Extracellular matrix and tissue engineering applications

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Received 10th December 2008, Accepted 15th April 2009
First published as an Advance Article on the web 19th May 2009
DOI: 10.1039/b822177d

The extracellular matrix is a key component during regeneration and maintenance of tissues and organs, and it therefore plays a critical role in successful tissue engineering as well. Tissue engineers should recognise that engineering technology can be deduced from natural repair processes. Due to advances in such distinct areas as biology, engineering, physics and chemistry and the possibility of using robotics to facilitate the search for new treatments, we can identify the basic principles and extrapolate them into tools to mimic the regenerative process. Ubiquitously distributed throughout the body, the extracellular matrix surrounding the cells plays a key instructive role, in addition to the previously recognised supportive role. In this review we will highlight the role of the extracellular matrix and discuss the latest technological possibilities to exploit the extracellular matrix in tissue engineering.

Extracellular matrix and its properties

"Tissue engineering is an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function".[1] These substitutes usually comprise a three dimensional (3D) scaffold providing support to cells, and growth factors to direct the differentiation of those cells.

In our body, the cell's direct environment is composed of an intricate 3D network of fibrillar proteins, proteoglycans and glycosaminoglycans (GAGs), collectively termed the extracellular matrix (ECM, Fig. 1A). It is the combination of cells and ECM which define the tissues in our body. For example, tissues like cartilage and bone are mainly constituted by ECM. In the first case, chondrocytes are entrapped in a highly hydrated ECM, whereas in the case of bone, ECM is highly mineralized conferring the rigidity typical of that tissue. In addition, collagen proteins secreted by cells in ligaments and tendons align along the long axis of the tissue, thus conferring resistance to load and strain. In contrast, collagen fibers in the intestine are arranged in a spiral, allowing contractions that will guide the bolus through.

For a long time considered a static entity providing only support to the tissues, we now know that ECM also plays a critical role in cell signalling and tissue homeostasis, *i.e.*, in maintaining a balance between anabolic and catabolic activities by which the turnover of a tissue is replaced by new ECM and cellular milieu.[2] ECM acts as a sensor, conveying information from the exterior of the cell to the inside and *vice-versa*. Cells are exposed to a myriad of different forces and the balance between internal and external forces will elicit a cellular response (Fig. 1B).[3,4] This phenomenon is easily visualized if we think of a tennis player: the bones in the arm that the player uses most frequently are usually thicker than the ones in the other arm. This means, that the external stimulus (hitting the ball) triggers a change in the inside (bone growth). Somehow, ECM provides the bone forming cells with a signal (so-called outside–in signalling) and the cell will respond accordingly by changing its gene

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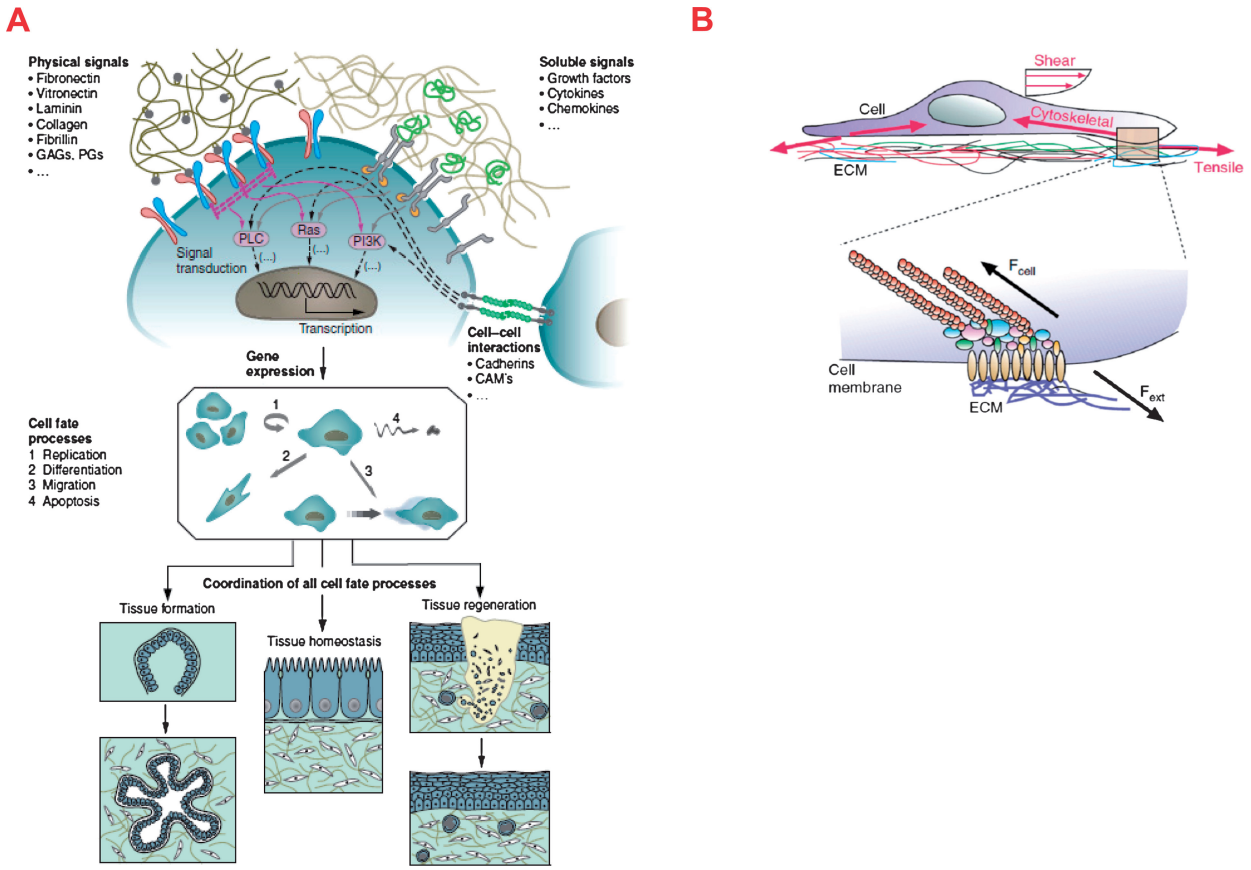
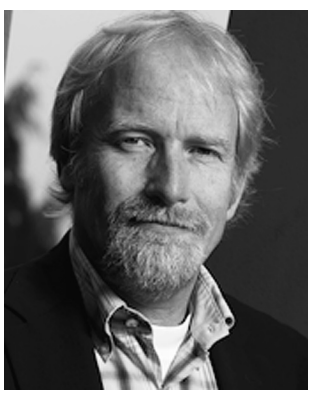


Fig. 1 A. Overview of the cell microenvironment. The cell's surrounding is composed of a highly hydrated environment containing physical and soluble signals, which can control signalling and activation of certain target genes. The activation of these genes will control the phenotype of the cell (Reprinted with permission from ref. 17 ©2005 Nature Publishing Group). B. Schematic representation of a cell and the forces applied to it. Note that ECM plays a key role in force transmission to and from the cell (Reprinted with permission from ref. 3 - *Journal of Cell Science*).

expression profile. The external signals perceived by a cell can be as different as shear stress due to fluid flow, tensile forces *via* binding to ECM with different molecular composition resulting in differences in stiffness, surface topography or cytoskeletally generated forces.[3,5] The way cells sense and respond to the stimulus provided by ECM is mainly *via* membrane receptors called integrins and/or mechanosensitive ion channels (for a comprehensive review see ref. 6 and 7).

The critical role of ECM in tissue formation and homeostasis is unveiled by mouse mutants for certain ECM proteins as well as some human disorders.[8] Diseases like osteogenesis imperfecta (OI), Ehlers–Danlos (ED) syndrome and epidermolysis bullosa (EB) are caused by mutations in genes encoding structural proteins such as collagen, resulting in a range of symptoms including skin blisters and erosions in the case of EB, to fatal outcomes such as disruption of blood vessels in ED syndrome.[9,10]



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Examples describing crosstalk between ECM and cells are reported in the literature. For example, it has been shown that during skin wound healing, a fibrin matrix is formed in the wound bed serving as a scaffold, allowing migration and proliferation of dermal fibroblasts (Fig. 2A).[11] Another example of cell–ECM crosstalk is matrix elasticity, which can control the differentiation of mesenchymal stem cells (MSCs) into different lineages. Soft matrices mimicking brain tissue are neurogenic, stiffer matrices mimicking muscle are myogenic and rigid matrices mimicking bone are osteogenic, indicating that ECM properties can guide gene expression and control cell fate (Fig. 2B).[12–15]

Overall, due to its 3D architecture, its mechanical properties and signalling potential, ECM is an interesting candidate for material scientist looking for appropriate 3D scaffolds with favourable biological properties.[16,17]

In this review we will first describe the composition of natural ECM and applications in tissue engineering based on natural ECM-derived materials. We will show the state-of-the-art of technologies aiming at substituting natural ECM and in the end we will identify the challenges for the future.

Natural ECM—nature's best scaffold?

Leonardo da Vinci was one of the first to comprehend that understanding nature is a pre-requisite to engineer solutions. Centuries later, tissue engineers striving to mimic ECM have to analyze and study natural ECM prior to designing scaffolds with similar characteristics.

The first mission is to identify the components of ECM. An obvious choice is to start with the most abundant protein: collagen. More than 20 genetically different types of collagen have been identified so far. Collagen molecules consist of three polypeptide α chains, each of them containing at least one repeating Gly-X-Y sequence, where X and Y are usually proline and hydroxyproline, respectively.[18,19] The three chains are supercoiled around a central axis in a right-handed manner to form a triple helix. Collagen molecules self-assemble into collagen fibrils, which form the collagen fibers after crosslinking. During the biosynthesis of collagen, the molecule undergoes several post-translational modifications, *i.e.* hydroxylation and glycosylation of particular residues. Depending on their structure and supra-molecular organization, collagens can be classified into fibrillar (accounting for 90% of all collagens) and

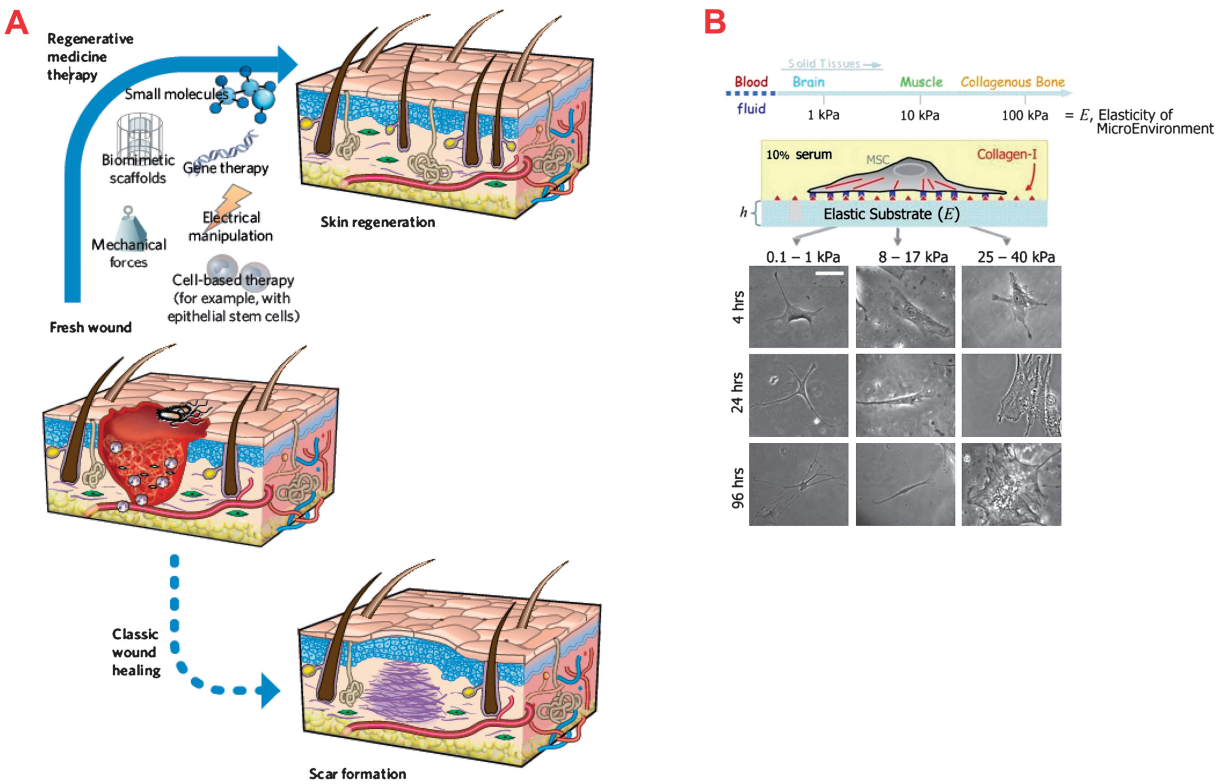

Fig. 2 A. Classical wound healing process. When skin is damaged, new tissue formation occurs followed by remodelling. Initially a scar is formed at the surface of the wound and new blood vessels appear. Fibroblasts migrate to the area and deposit a new disorganized collagen matrix which will be remodelled in a later stage. Alternatively, regenerative medicine provide us with tools to interfere with the classic healing process in order to avoid scar tissue formation and to recreate the original tissue with the same structure and function as the damaged tissue (reprinted with permission from ref. 11 ©2008 Nature Publishing Group). B. Matrix stiffness influences the differentiation of human mesenchymal stem cells. By controlling the level of crosslinking the stiffness of the gel can be adjusted to match that of ECM of the desired tissue. Depicted in the Figure are the differences in cell morphology, during time, for cells seeded on matrices with different stiffness. Note that for the stiffness typical of the brain ($E \sim 0.1$–1 kPa) cells present a branched neuron-like morphology, in the stiffness of the muscle ($E \sim 8$–17 kPa) cells showed an elongated morphology typical of myoblasts and on the stiffness of the bone ($E \sim 25$–40 kPa) cells showed a spread and cuboidal morphology typical of differentiated osteoblasts (reprinted and adapted with permission from ref. 13 ©2006 Elsevier).

non-fibrillar collagens. For example, fibrillar collagens provide torsional stability and tensile strength and can be found in tissues such as bone, cartilage or skin. In contrast, basement membrane collagens such as collagen type IV are more flexible, giving the basement membrane its typical characteristics.[18–22] In general, collagens are mainly seen as structural proteins although they contain small sequences responsible for binding to cellular receptors.

Elastin is a protein which can be found in ECM of tissues that have the ability of transiently stretching such as skin, oesophagus, lungs or blood vessels. Tropoelastin is the soluble precursor of elastin which, upon secretion to the extracellular space, can be stabilized by covalent crosslinking between the side chains of lysine, resulting in massive macro-arrays of mature elastin. Due to the extensive crosslinking there is a decrease in the solubility. The elastic properties of elastin have been attributed to the conformational entropy between the non-polar peptide sequences and lysine sequences which are extensively cross-linked.[23–26] In short, collagen and elastin may be considered as the bricks of ECM due to their contribution to the mechanical properties of ECM.

Another mode of action can be seen with the GAGs, which contribute to the gel-like characteristics of ECM. GAGs are long unbranched carbohydrated polymers consisting of repeating disaccharide units. These units are composed of one of two modified sugars—*N*-acetylgalactosamine or *N*-acetylglucosamine. They are responsible for growth factor sequestration and, due to their ability to retain water, they contribute to the characteristic appearance of ECM. When hydrated, GAGs are responsible for increase in tissue stiffness as they act as water pumps under mechanical loads. The reason for this can be due to water molecules binding to GAGs anionic groups as previously proposed.[27] In articular cartilage, this results in an osmotic pressure of 0.1–0.2 MPa that accounts for approximately 50% of static mechanical stiffness under compression.[28] Chondroitin sulfate A and B, heparin, heparin sulfate and hyaluronic acid are among some of the GAGs that can be found in ECM.[29–31] Chondroitin sulfate and hyaluronic acid contribute to frictional resistance against interstitial fluid flow. They are applied in cartilage tissue engineering as natural components of hydrogel-like scaffolds, because they can promote chondrocyte redifferentiation.[32–35] An extensive review of proteglycans is beyond the scope of this review and can be found elsewhere.[36] Cell attachment is another important role of ECM in many tissues. As such, some ECM proteins can be considered as the glue of ECM. Two of the most common proteins responsible for cell adhesion are fibronectin and laminin. Fibronectin (FN) is the second most abundant protein in ECM, where it is organized into a fibrillar network. It is a large glycoprotein dimer and each monomer contains three types of repeating units designated type I, II and III. In these units we can find functional domains responsible for interaction with cell surface receptors and with fibronectin itself. It can be found in two forms: soluble (in the blood plasma) or insoluble (present in ECM). Fibronectin is rich in Arg-Gly-Asp (RGD), a tripeptide important in cell adhesion *via* the $\alpha 5\beta 1$ integrins as well as in cell growth. Plasma fibronectin is involved in wound repair contributing to the formation of a provisory matrix whereas cellular fibronectin is incorporated into the fibrillar matrix secreted by the cell.[37–42] In addition to the RGD sequence other important sequences can be found in FN. For example, coating of materials with the FN fragment $FNIII_{7-10}$ or with the collagen mimetic peptide GFOGER enhanced osteoblastic differentiation in bone marrow stromal cells compared to uncoated materials.[43,44]

Laminin is a complex adhesion molecule especially found in the basement membrane of almost every tissue. It is composed of α, β and γ subunits arranged to form a large coiled-coil quaternary structure consisting of three short arms and one long arm.[38,45] It has globular domains at the end of its arms which mediate the interaction with other molecules. Laminin has been involved in cell differentiation, migration and proliferation and plays a critical role in angiogenesis.[46–48]

Fibrin is a fibrillar protein that plays a key role in the process of wound healing. It is formed by polymerization of fibrinogen in the presence of thrombin and subsequently undergoes cross-linking mediated *via* transglutaminases contributing to the clot formation during wound healing.[49–51] Fibrin matrices are currently used in clinics and they can be used as drug delivery systems for proteins such as bone morphogenetic proteins (BMPs), incorporation of bioactive peptides or as cell delivery systems.[52–56]

Use of naturally derived ECM proteins in regenerative medicine

All these ECM proteins have a common origin: the cell. Thus, the easiest way to obtain these proteins for tissue engineering applications is to allow cells to produce them and, after ECM deposition, to remove the cells. This is usually accomplished using detergents or other methods that will leave a decellularized matrix with most proteins in the native state.[57]

The use of decellularized matrix has revealed a pivotal role of ECM on cell fate. For example, laminin is the major component of an ECM-derivative widely used in tissue engineering—Matrigel®. Matrigel® is a solubilised basement membrane preparation derived from a mouse sarcoma cell line, a tumour rich in ECM proteins. Matrigel® is able to mediate endothelial differentiation and it is also used in invasion assays to analyze tumour progression. When human umbilical vein endothelial cells (HUVECs) are seeded on Matrigel® they adopt a tube-like structure, characteristic of the first steps of vessel formation, in contrast to HUVECs seeded in normal tissue culture plates.[58]

An elegant example where ECM influenced cell fate came from a study using ECM derived from embryonic stem cells. Cancerous pigment cells seeded onto ECM deposited by embryonic stem cells changed their gene expression profile from one typical of cancer cells to one resembling normal pigment cells. This exemplifies the ability of ECM to reprogram the fate of cells.[59–61] Similarly, bone marrow derived mesenchymal stem cells, grown on murine decellularized ECM from bone marrow cells, showed increased proliferation compared to normal culture. *In vivo*, mesenchymal stem cells (MSCs) expanded on ECM displayed enhanced bone and bone marrow formation.[62]

Lessons from basic science are already being applied in tissue engineering. For example, a bioartificial heart was generated by seeding cardiac and endothelial cells on ECM of a decellularized heart (Fig. 3).[63] Another application has been the coating of titanium with ECM prior to seeding of MSCs. Titanium meshes

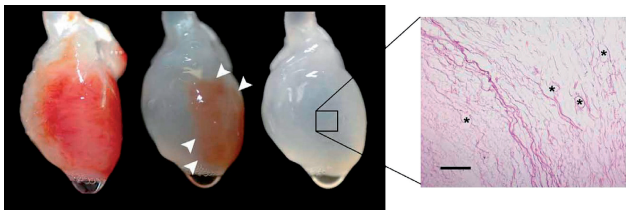

Fig. 3 Decellularization of whole rat heart. The Figure shows the sequential process of decellularization upon perfusion of the heart with a detergent. The inset shows that no intact cells or nuclei are present after the process and that vasculature conduits were preserved during the decellularization (asterisks) (Reprinted with permission from ref. 63 ©2008 Nature Publishing Group).

were coated with MSCs and decellularized after the cells had formed a confluent layer and had deposited ECM on the metal. Next, the material was re-seeded with MSCs and ECM-functionalized scaffolds showed enhanced calcium deposition compared to uncoated titanium.[64]

There is increasing evidence that ECM proteins also play an important role in stem cell niches. Stem cell niches are anatomical structures, including cellular and acellular components, which integrate local and systemic factors to regulate stem cell proliferation, differentiation, survival and localization. They are responsible for maintaining the self-renewal potential of stem cells and, at the same time, replenish the body with differentiated cells when necessary.[65] A critical role of ECM proteins in the stem cell niche has been demonstrated in the case of tendon tissue. Depletion of biglycan and fibromodulin, two ECM proteins, affected the differentiation of tendon stem/progenitor cells, thus impairing tendon formation *in vivo*.[66]

Some ECM-derivatives have found their way to the clinic. Acellular human dermal matrices generated by freeze-drying are used to treat skin wounds.[67] Another product commonly used in the clinic as a bone graft substitute is demineralized bone matrix (DBM). DBM is obtained by acid extraction of the mineralized component of bone, while maintaining the organic component comprised of collagen and non-collagenous proteins. Among these proteins, an important role in DBM osteoinductivity is played by the presence of BMPs, which contribute to the differentiation of local MSCs into the osteogenic lineage.[68–70]

Plagiarism allowed. Scientists attempt to copy nature.

An important strategy of tissue engineering is to enhance the natural regenerative capacity of the adult human body. It has been a long way from the stage of a single cell to the multicellular organism we are today. Tissue engineers cannot expect to mimic the complexity of a multicellular organism using a single technique. Rational combination of techniques will allow us to walk on a road that nature followed for thousands of years and, by analyzing ECM that constitute this multicellular organism, we can try to copy nature's best scaffold.

While designing ECM-inspired tissue engineering grafts one needs to take in consideration the advantages and limitations of the available techniques, such as decellularization of natural tissues, hydrogel polymerization and electrospinning. Such techniques will influence many properties such as mechanical, adhesion/signalling, architecture and remodelling. Implications on the above mentioned techniques and on their bias on the resulting ECM-inspired scaffolds are discussed hereafter.

Mechanical properties

To mimic the mechanical properties of ECM, three biomaterials/techniques are commonly used: hydrogels, electrospinning and rapid prototyping. Due to their capacity to swell and entrap water, hydrogels are considered as a logical choice to mimic hydrated ECM. In the presence of a crosslinking agent, these biomaterials form a crosslinked fibrillar network at the micro and nano scale dimensions when dissolved in an aqueous medium. Cells can be encapsulated in the liquid form of the biomaterial and are entrapped in the 3D fibrillar network during the crosslinking reaction (Fig. 4A and 4B). Increasing the percentage of the material dissolved results in hydrogels characterized by a network with increased stiffness but decreased pore size and nutrient diffusion properties.[71,72] Therefore, when designing hydrogel scaffolds for tissue engineering it is critical to maintain a balance between mechanical and diffusion properties to allow scaffold integrity and cell viability during tissue development.

Hydrogels that find applications in tissue engineering comprise natural and synthetic polymers, or combinations thereof, forming semi-interpenetrating networks. Among natural polymers, fibrin glue is successfully used in the clinics as a wound-repair scaffold.[73,74] Synthetic hydrogels are also considered, because their non-toxicity (biocompatibility) and bulk properties can be controlled during synthesis. Photopolymerizable hydrogels like poly(ethylene glycol diacrylate) (PEGDA) have found promising applications in cartilage regeneration (Fig. 4C).[75,76] Thermosensitive hydrogels such as poly(*N*-isopropylacrylamide) (PNIPAAm) and Pluronic® (polyethylene oxide–polypropylene oxide–polyethylene oxide block copolymers) are also interesting biomaterials for connective tissues, myocardic and bone tissue engineering.[77–79]

Electrospinning of polymers is gaining considerable attention because it allows the deposition of fibers with a nano- to micrometer resolution, which resembles ECM fibrillar composition.[80,81] These fibers can be produced using natural ECM proteins or synthetic polymers.[82–88] By varying the processing parameters, it is possible to achieve fibers with variable architecture and surface topology, which are known to influence cell adhesion.[89–91] Interestingly, when scaffolds are comprised of micro- ($\varnothing$ ~2–15 μm) and nanofibers ($\varnothing$ ~100–900 nm) cell–material interactions are enhanced and synthetic polymers gain further instructive properties for tissue regeneration compared to the same polymeric scaffolds made of larger diameter (>100 μm).[91–93] Thus, micro- and nano-scaled fibers may acquire some of the natural ECM functions.

Hydrogels and electrospun micro and nano fibrous scaffolds can mimic the physicochemical properties and the topographical and structural features of ECM, but unfortunately do not match, in most cases, the mechanical properties of the tissues to be regenerated. To do so, rapid-prototyping fabrication technologies might be a good alternative. It has been shown that with these techniques it is possible to generate scaffolds with

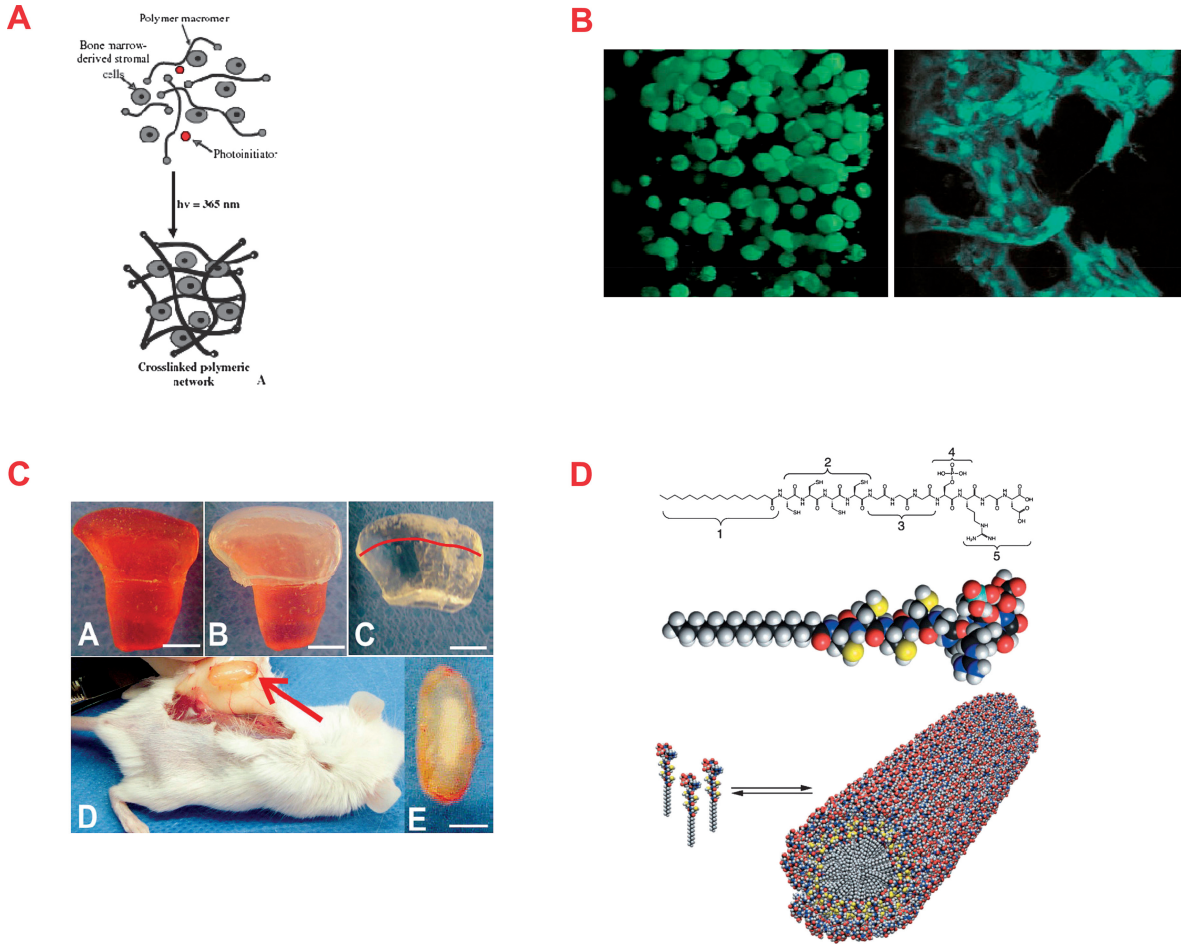

Fig. 4 A. Schematic diagram of the photoencapsulation process of cells in a hydrogel. Cells, a polymer macromer and a photoinitiator are combined and exposed to light in order to form a crosslinked polymeric network containing cells surrounded by a highly hydrated network (reproduced with permission from ref. 136 ©2005 Wiley-VCH). B. Changes in the morphology of human mesenchymal stem cells encapsulated in a functionalized degradable PEG gel. First, cells present a round morphology which will change to a more spread and tissue-like structure during time due to interactions between the cells and the hydrogel (from ref. 137, reprinted with permission from AAAS). C. Example of a tissue engineered hydrogel with a pre-defined shape and spatial separation of cell types. The hydrogel was designed to mimic the shape of the articular condyle and, in a two step process, mesenchymal stem cells committed either into the chondrogenic or in the osteogenic lineage were encapsulated. Upon implantation in an immunodeficient mouse the construct was recovered and the presence of bone and cartilage could be observed (reproduced from ref. 76 with kind permission of Springer Science and Business Media). D. Chemical structure of a peptide amphiphile with key structural features (1—long alkyl tail (hydrophobic), 2—four cysteine residues, 3—flexible linker consisting of three glycine residues, 4—phosphorilated serine to drive hydroxyapatite mineralization, 5—RDG adhesion ligand), the molecular model and finally the self-assembly scheme of the peptide amphiphile into a cylindrical micelle (from ref. 102 Reprinted with permission from AAAS).

modulable physicochemical and mechanical properties while maintaining a completely interconnected pore network for cell migration and nutrient diffusion.[91,94–98] These 3D structures can be optimized to match the mechanical properties of a number of soft and hard tissues like meniscal and articular cartilage.[96] However, rapid prototyping techniques are limited by the resolution of the main struts composing 3D scaffolds, which is confined to the order of hundreds of micrometers. As ECM has physical features well below this limit, ranging from the micron to the nano scale, perhaps the best solution to fabricate ECM-inspired scaffolds passes through the combination of the above mentioned biomaterials and techniques to create a structure with matching physicochemical and mechanical properties. Theoretical modelling elegantly disclosed properties of collagen molecules which can be crucial for the design of new materials. When the complexity of a biological system can be introduced in such models, scientist can gain insights to the mechanical properties which, for the time being, are only partially understood.[22]

Adhesion/signalling properties

When designing new materials, tissue engineers should achieve biofunctionalization of those materials in order to improve cell adhesion and, at the same time, provide biological cues able to recruit cells and control their activity. For example, peptides derived from two ECM proteins (laminin and N-cadherin) were incorporated in fibrin and tested for their potential application in nerve regeneration. Mixing these peptides with fibrin resulted in an 85% increase in the number of regenerated axons when compared with unmodified fibrin.[52] Similarly, laminin-derived

recognition sequences such as IKLLI or IKVAV preserved viability, reduced apoptosis and increased insulin secretion of β-cells.[99] Modification of the PEGDA backbone with RGD peptides, the peptide motif responsible for binding to the integrin family of adhesion receptors, was essential to achieve embryonic stem cell adhesion and their subsequent differentiation into the chondrogenic lineage.[100] In another example, a collagen mimetic peptide was used to endow biomaterials with properties to attract growth factors, such as vascular endothelial growth factor (VEGF). This resulted in improved morphological features of endothelial cells, indicative of tubulogenesis.[101]

A key issue in improving the biological properties of biomaterials is the density at which the biological moieties are presented to the cells. An important advance in this area has been achieved by using molecular self-assembly. For instance, functionalised synthetic amphiphilic peptides have been used to support differentiation of neurons, mineralization of bone hydroxyapatite, enamel formation and regeneration and adhesion of bladder smooth muscle cells to branched-peptide-amphiphile functionalized scaffolds, which open a new window of possibilities for tissue engineering.[102–110] Amphiphilic peptides possess a hydrophobic and hydrophilic region. Functional groups such as the RGD peptide can be incorporated in the molecule to provide control over biological functions (Fig. 4D).[102,104,105,111–115] It has been shown that tethered small molecules could directly influence the cell fate of human mesenchymal stem cells (hMSCs), with charged phosphate groups leading to osteogenesis and hydrophobic *t*-butyl groups inducing adipogenesis.[116] Another important aspect of biomaterial functionalisation is the difficulty to attach functional groups to the materials. Each moiety has to be combined with a material of interest in a separate process of chemical synthesis. Considering the enormous number of possible variables in material functionalisation, *i.e.* using peptide sequences or antibodies, streamlining this process is an important step towards bio-functionalisation and high throughput screening of biomaterials. An elegant approach to achieve this uses ureido-pyrimidinone (UPy) moieties, which form non-covalent hydrogen bonds strong enough to create mechanically viable biomaterials. In addition, a UPy-functionalised biomaterial can be functionalised with libraries of UPy-functionalised peptides by simple mixing. It has been shown that by using UPy-functionalized polymers in combination with UPy-modified biomolecules cell adhesive materials can be created.[117] Libraries of functionalised biomaterials can be screened on material arrays. For instance, robotic spotting technology has been applied to analyze combinations of ECM domains immobilized on a hydrogel surface. Thirty two different combinations of five different ECM molecules were tested for their capacity to maintain the phenotype of rat primary hepatocytes or to induce differentiation of mouse embryonic stem cells.[118,119]

Spatial organisation in ECM

In the previous paragraphs we have seen that it is possible to control the mechanical properties and molecular cues of biomaterials such that they mimic those of ECM of the tissue of interest. So far, both the properties and cell seeding have been homogenous throughout the material, whereas body tissues are complex structures in which multiple cell types and their respective ECM are highly spatially organized. For instance, an endothelial cell layer covers the inside of a blood vessel, smooth muscle cells surround the tube to support it, and an ECM separates the two cell types.[120] Evidently, engineering the correct spatial control of cells and ECM molecules within tissue engineered constructs is a challenge in which chemical science can create valuable tools. For instance, dielectrophoretic forces have been applied to control the spatial deposition of articular chondrocytes within photopolymerizable PEG hydrogels. Clusters of 3 to 18 cells each were created in a hydrogel and cluster size, dose-dependently, determined deposition of a cartilage matrix by the cells. This shows that micro-organization (a previously uncontrolled variable) influences cell behaviour (Fig. 5A).[121] Another technique which can be used to introduce heterogeneity in scaffold architecture is electrospinning. By changing the parameters during the deposition process, one can fabricate multi-layered scaffolds, each with unique properties as seen in tissues with a layered hierarchical architecture such as cartilage.[122] Increased complexity can be added by electrospraying different cell types at the same time as the electrospun fibers are deposited. This allowed, for example, the creation of tubular scaffolds seeded with smooth muscle cells, which maintained viability through a thickness of 300–500 μm, supporting successful vascular regeneration.[123]

Spatial organisation occurs not only at the tissue level, as demonstrated by the examples above, but also at the single cell level. For example, a muscle cell is elongated because this represents the most optimal mechanical properties, whereas lipid storage is most optimal in a spherically shaped adipocyte.[124,125] In these examples, form follows functions and technology to control cell shape will benefit cell function. However, biologists are increasingly aware of the fact that function can also follow shape. Several manuscripts describe that geometric presentation of ECM proteins to a cell is a powerful tool to influence cell function. A classical example of this is a study in which a poly-(dimethyl siloxane) (PDMS) stamp was used to create patches of fibronectin of variable sizes on a glass surface (Fig. 5B). Subsequently, hMSCs were allowed to adhere to the surfaces and it was found that depending on the patch size, the MSCs either differentiated into fat cells or bone cells (Fig. 5C and 5D).[125–128] Using similar technology, the choice between life and death of a cell was influenced by the shape the cell was forced into.[129] Even more delicate examples show that it is possible to deposit, with well-defined geometries, different ECM components at a subcellular resolution using automated printing techniques based on atomic force microscopy. Sub-cellular feature sizes of 6–9 micrometers were achieved using two components: fibronectin and a commercially available mixture of laminin/collagen type IV. The ratio between these components varied and affected cell alignment.[130]

Remodelling

As if mechanical and cell signalling properties and spatial organisation do not already provide sufficient engineering challenges, time is another variable in tissue function. Tissues are not fixed structures but undergo constant remodelling and a bio-mimetic approach for ECM engineering needs to address this aspect as

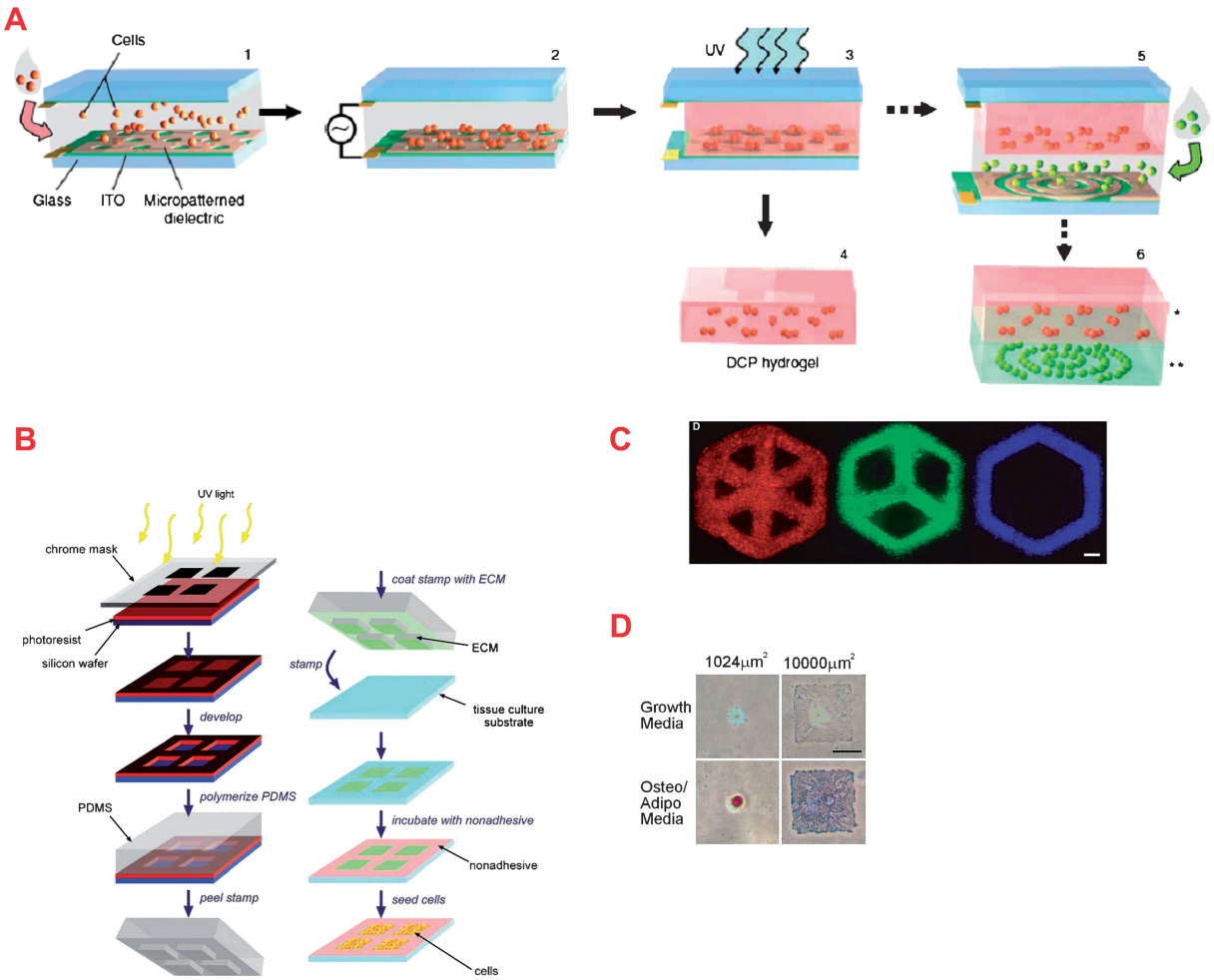

Fig. 5 A. Production of hydrogels with controllable incorporation of cells. Cells are localized using dielectrophoretic forces in micropatterned gaps of the dielectric layer. Exposure to UV light polymerizes the hydrogel thus keeping the cells embedding in a defined position. Once polymerized the gel can be removed and used for further applications. This process can be repeated using every time different cell organization schemes, cell types or hydrogel formulations (legend: DCP—dielectrophoretic cell patterning; ITO—indium tin oxide; reprinted with permission from ref. 121 ©2006 Nature Publishing Group). B. Scheme of ECM protein deposition onto a surface using microcontact printing. A PDMS stamp is produced using photolithography and ECM proteins are coated on the stamp and transferred to the substrate prior to treatment with a non-adhesive compound (reprinted and adapted with permission from ref. 126 ©2005 Elsevier). C. Examples of microscale patterns created using photopatterning. Viability of the cells in the different patterns is indicated by fluorescent dyes. Scale bar is 500 μm (reproduced from ref. 128 with permission of FASEB; permission conveyed through Copyright Clearance Center, Inc.). D. Controlling cell fate by cell shape. A fibronectin spot with a defined size was deposited and cells seeded on the protein were cultured in a mixed medium allowing differentiation either into adipocytes or osteoblasts. Cells that grew on the smallest fibronectin spot (1024 μm^2) became adipocytes whereas the cells grown in the larger spots (10 000 μm^2) became osteoblasts (reprinted and adapted with permission from ref. 125 ©2004 Elsevier).

well. Many ECMs are degraded by cell-secreted proteases, such as the matrix metalloproteinases (MMP) and serine proteases.[131,132] ECM remodelling is not only important for ECM maintenance but also to allow cell migration. Furthermore, during ECM remodelling, growth factors entrapped in the matrix will be released, which can act as morphogens controlling tissue formation. Proteases are highly specific and degrade ECM at defined peptide sequences. This has been used to design materials with specific proteolytic sites which allow ECM remodelling and new tissue ingrowth in the implant.[80,133,134] For instance, protease-functionalised hydrogels loaded with the bone inducing growth factor BMP-2 showed that the extend of bone formation depended on the proteolytic activity of the matrix.[134,135]

Challenges for the future

In this review we have highlighted the biological complexity of the ECM and have discussed engineering solutions to design ECM-mimicking scaffolds for tissue regeneration. Scaffolds from the next generation will not merely function as mechanical support, but act as instructive matrices that guide cells to correct tissue regeneration, growth and development. So far, efforts have focused on chemical modifications of the biomaterial backbone by inserting peptide sequences or on physical processing by downscaling the characteristic dimensions of the base elements (for example, fibers, struts and pore walls) forming the 3D scaffolds. As nature has provided us with multidimensional and multifunctional tissues, we should aim at mimicking this

complexity more closely. A possibility is to combine different scaffold fabrication technologies at different scales, to recreate scaffolds with physical and mechanical properties resembling those of ECM. This would also mean incorporating different biomolecules in different regions of the scaffold to hierarchically replicate the biological signals that govern tissue development and homeostasis. In a further effort to bring the worlds of biology and material chemistry together, it could be even envisioned creating chemically modified biomaterials that allow recruitment of cells and biological molecules *in situ*, thus preventing cell culture techniques *in vitro* before implantation. Possibilities are seemingly unlimited, as more and more biomaterials are synthesized every day and technologies are being developed. Since investigating each single combination would require extensive costs and resources using standard biological evaluation, the search for the most optimal ECM-like scaffold should pass through initial high-throughput screening. High content screening of cell–material interaction allows one to study the influence of thousands of biomaterials and ECM proteins on cellular activity. Nevertheless, the integration of mechanical and biological cues is still not optimal. When designing ECM-inspired scaffolds we need to overcome a major hurdle limiting their use for tissue engineering applications: nutrient diffusion limitations. A compromise between mechanical properties and diffusion capacity has to be achieved so the scaffold can have desirable mechanical properties without impairing diffusion of nutrients or by-products of the metabolism. Additionally, spatial control of ligands is a challenge for both biologists as well as material scientists as the decision on which biological cues and their spatial distribution can dictate cell fate. In that respect, degradation properties and the concomitant release of degradation products need to be carefully controlled in order to retain the properties of the scaffold, to avoid inflammatory responses as well as an increase in the local concentration of certain compounds capable of hindering cellular functions. Integration of different technologies can also be hampered by the need of combining different processing techniques which can result in incompatibility of building blocks or creation of fracture/degradation zones within the constructs. When considering the use of biological systems in an attempt to produce recombinant ECM proteins for incorporation in scaffolds one should be aware that most available systems are not capable of mimicking the complex post-translational modifications necessary to have a functional protein. Most likely, new systems to produce and isolate these proteins need to be developed.

We have witnessed the eras of genomics and proteomics but we envisage the era of materiomics to bridge the gap between natural and engineered tissue development.

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