Natural-origin materials for tissue engineering and regenerative medicine

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Abstract

Recent advances in tissue engineering and regenerative medicine have shown that combining biomaterials, cells, and bioactive molecules are important to promote the regeneration of damaged tissues or as therapeutic systems. Natural origin polymers have been used as matrices in such applications due to their biocompatibility and biodegradability. This chapter provides an up-to-date review on the most promising natural biopolymers, focused on polysaccharides and proteins, their properties and applications. Membranes, micro/ nanoparticles, scaffolds, and hydrogels as biomimetic strategies for tissue engineering and processing are described, along with the use of bioactive molecules and growth factors to improve tissue regeneration potential. Finally, current biomedical applications are also presented.

Keywords: Cell encapsulation; cryopreservation; composites; growth factors; hydrogels; hypothermal preservations; immunoisolation; membranes; micro/nanoparticles; natural polymers; proteins; regenerative medicine; scaffolds; tissue engineering.

LIST OF ABBREVIATIONS

ACE – Acemannan
AF- Annulus fibrosus
ALP- Alkaline phosphatase
BC - Bacterial cellulose
BMPs - Bone morphogenetic proteins
CA - Carrageenan
CaP- Calcium phosphates
CAV- Chitosan/aloe vera
CH/CS NPs - Chitosan-chondroitin sulfate nanoparticles
CS - Chondroitin sulfate
2D - Two-dimensional
3D - Three-dimensional
Da- Daltons
DS- Dermatan sulfate

ECM - Extracellular matrix

EGF- Epidermal growth factor

ESCs - Mouse embryonic stem cells

FGF – Fibroblast growth factor

GAGs- Glycosaminoglycans

GelMA- Methacrylated gelatin

GF- Growth factor

GG- Gellan gum

GG-MA- Methacrylated gellan gum

hBMSCs- Human bone marrow mesenchymal stem cells

hMSCs- Human mesenchymal stem cells

HAp -Hydroxyapatite

HA – Hyaluronic acid

HS- Heparan sulfate

HUVECs - Human umbilical vein endothelial cells

IGF- Insulin growth factor

IL- Ionic liquid

IVD- Intervertebral disc

MWCO - Molecular weight cut-off

MSCs - Mesenchymal stem cells

NP- Nucleus pulposus

PDGF- Platelet-derived growth factor

PDMS – Poly(dimethylsiloxane)

PEG – Polyethylene glycol

PL- Platelet lysate

PLGA- Poly(lactic-co-glycolic acid)

PRP- Platelet-rich-plasma

PVP- Polyvinylpyrrolidone

RP - rapid prototyping

TE – Tissue Engineering

TERM- Tissue engineering and regenerative medicine

TGF- Transforming growth factor

TMTD - Triazole–thiomorpholine dioxide

SF- Silk fibroin

SiNP - Silica nanoparticles

SRP- Stimulus-responsive polymers

PNIPAAm - Poly(N-isopropylacrylamide)

VEGF – Vascular endothelial growth factor

1. Introduction

Tissue engineering and regenerative medicine (TERM) is a multidisciplinary field that combines three fundamental tools, namely biomaterials, adequate cells and signaling molecules aiming to replace and regenerate damaged tissues. Current efforts focus on developing strategies that include 3D-based matrices to support cells, promoting their differentiation and proliferation envisioning the formation of new tissues. Such approaches will provide the production of hybrid constructs with adequate structural and mechanical properties that behave as bioresorbable temporary implants to induce the tissue regeneration or replace failing or malfunctioning organs.

Several materials have been proposed to be used in the processing of scaffolds, namely natural biodegradable polymers. Natural origin polymers derived from renewable resources, namely from algae, animal, plant, and microorganisms, offer the advantage of being similar to biological macromolecules, which biological environment is prepared to recognize and to deal metabolically with. Due to their similarity with the extracellular matrix (ECM), natural polymers may also avoid the stimulation of chronic inflammation or immunological reactions and toxicity, often detected with synthetic polymers. Therefore, the features of the natural polymers are the key players for designing therapeutic systems that can be used to deliver drugs or bioactive compounds efficiently to treat diseases or even to bioengineer functional tissues for organ replacement. This chapter provides an up-to-date review on the most promising natural origin polymers, namely polysaccharides (*e.g*. alginate, cellulose, chitosan) and proteins (*e.g*. collagen, silk fibroin, elastin) that have been proposed to be used in tissue engineering (TE) strategies, with some emphasis on their properties, and the strategies used for the processing such kind of materials into membranes, scaffolds and hydrogels. Moreover, materials containing cells and/or bioactive cells or even cell encapsulation into these materials that can be used in a non-invasive way are also discussed.

An overview of the properties, strategies and biomedical applications, namely cell encapsulation and intervertebral disc regeneration of natural polymers herein addressed is presented in Figure 1.

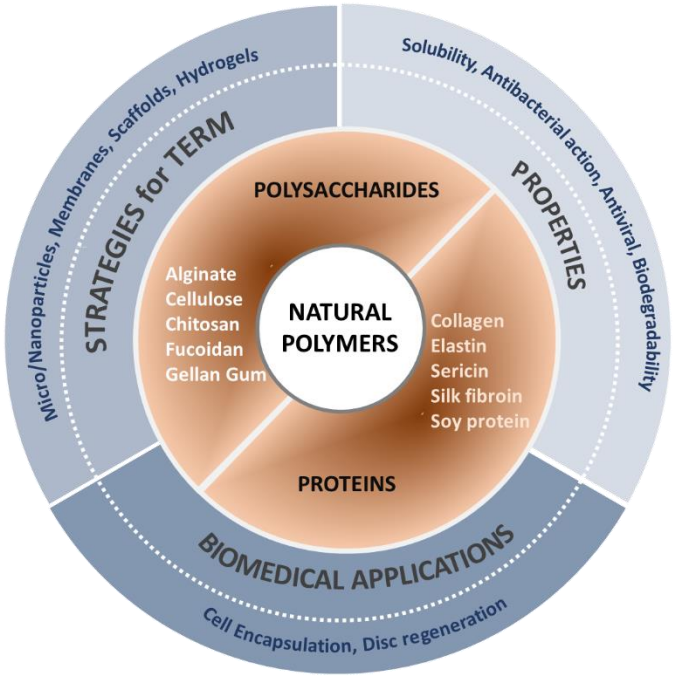

Figure 1: Overview of the properties, strategies and biomedical applications of natural polysaccharides and proteins for TERM.

2. **Polysaccharides and proteins**

2.1. **Polysaccharides**

Polysaccharides consist of repeating monosaccharide units linked by glycosidic bonds. Besides, polysaccharides can be obtained or synthetized from different abundant renewable resources in nature, namely algae (*e.g.* alginate, agarose, carrageenan, fucoidan, and ulvan), plant (acemannan, cellulose, and starch), microbial (bacterial cellulose, dextran and gellan gum) and animals (*e.g.* chitin/chitosan, chondroitin sulphate, glycosaminoglycans, heparin and hyaluronan).[1] As natural biopolymers polysaccharides are biodegradable, biocompatible and non-toxic.[1b] Furthermore, polysaccharides have a great number of reactive groups such as hydroxyl, carboxyl, and amino groups that make them easily chemically modified or physically blended.[2] These characteristics together with their intrinsic and biological properties suggested that polysaccharides are promising biomaterials. The physicochemical and biological properties of the mentioned polysaccharides are reviewed in the following sections.

2.1.1. Algae polysaccharides

Alginate is a linear polymer of β(1→4)-linked D-mannuronic acid and α(1→4)-linked L-guluronic acid. [3] Alginate is present in the cell wall of brown algae as different salt forms of alginic acid, namely calcium, magnesium and sodium salts.[3-4] Gelation is an important feature of alginates, and it depends on its molecular weight, molecular structure, and gelation agent concentration (*e.g.* calcium ions concentration).[2c, 3] For instance, alginate gels can be formed through hydrogen bonding at low pH or by ionic interaction with di- or polyvalent cations.[2c, 5] Besides controllable gelation, alginate is recognized by its biocompatibility, immunogenicity, and low toxicity. In TE, alginate is used in the development of hydrogels, micro/nanoparticles, and beads, for various applications including wound healing, protein delivery, and cell encapsulation.[3, 5] However, some drawbacks of alginate have been evidenced such as uncontrollable degradation profiles, inadequate mechanical properties and lack of cell recognition signals.[2a] Nevertheless, the application of conventional chemical modification on alginate including oxidation, graft copolymerization, sulfation, and esterification can enhance or tailor its properties, as reviewed previously.[2a, 2b]

Agarose is a linear polysaccharide found in seaweeds composed of (1→3)-β-D-galactopyranose-(1→4)-3,6-anhydro-β-L-galactopyranose unit. This neutral polysaccharide forms thermoreversible gels when solubilized in water.[6] In biomedical applications, the thermosensitive character of agarose has been combined with other biomolecules as chitosan, gelatin, and fibrin.[7] These approaches have been utilized in the formation of hydrogels that can be applied directly at the desired site; and in the fabrication of different bioengineered tissues, including skin and peripheral nerve.[7b, 8]

Carrageenan (CAs) are highly sulfated linear polysaccharides, present in red algae, with alternating repeating 3,6-anhydro-D-galactose and β-D-galactose 4-sulfated units. The number of sulfate groups per disaccharide unit in the structure determines the type of CAs that can be kappa (κ), iota (ι) and lambda (λ) distinguished in the presence of one, two or three sulfate groups, respectively. One of its main properties relies on the ability to form thermoreversible gels at room temperature in the presence of appropriate counterions. Recently, κ –CA-based materials have gained wide interest for TE applications namely as an injectable matrix for the delivery of living cells, bone regeneration, designing drug

delivery systems due to their intrinsic thixotropic behavior, the versatility of processing and resemblance to GAGs.[9] Moreover, the presence of functional groups (i.e. hydroxyl and sulphate groups) allows them to be easily modified, generating derivatives with improved properties and better stability.[9a, 9c]

Fucoidan is an anionic sulfated polysaccharide extracted from marine brown algae. Structurally, fucoidan is composed of a backbone of (1→3)- and (1→4)-linked α-l-fucopyranose residues; that may be organized in stretches of (1→3)-α-fucan or of alternating α(1→3)- and α(1→4)-bonded l-fucopyranose residues.[10] Moreover, fucoidan is a water-soluble polymer with several biological properties for TE approaches including anti-inflammatory, anti-oxidative, anticoagulant, antithrombotic, anti-tumor and reducing blood glucose.[11] In fact, the ability to combine fucoidan with other polymers like chitosan and gelatin and/or inorganic components, such as tricalcium phosphate and hydroxyapatite (HAp), has been explored for the production of matrices (*e.g.* micro/nanoparticles, fibers, and films) useful for oral delivery, burn wound healing and bone TE.

Ulvan is a sulphated polysaccharide isolated from green algae belonging to *Ulva sp.*[12] The composition of ulvan is variable, but rhamnose, uronic acid, xylose and sulphate groups are the main components of this polymer.[13] Recently, ulvan has been suggested as a starting material for designing many structures, namely nanofibers, membranes, hydrogels and 3D porous materials[13-14]. The applicability of these matrices may range from wound dressing to drug delivery and cell delivery. Moreover, ulvan can be easily modified through its functional groups (carboxyl, hydroxyl and sulphate groups) to achieve the needs for an envisaged biomedical application.

2.1.2. Animal polysaccharides

Chitin and chitosan are polysaccharides with great structural possibilities for the creation of the derivatives and matrices for different biomedical applications, including TERM.[15]

Chitin (β-(1-4)-poly-*N*-acetyl-D-glucosamine) can be found in shells of crustaceous such as crabs and shrimps.[15a] Furthermore, chitin can also be extracted from endoskeletons of cephalopods. Depending on its source, chitin can be characterized by α- and β-form, distinguished by strong and weak inter- and intramolecular bonds, respectively.[16] This polymer has appealing properties namely biocompatibility, tumor cell growth suppression, acceleration of wound healing and antimicrobial activity.[1a, 15a] These

properties have encouraged the production of chitin-containing products such as artificial blood vessels, tumor inhibitors, vascular implants and dressing for burns. Despite the promising biomedical use of chitin, its application has been limited due to its strong inter- and intra-molecular bonding between the polymer chain, which results in a lack of solubility in water and common organic solvents[1a]. Nevertheless, recent studies proposed that ionic liquids (ILs), defined as organic salts like 1-butyl-3-methylimidazolium acetate,[17] can be used not only as a solvent but also as reaction media of some polysaccharides, including chitin,[18] chitosan,[19] cellulose[20] and silk fibroin.[21] In particular, chitin/IL solutions can be used in the preparation of 2D and 3D-based matrices namely gels, films, sponges and micro/nanoparticles. Given the performance of the resulting chitin-based matrices, they may be applied in drug delivery, bone regeneration, gene delivery, and skin repair.[18a, 18c, 22]

Chitosan is the N-deacetylated derivative of chitin.[15a] Chitosan is recognized as an important biomaterial due to its intrinsic properties namely its polyelectrolyte and cationic nature, mucoadhesion, hemostatic action, film-forming ability, biodegradability and anti-microbial action.[1a] Moreover, chitosan is a versatile raw material for the designing of 2D and 3D-based matrices such as membranes, fibers, particles and composites at micro/nano level for applications in bone, skin and cartilage TE.[15a] The combination of chitosan with other polymers (natural or synthetic ones) and/or proteins are approaches frequently used to create blends and composites.[23] Besides, chitosan derivatives have been developed using several chemical and physical procedures. These chitosan-based materials have suitable properties and functionalities for biomedical purposes, for instance, dermal substitutes, cancer diagnosis, drug delivery and gene therapy.[15a] With the rise of nanotechnology, chitosan, together with other macromolecules, has also been fabricated into various bionanocomposites, providing alternative applications in regenerative medicine and drug delivery vesicles.[5, 24]

Glycosaminoglycans (GAGs) are linear, complex and polydisperse natural polysaccharides, composed of a repeating disaccharide unit formed by a hexose and a hexosamine. GAGs can regulate the action of proteins essential to bone and skin regeneration and modulate the biological performance of skin and bone precursor cells and their subsequent differentiation and gene expression. However, the precise action of GAGs varies according to their structural composition mainly on the degree of sulfation and polymer length. The sulfated GAGs such as chondroitin sulfate (CS), dermantan sulfate (DS), and heparan sulfate (HS) represent a group of bioactive polysaccharides

derived from animals. The characteristics of the mentioned GAGs together with their defined physical properties make them interesting material for TE. For instance, CS has functions in wound repair, response to infection, growth factor signaling, morphogenesis, and cell division, in addition to their conventional structural roles.[25] Moreover, many studies using shark-derived CS suggests that this type of CS may reduce allergic response and have an anti-allergic effect. CS and DS chains have interesting functions in central nervous system development, wound repair, infection, growth factor signaling, and cell division.[25]

Hyaluronic acid* or *hyaluronan (HA), is a water-soluble polysaccharide formed by a structure linear with a repeating disaccharide structure composed by α-1,4-D-glucuronic acid and β-1,3-N-acetyl glucosamine bonds. As a biomaterial, HA is suitable for a wide range of applications due to its biodegradability, biocompatibility, viscoelastic properties, nontoxicity, and nonimmunogenicity. Nonetheless, HA has low shape stability and poor mechanical properties. So, usually, HA is a key component in the preparation of 2D and 3D matrices such as membranes, hydrogels, and scaffolds that can be either chemically or physically modified or degraded controllably.[26] Hence, HA and HA-based materials serve as excellent tools for many biomedical needs such as orthopaedic, cardiovascular, pharmacologic and oncologic applications.[26-27]

2.1.3. Plant polysaccharides

Acemannan (ACE) *is a* -β(1→4)-acetylated polymannose present in the inner leaf gel of Aloe vera plant, a medicinal plant.[28] ACE has been described by its multifunctional properties such as immunomodulatory activity, antiviral, antioxidant and antibacterial actions.[28-29] Therefore, ACE acts as a bioactive molecule, exerting an immunostimulatory effect by activating macrophages,[30] induction of VEGF expression and wound healing actions.[29, 31] Recent studies demonstrated that ACE stimulated gingival fibroblast proliferation [32], dental pulp fibroblast proliferation,[33] periodontal tissue regeneration [34] and bone marrow stromal cell proliferation and differentiation *in vitro*.[31] These works suggested ACE as a therapeutical agent for tissue repair. However, further research studies are needed to validate and explain the action mechanism of ACE in healing.

Cellulose, the most abundant polysaccharide in nature, is constituted by β(1→4) linked-D-glucopyranose units. The polymeric chains are packed through hydrogen bonds and van der Waals forces forming fibrous structures called microfibrils. These nano structured

microfibrils with high structural strength and stiffness. This crystalline part is named nanowhiskers. Despite its great availability, cellulose has a high crystallinity degree and rigid intra/intermolecular hydrogen bonds which result in its insolubility in most solvents. To overcome these drawbacks, many chemical functionalizations based on hydroxyl groups, as e.g. esterification, graft copolymerization, and selective oxidation has been used to generate different cellulose derivatives.[2a] For instance, oxidized cellulose is used as an excellent hemostatic material in various surgical procedures; while other cellulose derivatives (in hydrogel and aerogel form) have been used in wound healing, 3D-cell culture, and drug delivery systems.[35] Besides, recent advances on the homogeneous modification and functionalization of cellulose have also been achieved using ILs, opening novel possibilities for its biomedical applications.[20] For example, Park *et al.* produced cellulose/chitosan composite nanofibers using 1-ethyl-3-methylmidazoliium acetate, which could be used as an antibacterial reagent to treat skin ulcers.[36] On a composite perspective, the use of cellulose crystals (*e.g.* nanowhiskers) is based on their high stiffness, being relevant as a reinforcement strategy in several systems such as hydrogels systems. [27b, 35]

Starch is a biopolymer composed of two major components, namely α-amylose and amylopectin. Starch can be found in many plant sources, such as corn, rice, potato, and wheat.[37] Depending on the source, the physico-chemical and functional properties of starch can vary. Chemical and physical modifications, and/or even combinations with other natural or synthetic polymers are some of the approaches applied onto starch to overcome its lack of processability. In TE, the starch-based materials *e.g.* 3D scaffolds, micro/nanoparticles, bone cement, and hydrogels, have been used as a carrier for controlled release of drugs.

2.1.4. Microbial polysaccharides

Bacterial cellulose (BC) can be produced by *Gluconacetobacter xylinus or Acetobacter xylinum*.[38] Although its similar molecular formula to plant origin cellulose, BC is characterized by a crystalline nano-fibrillar structure which creates a large surface area that can retain a large amount of liquid.[38a, 38c] BC has also been proposed to be used as an effective wound material due to its unique properties such as versatility, moldability *in situ*, biocompatibility, high water-holding ability, cost effect production and high mechanical strength in the wet state.[38b, 38c, 39] Furthermore, BC is used as wound dressing

composites with collagen type I,[40] chitosan [23a, 41] and aloe vera.[42] BC could be used in the production of scaffolds, transdermal applications and as a pharmaceutical excipient in drug delivery systems.[35, 43] In transdermal drug delivery, for instance, BC membranes could work bilaterally, both to deliver drug and absorb exudates.

Dextran is a biodegradable neutral bacterial exopolysaccharide consisting of repeating glucose subunits. Dextran is soluble in water and organic solvents, and this feature can be used in the preparation of different structures through blending dextran with bioactive agents or hydrophobic polymers. For instance, the interest in dextran hydrogels for soft TE has increased due to their great biocompatibility, the capability to reduce nonspecific protein adsorption and cell attachment. However, these properties are not sufficient for dextran hydrogels to fulfill their role to act as a TE scaffold. The conjugation of bioactive moieties such as RGD on dextran could facilitate tissue growth and regeneration.[44] Moreover, dextran can also be chemically modified to form spherical, tubular and 3D network structures.[45] Dextran has also been applied in nanomedicine. Dextran nanoparticles have superior aqueous solubility, high cargo capacity and intrinsic viscosity, and short storage period.[46] Therefore, the features of dextran made it suitable as nanodrug carrier, cell imaging system, and nanobiosensor.

Gellan gum (GG) is an anionic extracellular polysaccharide, composed by approximately, 60% glucose, 20% glucuronic acid, and 20% rhamnose as a repeating unit, and two acyl groups, acetate and glycerate bound to glucose residue adjacent to glucuronic. GG is recognized as a thermally reversible gel with excellent stability and high gel strength. However, in its high acyl form, GG produces transparent, soft, elastic and flexible gels, whereas the low acyl form results in brittle gels. These features have stimulated different approaches to use it as injectable hydrogels to regenerate and repair the damaged cartilage,[47] and chemically modified for invertebral disc (IVD) regeneration [48].

2.2. **Proteins**

Proteins are one of the versatile groups of macromolecules in living systems and play important functions in almost all biological processes. Proteins have also been used for the development of innovative materials for biomedical purposes mainly due to their (i) availability on a large scale and low cost; (ii) biodegradability since their degradation products are composed of amino acids that can be resorbed as nutrients; (iii) chemical

reactivity; and (iv) excellent biocompatibility. Given their unique properties, efforts have been expended in developing protein-based materials including films, capsules, foams, scaffolds, composites, and gels, through their association or not with bioactive compounds. These matrices have gained prominence as drug delivery systems, biosensors, and scaffolds for tissue regeneration. Moreover, the design approaches such as genetic engineering can be used for the synthesis of protein-engineered biomaterials, tailoring their properties (*e.g*. cell adhesion, elasticity, and biodegradability), and reducing the problems associated with those obtained from natural sources.

The features of the most promising proteins, namely collagen, gelatin, elastin, silk fibroin, sericin and soy protein used for TERM are described in the following paragraphs.

Collagen and gelatin

Collagen is the most abundant protein present in mammalian tissues (cornea, blood vessels, skin, cartilage, bone, tendon, and ligament) and is also the main component of the ECM. Gelatin is the partially hydrolysed form of collagen. Although sources of collagen and gelatin are bovine and porcine skin, recent studies have been shown their extraction from marine sources (*e.g*. marine sponges, fish skin) using simple protocols.[16] Both collagen and gelatin are the most preferred ECM proteins used in TE. They can be processed into fibers, films, and foams to engineer many tissues such as bone, cartilage, ligament, nerve and heart.[49] Depending on the biomedical purpose, improvement of the physical, chemical and biological properties of collagen can be achieved through its combination with other polymers. Similar to collagen, the use of gelatin in TE has been correlated with its appealing features such as cell-adhesive structure, low cost, and biocompatibility. Gelatin can be processed as composite scaffolds, nanoparticles, employed as a size-controllable porogen, and acted as surface coating agent.[50] Recent research has shown the proficiency of methacrylated gelatin (GelMA) for bioprinting and micro-structured patterned applications. The derived structures are suitable for bone, cartilage, cardiac, vascular tissues, drug, and gene delivery.[51]

Elastin is a vital protein component of the ECM present in many mammalian tissues, which require elasticity as part of their function,[52] including vasculature, skin and lungs. Mature elastin is an insoluble polymer composed of several tropoelastin molecules covalently bound to each other by cross-links. The nature of elastin itself has hindered the study of its properties and structure, mainly due to its insolubility in water and

backbone mobility. Elastin has inherent signaling properties that promote diverse responses, including chemotaxis, cell growth, and tissue homeostasis. Using the benefits effects of these characteristics, elastin and derivatives (tropoelastin and elastin derived peptides) can be used in the production of elastin-based materials and as surface coatings for polymers in order to improve cell adhesion and function on tissue engineered products. Elastin-like macromolecules are genetically engineered materials rooted in the repeating sequence of natural elastin.[53] They can be synthetized with a high degree of specificity and control, which is feasible by chemical methods. In fact, recombinant elastin-like polypeptides (ELPs) can be assembled into 3D matrices with tailored mechanical and thermal properties as well as unique functionalization opportunities using both enzymatic and genetic ways. Besides, incorporation of non-natural elements and inorganic materials in elastin-based materials could extend their application range, namely in diagnostic imaging and target therapeutic delivery.[54]

Silk are a class of proteins containing fibroin (70– 80%), the structural protein of silk fibers, and sericin (20–30%), the water-soluble glue-like protein that encased fibroin fibers. ***Silk fibroin*** (SF) is composed by glycine, alanine, and serine in different percentages. SF fibers are obtained from diverse sources such as non- and mulberry silkworm *Bombyx mori*, insects, and spiders.[55] SF possess good biocompatibility, elasticity, toughness, suitable mechanical properties and biodegradability with controllable degradation rates, make it a good biomaterial for TERM. SF is rich in β - sheet structures owing to hydrophobic domains thus influence its mechanical properties, biodegradation rate and the ability to support the cell adhesion and differentiation of MSCs Extensive studies have been carried out on the processing, blending and/or chemical modification of SF. It has also been combined with biomacromolecules as *e.g.* chitosan to form membranes, scaffolds, composites, micro/nanoparticles, nanofibers and hydrogels and/or with inorganic components like calcium phosphate (CaP) and HAp to generate composites.[56] These approaches have shown the benefits effects of SF-based structures for skin cartilage, bone, and ligament TE.

Sericin comprises a variable amino acid compositions such as serine, glycine, glutamic acid, aspartic acid, threonine, and tyrosine. This protein is considered a waste during silk processing. However, sericin exhibits attractive bioactive properties such as antioxidant, moisturizing ability, pH responsiveness and mitogenic effect on mammalian cells,[57] with potential applications in regenerative medicine. Sericin-based biomaterials such as sponges, hydrogels, micro/nanoparticles, composites, and films produced by ethanol

precipitation, crosslinking or blending with other polymers and/or with inorganic components [58] have been employed as dermal reconstruction and wound dressing for skin tissue repair, bone TE, and drug delivery.[59]

Soy protein is a globular protein isolated from soybeans. About 90-95% of the soy is a storage protein, with two subunits, namely 35% conglycinin (7S) and 52% glycinin (11S). Soy protein has advantages over the various types of natural proteins employed for biomedical applications, namely its low price, non-animal origin and relatively long storage time, and stability. Besides, the combination of its properties with a similarity to tissue constituents and a reduced susceptibility to thermal degradation makes soy protein a plant-derived macromolecule of interest in the biomedical field.[60] Membranes, fibers, microparticles and thermoplastics-based soy materials have been developed associating soy protein with other proteins (*e.g.* wheat gluten,[61] casein [62]), polysaccharides, such as cellulose,[63] chitosan,[64] and synthetic materials. The resulting matrices are helpful for tissue regeneration,[65] drug delivery system, and wound dressings.[1a, 64b]

2.3. **Bioactive agents and growth factors**

One of the significant challenges in TERM involves the production of matrices associated with bioactive signaling molecules that can promote cell adhesion, proliferation, differentiation, and metabolic activity for the *in vivo* regeneration process. These molecules are grouped in mitogens (that stimulate cell division), growth factors (with proliferation-inducing effects), and morphogens (that control generation of tissue form). In particular, growth factors (GFs) are signaling molecules capable of instructing cells, stimulating cellular growth, proliferation, healing and cellular differentiation in a biological environment. Given the critical role of GFs stability in cellular responses, of *in vivo* situations, natural polymers- based systems are good platforms as bioactive delivery substrates. These matrices could affect not only the encapsulation efficiency of the bioactive agents but also cell fate. Distinct strategies have been employed to prolong their release rate and increase their therapeutical effect in TE, namely (i) chemical immobilization of GFs into the polysaccharide- or protein-based matrices, and (ii) physical encapsulation of GFs in the delivery systems.[66] Examples of GFs and their carrier-based polymeric systems used in TE are presented in Table 1.

Platelet lysate (PL) has been increasingly explored in TERM as a natural and cost-effective resource of GFs and multiple proteins, and/or as a support for cell growth and differentiation. In fact, platelets have a wide range of cytokines such as the isoforms of

Insulin Growth Factor (IGF), Platelet-Derived Growth Factor (PDGF), Transforming Growth Factor (TGF), among others. Recent strategies for a controlled incorporation of PL have been developing using nanocoatings by layer-by-layer with marine origin polymers [67] (see Figure 2), and the production of biomimetic 3D hydrogel structure based on the assembly of chitosan-chondroitin sulfate nanoparticles (CH/CS NPs) carrying PLs.[67]

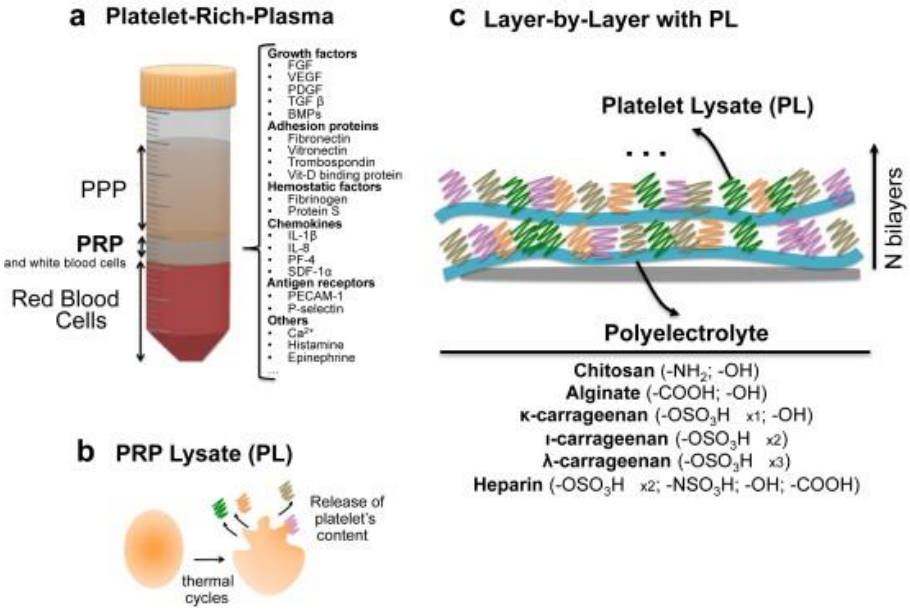

Figure 2. Main steps for the preparation of PL/Polysaccharides Layer-by-Layer assembled nanocoatings. a) Platelet isolation from human blood as Platelet-Rich-Plasma (PRP) and examples of bioactive proteins than can be found in the enriched protein cocktail; b) PL preparation: PRP activation by platelet disruption induced by thermal cycles for the release of the inner content and (c) Layer-by-Layer deposition combining PL with several PEs which respective functional groups and content are indicated. *Adapted from Ref [67] with permission.*

Table 1: Growth factors, their main functions and some examples of polymer-based carrier systems used in TE.

Growth factors	Main functions	Examples of polymer-based carrier systems	Ref
BMPs	Osteoblast cells differentiation and migration To induce MSCs to differentiate into bone.	Heparin-conjugated fibrin system	68
EGF	Regulation of epithelial cell growth, proliferation and differentiation	PLGA/gelatin hybrid nanofibrous scaffolds	69
FGF	Migration, proliferation and survival of endothelial cells	Gelatin sponge, Chitosan/fucoidan nanoparticles	24b, 70
IGF	Cell proliferation, inhibition of cell apoptosis	Collagen/GAG scaffold	71
NGF	Survival and proliferation of neural cells	Chitosan-β-glycerophosphate hydrogel	72
PDFG	Embryonic development, proliferation, migration, growth of endothelial cells	Gelatin hydrogels	73
TGF	Wound healing and increase cell proliferation and differentiation	Chitosan microspheres	74
VEGF	-Migration, proliferation and survival of endothelial cells. -Induce angiogenesis	Star-poly(ethylene glycol)-heparin hydrogel	75

Abbreviations: BMPs: Bone morphogenetic protein; EGF: Epidermal growth factor; PLGA: Poly(lactic-co-glycolic acid); FGF: Fibroblast growth factor; IGF: Insulin-like growth factor; NGF: Nerve growth factor; PDFG: Platelet-derived growth factor; TGF: Transforming growth factor; VEGF: Vascular endothelial growth factor.

3. Strategies for tissue engineering

Diverse strategies have been developed to engineer new tissues mimicking the natural tissue ECM to enhance cell-biomaterial interactions, and promote cell adhesion, proliferation, and differentiation ability. These strategies hold micro/nanoparticles, membranes, scaffolds, and hydrogels, with a specific design, controlled degradation rate, mechanical properties, and porosity for efficient gases, nutrients, and regulatory factors transport.

Conventional techniques such as foam replica method, solvent casting and particulate-leaching, freeze drying, gas foaming, and phase separation, are inexpensive and flexible to optimize physicochemical properties and have been used to produce structures. Rapid prototyping, supercritical fluid technology bioprinting, photolithography, microfluidics and electrospinning, as more sophisticated techniques, are also used for 3D structures and fibers, respectively, allowing the possibility of incorporating pharmaceutical agents. A brief description of such strategies is provided below.

Membranes

Membranes have been used in different TE applications, namely in wound healing, construction of drug delivery systems and diagnostic devices, due to its ease of manufacturing and self-application.[1a, 76] Membranes can be produced using solvent casting with or without porogen, freeze-drying technique, spin casting, and electrospinning.[77] Solvent casting are the technique commonly used in the preparation of polymeric membranes, owing the advantage of producing large and constant surface areas. This method consists in the polymer dissolution in an appropriate solvent and subsequent solvent evaporation. Additionally, drugs or specific molecules can be incorporated during the dissolution of the polymer or even on the casted film. The membrane structure and its properties are influenced by many experimental factors such as choice of solvent and non-solvent, polymeric solution composition, and humidity. Furthermore, instead of using pure polymer, two or more polymers can be mixed to form blends which are useful in the production of membranes with desirable properties. Many polymeric membranes have been produced using polysaccharides, proteins or their combinations envisioning biomedical TE applications. For example, Silva *et al.* developed blended membranes composed by chitosan and aloe vera (CAV) using the solvent casting technique.[15b] Results showed that the incorporation of AV into chitosan

provided blended membranes with better physical (roughness, degradation rate, wettability, mechanical properties) and cellular response (Figure 3) when compared to chitosan alone.

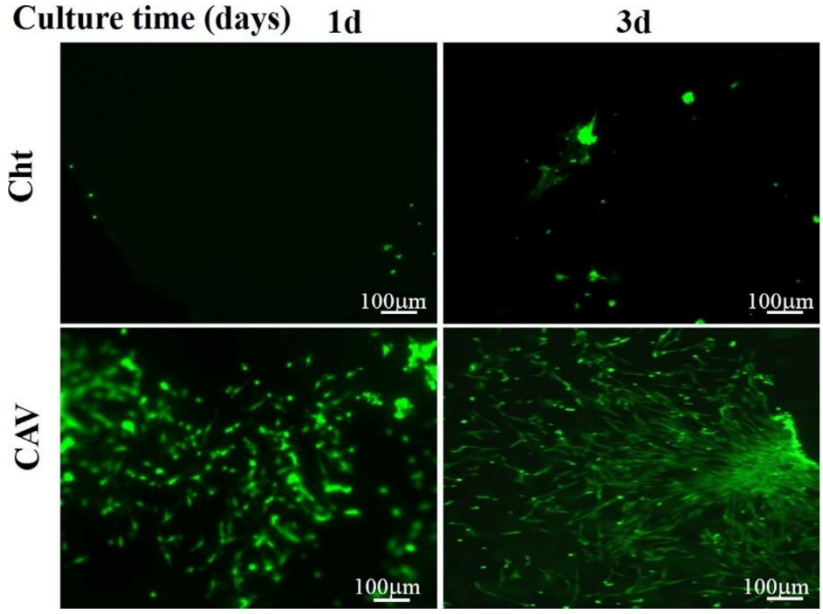

Figure 3: Calcein-AM staining of human dermal fibroblasts cells cultured for 1 and 3 days on the membranes (chitosan and chitosan/aloe vera, CAV). *Adapted from Ref [15b] with permission.*

The membranes surface properties, as *e.g.* roughness, wettability and surface energy, are important parameters that influence their biomedical application. Therefore, modification of the membrane surface is often needed to enhance the interaction of such material with the host or biological fluid and suppress the immune response. Various methods have been employed for modifying polymer surfaces including chemical modification, UV, gamma irradiation, and plasma surface modification. These modifications can determine the possible interactions of polymers with bioactive agents (*e.g.* growth factors, drugs). In plasma surface modification, the material is exposed to a partially ionized gas resulting in the formation radicals on the polymer surface that will modify the surface.[1a, 78] Also, the activation of the membrane surface can be used to create smart surfaces, where the wettability can be responsive to the change of external variables.[79] For example, surface properties of membranes can be modulated in response to an external stimulus (*e.g.* pH or temperature), and this characteristic could be useful to modulate the release of drugs.

A similar effect can be achieved through grafting of the monomer by chemical initiators using free radical polymerization methods. In that case, surface bound stimulus responsive polymers (SRP) can also be incorporated into membranes to create thermosensitive membranes.

3.1. Micro- and nanoparticles

Micro- and nanoparticles can be used in a large variety of TERM applications ranging from therapeutic delivery,[80] cardiovascular imaging,[81] to *in vitro* diagnosis,[82] incorporations in scaffolds or hydrogels,[83] or incorporated in biomaterials for the delivery of bioactive factors.[83a]

Micro- and nanoparticles have properties that can make them more or less useful for different applications, with particle diameters usually ranging from 1 to 100 µm, and 1 to 100 nm, respectively. The nanoscale dimension has unique physicochemical properties, such as ultra-small size, large surface area, and high reactivity when compared to the conventional microsized particles.[84] Nanoparticles are functionalized or surface-modified by capping certain functional groups on their surfaces, with specific recognition chemical moieties and enhanced efficacy, while simultaneously reducing side effects, due to properties such as targeted localization in tumors and active cellular uptake.[85] Various morphologies have been used to prepare nanoparticles, including liposomes, nanospheres, nanocapsules, micelles, and dendrimers,[80a, 86] which main characteristics are well documented in the literature.[87]

Numerous micro- and nanoparticle compositions have been used in tissue engineering, including natural and synthetic polymers,[23b, 88] inorganic/ceramic,[89] magnetic,[90] and gold nanoparticles.[91] For example, chitosan nanoparticles loaded with dexamethasone presented a significant improvement of osteogenic differentiation of hMSCs for bone tissue regeneration.[92] Park *et al.*[93] produced a hybrid composite scaffold using nanofibrous 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO)-oxidized bacterial cellulose and HAp with enhanced cell adhesion, migration, growth, mineralization, and differentiation for bone tissue regeneration. In a different study, mesoporous silica-based nanoparticles incorporating bone-forming peptide derived from BMPs-7 were developed to obtain a system for osteogenic factor delivery.[94] Results showed high cell proliferation and alkaline phosphatase (ALP) activity, making it a potential candidate for bone regeneration and bioimplant coating applications. A study by Chen *et al.*.[95] demonstrated that the presence of biphasic tricalcium phosphate microparticles incorporated into a

hyaluronic acid-*g*-chitosan-*g*-poly-*N*-isopropyl acrylamide hydrogel presented better cell proliferation and osteogenic differentiation than in the hydrogel alone.

Several methodologies have been presented in the literature for fabrication of polymeric micro/nanoparticles such as emulsification/solvent evaporation,[96] nanoprecipitation (also known as solvent displacement method),[97] salting out method,[98] ionic gelation,[24a] spray drying,[23b] sol-gel,[99] supercritical fluid technology,[100] microfluidics,[101] and electrospraying.[102] Manufacturing process should ideally allow the production of large quantities of particles with a narrow size distribution, with control of particle size, porosity and surface characteristics. Among them, the emulsion method is the most popular technique, which consists in the dispersion or dissolution of molecules into a polymer solution and emulsified to form micro/nano droplets that are further dried after solvent removal. However, the use of organic solvents is a disadvantage since it can lead to denaturation of protein-based drugs, thus increasing the variability in drug loading, encapsulation efficiency, size and surface morphology of nonhomogeneous particles.[103] To overcome these limitations, electrospraying technique has the potential to generate narrow size distributions of submicrometric particles, with limited agglomeration and high yields.[104] An example is presented in a study by Wang *et al*.[96] where it was fabricated a multiple-drug delivery system consisted of chitosan nanoparticles and polyvinylpyrrolidone (PVP) micro/nanocoating with core–shell structures, using ionic gelation and emulsion electrospray methods (Fig. 3). Results showed a successfully encapsulated dual model drugs, naproxen, and Rh.B, in the shell and core regions, with good release controllability.

Another processing method, often used by our group, for the fabrication of polymeric spheres, is based on the drop of the liquid precursor containing the polymer over superhydrophobic surfaces.[105] This method presents advantages over conventional emulsion and gelation techniques as the contact of the dispensed drops with an outer liquid environment is avoided. In addition, this process allows the encapsulation of living cells and/or therapeutic molecules for tissue engineering applications.[106]

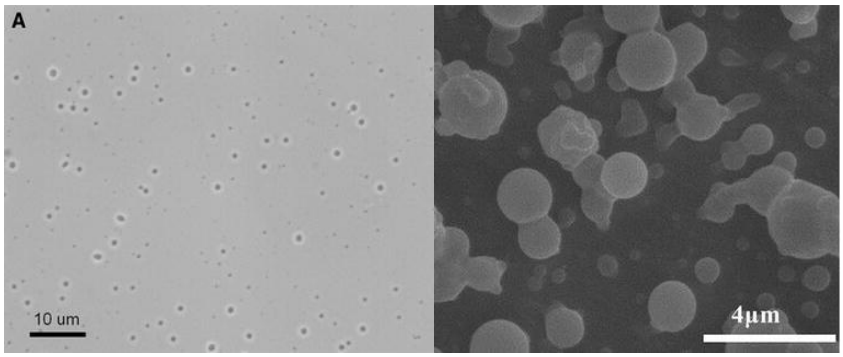

Figure 4: (A) An optical micrograph of the micro/nanoparticles, and (B) scanning electron micrograph of (naproxen/chitosan)/(Rh.B/PVP) core/shell micro/nanoparticles with naproxen/chitosan as the core and Rh.B/PVP as the shell. *Adapted from Ref.* [96] *with permission.*

3.3. Scaffolds

Scaffolds are defined as porous 3D support structures of the surrounding tissues mimicking ECM, with basic requirements, namely: (i) to promote cell-biomaterial interactions, cell adhesion, growth, and migration; (ii) to facilitate transport of mass, nutrients, and regulatory factors to allow cell survival, proliferation, and differentiation; (iii) controlled degradation that approximates the rate of tissue regeneration under the culture conditions of interest; (iv) possess adequate mechanical properties necessary to temporarily offer structural support until the formation of new tissue occurs, like tensile strength and elasticity; and (v) to elicit a minimal degree of inflammation or toxicity *in vivo*.[107] Moreover, instead of simple injection of cells to the defects, scaffolds have de advantage of allowing cell transfer into a defect site and to restrict cell loss.[108]

Scaffolds targeted for TE and regeneration have been developed using various natural and synthetic polymers, and bioactive inorganic materials, with specific pore size, porosity, surface-area-to-volume ratio, and crystallinity.[109] Composite materials combining a polymeric matrix and inorganic materials, as fillers, resulting in a single structure with significantly improved mechanical and biological properties, also appeared as a strategy for tissue engineering.[110] Special interest has been attributed to nanocomposites for bone tissue engineering and regeneration due to the nanosized features of the fillers which can intensely improve the tissue bonding capacity of the

polymeric matrices, that the individual materials cannot attain thus allowing the production of better biomaterials.[111]

Different technologies applied for scaffolds fabrication embrace foam replica method,[112] salt-leaching,[113] freeze-drying,[109, 114] phase separation,[115] gas foaming,[116] supercritical fluid,[117] rapid prototyping,[22] and electrospinning.[118] Barbani *et al.*[119] produced a gelatin/HAp nanocomposite scaffold with an elastic modulus similar to that of natural bone, using the freeze-drying technique. It was shown that HAp scaffolds supported the adhesion and proliferation of hMSCs onto the scaffolds. Recently, Martínez-Vázquez *et al.*[120] fabricated a 3D porous scaffold for bone regeneration and drug release capability, consisting of gelatine and Si-doped HAp through rapid prototyping technique, with adequate macroporosity for vascularization and microporosity that enables fluid exchange, as well possessing mechanical properties close to those of trabecular bone.

Our group has been developing mono- and bilayered porous structures for osteochondral tissue engineering applications. Yan *et al.*[109] developed a nanocomposite scaffold of SF and CaP using salt-leaching and freeze-drying methods, with superior mechanical properties and controllable porosity. The scaffolds supported new bone ingrowth, and no acute inflammatory was observed after 3 weeks of implantation. Based on their previous studies, the same authors fabricated bilayered scaffolds composed of an SF layer and an SF/nanoCaP layer, with different features (e.g. mechanical, chemical or morphology properties) for the simultaneous regeneration of the cartilage and the subchondral bone (Fig. 5).[121] It was shown that the scaffolds integrated well with the host tissues and allowed tissue ingrowth when implanted in rabbit knee critical osteochondral defects. Furthermore, the bilayered scaffolds supported cartilage regeneration in the top silk layer and encouraged large amounts of subchondral bone ingrowth and angiogenesis in the bottom silk/nanoCaP layer.

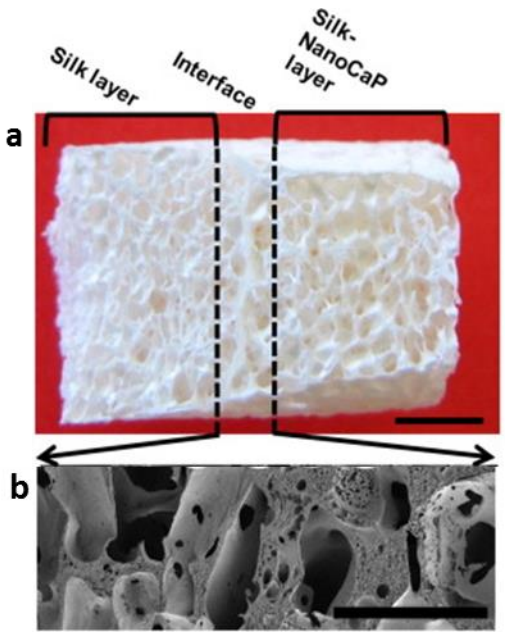

Figure 5: The interface of the bilayered scaffolds showing the different regions from the silk layer to the silk-nanoCaP layer: (a) Macroscopic image (scale bar: 3 mm), and (b) SEM image (scale bar: 500 μm). *Adapted from Ref[121] with permission.*

3.4 Hydrogel materials

Hydrogel-based materials, often classified as natural or synthetic polymers networks, are pre-formed by chemical or physical crosslinking of water-soluble precursors.[122] This class of biomaterials is an attractive candidate for building 3D structures that resemble the physical characteristics of the ECM because of their high water content, viscoelastic nature, and the easy diffusion of bio-macromolecules.[122-123] Hydrogels are polymer networks extensively swollen with water held together by (i) primary covalent crosslinks; (ii) hydrogen bonds; (iii) ionic forces; (iv) affinity or "biorecognition" interactions; (v) physical entanglements of individual polymer chains; (vi) polymer crystallites; (vii) hydrophobic interactions; or (viii) a combination of two or more of the previous interactions.[124] Among them, naturally derived polymers recently used in hydrogel structures include collagen type I,[125] alginate,[126] HA,[27b, 127] chitosan,[7a, 125b, 128] gelatin,[126b, 128-129] GG,[130] agarose,[7a] SF[131] and fibrin.[27c, 132] Hydrogels have recently emerged as a promising platform for 3D cell encapsulation and culture.[123a] Thus, their biomedical application depends to a large extent on their bulk structure. By controlling the chemical composition and crosslinking density, network structure, and pore size can be tuned and customized for a given application in TE.[123c] There are several forms to categorize

hydrogels, based on the preparation method, ionic charge or physicochemical structural features. Some of the most used fabrication techniques reported in the last years are highlighted in this section.

(1) Injectable hydrogels [133] have the potential to be minimally invasive delivered. Most of the hydrogels are injectable, and they can be formed via photopolymerization,[129, 134] which can be carried out under mild conditions in the presence of living cells. This allows homogeneous seeding of cells throughout the scaffold materials and formation of hydrogels *in situ.*[122] Alternative polymerization methods include the thermal polymerization,[133a, 135] by using alginate modified with glycidyl methacrylate and calcium as cross-linker to produce a thermal polymerizable alginate at physiological conditions. Human umbilical vein endothelial cells (HUVECs) were encapsulated in the modified hydrogels *in situ* at 37 ºC, revealing that cell viability and proliferation were unaffected by macromonomer concentrations, suggesting the potential application in the field of TE.[135] However, the lower mechanical properties of the developed hydrogels restrict the potential range of some TE applications. Hybrid injectable hydrogels show to be more complex systems that combine well-evolved biological mechanisms, such as high affinity and specificity of binding, with tailorable hydrogel properties (e.g., mechanical stability and environmental-responsive properties).[122] Research groups report the development of bioactive organic-inorganic hybrid materials as injectable hydrogel collagen, collagen-chitosan systems for bone tissue regeneration with silica nanoparticles (SiNP) prepared *in situ.*[133b] The *in vitro* studies on fibroblast cell viability indicated that the SiNP dispersed in the biopolymer matrix had a positive effect on cell viability. Recently, other study reports biohybrid temperature-responsive poly(N-isopropylacrylamide) PNIPAAm-Gelatin-based injectable hydrogel with good bioactivity as well as appropriated mechanical properties for cardiac TE.[136].

(2) Bioprinting comprises a group of biofabrication technologies for 3D constructs by precisely printing biocompatible materials, cells and biochemicals in predesigned spatial positions.[137] During the last years, a more interesting view is the printing and patterning in 3D of all the components that make up a tissue (cells and matrix materials) as a bioink to generate structures analogous to tissue. It can be found different printing approaches based on inject-based and extrusion-based systems that use a nozzle for the deposition of the hydrogel material.[137-138]

This implies that rheological properties, such as viscosity, shear forces and the mechanism by which the hydrogel is cross-linked to form a stable matrix, plays an important role allowing the ability of the hydrogel to be handled, deposit, stacked and retain volume over the time. Nevertheless, laser-based printers such as stereolithography, are nozzle-free systems that require different properties for the transfer of a hydrogel material from the laser absorbing donor slide to the recipient slide.[137, 139] These technologies have been successfully applied to fabricate biodegradable 3D constructs with complex architectures and heterogeneous composition. As shown in Figure 6, the use of bioprinting strongly depends on the development of novel biomaterials exhibiting fast crosslinking schemes and appropriate printability, cell-compatibility, degradability and biomechanical properties.[137, 140]

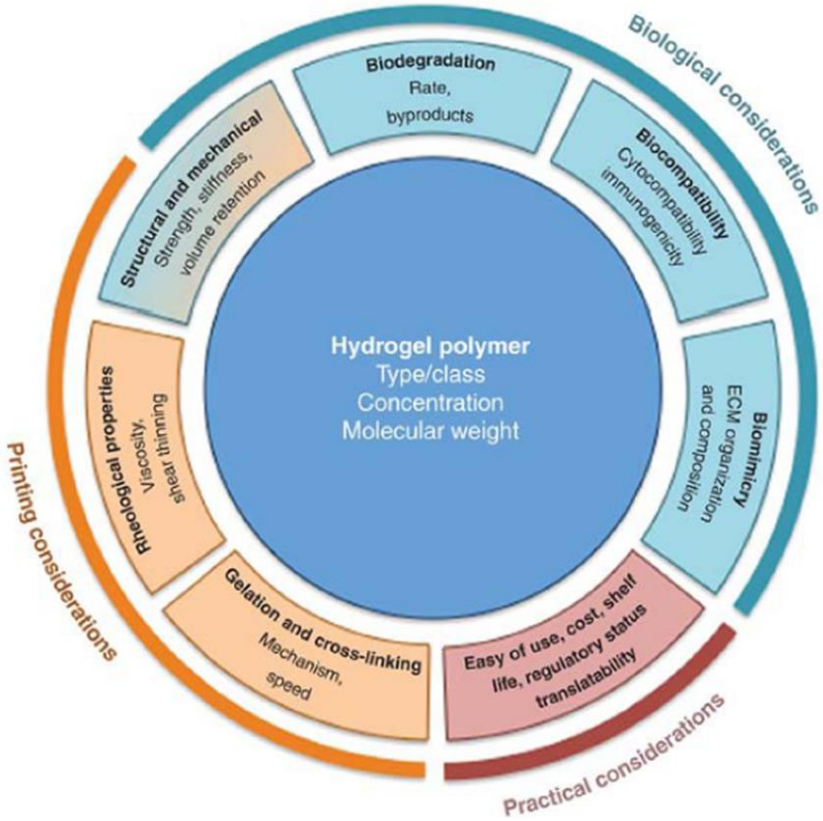

Figure 6: Concept map of the variables and relations to be considered when assessing a hydrogel for bioprinting applications. *Reprinted from Ref [137] with permission.*

Thus, photocrosslinkable hydrogels showed to be attractive materials for bioprinting since they provide fast polymerization under cell-compatible conditions and excellent spatiotemporal control over the gelation process.[139a] Since bioprinting uses a layer-by-

layer additive manufacturing approach is of high relevance that the hydrogel presents high viscosity and a fast cross-linking for the fabrication of 3D structures.

Also, after cells been deposit, the hydrogel composition may play an important role in supporting cell viability and proliferation. Figure 7, summarizes the typical process for bioprinting 3D tissues. Very few materials are available that fulfill requirements for bioprinting as well as provide adequate properties for cell encapsulation during and after the printing process. Typical natural materials previously referred in this section included in the hydrogels formulations are alginate and gelatin precursors.[139a, 141] In a recent study, these precursors were tuned with different concentrations of HAp. *In vitro* studies revealed that hMSCs mixed into the hydrogel precursor survived the printing process and showed high cell viability.[141] By adding factors other than HAp, these hydrogels could be used as a bioink for applications in microsphere deposition, drug release and TE applications.

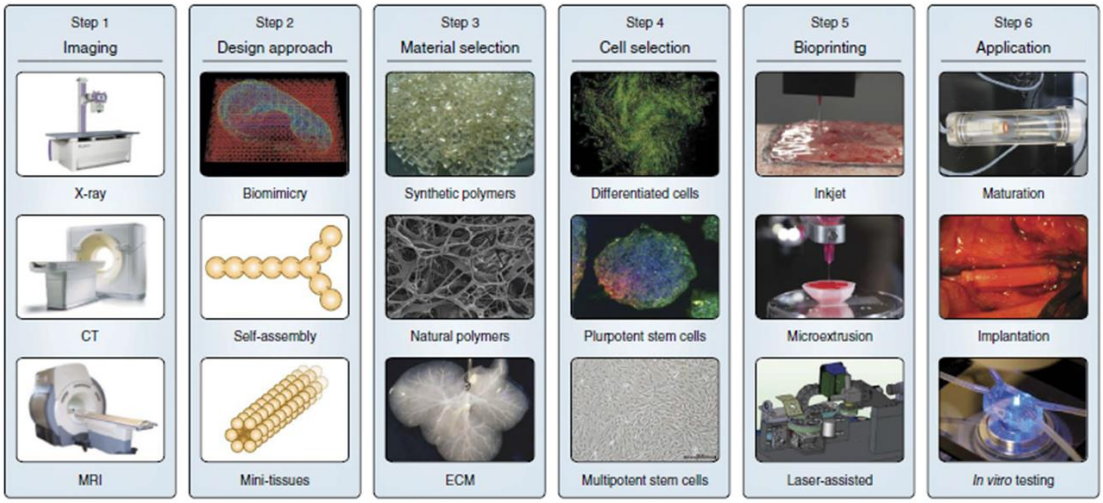

Figure 7: Typical process for bioprinting 3D tissues. Imaging of the damaged tissue and its environment can be used to guide the design of bioprinted tissues. Biomimicry, tissue self-assembly, and mini-tissue building blocks are design approaches used singly and in combination. The choice of materials and cell source is essential and specific to the tissue form and function. Common materials included synthetic or natural polymers and decellularized ECM. Cell sources may be allogeneic or autologous. These components have to integrate with bioprinting systems such as inkjet, microextrusion or laser-assisted printers. Some tissues may require a period of maturation in a bioreactor before transplantation. Alternatively, the 3D tissue may be used for in vitro applications. *Reprinted from Ref [142]with permission.*

(3) Photolithography and soft lithography techniques are used for cell encapsulation.[143] Both methods have also been developed by using a variety of other polymeric biomaterials such as polyethylene glycol (PEG), and alginate hydrogels.[144] Photolithography involves UV light through a photomask on to a substrate coated with a photoresist while; soft lithography includes a range of methods mostly in the field of microfluidics that is used to prepare microstructured elements from a polymeric material.[145] Soft lithography techniques include microfluidic patterning, stencil-assisted patterning, and micro-contact printing. Photolithography can provide a broad range of features, varying from micrometers to sub-microns (*e.g.*100 nanometres). Photolithography technique uses light or photons to transfer the geometric shapes of a mask to a light-sensitive surface. This fabrication enables to polymerize and cross-link polymers, resulting in a 3D scaffold. Advantages of this technique are the uniform cell encapsulation, the good special and temporal control of reaction kinetics, easy control of pore size by varying the selected polymer and its concentration. The main disadvantage is the use of multiple photomasks for multilayered scaffolds and the use of photo-cross-linkable polymers in the photolithography techniques. In addition, some challenges need to be solved such as the lack of resolution, economic viability and unsuitability of UV-sensitive biological materials for pattering.[146] Recent advances show the capability for producing environmentally triggered, self-folding, non-fouling and permeable hydrogel scaffolds, combined with nano- and micropatterned on both surfaces in a one-step photolithographic system.[143a] This was possible by combining serial hot embossing of sacrificial layers and photolithography. For each pattern, a mastering process was required in which quartz, silicon or PDMS stamps can be prepared, to be used in subsequent hot embossing and photolithography steps. The obtained patterned hydrogel films can be triggered consecutively allowing for successive rolling and unrolling depending on the aqueous pH. Hydrogel photolithography was also developing for integrating cells and sensing elements on culture surfaces (e.g. arrays of microwells for single cell capture and entrapment of enzymes inside hydrogel microstructures for local detection of cell metabolism). In both cases, PEG hydrogel lithography can be employed to control cell attachment, in the second approach hydrogel structures also carried enzymes and functioned as cell/sensors interfaces for diagnostics and tissue engineering.[147] Some of these approaches showed promising results not only by biomimetic structures with micropattern nanostructures but also a suitable biochemical

environment. However they are in early stages, and more developments and understanding should be applied to create robust clinical applications.

(4) Microfluidics technique deals with the handling of fluids in microenvironments, such as microchannels where the flow of fluids is laminar.[143c] Microfluidic patterning uses transient microchannels to flow coating agents over restricted areas of a surface.[145] Once the coating is complete, the micro-channels are disassembled, and the patterned surface can be used. Microfluidics has shown advantages for the synthesis of polymer particles and been used to produce hydrogel particles with a well-defined size, shape and morphology.[145, 148] Most importantly, during the encapsulation process, microfluidics can control the number of cells per particle and the overall encapsulation efficiency.[148] Using this microfabrication technique, it can be found the use of alginate and gelatin as substrates,[149] PEG [150] and typically made of PDMS.[150b, 151] Important advances have occurred on microfluidic bioassays, which incorporate hydrogel scaffolds into surface-accessible microchambers, driven by the strong demand for the application of spatiotemporally defined biochemical stimuli to construct *in vivo*-like conditions and perform real-time imaging of cell-matrix interactions.[152] One emerging use of microfluidic systems is the generation of shape-controlled hydrogels (i.e., microfibers, microparticles, and hydrogel building blocks) for various biological applications.[153] The diameters of the hollow and solid fibers can be manipulated by the flow rates.[153b] Furthermore, the microfluidic fabrication of cell-laden hydrogels is of great benefit for creating artificial scaffolds and should mimic the in vivo cellular environment.[154] Temperature-sensitive hydrogels obtained from natural materials (i.e., agarose, gelatin, collagen, and fibrin) have also been used to generate the microfluidic-based tissue architectures.

5. **Biomedical/Biotechnological applications**

5.1. ***Cell encapsulation***

TE construct comprises cells, GFs, and scaffolds. In a traditional TE strategy, cells are seeded on the surface of a porous 3D substrate with appropriate geometry and mechanical properties. In this scenario, cell-cell and cell-matrix interactions occur mainly in 2D. However, on *in vivo* conditions, these interactions happen in a 3D environment, with cells being embedded by the ECM. Bearing this in mind, researchers have been started to

encapsulate viable and functional cells into 3D environments that mimic the natural ECM.[155] Several methods have been used to encapsulate cells in 3D structures, from simple gravitational dripping to more complex ones as microtechnologies [156] or additive manufacturing.[157] This strategy has been shown interesting and promising results for different applications such as, (i) cell immunoisolation and drug delivery;[158] (ii) storage and transport;[159] and (iii) cell delivery.[160] (Figure 8)

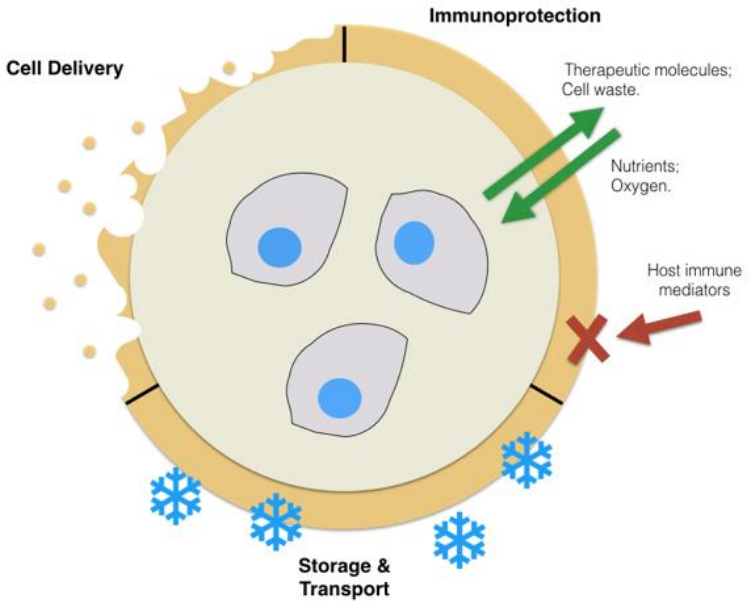

Figure 8: Schematic representation of the three applications of cell encapsulation technology: Cell delivery, Immunoprotection, and Storage&Transport.

Understandably, it is necessary to choose wisely the biomaterial. It should be biocompatible and must be permeable to oxygen, nutrients and metabolites. Naturally-derived hydrogels are attractive materials, as they have a similar structure to the ECM and can be processed under mild conditions, compatible with cells. Moreover, the low interfacial tension between the hydrogels and the environmental fluids results in low protein adsorption rate and cell adhesion, making them highly biocompatible. The soft and pliable nature of these gels reduce the mechanical irritation on the surrounding area, providing a high degree of permeability to nutrients and metabolites.[161]

Besides biocompatibility and porosity, encapsulation strategies may require other properties that are constrained by the final application of the construct. If the system is

designed for cell delivery, then the matrix should be biodegradable. On the other hand, if the final aim is the release of a therapeutic molecule secreted by entrapped cells, the matrix must be robust and non-biodegradable.

5.1.1. Immunoisolation and drug delivery

Cell encapsulation has been widely studied as a way to avoid the host immune response elicited by transplanted cells. The strategy implies the encapsulation of cells within an artificial compartment, confined by a semipermeable membrane. This membrane must isolate cells from the immune system response – immunoisolation - while permitting the diffusion of small molecules like glucose, oxygen, therapeutic molecules and waste products.[162] As a result, there is no need to use harmful immunosuppressant drugs after transplantation. Moreover, encapsulation technology also surpasses the shortage of donors as it allows not only allogenic but also xenogenic transplants to be performed.[163] When designing materials for this purpose one should bear in mind some properties that cannot be discarded. First, biomaterials should be selected to prevent cellular overgrowth on the encapsulated grafts while ensuring the free diffusion of nutrients, oxygen, and therapeutic molecules. Otherwise, the overgrowth of fibrous tissue would result in impaired diffusion profiles and subsequent cell death and graft failure. Secondly, they must be stable because the membrane cannot be disrupted after transplantation. Therefore, biodegradable materials with fast degradation kinetics should be avoided for the sake of capsule integrity and subsequent islet immunoprotection.[164] Moreover, materials should have an adequate mechanical stiffness and toughness to handle the forces imposed at the implantation site without disruption.[162] To improve those mechanical properties different geometries and chemical structures are being studied. These include the modification of the material with strengthening polymers, application of multiple layers,[165] reinforcement with polyester meshes and decrease of the capsule size.[164] Last, permeability is a central requirement in the design of cell encapsulation devices, which is tightly correlated with the performance of encapsulated. One important parameter to evaluate membrane permeability is the molecular weight cut-off (MWCO). It determines the minimum molecular weight measured in Daltons (Da), correspondingly minimum size, of a solute that is completely excluded by the semipermeable membrane. A MWCO of 50 to 150 kDa was reported as the most suitable for cell encapsulation purposes.[162, 164] Immunoisolation techniques have been studied as a treatment for different diseases, including heart infarction,[166] kidney and liver failure,[167] central nervous system

insufficiencies[168] and widely for Diabetes Mellitus.[165, 169] Inspired by the work of Lim and Sun (1980),[170] islet immunoisolation studies started to consider hydrogels to produce spherical semipermeable membranes (capsules), that host only one to a few islets. Their small size, usually less than 1 mm, and spherical shape enhances the diffusion of oxygen and nutrients across the membrane, promoting cell viability and function.[164] Other advantages of microcapsules include the mechanical stability *in vivo*, ease of manufacturing through different techniques and easy implantation by a simple injection.[169a] Among the various materials, alginate is the most studied for islet cell microencapsulation, although other materials as silk were also tested.[171] Similarly to Lim and Sun, other authors reported the successful application of alginate - poly-L-lysine - alginate microcapsules, using different diabetic models like a rodent, pig, and canine.[172] Human clinical trials were also performed with promising results.[169b, 172] though, researchers are still facing some complications, mostly regarding the lack of direct vasculature and the fibrotic overgrowth that occurs around the capsules. The lack of direct vasculature associated with encapsulated cells increases the probability of hypoxia, especially at the core of the device, then compromising the cell survival. Fibrotic overgrowth is normally a result of insufficient biocompatibility and has a negative impact on encapsulated islet function, which can lead to islet cell death.[173] To tackle this issue, Vegas *et al.*[174] used a modified alginate - triazole–thiomorpholine dioxide (TMTD) alginate – as a matrix to encapsulate human stem cell-derived β-cells (SC-β cells). While non-encapsulated SC-β cells were unable to restore a normoglycemic condition, TMTD alginate spheres have provided glycemic correction for over 70 days at all doses tested. Additionally, human C-peptide concentrations were higher when TMTD alginate was used. The immune response to these spheres was also quantified, and the results showed that TMTD alginate spheres had a lower number of cells related to an immune response to their surroundings as compared with other conditions, confirming a reduction in the fibrotic deposition. Further experiments as proteomics and histological processing have confirmed that the TMTD-based material was able to mitigate the fibrotic overgrowth while maintaining islets viable and functional. At last, long-term glycemic control was tested. For that, the created system was transplanted into mice and tracked for 174 days. The mice maintained the glycemic correction for the whole experimental period, with glucose levels similar to the wild-type, even when subjected to a glucose challenge. After six months, the capsules were retrieved, and no fibrotic overgrowth was noticed.

5.1.2 Storage and Transport

Cell-based therapies have grown substantially in recent years as a treatment for numerous diseases including cardiovascular disease, neurodegenerative diseases, cancer, liver disease, diabetes, skeletal disorders and eye diseases.[175] The pipeline from cell isolation to clinical practice implies a storage and transport step, meaning that cell preservation technology must evolve at the same pace to allow correct transport and storage.

The entrapment of living cells within a 3D matrix has been reported as a way to improve cells viability after storage, both on cryo- and hypothermic preservation.[159, 176] The presence of the biomaterial not only protects cells against the osmotic shock and mechanical stress, experienced during storage and recovery, but also provides cell membrane stabilization and supports the maintenance of cell-cell interactions.[176-177] Consequently, many researchers start to use and study encapsulation methods to store cells. For instance, alginate encapsulation has been investigated for its protective effect on the storage of rat hepatocytes,[177] recombinant baby hamster kidney cells,[178] hBMSCs, mouse embryonic stem cells (ESCs),[179] corneal epithelial cells[180] and adipose stem cells.[159]

5.1.2. Cell delivery

The encapsulation of cells inside 3D environments has also been shown promising results for tissue regeneration purposes, where the aim is to form tissue *in vivo* from a TE construct. Besides the aforementioned 3D structure, the encapsulation technique can simplify the handling process and allow a correct grafting of cells. Contrary to immunoisolation, in these cases the material should be biodegradable, allowing cell proliferation and ECM formation to restore the damaged organ. To be successful, the degradation rate should be adjusted to the tissue growth kinetics. Moreover, the degradation products must be cell-friendly to avoid adverse effects on encapsulated cells or in the host.[181]

Researchers are using different materials and strategies to encapsulate and deliver cells. Fibrous protein-based hydrogels, like silk, keratin, elastin, and collagen have been shown very promising on this topic.[182] Besides the structural, chemical and mechanical similarities with the ECM, they can be degraded by proteolytic enzymes once inside the human body.[182] Carbohydrate-based materials, including mixtures[183] or chemically modified[184] materials, as well as hydrogel composites,[123c] are also very promising since

the modifications can improve and help on fine tune their degradation rate,[183b, 185] according to with the desired purpose.

One interesting approach for cell delivery is the use of injectable cell-laden hydrogels.[184, 186] This strategy has called the attention of researchers mostly due to its non-invasive nature as well as the ease of handling and possibility to completely fill the defect. Recently, Liu *et al.*.[186a] developed a thermosensitive carboxymethyl chitin hydrogel that sets at 37 ºC and it is degraded in the presence of lysozyme or hyaluronidase. These hydrogels were able to promote cell proliferation and survival *in vitro* and *in vivo* studies showed tissue compatibility and good *in-situ* gel formation.

5.2. ***Application in Intervertebral Disc Regeneration***

Several polymers have been studied for intervertebral disc (IVD) regeneration, namely natural-origin polymers that can be used as hydrogel matrices or 3D porous scaffolds.[187] Despite the great advances using cell-free materials and growth factors, the use of acellular strategies combined with cells obtained from different sources is seen as the most promising research direction. Cell-based strategies directed to the regeneration of IVD focus mainly on the regeneration of the central nucleus (nucleus pulposus, NP), since it is in this tissue that disc degeneration initiates. The outer structure of the IVD, i.e. the annulus fibrosus (AF), suffers degeneration after the NP, mainly due to NP malfunction because of its loss of hydration and structure.[188] So when choosing the right biomaterial to carry cells to the NP, several properties must be taken into account, namely (Table 2):

Table 2. Biomaterial requirements as a cell carrier for IVD regeneration.

	Problem	**Solution**	**References**
1	AF should be left intact as much as possible during surgical procedure	Injectable material, so that only the area of the needle insertion in the AF is injured	[189]
2	The material must be able to polymerise *in situ* only after injection *	Polymerization can be achieved either by changes in pH, ion interaction, temperature or light exposure	[190,191]
3	The cell-loaded material should improve NP properties, such as disc height and biomechanical	The material's mechanical properties should be as close as possible to the mechanical properties of the native NP tissue	[192]

	function, for the cells to remain viable		
4	The biomaterial must provide an adequate environment for stimulation of NP cells phenotype	The material must be able to absorb at least 80% of water, which is the amount of water in the NP	[193]
5	The material should be biocompatible and biodegradable	The degradability rate must have the same timing has the tissue's rate of regeneration	[194]

*If the polymerised material can be injected or if the material does not polymerise *in vivo* it is very likely that the material will come right off through the needle hole, with cells included, on the first IVD loading.

With all those requirements in mind, hydrogels stand out as the best type of material to be used for NP regeneration (Figure 9). Hydrogels can withstand complex loading and allow the complete filling of irregular defects.[187] In the clinical setting, they possibly to be administered by using minimally invasive techniques, facilitating a good and faster surgical practice. Additionally, hydrogels present less risk of extrusion or migration, thus allowing to control the delivery of drugs or cells at the damaged site. Hydrogels for IVD repair may differ from physical crosslinking systems (e.g. ionic-crosslinked), which are obtained under mild conditions, to chemical crosslinking systems (e.g. photo- and enzyme-crosslinked) that present improved stability and mechanical properties.

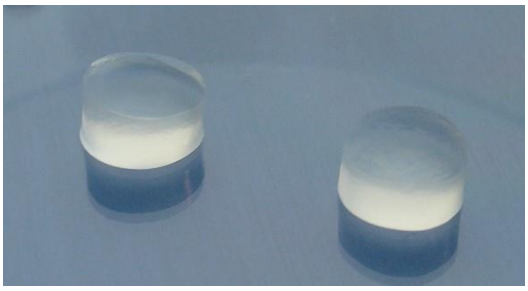

Figure 9: Ionically-crosslinked methacrylated GG (GG-MA) discs with a diameter of 7 mm and a height of 4 mm.

Several kinds of hydrogels have been studied for cartilage regeneration, as well as specifically designed for IVD TE strategies. There is a growing interest in natural-origin hydrogels, and besides their high biocompatibility, the economic aspect is another reason for it. The main reasons for why they are getting more attractive are their low toxicity and the wide range of possible TE strategies where they can be applied.[195] Additionally, as most of the naturally occurring hydrogels are extracted and not synthesized, this greatly

reduces its manufacturing cost. However, they also require purification processing, which sometimes involves using severely toxic solvents and reagents. Even so, in general, they are less expensive to produce than synthetic hydrogels.

Although natural-origin hydrogels offer a wide range of biological advantages, they lack the needed physical properties, such as solubility and adequate rate of degradation, which is too fast and does not allow the tissue to regenerate in such low time.[196] Some examples of natural-origin hydrogels being investigated for IVD TE are alginate, carboxymethylcellulose, chitosan, collagen, gellan gum, hyaluronan and silk fibroin.[48, 130b, 192, 195a, 195c, 197] The advantages and disadvantages of using each one of these natural-origin polymers in NP regeneration are indicated in Table 3.

Table 3. Natural-origin hydrogels used in IVD TE strategies – advantages and disadvantages specific for NP regeneration.

Natural Origin Hydrogels	**Advantages**	**Disadvantages**	**References**
Alginate	- Polymerization under mild conditions - *In situ* injectability - closely mimics NP mechanical and cell-adhesive properties	- Limited control over mechanical properties, swelling ratio and degradation profile - High variability between batches due to impurities presence - Immunogenic - Difficult to sterilize and handle - Lack of informational structure for positive cell response	197a,195c, 197b,197c,197d
Carboxymethyl cellulose	- Low-cost - FDA-approved - Commercially available in high-purity forms	- Lack of studies on IVD regeneration	197e,197f
Chitosan	- Hydrophilic surface - Bioactive - Cell adherent - Antibacterial activity - Non immunogenic	- Poor mechanical properties - Some level of toxicity (due to crosslinking agents)	197a, 195c, 197g,192

		- High variability between batches due to impurities presence	
Collagen	- Non- immunogenic - Piezoelectric properties - Bioactive	- Poor mechanical properties - High degradation rate - Some level of toxicity (due to crosslinking agents)	197a,195c,197h
Gellan Gum	- Non- immunogenic - Very low manufacturing cost - Stable in long-term - Mechanical performance and rheological behavior similar to the native NP tissue - Non-angiogenic when methacrylated	- Weak in physiological conditions in its native form due to the exchange of divalent cations by monovalent ones	48, 197a, 197i,130b, 197j
Hyaluronan	- Non-immunogenic - Easy control over polymer chain sizes - Bioactive	- Osteogenic - Cytotoxic in high concentration - Batch-to-batch variations	197a, 195c,197k, 197l
Silk Fibroin	- Good mechanical properties - Biocompatible - Controllable degradation rate - Can provide appropriate mechanical strength when combined with other polymers	- Can only be used to create composite hydrogels due to inducible formation of crystalline b-sheet structure	197m, 197n

6. **Final remarks and future trends**

Natural-origin polymers derived from renewable resources, namely from algae, animal, plant, and microorganisms, are appealing for tissue engineering and regenerative medicine applications due to its similarities with the extracellular matrix, specific degradation rates, chemical versatility, and good biological performance without toxicity or immunological reactions. A variety of applications for these type of materials includes cell encapsulation and intervertebral disc (IVD) regeneration. Biomimetic strategies for tissue engineering and processing are focused on micro/nanoparticles, membranes, scaffolds, and hydrogels, alone or in combination. Hydrogels are of particular interest because of their high water content, biodegradability and compatibility, and mechanical properties which resemble those of nonosseous living tissues. Many efforts have been made to improve the hydrogels potential. Injectable hydrogels, including mixtures of organic-inorganic systems, will continue to be improved and applied in areas for tissue enhancement and regeneration. Moreover, specific microfabrication techniques will be improved to spatially pattern the environment at microscale. One disadvantage of processing hydrogels is difficult to shape them in predesigned geometries. A challenge for tissue engineering is producing 3D vascularized cellular constructs of clinically relevant size, shape and structural integrity.

Conventional techniques used to fabricate structures include foam replica method, solvent casting and particulate-leaching, freeze drying, gas foaming, and phase separation. Rapid prototyping, supercritical fluid technology bioprinting, photolithography, microfluidics, and electrospinning, are more sophisticated techniques also used allowing the possibility of incorporating pharmaceutical agents and possibly cells.

Additive manufacturing such as bioprinting and bioink presents a high potential in combination with the design and imaging techniques. This includes the evolution and understanding of microfluidic devices whose controlled environments provide the cell culture with more life-like conditions than traditional cell culture methods. The innovations of these systems at the micro and nanoscale will open a wide range of applications, from fundamental research up to regenerative medicine.

The use of natural-derived polymers allows to better mimic the native ECM environment. Then, these molecules are very attractive for biomimetic strategies, including cell encapsulation. Nevertheless, their inherent properties are not enough to confer the perfect environment for cell growth and provide the proper mechanical properties. As a result,

they are commonly combined with other materials, both natural or synthetic, to obtain improved TE constructs. Thence, the development of new strategies or their translation to clinics are tightly dependent on the development and study of new biomaterials or combinations of well-established ones.

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