Chapter 13

Exploring the Extracellular Matrix to Create Biomaterials

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Additional information is available at the end of the chapter

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Abstract

The extracellular matrix (ECM) represents the framework of tissues and organs and is involved in cell differentiation and function. The study of ECM is challenging and required a combination of identification and imaging techniques to give a valuable scheme of its composition, organization, and finally function. The study of ECM enables to culture cells *ex vivo*, but cultures are restricted to two-dimensional surfaces, whereas in the meantime, material sciences were developing devices able to bring cell culture in a three-dimensional (3D) environment. This chapter presents basic techniques to investigate extracellular matrices composition and organization. Basic knowledge on ECM composition and organization should inspire material scientists to propose more biologically relevant materials. In a second time, we present strategies available to create *ex vivo* models of ECM and a series of examples of 3D materials that were engineered to investigate cell adhesion, phenotype, and differentiation in a biologically relevant microenvironment. The production of a gold-standard material is possible for a specific biological question, and it might be developed from an intelligible dialogue between material scientists, that bring engineering strategies, and cell biologists who implement the material design to meet the biological process that has to be investigated *ex vivo*.

Keywords: extracellular matrix, tissue organization, 3D materials, in vitro cell culture models, tissue engineering

1. Introduction

Multicellular organisms require a framework to delineate functional territories and to provide a shelf where the cells can attach to perform their specific functions. The extracellular matrix (ECM) represents this framework for tissues and organs and as such it is an important actor of

organisms' physiology. The most known examples of ECM-related tissues are the skin, where ECM act as a barrier against the outside environment, and the bones where ECM is strengthened by a mineral phase which allows the body to stand and to move. However, its apparent structural and mechanical properties have hidden more subtle roles of ECM in cell differentiation and function as ECMs are not restricted to load-bearing organs but are present and required in all types of tissues and organs. During the development of the embryo, neural crest cells lose their cell-cell adhesion properties toward cell–ECM interactions that allow them to move along the dorsal part of the embryo and reach their specific site of function and give birth to the future skeleton. Again, tissue remodeling, as observed during the healing processes, can release messenger molecules that were entrapped in the ECM, waiting for the right moment to trigger their signal and healing functions [1]. Some lack of knowledge on ECM functions remains mainly because of the challenge represented by its comprehensive study. Indeed, ECM is made of several high molecular weight proteins, proteoglycans, and polysaccharides molecules self-arranged into fibers and networks difficult to solubilize and individualize. Basic biochemistry techniques have led to the identification of the major components of ECMs such as collagens or laminins, but as the investigations are progressing, this results in the constant growing of the constituent members of collagen and laminin families and in the discovery of new ECM components with unknown functions [2]. Moreover, understanding the ECM not only means discovering new molecules but also to unravel their organization in the ECM network. So the study of ECM requires the combination of identification and imaging techniques to give a valuable scheme of its composition, organization, and finally function. Interestingly, unraveling ECM complexity meets one of the fundamental questions for biologists: how to recreate and maintain life outside a living organism (literally *ex vivo* but commonly referred as *in vitro*)?

The beginning of the 20th century aroused the possibility to dissociate cells from living tissues and to culture them *ex vivo*. This new technique has triggered the emergence of the new discipline of cell biology which has brought most of the knowledge that we possess today on cell proliferation, differentiation, metabolism, cell fate, and death. However, *ex vivo* cell cultures were restricted to two-dimensional (2-D) culture systems, originally on glass and subsequently on plastic dishes, occasionally supplemented by the coating of ECM molecules to favor cell adhesion. Parallel to the development of cell biology, the broad field of materials science was creating polymers and devices able to bring *ex vivo* cell culture to the third dimension, and to the 21st century. Dedicated to materials that interact with living tissues, the field of biomaterials encompasses several scientific disciplines, from physics and chemistry to biochemistry and medicine. Several types of three-dimensional (3D) materials have been engineered which may represent valuable tools for fundamental cell research, but a lack of knowledge on ECM structures have undermined their use for cell biology. On the other hand, cell biologists are not necessarily aware of the development and possibilities created by extensive research in the field of 3D biomaterials, and this partly compromises the expansion of 3D cell culture models.

In this chapter, we will present basic techniques involved in the investigation of extracellular matrices and data generated by their use to understand ECM composition and organization. Basic knowledge on ECM composition and organization should be useful for biomaterial

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scientists to propose more biologically relevant materials. Such methodologies are fully transposable for the characterization of biomaterials and 3D models of ECMs. In a second section, we will present a series of biomaterials that were engineered based on the investigation of ECMs composition and organization *in vivo* and could become suitable 3D cell culture models for mechanobiology, aging, cell migration, cell differentiation, and studies on pathologies and their treatments.

2. Exploring the ECM

Extracellular matrices are multimolecular three-dimensional (3D) networks made of a large variety of ECM-specific molecules and their compositions and organizations are tissue-specific. Exploring the ECM means (1) the determination of its distribution within the tissue and its relation to the cell content, (2) the identification and quantification of its composition, and (3) the characterization of the 3-D architecture of the ECM network [2]. ECMs contain similar biomolecules which can be organized in two main classes (1) proteins and glycoproteins and (2) proteoglycans and polysaccharides. Variation in the composition or in the amount of certain ECM molecules will change dramatically the physical properties of the ECM such as the tensile strength observed in the hard mineralized ECM in bones, the elasticity observed in dermis of the skin, or even the transparency in the cornea of the eye. The biochemistry of ECM components strongly influences the techniques used to investigate them. Light microscopy associated with histological staining is based on the differences of biochemical features of tissues (i.e., hydrophobicity, electrical charge, and molecular weight). Proteomics associated with mass spectrometry is a powerful tool to exhaustively identify proteins in a complex sample, but biochemistry of ECM proteins is particularly unfavorable to this method that need significant adaptation to be effective with ECM samples. Finally, electron microscopy is the ideal method to investigate the molecular and fibrillary organization of the ECM network.

2.1. Biochemistry of the main ECM components

2.1.1. Proteins and glycoproteins

A large diversity of proteins is found in ECMs where they are the principal component. They are classified either in structural proteins that are directly involved in the overall architecture of the ECM or in soluble factors that are globular proteins entrapped in the ECM network. Structural proteins are mainly fibrous, insoluble, and high molecular weight molecules, including collagens, elastin, laminins, and fibronectins. They are direct actors of the shape and the mechanical properties of tissues and organs and further possess the ability to auto-assemble among themselves as well as to interact with each other to form fibrillary network and complex 3-D architectures. Most of the ECM proteins have sequences recognized by cells for adhesions and some of them can bind specifically soluble growth factors or cytokines. These molecules present several posttranslational modifications like hydroxylation at Proline and

Lysine residues in collagens and O-glycosylation and N-glycosylation in laminins and fibronectin.

Collagens are found in all types of ECMs and are the main constituent of connective tissues like skin, bone, and tendons [3]. They belong to a large family of molecules with to date 28 members identified (numbered from collagen type I to type XXVIII). Collagens are trimeric proteins, made of the association of three alpha-chains specific to each type of collagens that assemble together to form a super-helix structure. For some collagen types several alpha-chains exist, leading to multiple isoforms of the same collagen molecule and raising the diversity and the complexity of the collagen family. In ECMs, collagens are organized in different supramolecular assemblies inherited from the specificity for each collagen types taking into account their amino-acid sequences and the 3-D folding of their tertiary structure [4]. Fibril-forming collagens include collagen type I, II, III, V, and XI. They assemble in large fibrils (up to 500 nm in diameter) that can merge to form collagen fibers of micrometric size. All ECMs contain fibrillary collagens. Connective tissues are characterized by an abundant ECM content made mainly by fibrils of collagen type I in dermis and bone, or of collagen type II fibrils in cartilage. Basement membranes (BM) are a specialized form of ECM mainly found in epithelial tissues and contain heterotypic fibrils combining collagen I and III or V [5]. Size and diameters of collagen fibrils are regulated by other ECM molecules like fibril-associated collagens or proteoglycans. Collagen fibrils and fibers are finally stabilized by covalent cross-links making these structures highly resistant to mechanical load and stresses. Network-forming collagens are mostly found in BM where collagen type IV is the most abundant. Collagen IV molecules assemble in a hexameric superstructure that propagate to form finally a 2-D network that is maintained by covalent crosslinks with methionine and lysine residues [6].

Laminins are large molecular weight (from 400 to 900 kDa), heterotrimeric glycoproteins and, along with collagen type IV, they are the main constituent of BM [7]. Even found in every BM, laminin is a large family of molecules, and their distribution among BM is tissue-specific. A laminin molecule consists of the association of one alpha, one beta, and one gamma chain. To date, 5 alpha, 3 beta, and 3 gamma chains have been identified which may be assembled in 16 different laminin molecules. All laminins share common structural features: a cross-shaped 3-D structure with one long and two shorts arms, di-sulfide bridges in-between the chains that maintain their association and the presence of several N-glycosylation on asparagine residues. Laminins auto-assemble in a network interlaced with the collagen type IV network. Directed toward the cells, laminins gives cues for cell adhesions through integrin receptors.

Elastin is organized in fibers closely linked to fibrillar collagens where it gives the elasticity to tissues and compensate the tensile strength of collagen fibers [8]. Elastin is secreted by cells as a 60–70 kDa monomeric soluble precursor, tropoelastin, which contains intermittent hydrophobic domains. Tropoelastin monomers auto-assemble to form elastin fibers that are stabilized by enzymatic cross-linking through Lysine residues and rendering the elastin network highly insoluble. Stacks of hydrophobic domains in the elastin network are responsible for its elastic properties and make elastin highly resistant to enzymatic degradation and solubilization in aqueous solutions.

Fibronectin is a large (500 kDa) dimeric glycoprotein made of the association of two nonidentical monomers linked by two disulfides bounds at their C-terminal extremities [9]. Diversity of the monomers is due to alternate splicing of the fibronectin mRNA, as fibronectin is encoded by only one gene. Fibronectin is expressed by several cell types and found in most of ECMs. It assembles through disulfide bridges in oligomers and finally in insoluble fibers possessing various diameters ranging from 10 nm to microns [10]. A soluble form made of the dimeric monomer may be also found to circulate in the blood. Fibronectin primary structure is arranged in several domains that specifically interact with collagens or with cells via integrins.

There are globular, soluble proteins associated with the ECM network of structural proteins. Among the globular proteins there are growth factors, cytokines, and ECM-specific proteolytic enzymes like matrix metalloproteinases (MMP). They play an important role in cell signaling and in the remodeling of the ECM network and finally in the overall biological activity of ECMs. They can be linked to structural proteins by labile interactions at specific biding sites or be trapped in the high molecular weight chains of the structural proteins and proteoglycan. However, they are not core proteins of the ECM network, and their biochemistry is similar to most of the other globular proteins.

2.1.2. Proteoglycans and polysaccharides

Polysaccharides found in ECMs of vertebrae are glycosaminoglycans (GAG) and are covalently linked to a core protein to form proteoglycans, except for hyaluronan representing the only "pure" polysaccharide of ECMs [11]. Even if this chapter focuses on mammalian ECMs, it has to be mentioned that polysaccharides are the main ECM components of invertebrates and plants represented by chitin and cellulose, respectively. Hyaluronan, equally called hyaluronic acid, has the particularity to be synthesized at the plasma membrane by three different Hyaluronan synthase enzymes and not inside the Golgi apparatus like all the other proteoglycans [12]. GAGs are linear, unbranched polysaccharides composed by tens to hundreds of disaccharides units. The combination of disaccharide units is highly heterogeneous, but can be specific for each individual chain. The disaccharide unit is made of glucosamine or galactosamine linked to another modified hexose, the most often to glucuronic acid, iduronic acid, or galactose. These monosaccharides are mainly modified by N-acetylation and N-sulfatation. The nature of the disaccharide unit and the types of modifications lead to the formation of different types of GAG, including chondroitin sulfate, dermatan sulfate, keratan sulfate, heparan sulfate, and hyaluronan. At physiological pH, GAGs chains are highly negatively charged due to the sulfate and carboxylic acid functions carried on modified hexoses. The net negative charge of GAGs make them highly hydrophilic, and thus, they play an important role in the hydration of ECMs [13]. High amounts of water associated with GAGs ensure some mechanical properties to ECMs, especially the resistance to compression as in the cartilage. Proteoglycans are abundant within ECM, but may be also found at the cell membrane or intracellularly. The most active part of the proteoglycans is the GAGs chain which can interact with growth factors, cytokines, cell receptors, and other constituents of the ECM. However, their core proteins also possess interaction sites that make proteoglycans highly versatile molecules inside ECMs [14]. Due to their interactions with ECM components, they

play a role in ECM organization, but their important role is to be a reservoir for growth factors and to anchor signal molecules that are released through specific enzymes in particular after injuries and favor wound healing.

2.2. Exploring the organization and the composition ECMs within tissues by histology

Histology is an old, but still a powerful technique to image the organization of tissues at the scale of the cells. Organs or tissues have to be fixed to stabilize their organization after removing them from the living body and to be embedded in a hard material for being sectioned into thin slices to allow light to pass through the tissue and so be visible by the lenses of an optical microscope. Usually, tissues are fixed in paraformaldehyde and embedded in wax (paraffin). Tissues can also be frozen-fixed in liquid nitrogen. This approach may help to avoid some prejudicial effects of chemical fixations and do not require an embedding step to slice the tissue. However, frozen sections give pictures with less contrast and sharp edges, therefore formalin-fixed paraffin embedded (FFPE) samples are preferred for routine examination. To be interpretable under light microscopic examination, the histological sections have to be stained to give some contrast to the different tissue and cells structures [15]. Numerous special stains have been developed to give a precise contrast to the organization and to the components of ECM, and this approach still keeps all its strength and efficiency to describe and accurately decorticate a tissue [16]. A significant further step in histology is the use of antibodies by the mean of immunohistochemistry (IHC) to specifically identify a target molecule, and at the same time, visualize its exact localization within the tissue [17]. However, IHC relies on the quality of the antibody, and it appears more difficult to obtain commercially reliable antibodies against ECM molecules than for intracellular molecules. A model of the accuracy and the potentials of histological techniques for investigation of ECMs are reported in a recent and thorough study on tooth root cementum using exclusively light microscopy techniques, with a combination of special stains, polarized light, and immunodetection [18].

2.2.1. Special stains for histology of ECMs

Masson's and Gomori's trichrome– they are ideal for connective tissues and in particular to stain fibrillar collagens. Trichrome staining indicates that three different dyes are used to discriminate the ECM materials from the cell cytoplasm and the nucleus. Selectivity of the dye for the different tissue structures is based mainly on size exclusion: a small dye will penetrate into low porosity elements while larger dye will penetrate preferably into the more porous structures. Gomori's trichrome stains collagen-based ECM in green, cell cytoplasm and noncollagenous tissue in pink to purple and cells nuclei in black to blue. Same discrimination of connective tissue comportments but with different colors is obtained with Masson's trichrome (collagen in blue, cytoplasm in red, and nucleus in black). This trichrome staining is longer and maybe more delicate to perform than the classical hematoxylin-eosin staining, however, this staining should be used each time when collagen-based samples (tissue or biomaterial) are investigated because of the high degree of contrast it enables compared to H&E [19].

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Von Kossa staining— It is used to reveal mineral deposits within tissues. Mineralisation of ECM is observed in bones or teeth but can also appear pathologically in blood vessels, skin, or cartilage. Von Kossa staining detects calcium phosphate deposits by its substitution with silver nitrate to form black precipitates. This method is robust and useful to follow mineralization of bone substitute materials or progressive differentiation of naive cells into the osteoblastic lineage. It can also discriminate calcium–phosphate based materials from organic compounds.

Picro-sirius—This staining method is specific of fibrillar collagens (type I–III), although it can slightly stain collagen type IV in BM. Sirius-red dye is an anionic molecule that arrange parallel to collagen molecules by interaction with basic (cationic) amino acids [20]. Collagenous tissue is stained in red while remaining tissue is pale yellow, and nuclei can be counterstained in black or deep blue if necessary. The parallel organization of picro-sirius is used to enhance the birefringence signal of collagen fibers observed under a polarized microscope. With the combination of polarized light and picro-sirius, it is possible to address the 3D orientation of the collagen fibers as the intensity of the birefringence depends on fibers orientation, with maximum birefringence intensity for fibers organized at 45°(+/–90°) toward the transmitted light axis, while no birefringence signal is observed with fibers organized parallel or orthogonal to the transmission axis [21,22]. Picro-sirius is also used to make quantitative analysis of the collagen content in tissues, in particular to address the degrees of fibrosis.

Verhoeff–Van Gieson—This coloration method stains preferentially elastic fibers which have a higher affinity for the dye than the rest of the tissue due to hydrophobic interactions. The elastic fibers are stained black or dark brown while collagen fibers are red and the rest of the tissue appears pale yellow. This coloration is particularly appropriate to reveal atrophy of elastic tissues in case of vascular diseases and to discriminate arteries from veins due to differences in the elastic fibers organization and content [23].

Alcian blue—This dye is a cationic molecule and interacts specifically with negatively charged polysaccharides like sulfated and carboxylated GAGs. Because the selectivity of the dye is based on the charge of GAGs it is of importance to ascertain the pH of the alcian blue solution, as the carboxylic acid groups are in their acidic neutral form at $pH < 2$ and therefore will not interact with the dye. This specificity allows the discrimination between the carboxylated and the sulfated GAGs, as the latter are negatively charged at $pH < 2$. Polysaccharide's rich tissues are stained in light blue, counterstaining with nuclear fast red, making cells cytoplasm and nuclei colored in pink-red. Alcian blue is routinely used to stain cartilaginous ECM due to their large content of GAGs [24].

Periodic Acid–Schiff (PAS)—This staining is also sensitive to polysaccharides and is used to stain BM due to their high content in glycoproteins and proteoglycans. Unlike alcian blue, PAS staining reveals both charged and neutral polysaccharides because this coloration is based on the oxidation of monosaccharides with periodic acid and subsequent reaction of newly formed aldehyde groups with the Schiff reagent dye. BM are finely stained in pink-purple color, whereas the cytoplasm and the nuclei are colored in shades of blue when counterstained with Harris' hematoxylin. This staining method is also used to reveal other polysaccharides-rich compounds, such as mucins and glycogen [15].

2.2.2. Immunohistochemistry of the extracellular matrix

IHC enables the identification of a specific component of the ECM and to image its distribution within the tissue [25]. The target molecule is recognized by an antibody that reacts to a specific epitope and then is visualized by light microscopy through a chromogenic enzymatic reaction (alkaline phosphatase or horseradish peroxidase) or through a fluorescent dye with a fluorescence microscope. The antibody is observed directly if the dye or the enzyme is linked to it, but most of the time it is detected indirectly by a labeled (by a fluorophore or an enzyme) secondary antibody which reacts to the first one through its Fc fragment. Frozen sections are more appropriate for Immunohistochemistry because they avoid the use of fixative that may alter the epitope, but frozen sections cannot be counterstained and so keeps the tissue organization around the epitope not visible [26]. In contrast, FFPE samples are well preserved and can be counterstained with different dyes after antibody incubation and detection. However, if the fixative (generally 4% paraformaldehyde in neutral buffer) preserves the morphology of the tissue, it can severely compromise the antigenicity of the target molecule, and then make immunodetection inefficient or inoperative. Paraformaldehyde fixative triggers intra- and intermolecular cross-linking of proteins by the formation of methylene bridges between amino acids residues [25]. It may also alter the molecular structure of polysaccharides, lipids, and nucleic acids. The degree of cross-linking will depend on the concentration and the pH of the fixative solution, as well as on the time and the temperature at what the fixation is performed. The formation of intra- and intermolecular cross-linking modifies the secondary and tertiary structures of proteins that lower the detection by antibodies because of the modification of the target epitopes [27]. In the early 1990s, an antigen retrieval (AR) method was introduced to recover the antigenicity of FFPE tissue sections impaired by the fixation treatment [28]. The AR method originally refers to the high-temperature processing of FFPE sections, but with the development of other methods it is nowadays a generic term for any kind of treatment used to recover the original antigenicity of the FFPE sections [29]. The rationale of AR is the breaking of fixative-induced cross-links and methylene bridges that enable a renaturation of the proteins and a partial recovery of the epitopes. However, it has to be noticed that the true mechanism of AR is not yet understood, and it remains an empirical technique that requires several positive and negative controls to avoid true- or false-positive reactions [30]. AR is performed with the use of heat (called heat-induced antigen or epitope retrieval) or enzymes (referred-to PIER for proteolytic enzyme-induced epitope retrieval) to break fixative cross-links.

In heat-Induced epitope retrieval (HIER), three parameters appear essential in the outcome of the AR: temperature and pH of the solution and time of incubation of the sections [29,31]. Classically, sections are incubated for 10–20 minutes at 95°C in a water bath. Microwave and steam-cookers are also used to heat sections and have shown good AR properties, although the control of the temperature is more delicate. The pH of the solution is a critical factor because some epitopes will be revealed only in acidic or in alkaline buffer. The most common acidic buffer is citrate used in a pH range of 3–6. The most used alkaline buffer is Tris supplemented or not with EDTA at pH 8–10 [29,31]. All pH, temperature, and time have to be checked carefully because extreme conditions will damage the tissue sections.

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Enzyme treatment is thought to break some of the fixative methylene bridges and to elicit the reconstitution of epitopes after a moderate digestion of proteins. It is generally performed with proteolytic enzymes such as pepsin, trypsin and proteinase K at a concentration of 0.05–1% for 10–30 minutes. Glycosidases, such as hyaluronidase, chondroitinase, and keratinase have shown valuable AR properties on polysaccharides-rich tissues and on glycosylated proteins [32]. The pH and temperature of the solution are adjusted to the optimal activity of the enzyme, and time of digestion and the concentration of the enzyme have to be carefully set to avoid overdigestion of the tissue sections which will lead to a loss of tissue structure and organization.

Success of immunohistostaining mainly relies upon the quality of the antibody. Compared to soluble proteins, only few antibodies against ECM molecules are commercially available [32]. The ECM proteins are highly conserved in mammals making difficult the immunization of animals to generate an efficient antibody. Some antibodies are raised from synthetic peptides (5–20 amino acids) chosen from the primary amino-acid sequence of the target protein, but the epitope generated could be irrelevant to the secondary and tertiary structures of the native proteins [33]. Polysaccharides are either not or very slightly immunogenic, making very difficult to generate antibodies against the sugar part of proteoglycans. Moreover, ECM proteins are organized into dense fibers structures or meshwork or bear high polysaccharides chains that hinder the access of the antibody to the epitopes. In conclusion, IHC of the ECM is a delicate technique but remains the best option to obtain a picture of individual ECM components distribution within the different compartments of a tissue. As an example among several ones, by the means of monoclonal antibodies raised against laminin chains alpha-4 and -5, beta-1 and -2, and gamma-1, it has been possible to elucidate the particular composition and organization of the basement membrane surrounding islets of Langerhans in human pancreas [34]. The identification of a duplex BM surrounding intra-islets vessels with a specific laminin composition for each of the two BM has led to the proposition of a double-basement membrane model of human islets of Langerhans clearly distinct from the organization of basement membrane surrounding islets in mouse [35].

2.3. Identification of ECM composition by proteomics with mass spectrometry

The proteomic strategy is based on the isolation of a complex mixture of proteins from cells, tissues, or a whole organism and their identification by mass spectrometry and genomic database. Mass spectrometers commonly used for protein identification are MALDI–TOF (for Matrix–Assisted Lazer Desorption Ionization—Time of Flight) and ESI-Q-TOF (for Electro Spray Ionization—Quadripole—Time of Flight) that have their own characteristics and performances but do not change the general flow-chart of the sample preparation and identification [36,37]. After extraction and purification, proteins are separated by 1D or 2D sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE), respectively to their molecular weight (1D) or by both their isoelectric point (pH which net charge of protein is neutral) and molecular weight (2D). Mass spectrometers only detect charged molecules with an accuracy and sensibility that depends on the ratio of mass over charge (m/z), so the proteins samples have to be hydrolyzed into peptides before mass spectrometry analysis to obtain

spectra at atomic resolution. Protein bands (1D) or spots (2D) are excised from the gel, hydrolysed into peptides by a proteolytic enzyme (frequently Trypsin), and loaded in the mass spectrometer to measure the exact mass of the peptides. Each protein from the original mixture is identified by matching the measured masses of their peptides with the expected masses of peptides calculated in silico from genomic database [37,38]. This technique allows a large-scale identification of components without the bias of predetermined molecular candidates as with antibody detection. It is thus possible in theory to have the exact protein composition of a tissue and follow its modification with time or diseases [39].

The total or relative amount of identified proteins can also be addressed. The SDS-PAGE migration pattern and intensity of protein band (1D) or spot (2D) give a "map" of the protein content of the target tissue or organ and can be used to identify particular band/spot that are modified in specific conditions, enabling discovery of new therapeutic targets [40]. Labeling methods exist to generate quantitative data with mass spectrometry. Samples of the control conditions are modified with nuclear isotopes 13C, 15N, or 18O, whereas the treated sample is left unmodified, and the relative abundance of both isotopic pics is compared [41]. A direct semiquantitative approach is also possible, with the mathematical integration of ion counts of the peptides identifying each protein to describe its relative abundance [42]. In the ECM analysis, relative quantification is a remarkable tool to identify the specific isoform of some multimeric ECM proteins, such as collagens and laminins, as the relative amount of each monomer will indicate under which form the ECM molecule is present in the tissue. To be more specific, collagen type V exists in the common heterotrimeric isoform $[\alpha1(V)]_2\alpha2(V)$ and a more scarce homotrimeric isoform $[\alpha1(V)]_3$. The relative amount of ion counts for the $\alpha1(V)$ chain over $\alpha2(V)$ chain will indicate if the $\alpha1(V)$ chain is associated only with $\alpha2(V)$ ($\alpha1$ chain signal twofold of $\alpha2$ chain signal) or if the investigated tissue contains both heterotrimeric $[\alpha1(V)]_2\alpha2(V)$ and homotrimeric $[\alpha1(V)]_3$ isoforms ($\alpha1$ chain signal >> twofold of $\alpha2$ chain signal). However, quantification by mass spectrometry can be restricted by the ionization properties of some proteins that will make them less detected and consequently under-represented in the final analysis. Nevertheless, this highlights the potentials of proteomics and mass spectrometry in the study of ECM proteins, as such characterization of ECM proteins isoform will require several antibodies (i.e., one per protein chain) to identify one isoform by western blot or IHC [43].

The most critical steps of a proteomic analysis are the purification of the protein mixture and their identification from database. ECM proteins have a high molecular weight and are tightly associated with each other by covalent cross links that make them mostly insoluble. An important point in the analysis of ECM by mass spectrometry proteomic will be the proper solubilization of the ECM [44]. The tissue has to be first carefully decellularized to purify the ECM and eliminate the remaining intracellular proteins. This step requires the use of a detergent like SDS and will eliminate from the ECM part of the loosely bounded proteins like remodeling enzymes or growth factors [40]. The purified ECM can be solubilized by a combination of physical, chemical, and enzymatic methods. A physical method is the mechanical breaking with a French press or grinding with mortar and pestle in liquid nitrogen. This step is important to homogenize correctly the purified ECM and make the following

solubilization treatment effective. Ultrasound can also be used, but this process yields heat that can denaturate and break the proteins creating smears instead of protein bands or spots during SDS-PAGE separation if temperature is not carefully controlled. Homogenized ECM can be solubilized with a chaotropic agent like concentrated urea or guanidium chloride [45]. These molecules are efficient for solubilization, but a too high concentration is not compatible with SDS-PAGE separation and can impair the trypsin digestion. Highly cross-linked collagen fibrils or elastin microfibrils can remain insoluble after chaotropic extraction. Partial digestion with proteolytic enzymes such as pepsin is also used to favor ECM solubilization, but again, it has to be done carefully to not hydrolyze the ECM sample before SDS-PAGE separation. Deglycosylation with glucosidase such as PNGase or chondroitinase can unravel parts of the dense polysaccharide network of proteoglycan and unleash trapped ECM proteins [45]. Moreover, deglycosylation is also favorable for further trypsin digestion and peptide identification from database. ECM proteins undergo several posttranslational modifications, such as hydroxylation, disulfide bridges, and glycosylation that can block digestive sites for trypsin, leading to inefficient peptide generation, or resulted in peptides of different masses than the expected masses from the genomic data base, leading to inappropriate identification of the protein. All these biochemical specificities of ECM proteins make proteomic discovery challenging and explain why only a few proteome of ECMs have been published so far. Nevertheless, this approach has a huge potential and consequently efficient solubilization and identification protocols are under development to make this technique more routinely usable in ECM and biomaterials characterization.

2.4. Three-dimensional organization of ECMs studied by electron microscopy

Electron microscopy gives higher spatial resolution than light microscopy with the use a shorter wave-length from an electron beam. With resolution at the nanometres scale, and below with high resolution microscopes, it gives access to the molecular structure of ECM proteins and can image their supramolecular organization (i.e., fibrils and fibers assemblies) that are hardly distinguishable with optical microscopes. Transmission electronic microscopes (TEM) are built on the same scheme as optical/visible-light microscopes and so, equivalent techniques and processing of samples are required for both type of microscopy. In TEM, the electron beam pass through the samples to give rise to a projected image on an electron-sensitive surface like a phosphorescent screen, on a silver-film plate to record the image or nowadays on CCD cameras. The electron beam requires a vacuum pressure and cannot pass through thick samples of several micrometers which both represent a challenge for biological samples that are mainly wet, thick, and soft materials [46]. Biological tissues have to be fixed, dehydrated, and embedded in hard material (epoxy resin) and sliced with a diamond knife ultra-microtome into hundreds nanometres slices to be investigated by TEM. The electron beam interacts poorly with low atomic numbers atoms, such as carbon, oxygen, and nitrogen found in biological samples, so sections are stained with heavy metal solutions (commonly tungsten in phosphotungstic acid, uranium in uranyl acetate, and lead in lead citrate) to give contrast [47]. Compared to histological staining, negative staining is more commonly used to prepare TEM sample to improve the contrast of organic materials: heavy metals dyes are absorbed by the background that creates contrast to the slightly stained specimen. The observation of ECM by

TEM is nearly concomitant of its apparition in the late 1930s. Native collagen fibrils extracted from tissues and stained negatively with phosphotungtsic acid present a typical cross-striated pattern with a series of dark and light bands, spaced with a regular period of 67 nm. These observations have allowed the establishment of the assembly model of collagen molecules into collagen fibrils, known as the quarter-stagger model from Hodge and Petruska (1963). This model proposes a lateral stacking of collagen molecules, creating overlaps that exclude phosphotungstic dye and appear light, and a longitudinal collinear succession of collagen molecule spaced with a constant gap filled by Tungsten dye and appears dark under electron beam [48]. The cross-striated pattern is characteristic of fibrillary collagen, that are collagen type I, type II, and type III. On the other hand, network forming collagen type IV do not present any bands on TEM but is seen as a meshwork of hexagonal structures [49]. The resolution (roughly 1–5 nm) of TEM allows analyzing single macromolecules deposited on carbon film and stained by rotary-shadowing, creating a 3D electron sensitive replica of the specimen [50]. This method has revealed the semiflexible rod structure of collagen molecules terminated by a globular C-term pro-peptide and the cross-shaped triple chain structure of laminin molecules. TEM is particularly accurate to measure length of ECM molecules and diameters of fibrils and fibers assembly. These last parameters are important when analyzing a tissue because ECM fibrils diameters appear to be tissue-specific and modification of their size can be induced by pathologies such as diabetes, fibrosis, cancer, or aging and consequently impair tissue organization and function [51]. Compact bone tissue which supports most of the load of the body and muscles anchorage has to resist strong mechanical solicitations, but it is surprisingly light in weight structure if compared to human-engineered buildings. Bone tissue is made of an abundant organic ECM, strengthened with a mineral phase, and has highly hierarchical structure with length scales ranging from meters to nanometers that give its overall mechanical properties [52]. The shaft of long bones is organized in cylindrical osteons formed by successive concentric lamellae, themselves constituted by compact assemblies of collagen fibrils. An oblique transverse section of successive concentric lamellae made with ultra-microtome and observed with TEM revealed coexistence of two patterns of organization for collagen fibrils [53]. One is an alternation of parallel and orthogonal fibrils, with a regular 90° shift of fibrils orientation from one lamellae to another. The second is seen under TEM as arced structures, as if collagen fibrils were bent in between two series of longitudinal fibrils. The arced pattern is the consequence of the oblique sections into succession of collagen fibrils rotating with a tiny and constant angle from one lamellae to another, creating the illusion of bend structures [54]. From these TEM observations, a twisted plywood model of collagen fibril organization in bone has been proposed. This particular constant angle twist recall the organization observed in some liquid crystal phase, and it has been suggested that collagen molecules could have a liquid crystal behavior and autoassemble in higher-scales structures [21,55]. This finally underlines the potentials of transmission electron microscopy (TEM) to address ECM architectures in tissues. As mentioned above, similar techniques and processing of samples used in optical microscopy are also applied with TEM. The different components of the ECM can be identified by immunolabeling with the same limitations for the necessity to retrieve antigens from the fixation and embedding processes. The antibodies are covalently linked to a gold

particle to be seen by TEM and multiple labeling is possible with the use of a specific size of gold particle for each antibody [30].

TEM, however, needs a very thin specimen and cannot directly image a 3D structure. Unlike TEM, scanning electron microscope (SEM) uses electrons reflected from the surface of the sample as signals for image generation and provides information on surface topography, fibrillar organization, porosity, and also atomic composition of a bulk sample [56]. Samples have to be dehydrated to enter the low-vacuum chamber of the microscope and coated with an electron conducting layer (commonly gold) to ensure an adequate contrast and avoid charging phenomenon on the sample which are deleterious for the quality of the image. To keep their native 3D structure intact, biological samples are usually dehydrated by ethanol treatment and to a critical point drying. This procedure has enabled the evaluation of collagen fibrils diameters and spatial organization in reconstituted collagen hydrogels [57]. In biological tissues, the higher proportion of cells compared to ECM can minimize the access to the ECM fibrillar network. By a gentle decellularization method, the cellular counterpart of the tissue is removed and the native ECM frame remains [58]. This process mainly keeps in their original shape the reticular fibers of collagen and elastin but degrade most part of the laminins and GAGs network.

3. Extracellular matrix-inspired biomaterials

The deep exploration of ECMs composition, organization, and biological functions associated with the development of methods to produce new biocompatible materials has enabled material scientists to recreate *ex vivo* some of the key characteristics of ECM [59]. This section focuses on how the structural and functional characteristics derived from the knowledge of the native cell microenvironment have been applied to design biologically relevant biomaterials. Different strategies currently exist to build 3D models of the ECM: tissue-derived ECM, use of natural or synthetic polymers, and formulation into hydrogel or porous 3D materials. Some biomaterials are designed to recreate the composition of the ECM and thus offering the right environment for studying cell adhesion and anchorage-associated cell phenotypes. Other materials are developed to recreate the 3D architectures of ECM, proposing fibrillary structures with similar organization and mechanical properties of native tissues. These examples represent preliminary attempts of *ex vivo* models of ECM that will most likely be improved and increase with an overcoming of technical hurdle faced by material scientists and with rising interest of cell biologists for 3D models that will ask for more refined and specific materials to answer fundamental questions on cell biology.

3.1. Strategies to engineer 3D models of ECMs

3.1.1. Tissue-derived ECM: the gold standard Matrigel®

A basement membrane-derived tissue isolated from Engelberth–Holm–Swarm (EHS) mouse sarcoma is commercially available under the brand name Matrigel® (BD Biosciences) and has

become widely used to evaluate cell migration, cancer cells behavior, and to create organoids *in vitro*. EHS-sarcoma produces a large amount of ECM rich in collagen type I, laminin-111, heparin sulfate proteoglycans (Perlecan), and Nidogen that are the main constituents of BM [60]. This basement membrane extract is liquid at 4°C and turns into a gel at 37°C under physiological pH and ionic strength. Matrigel® is currently the gold standard in most of the 3D assays performed in cell biology. Indeed, there is not yet any material in the market that is able to better reproduce the composition and partially the organization of BM, in particular because Matrigel® is obtained directly from animals and is not an *ex vivo* engineered ECM. It is a ready-to-use solution that allows user-defined utilization, even if manipulating Matrigel® requires skills and experience. The success of Matrigel® is also due to its biological activity that allows under normal conditions of culture the differentiation of several cell types and the formation of complex structures like vessels or mammary glands acinar structures [61,62]. Besides structural ECM molecules, Matrigel® contains soluble growth factors such as fibroblast growth factors (FGF), epidermal growth factor (EGF), and transforming growth factor-β 19 (TGF-β) and matrix metalloproteinases (MMPs) including MMP-2 and -9. The unique coexistence in an *ex vivo* substrate for cell culture of native and organized fibrous ECM proteins associated with soluble factors explains its genuine bioactivity. Concomitantly, this represents its main drawback to serve as an ECM model for 3D cell cultures. Because Matrigel® is produced and purified from an animal, there is a lack of control on its exact composition and a batch-to-batch variability of its content [63]. Moreover, the presence of growth factors in an unknown and uncontrollable amount can interfere (positively or negatively) with the intended parameters to be studied, like with the evaluation signaling molecule or a drug, and invalidate the use of Matrigel® in any experiments where the role of a growth factors in a cellular process would like to be addressed. The work of Edna Cukierman has demonstrated the dramatic changes in cell morphology in-between 2D and 3D cell culture systems with a massive reorganization of cell cytoskeleton and a modification of integrins expression [64]. This has increasingly recognized the importance of studying cells, in particular their adhesion and migration, within a 3D environment. Thus, Matrigel® should be considered with caution and with the full awareness of its limitations when it is chosen for a 3D model and so not creating a "black-box" during the switch of cell biology from 2D to 3D.

3.1.2. Biological polymers

Biological polymers were first used as a coating of tissue culture dishes to favor cell adhesion and spreading, and then incorporated into 3D materials under different forms (hydrogels, freeze-dried materials, and surface coating of bulk inorganic materials). Most of the biological polymers used in biomaterials are structural molecules derived from mammalian ECM such as collagens (type I–IV), elastin, fibronectin, laminin (mainly laminin-111), fibrin, and glycoaminoglycans (Hyaluronan, Chondroitin sulfate, and Heparan sulfate) [65]. They are classically purified from animals' ECM-rich tissues such as dermis and tendons (collagen type I, elastin), cartilages (collagen type II and GAGs), tumors (laminins and collagen IV) or directly from blood (fibrin and fibronectin). Nowadays, it is possible to obtain some of these molecules from DNA-recombinant sources. This allows to work with human ECM molecules, produced with a high degree of purity and free of many pathogens [66]. However, DNA-recombinant

production of multimeric high molecular weight molecules is still a challenge and it minimizes the number and the amount of molecules available from this source and raises dramatically their prices. Nonmammalian ECM molecules are also widely used in the design of biomaterials, mainly for their ability to self-assemble in 3D structures. Numerous materials are engineered in the form of hydrogel from chitin/chitosan (polysaccharide purified from fungi or arthropods exoskeleton), agarose or alginate (both polysaccharides purified from algae). Silk fibroin, a protein found in silkworm cocoon and spiders, is also used to produce fibrous materials [67]. Because of their origin, nonmammalian biological polymers lack many of the cell adhesion cues on their structures and should be most often supplemented with adhesive molecules or peptides to obtain a biologically active material. Their inherent abilities to form 3D materials remain their best interests. Last type of biological polymers usable in the design of 3D materials are growth factors (FGF, EGF, platelet derived growth factor [PDGF], and vascular endothelial growth factor [VEGF]) that give important biological signals to the cells and enzymes (MMPs, proteolytic enzymes) that can favor a remodeling and a progression with time of the material, like that is observed in the healing processes and during tumor invasion. However, as mentioned in the first section of this chapter, these molecules are not structural proteins and thus no 3D material can be raised from them. Nevertheless, both growth factors and enzymes represent essential molecules to reinforce the basic 3D scaffold in a relevant ECM model. For that purpose, there are strategies to incorporate these soluble molecules into a material and trigger their appropriate release at specific time points or location in the material [59].

3.1.3. Synthetic polymers

Synthetic organic polymers offer a large panel of creativity to produce 3D materials. They inherently lack basic biological activity but possess a great processing flexibility. They are easier to produce as well as purify in large quantities and finally, are free from animal contaminations. Synthetic polymers are suitable for many types of chemical modifications such as chemical grafting of adhesive peptides or incorporation of bioactive molecules and can be processed into 3D materials with many types of techniques (electrospinning, foaming, hydrogel, and sheets), some of them not bearable by biological polymers. The diversity of synthetic polymers used in biomaterials is large, including polyacrylamides, polyacrylates, polyethers (e.g., polyethylene glycol), polyesters (e.g., polycaprolactone), polyhydroxy acids (e.g., poly lactic acid, poly glycolic acid, and copolymers poly lactic-co-glycolic acid), polyfumarates, and polyphosphazenes [68]. Polyesters and polyhydroxy acids have both biodegradable properties with presence of hydrolysable bonds in their backbone, whereas polyacrylamides and polyacrylates are almost unbreakable under cell culture conditions. Chemically or genetically engineered peptides or protein-like polymers with amphiphilic or autoassembling properties offer a direct incorporation of bioactive cues into fibrillar materials. Inorganic materials such as metal (e.g., titanium, stainless, and cobalt) and mineral alloys (e.g., ceramics) are widely used as bone and dental substitutes, but their bulk and stiff structures associated with poor possibilities of chemical modifications make them rarely used as *ex vivo* engineered ECM [69]. Synthetic polymers are usually functionalized by chemical grafting of peptides which are recognized for cell adhesion such as the well-known Arg-Gly-Asp (RGD)

motif found in numerous ECM molecules (fibronectin, collagen, and vitronectin) and bind by several integrins. More specific ECM-derived peptides can also be grafted, such as DGEA and GFPGER sequences found in collagen I and IV, respectively, or IKLLI and YIGSR sequences from laminin alpha-1 and beta-1 chain, respectively. Functionalization strategies use the inherent properties of the synthetic polymers to form a 3D scaffold and to contain active chemical groups (amine, acid, or alcohol functions) sensitive to chemical reactions and so providing an ECM model with well-defined characteristics in order to answer a specific question. Synthetic polymers can also be functionalized with whole proteins such as growth factors or enzymes. Through the mediation of a peptide spacer incorporating a cleavage sequence, the release of the bioactive compounds may be triggered at the required time or progressively by the proper proteolytic action of the cells [70]. In conclusion, synthetic polymers represent an engineering solution to rationalized parameters tested in 3D model of ECM and can introduce a dynamic aspect into the system [71,72].

3.1.4. Hydrogels

Hydrogels are produced from polymers in solutions which are gelated into 3D materials that possess high water content. To some extent, hydrogels behave in the same way than proteoglycans in ECM and represent a valuable mimicry of soft-tissues ECM structures and physical properties due to its hydrated state. They are most often produced by mild, physiological conditions and, as such, are mainly compatible with cell encapsulation and culture, as water is the natural solvent for living organisms and biochemical reactions. The water content of the gels also facilitates the diffusion of low molecular weight nutriments, oxygen, and metabolic waste. Hydrogel can be produced from a large variety of polymers, including both natural and synthetic substrates, offering a large diversity of biochemical, physical, and mechanical properties. Depending on polymers characteristics, the gelation can be reversible or irreversible and triggered by different factors such as multivalent ions, chemical covalent cross-linking (including aldehyde fixatives), and physical phase-transition induced by temperature, pH, or concentration [73]. Covalent cross-linking can form hydrogel with almost all types of polymers in solutions, but the toxicity of cross-linking molecules must be considered, and furthermore, most of the cross-links are nondegradable by cells or in physiological conditions. Moreover, the gelating process can be deleterious for cells, and thus it will dictate the ways that cells are associated with the scaffold (i.e., before or after gelation). Mixing the cells with the polymers prior gelation allows a homogenous distribution of the cells inside the material, which could be more difficult to achieve when seeding the cells on an already formed hydrogel [74].

Natural polymer hydrogels can be made with collagen, fibrin, hyaluronan, alginate, and agarose. Collagen hydrogels are formed by pH neutralization of acid solutions that trigger the assembly of the collagen molecules into fibrils and fibers which will stabilize the structure of the gel. Mechanical properties of collagen hydrogels can be finely modulated by adjusting their concentrations [75]. Fibrin gels are formed following the same reaction that occurs in the body after an injury or an inflammatory response: fibrinogen is mixed in solution with thrombin, a serine protease, which hydrolyses the N-terminal peptide of fibrinogen to create fibrin

monomers that assembles into fibrils that will produce an interconnected fibrous hydrogel. Mechanical properties of fibrin gel are tuned by adjusting fibrinogen and thrombin concentrations. However, fibrin gels are highly sensitive to several proteases, which are secreted by cells, and a gel degradation regularly occurs in long-term cell cultures if no protease inhibitors are added in the culture media [76]. Hyaluronan hydrogels are formed by covalent cross-linking with hydrazide derivatives that react with the carboxylic acid groups of the polymer. They bear inherent biological properties and are susceptible for further chemical modifications which modulate these properties, such as the grafting of fibrous proteins or adhesive peptides. However, hyaluronan hydrogels are mechanically poor, and hyaluronan is more often incorporated into other materials than being the main frame of a hydrogel. Several different types of materials are produced from alginate hydrogels. Alginate polymerization is triggered by addition of divalent cations Ca2+, Mg2+, Ba2+, or Sr2+ that create bridges in-between negative charges of alginate monomers. Processing of alginate hydrogels enables an easy cell encapsulation, but a progressive diffusion and solution-exchange of divalent cation can undermine its structure. Agarose gels are formed by heating (near boiling temperature) of the solution that gelate with cooling. Different porous architectures and mechanical properties are obtained with modulation of agarose concentration.

Hydrogels can be formed with synthetic polymers including polyethylene oxide, polyvinyl alcohol or in a blended solution or copolymer association with poly lactic acid. Again, the interest of synthetic polymers to form hydrogels is the diversity and the reproducibility of materials that they are able to produce, with versatile biophysical, mechanical and biological properties. Poly ethylene glycol (PEG) polymers can be modified to allow *in situ* gelation by visible UV-light induction [77]. Photopolymerization is based on the interaction of light with photo initiators that create free radicals which react with the polymer and initiate cross-links. Compared to other type of chemical cross-linkers, photopolymerization is fast (second or minutes) and allows a spatial control of the polymerization. It works under physiological conditions and the radical species, even harsh for living species, are quickly removed by the polymerization process, making these materials more free of adverse chemical compounds as it may occur with aldehyde or hydrazide derivatives. Photopolymerization of PEG-based solutions was used to encapsulate chondrocytes that were cultured for 14 days with progressive deposition of a cartilaginous native ECM showing the biocompatibility of the process [77]. Synthetic peptides containing cell adhesion cues can also be designed for autoassembling into supramolecular structures able to form hydrogels. Engineered proteins produced by recombinant DNA can, in the same way, present adhesive domains and reactive groups such as thiols and amines for being the specific targets of polymerization reactants [72]. Finally, hydrogel-forming properties of synthetic polymers can be used to include biological polymers unable to form otherwise materials on their own and then offering a mixed inert 3D structures with biological functions.

3.1.5. Porous material

Hydrogels show a good biocompatibility due to their water content but present most often low mechanical properties, high degradation rates, and a compromised deep diffusion of large

molecules like proteins. To overcome these issues, porous materials with interconnected pore networks and surfaces or fibers to sustain cell adhesion as well as cell phenotype have been designed. These materials are discriminated in microporous scaffolds where embedded cells will mainly attach on the surface of pores of >100μm diameter and will more represent curved 2D surfaces, and in nanoporous scaffolds where pore structures are in the range of the cell diameter (roughly 10 μm) and represent more the native 3D environment of ECMs [78]. Nevertheless, microporous structures allow a more effective cell penetration and migration into the material while smaller-sized pores can represent a barrier for cell colonization of the material, and limit the cell interactions at the edges of the material [79]. This particular point of cell colonization represents one of the challenges with nanoporous materials, and definitely a limitation when directly compared with both the microporous and the hydrogel materials. Similar to hydrogels, porous materials can be prepared with both natural and synthetic polymers with a large panel of techniques including, but not restricted to electrospinning, phase separation, templating, and vapor-phase polymerization [79]. Electrospinning forms fibrillar materials with control on the fibers diameters, fibers alignment, and fibers interdistances that dictate the overall porosity of the material. A polymeric solution is injected through a capillary tube into a high electric field that forms a Taylor cone with application of the electrostatic forces. On the opposite side of the capillary, the collector of the electrospun fibers is charged or grounded. The electric field in-between capillary and collector accelerates the flow of the polymer solution that evaporates the solvent and generate coalescence of the polymers that finally form solid fibers arrays. This technique is mainly used with synthetic polymers (poly lactic/glycolic acid and poly caprolactone) but is also suitable for natural polymers such as collagen and silk fibroin [80]. Electrospinning produces thin materials not exceeding millimeters and with a high-packing density of fibers that can impair a deep colonization by cells, but intensive researches are presently conducted to overcome these issues [81]. Collagen porous materials can be formed by thermally induced phase separation leading to the production of the so-called "collagen sponges" due to their high porous structures. Acidic collagen solutions are frozen which induces a phase separation of collagen molecules from the water-based solvent that is then eliminated by lyophilization. Materials are then made of dense collagen walls with unstriated microfibrils [57] or with native cross-striated fibrils [82]. Size and interconnectivity of the pores can be modified by modulation of the length and temperature of the phase separation, by modulation of the collagen concentration or by mixing the collagen solutions with other natural polymers such as GAGs [82] or with synthetic polymers such as poly lactic acids. Freeze-dried collagen materials have weak mechanical properties and are often strengthened by the addition of GAGs, or cross-linked by chemical species (aldehydes) or dehydrothermal processes [83].

The various polymers and processes presented above enable the engineering of different types of 3D materials designed to study *ex vivo* a large variety of cellular phenomenon such as cell differentiation, drug response, tumor formation, cell migration, cell morphology and cytoskeleton organization, cell death and proliferation, tissue architecture, and coculture behavior of cells [84]. For that purpose, 3D materials are built either to mimic ECM composition, ECM organization, or ECM mechanical properties. We present here examples of such engineered

3D models of ECM with description of the material characteristics and cellular outcomes observed.

3.2. Three-dimensional models mimicking ECM composition

Recreating *ex vivo* the ECM composition in 3D materials offers the possibility to address in a relative more biologically relevant environment the specific function of one or several of its components on several cell mechanisms. It can be used to recreate cell adhesion and cell migration or to offer adhesive cues that are specially organized.

A minimal system has been designed with a gelatin hydrogel mixed with PEG and functionalized with RGD and PHSRN adhesion peptides to evaluate the inflammatory response of adhering immune cells [85]. Monocytes are circulating immune cells which are among the first cells to react at the site of an injury. Once they have migrated and attached to the site of aggression, they start to secrete cytokines and ECM remodeling enzymes. The hydrogel was built with PEG-diacrylate (PEG-da) photopolymerizable polymers mixed with gelatin monomers covalently grafted with PEG-RGD and PEG-PHSRN, two amino acid sequences found in fibronectin and recognized by several types of integrins. The hydrogel mixture was made with different ratios of Gelatin-PEG-peptides compared to the PEG-da polymers for the formation of hydrogels with different adhesive peptide densities (from 30 to 50% of gelatin-modified polymers). The study showed that monocyte adhesion and cytokine secretion reached a plateau when the gelatin-modified polymers concentration was above 40%, indicating that rising adhesive-peptides densities did not improve their accessibility for monocytes. The nature of the ligands has also an influence on monocytes behavior, with a higher level of MMP-9 expression when hydrogels are incorporating PHSRN peptides, while more interlekin-1β was secreted in RGD hydrogels. These data suggest a specific monocyte inflammatory response for each of these adhesive cues. This model can be developed and used, for instance, to study the different parameters that initiates the inflammatory response at the site of injury.

Metastatic cancerous cells escape the original tumor site to attach and invade a distant organ. The switch of the cellular microenvironment from primary tumor site to the metastatic organ can affect the metastatic cells response to therapeutics which was originally designed to act on the original cancerous cells in the specific microenvironment of the primary tumor site. Blehm and coworkers have engineered a 3D model of the ECM of the perivascular niche of the brain, a metastatic target of cutaneous melanomas, to address the effect of anti-MAPK therapeutics (ERK inhibitor and Darafemib) on metastatic cutaneous melanomas cells lines [86]. Cells were seeded in a huyaluronic acid-PEG-diacrylate hydrogel supplemented with purified full-length proteins, laminin-111 and fibronectin, or with RGD-cyclic peptides. Diminution of Darafemib drug efficacy is reported for one melanoma cell line in hydrogels supplemented with fibronectin, whereas laminin-supplemented hydrogels protected against ERK inhibition. With another cell line, drug efficacy was weakened for both therapeutics only on laminin-supplemented hydrogels, reinforcing the importance of the cellular microenvironment for drug testing.

Natural polymers, including ECM proteins, GAGs, and growth factors, with a high degree of purity were successively assembled to form molecularly defined materials evaluated by subcutaneous implantation as well as in a cartilage reconstruction model [87]. Collagen type I solution purified from bovine Achilles tendon was mixed with insoluble elastin, purified from equine ligamentum nuchae and homogenized in a grinder before molding and freeze-drying of the solution. This process formed a porous scaffold (collagen sponge-based material) with both collagen and elastin fibrils. Porosity of the material was controlled by the freezing temperatures, as higher rates of freezing provided materials with smaller pore diameter. Complexity and ECM mimicry of the material is extended by incorporation of chondroitin sulfate or heparan sulfate. Collagen-elastin material is soaked with either chondroitin or heparan sulfate solution, and GAGs are covalently bounded to the collagen-elastin fibers by carbodiimide cross-linking. The amount of GAGs effectively grafted to the materials is a function of GAGs structures and their respective reactiveness to the cross-linker, with a final grafting of 10% of the original heparan sulfate solution and 6% of the chondroitin sulfate solution. Finally, growth factors such as bFGF and VEGF were loaded into the Collagen-Elastin-GAG material to form a valuable model of the different classes of natural polymers found in ECMs. Incubation of growth factor solution with the material only allowed a partial adsorption as growth factors are rapidly lost after a few hours of cultures. However, heparan sulfate bears native biding sites for bFGF, enabling a more efficient loading of bFGF into the material and a progressive and extended release during cell culture. Two-weeks after subcutaneous implantation in rat, collagen-heparan sulfate materials showed a higher vascularization than collagen alone. After 10 weeks, collagen materials loaded with bFGF exhibited a lower cell invasion and vascularization than the collagen-heparan sulfate-bFGF material. Even evaluated *in vivo*, where cells are in a more positive environment than cultured *in vitro*, this study showed the possibility to engineer a complex material reproducing the different class of natural polymers found in ECMs. A critical parameter for the use of such porous material as an *ex vivo* ECM model is the possibility to seed evenly the cells within the material as compared to hydrogel-embedding. However, this can be overcome by creation and control of an open and interconnected porosity within the material.

3.3. Mimicking ECM organization and mechanics

Two-dimensional cell cultures on TCPS have been coated with ECM molecules to give more biologically relevant ligands for cell adhesion, spreading, migration, and expression of phenotypes. This has partly raised the idea that instead of coating, ECM-derived adhesive cues should be displayed in 3D to be more close to the cell environment, and we have presented above 3D materials that recreate ECM composition. However, this approach does not generally consider the reconstruction of the spatial organization of ECM that is of equal importance as the ECM composition [88].

The study of collagen-rich connective tissues such as skin, tendons, and bones by polarized-light microscopy and TEM revealed that type I collagen is highly structured, with a spatial geometry specific for each tissue. This spatial organization of collagen I in tissues can be reproduced *in vitro*, using the autoassembling properties of collagen molecules in acidic

solutions which will self-organize in different liquid-crystal organizations dependent on the collagen concentration [89]. Regulated evaporation of solvent raise progressively the collagen concentration and modify collagen molecules organization. These organized molecular textures are stabilized by neutralization of the pH of the solution and further triggering the assembly of soluble collagen molecules into insoluble cross-striated native collagen fibrils [54,90]. The supramolecular assembly of collagen molecules into collagen fibrils also induce the transition from the original collagen solution to a solid hydrogel structure (i.e., so-called "sol/gel transition") that retains the molecular tissue-like organization of collagen molecules. This leads to materials with collagen fibrils architecture which may mimic those observed in tendon, skin, and bones [21,91] but that also reproduce the mechanical features of collagen-rich tissues from dermis to bone, depending on the collagen concentration of the material [75]. Ordered collagen hydrogels have been used to study myofibroblasts migration as a model of wound-healing [92], behavior of human osteoblasts in long-term *in vitro* cultures [57,93] and also as a model to study the kinetics of noncollagenous ECM proteins secretion in relation to the biomineralization processes [94]. However, tissue-like organization of collagen fibrils resulted in mild (20–40 mg/mL) or high (>80 mg/mL) collagen concentrations, which mainly restricted cell seeding to the surface of the materials.

In the depth of a tissue, different ECM organization could be found, like in the skin with different ECM compositions, organizations, and properties of epidermis and dermis, or in articular cartilage from the GAGs-rich upper surface to the deeper interface with the underlying bone tissue. A layer-by-layer approach, based on successive freeze-drying of different natural polymer solutions to modulate material organization, was used to recreate the discrepancies in the ECM organization observed in the cartilage tissue [95]. The first layer intends to recreate main features of the deeper osteochondral tissue architecture. Collagen type I in acidic solution is mixed with hydroxyapatite, the mineral phase found in bones, and freeze-dried at a constant rate to obtain a dense porous structure. The subsequent porous material was cross-linked with carbodiimide to strengthen its structure. The second layer mimics the bone–cartilage interface and is made with an equal amount of mixture of the collagen type I, main collagen of bone tissue, and of the collagen type II that is the main collagen of cartilage tissue. Collagen I and II mixture is supplemented with hydroxyapatite at a final concentration five-times lower than on the first layer. This mixture is poured on the surface of the first layer and freeze-dried to finally form a two-layer material, with two distinct compositions and porous structures. The last layer mimics the articular cartilage tissue. This layer is made from a mixture solution of 25%/75% of collagen type I and II, respectively, and then further supplemented with hyaluronic acid to recreate the GAGs content of the articular cartilage. The last layer is again freeze-dried with time-prolonged freezing and drying steps to ensure the proper porous structure. The overall material is strengthened by a dehydrothermal process that creates amine-based cross-links. This process forms a material with three specific porous structures as well as a molecular composition and with the opened and interconnected porosity that allows an effective cell colonization of each of the three layers.

The specific composition and spatial organization of the ECM dictate its overall mechanical properties, that cells are able to sense through their integrin receptors and their cytoskeletons.

On 2D surfaces, it has been shown that cells are responsive to surface rigidity and that it influences the commitment of mesenchymal stem cells toward differentiation in a specific lineage [96]. Again, surface stiffness applies forces which are unevenly distributed on the cells (i.e., only located at the cell-surface interface). Developing a 3D material with tuned and controllable mechanical properties will generate a more biologically relevant environment to evaluate the role of ECM mechanobiology on cells functions and differentiation processes. To study the influence of mechanical stiffness on mesenchymal stem cells differentiation, a series of alginate gels with elastic modulus ranging from 2.5 to 110 kPa has been developed [97]. Mechanical properties of alginate gels are modulated by the percentage of alginate polymers in the final hydrogel. Because alginate is not sensitive to the degradation of hydrolytic enzymes of mammalian cells, the elastic modulus of the mechanical properties of the material are expected to remain constant all along the study (7 days of cell culture). RGD-peptides are covalently grafted to alginate polymers prior to hydrogel formation to give to the cells adhesive cues. The more rigid materials trigger mesenchymal stem cells differentiation toward bone lineage with an expression of the bone-related molecular markers such as alkaline phosphatase and osteopontin after 7 days of culture. On the other hand, alginate gels with the lower elastic modulus (softer material) triggers an accumulation of oil-droplets into stem cells, indicating adipose tissue differentiation. The density of RGD-peptides incorporated into these materials did not modify the cell fate related to the elastic modulus of the material, but induce a higher level of expression of the lineage markers for both bone and adipose-committed cells.

3.4. Conclusion: toward a gold-standard of 3D model of ECMs?

As mentioned at the beginning of this section, the ECM-derived Matrigel® represents currently the most often used material for 3D experiments in cell biology. Despite that Matrigel®-related drawbacks are of importance, Matrigel®is a widely and available model to investigate many fundamental questions in cell biology, from cell adhesion and tumor formation, to drug testing. We have presented in this chapter a large panel of techniques, methodologies, and engineering processes that allow the exploration of ECM organization and permit to recreate *ex vivo* some of their key features. At the conclusion of this chapter and after the review of several studies investigating various 3D materials, it appears that no material can represent the unique and ideal answer for all cell investigations in 3D [63]. A modular approach should be taken by rationalizing the biological question to be studied and the parameter of ECM intended to be recreated. Nevertheless, more and more complex materials are engineered that will finally be able to mimic simultaneously several key factors of ECM composition, architecture, or mechanical properties, and so enabling investigation of multiple parameters for cell biology experiments. An important drawback with engineered 3D materials is to create a "black-box" where undefined and uncontrollable parameters may influence the cellular outcomes to be investigated. To avoid part of this problem, rigorous attention should be paid on the purity of polymers used to build the material, in particular with biological polymers. The development of DNA-recombinant production of ECM proteins can overcome this problem, even if this will raise ultimately the cost of the final material. The structural characteristics of the final material (porosity, polymers distribution, and fibrils diameter) should be consistently reproducible and addressed. To do so, an "easy," meaning straightforward, process of the material should be

sought and preferred rather than a more complex multistep fabrication process. Biomaterial scientists propose continuously new design and approaches to engineer *ex vivo* ECMs. The production of a gold-standard material may become possible for a specific biological question. It might happen with the existence of a deep and intelligible dialog in-between material scientists, whom brings engineering strategies, and the cell biologists, that implement the material design to mimic the biological process that has to be investigated *ex vivo*. This collaboration may result in major advances for science and medicine.

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