

REVIEW ARTICLE

# Seaweed polysaccharide-based hydrogels used for the regeneration of articular cartilage

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## Abstract

This manuscript provides an overview of the *in vitro* and *in vivo* studies reported in the literature focusing on seaweed polysaccharides based hydrogels that have been proposed for applications in regenerative medicine, particularly, in the field of cartilage tissue engineering. For a better understanding of the main requisites for these specific applications, the main aspects of the native cartilage structure, as well as recognized diseases that affect this tissue are briefly described. Current available treatments are also presented to emphasize the need for alternative techniques. The following part of this review is centered on the description of the general characteristics of algae polysaccharides, as well as relevant properties required for designing hydrogels for cartilage tissue engineering purposes. An in-depth overview of the most well known seaweed polysaccharide, namely agarose, alginate, carrageenan and ulvan biopolymeric gels, that have been proposed for engineering cartilage is also provided. Finally, this review describes and summarizes the translational aspect for the clinical application of alternative systems emphasizing the importance of cryopreservation and the commercial products currently available for cartilage treatment.

## Keywords

Cartilage regeneration, clinical and commercial products, hydrogels, *in vitro* – *in vivo* applications, polysaccharide

## History

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## Introduction

Biological structures can be defined as open dynamic systems which interact and respond accordingly to changes in the environment. Therefore, to obtain a specific response from a target biological structure, it is necessary to create appropriate environments. A lot of work is being conducted to unravel the puzzle around structural and biochemical functions of the natural extracellular milieu that directs cell fate. Understanding the mechanisms of cell function, the typical response to generate matrix development and tissue growth, is critical for further advances in regenerating any damaged biological component. Also, a great deal of research has been focusing on the development of biological constructs that support cell proliferation based on specific interactions between the biomaterial interface domains and the cell receptors to mimic the physiological environment. The natural extracellular matrix (ECM) is a hydrogel-like structure itself, comprised of several different biopolymers, encompassing a wide range of biological, chemical, and mechanical properties (Aizawa et al., 2012). Generally,

hydrogels are used for cell growth and delivery, with the goal of developing *de novo* tissues and ultimately regenerating and integrating the functional engineered tissue equivalent within the body. In the last few years, a vast range of different hydrogels that mimic more closely the native ECM have been proposed for regeneration strategies, produced by different methodologies and materials, with varying properties and composition. The knowledge obtained to this date, indicates that there is not a single ideal hydrogel available that can meet the requirements for all possible applications and thus, one must select a specific matrix with unique properties akin to target regenerative purposes. The use of polysaccharides as supportive systems for tissue formation reveals an increasing tendency in the biomedical field (Tirtsa et al., 2005). Among all the naturally derived polymers, the carbohydrate based polysaccharides, composed of sugar-ring building blocks, are emerging as a front runner in cartilage tissue engineering (TE) applications (Guarino et al., 2012; Ko et al., 2010). The polysaccharides of marine algae origin, especially the ones of seaweed source, such as alginate, agarose and recently  $\kappa$ -carrageenan or ulvan, which will be presented in this review, play important roles in biomedical applications, contributing with specific properties. In cartilage tissue, the cells are anchored into a matrix network that hydrogel design intends to mimic. Once embedded in hydrogels, commonly used as encapsulation systems (Hunt & Grover, 2010), the cells renew and specialize due to spatial organization

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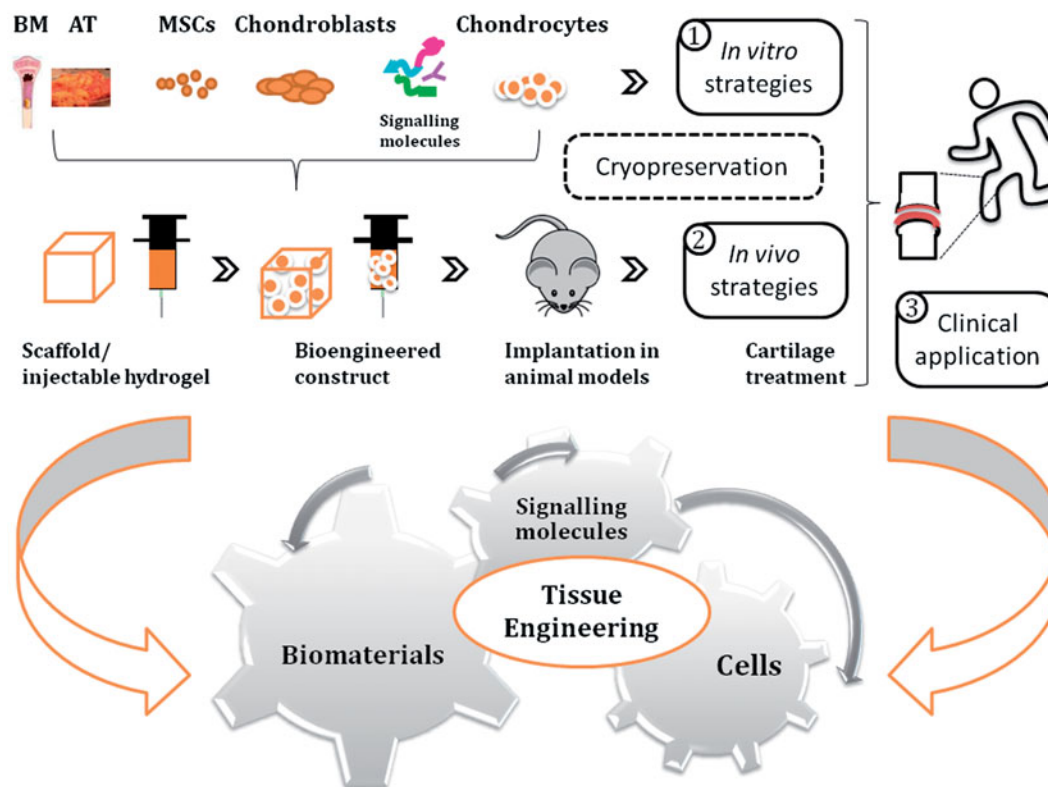


Figure 1. Schematic diagram showing multidisciplinary approach of cartilage tissue engineering (BM-bone marrow; AT-adipose tissue; MSCs-mesenchymal stem cells).

maintaining the round shape that characterizes specific cartilage cells phenotype (Abbott, 2003).

This review addresses the recent developments on the use of the above mentioned marine origin materials in studies envisioning the treatment and repair of cartilage defects. Several aspects are considered, namely the polysaccharide main characteristics/properties, *in vitro* and *in vivo* research and clinical/biomedical applications, with emphasis in cartilage TE (Figure 1).

### Cartilage structure, pathologies and tissue engineering therapies

In order to design adequate therapies for the regeneration of damaged cartilage tissue it is essential to know and understand its structure, function and properties, so as to mimic as closely as possible its native environment. Cartilage tissue is a stiff, dense and inflexible connective tissue typically deprived of blood vessels, aneural and with few cells (Vunjak-Novakovic & Freed, 1998). Chondrocyte specific characteristics include no cell-to-cell contacts, spherical shape, high individual metabolic activity and the ability to synthesize type II collagen. Furthermore, the cells in articular cartilage receive nutrition through a double diffusion barrier, survive on low oxygen concentration and hence depend on anaerobic metabolism (Bhosale & Richardson, 2008). Details about composition with the corresponding ultra-structure can be found in Supplementary Figure S1. In fact, considering the low cellularity and proliferative capacity of chondrocytes and due to specific characteristics such as being bound in lacunae with low migrating abilities to damaged areas

(Chhavi et al., 2011), cartilage tissue underlies an intrinsic inability to repair.

Damage of cartilage can be traumatic or degenerative and emerge as a result of a wide range of injuries or as an effect of another treated injury, having a great impact on the quality of life of thousands of people. Defects can be associated to partial and full thickness extension to the underlying bone and, based on the macroscopic changes of the articular cartilage, are categorized in grade I, II, III and IV (Suh et al., 1995). More detailed information on cartilage structure, diseases and treatment can be found elsewhere (Buckwalter & Mankin, 1998; Nestic et al., 2006).

Current strategies in the clinical field of cartilage repair progressed to what might be called the 3 ‘R’ paradigm: reconstruction, repair and replacement (Haleem & Chu, 2010). Cartilage regeneration strategies have evolved from marrow stimulation-based techniques to osteochondral transplantation and to cell-based repair techniques. Microfracture, autogenic (mosaicplasty) and allogenic tissue transplantation techniques show positive results for short term but low outcomes for longer times (Berthiaume et al., 2011; Gross et al., 2005; Lattermann & Romine, 2009). The four generations of the therapeutic approach consisting in the autologous chondrocyte implantation (ACI) intend to preserve the chondrogenic phenotype, maintain cellular viability and function (Gikas et al., 2009; Peterson et al., 2002). The 3rd generation of ACI matrix-induced autologous chondrocyte implantation (MACI) uses 3-dimensional (3-D) supports to achieve such requirements. Alternative cell sources have been investigated, particularly allogenic adult mesenchymal stem cells (MSCs) (Marquass et al., 2011). A summary description

of these cartilage repair techniques is detailed in Supplementary Table S1. Although these approaches offer good opportunities for the regeneration of cartilage defects, all current treatment options inflict some degree of tissue destruction before any therapeutic effect can be achieved. Consequently, the current available treatments based on “damage to heal approaches” (Rodrigues et al., 2011), have limitations, which might be overcome through emerging TE strategies (Figure 1). The potential advantages include the formation of a more reliable hyaline cartilage tissue, through the delivery of appropriate cell types embedded in a suitable hydrogel (Tuli et al., 2003) promoting enhanced integration with surrounding tissues (Hardingham et al., 2002). However, to achieve further improvements, minimally invasive procedures and innovative cell carrier concepts should be refined (Sittinger et al., 2004).

Furthermore, there is a lot of debate over the use of most suitable cell source in cartilage cell-based therapies. Ideally, a cell source should be easily available, enabling an excellent yield number, high proliferation capacity, stable phenotype/genotype, with no issues of immunogenicity or disease transmission risks, and no donor site morbidity. For cartilage TE, most of the published studies refer to the use of primary autologous chondrocytes (Brittberg et al., 1994; Wang et al., 2006) and adult stem cells (Noth et al., 2008; Raghunath et al., 2005). There are a number of pros and cons arguments in applying stem cells versus somatic, pre-committed cells for cartilage tissue engineering as summarized in Supplementary Table S2. Disadvantages associated with the use of chondrocytes are mostly related to the collection site, limited number of cells with low renewal capacity and the tendency to dedifferentiate Supplementary Table S2 (Jakob et al., 2001; Von Der Mark et al., 1977). Bone marrow-derived stem cells (BMSCs) (Kuroda et al., 2007) and adipose tissue-derived cells (ASCs) (Rada et al., 2009; Varma et al., 2007) have been frequently considered top candidates for cartilage TE, due to their ability to create functional cartilaginous tissues. Advantages of the two stem cell sources may be clearly identified in terms of collection procedures, cell number, proliferation capacity, while the major disadvantage include the need of growth factors supplementation (Supplementary Table S2). Some studies described a higher chondrogenic potential of BMSCs when compared to ASCs (Niemeyer et al., 2010) likely due to their role in bone formation, including endochondral ossification.

Another important issue is to determine the optimal number of cells (chondrocytes or stem cells) for a successful *in vivo* application. Although this is still under consideration, several studies suggest that higher cellular content induces better tissue repair (Gulotta et al., 2012; Iwasa et al., 2003; Watt, 1988). Commercially available sources of chondrocyte suspensions recommend a dose between  $0.5$  and  $2.0 \times 10^6/\text{cm}^2$  and  $2$  to  $3 \times 10^6$  for a  $4 \text{ cm}^2$  collagen membrane have been used in the clinical practice (Steinwachs et al., 2012). Moreover, the chondrocytes density in native cartilage tissue is around  $1.4 \times 10^7 \text{ cells}/\text{cm}^3$ , i.e. about 5–10% of the cartilage volume, but such cell density is difficult to replicate *in vitro* due to slow growth rates that differ depending on the cell type (Buschmann et al., 1992). The *in vitro* experiments have confirmed that growth factor

supplementation (Holland & Mikos, 2003) enhances the production of cartilage in tissue-engineered constructs (Blunk et al., 2002; Stoop, 2008). Clearly chondrogenesis is a complex process which involves not only biological growth factors, but also a carefully controlled time dependency (Bobick et al., 2009; Csaki et al., 2008). Furthermore, this process can be dramatically influenced by the 2D and 3D (two and three dimensional) environment in which cells are delivered/cultured. The fact that hydrogels provide 3D cellular microenvironments that can be tailored to stand physical, chemical and biological signals, has encouraged the development of engineered functional tissue equivalents based on such systems. In addition, the hydrogel structure protects the entrapped cells against the immune system of the host, simultaneously allowing the unhindered passage of nutrients, oxygen and secreted therapeutic factors or proteins (Zimmermann et al., 2007). The hydrogels design can vary from injectable systems to solid structure like discs, fibers, cylinder and capsules ranging from micro to macro dimensions.

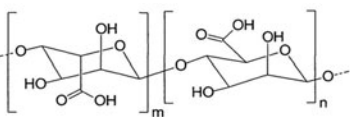
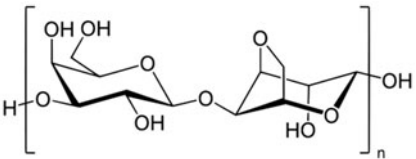
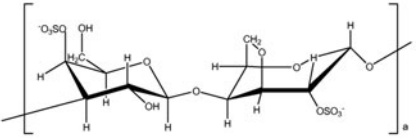
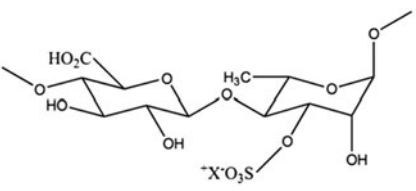
Several natural origin hydrogels sources including proteins (collagen, elastin, silk fibroin, fibrin), polysaccharides (chitosan, chondroitin sulphate, hyaluronic acid) and seaweed source (alginate, agarose, carrageenan, ulvan) have been extensively studied for cartilage repair (Malafaya et al., 2007). According to data collected from the literature, the reasons for using seaweed polysaccharides in regeneration applications lie in their intrinsic features, such as chemical similarity with native tissue components, non-harsh processing, variable degrees of hydrophilicity and biocompatibility (Oliveira & Reis, 2010). Natural materials obtained from algae are often preferred for biological applications since they are believed to elicit low immune response when choosing a potential biological application (Gomes et al., 2008). Most of them offer advantages concerning biocompatibility, which is of extreme importance for the integration with the surrounding tissues. Moreover, they are readily available, inexpensive and easy to fabricate into hydrogels, making them appealing choices for different biomedical application (Ko et al., 2010).

## Algae polysaccharides

### Detailed characteristics

Some algae polysaccharide based hydrogels, namely alginate and agarose have already been extensively studied for TE applications, but others such as carrageenan and ulvan are just starting to be investigated (Table 1). Even though hardly applied in the field of cartilage repair, these algae polysaccharides have recently registered increased attention in the biomedical research field. Such consideration is owed to the intriguing feature of the amounts of sulphate groups found in their structure, whose beneficial biological properties prompt scientists to focus on their use in the biomedical field (Silva et al., 2012). Certainly, the presence of sulphate groups in their structure/composition and the chemical affinity with mammalian glycosaminoglycans (GAGs) due to the similar chemical content, play important roles in the antiviral, anticoagulant, antioxidant and anticancer activity of these polysaccharides (Vera et al., 2011).

Table 1. Important properties of algae polysaccharide.

Polysaccharide	Seaweed type	Gelation	Degradation	Cell interaction	Ref.
Alginate 	Brown seaweed	Ionic (Ca <sup>2+</sup> )	Ion exchange; others	Low	Augst et al. (2006)
Agarose 	Red seaweed	Thermal	Non-degradable	Low	Varoni et al. (2012)
Carrageenan 	Red seaweed	Thermal and ionic (Ca <sup>2+</sup> /K <sup>+</sup> )	Ion exchange	Low	Campo et al. (2009)
Ulvan 	Green seaweed	Ionic (boric acid and divalent cations)	Enzymatic degradation	n.d.	Alves et al. (2013)

Modified from Hunt & Grover (2010) and Tan & Marra (2010).

n.d. – not determined.

The carrageenans are temperature dependent materials since they are soluble in water above 60 °C and gel upon cooling to temperatures between 30 and 40 °C, being designated as a physical hydrogels. The gelation of carrageenan is induced by the reversible temperature sensitive formation of intermolecular hydrogen bonds and involves a coil to helix conformational transition followed by helix aggregation (Mangione et al., 2003). The thermo-responsive solubility behavior and the gelation promoted by monovalent cations such as potassium ions, open perspectives to develop systems that gel at body temperature (Núñez-Santiago et al., 2011). They are characterized as high molecular weight polysaccharides with high sulfate-ester content, meaning higher levels of solubility and lower gel strength (Stanley, 1987). Ionically cross-linked hydrogels, such as carrageenan, normally undergo slow dissolution that can be shifted through chemical modification and the stiffness can be altered as well, allowing tuning of the mechanical properties (Daniel-da-Silva et al., 2009). Ulvan structure shows great complexity and variability as evidenced by the numerous oligosaccharide repeating structural units identified (Ray & Lahaye, 1995). The main constituents of ulvan are sulfated rhamnose residues linked to uronic acids, resulting in a repeated disaccharide unit  $\beta$ -D-glucuronosyl-(1,4)- $\alpha$ -L-rhamnose 3-sulfate, called aldobiouronic acid (Jaulneau et al., 2010). In aqueous solution, ulvan tends to form micro-aggregates, and the

limited number of functional groups available for chemical modifications hampers its potential versatility (Chiellini & Morelli, 2011).

The friendly gelation mechanism induced by temperature or ions clearly demonstrates the relevance of the use of such biopolymers in the field of TE. In particular, sulphated polysaccharides (carrageenan and ulvan) present a real potential for delivering products for therapeutic applications providing a valid alternative to mammalian glycosaminoglycans.

More details regarding seaweed type, gelation or degradation mechanism and the interactions of alginate, agarose, carrageenan and ulvan with cells are summarized in Table 1. These polymers are cell friendly due to the ionic nature of the cross linking process. Nevertheless, the cell interaction is low and the degradation is still not fully studied. For complete description of chemical structure, gelation process or other characteristics of algae polysaccharide, we recommend the reader to other existing publications (Gomes et al., 2013; Lahaye & Robic, 2007; Silva et al., 2012).

However, not every characteristic comes only as an advantage. Supplementary Table S3 summarizes pros and cons features reported for the polysaccharide hydrogels. As advantages, the cheap source and the easy gelling characteristic stand out, although the ionic degradation, low mechanical properties and low cellular interaction might be difficult



to overcome. For example, the lack of manufacture reproducibility on a large scale, batch-to-batch variability and also the presence of impurities in the final processed material due to the extraction process, constitute major concerns (Yang et al., 2001). These are time- and money-consuming processes and introduce variation in the biological response. For example, recent data suggests that contaminants during extraction processing are likely to cause an immune response but this issue is still being disputed, although such molecules can be removed by purification methods (Klöck et al., 1997; Orive et al., 2006). Other associated common weakness of the seaweed-based hydrogels are related to inadequate mechanical properties, which is a common trend (Drury & Mooney, 2003). Nonetheless, it is expected that a hydrogel structure will partially tolerate shock absorption and deformation, mimicking articular cartilage characteristics. Enhanced mechanical properties can be achieved by adjusting various parameters, including the concentration, the cross-linking density and the possibility of mixing such hydrogels with composite. In addition, introducing photo-cross-linkable parts, appropriate to the chemical structure of the hydrogels, can modify the stiffness of the structure but simultaneously compromise the viability of the cells (Jeon et al., 2009).

In terms of cellular response, in these natural algae polysaccharides, like many hydrogels, there is no integrin interaction between cells and the hydrogels matrix. Therefore, cells retain their rounded shape, likely to enhance chondrogenesis (Steward et al., 2011). On the other hand, long-term

integrin binding can lead to dedifferentiation and formation of fibrocartilage (Steward et al., 2011). However, being hydrophilic and binding to water, hydrogels provide few sites for the cells to attach, directly affecting cell viability and proliferation.

### ***In vitro* applications of hydrogels based on algae polysaccharide**

Considering all the above mentioned polysaccharide hydrogels characteristics, this section will review some of the most extensively studied algae polysaccharide-based hydrogels, such as alginate and agarose and some new candidates, like carrageenan and ulvan, for the culture of chondrocytes and differentiation of stem cells. In what follows, it will be provided an overview of different approaches and parameters including hydrogel concentration, different cell source and cell number, as well as the stimulation with various growth factors, envisioning application in cartilage regeneration. Table 2 summarizes the most recent studies concerning cartilage TE applications using algae polysaccharide hydrogels.

#### *Alginate*

Alginate continues to be the most widely used hydrogel for *in vitro* studies due to its easy production, effectiveness and low cost (Tables 1 and 2). Applications of alginate in the

Table 2. A summary of key studies from the current literature describing polymers hydrogels, cell types and cell densities used in laboratory cartilage tissue engineering applications.

Polymer concentration	Cell source	Signaling molecules	Cell density (cells/mL)	Ref.
<b>Alginate</b>				
1.2% (w/v)	Human chondrocytes	–	$2 \times 10^6$	Chia et al. (2005); Choi et al. (2006); Lee et al. (2007)
20 mg/mL; 2% (w/v)	Non-human chondrocytes	IGF-1, TGF- $\beta$ 1	$25\text{--}50 \times 10^6$	Coates & Fisher (2011); Gleghorn et al. (2007); Lee et al. (2007); Stevens et al. (2004b)
20 mg/mL	Human bone marrow MSCs	TGF- $\beta$ 3	$20 \times 10^6$	Ma et al. (2012); Xu et al. (2008)
2%; 1.5% (w/v)	Non-human bone marrow MSCs	TGF- $\beta$ 1 or Dex;	$20 \times 10^6$ ; $1\text{--}2 \times 10^6$	Bosnakovski et al. (2006); Coleman et al. (2007)
2%; 1.2% (w/v)	Human adipose tissue MSCs	TGF- $\beta$ 1; BMP-6	$10 \times 10^6$ ; $5 \times 10^6$	Awad et al. (2004); Estes et al. (2006)
<b>Agarose</b>				
2% or 3% (w/v)	Non-human chondrocytes	TGF- $\beta$ 3	$30\text{--}60 \times 10^6$	Beris et al. (2005); Lima et al. (2007); Rada et al. (2009)
2% (w/v)	Non-human bone marrow MSCs and chondrocytes	TGF- $\beta$ 3 or –	$10\text{--}60 \times 10^6$	Bian et al. (2010); Kelly et al. (2006); Mauck et al. (2003); Mauck et al. (2006)
2% (w/v)	Human bone marrow MSCs	TGF- $\beta$ 3	3, 6, and $9 \times 10^6$	Charles Huang et al. (2004); Finger et al. (2007); Pelaez et al. (2009)
2% (w/v)	Human adipose tissue MSCs	TGF- $\beta$ 1	$10 \times 10^6$	Awad et al. (2004)
2% (w/v)	Non human bone marrow & adipose MSCs	TGF- $\beta$ 1; TGF- $\beta$ 3	$10\text{--}15 \times 10^6$	Kisiday et al. (2008); Niemeyer et al. (2010); Sheehy et al. (2012); Steward et al. (2012); Thorpe et al. (2008)
<b>Carrageenan</b>				
0.8% and 1.2% (w/v)	Human chondrocytes	–	$2 \times 10^6$	Pereira et al. (2009)
1.5% (w/v) 2.5% (w/v)	Human adipose tissue MSCs; Human nasal chondrocytes;	TGF- $\beta$ 1	$5 \times 10^6$	Popa et al. (2012); Rocha et al. (2011)
2% (w/v)	ATDC5-chondrocytic cell line	–	$1 \times 10^6$	Popa et al. (2011)
<b>Ulvan</b>				
5%; 8% (w/v)	L929-mouse fibroblasts	–	$5 \times 10^5$ cells/structure	Alves et al. (2012c)

GF – Growth factor; TGF – Transforming growth factor; BMP-6/BMP-2 – Bone morphogenetic protein – BMP-6; Dex – dexamethasone; IGF-1 – Insulin-like growth factor; ES – embryonic stem; ATDC5 – mouse teratocarcinoma AT805-derived cell line.

cartilage TE field revealed that after an initial cell loss, chondrocyte maintained their typical chondrocyte phenotype (van Susante et al., 1995). To maintain the chondrocytic phenotype and the synthesis of ECM proteins, alginate has been used *in vitro* as a matrix for the three-dimensional culture of human articular chondrocytes from elderly patients (Carossino et al., 2007). Similar performance was reported in another study where human articular chondrocytes, embedded in alginate beads, showed enhanced collagen type II and aggrecan expression (Gründer et al., 2004; Vinatier et al., 2009). Moreover, the 3D alginate culture system was proven to be efficient in keeping high viability, chondrogenic phenotype and promoting the redifferentiation of articular chondrocytes (Choi et al., 2006; Lin et al., 2009). A different work showed that chondrocytes encapsulated in alginate maintain their viability and function due to the addition of microchannels to the polymeric hydrogels (Choi et al., 2007). In another study, the supportive alginate-based hydrogels provided an adequate environment to deliver chondrocytes and, when compared with monolayer culture, stimulated the deposition of sulphated glycosaminoglycans (sGAG) and collagen type II, but without promoting redifferentiation (Chia et al., 2005). Furthermore, alginate hydrogels have been used to expand and induce stem cell differentiation (Tortelli & Cancedda, 2009). Human MSCs encapsulated in alginate beads undergo chondrogenesis, demonstrated by the cells assuming a rounded morphology with lacunae. Another report showed the development of hyaline cartilage-like tissue since it was, positively stained for Safranin-O and other typical chondrogenic markers, namely COL2A1 and COL10A1 (Ma et al., 2003). Human MSCs showed a time-dependent accumulation of sGAG, aggrecan and type II collagen in this type of hydrogel (Xu et al., 2008). In other study, bone marrow-derived stem cells (BMSCs) encapsulated in alginate evidences an enhanced cartilaginous matrix accumulation over agarose (Coleman et al., 2007). Alginate has been demonstrated to improve the stability of the system, supporting sGAG and collagen II production as well as chondrogenic gene expression, when human BMSCs were entrapped in a blend that included fibrin in addition to alginate (Ma et al., 2012). Induction of chondrogenesis of stem cells, isolated from adipose tissue in alginate hydrogels, has also been achieved. After 2 weeks of *in vitro* culture the adipose-derived stem cells produced cartilage matrix proteins which show dependency of the physical environment and the culture conditions (Erickson et al., 2002). To overcome weaknesses like low mechanical and uncontrollable degradation properties, ionically cross-linked alginate hydrogels have been modified, with no toxic effects on the encapsulated MSCs, supporting chondrogenic differentiation (Ghahramanpoor et al., 2011). Recently, investigators have produced hybrid constructs mixing alginate with other materials or tailored alginate with synthetic adhesion peptides as ways of improving its properties (Bidarra et al., 2011; Connelly et al., 2007; Wayne et al., 2005).

### Agarose

Agarose has also been widely investigated in cartilage TE strategies (Table 2). For example, encapsulation within agarose hydrogels has shown to support rabbit articular

chondrocytes redifferentiation (Benya & Shaffer, 1982). Articular chondrocytes seeded on agarose hydrogels demonstrated enhanced chondrogenic matrix elaboration when cultured under physiological deformational loading, accelerating the formation of a cartilage-like tissue (Mauck et al., 2000). Compressive mechanical forces are transmitted to the embedded chondrocytes which respond by producing extracellular matrix proteins leading to increased stiffness of the newly developed engineered tissue (Bougault et al., 2008). The culture of murine chondrocytes embedded within agarose hydrogels has been shown to maintain the chondrocytic phenotype, allowing matrix deposition around chondrocytes and showing that chondrocytes sense and respond to mechanical forces (Bougault et al., 2009). The concept of developing functional TE systems centered on the use of bioreactor enabling physiologic-like loading, has also reported successful results with immature bovine primary chondrocytes but did not produce the same outcome when using adult canine primary chondrocytes (Lima et al., 2007). Moreover, studies suggest that the age of the cells plays an important role, showing that unpassaged cells can elaborate an inferior matrix as compared to passaged mature chondrocytes. The continuous supplementation of TGF- $\beta$ 3 in combination with mechanically loading prior to implantation also seems to result in an improved engineered tissue substitute (Bian et al., 2010). Agarose hydrogels have also been investigated in combination with mesenchymal stem cells, including human adipose-derived stem cells and bovine mesenchymal stem cells, for a variety of applications, including cartilage repair, as shown in Table 2. Agarose hydrogels are noted for their ability to promote and maintain the chondrogenic phenotype of bone marrow stem cells (BMSCs), with deposition of cartilage-specific biomacromolecules (Huang et al., 2009). Another work showed that the chondrogenesis of the stem cells was directly correlated with the number of cells used, meaning that higher cell density led to enhanced expression of cartilage-specific gene (Charles Huang et al., 2004). It has also been reported that BMSCs produced an ECM with lower mechanical properties than that produced by differentiated articular chondrocytes embedded in agarose hydrogel (Erickson et al., 2009; Mauck et al., 2006). Findings reported in other publications such as the induction of chondrogenesis in hMSC-seeded agarose constructs without TGF- $\beta$ , suggest that the application of hydrostatic pressure may initiate chondrogenesis faster than lower pressure (Finger et al., 2007). Although the exclusion of TGF- $\beta$ 3 from culture conditions has been reported to have a superior effect on the mechanical properties and also on the biochemical content (Huang et al., 2009), the functionality of cartilaginous tissues using MSCs from joint infrapatellar fat pad, encapsulated in agarose hydrogels, has resulted in robust chondrogenesis with TGF- $\beta$ 3 supplementation, as shown by another study (Buckley et al., 2010).

### Carrageenan

Carrageenan hydrogels have been mostly used as drug or growth factor delivery systems (Daniel-da-Silva et al., 2011; Rocha et al., 2011; Santo et al., 2009), immobilization of enzymes (Desai et al., 2004) and in pharmaceutical

formulations (Sipahigil & Dortunc, 2001). However, the characteristics and specific properties of these natural derived hydrogels concerning their potential for cartilage regeneration are poorly exploited in the literature (Pereira et al., 2009). Upon adding cations, the carrageenan solution rapidly forms a gel and can be used as *in situ* cell matrix delivery material, due to its mild cross-linking properties (Popa et al., 2011) which makes it extremely interesting to be applied in the biomedical field (D'Ayala et al., 2008). Nevertheless, carrageenan has been used for the encapsulation of human-adipose-derived stem cells (hASCs), human nasal chondrocytes (hNCs), and a chondrocytic cell line (ATDC5) showing potential for cartilage regeneration strategies (Popa et al., 2012). The findings from this study showed that hASCs embedded in  $\kappa$ -carrageenan hydrogels exhibited higher expression of typical cartilage markers (collagen type II and aggrecan) than human chondrocytes, providing further evidence for the advantageous use of hASCs as an alternative cell source in cartilage treatments (Table 2). In a subsequent study, hASCs encapsulated in  $\kappa$ -carrageenan hydrogel and cultured with medium supplemented with chondrogenic growth factors appeared to increase the stiffness and the viscoelastic properties of the hydrogel construct providing a versatile platform for cartilage TE (Popa et al., 2013a).

### Ulvan

Ulvans are the major constituents of green seaweeds cell walls (Ulvales, Chlorophyta) and are composed of rhamnose, glucuronic acid, iduronic acid, xylose and sulphate (Lahaye & Robic, 2007). These green algae can be prepared into different structure designs such as membranes (Alves et al., 2012b, 2013), particles (Alves et al., 2012a), hydrogels (Morelli & Chiellini, 2010) and 3D porous structures (Alves et al., 2012b). Due to the difficulties in identifying the chemical structure of algal sulfated polysaccharides, the relation between their structures and biological activities is not completely understood (Jiao et al., 2011). Despite ulvan chemical variability, biological properties have been frequently reported like antioxidant effects (Qi et al., 2010), anti-tumoral activity (Jiao et al., 2010), immunostimulating ability (Leiro et al., 2007). Antihyperlipidemic activities (Sathivel et al., 2008) or antiviral effects (El-Baky et al., 2009) have also been studied. Subsequent to all of these findings, ulvan polysaccharides are considered to have great potential for biomedical applications (Table 2). Nevertheless, relevant studies on ulvan are limited and their applicability may range from drug delivery (Alves et al., 2012b) to wound dressing or TE (Alves et al., 2012a, 2012c).

From the studies discussed above, it is possible to conclude that the redifferentiation of chondrocytes has been more successful using agarose hydrogels than alginate, although stem cells encapsulated in alginate displayed enhanced cartilaginous matrix accumulation over agarose (Chia et al., 2005; Coleman et al., 2007). Furthermore, these studies also showed that the addition of growth factors promoting stem cell chondrogenic differentiation increases the mechanical properties and the ECM content when using both alginate or agarose hydrogels construct. Moreover, the ECM produced by stem cells has lower mechanical characteristics compared to

the ECM synthesized by articular chondrocytes in agarose hydrogels (Erickson et al., 2009; Mauck et al., 2006).

When comparing alginate and agarose hydrogels as cell encapsulation systems, it is suggested that the gelation mechanism influence the interaction with cells, considering that the cells interfere with the hydrogen bond formation required for agarose gelation (Shoichet et al., 1996). The addition of cells did not decrease alginate strength as much as for agarose gels, indicating that the cells have an impact over the mechanical properties of the hydrogels. The chemical structure is also responsible for different cellular behavior, since alginates with high-M or G and intermediate-G content produce distinct cell growth. Also, the protein diffusion profile and the presence of ligands for cell attachment will affect the proliferation and chondrogenic differentiation of the cells when using alginate or agarose constructs. Overall, the studies presented suggest that agarose is more biologically active when compared to the inert nature of alginate (Awad et al., 2004).

Some advantages of carrageenan hydrogels over current systems include the thermo-reversible and ionic nature enabling mild condition for cell encapsulation, versatility of processing them into various shapes/formats or use as injectable systems administrated under physiological conditions. However, a significant temporal weight loss of the 3D carrageenan hydrogel was observed when exposed to environments rich in electrolytes, ions and cations, a behavior that has been commonly reported for other ionic hydrogels (Nicodemus & Bryant, 2008). Such behavior might be overcome through chemical modification. Ulvan hydrogel exposed versatility, enabling processing in different shapes; in addition, the presence of functional groups allow them to be easily chemically modified, although the unusual chemical composition and structure did not allow a standardized commercial form of this polysaccharide. Nevertheless, the potential of the ulvan hydrogel to be used in TE and regenerative medicine is still barely explored during *in vivo* studies on algae polysaccharide based hydrogels.

### ***In vivo* studies on algae polysaccharide based hydrogels**

As a first step in demonstrating the *in vivo* chondrogenic potential of tissue engineered constructs, researchers have used heterotopic animal models, typically consisting on the implantation of the bioengineered cartilage-like tissue in dorsal subcutaneous pouches of immuno-compromised nude mice (Reinholz et al., 2004). Although these experiments may indicate the biological construct performance under *in vivo* conditions, different results may be observed when the cartilage substitute is implanted into a cartilage defect.

### *Alginate*

Several *in vivo* studies have been conducted aiming at assessing the potential of alginate as a supportive matrix for different relevant cell types, including primary chondrocytes and stem cells from different origins, in various animal models (Table 3). Alginate seems to stimulate chondrogenesis as suggested by studies where chondrocytes were seeded and stimulated to produce a cartilage-like matrix, being



Table 3. A summary of key studies describing polymers hydrogels, cell types, densities and implantation time register in experimental animal models for cartilage tissue engineering applications.

Polymer concentration	Cell type	Formulation cells density	Animal model (defect)	Follow up time	Ref.
Alginate					
2% sodium alginate; 1.2%; 0.75%	Chondrocytes	10–50 × 10 <sup>6</sup> cells/mL	Subcutaneous implantation, mice; defect in rabbits;	8 weeks; 30 weeks; 6 months	Chang et al. (2001); Marijnissen et al. (2002)
2% alginate; 1.2% alginate beads	Chondrocytes	50 × 10 <sup>6</sup> cells/mL; 10 <sup>7</sup> /mL; 4 × 10 <sup>6</sup> cells/mL	Subcutaneous implantation, sheep/nude mice; defects 4 mm diameter mini-pigs	1 week; 5 weeks; 6 months;	Chang et al. (2003); Chawla et al. (2007); Kaps et al. (2002)
1.2%; 2% sodium alginate	Bone marrow MSCs	1 × 10 <sup>6</sup> cells/defect	Ø3 × 6 mm defects in rabbits; Ø4 × 5 mm defects in rabbit	3 months	Diduch et al. (2000); Lopiz-Morales et al. (2010)
2.2% barium alginate; 1.25% sodium alginate	Human and mouse ESCs	10 × 10 <sup>6</sup> cells/mL	SCID and BALB/c nude mice		Bai et al. (2010); Dean et al. (2006)
Agarose					
2%	Chondrocytes	–	Rabbit	18 Months	Rahfoth et al. (1998)
2%	Chondrocytes	30 × 10 <sup>6</sup> cells/mL.	Canine knee	12 Weeks	Ng et al. (2010)
0.6–1%	Periosteum	–	Rabbit knee	6 Weeks	Stevens et al. (2004a)
0.6%	Human bone marrow/adipose tissue MSCs	10 × 10 <sup>6</sup> cells/mL	Injection, muscles of athymic mice	5 Weeks	Xu et al. (2008)
2%	Bone marrow MSCs	20 × 10 <sup>6</sup> cells/mL	Subcutaneous implantation, nude mice	28 Days	Vinardell et al. (2012)

MSCs – mesenchymal stem cells; Ø – diameter; SCID – albino mouse strain with spontaneous severe combined immunodeficiency mutation; BALB/c – albino, immunodeficient inbred mouse strain; ESCs – embryonic stem cells.

subsequently implanted subcutaneously in nude mice for 8 weeks (Marijnissen et al., 2002). The same study suggests that the addition of alginate provided retention of the cartilage graft shape without any influence on the amount of cartilage matrix proteins produced per tissue wet weigh. The implantation of alginate with allogenic rabbit chondrocytes, (followed up for 6 months), provided complete *in vivo* reconstruction of a full-thickness articular cartilage defect (Fragonas et al., 2000). After 6 months, the implantation of alginate alone developed only a fibrous cartilage while the suspension of chondrocytes led to the recovery of a normal tissue structure. On the other hand, transplanted chondrocytes in an alginate gel implanted into full-thickness osteochondral defects in rabbits did not form repaired tissue and cell numbers decreased with time (Mierisch et al., 2003). The evaluation of chondrogenesis of human adipose tissue-derived stromal cells when cultured in alginate hydrogels and implanted subcutaneously in nude mice for up to 20 weeks (Beris et al., 2005), revealed significant production of cartilage matrix proteins, suggesting the beneficial use of alginate systems for cartilage regeneration, as they maintain stable cartilaginous phenotype with no sign of hypertrophy during 20 weeks. Also, using a rabbit model, full-thickness defects filled with alginate beads containing rabbit stromal cells remained viable, showing chondrogenic phenotype embedded in a positively stained proteoglycans matrix and occupying the defects within regenerated tissue (Diduch et al., 2000).

### Agarose

Agarose hydrogels laden with different cells types have been studied to assess their ability to induce the *in vivo* development of cartilage tissue (Table 3). One of these studies showed that, 18 months after the transplantation of allogenic chondrocytes in agarose hydrogels in rabbits, neo cartilage tissue was formed, exhibiting higher type II collagen and proteoglycan contents when compared to untreated defects. Additionally, control implants of agarose alone produced poor fibrous substitute tissue, insufficient healing and incomplete filling of the cartilage defects (Rahfoth et al., 1998). In another study, the implantation of chondral and osteochondral constructs based on primary or passaged (using growth factors) canine chondrocytes encapsulated in agarose, showed no gross or histological signs of rejection and excellent integration with surrounding cartilage and subchondral bone (Ng et al., 2010). However, when agarose constructs were seeded with stem cells and subcutaneously implanted in nude mice, they showed a significant decrease of sGAG content, while no significant change was observed using primary chondrocytes, indicating that the *in vitro* generated chondrocyte-like phenotype was transient (Vinardell et al., 2012).

### Laboratory to clinical application

Although the *in vivo* trials involving cell laden hydrogels have registered different outcomes, overall, the use of hydrogels in cartilage regeneration strategies produce positive results.

The clinical approach to any cartilage repair technique should be customized based on both patient-specific and lesion-specific factors (Haleem & Chu, 2010).



Table 4. Commercial products in cartilage tissue engineering.

Product name	Company	Product composition	Regulatory status	Form and Use	Ref.
Cartipatch	Tissue Bank of France, Lyon, France	Agarose-alginate hydrogel & autologous chondrocytes	Clinical trial phase is unclear	Chondral and osteochondral	Selmi et al. (2008)
Carticel, MACI	Genzyme Biosurgery, Cambridge, Massachusetts, USA	Type I/III collagens (porcine) and autologous chondrocytes	Completed phase III clinical trials; product not available	Sheet- articular cartilage injury	Bahaud et al. (1998); Bartlett et al. (2005); Cherubino et al. (2003)
Atelocollagen	Koken Co. Ltd. Tokyo, Japan	Type I collagen	Clinical trial phase is unclear	3D discs –articular cartilage injury	Adachi et al. (2007); Adachi et al. (2006)
CaReS	Arthro Kinetics, Esslingen, Germany	Type I collagen (rat-tail) and autologous chondrocytes	2007 Germany;	3D discs –articular cartilage injury	Maus et al. (2008)
Novocart 3D	TE TEC Tissue Engineering Technologies AG, Reutlingen, Germany	Collagen-chondroitin-sulfate based membrane and autologous chondrocytes	Clinical trial phase is unclear		Ochs et al. (2007)
Hyalograft® C	Fidia Advanced Biopolymers, Abano Terme, Italy	Benzyl ester of hyaluronic acid (roosters) and autologous chondrocytes	Commercial sale began in 2000; completed phase III clinical trials	3D discs –articular cartilage injury	Kon et al. (2009); Manfredini et al. (2007); Vijayan et al. (2012)
BioSeed-C	Biotissue Technologies, Freiburg, Germany	Polymer of polyglycolic/polylactic acid and polydioxanone and autologous chondrocytes	First clinical trials were in 2001; completed phase II clinical trials	3D discs –articular cartilage injury	Kreuz et al. (2009)

Adapted from Ahmed & Hincke (2010); Jacobi et al. (2011); Place et al. (2009). Status and descriptions of the clinical trials are available from the web site of U.S. National Institutes of Health, <http://clinicaltrials.gov/>.

The engineered graft has to fit completely into the damaged area, providing support and biological cues to restore the tissue function. A well-designed clinical model to regenerate cartilage should always consider the safety, efficiency and simplicity of the developed system, even if it is necessary to compromise the quality of the regenerated tissue (Chiang & Jiang, 2009). Additionally, to translate the research-scale production into routine clinical use, biological cartilage substitutes will need to demonstrate cost-efficiency ratios that are beneficial over other existing treatments and excellent reproducible results in order to perform reliable quality control and standardization (Pelttari et al., 2009).

One important development for the translation of TE products into the clinical/industrial scenario would be the production of “off the shelf” products, eliminating the waiting time and reducing the patient’s incapacity period, relying on the availability of the engineered grafts upon immediate clinical need. This could be achieved by common cryopreservation approaches, (Bhakta et al., 2009; Kuleshova et al., 2007) specifically designed and scaled up to generate ready-to-use engineered tissue substitutes. However, few studies have focused on tissue substitutes banking and storage technologies, addressing the impact of such process on hybrid constructs that contain a scaffold/carrier material and cells (Bhakta et al., 2009; Chen et al., 2011). Yet, there are studies that have reported the effect of cryopreservation in cell encapsulating systems (hydrogels) (Miyamoto et al., 2009; Popa et al., 2013b) using several different cryoprotectants, (Campbell & Brockbank, 2007; Thirumala et al., 2010,) and vitrification solutions (Kuleshova et al., 2007; Lawson et al., 2011), predicting that tissue-engineered products can be cryopreserved without prejudice of their functionality

It is already possible to find several hydrogels approved or under clinical trials for cartilage treatments (Table 4). The most well-known commercial product based on polysaccharides is a hybrid medical grade agarose-alginate hydrogel, named Cartipatch® (Company/Responsible – TBF Genie Tissulaire, Mions, France). This product was tested for implantation of autologous chondrocytes in chondral and osteochondral human defects. After a minimum follow-up of two years, patients with lesions larger than 3 cm<sup>2</sup> improved significantly more than those with smaller lesions. Also, in 8 out of 13 patients, hyaline cartilage-like repaired tissue was predominantly observed (Selmi et al., 2008). In a phase III clinical trial, an alginate gel suspension (Curis Inc., Lexington, MA) was used, injecting autologous chondrocytes harvested from the ear cartilage for the treatment of pediatric patients with vesicoureteral reflux. As seen in Table 4 the majority of commercial tissue engineering products developed for cartilage regeneration are collagen-based hydrogels, since collagen is a major component of ECM and due to the presence of bioactive domains in its structure, which regulates important processes during chondrogenesis (Ahmed & Hincke, 2010). However, other clinical products are being used to promote cartilage repair namely, hyaluronate (hyaluronic acid) or synthetic polymers. Although there is an extensive range of products being studied for cartilage treatments, only few of them are approved for clinical trials.

Ultimately, the essential biopolymers properties that prevent the industrial scale-up and clinical applications of these hydrogels are the existing batch-to batch differences, the inferior mechanical characteristics and low cell interaction.

### Summary and future directions

Research and knowledge on polysaccharide-based hydrogels for TE application is continuously growing and there are many questions that instigate the attempts to use them in cartilage regeneration therapies. To mimic the structural (biochemical and histological characteristics) and functional (compression properties) complexity of native cartilage is a challenging objective to accomplish. Consequently, every attempt to achieve this aim brings scientists closer in the process of assembling the necessary knowledge to repair damaged cartilage tissue.

The knowledge and research, so far, supports that natural cell-carrying matrices based on algae polysaccharide are suitable for engineering cartilage. Many of these hydrogels can be combined well with the cells and can be designed as injectable systems, setting *in situ* and filling any shape and size of the recipient defect. All of these available data need to be compiled in order to develop clinical relevant solutions for cartilage regeneration, considering that the functionality of these hydrogels in the laboratory and animal experiments has been established.

For *in vitro* applications, the developed tissue engineered constructs show promising results, the potential value for the *in vivo* treatment of cartilage defects remains uncertain, as set-up parameters of the performed *in vitro* and *in vivo* studies vary to a great extent. Furthermore, *in vitro* manipulation of either chondrocytes or stem cells used is required in most of the currently available approaches. In fact, culturing the cells prior to *in vivo* implantation allows creating a mature cartilage and the presence of matrix around the cells is known to enhance donor cell retention at the repair site (Berthiaume et al., 2011). However, the manipulation of the cells *in vitro* increases concerns on how cells may be affected and increases the complexity of the treatment, compromising the safety of the procedure and the associated cost. Despite the advantages of using polysaccharide hydrogels in cartilage TE, some concerns remain which results mainly from insufficient characterization of the cell hydrogel construct or the level of repaired tissue formation and from too short follow-up periods. Findings have demonstrated that cell density is likely a key parameter to consider in TE design, since cells could not develop cell-cell contacts or express cartilage-like tissue component without some minimal cell density which can vary with the source of cells (Troken et al., 2007). Also, cells respond differently to substrate rigidity. Therefore, the increase in polymer concentration or stiffness involves consequences on the cell morphology, cytoskeletal structure and on stem cell differentiation (Engler et al., 2006; Yeung et al., 2005). Although current cartilage TE strategies investigate a wide range of hydrogel materials in combination with a large variety of cell sources, the existing data demonstrates that the optimal hydrogel type has not been determined so far, while outcomes also depend on the cell type – stages of differentiation and culture conditions (Renth

& Detamore, 2012). Nevertheless, hydrogels based on marine origin polysaccharides are still commonly used and each individual natural polymer has strengths and weaknesses to its use and results can vary depending on the application.

Besides these comments, consideration should be given to the lesion location and damage size, activity level and patients' age. These are influencing parameters in choosing the right cartilage repair techniques and controlling the outcome of the treatment. More clinical trials are needed to find definitive answers and to develop procedures that relieve patient pain and produce a durable replacement for damaged cartilage. Until the present day, no treatment has been shown to completely regenerate or restore articular cartilage/subchondral bone to its normal status, therefore articular cartilage repair remains under intense investigation and the cure is yet to be defined. The newer generation of repair/regeneration techniques has shown some promise, but the long-term outcome is still unknown.

Furthermore, the extended variety of existing polysaccharides and their inherent features opens a wide range of opportunities for synergistic fabrications of new multifunctional biomaterials (Shull, 2012; Sun et al., 2012). The development of strategies aiming to overcome hydrogels limitations is gaining increasing interest. Such alternatives encompass the fabrication of blended systems, or chemically and/or physically modification of their original structure. Modifying the hydrophobic properties of the polysaccharides to self-associate, reducing the molecular weight by depolymerization and changing the sulfation content are ways to improve polysaccharide-hydrogels properties (Lawson et al., 2011). The addition of interest signaling molecules is another way to confer a better outcome performance to the overall tissue engineered constructs (Freyria & Mallein-Gerin, 2012).

The opportunities and trends regarding the use of natural based hydrogels are currently directed towards strategies for chemical modification through photopolymerization, allowing temporal and spatial control of various features (Jeon et al., 2009) and to technologies to conjugate peptides (Yamada et al., 2010).

### Final remarks

The purpose of this review is to detail a range of the most commonly used biomaterial hydrogels based on algae polysaccharides for transplantation of chondrocytes or stem cells in a cartilage regeneration strategy approach. The progress from the *in vitro* experimental cartilage TE research, using relevant cells types, to the *in vivo* scenarios was described throughout the demonstration of applicable results. Ultimately, some hydrogels have already been submitted through clinical trials, showing that it is possible to maximize the generated knowledge and attain the fabrication of medical products that make use of these systems.

With regard to their prevalence in the cartilage TE field, it is easily identified that alginate is the most commonly used polysaccharide followed by agarose, whereas polysaccharide  $\kappa$ -carrageenan and ulvan have only recently been proposed, reflected by the few found published reports. It is clear that these biopolymers provide a versatile class of hydrogels that

may found widespread application in the field of regenerative medicine.

## Declaration of interest

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