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Review

Chitosan, hyaluronan and chondroitin sulfate in tissue engineering for cartilage regeneration: A review

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ABSTRACT

Injection of hyaluronan into osteoarthritic joints restores the viscoelasticity, augments the flow of joint fluid, normalizes endogenous hyaluronan synthesis, and improves joint function. Chitosan easily forms polyelectrolyte complexes with hyaluronan and chondroitin sulfate. Synergy of chitosan with hyaluronan develops enhanced performances in regenerating hyaline cartilage, typical results being structural integrity of the hyaline-like neocartilage, and reconstitution of the subchondral bone, with positive cartilage staining for collagen-II and GAG in the treated sites. Chitosan qualifies for the preparation of scaffolds intended for the regeneration of cartilage: it yields mesoporous cryogels; it provides a friendly environment for chondrocytes to propagate, produce typical ECM, and assume the convenient phenotype; it is a good carrier for growth factors; it inactivates metalloproteinases thus preventing collagen degradation; it is suitable for the induction of the chondrogenic differentiation of mesenchymal stem cells; it is a potent means for hemostasis and platelet delivery.

Contents

Abbreviations: ATDC5, murine chondrogenic cell line; BMP, bone morphogenetic protein; CM, chitin carboxymethyl chitin; DA, degree of acetylation; DD, degree of deacetylation; DPBS, Dulbecco's phosphate-buffered saline; ECM, extracellular matrix; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide.HCl; FGF, fibroblast growth factor; GAG, glycosaminoglycan(s); IL1, interleukin 1; MMP, matrix metalloproteinase(s); MSC, mesenchymal stem cell(s); MW, average molecular weight; NHS, N-hydroxysuccinimide; NO, nitric oxide (chemical formula); N/P, molar ratio of the amino group of chitosan to phosphate group of DNA; OA, osteoarthritis; pEGFP, plasmid with enhanced green fluorescent protein; PGE2, prostaglandin E_2; PLGA, poly(lactide-co-glycolide); PNIPAM, poly(N-isopropylacrylamide); RTPCR, real time polymerase chain reaction; SOX9, sex determining region Y box 9; TGF-β, transforming growth factor beta; TNFα, tumor necrosis factor alpha.

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1. Introduction

The hyaline articular cartilage covers both ends of opposing bones in synovial joints and forms the bearing surface that allows for a nearly frictionless articulation; it withstands the demand of repetitive loading with no wear, even with recurring loading cycles largely exceeding the body weight of the individual. The anisotropic, nonlinear, inhomogeneous and viscoelastic properties of the cartilage have been attributed to its complex structure and composition (Makris, Hadidi, & Athanasiou, 2011; Mow, Ratcliffe, & Poole, 1992). In fact, friction between the opposing joint surfaces is minimized by the excellent lubrication that allows for effortless motion under the mechanical stress. Each diarthrodial joint is enclosed in a strong fibrous capsule which is lined on the inside with a synovial membrane, a metabolically active layer that releases the viscous synovial fluid. The latter contains several components of the hyaline cartilage such as hyaluronan, proteoglycans (mainly aggrecan), chondroitin sulfate and keratan sulfate.

1.1. Characteristic properties of the articular cartilage

The cartilage lacks a spontaneous wound repair capacity owing to limited proliferating potential of chondrocytes and their catabolic response to pathological mediators; the avascular nature prevents immigration of regenerative cells except when the lesion provides access to bone marrow. Vascularization is present in *ca.* 10–30% of the meniscus at 10 years of age, but only the peripheral 10–25% of the meniscus at maturity age contains blood vessels and nerves (Clark & Ogden, 1983). Later on, two regions of the meniscus can be distinguished: the outer, vascular/neural region, and the inner, completely avascular/aneural region (Arnoczky & Warren, 1982). Therefore, in adult patients and animals, the injured cartilage tissue is unable to heal spontaneously.

The meniscus is highly hydrated (72 wt% water), with the remaining 28 wt% comprised ECM and cells: in general, collagens make up the majority (75%) of this organic matter, followed by GAG (17%), DNA (2%), adhesion glycoproteins (<1%), and elastin (<1%). Said percentages might vary depending on age and injuries. Aside from collagen, another fibrillar component is elastin: mature and immature elastin fibers are present in very low concentrations (<0.6%) in the adult meniscus (Herwig, Egner, & Buddecke, 1984; Proctor, Schmidt, Whipple, Kelly, & Mow, 1989).

The transport of nutrients and metabolites to and from the cartilage occurs mainly *via* the synovial fluid; nutritional supply may also occur *via* the subchondral bone as a second route (Arkill & Winlove, 2008; Pan et al., 2009). The chondrocytes within the cartilage are exposed to hypoxia (oxygen concentrations ranging from 1 to <5%) since the distance to the supplying vessels of the synovial membrane is exceptionally large compared to other tissues. Chondrocytes, however, sustain the hypoxia by making use of an anaerobic energy metabolism. Superficial chondrocytes consume only little of the oxygen available that therefore diffuses to chondrocytes at deeper levels.

Collagen-I and -III genes, which pertain to fibrous tissue phenotypes and are commonly observed in cartilage formation *in vitro*, and in cartilage regeneration *in vivo*, are repressed in hypoxia *via* specific factors. Beneficial effects of hypoxia on chondrocyte-specific gene expression, matrix deposition, and metabolism were observed in cultures of primary chondrocytes and cartilages across several species. The expansion of human articular chondrocytes at 5% O_2 is detrimental for chondrogenesis in agreement with experiments using bovine chondrocytes which suggest that 1.5% but not 5% O_2 could improve the chondrogenic differentiation (Coyle, Izzo, & Chu, 2009; Egli, Bastian, Ganz, Hofstetter, & Leunig, 2008).

The matrix is considered as a porous network of solid components with interconnecting channels. The elasticity of the tissue is due to proteoglycans, heavily glycosylated proteins that constitute a major component of the meniscal ECM (S.Y. Lee et al., 2009; Taylor & Miller, 2006; Yanagishita, 1993). They are made of a core protein decorated with GAG, that in normal human meniscal tissue are chondoitin-6-sulfate (60%), dermatan sulfate (20–30%), chondroitin-4-sulfate (10–20%), and keratan sulfate (15%); aggrecan is the main large proteoglycan of the meniscus (Fig. 1). They enable the meniscus to absorb water, whose confinement supports the tissue under compression. Aggrecan, composed of a ~300 kDa core protein, is a member of the hyaluronan-binding proteoglycan family: in fact it associates non-covalently with hyaluronan to form >200 MDa aggregates. In the cartilage, these aggregates are a dense hydrated gel enmeshed within a network of reinforcing collagen fibrils.

Electrostatic repulsion between the anionic GAG of aggrecan is the major contribution to the equilibrium compressive modulus of cartilage. Aggrecan exhibits exceptional insensitivity to calcium ions in the physiological ion concentration range and beyond. This property allows aggrecan to play a role of ion reservoir that can mediate calcium metabolism in cartilage and bone (Chandran & Horkay, 2012; Horkay, Basser, Hecht, & Geissler, 2011). The main adhesion glycoprotein present in the human meniscus is fibronectin, an indispensable component of the meniscus matrix, as it serves as a link between ECM and cells. Moreover, the ECM composition includes growth factors, and controls cell proliferation and differentiation for homeostasis and regeneration of damaged tissue.

1.2. Major challenges and new approaches

Damage to the articular cartilage occurs frequently as a result of sport-related injury, disease, trauma and tumor: failure to treat damaged cartilage may lead to osteoarthritis, a

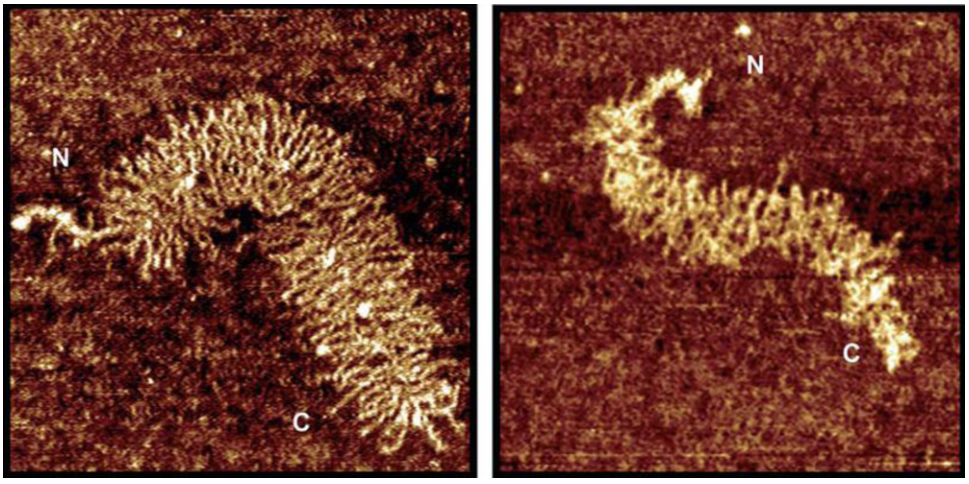

Fig. 1. Atomic force microscopy image of (left) fetal aggrecan monomer obtained from epiphyseal cartilage in the load-bearing region of an articular joint, and (right) fetal epiphyseal and mature nasal aggrecan monomer. In confined compression, the equilibrium modulus of human articular cartilage is *ca.* 600 kPa whilst that of human nasal cartilage is 233 kPa, owing to the fact that aggrecan from the load-bearing cartilage has a denser chondroitin sulfate-GAG brush region, longer chondroitin sulfate chains, and a greater stiffness, when compared to the non-load-bearing nasal cartilage.
Source: from Ng et al. (2003). Elsevier Science, San Diego.

disease affecting elderly people, and more and more diagnosed in young individuals as well. Although the latter is a broad clinical syndrome, it is characterized by the progressive loss of articular cartilage and chondrocytes within the synovial joints, thus cells must be replenished. Repair procedures can be broadly categorized into four groups: arthroscopic lavage and debridement (very small defects), marrow stimulating techniques, osteochondral autografts and allografts and, more recently, cell implantation.

The isolation of a sufficient number of undifferentiated meniscal cells from an injured meniscus is most difficult, and the search for alternative cell sources has led researchers to use allogeneic cells for meniscus tissue engineering, based on the positive outcomes of allogeneic articular, auricular, and costal chondrocytes in lesions of the avascular zone of the meniscus, in an animal model (Weinand, Peretti, Adams, Randolph, et al., 2006). Experimental surgery showed that in pigs, for example, both allogeneic and autologous cell-seeded scaffolds after 12 weeks were capable of promoting healing (Weinand, Peretti, Adams, Bonassar, et al., 2006). These facts suggested that the use of allogeneic cells was feasible and that certain difficulties with autologous cells could be overcome.

On the other hand, the surgical stimulation of bone marrow involving drilling or microfracture in the base of the debrided cartilage lesion induces subchondral bleeding and generates conduits that permit bone marrow stem cell migration into the cartilage lesion. This would appear to be a favorable aspect, however in skeletally mature human patients and animal models, this surgical approach usually results in the formation of a repair tissue predominantly composed of fibrous tissue or fibrocartilage with weak biomechanical properties compared to the desirable hyaline articular cartilage (Abarrategi et al., 2010; Brittberg & Gersoff, 2010; Cancedda, Dozin, Giannoni, & Quarto, 2003).

Thus, the failure to obtain hyaline cartilage rather than fibrocartilage stimulated research in the field of cartilage engineering. For this purpose, cells harvested from patients are grown *in vitro* with the aid of scaffolds, biochemical factors, and mechanical stimulations: the intention is to provide a tissue construct that once implanted into a cartilage defect, sustains the physiological challenges and integrates into the recipient site with all desirable characteristics. This is why chemical, biochemical and histological aspects become important.

In fact, with the development of tissue engineering, the partly artificial reconstruction of the cartilage provides a new approach to its repair: scaffolds are used because they provide highly desirable 3D environments for cell growth and consequent production of cartilaginous tissue. The manufacture of scaffolds requires chemical knowledge for the choice of their constituents, and biochemical knowledge for the optimization of their behavior.

Cartilage-specific extracellular matrix components such as collagen-II and GAG play the crucial role of regulating the expression of the chondrocytic phenotype, and supporting chondrogenesis both *in vitro* and *in vivo*. This means that the chosen biomaterials should provide the appropriate biochemical and biomechanical environment needed for cells to regenerate a long-lasting hyaline cartilage at the defect site. For example, in a hydrogel containing chitosan and hyaluronan with entrapped primary chondrocytes and meniscal cells, the mechanical deformation applied at 15% tensile strain, 0.5 Hz, and 10 min per day for 43 days resulted in substantial GAG increase and collagen production (all > 31%) over static controls, while not significantly affecting cell proliferation and viability (Beris, Lykissas, Papageorgiou, & Georgoulis, 2005; J.P. Chen, Liao, & Cheng, 2011; Suh & Matthew, 2000; Getgood, Brooks, Fortier, & Rushton, 2009; Hunziker, 2002; Marimuthu & Kim, 2009; Puppi, Chiellini, Piras, & Chiellini, 2010; Spiller, Maher, & Lowman, 2011).

1.3. Chemical and biochemical characteristics of the materials

Cartilage engineering requires crucial combinations of cells, factors, and biomaterials endowed with specific chemical and biochemical characteristics (Chung, Erickson, Mauck, & Burdick, 2008; Vinatier, Bouffi, et al., 2009; Vinatier, Mrugala, et al., 2009) as well as two phases of cell/tissue culture: in the first phase, cells increase their number, whereas in the second phase the cells are induced to their specific cartilaginous phenotypes. Because primary chondrocytes expanded in cell culture lose their capacity to form cartilaginous matrix in subsequent chondrogenic cultures, the media are supplemented with growth factors, mainly FGF-2 and TGFb. The delivery of bioactive compounds in cartilage engineering has been reviewed by Lee and Shin (2007).

The scaffold material has to be resorbed enzymatically: it should give way to the new tissue. Inflammatory reactions by degradation products, or immune responses by the natural materials

should not be generated within the newly formed tissues. The natural materials suitable for scaffold preparation include proteins such as collagen, elastin, keratin and fibroin; polysaccharides such as chitosan, hyaluronan, and alginate; polyesters, such as polyhydroxybutyrate. These materials, once processed into scaffolds, contribute to impart features of pore architecture, namely pore size, shape, and distribution; elasticity, including modulus and time-dependent deformation; the surface energetic parameters such as hydrophobic–hydrophilic balance and molecular mobility. Moreover, chemical functionality, environmental responsiveness with respect to pH, stress and temperature; the surface micro- and nano-topography; biodegradation mechanisms, and metabolism of degradation products are all important aspects.

In vivo, the cell-tissue constructs are exposed to the harsh conditions of a synovial joint subjected to load, hypoxia, and the synovial fluid. The latter is an ultrafiltrate from blood plasma without fibrinogen, supplemented with products from synovial lining cells and chondrocytes. The main difference from serum is the presence of *ca.* 2 mg/ml hyaluronan, and a protein content as low as *ca.* 30 mg/ml. As in blood plasma, a complex mixture of growth factors and cytokines is found within the synovial fluid that stimulates or suppresses chondrogenic and antichondrogenic actions (Punzi, Calo, & Plebani, 2002). It can be considered as a tool in cartilage repair whereby cells are obtained from the patient, seeded on a scaffold and implanted directly at the place of a cartilage defect (Egli, Wernike, Grad, & Luginbuhl, 2011).

The conceptual approach to cartilage regeneration evolved during the last decade. The first-generation technology (autologous chondrocyte transplantation) involved the transplantation of *in vitro* expanded chondrocytes to cartilage defects. The second generation involves the seeding of chondrocytes in a 3D scaffold, and has several advantages: *in vitro* pre-differentiation of cells, arthroscopic implantation, and implant stability.

Embryonic stem cells, induced pluripotent stem cells, amniotic fluid derived stem cells, or autologous adult stem cells represent today an attractive alternative to recombinant cell lines and primary cells, particularly when in polysaccharide gels (Green, Li, Milthorpe, & Ben-Nissan, 2012; Hook, 2011; Wang et al., 2010). The environment of stem cells in culture, including the material substrate has a powerful influence in determining their performance. Control of hydrophilicity and molecular mobility becomes important because once cells are seeded into a scaffold, they should adhere to its surface. Ideally, MSC could be harvested from the patient prior to surgery, expanded *in vitro*, and then implanted into the same patient. Human and bovine MSC undergo chondrogenesis in hyaluronan hydrogels, supporting upregulation of chondrocyte-specific genes and producing cartilage-like matrix rich in aggrecan and collagen-II (I.L. Kim, Mauck, & Burdick, 2011; Pittenger et al., 1999). They represent an autologous supply of cells which can be easily harvested from different tissues, including synovium, skeletal muscle, periosteum, bone marrow, and adipose tissue (Da Silva et al., 2011; Merceron et al., 2008). Bone marrow-derived MSC have generally shown superior chondrogenesis when compared to MSC from adipose tissue. The superiority of synovium-derived MSC over stem cells from other human tissues was demonstrated in comparative studies, in particular with respect to the chondrogenic potential. In fact, Li et al. (2012) indicate that adipose stem cells transfected with insulin-like growth factor benefit articular cartilage treated with chitosan-gelatin by inducing chondrogenesis and matrix biosynthesis.

1.4. Scope of the present review

In consideration of the above, the scope of the present review is to identify the lines of applied research that are now consolidating quite significant advances made with hyaluronan during the last decade, and that today involve most often chitosans and modified chitosans either alone or in conjunction with other polysaccharides. The novelty of these aspects is underlined by the fact that they are totally absent in the otherwise exhaustive review article on the articular cartilage repair authored by Hunziker (2002). While the complexity of the cartilage constituents is astonishing and certainly cannot be reproduced in an artificial construct, emerging results show that chitosan can strongly enhance the performances of hyaluronan in regenerating/repairing the cartilage *in vitro* and *in vivo*. This review therefore intends to convey to the reader detailed chemical information on preparative chemistry and pre-clinical applications of chitosan, optionally in conjunction with hyaluronan, specific factors and other biopolymers such as chondroitin sulfate. Because said materials are intended for interaction with chondrocytes and with undifferentiated cells, full knowledge of their capacity to enhance/depress key cellular functions, as well as to preserve/change phenotype, is indispensable.

2. Chitosan

Basic information on chitins and chitosans is amply available to the reader: besides the most recent review article celebrating the bicentennial of the discovery of chitin, the first polysaccharide known (Muzzarelli et al., 2012), interdisciplinary documentation can be found in Chen (2008), Jollès and Muzzarelli (1999), Kasaai (2009), Kennedy and White (1983), Keong and Halim (2009), Kim et al. (2008), Kumar, Muzzarelli, Muzzarelli, Sashiwa, and Domb (2004), Kurita (2006), Muzzarelli (1977, 2011a), Muzzarelli, Jeuniaux, and Gooday, (1986), Muzzarelli and Muzzarelli (1998, 2009), Neville (1993), Pillai, Paul, and Sharma (2009), Rudall and Kenchington (1973), Sashiwa and Aiba (2004), Stankiewicz and VanBergen (1998). Chemical and technological advances relevant to the present title matter, namely biochemical exploitation of chitosan, absence of allergenicity, nanochitosans, formulations of biopharmaceuticals, stem cells for bone regeneration, were reviewed by Muzzarelli (2010, 2011b, 2011c, 2011d, 2011e).

The review article by Balakrishnan and Banerjee (2011) besides chitosan covers hyaluronan, chondroitin sulfate, alginate, agarose, fibrin, collagen and silk; moreover various hybrid scaffolds and composites are dealt with (but unfortunately the bibliography does not include articles more recent than 2007). They underline that injection of chitosan solution into the murine knee joint caused a significant increase in the density of newly formed chondrocytes, suggesting that it could facilitate the healing of the cartilage. Chitosan promotes attachment, proliferation, and viability of mesenchymal stem cells, and thanks to these promising features, chitosan and its derivatives are considered as very interesting biomaterials. Most of the studies have been short term and were conducted on animal models using animal cells. Few works are reported on human chondrocytes/mesenchymal cells seeded onto hydrogels for cartilage engineering. The focus of all those studies is on the biosynthesis rate of matrix components such as proteoglycans and collagen, and the macroscopic mechanical properties of the constructs.

In a further review article, Jayakumar et al. (2011) put emphasis on chitin instead of chitosan, and the fact that most of the researchers working in regenerative biotechnology attempt to create tissue replacements by culturing cells onto synthetic porous 3D polymeric scaffolds: this approach is currently regarded as the best one because it creates and maintains channels that facilitate progenitor cell migration, proliferation and differentiation. Chitin scaffolds have been widely used in tissue engineering due to their non-toxic, biodegradable and biocompatible nature. The advantage of chitin as a tissue engineering biomaterial lies in the fact that it

can be easily processed into hydrogels and xerogels (Dutta et al., 2011).

Chitosan possesses a number of peculiar properties that make it unique among polysaccharides and biomaterials, summarized as follows: chitosan has found commercial applications in the area of wound bandages, as an hemostatic capable to stop severe bleeding and suitable for use in emergencies and on battle fields, thanks to redundant chemical and biochemical mechanisms of clot formation (Millner, Lockhart, & Marr, 2010; Muise-Helmericks, Demcheva, Vournakis, & Seth, 2011). It exhibits exceptionally good versatility in drug delivery (Saboktakin, Tabar, Tabatabaie, Maharramov, & Ramazanov, 2012; Sonia & Sharma, 2011; Sridhar et al., 2011), as a vehicle of DNA (Y.K. Kim et al., 2011; Peng et al., 2011; Saranya, Moorthi, Saravanan, Devi, & Selvamurugan, 2011), in the reconstruction of nerves (Cho & Ben Borgens, 2012) and for targeting tumors (Vinsova & Vavrikova, 2001; Wang et al., 2011).

Chitosan exhibits even more surprising biochemical actions: it is recognized as an immune adjuvant (C.A. Da Silva et al., 2010) exempt from toxicity (Waibel et al., 2011); reportedly it inactivates matrix metalloproteinases, that promote collagen degradation (Li et al., 2011; Liu, Qiu, Chen, Peng, & Du, 2005) and interacts with growth factors in such a way as to retain them in the site under treatment; when the degree of acetylation is close to 0.2, chitosan is also susceptible to partial and slow depolymerization by lysozyme so that its permanence in a living tissue is longer than that of hyaluronan, but reasonable resorption times can be optimized. The sustained release of glucosamine oligomers provides building blocks that enter the peptidoglycan and hyaluronan synthesis pathways (Liu, Liu, Li, Du, & Chen, 2011; Muzzarelli, 1993); reportedly, chitosan is quite suitable for the induction of the chondrogenic differentiation of the human bone marrow mesenchymal stem cells, thus qualifying for a key role in exploitation of the latter (Schwartz, Griffon, Fredericks, Lee, & Weng, 2011). Chitin and chitosan preserve the round morphology of chondrocytes and their capacity to synthesize cell-specific extracellular matrix.

The only limitation of chitin used as a scaffold for bone and cartilage seems to be the poor mechanical strength and elasticity. However, the mechanical properties can be improved with the addition of genipin, the safest and most biocompatible crosslinker, or bioinorganics such as hydroxyapatite and bioactive glass ceramic; in fact Mwale, Wertheimer, and Antoniou (2009) showed that Protasan®, a biomedical grade chitosan, once crosslinked with 5% genipin is a promising hydrogel for disc and cartilage engineering, as confirmed by Yan et al. (2010) and by Hrabchak et al. (2010) who found that genipin, used in the treatment of an osteochondral knee defect, was biocompatible and non-immunogenic.

2.1. Chitosan scaffolds

2.1.1. Advantages of chitosan in the preparation of scaffolds

In their review article concerning the applications of chitosan-based biomaterials in cartilage engineering, Suh and Matthew (2000) underlined a number of positive characteristics of chitosan, among which the physiological depolymerization in the presence of ubiquitous lysozyme (a most convenient feature because scaffold must be temporary), minimal foreign body reaction, absence of chronic inflammatory response, absence of fibrous encapsulation, favorable chemoattractive and immuno-enhancing effects, accompanied by excellent technological features that permit to process it into tailor-made porous or nanofibrillar structures, as recently described by Muzzarelli (2011b). Its filmogenicity permits to obtain porous structures by freezing and lyophilizing chitosan solutions, for use in cell transplantation and tissue regeneration: chondrogenic cell adhesion and proliferation onto these structures has been reported. Early measurements indicated that a lyophilized scaffold had apparent density 0.31 g/ml, total pore area 0.8 m^2/g, porosity 81%, and pore diameter in the range 40–100 μm with prevailing value of 67 μm (Nettles, Elder, & Gilbert, 2002; Oliveira, Amaral, Barbosa, & Teixeira, 2009). Biologically active chitosans obtained *via* entrapment or chemical immobilization of oligopeptides and proteins, and the associated strategies to develop them for tissue engineering, were reviewed by Jiang, Kumbar, Nair, and Laurencin (2008).

Scaffolds made of either pure chitosan, or pure β-chitin, or 3:1, 1:1 and 1:3 admixtures of β-chitin + chitosan, showed the same efficiency in supporting chondrocytes (*ca.* 98%), and the same concentration of generated chondroitin sulfate. The content of hydroxyproline in the β-chitin sponge was significantly greater than in other sponges at week 4 post-culture. Based on histochemical and immuno-histochemical findings, the cartilage-like layer in the chondrocyte-bearing sponges was similar to hyaline cartilage; however, only in the pure β-chitin sponge the collagen-II was closer to normal rabbit cartilage (Suzuki et al., 2008).

The formation of chitosan scaffolds takes place also upon addition of n-butanol to aqueous chitosan solutions: the initial adhesion and the proliferation rate of human dermal fibroblasts in the scaffold were nearly twice the control, after 3 days of culture, mainly because the specific surface area was large enough for cell attachment and tissue growth (Chun et al., 2008).

An example of the versatility of chitosan in terms of filmogenicity and pore formation is provided by Zhong et al. (2011) who adopted the emulsion technique followed by lyophilization to fabricate chitosan-poly(ε-caprolactone) xerogels. Fig. 2 shows that the choice of the freezing temperature and the composition

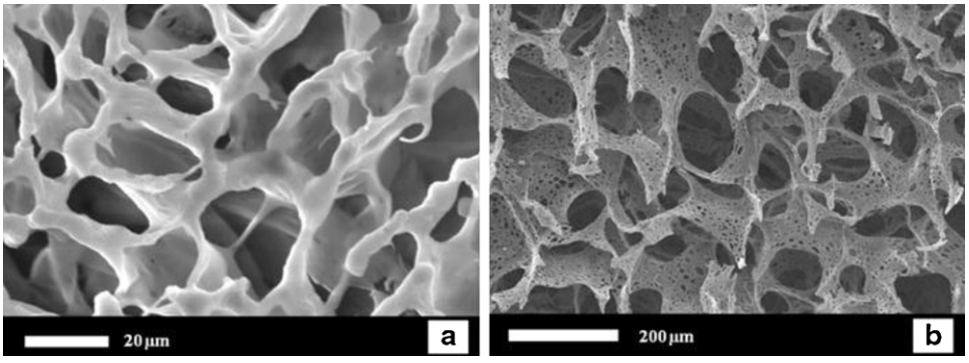

Fig. 2. SEM images of chitosan xerogels containing 25 wt% poly(ε-caprolactone) freeze-dried after being frozen at [a] −196 °C (liquid nitrogen), and [b] −20 °C. The major feature in [b] is the formation of fine pores (less than 10 μm) in the macropore walls, generated by the evaporation of organic solvents during freeze-drying.
Source: from Zhong et al. (2011). Springer, Dordrecht.

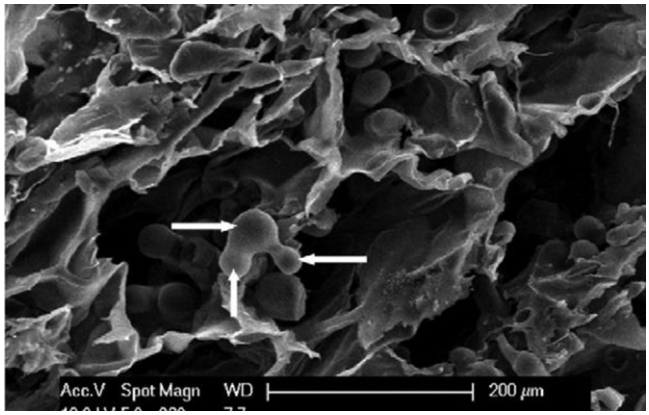

Fig. 3. SEM micrographs of chitosan-poly(ε-caprolactone) aerogel (PCL content *ca.* 42 wt%) housing TGFβ1-loaded microspheres [arrows and elsewhere] made of chitosan and 2.5 wt% genipin.
Source: From Wu et al. (2011). Elsevier Science, Oxford.

of the emulsion permits to prepare scaffolds made up of thin and porous films.

2.1.2. Incorporation of growth factors in chitosan scaffolds

From a porous freeze-dried chitosan scaffold incorporating TGF-β1-loaded microspheres, and used for the treatment of cartilage defects, TGF-β1 was released in a sustained fashion, and promoted chondrocyte proliferation and matrix synthesis (Kim et al., 2003). In the study by Wu et al. (2011), TGF-β1 was loaded into chitosan microspheres crosslinked with genipin, that were then incorporated into porous chitosan-poly(ε-caprolactone) scaffolds (Fig. 3). It was expected that TGF-β1 could be released from these scaffolds over a long period of time without significant bursts whereas the scaffolds have desired compressive strength in wet state. Quen et al. (2009) promoted new hyaline cartilage formation in the sheep with the use of TGF-β3.

BMP signaling is involved in the proper development of many components of the skeletomuscular system including cartilage. BMP belongs to the transforming growth factor-β gene superfamily that comprises *ca.* 30 structurally related members. Similar to TGF-β, BMP signal through trans-membrane serine/threonine kinase receptors. The N,N-dicarboxymethyl chitosan associated with bone morphogenetic protein-7 was also found to repair femoral articular cartilage lesions in the rabbit, indicative of synergism of their respective biological properties (Mattioli-Belmonte, Gigante, et al., 1999; Mattioli-Belmonte, Nicoli-Aldini, et al., 1999). Indeed, BMP-7 enhanced the *in vivo* proliferation of chondrocytes, leading to partial healing of the articular surface of the lesions. Thus, a connection was established between chitosan and larger proliferation of fibro-vascular tissue, that is the initial process of ossification, and therefore chitosan was considered a crucial carrier for BMP-7, the latter being approved by FDA for clinical use as well as BMP-2 (Kamiya & Mishina, 2011; Seeherman & Wozney, 2005).

2.1.3. Physical stimuli for the correct cellular response

Along the lines set by Xia et al. (2004), rabbit chondrocytes were cultured in chitosan–gelatin scaffolds for 3 days before dynamic compression. The seeded constructs were subjected to short-term (3 or 9 h) or long-term (6 h/day for 3 weeks) cyclic compression with 40% strain and 0.1 Hz. The expression of collagen-II and aggrecan was upregulated after 3 h of compression in comparison with the free-swelling samples. Moreover, long-term culture under dynamic compression facilitated cellular proliferation and deposition of GAG (Wang, Chow, Lai, Liu, & Tsai, 2009). By mimicking the combination of collagen and polysaccharide in the ECM, Li, Matsuda, Bao, Teramoto, and Abe (2010) manufactured a porous hybrid scaffold for cartilage engineering. The stem cell line ATDC5 on chitosan:gelatin 1:4 showed higher differentiation rate than on other films and culture dishes.

Synovium-derived stromal cells were coupled with chitosan–collagen-I: scaffolds and then manufactured *via* freeze-drying and cross-linking with carbodiimide (Gong et al., 2010). Those cells were cultured onto said scaffolds, incubated in serum-free chondrogenic medium with sequential application of TGF-β1 and bFGF for up to 21 days and then implanted into nude mice. The DNA content of the cells in the porous scaffolds increased at 1 week. The constructs *in vitro* and the implants were examined histologically, and the implants with positive collagen-II were tested immunohistochemically. RT-PCR of the implants indicated that aggrecan and collagen-II were expressed.

2.1.4. Significance of the hybrid scaffold composition

Han, Wei, Wang, and Song (2009, 2010) evaluated the effect of hybrid microspheres composed of TGFβ1-loaded gelatin and chitosan on the enhancement of the differentiation of adipose-derived stem cells (ASC) into chondrocytes in pellet culture. Quantitative PCR was used to analyze the expression of collagen-II and aggrecan. Proliferation of ASC was higher in the TGFβ1-loaded hybrid than in the TGFβ1-loaded gelatin. The GAG content and the gene expression of collagen-II and aggrecan were significantly higher in the loaded chitosan–gelatin than in the loaded gelatin. Thus the enhanced differentiation of ASCs by the TGFβ1-loaded chitosan–gelatin microspheres provides an easy and effective way to construct cartilage.

The silk fibroin–chitosan 1:1 scaffold supported cell attachment and growth, and chondrogenetic phenotype; it showed higher compressive strength and modulus over its individual components (Bhardwaj & Kundu, 2011; Bhardwaj et al., 2011) in agreement with Chung and Chang (2010). Cell number, matrix amount, and expression of genes specific for chondrogenesis were improved after culture of MSC in chitosan constructs coated with collagen-II, but not when reacetylated chitosan was used (Ragetly, Griffon, & Chung, 2010; Ragetly, Griffon, Lee, & Chung, 2010). Gelatin associated to chitosan in the form of microspheres was also used in conjunction with adipose-derived stem cells for cartilage regeneration (Han et al., 2010).

Kuo and Hsu (2009) investigated chitosan and poly(ethylene oxide) (PEO) composites for the culture of bovine knee chondrocytes: PEO and chitosan with various weight ratios were crosslinked and freeze-dried. The range of pore diameters was 200–400 μm, and the porous surface of the PEO–chitosan scaffolds were chemically modified with human fibronectin for accelerating cell adhesion and growth. A high content of PEO generated high porosity, moisture content, ductility, biodegradation rate, and cell viability, as well as low Young's and compression moduli. High levels of PEO, human fibronectin, and extracellular calcium were favorable to the chondrocytes, as indicated by the enhanced amounts of cells, glycosaminoglycans, and collagen. Genipin-crosslinked scaffolds comprising PEO and chitosan with pore size *ca.* 200–250 μm and pore surface modified with the peptide CDPGYIGSR favored the production of cartilage in the constructs because said sequence was recognized by the receptor on mammalian cells (Fig. 4). The surface peptide also avoided the generation of hypertrophic chondrocytes (Kuo & Wang, 2011).

Tigli and Gumusderelioglu (2009a, 2009b) originally investigated the suitability of chitosan scaffolds loaded with BMP6 in both stationary and dynamic conditions for cartilage engineering. They also observed that alginate–chitosan scaffolds can promote chondrocyte proliferation and retain cell functionality and phenotype significantly compared to alginate scaffolds. Further to work by Hsu et al. (2004) on chitosan–alginate–hyaluronate

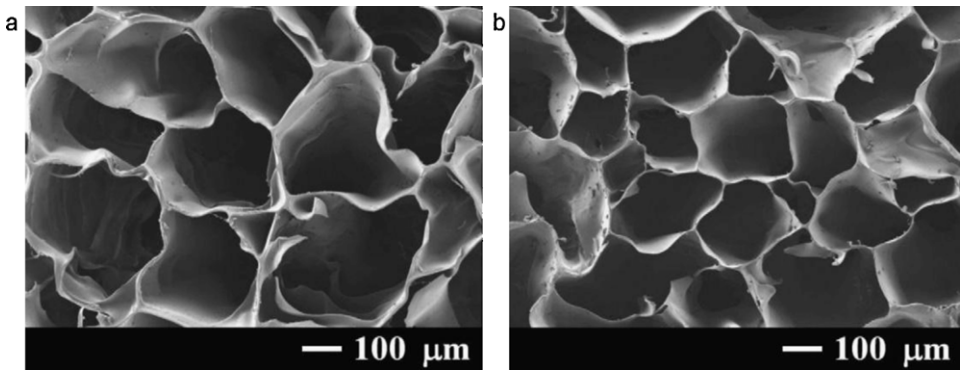

Fig. 4. SEM micrographs of the CDPGYIGSR-coated chitosan–PEO scaffolds. (a) PEO:chitosan = 1:4; (b) PEO:chitosan = 1:3. The pores in this size range are the most suitable for the growth of chondrocytes, and the presence of the peptide favors the production of cartilage.
Source: from Kuo and Wang (2011). Elsevier Science, Amsterdam.

complexes modified with an RGD-containing protein, Tigli and Gumusderelioglu (2008) linked covalently the cell adhesion factor arginine–glycine–aspartic acid (RGD) or the epidermal growth factor (EGF) to chitosan with the intention of enhancing cell attachment and proliferation of ATDC5 murine chondrogenic cells. After 4 weeks of incubation, the chondrogenesis within plain chitosan, RGD-chitosan and EGF-chitosan was quantified as 7.5, 9.7 and 10.2 mg GAG/g, respectively. The cell density in chitosan scaffolds was quantified on the basis of their DNA contents: the chondrocytes cultured on chitosan, RGD-chitosan and EGF-chitosan for 4 weeks *in vitro* reached 1.95×10^7, 2.23×10^7 and 2.30×10^7 cells respectively in each 3-mg scaffold. After 14, 21 and 28 days of cultivation, the EGF-chitosan had the highest DNA content, thus it was indicated as the best one. According to Tsai et al. (2011) a more refined method for linking peptides to chitosan can be adopted: for example, equimolar quantities of the RGD peptide, EDC and N-hydroxy-2,5-pyrrolidinedione were dissolved in N-dimethylformamide and then mixed with 2% chitosan solution, to be optionally photo-crosslinked. After 10 days of culture, the number of osteoblasts in the RGD-chitosan scaffolds increased by 50% compared to the control.

Cellular responses of mesenchymal stem cells derived from human embryonic stem cells, were assessed for chondrogenic potential in 3D culture by Tigli et al. (2009) who were motivated by the need to determine suitable cell sources for the *in vitro* formation of cartilage. For this purpose, freshly lyophilized fibroin or chitosan scaffolds were rehydrated, aspersed with BMP6 in DPBS and lyophilized again. The cells were differentiated in said scaffolds in the presence and absence of BMP6, along with the standard chondrogenic differentiating factors such as TGFβ, insulin and dexamethasone. The differentiation of all of the cell sources in the fibroin and chitosan scaffolds was made evident by the upregulation of mRNAs for cartilage-related genes (*AGC*, *Col-II*, *Col-X* and *Sox-9*). The upregulated *AGC*, *Col-II* and *Sox-9* genes in embryonic stem cells and MSCs suggest enhanced potential for these cell sources compared to chondrocytes. MSCs derived from embryonic stem cells showed unique characteristics, with preserved chondrogenic phenotype in both scaffolds with regard to chondrogenesis, as determined by RTPCR, histological and microscopical analyses. After 28-day cultivation, said MSC were prone to chondrogenesis. These results suggest that among the variables considered in the study, the human embryonic stem cell-derived MSC were the preferable ones, and BMP6 was important for cell differentiation in chitosan scaffolds. The authors admit that the pore size (*ca.* 100 μm) was possibly somewhat small despite the interconnections, and in fact Yamane et al. (2005, 2007) while investigating similar hyaluronan–chitosan polyelectrolyte complexes, reported that the 400 μm pore size was better (Table 1).

2.1.5. Scaffolds with expanded specific surface area

An expanded specific surface area of the scaffold can be obtained with new preparative techniques such as electrospinning, a process that gained attention in the last quinquennium, and that generates chitosan fibers with diameters in the nano range, which may be fabricated layer-by-layer, or can be of hybrid nature (Bhardwaj & Kundu, 2010; K.Y. Lee, Jeong, Kang, Lee, & Park, 2009; Sasmazel, 2011; Sonina, Uspenskii, Vikhoreva, Filatov, & Gal'braikh, 2011). Nano- and microfibrous double-face supports were fabricated with chitosan by electrospinning nanofibers onto a microfibrous mesh, that provided a significantly greater microenvironment for chondrocytes to proliferate and produce GAG as compared with only microfibrous 3D support. The chitosan nanofibrous surface facilitated cellular attachment and proliferation, and efficiently prevented phenotypic changes of chondrocytes (S.Y. Lee et al., 2009). Subsequent reviews describe design criteria and fabrication methods to be considered in cartilage biochemical engineering (Klossner, Queen, Coughlin, & Krause, 2008; Jayakumar, Prabaharan, Nair, & Tamura, 2010; Venugopal, Low, Choon, & Ramakrishna, 2008).

Iwasaki et al. (2004) demonstrated that alginate–chitosan fibers: (1) adhered better to chondrocytes in comparison with plain alginate fibers; (2) favored the characteristic round morphology of the chondrocytes; (3) promoted the production of dense fibers of collagen-II by the chondrocytes. More recently, Iwasaki et al. (2011) published a review of the data relevant to chitosan–hyaluronan spun fibers used as a scaffold for cartilage regeneration in the rabbit knee (hyaluronan content 0.04–0.07%). Nehrer et al. (1997) long before clarified the effects of scaffold pore sizes (20–86 μm) on chondrocyte behavior using collagen sponges: the cells cultured on the material with small pore diameter lost the chondrocytic morphology over time, whilst they kept it in suitably cross-linked chitosan (Senkoylu et al., 2001). Frenkel et al. (2005) implanted in rabbits a preformed xerogel made of the polyelectrolyte complex hyaluronan–chitosan (respective MW 1680 and 950 kDa). The degree of bonding to the host, the structural integrity of the hyaline-like neocartilage, and the reconstitution of the subchondral bone were greater than in controls; cartilage in the treated sites stained positive for collagen-II and GAG. It should be remarked that plain collagen scaffolds are not recommended for practical use owing to fast enzymatic degradation and poor mechanical strength (Zhu et al., 2009). By using poly(ethylene-glycol)-terephthalate-poly(butylene terephthalate) scaffolds with different pore sizes, Malda et al. (2005) demonstrated that scaffolds with larger pores (in the 182–525 μm range) significantly increased the GAG production of cultured chondrocytes.

The typical pore diameter of the chitosan scaffolds prepared by Kuo and Leou (2010) was 250 μm, indicating a void space suitable

Table 1
Effects of pore size in chitosan-based hybrid scaffolds (0.07% hyaluronan) on gene expression of cartilage specific extracellular matrix and glycosaminoglycans produced by chondrocytes after 28-day cultivation.

Pore size ($n = 5$)	Type II collagen	Type I collagen	Type II/type I ratio	Aggrecan	GAGS (μg/sample)
100 μm	0.66 ± 0.08	0.61 ± 0.11	1.12 ± 0.35	0.79 ± 0.05	35.9 ± 2.8
200 μm	0.67 ± 0.13	0.44 ± 0.10	1.63 ± 0.48	0.64 ± 0.14	45.2 ± 3.1*
400 μm	0.79 ± 0.08	0.46 ± 0.19	1.95 ± 0.78*	0.67 ± 0.16	56.2 ± 5.9*,**

Modified from Yamane et al., 2007.
Mean ± standard deviation. Values under type II collagen, type I collagen, and aggrecan, are mean normalized ratios (experimental integrated density/GAPDH integrated density) of mRNA. Those under glycosaminoglycans are the mean contents of each sample.
* $p < 0.05$ *vs.* 100 μm.
** $p < 0.05$ *vs.* 200 μm.

for chondrocyte growth. After deposition of hydroxyapatite on the pore surfaces of the scaffolds, the characteristic diameter of needle-like hydroxyapatite crystals was 85 nm. Over 4-week cultivation of bovine knee chondrocytes in the hydroxyapatite–chitosan scaffolds supplemented with pituitary extract with TGF-β1 (*ca.* 33 ng/ml), the constructs revealed newly formed mature cartilage, and the chondrocytes, GAG and collagen increased linearly with the concentration of TGF-β1.

As shown by many authors, control over the porosity of chitosan xerogels optionally containing hyaluronan can be exerted quite easily; in all cases however the planning of a scaffold should take into consideration *ab initio* a number of aspects relevant to material science, chemistry, biochemistry, orthopedics and surgery.

2.1.6. Chondrogenic differentiation induced by insulin or carboxymethyl chitin

Because insulin is a potent bioactive substance known to induce chondrogenic differentiation, various formulations of insulin-loaded chitosan were developed as a model for cartilage and osteochondral tissue engineering. The insulin encapsulation efficiency was high, up to 87.2 ± 1.6% for loading of 5%. The effect on pre-chondrogenic ATDC5 cells was investigated for 4 weeks by studying the influence of these release systems on cell morphology, DNA and glycosaminoglycan content, histology, and gene expression of collagen types I and II, Sox-9, and aggrecan assessed by RTPCR. The 5% insulin-loaded system was the most effective tested formulation in promoting chondrogenic differentiation (Malafaya, Oliveira, & Reis, 2010).

Chitin and chitosan derivatives too are interesting: after the pioneering work with N-carboxybutyl chitosan, that was found suitable for the repair of the meniscus in adult rabbits (Muzzarelli, Bicchiega, Biagini, Pugnaloni, & Rizzoli, 1992), carboxymethyl chitin has been recently reported to promote chondrogenesis by inducing the production of growth factors by immune cells. The murine pluripotent cell line C3H10T1/2 was maintained as a micromass culture in the CM-chitin conditioned medium that induced RNA expression of the chondrogenic factor Sox9, and the aggrecan. The tissues cultured in the presence of CM-chitin at day 21 were clearly stained by Toluidine blue or Alcian blue (histological staining) and collagen-II antibody (immuno-histological staining), showing the expression of acidic GAG and collagen-II. These results indicate that CM-chitin is a potent generator of chondrogenesis mediated by the induction of TGF-β1 in immune cells (Kariya et al., 2010).

Finally, it is worth mentioning an elegant work by J. Chen et al. (2011) who demonstrated that the bilayered gene-activated osteochondral scaffold consisting of plasmid TGF-β1-activated chitosan-gelatin as a chondrogenic layer, and plasmid BMP-2-activated hydroxyapatite-chitosan–gelatin as an osteogenic layer, induced MSC present in each layer to differentiate into chondrocytes and osteoblasts *in vitro*, respectively, while supporting the regeneration *in vivo* of the articular cartilage and the subchondral bone. Evidence was provided that multi-tissue regeneration through the combination of biomimetic and multi-phasic scaffold design, spatially controlled and localized gene delivery system and multi-lineage differentiation of a single stem cell population represents a promising strategy for promoting the development of complex tissues (Shao, Hutmacher, Ho, Goh, & Lee, 2006).

2.2. Injectable chitosan hydrogels

The chemical functionalization of hydrogels has been pursued to create native-like microenvironment for cells. Another principal advantage of hydrogels, the so-called "smart" biomaterials, is their ability to reversibly gel in response to temperature, pH, ionic strength, ultrasound, and electric field (Soppimath, Aminabhavi, Dave, Kumbar, & Rudzinski, 2002).

Injectable hydrogels, formed *in situ* after injection at the defect site, are suitable for minimally invasive procedures. Moreover, the incorporation of cells and growth factors can be readily made, and the gels assume any shape to conform with the surrounding tissue.

Early works indicated that the chitosan gels delivered to rats *via* intra-articular injection generated thicker epiphyseal cartilage in the tibial and femoral joints, together with chondrocyte proliferation (Hasegawa et al., 2008; Lu, Prudhommeaux, Meunier, Sedel, & Guillemin, 1999; Nolte & Klimkiewicz, 2003). While these hydrogels can be readily degraded by lysozyme, *in vitro* culturing of chondrocytes in glycolamido chitosan gels revealed that after 2 weeks the cells were viable and retained their round shape. Thus the gelation process does not compromise cell viability; sufficient mass transport of nutrients and oxygen to the cells inside the glycolamido chitosan gel takes place. The enzymatically crosslinked chitosan hydrogels have better biocompatibility toward chondrocytes as compared to other injectable hydrogel systems based on chitosan methacrylate (Jin et al., 2009). A recent example of sustained release of BMP-2 from an injectable chitosan gel is the study made by S. Kim et al. (2011) who examined the responses of pre-osteoblast mouse stromal cells and human embryonic mesenchymal cells to BMP-2.

2.2.1. Chitosan glycerophosphate hydrogels

The chitosan glycerophosphate hydrogels are attracting much attention since the early research works by Chenite et al. (2000); the possibility of tuning the gelling ability as well as the mechanical properties of these hydrogels was recently demonstrated by Qiu et al. (2011). A thermo-responsive chitosan hydrogel was prepared by Hoemann, Sun, Légaré, McKee, and Buschmann (2005) who used medical grade chitosan hydrochloride. The powder was dissolved in distilled water at 2% and then sterilized in an autoclave. The chitosan solutions were mixed with concentrated stock solutions of filter-sterilized Na β-glycerophosphate to yield a chitosan-glycerophosphate solution with 1.6% chitosan with final pH 6.8. Chitosan hydrogels were formed by mixing said solution with hydroxyethyl cellulose. About 4×10^7 chondrocytes were mixed with 1 ml chitosan hydrogel, poured into a cell culture plate, and then incubated at 37 °C for 10–15 min for gelation. Likewise, Hao

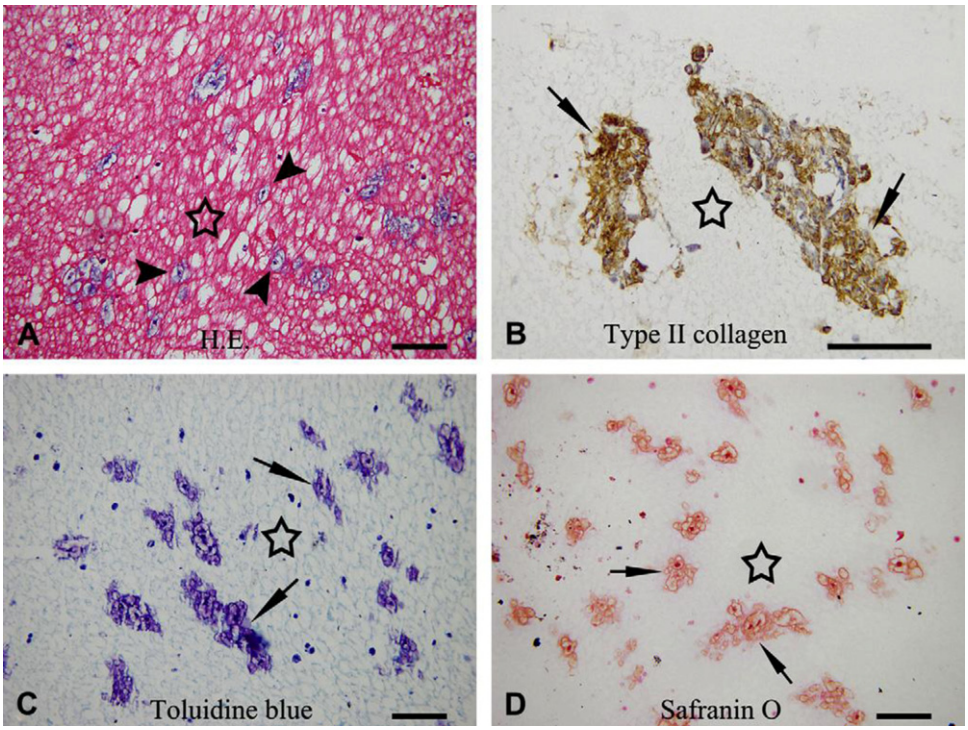

Fig. 5. Histology and immunohistochemistry of chondrocytes cultured in chitosan glycerophosphate hydrogels for 3 weeks. The chondrocytes in the chitosan hydrogels accumulated pericellular sulfated GAG-containing matrix. (A) Hematoxylin and eosin staining; (B) type II collagen immunohistochemical staining; (C) toluidine blue staining; (D) safranin-O staining. Star: chitosan hydrogel; Arrowhead: cell nucleus; Arrow: matrix of the chondrocytes. Bar length 100 μm.
Source: from Hao et al. (2010). Elsevier, Oxford.

et al. (2010) used chitosan hydrogel as a scaffold for chondrocytes for repairing cartilage defects in the sheep. Thermo-responsive chitosan hydrogels were prepared by combining medical grade chitosan β-glycerophosphate (10 ml) and hydroxyethyl cellulose (2.5 ml) both filter sterilized. The cartilage was reconstructed *in vitro* by using said hydrogels. The staining showed that >90% chondrocytes remained viable in the chitosan matrix after being cultured for 1 day *in vitro*. After implantation *in vivo*, the hydrogel conformed to the shape of the defects, thus avoiding liquid chitosan runoff into the articular cavity (Chung & Burdick, 2008). The reconstructions cultured for 1 day were transplanted to the freshly prepared defects of the articular cartilage of sheep. At 12 and 24 weeks after transplantation, the grafts were extracted and analyzed histologically and immuno-histochemically (Fig. 5). The chondrocytes in the reconstructed cartilage survived and retained their ability to secrete matrix when cultured *in vitro*. Once transplanted *in vivo*, the reconstructed specimens repaired the cartilage defects completely within 6 months. The implantation of chitosan hydrogels without chondrocytes also helped repair cartilage defects in the same time (Hao et al., 2010).

According to Song et al. (2010), a hydrogel composed of chitosan, glycerophosphate and collagen when compared to hydrogels composed of only chitosan and glycerophosphate, has better cellular compatibility and more uniform pore structure to support cellular growth. It should be added that these hydrogels are prone to the regeneration of bone: in fact thermo-sensitive chitosan–collagen–glycerophosphate hydrogels were proven to sustain *in vivo* osteogenesis after being loaded with pre-osteodifferentiated canine BMSC *in vitro* (Sun et al., 2011).

The chitosan glycerophosphate hydrogel was used as a controlled release system of ferulic acid for the regeneration of nucleus pulposus. In the latter, proteoglycans (core protein with attached chondroitin sulfate and keratan sulfate) and collagen-II provide mechanical support to the disc. In the course of degeneration, a decrease in the content of chondroitin sulfate results in a decrease of water content that affects the capability to offset the external stress. Overproduction of oxygen radicals can directly damage the cell and degrade the ECM; moreover, the cells change their phenotype and become unable to keep anabolism and catabolism of the extracellular matrix in balance because matrix metalloproteinases, apoptotic factors and inflammatory cytokines accelerate the degenerative process of the intervertebral disc. The antioxidant ferulic acid can be a remedy if delivered with the chitosan glycerophosphate–gelatin hydrogel (Cheng, Yang, & Lin, 2011).

2.2.2. Solidification of chitosan glycerophosphate with blood

Because subchondral drilling initiates a cartilage repair response involving formation of chondrogenic foci in the subchondral compartment, Chevrier, Hoemann, Sun, and Buschmann (2011) characterized these sites. Rabbits were submitted to bilateral cartilage defects bearing four subchondral drill holes. One knee per rabbit was treated by solidifying a chitosan glycerophosphate-blood implant over the defect. The animals were observed at 1–56 days post-surgery: chondrogenic foci were categorized as nascent, mature or resorbing based on global morphology, while the timing of foci appearance and foci position relative to the articular surface were recorded. They were also characterized using GAG histostaining, collagens type I, II, and X immunostaining, and polarized light microscopy for the collagen structure. Patched immunostaining was used to identify pre-hypertrophic chondrocytes and Ki-67 immunostaining was used to identify proliferating cells. By this approach the temporal and spatial modulation of the chondrogenic foci in subchondral microdrill holes became possible with the aid of chitosan glycerophosphate-blood implants (Chevrier et al., 2011).

The solidification mechanism of said chitosan hydrogel in the presence of blood was studied with a view at a most rapid gel formation (Hoemann et al., 2007; Marchand, Rivard, Sun, & Hoemann, 2009). Chitosan β-glycerophosphate (obtained from medical-grade

chitosan, sterile solutions 2.05 w/v%, 80% degree of deacetylation, 1200–2000 mPa s, pH 5.6, <100 endotoxins units/ml and <5 ppm heavy metal content) when mixed with whole blood solidifies over micro-fractured or drilled articular cartilage and elicits a more hyaline cartilage; for clinical use, rapid *in situ* solidification is preferred. Therefore, Marchand et al. (2009) investigated the solidification of chitosan glycerophosphate-blood implant with or without added clotting factors by thrombo-elastography (clotting time and clot tensile strength). Serum was analyzed for the onset of thrombin, platelet, and FXIII activation. *In vivo* solidification of chitosan glycerophosphate-blood mixtures, with and without clotting factors, was evaluated in 41 microdrilled cartilage defects of adult rabbits. Chitosan–glycerophosphate–blood clots solidified with high initial viscosity and minor platelet activation followed by the development of clot tensile strength concomitant with thrombin generation, burst platelet and FXIII activation.

Whole blood and chitosan–glycerophosphate–blood clots developed a similar final clot tensile strength, while polymer–blood clots showed a unique, sustained platelet factor release and greater resistance to lysis by tissue plasminogen activator. Thrombin, tissue factor, and recombinant human activated factor VII (rhFVIIa) accelerated chitosan–glycerophosphate–blood solidification *in vitro*. Pre-application of thrombin or rhFVIIa + tissue factor to the surface of drilled cartilage defects accelerated implant solidification *in vivo* ($P < 0.05$). The authors concluded that chitosan glycerophosphate–blood implants solidify through coagulation mechanisms involving thrombin generation, platelet activation and fibrin polymerization, leading to a fibrin–polysaccharide clot scaffold that resists lysis, and more stable than controls. Higher viscosity of blood + polymer mixtures could be related to chitosan precipitation or gelation at blood pH which is above the chitosan pK_a, to early platelet activation, or to the agglutination of red blood cells by chitosan that protected the clot against lysis (Marchand et al., 2009). These data are supported by Lord, Cheng, McCarthy, Jung, and Whitelock (2011) who showed that chitosan alone promotes both platelet adhesion and activation: when proteins are adsorbed to chitosan they modulate activation. Further, microdrilled cartilage defects, treated with thrombin-solidified chitosan/blood implants, regenerate a more hyaline, stable, and structurally integrated osteochondral unit than drilled controls (Marchand et al., 2012).

Platelet adhesion to chitosan on the other hand was found to be enhanced in the presence of adsorbed plasma and ECM proteins, while platelet adhesion to protein-coated chitosan was found to be mediated by integrins, with the main integrin involved being $\alpha_{IIb}\beta_3$ as widely reported. Therefore plasma and ECM proteins have a role in promoting platelet adhesion to chitosan while modulating the activation of bound platelets, which has important positive downstream effects on wound healing, as reported.

3. Hyaluronan

A relevant point of similarity between hyaluronan and partially deacetylated chitin is the GlcNAc repeating unit present in both. Hyaluronan is composed of units of glucuronic acid (GlcUA) and *N*-acetylglucosamine joined alternately by β-1,3 and β-1,4 anhydroglycosidic bonds. Hyaluronan is a key compound in the ECM of cartilage, thanks to its hydrogel-like nature, extremely high molecular weight, and moderate anionicity (Almond, 2007; Garg & Hales, 2004; Hillel, Shah, & Elisseeff, 2007; D.D. Kim, Kim, & Son, 2011; Kogan, Soltes, Stern, Schiller, & Mendichi, 2008; Kong & Mooney, 2005; Laurent, 1998; Milas & Rinaudo, 2005; Tamai et al., 2003).

Hyaluronan contributes to the elasticity and viscosity of the synovial fluid, and acts as a fluid shock absorber while maintaining the structural and functional characteristics of the cartilage matrix. This highly viscoelastic polymer fills the intercellular space between the collagen fibrillar network, surrounding all of the cells, the blood and lymph vessels and the neural elements in the fibrous tissue of the joint. It also inhibits the formation and release of prostaglandins, induces proteoglycan aggregation and synthesis, and modulates the inflammatory response (Adams et al., 1995; Gomis, Miralles, Schmidt, & Belmonte, 2009).

Hyaluronan is produced industrially with the aid of *Streptococcus* sp., nevertheless that approach is facing a growing concern due to the risk of accompanying endotoxins. In this light, the recombinant hyaluronan production has attracted increasing interest, and Novozymes has produced it with recombinant *Bacillus subtilis* (Widner, Behr, & Von Dollen, 2005). In their review, Liu et al. (2011) report history, current markets, and production of hyaluronan by *Streptococcus zooepidemicus* and recombinant systems, along with the challenges facing the production with microbial cells. While *S. zooepidemicus* remains the current microbial strain in the industrial production of hyaluronan, the recombinant hyaluronan production has emerged as an attractive alternative. Bacteria used as hosts include *Bacillus* sp., *Lactococcus lactis*, *Agrobacterium* sp., and *Escherichia coli*, that produce hyaluronans of different chain length in a mixture, thus a monodisperse product still represents a challenge. However, hyaluronans with relatively low MW (>10 kDa) have good viscoelasticity, moisture retention, and mucoadhesion, *i.e.* qualities desirable in ophthalmology, orthopedics, wound healing, and cosmetics. Nonetheless, for the purpose of cartilage regeneration, much higher MW values in the range 700–6000 kDa are necessary. Interestingly, Yamada and Kawasaki (2005) indicated that it is possible to produce simultaneously hyaluronan and chitin by the *Chlorella*-virus system.

A large number of different preparations of hyaluronan are commercially available in Europe and USA: each is produced from either rooster combs or by recombinant biotechnology and differs by molecular weight and hence residence time in the joint, and rheological properties. Low molecular weight products such as Sinovial® (800–1200 kDa) and Hyalgan® (500–730 kDa) differ from the high molecular weight hyaluronans because the latter are cross-linked, for example Synvisc® with 6000 kDa (Gigante & Callegari, 2011).

With the knee osteoarthritis patients increased by 26% from 15 million in 2000 to 19 million in 2010, the demand for visco-supplements is expected to escalate. In the US, the first single-injection hyaluronan for visco-supplementation, Synvisc-One®, was approved in early 2009. Likewise, the global market for dermal fillers is booming: nowadays there are almost 100 different dermal fillers on the market for aesthetic plastic surgery and about half of them are based on hyaluronan (Liu et al., 2011).

The continuous rise in the cost of the raw materials necessary for the operation of fermentation reactors and for the optimization of the biosynthesis of hyaluronan, weakens the commercial competitiveness of microbial hyaluronan production, and thus it is necessary either to find cheaper products for the fermentation, or to surrogate part of the hyaluronic acid with chitosan that performs equally well in the main applications, as demonstrated in the previous sections (Liu et al., 2011).

Visco-supplementation refers to the concept of synovial fluid replacement with intra-articular injections of hyaluronan mainly for the relief of pain associated with osteoarthritis (Balazs & Denlinger, 1993; Marshall, 2003). This treatment was approved by the US Food and Drug Administration in 1997. It is included in the guidelines for the treatment of knee OA of the American College of Rheumatology; the Orthopedic Consensus Conference made similar recommendations (Watterson & Esdaile, 2000)

For example, Sun, Chou, Hsu, and Chen (2009) studied 5 injectable hyaluronans, *i.e.* Hyalgan®, Supartz®, Orthovisc®, Synvisc®, and Euflexxa®, the latter being from bacterial fermentation and the other four from rooster combs; all of them are in the

form of sodium hyaluronate but Synvisc® is chemically modified. Cross-linked hyaluronans are called hylans. These products differ in their molecular weight (in the range 500–6000 kDa), dosing instructions, biochemical characteristics, and possibly clinical outcomes. So far there is no consistent evidence from clinical studies that documents the superior efficacy of one product over another. However, a review of the clinical research on viscosupplementation of the knee in patients and animals has been published by Edouard et al. (2011)Edouard, Rannou, and Coudeyre (2011) who highlighted the clinical improvements promoted by injections of hyaluronan after surgery.

Any degradation of endogenous hyaluronan *in vivo* is associated with vulnerability of the articular cartilage. While OA leads to a reduction in average molecular size and concentration of hyaluronan in the synovial fluid, the injection of hyaluronan into osteoarthritic joints is claimed to restore the viscoelasticity of the synovial fluid, augment the flow of joint fluid, normalize endogenous hyaluronan synthesis, inhibit hyaluronan degradation, reduce joint pain, and improve joint function.

The review by Gigante and Callegari (2011) mentions that in knee OA, visco-supplementation with 3–5 weekly intra-articular hyaluronan injections diminishes pain and disability, generally within one week and for up to 3–6 months. Visco-supplementation making use of Sinovial® is a valuable treatment, particularly when other therapies are contraindicated or have failed. Intra-articular injections of hyaluronan are approved worldwide for the mitigation of pain associated with OA of the knee.

Hyaluronans have comparable efficacy as non-steroidal anti-inflammatory drugs, but no gastrointestinal adverse events, and their benefits last longer than with intra-articular corticosteroids. In mild/moderate hip OA, intra-articular injections of hyaluronan ameliorate pain and function, generally for up to 3 months with no serious adverse events. Intra-articular injections of low/medium MW hyaluronans relieve pain and improve the function of the knee, shoulder, and the carpo-metacarpal joint of the thumb. Hyaluronans in most commercial preparations have the same structure as endogenous hyaluronan, although high molecular weight products containing cross-linked hyaluronan (hylans) with MW 6000 kDa exhibit greater elastoviscosity and intra-articular dwell-time.

Clinical efficacy is maintained for several months despite the half-life of intra-articular hyaluronan being only a few days. Hyaluronans are generally administered as a weekly injection over a course of 3–5 weeks: the pain relief obtained generally lasts several months. It should be kept in mind that the hyaluronan macromolecules are quite vulnerable in the presence of hyaluronidases and in unfavorable media, and that their cosmeceutical applications often give unsatisfactory results owing to prompt degradation. Therefore, the lasting action of hyaluronans injected in the knee is justified by stimulated *de novo* synthesis or, even better, by stimulated proliferation of chondrocytes. It has been suggested that exogenous hyaluronan induces the biosynthesis of hyaluronan by stimulating the regenerative process within the joint. Indeed, in studies of synoviocytes from joints of patients, exogenous hyaluronan was related to *de novo* synthesis of hyaluronan *in vitro* (Dixon, Jacoby, Berry, & Hamilton, 1988; Maheu, Ayral, & Dougados, 2002). Also, according to Jansen et al. (2008), rabbits were treated with hyaluronan after receiving partial-thickness articular cartilage wounds in the medial femoral condyle. Two days postoperatively, in hyaluronan-treated cartilage the percentage of dead cells was 6.7%, which was significantly lower compared to 16.2% in saline-treated cartilage. After 3 months the percentages of dead cells in both groups were statistically similar. Hyaluronan treatment resulted in significantly higher ^{35}S-sulfate incorporation compared to controls. These results suggest a potential role of hyaluronan in preventing cell death consequent to damages to the cartilage.

3.1. Scaffolds containing both chitosan and hyaluronan

Thanks to its cationic nature, chitosan forms polyelectrolyte complexes with a large variety of anionic polysaccharides, among which hyaluronan (Muzzarelli, Stanic, Gobbi, Tosi, & Muzzarelli, 2004). Scaffolds of chitosan and hyaluronan in different weight ratios have been prepared, based on the hypothesis that hyaluronan could enhance structural and biological properties of the chitosan scaffolds: they were found to be non-cytotoxic and to promote cell adhesion. The incorporation of hyaluronan enhanced cartilage extracellular matrix production as observed after safranin-O and Alcian blue staining (Correia et al., 2011). Likewise, chitosan–hyaluronan membranes with seeded keratinocytes indicated that chitosan and hyaluronan were compatible at the blending ratios of interest; they had homogeneous structure, suitable chemical properties and biocompatibility with cells, which implied that the polyelectrolyte complex could be used not only as a membrane for corneal cell culture *in vitro* (Yao & Wu, 2011), but also as a crosslinked injectable hydrogel for cartilage regeneration (Tan, Wu, Lao, & Gao, 2009), besides as a scaffold with large surface area for dental pulp regeneration (Coimbra et al., 2011). Mature cartilage constructs regenerated with chitosan-hyaluronan scaffolds were implanted into 5-mm diameter osteochondral defects in the patellar groove of rabbits. At 12 weeks after implantation, the reparative tissues consisted of hyaline-like cartilage with stable fusion to native cartilage and normal reconstitution of subchondral bone. The histological score was higher than for controls in terms of hematoxylin and eosin staining, and collagen-II immunohistochemical staining. Biomechanically, the compression modulus of reparative tissue at 12 weeks postoperatively was comparable to that of normal articular cartilage (Kasahara et al., 2008).

Articular cartilage repair with tissue-engineered hyaline cartilage reconstructed in a chitosan-hyaluronan scaffold by costal chondrocytes was obtained by Lee, Choi, Kim, Kim, and Son (2011) who partially re-acetylated the porous chitosan scaffolds with acetic anhydride to DA 0.60 to enhance hydrophilicity while maintaining structural integrity and porosity. Said scaffolds with or without hyaluronan coating were used for cartilage reconstruction *in vitro*: the chondrocytes within the scaffolds re-differentiated to hyaline cartilage-like constructs, featuring GAG and collagen-II expression and presence of lacunae. The obtained hyaline cartilage was transplanted to full thickness cartilage defects made on the patellar grove of rabbit knee, and was evaluated by immunohistology and Wakitani's scoring: hyaline cartilage and subchondral bone were restored, as opposite to the fibrocartilage formed in the control.

Gene therapy treats the causes of osteoarthritis by targeting specific pathological mechanisms; however, the transfection efficiency of hyaluronan–chitosan nanoparticles in chondrocytes was found inadequate (Zhang et al., 2006). As an advancement in this area, Lu et al. (2011) reported on hyaluronan–chitosan nanoparticles as delivery vectors capable of transferring exogenous genes into primary chondrocytes for the treatment of joint diseases; the pEGFP was incorporated into said cationic particles (Fig. 6). Transfection efficiency was maximized for the medium pH *ca.* 6.8, N/P ratio 5, plasmid concentration of 4 μg/ml, and chitosan MW 50 kDa. The average viability of cells transfected with said nanoparticles was over 90%.

It is reasonable to make use of collagen to accompany chitosan and hyaluronan in the preparation of scaffolds. Collagen-II and hyaluronan are the two major components of the native ECM: both provide the associated tissues with tensile strength, and also serve for cell adhesion and growth. Lin et al. (2009) identified the optimum ratio 9:1:1 for the manufacture of collagen–hyaluronan–chitosan scaffolds. The mean pore diameter was in the range 120–182 μm and decreased

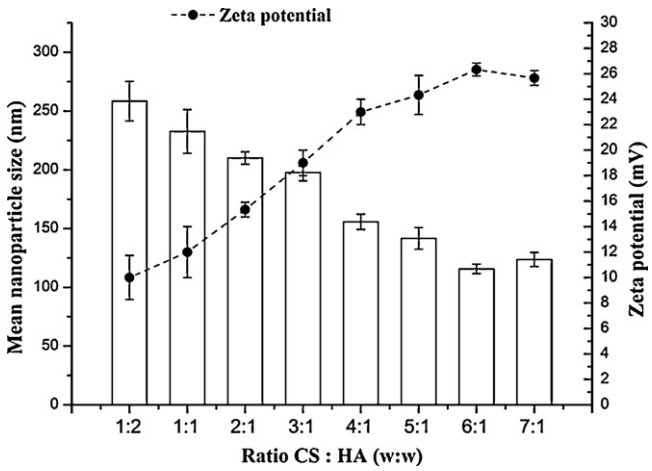

Fig. 6. Hyaluronan of low MW (<10 kDa) was used at pH 5.5 and at the constant concentration of 11.25 μg/ml, and the chitosan concentration varied (from left to right): 5.625, 11.25, 22.5, 33.75, 45, 56.25, 67.5, and 78.25 μg/ml. The size and zeta potential of the nanoparticles were dependent on the composition, with a decrease in size and an increase in the zeta potential with increasing chitosan content. At this pH (5.5) all zeta potential values are in the positive range, owing to the prevailing cationic nature of the polyelectrolyte complex.

Source: From Lu et al. (2011). Elsevier BV, Amsterdam.

as the chitosan quantity in the mix increased; all scaffolds showed high pore interconnectivity, and could bind 35–40-fold of physiological fluid without loosing shape and stability. The enzymatic depolymerization assay demonstrated that the presence of chitosan can markedly improve the biostability of the collagen–hyaluronan–chitosan scaffolds under EDC treatment. Based on the amount of 4-hydroxyproline and reducing sugar released from the various collagen–hyaluronan–chitosan scaffolds degraded by collagenase and lysozyme for 1, 3, 5 and 7 days, a steady increase in the biostability of the scaffolds was observed when the chitosan concentration was increased. Absence of chitosan meant extremely high susceptibility to hydrolysis: this implies that resistance to collagenase increased along with the dose of chitosan.

With the intention of increasing the compressive modulus, microspheres were incorporated into scaffolds: for example poly(lactide-co-glycolide)-based microspheres were prepared with gelatin powder and Na hyaluronate in water: then, at room temperature, the chitosan acetate solution was added. The PLGA accounted for weight ratios 30, 50 and 70% of the total polymer mass. The system was then crosslinked with EDC before being frozen and freeze-dried. Chondrocytes proliferated and secreted extracellular matrix at the same level as in the control gelatin-chitosan-hyaluronan scaffold provided that the PLGA weight ratio did not exceed 50%, *i.e.* the porosity was adequate (Tan, Chu, Payne, & Marra, 2009). In chitosan–hyaluronan films manufactured layer-by-layer, polyacrylate when deposited sparsely between the layers, disturbs the soft hyaluronan, and forms diffusion barriers. It imparts more mechanical strength to the rather soft hyaluronan–chitosan films. Likewise, chitin nanofibrils $255 \pm 56 \times 31 \pm 6$ nm were incorporated into hyaluronan–gelatin (Hariraksapitak & Supaphol, 2010). As for polyacrylate, it is still unclear whether the mechanical properties are improved without impairing their biocompatibility (Salomaki & Kankare, 2009).

Tan et al. (2010) focused on fabrication and characterization of biomimetic surfaces with the aid of hyaluronan–chitosan multilayers, endowed with a surface of major extracellular matrix components of native meniscus, namely collagen-I and -II or chondroitin sulfate. The surface modification involved a hyaluronan solution incubated with EDC/NHS for 15 min at room temperature followed by deposition of chitosan solution to a total of 3.5 bilayers: the outermost hyaluronan layer contained EDC/NHS-activated carboxylic groups ready for covalent reaction with collagen or chondroitin-6-sulfate. This multi-layered material was chemically stable because all 3 polysaccharides were covalently linked to each other, and therefore it was suitable as a biomimetic material for phenotype-stable meniscal cell expansion. In fact, dedifferentiation of meniscal cells (that normally occurs during monolayer expansion) could be avoided by culturing the cells on said chondroitin sulfate surfaces, as indicated by increased collagen-II and aggrecan gene expression, proteoglycan production, and abundant lipid vacuoles evident over an extended culture period.

4. Chondroitin sulfate scaffolds with chitosan and other polymers

Associations of chondroitin sulfate with hyaluronan and GlcNAc are known to repair cartilage after surgery because chondroitin sulfate increases the synthesis of hyaluronan, glucosamine and collagen-II, while inhibiting ECM degrading enzymes (Henson, Getgood, Caborn, McIlwraith, & Rushton, 2012). Chitosan–chondroitin sulfate nanoparticles are recognized as vehicles for protein drug delivery *via* endocytosis (Yeh, Cheng, Hu, Huang, & Young, 2011); they can be reinforced by calcium chelation (Hyland, Taraban, Hammouda, & Yu, 2011). A hydrogel containing 40% chitosan and 60% chondroitin sulfate was studied to be used as a chondroitin sulfate carrier. The polyelectrolyte complex presented macromolecular reorganization at pH values ranging from 6 to 12; the lowest zeta potential values occurred at pH 1 and 9 but remained positive over the entire pH range (Piai, Rubira, & Muniz, 2009). The effect of chondroitin sulfate in patients with osteoarthritis is possibly the result of the stimulation of the synthesis of proteoglycans and the decrease in catabolic activity of chondrocytes by inhibiting the synthesis of proteolytic enzymes and other factors that contribute to cartilage matrix damage and cause the death of these cells. Chondroitin sulfate also exerts anti-inflammatory activity. The comparison of the effects of chondroitin sulfate of different origins and levels of purity on human osteoarthritic cartilage revealed the existence of disparate effects, the positive ones being possibly due to its contribution to a proper balance between anabolism and catabolism in the articular tissues (Kwan Tat, Pelletier, Mineau, Duval, & Martel-Pelletier, 2010; Martel-Pelletier, Kwan Tat, & Pelletier, 2010).

Intelligent biopolymer systems with *in situ* gel-forming capability are of increasing importance in the development of therapeutic implants and drug delivery systems. Poly(N-isopropylacrylamide) is a thermoresponsive polymer exhibiting reversible sol-to-gel phase transition in water, where it is soluble below its lower critical temperature (*ca.* 30 °C). Poly(N-isopropylacrylamide) end-capped with a carboxyl group (PNIPAM-COOH) was grafted to chitosan for synthesizing thermo-reversible chitosan-*g*-poly(N-isopropylacrylamide), further grafted with hyaluronan to form hyaluronan-*g*-chitosan-PNIPAM (Table 2) (Chen & Cheng, 2009). Overall, rabbit articular chondrocytes and meniscal cells cultured in hyaluronan-*g*-chitosan-PNIPAM hydrogels showed beneficial effects on cell phenotypic morphology, proliferation, and differentiation. This should be a favorable consequence of the change of surface potential from positive to moderately negative (−9.35, Table 2).

In fact, the surface potential values of pure hyaluronan and pure chitosan were -47.8 ± 1.7 mV and $+11.3 \pm 0.3$ mV, respectively. Progressive tissue formation was demonstrated by monotonic increase in extracellular matrix amounts and mechanical properties (Chen & Cheng, 2009). Likewise, chitosan-poly(butylene succinate) scaffolds were assayed by using bovine articular

Table 2
Properties of PNIPAM-COOH, chitosan-g-PMIPAM and hyaluronan-g-chitosan-PNIPAM.

Polymers	[a]Grafting ratio	[b]MW (kDa)	Yield (%)	Potential (mV)
PNIPAM-COOH	–	21	73	−3.26 ± 0.39
Chitosan-g-PNIPAM	46	1120	67	+8.64 ± 0.56
Hyaluronan-g-chitosan-PNIPAM	27	32200	94	−9.35 ± 1.17

(Modified from Chen & Cheng, 2009).
All experiments were conducted at room temperature with 1% (w/v) polymers in water.
[a] Number of PNIPAM-COOH chains grafted onto each chitosan macromolecule, or the number of chitosan-g-PNIPAM chains grafted onto each hyaluronan macromolecule.
[b] Calculated from end-group titration or from grafting ratio, and expressed in kDa.

chondrocytes: thanks to suitable pore size and geometry, the formation of cartilagineous tissue took place. Cells colonized the entire scaffolds and were able to produce ECM; GAG production was enhanced by stirring (M.L.A. Da Silva et al., 2010).

5. Conclusion

The keys to the comprehension of the exciting advances made during the last quinquennium are the chemical manufacture of porous scaffolds made of hyaluronan and chitosan, the biological functionality imparted by growth factors, and the tissue generation achieved *via* induced stem cells from a variety of sources. The engineered functional hyaline cartilage that meets specific mechanical requirements, and the study of cell populations capable of synthesizing ECM with appropriate composition and structure made it possible to circumvent the obstacle represented by the fibrous nature of the repair cartilage obtained in early therapeutic approaches.

The planning of a scaffold today takes into consideration aspects relevant to chemistry, biochemistry, material science, orthopedics and surgery. Porous chitosan xerogels are easily manufactured with advanced technologies that permit control over surface area and pore size, two key parameters for maximum compatibility. Scaffolds with or without hyaluronan were used for cartilage reconstruction *in vitro*: the chondrocytes within the scaffolds re-differentiated to hyaline cartilage-like constructs, featuring presence of lacunae and expressing GAG and collagen-II. The hyaline cartilage and subchondral bone can be restored, as opposite to the fibrocartilage formed in the control.

The implantation of chitosan hydrogels without chondrocytes also helps repair cartilage defects: this surprising finding parallels the documented capacity of hyaluronan hydrogels to stimulate cartilage growth for a relatively long period, notwithstanding their susceptibility to depolymerization by hyaluronidases. In fact, the injection of hyaluronan into osteoarthritic joints is reported to restore the viscoelasticity of the synovial fluid, augment the flow of the joint fluid, normalize endogenous hyaluronan synthesis, inhibit hyaluronan degradation, reduce joint pain, and improve joint function. Clinical efficacy is maintained for months despite the half-life of intra-articular hyaluronan being only a few days. The lasting action of hyaluronans injected in the knee is justified by stimulated *de novo* synthesis or by stimulated proliferation of chondrocytes.

Chitosan exhibits a number of properties favorable for use in cartilage regeneration and repair, particularly when in admixture or chemically linked to fibroin, gelatin and collagen, or combined with safe man-made polymers such as PEO and PCL, or in the form of polyelectrolyte complexes with hyaluronan and chondroitin sulfate, as testified by many research groups.

One of the recent approaches involves the seeding of chondrocytes in a hyaluronan scaffold, resulting in pre-differentiation of cells *in vitro*, suitability for arthroscopic implantation, and implant stability. On the other hand, chitosan induces the chondrogenic differentiation of the human bone marrow mesenchymal stem cells; it preserves the round morphology of chondrocytes, and their capacity to synthesize cell-specific extracellular matrix. Thanks to the inherent high biochemical significance of chitosan, gene therapy supported by the use of chitosan–hyaluronan hybrid vectors will certainly develop, in consideration of the excellent transfection efficiency so far obtained.

Bioreactors represent an attractive tool to enhance the biochemical and mechanical properties of the engineered tissues by providing adequate mass transfer and physical stimuli. Concaro, Gustavson, and Gatenholm (2009) expressed the view that development of automatic culture systems and non-invasive monitoring of matrix production will take place soon, and will improve the qualitative and economical aspects of said biochemical products.

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