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Cell Encapsulation Systems Towards Modular Tissue Regeneration: From Immunoisolation to Multifunctional Devices

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In the primordial cell encapsulation systems, the main goal was to treat endocrine diseases avoiding the action of the immune system. Although lessons afforded by such systems were of outmost importance for the demands of Tissue Engineering and Regenerative Medicine, the paradigm has recently completely changed. If before the most important feature was to mask the encapsulated cells from the immune system, now it is known that the synergetic interplay between immune cells and the engineered niche is responsible by an adequate regenerative process. Combined with such immuno-awareness, novel or non-conventional emerging techniques are being proposed developed the new generation of cell encapsulation systems, namely layer-by-layer, microfluidics, superhydrophobic surfaces, and bioprinting technologies. Alongside with the desire to create more realistic cell encapsulation systems, cell-laden hydrogels are being explored as building blocks for bottom-up strategies, within the concept of modular tissue engineering. The idea is to use the well-established cell friendly environment provided by hydrogels, and create more close-to-native systems owning high heterogeneity, while providing multifunctional and adaptive inputs.

1. Introduction

The primordial cell encapsulation systems were designed to treat endocrine diseases, while protecting and masking the encapsulated cells from the immune system.[1,2] Aimed to provide long-lasting solutions for multiple cell dysfunctions,[3–5] such immuneprivileged systems prompted the beginning of the bioartificial organs era with the creation of the first fully artificial pancreas.[6] Another remarkable milestone, is the work of Lim and Sun [7] with the microencapsulation of pancreatic islet cells in alginate spherical hydrogels for transplantation in diabetic rats. Analyzing the historical root of hydrogels encapsulating cells, it is easy to understand why most of the studies reported in the literature are focused in the treatment of endocrine diseases, mainly diabetes mellitus through the encapsulation of pancreatic islets. This is driven by the high number of diabetic patients and the recognized benefits of cell encapsulation strategies.[8] Although these studies did not foresee the applicability of each cell-containing microcapsule as building blocks for modular tissue engineering (TE), they belong to its historical roots. Put simply, the primordial cell encapsulation systems were cell-containing blocks packed to create an artificial structure with biological activity. The bioactive molecules released to target an endocrine disease were dependent on the type of cells encapsulated. However, such artificial biologically active structures lack heterogeneity, and thus do not reassemble tissue-like structures. Native tissues are composed by heterogeneous mixtures of cell phenotypes or morphologies (e.g. the particular case of cartilage tissue with only one cell type, chondrocytes, but with depth-dependent morphology). As such, although current strategies for creating modular tissues draw from the primordial cell encapsulation systems, the paradigm has significantly change with the evolution of the tissue engineering and regenerative medicine (TERM) field. One of the strategies of the TERM field is to create engineered tissues through the bottom-up assemble of microstructural functional units.[9] This strategy, termed as modular TE, is based on the concept that the replication of functional units may aid in the reconstruction of the heterogeneity of native tissues, and thus accelerate tissue healing. As such, when aiming tissue regeneration, cell encapsulation systems must be capable of facilitating the reconstruction of this heterogeneity upon implantation into a lesion site. These modules can be created in multiple ways,[10] but the present review will focus on the use of hydrogels produced by novel or non-conventional techniques, namely layer-by-layer (LbL), microfluidics, superhydrophobic surfaces, and bioprinting. Hydrogels are the gold standard material of most cell encapsulation systems proposed for TERM, mainly due to their highly hydrated 3D environment, which resembles the tissue-like elasticity of the native extracellular matrix (ECM), and maximizes the diffusion of essential molecules for cell survival. Moreover, hydrogels allow mild processing conditions and can be easily functionalized to enhance cell-material interactions.[11–14] Despite the established applicability of hydrogels as cell encapsulation systems for TE, it is only recently that researchers have begun to explore its potential to modulate the immune response, with significant clinical impulse observed in the past decade itself.[15,16] Of note, such immunomodulation is completely distinct from the classical long-term immunoprotective feature of the primordial cell encapsulation systems. It is true that the encapsulation matrix mediates the interaction with the host environment, and it may find great applicability during the acute inflammatory phase following any tissue damage. But then, it is desirable to occur the degradation of the encapsulation matrix balanced with the newly deposited ECM in order to promote a proper vascularization and tissue integration that dictate the success of the biomaterial implanted. Therefore, immunoprotection is no longer a requisite if tissue regeneration is aimed, but hydrogels with immunomodulatory properties allow to control the immune response upon implantation while promoting tissue regeneration.

In the present review, we intend to bridge the path of cell encapsulation systems from immunoisolation devices to treat endocrine diseases to multifunctional-engineered systems that aim to stimulate the regeneration of damaged tissues. A brief discussion of this path will be given to highlight the new immunomodulatory biomaterials being proposed, while emphasizing the use of novel or non-conventional emerging techniques that are contributing to the development of the next generation of such systems. Subsequently, we will highlight the applicability of cell encapsulation systems for creating modular engineered tissues that can effectively direct the formation of larger and clinically-relevant tissues using bottom-up TE principles.

2. *"Open"* vs. "*closed*" scaffolds: limitations, advantages and practical considerations

Independently of the type of strategy used, all TERM strategies aim to regenerate living, healthy, and functional tissues, either partially by tissue grafts or even a total replacement of a severely damaged organ. In the perspective of using scaffolds for TERM, there are two main strategies: *(i)* scaffolds with adhered cells that contact directly with the host environment, here termed as "*open*" scaffolds, or *(ii)* scaffolds at which core cells are encapsulated, here termed as "*closed*" scaffolds that comprise cell encapsulation strategies (**Figure 1**). The designation of "*open*" or "*closed*" scaffolds is thus respectively related to the direct contact or isolation between cells and the surrounding environment. In "*open*" scaffolds the production methods are not limited by the presence of cells, thus can include the use of precursors and harsh solvents and/or reactants, as long as the cytocompatibility of the obtained scaffold is assured, including its degradation products. On the contrary, the production methods of "*closed*" scaffolds must ensure mild conditions since cells are present before the processing of the biomaterial. This significantly impairs the available spectrum of technologies and biomaterials that can be applied to produce closed scaffolds. Nevertheless, "*closed*" scaffolds can offer several advantages compared to "*open*" scaffolds. "*Closed*" scaffolds *(i)* enable the creation of privileged and controlled microenvironment for cells; *(ii)* allow minimal invasive implantation by *in situ* injection, and thus the hydrogel can be easily fixed into injured sites with variable geometries without requiring glue or sutures;[17] and *(iii)* facilitate the incorporation within a single structure of multiple compartments or elements with distinct functions, which confers multifunctionality to the engineered system. Additionally, "*closed*" scaffolds can also maximize cell interactions (Figure 1-red dots) by allowing direct cell-cell contact in the particular case of liquefied systems.

3. Critical properties of hydrogels for cell encapsulation

The application of cell encapsulation principles to be used in the regeneration of tissues brought several advantages to the TERM field as compared to conventional strategies that mainly use porous scaffolds. Knowing that the key element in TERM is to combine cells with instructive biomaterials to ultimately regenerate damaged tissues, it is easy to understand that the function of the engineered device is not limited to the protection of cells, as it is its main function when cell encapsulation is applied to treat endogenous diseases. Therefore, the development of cell encapsulation devices that aim to promote tissue regeneration should be carefully pondered before its conception, in order to meet all the complex requirements of the field.

3.1 Mild and sterile conditions

The selection of the type of methodology to produce cell encapsulation systems,[18] as well as the type the biomaterials that compose the encapsulation matrix is restrained by the imperative preservation of the viability of the encapsulated cells. Allied to a number of appealing features, such as their structural resemblance to many natural biological tissues, viscoelasticity, and high-water content, hydrogels have become the most favourable material used for cell encapsulation. Since the first hydrogel application in cell encapsulation when Lim and Sun developed calcium alginate microcapsules for islet encapsulation,[19] both synthetic and naturally derived cell encapsulation matrixes have been developed. Most of the previous research has been focused on natural-derived hydrogels, such as alginate, chitosan, pectin, agarose, gelatin, collagen, fibrin, hyaluronic acid, and gellan gum.[20] Their main advantages are: *(i)* their resemblance with the ECM of native tissues, originating in most cases native-like responses under physiological conditions (e.g. biodegradation), *(ii)* abundance, including the vast low-cost resources provided by the marine environment,[21] and *(iii)* their ability to produce hydrogels at mild condition. In particular, some natural polymers have an intrinsic cell adhesion ability due to the presence of cell-binding domains, and thus the functionalization of the encapsulation matrix to allow the adhesion of cells is not required;[22] although this is not applicable for the most widely used natural polymer in cell encapsulation, namely alginate. On the other hand, the main limitations of natural-derived hydrogels are the batch-to-batch variations, and the presence of impurities and contaminants. As an alternative, synthetic polymers have been employed, such as poly(ethylene glycol) (PEG), and poly(vinyl alcohol), which are commonly used in combination with natural polymers to produce hydrogels for cell encapsulation.[18,20,23–26] The most widely used method to produce a viable cell encapsulation matrix is to incorporate cells in a water-based solution of a hydrogel precursor (sol flowing phase) followed by crosslinking commonly triggered by thermal, ionic, or light. Particularly, crosslinking via the interaction of the polymer chains with ions (ionotropic gelation) is the most common method, since via temperature change or ultraviolet (UV) exposure can jeopardize multiple cellular processes. By ionotropic gelation, the solutions can be easily sterilized by filtration or autoclaved, and subsequently processed under mild conditions. The most commonly used method is the ionotropic gelation of alginate in calcium chloride, but also many other ionotropic gelation with different materials have been proposed, such as potassium chloride for carrageenan,[27] and calcium carbonate/D-glucono-δ-lactone for pectin hydrogels.[28]

3.2 Permeability and mass transfer

Once processed and formed, the cell encapsulation matrix will exhibit different transport properties depending on its structure, chemical composition and, in the particular case of hydrogels, the degree and type of crosslinking. Only an adequate mass transport will allow an efficient permeability of essential molecules through the entire matrix, thus ensuring the viability of encapsulated cells. Permeability and mass transfer can thus dictate the successful of cell encapsulation strategies. If desired, either by the presence of a protective enveloping membrane or by tuning the permeability properties of the matrix itself, the entrance of immune cells or resultant redox molecules can be blocked. In fact, even in the absence of a membrane, the hydrogel matrix can act as a mechanical and/or chemical barrier towards in- and out-flowing molecules. The efficiency of the barrier is application dependent, as well as being intrinsically connected with other parameters such as the stability of the cell encapsulation matrix, as further discussed. Nevertheless, in all the encapsulation strategies proposed for tissue regeneration, while the interaction with immune cells can be controlled (see section **4. Immunomodulation in cell encapsulation systems**), they all must ensure an appropriate exchange of essential molecules for cell survival, such as nutrients, oxygen, metabolites, and waste products. Additionally, also the exchange of important signalling biomolecules must be ensured, either between the encapsulated cells (inward diffusion) or between encapsulated and neighbouring cells (outward diffusion). Additionally, in order to promote the integration of the implanted cell encapsulation device with the host tissue, it is desirable that the device should be permeable to the in-growth of blood vessels, except when regenerating avascular tissues such as cartilage. Therefore, either existing or not the presence of an external membrane, the permeability of essential molecules ensuring cell survival across the entire 3D structure is of great importance in the design of the encapsulation matrix. For the particular case of modular TE, the interstitial void spaces between the hydrogel building blocks can facilitate the diffusion exchange by facilitating the ingrowth of the recruited blood vessels. It is important to highlight that the diffusion of essential molecules for cell survival will have a delay when comparing encapsulated cells localized in the core with the ones in the border of the matrix. The same is applied for hydrogel blocks localized in inner regions of the 3D construct. This directly influences the size and/or geometry of the building blocks. Consequently, the majority of cell encapsulation building blocks are typically limited to a diameter of 400 µm, since 200 µm is reported as the maximum diffusion distance of oxygen and nutrients from blood vessels to cells.[29] With the increasing techniques to fabricate innovative and complex hydrogel-based systems for modular TE, other geometries have been progressively proposed (see section **6. Cell encapsulated building blocks to generate complex functional systems**).

The diffusion and permeability of hydrogels depends on at least three factors, namely *(i)* the obstruction effect caused by the presence of impenetrable slowly moving polymer chains that increase the path length for diffusion, *(ii)* the hydrodynamic drag at the polymer interface due to polymer-solvent and polymer-solute bonds during the solute diffusion, and *(iii)* the residual charges of the matrix, presence of counter ions, hydrogen bonds, polar and hydrophobic interactions, which will affect the transportation of solutes exhibiting similar interactive groups (especially essential in the transport of biological molecules).[30] The mass transportation is driven by two main forces, namely *(i)* by pressure gradient (convective), and *(ii)* by concentration gradient (diffusive). In most hydrogels, the transport of solutes occurs by diffusion. In terms of solute diffusion depending on their pore size, hydrogels can be divided into three different classes, namely *(i)* macroporous, with pores >0.1 µm, which the transport occurs mainly by convection *(ii)* microporous, with pores ranging from 0.005 to 0.02 µm, which are in most cases smaller than the solute resulting in hindered diffusion, and *(iii)* nonporous hydrogels, the most commonly used in cell encapsulation, in which solute transport occurs only by diffusion through spaces between macromolecular chains. The different parameters affecting the permeability of cell encapsulation systems using hydrogels have been detailed discussed.[31] Additionally, some cell encapsulation strategies are composed by membranes that surround the encapsulation matrix or directly the encapsulated cells (conformal coating). Although multilayered cell encapsulation systems have been commonly used to allow immunoisolation,[32–34] they have been poorly explored in tissue engineering aiming the regeneration of tissues. This is because in tissue regeneration the immunoisolation feature is not required, although the presence of multilayers can confer other functionalities and advantages to the hydrogels. For example, the properties of each membrane can be independently controlled, and the stability and permeability of the system can be easily customized by varying the number of the multilayers. Multilayered cell encapsulation systems can be obtained by the sequential adsorption of oppositely charged polyelectrolytes using the mild conditions of the LbL technology,[35,36] as further discussed in section **5. Novel and nonconventional technologies to produce cell encapsulation systems**. Of note, in hydrogels assembled for modular TE, the influence of the presence of a membrane surrounding each building block on the permeability and diffusion properties must be carefully evaluated, since the different extent of material heterogeneity between the encapsulation matrix and the membrane might create fluctuations of transport properties across the different 3D structure. An interesting approach is the assembled of multilayered and liquefied capsules by the action of cells cultured on the outside environment. Simultaneously, at the core, 3D microaggregates of encapsulated cells and surface-modified microparticles were developed, in a concept termed has 3D+3D bottom-up TE.[37]

3.3 Stability

The stability of the cell encapsulation matrix is related with its physical features, namely the mechanical resistance that it can support without disruption of its integrity or chemical features related with their ability to maintain its structure without being dissolved by physiological chelator agents or degraded. Either physical or chemical instability would lead to the premature release of the encapsulated cells and other materials of interest, releasing its content to other regions of the body rather than the implantation site. Increasing the stability of the encapsulation matrix can be performed by different approaches that could, however, influence other important parameters for cell survival. Developing systems with both adequate mass transportation and mechanical stability remains a key challenge in cell encapsulation technology, as they are often inversely related.[31] Since alginate is the most widely applied hydrogel in cell encapsulation systems, efforts have been made to increase its stability. The first approach was to coat alginate beads with the oppositely charged polyelectrolyte PLL,[19] but other polymers are being employed to construct improved biocompatible membranes, mainly using the LbL technology section (see section **5. Novel and nonconventional technologies to produce cell encapsulation systems**).

Another possibility is to increase the crosslinking density of the cell encapsulation matrix. The simplest example for the case of alginate systems is the use of barium instead of calcium as the gelling divalent agent, or alginates richer in guluronic acid units. However, it may lead to encapsulation matrixes with lower swelling capabilities and decreased mesh sizes, thus influencing the permeability of the construct. Crosslinking control will also result in matrices with distinct stiffness that will play an important role in cell behaviour, namely on the ability of stem cells to differentiate into specific lineages.[38–41] For example, different osteogenesis levels could be obtained by controlling the viscoelastic properties [42] or the stress-stiffening effect of hydrogels.[43] Different groups are increasingly exploring the use of precise chemical routes to produce intrinsically robust hydrogels. A successful strategy was proposed by incorporating in alginate hydrogels 2,6,6-tetramethylpiperidine-1-oxyl (TEMPO)-mediated oxidized bacterial cellulose (TOBC). TOBC and alginate participate in the ionotropic gelation with calcium ions.[44] The carboxyl groups available on the surface of TOBC provided the possibility of participating in the construction of an alginate-based composite and played crucial roles in the structural, mechanical and chemical stability of the formed hydrogel. The encapsulated NIH 3T3 cells remained viable and proliferative. Others, proposed robust hydrogels with adequate permeability inspired by the role of glycosaminoglycans in providing rigidity to the ECM due to their rigid sugar units and hydrophilic groups.[45] Inspired by that, a polysaccharide containing multivalent methacrylate groups and hydrophilic groups was incorporated into a hydrogel to control its stiffness over a broad range, while controlling the swelling ratio. This was achieved by the chemical crosslinking between methacrylic alginate and PEG-dimethacrylate (PEG-DMA). The increase of gel stiffness resulting from the incorporation of methacrylic alginate into a PEG-DMA hydrogel was related to the high chain rigidity of alginate as well as the multivalent methacrylate groups. In parallel, multiple hydroxyl groups of methacrylic alginate thermodynamically counterbalanced kinetic limits of osmotic water entry. Results showed that the chemical crosslinking of PEG-DMA allowed controlling the hydrogel stiffness without compromising its permeability, as demonstrated by the suitable viability of encapsulated neural cells (PC12 cells). In another study, alginate and PEG were also incorporated into interpenetrating network structures, improving significantly the toughness and elasticity of the final hybrid construct, without compromising the viability of encapsulated mesenchymal stem cells (MSCs).[46] The resultant toughness relied on two mechanisms, namely the reversible calcium ions crosslinking of alginate that dissipated mechanical energy, while the covalent crosslinking of PEG maintained elasticity under large deformations. Besides alginate, other polymers have also been employed to produce hydrogels with enhanced mechanical properties and encapsulated cells by the use of precise chemical routes.[47] For example, two chitosan derivatives, namely low molecular weight methacrylamide and medium molecular weight, were mixed with a photoinitiator (I2959) and the weak base β-glycerophosphate.[48] To produce double-network ultra-tough hydrogels a sequential dual-crosslinking was performed, first by using UV-light exposure for the methacrylamide chitosan, and then by immersion in a solution containing negatively-charged tripolyphosphate (TPP) for the ionic crosslinking of medium molecular weight chitosan through their positively charged amine groups. This strategy allowed to create tough hydrogels that were able to withstand an impressive compressive stress in the same order of magnitude as the ones found in native load-bearing soft tissues, with fast recover ability of their mechanical properties upon unloading, while allowing cell encapsulation.

3.4 Degradation

There are a number of factors influencing the degradation of hydrogels, which can be mediated by *(i)* the chemistry of its matrix and the density of degradable groups, *(ii)* the presence and biological activity of cells, including the deposition of ECM and metabolite products, and *(iii)* environmental triggers in the *in vivo* physiological environment of the host. Considering the first referred circumstance, in physically crosslinked hydrogels the gelation is reversible. Using the example of the ionotropic gelation of alginate with calcium chloride, events such as the presence of electrolytes or the deposition of a newly formed ECM, can lead to the exchange of divalent calcium ions for monovalent cations, leading to the dissolution of the polymer chains or to the disruption of hydrogen bonds. In many cases, hydrogels are engineered to degrade by hydrolysis and/or enzymatically by adding specific degradable sequences within its chemical structure. This allows to control both the degradation rate and profile. Different to the commonly used "*open*" scaffolds composed of pores in which cells can deposited ECM prior to the scaffold degradation (see section **2. *"Open"* vs. "*closed*" scaffolds: limitations, advantages and practical considerations**), in cell encapsulation systems the degradation of the matrix has to occur to provide space for the deposition of newly ECM by the encapsulated cells. Therefore, the formation of the new tissue and the degradation of the hydrogel matrix are intrinsically linked in cell encapsulation strategies. If the degradation of the hydrogel occurs too quickly, i.e. prior to an appropriate deposition of ECM, the encapsulate cells will be deprived from the physical support required for different cell anchorage processes. On the other hand, if the degradation occurs too slowly, the deposition of ECM will occur in the pericellular regions, since those are the regions at which the diffusion of essential molecules for cell survival is higher, leading to a heterogeneous distribution of ECM in the hydrogel as well as cell necrosis at the core of its structure, a very common drawback in 3D systems, and particularly in cell encapsulation systems. It is thus highly desirable that degradation of the encapsulation matrix must match the formation of the new tissue. For that, one of the options is to control the chemical degradation of hydrogen bonds through the chemistry of the degradable linker. An example is the controlled degradation of PEG by combining slowly and rapidly degrading linkers such as polycaprolactone (PCL) and poly(L-lactic acid) (PLLA), respectively.[49] While these methods are frequently used nowadays, alternative attractive possibilities involve the use of cell-mediated degradation.[50–53] Through enzymatic cleavage or degradation, the cells can direct the time line of degradation and adjust their surrounding environment as needed for cellular growth, matrix deposition, and matrix re-organization. Techniques to achieve cellular-mediated matrix degradation can be performed either by using hydrogels from natural biopolymers, such as hyaluronic acid which degrades by hyaluronidases activity [54,55] or thiol-ene pectin hydrogels by collagenase type II [50] or to program short amino acid sequences into the hydrogel network, which are susceptible to enzymatic cleavage.[52] The incorporation of short peptide sequences in hydrogels present several advantages for TE applications, including specificity for cell binding, and the development of cell instructive 3D systems on a large scale.[56,57] Examples of enzymatically degradable segments used in cell encapsulation strategies are polysaccharide-based systems composed of ECM proteins, such as collagen, fibrin, fibronectin and laminin proteins, and peptide-based linkages that have specific cleavage sites for degradation by enzymes, such as elastase, plasmin or matrix metalloproteinases (MMPs).[57,58] Longer enzyme-cleavable chains can also be used as connecting points for hydrogel formation, such as fibrinogen.[59,60] Although enzymatically degradable single-phase hydrogel materials offer elegant control over the cellular invasion, cell confinement within these systems remains strongly coupled to matrix elasticity, and enzyme-mediated changes to local mechanical properties may be difficult to control in a pre-determined manner. Taking advantage of controlling the degradation of hydrogels, an interesting work was proposed using void-hydrogels.[61] Void-forming hydrogels were obtained by encapsulating sacrificial gel porogens composed by oxidized hydrolytically labile alginate within a high molecular weight alginate hydrogel, which has thus a slow degradation rate. The rate of pore formation was controlled by the rate of porogen degradation and cell migration and proliferation within pores. Remarkably, this strategy allowed decoupling the pore formation from the elasticity of hydrogels, while controlling MSCs osteogenesis *in vitro.*

3.5 Biotolerability

While some groups claim that biocompatible polymers for cell encapsulation are available, others doubt whether such materials can ever be designed. This paradox is explained by the interpretation of the expression *“appropriate host response”* from the original definition of biocompatibility. During the emerging of artificial organs field, the definition of biocompatibility emerged as *“the ability of a biomaterial to perform with an appropriate host response in a specific application”*.[62] An “appropriate host response” included the fibrotic capsules formation surrounding the implant. However, for cell encapsulation systems, defining an appropriate host response is more complex because any inflammatory response against the implanted system is potentially harmful to the encapsulated cells. This is because if immune cells led to the formation of a fibrotic capsules surrounding the encapsulation system, the diffusion of essential molecules for cells survival and the exchange of therapeutic molecules will be jeopardized (we also recommend the reading of[63] for a deep understand of the interaction between the physicochemical properties and the biological responses in cell encapsulation systems). Encapsulation systems developed for tissue regeneration applications are not meant to prevent immune responses as cell encapsulation strategies designed to treat endocrine diseases were. When implanting encapsulation systems aimed for tissue regeneration, the immune system will be inevitably activated due to leakage of antigens, protrusion of cells due to their proliferative ability, and to native responses associated with the surgery. Therefore, the current leading opinion is that cell encapsulation systems for tissue regeneration should preferably elicit a minimal immune response to avoid cellular overgrowth surrounding the capsules. With that, emerges the term biotolerability as *"the ability of a material to reside in the body for long periods of time with only low degrees of inflammatory reactions"*.[64] The current leading opinion is that, rather than "*an appropriate host response*" of the term biocompatibility, the biotolerability concept of "low degrees of inflammatory reactions" is more appropriate in the cell encapsulation field. Within the broad spectrum of biomaterials available that induce different appropriate foreign body responses, other factors such as chemical modifications,[65] and the dimension of the encapsulation matrix[66] also influence immune-mediated reactions. Combinatorial methods were proposed to study the immunological *in vivo* outcome of different biomaterials,[67] or with a large library of the same biomaterial, namely alginate hydrogels, but with variable dimensions and chemical modifications.[65] The interaction of hydrogels with the immune system is detailed discussed in the following section.

Ultimately, for a cell encapsulation strategy to be successfully applied in TERM, a number of practical factors must be also considered in addition to the critical properties discussed above. The process must allow low-cost production, scale up manufacturing (e.g. encapsulation process in the order of seconds to minutes), and easy handling and implementation. Such characteristics are fundamental to facilitate the market acceptance by the target community, including the appliers, surgeons; those who will receive the treatment, patients; those who will commercialize it, the healthcare providers; and ultimately those who will allow such flow of events, namely the ethical authorities, such as the FDA and equivalents. The incorporation of all these considerations into the early stages of the design and conception of the cell encapsulation system will significantly accelerate the translation of such hydrogels from the bench to the clinics.

4. Immunomodulation in cell encapsulation systems

For a long time, the majority of biomaterials were designed to be biologically inert, in order to avoid an acute inflammatory response that would end with the formation of a collagenous fibrotic capsule surrounding and isolating the implant.[62] Nowadays, it is well-known that a proper tissue healing involves a well-regulated set of immune responses. Either by trauma or simply by the implantation of a biomaterial, such inflammatory response starts with the rapid arrival of cells from the innate immune system to the injury scene. Among all immune cells, macrophages tend to be fundamental during all stages of the tissue repair process. In response to factors present in the local tissue environment, recruited and resident macrophages mediate multiple cellular events, namely proliferation, angiogenesis, and the deposition of ECM. Only an efficient and precise timely switch from proinflammatory (“M1”) to regenerative (“M2”) macrophage phenotype results in a tissue remodeling cytokine release, which appears mandatory to tissue healing.[68] Therefore, the paradigm has completely changed, and nowadays the interplay between the two fields of immunology and TERM is a hot topic among the scientific community. **Figure 2** schematically represents the different phases of the actuation of the immune system following the implantation of a biomaterial into a lesion site. Knowing that immune system plays a central role in tissue regeneration has contributed to the design of a new generation of smart biomaterials able to modulate the action of the immune cells towards tissue remodeling and regeneration. Furthermore, the availability of methods for specific blood-derived monocytes isolation ($CD14^+$) and their differentiation into desired phenotypes, such as macrophages or dendritic cells, has led to an emergent development of engineered strategies with autologous cells.[69] Such strategies are mainly focused in co-culture studies of immune and stromal cells encapsulated within hydrogels, due to a number of appealing features, namely the resemblance of the native scenario of tissue regeneration.[70] Particularly, regulatory macrophages are essential in TERM systems, since they are involved in neovascularization, granulation tissue removal, and new ECM components synthesis. Therefore, over the last few years, novel stimuli-responsive encapsulation strategies aimed to dictate the kinetics of macrophage polarization and hence, a more realistic tissue regeneration process, have been validated in numerous 3D cell encapsulation systems.

4.1 Interaction of hydrogels with the immune system

A healthy immune system is able to protect the host by recognizing and eradicating pathogens and other foreign molecules. In the classical design of biomaterials, the main impetus was to avoid an immunological response. In fact, following implantation of a biomaterial, a set of adverse immune reactions can occur. After material-immune system interaction, a provisional matrix on material surface is formed, resultant from the blood and interstitial fluid proteins precipitation and adsorption.[71,72] The adsorption of proteins, such as albumin, vitronectin, and fibronectin, have an influence on desired cell migration and attachment and subsequent interplay between them and the material. Moreover, the blood-based transient matrix sustains the release of bioactive compounds, which are crucial for the subsequent inflammatory response. The acute inflammatory reaction is initiated in a sequential fashion, mainly driven by neutrophils (polymorphonuclear leukocytes, PMNs) and mast cells. While PMNs release proteolytic enzymes and reactive oxygen species (ROS) in an effort to degrade the biomaterial, the degranulation of mast cells leads to the secretion of histamine, growth factors, and inflammatory cytokines and chemokines, increasing the intensity of the immune response.[73–75] Additionally, the released chemotactic agents induce the recruitment and further differentiation of monocytes into M1 macrophages, in an attempt to increase antimicrobial and phagocytic responses.[76,77] The migration and activation of lymphocytes are also involved in the cascade of the immune response, resulting on the production of pro-fibrotic factors, including interleukin (IL)-4, IL-13, and transforming growth factor (TGF) β. The prolonged presence and stimulation of mononuclear cells, i.e. monocytes and lymphocytes, surrounding the implanted biomaterial, give rise to a chronic inflammatory phase.[78,79] If the material is biotolerable, this chronic phase is typically of short duration, and subsequent remodeling and regenerative responses are identified with fibroblasts recruitment and new healing tissue formation.[80] However, the exposure of biomaterials to host cells can trigger a foreign-body reaction (FBR), which if not controlled, may lead to end-stage tissue fibrosis and scarring. An indicative of FBR is the large presence of macrophages and foreign body giant cells (FBGCs). FBGCs arise from macrophages that adhere to the transient matrix on material surface and fuse to form these multinucleated cells, since the biomaterial is too large to be internalized by cells. In later stages of FBR, if the excessive inflammatory response to biomaterials continues, it may originate a fibrotic response.[16,75] Fibrosis occurs mainly if the host environment fails to naturally and sequentially stimulate the polarization of macrophages into a M2 pro-healing phenotype. An imbalanced M1/M2 ratio, which can occur due to the prolonged presence of M1 macrophages leading to a delayed switch towards the M2 phenotype, can induce the release of fibrosis-enhancing cytokine pattern by M2 macrophages. [81,82] Briefly, M1 macrophages are able to metabolize arginine into *(i)* nitric oxide (NO), which can be further metabolized to downstream reactive nitrogen species, and *(ii)* citrulline, which can be reused for efficient NO synthesis via the citrulline-NO cycle. On the other hand, M2 macrophages are able to hydrolyze arginine to ornithine and urea through the expression of the enzyme arginase. Therefore, the imbalance M1/M2 ratio is directly correlated with the arginase pathway, which limits the availability of arginine for the NO synthesis, and ornithine can thus downstream the pathways of polyamine and proline syntheses. Polyamine and proline are key mediators for cellular proliferation and tissue repair. Since both arginine metabolic pathways cross-inhibit each other, the M1/M2 polarization can thus lead to the formation of a fibrotic capsule around the implanted biomaterial, impairing a proper interaction with the host. Consequently, the tissue integration of the biomaterial, and the subsequent tissue regeneration, key indicators of the success of the implantation of the biomaterial, is inversely related with the extent of FBR.[75]

One important variable for the interaction of the immune system with the implanted biomaterial is its physicochemical composition. The main challenge is to design a biomaterial that allows not only the regeneration of the target tissue, while simultaneously controlling intra- and intercellular mechanisms of the recruited immune cells. To achieve such goal is imperative to fully understand the immunological profile of the biomaterials.[83] Hydrogels are the most widely explored systems for cell encapsulation strategies aiming the regeneration of tissues, due to their high-water content, good biotolerability, and similarity with the ECM of native tissues.[84,85] Hydrogels can be produced from natural-derived polymers, such as alginate, chitosan, hyaluronic acid, and collagen,[84] or in combination with synthetic polymers, such as PEG, PCL, polyacrylamide, and poly(vinyl alcohol), among others.[23,24] Overall, natural-derived polymers are known to improve the biotolerability of the implant, since they are composed by low immunogenicity structures and induce a type-2-like immune response with the upregulation of genes involved with damage-associated molecular pathways. On the other hand, synthetic materials can display a more exacerbated inflammatory reaction by the recruitment of a high portion of neutrophils, despite efforts to mitigate this response by tuning their chemical and topographical surface characteristics.[86–88] Moreover, natural-derived polymers induce a positive innate immune response with a constructive remodeling phenotype, a crucial gateway for tissue repair and regeneration. Alginate has been the material of choice for encapsulation, but batches of this natural polymer need to be standardized to present minimum endotoxin and proteins contents. In this regard, ultrapure alginate and other polymers are being commercialized. A classic example of a natural-derived highly biotolerable hydrogel is collagen. Collagen hydrogels are reported to induce a mild inflammatory response, mostly guided by macrophages, while prompting the deposition of newly generated host ECM.[89,90] In an attempt to improve one of the main disadvantages of natural-derived hydrogels, namely their poor mechanical properties, collagen hydrogels were modified with glutaraldehyde and an aminosilane. *In vivo* results showed that such silicified collagen hydrogels recruited more new blood-vessels from the host compared to collagen hydrogels modified only with glutaraldehyde. Importantly, only collagen hydrogels lacking the aminosilane induced a FBR, and the consequent fibrous capsule.[91] Chitosan has also been shown to generate a pro-inflammatory response by dendritic cells, while avoiding the proliferation of T lymphocytes and timely polarizing the phenotype of macrophages into a remodeling state.[92] The immunomodulatory properties of hydrogels could be improved with the encapsulation of MSCs. Besides low immunogenicity, MSCs are able to change the phenotype of native immune cells that infiltrate the biomaterial. Due to their ability to play both enhancing and inhibiting roles on immune cells, MSCs are very attractive for TE approaches.[93–99] MSCs encapsulated in PEG hydrogels were shown to down-regulate the response of M1 macrophages, and hence decreased the fibrotic response of the FBR upon subcutaneously implantation in C57BL/6 mice for 28 days.[95] Additionally, the encapsulation of MSCs is linked with the suppression of the allogeneic lymphocyte activity, as well as with M2 macrophages recruitment and polarization.[100,101] Of note, such studies used autologous MSCs. To date, any study showed the immune privileged feature of allogeneic MSCs, and their advantage compared to the use of autologous MSCs.[94,102] In fact, there are studies reporting adverse side effects following intra-articular injection of allogeneic MSCs in equine models, such as synovial cellularity and the formation of FBGCs.[103,104] The immunomodulatory feature of MSCs can be enhanced using TE strategies for cell delivery, such as cell encapsulation systems using biomaterials. Such biomaterials can also contain within their polymeric matrix specific bioactive growth factors enabling a local delivery, and thus directly modulating the cellular infiltration around the implanted scaffold. For example, the conjugation and sequential release of immunomodulatory cytokines were shown to control macrophage phenotype with resulting effects on scaffold vascularization.[105] Firstly, M1 macrophage response was enhanced by the presence of interferon γ, an inflammatory cytokine, physically adsorbed onto the scaffold. Then, IL-4 attached via biotin and streptavidin binding, was continuously released to polarize macrophages into the M2 remodeling phenotype. This strategy allowed the host macrophages to achieve a greater vascularization and healing, following murine subcutaneous implantation.

Indeed, it is clear that the immune system plays a key role in tissue repair and regeneration process. Therefore, the integration of a functional biomaterial is facilitated when the crosstalk with the host immune cells is well-established. Furthermore, using biocompatible biomaterials in combination with autologous MSCs, which besides their well-described advantages for TE applications, namely ease of isolation, manipulability and multilineage differentiation potential, would also avoid the use of immunosuppressant drugs.[106] The administration of such drugs are related with several adverse side effects known to down-regulate the immune response.

Moreover, numerous studies have been shown that glucocorticoid-based anti-inflammatory treatments are related with the reduction of inflammatory cytokines, and hence jeopardize the healing of the injured tissue by delaying the clearance process, and decreasing the angiogenesis capability and cell proliferation.[107–109] In summary, immunomodulatory biomaterials should be able to control the immune environment surrounding the implantation site by eliciting a pro-regenerative immune response rather than avoiding the initial inflammatory reaction.

4.2 Immunomodulatory encapsulation strategies for tissue regeneration

Immune cells have been identified as potential targets to integrate tissue engineered constructs and supplement or ameliorate a desired event, such as regeneration and vascularization. Several studies have attempted to prove that the incorporation of macrophages is feasible and can actually improve the proposed TERM strategies. Some of these approaches are discussed hereafter.

A hyaluronic acid hydrogel encapsulating MSCs led to an anti-inflammatory polarization of monocyte-derived macrophages (MDM) cultured in the external environment. Therefore, not only the inert feature of most biomaterials is a utopic scenario, as in fact, it can be beneficial to the healing process.[110] Such beneficial biological outcome was enhanced when MSCs and MDM were co-cultured within the same hydrogel, leading to the production of bioactive molecules involved in collagen homeostasis, cell adhesion, angiogenesis, immunosuppression and tissue repair.[111] Alternatively, a photoresponsive hyaluronic hydrogel combined with Arg–Gly–Asp (RGD) adhesive peptide was engineered to control the immunomodulatory crosstalk of encapsulated macrophages. Here, results shown that the RGD peptide can activate macrophage $\alpha v\beta 3$ integrin, and hence, enhance an anti-inflammatory “M2” macrophage polarization.[112] Otherwise, macrophages encapsulated in two different hydrogels in the presence of IL-4, which chemically polarizes macrophages into a regenerative “M2” phenotype, had the most distinguishing reactions. Whereas macrophages encapsulated in gelatin methacryloyl (GelMA) hydrogels, were driven into a regenerative profile, macrophages that were encapsulated in poly(ethylene glycol) diacrylate (PEGDA) hydrogels, expressed a more proinflammatory "M1" phenotype.[113] Additionally, a reduced availability of soluble tumour necrosis factor (TNF)-α following a pro-inflammatory stimulation was observed in monocytes entrapped in GelMA hydrogels. These results indicate that under pro-inflammatory conditions, GelMA can be potentially characterized with anti-inflammatory properties.[114] When cultured on polyethylene terephthalate coated with collagen (PET/Col), macrophages expressed a pro-inflammatory "M1" profile, while on polypropylene (PP), these immune cells expressed a regenerative "M2" phenotype.[115] In order to create a wound healing model, a co-culture of macrophages and adipose-derived stromal cells (ASCs) were encapsulated in PET/Col and PP hydrogels. Results suggested that such hydrogels influenced the process of tissue regeneration by guiding the polarization of macrophages. In fact, genes involved in proliferation, vasodilation and collagen deposition, such as COX2 and PTGS2, were differentially expressed by ASCs when co-cultured with macrophages.[116]

An *in vitro* model was created using gelatin hydrogels to assess the relevance of resident macrophages in engineered tissues aiming regeneration.[117] For that, non-polarized monocytes, polarized macrophages with "M1" or "M2" stimuli, or incoming cells were combined. The incoming cells formulation encompassed the co-encapsulation of non-polarized THP-1 (human monocyte cell line), fibroblasts, and endothelial cells (ECs). Results show that hydrogels encapsulating macrophages were able to recruit more ECs and fibroblasts, which are key elements in the wound healing process, compared to non-polarized monocytes. Furthermore, after an initial characteristic proinflammatory phenotype of the wound healing, the microenvironment has become more pro-regenerative through the release of IL-1RA, CCL-18 and IL-4 cytokines. To recreate an artificial homeostasis of wound regeneration in the gelatin hydrogels via paracrine and cell-cell contact effect, macrophages were co-encapsulated with ECs or/and fibroblasts. Firstly, macrophages in co-culture with fibroblasts significantly enhanced cell proliferation and cytokine secretion, creating a more stimulating microenvironment in the encapsulation system. When macrophages were co-cultured with ECs, a favourable microenvironment for angiogenesis was created with the up-regulation of IL-6 and IL-1RA. Finally, to recreate the actual *in vivo* microenvironment, a tri-culture of macrophages, fibroblasts and ECs encapsulated in the gelatin hydrogels resulted in a denser like-tissue structure. Moreover, macrophages affected the angiogenic and proliferation secretory environment by significantly boosting the release of activin, IL-6 and IL-8.[118] Likewise, after the encapsulation of macrophages and ECs on a 3D PEG-based system, macrophages were capable to influence vessel formation inside of the hydrogel. In particular, macrophages were shown to associate with ECs in a pericyte-like manner, as well as, bridging between endothelial structures in a cell-chaperoning fashion.[119]

In particular, the complex role of immune cells in musculoskeletal diseases has motivated the development of the osteoimmunology field. The study of the interactions between MSCs and macrophages in gelatin/PEG matrices demonstrated that although the co-culture attenuates chondrogenic differentiation, it actually enhances osteogenic and adipogenic differentiation.[120] Otherwise, transglutaminase cross-linked gelatin (TG-gel) for 3D culture was used to study how stiffness-tuneable matrices can affect macrophage induced osteogenesis. Here, despite the high-stiffness harnessed MSC osteogenic differentiation, macrophages presented a pro-inflammatory phenotype. However, when macrophages and MSCs were encapsulated in the same type of TG-gel, the gap of osteogenesis levels between low and high stiffness matrices was narrowed. In fact, both stiffness systems showed mineralized nodules development and enhanced alkaline phosphatase activity.[121] Overall, accumulating evidence indicates that dimensional and mechanical parameters of cell encapsulation systems facilitate the polarization of macrophages towards a target phenotype commitment, and thus, it should be considered when designing hydrogels as cell encapsulation systems aiming the regeneration of tissues.

4.3 Challenges of immunomodulatory hydrogels aiming tissue regeneration

One of the most critical parameters in TE strategies is the biotolerability character of the biomaterials. Biotolerability is a controlled low degree inflammatory reaction, in which the host immune cells tolerate the implanted biomaterial for long periods of time.[64] As aforementioned, it is essential that a biomaterial stimulates tissue repair and regeneration without eliciting a FBR. However, in strategies such as cell encapsulation, the definition of an appropriate host response is more complex. Here, any inflammatory reaction against the implanted biomaterial can be destructive for the encapsulated cells, because this response is associated with the diffusion of harmful cytokines, leading to cell death and further failure of the cell encapsulation system.[64,122] Additionally, the presence of cells within the biomaterial also can elicit a more intense immune reaction. Not only the biomaterial composition and processability should be compatible with the encapsulated cells, as such cells should be tolerated by the immune system of the host.

A major challenge dictating the hydrogels biotolerability is the presence of endotoxins. Prior to implantation, a highly pure and sterile polymer is required. Low levels of endotoxins can induce severe inflammatory responses, leading to inadequate integration of the cell encapsulation system.[123,124] Occasionally, other adverse effects are observed on natural-derived hydrogels. Due to allogeneic/xenogeneic properties of the natural materials, enzymatic and hydrolytic degradation can occur *in vivo*.[125] Additionally, it is noteworthy that most of monocytes and macrophages used for biocompatibility tests are derived from leukemia, such as THP-1 and HL-60 cell lines isolated from acute monocytic leukemia, or lymphomas, such as the U-937 cell line obtained from the pleural effusion of a 37-years old patient with histiocytic lymphoma. The differences between the human blood–derived monocyte and the cell lines should be better understood, and the tumour-derived cells used for the *in vitro* assays must be chosen accordingly to the intended use of the medical device.[126]

5. Novel and nonconventional technologies to produce cell encapsulation systems

To face the demanding requirements of cell encapsulation systems aiming the regeneration of tissues, different technologies are being proposed. Herein, we highlight the contribution of LbL, microfluidics, superhydrophobic surfaces, and 3D bioprinting technologies to produce the next generation of cell encapsulation systems (**Figure 3**). LbL is proposed for the build-up of a multilayered membrane surrounding the cell encapsulation matrix. The membrane is formed due to the electrostatic interaction of oppositely charged polyelectrolytes. Microfluidics allows the homogenous production of microgels encapsulating multiple or single cells. Due to the repellence properties of superhydrophobic surfaces, spherical hydrogels can be produced using different bath-free crosslinking methodologies, such as by placing a drop on top of the previously formed droplet (drop-on-drop) or by photopolymerization with UV light. 3D bioprinting allows the production of clinically relevant structures using bioinks encapsulating individualized cells, cellular aggregates or combining cells with microcarriers. The different cell encapsulation systems described in the literature using such techniques are highlighted in the following subsections.

5.1 Layer-by-layer

Since its introduction,[127] LbL has become one of the mostly used techniques to coat with multilayers the surface of biomaterials. The main advantages of the LbL technique are the ability to provide a reliable, easy, versatile, environment friendly, and cost-effective way of coating and consequently modifying surfaces.[36] The principle of the technique is based on the sequential adsorption of a wide range of polyelectrolytes.[35]

In particular, LbL has been widely applied to produce polymeric multilayered capsules (PMCs). PMCs are fabricated through sequential deposition of polymers in the surface of a sacrificial core, which is subsequently eliminated.[128,129] The obtained nanometer thin membrane, composed by a few or several multilayers, is "permselective" allowing the diffusion of water, ions or other relevant bioproducts (e.g. nutrients, oxygen, metabolites, and waste products), while excluding larger components (e.g. high immune components and cells). Additionally, the sequential fabrication procedure combined with multiple post-processing modifications (e.g. incorporation of molecules or micro/nanoparticles, elimination or solubilization of the core) allows a precise fine-tuning of the system properties.[130] PMCs have been used in a wide range of biomedical applications, such as in imaging,[131] drug delivery,[132–134], biosensors,[135,136], synthetic vaccines,[137] nanoreactors,[138] catalysts,[139] cell coating,[140] and many others. More recently, these properties have put PMCs under attention in the field of cell encapsulation.

The most appealing features that the LbL technique could offer to the cell encapsulation field are *(i)* the possibility to be performed at room temperature or at 37ºC and in mild conditions, assuring the ideal conditions for cell viability, *(ii)* it is a aqueous-based procedure compatible with a broad range of natural and synthetic polyelectrolytes as well as with biomolecules, *(iii)* 3D structures, including those with complex shapes and irregular topographies, can be easily coated with multilayers, *(iv)* it offers precision control over the composition and thickness of composite membranes through control over the number and nature of layers deposited, allowing to tune the semipermeability of the membrane, and *(v)* it allows increasing the complexity of the encapsulation system by adding new functionalities and capabilities - the multilayered membrane can act as drug reservoirs or include biological functional components, such as proteins, enzymes, antibodies, and peptide sequences that elicit specific biological responses.[141–143] Therefore, LbL can be performed in cell encapsulation strategies by using the jellified hydrogel matrices as templates to build an engineered membrane over its surface, which in tissue regeneration its presence is not compromised to the immunobarrier role. While being a very promising technique, with the possibility of open new prospects to the function of semipermeable membranes, LbL has been poorly explored in 3D cell encapsulation systems for tissue regeneration. The main exploited field of the LbL technique in cell encapsulation is within the single-cell encapsulation, which although being a promising instrument for engineering cells with enhanced properties is not on the scope of the present review (we recommend the reading elsewhere[144,145]). We believe that this is mainly related to its inherent time-consuming aspect of LbL. Therefore, to the effort required to obtain an engineered cell encapsulation matrix, then adding the time-consuming task of the construction of a multilayered membrane has to be carefully pondered. This aspect is correlated to the viability of the encapsulated cells, limiting the number of layers composing the membrane. Clearly more efforts should be made to decrease the processing time by, for example, reduce the adsorption time of the layers (avoiding the adsorption until equilibrium or increasing the polyelectrolyte concentrations) or by using other assembly mechanisms between the layers, such as fast chemical reaction towards robust covalent bonds formation.

LbL technology was combined with the ionotropic gelation of alginate to produce a liquified cell encapsulation strategy.[146] Alginate hydrogel spheres were used as cell encapsulation templates for the construction of the LbL membrane. Once the LbL membrane was built, the alginate core was liquified by chelation of the calcium ions through ethylenediaminetetraacetic acid (EDTA) treatment. The multilayered membrane was built due to the electrostatic bonds between chitosan and alginate polyelectrolytes. Results showed that mechanical strength of the capsules and the viability of the encapsulated cells were affected by the different number of layers employed. More recently, a liquified cell encapsulation strategy was developed combining the ionotropic gelation of alginate, LbL assembly, and the co-encapsulation of stromal cells with surface modified PLLA microparticles.[147–150] In this concept, by providing to the encapsulated cells surface functionalized microparticles as solid cell adhesion sites, the viability of the encapsulated cells could be enhanced. Additionally, the number of layers was increased to 12 layers without compromising cell viability, and the mechanical strength of the capsules was improved by using a three-component polyelectrolytes assembly, namely poly(L-lysine), chitosan, and alginate. Importantly, the LbL technique allowed creating an encapsulation strategy in which cells are encapsulated in a liquid environment. This allowed to confer freedom for the encapsulated cells to freely self-construct their 3D organization, while providing an appropriate diffusion of essential molecules for cell survival, a major concern in cell encapsulation strategies. However, maximizing the core dissolution to achieve an excellent diffusion required the introduction of solid cell adhesion spots, provided by the PLLA microparticles. Consequently, this innovative cell encapsulation strategy allowed capsules to have a much higher diameter (of ca. 2 mm) rather than the established 400 µm for cell encapsulation matrices, without observation of a necrotic core. The proposed capsules were proposed as an alternative methodology to the commonly used "*open*" scaffolds, since here cells are also adhered at the surface of a subtract but then the system is wrapped by a membrane, being physically isolated from the environment but without being embedded in an elastic matrix as usually observed in "*closed*" scaffolds. The successful of this strategy boosted its application to the encapsulation of stromal cells for bone[148,149] and cartilage[147] TERM applications. Such system was already validated *in vivo* for bone TE.[149] We recently proposed to combine the technology to produce liquefied and multilayered capsules with the bioelectrospraying technology for the production of microcapsules at high rates.[150,151] Additionally, we also proposed the development of multilayers surrounding liquified capsules encapsulating cells as an assembly methodology to construct 3D macrostructures from a bottom-up approach.[152]

Alternatively to the diffusion-driven kinetics of classical LbL assembly, which consists of dipping the substrate in the polymer solution, recent advances in LbL assembly technologies have explored other driving forces (e.g. dewetting, centrifugation, immobilization, spinning, spraying, atomization, electrodeposition, magnetic assembly, microfluidics, among others[145]). Besides many other advantages, some of those different assembly technologies are also able to solve the time-consuming feature of the process. Additionally, there is now a growing realization that the assembly method not only determines the process inherent properties, but also directly affects the physicochemical properties of the membrane built. However, the applicability of such LbL assemblies has not been tested yet in cell encapsulation devices. Nonetheless, we anticipate that its extrapolation and application will have a tremendous impact on the field. Besides the mechanical protection and mass transfer control, multilayers could also confer new features and elicit specific functions. For example, they could integrate inorganic elements to enhance bioactivity,[153] magnetic-responsive nanoparticles to control and manipulate its movement,[147] and light-responsive multilayers[154] or containing gold nanoparticles for light-activated disruption.[155] Moreover, they could include or expose biochemical elements to exhibit specific bioinstructive characteristics,[156] such as growth factors to stimulate cell differentiation.[143,157,158]

Besides using the LbL methodology to surround cell encapsulation matrices, conformal coating, in which cells are directly coated with ultrathin (2-100 nm) protective soft shells, are also an application example of LbL in cell encapsulation.[159,160] The great advantage that conformal coating brought to the cell encapsulation field was the possibility to improve cell functionality and viability using a very simple technique rather than adding soluble factors to the culture medium, as commonly used in *in vitro* culture, or other complex techniques such as genetic manipulation. For example, the multilayers can improve their mechanical stability, to protect them against phagocytosis by masking the cells surface from immunological agents, provide chemical resistance to aggressive environments, and to supply cells with additional instrumentation for their functionality, as similarly above discussed for cell encapsulation matrices. The production of ultrathin protective shells directly surrounding cells by LbL technology can be performed through *(i)* synthetic polyelectrolyte shells, in which due to the cell surface negatively charge at physiological pH, the shell assembly begins with the deposition of a polycation, then a polyanion is deposited, and so on, until the planned shell architecture is realized, *(ii)* synthetic hydrogen-bonded shells, in which the assembly occurs via non-covalent hydrogen-bonding interactions and their micromechanical properties can be controlled by changing pH, ionic strength, light conditions, slat concentration, or temperature; additionally, the degradability of the shells can be tuned by adjusting conditions that result in a controllably disassembled, and *(iii)* natural proteins, including hemoglobin, bovine serum albumin, and human serum albumin (the different LbL protective shells have been detailed reviewed elsewhere[159]). The presence of protective shells has been poorly explored in the field of cell encapsulation towards tissue regeneration, since usually the living organisms coated are mainly bacterial and yeast cells. However, the application of conformal coatings on such living organisms has allowed improving one of the main drawbacks of the LbL technique in cell encapsulation, which is related with its extensive time-consuming. In an innovative study, it was eliminated the adsorption step required between polyelectrolytes,[161] thus significantly decreasing the required time to produce a thin membrane. For that, during the polycation adsorption, the surface potential of each layer was permanent monitoring. When the surface recharging process to positive is completed, the polycation solution is immediately replaced by the polyanion solution until negative surface charge saturation is reached, and so on.

Table 1 summarizes the polyelectrolytes, encapsulation matrix, type of encapsulated cells, and the TERM application of examples of cell encapsulation systems aiming tissue repair using the

LbL technique.

5.2 Microfluidics

Micro-technologies in cell encapsulation allow a high degree of control over the morphological and dimensional (size and shape) properties of the encapsulation matrices. It is also possible to encapsulate cells in different geometries, such as spherical hydrogels and in fibers, in a short time. The microspheres and spheres can also be used to construct complex geometries through assembly into larger architectures mimicking the structure of tissues and organs, as discussed in section *5. Cell encapsulated building blocks to generate complex functional systems*. In the case of microspheres, microfluidics is mainly performed by flow-focusing or T-junction. In flow-focusing, microspheres are formed by intercepting the core solution with a sheath stream flowing, while in T-junction microspheres are formed by permitting the core fluid to be swept away by one sheath stream in one direction.[162] Typically, an aqueous alginate solution is emulsified in an oil phase and crosslinked ionically with divalent ions, immediately upon contact of the two solutions.[163] However, the gelation process is poorly controlled and, consequently, clogging and polydispersion are often observed.[164,165] To overcome these problems, different studies had proposed the use of calcium carbonate ($CaCO_3$) nanoparticles,[166,167] which allow to deliver calcium ions to the alginate solution without inducing unintended gelation prior to drop formation. $CaCO_3$ nanoparticles are dispersed in the alginate matrix to avoid premature gelation. After drop formation, nanoparticles are dissolved under acidic conditions after drop formation. The main drawback is the nonhomogeneous microspheres due to the heterogeneous distribution of calcium ions. Other similar techniques use calcium chloride or acetate particles dispersed in the oil phase to initiate the crosslinking process, which are subsequently dissolved in the emulsion droplet.[168,169] However, the same drawbacks of inhomogeneous calcium distribution and clogging are observed. Therefore, the new generation of microfluidics system to develop microspheres as cell encapsulation systems are focused in controlling the crosslinking process to produce homogenous microspheres with reliable and precisely tunable properties, which is of great importance in TERM, stromal cell research, and disease treatments.[170–172] Alternatively, the generation of alginate microspheres via coalescence of separate droplets containing alginate and calcium chloride has been proposed.[173] However, mixing inside the coalesced droplets still results in heterogeneous microspheres since crosslinking occurs before a homogeneous distribution of calcium ions can be achieved. The fabrication of monodisperse alginate microspheres with structural homogeneity via droplet-based flow-focusing microfluidics was successfully developed.[174] The solution to overcome the above-mentioned drawbacks observed was to deliver calcium ions by a solution containing water-soluble calcium mixed with the chelator EDTA. By chelating the calcium ions with the EDTA, the ions remained in solution while being inaccessible to the alginate chains. After drop formation, acetic acid is added to the continuous phase to dissociate the calcium-EDTA complex, which results in the release of calcium ions. The free calcium ions react with the alginate chains in a highly controlled fashion, reticulating the alginate microspheres. Results demonstrated that the proposed gelation process was suitable for the encapsulation of living MSCs.

Besides microspheres, microfluidics technique has been also used to fabricate long hydrogel microfibers. These microfibers were generally prepared by embedding dispersed cells directly within a hydrogel precursor, such as alginate,[175–180] chemically modified gelatin,[181,182] and supramolecular hydrogels.[65] Ideal platforms to mimic the complexity of biological systems are fiber-based systems.[162,175,183] Of note, the fabrication process of such systems must withstand the use of proteins and other soft materials, incompatible with the processing conditions of the conventional spinning techniques to produce fibers. As already mentioned, the processing of such biological materials in cell encapsulation systems requires aqueous conditions with precisely tuned temperature and pH in order to do not jeopardize the viability of the encapsulated cells and the incorporated bioactive molecules. These processing limitations have impaired the successful outcome of the traditional fiber spinning processes. Consequently, a new fiber spinning methodology by microfluidics technique was proposed. Microfluidic-spinning methodology has been employed to produce fibers in a microchannel using the coaxial flow of a pre-polymer and the crosslinking agent.[162] It is similar to the wet spinning,[184] but the bath is substituted by a direct supply of the crosslinking agent through the coaxial flow. Microfluidics spinning is thus the most suitable fiber formation technique for cell encapsulation because it does not require high voltage or temperatures, fibers can be fabricated continuously, and allows a precise control over the diameter of the fibers only by regulating the flow rate, which can be tuned from a few microns to a few hundred microns, and a wide diversity of cells can be encapsulated without incurrent significant damage to cells.

Table 2 summarizes the type of chip, encapsulation matrix, type of encapsulated cells, and the TERM application of examples of cell encapsulation systems using microfluidics.

5.3 Superhydrophobic surfaces

Superhydrophobic (SH) surfaces have a unique chemistry and nano/microstructure organization. They can be universally found in nature, such as in the classical example of lotus leaf, but also in many others.[185,186] SH have inspired biomimetic designs for controlling surface wettability for TERM, namely in microfluidics, drugs and/or cell encapsulation, and cell spheroids formation as discussed in different studies.[187–192] Inspired by the rolling of water drops on the lotus leaf, SH surfaces with water contact angles higher than 150º have triggered increasing interest in the scientific community for their application in the biomedical field,[185,190,193] such as to produce cell spheroids in an innovative hanging drop methodology.[194,195] Of particular interest to the present review, SH surfaces have been also used as an alternative methodology to produce spherical objects for cell encapsulation. Usually, cell encapsulation systems imply the use of two solutions: one loaded with the encapsulation materials and the other comprising the precipitation/crosslinking bath. It is precisely here that SH bring a great advantage for encapsulation systems: its major advantage is the high encapsulation efficiency of cells and bioactive molecules, which is of almost 100%, by eliminating the need of a crosslinking bath. As first reported,[189] the process involves the dispensing of a polymeric solution loaded with cells on its surface, which leads to spherically shaped droplets due to the repellence properties of the surface, and, subsequently, the liquid droplets are crosslinked under mild conditions, originating cell encapsulated hydrogel spheres. The crosslinking process is often performed by dispensing another drop on top of the previously formed droplet (Figure 3-drop-on-drop) or by photopolymerization (Figure 3-UV light). SH polystyrene surfaces were further explored to produce alginate spheres encapsulating MSCs and fibronectin.[196] The alginate drops on the top of the SH polystyrene surfaces were crosslinked by adding a small amount of calcium chloride at the top of each droplet. Similarly, the same authors developed a thermoresponsive chitosan-based cell encapsulation system.[197] Briefly, the acidic chitosan solution was first neutralized with β-glycerophosphate (βGP), and to crosslink the matrix sodium tripolyphosphate was added at the top of each droplet. After incubation at 37ºC, a second gelation step occurred due to the thermoresponsive ability conferred by adding βGP to chitosan, while the pH-responsive behavior of chitosan was maintained. Using the same type of SH surfaces, the authors also proposed a multicompartmentalized “onion-like” hydrogel[198]). Methacrylated dextran (DEX-MA) solution containing a photoinitiator and calcium chloride was dispensed on SH surfaces and then crosslinked under UV light. Then, a solution of sodium alginate containing mouse fibroblast cell line L929 cells was dispensed on the top of the spherical DEX-MA hydrogels. Subsequently, the alginate outer layer was crosslinked by the release of calcium ions previously immobilized in the core of the DEX-MA hydrogels.

Other types of SH surfaces were also explored to produce cell encapsulation systems. For example, glass SH surfaces produced by chemical vapor deposition were used to develop a hierarchical system encapsulating cells and/or drugs.[199] For that, microdroplets of DEX-MA containing the cytocompatible photoinitiator I2959 and L929 cells were dispensed through a 350 µm diameter nozzle on the top of the developed glass SH surfaces. After UV crosslinking, the obtained microspheres were encapsulated in alginate spheres using again the same SH surfaces. With this simple and low-cost technique, authors were able to produce hierarchical (micro-in-macro) encapsulation systems.

In order to propose encapsulation systems using SH surfaces but that would be not limited to the spherical shape, SH surfaces with wettable spots were developed. The idea is to vary the hydrogel geometries by varying the geometry of the wettable regions. Those SH surfaces were combined with ultra-rapid production of multi-shaped hydrogels.[200] Based on the co-existence of superhydrophobic-superhydrophilic patterns, also called “discontinuous dewetting”, the authors were able to produce arrays of droplets containing maleimide-polyvinyl alcohol encapsulating HeLa cells. Using this technique, the droplets of future hydrogels are instantly formed as the liquid moves along the superhydrophilic-superhydrophobic patterned surface. Remarkably, any HeLa cells were detected on the superhydrophobic regions, thus all cells were encapsulated within the microgels.

Table 3 summarizes the substrate and respective treatment to produce superhydrophobic surfaces, the encapsulation matrix, the geometry, the type of encapsulated cells, and the TERM application of examples of cell encapsulation systems using superhydrophobic surfaces.

5.4 3D Bioprinting

One of the main disadvantages of processing hydrogels for cell encapsulation systems is the difficulty to shape them in predesigned geometries to mimic the complex microenvironments of natural tissues. To solve this specific drawback, different rapid prototyping (RP) techniques have emerged to produce cell encapsulation systems with complex 3D structures.[201] 3D computer models shape the external design that will dictate the final structure, and such models can either be designed by Computer-Aided Design, known as CAD software, or by modelling imaging data (e.g. computer tomography and magnetic resonance imaging). In fact, this is one of the greatest advantages of RP, namely the direct fabrication of patient-specific structures independently how complex is the geometry of the defect.[202,203] In the context of TERM, RP techniques can be divided in two main strategies, namely *(i)* scaffold-based TERM systems, in which is assumed that cells require a 3D structure acting as a cell guide and supporting template that mimics the natural environment of the tissue to regenerate[204,205] or *(ii)* scaffold-free TERM systems, in which cell-cell interactions and self-organization are the main key points of the system to regenerate the damaged tissue.[206] While scaffold-based TERM strategies emphasize the role of biomaterials as a supporting structure to guide cell function, minimizing the self-assembly and self-organization capability of the encapsulated cells, scaffold-free TERM reverses the importance of both contributions. A primary classification of the scaffold-based RP techniques supporting biomedical applications can be made hinged on the working principle, namely laser-based (photopolymerized hydrogels), nozzle-based (pre-polymers by dint of extrusion/deposition), and printer-based (powder beds and deposition of a binder that fuses the particles or directly depositing material using inkjet technology) systems. Although the wide diversity of scaffold-based TERM systems produced by RP technologies for biomedical applications, only some of them are compatible with the processing of hydrogels. Additionally, among of them, the number of cell compatible RP technologies allowing mild processing is still reduced, thus are unable to produce cell encapsulation systems. Different studies using the referred scaffold-based RP techniques have been proposed, as reviewed elsewhere,[201] including a detailed discussion of the advantages and limitations of each technique. On the other hand, scaffold-free RP techniques are based on the principle that cells and tissues do not require an engineered biomaterial, due to their ability of self-assembly (the autonomous organization of components without externally manipulation) and self-organization. Based on the implementation of RP technology, a fascinated perspective on scaffold-free TERM systems emerged, termed as bioprinting.[207,208] Bioprinting emerged as the process of creating 3D structures using a "bioink", which was basically individual cells or spheroids dispersed in a "biopaper", i.e. hydrogels. Currently, bioprinting is a hot topic in the TERM field, and also encompasses the use of other supporting materials by combining bioprinting with other techniques, such as microcarrier[209] or melt-electrowriting technologies.[210] The main advantage that bioprinting brought to the TERM field was the ability to produce custom-made cell encapsulation systems for personalized treatment, while allowing the precise positioning of cells and biologics in an automated fashion. With this technology clinically-relevant 3D structures can be developed in a spatially controlled manner with high precision over the shape, size, and cell location across the entire hydrogel 3D structure. The main techniques currently used in bioprinting are *(i)* laser-based, *(ii)* droplet-based, including inkjet, electro-hydrodynamic jet, acoustic-droplet-ejection, and micro-valve, and *(iii)* extrusion.[211] Using droplet-based or extrusion-based technologies, the bioink objects, typically with spherical or cylindrical shape and composed of single or multiple cell types, are deposited in well-defined topological patterns into biopaper sheets. Then, the obtained construct is transferred to a bioreactor and the assembled bioink objects are fused. After that, the biopaper can be removed, if required. Laser-based bioprinting utilizes a laser pulse directed via mirrors onto a bioink layer above the substrate. Bioprinters can be classified in *(i)* nozzle-based, which can be further divided into intermittent drop-wise printers, such as inkjet printers (both thermal and piezoelectric), and continuous robotic dispensing printers, and *(ii)* dropwise nozzle-free, which is based on laser-induced forward transfer printing techniques.[212] In the case of inkjet technology, individual or small cell clusters are printed. Despite the advantageous speed, versatility and cost, high cell densities are difficult to obtain and considerable cell damage is induced.[213,214] On the other hand, extrusion-based bio-printers are more expensive but offer a more "mild approach" towards cells.[201] Common hydrogels proposed for bioprinting obtained from natural polymers, such as collagen,[215] hyaluronic acid,[216] chitosan,[217] gelatin,[218] and alginate.[219] To be suitable for bioprinting, a hydrogel must be viscous enough to keep its shape during printing and must have crosslinking abilities allowing the maintenance of the 3D structure after printing. Crosslinking can occur by temperature change,[215] photopolymerization,[220–223] and ionic crosslinking.[219] A common challenge when bioprinting hydrogels is that the printed shapes tend to collapse due to low viscosity. The viscosity of alginate, the most widely used polymer in cell encapsulation, can be increased by varying the concentration and molecular weight.[224] However, it has not been sufficient for achieving shape fidelity while printing.[219] To increase the structural accuracy hydrogels are often printed in combination with other materials. In such cases the printability of alginate has been improved by the addition of gelatin,[225] or by printing with a supporting sacrificial polymer.[178] When combined with nanofibrillated cellulose, alginate was successfully proposed to produce anatomically shaped cartilage structures.[219] Human ears and sheep meniscus were bioprinted, encapsulating human chondrocytes. The complex cell encapsulation devices retained its shape during the 7 days of *in vitro* culture, while assuring the viability of the encapsulated cells. Composite bioinks have also been proposed to combine the above-referred advantages of bioprinting in constructing clinically-relevant structures with biological cues.[209] Aggregates of MSCs and PLLA microparticles were produced via static culture or spinner flask expansion, and further encapsulated in gelatin methacrylamide-gellan gum bioinks. Such hybrid bioink was successfully proposed to construct bilayered osteochondral models.

Table 4 summarizes the encapsulation matrix, geometry, type of encapsulated cells, and the TERM application of examples of cell encapsulation systems using bioprinting.

6. Cell encapsulated building blocks to generate complex functional systems

The assembly of 3D scaffolds into building blocks is a strategic idea to overcome the main drawbacks of TE approaches. Issues related with nutrients and waste diffusion, limited to a size of ca. 200 µm, inhomogeneous cell distribution, as well as, the manipulation of biomaterials microenvironment in space and time, can be solved with a modular approach. Larger number of identical 3D engineered structures with smaller volumes can be assembled to create complex functional tissues, while being structured across multiple length scales. In fact, the native tissues are characterized by repetitive functional units, which include heterogeneous types of cells and ECM, organized in a multiscale fashion.[226] Furthermore, the development, maintenance, and function of the tissue is regulated by the tissue form and architecture.[227,228] Inspired by that, different tissue building blocks are envisioned to recreate larger tissues with specific microarchitectural features, while focus on an adequate multicellular geometry to promote a proper remodeling. Such modular systems can be created by cell-sheets generation,[229] cell aggregation assembly,[230] and by encapsulation of cells in microgels, subject that we will deepen bellow. The assembly of these cell encapsulation systems as building blocks by bottom-up approaches are discussed in terms of their variable 3D modular structures, comprising spherical, fiber-shaped, and multi-shaped complex structures. **Figure 4** shows examples of multi-shaped cell-laden hydrogels, which can be further assembled into clinically-relevant 3D structures, based on the concept of modular TE.

6.1 Spherical systems

Spherical microgels are the most widely used geometry for modular TE. This type of shape offers an optimal surface-to-volume ratio, providing an efficient mass exchange, thus favoring a long-term cell viability. The micrometric size of such microgels allows minimal invasive implantation procedures, while protecting cells from shear force damage during injection.[231] However, the main limitation is that dispersed spherical microgels cannot mimic the higher order structure of native tissues. For that, several research groups have been using spherical cell encapsulation systems as building blocks for bottom-up TE strategies. For example, the assembly of MSCs-encapsulated microgels assembled by covalent crosslinking was proposed for cartilage repair.[232] Here, the 4-arm poly(ethylene glycol)-N-hydroxysuccinimide crosslinker not only allowed the assembly of MSCs-encapsulated microgels, but also induced spontaneous adhesion between the assembled construct and an *in vitro* tissue mimetic model. Furthermore, such tissue mimetic model provided physical and biological cues for MSCs chondrogenesis, leading to the production of a mature hyaline cartilage structure. Similarly, spherical microgels encapsulating L929 cells were assembled in 3D macrostructures but using another assembly technique, namely the LbL technology.[152] After the nanometer multilayered coating, the cell-laden template was liquefied by chelation, using EDTA treatment. Such technology provided a bottom-up assembly in a scalable manner of individual compartments for cells, while the liquefied core improved the ability of the construct for long-term cell survival. Using the self-healing-driven assembly (SHDA) strategy, microgels were combined with macrogels to facilitate the fabrication of various programmed materials toward biological tissues.[233] Smart macro- and microgels, fabricated by a controllable and continuous microfluidic technique, were used as building blocks. Then, driven by the inherent hydrogen bonds or supramolecular interactions between the gels, linear, planar, and 3D structures were assembled. To enhance cell spreading and proliferation, specific cellular adhesion recognition sites using the well-established RGD peptide sequence were added into the spherical hydrogels. After the encapsulation of co-cultures of 3T3 and L929 cell lines, cell proliferation and migration across the hydrogel boundaries were detected to interact with neighboring cells. Single-cell-laden microgels find also great applicability as interesting building blocks for modular TE strategies. The encapsulation of single cells not only allows a precise microscale control of tissue assembly, but also enhances the ability of cells to respond to exogenous stimuli.[233] Single-cell microgels incorporated in injectable hydrogels were projected to engineer multifunctional tissues via a modular approach.[234] This strategy was leveraged to incorporate immunoprotective single-cell-laden microgels within a proangiogenic macrogel. Basically, the uncoupled micro- and macroenvironments, which are independently tunable, were designed to create biomaterials with the multifunctionality typically found in native tissues.

6.2 Fiber-shaped system

Fiber-shaped systems have attracted attention due to their unique and useful advantages. This long, thin and flexible structures are being used as building blocks to facilitate higher-order assemblies such as nanoscale materials,[235,236] and textiles.[237] Cell-laden fibers are also being proposed to resemble hierarchical structures of the human body, such as blood vessels,[178] muscle fibers,[238] and osteons from cortical bone.[239] These microfibers can be composed exclusively by cells,[240] or in cell-embedding hydrogels mostly generated by microfluidics technique. However, most of the hydrogels used are not composed by natural ECM proteins, and thus, are insufficient to reconstruct the tissue microenvironment. Therefore, an ECM-based encapsulation system, named meter-long core shell hydrogel microfibers, was proposed with natural ECM proteins and cells to reconstitute the intrinsic cellular morphologies and functions of native tissues.[175] Additionally, the proposed microfibers were assembled into macroscopic structures, by weaving and reeling, demonstrating higher-order assembly constructs with various spatial patterns. The generation of 3D vascular networks is one of the major challenges of TE. Inspired by that, the assembling of cell-laden fibers in a spatially defined manner was proposed to form pre-vascularized adipose and hepatic tissues.[241] Chitin- and alginate-based fibers were assembled through interfacial polyelectrolyte complexation. Then, the micropatterned niche was created by assembling a central ECs-laden fiber, surrounded by parenchymal cells-laden fibers. Finally, the tertiary structure construct was obtained by spooling and layering the repeat unit (secondary structure). Interestingly, *in vivo* studies show the ability of the patterned constructs to anastomose with the host, leading to vascularized tissues. Microfibers produced by microfluidics were also proposed as engineered osteons from cortical bone.[239] A biomimetic osteon-like structure was obtained by the encapsulation of ECs in a middle layer, surrounded by an outer layer encapsulating human osteoblasts. Cell-laden microfibers were assembled into braided strand, helical tube, knot, and woven structures. The achieved double-layer hollow microfibers, exhibited not only a robust cell growth, but also up-regulated gene expression.

6.3 Multi-shaped complex structures

Frequently, to promote a proper tissue remodeling as well as to simulate a certain tissue functionality, the fabrication of multi-shaped complex 3D structures is proposed. Bottom-up approaches to build vascular-like microchannels using cell-laden microgels have been widely used.[242] Generally, most of vascularized TE systems are designed as cell-free or cell-laden bulk hydrogels via microfluidics. An array of microgels, encapsulating ECs and smooth muscle cells, with a particular architectural design, were sequentially assembled in a controlled manner. The microchannels of each microgel were assembled, to create an interconnected network mimicking the bifurcating structure of the native vasculature. Others have engineered the heart tissue using ring shaped molds.[243] Native rat cardiomyocytes were mixed with collagen or Matrigel and then, assembled with other engineered cardiac bands on a cycling stretching device. Results show that the obtained cardiac grafts significantly improved cardiac function in rat myocardial infarct models, improving cell alignment and supporting contractile function of infarcted hearts.[244] A different microgel-shaped structure was proposed for the fabrication of 3D multilayer hepatic lobule-like tissues.[245] The drive was based on the fact that only an appropriate architectural organization of hepatocytes allows a proper functioning of the liver. Therefore, a new method for forming hepatic lobule-shaped microtissue was proposed using poly(L-lysine)/alginate spherical microgels encapsulating rat liver cells. Using a repetitive one-step micromanipulator system, four-layered hepatic lobule models were feasibly constructed, demonstrating the applicability for *in vitro* artificial liver fabrication.

To precisely control the assembly process, the microgels can be functionalized in more complex approaches. A molecular recognition-assisted self-assembly strategy was proposed through the surface functionalization of cube-shaped gels with single-stranded segments of DNA.[246] Acting like sequence-specific glue, this strategy can control the assembly of microgels to create complex microarchitectures. Another strategy is to use “magnetoceptive” hydrogel subunits that self-assemble into 3D structures. The assembly of cell-laden microgels was achieved through the paramagnetism of free radicals as a driving mechanism. Under a permanent generated magnetic field, complex heterogeneous structures could be built.

7. Conclusion

Lessons afforded by the primordial cell encapsulation systems were of outmost importance for the evolution path that culminated in the current hydrogels encapsulating cells for tissue regeneration. The complexity of the current cell-laden hydrogels is being enriched, due to their recognize potential as modules for bottom-up TE. Consequently, the new generation of hydrogels for cell encapsulation possess a hierarchical and highly complex organization, including smart and adaptive matrices with adequate environmental signals able to mimic the regeneration process of native tissues. Given the importance of physicochemical cues of the hydrogel matrices on cell behavior, we believe that a fine control over chemical and mechanical cues might boost their application towards the clinics. For example, by designing hydrogels with controlled stiffness, fabricating proteolytically degradable hydrogels, or by enriching the cell encapsulation matrices with bioactive molecules, such as soluble particles derived from decellularized tissues, will boost cell encapsulation systems to the next level. Importantly, the process must allow low-cost production, scale up manufacturing, and easy handling and implementation. Such characteristics are fundamental to facilitate the market acceptance by the target community, including the appliers, surgeons; those who will receive the treatment, patients; those who will commercialize it, the healthcare providers; and ultimately those who will allow such flow of events, namely the ethical authorities, such as the FDA and equivalents. Additionally, to ensure a successful translation into the clinics, issues related to long-term cell viability, tissue integration, and risk of FBR due to an acute immune response, should be carefully evaluated. In fact, using the well-established 3D and highly-hydrated environment of hydrogels for cell encapsulation, new technologies are being increasingly proposed for the development of systems with tissue-like complexity. But, technologies how to assemble such units are mandatory One of the major challenges on using cell encapsulation systems as building blocks for modular TE is the spatial and time resolution to create 4D realistic modules that precisely tune the heterogeneity of native tissues. Combining bioprinting with technologies to enrich the multifunctionality of the encapsulation matrix, and with more sophisticated assembly-techniques able to generate 3D structures with tissue-like complexity are mandatory. Such automated techniques might rely on biomaterials with self-assembly or stimuli-responsive (e.g. acoustic, light, pH, temperature) capability. Additionally, the development of highly ambitious bioinks possessing print-fidelity and biological cues to guide cell behavior are also required. While this more engineer atmosphere is under the spotlight of the scientific community, also the importance of the immune system contribution into the regenerative process cannot be depreciated, and thus must be considered when designing novel TE systems. To achieve such ambitious goal the *cliché* of a "multidisciplinary team requisite" was never so demanding in the TERM field as today.

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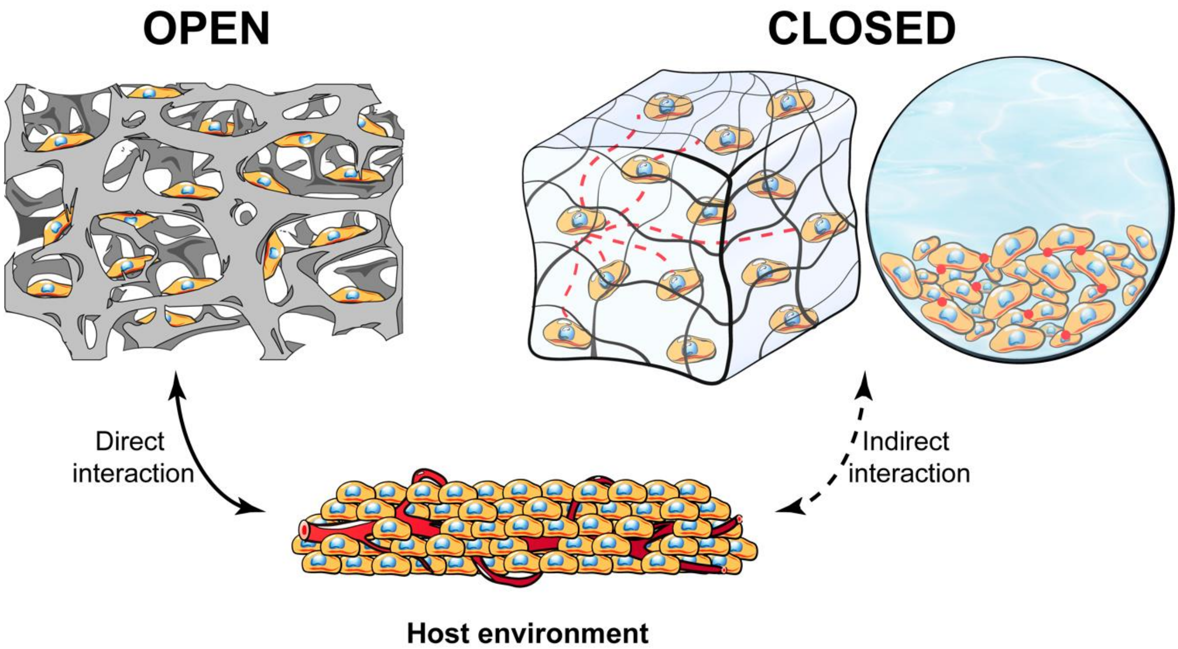

Figure 1. Interaction of tissue engineered scaffolds with the host environment. In "open" scaffolds, there is a direct interaction between the cells seeded at the surface of the biomaterial and the external environment. In "closed" systems the encapsulation matrix mediates this interaction, and provides an indirect interaction with the external environment. If the matrix is

liquefied, cell-cell interactions are maximized due to the direct contact between the encapsulated cells (red dots), contrary to the indirect cell signaling in crosslinked matrices (dotted red lines).

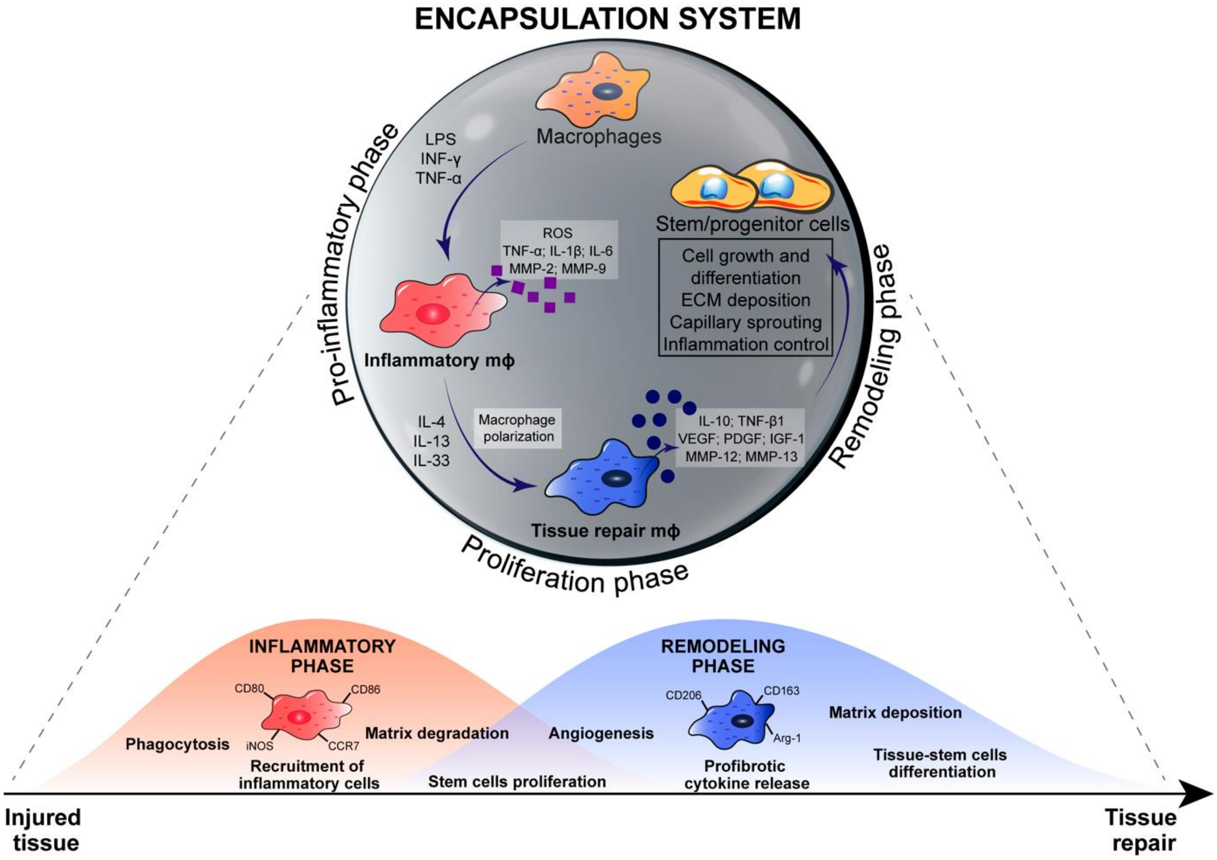

Figure 2. Upon the implantation of a biomaterial into a lesion site, a cascade of immune-related processes towards tissue repair occurs. As example, a stem-cell encapsulation system is represented.

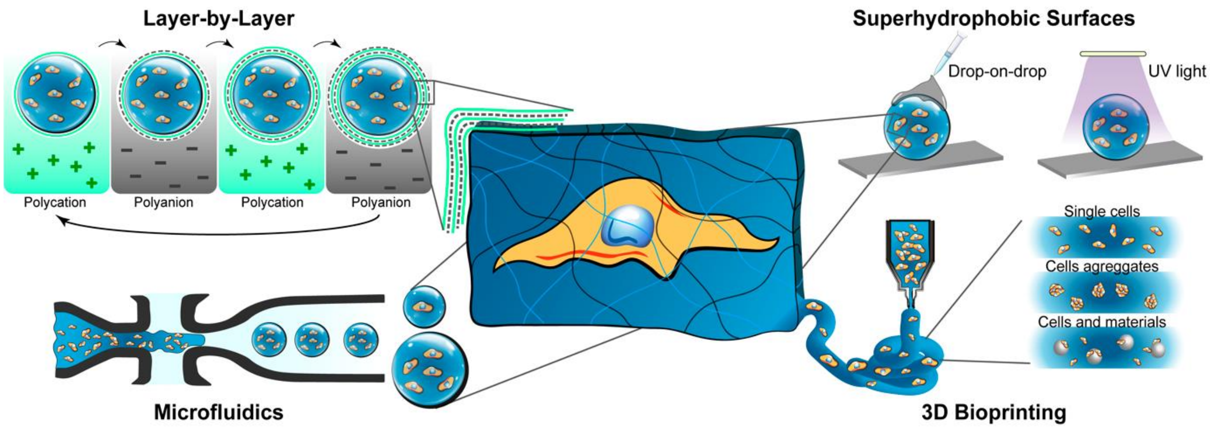

Figure 3. Layer-by-layer, microfluidics, superhydrophobic surfaces, and 3D bioprinting as novel or unconventional technologies to produce innovative cell encapsulation systems.

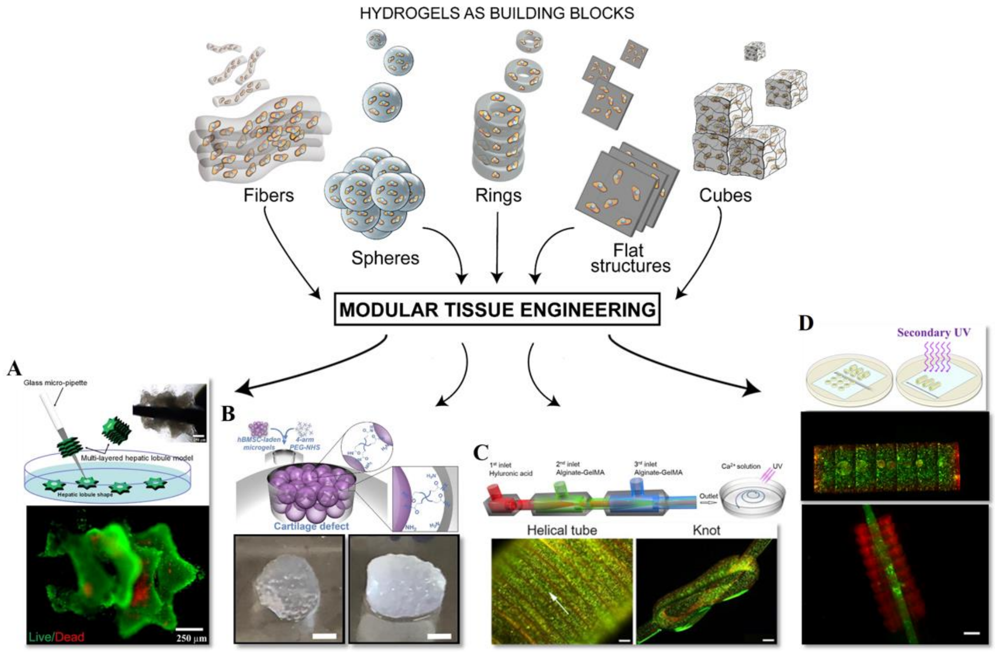

Figure 4. Multiple-shaped hydrogels, including fibers, spheres, rings, flat structures and cubes, as cell encapsulation building blocks for modular tissue engineering. **(A-D)** Such hydrogels can be further assembled by modular tissue engineering to produce clinically-relevant 3D structures with close-to-native heterogeneity. **(A)** Flatted poly(L-lysine)/alginate spherical microgels encapsulating rat liver cells. Flatted structures were assembled into 4-layers to construct 3D hepatic lobule-like tissues. Reproduced with permission.[245] Copyright 2017, Elsevier. Living cells are stained in green and dead cells in red by LiveDead fluorescence assay. Scale bar is 250 µm. **(B)** Spherical microgels of 4-arm poly(ethylene glycol)-N-hydroxysuccinimide encapsulating mesenchymal stem cells. After crosslinking, microgels spontaneously adhered, and originated a 3D model for cartilage repair. Scale bars are 1 mm. Reproduced with permission.[232] Copyright 2019, Elsevier. **(C)** Double-layer hollow microfibers production after calcium-alginate reaction and UV exposure for gelMA crosslinking. Fluorescence images (microballoon) of the produced woven structures, namely in helical tube and knot conformations. Scale bars are 200 µm. Reproduced with permission.[239] Copyright 2016, Elsevier. **(D)** Chitin- and alginate-based microfibers assembled by polyelectrolyte complexation to create pre-vascularized adipose and hepatic tissues. Central fibers encapsulating endothelial cells were surrounded by parenchymal cell-laden fibers. The tertiary structure construct was obtained by spooling and layering the repeat unit (secondary structure). Reproduced with permission.[242] Copyright 2011, John Wiley and Sons. Scale bar is 500 µm.

Table 1. Examples of cell encapsulation systems for Tissue Engineering and Regenerative Medicine (TERM) using the layer-by-layer technique. The examples cover the type of polyelectrolytes used to produce the different cell encapsulation systems, the biomaterials

employed to produce the cell encapsulation matrix, the type of encapsulated cells, and the TERM application.

MEMBRANE COMPOSITION	ENCAPSULATION MATRIX	GEOMETRY	ENCAPSULATED CELLS	TERM APPLICATION	REF.
Alginate and chitosan	Alginate	Fibers	Human kidney 293	Vascular	[176]
			L929	General	[247]
		Spheres			[146,152,248]
Alginate, chitosan and PLL					[249]
			hASCs and hAMECs	Bone	[148,149]
Alginate, chitosan, PLL and MNPs			hASCs	Cartilage	[147]

$CaCl_2$ – Calcium chloride; PLL – poly(L-lysine); hASCs – Human adipose-derived stem cells; hAMECs – Human adipose-derived microvascular endothelial cells; MNPs – Magnetic nanoparticles.

Table 2. Examples of cell encapsulation systems for Tissue Engineering and Regenerative Medicine (TERM) using microfluidics. The examples cover the type of chip used to produce the different cell encapsulation systems, the biomaterials employed to produce the cell encapsulation matrix, the type of encapsulated cells, and the TERM application.

TYPE OF CHIP	ENCAPSULATION MATRIX	CONTINUOUS PHASE	GEOMETRY	ENCAPSULATED CELLS	TERM APPLICATION	REF.
T-junction with a downstream entrance	Alginate containing $CaCO_3$ nanoparticles in RPMI medium	Corn oil and lecithin Acetic acid (downstream entrance)	Spheres	Jurkat cells	General	[166]
Flow-focusing	Alginate and calcium-EDTA complex	Fluorinated carbon oil (HFE7500) and a biocompatible		MSCs		[174]

		surfactant (PFPE/PEG)				
Spinning chip (multiple inlets)	Alginate and chitosan	$CaCl_2$	Fibers	Primary rat hepatocytes and L929 fibroblasts (alone or co-cultured)		[177]
Capillaries	Alginate			Human fibroblasts	Nerve or muscular	[179]
				Mouse ESCs	General	[182]
Y-junction (3 inlets)				HIVE-78	Vascular	[178]
Micro nozzle array				HEK 293		[176]
Axisymmetric with uniform depth	Alginate with or without propylene glycol alginate			NIH 3T3 and HeLa	Hepatic, vascular and musculoskeletal	[180]
Three-phase coaxial flow (5 inlets)	Gtn-HPA/HRP	Gtn-HPA diluted in H_2O_2 (middle) PBS (outter)		MDCKs	Vascular, nerve, kidney	[181]
		PBS and H_2O_2 (inner) Polysulfone dissolved in NMP (outter)		HPTCs		

$CaCl_2$ – Calcium chloride; $CaCO_3$ – calcium carbonate; DI – deionized water; EDTA – ethylenediaminetetraacetic acid; ESCs – embryonic stem cells; Gtn-HPA – gelatin-hydroxyphenylpropionic acid; HeLa – cell line isolated from human cervix epitheloid carcinoma; HEK 293 – human kidney (embryonic) cell line; HIVE-78 – vascular cell line; HPTCs – human proximal tubule cells; L929 – fibroblast cell line isolated from mouse adipose tissue; MDCKs – Madin-Darby canine kidney cells; MSCs – mesenchymal stem cells; NIH 3T3 – embryonic fibroblast cell line; NMP – polysulfone and N-methyl-2-pyrrolidone; PBS –

phosphate buffer saline; PFPE – oligomeric perfluorinated polyethers; PEG – poly(ethylene glycol); polyNIPAAm – Poly-(N-isopropyl acrylamide); HRP – horseradish peroxidase; H_2O_2 – hydrogen peroxide.

Table 3 – Examples of cell encapsulation systems for Tissue Engineering and Regenerative Medicine (TERM) using superhydrophobic surfaces. The examples cover the substrate used to produce the superhydrophobic, the biomaterials employed to produce the cell encapsulation matrix, the geometry, the type of encapsulated cells, and the TERM application.

SUBSTRATE/TREATMENT	ENCAPSULATION MATRIX	GEOMETRY	ENCAPSULATED CELLS	TERM APPLICATION	REF.
Polystyrene/THF, ethanol, Ar-plasma and PFDTS	Alginate and fibronectin	Spheres	rMSCs	Bone	[196]
	Alginate		L929	General	[189]
	Chitosan/β-glycerophosphate				[197]
Copper/NH_4OH, PFDTS and ethanol	Alginate and DEX-MA				[198]
Glass/soot coated with paraffin candle,TEOS, and ammonia; calcination, and silane by CVD					[199]
Glass/HEMA-EDMA photographed with PFPMA (quartz chromium photomask)	MI-PVA	Crosssections with variable geometries: circles, triangles, hexagons, and squares	HeLa		[200]

Ar – argon; CVD – chemical vapor deposition; DEX-MA – Methacrylated dextran; HeLa – cell line isolated from human cervix epitheloid carcinoma; HEMA-EDMA – poly(hydroxyethyl methacrylate-co-ethylene dimethacrylate); L929 – fibroblast cell line isolated from mouse adipose tissue; NH_4OH – ammonium hydroxide; MI-PVA – maleimide-functionalized polyvinyl alcohol; PFDTS – 1H,1H,2H,2H-perfluorodecyltrimethoxysilane; PFPMA – 2,2,3,3,3-pentafluoropropyl methacrylate; rMSCs – mesenchymal stem cells isolated form the bone marrow of Wistar rats; THF – tetrahydrofuran.

Table 4 – Examples of cell encapsulation systems for Tissue Engineering and Regenerative Medicine (TERM) using bioprinting. The examples cover the type of biomaterials employed to produce the cell encapsulation matrix, the geometry, the type of encapsulated cells, and the TERM application.

TECHNOLOGY	ENCAPSULATION MATRIX	GEOMETRY	ENCAPSULATED CELLS	TERM APPLICATION	REF.
Electromagnetic jet	Alginate and nanofibrillated cellulose	Human ear and sheep meniscus	hNCs	Cartilage	[219]
Additive manufacturing	Alginate	Human ear	ASCs		[250]
	HA/Dex-HEMA	Free form	Chondrocytes		[222]
	Collagen type I		HFF-1 and HaCaT	Skin	[215]
LIFT	Alginate Nano-sized hydroxyapatite		Eahy926	General	[251]
	Alginate/glycerol Matrigel^{TM} Fibrin		Eahy926		[252]
Microextrusion	Alginate/gelatin		Myoblasts	Myocard	[225]
	Methacrylamide gelatin/CBD-BMP-2		BMMSCs	Bone	[218]
	gelMA	Hollow fibers	HNDFs and C3H/10T1/2	Vascular	[221]
Inkjet printing	Fibrin		hMVEC		[253]
	HA-MA:GE-MA/PEGDA		HepG2/C3A Int-407 NIH 3T3		[254]
Microcapillary	CMHA-S:Gtn-DTPH/TetraPAc8/TetraPAc13				[255]
	gelMA		HepG2		[220]

ASCs – adipose-derived stem cells; BMMSCs – bone marrow mesenchymal stem cells; CBD-BMP-2 – collagen binding domain-bone morphogenic protein 2; CMHA – thyolated hyaluronic acid derivative; C3H/10T1/2 – cloned murine embryo fibroblast cell line; Dex-HEMA – hydroxyethyl-methacrylate-derivatized dextran; Eahy926 – Endothelial cell line; GE-MA - methacrylated ethanolamide derivative of gelatin; gelMA – methacrylated gelatin; Gtn-DTPH – thiolated gelatin (available as Gelin-S, Glycosan Byosistems); HA – hyaluronic acid; HaCaT – human keratinocytes cell line; HA-MA – methacrylated hyaluronic acid; hECs – human endothelial cells; HepG2 – human liver cells isolated from a hepatocellular carcinoma; HepG2/C3A – clonal derivative of HepG2; HFF-1 – fibroblasts; hMVEC – human microvascular endothelial cells; Int-407 – HeLa derivative cell line isolated from human cervix; hNCs – human nanoseptal chondrocytes; HNDFs – human neonatal dermal fibroblasts; dermal IR – infrared; LIFT – Laser Induced Forward Transfer; NIH 3T3 – embryonic fibroblast cell line; PEG – poly(ethylene glycol); PEGDA - poly(ethylene glycol) diacrylate; TetraPAc8 - tetra-acrylate derivatives of four armed PEG 2000 chains; TetraPAc13 - tetra-acrylate derivatives of four armed PEG 3400 chains.

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