Chapter 6

INJECTABLE HYDROGELS FOR BIOMEDICAL FORMULATIONS

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ABSTRACT

Regenerative medicine, a new paradigm stemming from technologies such as tissue engineering and the controlled release of therapeutic molecules, is revolutionizing the clinical practices. The sophisticated smart materials used in these biomedical applications must meet strict criteria, namely convenient mechanical properties and degradation rate, biocompatibility, porosity and interconnectivity, functional properties related to the interaction with cells and the release of pharmaceuticals, etc.

This review will focus on injectable hydrogels, whose great interest in the clinical perspective is, to a large extent, due to the minimal invasive manner through which they can be implanted in the human body. The injectable hydrogels share the same general advantages as those of the hydrogels, with the additional benefit of being able to fill irregular shaped defects doing so in a minimal invasive manner. The materials and the challenging requirements for the design of injectable hydrogels and their applications in the biomedical field will be addressed.

1. INTRODUCTION

The advent of tissue engineering has emerged as a promising approach to circumvent the limitations of the existing therapies for the treatment of tissue loss or organ failure, serving the challenging task of producing tissues substitutes that might restore, maintain or improve the structural features and physiological functions of natural living tissues [1-3]. The strategy underlying the creation of new tissues includes the isolation and cultivation of cells within

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suitable scaffolds to support their three dimensional growth, promoting the cell adhesion, growth and differentiation through the incorporation of bioactive molecules [4,5]. The possibility for long term support of the proliferating cells using growth and differentiation factors, turned the controllable release systems into the fusion step in the development of a new generation of biomedical tools [6]. The *scaffold* provides the necessary support for cells to attach, proliferate, maintaining their differentiated state and can even define the overall shape of the tissue-engineered transplant. The seeding of the cells into the scaffolds can be performed using two distinct strategies. The temporary support can be required for an *in vitro* maturation stage, serving as an adhesive substrate providing physical support for the cells, before being implanted. In another approach, the scaffold is implanted to fill a void in the damaged tissue, already carrying cells or being then subsequently seeded, to stimulate the new tissue formation. In the later strategy, the scaffold can either be formed before implantation or directly injected to form the three dimensional structure *in situ*. This review will focus on injectable hydrogels, the materials and the challenging requirements for their design and their applications in the biomedical field.

1.1. Hydrogels as Scaffolds

The development of biomedical devices has focused on the design of three-dimensional structures made from natural or synthetic materials, termed scaffolds. Among them, hydrogels are receiving an increasing attention, as several biomedical applications require materials that possess a jelly consistency, which can set and be molded into a desired shape under physiological conditions. Hydrogels are a class of hydrophilic polymeric scaffolds, with appealing features from the perspective of biological mimicking. They have a good biocompatibility, degradability and appropriate mechanical properties, allowing for a favorable controlled interaction with living systems. The importance of hydrogels in biomedical applications was first reported in the late 1950s, with the development of poly(hydroxyethyl mehacrylate) (PHEMA) gels as a soft contact lens material [7]. Nowadays they are used in numerous applications, including ophthalmic devices, biosensors, biomembranes, to support and promote tissue regeneration, and as attractive systems for the controlled release of pharmaceutically active molecules.

1.2. Rational for the Clinical Need of Injectable Formulations

All therapies would benefit from minimal surgical procedures which might decrease patient morbidity. From this perspective, the use of injectable scaffolds has recently become of great interest, to a large extent due to the minimal invasive manner through which they can be implanted in the human body, associated to improved patient compliance. Other advantages are lower risk of infection and reduced scar formation. The injectable hydrogels share the same general advantages as those of the hydrogels with the additional benefit of being able to fill irregular shaped defects, avoiding the need for patient specific prefabrication [8]. In addition, the hydrogel components being in solution before gelation, a more homogeneous distribution of bioactive molecules or cells may be obtained [9-12]. These

injectable formulations are, hence, promising matrices not only for tissue regeneration, but also to serve as controlled release devices for local drug delivery [13,14]. Recent works report the use of injectable hydrogels as lumen-fillers of guidance channels for nerve regeneration, gathering the function of providing a 3D matrix on which cells can adhere, proliferate and differentiate, with the ability of entraping biomolecules (namely neurothophic factors), protecting them from degradation, while also controlling the release rates, resulting in a leading outcome for nerve repair [15-20]. Cells are being increasingly exploited as alternative drug delivery devices, by acting as drug depots enabling the delivery of therapeutic molecules over an extended time period. Stem cells, progenitor cells, and lineage-committed cells are thus being considered as a new generation of drug depots for the sustained release of therapeutic biomolecules. The entrapment of cells in hydrogels, while providing a physical barrier to protect the cells from hostile extrinsic factors, must simultaneously improve the secretion of therapeutic proteins from cells [21]. The cell-material interactions and the mechanical properties of the hydrogel may have an impact on the profile of protein expression, and thus current work in the bio-materials and biomedical fields use gene expressions of cells and their responses to their 3D environments to measure the performance of these tissue-engineering constructs [22,23].
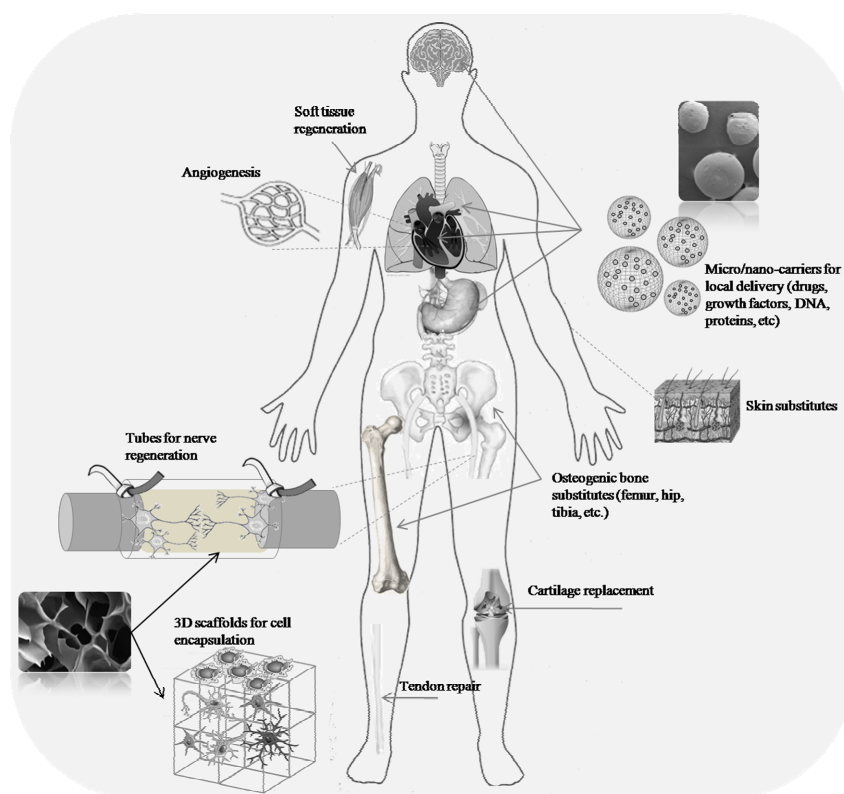

Figure 1. Injectable hydrogel applications for regeneration of various tissues. Examples include scaffolds delivering angiogenic growth factors to induce blood vessel formation, scaffolds seeded with osteoblasts/chondrocytes for bone and cartilage regeneration, growth factor and other bioactive molecules releasing systems for local delivery, and porous conduits-fillers for cell encapsulation and nerve regeneration.

2. MATERIALS

The development of biomaterials for medical applications has focused on the design of biomimetic materials that might be able to interact with surrounding tissues by biomolecular recognition, to make them capable of eliciting specific cellular responses, mediated by specific interactions [24-26]. The selection of a scaffold material is both critical and difficult. A wide variety of biomaterials, both synthetic and natural, is currently available (Table 1). Naturally derived and recombinant biomaterials that combine the beneficial aspects of both natural and many of the desirable features of synthetic materials have been designed and produced (Figure 2). In general, some authors claim that the latter offer some advantages, since they can be tailored to give a wide range of properties and more predictable and reproducible results that the materials derived from natural sources [27-29]. However, natural biomaterials are more likely to induce the appropriate biological response which is fundamental in view of its biomedical application (Figure 2).

An interesting concept suggests the use of entirely autologous hydrogel systems, based on the use of plasma proteins and relying on hemostasis to trigger the gelation process. In this approach, the host plasma is co-injected at the site of interest with culture medium with high free calcium concentration, thus leading to clot formation and an excellent interaction with cells and tissues [30]. A similar approach was described by Yunsong Liu and colleagues, consisting of a novel injectable tissue-engineered bone composed of human platelet rich plasma and human adipose-derived stromal cells, co-injected with thrombin to start gelation [31].
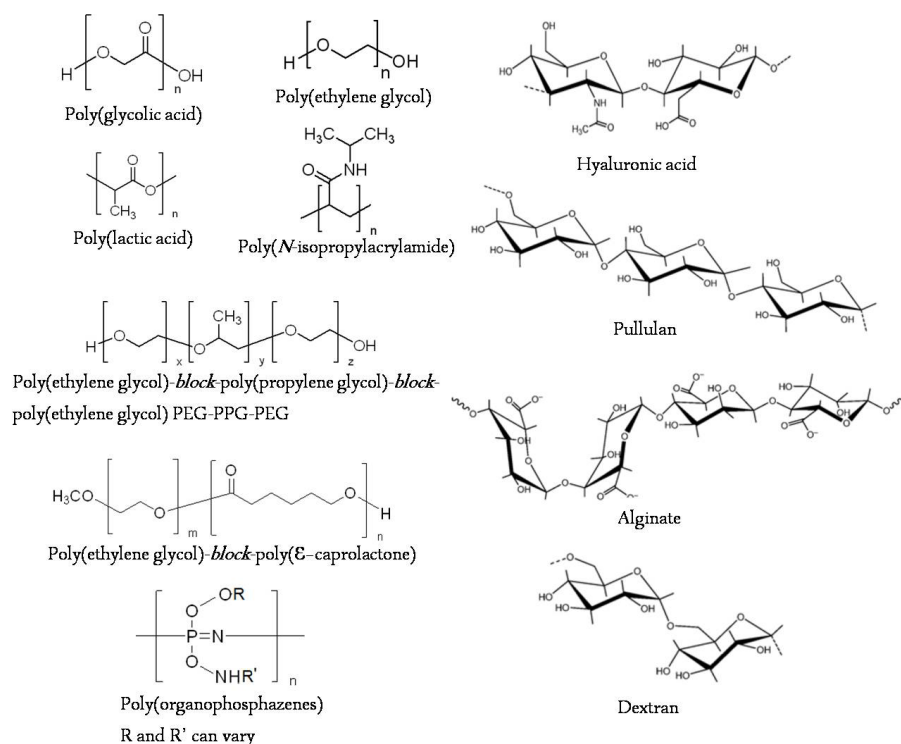

Figure 2. Chemical   structures of selected natural and synthetic materials.

Table 1. Biomaterials currently available as injectable scaffolds.

	Material	Solidification mechanism	Medical applications	References
Inorganic materials	Calcium phosphate	Ceramics setting	Bone/other mineralized tissues regeneration	206-208
Natural organic materials	Collagens	Thermal gelation	Soft tissue regeneration Controlled drug delivery Cell encapsulation Cell culture surfaces	69, 97, 212
	Hyaluronic acid (HA) and derivates	Photo crosslinking		106-108, 184, 184, 206-208
	Alginate	Photo crosslinking/Ionic gelation		19, 35, 60, 68, 99-112, 122-128, 182, 187, 188, 202, 214
	Starch based polymers	Chemical crosslinking Radical Polymerization		57, 77, 83, 84, 99, 100, 103
	MethylCellulose	Thermal gelation		11, 59, 176, 183, 229
	Chitosan	Thermal gelation		19, 61, 101, 103, 112, 178, 187-189, 193, 209.214
	Pullulan	Chemical crosslinking		123, 124, 187
	Blood plasma proteins	Enzymatic (hemostasis) gelation		30, 31, 161. 173, 174, 203
Synthetic materials	Poly(glycolic acid) (PGA) and copolymers	Photocrosslinking Thermal gelation	Bone and cartilage repair Ophthalmic applications Artificial skin Controlled drug delivery Cell encapsulation Cell culture surfaces	202-208
	Poly(lactic acid) (PLA) and copolymers	Photo crosslinking		114, 145
	Poly(vinyl alcohol) (PVA)	Photo crosslinking		44
	Poly(ε-caprolactone) (PCL)	Photo crosslinking		
	Polyanhydrides	Photo crosslinking		
	Poly(N-isopropylacrylamide) (PNIPAM) and copolymers	Thermal gelation		12,45,94,114-117
	Poly(ethylene glycol) (PEG) and copolymers	Enzymatic crosslinking Michael-type addition Photo crosslinking Self assembly		62,72,81
	Poly(propylene fumarate) (PPF)	Photo crosslinking Radical polymerization		54, 74, 215, 216
	Polyurethanes	Chemical crosslinking		68,214,217-223

3. PHYSICAL, CHEMICAL AND BIOLOGICAL PROPERTIES IN DESIGNING INJECTABLE HYDROGELS

The requirements for a scaffold suitable for a biomedical application are complex; however, the following basic characteristics must be addressed to bring about the desired biologic response [32]: (1) The scaffold should be biocompatible. Neither it nor its degradation products should induce any adverse response or toxicity. (2) The scaffold should possess the appropriate mechanical properties, to provide the correct environment, matching the intended site of implantation. (3) Additionally the scaffold should be made from material

with controlled biodegradability or bioresorbability, so that tissue will eventually replace the scaffold. (4) It should have an interconnected pore network, enhancing the diffusion rates, improving oxygen and nutrient supply and waste removal, thereby facilitating the vascularization. (5) Furthermore an appropriate surface chemistry should favor cellular attachment, differentiation and proliferation. (6) The additional characteristic of injectability is an essential issue regarding its final application as an injectable formulation. The scaffold should be easily processed into a variety of shapes and sizes as well as easily sterilized [1,3,9,33-41]. The speed of gel formation is also very important; it should be slow enough as to allow the mixture of the gel components (including cells and active molecules), but fast enough to be of pratical use.

3.1. Biocompatibility

Apart from favorable physico-chemical and mechanical properties, the most important requirement for an injectable hydrogel to be used in medical applications is its biocompatibility in a specific environment, the absence of an exacerbate inflammatory reaction, together with the non-cytoxicity of its degradation products. Most of the toxicity problems associated with hydrogels is associated to the unreacted monomers, oligomers and initiators that leach out during application. These leachables can exhibit varying levels of reactivity and consequently toxicity. In addition, it is also necessary to consider the potential toxicity of the degradation products. Therefore, the knowledge of the degradation processes and the effects that the by-products might have is crucial for long-term success of the hydrogel application [42-45]. Moreover, to prevent infections, the injectable precursors should be sterilized before injection, and the sterilization process itself, should not have significant impact on the chemical properties of the resulting hydrogel [45,46].

3.2. Mechanical Properties

Mechanical properties of hydrogels are very important for pharmaceutical applications and shall match, as much as possible, those of the tissue to be regenerated, since they can have important effects on cellular phenomena such as cell adhesion or even gene expression [47,48]. The scaffold should be stable in the body after injection and solidification, providing the correct mechanical strength to support cellular growth and, at the same time, to withstand biomechanical load. In the case of a drug delivery device, the integrity of the system is crucial, during the lifetime of the application. The system must protect a sensitive therapeutic agent, such as protein, maintaining its integrity until it is released out of the system. The mechanical properties are closely related with the swelling ability and the chemical structure of the matrix. Changes in the crosslinking density of the hydrogels have been routinely applied to achieve the desired mechanical properties, ultimately affecting both the diffusion and the release profiles [49,50].

The mechanical stiffness of hydrogels may furthermore influence the viability and function of encapsulated cells via integrin-ligand bonds. The stiffness of biomaterials regulates the cellular activities of adherent cells, including proliferation, apoptosis, and

differentiation. However, relatively little research has focused on the possibility of regulating the cell's ability to secrete therapeutic molecules through the mechanical properties of a 3D hydrogel matrix. Tuning of mechanical properties of cell-encapsulated hydrogels to an optimal level may improve the cell's ability to secreted drug molecules and also protect cells from external hostile environments [21].

3.3. Degradation Kinetics

The desired kinetics for hydrogel degradation depends on the final application. Degradation is essential in many small and large molecule release applications and in functional tissue regeneration. Ideally, the rate of scaffold degradation should be adequate for the controlled release of bioactive molecules or reflect the rate of new tissue formation. Degradation of hydrogel may occur according to different pathways: hydrolysis, enzymatic cleavage and dissolution. Most of the synthetic hydrogels are degraded through hydrolysis of ester linkages. As hydrolysis occurs at a constant rate, the degradation can be manipulated by the composition of the material, the crosslinking, the molecular weight, morphology and porosity and other factors, such as the pH of the surrounding milieu [51-56]. In addition, the degradability also plays a critical role in determining overall diffusion rates and release profiles; the degradation of crosslinks, by increasing the mesh size of the hydrogel, allows for a facilitated diffusion of the entrapped molecules [57-60].

3.4. Injectability and Solidification Process

For injectable hydrogels, the solidification process will occur *in vivo*. Frequently, at the time of the injection, the precursor is carrying cells and/or bioactive molecules. It is, therefore, essential that the process occurs in a way that is compatible both with the surrounding tissues and with the encapsulated cells. In general, the injectability of a scaffold is related to its rheological properties, the solidification rates being determined by the structure/composition of the formulations and their processing conditions. Some approaches use polymers dissolved in organic solvents, which precipitates after injection as they are insoluble in physiologic fluids [61]. However, organic solvents or harsh processing conditions, such as high setting temperatures, should be avoided, as they may be potentially harmful for cells. Ideally, the solvent used should be physiological saline, cell culture medium or a biologically compatible organic solvent [62,63]. In addition, a commitment in the solidification time must be achieved. It should be, at the same time, short enough to allow for a homogeneous distribution of the cells and/or bioactive molecules and guarantee the hydrogel cohesivity at the injection site, and still sufficient to a proper surgical handling. In this context, thermally or photochemically activated polymerization and crosslinking methods are preferable for a fast and mild solidification procedure. In these cases, the chemical composition and concentration of the macromonomers, the initiators and the crosslinking parameters, are the most important factors influencing the injectability of the scaffold [62,64-67]. Environmentally sensitive polymers have also been paid special attention as they can be tailored to provide solidification at physiologic temperature and pH, in desirable time frames.

3.5. Functionality

The hydrogel scaffold should provide the structural and chemical support for two main functional goals: 1) in tissue engineering applications, to allow cells to proliferate, differentiate and migrate within the scaffold and 2) in both tissue engineering and controlled release applications, to store and release, in a controlled way, bioactive molecules, for instance growth factors. The structure of the scaffold, namely the porosity and interconnectivity of the pores, is of paramount relevance regarding cell proliferation and tissue regeneration. The availability of chemical structures (e.g. peptides), functioning as anchors where the cells attach, may determine the viability of the cells [68]. The chemical and physical properties of hydrogels often regulate the activities of adherent cells, including proliferation and differentiation, performing like a natural extracellular matrix. In a similar manner, the hydrogel properties may also regulate the cellular secretion level of therapeutic molecules by activating the desired cell signaling and subsequently stimulating the gene expression level. Various nanoscale and microscale techniques will probably provide significant benefits in modulating individual properties of hydrogels to continuously control the cellular response [69].

4. MAIN TYPES OF INJECTABLE HYDROGELS

4.1. *In Situ*-Gelling Materials

For the majority of applications, the solidification process of the injectable hydrogels occurs *in situ* (Figure 3, Table 2). The mechanisms underlying this process directly affect the kinetics of the gelation and the stability of the resulting scaffold [70]. Typical *in situ* solidification mechanisms include thermally or photoinitiated chemical polymerization/crosslinking, thermal gelation, ionic crosslinking, self-assembly and Michael-addition reactions.

4.1.1 Chemical Gelation

4.1.1.1. Thermally or photoinitiated chemical polymerization/crosslinking

Thermal or photo activated chemical polymerization/crosslinking are conventional approaches to obtain hydrogels from precursors with functional groups [20,62,71-78]. In this mechanism, radicals produced by an initiator or photoinitiator, react with the functionalized monomers bearing multi residues, in a so-call chain-reaction polymerization, commonly known as radical polymerization. Free radicals can, however, directly react also with cellular components such as cell membranes, proteins and DNA, thereby directly inducing unwanted cellular damage, or indirectly via formation of reactive oxygen species (ROS). Despite the use of the exogenous defenses against oxidative damage and intracellular anti-oxidants to quench ROS, exposure to ultraviolet A (UVA) radiation can induce the formation of ROS. Adverse effects of photopolymerization on viability and cell cycle progression of exposed multipotent stromal cells monolayers has been demonstrated. However, the viability of

hydrogel encapsulated cells was not adversely affected, likely due to the exposition to a lower amount of reactive species available for cell-damage [79]. In tissue engineering applications, the most commonly used functional groups are (meth)acryloyl [71-73,80-86], styryl [87,88] and fumaryl [56,89]. The solidification process is determined by a number of factors including reactivity, functionality, concentration and molecular weight of the precursors, intensity of visible or UV light, temperature, reaction time, as well as the type and concentration of the initiator. Recent advances in photopolimerizable hydrogels, include the use of hyperbranched methacrylated polyglycerol, phosphoester-based polymers and copolymer networks containing lactic and caproic acid segments, with successful application in the tissue engineering field [90-93]. When compared with the thermal activated systems, the photo initiation can be, sometimes, disadvantageous, as some areas show limited capacity of light penetration, restricting its application [86]. Due to this problem, water-soluble redox initiation systems, such as N,N,N´,N´-tetramethylethylenediamine and ammonium persulfate, have been successfully developed by several groups for the production of a variety of hydrogels and aplications [94,95].

4.1.1.2. Chemical crosslinked gels

Concerning the use of reticulating agents free of initiators, such as glutaraldehyde, polyepoxides and isocyanates, the major concern relates to its toxicity, potentially harmful for the human beings [96,97]. Agents that crosslink without incorporation, by activating the carboxylic acid residues in biopolymers, such as acyl azides and carbodiimides, are considered less toxic. The use of potentially less toxic reagent, adipic acid dihydrazide, have been firstly reported by Bouhadir et al. as crosslinking agent for oxidized poly(aldehyde guluronate) [98]. Subsequent studies have successfully applied the same approach to produce injectable *in situ* forming scaffolds from oxidized alginate, gelatin and dextran for cell encapsulation and drug delivery, avoiding the toxicity problems associated with the initiators [99-102]. Due to toxicity concerns, the use of polymeric systems that react without the use of initiators neither crosslinking agents is very attractive. One of such systems was recently introduced by Lihui Weng and colleagues [103]. Oxidized dextran and N-carboxyehtylchitosan readily react under physiological conditions, solidification occurring in a time scale of 1 to 6 minutes, depending on the degree of oxidation of dextran. These gels were successfully used to encapsulate cells and also in wound regeneration assays.

4.1.1.3. Michael-type addition reaction

Hydrolytically degradable PEG-peptide hydrogels, have been produced through a conjugate addition reaction (also termed Michael-type addition reaction) between multiacrylated compounds and dithiols [104-106]. The Michael-type addition reaction can be carried out at physiological temperature and pH without requiring organic solvents. The ester in the conjugate addition product is susceptible to hydrolysis, rendering the material degradable. In addition, during the degradation of the hydrogel, the formed products are primarily neutrally charged, due to the high molecular weight of the PEG multiacrylate in the hydrogel precursor. These properties make this hydrogel formation and degradation mechanism rather suitable for the encapsulation and release of chemically functional, biologically labile agents like protein drugs. The architecture of the networks, namely the

gelation rates and the mechanical characteristics of the resultant hydrogels, can be tailored by a number of factors, such as the functionality, molecular weight and concentration of the precursor macromonomers, the preparation conditions including the stoichiometry of the reactive groups and the pH during cross-linking. Recent studies refer the production of *in situ* crosslinked hyaluronic acid-poly(ethylene oxide) hydrogel for bone regeneration [107,108].
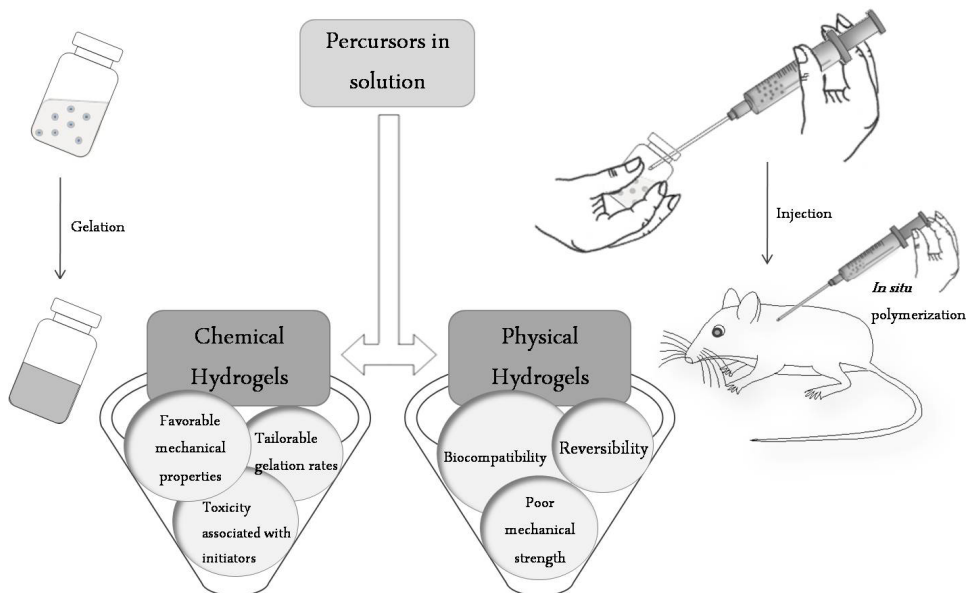

Figure 3. Schematic representation of an injectable hydrogel system. The percursors can be dissolved in water or phisiological buffer solutions. Cells or bioactive molecules can be incorporated in solution before injection. The gelation occurs after injection and the implantation of the biomaterial can be carried out in a minimal invasive manner. Chemical hydrogels are often highly versatile and the resulting networks possess superior mechanical strength. However, toxic chemical agents are often employed in its formulations, adversely affecting cells and bioactive molecules during solidification. Physical crosslinking can overcome these limitations, as the initiatores are avoided, but the resultant networks usually possess limited mechanical properties and stability.
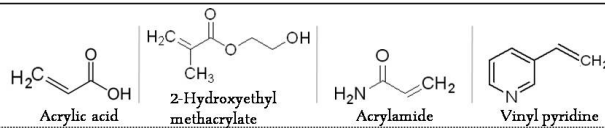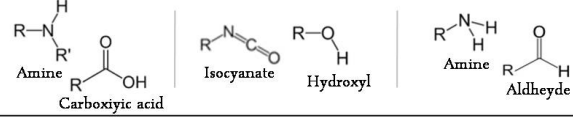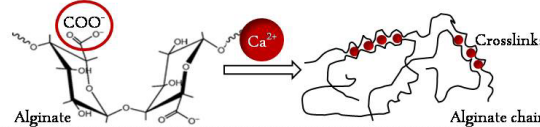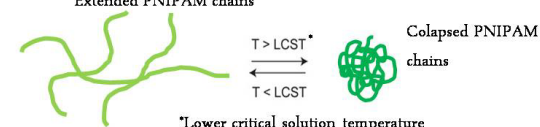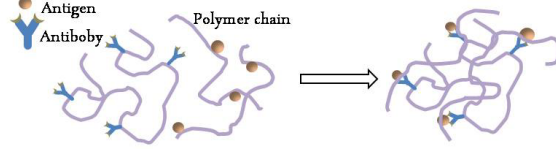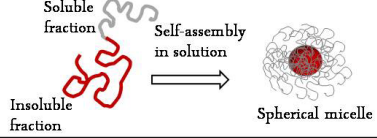
4.1.2 Physical Gelation

4.1.2.1. Thermal gelation

Injectable physical hydrogels constitute promising alternatives to chemical hydrogels. Some polymer solutions have the ability to undergo gelation in response to a change in temperature. The major advantage in using physical gelation is that this process does not require any chemical reaction, therefore avoiding the biocompatibility problems associated with residual initiators or monomers. In addition, the gelation point can be set at a temperature close to the human body, so that they can be injected in a liquid form before solidifying inside the body. Both natural-based and synthetic thermogelling polymer systems have been developed and tested in tissue engineering [109,110]. Among the natural polymer-

based hydrogels that have been reported are cellulose derivatives [11], laminin [111], chitosan and gelatin [112,113]. As the most intensively investigated thermosensitive synthetic polymers are N-isopropylacrylamide-based copolymers, poloxamer (Pluronics®) polymers and PEG-based block copolymers [12,45,94,114-117]. These materials exhibit a sol–gel transition, as the temperature is increased above their lower critical solution temperature (LCST), which is due, in general, to the drastic solubility differences below and above that critical temperature. The gelation is related to the chain entanglement and the gradual chain collapse as the temperature increases [118,119]. The transition temperature underlying the gelation process can be further tuned by changing the polymer concentration and/or molecular weight, and the composition of the copolymers. Once the gels are formed, they do not change their water content and the gelation is reversible without appreciable hysteresis. Besides the advantage of being free of chemicals, thermogelling formulations offer the additional benefit that the low temperature used when mixing polymers and drugs before injection, protects them from denaturation or aggregation, the same occurring with cells [120,121].

Table 2. Hydrogel classification based on the solidification mechanism

4.1.2.2. Ionic crosslinking

Hydrogels formed through ionic crosslinking belong to the group of *stimulus*-responsive scaffolds. It is well known that aqueous solutions of alginate can form hydrogels in the presence of di- or trivalent cations and have shown excellent potential in a variety of biomedical applications, including scaffolds for tissue engineering or carriers for drug delivery systems [122]. Commonly derived from seaweed, alginate is a linear polysaccharide consisting of β-D-mannuronic acid (M) and α-L-guluronic acid (G) monomers that are arranged in blocks of G and M in varying proportions and sequential arrangements [122,123]. The gelation process can be easily controlled by the cation type and concentration, as well as by alginate composition and concentration, and gelation temperature [124]. The drawback associated with the use of these systems is that the ionically crosslinked hydrogels have a tendency to swell and eventually dissolve in physiological environment, with the mechanical properties and the overall dimensional stability being compromised because of loss of crosslinking ions when in the presence of calcium chelators (e.g. phosphates), monovalent ions (e.g. K^+, Na^+, etc.), and noncrosslinking divalent ions (e.g. Mg^{2+}), which are often present in tissue culture medium and other biological solutions [125]. Alginate hydrogels are commonly used as extracellular matrix analogues, with excellent biocompatibility and biointeractive behavior [126,127]. In spite of these results, various efforts have been made to stabilize alginate crosslinks, such as the use of barium- and copper-crosslinked or covalently crosslinked alginate gels, which are relatively stable in aqueous solutions. However, both the cations and the reagents involved in the covalent crosslinking reactions are often cytotoxic. Alternatively, promising results have been described by Kuo and Ma, which achieved dimensional stability via controlling the calcium ion concentration of a culture environment [128].

4.1.2.3. Self-assembly

Self-assembling systems do not use chemical crosslinking agents or initiators, preventing the biological systems to be exposed to these potentially cytotoxic chemicals, which represents a major advantage of the self-assembly strategy [129]. Unfortunately, due to the lack of covalent crosslinking, hydrogels formed this way, often lack the mechanical strength that can be achieved through the conventional methods of chemical crosslinking. The self-assembled systems can be unable to withstand the, sometimes, great mechanical load or tension of tissue engineering applications. On the other hand, it is not possible to fine-tunethe release profiles, as the crosslinking density cannot be adjusted in many self-assembled systems [130,131]. Two major strategies are currently used in the production of these systems are the phase segregation and the use of amphiphiles. In the case of phase segregation, as the polymer phase is water insoluble, the injection into an aqueous environment results in exchange of the injected solvent with water from the surrounding environment, leading to precipitation of the polymer phase [131-133]. The amphiphiles have both hydrophilic and hydrophobic domains, such that the macromolecules self-assemble to maximize interactions between the hydrophilic domains and the environment [134-142]. The hydrophilic domain is often a peptide specific for cell integrins, thus allowing a control over the interaction with the surrounding living tissue or the encapsulated cells. In addition, under the principles of the self-assembly, other conformations have been developed, such as self-assembling micelles

containing hydrophobic drugs within their hydrophobic core [143]. The control of the relative molecular weight and relative hydrophilic/hydrophobic character of the domains provides a simple way to obtain fibres, sheets or spheroid structures.

4.2. Pre-Gelated Composite Materials (Micro and Nanoparticles)

Despite the great attention paid to the *in situ* gelation processes, a wide variety of composite materials is available as injectable systems, most of them finding application in the drug delivery field . The most common carrier materials are PLGA [144,145] and gelatin [146], and the majority of formulations refer to degradable micro- or nanoparticles or spheres that contain the drug to be delivered [95,147-153]. The injectability of the particle containing systems depends on the particle concentration and the type of injection system used, and must be carefully chosen in order to obtain an effective delivery of the composite in the appropriate location with a reasonable amount of force needed for injection [154-160].

5. SMART AND BIOMIMETIC DEVICES

Recent advances in the development of hydrogels encompasses its modification, at a surface or bulk level, so that they will selectively interact with cells through specific biomolecular recognition, thus, being rendered 'biomimetic'.

Early studies reported on the use of long chains of ECM proteins such as fibronectin (FN), vitronectin and laminin for surface modification, with successful promotion of cell adhesion and proliferation [161]. More recent trends leave behind the long chain proteins, using instead short peptide fragments and antibodies as signaling domains, which are more stable during the modification process and can be massively synthesized. The most commonly used peptides for modification are Arg-Gly-Asp (RGD), the signaling domain derived from fibronectin and laminin, Tyr-Ile- Gly-Ser-Arg (YIGSR), Arg-Glu-Asp-Val (REDV) and Ile-Lys-Val-Ala-Val (IKVAV) [64,162-167].

The functionalization with these bioactive molecules, serving the purpose of mimicking ECM, allows for the modulation of cellular functions such as cell attachment, proliferation, and differentiation.

There are some situations, namely in controlled delivery, which require the use of 'intelligent' hydrogels. Hydrogels have a structure that can be tethered, allowing for control of drug diffusion, the sensitivity to its environment, or the recognition of a specific target by incorporation of functional groups in the matrix. A specific feature includes the incorporation of enzymatically or otherwise cleavable sequences, allowing the degradation to be modulated by the presence of enzymes or other compounds that specifically recognize cleavage sites within the hydrogel, which is a very fine approach in hydrogels designed for controlled release, since the cleavage of the sequence releases the bioactive agent to which is linked [168].

Another typical example is a hydrogel prepared by grafting an antigen and the corresponding antibody to the network structure. The binding between the antigen and the antibody introduces extra crosslinks in the network. When the hydrogel is in contact with free

antigen solutions, competitive binding of the diffused free antigen triggers a change in the hydrogel volume owing to the breaking of the noncovalent crosslinks.

The possibilities offered by a precise molecular design, confers these smart devices precise bioresponsive behaviour, not only in improving cell-material interactions, but also in the release properties and specific targeting.

Taking the nanoparticles as an example, recent works report the covalent attachment of drugs via cleavable linkers [169,170]. These responsive particles presented an oxidation-sensitive bulk and PEG outer layer with RGD-containing peptide sequences [171]. The presence of an oxidative environment as sign of inflammatory reactions, allows for the release to be driven in an inflammation-sensitive fashion. In addition, the pegylated surface prolonged the circulation time in body fluids.

5.1. Autologous and ECM-Like Hydrogels

Research on the design of scaffolds for regenerative medicine is shifting away from the use of inert synthetic materials towards an increasing emphasis on interactive scaffolds, which can influence cell adhesion or phenotype expression through selective ligand presentation or targeted degradation. A family of peptide-amphiphile (PA) molecules that self-assemble into high-aspect ratio nanofibers under physiological conditions, and can display bioactive peptide epitopes along each nanofiber's periphery, has been developed in an attempt to design suitable bioactive scaffold materials that can act as artificial extracellular matrices [140].

ECM hydrogels have been utilized as vectors for cellular delivery using a mixture of ECM components associated with cell adhesion and recognition, such as collagen, keratin, elastin and fibrin, which have been used for tissue engineering applications such as nerve conduits. These components provide an ideal substrate for cellular delivery and *in vivo* culture [69].

Recent applications of injectable fibrin hydrogels include the incorporation of the biological activity of ECM proteins, such as fibronectin, vitronectin, laminin, and collagen. The peptide domains responsible for the biological activity of these proteins are synthesized for covalent cross-linking to the fibrin hydrogel through a transglutaminase-catalyzed reaction, in which the bioactive domain peptide is coupled to a transglutaminase substrate sequence (NQEQVSP) to generate a bifunctional peptide or bi-domain peptide. Moreover, as an extension of this concept, a heparin-binding domain can be synthesized for subsequent crosslinking to fibrin [172].

A further development of the biomimetic approach relies on the production of hydrogels on entirely autologous materials and mechanisms. Gels are derived from autologous host plasma, thus providing a totally natural material for cell expansion and implantation, overcoming potential complex histocompatibility issues associated with organic implantable biomaterials. Furthermore, the exploitation of enzymatic mechanisms such as hemostasis allows for the gelification of materials without any chemicals [30]. Geuze and colleagues have recently used a similar cell-based bone-tissue engineering strategy, in which an injectable platelet gel, obtained from a platelet and leukocyte-rich plasma mixed with trombin and combined with bone marrow stomal cells (BMSCs), was used to promote bone formation [173]. In another study, Yamada and co-workers also used an injectable platelet-rich plasma

scaffold with immobilized osteogenic differentiated BMSCs for bone regeneration in maxillary sinus augmentation [174]. The same approach was attempted by Cheng et al., using similar scaffolds as bone grafts substitutes, in the repair of cranial deffects in rabbits, with promising results [175]. Additionally, injectable mixtures of hydroxyapatite/tricalcium phosphate particules, fibrin and *ex vivo*-expanded BMSCs, could assemble into mature bones with histologic and mechanical properties similar to standard bone transplants (Mankani et al. 2008 Tissue Engineering). The use of an injectable cellulose-based hydrogel containing autologous chondrocytes was successfully used for the repair of articular cartilage [176].

6. TISSUE ENGINEERING AND DELIVERY APPLICATIONS

The injectable formulations are currently being applied in research areas encompassing sustained drug [109,120,177-179], cell [152] and gene delivery [153,180,181], vaccination [182], tissue adhesion prevention [77,78,183,184], soft and mineralized tissue regeneration or promotion of angiogenesis. Generically, the problem underlying the delivery of bioactive molecules in a given location lies in the short half-life and easy diffusion of these compounds *in vivo*, giving rise to the need of an appropriate system which enhances the delivery efficacy. In this context, injectable hydrogels have been studied as such a delivery vehicle due to their easy preparation and handling.

The subcutaneous delivery of hidrogels loaded with dendritic cells successfully triggers an immune response. It has been demonstrated that antigene-loaded dendritic cells, after a prolonged time period at a defined site, behaving as "vaccination nodes", led to the recruitment of activated antigen-specific T cells, thus showing as a promising immunotherapy tools [182].

Hafeli and coleagues have recently developed a radiopharmaceutical system that consists of an injectable gel to be applied in brain tumors to deliver high focal doses of radiation [185,186]. The gel, which strongly adheres to tissue in the treatment area, consists of fibrin containing the β-emitters rhenium-188 and rhenium-186 in microsphere-bound form. This gel provides an effective method of delivering high doses of local radiation to tumor tissue, particularly to wet areas where high adhesive strength and long-term radiation (with or without drug) delivery are needed. Normal brain tissue can be spared and the application of radioactive fibrin gel may be possible even in critical areas such as near the optical nerve.

6.1. Angiogenesis

Angiogenesis – a key process in tissue regeneration – is a challenging task achieved through the controlled release of vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF). In this context, several hydrogels have been developed such as alginate/heparin microparticles, alginate microspheres or beads, fibrin, sodium hyaluronate, chitosan and PLGA millicylinders[187-194]. Recent works by Leroyer et al. report the use of microparticles containing CD40 ligand+ from human atherosclerotic plaques to stimulate endothelial proliferation and angiogenesis [195,196].

6.2. Cartilage Regeneration

For the purpose of cartilage regeneration, the injectable scaffolds have also found a great application. Since during cartilage repair, extracellular matrix formation is affected by the properties of the scaffolds, such as swelling ratio, compression modulus, degradation rate and cell seeding density, a careful control over the crosslinking density and structure of the macromonomers is, thus, necessary [81,197]. Various systems have been developed fitting these requirements, including oligo(poly- (ethylene glycol) fumarate) [147], poly(N-isopropylacrylamide) copolymers [198-201], poly(ethylene oxide) [62,72,81] alginate, fibrin [197], PLGA-g-PEG [45,114], pluronics [202-205], calcium phosphate/hyaluronic acid composites [206-208] and chitosan [209-211]. A hydrogel composite of collagen-coated polylactide microcarriers with chitosan, formed at neutral pH and body temperature, was recently used as an injectable scaffold for cartilage regeneration [212]. The Antonios Mikos group investigated the development of an injectable, biodegradable hydrogel composite of oligo(poly(ethylene glycol) fumarate) (OPF) with encapsulated rabbit marrow mesenchymal stem cells (MSCs) and gelatin microparticles (MPs) loaded with transforming growth factor-b1 (TGF-b1) for cartilage tissue engineering applications [213]. Rabbit MSCs and TGF-b1-loaded MPs were mixed with OPF, a poly(ethylene glycol)-diacrylate crosslinker and the radical initiators ammonium persulfate and N,N,N',N'-tetramethylethylenediamine, and then crosslinked at 37 ºC for 8 min to form hydrogel composites. The authors demonstrated the viability of MSC in the hydrogel and the up-regulation of cartilage relevant genes.

6.3. Bone Regeneration

Injectable scaffolds have also been extensively investigated for applications in bone and soft tissue regeneration. Injectable materials have been successfully used as osteogenic bone substitutes [214]. Recent advances in chemistry, molecular biology, physiology, and biomaterials science have translated into the development of novel synthetic, injectable bone graft substitutes. These materials may offer several advantages over the traditionally used autografts or allografts. The flowable nature and *in situ* polymerization of these materials allows filling defects of any shape. In addition, the clinician can fill the defect via minimally invasive procedures and avoid the morbidity associated with traditional open surgical exposures. Important intrinsic features of synthetic bone substitutes include material composition, mechanical strength and stiffness, biocompatibility, safety, and degradation time. An essential characteristic of the synthetic graft is its microarchitecture. For optimum bone ingrowth, the material must possess a system of interconnected pores that allows cellular migration, deposition of extracellular matrix, and the diffusion of nutrients and waste products [215].

Poly(propylene fumarate) (PPF) has been investigated as an injectable, biodegradable scaffold for orthopedic applications. The foaming technique created a porous, interconnected scaffold, demonstrating that clinically useful polymers can be fabricated for use in various bone tissue engineering applications [215].

Adhikari et al. developed a two-part injectable prepolymer system (crosslinked polyurethanes) [216]. The mixture, with incorporated tricalcium phosphate, remains

injectable for up to 10 minutes, and gradually increases viscosity. Sheep studies demonstrated that the injected polymers did not cause any adverse reaction and evidence of new bone growth and gradual degradation of the polymers was observed up to 6 months.

Injectable scaffolds have also been extensively investigated for applications in bone and soft tissue regeneration. A large variety of injectable materials is being successfully developed as osteogenic bone substitutes [68,214,217-223].

6.4. Neural Regeneration

Injectable formulations have found their ultimate function in promoting the axonal rewiring in spinal cord injuries [224-227].

Peptide amphiphile (PA) molecules that self-assemble *in vivo* into supramolecular nanofibers were used as a therapy in a mouse model of spinal cord injury (SCI) [228]. Because self-assembly of these molecules is triggered by the ionic strength of the *in vivo* environment, nanoscale structures can be created within the extracellular spaces of the spinal cord by simply injecting a liquid. The molecules are designed to form cylindrical nanofibers that display to cells of the spinal cord the laminin epitope IKVAV. IKVAV PA nanofibers are known to inhibit glial differentiation of cultured neural stem cells and to promote neurite outgrowth from cultured neurons. In this work, *in vivo* treatment with the PA after SCI reduced astrogliosis, reduced cell death, and increased the number of oligodendroglia at the site of injury.

Another recent work reported the use of injectable liquid agarose and methylcellulose hydrogel combinations, which polymerize once exposed to physiological temperatures naturally [229]. Furthermore, *in vitro* experiments suggested that the application of the hydrogel did not negatively affect neurons spared from the initial injury. Therefore, these hydrogel blends could prove to be beneficial as a component of a multi-faceted neuronal treatment, through providing a mechanism for drug delivery and anchoring scaffolding for directed regenerating of neurons through an injury site.

7. FINAL REMARKS

In the past decade one assisted to the rising of a wide range of injectable formulations developed via chemical or physical processes. Injectable systems offer the advantages of filling irregular shaped defects, simple incorporation of cells and/or pharmaceutically active agents, doing so with limited surgical invasion. Chemical cross-linking is highly versatile for the preparation of injectable scaffolds, and the resulting networks possess superior mechanical strength. However, toxic chemical agents are often employed in the formulations, adversely affecting cells and bioactive molecules during solidification. Physical crosslinking can overcome these limitations, but the resultant networks usually possess limited mechanical properties and stability. Therefore, careful selection of precursor formulation and appropriate crosslinking methods are crucial to the preparation of injectable hydrogels. In light of the rapid development of regenerative medicine, the demand for new systems that can fulfill its

challenging requirements is increasing. Likewise, novel injectable hydrogels are welcomed and will have to be tailored to fit specific future applications.

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