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.

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

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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