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REVIEW

Cell-Laden Hydrogels for Tissue Engineering

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This article reviews cell-laden hydrogels focusing on their impact and recent trends in tissue engineering. Tissue engineering aims to develop functionalized tissues and organs for repair and regeneration of defective body parts with help of cells and engineered matrices called scaffold. Scaffold plays a key role in tissue engineering as a supporting system to accommodate cell attachment, proliferation, migration and differentiation into a specific tissue. Scaffolds in the form of hydrogels are widely used as a support system for engineering tissues owing to their functional properties such as biocompatibility, matching physical, mechanical and chemical properties to the native niche, providing microenvironment for cells to grow and infiltrate into three-dimensional (3D) space and for providing adequate nutrient and oxygen supplies. To optimize the scaffold properties and its compatibility with native niche, cell-laden hydrogel is an appealing option that helps engineering potential tissue constructs with biomimetic structure and function. In this article, therefore, we review cell-laden hydrogels and their applications in tissue engineering with special emphasis on different types of gel scaffolds and their functional properties. Recent trends in hydrogel-based scaffolding systems, especially stem cell-laden hydrogels, gradient hydrogels, and their potential in engineering cells and tissues are also discussed. The review is expected to be useful for readers to gain an in-sight on the cell-laden hydrogel as a promising scaffolding system for tissue engineering applications such as bone, cartilage, cardiac and neural.

Keywords: Scaffolds, Hydrogels, Microenvironment, Gradients, Cell Encapsulation, Stem Cells, Tissue Engineering.

CONTENTS

1. INTRODUCTION

Tissue engineering is an emerging area of regenerative medicine with a goal of repairing or regenerating the functions of damaged tissues and organs, which fails to heal spontaneously by themselves with the help of cells and engineered matrices called scaffold.[1–3] Tissue engineering is a multidisciplinary subject that integrates the principles and concepts of biomaterial sciences, biological sciences and bioengineering, and drives the progress in diagnostics, monitoring, discovering implantable materials/devices

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and engineered tissue grafts for the benefit of human health care. Though conventional tissue or organ transplantations save millions of lives, they have their own limitations too, which include compromised biocompatibility, limited biofunctionality, shortage of donated organs, donor site morbidity and immune rejection. As per the report of United States' Organ Procurement and transplantation network (OPTN), reported in the year 2014, there are about 123,000 patients currently in need of organ transplantation.[4] It is also stated that 18 of these patients die every day while waiting for the donors. In January 2014, 2,401 transplants were done from 1,209 organ donors, which are considerably far from the number of people who are waiting for the transplantation procedure.[5] The inadequate supply of transplants triggers the development of artificial tissue-engineered grafts with efficient tissue regenerative properties and reduced immunosuppressive effects. Tissue engineering approaches have been introduced for overcoming these limitations by employing recent advancements and development of patient-specific tissue grafts to mimic the functional properties of native tissue that could be transplanted back to the patient with a minimal surgical intervention and maximum host tissue integrity.

The concept of tissue engineering, in particular scaffold-based tissue engineering, involves culturing of isolated cells from the patient or donor into a scaffolding system that support the growth and function of the isolated cells into a specific tissue which could be grafted back to the defective site of the patient where tissue regeneration is required (see Fig. 1).[6] The key components, which determine the success of tissue engineering, include cells, engineered matrices (scaffolds) and cell-matrix interactions. Scaffold plays a key role in tissue engineering by providing a structural support for the cells to accommodate and guide their growth in the 3D space into a specific tissue or organ. In the view of biology, cells in the human body live in a complex mixture of pores, ridges and components of micro and nano-featured gelly kind of extracellular matrix (ECM) environment, which are all play a vital role in facilitating cell-matrix interactions and cell–cell communications upon implantation of the graft.[7–9] Therefore, in order to mimic the native environmental conditions of a defective tissue, scaffolds with ability to facilitate cell-matrix interactions and cell–cell communication signals have been emerged. Currently, many materials are being tested as a tissue scaffold. Scaffolds in the form of hydrogels could mimic the native tissue environment as an encouraging niche for regulating cell attachment, proliferation, synthesis of ECM proteins, cell-matrix interactions, cell–cell communications and corresponding functions.[10–13] Hydrogels are cross-linked form of polymer networks with hydrophilic characteristics. Hydrogels have received much attention for tissue engineering applications because of their dynamic functional properties and the best mimicking abilities of native ECM. To further increase the efficiency, incorporation of various growth factors, bioactive molecules and nano-fillers into these matrices as biological signals could be introduced to promote the desired differentiation lineage of cells[14, 15] as shown in Figure 2.

Hydrogel scaffolds encapsulating or entrapping cells within their cross-linked polymer network structure is an approach to mimic the native tissue-like structure and function, which is called cell-laden hydrogel. Among the hydrogel-based scaffolding systems, cell-laden hydrogels are the most recent and popular choice as carriers for site-specific cell-delivery within the body. Cells encapsulating hydrogels could be used for the generation of 3D tissue engineering structures. These cell-laden hydrogel systems can address several challenges associated with conventional scaffolds, such as inability to control the complex cellular interactions in the scaffolds and the lack

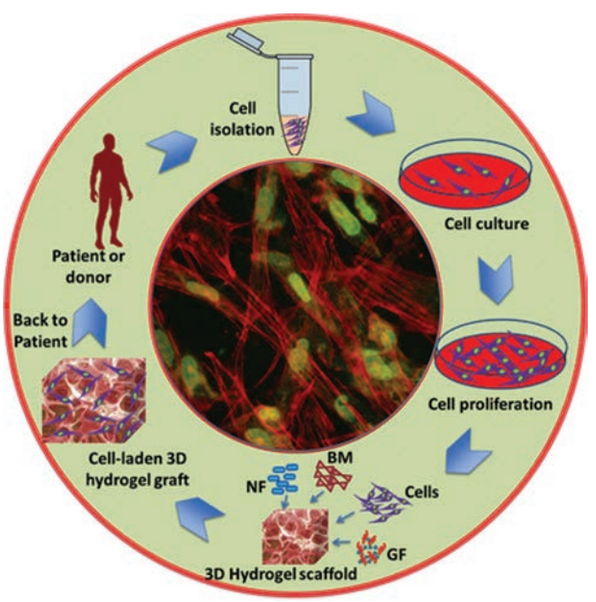

Fig. 1. Schematic representation of concept of scaffold-based tissue engineering. NF, BM and GF denote nano-fillers, bioactive molecules and growth factors, respectively.

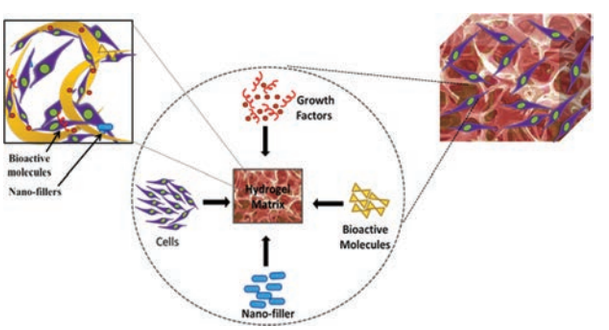

Fig. 2. Schematic illustrations for major components of tissue construct. The left panel highlights cell-material interactions with nano-fillers, bioactive molecules and growth factors, and the right panel shows the bulk cell-laden 3D hydrogel graft.

of vascularization. Studies have suggested that the cell-laden hydrogels provides a viable solution for cell seeding, oxygen delivery and mass transfer in large 3D cell and tissue engineering. For example, Chen et al. demonstrated a stable even coverage of cells on the surface of the hydrogel filaments as a preliminary microvasculature network.[16] Commonly used methods of 3D cell-culture are cell spheroids, microspheres, *in situ* forming hydrogels and preformed porous scaffolds.[17–19] Among them, spheroids and microspheres lack integral structures and have difficulty in providing sufficient perfusion and mass transfer with increasing size. Similarly, preformed porous scaffolds, for large constructs show inefficiency in cell seeding and distribution.[20] Cell's ability to adhere to the surface of the scaffold is prime factor for retention of cells in preformed porous scaffolds; therefore less adhesive cells may be lost during perfusion. Additionally, cells are exposed to non-physiological shear forces if perfusion is not well controlled,[21,22] but they can be protected from hydrodynamic forces if perfused over the surfaces. To further standardize the conditions, cell-laden hydrogels has been developed with many modifications such as porous cell-laden hydrogels, stimuli-responsive cell-laden hydrogels, stem cell-laden hydrogels etc., to make these systems more efficient with native ECM mimicking properties and in providing right environmental cues. These systems have been discussed in detail with experimental examples in preceding sections. Due to the several merits associated with cell-laden hydrogels in terms of structure and functions that mimics the native ECM, these kinds of hydrogels have applications in different areas such as tissue engineering, drug delivery, gene delivery, immunoisolation microcapsule systems and scalable bioreactors. Although cell-laden hydrogels are well established and proven to be useful, there are still a few challenges to build tissue constructs with biomimetic architecture and function. In addition, cellular rearrangement within cell-laden scaffolds often does not resemble the biomimetic structure of the native tissue, which hinders proper cell-microenvironment interactions, cell phenotype preservation and cell differentiation.[23]

Considering the aforementioned impact of hydrogel-based systems, in this review, the authors have focused their attention on cell-laden hydrogels as a potential tissue graft and analyzed their merits and demerits in the context of tissue engineering. Synthesis, properties and applications of the hydrogels have been discussed along with current trends in hydrogel systems such as stem cell-laden and gradient hydrogel systems, which could be used in interface tissue engineering (ITE), a new subset of tissue regenerative medicine. For the benefit of readers, basics of scaffold-based tissue engineering, different types of hydrogels, and key mechanisms involved in the cell-laden hydrogels are also briefly discussed. The authors do not suggest that this is the only material of promise for scaffold-based tissue engineering, but the key intention is to stimulate research on cell-laden hydrogels and to formulate them as promising synthetic ECM to modulate cellular growth and functions for tissue engineering applications.

2. HYDROGEL SCAFFOLDS

Hydrogels represent a class of biomaterials that are widely used in tissue engineering and regenerative medicine as a scaffold due to their structural and physicochemical functional properties. Hydrogels are cross-linked form of polymer networks with hydrophilic characteristics. They exhibit a high degree of swelling in aqueous environments due to their insoluble 3D networks.[9] Hydrogels are often used in cell culture for tissue engineering and drug discovery applications. With hydrogels, the cells are cultured generally in two ways: (i) on the gels or (ii) in the gels. In 'on the gel' condition, hydrogel provides a substratum for the attachment and proliferation of the cultured cells and cells grow in a 2D fashion, whereas 'in the gel' condition of cell culture leads to encapsulation of cells inside the 3D gel network in order to mimic natural tissue microenvironment. The highly swollen state of the hydrogels facilitates transport of nutrients into and cellular waste out of the gel. They provide a temporary support for the cells to attach, grow, proliferate, migrate, and differentiate into a specific tissue, facilitating its retention and distribution within the region of desired tissue growth for supporting vascularization, neo-tissue formation, and remodeling of niches with efficient mass transport.

2.1. Properties of Hydrogels

Hydrogels that are used for cell culture and tissue engineering should have some basic properties in order to use them as a scaffolding material.[24] The properties of the hydrogels can be tuned according to the application and requirement of the scaffolds. Some of the important properties of hydrogel scaffolds are highlighted in Table I.

Biocompatibility is one of the important properties of hydrogels regarding tissue regenerative applications.[25] It means that the gel should not provoke any adverse reaction, rejection or immune response upon implantation. It should be biologically compatible to the host tissues. Degradability is another important feature of hydrogel scaffolds. Degradation process of hydrogels should not produce toxic or non-degradable products i.e., components formed should be metabolized into harmless products or they should be able to excrete from the body. Degradation rates of these hydrogel scaffolds should be directly proportional to the rate of regeneration of a new tissue with biomechanics quite similar to the replaced tissue. Some hydrogels shows dissolution of their hydrophilic polymer chains in an aqueous phase that can be avoided by incorporating physical or chemical crosslinks into the structure to improve gelation.[26] Sometimes, unstable bonds are

Table I. Basic properties of hydrogels for tissue engineering application.

S. no.	Properties	Description
1.	Mechanical strength	Mechanical property of gels can be tuned using polymer concentration, mesh size, porosity and crosslinking density
2.	Swelling behavior	Shows swelling property which can be used for releasing biomolecules, cells or drugs
3.	Tissue-like microenvironment	Mimicking the dynamic nature of native ECM Biochemical and biophysical cues could be incorporated to modulate cell behavior
4.	Mass transportation	Supports continuous exchange of nutrients, proteins, gases and waste products into, out of and within the hydrogel
5.	Degradability	Rate of degradation should be match with rate of tissue regeneration Biodegraded product should not be toxic Controllable degradation kinetics
6.	Biocompatibility	Should be biologically compatible with host and surrounding tissues Should not provoke any rejection or immune response Should supports host tissue integration
7.	Cell-compatible crosslinking	Effect of crosslinking reaction on cell viability or protein bioefficacy can be modulated
8.	Surface modification	Incorporation of chemical functional groups and biological ligands are feasible Surface properties can be altered that contribute to better cell-matrix interactions
9.	Stimuli-responsiveness	Crosslinking could be controlled by external stimulus such as light, pH, ionic strength etc.
10.	pH adjustability	pH can be tuned by changing reaction conditions and fabrication methods pH can be tuned to physiological pH suitable for *in vivo* studies.

intentionally introduced in the gels to develop biodegradable hydrogels for different applications,[25] whose degradation behavior can be regulated either by enzymatically or chemically via hydrolysis.[27] Degradation of hydrogels can be tuned by either chemical or physical crosslinking or sometimes both to create 3D structures of polymer networks in aqueous environments. In physical crosslinking, the physical interactions between polymer chains prevent dissociation of the hydrogel, while in chemical crosslinking covalent bonds between polymer chains create stable hydrogels. Physically-crosslinked hydrogels employ mild reaction conditions and changes physical or environmental conditions such as pH, temperature, ionic interactions, hydrogen bonding, and protein interactions. Chemically-crosslinked gels have been obtained by radical polymerization, chemical reactions, energy irradiation, and enzymatic crosslinking. Chemical crosslinkers create unwanted free radicals and toxic ions within the system that can degrade the embed proteins or bioactive factors thus compromising on cell compatibility and viability. Hence exclusion of chemical crosslinkers is the main biocompatibility factor in these types of hydrogels.[28] Various injectable hydrogels based on alginate, collagen, agarose, hyaluronic acid (HA) and chitosan have been synthesized by using physical crosslinking approaches for engineering different tissues.[29] These gels can be confined in damaged site thus eliminating the need of invasive surgery. However, low mechanical properties of physically-crosslinked hydrogels may limit their tissue engineering applications, particularly in the regeneration of load bearing tissues. Whereas, chemically-crosslinked gels have higher mechanical properties as compared to their physically-crosslinked counterparts but they exhibit cytotoxicity due to residual chemical crosslinkers, organic solvents and photoinitiators. Some examples of chemically crosslinked gels for tissue engineering applications include poly(2-hydroxyethyl methacrylate) (PHEMA), polyacrylamide, glutaraldehyde (GA) crosslinked polyvinyl alcohol (PVA), elastin, chitosan, UV crosslinked methacrylated gelatin and elastin, and transglutaminases crosslinked fibrinogen hydrogels.[30] Porosity is another highlight of hydrogels that help in efficient mass transfer within the scaffold and facilitates cellular infiltration in 3D space. Surface properties that can enable cell attachment, growth, proliferation, and differentiation as well as ECM deposition, optimum structural properties in terms of pore size, porosity, pore interconnectivity, and processing compatible to fabricate 3D complex shapes in a well-controlled and reproducible manner are some of the other requisite properties for the optimization of hydrogel scaffolds. These properties make hydrogels a potential scaffolding system for tissue engineering.

On the other hand, hydrogels have their own limitations to serve as an ideal scaffolding material for tissue engineering due to their poor mechanical properties, which could be attributed to random alignment of polymer chains and high water content within the structure. However, the mechanics of hydrogels could be modulated. Interestingly, cells are reported to improve mechanical strength of the hydrogel construct through the reorganization of polymer chains, production of ECM products and the application of intrinsic strains.[31] Hydrogels have been used extensively to prevent adhesions due to their relative lack of cell adhesiveness.[32] Consequently, cell adhesion proteins are needed to incorporate into hydrogels to promote cell adhesion.[33] Degradation of hydrogels generally occurs by hydrolysis; however, enzymatically degradable hydrogels have also been reported.[34] Though the use of hydrogels has successful records but they are also associated with clear limitations including donor site morbidity, shortages in supply of nutrients and waste removal, immunologic

reactions and poor integration.[35] Overall, however, hydrogels are a good choice for culturing and delivering cells and their physical, chemical and biological properties can be tuned to specific cell type in order to engineer functional tissues and organs

2.2. Types of Hydrogels

Hydrogels are different types, depending upon their structure, property and function. The use of different types of hydrogels in tissue engineering purely depends on its application of interest. This is because each cell- and tissue-type is unique in its functional properties and thus their cell-material interactions are significantly varied upon implantation. Therefore, choice of hydrogel scaffolds for a particular cell or tissue engineering application requires defined physical, mechanical, chemical and biological properties. In the following section, various types of hydrogel scaffolds (see Fig. 3) have been discussed along with their major advancements, properties, research status and area of application in tissue engineering.

2.2.1. Elastomeric Hydrogels

Hydrogels have water content and mechanical properties comparable to soft tissues. Significant efforts have been made to mimic elastic properties of soft tissues by engineering elastomeric biomaterials that can extend under stress conditions. Due to the high strechability of native tissues, thermoplastic polymers with elongation break of less than 3% fail to replicate the innate tissue elasticity by undergoing plastic deformation under variable loading.[36] This observation shows inability of elastomeric systems to mimic non-uniform elasticity of native tissue. For example, most of the native tissues display strain stiffening and are responsive to applied strain, which cannot be easily obtained by elastomeric systems.[37] To overcome this limitation, many groups are trying to develop responsive hydrogels for biomedical applications.[38] As a solution to this problem, several elastin-based hydrogels have been synthesized from solubilized elastin for engineering different types of tissues such as skin,[39] cartilage,[40] and blood vessels.[41] For example, α-elastin hydrogels have been fabricated through chemical crosslinking approaches using various types of crosslinking agents.[39–41] Highly porous and elastic hydrogels were also engineered by crosslinking α-elastin with GA[42] and hexamethylenediisocyanate (HMDI)[39] under high pressure carbon dioxide (CO_2). The fabricated hydrogels facilitated the attachment, infiltration and growth of 3T3 fibroblasts within the 3D structure of the hydrogels.[42] Additionally, the combination of α-elastin with PCL promoted chondrocyte adhesion and proliferation.[40] Regeneration of cartilage tissue has also been achieved by using composite hydrogels containing K-elastin, alginate, and collagen.[43] In a study, chondrocytes isolated from porcine and human were embedded inside the hydrogel composite and subsequently implanted into nude mice. After 12 weeks of implantation, cartilage-specific components including proteoglycans, collagen, and elastin fibers were formed within the engineered tissues which closely mimicked the native articular cartilage.[43] Despite its extensive use in tissue engineering, animal-derived soluble elastin have certain limitations related to its heterogeneous mixture of peptides which are partially crosslinked and provideinadequate cell binding sites.[44] In addition, the clinical use of animal-derived proteins is often restricted due to the risk of pathogen transfer and immunological rejection.[45]

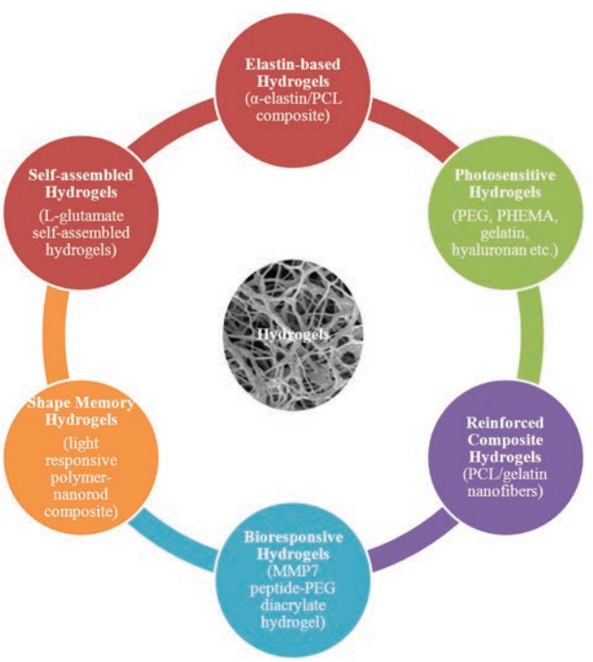

Fig. 3. Different types of hydrogels applicable for tissue engineering.

2.2.2. Photosensitive Hydrogels

Photosensitive hydrogels are defined as the hydrogels exhibiting a light-induced reversible change of chemical structures and physical properties i.e., it can be generated or degraded by light exposure. Ionic interactions, pH stimulation and light exposure are the commonly used approaches for the crosslinking and degradation of hydrogels.[46] Photosensitive hydrogels have been extensively used for a wide range of tissue engineering applications which can be prepared by mixing a photocurable hydrogel precursor with a photoinitiator and then exposed to light that initiates the crosslinking reaction.[47] Although a range of light wavelengths can be used, UV light is most commonly used to induce the photoinitiator to generate free radicals. The activated functional groups then form covalent bonds with free radicals to create crosslinked networks.[48] Subsequently, unreacted polymer is washed out upon completion of the crosslinking process. Photosensitive hydrogels offer a number of advantages over other

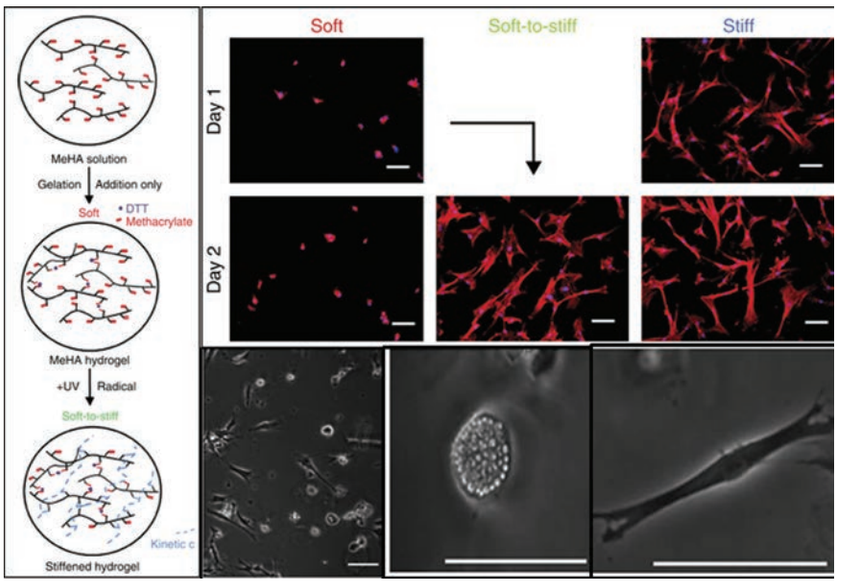

Fig. 4. Sequential crosslinking process of MeHA and effect of *in situ* stiffening caused by polymerization on cell spreading (Confocal and SEM images). Reprinted with permission from [64], M. Guvendiren, et al., Stiffening hydrogels to probe short-and long-term cellular responses to dynamic mechanics. *Nat. Commun.* 3, 792 **(2012)**. © 2012, Nature Publishing Group.

types of crosslinking schemes. For example, they enable controlled spatial crosslinking of the hydrogel to control the microarchitecture of the resulting material,[49] which can be used to modulate cellular behavior such as adhesion, proliferation, migration, and differentiation.[50] In addition, photocrosslinking is a simple, rapid, and cost effective technique.[47] Despite of their attractive features, photosensitive hydrogels also entangle some drawbacks such as the formation of free radicals upon UV exposure that could lead to DNA damage and impaired cellular function.[47] Additionally, *in vivo* gelation of photocrosslinkable hydrogels is challenging due to the limited light penetration through the tissues.

Materials with both synthetic and natural origins have been modified with photocrosslinkable functional groups.[51] For instance, PEG[52] and PHEMA[53] were chemically modified by methacrylate groups to synthesize photocrosslinkable hydrogels. Similarly, naturally-derived materials, such as alginate,[54] dextran,[55] agarose, heparin,[56] hyaluronan, chitosan,[57] collagen,[58] and gelatin[59] were methacrylated to yield photocurable gels. These photocrosslinkable hydrogels were used as robust 3D environments to engineer biomimetic cell-laden hydrogels for different tissue engineering applications. For instance, macrophages,[52] human umbilical vein endothelial cells (HUVECs)[51] and hepatocytes[60] were tested for their cellular response within photo-crosslinked gels based on PEG, gelatin, and HA. Results of the study showed that encapsulated mammalian cells (fibroblasts, hepatocytes, and macrophage) responded positively to RGD modified PEG hydrogels with enhanced spreading of encapsulated fibroblasts over a 24-h period in culture and all encapsulated cells remained viable in hydrogel microstructures for a period in excess of 1 week in culture.[52] Interesting results were obtained from the cellular response study on HA-gelatin hybrid hydrogels showing enhanced cell spreading within hybrid structures on addition of GelMA into methacrylated hyaluronic acid (HAMA),[51] concluding that cellular responses can be optimized within the scaffold by integrating GelMA and HAMA in a hybrid photo-crosslinked hydrogel. In an interesting study, gel was crosslinked by a Michael-type addition reaction with dithiothreitol (DTT) and then its mechanical stiffness was tuned by additional UV crosslinking which resulted in substrate stiffness that can affect differentiation of MSCs seeded onto hydrogel surface (see Fig. 4).[61]

In addition to conventional and mechanically tunable systems, inter-penetrating networks (IPNs) can also be synthesized through photo-crosslinking. For example, Hago et al. showed a new route to develop photo-crosslinked alginate IPN macromeres and studied their biodegradation rates, biocompatibility and mechanical properties.[62] The results showed no cytotoxicity and excellent cyto-compatibility for fibroblasts L929 cells holding promise for biomedical purpose.[63] Advanced techniques such as micro-patterning have also been studied for cellular behavior modulation with photo-crosslinked gels. It is reported that micro-patterned gelatin-based hydrogels enable guidance and alignment of different cell types, such as 3T3 fibroblasts, C2C12 skeletal muscle cells, cardiac side population (CSP) cells, and HUVECs.[62] Similar to photocrosslinkable functional groups, photodegradable hydrogels from synthetic sources can be fabricated by incorporating photodegradable functional groups such

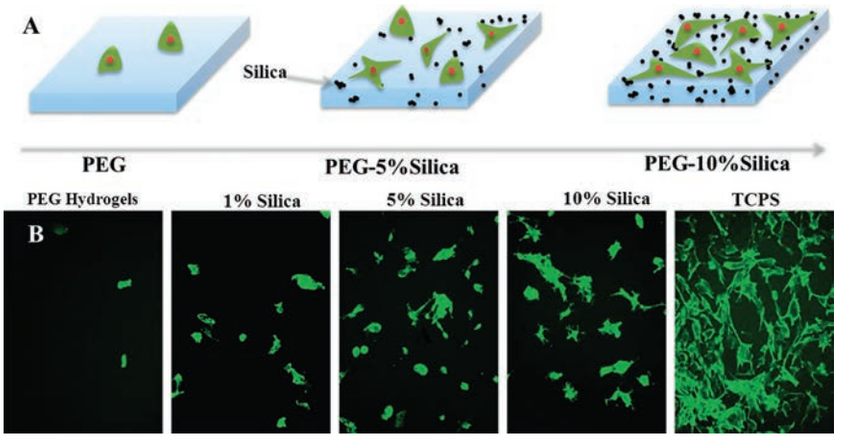

Fig. 5. Effect of silica in photocrosslinked nanocomposite hydrogels from PEG and silica for cell adhesion, spreading and proliferation. Addition of small amounts of silica enhances cell adhesion in comparison with control PEG hydrogels. TCPS stands for tissue culture polystyrene. Reprinted with permission from [71], A. K. Gaharwar, et al., Photocrosslinked nanocomposite hydrogels from PEG and silica nanospheres: Structural, mechanical and cell adhesion characteristics. *Mater. Sci. Eng. C* 33, 1800 **(2013)**. © 2013, Elsevier.

as nitrobenzylether,[64] poly(*t*-butyl acrylate),[65] 4-[4-(1-Hydroxyethyl)-2-methoxy-5-nitrophenoxyl butanoic acid[66] and bis(4-(dimethylamino)phenyl)(4-vinylphenyl)methyl leluco cyanide.[67]

2.2.3. Reinforced Composite Hydrogels

Reinforced composite hydrogels can be defined as hydrogels mixed with other materials within their matrix to develop inhomogeneity and enhance their chemical or physical properties that modulates biological functions of the encapsulated cells. It involves incorporation of different entities to create composite hydrogel matrices with improved properties. Since single polymer crosslinking cannot hold all the required physical, mechanical and biological properties, reinforced composite hydrogels emerged as a possible solution. These strategies include incorporation of secondary polymers as well as various nanostructures into the core hydrogel (see Fig. 5).[68] Polymer composite hydrogels such as alginate-composite hydrogels prepared via physical blending are commonly used in various bioengineering applications.[69] In this alginate-composite system, researchers have incorporated ECM proteins such as collagen orfibronectin or any cell-responsive synthetic polymers (e.g., polylysine) to regulate their cell-interactive properties.[70] In addition, mechanical properties of the alginate hydrogels were enhanced by incorporating other natural or synthetic polymers (e.g., chitosan, PVA and poly(acrylic acid)) into the system.[71] There are other types of polymer composite hydrogels, which utilize more elaborate strategies for incorporating a secondary polymeric network, such as hybrid networks, IPNs, and semi-IPNs.

Nanocomposite hydrogels are another type of reinforced hydrogels that have gained much attention with the rapid development of nanotechnology. Nanoparticles (NPs) can be engineered from a variety of sources (e.g., polymers, minerals, metals, and semiconductors) and into different shapes (e.g., spheres, rods, shells, wires, and tubes).[72] In addition, chemical modification strategies are available to further modulate the properties of NPs.[73] For example, Hou et al. reported synthesis of thermo responsive nanocomposite hydrogels comprised of a PNIPAAm hydrogel matrix and polysiloxane colloidal NPs via *in situ* photopolymerization method. They mentioned that due to incorporation of NPs, an increase in modulus as well as in the rate of deswelling was observed along with effective detachment of mouse smooth precursor cells (10T1/2) from nanocomposite hydrogel surface due to swelling effect.[74] Due to diverse array of NPs with distinct physical and chemical properties, research efforts are being made to incorporate various types of NPs into hydrogel systems to create reinforced nanocomposite hydrogels. Various types of NPs have been employed including mineral, polymeric, metallic, magnetic and carbon-based NPs. Bionanocomposite hydrogels are also a popular choice for modulating physical and chemical behavior of scaffolds. For example, Jia et al. reported synthesis of bionanocomposite hydrogels from bacterial cellulose (BC)/chitosan and evaluated their biocompatibility for tissue engineering applications.[75] The results of this study suggested BC/chitosan scaffold promotes the growth and proliferation of fibroblast and keratinocyte cells.[75]

Besides NPs, recently many researchers have reported the use of nanofibers as the filler material for reinforced hydrogel scaffolds due to their high surface area to volume ratio and other functional properties such as tunable mechanical strength and cellular compatibility. Incorporation of hydrogel with nanofibers can reinforce the strength of the hydrogels and the presence of nanofibers may potentially improve or influence cell activity in the resultant composite.[76] There are a bunch of methods reported for fabricating this type of advanced

hydrogels, as Sakai et al. reported the synthesis of nanofiber membranes compatible with solution state of hydrogels.[73] These membranes can be unraveled (by manually tearing of the membrane) and mixed with the solution to make nanofiber reinforced composite hydrogel.[73] Another innovative method reported is to incorporate layers of nanofibers in hydrogel in a layer-by-layer assembly. Ekaputra et al. constructed a hybrid nanofiber and hydrogel 3D structure by having electrospinning and electrospraying of hydrogel simultaneously.[76] In a study by Kai et al. a nanofiber reinforced composite hydrogel was fabricated by incorporating electrospunPCL/gelatin 'blend' or 'coaxial' nanofibers into gelatin hydrogels. Morphological, mechanical, swelling and biodegradable properties of nanocomposite hydrogels were studied and concluded that the moduli and compressive strengths are higher in these composites than pure gelatin hydrogels. Bone marrow mesenchymal stem cells (BM-MSCs) were used for biocompatibility evaluation of nanofiber reinforced hydrogels by studying cell proliferation and immunostainning. The results showed that the nanocomposite hydrogels with PCL/gelatin 'blend' nanofiber (PGB25) resulted into enhanced cell proliferation, indicating that the 'nanocomposite hydrogels' could provide necessary mechanical support and could be used as cell delivery system for tissue regeneration applications.[77] In another notable study, a combination of three approaches for scaffold design was investigated.[76] That is, selective leaching of water-soluble fiber phase (PEO or gelatin), the use of micron-sized fibers (mPCL/Col) as the scaffold, and a combination of micron-sized fibers with co-deposition of hyaluronic acid-derivative hydrogel, Heprasil. All the three scaffolds supported attachment and proliferation of human fetal osteoblasts. The results of the study were encouraging in a way that better cell penetration results obtained with mPCL/Col microfibers and effect was more pronounced when Heprasil regions were present, therefore further emphasizing on the better cyto-compatibility and material properties of nanocomposite reinforced systems than conventional hydrogels.[76]

2.2.4. Shape Memory Hydrogels

Shape memory hydrogels (SMHs) are a class of smart hydrogels that are capable of varying their shapes when exposed to an external stimulus such as temperature or pH. Thermal stimulation is the most studied variable to modify polymers and hydrogels that can cause largest conformational and structural responses. The type of bonding dominating in SMHs is supramolecular bonding that utilizes hydrogen bonds, van der Waals interaction, π–π interactions, or metal complexes to provide conformational reversibility to the SMHs systems. These interactions serve to build up network chains from non-covalent interactions between monomers and polymer chains.[78] One of the advantage of SMHs is that one can control the polymer variables like wettability,[79] swelling capability,[80] permeability,[81] and sol–gel transition properties[25] by providing different combinations of non-covalent bonds to the SMHs system. While completing polymerization action, SMHs merge transient, reversible and non-covalent physical bonds with stable chemical bonds.

When SMHs are subjected to heat upto a critical temperature (T_T), the physical crosslinks starts to dissociate and show network deformation and thus leaving behind covalent crosslinks that are solely responsible for an elastic response.[82] This deformation of physical crosslinks is reversible and shows association of crosslinks once the system temperature starts to decrease below T_T leading to hydrogel deformation and further locking the system. Once the association is completed, again if the temperature goes above T_T system would result in dissociation of physical crosslinks owing to their reversible behavior. The supramolecular interactions allow these networks to associate and dissociate in response to a thermal simulation by releasing stored elastic energy of the permanent crosslinks, and restoring the hydrogel back to its original shape.[83] Since SMHs can be controlled by an external stimulus, temperature-responsive SMHs can be used as delivery vehicles of multiple bioactive molecules or growth factors due to their unique self-healing properties.[84] Once the SMH is stimulated, it can modify its shape and selectively attract or release a pre-determined set of biomolecules. For example, Ozadin-Ince et al. developed a coaxial nanofilm with a hydrogel core and a p(*tert*-butyl acrylate-co-diethylene glycol divinyl ether) shape memory shell to form temperature activated nanotubes using initiated chemical vapor deposition.[85] The temperature response of the coaxial nanofilm was studied through the release time measure of encapsulated and adsorbed fluorescent dye from the hydrogel layer. These systems showed burst release profile of the fluorescent dye due to stress applied by shape memory outer layer polymer when activated with increased temperature.[85]

Temperature-responsive SMHs can be used as smart hydrogels for cells and growth factor encapsulation. For example, Wang et al. developed a biodegradable, partially crosslinked alginate hydrogel with shape-memory properties at body temperature for minimally invasive surgical applications.[86] 90% of recombinant insulin-like growth factor-1 (IGF-1) that was encapsulated in these hydrogels was released over several days *in vitro*, allowing skeletal muscle cell survival, proliferation, and migration within the scaffold over a 28-day period. Recently, SMHs with tunable mechanical characteristics have shown promise as injectable hydrogels. For example, Bencherif et al. developed injectable macroporous alginate scaffolds with well-defined shape-memory properties (see Fig. 6).[87] These injectable hydrogels were highly compressible and could withstand reversible deformations up to 90% strain upon *in vivo* injection by using a conventional needle–syringe

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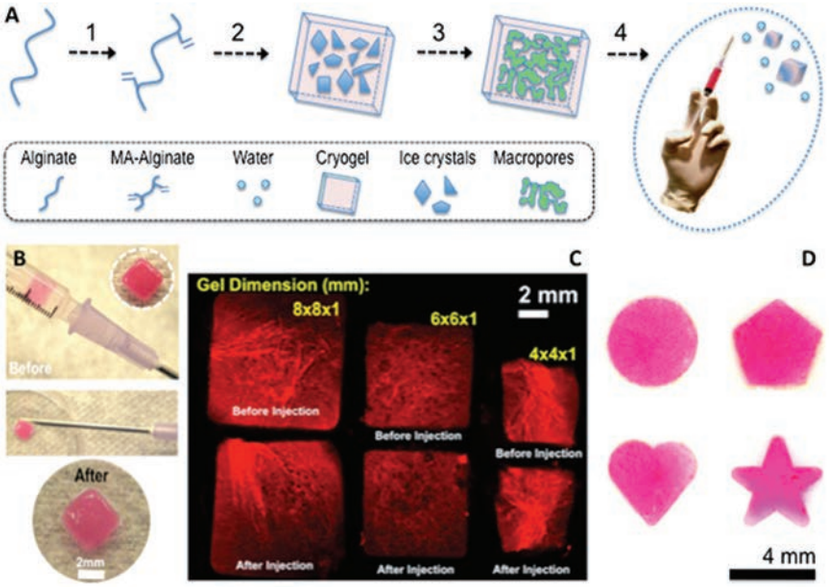

Fig. 6. Shape-memory alginate hydrogels fabricated using cryogelation process; (A) overview of process of cryogelation; (B) images of cryogel in a syringe and shape recovery after injection; (C) fluorescent images of rhodamine labeled cryogel exhibiting geometric restoration; (D) different shapes of hydrogels prepared by cryogelation. Reprinted with permission from [90], S. A. Bencherif, et al., Injectable preformed scaffolds with shape-memory properties. *Proc. Natl. Acad. Sci. USA* 109, 19590 **(2012)**. © 2012, National Academy of Sciences, USA.

technique. They also demonstrated long-term release of biomolecules such as BSA *in vivo* as a carrier and resulted enhanced survival, higher local retention of bioluminescent reporter cells and extended engraftment of transplanted cells at the injection site compared with a standard injection technique, thus promising a future for cell therapy.[87]

SMHs are also being used in drug delivery. A near-infrared light responsive polymer-nanorod composite with a T_T in the range of body temperature was employed for the controlled release of anti-cancer drugs such as doxorubicin.[88] *In vitro* studies on these composite microspheres demonstrated a $\sim$90% reduction in the activity of cancerous T6–17 cells when the release of doxorubicin was triggered from microspheres exposed to near-infrared light. Due to their high surface area, the microspheres facilitated cumulative release of drug.[88] Based on the experimental examples discussed in this section, and other reported literatures, SMHs are good choice for tissue engineering applications.

2.2.5. Self-Assembled Hydrogels

Self-assembled hydrogel are composed of amphiphilic molecules with hydrophobic groups to promote aggregation and hydrophilic groups to support solubility as competent hydrogelators. Functional group on gelator molecule assists physical gelation of a solvent that forms supramolecular gels by maintaining a balance between crystallization and solubilization. The whole structure of a self-assembled gel is controlled by the properties of these amphiphilic molecules. Therefore, an intensive research is going on in designing amphiphiles specifically to control self-assembly and gelation (formation of entangled fibril network) by environmental stimuli such as temperature, pH, ionic strength or an additive (enzymes or multivalent cations) to form a functional hydrogel system. In a study reported by Kimizuka et al. light-harvesting supramolecular self-assembled receptor hydrogel from cationic *L*-glutamate derivatives was developed that can bind to anionic fluorophores by moderate electrostatic and van der waals interactions resulting into a spontaneous reaction between polymer chains.[89] Enzyme-triggered molecular self-assembly have also been explored for the formation of supramolecular hydrogels.[90, 91] For example, Xu et al. reported hydrogels with high drug delivering capacities, enzyme detection activity and cell fate control. They used phosphatase and β-lactamase to control the dephosphorylation, hydrolysis and molecular self-assembly resulting into nanofibers and supramolecular hydrogels.[92, 93]

Current interest in supramolecular hydrogels includes designing of amphiphiles, artificial proteins and carbohydrates for exploring self-assembling property of hydrogels. Pochan et al. reported de novo a peptide with 20 amino acid-long sequence (without a hydrophobic tail) that can fold into a β-hairpin upon heating due to self-assembly and further can assemble into fibril structures and show hydrogelation.[94] In contrast to macro-hydrogel structures, Zhang et al. reported aligned monodomain hydrogels by molecular self-assembly of peptide-based small molecules on cooling.[95] After heat treatment water molecules formed lamellar plaques in filamentous textures that can be employed as spontaneous template for

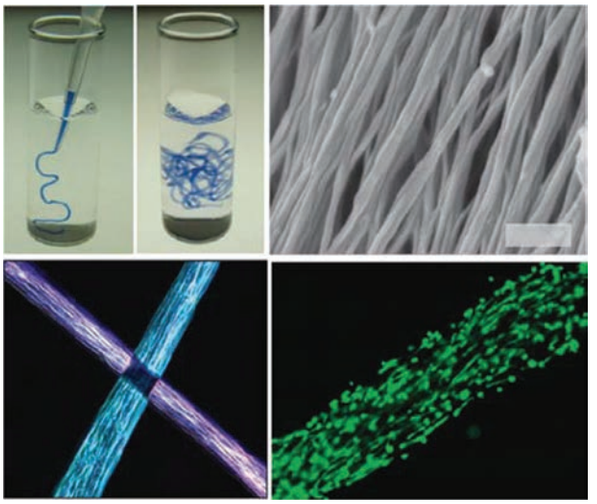

Fig. 7. Self-assembled monodomain gels with long aligned filaments of bundled nanofibers of peptide amphiphiles. (Top) a peptide amphiphile solution colored with tryphan blue injected into PBS after heat treatment. (Bottom) Optical polarizing micrograph of two stripe hydrogels and fluorescence image of aligned calcein-labeled cells cultured on string. Reprinted with permission from [98], S. Zhang, et al., A self-assembly pathway to aligned monodomain gels. *Nat. Mater.* 9, 594 **(2010)**. © 2010, Nature Publishing Group.

long-range alignment of bundled nanofibers (see Fig. 7). These strings of aligned nanofibers were used to direct the orientation of human mesenchymal stem cells (hMSCs) by dispersing in preheated solutions in 3D environment. Both the cell bodies and filopodia were found to be aligned with the nanofiber bundles in the extracellular space. The effects on hMSCs orientation possibly resulted from the contact guidance along the preferentially oriented matrix, thus emphasizing their tissue engineering applications.[95] Therefore, self-assembled hydrogels can be considered as a notable scaffolding system for modulating cellular behaviors.

2.2.6. Bioresponsive Hydrogels

Bioresponsive hydrogels are the 'smart' biomaterials that can change properties in response to selective biological recognition events. When these hydrogels are exposed to a biological target (nutrient, growth factor, receptor, antibody, enzyme or whole cell), molecular recognition events trigger changes in molecular interactions that translate into macroscopic responses, such as swelling/collapse or solution-to-gel transitions. For better cell-material interactions, ligands with biomolecule specific binding sites should be incorporated into scaffold systems to make them tailor-made for providing biological and environmental cues to the cells within scaffold. Many research groups are trying to design precisely positioned bioactive ligands such as cell-adhesive tripeptide, Arg-Gly-Asp (RGD) that can instruct cell behavior at cell-material interface. RGD attached to the surface of the scaffold guide the cells by providing receptors or ligands for the cell-surface proteins, such as integrins.[96] Laminin-derived peptide Ile-Lys-Val-Ala-Val (IKVAV) can also be incorporated into a hydrogel to trigger stem cell differentiation toward neuronal cells.[97] Similar to peptides, polysaccharides may also be used as bioactive ligands to direct cell behavior. For example, Ranjangam et al. have incorporated heparin into peptide amphiphileassemblies to control angiogenesis.[98] Collectively all these types of biomaterials that contain biological instructions are named as bioactive molecules that can be incorporated within the scaffold system.

Bioresponsive hydrogels are capable to perform controlled release of biological factors, changes in mechanical properties and tunable degradation rates with natural extracellular matrix. All of these properties make these intricate design systems in immediate demand for tissue engineering applications. These types of hydrogel systems can provide biomechanical stimuli to the cells to induce mutual constructive responses.[99] Bahney et al. supported this fact by developing a bioresponsive hydrogel with cell-mediated degradation that can induce chondrogenic differentiation of hMSCs.[100] Matrix metalloproteinase 7 (MMP7) was identified as an enzyme with a temporal expression pattern that corresponded with cartilage development. By embedding MMP7 peptide substrates within a PEG diacrylate hydrogel, a MMP7-sensitive hydrogel with distinct degradation rates was synthesized. A comparison between bioresponsive system, non-degradable scaffolds and photoencapsulated hMSCs constructs was performed which showed more extensive collagenous matrices production in MMP7-sensitive bioresponsive hydrogels than other two systems. Furthermore, these changes translated into an increased dynamic compressive modulus. This type of bioresponsive scaffolds can be proposed as a mean to improve matrix deposition and biomechanical properties of neocartilage by delivering cells and contributing to structure assembly of the cartilage matrix.[100]

Cell behaviors such as migration can also be controlled using bioresponsive hydrogels. Bioresponsive hydrogels possesses ECM-mimicking properties to some extent that permit cell migration within the scaffold. This finding was supported by the work of Hubbell and co-workers.[101] Crosslinking of PEG-based hydrogels using oligopeptides was performed which were found to be cleavable by matrix metalloproteinases (MMPs) to form a substrate for cell infiltration. As stated before, MMPs are a family of enzymes that have many roles including the breakdown of ECM molecules during tissue remodeling and disease. Therefore, the integration of MMP-cleavable sites is a logical approach toward ECM mimics. In another study, potential of hydrogels in bone tissue engineering was studied by incorporation of integrin-binding domains (Arg-Gly-Asp-Ser-Pro) via PEG-linkers against human fibroblasts with bone morphogenetic protein-2 (BMP-2) loaded hydrogel. BMP-2 is known to be involved in bone formation.

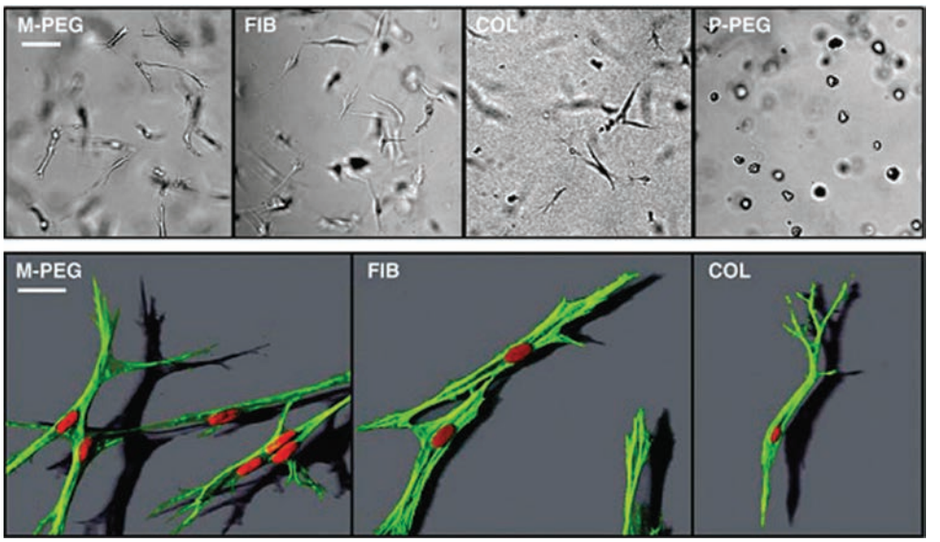

Fig. 8. Bioresponsive MMP sensitive PEG hydrogels allow spindle-shaped cell morphologies similar to natural fibrin (FIB) and collagen (COL) gels, whereas plasma sensitive PEG hydrogels (P-PEG) inhibits cell spreading. Reprinted with permission from [105], G. P. Raeber, et al., Molecularly engineered PEG hydrogels: A novel model system for proteolytically mediated cell migration. *Biophys. J.* 89, 1374 **(2005)**. © 2005, Elsevier.

The fibroblasts were observed to cause a local breakdown of hydrogel crosslinks via secreted MMPs. An assessment of the degradation behavior of MMPs and the cell invasion of provisional matrices revealed that the healing response *in vivo* depends on the enzymatic sensitivity of the matrix. Raeber et al. subsequently tested the suitability of two proteolytically degradable PEG hydrogels with cell migration in 3-D to mimic natural ECM.[102] The results indicate that migration in M-PEG (PEG hydrogel crosslinked with MMP-sensitive sequence) gels is highly sensitive to MMP modulation (see Fig. 8). The ability of a gel to respond to a single class of enzyme provides base for an effective communication between cells and the matrices.

Bioresponsive hydrogels are also employed for growth factor delivery. For example, Hall et al. designed a 3D fibrin hydrogel scaffold that act as a depot/release system for growth factors to aid in angiogenesis.[103] The fibrin matrices are modified by covalently adding receptor-binding sites of integrin $\alpha_v\beta_3$. *In vivo* analysis of angiogenesis reveals that a denser capillary network was formed in receptor-stimulated matrices than native fibrin networks. On comparing these results with additional growth factors for co-stimulation, similar results that are in coherence with either receptor or growth factor embedded were obtained. To combine the advantages of synthetic and natural systems, peptide amphiphiles (PAs) can be considered. Jun et al. described a strategy to regenerate dental tissues using PA molecules that form rigid, cell-responsive fibrous nanostructures and incorporate biological epitopes as the peptide part of the molecule.[104] Along with other bioresponsive molecules, proteases are found to play an essential role in a large number of biological processes such as wound healing and cell differentiation making them critical for incorporation into scaffold systems for the tissue regeneration.

All these hydrogel types provide better opportunities to increase cell-material interaction and ECM remodeling. To further enhance the biocompatibility and niche mimicking properties of these hydrogels, cell-laden hydrogel scaffolds have been introduced to yield significant differences in cellular response to exogenous cues as compared to monolayer culture. As embedding cells within 3D hydrogel can allow for their immobilization for better replication of spatiotemporal presentation of cells to each other throughout the developmental process. These advanced scaffolding systems are being discussed in the preceding sections with their different types, properties and advantages over the conventional hydrogel systems in the context of tissue engineering.

2.3. Fabrication of Hydrogels

The method of fabrication of hydrogel scaffolds plays a vital role in imparting ECM-mimicking functional properties and tissue-like environment. Hydrogel scaffolds that can be fabricated with optimum mechanical properties generally gives negative results for cell encapsulation and proliferation, while constructs with high porosity and uniform cell distribution results into being mechanically weak.[105] Thus, a need to combine structural stability with high cell density while maintaining tissue-like environment for accelerated tissue formation has aroused. Over the past decades, many research groups have published an amalgamation of various engineering techniques for the fabrication of hydrogel scaffolds with controlled architecture.[106] In this section, various fabrication methods for the betterment of geometrical features as well as the distribution of

cells and biomolecules within the hydrogel scaffolds have been discussed

2.3.1. Micro-Fabrication Techniques

Micro-fabricated hydrogels can be developed by employing photolithography or photo patterning approaches.[107] In photolithography, a mask is specifically designed to implement patterns over the hydrogel constructs. Alternative transparent and opaque areas are introduced in the mask according to the pattern for controlling the crosslinking of hydrogels. Upon light irradiation, transparent mask areas exposed to UV light for crosslinking to form micro patterns whereas the remaining unexposed parts are shielded from crosslinking and subsequently washed out in the washing step.[108] Visible or UV light can react with certain light-sensitive compounds called photoinitiators to form crosslinked hydrogels *in vitro*, *in vivo* or *in situ*. Photopolymerization allows spatial as well as temporal control over polymerization by controlling their curing rates from less than a second to a few minutes at room or physiological temperatures. Photopolymerized hydrogels exhibit minimal heat production, as well as the ability to form complex shapes that adhere and conform to the defect site which makes them exceptionally important for the field.[109] Although biological systems put constraints on the use of photopolymerization *in vivo*, owing to the limits of acceptable temperatures, pH, as well as toxicity of most monomers and organic solvents, but can be overcome by the use of mild polymerization conditions (i.e., low light intensity and organic solvent levels, short irradiation time, and physiological temperature).[109]

Along with photocrosslinkable systems, other methods have been also developed including enzymatic[110] and thermo sensitive systems to avoid the use of potentially cytotoxic UV light and free radicals. For example, Ferruti et al. found amphoteric poly(amidoamine) (PAA)-based hydrogels containing carboxyl and amino groups in their repeating units as a potential scaffold material because of its cyto-compatibility with fibroblasts as well as non-cytotoxic degradation products, but their mechanical properties needed further improvement.[111] The group further modified the PAA hydrogels by introducing side guanidine groups to improve cell adhesion and proliferation and found improved mechanical properties when a second PAA carrying primary amino group was used as a crosslinking agent, suggesting that mechanical properties can be tuned for PAA or acrylate-based photocrosslinkable hydrogels by incorporating crosslinking agents with considerable cyto-compatibility for cell growth. Some examples of these hydrogels includes PEG diacrylate (PEGDA), PEG-dimethacrylates (PEGDM), methacrylated gelatin (GelMA), methacrylated HA and methacrylated tropoelastin(MeTro).[106] Similar to chemical and physical crosslinks, hydrogels are quite often modified with cell adhesion peptidesin order to enhance the cell attachment and growth.[112] For example, Sannino et al. combined the photocrosslinking reaction with a foaming process to induce an interconnected porosity within PEG-based hydrogels that had been modified with peptide sequences for enhancing cell adhesion.[113] Though this method is remarkably successful, it also includes some limitations such as uncontrollable crosslinking depth over hydrogel layer, resolution of the features depends on the quality of photomask and aspect ratio.

Soft lithography and molding are other popular techniques for the synthesis of micro-fabricated hydrogel scaffolds. Micro-fabricated hydrogel scaffolds can be molded like conventional molding techniques but with a replacement of elastomeric molds such as polydimethylsiloxane (PDMS) due to their biocompatibility and hydrophobic surface properties. To further facilitate the detachment of crosslinked hydrogels from the mold, a temperature responsive hydrogel poly(*N*-isopropylacrylamide) (PNIPAAm) coating over mold surface have been introduced.[114] Both physically and chemically cross-linkable hydrogels can be fabricated using this method.

Rapid-prototyping is a recently developed additive-based fabrication technique with sequential delivery of material or energy to form a scaffold. It is an automated system where scaffolds are designed using a CAD software and then converted to sliced models which can be fabricated using laser-based systems through an additive process. Stereolithography (SLA), digital light projection (DLP) and two photon polymerization techniques are the other popular laser-based systems currently being employed for the micro-fabrication of hydrogel scaffolds.

2.3.2. Wetspinning and Microfluidic Spinning

Fibrous structure play an important role in mimicking native ECM for regeneration of tissues emphasizing on the importance of fibrous constructs. In fact, the native ECM is by itself made of micro and nano-featured fibers and porous structure in the network of gelly-like environment. Hydrogel fibers with high porosity and high surface area to volume ratio can be fabricated using wetspinning and microfluidic spinning methods. Microfluidic fiber spinning involves arrangement of two or more parallel streams of polymer solutions and a sheath flow in a microchannel which will further lead to hydrogel formation down the stream by chemical, optical or thermal crosslinking. Precise control on single or multiple fibers shape, size, cell distribution and chemical composition is feasible through this approach. Alginate, gelatin/hydroxyphenylpropionic acid (Gtn-HPA) and NIPAm are the commonly used materials for the fabrication of fibers in a microfluidic platform.[115] But these polymers lack components of native ECM thus limiting the cell–cell interactions. Similar to microfluidic spinning, wetspinning includes injection of a pre-polymer solution into one or multiple coagulation baths through a syringe pump or by using pressurized air.

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Various biocompatible materials have used for hydrogel fiber synthesis through this method such as alginate, collagen/alginate composite, collagen, chitosan and starch/PCL composite suitable for tissue engineering applications.[106]

3. CELL-LADEN HYDROGEL SCAFFOLDS

Hydrogels that are encapsulated or entrapped with cells in their 3D cross-linked networks of hydrophilic polymers are called cell-laden hydrogels. Cell-laden hydrogels serve as a scaffold for engineering cells, tissues and organs. It has several advantages over conventional gels for tissue engineering and delivering of cells at the defective site. This is because of its 3D tissue-like microenvironment with tunable physical, mechanical and chemical properties. There are different types of cell-laden hydrogels such as porous, stimuli-responsive and stem cell-laden hydrogels, which will be discussed in the later sections. Cell-laden hydrogels are commonly made by mixing of cells in pre-polymer solution followed by gelation (see Fig. 9). In a typical photopolymerizable cellladen hydrogel system, cells are combined with the hydrogel precursors along with photoinitiator, poured into micromolds and allowed to polymerize using incubation or UV light. The presence of cells within the gels limits the applicable temperature range resulting in relatively long crosslinking reaction periods. Fast crosslinking reaction corresponds to rapid gel viscosity increase that compensates cells tendency to settle down and supports uniform cell distribution in 3D fashion within the microstructure. Hence this method provides temporal control over reaction to generate uniformly populated 3D constructs. An alternative approach creates cell-laden hydrogels by directly passing UV light through a patterned mask containing specific shape and size, which when optimized, only polymerizes the hydrogels where the UV light is able to penetrate the mask. Considering the crosslinking basics, the cell-laden hydrogels have been reported to synthesize by modifying thermal or photo-crosslinking processes with advanced techniques for the optimization of properties. Various fabrication techniques for the cell-laden hydrogels have been briefly discussed in the following sections.

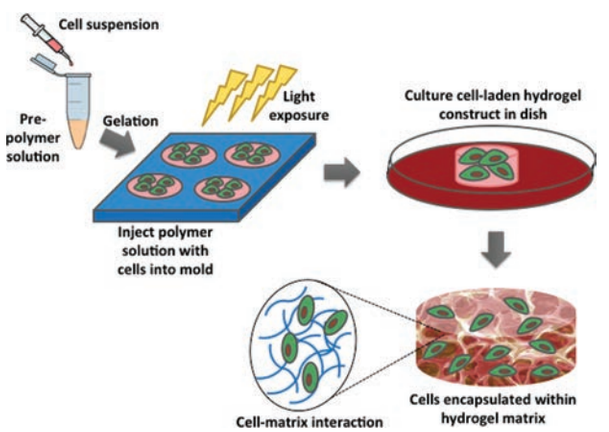

Fig. 9. Schematic diagram representating the fabrication of cell-laden hydrogels.

3.1. Photopatterning

Photopatterning is a popular method for the fabrication of cell-laden constructs or patterning of hydrogels with various cell types. In addition to the conventional photocrosslinking of the hydrogels, it allows precise spatial control over the cellular microenvironment. Yeh et al. reported a technique to encapsulate live cells in 3D microscale hydrogels (also called microgels) of controlled shapes and sizes in the form of harvestable freestanding units.[116] Cells were suspended in methacrylated hyaluronic acid (MeHA) or poly PEGDA hydrogel precursor solution containing photoinitiator, micromolded using a hydrophilic PDMS stamp, and crosslinked using UV radiation. By controlling the features on the PDMS stamp, the size and shape of the molded hydrogels were controlled. Cells within microgels were well distributed and remained viable. These shape-specific microgels could be easily retrieved, cultured and potentially assembled to generate structures with controlled spatial distribution of multiple cell types.[116] Similar approach has been used for biomolecule patterning within photocrosslinkable hydrogels and spatial patterns with different mechanical properties to direct cellular activity, migration and differentiation. Though it is easy-to-use technique but longer exposure time is found to affect cellular viability and activity or sometimes results into phenotypic variation. To control the cell distribution it requires multiple photomasks, which is challenging due to need for regular alignment of photopatterns with masks before each exposure.

3.2. Directed Assembly Technique

Directed assembly technique has been developed for combining smaller gel building blocks to mimic the complexity of native tissues. This modular approach utilizes specific microarchitectural structures (building blocks) to engineer macro-size biological tissues that could be planar structures stacked together to generate 3D constructs or fibers assembled to form a tissue construct. Directed assembly of tissue module is one of the attractive methods for the fabrication and assembly of these building blocks (tissue modules). In this method, centimeter-sized tissues are made from small modules by manipulating their surface energy in contact with a hydrophobic surface. Many tissue modules[117] are reported to generate macroscale tissues from microscale functional units made of cell-laden hydrogels.[118] For example, Nichol et al. reported GelMAcell-responsive hydrogels used for creating cell-laden microtissues and microfluidic devices.[119] Its mechanical and hydration properties were tunable depending upon the applications by modifying methacrylation degrees and gel concentrations. Cells showed spontaneous, proliferation, elongation, and migration when encapsulated and

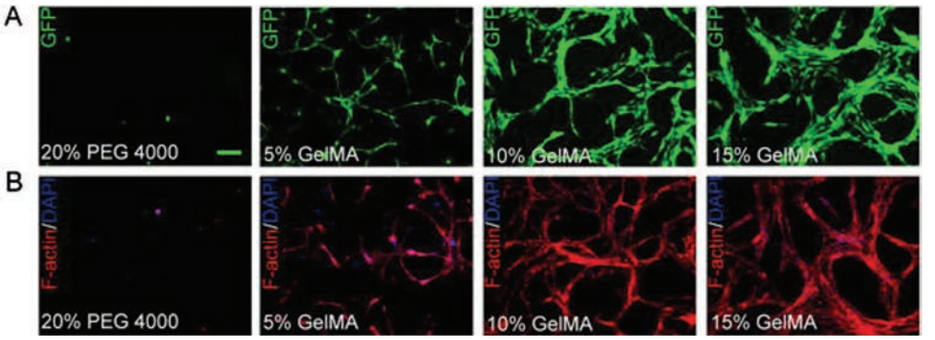

Fig. 10. Fluorescence images of HUVEC cells (cells on the gel) adhered to GelMA of all macromere concentrations exhibiting cell adhesion, proliferation and migration, but did not adhere to PEG 4000 as demonstrated by endogenous GFP (A) and rhodamine-labeled phalloidin/DAPI staining for F-actin/cell nuclei (B) on day 5 of culture (scale bar = 200 μm). Reprinted with permission from [111], J. W. Nichol, et al., Cell-laden microengineered gelatin methacrylate hydrogels. *Biomaterials* 31, 5536 **(2010)**. © 2010, Elsevier.

seeded in hydrogels (see Fig. 10).[119] Figure 11 shows fluorescent images of cells seeded within these microgel channels having potential for perfusable microvasculature. In general, modular approaches allow a precise control over the population and distribution of different cell types within the construct. The lack of scalability and low mechanical properties of the fabricated constructs is the few challenges that need to be resolved.

As an alternate to modular approaches, Shin et al. reported a double-network (DN) strategy that could be used to engineer strong hydrogels encapsulating cells.[120] They synthesized DN hydrogels by a two-step photocrosslinking using gellan gum methacrylate (GGMA) for rigid and brittle first network, and GelMA for the soft and ductile second network. The resulting DN hydrogels exhibited the compressive failure stress of up to 6.9 MPa, which is comparable to the strength of cartilage. DN hydrogels with a higher mass ratio of GelMA to GGMA exhibited higher strength, which shows potential in developing even stronger DN hydrogels in the future. 3D encapsulation of NIH-3T3 fibroblasts showed cell compatibility of DN formation process, which provides an insight for regeneration of load-bearing tissues.[120] However, the DN strategy needs to be modified according to the different microscale hydrogel tissue modules assembly methods for the regeneration of tissues and organs.

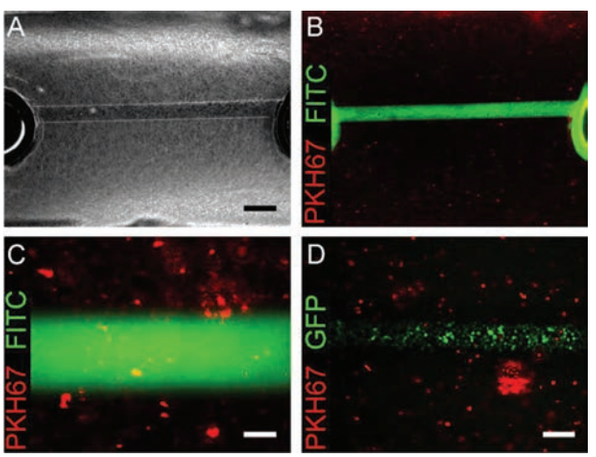

Fig. 11. Fluoresence images of cell seeded within the cell-laden GelMA microfluidic channels that were 300 μm in diameter. (A) and (B) have PKH67 labeled 3T3 fibroblasts, and (C) and (D) have GFP-HUVEC cells demonstrating attachment within endothelial-lined perfusable microvasculature microgels. Reprinted wth permission from [111], J. W. Nichol et al., Cell-laden microengineered gelatin methacrylate hydrogels. *Biomaterials* 31, 5536 **(2010)**. © 2010, Elsevier.

3.3. Microfluidics

Along with the hydrogel and microscale technologies, microfluidic device can also potentially be used to facilitate the exchange of nutrients and soluble factors in 3D tissue constructs.[121] Microfluidic device requires minimal reagent consumption, allow for the laminar flow of fluids, and may be used for high-throughput analysis. PDMS molds are usually used as they are non-toxic to cells along with poly(DL-lactic-co-glycolide) (PLGA) and poly(glycerol sebacate) (PGS) to engineer microvasculature within synthetic scaffolds.[122] In certain works, endothelial and hepatocyte cells were seeded within the complex microfluidic channels togenerate patterns for blood vessels and liver constructs for tissue engineering.[123]

Recently, calcium alginate[121] and gelatin[124] hydrogels have been used to fabricate microfluidic devices with cells seeded on the surface of microchannels. Ling et al. reported a fabrication technique for microfluidic channels from cell-laden agarose hydrogels.[125] Molten of agarose was molded against SU-8 patterned silicon wafer as per standard soft lithography protocols to fabricate sealed and water-tight microfluidic channels. For cell-laden microfluidic hydrogel channels, 6% Agarose molten solution (autoclaved/sterilized to dissolve in PBS) cooled to 70 °C and mixed with equal volume of cell suspension with defined cell density to yield a 3% agarose mixture loaded with cells. This master solution of molten agarose and cells was then poured onto silicon master and allowed for gelation for 2 hr at 25 °C in sterilized conditions.

For microchannel base, a thin flat slab of agarose was developed. Agarose molds were gently peeled from the silicone masters and trimmed to a suitable shape. In addition, a metal feeder wire of upto 2 cm length was used as a guide for insertion of the flexible polyethylene tubing to generate holes for inlets and outlets, which was pressed against the feeder wire to force the wire out of the hole and place tubing correctly. Finally, molded agarose surfaces were heated at 71 °C for 3 s and pressed against another surface-heated agarose slab to form sealed microfluidic channels. Channels of different dimensions were generated and it was shown that agarose, though highly porous, is a suitable material for microfluidic channels. Cells embedded within the molds were well distributed and media pumped through the channels allowing exchange of nutrients and waste products. They demonstrated the importance of a perfused network of microchannels for delivering nutrients and oxygen to maintain cell viability in large hydrogels.[126] Du et al. reported a sequential assembly of cell-laden hydrogel constructs to engineer vascular-like microchannels.[127] They sequentially assembled microengineered hydrogels (microgels) into hydrogel constructs with an embedded network of microchannels. Photolithography were used to fabricate arrays of microgels with predefined internal microchannels and assembled into 3D tubular construct with multi-level interconnected lumens. This technique holds promise as a biofabrication method, which does not influence cell viability within the microgels. Endothelial cells and smooth muscle cells were incorporated into an assembled construct with a concentric microgel design.[127] Demonstrating a completely innovative approach, Chiang et al. reported an integrated platform combining digital microfluidic and photo-patterning techniques to manipulate and fabricate biomimic cell-laden hydrogels.[128] They combine electrowetting-on-dielectric (EWOD) and photo-patterning techniques on a single platform. Different hydrogels cured from individually-driven polymer droplets provide 3D microenvironment for *in vitro* cell culture and actively drive polymer droplets, assemble the hydrogels and arrange the cells inside the hydrogel.[128]

Currently, flow lithography and microfluidic fiber spinning techniques are the newly emerging fabrication methods for cell-laden hydrogel constructs. As discussed in Section 2.3.2, microfluidic fiber spinning technique accompanies drawbacks such as lack of natural ECM components. Core–shell fibers came up as a solution to this problem with ECM proteins in the core and alginate hydrogel in the shell. It employs a double co-axial laminar flow microfluidic device system where a stream of ECM protein was generated in a core flow surrounded by sodium alginate prepolymer and a sheath calcium chloride flow. The diffusion of ECM proteins was reported to be minimized by the calcium alginate shell during their gelation and thus reconstitute intrinsic morphologies and functions of living tissues. Moreover, this technique allows the incorporation of cells and chemicals in single-and multi-layer fibers during the manufacturing process but limiting its use due to low mechanical properties of the fabricated fibers.

3.4. Bioprinting

Bioprinting of hydrogels at precise 3D architectural arrangements represents an innovative approach for engineering biomimetic tissue constructs. Bioprinting includes sequential deposition of solid layers for the precise development of complex structures. In bioprinted tissues, cells can be either embedded within biologically relevant hydrogels or printed free of scaffold support with high cell viability. Hasan et al. has reported an innovative and unique platform that prints a 3D smooth muscle cells (SMCs) patch consisting of multiple cell-laden hydrogel layers.[129] The developed bioprinting platform allows high throughput patterning of SMCs encapsulated in collagen hydrogel droplets, microscale spatiotemporal droplet placement control, printing of 3D cell-laden hydrogel structures and cell seeding uniformity. This layer-by-layer 3D tissue epitaxy is a powerful approach to treat diverse diseases such as cancer, loss of tissue function or organ failure.[130] The major drawback of this approach is poor or inadequate material-printing device and substrate combinations, as well as the relatively small size of the printed construct. Campos et al. hypothesized that cell-laden hydrogels can be printed when submerged in perfluorotributylamine ($C_{12}F_{27}N$), a hydrophobic high-density fluid.[131] Human mesenchymal stem cells and MG-63 cells were encapsulated into agarose hydrogels, and subsequently printed in high aspect ratio in three dimensional structures that were supported in high density fluorocarbon. The results showed that the cells encapsulated within 3D hydrogel constructs remain viable while printing 3D structures with various shapes and sizes were manufactured and remained stable for more than six months. The compressive strength values of the printed gels consequently increased during the two weeks in culture.[131] Along with these cell-laden scaffolds in tissue engineering, a number of new approaches have been utilized by other groups serve great importance to regenerative medicine field. All the fabrication methods discussed above allow the cell-laden hydrogel constructs to optimize their structural properties and functions. To further explore different properties of cell-laden hydrogels, in the following sections, we have included some innovative and current trends used in cell-laden scaffolds with their research potential and promising properties.

4. POROUS CELL-LADEN HYDROGELS

Porosity is an essential characteristic of tissue scaffold as it allows cells to infiltrate in 3D space. Porous structures in hydrogels are potentially useful for mimicking native tissues by improving protein transport and diffusion in gels. To synthesize the porous scaffolds, several methods

have been developed. For example, Cordell et al. stated a method in which a colloidal suspension was used to create pores within hydroxyapatite scaffolds.[132] The mechanical bending and compression analysis of this scaffold confirmed about the strength of the bulk microporous scaffold with smaller micropore sizes was higher than scaffold with larger micropore sizes. These results were found to be in agreement with reported results for other porous materials.[133] Poly(methyl methacrylate) (PMMA) beads were also reported as porogento generate microporous structures within fibrin scaffolds.[134] However, the drawback of this method is the use of toxic chemical processes while removing the PMMA beads from the scaffold, which ultimately results into micropores.[135] Porogen and its leaching method is thus an important parameter for microporous cell-laden hydrogels. Sucrose holds good characteristics for this and has been used to create particles and pores within PLGA sponges during a gas foaming process.[136] In addition to sucrose crystal leaching method, salt crystals have also been used to create interconnected pores within polymeric scaffolds.[137] The method of fabrication employed for these porous PLGA scaffolds are solvent casting/particulate leaching and gas foaming using a suitable porogen (usually salt). Fusion of porogen in the solvent casting process found to pronounce pore interconnectivity and mechanical properties of polymeric scaffolds and thus poresize could be controlled by using porogenmicroparticles (see Fig. 12). Overriding the inability of these scaffolds to directly encapsulate cells within the materials and avoiding use of toxic chemicals becomes one of the major requirements for their development emphasizing on biocompatible modifications in conventional scaffolds. In a study by Park et al. the authors introduced cell-laden agarose gel system containing engineered constructs with a microvascular structure and micropores that are created by dissolving sucrose crystals without any organic solvents.[138] They developed a porous cell-laden agarose fluidic device and characterized the physical and mechanical properties of agarose gels with various micropores. Hepatic cells were encapsulated within gels studied for their corresponding diffusion profile in microchannels. Cell viability in hydrogels with 100 wt% sucrose formulation was found to be higher than that in hydrogels with 0 wt% sucrose formulation at all distances from the medium perfusion channel.[138] Synthetic polymer scaffolds of PEGDA can be formed under physiological conditions through the process of photopolymerization which allows uniform encapsulation of cells.[139] Photopolymerization utilizes light energy to dissociate initiator molecules into free radicals that can react with monomers functionalized with double or triple chemical bonds of acrylate groups in PEGDA that will lead to propagation of radical chain polymerization. Though this method is the most common and easiest to perform it causes unintended formation of free radicals that can damage cell membranes, proteins and DNA.

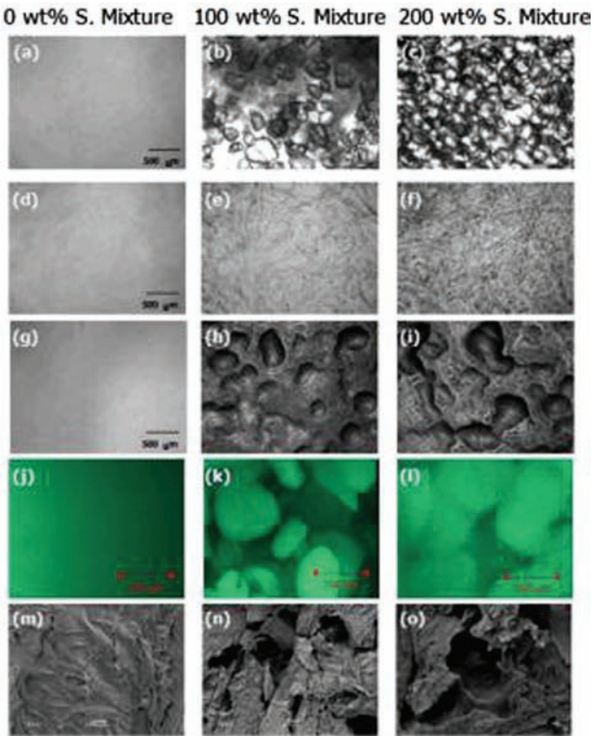

Fig. 12. Images of microporosity: Phase contrast images of sucrose crystals (0–200 wt%, (a)–(c)), sucrose crystals dissolved within agarose gels (d)–(f), and cross-section images of agarose gels containing micropores (g)–(i). Confocal microscope images (j)–(l) and SEM images (m)–(o) of microporosity within agarose gels. Reprinted with permission from [130], J. H. Park, et al., Microporous cell-laden hydrogels for engineered tissue constructs. *BiotechnolBioeng* 106, 138 **(2010)**. © 2010, John Wiley and Sons.

5. STIMULI RESPONSIVE CELL-LADEN HYDROGELS

Considerable effort has been devoted for controlling the release of cells and degradation rate of the scaffold system. Scaffolds made up of stimuli-responsive materials have been developed to release their encapsulated cells and bioactive molecules in their host tissue microenvironement. The *in situ* formation of cell/scaffold construct allows the delivery of encapsulated cells, nutrients and growth factors at the defective site using minimally invasive techniques. The method involves mixing of stimuli-responsive polymer with cells at room temperature and then pre-gel solution is injected into the body. Upon injection, due to the external stimuli, the polymer forms a physical gel leading to cell encapsulation within 3D matrix. In a study by Ratner et al. they used templating methods to produce crosslinked PNIPAAm hydrogels with a defined pore size.[140] They found that switching of pore size with temperature can modulate encapsulation of cells within the gel.[140] In another interesting study, Han et al. reported the formation of a dynamic hydrogel as a tissue-engineering scaffold with tunable macroporosity using stimuli-responsive porogens of gelatin, alginate

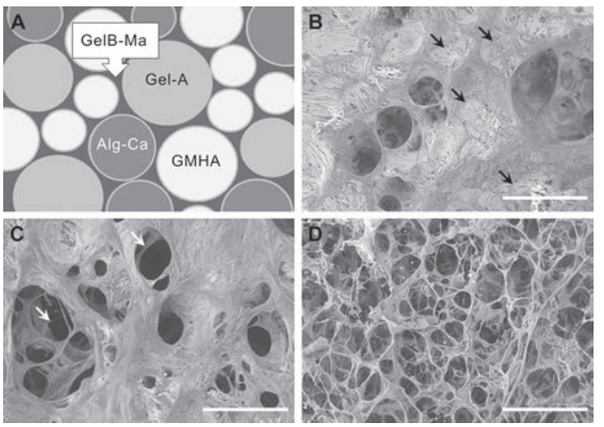

Fig. 13. SEM images of stimuli-responsive tri-porogen-based scaffolds upon sequential porogen removal: (A) Illustration; (B) Type A gelatin porogen removed by temperature stimulus (37 °C incubation); (C) Alginate-calcium porogen removed by chelation stimulus (EDTA/disodium citrate), and (D) Hyaluronic acid porogen removed by enzymatic stimulus (hyaluronidase). Scale bars: 200 μm. Reprinted with permission from [133], L. H. Han et al., Dynamic tissue engineering scaffolds with stimuli-responsive macroporosity formation. *Biomaterials* 34, 4251 **(2013)**. © 2013, Elsevier.

and hyaluronic acid (see Fig. 13).[141] These porogens were embedded within 3D hydrogels made up of type-B gelatin that encapsulate bovine chondrocytes. They reported the degradation of porogen can be controlled by specific stimuli including temperature, chelating and enzymatic digestion which lead to sequential formation of macropores within hydrogels to enhance cell proliferation and ECM production over time. The results of this study showed effective cell release from alginate porogen with high cell viability, cell proliferation and spreading throughout the 3D hydrogel. In addition, dynamic pore formation was also found to contribute for increased type II and X collagen production by chondrocytes and thus provide a platform for a tool to create stimuli-responsive scaffolds for wide spectral tissue engineering application and to study cell responses for dynamic niche properties.[141]

6. STEM CELL-LADEN HYDROGELS

Stem cells can be used as anexcellent cell source for tissue engineering and regenerative medicine applications.[142] Unlike other types of cells in the body, stem cells are unspecialized cells that are capable of self-renewal for longer period yet maintain their capacity to differentiate into multiple specialized cell types when exposed to specific induction cues. Techniques for culturing and regulating human stem cells could lead to unprecedented regenerative treatments. Many tissues are comprised of cells that typically do not undergo extensive self-replication, such as cardiomyocytes in cardiac tissue thus the ability to control and dictate stem cell behavior is crucial for the future success of engineered tissues. Stem cells can reside in niches that are unique to the tissues and organs containing highly ordered microarchitectures, cellular compartmentalization and arrangement.

Employing stem cell-laden hydrogels as an investigative model for determining and controlling cell behavior is a beneficial approach for driving stem cell differentiation and to function down specific lineages that can be advantageous for use in a variety of tissue engineering and regenerative medicine applications. Stem cells with hydrogels can be used as micro-tissue constructs with specific mechanical properties and geometries. On combining these scaffold constructs with microfluidic patterning and micromoulding techniques, cells can be arranged, shaped, layered and made to increase permeation for oxygen, nutrients and other water-soluble metabolites to exactly mimic the natural organization of cells in native tissues. Bahney et al. reported about the non-toxic conditions for photoencapsulation of human mesenchymal stem cells (hMSCs) in PEGDA scaffolds using a visible light photoinitiator system composed of eosin Y, triethanolamine and 1-vinyl-2-pyrrolidinone. This system showed increased viability of encapsulated hMSCs and a more tightly crosslinked network in one-third the time of UV polymerization with Irgacure 2959 (See Fig. 14).[143]

In contrast to natural proteins, many groups have used synthetic peptide sequences designed to self-assemble into a hydrogel under certain environmental conditions.[144] One strategy was based on an amphiphilic peptide containing positively charged lysine residues.[145] When the peptide solution was mixed with cell culture medium containing a high concentration of electrolytes, the lysine residues become shielded, causing the hydrophobic regions of the peptide to rapidly assemble and form a crosslinked hydrogel. Under shear stress, the crosslinked gel undergoes shear thinning resulting in a viscous gel and recovers fully when unloaded. They demonstrated a cell-laden gel encapsulated with MSCs to be delivered *in situ* through a syringe.[145] Similar reports were obtained with elastin-like peptide (ELP). Human adipose-derived adult stem cells were encapsulated in ELP hydrogels and it was found that ELP environment promoted chondrogenic differentiation without the addition of chondrogenic medium which infers that hydrogel matrix provide some cues to induce stem cell differentiation.[146]

Stem cell-laden hydrogels have many advantages over conventional scaffolds that can be employed in different tissue regeneration application. In an interesting study, Fedorovich et al. have studied the effect of calcium phosphate (CaP) particles on osteoinductive potential of cell-laden hydrogel-CaP composite matrices.[147] They included apatitic nanoparticles in Matrigel constructs and studied viability of embedded multipotent stromal progenitor cells *in vitro*. Furthermore, they investigated the *in vivo* osteoinductive potential of cell-laden Matrigel containing apatitic nanoparticles after subcutaneous implantation in immunodeficient mice and compared with composites

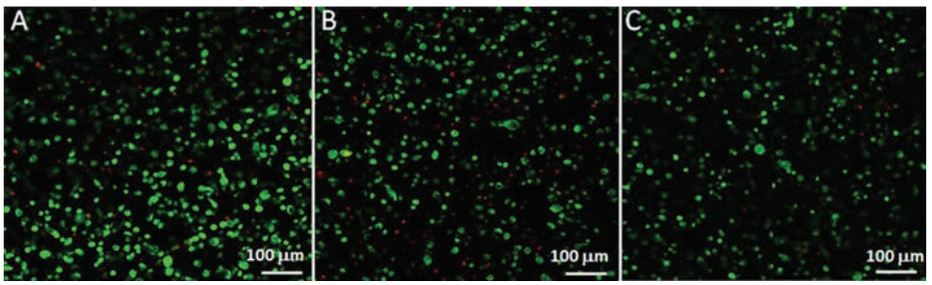

Fig. 14. Cytotoxicity of visible light initiation compared to UV light initiation by live-dead staining of hMSCs 48 h after photoencapsulation in a 10% PEGDA scaffold using (A) I2959, (B) 0.75% TEA and 0.1 mM eosin Y and (C) 0.1% TEA and 0.01 mM eosin Y. Reprinted with permission from [135], C. S. Bahney, et al., Visible light photoinitiation of mesenchymal stem cell-laden bioresponsive hydrogels. *Eur. Cell. Mater.* 22, 43 (**2011**). © 2011, AO Research Institute Davos.

containing osteoinductive biphasic calcium phosphate (BCP) microparticles. Finally, they concluded that apatitic nanoparticles were osteoinductive and induced osteoclast activation without bone formation whereas BCP particles were found to be more effective in inducing bone formation at ectopic location.[147] Similar to bone application, Chung et al. studied the influence of 3D HA microenvironments on chondrogenic potential of MSCs for cartilage applications.[148] As HA is a native component of cartilage, MSCs may interact with HA via cell surface receptors that influence stem cell differentiation. *In vitro* and *in vivo* cultures of MSC-laden HA hydrogels supports chondrogenesis. To assess the influence of scaffold chemistry on chondrogenesis, HA hydrogels were compared with inert PEG hydrogels and showed enhanced expression of cartilage-specific markers. Differences between HA and PEG hydrogels *in vivo* were most noticeable for MSCs and polymer alone, indicating that hydrogel chemistry influences the commitment of MSCs to undergo chondrogenesis (e.g., ~43-fold up-regulation of type II collagen of MSCs in HA over PEG hydrogels). Although this study investigated only early markers of tissue regeneration, these results emphasize the importance of material cues in MSC differentiation.[148] Therefore, based on the experimental examples discussed in this section, and other reported data, stem cell-laden hydrogels may be considered as a potential candidate for scaffold-based tissue regenerative applications.

7. APPLICATIONS OF CELL-LADEN HYDROGEL SCAFFOLDS

Cell-laden hydrogels are widely used in engineering cells, tissues and organs. This is because, they offer numerous attractive features such as hydrated tissue-like microenvironment for cell and tissue growth and ease of cell transplantation. Table II summarizes applications of different types of cell-laden hydrogel systems with their unique properties. In the preceding sections, different tissue engineering applications of cell-laden hydrogels are briefly discussed.

7.1. Bone Tissue Engineering

Conventional hydrogels that can be used as injectable or in organ printing are reported to lack appropriate stimuli to direct osteogenic differentiation in encapsulated multipotent stromal cells causing inefficient bone formation within the matrix. To overcome this problem, Fedorovich et al. studied the effect of CaP nanoparticles for the osteoinductive potential of cell-laden hydrogel-CaP composite matrices in comparison with composites containing osteoinductive biphasic calcium phosphate (BCP) microparticles.[152] They concluded that apatitic nanoparticles were found to be osteoinductive and induced osteoclast activation without bone formation whereas BCP particles were found to be more effective in inducing bone formation at ectopic location.[152] To further resolve the problems, the group tried to fabricate a suitable osteochondral implant with optimum tissue formation and integration properties. They characterized the use of a 3D fiber deposition (3DF) technique for the fabrication of cell-laden, heterogeneous hydrogel construct as osteochondral grafts. Fluorescently labeled human chondrocytes and osteogenic progenitor cells were encapsulated and printed in alginate hydrogels yielding scaffolds with both cell types located in different parts. The results of this study confirmed that the scaffolds of varying porosity and elastic modulus can be obtained with high cell viability, by changing fiber spacing or angle of fiber deposition, while printing that support distinctive tissue formation both *in vivo* and *in vitro* at different locations within one construct.[153]

7.2. Cartilage Tissue Engineering

Similar to bone applications, many research groups are exploiting cell-laden hydrogels for chondrogenic applications. As mechanical forces play an important role in regulating cartilage development from MSCs, these forces can be employed to develop engineered constructs with enhanced mechanical properties. Huang et al. reported the effect of long-term dynamic compression on MSC-seeded constructs and observed the influence and phenotypic transitions by varying pre-culture duration, loading regimes and inclusion of TGF-β3 during loading.[149] They found that loading initiated before chondrogenesis

Table II. Applications of cell-laden hydrogel scaffolds.

S.no.	Hydrogel scaffolds: Type	Hydrogel scaffolds: Material	Property	Application
1.	Stem cell-laden hydrogel	MSCs-seeded constructs with varying TGF-β3 levels	Enhanced matrix distribution and mechanical properties due to dynamic compressions	Cartilage tissue engineering and chondro-inductive processes[149]
		MSC-laden hyaluronic acid hydrogels	Provides material cues in stem cell differentiation and regulate scaffold surface and cell surface receptors interaction	Chondrogenic differentiation and cartilage regeneration[150]
		MSC-laden 2-RGD functionalized hydroxypropylmethacrylamide hydrogels	Atrophy prevented and hydrogels were infiltrated with axons myelinatedschwann cells	Spinal cord injury repair and neural tisuue engineering[151]
2.	Composite cell-laden hydrogel	Multipotent stromal cell-laden Matrigel with CaP nanoparticles	Osteoinductive in nature and shows osteoclast activation, but without bone activation.	Bone regeneration application and organ printing.[152]
3.	Hetrogenous cell-laden hydrogel	Alginate hydrogel encapsulated with human chondrocyte and osteogenic progenitor cells	Employed 3D fiber deposition technique to print two types of cells in different compartments of same scaffold	Osteochondral grafts for osteochondral defect healing[153]
4.	Porous cell-laden hydrogel	Hepatocarcinoma cell line (HepG2) encapsulated in porous alginate hydrogels	Shows enhanced mass transfer of nutrients, oxygen and waste removal	Tissue engineering and regenerative medicine[154]
5.	Microstructural cell-laden hydrogel	Methacrylated pre-polymer solution laden with continuous cell-line and primary BMSCs	Cell compatible photocrosslinking method formed biocompatible 3D microgels	Tissue engineering constructs[155]
6.	Bilayered cell-laden hydrogel	PNIPAAM implantable hydrogels loaded with neonatal rat cardiomyocytes	Temperature-sensitive implantable hydrogels	Catheter-based delivery systems for cardiac tissue engineeing[156]
7.	Biomimetic cell-laden hydrogel	Matrigel loaded with neonatal cardiomyocytes and seeded within collagen foams	*In vivo* constructs showed transition to functional cardiac muscle tissues using pulsatile electrical stimulation	Cardiac tissue engineering[157]
8.	Stimuli-responsive cell-laden hydrogel	Type-B gelatin hydrogels embedding three types microspherical biomaterial based porogens with neonatal bovine chondrocytes	Sequential formation of macropores to enhance cell proliferation and ECM production over time	Stimuli-responsive cell delivery vehicles for tissue engineering[141]

decreased the functional maturation of the construct although chondrogenic gene expression increased. In contrast, loading initiated after chondrogenesis and matrix elaboration improved the mechanical properties of MSC-seeded constructs but only at specified TGF-β3 levels and loading parameters. These findings support that dynamic compressive loading initiated after a sufficient period of chondro-induction at sustained TGF-β3 exposure enhances matrix distribution and mechanical properties of the construct improving their chondrogenic potential.[149] Material cues in stem cell differentiation microenvironment can potentially alter the interactions between scaffold surface and cell surface receptors. Chung et al. studied the effect of 3D HA microenvironments on MSCs chondrogenesis.[150] HA being a native cartilage component may allow MSCs to interact via its cell surface receptors and thus influence stem cell differentiation. This fact supported by the *in vitro* and *in vivo* cultures of MSC-laden HA hydrogels by expressing more cartilage-specific markers that found to support chondrogenesis in comparison with relatively inert PEG hydrogels. They observed a 43-fold up-regulation of type-II collagen of MSCs in HA over PEG hydrogels, thus holding promise for chondrogenic applications of cell-laden hydrogels.[150]

For evaluating the clinical potential of hydrogels for cartilage repair, Chung et al. designed a study for feasibility and efficacy of articular cartilage repair using composites of human umbilical cord blood derived mesenchymal stem cells (hUCB-MSCs) and four different hydrogels (group A, 4% hyaluronic acid; group B, 3% alginate: 30% pluronic (1:1, v/v); group C, 4% hyaluronic acid: 3% alginate: 20% pluronic (2:1:1, v/v) and group D, 4% hyaluronic acid: 3% alginate: 20% pluronic: chitosan (4:1:1:2, v/v).[158] They implanted these composites into right knee defect in each group of rats and a control hydrogel without hUCB-MSCs cells for 16 weeks. The results of this study confirmed that group A (4% hyaluronic acid hydrogel) showed superior cartilage repair grossly, histologically and achieved a better cellular arrangement and collagen organization pattern that mimics adjacent uninjured articular cartilage (see Fig. 15). Thus supporting HA hydrogel and hUCB-MSCs composites for clinical cartilage applications delimits immunological rejection and suppress its drawbacks.[158]

7.3. Cardiac Tissue Engineering

In addition to other applications cell-laden hydrogels have also been used for cardiac tissue engineering applications.

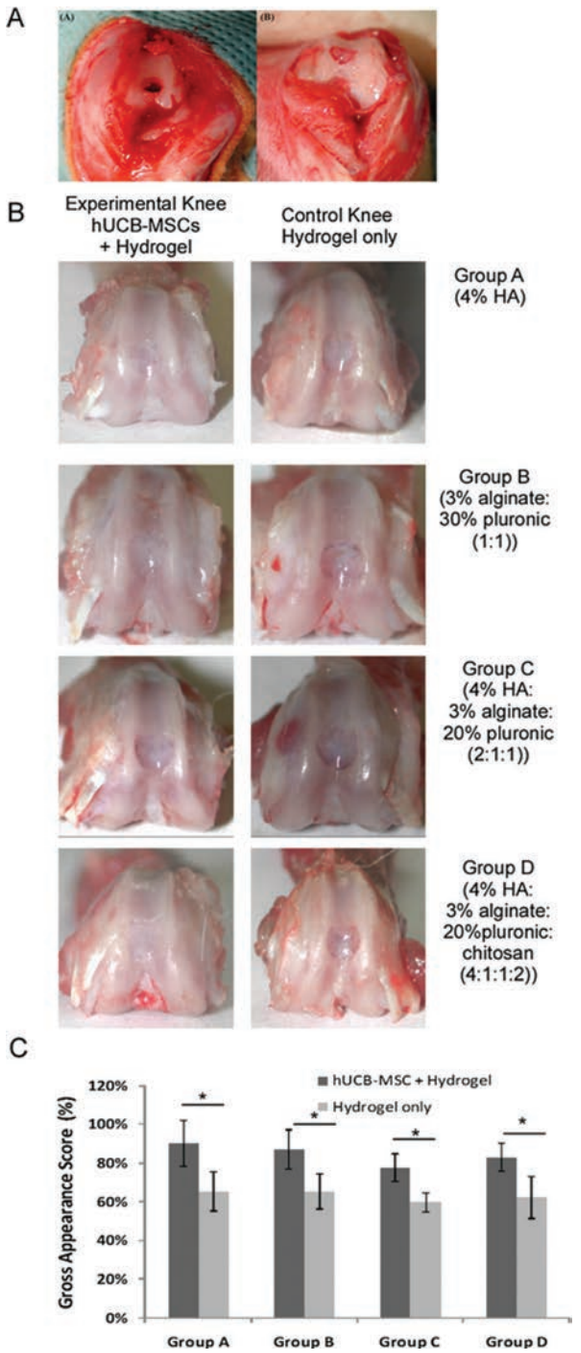

Fig. 15. Articular cartilage defects in a rat model and gross appearance and scoring result at 16 weeks post-transplantation. (A) Gross photos of articular cartilage defects in a rat model transplanted with composites of hUCB-MSCs and different hydrogels; (B) Gross findings of repair tissue at articular cartilage defects in a rat model after 16 weeks of implantation showing transplanted knees repaired to almost normal level and border regions between repair and normal tissue were less distinct than control knees; (C) Gross appearance scores of experimental and control knees in four different hydrogel groups. Reprinted with permission from [151], J. Y. Chung, et al., Comparison of articular cartilage repair with different hydrogel-human umbilical cord blood-derived mesenchymal stem cell composites in a rat model. *Stem. Cell. Res. Ther.* 5, 39 (**2014**). © 2014, BioMed Central.

A bilayered hydrogel from a temperature-responsive PNIPAAM and non-responsive polymer was generated to investigate its potential use as an implantable cell-loaded delivery system. Neonatal rat cardiomyocytes were cultured on the hydrogels until they reached confluence. Subsequently, temperature was lowered, and the hydrogel sheets were curled/rolled into tubes owing to the difference in swelling properties of the bilayered polymers, resulting in the formation of cell-laden hydrogels for catheter-based delivery systems.[156]

Decellularized cardiac tissues have also attracted significant attention for cardiac regeneration. In an interesting study, pig myocardial ECM was used to synthesize hydrogels.[156] The ECM material was isolated by processing decellularized porcine heart muscle. After characterization of the structure and composition of the ECM hydrogels, their potential to promote vascularization was investigated using endothelial cells (ECs) and smooth muscle cells. In addition, the ECM hydrogels supported high cardiomyocytes viability. Decellularized ECM-based hydrogels have strong potential for *in vivo* studies, particularly for the delivery of cell-laden gels into the myocardium via syringe or catheter-based approaches. In a report by Zhang et al. they mentioned fabrication of tissue engineered cardiac patch for the maturation of human embryonic stem cell–derived cardiomyocytes (hESC–CMs) (see Fig. 16).[159] The results of this study confirmed the uniform alignment of hESC-CMs within the patch by locally controlling the direction of passive tension. Higher conduction velocities (CVs), longer sarcomeres and enhanced expression of genes involved in cardiac contractile functions were observed in hESC-CMs 3D patches. The CVs in cardiac patches increased with cardiomyocyte purity, reaching 25.1 cm/s in patches constructed with 90% hESC-CMs. Maximum contractile force amplitudes and active stresses of cardiac patches averaged to 3.0 ± 1.1 mN and 11.8 ± 4.5 mN/mm2, respectively with contractile force per input cardiomyocyte averaged upto 5.7 ± 1.1 nN/cell resulting into a negative correlation with hESC-CM purity. This study demonstrated advanced levels of hESC-CM maturation after 2 weeks of 3D cardiac patch culture for future cell therapy studies.

7.4. Neural Tissue Engineering

Neural tissue engineering approaches also require development of engineered 3D constructs to provide guidance conduits to cell-based therapies. Schwann cells play a key role in peripheral nerve regeneration by forming oriented paths for axonal regeneration. Suri et al. encapsulated schwann cells within engineered collagen and HA IPN hydrogels with and without laminin as a 3D culture system.[160] The results of this study exposed that encapsulation of cells in 3D hydrogel constructs did not affect the cell viability and underwent spreading and proliferation with increased cell numbers. Cells were also observed to align

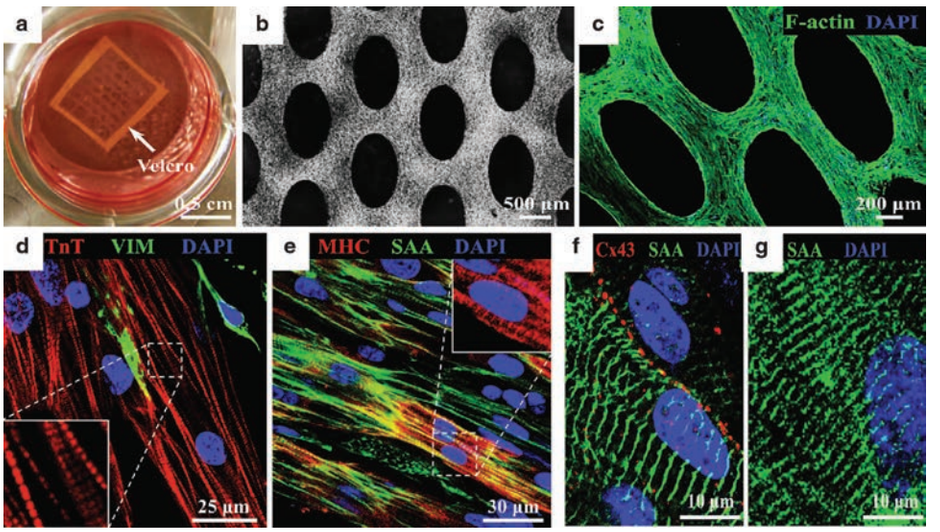

Fig. 16. Functional cardiac patches fabricated using human ESC-derived cardiomyocytes. (a) Functional cardiac patches fabricated using human ESC-derived cardiomyocytes after 2 weeks of *in vitro* culture; (b) micro-fabricated gels contained elliptical pores; (c) aligned human ESCs imunostained with cardiac markers i.e., (d) troponin T (red); (e) myosin heavy chain (red); (f) connexin-43 (red) and sarcomeric a-actinin (green) within the hydrogels after 2 weeks of *in vitro* culture and (g) immunostaining for sarcomeric a-actin (green) on 2D gels, demonstrating decreased sarcomere length in 2D compared with 3D constructs. Reprinted with permission from [152], D. Zhang, et al., Tissue-engineered cardiac patch for advanced functional maturation of human ESC-derived cardiomyocytes. *Biomaterials* 34, 5813 **(2013)**. © 2013, Elsevier.

parallel to each other in structures like Bands of Bunger and actively secrete increased levels of nerve growth factor and brain-derived neurotrophic factor on addition of laminin within hydrogels. In co-culture of dissociated neurons with schwann cells, neurons were able to extend neurites and some neurites were observed to follow schwann cells. This study emphasizes on the promise of schwann cells-laden hydrogels for mimicking ECM and supporting nerve regeneration therapies.[160] In another notable study, an attempt was made to repair spinal cord injury (SCI) in rat models by Hejčl et al.[151] They studied whether it was feasible to bridge a chronic lesion by implanting a hydrogel based on 2-hydroxypropyl methacrylamide functionalized with RGD (Arg-Gly-Asp) sequences either alone or seeded with MSCs. With respect to the control group, the results showed an improvement for the rats implanted with the MSC-laden hydrogels; furthermore, in this case tissue atrophy was prevented, the hydrogels were infiltrated with axons myelinated with schwann cells, and MSCs were still present in the hydrogels for 5 months after implantation. A slight tendency to improvement was also observed for the rats implanted with the hydrogel alone, but it was not statistically significant. Taken together, these results support the therapeutic potential of synergistic approaches integrating cells and suitable hydrogels for different tissue engineering applications

8. RECENT TRENDS IN HYDROGEL SCAFFOLDS: GRADIENT HYDROGELS

With the advancement in biomaterials and bioengineering, more opportunities have been developed to understand and mimic the cellular microenvironment and study the interactions at the cell-material interface. Gradient hydrogels are a class of scaffolding system, which are recently introduced to tissue engineering. Gradient hydrogel can be defined as a hydrogel possessing a gradual spatiotemporal change in at least one property.[161] The gradient biomaterials have capability to mimic the graded tissue microenvironment such as at the interface of soft-to-hard tissues. These gradients were successfully applied to control cellular fate and function. Gradient biomaterials contain anisotropy in properties such as composition, structure, mechanics and biomolecularproperties. Different scaffolding systems till now have been reported with gradient systems including nanofibers but since hydrogels mimic the ECM and their chemistry, cross-linking density and response to environmental stimuli (e.g., heat, light, electrical potential, chemicals and biological agents) may be manipulated, they can be designated as ideal systems for producing tailored 3D cellular microenvironments. Gradient hydrogels exhibit a continuous spatial change in a given property and allow a continuum of these property values to be tested on a single biological sample enabling high-throughput screening of cell-material interactions making them capable to replicate *in vivo* physical and chemical cellular microenvironment gradients.

In this section, new innovative approaches to modify hydrogels suitable for tissue engineering applications, in particular interface tissue engineering (ITE), are being discussed. A schematic of gradient hydrogel system for ITE is shown in Figure 17. The different approaches and types of

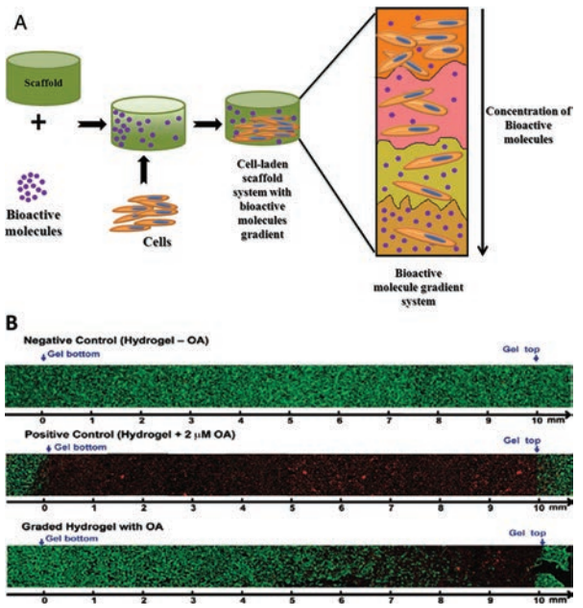

Fig. 17. (A) Schematic representation of cell-laden gradient hydrogel scaffolds for interface tissue engineering applications. (B) Fluorescent micrographs of MC3T3 cells cultured on PEGDA hydrogels with various concentrations of toxin (OA). Negative control i.e., without OA, positive control i.e., with 2 μM OA and with an OA concentration gradient for 24 hours. Dead cells are marked in red by ethidium bromide and live cells marked in green by calcein-AM.[162] Reprinted with permission from [154], S. Ostrovidov, et al., Controlled release of drugs from gradient hydrogels for high-throughput analysis of cell-drug interactions. *Anal. Chem.* 84, 1302 **(2012)**. © 2012, American Chemical Society.

hydrogels discussed are inspired from fundamental biology of the cellular microenvironment to resemble or mimic ECM by controlling the various bioactive cues such as adhesion molecules and growth factors.

Molecular concentration gradients of growth factors, chemokines and cytokines are proved to play an important role in many biological phenomena such as chemotaxis,[163] morphogenesis and wound healing.[164] The ECM's mechanical properties also signal cells to induce a structural rearrangement of the cytoskeletal and immobilized proteins to generate a mechanotransduction response.[165] In addition, gradient's properties provide a way to mimic the properties of the ECM surrounding cell which are mechanically connected to different concentrations of different cells such as bone-cartilage interfaces and dentino-enamel junctions.[166] Various technologies have been developed to create spatiotemporal gradients and complex biomaterials to rapidly screen cell-biomaterial interaction[167] and to study cellular processes such as migration and angiogenesis *in vitro*[168] which can be employed in drug delivery[169] and tissue engineering[170] applications.

Peptide gradient gels are investigated for use in controlling cellular functions. For example, Guarnieri et al. studied the effect of covalently immobilized RGD peptide gradients on PEGDA hydrogels with their cell behavior.[171] The results of this study suggest that cells recognize the RGD gradient and adhere onto it assuming a stretchable shape. Moreover, cells tend to migrate in the direction of the gradient. The speed of migration of cells on the hydrogel with uniform RGD distribution was found to be higher. Speed of migration increases with increasing RGD gradient steepness supporting the augmentation of bias speed component of the mean squared speed i.e., the drift of the cell population migrating on the anisotropic surface provided by the RGD gradient.[171] In another interesting study, Turturro et al. engineered PEGDA hydrogel scaffolds with gradients of mechanical properties and immobilized biofunctionality using perfusion-based frontal

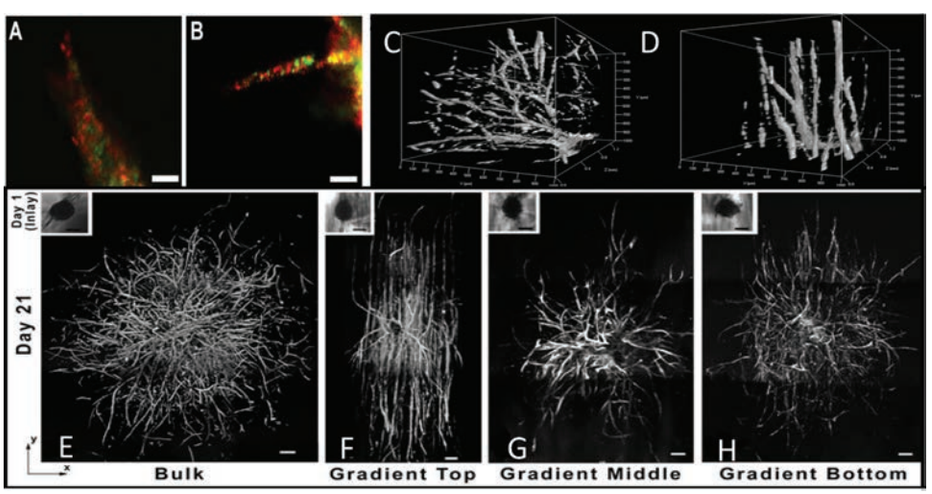

Fig. 18. Vascular sprout invasion within PEGDA hydrogels with gradient of PBFP. (A) and (B) confocal images of fluorescently labelled endothelial cells (red) and smooth muscle cells (green) within matrix, 3D vascular sprout invasion in bulk control gels (C) and PBFP gradient hydrogels (D), flattened 3D mosaic renderings of co-culture aggregates in (E) bulk control gels, (F) top, (G) middle and (H) bottom regions of PFBP gradient hydrogels. Reprinted with permission from [164], M. V. Turturro, et al., MMP-Sensitive peg diacrylate hydrogels with spatial variations in matrix properties stimulate directional vascular sprout formation. *PLoS One* 8 **(2013)**. © 2013, PLOS.

photopolymerization (PBFP).[172] The controlled delivery of a buoyant photoinitiator, eosin Y, through a glass frit filter results in the formation and subsequent propagation of a polymer reaction front that is self-sustained and able to propagate through the monomeric mixture. Propagation of this front results in monomer depletion, leading to variations in cross-linking, as well as spatial gradients of elastic modulus and immobilized concentrations of the YRGDS cell adhesion ligand within PEGDA hydrogels. Furthermore, the magnitudes of the resulting gradients are controlled through alterations in polymerization conditions (see Fig. 18).[172]

The effects of VEGF and matrix composition on 3D endothelial cell invasion have been investigated in natural scaffolds such as collagen[173] and semi-interpenetrated networks of collagen and HA.[174] Directed endothelial cell invasion within porous collagen scaffolds has been found to occur due to gradients of immobilized VEGF, while endothelial cell sprout formation has been shown to be guided towards regions of decreased HA concentration in collagen-HA interpenetrating networks with observed 2-fold increases in sprout length as compared to that occurring in isotropic matrix controls.[174] These studies strongly suggest that gradients of matrix cues lead to enhanced and directed endothelial cell behavior and play a critical role in inducing vascular sprout formation. Based on the experimental studies discussed in this section, and other reported data, advanced capabilities of gradient hydrogel systems in recapitulating native tissue environment are proved to be supportive by providing a gradient of properties for heterotypic tissues regeneration.

9. CONCLUDING REMARKS

Hydrogels are being considered as a promising scaffolding system that supports cell adhesion, proliferation and differentiation by providing requisite physical, mechanical and chemicals cues for the growth of cells and their subsequent development into tissues and organs for regenerative medicine applications. Hydrogel design and synthesis approaches play a significant role to impart the microenvironment of native tissue, which is essential to achieve the ultimate goal of development of biological substitutes for damaged tissues and organs. Only certain aspects of tissue properties such as physiochemical properties are taken as the main focus while designing conventional hydrogel systems for tissue engineering applications, which results into unsuccessful replicas of native environment's complexities. Thus, the development of cell-laden hydrogels with high biocompatibility, biodegradability, tunable patterning properties and efficient mass transfer properties are of great importance. Cell-laden hydrogels encapsulate cells in a spatially and temporally controlled manner with suitable cell sources and bioactive molecules in order to mimic native tissue microenvironment, which paves a way towards engineering functional tissues and organs. Limitations such as biocompatibility, tuned mechanical properties, porosity and permeability content to permit ingress of cells and nutrients, appropriate surface structure and cell-attachment sites on the scaffolds, could be overcome with cell-laden hydrogel-based approaches and cells could be efficiently delivered at the defective site of tissues too. Stem cells are a recent addition to hydrogel systems and its being extensively investigated in tissue engineering because of its self-renewal and differentiation potential. The use of stem cells within the hydrogels would employ the regeneration potency of stem cells within the *in vivo* microenvironment or *in vitro*. And thus cell-laden hydrogels loaded with stem cells can be proved as a potential scaffold material for the development of tissue engineering and regenerative medicinal tools and techniques. Though the positive impact of these biomaterial scaffolds are proved by numerous research groups but still the requirement of hydrogels has not yet optimized which require much attention in future. The major challenges concerned with cell-laden hydrogel scaffolds are the homogeneous cell encapsulation and optimization of porous structure within the gel system. These limitations can be resolved with more sophisticated fabrication techniques and material modification processes. So far, as per literature survey, cell-laden hydrogels are often used to repair homotypic tissues. In the view of regeneration of heterotypic tissues, cell-laden hydrogels lack the heterogeneity within their structures to mimic the native tissue microenvironment or niche. Though each tissue has its own structure and functional properties, they are all interconnected and inter-dependent with each other to maintain physiological and metabolic events within their niches. This cross-talk protrudes anisotropy within the niches of each tissue. This anisotropy within the diverse tissues and organs proved to be the main hurdle in optimization of scaffold system conditions and parameters. As a solution to this, the field ITE has emerged to regenerate functional tissues at the interface of different types of tissues, such as soft-to-hard tissues. ITE deals with tissue interfaces, which are heterogeneous structure and properties in a gradient manner. It helps in the development of complex biomaterials with biomimetic gradient properties in terms of composition, structure, mechanical and other functional properties. In this regard, gradient systems are proved to beoptimal scaffold systems for engineering graded tissue interfaces for multiple types of cell growth and subsequent tissue development. Cell-laden hydrogel gradient system could then be exploited as complex biomaterials with biomimetic gradient properties in terms of physical, mechanical, chemical and biological cues to promote heterotypic cellular growth, differentiation and interaction. This is an exciting time to be involved in cell-laden hydrogel scaffold engineering in order to formulate them as a clinically promising synthetic ECM for tissue repair and reconstruction, with great challenges and also great expectations ahead.

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REVIEW