Engineering cell-derived matrices: from 3D models to advanced personalized therapies[†]

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Abstract

Regenerative medicine and disease models have evolved in recent years from two to three dimensions, providing *in vitro* constructs that are more similar to *in vivo* tissues. By mimicking native tissues, cell-derived matrices (CDMs) have emerged as new modifiable extracellular matrices for a variety of tissues, allowing researchers to study basic cellular processes in tissue-like structures, test tissue regeneration approaches, and model disease development. In this review, different fabrication techniques and characterization methods of CDMs are presented and examples of their application in cell behavior studies, tissue regeneration, and disease models are provided. In addition, future guidelines and perspectives in the field of CDMs are discussed.

1. Introduction

A shortage of donors, little success in traditional tissue engineering approaches, and disease complexity are currently major obstacles in basic and clinical research, leading to the requirement of alternative sources of tissue substitutes and disease models. These structures must mimic native tissues to identify the biological mechanisms governing cell metabolism, disease development, and healing. These processes involve complex and tissue-specific networks of molecules that drive cell adhesion, migration, proliferation, and differentiation, as well as tissue morphogenesis and homeostasis [1,2]. The extracellular matrix (ECM) are three-dimensional (3D) environments that interact with cell surface receptors and growth factors, activating cell signaling cascades and gene transcription that in turn regulate the dynamic remodeling of the ECM [3].

Numerous strategies ranging from the use of decellularized native tissues, which are processed to remove cells, to the engineering of ECM analogs have been developed. The type, availability, and complexity of the tissue influence the approach used.

The use of decellularized tissue such as ECM is one of the most promising options in tissue engineering. The main advantage of using decellularized ECM (dECM) from native tissues is that it keeps its complex tissue-specific architecture and vascular network. A wide variety of tissues and organs have already been used to obtain native decellularized scaffolds (e.g., heart [4], blood vessels [5], lungs [6], skin [7], and liver [8], among others). However, the restricted availability of tissues and organs for decellularization and the correct repopulation of the whole structure are the main limitations of this strategy. Furthermore, native tissues are appealing for their biological composition and some approaches remove their 3D structures during processing (i.e., as powders to develop hydrogels [9–11] or microparticles [12]). The use of dECM from other individuals (allografts) or other species (xenografts), even if the original structure is not

maintained, has potential limitations such as immunogenicity, the possible presence of infectious agents, and batch-to-batch variability. Other drawbacks include difficulties in reseeding cells homogenously due to the geometry and porosity of the ECM and the inability to completely specify and characterize the bioactive components of the material [13].

To overcome these issues, cell-derived matrix (CDM) scaffolds have been produced *in vitro* directly from the patient's cells, providing a personalized strategy with unlimited availability as well as avoiding pathogen contamination or immunogenicity as cells can be screened and kept in a pathogen-free environment [14]. Furthermore, synthetic templates are used for guiding and supporting cells during the formation of CDMs. Since matrix tri-dimensionality exerts different responses in cells compared to 2D cultures [3,15], cells are seeded in 3D scaffolds that closely resemble native tissue dimensionality. Several biomaterials such as microencapsulated collagen fibers [16], titanium fiber mesh [17,18], electrospun polycaprolactone (PCL) microfibers [19], β-tricalcium phosphate particles [20], and poly(lactic-co-glycolic acid) (PLGA) meshes [21] have been used for that purpose. CDMs offer a better alternative to decellularized native tissues as they can be bioengineered. The use of different cell types, including autologous cells, can change the composition and structure of the extracellular matrices produced. Moreover, these cells can be modified genetically, biochemically, or mechanically to tailor the biochemical and mechanical properties of the CDMs produced. Other advantages of CDMs include the possibility of combining different ECMs derived from different cell types separately or the development of an ECM from the co-culture of different cell types.

The ability to produce a myriad of microenvironments can help address basic biological questions about cell-matrix interactions, cell phenotype induction, and tissue morphogenesis, as well as assess clinical questions regarding tissue regeneration, disease progression, modeling, and treatment.

In recent decades, emerging techniques have allowed the generation of CDMs following bottom-up approaches, where small units are precisely assembled into lager scaffolds [22–24]. Some of these techniques include bioprinting [25], self-assembled polymers [26], and modular aggregation using bioreactors [27].

CDM research is a growing field involving the study of its fabrication processes, the best cell types for its production, as well as its characterization. There are currently only a few applications for CDMs, but they are expected to grow in the coming years. In CDM production, different variables must be considered to mimic the composition, structure, and mechanical properties of the target tissue, such as (1) the cell type used to produce the CDM, (2) the production method and conditions, and (3) post-production manipulation or application. The field is still in its infancy and needs to be explored. Therefore, in this review, we aimed to compile the latest information on CDMs, providing a complete view of their relevance and details on their fabrication, characterization, and potential uses.

2. Fabrication methods of CDMs

The final aim in the production of CDMs is to obtain a functional and feasible CDM-based hydrogel that mimics the biochemical and physical characteristics of the original ECM of a native tissue. The type of morphology and structure of the CDM is very important in regenerative medicine and disease modeling applications. However, their manipulation invariably drifts into some loss of the original properties. This is a significant disadvantage in any dECM and CDM produced, especially with regards to tissue repair with minimal load-bearing, as well as critical size defects, where long-term mechanical support is required during the regeneration of the newly formed tissue. In this case, combination with other supporting polymeric or stronger hydrogel materials may be required. ECM stiffness can be increased using chemicals like genipin or glutaraldehyde. Although these compounds are generally used

to stiffen collagen or gelatin-based constructs, they can also exert cytotoxic effects, thereby affecting cell behavior [28–30].

The decellularization process used is a key step in the production of all CDMs. The decellularization methods are similar to the ones used for tissue and organ decellularization, where the protocol chosen can alter ECM properties. Decellularization has been widely reported to alter the native 3D architecture of the ECM, thereby affecting its mechanical and biological properties. There is no standardized decellularization process, with many techniques combining physical, chemical, and enzymatic methods. Many studies suggest that chemical decellularization can keep the native fibrous structure of the ECM compared to physical methods [31,32]. In our experience, the combination of chemical and enzymatic (for DNA degradation) methods has shown the best results in removing cells and maintaining protein composition and ECM structure. This contrasts with the findings of Lu *et al.,* who concluded that only two methods, freeze-thaw cycling with aqueous ammonia and Triton X-100 treatment with a 1.5 M-KCl osmotic shock, successfully removed cellular components among the seven processes that were studied [33]. There is a need for consensus on the decellularization method used as this is paramount in being able to compare results from different studies and to maintain the biochemical composition and mechanical properties of the CDMs as much as possible. The production of any templated CDM hydrogel also involves three key steps: (1) elimination of the template, which is optional and strongly depends on the nature of the guiding material; (2) dissolution of the CDM by partial digestion of the ECM collagen fraction; and (3) temperature and/or pH-controlled neutralization to induce spontaneous recovery of the intramolecular inter-chain bonds of the ECM collagen backbone to produce a homogeneous gel [34]. Common methods involve the use of pepsin or urea, together with hydrochloric acid. The gel is then neutralized to physiological pH and ionic strength, and stored at a low temperature (pre-gel)

until its application at physiological temperatures, when self-assembly occurs to form a gel (ECM gel) [34].

To date, different methods have been developed to produce adequate amounts of ECM by culturing different cell types. The fabrication method is critical regarding the desired ECM properties and three-dimensionality. Furthermore, the materials used as a support or template for matrix production clearly influence the composition of the ECM produced. This not only increases the range of possibilities enormously, but also requires a strict control of the composition, rigidity, and elasticity of the ECM produced.

2.1. Supporting biomaterials for CDM production

Several biomaterials and processing methods have been used to produce efficient and functional CDMs. These will be discussed in the following sections and are summarized in **Table 1** and **Figure 1.**

2.1.1. 2D surfaces to produce cell sheets

In the early 2000s, Okano and collaborators [35,36] developed a technology that allowed the immediate assembly of cell sheets to form an autologous deposited ECM using a thermo-responsive polymer such as poly(N-isopropylacrylamide) (PIPAAm). The method consists of culturing and expanding cells on a PIPAAm-coated surface. Once enough ECM has been secreted, the culture is cooled below 32°C, at which temperature the PIPAAm becomes hydrophilic, eliciting its rapid hydration and inducing the release of the ECM together with the cells that can then be used in several clinical applications for tissue regeneration. It is a highly effective method that does not damage the newly produced ECM by avoiding the use of proteolytic enzymes while maintaining inherent growth factors. Another method is the covalent immobilization of a tissue onto SiO_2 glasses using poly(octadecene-alt-maleic anhydride)

(POMA), which has a strong affinity for amines from proteins like fibronectin (FN) [37]. Further decellularization allows either the reseeding of the CDMs in patients or with other cell types, or to use them acellularly for applications in regenerative medicine [38,39]. The previously described decellularization approaches can be used to produce several micron-thick sheets of ECM mimicking native tissue properties based on the cell source [40]. Prewitz *et al.* demonstrated that mesenchymal stem cell (MSC)-derived ECM coatings can act as an *ex vivo* niche for human hematopoietic stem cell (hHSC) expansion, generating up to a three-fold increase in the number of cells compared to conventional plasma-treated tissue culture plates (PTP) [37]. Furthermore, CDM sheets can be used as patches to deliver growth factors and other molecules that can act as chemoattractants for cell recruitment in tissue regeneration [39,41–43].

2.1.2. Microparticle-based templates

A simple but a very effective and versatile way of introducing three-dimensionality into cell cultures is to use microparticles as 3D templates. This approach is based on a greater specific surface area for cell adhesion compared to conventional culture plates, affecting the quality of the CDM produced. For example, Tour *et al.* seeded primary rat calvaria osteoblasts and dermal fibroblasts separately onto hydroxyapatite (HA) microparticle scaffolds, which were then co-cultured for CDM production [31]. The decellularized CDM-HA construct was then implanted in a rat calvarial defect, resulting in better bone growth compared to pure unmodified HA scaffolds. Remarkably, the spherical shape of the microparticles strongly regulates the CXCR4 receptor. The SDF-1α/CXCR4 axis is a key target in chemotactic and regenerative processes, enhancing stem cell recruitment. It has been reported that MSCs cultured on PLA microparticles show increased expression of this factor and its receptor, promoting cell engraftment onto the microcarriers and increasing ECM production [44]

In several studies, microparticles have been used as carriers to encapsulate cells together with their own ECM or just pure CDM [45,46]. Cheng and coworkers [16] were able to encapsulate porcine chondrocytes within rat tail collagen type I microspheres, promoting the creation of chondrogenic ECM and preserving about 40% of the glycosaminoglycans (GAGs) compared to the control group. hMSCs seeded in this construct acquired a chondrogenic phenotype without supplementing media. Similarly, Yuan *et al*. also encapsulated primary nucleus pulposus cells (NPCs) in collagen microspheres to produce a CDM and evaluated its composition by proteomic analysis [46]. Human dermal fibroblasts (hDF) reseeded in NPC-derived matrices acquired a less fibroblastic phenotype and were characterized by an increased expression of collagen type II and the non-chondrogenic NPC marker CA12.

Our group is currently developing different CDM production methods with microparticle-based technology. By changing the polymer type, modifying functionalization with various proteins, or using different cell sources (hDFs or MSCs; **Figure 2**), we aim to obtain CDMs with different compositions, structures (matrix alignment), and mechanical properties. These specific CDMs can be used for different tissue regeneration and disease modeling applications. As shown in **Figure 2**, fibroblast-based CDMs (**Figure 2A-C**) deposit lower amounts of ECM compared to MSC-based CDMs (**Figure 2D-F**), but fibroblast-based CDMs show better cell and ECM alignment than MSC-based CDMs (**Figure 2A-B** and **2D-E**).

2.1.3. Electrospinning

Electrospinning is a useful technology for engineering nanofibrous scaffolds with biomimetic structural properties [47–49] that reproduce ECM structures with the best benefit-cost ratio. This process can be applied as a template for the growth and remodeling of the ECM [50,51]. Interestingly, Zhou *et al*. reported that CDMs promoted peripheral nerve repair when L929 mouse fibroblast cells from adipose tissue were seeded onto ~10 $S \cdot cm^{-1}$ conductive mats

combining electrospun poly(L-lactic acid) (PLLA) fibers and electrochemically deposited polypyrrole (PPy) nanoparticles. When rat PC12 cells were seeded onto the combined PLLA/PPy/ECM decellularized scaffolds (containing laminin, fibronectin, and collagen), their neurites were seen to differentiate and protrude [52]. Furthermore, the cells showed better adhesion, neurite-bearing, and alignment rates on the CDM scaffolds than on the conductive scaffolds without the ECM.

2.1.4. 3D printing

Organ-derived dECM inks and bioinks have emerged as arguably the most biomimetic bioinks, although this technology is still in its early stages. dECM bioinks for bioprinting, together with cells, have been obtained from various animal organs such as the heart, liver, skeletal muscle, adipose tissue, cartilage, vascular tissue, and skin, each containing specific growth and differentiation factors.

Acellular 3D printing is a very useful tool for the printing of 3D scaffolds using an ink based on a dECM or CDM. It can also be used as a template in CDM production. Cells can be cultured directly on the polymer surface, which in turn stimulates the deposition of ECM to form a 3D cell-ECM construct, or they can be mixed with the ink to be printed together. 3D macroporous scaffolds allow efficient colonization by providing a large surface area as well as space for cell migration and colonization. These properties promote the formation of an ECM coating for subsequent decellularization. Using this technique, the ECM secreted by the cells presents several advantages over the ECM collected from mature tissues, such as the possibility to bioprint ECM layers that generate a transitional gradient through all stages in cell differentiation. In addition, once decellularized, the resulting ECM can act as a support for the culture of other different cell populations, enabling the cell phenotype to be modulated. One of the most used progenitor cells for this purpose are MSCs, which are commonly investigated for

use in ECM-coated substrate production due to their high proliferative potential, their ability to differentiate into multiple cell types, and their large production and remodeling of the ECM [53].

Pati *et al*. combined MSC-derived mineralized ECM from human nasal inferior turbinate tissue with 3D-printed hybrid scaffolds composed of PCL, PLGA, and beta-tricalcium phosphate (TCP) [54]. The hMSCs were seeded again onto the scaffolds coated with the ECM to assess their capacity for osteoblast differentiation and bone formation, which significantly increased not only *in vitro* but also *in vivo*, demonstrating that the properties of the ECM can be preserved and mechanically supported with a biocompatible and biodegradable scaffold.

3D bioprinted constructs have been also used for other applications such as tissue regeneration and disease modeling. The use of dECM bioinks has been reviewed elsewhere [55–57]. CDMs can be printed in combination with cells. In an example of efficient bioprinting, Lee *et al*. printed MC3T3-E1 preosteoblast cell-derived ECM [58]. They observed that mechanical resistance and ink viscosity increased with the ECM culture production time, which was associated not only with a more mature ECM, but also with an increased cell number. In that proof-of-concept study, decellularization was omitted and only the aerosol of the alginate cross-linker $CaCl_2$ solution was required for proper printing. Culturing of the resulting scaffolds was successful in terms of osteoblast differentiation, especially for the 3-day-cultured ECM. Longer culture times increased viscosity and mechanical resistance so much that it made printing too difficult. The study demonstrated that a stiff substrate is not required for a properly mineralized ECM. It is also worth noting that this is, as far as we know, the only study applying CDMs as a bioink for bioprinting, indicating that this technique is ambitious, and it is paramount to achieve a collagen-rich CDM that gives rise to a consistent gel for bioprinting. Therefore, further information is needed on the two main factors that ensure ECM hydrogel formation *in vitro*, collagen kinetics and basement membrane assembly. Moreover, the proportions of the other ECM components (such as GAGs, proteoglycans, fibronectin, and minor collagens) can

also influence collagen polymerization. The use of CDMs as bioinks might benefit from blending with different well-known ink materials (i.e., alginate, GelMA, and PEGDMA) and the incorporation of PCL as a supporting material [59].

The many possibilities offered by CDMs as bioinks are yet to be explored. CDMs are an excellent alternative to dECM due to their versatility and the ability to use patient-derived cells as a source of CDMs. However, the CDMs have to be rich in collagen, as the viscosity of bioinks is greatly influenced by the amount of collagen and its crosslinking. Moreover, processing CDMs for bioink production leads to a loss of their original tissue-like architecture and protein structure, which might affect cell behavior.

2.2. **Tailoring the properties of CDMs**

The properties of an ECM resulting from a cell culture can be modulated to some extent by adjusting different factors such as oxygen concentration [60], the mechanoinductive environment of the template substrate [61] or topography [62], biomolecules or macromolecular crowders (MMC), cell type, and the culture method (**Table 2**). These parameters are known to affect cell phenotypic changes [63–65] eventually. Tumor cells are also a source in ECM production and can be used to create cancer models *in vitro*, as will be discussed later in this review in section 4.3.1.

Templates for CDMs may or may not be used in their preparation (see section 2.1). Furthermore, the resulting ECM can be chemically modified by covalent crosslinking [66,67].

2.2.1. Cell type

Different CDMs can be created by using different types of cells, which secrete specific ECMs to meet the biological needs of each type of native tissue [68–71]. Composition and production rates are influenced not only by the cell linage, but also by the age, source, and method of cell extraction. Silva and co-workers [68] seeded different human cell types, such as chondrocytes,

bone marrow stem cells (BMSC) and synovial mesenchymal stem cells (SMSC), to characterize the different decellularized CDMs obtained in terms of the presence of ECM proteins (collagen type I, fibronectin, and laminin), GAG composition, and disaccharide sulfation patterns. All the CDMs had a similar architecture composed of fibrillary networks, but had varying concentrations of proteins. For example, the BMSC-derived ECM and chondrocyte-derived ECM showed a lower expression of laminin compared to collagen type I and fibronectin. Furthermore, GAG composition was affected by the decellularization protocol. Therefore, by providing a precise 3D environment, culture conditions, and cell behavior can be modified to regulate specific proteins. Cell cross-talking and co-cultures cell ratios can influence the ECM composition[72] as well. In a previous study by our group, CDMs developed with hDFs or MSCs seeded on PLA microparticles (**Figure 3**) displayed a different protein expression profile based on the protein used in the functionalization steps. For instance, collagen type I-functionalized PLA microparticles induced fibroblasts to downregulate this protein expression (**Figure 3A**), while MSCs expressed high amounts of collagen type I when fibronectin-functionalized microparticles were used (**Figure 3B**).

Hoshiba *et al.* [73] investigated different CDMs from chondrocytes, fibroblasts, and MSCs to observe how reseeded chondrocytes behaved in terms of adhesion, proliferation, and differentiation. Surprisingly, although adhesion of chondrocytes onto the chondrocyte-derived ECM was the greatest, they showed the lowest proliferation rate. The reason for this seemed to be the higher content of fibronectin in the ECMs derived from the fibroblasts and MSCs. The authors also observed that while relevant contents of collagen type II are present in the cartilage, they produced collagen type I. The difference was attributed to the earlier differentiation stage of the chondrocytes compared to native tissue, and the difference between *in vivo* and *in vitro*, indicating that cultures should progress towards more close-to-real conditions to proper mimic natural tissue composition.

2.2.2. Cell modification

Induced pluripotent stem cells

Since somatic cells can be dedifferentiated or reprogrammed to increase their potential, they are one of the most important and versatile cell sources. The fact that patient-specific induced pluripotent stem cells (iPSCs) can be obtained for further applications in tissue engineering was a turning point in the field [74]. However, their real potential is still unknown. Although they still cannot match their adult equivalents in efficiency, their versatility is greater. There are already studies indicating important differences in the CDMs produced with iPSCs. For example, Shamis and colleagues [75] found that fibroblast-derived iPSCs produced an ECM richer in type III collagen than that produced with normal control fibroblasts. Shtrichman *et al.* used human iPSCs from hair follicle keratinocytes to produce a tailored ECM [51]. The use of these cells allows the application of the resulting CDMs in precision medicine.

Transfection and RNA interference

By modulating the expression of ECM-related genes, cell transfection offers the opportunity to regulate the resulting ECM and, therefore, the behavior of the seeded cells. This has been applied in the reproduction of hepatic tissue as it is currently one of the biggest engineering challenges. Grant *et al*. overexpressed fibronectin in human urinary bladder epithelial 5637 cells by transfecting them with the human fibronectin gene (FN1) through the retroviral expression vector PJ1520 [76]. These cells, seeded on electrospun PLA fibers, were able to successfully produce a CDM layer containing more adhesive fibronectin than that generated by untransfected CDMs. After decellularization, they seeded HepG2 hepatocytes derived from a hepatocarcinoma cell line (the most typical type of primary liver cancer in adults) onto the combined CDM layer/electrospun scaffold. The authors observed that the CDM-PLA scaffolds

from transfected cells altered the expression of albumin, a key marker of liver cell differentiation, as well as of other key hepatic genes.

CDM composition can also be modified by RNA interference (RNAi). An example of RNAi is microRNA (miRNA), which is essential in many processes and cell signaling cascades such as the secretion and remodeling of ECMs. Hence, miRNAs can modulate the amount and structure of ECM [77] components like nephronectin, collagen type I, II, III, fibronectin, versican, and other proteoglycans. They can also regulate other molecules such as cytokines, growth factors, integrins, and non-integrin ECM receptors. Furthermore, miRNAs can modify ECM degradation and remodeling by interacting with matrix metalloproteinases (MMPs) and their inhibitors (TIMPs).

Short hairpin RNA (shRNA) is another RNAi method that overcomes the main limitation of the short lifespan of synthetic small interfering RNA (siRNA). Tumbarello *et al*. used shRNA to regulate transforming growth factor-beta induced (TGF-βI), ECM deposition, and paclitaxel response in ovarian cancer cells, influencing desmoplasia and drug efficacy in ovarian cancer [78]. Another study reported the knockdown of collagen type VI in CDM production with shRNA [79]. Collagen type VI is a non-fibrillary collagen that acts transversely, helping to fix and assemble the ECM in tissues such as the dermis of the skin, consequently affecting dermal cell functions. Theocharidis and collaborators knocked down COL6A1 expression in fibroblasts through lentiviral transduction with three different shRNA constructs and a non-targeting control (NTC). They then produced CDMs from normal primary and knockdown HCA2 fibroblasts. The primary fibroblasts seeded in the CDM produced with the knockdown cells showed greater cell polarization and motility than those seeded in the CDM obtained with control cells. Senthebane and coworkers reported that CDMs depleted in collagen and fibronectin through siRNA increased the sensitivity of tumor cells to chemotherapeutic drugs by around 30-50% compared to control and also reduced cell migration and the formation of

tumor cell colonies [80]. They found that collagen and fibronectin have crucial roles in the survival, migration, and chemotherapeutic resistance of esophageal tumor cells.

Thus, RNAi strategies provide an innovative way of modulating the features of CDMs. At present, we speculate that they could be a novel approach in the production of cell-based constructs.

2.2.3. Macromolecular crowding

The specific properties and high complexity of native tissue microenvironments are difficult to reproduce in the laboratory. The CDMs produced to date do not fully mimic native ECMs and the production rate is also very low. Macromolecular crowding (MMC) has been proposed as a potential strategy to improve the generation of cell-derived microenvironments [81].

MMC is a biophysical phenomenon that increases the density of media such as that in the cytoplasm. Cells act as full compartments that are surrounded by a concentrated medium of macromolecules. These mixtures move and exclude part of the medium that causes steric hindrance due to the inability to cross the cell [82,83]. MMC increases viscosity and, in general, reduces diffusivity. Furthermore, it affects the nature of all the species involved, such as reactivity rates, enzyme kinetics, metabolon stabilization, diffusion of molecules, oligomerization, micro-compartmentalization, protein conformation and aggregation, as well as interactions with the environment affecting pH, ion concentrations, and ionic strength[82,84]. It also influences cell signaling and gene expression by limiting diffusion, with many of these changes in the order of magnitudes [81].

MMC can regulate ECM features in two ways: first, by modifying cell behavior and ECM expression and, second, by modifying the cell media during ECM synthesis. MMC also increases the expression of C-propeptide-promoting proteins, elevates enzyme catalytic

activity, stabilizes substrate-enzyme interactions, and enhances protein aggregation and polymerization in fibroblasts to increase the production and maturation of collagen type I [85], which is a key protein of fibroblast-produced ECMs. Moreover, Dewravin *et al.* suggested that hydrogel structures created in environments with MMC have increased mechanical stiffness [86].

Interestingly, MMC elicits a huge increase in the production rate of CDMs. In addition, it can promote the creation of defined microenvironments that direct cell fate and differentiation [87,88]. These CDMs can resist the process of decellularization and still form a proper microenvironment. The most common compounds used for MMC that have been shown to increase CDM production rates are non-cooperative proteins, such as bovine serum albumin (BSA) [89], artificial polymers, such as PEG, polystyrene, and polyvinylpyrrolidone (PVP), and natural hydrophilic polysaccharides (PS), such as dextran and cellulose derivatives (hydroxypropyl cellulose and ficoll) [82,90]. However, further studies are required to assess the best options for each requirement. It has been suggested that the size of the compound for MMC has a significant effect on the ECM obtained. Sharp *et al*. reported that the smaller the molecular size of the MMC compound, the more efficient the crowding of the medium, which affects cell behavior and the amount of ECM deposited [91]

Ficoll not only increases the production of collagen type I, but also significantly improves the assembling of fibronectin into the collagen backbone of the ECM as well as the rate of fibrillogenesis in the collagen type I assembly process [92]. Macromolecular polydispersity and negatively charged polysaccharides modulate ECMs [81] by enhancing collagen type I deposition. Zeugolis *et al.* suggested the use of mixtures of MMC compounds with different molecular weights and concentrations [93]. In a previous study by our group, MMC significantly increased protein deposition in 2D and 3D cultures as well as overall CDM stiffness (fiber crosslinking; unpublished results) compared to non-MMC-supplemented cultures.

2.2.4. Other variables

Additional parameters affecting CDM composition, architecture, and production rate have also been studied. The different components of the culture media [94–96] can influence fiber orientation, elastic properties, and ECM composition. For instance, ascorbic acid has been reported to increase the deposition of collagen type I from human pulmonary fibroblasts [97], while serum proteins have been observed to be adsorbed onto the expressed ECM, masking other proteins like fibronectin, vitronectin, and BSA. Differences between culture media can also modify the resulting ECMs. Jeon *et al*. obtained different results depending on the culture media (normal or osteogenic) used to create primary rat osteoblast-derived ECMs [98]. After re-seeding the same osteoblasts in the different decellularized ECMs, the ECM secreted in the osteogenic medium stimulated a more osteogenic differentiation than that produced in the expansion medium.

Regarding the effects of cell crosstalk on the properties of secreted ECMs [99], the addition of an exogenous ECM such as Matrigel® also affects ECM production [100] due to the growth factors in Matrigel®. Wang *et al.* modified a HUVEC-derived ECM with a peptide (QK) mimicking vascular endothelial growth factor (VEGF) [101]. The peptide simulated the helical VEGF structures at the binding site of VEGF and showed a high affinity for the VEGF receptor (VEGFR). Wang *et al.* observed that the angiogenic response of the covalently linked peptides was better compared to Matrigel® samples and the other conditions, and did not affect cell adhesion and proliferation. A similar process was developed by Ruff *et al*. [66]. By engineering metabolic oligosaccharides, they incorporated an azide-functionalized sugar analog into the extracellular glycans of fibroblast cell cultures, allowing reactions to occur with bioactive cues through biorthogonal click reactions with alkyne-modified molecules or surfaces. They also created an option to covalently incorporate signaling cytokines, growth factors, or antibiotics into the ECM by click-chemistry for future studies.

3. Characterization of CDMs

3.1. CDM morphology

In addition to the mechanical and biochemical characteristics of CDMs, the morphological properties of the cells as well as of the cell-deposited proteins have a large impact on mimicking native tissues and on cell behavior. Different tissues, such as muscle, tendon, cardiac, and vascular tissues [102,103], display highly aligned collagen fibers in their ECMs, which help to align cells along these fibers and also regulate processes like proliferation, migration [104], and force distribution of impulse transmission along the tissue. However, some tissues like skin tissue present a randomly oriented ECM structure. There are several techniques to determine and quantify fiber alignment/misalignment, the most widely used being scanning electron microscopy (SEM) [105,106]. However, sample processing can alter CDM structure, especially during drying when water is eliminated and in immunofluorescence staining techniques [62,107]. These techniques together with software tools such as ImageJ (FibrilTool, Orientation J), MATLAB (CytoSpectre, CurveAlign), and CT-FIRE [108] allow the quantification of protein fiber orientation and provide angle distributions to determine alignment or misalignment in CDMs.

3.2. Mechanical characterization

It is becoming widely known that the passive mechanical properties of a biomaterial or scaffold are critical in cell signaling and can influence subsequent cell behavior and the remodeling of the biomaterial or ECM.

Biological tissues are intrinsically viscoelastic and exhibit stress relaxation [109,110]. Therefore, the following two properties are particularly interesting: (1) stiffness or flexibility, which are

sensed by cells and can modify their phenotype (mechanotransduction); and (2) viscoelasticity, which indicates the capacity of the seeded cells to remodel the supporting CDM. In other words, the same cell can mold the CDM while creating a new ECM and degrading the previous one. Stiffness directly depends on the modulus of elasticity or Young's modulus (E) and quantifies the resistance to deformation. The values of Young's modulus for different human tissues range from 0.1 kPa to 1 GPa [111]. Generally, these values remain more or less constant in different individuals under normal and healthy physiological conditions, with few changes occurring with age, thus indicating a mechanical homeostatic balance [112].

The mechanical characterization of CDMs can be approached from two perspectives:

1. Mechanical properties at the macroscale, which show the properties of the whole structure and indicate how manageable the material will be to avoid collapse. It is related to the chemical composition and structure of the ECM backbone.
2. Mechanical properties at the micro- and nanoscale, which are more local and related to what cell integrins and other receptors sense. Cells sense, transduce, and respond locally to external mechanical cues [112].

Both are complementary and there is no specific method to measure the mechanical properties of CDMs. Instead, a combination of methods is used to compare these tissue-like structures [113].

Tensile strength and compression tests are very useful for a rapid macroscopic evaluation of the E modulus using a low range mechanical cell (~5 N) and a uniaxial tension universal testing machine. Compression tests require lower amounts of sample and the controlling of environmental conditions is less demanding, with tests performed in solution at 37ºC. However, the elastic and plastic areas are difficult to separate [114], which is even more difficult when sample porosity is not controlled or known [115]. Tensile strength tests require larger amounts

of sample and a slightly complicated set-up, but it is easier to obtain the E modulus with less uncertainty. Thus, it is recommended to perform both tests together, with stress relaxation measurements to understand the fundamental cell–ECM interactions and mechanotransduction [110]. It should be noted that the moduli obtained with the tensile strength and compression tests will probably differ.

Dynamic mechanical analysis (DMA) is also recommended. It is similar to a compact version of a mechanical testing machine in which sinusoidal stress is applied at a controlled frequency and temperature (best way to obtain the glass transition temperature, T_g). However, measures in liquid media are complicated [115], and specialized set-ups and expensive holders are required. This method assesses the storage modulus (E'), which gives a direct measurement of elasticity, and the loss modulus (E"), which measures the energy dissipated as heat and is associated with the viscous response of the biomaterial. Both parameters are used to determine the elasticity, viscoelasticity, and viscous characteristics of CMDs.

Rheology is probably the quickest method to mechanically characterize fluid CDMs as it is sensitive and requires a low amount of sample. Furthermore, it distinguishes between the different levels of stiffness of the gel, and also measures glass transition temperatures, molecular weight, and sample homogeneity [116]. Small-amplitude oscillatory shear (SAOS) is the most widely used rheological technique that involves a rotational rheometer with temperature control. This is useful in evaluating viscosity, viscoelasticity, stiffness, and the sol-gel transition in both Newtonian (viscosity is only dependent on temperature) and non-Newtonian (viscosity depends on the shear strain rate as well) gels, and is especially useful in 3D printing and bioprinting [57], ink crosslinking, and depositions. The main parameters that are evaluated in the linear elastic, viscoelastic, and viscous regimes are the shear storage modulus (G') and the shear loss modulus (G").

Perhaps the method that has revolutionized the small-scale analysis of mechanical features is the atomic force microscopy (AFM) nanoindentation technique [117–119]. Its efficiency in obtaining information, versatility in adapting to different conditions and measurement types, and apparent simplicity in terms of sample preparation makes this technique highly reliable with a lot of potential, especially when the amount of material is low, and it is soft and/or fragile. AFM works as a real nanoindenter, acting in the elastic zone if possible. A complex map of several square microns of the E modulus can be obtained on flat surfaces. However, tissues and CDMs are usually quite rough, which makes the measurements complicated. Therefore, research groups have developed several coating and adhesion techniques to flatten substrates [120], using sophisticated *ad-hoc* platforms to immobilize samples, for example, with the aid of a mesh [121]. The AFM tip material and geometry are other variables to consider in AFM nanoindentation. Stiff or soft tip materials are available, while the geometry can be pyramidal, cylindrical, or spherical and large or small. Large geometries allow the averaging of rough surfaces, but this affects resolution.

3.3. Biochemical characterization

Alongside the characterization of mechanical properties, analysis of the biochemical properties of CDMs is very important. CDM composition is critical in developing tissue and disease models, as the properties of CDMs must match those of native tissues to mimic *in vivo* conditions as closely as possible. Therefore, we have reviewed some of the techniques used to provide more information about the qualitative and quantitative composition of CDMs (**Table 3**).

After decellularization, the removal of cellular components is assessed to confirm that this process has worked. Generally, fluorescence probes such as DAPI (4′,6-diamidino-2-phenylindole) [122] and Hoechst 33258 [123] are used to determine the presence of cell nuclei.

DNA remnants are usually quantified using an ultrasensitive fluorescent nucleic acid stain for quantitating double-stranded DNA (dsDNA) [124]. Other cell components such as F-actin and cell membranes are detected using Alexa Fluor 488-phalloidin or DiI (1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate) stains, respectively [14].

Immunofluorescence, immunocytochemistry, and immunohistochemistry are widely used to detect and localize the presence of specific ECM proteins and to characterize CDMs. Collagen types I, II, III, IV and X, fibronectin, biglycan, decorin, versican, and laminin have been reported to be present in CDMs after decellularization [46,125–129].

Other techniques can also be used to examine specific protein contents. For example, Alcian blue staining visualizes proteoglycan deposition [130], while Picrosirius red staining analyzes the presence and spatial distribution of collagen fibers, specifically collagen types I and III [131]. The amount of these extracellular components can be quantified by a variety of biochemical assays. GAGs are measured using a dye that binds to these proteins (Blyscan) [130], while elastin and collagen contents are quantified with colorimetric assays [132]. Other ECM proteins such as collagen type I and fibronectin have been quantified by in situ enzyme-linked immunosorbent assays (ELISA) [133].

SDS-PAGE and either Coomassie, silver, or Ponceau staining have been widely used to identify the total protein profile of CDMs [134]. As well as detecting the presence of a particular protein, Western blots can also quantitatively measure the concentration of these extracellular proteins [135]. However, CDMs sometimes show highly complex compositions, protein crosslinking, and overall mechanical properties. As a result, digesting these matrices can involve an intricate procedure that breaks down fibrillar ECM proteins without degrading samples and uses appropriate buffers that do not interfere with protein quantification and SDS-PAGE/Western blot procedures.

Mass spectrometry can also be used to determine the qualitative composition of CDMs. Again, due to the complex properties of CDMs, it is important to develop efficient digestion protocols using enzymes (trypsin, pepsin, and collagenases) and buffers (RIPA buffer, urea, sodium dodecyl sulfate, and dithiothreitol) that effectively break down protein chains without degrading their structure [136,137]. Different studies have analyzed CDM proteins using nano-liquid chromatography-tandem mass spectrometry (nLC-MS/MS) after efficient decellularization and scaffold template removal [46,135,138].

The diverse characterization methods reviewed here highlight the heterogeneous nature of CDMs. This heterogeneity, marked by the enormous range of possibilities in CDM production, emphasizes the essential requirement of designing CDMs based on the tissue or structure being mimicked. Thus, CDM characterization must be carefully adapted to study every feature of CDMs that resemble those of native human tissues and organs.

4. CDMs as 3D models

4.1. Models to study cell processes

An increasing number of studies are using CDMs to study basic processes such as cell migration and proliferation, as 3D microenvironments can mimic native tissues [139] and offer a realistic picture of what happens in the body. The potential to produce CDMs through different approaches and culture conditions makes this approach even more promising. CDM characterization is crucial in understanding the variety of matrices produced by different cells and culture methods, as the specific composition, architecture, and mechanical properties of CDMs affect cell behavior [140,141]. Therefore, the production and characterization of CDMs introduce a new set of possibilities to study gene expression profiles, tissue morphogenesis, and the forces or tractions between cells and the microenvironment [142,143].

CDMs will allow researchers to study how their three-dimensionality and structural properties, combined with their biochemical characteristics, affect basic processes such as cell polarity, morphology, migration, proliferation, and differentiation. This will be important in changing some of the paradigms established by studies using two-dimensional cell cultures.

4.1.1. Cell adhesion

One of the most widely reported differences between 2D and 3D models involves the effects of cell polarity and morphology on cell adhesion [15,144]. In 2D cultures, fibroblasts, for example, are artificially polarized, showing morphological differences between the dorsal and basal parts of the cell [145]. Additionally, 2D cultures or hydrogels with low compliance induce a loss or change in cell polarity, which can correlate with changes in other functions such as cell morphology, migration, tissue morphogenesis, and the sensing of growth factors and signaling cues [146–149]. On the other hand, 3D cultures in CDMs and dECMs preserve cell polarity and morphology by providing structured matrices that mimic native tissues (in architecture, distribution, and composition), [150] which in turn increase cell adhesion through multiple focal adhesions [151], as has been observed *in vivo* [152,153]. Focal, fibrillar, and nascent adhesions involve integrins, vinculin, paxillin, focal adhesion kinase (FAK), and tensins. In 2D cultures, stiff glass or plastic substrates increase cell adhesion strength and, consequently, their protein composition [154–156]. By contrast, 3D environments are much softer, with focal and fibrillar adhesions not requiring as many mechanosensitive proteins and thus being much more sensitive to the physical microenvironment [156]. Moreover, cell-matrix adhesions have been shown to be more effectively mediated in CDMs or tissue-derived matrices compared to 2D or hydrogel cultures due to the matrix composition, fibrillar protein concentration, and an increased ability of cells to exert contractile forces on the matrix [151,157,158].

4.1.2. Cell migration

Cell migration is strongly influenced by the substrate on which cells grow. The factors involved in cell migration are directly connected to tissue morphogenesis, homeostasis, remodeling, and repair [159,160]. CDMs can mimic the biochemical composition, three-dimensionality, and mechanical properties of *in vivo* tissues, thereby allowing the study of cell migration in 3D cultures. ECM mechanical properties such as viscoelasticity, stiffness, and elasticity are important factors affecting migration [2], which depends on the elastic tensile behavior of the matrix [161,162]. Another important parameter affecting cell migration is the fibrillary composition of native tissues. Collagen and fibronectin fibers generate an interconnected porous network that cells use to migrate along, a process that is limited by multiple cell adhesions in all directions, matrix topography, and the availability of matrix ligands for cells to adhere to [2,149]. CDMs are a suitable model for studying cancer cell invasion [150] and cell responses to external stimuli such as tissue defects or ECM structure [163]. CDMs can also potentially drive the directional migration of morphologically elongated cells through spatially oriented fibers produced by fibroblasts [2,159]. In cell migration studies using 2D cultures, the 2D substrates are often coated with fibronectin and present significant differences in composition, physical characteristics, and mechanical properties compared to 3D models. The same issue occurs when using hydrogels, with their non-complex composition and low fiber content hindering cell adhesion and migration. These differences between 2D cultures, hydrogels, and 3D CDMs affect cell phenotype, adhesion, and migration. For instance, a lack of matrix alignment results in the loss of migration directionality provided by the fibers in human tissues, while substrate stiffness can affect migration speed [157,164].

4.1.3. Cell viability and proliferation

Cell viability and proliferation have been studied for many years on synthetic 2D substrates. Recent scientific advances have allowed researchers and clinicians to study the same processes in structures that are more similar to *in vivo* tissues [149,165,166]. Due to the limitations of natural and synthetic hydrogels [157], CDMs and decellularized tissues appear to be the best models to study cell proliferation given their ability to mimic native tissues. In 2D culture plates, differences in cell proliferation have been observed in different cell types between plastic plates coated with ECM proteins and those that are not coated [150,151]. Lai *et al*. demonstrated that MSCs cultured in their own ECM showed increased proliferation and maintained their undifferentiated state compared to those cultured on 2D plastic substrates [167]. Yamada and Cukierman suggested that the ventral and dorsal cell contacts with the ECM restored a normal 3D cell morphology [148]. This resulted in an increase in focal adhesions containing integrins, vitronectin, fibronectin, and tenascin C, which subsequently promoted migration and proliferation [168,169].

4.1.4. Cell differentiation

Cells undergo differentiation in the presence of certain stimuli. This process involves major changes in cell shape, size, and metabolic activity. The mechanisms underlying cell differentiation have been a topic of interest for a long time, but many questions remain unanswered [170]. Similar to the other cell processes, the 3D architecture, environmental signaling cues, complex matrix composition, [167] and mechanical properties are key aspects in cell differentiation. The potential of CDMs and decellularized tissues in studying cell differentiation depends on their reliability, versatility, and similarity to native tissues.

Considering their regenerative potential, MSCs have been used to produce CDMs to keep cells undifferentiated and preferably promote their proliferation once implanted. Following

implantation, differentiation is induced [171,172]. CDMs can also be used to induce the differentiation of MSCs into certain cell types, with the composition and mechanical properties of the CDMs playing key roles in this differentiation, as described previously. By targeting these properties, MSCs can be differentiated into cells from a different embryonic layer. For example, Aizman *et al*. differentiated MSCs (originated in the mesoderm) into neurons (from the ectoderm layer) in well plates coated in an MSC-derived (SB623) CDM, highlighting the importance of substrate composition [151,173]. MSCs can also be regulated and driven towards differentiation by substrate three-dimensionality, composition, signaling molecules, or by co-culturing with other cell types [21,148].

Stiffness is crucial for differentiation. In native tissues, cell differentiation correlates with increasing tissue stiffness [169], although this differs significantly among cell types. In addition, the combination of matrix stiffness and signaling cues can induce mesenchymal cell differentiation into different cell types [29]. It has been postulated that in viscoelastic materials such as biological tissues, cells show a larger spreading area in ECMs with low crosslinking, even stopping the start of differentiation to avoid undesired pathologies. Interestingly, soft CDMs have been reported to promote adipogenesis, while stiff matrices have been linked to chondrogenesis/osteogenesis [30].

In addition to the already mentioned differences in cell processes, heterogeneous cell properties amongst different cell types can have a strong impact on culture methodologies. Depending on the cell type, CDM requirements can vary significantly. Therefore, inadequate CDM properties can greatly affect cell behavior, leading to unexpected results. For instance, several studies have stated that fibroblasts need some mechanosensitive requirements to correctly develop their normal functions [174,175]. In matrices with low mechanical loads, fibroblasts can adopt a resting phenotype, resulting in cell quiescence and, eventually, apoptosis. By contrast, in matrices with high mechanical loads, fibroblasts are activated, and show increased focal adhesions and cell

proliferation [159]. Liver tissue is also strongly affected by changes in stiffness in diseases like fibrosis and cirrhosis. In these pathologies, tissue stiffening induces the differentiation of quiescent hepatic stellate cells into myofibroblast-like cells [176]. Some cells such as neutrophils and myocytes present no preferences for substrate stiffness [169]. Meanwhile, tissue stiffening has been linked to cancer and malignancy (**Figure 4**), while stiffer substrates in combination with specific growth factors or signaling cues have been reported to induce fibroblast activation or differentiation into myofibroblasts [159,177].

ECM three-dimensionality as well as its complex and specific architecture provide cells with biochemical and mechanical signaling cues, which greatly affect cell behavior and further increase culture complexity. The properties of the ECMs mimicking those of *in vivo* tissues are crucial for cells to maintain their phenotypes and behave as they do in their native tissues [15]. To study these environment-dependent behavioral differences, CDMs are promising platforms that effectively reproduce *in vivo* tissue conditions *in vitro,* providing powerful tools to understand cell behavior. Although CDMs developmental stage, they also represent an excellent way to evaluate the regenerative and healing capacity of therapies or drugs by providing the correct physical and chemical signals to guide cell fate.

4.2. Tissue models for regenerative medicine and implants

When studying cell processes in 3D environments mimicking *in vivo* tissues, it is crucial to understand the changes that cells undergo in tissue malfunction and regeneration. Thus, CDMs are potentially useful models to study and identify these changes. In this section, we will discuss the CDMs used in regenerative medicine approaches and those applied in disease progression studies.

The application of CDMs as biological scaffolds in tissue regeneration is still in its infancy. Nevertheless, there is increasing interest in the production of customizable ECM components

to overcome the shortage of donor-derived ECM bioscaffolds and the issues regarding animal-sourced biomaterials.

The use of CDMs as real implants has not been implemented yet due to the novelty of the approach and the poor mechanical properties of CDMs. Moreover, since the production of CDMs is still at the laboratory level, scalability and mass production are still lacking to respond to the need for tissue implants. A thorough automated process will help to increase CDM production. Furthermore, methods characterizing the matrices, as described in section 3, need further advances to fully and reproducibly assess the composition and mechanical properties without affecting the native structure of CDMs. There have been several attempts to use CDMs as tissue implants, which are detailed below.

4.2.1. Skeletal tissue

Skeletal CDMs are one of the most studied matrices, with cartilage CDMs being widely investigated [178]. Several studies have assessed the effects of the matrices produced by synovium-derived stem cells (SDSCs), BMSCs, NPCs, infrapatellar fat pad-derived stem cells (IPFSCs), and chondrocytes in cartilage regeneration. Matrices produced by less differentiated cells can reprogram cartilage cells for use in cartilage regeneration treatments. This was proven after injecting these cells into pig knees with cartilage defects, which led to the CDMs enhancing cell proliferation and the chondrogenic potential of SDSCs. Furthermore, collagen type II and sulfated GAG expression was reported to be increased following implantation to resolve partial-thickness cartilage defects.

Jin *et al.* implanted CDM scaffolds *in vivo* with chondrocytes. After 3 weeks, all the implanted CDMs resembled normal cartilage [179]. Porcine chondrocytes were used to produce matrices by freeze-drying supporting rabbit chondrocytes, which maintained their characteristic phenotype and produced cartilage ECMs *in vivo*. The authors highlighted that CDMs

maintained their volume during cultivation, ensuring a perfect fit to resolve chondrogenic defects.

Li *et al*. fabricated cartilage CDM membranes produced by porcine chondrocytes. After retrieval and decellularization, the membranes were used to form a multilayer structure with a thickness of 30–60 μm. The implantation of these membranes alongside bone marrow stimulating therapy to address an articular cartilage defect improved cartilage regeneration in a canine model. The authors linked the improvement to the actions of the membranes in preserving blood clots and maintaining the MSCs therein. Cartilage tissue formation was faster in decellularized CDM membranes with an increased hyaline-like characteristic [180].

In summary, CDMs have been demonstrated to be more effective in cartilage regeneration than tissue dECMs and synthetic scaffolds coated with ECM (reviewed by Sun *et al*. [181]).

Several approaches have been developed for using CDMs in bone regeneration. For instance, Datta *et al*. produced CDMs using rat MSCs and seeded them onto titanium fiber mesh scaffolds in a flow perfusion bioreactor. Previous *in vitro* results had demonstrated osteogenic differentiation of the MSCs [182]. However, decellularized constructs implanted intramuscularly in a rat model did not induce bone formation [183]. Although mineralized deposits were observed in the ECM produced *in vitro*, this was not enough to stimulate endogenous cell recruitment to elicit osteogenesis *in vivo*. Thus, the CDM had no osteoinductive properties, but it improved implant vascularity.

Lu *et al*. fabricated a knitted PLGA mesh and used it as a sacrificial template for autologous ECM scaffolds [184]. Human BMSCs, normal human articular chondrocytes (NHAC), and normal human dermal fibroblasts (NHDF) were used to prepare their respective CDM scaffolds, demonstrating high specificity of the produced ECMs depending on the cell type used. Mice

fibroblast-CDM scaffolds were implanted in mice, which demonstrated excellent biocompatibility and minimized undesirable host tissue responses.

A methodology was recently developed to overcome several issues related to the density and lack of porosity of CDMs that inhibit cell infiltration. Zhu *et al*. developed a matrix by subcutaneous implantation of sacrificial templates [185]. PCL microfibers were used to produce microchannels in CDMs *in vivo*. After sacrificial scaffold removal, samples were decellularized and the fibers removed. The CDMs produced were then implanted into a rat tibialis anterior muscle with volumetric muscle loss, sciatic nerve, and abdominal artery with a critical size defect. The implantation resulted in a regenerated vasculature and innervated neomuscle tissue, vascularized neo-nerve, and pulsatile neo-artery with functional integration. Thus, the CDMs elicited host endogenous recruitment, vascularization, and tissue innervation, overcoming some of the bottlenecks in current tissue engineering strategies.

4.2.2. Cardiovascular tissue

One of the most popular applications of CDMs is in cardiovascular tissues due to the limited availability of cadaveric allogenic tissues and issues related to cross-contamination when using xenogeneic tissues. Prof. Okano, a pioneer in using cell sheets (described in section 2.1.1) and their deposited ECM, used cell sheets in a rat myocardial infarction model, which significantly improved cardiac function compared to cell injections, thus demonstrating the benefits of ECMs produced by cells *in vitro* [186]. Several clinical trials are currently underway. Similar uses of cell sheets have been tested in the liver, pancreas, and cornea [43].

Other strategies have been developed for engineering blood vessels. Wystrychowski *et al*. produced a fibroblast-derived vessel using three allogeneic lifeline grafts from Cytograft Tissue Engineering, Inc. (CA, USA) [187]. The graft produced was stored at -80°C for 9 months and devitalized, without endothelial cells or any other cultured cells after thawing. Only the

fibroblasts that produced the graft were in the construct, but they did not survive the freezing process. This was the first clinical use of an induced CDM for blood vessel regeneration.

Another study tested CDMs grown on a polymer scaffold for small vessel regeneration in 60 patients with end-stage renal disease. Polyglycolic acid (PGA) polymer scaffolds were seeded with smooth muscle cells from donors, which were cultured in a perfusion bioreactor for 8 weeks before decellularization. Patients were followed up for 16 months. The CDM hybrid scaffolds showed long-lasting performance and provided safe and functional hemodialysis access [188]. In another study, a cell-derived vascular graft was produced by seeding fibroblasts from a donor in a fibrin gel with a tube shape[189]. After decellularization, the construct was grafted as a pulmonary artery in a growing young lamb. Normal tissue growth was observed, and the graft maintained physiological strength and stiffness, showing excellent lumen endothelization and extensive colonization by mature smooth muscle cells. Furthermore, proper elastin and collagen deposition was maintained and there were no signs of undesired mineralization, deformation, or blockage.

CDMs have also been applied to produce human tissue-engineered sinus cardiac valves (hTESVs) [190]. Clinically relevant off-the-shelf valves were produced using human neonatal dermal fibroblasts (hDF), with different concentrations of transforming growth factor-β1 (TGF-β1) used to induce matrix production. After decellularization, *in vivo* evaluation was performed in a translational sheep model. Although only three animals were implanted and follow up was limited to 4 h, the results indicated that this was a safe and promising approach for producing heart valves.

4.2.3. Skin wound healing

Lung human fibroblasts have been used to produce CDMs. This type of decellularized CDM was combined with collagen, several angiogenic growth factors (VEGF, bFGF, and SDF-1),

and HUVECs for use in an *in vivo* skin wound healing model, which resulted in more effective re-epithelialization, hair follicle regeneration, higher viability of the transplanted cells, and improved neovascularization [191]. A similar study combining an antibiotic and a skin patch derived from decellularized human lung fibroblast-derived matrix (hFDM) demonstrated reduced bacterial infections and increased wound healing in mice [192].

Adipose ECM and the injection of fibroblasts differentiated from adipose-derived stem cells (ADSCs) have been demonstrated to be more effective in wound healing than just ADSCs alone [193]. The combination of polymeric scaffolds with human CDMs has also been applied. An electrospun fibrous membrane based on poly(L-lactide-co-caprolactone) (PLCL) and a decellularized hFDM delivering HUVECs in a mouse wound healing model led to faster wound closure, increased neovascularization, a nearly normal thickness of the epidermis, and hair follicle regeneration [194].

Although dECMs have been used in some tissues like hepatocytes and nerve cells, CDMs have not yet been tested due to difficulties associated with low cell expansion. Approaches are currently underway to apply matrices derived from MSCs or fibroblasts to nervous, hepatic, and renal tissues.

In conclusion, despite promising findings in musculoskeletal innervation, vascularization, and cartilage regeneration, there are still significant limitations regarding CDM use. The poor mechanical properties of CDMs hamper their use as skeletal implants or replacements, which are required to have high resistance to loads, and fatigue. Combining CDMs with scaffolds could overcome this issue. CDMs show great potential in cardiac, vascular, and skin tissue regeneration as well as in wound healing. Nonetheless, we believe that research must focus on the production of CDMs rich in biological cues that can promote cell recruitment and proliferation in damaged areas over a sustained period. Cell recruitment is crucial in

regenerating internal tissues and organs. Therefore, the successful application of CDMs in this field could dramatically decrease the need for transplantation. Another limitation to overcome is CDM scalability as large amounts of CDM sheets are needed in applications such as those used in severely burnt patients.

4.3. CDMs as disease models for advanced personalized therapies

CDMs are also being used to study the mechanisms underlying diseases to identify potential therapeutic targets as well as screen different therapies to develop personalized treatments [195]. Their ability to mimic *in vivo* tissues makes CDMs excellent candidates to study how diseases arise, develop, and progress in patients. These *in vitro* experiments will allow the study of complex cell behaviors as well as the heterogeneity and changing nature of some diseases. Ultimately, CDMs as disease models could be used as drug screening platforms either to test new drugs or to screen for new treatments for personalized medicine.

4.3.1. Cancer

Damianova *et al.* studied the effect of CDM three-dimensionality on ERK1/2 activation via Src/Ras/Raf pathways. ERK1/2 regulates cell processes such as proliferation, differentiation, and death and is present in about a third of human cancers. The sustained activation of ERK1/2 through Src in CDMs is similar to that observed in *in vivo* tissues, demonstrating differences in signal transduction between 2D and 3D cultures [196]. Senthebane *et al.* used CDMs to assess the role of ECM proteins in cancer cell responses to chemotherapy. They cultured esophageal cancer cells in fibroblastic CDMs and found that activated MEK-ERK and PI3K-Raf signaling in CDMs affected cell apoptosis. This indicated that the ECM protected against anti-cancer substances through its complex 3D architecture and the activation/downregulation of signaling pathways. By contrast, the poor structural complexity of 2D cultures allows drugs to successfully kill cancer cells. Consequently, new treatments targeting ECM proteins have been

proposed for chemoresistant tumors [80,197]. Gulvady *et al.* studied the role of the focal adhesion protein Hic-5 in cancer cell morphology, migration, and plasticity in CDMs. They highlighted the important role of this protein in regulating cancer cell phenotype, migration, and invasion in 3D. By contrast, Hic-5 expression did not correlate with cell morphology and migration in 2D [198]. Franco-Barraza *et al.* developed a protocol to prepare CDMs from cancer-associated fibroblasts (CAFs) and proposed that they could be used to study whether they maintained an active phenotype in any fibroblastic cell compared to CDMs from normal fibroblasts [199]. Caley *et al.* developed osteoblastic and fibroblastic CDMs to study cell plasticity and migration in prostate cancer [200]. They demonstrated that the tumor-associated collagen receptor Endo180 and collagen crosslinking by lysyl oxidase (LOX) were crucial for cancer cell invasion and proposed that these proteins could be targets for metastasis-limiting treatments. Hoshiba and Tanaka studied the importance of generating CDMs from appropriate tissues to obtain specific and individualized cancer models that effectively mimicked *in vivo* events such as tumor chemoresistance for use in the development of more reliable chemoresistance assays and new treatments [201]. Meanwhile, Cai *et al.* compared the different ECMs obtained from hBM-MSCs, fibroblasts, osteoblasts, and MG-63 osteosarcoma cells [202]. The adhesion, spreading, and proliferation of MSCs and MG-63 cells seeded in the different matrices were evaluated. As expected, the composition of the ECM varied with the cell source, showing different effects on cell morphogenesis, attachment, and proliferation. Interestingly, both MSCs and MG-63 cells showed lower adhesion and spreading on osteosarcoma-derived ECMs, while MG-63 cell proliferation was inhibited in all the CDMs produced by non-cancerous cells. In another study, the breast cancer cell lines MCF-7, MDA-MB-231 (metastatic cell line), and MCF10A (non-tumorigenic cell line) were chosen to create different CDMs and study the different steps of *in vitro* breast tumorigenesis and angiogenesis [203]. The CDMs had to be assisted with fibroblast co-cultures to produce enough deposited ECM components for the studies. The capillary structures grown in the ECM derived from metastatic MDA-MB-231 cells had an intricate

organization and showed the highest occupancy of the assessed area compared to those cultivated in the other ECMs.

4.3.2. Hepatic disease

CDMs have been generated to mimic liver structures for the study of liver metabolism and disease. Guo *et al.* studied endothelial CDMs as promoters of hepatocyte-like cells obtained from adipose-derived stem cells (hASC-HLCs) by investigating the interactions between ECM signals and different transcription and nuclear factors. They used this as a model to study interactions between hepatocytes and non-parenchymal CDMs, as well as for hepatotoxicity testing and for diseases such as liver fibrosis [204]. Hoshiba reviewed the potential of CDMs to maintain hepatocyte-specific functions and the importance of adjusting the properties of CDMs according to the needs of tissues [95]. These matrices are expected to be used in 3D hepatocyte cultures to assess their phenotype in health and disease.

4.3.3. Neurodegenerative disease

CDMs could effectively regulate neural cell adhesion and proliferation to treat neurodegenerative diseases. By providing nutrients and growth factors, CDMs could promote a complex system of neurons, astrocytes, and oligodendrocytes that can be implanted into brain lesions or be applied in regenerative medicine or used as disease models [173]. One study compared the mechanical properties of glaucomatous ECMs to those of non-glaucomatous ECMs, studying the bidirectional interaction between trabecular meshwork cells and their microenvironment [205].

4.3.4. Cardiovascular disease

In cardiovascular disease, CDMs have been used to expand and differentiate c-kit cells for the regeneration of infarcted or injured areas of the heart. CDMs from MSCs upregulate cardiac

gene expression in c-kit cells, promoting their survival and functions after *in vivo* transplantation [39].

Research in different diseases has evolved from using 2D substrates to 3D environments to identify the mechanisms underlying diseases and their progression. We believe that CDMs are excellent platforms to model the different aspects of a disease and identify possible treatment targets, given that CDMs can mimic most of the properties of native tissues and provide cells with a complex environment that is as close as possible to *in vivo* conditions. We hypothesize that CDMs will be developed for a wide range of tissues to study diseases such as cancer, neurodegenerative disorders, and cardiovascular diseases. This could also be used for research in personalized medicine, with each patient's disease studied in detail to provide more specific and less toxic treatment to improve prognosis [40].

5. Future perspectives

Regularly used 2D cultures are becoming obsolete as they fail to reproduce the complexity of tissue environments. A reliable ECM that mimics native tissues or emulates the pathophysiological state is needed. CDMs have already demonstrated that this approach is feasible and reproducible. The development of reliable CDMs will advance the field of regenerative medicine and research on diseases, shortening the gap between *in vitro* and *in vivo* experiments and improving the translation of treatments from animal models to clinical trials in humans.

CDMs are still in the developmental stage and present some significant limitations. There is a need to improve the composition and mechanical features of CDMs. Therefore, new fabrication strategies must be developed to produce CDMs with specific and modifiable properties as well as with non-aggressive processes that will preserve their composition and structure and allow remodeling by cells. Genetically modified cells and MMC can help accomplish this objective.

Biomaterials as templates in combination with CDMs or with specific characteristics (conductive/optical/magnetic materials) could also be used to overcome the limitations associated with current CDMs. On a higher level, a combination of different CDMs to create multi-organ or multi-tissue platforms for tissue repair or modeling will be essential.

Finally, to translate CDMs from the bench to clinical use, industrial scalability in terms of standardization, costs, time, sterilization, and storage is crucial. By addressing these current limitations, CDMs have the potential to become very useful for tissue regeneration and disease modeling. The ability to generate CDMs from different types of cells and tissues supports their use as a gold standard 3D platform for personalized *in vitro* drug screening. Further advances in state-of-the-art approaches in CDM technology will be important in understanding complex physiological and pathological processes, which will improve diagnostics and personalized therapy. CDMs could also be applied in other fields such as pharmaceuticals, patient-specific drug screening, biomolecule production, and *in vitro* food production. For example, cell-based meat production could have a huge impact on global society, with significant environmental implications.

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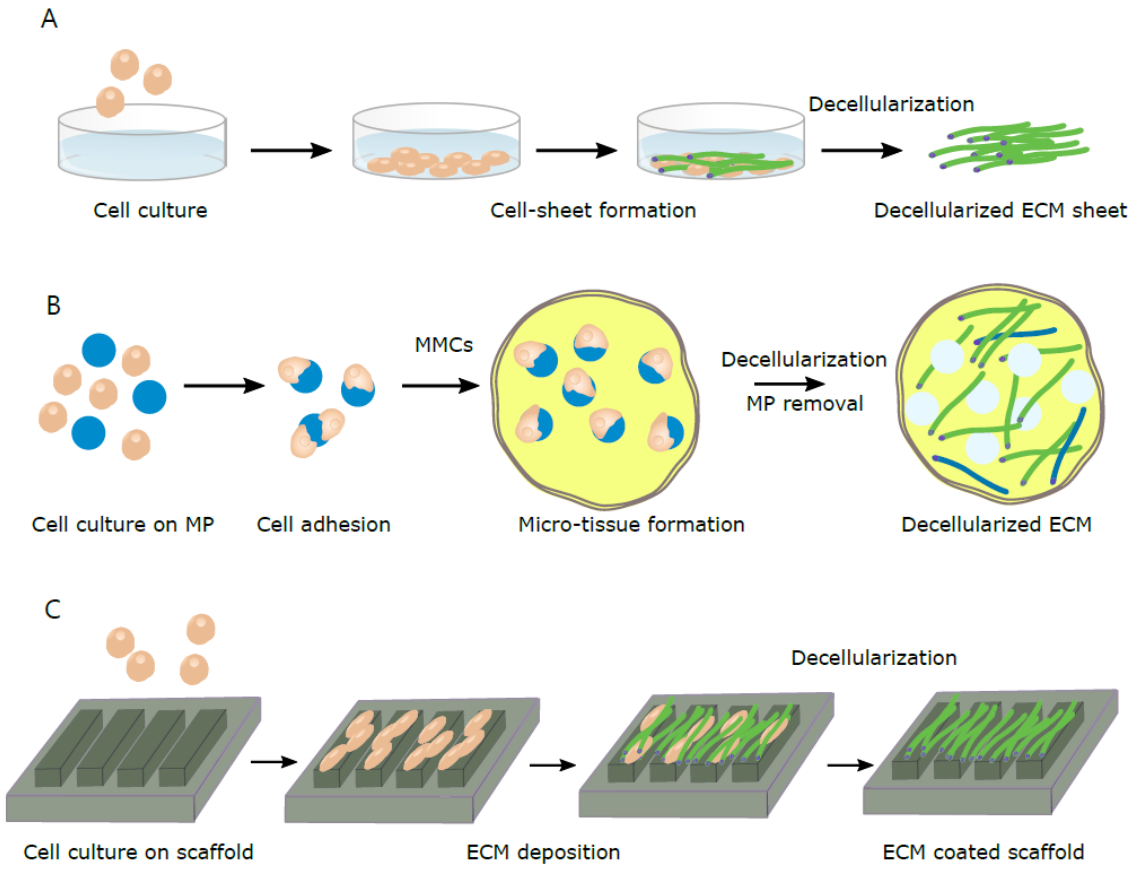

Figure 1. Biomanufacturing strategies of cell-derived ECM. Cells are cultured to form (A) cell sheets, (B) decellularized microtissues applying microparticle-based templates, (C) tissues on a 3D substrate's surface followed by decellularization.

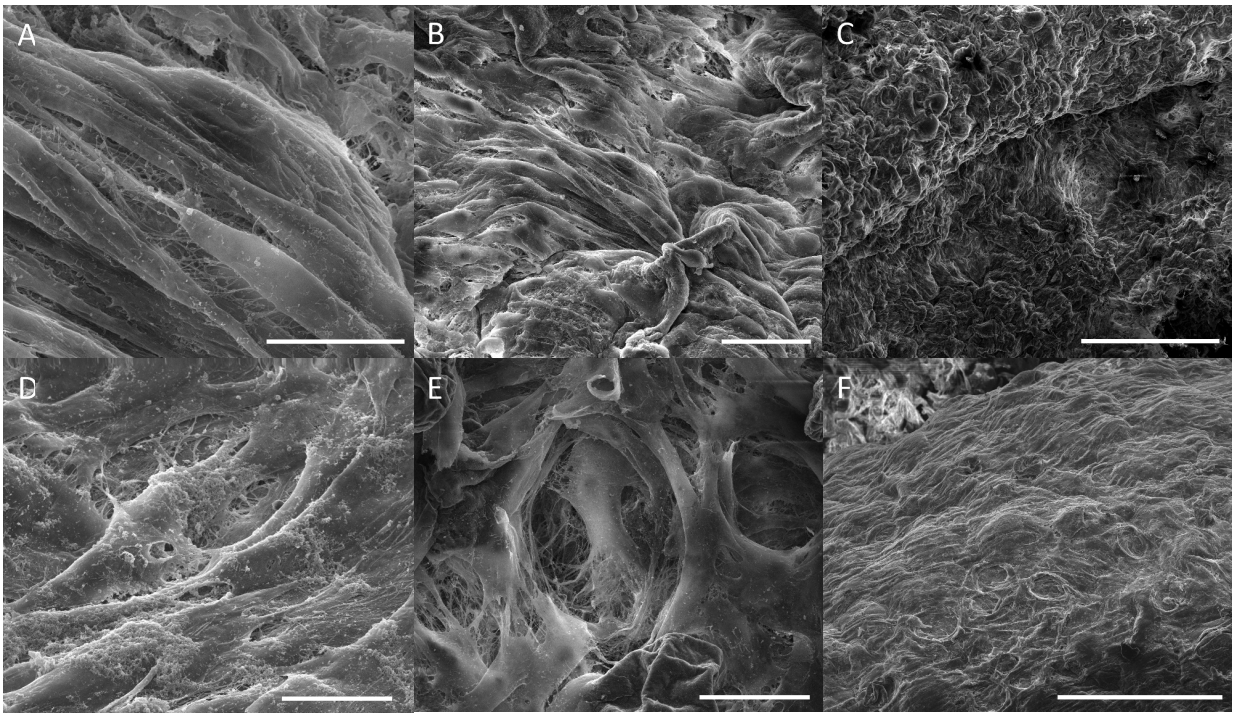

Figure 2. SEM images of fibroblast/MSCs-produced CDMs. A-C) Human dermal fibroblast produced CDMs. Magnifications of CDMs developed using microparticles as 3D scaffolds. Scale bars: A=20µm; B=50µm; C=500µm. D-E) MSCs-produced CDMs. Magnifications of CDMs developed using microparticles as 3D scaffolds. Scale bars: A=20µm; B=50µm; C=300µm.

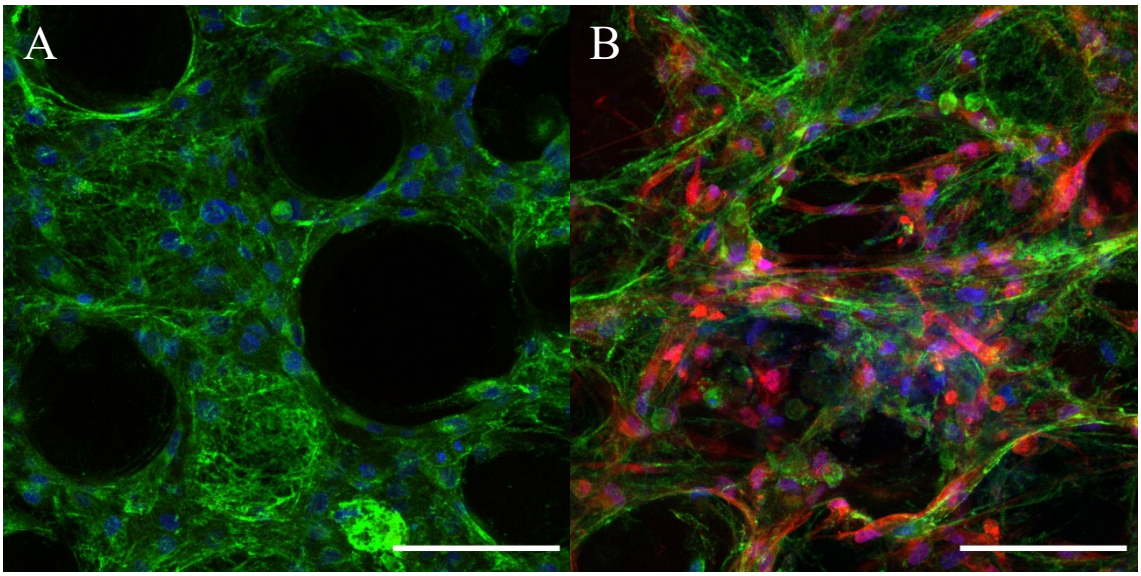

Figure 3. Immunofluorescent staining of fibroblasts/MSCs produced CDMs. A) Human dermal fibroblast produced CDM. B) MSCs-produced CDMs. Fluorophores: Red-Collagen type I; Green-Collagen type III; Blue-DAPI. Scale bars = 100µm.

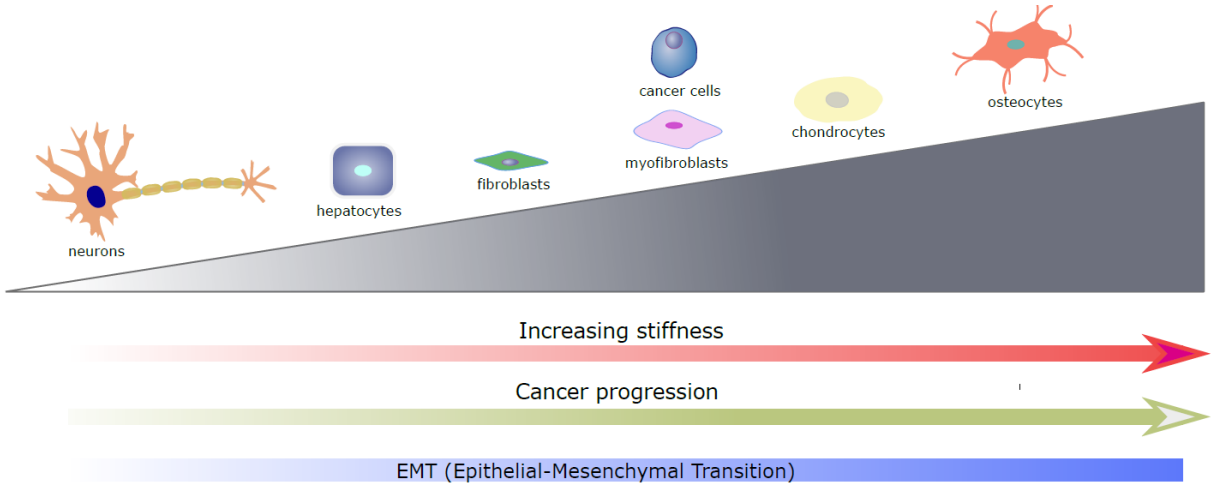

Figure 4. The effect of matrix stiffness in cell phenotype, function, and cancer progression.

Table 1. Summary of the main methods to produce CDMs.

Processing method	Culture template	Cell type for CDM synthesis	Combined material	Outcome	Reference
Microparticles	Hydroxyapatite microparticles	rat primary calvaria osteoblasts and dermal fibroblasts	Same hydroxyapatite microparticles	• Improve osteogenesis in bone • Template sphericity have a strong effect regulating chemotactic CXCR4 receptor	[31]
	No template	hBM-MSCs	PLGA microspheres-based scaffold and bone marrow aspirate (BMA)	• Attraction of cells from BMA • Reduction of CD45+ myeloid cells • Increase of CD31+CD45−endothelial cells • Increase of 143-fold increase in the MSCs number	[45]
	Encapsulation in rat tail collagen type I microspheres	porcine chondrocytes		• Seeded hMSCs acquired a chondrogenic phenotype without supplemented media	[16]
	rat tail collagen type I microspheres	primary nucleus pulposus cells (NPCs)		• Seeded human dermal fibroblasts change their phenotype • Increase of the expression of collagen II and the non-chondrogenic NPC marker CA12	[46]
Cell sheets	PIPAAm-coated surface	Multiple		• High viability and long-term engraftment of cell sheets	[43]
	poly(octadecene-*alt*-maleic anhydride) (POMA)/fibronectin coated plates	hBM-MSCs		• ECM coatings can work as *ex vivo* niche for hHSCs	[37]
Electrospinning	Electrospun PCL mats	hDF	Electrohydrodynamic deposited PCL fibrous mats	• Improved the proliferation of reseeded hDF compared to the control.	[50]
	Silk fibroin electrospun fibers	Mesenchymal adipose stem cells	Same Silk fibroin	• Improve in the angiogenesis process when HUVECs were co-cultured on the non-decellularized meshes.	[49]

			electrospun fibers		
	PCL and PCL/PLGA electrospun nanofibers	mesenchymal progenitor cells (MPCs) derived from human embryonic stem cells (hESCs) and human-induced pluripotent stem cells from hair follicle keratinocytes (HFKTs -hiPSCs)	Same nanofibers	• Construct was feasible, biocompatible and biodegradable when subcutaneously implanted in SCID beige mice and Sprague-Dawley rats	[51]
	PCL electrospun mats	bovine chondrocytes	Same PCL fibers	• PCL/ECM Scaffolds with the addition of TGF-b1 better supported chondrogenic differentiation	[19]
	Electrospun (PLLA) fibers and electrochemical deposited polypyrrole (PPy) nanoparticles	L929 mouse fibroblasts cells	Same fibers and nanoparticles	• Rat PC12 cells onto the combined PLLA/PPy/ECM scaffolds could differentiate to protrude neurites. • Evidence of a better cell adhesion, neurite-bearing and alignment rates on conductive scaffolds combined with the CDM	[52]
	Electrospun PLGA	human adipose stem cell (hASCs)	Same PLGA fibers	• L929 mouse fibroblasts cells showed excellent survival and proliferation • Wound healing was improved in a full-thickness skin excision mouse model	[48]
3D printing	PCL, PLGA, and beta-tricalcium phosphate (TCP).	human nasal inferior turbinate tissue MSCs	Same scaffold	• Same seeded MSCs Increase amount of produced ECM *in vitro* and *in vivo*, • Properties of the ECM can be preserved and mechanically supported	[54]
Bioprinting		Preosteoblasts MC3T3-E1		• Mechanical resistance and ink viscosity increased with the ECM culture time associated not only to a more mature ECM, but to an increase in cell number	[58]

- Longer culture times increased viscosity and mechanical resistance

Table 2. Methods to tailor CDMs' properties.

Method	Main Outcomes	Reference
Different cell type, cell passage, and age of the cell source	• Production of a unique and particular CDM • Influences composition and production rate • Modification of the adhesion, morphogenesis and proliferation of seeded cells on the produced CDM • Influence differentiation through the modification of the CDM signal-based stimuli • Modulation of the CDM's collagen fibers package • Modulation of the angiogenic resultant capillaries structure and distribution	[6,24,77–81]
Use IPSCs	• Control and modulation of the CDM's composition	[42,82]
Genetically transfected cells and RNA interference	• Adjust and modulation of the CDM's composition (nephronectine, collagen I, II, III, IV, fibronectin, versican and other proteoglycans, different cytokines and GF, and different integrins and non-integrin ECM receptors) • Modification of the mechanical properties • Modification of the ECM's degradation and remodeling by the interaction with matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) • Regulate ECM's signaling concentration and response	[83–88]
Macromolecular crowders	• Relevant increase amount of CDM produced with higher stiffness • Enhances collagen type I deposition • Creation and modulation of defined microenvironments to direct cell fate and cell differentiation • Improves the assembling of the CDM proteins to collagen-backbone at the CDM	[89–93]
Culture media composition	• Influences fiber orientation, elastic properties and CDM composition • Increases the deposition of collagen type I from human fibroblasts • Serum proteins can be adsorbed to the ECM masking other proteins • ECM-control of differentiation	[5,94–97]

Cell cross-talking and/or addition of an exogenous ECM	• Modifies the quality of the expressed CDM • Modification of concentration and distribution of the CDM's components • Control of the seeded cell fate	[98–100]
Introduction of ECM signaling	• Better angiogenic response • Modulation of the ECM signals to control the cell fate	[75,101]
O_2 concentration, the mechanoinductive environment of the template substrate and topography	• Adjust and modulation of the CDM's composition	[69–71]

Table 3. Biochemical characterization of CDMs

Characterization method	Purpose	Reference
DAPI (4′,6-diamidino-2-phenylindole)	Nuclei staining	[122]
Hoechst 33258	Nuclei staining	[123]
Quantitating double-stranded DNA (dsDNA)	DNA quantification	[124]
Alexa Fluro 488-labeled phalloidin	F-actin staining	[14]
DiI (1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate)	Cell membrane staining	[14]
Immunofluorescence	Protein staining	[46,125–129]
Immunohistochemistry	Protein staining	[46,125–129]
Immunocytochemistry	Protein staining	[46,125–129]
Alcian Blue staining	Acidic polysaccharides staining	[130]
Picrosirius red	Collagen types I and III staining	[131]
Blyscan	Glycosaminoglycans staining	[130]
Colorimetric assays	Collagen and elastin quantification	[132]
Enzyme-linked immunosorbent assay (ELISA)	Protein quantification	[133]
SDS-PAGE (Coomassie, silver, Ponceau staining)	Protein profile identification	[134]
Western blot	Protein profile identification	[135]
Mass spectrometry	Protein profile identification	[136,137]
Nano-liquid chromatography tandem mass spectrometry (nLC-MS/MS)	Protein profile identification	[46,135,138]

Author biographies and photographs

Gerard Rubí-Sans completed his B.Sc. in biosystems engineering at the Technical University of Catalunya (UPC) in Spain. Then, he received his M.SC. in Bioengineering from Chemical Institute of Sarrià – Ramón Llull University in Barcelona. Currently, he is now working on his Ph.D. at the Institute for Bioengineering of Catalonia (IBEC) in the group of Biomaterials for regenerative therapies, under the supervision of Prof. Elisabeth Engel. His research interest is focused on the development of three-dimensional *in vitro* cell-derived matrices for disease modeling.

Oscar Castaño received his B.Sc. degree in chemistry from the UB and carried out his Ph.D. in material science at ICMAB. He joined Prof. Planell's and, later, Prof. Engel's group at IBEC in 2007 to work on the design and development of biomaterials for tissue engineering with a "Ramon y Cajal" tenure-track contract from the Spanish Ministry. In 2014, he was awarded as the Best Young Biomaterials Researcher in Spain by CIBER-BBN-YSF. Currently, he is an associate professor at the UB. His research interests cover new approaches to structure and process 3D biomaterials as tissues, and fundamental bioengineering applying organ-on-a-chip technology.

Elisabeth Engel studied biology at the UB in Barcelona. She carried out her Ph.D. in Bone metabolic diseases and graduated *summa cum laude*. She obtained a post-doctoral grant from the Spanish Ministry to start a cell biology lab Prof. Planell's group at the UPC in Barcelona. Since then, the group has focused its research on regenerative therapies highlighting the effect of biomaterials' properties in changing the tissue microenvironment to induce endogenous tissue engineering. She became PI of the group of Biomaterials for Regenerative at IBEC in Barcelona in 2012. She is also a full professor at the Materials science department at UPC.

Table of contents

The engineering of cell-derived matrices has already evidenced to be a feasible and reproducible approach. Beyond the 2D to 3D transition considering the *in vivo* tissue as inspiration, cell-derived matrices have the potential to become a serious alternative for tissue regeneration and disease modeling. Their feasibility and plasticity towards varied tissue-platforms supports their use as the next *in vitro* gold standard.

Keywords: cell-derived matrices, extracellular matrix, biomaterials, 3D dimensional models, personalized therapies

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Title: Engineering cell-derived matrices: from 3D models to advanced personalized therapies