Biomacromolecules

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Review

3D Extracellular Matrix Mimics: Fundamental Concepts and Role of Materials Chemistry to Influence Stem Cell Fate

Julien Nicolas, Sofia Magli, Linda Rabbachin, Susanna Sampaolesi, Francesco Nicotra, and Laura Russo*

Cite This: *Biomacromolecules* 2020, 21, 1968−1994

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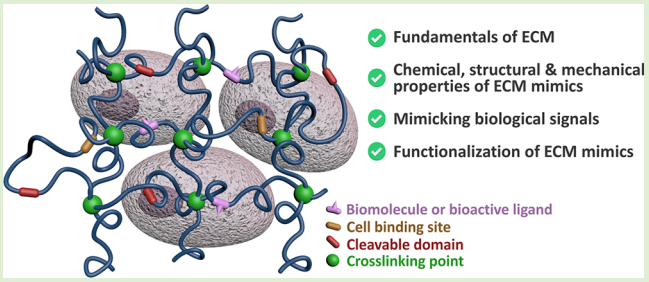
ABSTRACT: Synthetic 3D extracellular matrices (ECMs) find application in cell studies, regenerative medicine, and drug discovery. While cells cultured in a monolayer may exhibit unnatural behavior and develop very different phenotypes and genotypes than *in vivo*, great efforts in materials chemistry have been devoted to reproducing *in vitro* behavior in *in vivo* cell microenvironments. This requires fine-tuning the biochemical and structural actors in synthetic ECMs. This review will present the fundamentals of the ECM, cover the chemical and structural features of the scaffolds used to generate ECM mimics, discuss the nature of the signaling biomolecules required and exploited to generate bioresponsive cell microenvironments able to induce a specific cell fate, and highlight the synthetic strategies involved in creating functional 3D ECM mimics.



1. INTRODUCTION

In vitro cell cultures are widely used in different key biomedical applications such as cellular and organismic biology, drug discovery, or regenerative medicine. To date, most of the cell cultures and cell-based assays have been performed in 2D layers on polymer or glass dishes, yielding the fundamental knowledge of the biological phenomena that are responsible for healthy and pathological states. Until recently, cell biology principles, drug activities, cell responses to endogenous and exogenous perturbations, mechanisms involved in cell development,[1] and tissue morphogenesis have been determined by 2D cell culture studies. Despite 2D cell cultures allowing the study of the correlation between cell functions and some components of the microenvironment,[2] such a bidimensional cell environment is obviously unnatural. Consequently, it likely induces different cell behaviors with respect to the natural three-dimensional (3D) microenvironment, lacking most of the interactions occurring in the native 3D tissue. Indeed, in 2D cultures, many cell types develop different phenotypes and genotypes with respect to what happens *in vivo*.[3,4]

Cell macro- and microenvironments are involved in the modulation of complex signaling pathways that direct cell fate. It is now clear that, due to the interactions with extracellular matrices (ECMs), phenotype can supersede genotype.[5,6] Consequently, if the cell microenvironment present *in vivo* can be mimicked *in vitro*, it is possible to regulate the cell behavior, influencing cell survival, shape, migration, proliferation, and differentiation, thus leading to the morphology and physiology that occur *in vivo*.[7] This mimicry can be generated by 3D cell aggregates or as suspensions of cells in 3D hydrogels made of ECM proteins.[8−11] Different environmental factors contribute to the change of behavior of cells in 3D cultures versus 2D monolayers. Cells and extracellular matrices are characterized by an "outside-in" as well as an "inside-out" signaling process, dynamically modulated by molecular and geometrical requirements. Changes in the ECM are detected by cell receptors, which provide signals that finally determine gene expressions.[8,9,12] Differences in the composition of the extracellular space surrounding the cells influence both the topology of cell−cell and cell−matrix contacts at the cell surface and the distribution of the signaling biomolecules.[13−15] For example, a tensioned ECM will induce a stretching of both the cytoskeleton and the nucleus of the cells, while compressed ECM will result in altered local charge density and ion concentrations, which affect the ion channels.[16] Cell growth and differentiation, both *in vitro* and *in vivo*, are strongly influenced not only by mechanical but also by biomolecular stimuli.[17−21] Cells cultured in 3D are characterized by different interactions with both the ECM components and the other cells; these interactions influence cell organization and cell regulatory pathways.[22] Starting from these observations, it is clear that an accurate study of the different mechanisms

Received: January 11, 2020
Revised: March 29, 2020
Published: March 31, 2020

https://dx.doi.org/10.1021/acs.biomac.0c00045
Biomacromolecules 2020, 21, 1968−1994

involved in disease progressions and the design of efficient cell culture models for biomedical purposes require the use of 3D ECM mimics. In this Review, we will first present the fundamentals of ECM and then cover the most relevant findings on ECM mimics developed as 3D cell constructs, with emphasis on synthetic strategies to control the morphology, the physical properties, and their functionalization with the different biochemical cues required for cell fate regulation.

2. ECM AND CELL MICROENVIRONMENT: TAKING INSPIRATION FROM NATURE

For a long time, the ECM has only been considered for its structural role, providing stability and support to surrounding cells. However, it is now well-established that it has also fundamental functional roles, notably by producing a myriad of dynamic signals that influence the cell fate.[23] The biochemical and structural variability of the ECM, together with its dynamic and multifactorial nature, exert a functional role. Despite crucial advances in the field of ECM research, more work is needed to fully understand the different mechanisms regulating the cell−ECM interaction.

Each tissue has a certain amount and spatial distribution of macromolecular components and biochemical motifs, which are strongly related to some specific functions.[21,24] Unravelling the effects of specific ECM components on cell fate and clarifying recognition mechanisms are complicated by the fact that the ECM structure and composition are not static. The cells themselves secrete ECM components, such as interactor proteins and tethered and soluble factors, which dynamically remodel the ECM.[25] This dynamic environment must be analyzed from both physical and biochemical point of views. The ECM physical properties modulate several adhesion-related cell functions, such as cell cycle, cell adhesion, cell proliferation, and cell polarity and migration. The ECM biochemical composition, made of different signaling biomolecules (effectors), also influences the cell fate through interactions with cell-surface receptors, resulting in bidirectional signaling cascades that control cell development.[26] These properties are not independent; they are indeed interconnected and related to the ability of the ECM to communicate with cells through molecular recognition events, mechanical transduction, electrical sensing, and soluble molecule transfer.

ECM changes in pathological states may result from (a) variations of the composition and/or organization of the ECM network due to either altered synthetic processes or eventual degradation of one or more ECM components, (b) variations of an individual ECM component due to altered post-translational modifications, or (c) different spatial arrangements via covalent or non-covalent modifications.[27] It is now clear that changes in the nature, concentration, and topology of specific ECM components strongly impact the biochemical and morphological nature of the ECM, leading to a disorganization that results in a failure of homeostasis and function of the organ.

2.1. ECM Composition.

The ECM of mammals is composed of about 300 interconnected proteins and is well organized in 3D structures.[28] In 1984, the term "matrisome" was proposed by Martin and co-workers to obtain a picture of the functional components of the basal membrane. This definition has then been extended to the structural proteins of the ECM.[29] On the basis of this matrisome classification, the ECM comprises several components, among which include fibrous proteins, glycoproteins, and proteoglycans. Beyond the core matrisome, other groups of proteins are also associated with the core constituents, modulating both the ECM structural organization and biochemical role. These ECM-affiliated proteins include, for example, mucins, lectins, semaphorins, and plexins. Here, we will focus our attention on some examples of ECM components by looking at specific molecular interactions and recognition events that occur between components and other less known protein partners.

2.1.1. Collagen. Collagen is one of the major ECM constituents and represents the most abundant class of proteins in mammals (~30% of the total). Collagen functions are linked to many biological mechanisms involved in homeostasis maintenance and tissue development.[30−32] It was long believed that the main function of collagen was "just" structural, but now it is clear that it has also a role at the biomolecular level, as collagens dictate differential cell signaling mechanisms, spanning from cell adhesion and survival[33] to cell differentiation[34] and paracrine signals induction.[35,36] To date, more than 28 different collagen forms have been identified, which vary on the basis of tissue and cell specificity, and exert a structural role for cell adhesion. Each collagen form is made of homotrimers and heterotrimers that are composed of three α-chains basic motifs. All forms are characterized by a well-known triple helix structure, a right-handed helix of three α-chains. The chains contain one or more regions presenting the repeating amino acid motif Gly-X-Y, where X and Y can include any amino acid. The rod-like domain of the protein is responsible for the self-assembling and the interactions with cell receptors and other components of the ECM.[30] Because of its involvement in major tissues and organs in the body, collagen is one of the most used biopolymers in tissue engineering and 3D cell models.[36,37] Despite the advantageous *in vitro* and *in vivo* bioactivity of collagen-based hydrogels and biomaterials, the major issue for the clinical translation and the therapeutic use still remain related to the fact that collagen is of animal origin (i.e., bovine, porcine-derived collagen type I), with potential pathogenic content (disease transmission).[38] To overcome this limitation, recombinant methods are under study to produce controlled and safe collagen or derived materials.[39−41]

2.1.2. Non-Collagenous Glycoproteins. Several families of proteins with diverse origins and functions constitute the noncollagenous glycoproteins of the ECM. Among them, adhesive glycoproteins are a class of ECM macromolecules that exist in several variant forms, with multiple binding domains able to interact with collagen and proteoglycans, and to bind cell surfaces.[31,42] Representative adhesive glycoproteins are fibronectin (FN), laminin (LM), vitronectin (VN), thrombospondins (TSPs) and tenascin (TN). Most of the ECM glycoproteins interact with integrins and other receptors expressed at the cell surface, and with collagen or other components of the ECM.[43] Their role and differential expression in pathological and healthy conditions span across a wide range of tissues and between different cell populations. Here we provide just a brief overview on the structures and functions of FNs and LMs families, employed and currently under study as glycoproteins involved in stem cell modulation.

Fibronectins represent one of the main family of ECM glycoproteins. Different fibronectins have been extensively studied for their multiple roles in dictating stem cells adhesion, cell fate and cell−ECM interaction.[44,45] For this reason,

https://dx.doi.org/10.1021/acs.biomac.0c00045
Biomacromolecules 2020, 21, 1968−1994

fibronectin is often employed as coating for cell culture in microfluidic devices,[46] in 2D cultures[46] or in 3D scaffolds.[47,48]

Another important class of ECM glycoproteins are represented by LMs. Fifteen different LMs are known today as major components of basement membrane (BM).[49] LMs exploit their multifunctional roles in the ECM with the other macromolecular components, where they are involved in cell adhesion, differentiation and control of cell function. The multiple role of LMs is linked to the structural organization and the posttranslational modifications. Thanks to their heterotrimeric structure, characterized by the interaction between α-chain, β-chain and γ-chain, LMs can link and entrap growth factors and expose oligosaccharides involved in cell signaling.[50,51] LMs are also involved in the physical organization of tissue areas by modulating the interaction between cells and cell−ECM and in neurites outgrow in the nervous system.[51−54] As for fibronectins, also LMs were employed in the entire form or as motif as coating, 3D scaffolds components or hydrogel to mimic ECM composition and to induce specific cell fates and functions.[55−62]

2.1.3. Proteoglycans and Glycosaminoglycans. Proteoglycans and glycosaminoglycans (GAGs) constitute another important class of biomacromolecules of the ECM. Proteoglycans are composed of a core protein covalently linked to a long, linear polysaccharide, made of disaccharidic, anionic GAGs repeating units.[32,43,63,64] Proteoglycans are classified into subtypes on the basis of: (i) the structure of the GAG chains, their distribution and density along the core protein; (ii) their cellular or subcellular location and (iii) their genetic homology. The most representative GAGs are keratin sulfate, chondroitin sulfate, heparin sulfate, dermatan sulfate and hyaluronan. The primary biological function of proteoglycans comes from their hydrodynamic and swelling properties. Indeed, GAGs bind water, thus providing hydration and compressive resistance. It is now well recognized that GAGs are involved in different biological processes such as tumor progression, angiogenesis and cell development.[65] Polysaccharide-based biopolymers are key molecules in synthetic ECM, due to their ability to interact with other ECM components and cell-surface receptors. Examples of proteoglycans extensively expressed in ECM, and involved in the control of cell fate, are heparan and chondroitin sulfate proteoglycans (HSPGs and CSPGs), decorin, byglican and versican. Heparan and chondroitin sulfate proteoglycans, expressed in ECM and in stem-cell niches, modulate their action by interacting with other ECM components, dictated by the molecular weight and the sulfation pattern. Their biomolecular roles are also integrated with the stiffness of the hydrogel-like structure of the niche. Decorin and byglican are both leucin-rich proteoglycans with a pivotal role in ECM regulation and signaling.[66] They show differential expression of sulfated oligosaccharides depending the tissue in which they are expressed (i.e., chondroitin sulfate in bone, dermatan sulfate in soft connective tissues[67]). Decorin interacts with ECM components, growth factor and multiple cell-surface receptors.[68] The interaction of decorin with collagen, VEGFR2 and EGFR is involved in stem cell regulation and differentiation for various tissues and organs. In particular, decorin showed differentiation potential in the hematopoietic niche, in neuronal differentiation and in kidney and tendon regeneration. Byglican is also important for collagen integrity and functional structure, and cell−ECM interaction.[69]

2.2. Biochemical and Structural Regulation through ECM Components and Cell-Surface Receptors. Most of the studies on ECM architecture have been focused on macromolecular organization of its components. However, its role is also strongly influenced by the many molecular interactions with cell-surface receptors.[24−26] One of the most characterized functions of ECM is to provide an adhesive and structural substrate to which integrins and other adhesive cell receptors can bind. These interactions are involved in the activation and regulation of pro-survival signaling cascades. Moreover, other bioresponsive molecules, either tethered or in soluble form, are able to provide signals that modulate not only cell adhesion, but also cell differentiation and cell development through cell−cell and cell−ECM interactions.[65] It has been demonstrated that these interactions are mediated by interactions between ECM components and both soluble and cell-surface receptors.[70,71] Yet we still have an incomplete vision and understanding of these interactions, because of their complexity and their synergistic or antagonistic interplay *in vivo*. Furthermore, the structural features, mechanical assembly and physical stimuli of the ECM in general are synergistically involved in cell microenvironment regulation. The ECM is dynamically produced by cells in the tissue environments. The cells are able to release differential components in relation to the nature of the tissue, the age of the individual and the health of pathological state. On the other hand, the properties of the ECM in terms of molecular composition and stiffness, can influence the cell behavior.[72,73] The microenvironment components are produced by the different cell populations, the matrix itself is then able to take an active role, by regulating the production of new ECM and consequently modulate the cell fate, using different biochemical and mechanical pathways.[28,31,74−76] Several studies have studied and partially characterized the functional role of ECM in the regulation and the development of the stem-cell niche. We know that both embryonic and adult niches are related to ECM biochemical and physical interactions.[6,66] The knowledge of factors that regulate ECM production in stem cells niche has a tremendous potential for tissue regeneration and to understand the cause of pathological events. For instance, mesenchymal stem cells (MSC) in 3D collagen hydrogels produce paracrine and angiogenic factors used in the remodeling of new synthesized ECM.[35] In pathologies such as tumors, inflammation, fibrosis, different ECMs[77,78] in terms of component gradients,[79] post-translational modifications,[80,81] and ECM affiliated proteins are generated by cell populations.[82−84] In summary, the biochemical composition and mechanical stretch of the microenvironment are the result of a dynamic process that actively participates to the regulation of cell functions and physiology.[85,86] The mechanical properties of ECM also influence the cell fate. The stiffness of the ECM is heavily involved in cell differentiation and viability, and the changes in stiffness are not just a consequence of pathological states, but they also play an interactive role in tissue homeostasis or in disease progression.[87−89]

2.2.1. Receptors Involved in Cell−ECM Communication. Integrins are the most studied cell receptors. They are involved in cell adhesion and control many cell functions. They perform their actions by binding ECM glycoproteins such as FN, LM, and collagen, among others.[90] The specificity of integrin-ligand interaction is dictated by different integrin isoforms of their α and β chains. It is now clear that integrins mediate cell adhesion by multiple interactions with ECM molecules. There

https://dx.doi.org/10.1021/acs.biomac.0c00045
Biomacromolecules 2020, 21, 1968−1994

is evidence that this mechanism is also involved in cell deregulation processes, such as cancer progression and inflammation.[91] In addition to adhesion events, integrins are also involved in signaling processes, like regulation of stress transmission and bidirectional signaling.[92] The rapid and efficient transduction pathway mediated by integrins is known as "Inside-Out" signaling and it is involved not just in cell adhesion or migration, but also in many pivotal cell processes that affect also cell viability and differentiation by regulation of different signal pathways.[93,94]

Integrins' activity is mediated by other cooperative nonintegrin receptors, and by post-translational modifications mainly phosphorylation and glycosylation. Furthermore, a wide range of nonintegrin putative receptors exploit their activities by interaction with the cell microenvironment, but a limited number of studies were focused on the role and molecular recognition processes of these receptors.[32,43] Nonintegrin receptors include, among others, transmembrane discoidin domain receptors, dystroglycan, syndecans, CD44, and lectins; all of them being involved in cell−ECM communication and in cell fate regulation.

Discoidin domain receptors (DDR) DDR1 and DDR2 are members of the receptor tyrosine kinase family. They can bind different collagen types playing important roles in embryo development. DDRs link distinct amino acid sequences of collagen and are involved in ECM remodeling, influencing cell survival, migration, proliferation and differentiation. It has been observed that the deregulation of DDRs functions is associated with various human diseases progressions, such as cancer, fibrosis and arthritis.[95]

Dystroglycan is a glycosylated receptor for ECM proteins such as the basement membrane proteins LM, agrin, and perlecan, and the trans membrane proteins neurexins. The interaction of this receptor with ECM proteins is glycosylation-dependent; the binding likely involving the carbohydrate side chains of dystroglycan.[96] Mutations in dystroglycan, or its associated proteins, result in various forms of muscular dystrophy, due to the loss of the connection with the basement membrane surrounding the muscle cells.

Syndecans are a family of transmembrane proteoglycans composed of a cytoplasmic domain, a hydrophobic membrane domain, and an extracellular domain. Syndecans are able to bind not only FN, collagen, and TSPs but also β fibroblast growth factor (βFGF). Colocalization of both ECM molecules and growth factors at the cell surface makes syndecans unique molecules capable of assembling signaling complexes in combination with other receptors and interacting with several ligands, including ECM glycoproteins.[97] Tissue morphogenesis and cell specialization are induced by the expression of different syndecans.[98,99] Syndecans act as cell-surface receptors, providing a signal cascade inside the cell via the presentation of a ligand to other receptors upon ectodomain shedding and as soluble effectors. In conclusion, syndecans regulate the cell fates in terms of adhesion, proliferation, migration, and differentiation.[100]

Glycoprotein I (CD44) is another nonintegrin receptor widely studied for its differential expression during cancer progression and in inflammation. CD44 is a multifunctional *N*- and *O*-glycosylated protein present at the cell surface, which interacts with GAGs.[101] The affinity of CD44 for GAGs is linked to post-translational modifications, like glycosylation, which are specific to the cell line and the growth conditions. CD44 regulation has a pivotal role in several cellular processes, including tissue development, neuronal axon guidance, immune regulation, and hematopoiesis. Interestingly, CD44 can also trigger hyaluronan metabolism and can be itself involved in the regulation of the pericellular hyaluronan matrix, thereby providing another mechanism by which adhesion and deadhesion to the ECM is influenced.[102]

Lectins are carbohydrate-recognizing proteins expressed in the plasma membrane or secreted in the extracellular space. They also interact with the ECM itself and influence its properties. Lectins are involved in microenvironment remodeling, a process that occurs via an interaction with cell receptor or with ECM components.[28,103] There are several types of lectins, but the most representative ones involved in ECM−cell interaction and ECM remodeling are classified as C-, I-, P- and S-type. All lectins are characterized by a well-defined carbohydrate recognition domain (CRD), and each one has a specific recognition ability toward a certain type of glycan and has a different role in cell development.[104] C-type lectins, involved in cell adhesion,[103] specifically recognize carbohydrates in a calcium-dependent manner, which means that the sugar-receptor interaction occurs via complexation of calcium ions. I-type lectins are also involved in cell adhesion,[103] whereas P-type lectins regulate intracellular shuttling and ECM degradation. S-type lectins, also termed galectins, are involved in numerous biological functions, such as promotion of cell−cell adhesion, induction of metabolic changes, and even apoptosis.[105−108] Integrin and lectin complexes act as cell modulators able to control signaling cascades through cytoskeletal components. Galectins are one of the most representative families of lectins, classified into galectin-1 and galectin-3 subgroups. They are involved in a variety of cellular events such as cell adhesion and migration,[109] cell growth, and cell differentiation.[110,111] Galectin-1 is secreted in the extracellular medium by many cell types, including malignant and mesenchymal stem cells; the content of galectin-1 in the ECM affects cell development.[112] Galectin-3 and galectin-8 are considered as matricellular proteins with both pro- and anti-adhesive activities, depending on the cell type or their extracellular concentration.[113,114] Galectin-3 is also able to modulate the adhesion of different cell types to the ECM.[115] Galectin-8 has high affinity to cell receptors, in particular integrins and CD44 variants. As a general starting point, it is well-established that the *N*- and *O*-glycans and their protein interactors have key physiological roles, not just in cell−cell communication but also in cell−ECM interactions. It is now clear that the comprehensive understanding of these events can spread light to the biomolecular basis of numerous pathologies.[115−117]

Glycoprotein IV (CD36) is a glycosylated 88 kDa integral membrane protein, also commonly referred to as GPIV or FAT (fatty acid translocase). CD36 is a scavenger receptor operating in high-affinity tissue uptake of long-chain fatty acids (FAs). Under excessive fat supply, CD36 contributes to the accumulation of lipids and to metabolic dysfunction. The glycoprotein CD36 is expressed on different cell types, including endothelial cells, macrophages, and myocytes. It has a high affinity for collagen-I and IV, and these interactions are involved in cell adhesion and signaling, modulating multiple cell functions in different cell types.[76,118]

A plethora of physiological functions of the extracellular compartment have been assigned to ECM receptor proteins, and more are yet to be discovered and characterized. Table 1 reports the most representative receptors involved in cell−

https://dx.doi.org/10.1021/acs.biomac.0c00045
Biomacromolecules 2020, 21, 1968−1994

Table 1. Representative Receptor Involved in Cell−ECM Communication

ECM receptors	ECM interactors	cellular functions	references
integrins	FN, LM, collagen, soluble galectins, and several matrix glycoproteins	cell adhesion, regulation of stress transmission and bidirectional signaling, and angiogenesis	90, 91, 119
discoidin domain receptors (DD1 and DD2)	different fibrillar collagen types	embryo development, cell migration, cell survival, proliferation and differentiation, and remodeling of extracellular matrices	95, 120
syndecans	collagens, FN and TSP, βFGF, VEGF, βTGF, and PDGF	growth-factor receptor, activation, cell-adhesion, cell−cell communication, cell proliferation, differentiation, and adhesion and migration.	98−, 100, 121
dystroglycan	LM, agrin, and perlecan in basement membranes and neurexins transmembrane	cell development, basement membrane formation, epithelial morphogenesis, membrane stability, cell polarization, and adhesion and migration	122, 123
lectins	integrins, FN, LM, TSP and VN, and other glycoproteins and GAGs	cell adhesion and migration and cell growth, apoptosis, and differentiation	110−, 112, 124, 125
CD44	GAGs	cellular motility and cell−cell and cell−ECM adhesions	101, 111, 126
CD36	collagen	fatty acid uptake, cell adhesion, and angiogenesis	127, 128

ECM communication, together with their ECM interactors and the cellular functions exerted.

2.2.2. Recognition Motifs and Bioactive Molecules. The interaction between ECM components and their receptors is finely controlled at the molecular level by different signals and recognition motifs. The ECM directs essential morphological organization recognition motifs that bind growth factors, bioactive molecules, and cell-surface receptors.[129,130] Exposure of these bioactive signaling molecules, by direct interaction with cell receptors or by indirect modification of ECM components affecting their structural organization, has shown to be an important regulatory factor in ECM remodeling and cell processes, including cell-adhesion or angiogenesis. The bioavailability of these molecules is spatially and temporally regulated and can elicit signal transduction events or regulate gene transcription. One of the most explored recognition motifs found in the ECM is the adhesion peptide Arg-Gly-Asp (RGD). This sequence, ubiquitous in ECM-proteins, interacts with integrins, thus promoting cell adhesion.[43] Different peptides and peptidomimetics containing the RGD sequence, linear or constrained in a cyclic structure, have been generated and extensively studied for their capacity to regulate cell adhesion and migration. Some of them have also found applications as therapeutic agents in cancer research, in tissue engineering, or even as diagnostic agents.[131] Other well-known examples of ECM peptide sequences that are able to stimulate cell adhesion through integrin interaction are the LM-derived motifs IKVAV and YIGSR and the collagen six amino acid motif GVMGFO (O = hydroxyproline).[132] Other short peptide sequences, mainly derived from circulating proteins, are currently being studied for their capacity to govern cell fates, and the research of new peptide sequences is still ongoing.[133−135]

Many ECM proteins possess binding sites for both cell adhesion motifs and growth factors, allowing controlled local availability of growth factors to cell receptors. Growth factors can be tethered to ECM components or released as soluble factors to enhance their activity or alternatively to protect them from degradation. The interaction between the ECM and growth factors is bidirectional. Growth factors, such as platelet-derived growth factor (PDGF) and vascular endothelial growth factors (VEGFs) are involved in the formation of blood vessels, which results in tissue vascularization, an essential process for tissue regeneration.[136−138] Other interactions, including ECM binding with integrins expressed at the cell surface, modulate growth factor expression[139] and promote different processes such as angiogenesis induced by VEGF. Other soluble ECM components, such as matrikines, can bind cell-surface receptors or insoluble ECM components, modulating several cell functions. ECM components contain binding sites for growth factors so that they act as reservoir systems that maintain the availability of growth factors during cell development, controlling also the gradient of bioactive factors useful for cell signaling.[75] For instance, fibroblast growth factors (FGFs) and VEGFs are bounded to heparan sulfate proteoglycans (HSPG) and can, when needed, be released as soluble factors by the hydrolytic enzyme heparinase.[43]

HSPGs accommodate FGFs that interact with their receptors using heparin sulfate (HS) as a cofactor. This complex remains linked and available to cell-surface receptors during the signaling process.[140] Cell signaling activities are often modulated by the binding between growth factors and the ECM through the recognition of specific motifs of ECM proteins.[75] The ECM proteins FN and VN are also able to interact with and regulate the morphogen hepatocyte growth factor (HGF) when complexed with Met (the HGF receptor) and integrins, thus enhancing cell migration.[141] Similarly, FnIII, which is present in both FN and tenascin-C, binds VEGF and potentiates cell signaling across its receptor VEGFR2. FnIII is also present in ECM-associated proteins, such as anosmin-1, where it acts as coligand of FGFR1 to modulate its activity.[142,143]

As mentioned before, it is well established that growth factors can act under their free and linked forms. The ability of the ECM to present or release growth factors at different moments of the cell life confers the ECM with a controlling role in the cell development. The EGF linked to an ECM component cannot be internalized and degraded, but will instead provide sustained signaling. Compared to the EGF soluble form, the tethered form is more potent in its ability to promote DNA synthesis, MSC survival, and osteogenic differentiation of multipotent marrow stromal cells.[144] On the other hand, when soluble EGF is added to cells cultured on tethered EGF, osteogenic differentiation is reduced. Soluble EGF is involved in downregulation of epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 (HER2), modulating tethered EGF/EGFR interactions.[145] Similarly, in FN-null fibroblast, signaling of platelet-derived growth factor BB (PDGF-BB) is improved when the FN domains (FnIII) are tethered. The interaction with VEGF, PDGF-BB, EGF-2, and TGF-β1 domains occurs via specific peptide sequences.[141] These data demonstrate that tethered

https://dx.doi.org/10.1021/acs.biomac.0c00045
Biomacromolecules 2020, 21, 1968−1994

growth factor availability in the extracellular medium is finely tuned to control and induce cell signals that are different compared to their soluble forms. In this context, the ECM represents an organizing center of the signaling complex to finely tune the cell-surface interactions.

Glycans in the ECM cover a fundamental role regarding both their mechanical and biochemical properties. GAGs and proteoglycans are at the basis of higher order ECM structures in cell media. HSPGs are components of both cell surfaces and the ECM. They control angiogenesis, embryogenesis, and homeostasis, and in this way, they modulate cell growth and differentiation.[146,147] HSPGs contain one or more covalently attached heparan sulfate GAG chains. The different groups of HSPGs are classified according to their location. Other proteoglycans are involved in wound healing and cell development. For instance, chondroitin sulfate (CS) is responsible for neuronal regeneration and homeostasis.[148] Another important component of the ECM involved in cell fate regulation is hyaluronic acid (HA). HA has been extensively studied as a major component of the ECM. It provides hydration and compression strength and regulates cell adhesion, proliferation, and differentiation, playing an active biochemical role in different tissues (i.e., in the nervous system, skin, and cartilage).[149−152] The biological function of HA is strongly related to its molecular weight (MW): low MW HA fragments (up to 700 kDa) are able to induce angiogenesis whereas high MW HA fragments limit it.[153−155] Poly(sialic acid) (PSA) is another polysaccharide present in the ECM involved in several physiological processes, such as neural cell differentiation and organogenesis.[153−155]

Glycans involved in ECM−cell interaction are not only polysaccharides. Smaller oligosaccharides or even monosaccharides present in the ECM and at the cell surface have an important role in regulating the cell fate. It is generally admitted that the signaling glycans are specific requirements of cells, exerting their role solely at the cell surface. Consequently, the glyco-components of ECM proteins have been largely neglected. Nevertheless, ECM proteins, such as collagen, VN, LM, and FN, undergo dynamic glycosylation, and we have the first evidence that this event influences the interaction with cells and consequently the cell fate.[81] These glycosylation processes are often variable across different species and are strongly dependent on several factors (e.g., age, pathology, diet, etc.). Recent observations indicate that *N*- and *O*-glycosylation of ECM proteins influences the binding capacity to cell receptors and, therefore, the impact on cell fate. For example, glycosylated collagen or LM can interact with the cell surface integrins, an event strongly related to pathological states and malignancies (i.e., several cancers).[54,156−159] *N*-Glycosylation of LM 332 has an impact on cell spreading and adhesion. *O*-Glycosylation of FN regulates the expression of mesenchymal markers and cell adhesion in the epithelial mesenchymal transition process. The interaction of glycosylated ECM proteins with lectins present in the extracellular space, such as some galectins,[112] contributes to the structural organization and functional role of the matrix itself.[21] The discussion above is visually summarized in Figure 1.

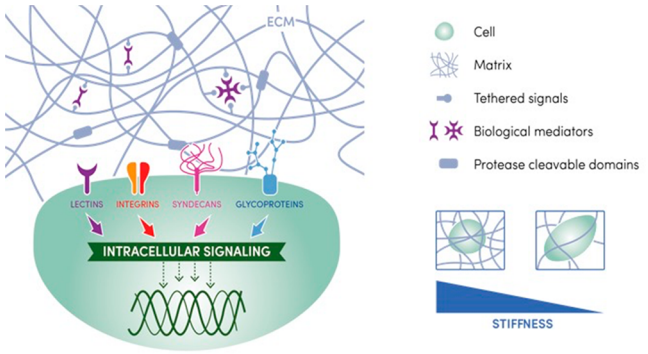


Figure 1. Graphical representation of ECM−cell microenvironment. (Left) Cell and schematic cell receptors involved in cell−ECM interactions, ECM tethered signals, ECM affiliated proteins, and extracellular interactors (biological mediators). (Right) Different actors involved in ECM−cell signaling and graphical representation of the role of ECM physical properties in cell morphology and shape.

3. ECM MIMICS

The importance of the ECM, its physical and biochemical properties in so many fundamental cellular processes, has stimulated interest in the development of a myriad of tailored tissue-culture models. These models allow for the studying of the interplay between the ECM biochemical and biophysical properties, understanding of the molecular mechanism of cellular behaviors regulated by ECM properties, and developing of ECM mimics for cell cultures exploitable for biomedical applications.

Cell culture studies exploit two different types of scaffolds: reconstituted matrices containing biomacromolecules isolated from animal tissue or synthetic ECM mimics.[160] Both systems can be implemented by surface coatings to promote cell adhesion or utilized to generate 3D scaffolds for embedding cells in a more *in vivo*-like environment.[9,160−164] A significant advantage of synthetic ECM is the possibility to tune certain biophysical parameters, such as the mechanical properties or the permeability of the matrix, in order to investigate their influence on the cell fate. ECM mimics have been synthesized from different polymers of both natural and synthetic origin (Figure 2).[12,160−163,165,166]

Among natural materials, several proteins and polysaccharides, such as collagen, gelatin, FN, HA, CS, or PSA, have been selected[167,168] with the aim to generate new functional tissues or more affordable models for 3D cultures.[169] Synthetic materials have some advantages compared to natural counterparts; they are more easily available and can be more easily modified and formulated with different stiffness, covering also hard tissue engineering applications (i.e., bone). Examples of synthetic polymers that have found application as scaffolds for cell seeding and tissue regeneration are polyethylene glycol (PEG), polycaprolactone (PCL), poly(lactic acid) (PLA), and poly(glutamic acid) (PGA). The main disadvantage of using synthetic polymers as building blocks for ECM mimics is their inability to provide the biochemical signals needed to "communicate" with the cell.[170] To overcome this limit, synthetic polymers can be functionalized by adding signaling biomolecules, such as peptides, growth factors, and glycans.

3.1. ECM Mimic Applications. ECM mimics find application in cell biology, as models to study drug biodistribution in specific tissues, and in regenerative medicine. In such applications, a significant improvement comes from the development of 3D scaffolds and 3D bioprinting.

3.1.1. Cell Biology and Drug Discovery. In cell biology, the 3D spatial organization allows the architecture of the natural cell microenvironment to be best mimicked, which significantly impacts the cell development in physiological and pathological

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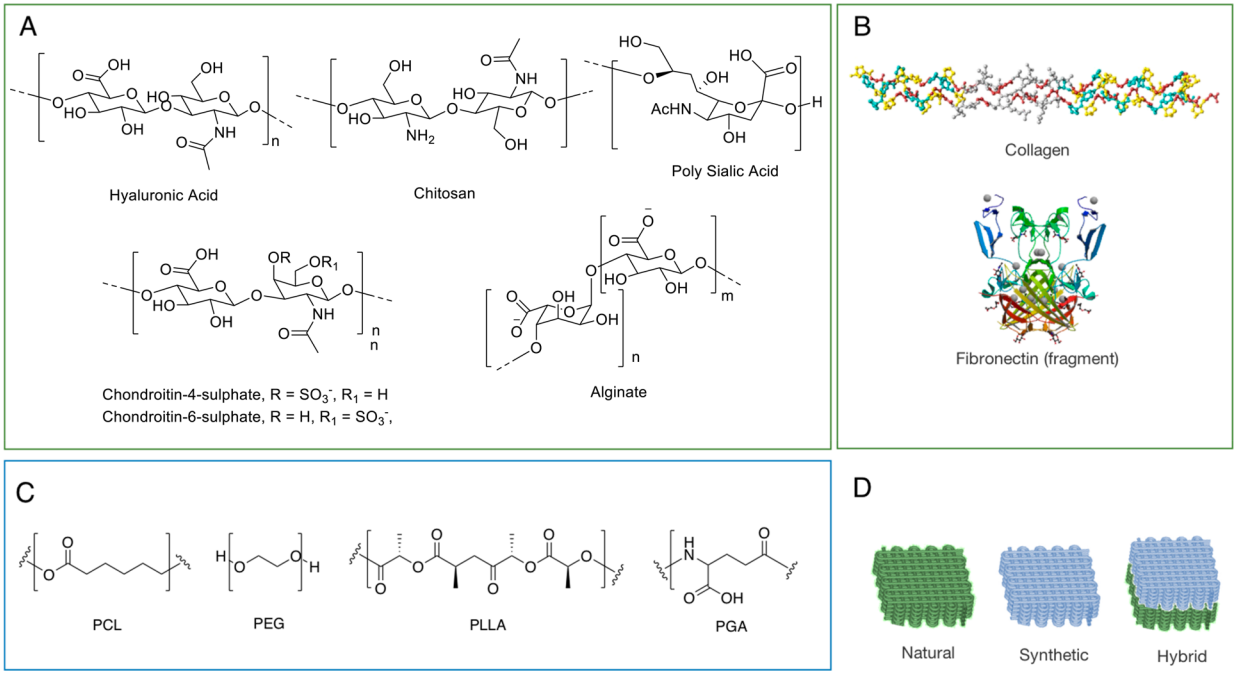

Figure 2. (A and B) Examples of natural polymers (collagen PDB ID, 1BKV;[171] fibronectin (fragment) PDB ID, 3M7P[172]) and (C) synthetic polymers. (D) Representation of natural, synthetic, or hybrid scaffolds. Modified from Sgambato et al. "Bioresponsive Hydrogels: Chemical Strategies and Perspectives in Tissue Engineering".[173] Licensed under CC BY 4.0.

states. This requirement is a major advantage in tissue engineering and enables the development of tissue models with personalized features that can be used in pharmacokinetic studies as an alternative to animal models. This is not only an ethically preferable "animal free" approach but also potentially more accurate, providing data closer to what would be obtained in humans. The nature of the cell microenvironment strongly influences the pharmacokinetics of a specific drug and, therefore, the pharmacological response. The activity and toxicity of well-established drugs have been compared in 2D and 3D cultures, showing very different drugs efficacies.[8,174]

The combined progress in biomaterial research, 3D bioprinting technologies, and stem cell cultures permits the reproduction of *in vitro* the *in vivo* tissue organization and structure and finally will allow the generation of artificial functional organoid tissues.[175,176]

Synthetic 3D cell niches can be "tailored" with specific structures, stiffness, and bioresponsive molecular cues in order to mimic a specific human tissue in typical physiological conditions or those affected by different pathologies.

3.1.2. Tissue Engineering. Tissue engineering is a multidisciplinary research based on advancements in chemistry, materials science, and cell biology. The combination of these fields provides new opportunities to generate tissues and even organ substitutes for clinical applications.[177−181] Tissue regeneration is based on two main pillars: the proper stem cell and the 3D artificial microenvironment (ECM mimic) in which they are cultured. The ECM mimic must possess the requirements needed to "induce" differentiation in order to generate the desired tissue. Biomaterials for tissue engineering require bioactive motifs to induce specific biological signals and cells.[175] They must be chosen on the basis of the nature of the organ to be regenerated and its functional role. Other parameters, including age or comorbidities, must also be taken into consideration. Two different applications of ECM mimics in tissue engineering must be considered: (i) the use of a scaffold for *in vitro* culture of cells that will be subsequently transplanted and (ii) the use of a scaffold as a biomaterial to be implanted *in vivo* to generate *in situ* new functional tissue.[182] Personalized biomaterial scaffolds are under study in order to provide them with tailored biological properties once implanted into the body.[176,182,183]

4. SYNTHETIC STRATEGIES TO MIMIC EXTRACELLULAR MATRICES

In order to build up a solid strategy to generate ECM mimics for both tissue engineering and cell studies, nature remains the best source of inspiration. Both structural features and biochemical properties of the natural cell microenvironment must be taken into consideration.[177,178,180,181,184]

4.1. Mimicking Stiffness and Geometry. Composition, stiffness, and topological structure of the ECM scaffold are all critical to its function and affect the cellular interaction with the material. The mechanical properties of the ECM influence embryo development, whereas tissue stiffness affects organ development. Furthermore, the stiffness influences a variety of cellular properties, such as cell adhesion, spreading, proliferation, differentiation, and apoptosis. Tissues in the body are composed of different ECM components and cells, with a controlled organization that makes each organ different to the others in terms of their response to mechanical stimuli. On this basis, organs have stiffness values that comply with their physiological and functional roles (Figure 3).

It is now evident that ECM stiffness plays a crucial role in tissue development and in pathologies. Tissue injuries result in imbalanced homeostasis, which influences the tissue function.[31,89,185−187] As a consequence of pathological states, the mechanical properties of the tissues are altered; for example, fibrous tissues become usually stiffer than the original tissues. An increase of ECM production is essential for wound-healing

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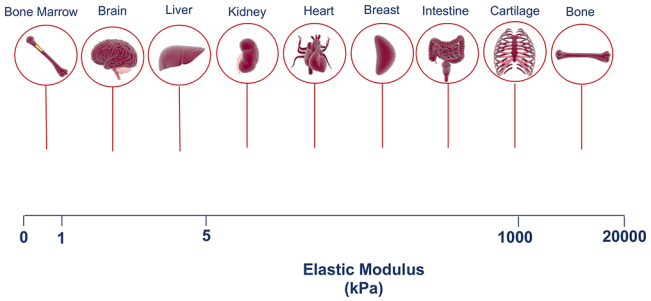

Figure 3. Organ-specific stiffness values. Adapted with permission from ref 185. Copyright 2015 Taylor & Francis.

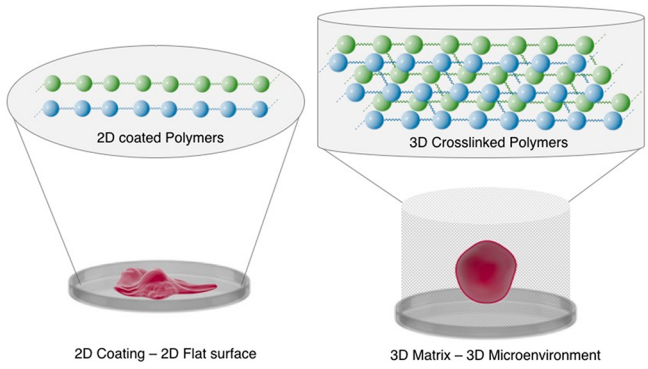

Figure 4. 2D coated polymers and 3D cross-linked polymers.

processes, but an excess of matrix deposition leads to tissue dysfunction, as observed in fibrotic diseases. Tumor progression, chronic inflammation, and disorders also result in changes in ECM production.[77] One of the most studied tissue models is the tumor microenvironment, where the ECM is continuously remodeled, leading to an increase of stiffness. Tumor initiation/progression and fibrosis share a common mechanism, which involves the increased production of transforming growth factor beta (TGF-β) from myofibroblasts, derived from fibroblast dedifferentiation. TGF-β acts following two mechanisms: (i) by binding its own receptor, (TGFβR1) it is responsible for SMAD pathway activation, which leads to collagen and fibronectin family genes overexpression, and (ii) TGF-β interferes in ECM enzymatic degradation by inhibiting MMPs.[188] In addition to its involvement in cancer progression, lysyl oxidase also plays a crucial role in the increase of ECM stiffness under pathological conditions by inducing inter- and intra-cross-linking among ECM proteins by oxidative deamination,.[189,190] In addition to the stiffness, the architecture of the ECM has also a major impact on cell response. Cell adhesion and migration are strongly influenced by the geometry of the scaffold. Different geometries of the ECM mimics influence cell migration based on their structural organization and density. For instance, thin networks reduce cell migration, whereas larger ones can result in an increase of cell migration across the scaffold.

The architecture of ECM mimics can be designed and produced, exploiting both physical and chemical cross-linking strategies, in order to obtain the most suitable biomaterials for a specific tissue formation.[191] 3D polymer networks can be formulated in smart hydrogels[192] or scaffolds to mimic specific extracellular microenvironments. With this aim, natural, synthetic, and hybrid polymers have been cross-linked from different methodologies to control geometries and matrix stiffness and consequently to induce the required differentiation of the encapsulated cells (Figure 4).[191]

With respect to traditional 2D cell cultures, cells encapsulated in 3D scaffolds will have a more "nature-like" interaction with ECM mimics, providing more adapted intracellular signals to the physiological conditions. Therefore, to finely tune the cell fate and to guide the tissue formation, ECM mimics must be organized in 3D structures[193] composed of natural or synthetic polymers and decorated with bioresponsive molecules.[170] As a matter of fact, 3D ECM mimics can be produced using different polymers (both natural and synthetic) interconnected by physical and ionic interactions and even covalent linkages.[12] Cross-linking strategies are widely used to control the stiffness and structural organization of the final 3D scaffold and must be performed without affecting the cell viability. Cells embedded into natural biopolymers can take advantage of signaling motifs already present inside the matrix, whereas synthetic polymers lack signaling motifs able to modulate the cell fate. Chemists have developed a variety of chemoselective ligation strategies to conjugate biomolecules.[194] Some chemoselective ligations, such as bio-orthogonal click reactions, find application in 3D biomaterials to cross-link the constitutive biopolymers and to link the bioresponsive molecules.[173,183,191] Some examples of bio-orthogonal click reactions employed in hydrogel cross-linking and bioconjugation are represented in Table 2. In general, one of the two functional groups involved in the chemoselective reaction is introduced in the polymer (if not already present) and exploited in a reaction with a complementary functional group present in a bifunctional linker. The goal is to obtain a controlled cross-linking process during cell encapsulation without affecting the cell viability.[192]

To this purpose, several different chemoselective reactions have been developed to generate smart hydrogels for cell encapsulation.[191] The major problem consists of maintaining the cell viability during the cross-linking procedures, which means that experimental conditions (e.g., temperature and pH) and potential side products must be carefully controlled. The reactivity and the biocompatibility of a variety of bio-orthogonal click reactions have been well-studied. Among those ligation strategies, the most widely used are the thiol−ene, thiol−yne, and thiol−Michael reactions, together with the Diels−Alder and the strain-promoted azide−alkyne cycloaddition (SPAAC) reactions.[191] The best conjugation strategies depend on several different factors: (a) the nature of the selected polymer, (b) the cell line to be employed,[12] and (c) the formulation strategy to generate the 3D model.[224] The different reactivities of natural polymers like polysaccharides or proteins are just one of the limitations to overcome. They must be functionalized with "unnatural" functional groups, suitable for subsequent "click chemistry", in order to create efficient cross-linking to generate the 3D ECM mimics under biocompatible experimental conditions without the formation of side-products. With this aim, Michael and Diels−Alder additions onto maleimide have been extensively employed in the past few years, considering that these reactions can occur under cell-compatible conditions and require neither UV light nor photo initiators.

Michael addition is one of the most widely used strategies in biomaterial design and synthesis.[223,225,226] Michael addition with thiols has been employed in the development of many hydrogels for cell encapsulation and tissue engineering.[193,227,228] The high efficacy in mild experimental conditions

https://dx.doi.org/10.1021/acs.biomac.0c00045
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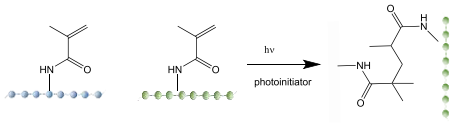
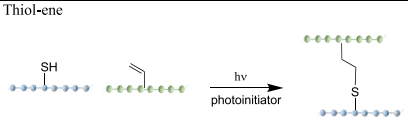
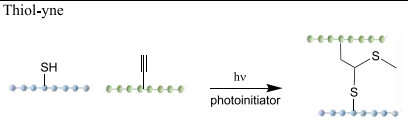
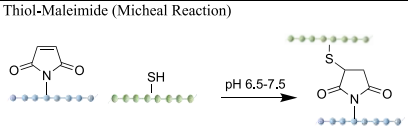
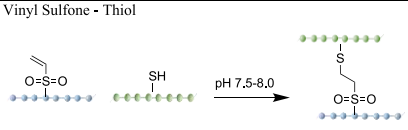
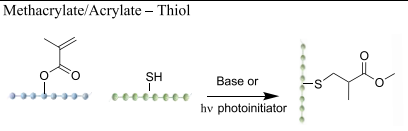
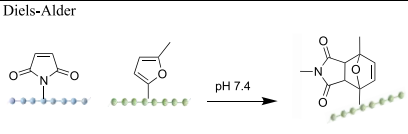
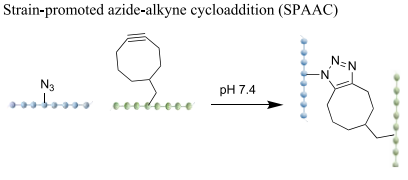
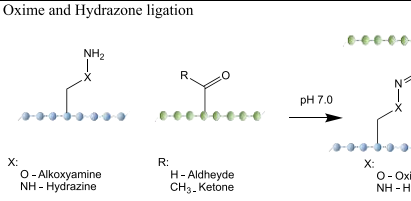
Table 2. Examples of Chemical Strategies Employed in Bioconjugation or Hydrogel Crosslinking[a]

Reaction	Refs.
Chain photopolymerizations - Methacrylation 	[196–199] and many others.
Thiol-ene 	[200–207]
Thiol-yne 	[208,209]
Thiol-Maleimide (Micheal Reaction) 	[210,211]
Vinyl Sulfone - Thiol 	[212,213]
Methacrylate/Acrylate – Thiol 	[214–216]
Diels-Alder 	[217–220]
Strain-promoted azide-alkyne cycloaddition (SPAAC) 	[47,206,221]
Oxime and Hydrazone ligation 	[222,223]

[a]Adapted with permission from ref 195. Copyright 2019 Elvesier.

and the orthogonal nature of the reaction are strong assets for all applications related to biomedicine.[229] Also, Diels–Alder has been employed to produce biomaterial conjugated with bioactive factors[230] of to control hydrogel networks of natural and synthetic biomaterials.[217,218,231] The Diels–Alder reaction proceeds under mild conditions with high yields, no side products, or reagent contamination. In typical Diels–Alder reactions, an electron-rich diene reacts with an electron-poor dienophile. The maleimide moiety is a dienophile widely used in Diels–Alder chemoselective reactions.[232] Interestingly, once the maleimide function is inserted in a biomaterial, it becomes suitable for both Michael and Diels–Alder chemoselective ligations. As far as the diene moiety is concerned, furan, methylfuran, cyclopentadiene, and photogenerated ortho-quinodimethanes (photoenols) have been used to functionalize the biopolymers for subsequent Diels–Alder conjugations with maleimide.[217,233]

In both Michael and Diels–Alder addition, the possibility to modulate the coupling kinetics using functional groups with different reactivities has been studied. It allows for the exploitation of different formulation methodologies in the hydrogel fabrication. Cross-linking kinetics are very important for an efficient 3D printing process and to guarantee high cell viability and homogeneous cell encapsulation. Typical Michael acceptors show different kinetic profiles (Figure 5a). In Diels–Alder reactions, an *s-cis*-diene is required. Therefore, all the structural requirements that induce or favor the formation of *s-cis* isomers will fasten the cycloaddition reaction. The most used Michael acceptors/dienofiles and dienes are ranked in Figure 5 in decreasing reactivity order.

Oxime and hydrazone ligations are based on the chemoselective reaction between an aldehyde or a ketone with an alkoxyamino ($H_2N–O–R$) or a hydrazino ($NH_2–NH–R$) derivative. Given that proteins do not contain aldehydes and ketones, this coupling strategy represents a very attractive methodology.[222,223] Oxime/hydrazone ligations result in site-specific conjugations that do not affect the protein structures, thus allowing for polymer functionalization while maintaining the protein bioactivity and biocompatibility.

The study of the cell response to specific geometrical, mechanical, or morphological physical features and other general physical features of the ECM needs technologies and polymeric materials that allow for the generation of patterned structures with controlled spatial organization. With this aim, several synthetic materials have been developed.[234,235] Polyisocyanopeptides were functionalized with oligo(ethylene glycol) side chains to obtain responsive polymers with helical hierarchical architectures, in which stiffness and molecular interactions can be tuned to formulate transparent hydrogels.[236] Thermoresponsive worm-like synthetic hydrogels have also been developed through reversible addition–fragmentation chain-transfer (RAFT) polymerization of styrene in the presence of poly(*N*-isopropylacrylamide) (PNIPAM). The hybrid features of the final biomaterials, characterized by a hard polystyrene core surrounded by PNIPAM chains, were employed with PNIPAM-grafted vitronectin fragments to allow for the formation and the maintenance of human embrionic bodies (hEB).[237] PNIPAM has also been cross-linked to poly(diacetylene) bis-urea bolaamphiphile (PDA) fibers to generate thermoresponsive hydrogels with tailored mechanical properties.[238] Poly(α-hydroxy) esters, including their copolymers and hybrid derivatives, have also been extensively employed and studied to control the morphological and

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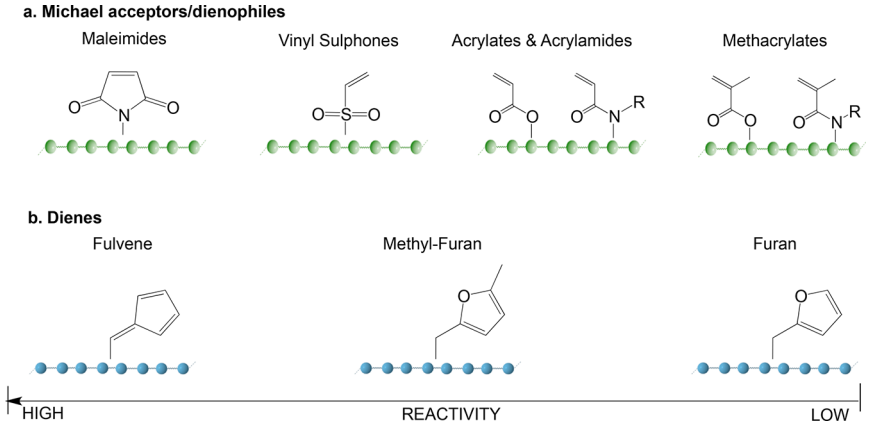


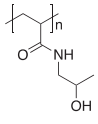
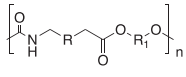
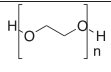
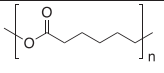
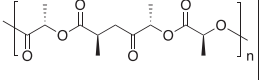
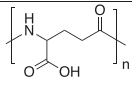
Figure 5. (A) Reactivity of the most used Michael acceptors/dienophiles, presented in decreasing order of reactivity. (B) Differential reactivity of the dienes employed in the Diels–Alder reaction for hydrogel fabrication and cell encapsulation. Adapted with permission from ref 229. Copyright 2014 American Chemical Society.

mechanical properties of the microenvironment in stem cell control and tissue engineering applications.[239,240] The representative examples of poly(α-hydroxy) esters employable for 3D ECM mimics include PCL, PGA, and PLA. Copolymers made of conductive poly(3,4-ethylenedioxythiphene) grafted with D,L-PLA (PEDOT-*co*-PDLLA) were synthesized and tested with embryonic stem cells (ESCs) to obtain biodegradable biomaterials with electric properties.[241] Many other biomaterials based on poly(α-hydroxy) esters[242] and derived copolymers have been synthesized and investigated, taking advantage of their approved use in clinical fields and their processability by different fabrication strategies.[243−246] Some examples of synthetic polymers employed for stem cell encapsulation are reported in Table 3.

4.2. Mimicking Biochemical Signals. Cell events such as adhesion or subsequent differentiation and proliferation are the result of interactions between the receptors expressed on the cell surface and their counterparts founded on close cells and ECM.[24,28,29,31,70,84,99,103,285] These events, mediated by effector proteins, growth factors, and post-translational protein modifications such as phosphorylation and glycosylation, play a fundamental role in cell−cell and cell−ECM communication and inconsequent signal transduction to the nuclei.[21,25,26,193] To mimic cell−ECM biomolecular recognition and communication events, different approaches have been developed to enrich ECM mimics with bioactive molecules, such as small peptides, glycans, and even proteins or polysaccharides, producing bioactive materials. Considering the dynamic nature of the ECM and cell microenvironment, the bioresponsiveness is also a key feature of ECM mimics. The functionalization of polymers with biomolecules and cross-linkers, which are cleavable with specific stimuli (e.g., pH, temperature, light, or enzymes), allows for the modification of the hydrogel properties in a specific biological environment.[286]

The functionalization of polymers with bioactive molecules can be performed by click chemistry, as described before. The process can exploit functional groups already present in polymer chains, or more often, the introduction of a functional group that is more suitable for the chemoselective ligation may be needed. The functionalization of polymers with bioactive molecules has, however, some constraints that must be considered in the synthetic strategy: (i) to be "active", the attached molecule must be correctly exposed in order to be recognized by cell-surface receptors, and (ii) the conjugation must also be performed in mild conditions, like in click reactions, to avoid a detrimental effect on the "bioactivated" polymers and the formation of toxic side products. Many of the methods used for cross-linking strategies (Table 2), have also been employed to decorate biomaterials with bioactive molecules.

Table 3. Selected Examples of Synthetic Polymers Employed to Mimic ECM Architecture and Physical Behavior

Name	Structural Motif	Cells and Refs
PNIPAM, copolymers and hybrid polymers		hESCs[247,236], enteroids[248], CHs [249], MSCs[250−251]
HPMA polymers and copolymers		MSCs[252,253] and BMCs[254]
Polyurethanes biodegradable polymers		MSCs[255,256], CD34$^+$ cells[257], ESCs[258], NSCs[259−261], ADSCs[262,263]
PEG polymers and copolymers		b-cells [264], hPSC[265], hMSCs[266−271] ADSCs[272]
PCL polymers and copolymers		CHs and BMSCs[273,274]
PLLA polymers and copolymers		ADSC[275], MSCs[271,276−278]
PGA polymers and copolymers		SCCs[279],NSCs [280],MSCs[277,281−283], IPS [284]

4.2.1. Bioresponsive Polymers and Hydrogels. Bioresponsive polymers have recently been developed to produce scaffolds that mimic the mechanical, biophysical, and adaptive properties of native ECMs via the establishment of direct interactions with cells.[2,287] These materials contain specific active sites that, under proper stimulation by biochemicals or

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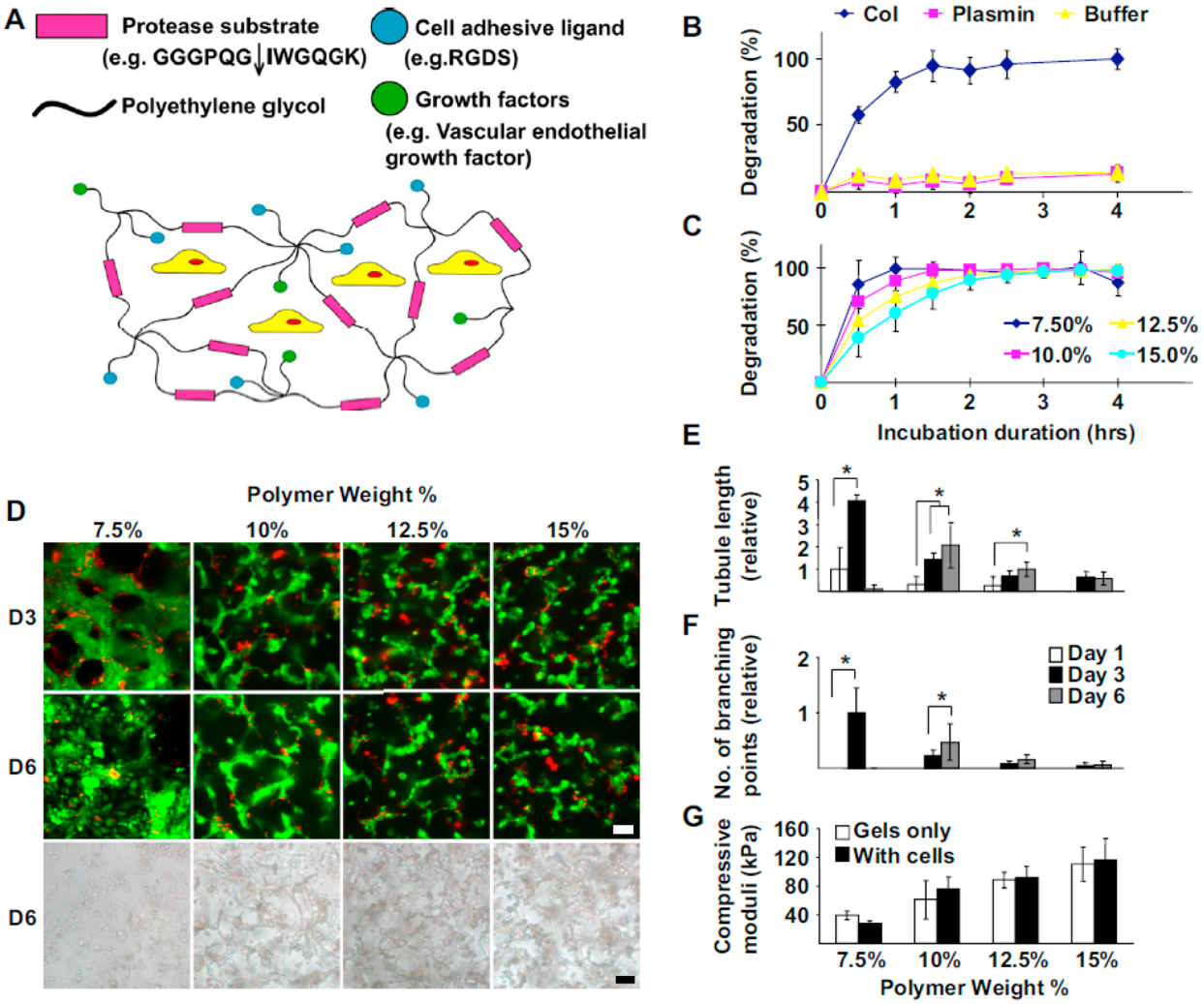

Figure 6. (A) Schematic representation of MMP-sensitive PEG hydrogels. (B) Hydrogel degradation by collagenase (MMPs) but not by plasmin or buffer solutions. (C) Hydrogel degradation profiles as a function of the polymer weight percentages in the presence of collagenase. (D) Confocal and bright field images of HUVECs and 10T1/2 cells cultured for 3 and 6 days in hydrogels with varying polymer weight percentages. (E) Total tubule length formed. (F) Number of branching points after 6 days of culture with varying polymer weight percentages. (G) Compressive moduli ranging from 30 to 110 kPa were measured in hydrogels with and without encapsulated cells. Scale bars = 50 mm. Reprinted with permission from ref 295. Copyright 2010 Elvesier.

cells, undergo macroscopic transitions such as localized or bulk changes in properties. This is of utmost importance to develop dynamic ECM mimics able to respond to the transient changes of cells (e.g., spreading, migration, or signaling) and tissues (e.g., pathology or wound) and direct cell behavior.

Bioresponsive hydrogels have been engineered to allow the modulation of ECM mimic properties. For instance, to allow the spread and proliferation of encapsulated cells, degradable hydrogels have been engineered by incorporating hydrolytically or cell-mediated enzymatic degradable moieties.[287] However, because hydrolytic degradation is not representative of the various dynamic processes taking place in native ECMs,[267] materials able to respond to cell-mediated cues were preferred. This was achieved by embedding cross-linkers into the polymer matrix that degrade under the action of plasmins or cell-secreted matrix metalloproteinases (MMPs).[288] MMPs are cell-secreted enzymes that are relevant in such a context, as they can degrade ECM molecules during tissue remodeling and disease. A typical example is the use of MMP-sensitive oligopeptide cross-linkers in PEG-based hydrogels. In particular, Hubbell and co-workers reported Human fibroblasts that could spread by degradation of MMP substrate GPQG↓IAGQ sites (where ↓ denotes the peptide cleavage site) after cell-mediated release of MMPs.[289,290] The application of this system to bone tissue engineering was then investigated by loading the hydrogel with bone morphogenetic protein-2 (BMP-2), which is known to be involved in bone formation. In a similar fashion, other PEG-based hydrogels[291] embedding MMP-sensitive sequences were designed. In other studies, cross-linked cell adhesive and proteolytically degradable PEG hydrogels[289,291−293] were obtained from the reaction between multiarm PEG derivatives and RGD-containing peptides as well as enzymatically degradable peptide sequences.

Mechanical properties play an important role in the evolution of cells. For instance, by tuning the hydrogel modulus, it was shown that cell proliferation can be manipulated irrespectively of the sensitivity of the PEG matrix to proteolysis and the presence of cell adhesion motifs.[294] Mechanical properties of bioresponsive hydrogels can be readily adjusted by changing their different structural

https://dx.doi.org/10.1021/acs.biomac.0c00045
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parameters. The most representative example is certainly the influence of the cross-linking density, which can drastically influence cell fate and dictate 3D cell behavior. Indeed, in MMP-sensitive PEG hydrogels, the formation of tubules was witnessed for intermediate PEG contents, whereas their complete regression was obtained with less cross-linked PEG matrices[295] (Figure 6). Also, the stiffness of the hydrogel[296] and its sensitivity to proteolysis can be simultaneously changed by varying the weight fraction of MMP-sensitive sites, resulting in the manipulation of hydrogel degradation and the formation of functional blood vessels.

However, MMP-sensitive substrate sites exhibit relatively slow degradation kinetics that may limit cellular infiltration within the scaffold. Also, these peptides can also be cleaved by a variety of different MMPs, which could result in selectivity issues. Therefore, recent solutions to circumvent these limitations have been proposed, including (i) the use of peptide substrates with increased catalytic activity[292,297] to enhance proteolytic degradation of PEG-based hydrogels or (ii) the increase of the spatial presentation of such signaling molecules within the hydrogel.[298]

Interestingly, bioresponsive hydrogels as ECM mimics have also been obtained from other polymer scaffolds such as poly(*N*-isopropylacrylamide-*co*-acrylic acid) (P(NIPAAm-*co*-AA)) cross-linked with MMP-13/collagenase-3-degradable peptide sequences.[299] Increased cell migration was observed in MMP-degradable P(NIPAAm-*co*-AA) hydrogels compared with nondegradable counterparts, thus emphasizing the benefit of bioresponsiveness.

Since hydrogel degradation is irreversible in these systems, the long-term use of such ECM mimics is therefore limited. More advanced ECM mimics able to exhibit reversible modulation of their properties have therefore been developed. This was achieved by designing hydrogels with reversible/adaptable cross-linking chemistries,[300] thus leading to dynamic nondegradable materials. Two main strategies have been investigated: covalent and noncovalent adaptable hydrogels. For the former, biocompatible hydrogels comprising bound hydrazine[301−306] and imine[307−309] have been successfully used to encapsulate cells while maintaining their function and a good cytocompatibility. For instance, a hydrazine-linked PEG-based hydrogel was able to mimic the modulus and stress relaxation properties of different biological tissues,[302] thus making it a valuable tool for designing viscoelastic scaffolds and for studying cellular responses to scaffold elasticity (Figure 7). A similar material was mixed with collagen and resulted in cardiac tissue with enhanced mechanical properties from encapsulated cardiomyocytes compared with collagen alone.[305] As for noncovalent adaptable hydrogels, they are usually made of cross-linked points based on weaker interactions than covalent linkages, such as calcium coordination,[310−312] host−guest interactions,[313−315] hydrogen bonding,[316] and others.[317−320]

Bioresponsive polymer matrices have also been engineered to display bioactive ligands (e.g., growth factors) in a dynamic fashion to modulate the chemical environment of the cells to mimic native ECMs. This is of high importance, as the spatiotemporal presentation of growth factors to cells is connected to numerous *in vivo* processes.[321] To avoid side-reactions and ensure precise on demand ligand presentation, biorthogonal chemistry has been developed.[322,323] In this field, light-based chemistries have been extensively used to conjugate and release ligands to hydrogels, given their mild and rapid

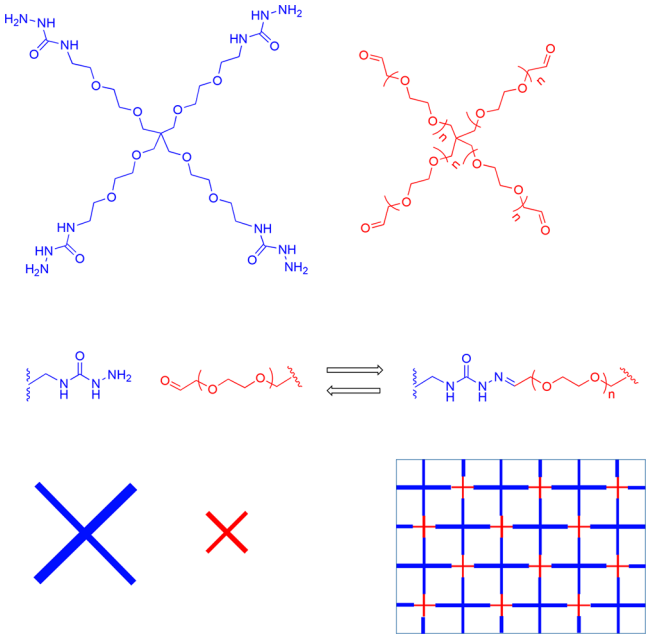

Figure 7. Structure of hydrazine-linked PEG-based hydrogel showing reversible gelation. The blue cross-structure represents the hydrazide-PEG star, the red cross-structure represents the aldehyde-PEG star, and the blue−red network shows a graphical representation of the final cross-linked structure. Adapted with permission from ref 300. Copyright 2012 John Wiley and Sons.

reaction conditions.[206,301,324−329] Also, the use of light ensures accurate spatiotemporal control in both 2D and 3D geometries. Reversible ligation strategies involving light have been utilized to link small molecules (e.g., dyes, peptides) onto polymers by using click chemistry-based photoreversible patterning strategies. For instance, RGD or NTA-amine functionalized to the NHS moiety can be conjugated through copper(I)-catalyzed alkyne−azide cycloaddition (CuAAC) to azide groups localized on prefunctionalized hydrogels via a photocleavable nitrobenzyl linker, which contains a photocleavable group, leading a photochemical release[330] (Figure 8). This concept can also be applied to larger entities, such as proteins, for instance, via the use of a prefunctionalized

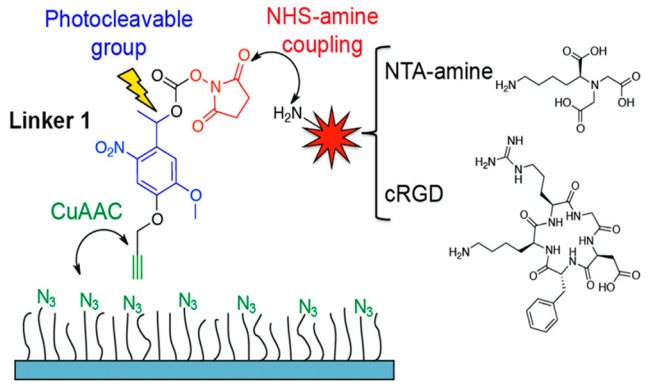

Figure 8. Hydrogel functionalized to bioactive molecules through a photocleavable linker strategy can be used for the local release of bioactive molecules with light. Reprinted from Wegner et al. "Photocleavable Linker for the Patterning of Bioactive Molecules".[330] Licensed under CC BY 4.0.

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hydrogel able to react through a click-type reaction with proteins,[331] such as the stem-cell differentiation factors sonic hedgehog (SHH) and ciliary neurotrophic factor (CNTF) (Figure 8).

To avoid denaturation and loss of activity from fragile macromolecules caused by free radicals, an alternative strategy relying on an enzyme-catalyzed reaction with the hydrogel by avoiding light exposure has been reported.[332] Sequential light-mediated reaction procedures have also been investigated to position and release different proteins using the same chemistry.[329] For instance, the Michael-type addition reaction has been successfully applied to reversibly photopattern bioactive peptides into hydrogels, even after numerous cycles thanks to the double-bond regeneration.

Dynamic ligand presentation can also be achieved by noncovalent approaches. Among the most used pathways, the hybridization of leucine zippers[333] offered a clever solution to reversibly display RGD-containing peptides into PAAm-based hydrogels (Figure 9). Bioactivity could be turned off by

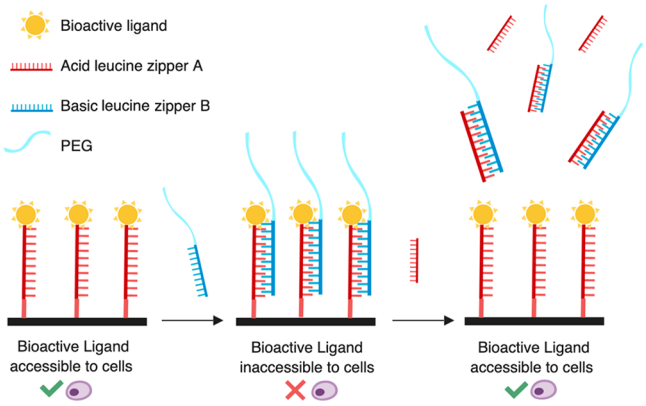

Figure 9. Molecular design of bioactive surfaces capable of dynamically and reversibly regulating immobilized ligands via hybridization of leucine zippers. Adapted with permission from ref 333. Copyright 2010 American Chemical Society.

the addition of a PEG chain with a complementary leucine zipper and turned on again by introducing a competing peptide able to displace the PEG moieties. Reversible exposition of cell adhesion sites was also achieved by folding/unfolding via aptamer hybridization.[334]

Short oligopeptides, derived from or inspired by ECM proteins, have been widely studied as bioresponsive and bioactive molecular cues. Numerous bioactive sequences have been selected, synthesized, and grafted to polymer chains to induce specific biological signals. Today, a wide range of sequences are available to induce different biological responses. They are selected on the basis of the tissue (cell populations) and the biological signal of interest and also on the basis of the polymers employed in the design of ECM mimetics.[335,336] The development of screening strategies to select and decipher the sequences that must be employed in the design of new synthetic materials mimicking ECM is of great interest for both cell biology studies and tissue engineering applications.[132] Some examples of peptide sequences employed in dynamic bioactive bioresponsive hydrogels are summarized in Table 4.

The RGD sequence, discovered in the FN domain III10 (which binds $\alpha 5\beta 1$ and $\alpha V\beta 3$ integrins),[337] is the most famous peptide involved in cell adhesion processes. RGD-containing sequences were also found in LM, fibrinogen, VN, fibronectin,

Table 4. Selected Peptide Sequences

sequences/motifs	receptor/stimuli
RGD and cyclic peptides	integrins $\alpha 5\beta 1$, $\alpha v\beta 3$/cell adhesion
IKVAV	integrin $\alpha v\beta 3$/neuronal differentiation
YIGSR	integrins $\alpha 1\beta 1$, $\alpha 3\beta 1$/angiogenesis, epidermal development of skin, and inhibition of tumor growth and metastasis
FRHRNRKGY	heparin/human osteoblast cell adhesion
CGG-QPPRARITGYII	integrin $\alpha 4\beta 1$, syndecan

bone sialoprotein, tenascin, osteopontin, and in some collagen types.[338,339] RGD-containing peptides affect the biological functions of human induced pluripotent stem cells (IPSs) in different ways (e.g., cell attachment, self-renewal, and pluripotency), suggesting that the whole sequence is involved in the modulation of cell behavior.[340] As previously reported, it has been observed that changes in the RGD peptide conformation from linear to cyclic significantly modify the specificity of integrin recognition, stimulating different cellular responses, such as differentiation and/or phenotype maintenance, depending on the conformation.[341] Other peptides used to obtain bioresponsive and bioactive hydrogels are IKVAV or MMP-sensitive sequences.[342−345] IKVAV is a LM-derived peptide, and it has been extensively studied for its ability to induce neuronal differentiation and to improve neurite outgrowth in cortical neurons;[346] it has been linked to different hydrogels, like hyaluronic acid,[347,348] polylysines,[349] and polyacrylamide.[350] Another LM-derived sequence is YIGSR, which, when linked to different polymers and hydrogels, is able to induce angiogenesis, promotion of skin development, and inhibition of tumor growth and metastasis.[351] FRHRNRKGY is a peptide derived from VN that interacts with heparin, and the sequence is specifically involved in human osteoblast adhesion.[352] Other sequences were identified from ECM proteins and tested for their activity to induce cell fate under their free form or when linked to material surfaces or hydrogels.[132]

4.2.2.2. Glycans. In addition to small peptides, glycans also exert a relevant role to control and guide cell adhesion or other cellular processes. The role of glycans in a broad range of recognition phenomena of biological relevance has been well established. It is not surprising, therefore, that glycans also exert a relevant role in the cell microenvironment, influencing the cell fate. In the complex cell microenvironment, several glucosaminoglycans (GAGs) and proteoglycans play a fundamental role. Therefore, a wide number of hybrid GAG-based materials are currently under investigation.[353] Some polysaccharides present unique hydrogel properties, making them very suitable as a biomaterial for cell cultures. Polysaccharides or polysaccharide−protein and polysaccharide−polymer hybrids, such as hyaluronic acid−collagen or chitosan−collagen, have found application in human stem cell culture and tissue engineering.[354−356] Several cross-linking and functionalization strategies have been investigated to formulate hybrid materials but also to study the effect of polysaccharidic chains as signaling molecules.[356]

Hyaluronan, polysialic acid, heparin, heparan and chondroitin sulfate, chitosan, and cellulose (Figure 10) are the most widely used polysaccharides that gave rise to a wide range of smart biomaterials for tissue engineering and cell culture.[357]

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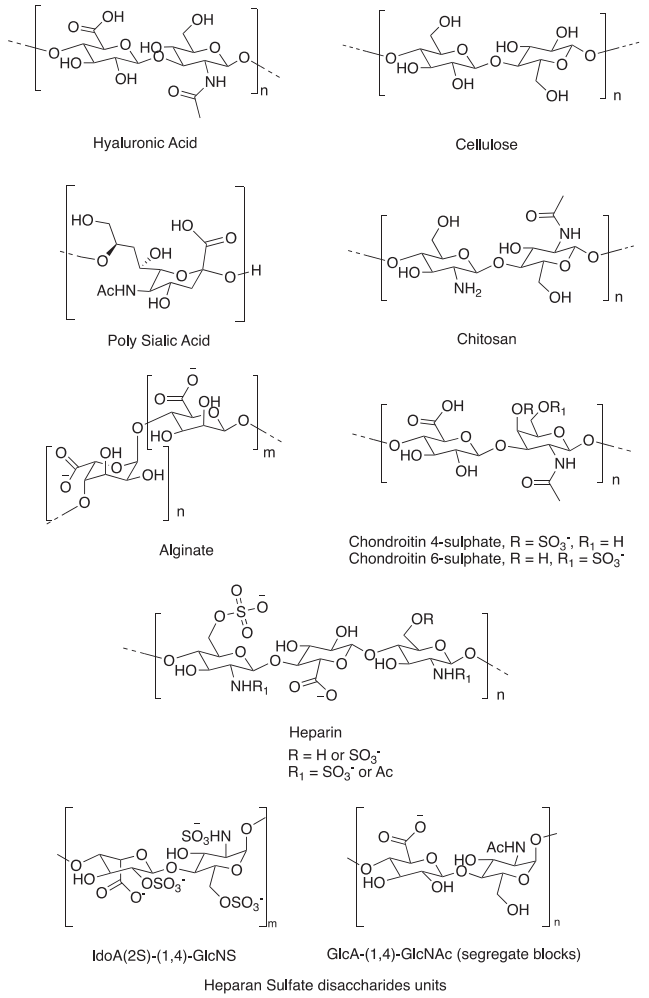

Figure 10. Polysaccharides employed in hydrogels for cell cultures and tissue engineering applications.

Composites of hyaluronic acid or chondroitin sulfate with ECM structural proteins, such as collagen, have been successfully generated and exploited to induce osteochondral or skin differentiation.[358−361] Polysialic acid (PSA) has been adsorbed and/or linked to other natural or synthetic polymers, such as hyaluronic acid (natural polysaccharide), polylysine and polyornithine (synthetic polypeptides), or LM and gelatin (proteins). The obtained composites were used for neuronal differentiation in both tissue engineering and cell culture studies.[362−367]

Alginate is another widely used polysaccharide for tissue adaptation, such as cell encapsulation, and for use as a 3D matrix.[368] Several alginate-based hydrogels have been developed as cell-instructive hydrogels. One of the most explored applications of alginate is the encapsulation of beta islets for pancreatic tissue engineering applications.[369]

After having defined the role of polysaccharides, which are widely used as the main components of the hydrogel skeleton for cell cultures, the role of mono- and oligosaccharides in signaling to induce and control the cell fate is introduced below.[210,370]

The functionalization of protein-based or synthetic polymers with monosaccharides or oligosaccharides has also been widely used to improve their properties as functional biomaterials.[210] End-functionalized glycopolymers as mimics of chondroitin sulfate have been synthesized and investigated to study the effect of the sulfation pattern and multivalency on protein recognition (Scheme 1a).[371] Proteoglycan mimics have been produced using thiolated hyaluronic acid functionalized with a bifunctional *N*-β-maleimidopropionicacid hydrazide bifunctional linker and have subsequently been employed to graft chondroitin sulfate and heparin (Scheme 1b).[372] Other synthetic or natural polymers have also been employed as backbones to produce aggrecan-like structures mimicking PGs of the ECM.[373,374,356,375,376]

Typical chemoselective functionalization strategies employed to functionalize hydrogels and biomaterials with unprotected glycans are reported in Scheme 2.

Reductive amination is one of the most extensively employed procedures for protein glyco-conjugation. In the field of biomaterials, reductive amination has been exploited to perform the functionalization of collagen matrices with several di- and oligosaccharides.[377] For example, the functionalization of collagen with α-D-glucose by reductive amination involving the lysine residues and maltose provided interesting results, influencing the fate of F11 neuroblastoma cells. The exposed α-glucose residue indeed induced morphological and functional differentiation of nonfunctional F11 neuroblastoma cells. The glycosylation of collagen with sialylated epitopes influence osteochondral regeneration.[378] Collagen functionalized with Neu5Acα2−3-Galβ1−4Glc residues induced up-regulation of chondrogenesis markers, whereas Neu5Acα2−6-Galβ1−4Glc residues up-regulated the expression of osteogenesis markers.[379]

Thiolated collagen matrices have been functionalized with α-allyl-D-glucoside and β-allyl-D-galactoside by thiol−ene reaction. The choice of α-glucose and β-D-galactose to glycosylate collagen is dictated by the fact that the two sugar epitopes are exposed in the native collagen glycosylation pattern in the ECM. Both glycosylated collagen matrices are able to induce cartilage repair in osteoarthritic mice.[380] Other biomaterials decorated with simple glycan motifs have been generated and employed as tools for glycomic studies,[381] for 3D liver cell culture,[382−385] or for improving MSCs adhesion on polymeric scaffolds.[386,387]

4.3. Artificial ECMS: New Perspectives with 3D Printing and Bioprinting. 3D printing is becoming a widely accepted manufacturing technique in biomedicine. It promises patient-specific personalized tissue design, on-demand fabrication, and high reproducibility.[388−390] The main applications span dentistry, medical devices, tissue engineering, engineered tissue models, and drug formulation.[391]

Biomaterials for 3D printing can be used alone (inks) or can encapsulate living cells/spheroids (bioinks for 3D bioprinting). In both 3D printing and 3D bioprinting for cell culture, the biomaterials used in the processes must meet usual biomaterial requirements: biocompatibility, controlled biodegradability, biofunctionality, controlled mechanical properties, etc. Consequently, most research efforts are devoted to the development of 3D printing protocols exploiting already approved biomaterials, which include natural, synthetic, and hybrid polymers. The research efforts are devoted to modulating their chemical and biological features and to controlling the printability window and cell encapsulation capacity during the bioprinting process.[392] Hydrogel inks formed by the natural biopolymers agarose, alginate, and hyaluronic acid and semisynthetic PEG-RGD-functionalized gelatin have been used in bioprinting and shown to have good cell compatibil-

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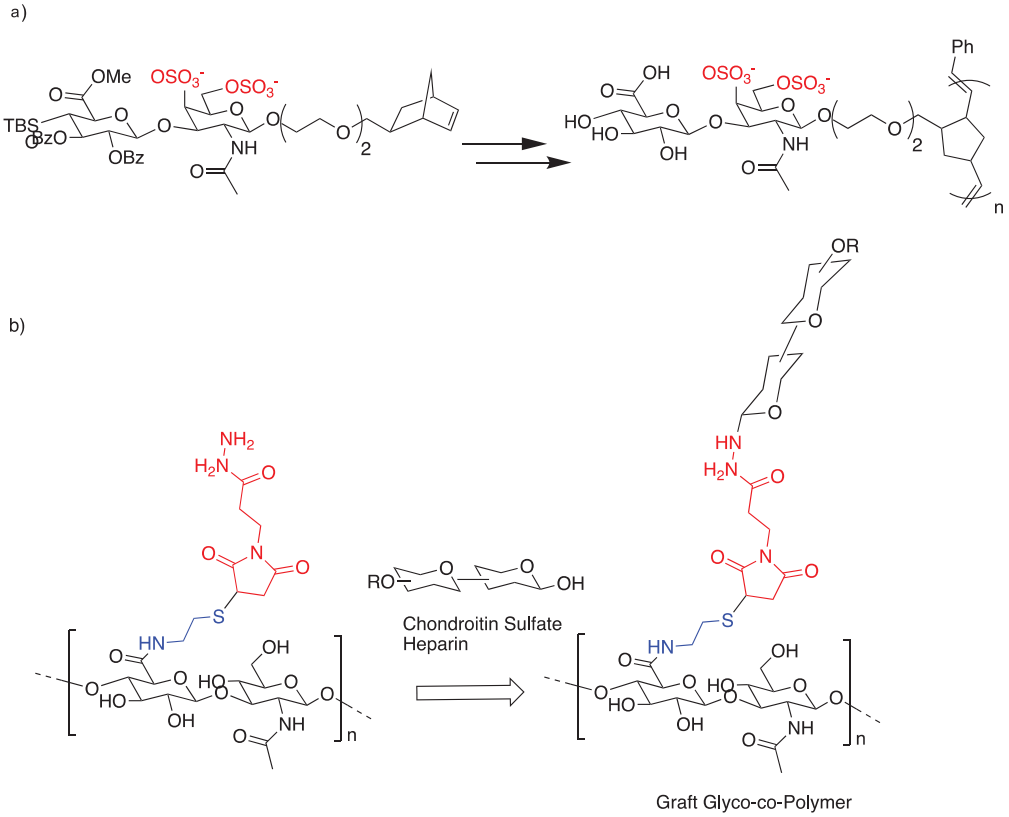
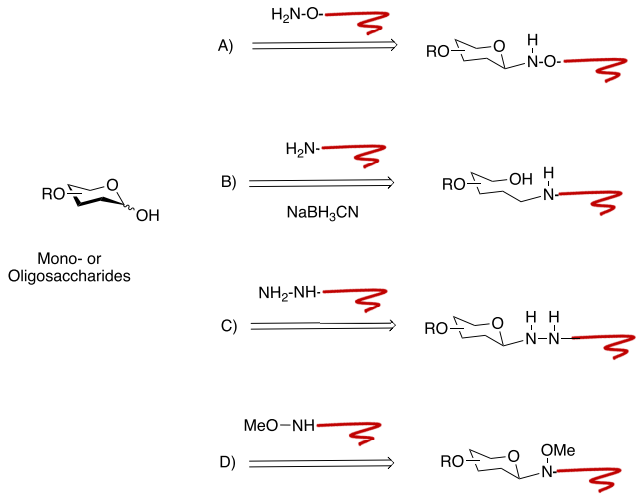
Scheme 1. Two Strategies to Generate Synthetic Glycopolymers



Scheme 2. Chemoselective Strategies Employed with Unprotected Natural Oligosaccharides: (A) Hydroxylamine, (B) Primary Amine by Reductive Amination, (C) Hydrazine, and (D) Oxyamine Linkers



ity.[389,390,392,393] Other natural materials, such as fibrinogen and collagen, possess both cell-binding and cell-degradable sites but suffer from poor mechanical properties. To improve them in terms of mechanical properties and imparted printability, synthetic polymeric components have been inserted and cross-linked. In terms of printability, thermoplastics are one of the most common polymers for 3D printing, inside and outside of the medical field. These materials include PLA,[394] poly(lactic-*co*-glycolic acid) (PLGA),[394] PCL,[395,396] and all other biocompatible polymers.

The main advantage of the use of 3D bioprinting to generate ECM mimics consists of the possibility of personalized design and precise fabrication, critical requirements for tissue engineering. 3D bioprinting technology allows for the build up of a tissue construct by using the layer-by-layer technique with accurate spatial control of the embedded cell populations, biomaterials, and grown factors. The combination of proper biomaterials and both multipotent and mature cells could be applied to reproduce the phenotype of each tissue. Beyond setting up the 3D bioprinting process, by adapting different parameters such as the nature of the injector, cell lines, microextrusion, and pressure, the implementation of the vascularization represents the main bottleneck.[390,397] To date, only a few vascularization-free tissues have been fabricated by 3D bioprinting, such as cartilage and skin.[398−401] The capacity to induce vascularization in a synthetic 3D ECM mimic would surely open crucial perspectives in regenerative medicine.

4.4. Decellularized Extracellular Matrix. In recent years, decellularized extracellular matrices (dECM) have been considered as a valid alternative for *in vitro* 3D cultures and tissue engineering strategies. The decellularized matrices are usually obtained from animal-derived organs in which cell populations are eliminated to obtain the bioactive ECM with natural components and morphology.[402] Decellularized organs and tissues show an impressive capacity to host stem cells and induce their differentiation. However, there are some limitations in terms of availability, variability, and potential immunogenicity due to post-translational modification of the tissues (i.e., glycosignature).

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The decellularization process often involves physical, enzymatic, or chemical processes selected on the basis of the tissue or organ of choice. Irrespective of the organ or tissue, the treatment affects the physical properties, the biochemical composition of the matrix, and, consequently, the "host properties".

Artificial ECMs made by decellularized material on synthetic supports have been investigated. Pati and co-workers employed porcine-derived dECMs (cartilage, heart, or adipose) using PLC as a polymeric support.[403] Different strategies have been followed by other authors who developed dECMs cross-linked with gelatin or hyaluronic acid as natural biopolymers to obtain 3D models with tunable stiffness to better mimic soft tissues properties.[404] A cardiac tissue was also developed *in vitro* by combining porcine heart dECMs with human cardiac progenitor cells, where the desired bioprinting stiffness was tuned by sequential VB2/UVA and thermal cross-linking.[405] In light of these results, decellularization of cell cultures has also gained interest as an alternative to full-organ or tissue decellularization. This method has the advantage of producing matrix entirely derived from one single cell population, whereas the matrix derived from tissues are the product of different cell types. Thus, the decellularization of cell cultures enables the study of only the components of interest secreted by a single type of cell. For instance, several studies have been carried out investigating MSCs' ECM effect during *ex vivo* expansion to study the effect of dECM on cell MSC cell populations.[406]

Overall, decellularization methods offer promising avenues in molecular biology and in cell studies in general, but oftentimes, these methods have limitations and still need further development in terms of the protocols and formulation strategies to maximize the translation and the investigation of the ECM effects. The major challenge of these approaches is to find the right balance between structural tissue maintenance and cellular removal. Other limitations are related to the scale-up for further translation.[407]

5. CONCLUSION AND FUTURE PERSPECTIVES

Artificial ECMs are promising tools with important perspectives and applications in biomedicine, which may result in significant improvements in stem cell therapies and in more efficient pharmacological studies that avoid animal models. From a scientific point of view, a predictive understanding of how the physical and biochemical properties of ECMs induce different cell fates and how such properties are related to pathologies, age, diet, or environmental changes is of huge relevance. This knowledge will clear the way for personalized therapies.

To reach these ambitious goals, tailor-made synthetic 3D ECMs must be developed, in which the different structural and biochemical parameters influencing the cell fate can be finely tuned. Progress in the knowledge of the nature of ECMs in different tissues, different pathologies, and even different individuals, together with the capacity to generate increasingly sophisticated smart 3D biomaterials, may assist in the generation of artificial organs.

AUTHOR INFORMATION

Corresponding Author

Laura Russo − *University of Milano-Bicocca, Department of Biotechnology and Biosciences, 20126 Milan, Italy;* orcid.org/0000-0002-5914-766X; Email: laura.russo@unimib.it

Authors

Julien Nicolas − *Université Paris-Saclay, CNRS, Institut Galien Paris-Saclay, 92296 Châtenay-Malabry, France;* orcid.org/0000-0002-1597-0873

Sofia Magli − *University of Milano-Bicocca, Department of Biotechnology and Biosciences, 20126 Milan, Italy*

Linda Rabbachin − *University of Milano-Bicocca, Department of Biotechnology and Biosciences, 20126 Milan, Italy*

Susanna Sampaolesi − *University of Milano-Bicocca, Department of Biotechnology and Biosciences, 20126 Milan, Italy*

Francesco Nicotra − *University of Milano-Bicocca, Department of Biotechnology and Biosciences, 20126 Milan, Italy*

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.biomac.0c00045

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Funding

This work was supported by the European Union's H2020-NMBP-15−2017-GA-760986 Integration of Nano- and Biotechnology for beta-cell and islet Transplantation (iNanoBIT), Italian Ministry of Health (Grant No. RF-2016-02362946), POR-FESR 2014−2020 Innovazione e Competitività. Progetti Strategici Di Ricerca, Sviluppo E Innovazione, Azione I.1.b.1.3-IMMUN-HUB, Sviluppo di nuove molecole di seconda generazione per immunoterapia oncologica.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We acknowledge the graphic design support of Mr. Davide Perrone.

ABBREVIATIONS

ECM, extracellular matrix; 3D, three-dimensional; GAGs, glycosaminoglycans; DDR, discoidin domain receptors; bFGF, basic fibroblast growth factor; CRD, carbohydrate recognition domain; CD36, glycoprotein IV; CD44, glycoprotein I; FAs, long-chain fatty acids; DD1 and DD2, discoidin domain receptors; RGD, Arg-Gly-Asp motif; PDGF, platelet-derived growth factor; VEGFs, vascular endothelial growth factor; FGFs, fibroblast growth factors; HSPG, heparan sulfate proteoglycans; HS, heparin sulfate; FN, fibronectin; LM, laminin, VN, vitronectin; HGF, morphogen hepatocyte growth factor; VEGFR, vascular endothelial grow factor receptor; MSC, mesenchymal stem cell; EGFR, epidermal growth factor receptor; HER2, human epidermal growth factor receptor 2; PDGF-BB, platelet-derived growth factor BB; CS, chondroitin sulfate; HA, hyaluronic acid; MW, molecular weight; PSA, poly(sialic acid); PEG, polyethylene glycol; PCL, polycaprolactone; PLA, poly(lactic acid); PGA, poly(glutamic acid); TGF, transforming growth factor; hESC, human embryonic stem cells; BMC, bone marrow cells; ESCc, embryonic stem cells; NSCs, neural stem cells; ADSC, adipose-derived Stromal Cells; hPSC, human pluripotent stem cells; BMSCs, bone marrow-derived mesenchymal stromal cells; CH, chondro-

https://dx.doi.org/10.1021/acs.biomac.0c00045
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cytes; IPS, induced pluripotent stem cells; MMPs, matrix metalloproteinases; P(NIPAAm-*co*-AA), poly(*N*-isopropylacrylamide-*co*-acrylic acid); PAA, poly(acrylic acid); PLGA, poly(lactic-*co*-glycolic acid)

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