• Potentially associated with high costs • Does not recapitulate the natural variety of matrix molecules • Spatially homogeneous presentation of matrix molecules
Hydrogels derived from decellularized matrix	• Natural concentration/variety of most matrix molecules • Cost-effective method to provide numerous matrix molecules • Able to conformally fill irregular tissue defects	• Limited control over physicochemical properties • Spatially homogeneous presentation of matrix molecules • Risk on disease transmission, if not autologous • Potential batch-to-batch variation • Potential donor source limitation
Decellularized intact tissues as matrices	• Natural concentration of most matrix molecules • Biomimetic spatial presentation of matrix molecules • Cost-effective method to provide numerous matrix molecules	• Difficult to effectively reseed and organize cells within biomaterial • Limited control over physicochemical properties • Processing methods can damage natural extracellular matrix • Unable to conformally fill irregular tissue defects • Risk on disease transmission, if not autologous • Potential batch-to-batch variation • Potential donor source limitation

function and the ECM provides a mechanically stable microenvironment.

In conclusion, the ultimate decision of which one of these approaches will be most suitable for a specific therapy strongly correlates with the desired balance between control over physical and mechanical properties, required ECM amount, spatial organization of the ECM and the envisioned application method (Table 1).

Future challenges and perspectives

Conventional hydrogels based on matrix-like polymer networks allow for well controlled tuning of physical and mechanical characteristics. However, the incorporation of a wide variety of bioactive stimuli at appropriate concentrations to achieve proper tissue-like function has remained a major challenge and area of intensive research. Hydrogels containing decellularized ECM present a natural selection of stimulating matrix molecules, but at unnaturally low densities. Hydrogels derived from ECM display a natural variety of matrix molecules at appropriate concentrations, but provide little control over their physical and mechanical characteristics. Although hydrogels derived from digested decellularized tissues lack the desired mechanical properties, the primary and to some extent the secondary structure of the original ECM is preserved. Therefore, hybrid matrices based on synthetic gels for tuning physico-mechanical properties and digested tissue gels for controlling cell function are very promising as cellular scaffolds in regenerative medicine. Hydrogels of decellularized intact tissues retain both the composition and complex nano- and microstructures of the natural tissue. This provides an exquisitely biomimetic microenvironment for soft tissue repair and regeneration, but offers challenges with regards to effective cell seeding and is currently not compatible with minimally invasive strategies. Regardless, with the exception of decellularized intact tissues, it has remained a true challenge to provide the appropriate stimuli at the appropriate place. Instead, ECM and associated growth factors are typically presented in a homogeneous and unnatural manner. Micro- and nanoscale techniques are expected to generate the intricate patterns of cells, growth factors, and complex ECM structures that are essential for the function of natural tissues. Hybrid matrices combined with microscale technologies are therefore expected to lead to the development of biomimetic matrices with balanced and tunable physico-mechanical, biochemical, and cellular properties for applications in regenerative medicine.

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