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Engineering Hydrogels for Modulation of Material-Cell Interactions

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Hydrogels are a recurrent platform for Tissue Engineering (TE) strategies. Their versatility and the variety of available methods for tuning their properties highly contribute to hydrogels' success. As a result, the design of advanced hydrogels has been thoroughly studied, in the quest for better solutions not only for drugs- and cell-based therapies but also for more fundamental studies. The wide variety of sources, crosslinking strategies, and functionalization methods, and mostly the resemblance of hydrogels to the natural extracellular matrix, makes these three dimensional hydrated structures an excellent tool for TE approaches. The state-of-the-art information regarding hydrogel design, processing methods, and the influence of different hydrogel formulations on the final cell-biomaterial interactions are overviewed herein.

1. Introduction

Since their introduction in the 1960s, hydrogels have been studied for a plethora of applications.[1,2] Tissue Engineering (TE) field is one of the research areas in which hydrogel development and application retrieve fascinating results,[3–6] either from simple hydrogel networks or when using the more advanced stimuli-responsive hydrogels.[7] These three dimensional (3D) networks are mainly formed by hydrophilic polymers that can be of natural or synthetic nature, or even a mixture of both, with a rather significant number of possible combinations. Such a volume of options translated into a wide range of hydrogel formulations already described, and a lot more would undoubtedly be uncovered, considering the potential of these structures. As a result, hydrogels have been classified according to several factors, as illustrated in **Figure 1**. These include the source of the polymeric network, the ionic charge of the resulting gel, as well as the crosslinking and preparation methods used to obtain the 3D structure. Additionally, the hydrogels can be classified according to their behavior after preparation, particularly regarding their response to chemical, biochemical and/or physical stimuli, and degradability.

The variety of possible hydrogel formulations turned these biomaterials into a highly interesting tool for tissue regeneration. Besides the type of polymer, or mixture used, other levels of complexity and/or functionality can be obtained. Most of the hydrogel precursors can be further functionalized, using different types of chemistries to add specific biological ligands or responsive motifs.[8,9] As a result, hydrogel applications have been growing in the last few years, from cell delivery approaches to more fundamental studies, as cell-material interactions or mechanobiology.

It is well known that the external environment profoundly influences cell behavior. That is valid not only when cells are embedded by their natural ECM, but also when biomaterials are present. This interaction occurs at a multiscale level, meaning that hydrogel designing must consider both bulk and microscale properties of the final hydrogel. In this review, we aim to highlight the most recent hydrogel engineering developments and how these biomaterials can influence and tune cell behavior.

2. Hydrogel Preparation

Most of the polymers used as hydrogel precursors are highly soluble in water, mostly due to the abundant presence of hydrophilic groups as $-NH_2$, $-COOH$, $-OH$, or $-SO_3H$. To form the 3D network, it is necessary to propitiate the conditions to occur a sol-gel transition, forming a gel non-flowing phase. Although the constituent polymers show a high affinity to water, their dissolution can be prevented by the crosslink between their monomers, using chemical or physical approaches, as schematically represented in **Figure 2**. Physically crosslinked hydrogels are obtained by taking advantage of reversible intermolecular interactions. Those can be of different natures, being the ionic/electrostatic, and hydrophobic interactions the most common, and therefore will be discussed below. Besides those, hydrogen bonding,[10–12] metal coordination,[13,14] or host-guest interactions[15,16] can also be used as physical approaches for hydrogel crosslinking. As physical hydrogels are highly dependent on the polymer's intrinsic properties, it is harder to fine-tune the final hydrogel assets. In this regard, chemical hydrogels allow superior control of the mechanical properties and degradation profile, as well as

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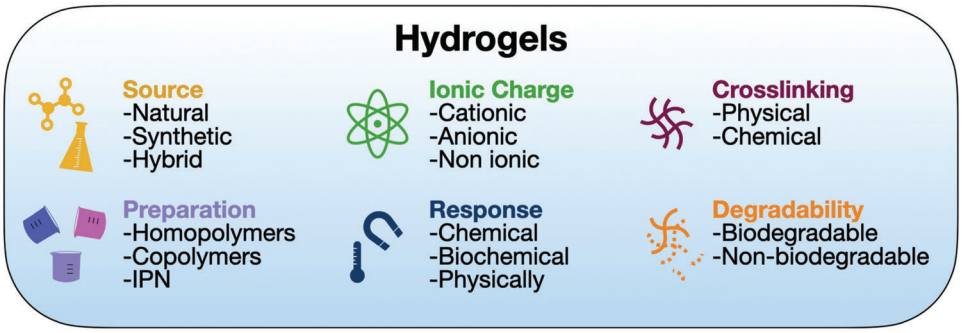

Figure 1. Hydrogel classification categories. Hydrogels can be categorized according to their polymer source, preparation method, final ionic charge, responsive behaviors, type of crosslinking, and degradability.

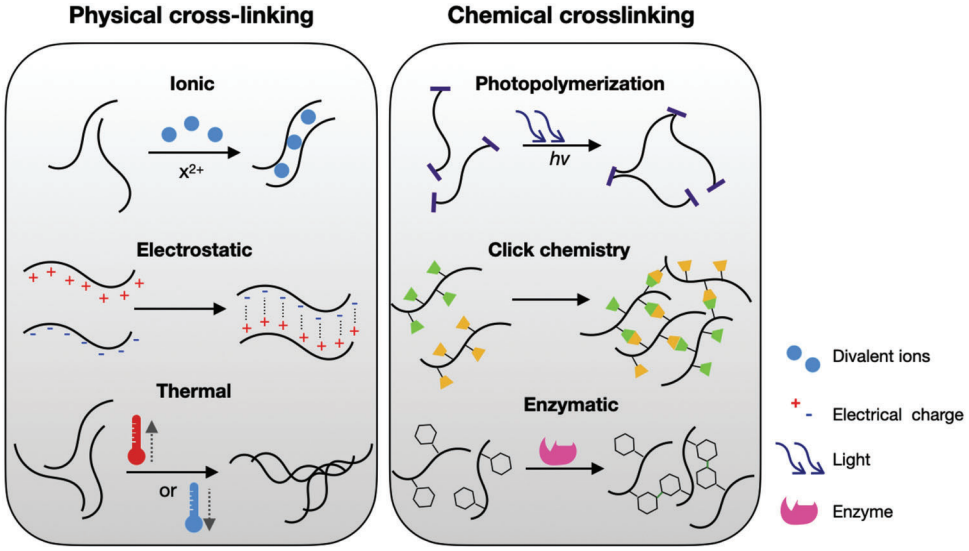

Figure 2. Schematic representation of the most widely used crosslinking strategies for hydrogel formation. These include physical crosslinking methods, as ionic crosslink, electrostatic interaction, and thermal-induced crosslink; and chemical crosslinking, including photopolymerization, click chemistry reactions, and enzyme-catalyzed crosslink.

spatiotemporal resolution.[17] The crosslink occurs due to the formation of covalent bonds amongst the polymeric backbone, which are stronger than those observed in physically crosslinked gels. Chemical crosslinks include photopolymerization, enzymatic crosslink, "click" chemistry,[17–19] and also dynamic covalent bonding, as already recently reviewed by others.[20,21]

More than allowing the formation of a 3D network, the crosslinking strategies can play an important role in the final mechanical and biochemical properties of the hydrogel.[22] Also, when cell and/or drug entrapment is envisaged, it must be assured that the whole process uses mild conditions that are compatible with the therapeutic agents used. Indeed, it is preferred to prepare hydrogels using conditions similar to physiological ones, although some crosslinking strategies cannot meet these criteria. In such cases, care must be taken to minimize the time that cells are exposed to the harmful agent. While chemical crosslinking offers superior control and flexibility over the hydrogel formation process, physical crosslinking does not need external chemical agents, being therefore considered a safer approach for biomedical applications.[23] These mechanisms can also be combined in order to obtain improved hydrogels.[24–26] Regardless, the tradeoff between safety and efficacy must be considered when designing hydrogels to achieve a successful and functional formulation.

2.1. Physical Crosslinking

2.1.1. Ionic/Electrostatic Interactions

Ionic/electrostatic interaction is routinely used to obtain physically crosslinked natural-based hydrogels. Most of the natural polymers are charged at neutral pH, either due to the presence of carboxylic (alginate, gellan gum (GG), or hyaluronic acid) or amine (gelatin and chitosan) groups on their backbone.[27] When these polymers interact with molecules of different charges, the charged groups become shielded, decreasing water-polymer interactions, which leads to the formation of an insoluble complex. For example, alginate has a high affinity to alkaline earth cations, such as Ba^{2+}, Sr^{2+}, or Ca^{2+}.[28] The affinity of alginate molecules to these ions is not equal, with Ba^{2+} showing the greatest one, meaning that distinct hydrogel properties can be obtained only by

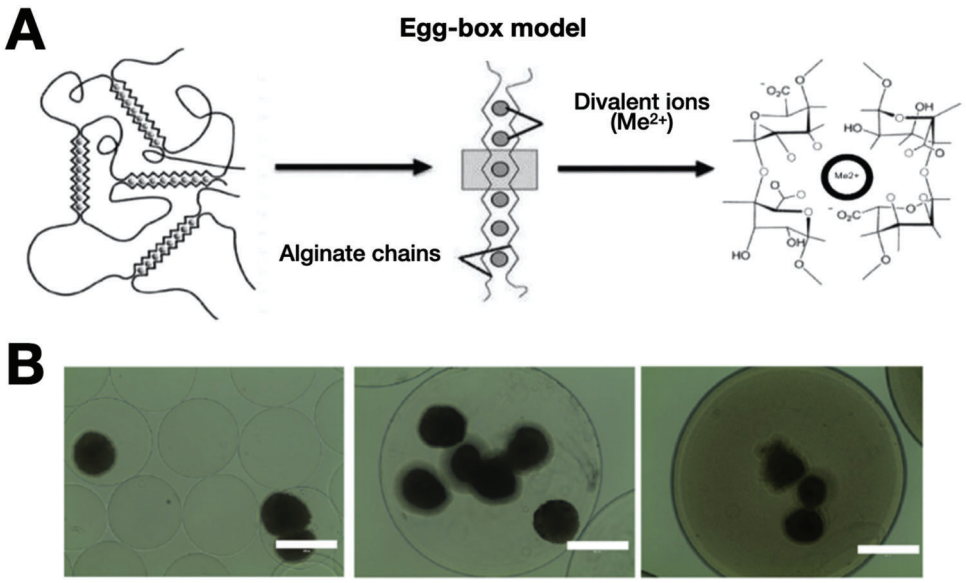

Figure 3. Hydrogel crosslinking using ionic interactions. A) Drawing of the egg-box model for alginate crosslinking. Divalent ions, typically Ca^{2+}, are enclosed within the alginate polymeric chains, forming an "egg-box"-like structure. Adapted with permission.[29] Copyright 2019, Elsevier. B) Representative bright-field images of alginate spheres containing stem cell-derived β-cells as a therapy approach for Type 1 Diabetes Mellitus (scale bars: 400 µm). Reproduced with permission.[34] Copyright 2016, Springer Nature.

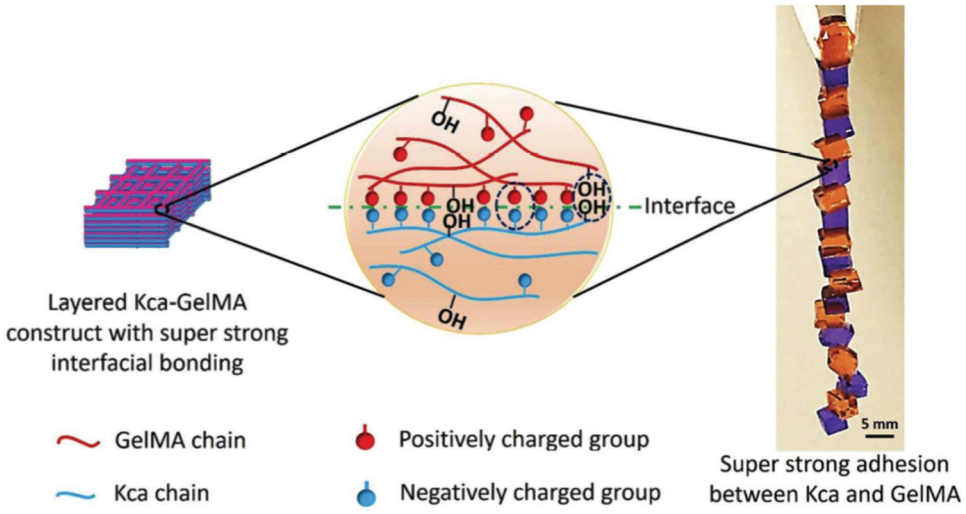

Figure 4. Electrostatic interaction between polyelectrolytes of opposite charge allows the formation of multilayered constructs. The drawing shows the interaction between κ-carrageenan (Kca) and gelatin methacrylate (GelMA), which permits the 3D printing of polymeric layers due to the strong interface bonding. Reproduced with permission.[40] Copyright 2018, American Chemical Society.

changing the ionic crosslinker.[29,30] Upon interaction, the ionic components are enclosed in an egg-box structure formed between two chains of different alginate molecules, thus leading to hydrogel gelation (**Figure 3**).

GG is another example of natural-derived polymers that also form stable gels in the presence of divalent ions. Although GG has a thermo-responsive behavior, which is discussed below, stable GG hydrogels are only obtained when ions are present as crosslinkers.[31] Similar to alginate, the gelation process is affected by the chemical nature of the ions used as crosslinkers. Monovalent cations, such as Na^{2+} or K^{+}, induce a mild GG gelation via screening effect. On the other hand, divalent cations such as Ca^{2+} or Mg^{2+}, lead to GG aggregation through the abovementioned screening effect, but also due to the bonding of two carboxylate groups present on its glucuronic acid groups.[32,33]

The electrostatic interaction can also occur between two polyelectrolytes of different charges, forming a polyelectrolyte complex (PEC) hydrogel. In this regard, alginate and chitosan are often used to prepare hydrogels via this technique, considering its inherent opposed charged nature (**Figure 4**). Polyelectrolyte complexation can be used to prepare stable structures with different geometries, including macromolecular complexes,[35–38]

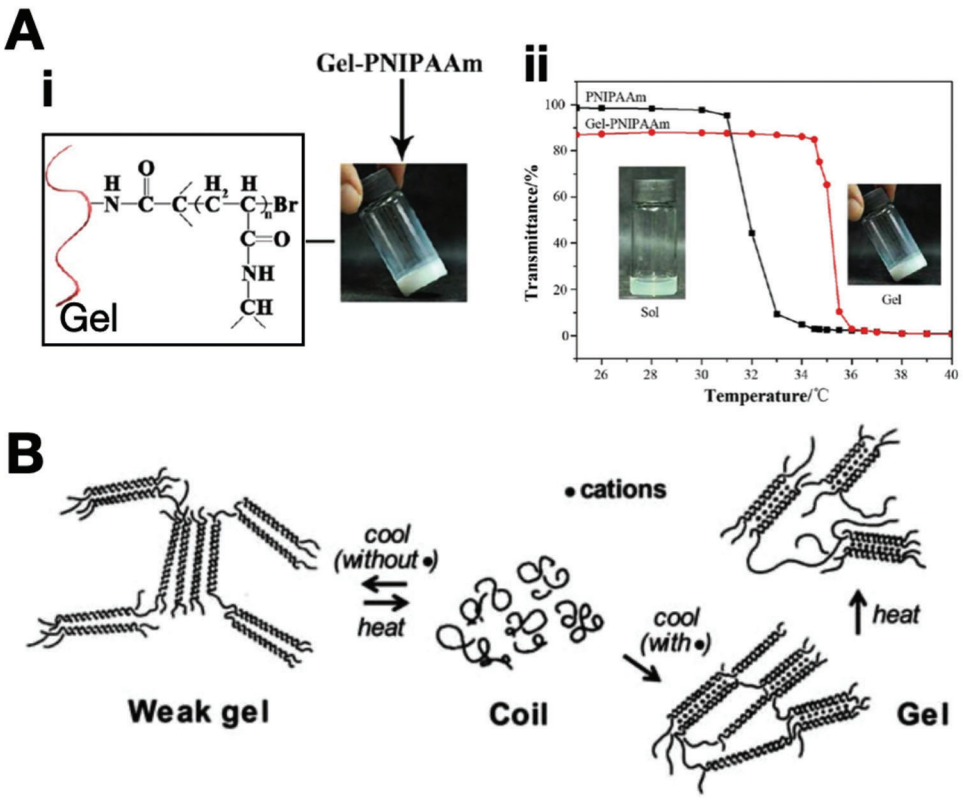

Figure 5. Thermo-responsive hydrogels. A) Gelatin-PNIPAAm hydrogel i) schematic representation of the polymer precursor and ii) phase transition analysis of the Gel–PNIPAAm aqueous solution using UV–vis spectrophotometry at 350 nm. The increase of the gelation temperature to near physiological conditions allowed the use of this hydrogel as an injectable carrier for stem cells for the treatment of a bone cranial defect. Adapted with permission.[62] Copyright 2015, American Chemical Society. B) Schematic representation of GG crosslink process, according to Robinson et al.[63] GG is thermo-sensitive, forming a weak gel upon cooling. A strong gel is only formed in presence of cations, that stabilize the GG double-helices. Reproduced with permission.[64] Copyright 2013, Royal Society of Chemistry.

multilayered polyelectrolyte constructs,[39,40] PEC fibers,[41] and bulk hydrogels.[42] Although the interaction is mainly driven by electrostatic bonds, it can also include inter-macromolecular interactions such as hydrogen bonding, van der Waals forces, hydrophobic, and dipole interactions.[43]

2.1.2. Thermal Induction

Thermally driven gelation is another common approach to preparing hydrogels, as many polymers of natural and synthetic nature are sensitive to temperature. The physical entanglement of the polymeric network occurs due to hydrophobic interactions, as a result of increasing or decreasing temperature.[44] However, thermo-responsive polymers can only be used for cell encapsulation if the sol-gel transition occurs near-physiological values. Therefore, polymers with transition temperatures near 37 °C are preferred for such approaches. Particularly, minimally invasive procedures that rely on injectable formulations into the body, take particular advantage of hydrogels with thermal-driven gelation.[45–47]

If a polymer forms a gel when heated, the temperature where the sol-gel transition occurs is the lower critical solution temperature (LCST). Below this temperature, the polymeric network is soluble in water, and no gel is formed. The synthetic polymer poly(N-isopropylacrylamide) (PNIPAAm) is a typical example of this kind of polymer,[48] and has been applied for different TE approaches.[49–52] The sol-gel transition of PNIPAAm occurs around 32 °C,[53] although LCST can be modified by copolymerization of PNIPAAm with other polymers, widening its range of applications.[54–56] On the other hand, for polymers that gel upon cooling, the crosslink occurs below the upper critical solution temperature (UCST), as the polymer starts to pack in physically rigid polymeric backbones. This is the case for most natural thermo-responsive polymers, such as gelatin or GG (**Figure 5**). Both polymers crosslink due to a network re-organization from a random coil to helix, but their UCSTs are considerably different. The sol-gel transition of gelatin occurs around 25 °C, meaning that it dissolves at body temperature. Thus, gelatin is often combined with other polymers, or chemically crosslinked, to increase its UCST.[57] However, other works take advantage of this low transition temperature to obtain hydrogels using room temperature conditions. That is particularly useful in bioprinting, as the thermal gelation of gelatin prevents a premature loss of structures' shape.[58,59] GG, by its turn, has a UCST far above physiologic conditions, limiting its dissolution at room temperature, as well as the preparation of hydrogels with encapsulated bioactive agents.[60] As an alternative, methacrylated GG (GG-MA) is

water-soluble at room temperature[61] and can be used as a substitute for low acyl GG when high temperatures cannot be employed.

2.2. Chemical Crosslinking

2.2.1. Photopolymerization

Photo-activated hydrogels have been widely used in the field of TE, mostly to rapidly prepare cell-laden hydrogels.[65] This approach often requires chemical modification of the backbone polymer to include functional photo-responsive groups, as (meth)acrylates. Also, it is necessary the presence of a photoinitiator to start the reaction.[61,66] Different molecules are available as cytocompatible radical photoinitiators. The majority of them are UV light-sensitive, such as 2-hydroxy-4-(2-hydroxyethoxy)-2-methylpropiophenone (Irgacure 2959).[67,68] However, the putative DNA damage caused by UV light[69] has driven biomedical photochemistry to the use of visible light-sensitive photoinitiators, including lithium acylphosphinate (LAP),[70,71] riboflavin,[72] or ruthenium.[73,74] Lim et al.[75] recently showed that the photocrosslinking of cell-laden hydrogels using ruthenium/sodium persulfate induces less adverse effects on human articular chondrocytes compared to hydrogels prepared with LAP or Irgacure 2959.

Regardless of the photoinitiator nature, polymerization is triggered when these molecules are exposed to light, forming free radicals that, in turn, can react with the modified polymer. As a result, new covalent, intermolecular bonds are formed, and the crosslink occurs. The polymerization process can proceed following two different pathways, depending on the functional groups available at the backbone polymers. The most common polymerization method is the free radical chain-growth polymerization, where the formed radicals interact with the vinyl bonds of the (meth)acrylates groups.[22] Although it is possible to obtain hydrogels with tunable mechanical properties and degradation rates, the polymerization rate is inhibited by the presence of oxygen.[76] When oxygen is present, free radicals can interact with it, forming peroxy radicals that do not contribute to the reaction.

On the other hand, free radical step-growth reactions occur by the interaction of thiols with acrylates/enes, which are insensitive to the presence of oxygen.[77,78] Additionally, the thiol-acrylate reaction involves a supplementary propagation step, since the acrylate groups can also react with the carbon-based radicals. This mixed-mode polymerization can be easily adjusted by changing the thiol:acrylate ratios, resulting in tunable hydrogel properties.[77] Considering the advantages of this system over the conventional free radical polymerization, different polymers were already modified to contain thiol and acrylate/ene groups. As a result, thiol-acrylate and thiol-ene chemistries have been widely and successfully used to prepare biocompatible hydrogels from different polymers, including polyethylene glycol (PEG),[79–81] hyaluronic acid (HA),[82,83] gelatin,[84,85] and alginate.[86] One promising application of these photo-reactions is the biofabrication field.[87,88] As an alternative to the free-radical chain-growth crosslinking strategies, the step-growth polymerization of thiol-ene results in a more homogenous network that can be easily tailored and modified by the addition of any thiol-containing biomolecules, as schematically represented in **Figure 6**. A notable example of this application is the modular thiol-ene alginate bioink recently developed by Ooi et al.[86] Using this method, the authors were able to bioprint using lower alginate concentrations, maintain high cell viability, and easily incorporate cell adhesive motifs.

2.2.2. "Click-Chemistry"

"Click-chemistry" reactions hold significant advantages for biomedical applications, including very high yields under mild conditions, fewer and innocuous by-products, high specificity, and great selectivity.[89,90]

Diels–Alder (DA) reaction, Michael addition, and Schiff base linkages[91–93] are some of the "click chemistry" methods often used for hydrogel crosslinking. DA reaction relies on the interaction between a diene and a substituted alkene, which are not present in most of the suitable polymers. Although it requires a previous modification of the backbone polymers, it has gathered a great deal of attention over the past years.[94] That is highly related to the selectivity, efficiency, and thermo-reversibility associated with these reactions. Also, DA reactions do not require the addition of any catalyst, which poses an additional advantage for this system.[95] As said, DA chemistry requires a polymeric functionalization, which typically includes the addition of furan and maleimide groups.[96] For example, HA has been modified with furan for further crosslinking with dimaleimide PEG.[97] The reaction leads to the formation of a tunable hydrogel, where mechanical and degradation properties can be controlled by the furan to maleimide molar ratio. Although promising, the resulting gels are only compatible with 2D culture systems, as the crosslink involves a sub-physiologic pH of 5.5. To tackle this issue, furan-HA can be substituted by the electron-rich hyaluronan-methylfuran,[98] as it accelerates the DA reaction at physiologic pH (**Figure 7**A). Using such conditions, cells can be successfully encapsulated into the hydrogel's matrix while taking advantage of DA reaction properties.

By its turn, Michael-type reactions occur via the addition of a nucleophile Michael donor, and an electrophilic carbon-carbon double bond conjugated with a carbonyl group that behaves as a Michael acceptor (Figure 7B). Examples of Michael donors are thiols and amines, but thiol-based molecules are usually preferred, considering the higher nucleophilicity and selectivity at physiological pH and temperature. On the other hand, Michael acceptors are more variable amongst the hydrogel preparations reported in the literature, including acrylates, acrylamides, vinyl sulfones, and maleimides.[99] This reaction is compatible with aqueous environments, room temperature, and physiological pH, making it suitable for biomedical hydrogel applications, including cell encapsulation and injectable formulations.[98,100] As an example, thiolated gelatin was already used in combination with different PEG-modified molecules (PEG-maleimide, PEG-acrylate, and PEG-vinyl sulfone) to prepare bioinks.[101] Interestingly, the gelation time could be tailored by changing the Michael acceptor of the system, with PEG maleimide conferring the fastest gelation (<30 s) while PEG-vinyl sulfone the slower (>10 min). Hydrogels made from modified PEG,[102,103]

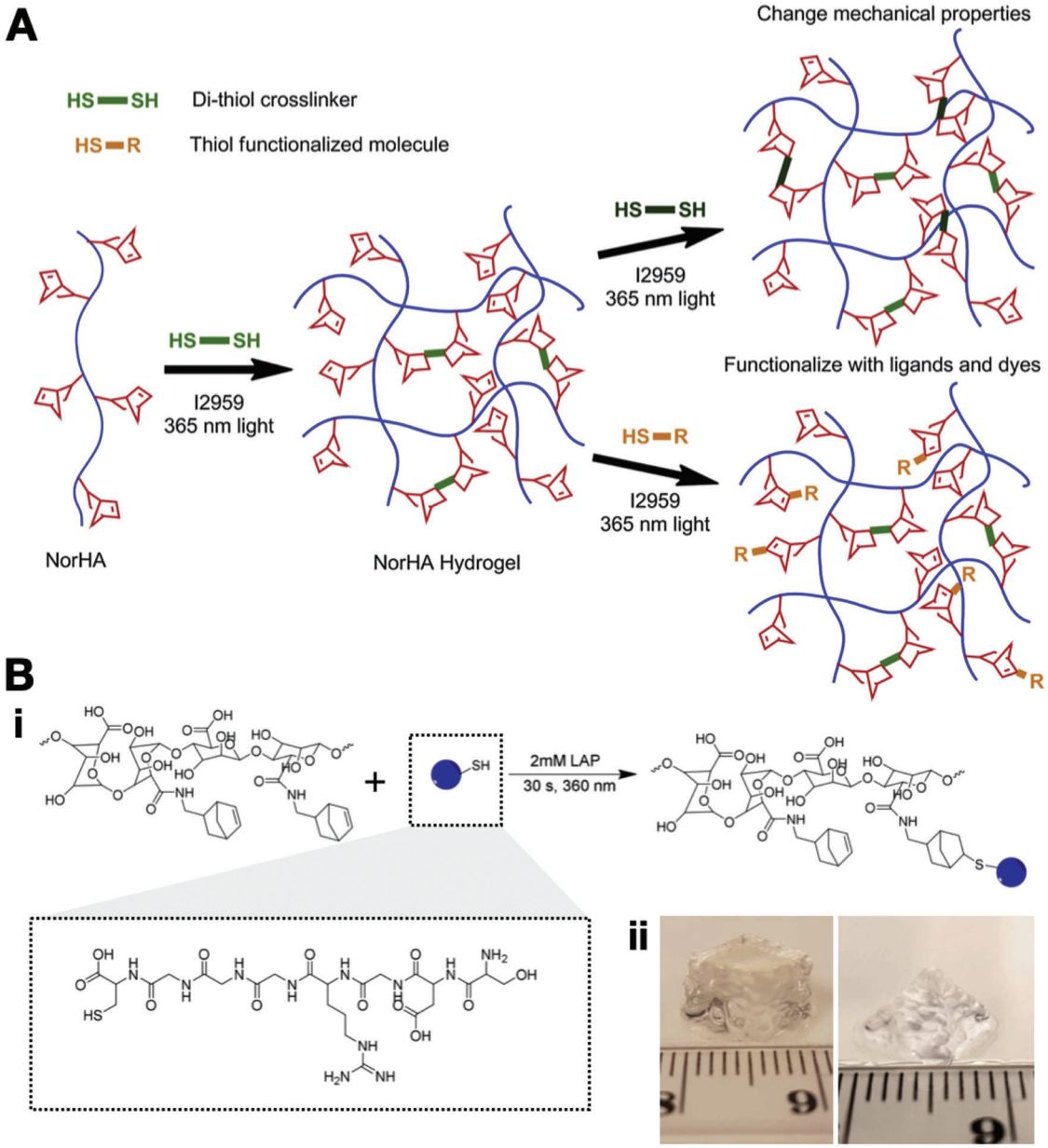

Figure 6. Photopolymerization strategies. A) Schematic drawing of photopolymerization crosslink of norbornene-modified HA (NorHA). Hydrogel is formed via light-initiated thiol-ene reaction between a di-thiol and NorHA. The resulting hydrogel can be further modified with mono- and/or di-thiols, allowing the addition of functional motifs (R) to the network. Adapted with permission.[82] Copyright 2013, Elsevier. B) Thiol-ene alginate hydrogels. i) Norbornene alginate reacts with RGD sequences containing thiol groups (CGGGRGDS), using LAP as photoinitiator. ii) The resulting 3D bioprinted structures. Adapted with permission.[86] Copyright 2018, American Chemical Society.

chitosan,[104] and HA[105] are some examples of the applicability of this reaction in TE.

At last, Schiff base linkages generate imine linkages upon the interaction of amino and aldehyde groups, being a great choice for in situ hydrogel crosslinking. Since there is a dynamic equilibrium between the components of this reaction, the imine linkages are considered as pseudo-covalent bonds, conferring a self-healing characteristic to the hydrogels obtained via this route, useful for injectable formulations (Figure 7C).[106] Oxidized polymers are great tools for this type of chemistry, as they have multiple aldehyde groups available to react with materials having amino groups.[107] The oxidation of HA using sodium periodate, as an example, allows the crosslink of this natural polymer via Schiff base reactions, thus avoiding the need to use chemical crosslinkers. Oxidized HA can then be crosslinked with amine-rich glycol chitosan, to prepare an injectable formulation compatible with cell encapsulation.[108] Besides oxidation, previous functionalization of the polymers with aldehydes and amines has also been used to prepare Schiff base hydrogels.[109,110]

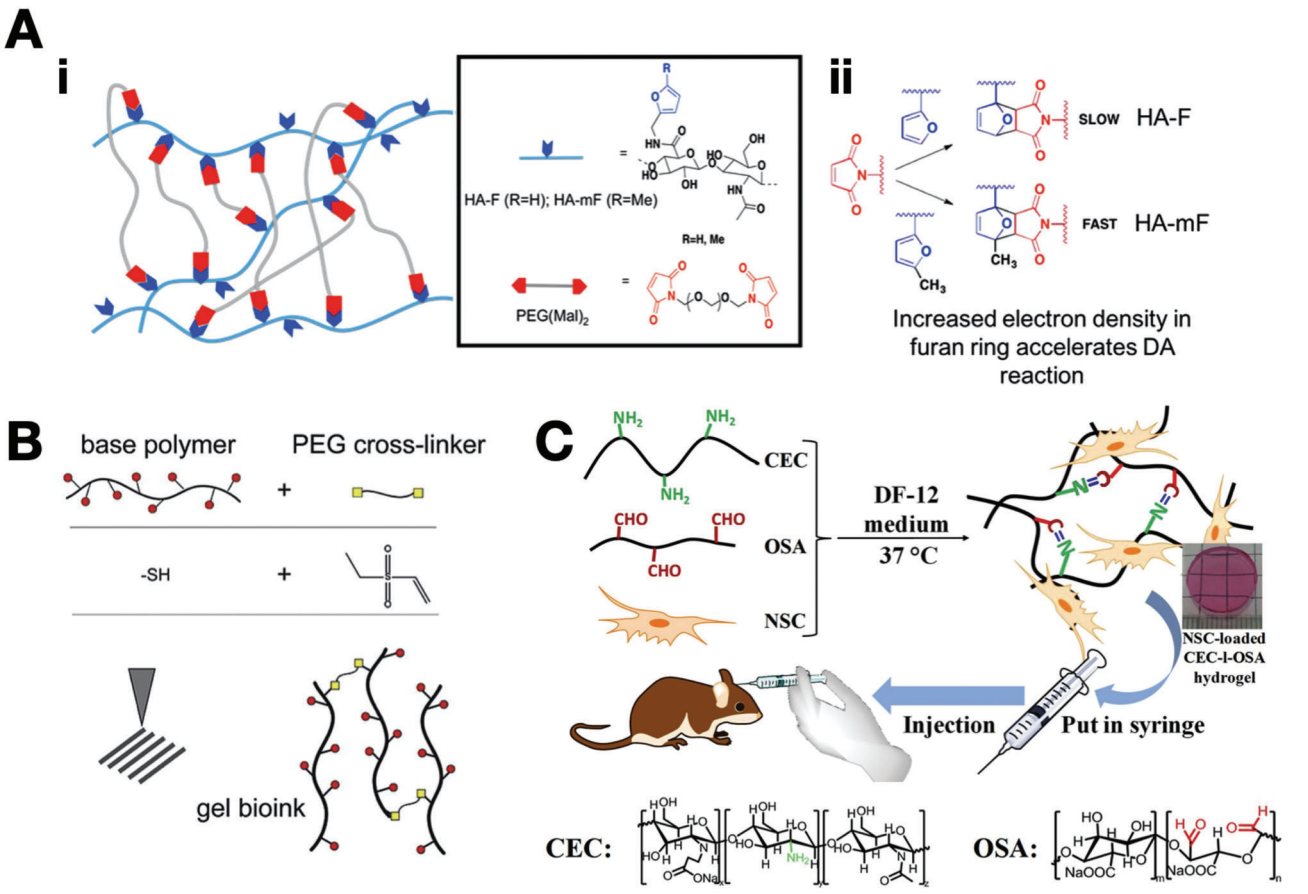

Figure 7. Overview of the discussed chemical crosslinking chemistries. A) i) DA reaction between furan-HA (HA-F) and maleimide-modified PEG (PEG(Mal)). ii) The reaction rate can be tailored by changing the furan group. In this case, furan functional group was switched by methylfuran, which accelerated the reaction. Adapted with permission.[98] Copyright 2018, American Chemical Society. B) Example of Michael-type reactions between a thiolated base polymer and a vinyl sulfone-modified PEG crosslinker to prepare bioinks. Adapted with permission.[101] Copyright 2019, Elsevier. C) Use of N-carboxyethyl chitosan (CEC) and oxidized sodium alginate (OSA) to prepare Schiff base hydrogels. The dynamic imine bond formed between the amino and aldehyde functionalities allows the application of this hydrogel as injectable material envisioning cell delivery into the central nervous system. Adapted with permission.[106] Copyright 2016, Springer Nature.

2.2.3. Enzymatic Crosslinking

The use of enzymes to chemically crosslink hydrogels is gathering a great deal of attention, as these molecules can be considered as "green-catalysts" of hydrogel formation. One of the main advantages of enzyme-based systems is the substrate specificity that reduces the occurrence of toxic side reactions. The possibility to control hydrogel formation kinetics, the relatively fast gelation, and the resulting strong covalent bonding also contribute to the increasing interest in this strategy.[111] As a result, different enzyme-mediated methods have been developed until now, using different types of enzymes and often using Nature as inspiration.

Transglutaminases, which are part of the transferase's family, are widely used in the TE setting. These thiol enzymes catalyze the formation of covalent bonds between the γ-carboxamide group of glutamines and a free lysine amine group. The gelation occurs in 5 to 20 min, and the resulting bonds are greatly resistant to proteolysis, making it possible to obtain stable networks.[112] A remarkable example of transglutaminase-catalyzed reactions is the formation of fibrin clots from soluble fibrin, the "biological glue" formed during blood coagulation. The enzymatic activity of transglutaminases has already been quite explored, mostly for the crosslink of various peptides, such as collagen or gelatin,[113–115] or modified polymers carrying the transglutaminase substrates.[116,117]

Horseradish peroxidase (HRP) is the most used oxidative enzyme in TE, when crosslinking is envisaged. As an oxidative enzyme, HRP catalyzes the oxidation of aromatic proton donors (such as phenols, anilines, or amines), resulting in the coupling of the formed reactive species, using H_2O_2 as a cofactor. The specificity of HRP makes it only interact with polymers that have hydroxyphenyl groups, such as polymers containing tyramine, tyrosine, or 4-hydroxyphenyl acetic acid. While some polymers naturally present the groups mentioned above, others need to be functionalized for further HRP-mediated crosslink. That includes gelatin,[118,119] PEG,[120,121] HA,[122–124] alginate,[125,126] among others. A typical example of HRP-mediated crosslink is the hydrogels prepared from silk fibroin (SF),[127,128] obtained by the chemical crosslink between two tyrosine residues (**Figure 8**). The resulting hydrogels are elastic, with tunable

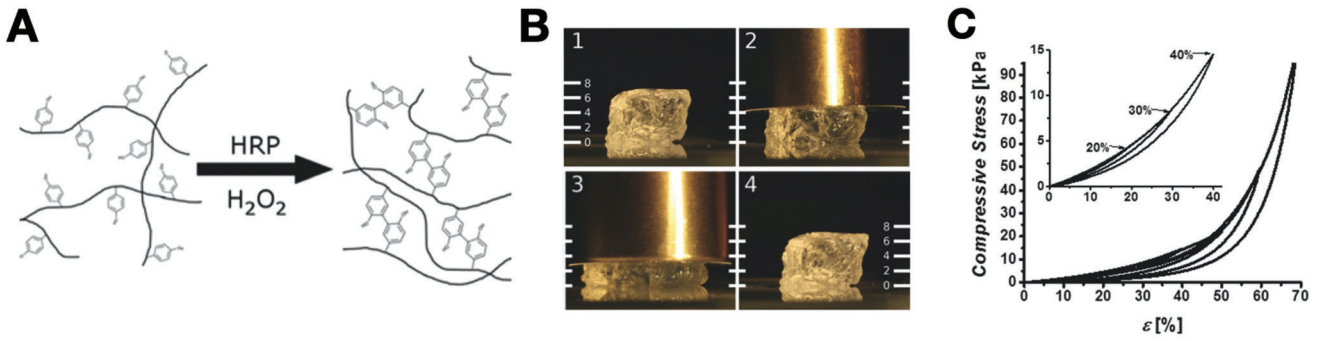

Figure 8. Enzymatic crosslink. A) Molecules rich in phenolic groups can be crosslinked using HRP enzyme, in the presence of H_2O_2. B) Silk hydrogels obtained by HRP-mediated crosslink show an elastic behavior and superior mechanical properties. After ≈50 % compression, under 50 g (2) and 100 g (3) brass weights, the hydrogel shows complete recovery (4). Scale is in millimeters. C) Cyclic compression curves of hydrogels showing excellent recovery below 70 % strain, and complete recovery below 40 % strain (inset). Adapted with permission.[128] Copyright 2014, Wiley.

mechanical properties,[128] paving the way for a wide range of applications from fundamental studies[129] to biofabrication.[130] Moreover, this chemical crosslink can be combined with the SF physical crosslink, causing a conformation change from random coil to β-sheet. Such "dual crosslink" greatly enhances the hydrogel's mechanical properties, particularly its compressive modulus, which can be as high as 3 MPa.[131]

Besides the different crosslinking methods that can be used to achieve different hydrogel properties, further advancements include the addition of a secondary network to expand their functionality to several applications such as TE, drug delivery, and the development of in vitro disease models.[132,133] These interpenetrating polymer network hydrogels allow not only to better mimic the ECM, but also to have stimuli-responsive hydrogels with improved mechanical properties.

3. Material-Cell Interactions

As mentioned before, hydrogels can be prepared from different sources, with different properties, and further modified using several different approaches. Consequently, hydrogels can display different assets that must be tailored for the final desired application. The following section discusses these macroscale properties and how they fit into different TE strategies, as schematically represented in **Figure 9**.

3.1. Hydrogel Stability

The growing interest in stem-cell-based therapies led to an increased effort to improve the final stem cell fate. In this regard, hydrogels are considered an ideal tool to help cell survival and correct placement inside the body, and also a promising strategy to mitigate some of the drawbacks associated with cell storage and distribution that are often required prior to therapy.[134–136] Hydrogels can then be used as carriers, improving cell function and viability, while providing protection from the hostile environment found in diseased or wounded tissues.[137]

Typically, encapsulated cells are delivered by injection, using minimally invasive techniques.[138] Shear-thinning materials, compatible with reversible crosslinking strategies, are thence preferred for this kind of approach.[139,140] Being shear-thinning, hydrogels exhibit a liquid-like behavior under the shear stress created by the injection procedure, thus allowing cell delivery. After injection, the reversible nature of the crosslink leads to the recovery of the hydrogel mechanical properties, assuring a homogeneous encapsulation that prevents leakage of its cargo to the surrounding media.

Besides being essential for the initial cell retention in the desired place, hydrogel fate must be compliant with the final purpose of the cell-based strategy. While degradation is necessary in some cases, other approaches require stable hydrogels capable of protecting transplanted cells for long periods.

TE strategies often rely on the implantation of exogenous therapeutic cells to regenerate the damaged tissues, either by direct action of the therapeutic cells or by a paracrine effect via cell's secretome.[141–145] To be successful, these approaches trust on cell proliferation and migration from the hydrogel to the surrounding environment, as well as de novo ECM synthesis, combined with a sustained degradation of the biomaterial. At last, the tissue is repaired, due to de novo ECM synthesis combined with the therapeutic effect of encapsulated cells. Therefore, it is rather important that the hydrogel degrades at a suitable rate, compatible with sustained cell migration or new ECM formation. Such timing poses a particular challenge in the field and can be tackled by modifying the crosslinking strategy or the polymers used to prepare the hydrogels.[146,147] Similarly, the effect of the employed hydrogel on cell's secretome must also be considered. As reported by Silva et al.,[148] it is possible to modulate the secretome by fine-tuning the encapsulating hydrogel, for example, by the addition of motifs naturally present on the ECM.

Hydrogels prepared from biodegradable materials,[141] or modified polymers containing degradable groups,[149,150] are often used for cell delivery approaches due to their inherent degradability. In such cases, the primary degradation mechanism is enzymatic degradation and/or hydrolytic degradation. Hydrogels designed to be degraded via enzymatic degradation take advantage of the enzymatic pool existent in the body, particularly proteases such as matrix metalloproteinases (MMPs). Although some polymers are naturally degraded by such enzymes, as gelatin,[151] others need to be modified to include enzyme-labile motifs.[152,153] The typical example of modified hydrogels for proteolytical degradation purposes are PEG-based hydrogels,[154–157] but others,

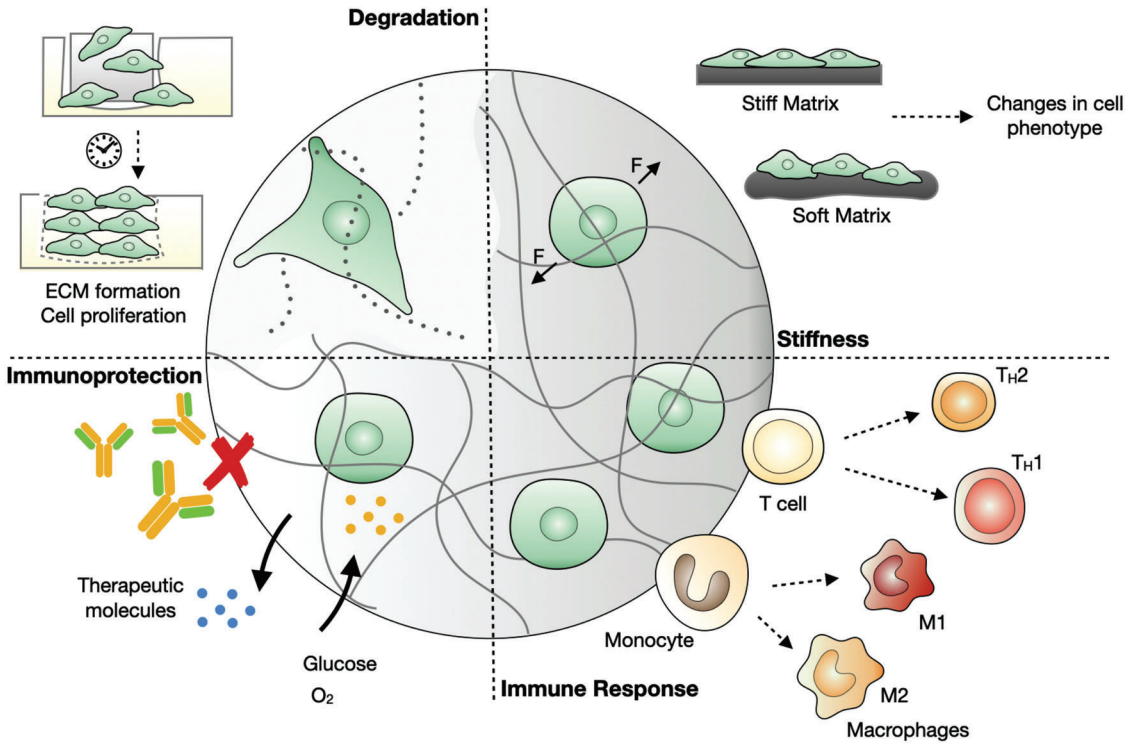

Figure 9. Schematic overview of the material-cell interactions discussed in this review. Degradable hydrogels are preferred for cell delivery approaches, where cell proliferation and migration are intended, leading to in situ ECM formation. However, the hydrogel can act as an immunoprotective device, displaying a semi-permeable behavior, where only small molecules enter the hydrogel space, a strategy suitable for cell encapsulation purposes. Upon interaction with the host, the hydrogels elicit an immune response that can be tailored to favor a pro-regenerative phenotype. At last, the mechanical properties of the hydrogel are also important as they greatly influence cell fate and function.

including natural polymers, have already been modified for the same end. GG, as an example, was modified to include divinyl sulfone groups, able to react with dithiol peptide crosslinkers sensitive to MMP-1.[158] The resulting gels showed a bioresponsive behavior, with promising results for vascularization as endothelial cells could polarize on these matrices, but not on unmodified hydrogels. Alginate[159–161] and HA[162,163] are examples of other polymers modified to promote cell-mediated degradation.

On the other hand, immunoisolation strategies require full protection of the biological material from the host immune system. This technology has been largely developed as a treatment for Type 1 Diabetes Mellitus, where only insulin-producing cells are transplanted into the host.[164] The rationale implies the transplantation of therapeutic relevant cells in the absence of immunosuppression. Therefore, the biomaterial of choice must be stable for long periods, avoiding the interaction between the host immune cells and the transplanted material. However, the material-tissue interface must be semipermeable, allowing the free diffusion of small nutrients and therapeutic molecules between encapsulated cells and the surrounding environment. In this regard, microencapsulation using alginate has been the most studied strategy, with several works reporting the feasibility of this material as an immunoprotective matrix.[165,166] Interestingly, it was already shown that alginate immunoprotective properties depend not only on the alginate type, but also on the ion used to perform the ionic crosslink. Microcapsules prepared with barium are typically more stable and biocompatible than those prepared with calcium, meaning that barium should be preferred for this type of application.[167]

Amongst the latest works on immunoisolation, the work of Vegas et al.[34] has shown how alginate modification can improve the immunoisolation capacity of the hydrogel matrix. By functionalizing the alginate with specific peptides, it was possible to engineer microcapsules capable of mitigating the foreign-body response in mice models. Without any immunosuppression, the authors could maintain and correct the glycemic values for a period of up to 174 days, the moment when the implants were retrieved. Although the work published by Vegas et al. is undoubtedly a significant step towards a clinical application, other strategies and materials have been reported during the last years for immunoprotection purposes, as summarized in **Table 1**.[168–180]

An et al.[170] engineered an interesting approach for immunoisolation of islets cells, the TRAFFIC system (**Figure 10**). Instead of spherical microcapsules, the authors prepared alginate threads, using nylon sutures as templates and spider silk as inspiration. The aim was to modify the thread with a nanoporous polymeric coating to mimic the capillary-enabled water collection and retention observed in certain spiders. The nanoporous coating served as a $CaCl_2$ reservoir that later was used to crosslink a thin alginate layer around the whole thread. The resulting threads were more mechanically robust than bare alginate fibers and easily handled, including laparoscopic implantation and retrieval. It was also verified that the device provides immunoprotection to encapsulated islets for up to 1 month, similar to neat alginate

Table 1. Recent strategies for immunoisolation.

Hydrogel	Crosslink	Structure	Cell-type	Main Conclusions	Ref.
Thiolated HA/thiolated gelatin	Michael addition	Bulk hydrogel	Canine and rat islets	Semi-permeable matrix due to thermodynamically favorable interactions between hydrogel and dextran. Allogeneic transplant of islets into rats reversed diabetes up to 18 months.	[171]
Alginate	Ionic crosslink	Bulk hydrogel	Rat and human islets	Suture threads coated with alginate. The threads improve mechanical strength allowing a facile retrieve of the construct.	[170]
Alginate	Ionic crosslink	Microcapsules	Human and murine islets	Comparison between free and microencapsulated islets. Small microcapsules caused only a slightly delayed insulin response compared to unencapsulated islets. Larger capsules decreased the total amount of insulin released.	[172]
Alginate	Ionic crosslink	Microbeads	-	Microbeads with intermediate G alginate or sulfated alginate, crosslinked with calcium and barium ions, display a lower pericapsular fibrotic overgrowth in immunocompetent mice (C57BL/6JRj) as compared to microbeads prepared with alginate with a high-G content.	[179]
Alginate and methacrylated chitosan	Ionic and photo-crosslink	Microcapsules	Porcine islets	Good islet viability and function after 1 month of in vitro culturing. Improved biocompatibility over APA capsules.	[173]
Alginate-pluronic F127 and pectin	Ionic crosslink	Bulk hydrogel	MIN6	Constructs with pectin were able to support MIN cells' survival, even under inflammatory stress (exposure to pro-inflammatory cytokines) and inhibit the activation of Toll-like Receptor 2/1.	[180]
PEG	Photo-crosslink	Bulk hydrogels	Mouse embryonic fibroblasts	Comparative study to assess the effect of functional end groups of multi-arm PEG on hydrogel stability and host immune response. PEG-vinyl sulfone evidenced an attenuated immune response,	[174]
PEG	Michael addition	Bulk hydrogels	Rat islets	Long-term stability in vitro and in vivo. Functionalization of PEG with RGD improved insulin responsiveness. Addition of a vasculogenic, degradable hydrogel layer enhanced islet viability in vivo.	[175]
PEG vinyl sulfone	Michel addition and photo-crosslink	Core-shell hydrogel	Mice ovaries	Proteolytically degradable hydrogel core and non-degradable shell. Ovaries encapsulated within the core, in conductive environment for tissue development. Non-degradable shell protected the tissue from immune response.	[176,177]
PEG diacrylate (575 and 3500 Da)	Photo-crosslink	Ultra-thin coating	Jurkat	Films with 100–200 nm thickness. 10 and 20 kDa molecules are blocked, but 4 kDa move freely.	[178]

fibers. Overall, the possibility to easily implant and retrieve the system, allied with the immunoprotection feature, paves the way for future clinic applications of this strategy.

3.2. Mechanical Properties

The importance of ECM on cell fate has already been established, and it is now known that matrix mechanical properties significantly affect how cells proliferate, migrate, and even maintain their normal phenotype.[181,182] In this regard, the mechanical properties of hydrogels must be considered when designing hydrogels for TE, to recapitulate the correct mechanical information.[183] Dynamic modulation of hydrogels' stiffness using various stimuli, such as light, temperature, and pH can be seen as a strategy to have better control over cell behavior and fate.[184]

Cells can sense and interact with their external mechanical environment via integrin-mediated focal adhesion signaling.[185] The mechanical resistance of the ECM to cell-generated forces dictates the stability of the focal adhesion complexes, which likely activate mechanoresponsive signaling pathways. Such information, which is converted into a biochemical signal through mechanotransduction, ends up changing the gene expression of resident cells by different pathways, as schematically represented in **Figure 11**.[186]

These include the FAK-RhoA-Rho kinase cascade,[187] a pathway that starts with the phosphorylation and activation of RhoA

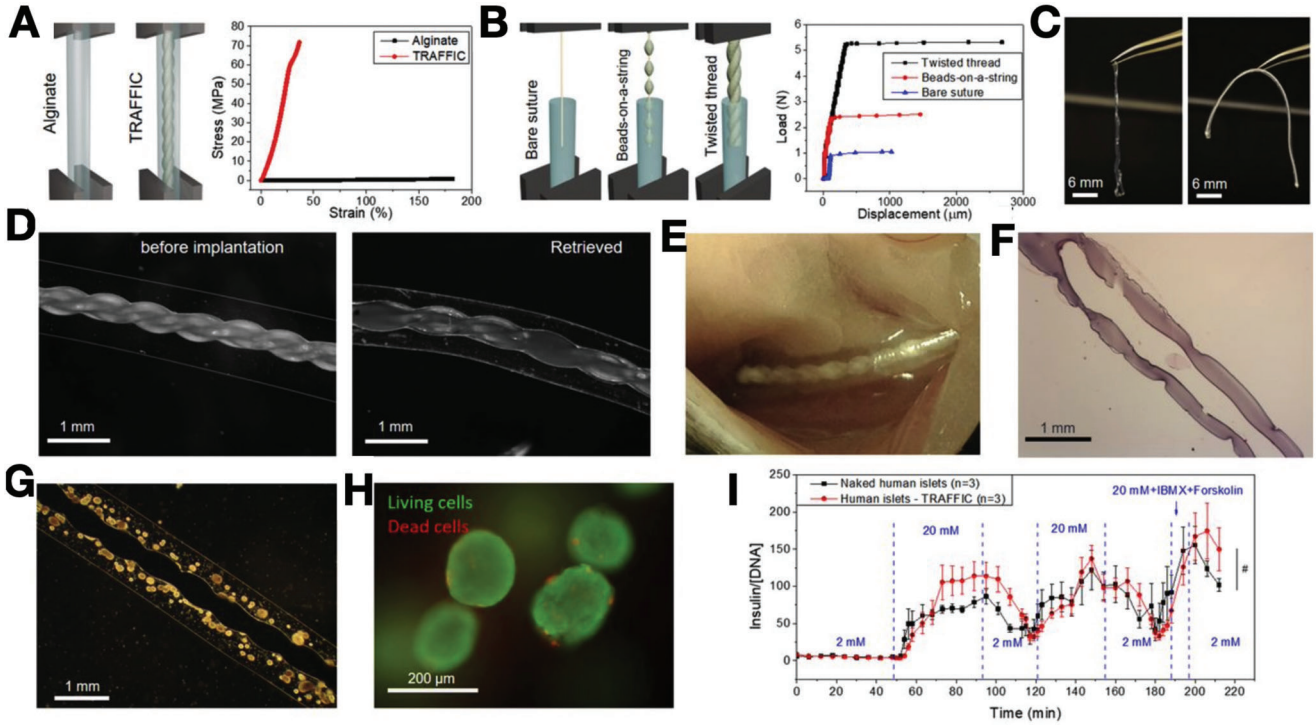

Figure 10. Overview of the TRAFFIC system. A) Schematic representation and plot of the strain-stress measurement, comparing bare alginate fibers and TRAFFIC; B) Schematic representation and results of the load-displacement measurement, comparing bare alginate fibers with bead-on-a-string configuration and the twisted thread (TRAFFIC); C) Handling of neat alginate fiber (right) and a TRAFFIC device (left); D) Microscopic images comparing the structure of TRAFFIC before and after 7-month implantation in mice; E) TRAFFIC device inside the intraperitoneal cavity of a mouse; F) Hematoxylin and eosin staining of retrieved; G) Human islet cells encapsulated in the alginate layer; H) Live (green)/dead (red) staining of encapsulated human islets; I) Results of a dynamic perfusion test for glucose-stimulated insulin secretion comparing naked islets with encapsulated ones. n = 3, mean ± SEM (standard error mean), #$P > 0.05$. Reproduced with permission.[170] Copyright 2018, National Academy of Sciences.

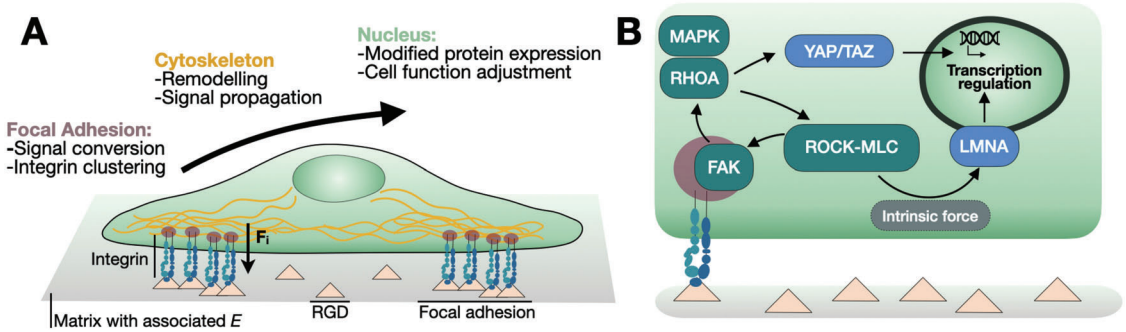

Figure 11. Schematic representation of the mechanotransduction phenomena. A) Cell-material interaction via focal adhesions starts a signaling cascade that travels through the cytoplasm to the nucleus. B) Overview of the signaling cascade triggered by cell mechanosensing.

and Mitogen-Activated Protein Kinases (MAPK) by focal adhesion kinases (FAK). As a result, mechanosensitive transcription factors are translocated into the cell nucleus, altering the gene expression as a response to the mechanical environment. For example, YAP (Yes-associated protein) and TAZ (transcriptional coactivator with PDZ-binding motif) are emerging as universal control systems for mechanosensing, in 2D and 3D conditions, as well as in a wide range of elastic and viscoelastic stimuli.[188,189] As mediators of mechanical cues, the translocation of YAP/TAZ into the nuclei occurs when cells are in contact with stiff substrates.[188] This translocation is independent of the Hippo/LATS cascade but depends on the aforementioned FAK-RhoA-Rho kinase cascade.[190] For example, Silver et al.[189] demonstrated that elevated hydrogel stiffness promotes migration and proliferation of resident muscle stem cells (MuSCs), and this behavior was related to accumulation of YAP and TAZ in the nucleus. By using pre- to post-injury stiffness hydrogels, the authors demonstrated that continuous exposure to enhanced stiffness triggers mechanotransduction signaling, while maintaining activated and proliferating MuSCs.

The mechanical signals can also be directly transmitted to the nucleus. Lamina proteins, such as laminin A (LMNA),

physically connect the nucleus with the cytoskeleton through the LINC ("linker of nucleoskeleton and cytoskeleton") complex.[191] The mechanical signals can then reach the nuclear structure, thus affecting chromatin structure and gene expression.[192]

Regarding hydrogel design, the mechanical properties are often studied considering the biomaterial elasticity and its stiffness. To that end, hydrogels with different stiffnesses are typically obtained by systematically changing the polymer concentration or crosslinking density.[193–197] Such combinations resulted in hydrogels with elastic modulus (E) ranging from Pa to MPa,[198] that can be used as platforms in order to improve TE strategies[199] but also to study the mechanotransduction phenomena.[200] **Table 2** summarizes the latest studies on this subject.[189,193–197,199–231]

Pelham et al.[201] demonstrated that substrate flexibility affected cell morphology and locomotion, and recent works have been strengthening their results by showing the impact of substrate stiffness on mesenchymal stem cells (MSC) migration.[204] The latest data suggests that most of the cell behavior is influenced by mechanotransduction, including migration, proliferation, differentiation, and even the immunomodulatory effect.[186,226,232–234] Although, it was the seminal work of Engler et al.[202] that clearly demonstrated the importance of matrix stiffness on stem cell differentiation (**Figure 12**). Polyacrylamide gels with defined stiffnesses were obtained by varying the concentration of the crosslinker (bis-acrylamide) and used as a platform to study the differentiation of MSCs. They found that lineage specification of MSCs can be directed by matrix stiffness and that matrices with tissue-like stiffness induced stem cells to differentiate into analogous specific cell lineages. It was observed that soft substrates with a brain-like stiffness ($0.1<E<1$ kPa) induced neurogenesis, while stiff substrates with an elastic modulus of 25–40 kPa promote osteogenesis. These results shed light on the idea of stem cell pre-commitment into a specific lineage using biomaterials to overcome an inhospitable in vivo environment.

Undoubtedly, Engler's work had a great impact on Mechanobiology, and a great deal of attention has been given to this matter since then. However, it has been noticed that time-dependent mechanical properties can also impact the cell-matrix interaction. Therefore, a lot of work has been developed recently in order to engineer viscoelastic hydrogels, that better recapitulate the ECM properties, and allow to study the influence of time-dependent deformations on mechanotransduction. Contrarily to what occurs in pure elastic substrates, in hydrogels that exhibit stress relaxation properties, the resistance to cell-induced forces decreases over time, changing the way the cell perceives its surroundings (**Figure 13**A).

Chaudhuri et al.[207,235] successfully designed a set of hydrogels with similar initial elastic modulus but a wide range of stress relaxation rates, using the same concentration of the backbone polymer. Their results showed that the relaxation times greatly influence the behavior of encapsulated MSCs, as hydrogels with faster relaxation times enhanced cell spreading, proliferation, and osteogenic differentiation. The authors claimed that such effect was transduced via integrin-based adhesions, local clustering of Arg-Gly-Asp ligands (RGD), actomyosin contractility, and YAP translocation into the nucleus. For this study, the authors used RGD-modified alginate of different molecular weight, as well as PEG spacers, to modulate the relaxation times, as shown in Figure 13B. The formed hydrogels showed a decrease in stress relaxation time by lowering the molecular weight and introducing PEG spacers. Since the stress relaxation properties are independent of initial elastic modulus and matrix degradation, it is possible to mimic the viscoelastic behaviors of living tissues. Other strategies can also be used to tune the viscoelasticity and relaxation times of hydrogels, always taking advantage of physically associative or reversible covalent chemical bonds. For instance, boronate-based hydrogels allow the formation of synthetic matrices with different viscoelasticity, which is proven to be useful as a platform for studying the mechanotransduction phenomena.[213,214] The faster association and dissociation dynamics of boronates, as compared to other reversible bonds, permit the study of events triggered by short relaxation times. Moreover, by changing the boronic acid derivatives, it is possible to finely tune the relaxation dynamics (Figure 13C). As an example, the use of 2-fluorophenylboronic acid (FPBA), 1-hydroxy-1,3-dihydrobenzo[c][1,2]oxaborole (m-boroxole, BX), or a Wulff-type o-amino-methylphenylboronic acid (WBA), considerably changed the relaxation time of the resulting hydrogels. That can be attributed to the different binding constants, as the strongest bonds (using FPBA) had the slowest relaxation as opposed to the weakest WBA-based bonds, which showed a faster relaxation time.[213]

Another relevant consideration that must be highlighted when considering matrix stiffness is its impact on MSCs differentiation capacity. Taking advantage of the photoinduced softening of modified PEG hydrogels, it was possible to study the "mechanical memory" of MSCs, as depicted in **Figure 14**.[205,206] Surprisingly, chromatin organization and histone acylation states induced by the rigid matrix can be reversed if cells are cultured only for 1 day prior to softening; after a longer culture period, like 10 days, those changes became irreversible. Indeed, as depicted in Figure 14B(iii), cell culture on a stiff hydrogel for 1 day does not affect the YAP and RUNX2 response to in situ softening, with MSCs demonstrating a transient and fully reversible activation of YAP and RUNX2. After 7 days of culture on stiff hydrogels, YAP and RUNX2 response to in situ softening revealed a partial reversible activation of these markers on MSCs (Figure 14B(iv)). However, MSCs cultured for 10 days on a stiff hydrogel induced an irreversible activation of YAP and RUNX2, as these markers persisted at active levels significantly above basal levels for soft hydrogels (Figure 14B(v)). This mechanical dosing gives more insight into the deleterious effect of long-term MSCs expansion, a step typically crucial to obtain therapeutic relevant cell numbers and is typically performed using rigid plastic flasks.[205]

Although the bibliography discussed until now focuses mainly on MSCs, the effect of the mechanical properties on cell function has been shown to be valid for other cell types as myoblasts,[211] vascular progenitor cells,[236] dental pulp stromal cells,[217] podocytes,[227] skeletal MuSCs,[189] to name a few. Also, it is important to stress that the mechanotransduction phenomena cannot be pictured only from the stiffness point of view. Besides the previously mentioned time-dependent properties, that is, viscoelasticity, also the cell-ligand density,[224] presence of different ECM proteins,[225] and cell volume/density,[222] play an important role in the final cell response and must be taken into account.[209] The type of platform where cells are seeded into, that is, 2D versus 3D environment, is also an important variable to be considered. While 2D platforms are routinely done in almost

Table 2. Summary of the latest works addressing the effect of hydrogel mechanical properties in the mechanotransduction phenomena.

Hydrogel	Elastic Modules [kPa]	Properties	Cell Type	Main Effect	Main Conclusions	Ref.
Polyacrylamide coated with type 1 collagen	0.015–0.07	2D Elastic	3T3 (fibroblasts) NRK (epithelial)	Cell adhesion	Stiffer substrates promote cell adhesion, by phosphotyrosine action on cell adhesion sites. Flexible substrates promote highly dynamic adhesion points.	[201]
Polyacrylamide coated with type 1 collagen	0.1–40	2D Elastic	MSCs	Cell differentiation	Soft matrices that mimic brain are neurogenic, stiffer matrices that mimic muscle are myogenic, and comparatively rigid matrices that mimic collagenous bone induce osteogenic differentiation	[202]
RGD-modified alginate	2.5–110	3D Elastic	MSCs	Cell adhesion	Cell-traction needed to sense and bind to cell adhesion motifs. RGD clustering was maximized in matrices of intermediate rigidity.	[203]
Polyacrylamide coated with fibronectin	1–160	2D Elastic	MSCs	Cell migration	MSC durotaxis is dependent on the stiffness gradient and not only on stiffness.	[200,204]
Photodegradable PEG	2–30	2D Elastic	MSCs	DNA modification	MSCs retain mechanical information from past physical environments. Mechanical dosing for long periods causes constitutive alterations.	[205,206]
RGD-modified alginate	9–17	3D Viscoelastic	MSCs	Cell migration, proliferation, and differentiation	Faster relaxation times enhance cell spreading, proliferation, and osteogenic differentiation of MSC.	[207]
Self-assembling peptides	1.5–9	3D Viscoelastic	MSCs	Hydrogel design	System provides control over matrix stiffness and binding sites.	[208]
RGD-modified alginate	3–30	3D Elastic	MSC and CNPCs	Transcriptome alterations	Cells encapsulated in hydrogels with different stiffness, stress relaxation, and adhesion ligand density. RNA-seq analysis used to study the effect of each parameter on cells' transcriptome.	[193,209]
Gelatin	2–24	2D Elastic	TDSC	Cell differentiation	Tenogenic, chondrogenic, and osteogenic lineages were inhibited on stiff hydrogels, due to FAK and ERK1/2 phosphorylation.	[210]
PEG DBCO	9–30	3D Elastic	C2C12	Cell differentiation; disease model	Mimicking of physiologic stiffening associated with muscle disease and aging. Stiffen matrices hamper spreading, with YAP being concentrated in the cytoplasm.	[211]
Dextran	6–109	3D Elastic	EPCs	Cell differentiation	Matrix stiffness regulates the differentiation of EPCs during vasculogenesis. Stiffer gels induced higher vascularization, using the Ras/MEK signaling pathway.	[195]
Matrigel/alginate	0.09–0.93	3D Elastic	A549 (lung adenocarcinoma)	Cell migration and proliferation	Stiffer conditions enhanced cell proliferation and invasiveness.	[212]
PEG-diacrylate	10–60	2D Elastic	DP	ECM mimicking	Softer hydrogels are better to mimic the native environment of DP cells.	[196]
RGD-modified methacrylated ha	0.2–4.5	2D Elastic	MDA-MB-231Br	Cell adhesion, spreading, proliferation and migration.	Cell adhesion, spreading, proliferation, and migration significantly increased with the hydrogel stiffness. Response is mediated by focal adhesion kinase-phosphoinositide-3 kinase pathway.	[197]
PEG boronate	27–60	2D and 3D Viscoelastic	MSCs	Hydrogel design	Cytocompatible fast relaxing hydrogels, that maintain structural and mechanical properties under cell culturing conditions.	[213,214]

(*Continued*)

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Table 2. (Continued).

Hydrogel	Elastic Modules [kPa]	Properties	Cell Type	Main Effect	Main Conclusions	Ref.
HA/collagen	0.008–1.5	3D Viscoelastic	MSCs	ECM mimicking	Hydrogel captures the viscoelasticity and fibrillarity of ECM in tissues.	[215]
Polyacrylamide coated with fibronectin	3–35	2D Elastic	ASCs	Hydrogel design	High-throughput system allows to study 54 different extracellular matrix types of defined stiffness, shape, and area.	[216]
Polyacrylamide with hydroxyapatite microparticles	3–75	2D Elastic	DPSCs	Cell differentiation	Cells are affected by the mechanical properties of the hydrogel. Relatively stiff substrates (>75 kPa) may be needed for significant mineralization.	[217]
Polyacrylamide	16–19	2D Viscoelastic	3T3 (fibroblasts)	Hydrogel design	Method to prepare soft viscoelastic solids, with viscoelastic properties that can be tuned to closely mimic soft tissues.	[218]
Adamantane norbornene-modified HA/ß-cyclodextrin-modified HA; polyacrylamide	0.1–10	2D Viscoelastic	MSCs and 3T3 (fibroblasts)	Hydrogel design	Mathematical modulation of dynamics of focal adhesions.	[219]
Polyacrylamide containing azobenzene	2–8	2D Elastic	MSCs	Hydrogel design	New platform to study mechano-signaling in cells responding to dynamic changes in stiffness.	[220]
Collagen-coated methacrylated HA	0.150–3	2D Elastic	Mammary epithelial cells	Cell signaling pathways	Stiffness-dependent responses are modulated by TGFβ and YAP signaling.	[221]
Fibronectin-coated methacrylated HA	5–23	3D Elastic	MSCs	Hydrogel design (stiffness vs volume)	3D microniches with varying stiffness and volume. Focal adhesion formation, stress fiber organization, and YAP/TAZ activity is not only regulated by substrate stiffness and is sensitive to cell volume.	[222]
Fibronectin-conjugated polyacrylamide	0.15–10	2D Elastic	ASCs	Vascularization	Soft substrates enhance ROS expression and the production of pro-angiogenic factors.	[194]
RGD – modified alginate	1–20	3D Viscoelastic	MSCs	Cell migration	Cells seeded on stiffer matrices expressed low laminin a/c and decreased nuclear stiffness. Nuclear deformation is then used to help cell migration.	[223]
Functionalized polyacrylamide	3–38	2D Elastic	Embryonic cells	Hydrogel design (stiffness vs Biochemical cues)	Modification with different cell ligands to study the impact of biochemical cues on mechanotransduction. In intermediate concentration of biochemical cues, stem cell mechanotransduction depends on substrate stiffness. For low or very high concentrations biochemical cues override the effect of substrate stiffness. Regardless of substrate stiffness, low ligand concentration results in low nuclear YAP while high ligand concentration leads to high nuclear YAP.	[224,225]

(*Continued*)

Table 2. (Continued).

Hydrogel	Elastic Modules [kPa]	Properties	Cell Type	Main Effect	Main Conclusions	Ref.
RGD-modified alginate	1–15	3D Elastic	MSCs, ASCs and UCSCs	Hydrogel optimization for cell differentiation	Platform allowed to establish the optimal combination of parameters for maximal differentiation, which varied with lineage and cell type.	[199]
Polyacrylamide	0.5–200	2D Elastic	MSCs	Cell paracrine function	Model to explore the effects of substrate stiffness on the paracrine function. Soft substrates elicit a higher production of immunomodulatory and trophic factors.	[226]
Hydrolyzed polyacrylamide	0.6–44	2D Elastic	Podocytes	Cell culture and ECM mimicking	Podocytes cultured on hydrogel with stiffness near the elasticity of the glomerular basement membrane (0.9 to 9.9 kPa) provide the best conditions for podocytes, with a normal regulation of podocin.	[227]
Anthracene-functionalized PEG	10–50	2D Elastic	C2C12 and cardiac fibroblasts	Cell signaling pathways	Dynamic stiffening gels induce NFAT translocation into the nucleus of cardiac fibroblasts, which does not happen when using static hydrogels.	[228]
Hydrazone crosslinked PEG	60	3D Viscoelastic	Chondrocytes	ECM mimicking	Viscoelastic hydrogel with different percentages of alkyl-hydrazone and benzyl-hydrazone. The incorporation of adaptable alkyl-hydrazone bonds improves neotissue formation.	[229]
Polyacrylamide hydrogel crosslinked with bis-acrylamide	0.75; 160	2D elastic	HNES1; cR-H9; hPSC; MEF	ECM mimicking	By tailoring the strength of ECM tethering to hydrogels of different stiffness it was possible to infer about the role of ECM proteins and stiffness on cell culture. ECM protein type and adhesion strength, along with substrate stiffness, are important factors for pluripotent cell type culture.	[230]
PEG-based hydrogels via strain-promoted azide/alkyne cycloaddition	1–32	2D viscoelastic	C2C12	Cell signaling	Dynamical stiffening of hydrogels used to model skeletal muscle mechanical changes. BAG3 chaperone protein plays a key role in the transduction of matrix stiffens signaling by redistributing YAP and TAZ subcellular localization to the nucleus,	[231]
PEG-DBCO PEG-N_3 (strain-promoted azide-alkyne cycloaddition)	2–32	2D 3D	C2C12 Myofibers	Cell signaling	Prepared hydrogels allowed mimicking the stiffness of pre- to post-injury skeletal muscle microenvironment. Elevated stiffness was found to enhance the migration and proliferation of muscle stem cells by localizing YAP and WW domain-containing transcription regulator 1 to the nucleus.	[189]

NRK – Normal rat kidney epithelial cells; CNPCs – cortical neuron progenitor cells; TDSC – tendon-derived stem cells; DBCO – dibenzocyclooctyne; C2C12 – mouse myoblast cell line; EPCs – endothelial progenitor cells; DP – dermal papilla; MDA-MB-231Br – brain metastasizing variant of the triple-negative breast cancer line MDA-MB-231; ROS – reactive oxygen species; DPSCs – dental pulp stromal cells; TGFβ – transforming growth factor UCSCs – umbilical cord-derived stem cells; NFAT – Nuclear factor of activated T-cells; HNES1 – Human embryo-derived naïve pluripotent cells; cR-H9 – chemically reset naïve cells from a H9 background; hPSC – human pluripotent stem cells; H9C2 – rat myoblast cell line.

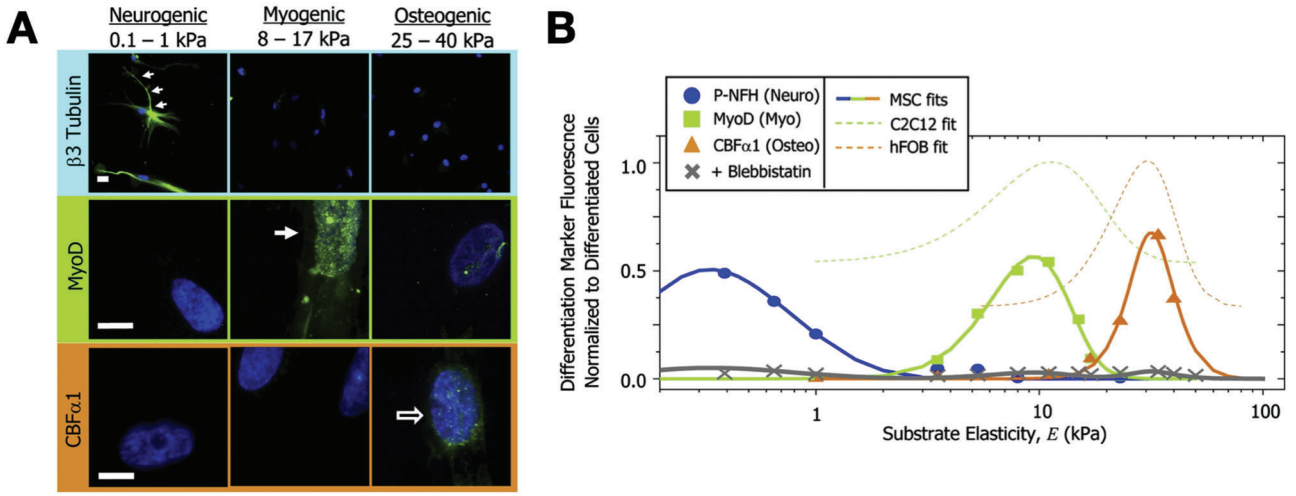

Figure 12. MSCs response to substrate stiffness. A) Fluorescence microscopy images showing the expression of a neuronal cytoskeletal marker (β3 tubulin), a muscle transcription factor (MyoD1), and an osteoblast transcription factor (CBFa1, core-binding factor alpha 1) in substrates of different stiffness. β3 tubulin is expressed in cells cultured on soft matrices but not on intermediate or stiff substrates. By its turn, MyoD1 is upregulated only in MSCs cultured on matrices with intermediate stiffness. At last, the presence of CBFa1 is noticed to be expressed only on stiff gels. Scale bar is 5 µm. B) Lineage specification, assessed by the fluorescent intensity of differentiation markers, is maximum when cells are cultured in matrices with a stiffness typical of each tissue type. Blebbistatin blocks all marker expressions in MSCs. Adapted with permission.[202] Copyright 2006, Elsevier.

every TE laboratory, this model does not totally recapitulate the in vivo environment of cells. On the other hand, 3D cell culture models such as cell-laden hydrogels, can better mimic the natural ECM environment giving a better insight into cell organization, gene expression levels, and allows a more accurate representation of response to mechanical stimuli of cells.[237,238]

3.3. Immune Response

Host immune responses often lead to an engraftment impairment or even rejection upon hydrogel in vivo implantation. Because of that, the immune system was for long considered a foe of TE strategies, and a substantial amount of work was developed to inhibit such unwanted immune responses.[239] Nevertheless, the interplay between the immune system and biomaterials is more intricated than initially thought. In fact, some of the immune system effectors generate a positive outcome on tissue healing and regeneration. As a result, the immune system is now considered a regulator between tissue regeneration and rejection.[240] More than suppressing its action, researchers are now focused on modulating the immune response to have a balanced spatiotemporal expression of the different immune effectors, yielding a successful in vivo implantation.

The paradigm shift occurred using Nature as a great source of inspiration. Recently, it was found that macrophages play a key role in the regeneration of salamander limbs after injury.[241] Later, the role of these cells on neonatal heart regeneration was unveiled, strengthening the importance of macrophages in the regeneration context.[242] This pivotal role arises mostly from the plasticity of these cells, which can be polarized in two different states, depending on the stimuli received. As part of the innate immune response, macrophages are immediately triggered after body exposure to a biomaterial. Macrophage phenotype can be broadly classified as M1 or M2, where M1 macrophages are linked with a pro-inflammatory function and M2 with anti-inflammatory and wound-healing action.[243] Upon activation, macrophages can stimulate T cells from the body's adaptive immune system, orchestrating a mutually dependent immune response that dictates the final biomaterial's immune environment. M1 macrophages boost T_H1 cells, which in turn stimulate the differentiation of more M1 macrophages, starting a feedback process that promotes a pro-inflammatory phenotype. On the other hand, M2 macrophages stimulate T_H2, cells that mediate a response towards tissue regeneration and wound healing. The final host immune response, inflammatory or pro-regenerative, will highly depend on the ratio of each of the differentiated/polarized cells (**Figure 15**A).[244]

Thus, more than suppress the immune response, biomaterials should be designed to adjust the M1:M2 ratio, and consequently the T_H1/T_H2 response, towards the later. For that, it is important to consider several aspects of the biomaterial of choices, such as the hydrogel mechanical properties, or the biomaterial type. As an example, the response triggered by HA is highly dependent on its molecular weight.[245] While high molecular weight HA typically exerts an anti-inflammatory effect, low molecular weight HA fragments are known to promote a T_H1, pro-inflammatory response.[246,247] Regardless, hydrogels can be further modified to modulate the immune response. The presence of hyaluronic microsphere hydrogels modified with anti-Fas molecules (Anti-Fas HA) decreased T cell viability, as compared to a media control. Besides HA is known for its immunomodulatory properties, vinyl sulfone-modified HA (VS-HA) hydrogels did not elicit the same toxic effect on T cells, as depicted in Figure 15B. Soluble anti-Fas (sAnti-Fas), was also able to decrease cell viability but to a lesser extent.[248] Biomaterials containing methacrylic acid motifs can also bias macrophages into a regenerative pro-phenotype. Recently, PEG-hydrogels

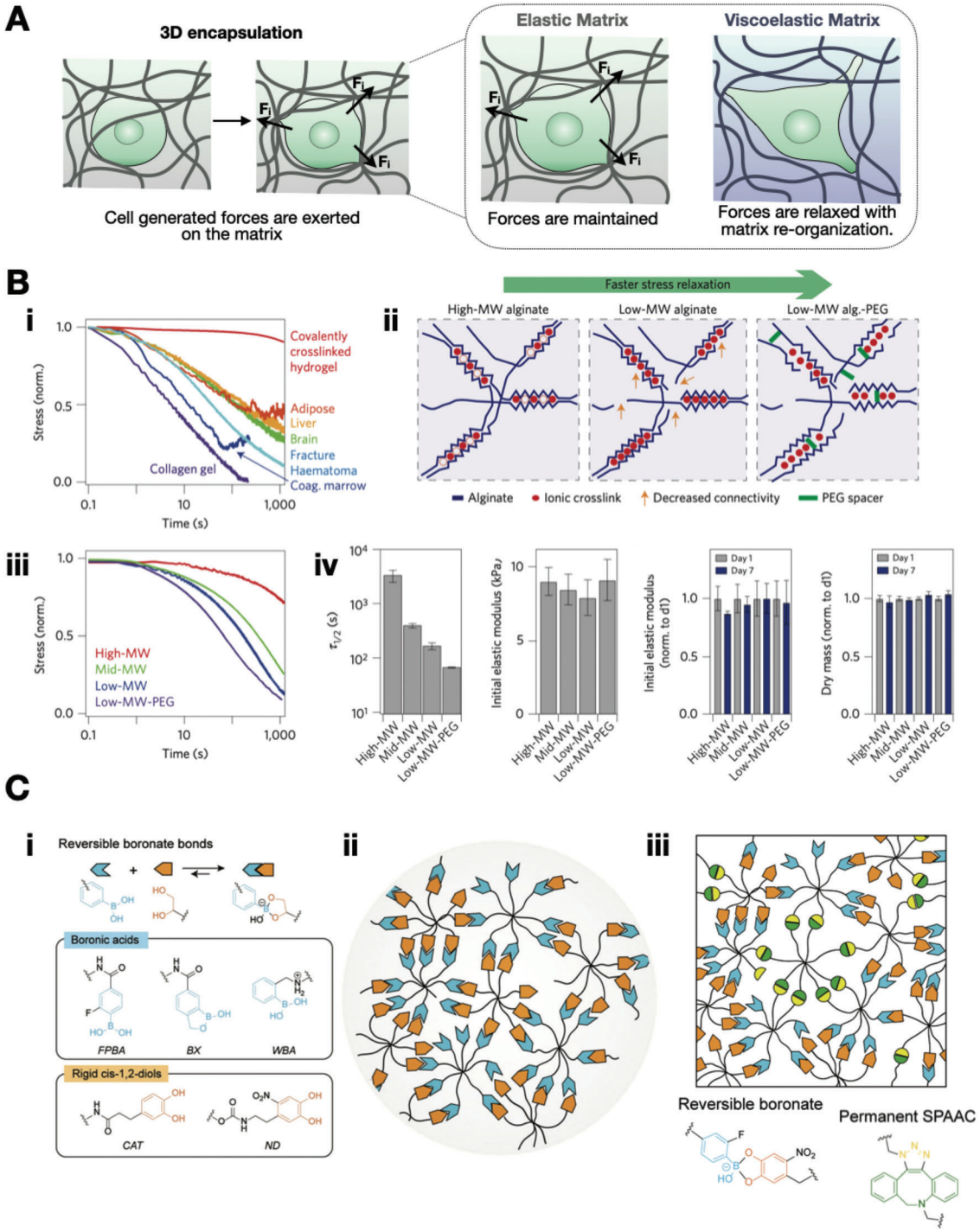

Figure 13. Elastic versus viscoelastic matrices on mechanotransduction. A) Schematic representation of the cell rearrangement on elastic and viscoelastic matrices. B) Viscoelastic alginate hydrogel prepared with different polymer molecular weights and PEG spacers. i) Stress relaxation tests using various rat tissues, an initial fracture hematoma (human), a collagen gel, and a polyacrylamide crosslinked gel. ii) Schematic representation of the approach used to increase the rate of stress relaxation. iii) Stress relaxation tests using the different alginate hydrogel formulations. iv) Plotted values of the time needed for the stress to relax to half of its original value ($\tau 1/2$); the initial elastic modulus measurements; Elastic modulus of the gels after 1 and 7 days in cell culture conditions, normalized by the value at day 1; dry mass of alginate hydrogels after 1 day or 7 days in culture normalized by the value at day 1. Data are shown as mean ± SD. Reproduced with permission.[207] Copyright 2016, Springer Nature. C) Adaptable fast relaxing boronate-based hydrogels. i) Reaction scheme illustrating the reversible formation of boronates and chemical structures of the different boronic acid derivatives used to modify the material relaxation time. ii) Schematic drawing of the polymeric network based on 8-arm PEG functionalized with boronates and cis-1,2-diols. iii) Representation of the hydrogel network with permanent azide-alkyne cycloaddition (SPAAC) bounds and reversible boronate bonds. Adapted with permission.[213] Copyright 2018, Wiley.

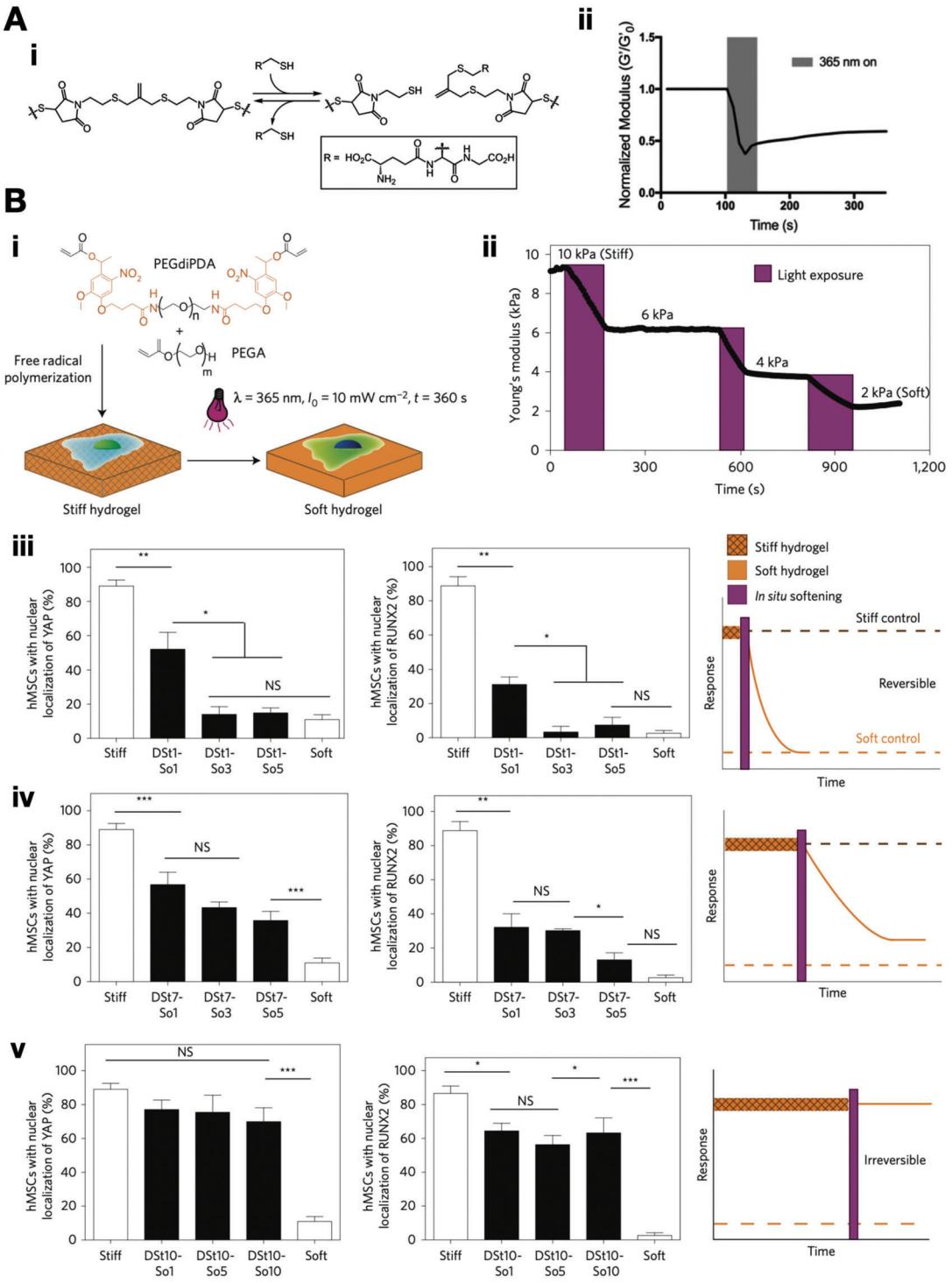

Figure 14. Time of exposure to stiff microenvironments affects MSCs fate. A) Photo-responsive hydrogels with stiff-to-soft transition. i) Mechanism that leads to the change of mechanical properties, where crosslink fragmentation is achieved through radical-mediated addition of gluthathione to the allyl sulfide crosslinker. ii) Normalized modulus of a hydrogel before and after light exposure (365 nm) in the presence of LAP. Reproduced with permission.[206] Copyright 2018, Wiley-VCH. B) Photodegradable hydrogels with phototunable substrate modulus to study the reversibility of mechanical dosing. i) hydrogels prepared via free-radical polymerization of PEGdiPDA, a photodegradable crosslinker, and monoacrylated PEG. Stiff to soft transition occurs upon light exposure at 365 nm, in presence of LAP. ii) Changes in the young modulus with light exposure times. iii) Cell response after culture on a stiff hydrogel for 1 day followed by in situ softening, by expression of YAP and RUNX2 (Runt-related transcription factor2). iv) Cell response after 7 days of culture on stiff hydrogels, followed by in situ softening, by expression of YAP and RUNX2. v) Cell response after 10 days of culture on stiff hydrogels followed by in situ softening, by expression of YAP and RUNX2. Data presented as mean±SEM (standard error mean). NS, not significant; $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. Adapted with permission.[205] Copyright 2014, Springer Nature.

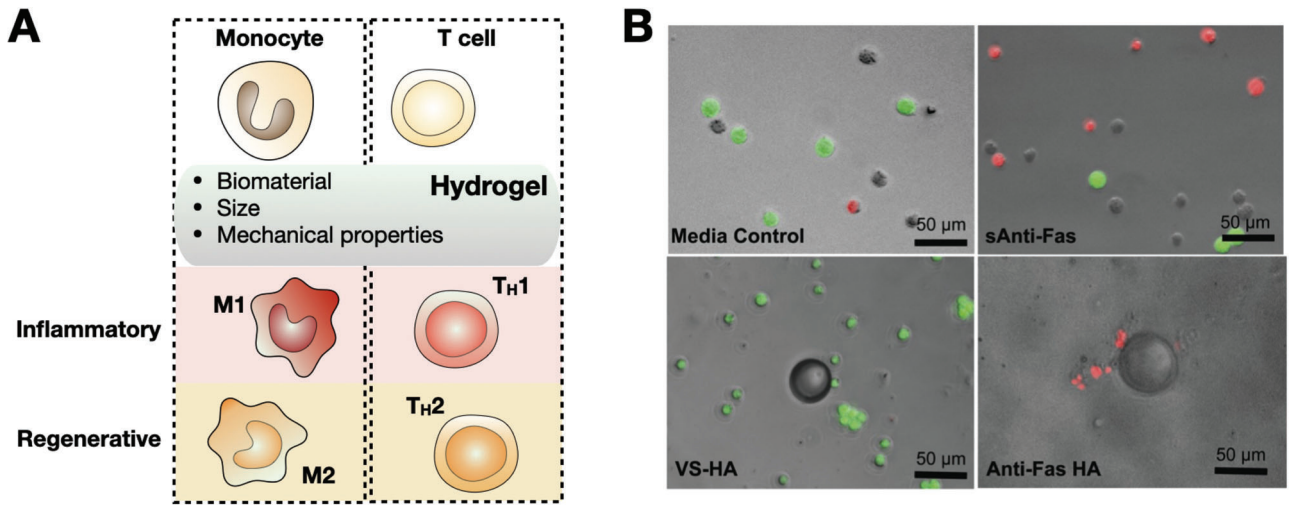

Figure 15. Host immune response to hydrogels. A) Monocyte and T cell polarization is highly influenced by hydrogel properties, such as the biomaterial source, hydrogel size, and mechanical properties. Upon exposure to hydrogel material, monocytes can differentiate in M1 or M2 macrophages, and T cells polarize into T helper 1 (T_H1) or T helper 2 (T_H2). B) Representative merged bright field and fluorescent images for T cells cultured for 48 h, in the presence of soluble anti-Fas (sAnti-Fas), vinyl sulfone modified HA (VS-HA) microsphere hydrogels, or hyaluronic microsphere hydrogels modified with anti-Fas molecules (Anti-Fas HA). Adapted with permission.[248] Copyright 2017, Wiley Periodicals, LLC.

functionalized with methacrylic acid showed promising results in a skeletal muscle implant, where they increased the expression of IL-10, TNFα, and M2 macrophage markers.[249]

Dendritic cells also have a preeminent role in the immune response, via antigen-presentation to naïve T cells.[250] Similar to macrophages, dendritic cells' activation is correlated with the nature of the biomaterial[250] as well as its source.[251] Park et al. studied the effect of different biomaterials on dendritic cell maturation. Their study revealed that high molecular weight HA inhibited dendritic cell maturation, contrary to chitosan or poly(lactic-co-glycolic acid).[252] Considering the tight relationship between dendritic cell activation and T_H1-response, it is highly expected that materials capable of activating dendritic cells would elicit a strong inflammatory reaction.[250]

The last paragraphs considered the biomaterial per se and how its inherent properties might interfere with the host immune system. Nevertheless, it is virtually possible to combine any molecule of interest to modulate the immune system towards a regenerative response.[253] As an example, HA hydrogels have been functionalized with anti-Fas molecules to improve the survival of neural stem cells.[248] Despite the natural anti-inflammatory properties of HA, the presence of anti-Fas elicits T cell death upon contact with the hydrogel, decreasing its viability to 65% as compared to control media (Figure 15B). Addition of zwitterionic elements, such as phosphorylcholine, can also be used to prevent the immune response.[254,255] The strong electrostatic interactions between zwitterions and water molecules hinder the water displacement needed for protein binding on the material surface, thus affecting the material recognition by the immune system. Besides the source of the backbone polymer and final hydrogel size,[256] the material's mechanical properties[254] can also affect the final immune response.

4. Concluding Remarks and Future Trends

Hydrogel development opened up a new era for many scientific fields, including TE and Regenerative Medicine. These hydrated 3D structures allow to better mimic the natural extracellular matrix (ECM) conditions of different tissues, meaning that hydrogels are a suitable choice for recreating the in vivo cell microenvironment. Thence, hydrogels are a great alternative to the unrealistic 2D culture conditions found on plastic surfaces, conferring a more realistic scenario.

And if it is true that several methods are available to prepare and modify hydrogel's properties, it is also important to stress how these changes and preparation methods can affect the final cell-material interaction. Changes in parameters such as polymer source or crosslink strategies, can lead to drastic changes in the final hydrogel outcome. Indeed, the possibility to dictate cell fate by tailoring the physical and chemical properties of the biomaterial has raised a great deal of interest. Undoubtedly, the crosslinking method plays an important role when envisioning TE applications. Often, it must comply with physiological parameters as well as with the cell's delicate nature, which is not compatible with some of the developed methods. Rapid crosslinking periods using cell-friendly materials are typically desired and can be achieved using physical and chemical approaches. Moreover, the combination of both methods may endow hydrogels with further stability and functionality. Mainly, crosslinking methods that rely on dynamically reversible bounds are of great interest for the development of self-healing hydrogels. Indeed, the higher durability and stability of self-healing hydrogels, capable of shape recovery upon damage, are rather interesting features, and in the future, self-healing gels will probably change the current TE paradigm.

On the other side, the impact of this delicate and complex relationship between cells and microenvironment must be seriously considered. It is then necessary to think carefully about the design of a hydrogel, making sure that the gel meets the criteria of the intended applications. Besides studying the cyto- and biocompatibility of the produced hydrogels, the study of the long-term hydrogel functionality is of utmost importance. For example, a highly biodegradable hydrogel cannot be applied in immunoprotective approaches, and vice-versa, even if the hydrogel has shown great biocompatibility in previous studies. Regarding degradable hydrogels, most of their uses rely on a time-dependent degradation process that must be similar to the regeneration timeframe. Therefore, the field is focused on improving the network kinetics to fine-tune the degradation process, while avoiding the formation of deleterious hydrogel by-products.

On the other hand, the design of immunoprotective hydrogels is now focused on the development of new materials and/or chemistries that may better modulate the host immune response. Certainly, the last few years were prolific in the development of new, improved strategies with extraordinary results. Nevertheless, together with nutrient and oxygen diffusion, the adverse immune response is still the main bottleneck for the translation of this promising technology from the bench to the bedside, and therefore the efforts to improve this strategy will continue.

Considering the close material-cell relationship, the latest studies show that this impact starts as soon as cells contact with the biomaterial and sense the mechanical properties of their environment. Such contact can be irreversible and modify the cellular behavior to a great extent, and therefore it must be entirely understood to avoid unwanted cell responses. However, one can also use the material's mechanical properties to easily instruct stem cells into defined cell lines, or secretory functions, which are advantageous when designing TE solutions.

Regardless, hydrogel development is a bubbling field, and the new advances and designs brought new challenges. The possibility to engineer hydrogels that specifically respond to environmental cues paved the way for more targeted and active solutions. It is now possible to design dynamic materials, capable of responding to microenvironmental cues, as a native ECM. Such an exciting opportunity has driven several researchers to pursue new and improved hydrogels that can dynamically change over time, as a response to an external stimulus or to the environment of the host diseased tissue. Certainly, dynamic hydrogels that can undergo temporal and spatial changes hold great potential for future TE applications. Therefore, it is highly expected that an increase in the number of works related to new chemistries and engineering methods to precisely control the hydrogel fate and, consequently, cell responses.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

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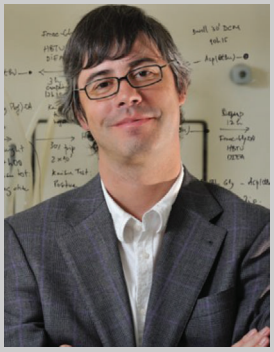

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